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Dissecting CD8+ NKT Cell Responses to Listeria Infection Reveals a Component of Innate Resistance

Sergey S. Seregin,* Grace Y. Chen, † and Yasmina Laouar*

A small pool of NK1.1+ CD8+ T cells is harbored among the conventional CD8+ T cell compartment. Conclusions drawn from the analysis of immune responses mediated by cytotoxic CD8+ T cells are often based on the total population, which includes these contaminating NK1.1+ CD8+ T cells. An unresolved question is whether NK1.1+ CD8+ cells are conventional T cells that acquire NK1.1 expression upon activation or delineation into memory phenotype or whether they are a distinct cell population that induces immune responses in a different manner than conventional T cells. To address this question, we used the Listeria monocytogenes model of infection and followed CD8+ NK1.1+ T cells and NK1.1− CD8+ T cells during each phase of the immune response: innate, effector, and memory. Our central finding is that CD8+ NK1.1+ cells and conventional NK1.1− CD8+ T cells both contribute to the adaptive immune response to Listeria, but only CD8+ NK1.1+ cells were equipped with the ability to provide a rapid innate immune response, as demonstrated by early and Ag-independent IFN-γ production, granzyme B expression, and degranulation. More importantly, purified conventional CD8+ T cells alone, in the absence of any contaminating CD8+ NK1.1+ cells, were not sufficient to provide early protection to lethally infected mice. These results highlight the role of CD8+ NK1.1+ T cells in mounting early innate responses that are important for host defense and support the therapeutic potential of this subset to improve the effectiveness of protective immunity. The Journal of Immunology, 2015, 195: 1112–1120.

The immune system is composed of a rapid, Ag-independent innate arm and an Ag-specific delayed adaptive arm. CD8+ T cells are an integral part of classical Ag-dependent immunity against a variety of viral and bacterial pathogens. Development of CTL activity is a process that takes days (1, 2). To acquire full functional competence, CD8+ T cells must be activated by the innate immune system, enter a phase of replication, and differentiate into effector cytotoxic T cells that provide long-lasting protection. However, although the adaptive CD8+ T cell response is developing, innate immune cells, especially NK cells, play a critical role in eradicating infected cells, often using similar strategies as CD8+ T cells: the production of IFN-γ, perforins (3−5).

Recent reports suggested innate capabilities within the CD8+ T cell population (6, 7). Specifically, memory CD8+ T cells were shown to secrete IFN-γ in response to IL-12/IL-18 stimulation in an Ag-independent manner (8). Memory CD8+ T cells also were shown to contribute to innate immune responses and early protection from pathogen re-encounter (9−11). In these studies, Listeria-primed memory CD8+ T cells were able to secrete IFN-γ and granzyme B (GzmB) upon reinfecction with Listeria in an Ag-independent manner (9, 10, 12−14). Ruiz et al. (13) identified this population of memory CD8+ T cells as NK1.1+ CD8+ T cells. Similarly, lymphocytic choriomeningitis virus−primed memory CD8+ T cells were able to rapidly produce innate IFN-γ upon infection with murine CMV in an Ag-independent manner (15). However, these innate capabilities were identified in the context of prior pathogen exposure.

Although the existence of CD8+ NK1.1+ cells (most often termed CD8+ NKT cells) has been reported for more than two decades, their legitimacy as a distinct T cell subset is still under debate (16−23). To compare and contrast its function with that of the conventional CD8+ T cell (NK1.1−) compartment, we used a L. monocytogenes infection model and examined the kinetics of responses by both populations during infection. This model of infection has a well-established pattern of Ag-specific CD8+ T cell adaptive immune responses in mice required for bacterial clearance, but it also allows the study of innate immune responses to control bacterial burden during the early phase of infection (24−27). In this study, we show that CD8+ NKT and conventional NK1.1− CD8+ T cells both contribute to the adaptive response to Listeria infection; however, only CD8+ NKT cells had the ability to produce rapid innate immune responses, as demonstrated by early and Ag-independent proliferation, IFN-γ production, GzmB expression, and degranulation. Importantly, when conventional CD8+ NK1.1− T cells were adoptively transferred into immunodeficient mice, these cells were inferior to NKT cells in protecting mice against early infection. Thus, we propose that, in naive mice, a subset of CD8+ T cells that expresses NK1.1 has innate capabilities that are critically important for early host defense against initial infection. Accordingly, we propose that the pattern of NK1.1 expression in CD8+ T cells is similar to the pattern of CD25 expression in CD4+ T cells (28), with both constitutive and acquired expression yielding two subsets of CD8+ T cells that have distinct functions during the course of an immune response.

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Abbreviations used in this article: BHI, brain-heart infusion; Erm, erythromycin; FSC, forward scatter; GzmB, granzyme B; iNKT, invariant NKT; LB, Luria Broth; LM-OVA, L. monocytogenes expressing ovalbumin; SSC, side scatter.

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Materials and Methods

Animal procedures

Adult C57BL/6 wild-type, Rag2<sup>−/−</sup>, Rag2<sup>−/−</sup>/γc<sup>−/−</sup>, and CD14<sup>−/−</sup> mice were purchased from Taconic. All mice were housed in a specific pathogen-free room; all *Listeria*-infected mice were housed in a specific Animal Biosafety Level 2 facility. For i.v. infections, mice were anesthetized i.p. with ketamine (80 mg/kg) and xylazine (10 mg/kg) in 200 µl PBS. Intravenous infections were performed retro-orbitally. Blood and tissue samples were collected and processed at the indicated time points in accordance with University of Michigan Animal Care and Use Committee guidelines, and approval to use mice was granted by the University of Michigan in accordance with the National Institutes of Health requirements for the care and use of animals. Care for mice was provided in accordance with Policy on Humane Care and Association for Assessment and Accreditation of Laboratory Animal Care International standards.

*L. monocytogenes* infection

*L. monocytogenes* expressing ovalbumin (LM-OVA) strain 10403s was a kind gift from Mary O’Riordan (University of Michigan). LM-OVA was grown in brain-heart infusion (BHI) or Luria Broth (LB) medium with 5 µg/ml erythromycin (Erm) (30). The dose and route of LM-OVA infection for priming and the prime/boost regimen were established previously (29, 31, 32). We collected bacteria in a mid-log phase and injected 10<sup>5</sup>, 10<sup>6</sup>, or 2 × 10<sup>5</sup> CFU/mouse i.v. The infection dose was determined based on the following formula: CD<sub>50</sub> of L. *monocytogenes* was 1.2 × 10<sup>2</sup> bacteria/ml. The dose was validated retrospectively on BHI or LB agar plates + 5 µg/ml Erm. LM-OVA burden was calculated using CFU determination, as detailed previously, by culturing serially diluted homogenized spleen and liver on BHI/Erm or LB/Erm agar plates (27, 33).

In vivo treatment

Where indicated, mice were treated with 2 mg BrdU (Sigma-Aldrich) for 3 d (once a day) or with 4 mg/kg polynosinic-polycytidylic acid (GE Healthcare) once (i.p., in 200 µl PBS).

Lymphocyte isolation

Single-cell suspensions of spleen, liver, and PBLs were prepared in RPMI 1640 supplemented with 5% FCS. Cells were passed through a nylon mesh (70 µm), RBCs were lysed, and cells were counted and stained. Liver lymphocytes were prepared by perfusion and then crushed through a nylon mesh. Liver cells were passed through a 40%/70% Percoll gradient and centrifuged at 2000 rpm for 20 min at room temperature. Cells were harvested from the interface, counted, and stained.

Cell staining and flow cytometry

All cell suspensions were treated with 2.4G2 and then surface stained with the following fluorochrome-conjugated Abs: CD3 (145-2C11 or 500A2), CD8 (53-6.7), CD4 (RM4-5), NK1.1 (PK136), CD49b (DX5), CD127 (A73-20C7), CD132 (43G), CD19 (id3), CD244 (m2B4), CD27 (LG779), CD44 (IM7), CD62L (MEL-14), CD94 (18d3), MHC class II (M5/114.15.2), Ly49A (YEL/148.10.6 or A1), Ly49A/D (12A8), Ly49C/1 (5E6), Ly49D (4E5), Ly49G (AT-8), Ly49H (3D10), Ly49I (YLI-90), NKGA2/C/E (20d5), NKGD2 (CX5), NKp46 (29A1.4), CD69 (H1.2F3), and CD107a (1D4B). For intracellular cytokine staining, cells were incubated for 4 h at 37°C in the presence of protein transporter inhibitor GolgiStop (BD Biosciences), surface stained, treated with Cytofix/Cytoperm buffer (BD Biosciences), and then stained with fluorochrome-conjugated Abs against IFN-γ (XMG1.2) and GzmB (GB11). BrdU staining was performed using a BrdU staining kit (BD Biosciences), according to the manufacturer’s instructions. All Abs were purchased from BD Biosciences, eBioscience, or BioLegend. Allophycocyanin-conjugated CD1d-PBS57 tetramer and PE-conjugated H2K<sup>b</sup>-m3G1211 tetramer were obtained from the National Institutes of Health Tetrramer Core Facility. PE-conjugated H2K<sup>b</sup>-SIINFEKL (OVA) tetramer was from BD Biosciences and MBL International. Cells were acquired on a FACS Canto or FACS Aria III flow cytometer (BD Biosciences) using FACSDiva software, and data were analyzed using FlowJo software (TreeStar). All FACS analyses were performed after excluding the contamination of doublets. Briefly, samples were gated by comparing forward scatter (FSC)-W versus FSC-H; to exclude doublets, events with a high FSC-W profile were gated out of the total sample population. The remaining population was examined based on its side scatter (SSC)-W/SSC-H profile, and any SSC-W<sup>high</sup> events were similarly excluded. When indicated, cells were sorted on a FACS Aria III at the flow cytometry core facility at the University of Michigan.

IFN-γ secretion assay

Total splenocytes from uninfected or LM-OVA–infected mice were isolated at the indicated time points and stimulated ex vivo in the presence of GolgiStop with a combination of recombinant mouse IL-12 (5 ng/ml; PeproTech) and IL-18 (25 ng/ml; R&D Systems) for 4 h or for 24 h in the presence of OVA (4 µg/ml) or OVA (4 µg/ml) peptides. Following incubation, cells were surface stained, fixed and permeabilized, and stained for intracellular IFN-γ.

Adaptive transfer experiments

Sorted populations of conventional CD8<sup>+</sup> T cells (CD3<sup>+</sup> NK1.1<sup>−</sup> CD1d-tetramer<sup>−</sup> CD8<sup>+</sup>), NKT cells (CD3<sup>+</sup> NK1.1<sup>−</sup> CD1d-tetramer<sup>+</sup>), or CD8<sup>+</sup> NKT cells CD8<sup>+</sup> CD3<sup>+</sup> NK1.1<sup>−</sup> CD1d-tetramer<sup>−</sup> CD8<sup>+</sup> CD3<sup>+</sup> NK1.1<sup>−</sup> CD1d-tetramer<sup>−</sup> were injected i.v. into Rag2<sup>−/−</sup>/γc<sup>−/−</sup> recipient mice (100,000 cells/mouse). Three days posttransfer, Rag2<sup>−/−</sup>/γc<sup>−/−</sup> recipient mice were infected with 100,000 CFU LM-OVA and monitored for survival for 10 consecutive days.

Statistical analysis

Statistically significant differences were determined using two-way ANOVA with a Bonferroni post hoc test (time × cell population) or by one-way ANOVA with a Student–Newman–Keuls post hoc test (p value < 0.05). Survival curve comparison was performed using the Mantel–Cox log-rank test. Graphs show mean ± SEM. GraphPad Prism 6 software was used for statistical analysis.

Results

Conventional CD8<sup>+</sup> NK1.1<sup>−</sup> T cells and CD8<sup>+</sup> NKT cells both contribute to the adaptive response to *Listeria infection*

CD8<sup>+</sup> NK1.1<sup>−</sup> cells are a subset of cells from the NKT compartment that are CD1d-tetramer<sup>−</sup> and, therefore, are distinct from invariant NKT (iNKT) cells (Supplemental Figs. 1, 2). To determine whether this cell population (CD8<sup>+</sup> NKT cells) has functions distinct from those of conventional CD8<sup>+</sup> NK1.1<sup>−</sup> T cells (conventional CD8<sup>+</sup> T cells), we studied both cell populations (CD8<sup>+</sup> NK1.1<sup>−</sup> and CD8<sup>+</sup> NK1.1<sup>−</sup>) during the host response against *L. monocytogenes* infection, which requires both innate and adaptive immunity for effective bacterial clearance. To examine both effector and memory T cell responses, we used a typical prime/boost LM-OVA infection regimen (29, 31, 32), with the first dose administered at day 0 (10<sup>2</sup>–10<sup>5</sup> CFU), followed by a second high dose (2 × 10<sup>5</sup> CFU) at day 42 (Supplemental Fig. 3). Kinetic studies of conventional CD8<sup>+</sup> NK1.1<sup>−</sup> T cell numbers in the spleen, liver, and blood from LM-OVA–infected mice showed peak Ag-specific responses consistent with the development of effector (day 7 postprime) and memory (day 7 postboost) Ag-specific CD8<sup>+</sup> T cell responses, as indicated by H2K<sup>b</sup>-SIINFEKL tetramer (OVA tetramer) positivity (Fig. 1A–E). Likewise, we found that CD8<sup>+</sup> NK1.1<sup>−</sup> T cells were capable of mounting OVA-specific T cell responses with similar kinetics as their counterparts from the conventional CD8<sup>+</sup> T cell compartment, with ∼6% of conventional CD8<sup>+</sup> T cells and ∼8% of CD8<sup>+</sup> NKT cells positive for OVA-tetramer staining on day 7 (peak of the effector response) (Fig. 1A–E). These results were observed in the spleen, liver, and blood of infected wild-type mice (Fig. 1C, 1E), as well as in CD1d<sup>−/−</sup>/γc<sup>−/−</sup> mice lacking iNKT cells (Fig. 1D). Of interest, we found that a high dose of infection (i.e., 10<sup>7</sup> CFU) similarly primed the two CD8<sup>+</sup> T cell compartments, whereas a low dose of infection (i.e., 10<sup>5</sup> CFU) efficiently primed CD8<sup>+</sup> NKT cells but not conventional CD8<sup>+</sup> T cells, suggesting an increased sensitivity to priming in CD8<sup>+</sup> NKT cells (Fig. 1A, 1E). However, after rechallenge, the pool size of OVA-tetramer<sup>+</sup> CD8<sup>+</sup> T cells increased similarly in both T cell populations, indicating similar outcomes of the memory response (Fig. 1C, 1E). To further confirm these observations, we used another tetramer, Lema
Listeria specific N-formylated peptide LemA; f-MIGWII), which is known to detect Listeria-specific T cell responses (34) (Fig. 1F). Although this tetramer was not ideal to study Listeria-specific T cell memory responses, the results during the effector phase confirmed the efficient priming of both CD8+ T cell subsets, with a slightly higher frequency of LemA-specific T cells among CD8+ NKT cells compared with conventional CD8+ T cells (Fig. 1F). Of note, the absolute numbers of tetramer+ (e.g., OVA or LemA) cells were higher for conventional CD8+ T cells with a similar profile for both tetramers (Fig. 1C, 1F, Supplemental Fig. 4), likely reflecting the much larger pool size of the conventional CD8+ T cell compartment compared with the NKT cell compartment (~50 times) (Supplemental Fig. 1).

Likewise, the patterns of memory T cell responses (35, 36), as indicated by the central (CD62L+ CD127+) and effector (CD62L− CD127+) memory phenotypes, were not dramatically different in conventional CD8+ NK1.1+ T cells and CD8+ NKT cells (Figs. 1B, 2A, 2B), with some minor, but statistically significant, differences in the conversion of CD8+ NKT cells to central memory phenotype at day 7 postinfection. However, CD8+ NKT cells were of similar phenotype as conventional CD8+ T cells at later time points (Fig. 2A, 2B). Consistently, we found that IFN-γ release in response to ex vivo stimulation with OVA peptide was equally robust in CD8+ T cell and CD8+ NKT cell compartments during the effector and memory phases (Fig. 2C). Likewise, equivalent levels of GzmB expression were observed in both CD8+ T cell subsets at both phases of the adaptive response mounted against Listeria (Fig. 2D). Collectively, our data suggest that conventional CD8+ NK1.1+ T cells and CD8+ NKT cells have similar adaptive responses to Listeria infection.

CD8+ NKT cells can provide innate responses against Listeria infection

The kinetics of OVA tetramer staining are consistent with the absence of Ag-specific OVA-tetramer+ T cells early during LM-OVA infection of naive mice in either conventional CD8+ T cell or CD8+ NKT cell populations (Figs. 1, 3A). Accordingly, we chose to examine early responses to LM-OVA infection in both CD8+ T cell subsets (CD8+ NK1.1+ and CD8+ NK1.1−) on day 3 postinfection. This included analysis of early T cell activation (CD69), T cell expansion (BrdU), innate IFN-γ production, cytotoxicity

**FIGURE 1.** CD8+ NKT cells mount an Ag-specific T cell response to Listeria infection with kinetics similar to that of conventional CD8+ T cells. (A) Spleen and blood (PBL) of LM-OVA–infected mice (103 or 105 CFU) were analyzed on day 7 postinfection. Plots show the frequency of OVA tetramer+ cells among gated NK, iNKT, and CD8+ T cells. For NKT cells, we show the distribution of OVA tetramer versus CD8. (B–F) Mice were infected with 104 CFU of LM-OVA on day 0 and rechallenged with 2 × 105 CFU on day 42, as indicated by arrows. (B) Distribution of CD8 versus OVA tetramer staining among gated NK, iNKT, T, and NKT cell subsets. Kinetics of frequency (C and D) and numbers (E) of OVA tetramer+ cells among NK, iNKT, and CD8+ T cells from infected wild-type (C) and CD1d−/− (D) mice. (E) Frequency of CD8+ NKT cells in spleen, liver, and blood from mice infected with 104 or 105 CFU LM-OVA. (F) Kinetics of frequency of LemA-tetramer+ cells among NK, iNKT, and CD8+ T cells in spleen and liver. Data in (D) are representative of two independent experiments (n = 4; mean ± SEM). Data in (C), (E), and (F) (mean ± SEM) are representative of one of three independent experiments, n = 3 for each (for days 0, 7, 49); data are representative of one of two independent experiments, n = 3 for each (for days 3, 14, 42, 45, 70). *p < 0.05, **p < 0.01 NKT versus conventional CD8+ T cells.
(GzmB), and degranulation (CD107). Analysis of the spleen, liver, and blood showed expression of CD69 on all lymphocyte subsets on day 3, indicating early activation (Fig. 3B). However, despite early activation of CD8+ T cells from both compartments, only CD8+ NKT cells exhibited robust expansion (BrdU+) at day 3 postinfection in the absence of previous Listeria exposure (Fig. 3C), which correlated with a more robust CD69 expression in CD8+ NKT cells (Fig. 3B). However, the absolute numbers of CD69+ conventional T cells on days 0–3 postinfection were slightly greater than those for CD8+ NKT cells, likely due to the increased pool size of the conventional CD8+ T cell compartment (~50 times, Supplemental Fig. 1).

Next, we compared CD8+ NKT cells and conventional NK1.1+CD8+ T cells for their ability to exhibit rapid cytotoxicity against Listeria infection (Fig. 4). Expression of GzmB (Fig. 4A) and the degranulation marker CD107 (Fig. 4B) was examined at day 3 postinfection. Results revealed significantly greater expression of GzmB in CD8+ NKT cells compared with conventional CD8+ NK1.1+ T cells, suggesting an ability of CD8+ NK1.1+ T cells to rapidly acquire cytotoxic activity early during Listeria infection (Fig. 4A). These results were further confirmed by efficient degranulation of CD8+ NKT cells, as indicated by the significantly increased frequency of CD107+ cells on day 3 postinfection compared with conventional CD8+ NK1.1+ T cells (Fig. 4B), which typically require more time to acquire full functional competence (i.e., 6–7 d) (1, 2). Notably, the early potent cytotoxicity among CD8+ NKT cells is Ag independent, as indicated by the lack of OVA tetramer staining on days 0–3 postinfection (Fig. 4). Expression of GzmB (Fig. 4A) and the degranulation marker CD107 (Fig. 4B) was examined at day 3 postinfection. Results revealed significantly greater expression of GzmB in CD8+ NKT cells compared with conventional CD8+ NK1.1+ T cells, suggesting an ability of CD8+ NK1.1+ T cells to rapidly acquire cytotoxic activity early during Listeria infection (Fig. 4A). These results were further confirmed by efficient degranulation of CD8+ NKT cells, as indicated by the significantly increased frequency of CD107+ cells on day 3 postinfection compared with conventional CD8+ NK1.1+ T cells (Fig. 4B), which typically require more time to acquire full functional competence (i.e., 6–7 d) (1, 2). Notably, the early potent cytotoxicity among CD8+ NKT cells is Ag independent, as indicated by the lack of OVA tetramer staining on days 0–3 postinfection (Fig. 4).

Finally, we assessed the contribution of conventional CD8+ T cells and CD8+ NKT cells in the early production of IFN-γ (Fig. 4A, 4C). In this case, cells from untreated or infected (day 3 postinfection) mice were examined for intracellular expression of IFN-γ in response to the cytokine stimulus IL-12/IL-18. We found that CD8+ NKT cells were as potent as iNKT and NK cells in producing IFN-γ in response to IL-12/IL-18 alone (Fig. 4C). In sharp contrast, conventional CD8+ T cells exhibited poor IFN-γ production in response to IL-12/IL-18 stimulation (Fig. 4C). Similarly, CD8+ NKT cells, but not CD8+ NK1.1+ T cells, harvested from 3-d–infected mice and restimulated ex vivo with IL-12/IL-18 produced significant levels of IFN-γ early during Listeria infection (Fig. 4C). After the early innate phase, the expression of IFN-γ in both the CD8+ NKT and conventional CD8+ T cell populations peaked at day 7 post-Listeria priming or boost. Importantly, the absolute number of IFN-γ–producing conventional CD8+ T cells was still significant, likely due to the much larger CD8+ T cell compartment size compared with the NKT cell compartment (Supplemental Fig. 1), and confirms previous results that demonstrate the responsiveness of conventional CD8+ T cells to IL-12/IL-18 stimuli (6). Based on the ability of CD8+ NKT cells to produce IFN-γ and GzmB in an Ag-independent manner, as well as to degranulate at a very early stage in LM-OVA infection, our data strongly suggest that CD8+ NK1.1+ cells can provide innate functions during Listeria infection.

Conventional NK1.1+ CD8+ T cells alone are not sufficient to provide early protection to lethally infected Rag-/-γc-/- host mice

Based on the above results, we questioned whether conventional NK1.1+ CD8+ T cells alone (i.e., without contaminating CD8+ T cells...
NKT cells) are capable of providing protection against *Listeria* infection. To this end, we transferred similar numbers (10^5 cells) of sorted conventional CD8^+ NK1.1^-T cells (Supplemental Fig. 1E) or CD1d-tetramer^- NKT cells (Supplemental Fig. 1C) into Rag2^-/- g-recipient mice, which lack functional T, B, and NK cells, and assessed their effects on mice survival for 10 d post-infection (Fig. 5A). Because the early response to *Listeria* infection (as measured by T cell activation and proliferation, IFN-γ production, cytotoxicity, and degranulation) was not significantly different between the CD8^+ NKT and CD8^- NKT cell subsets (Figs. 3, 4), we first chose to transfer total NKT cells to overcome the limiting numbers of CD8^+ NKT cells despite the pooling of mice donors. *L. monocytogenes*-infected Rag2^-/- g- mice adoptively transferred with NKT cells showed a 12-fold reduction in bacterial burden in the livers compared with infected mice that received no cells. However, infected Rag2^-/- g- mice adoptively transferred with conventional CD8^+ NK1.1^- T cells showed no reduction in bacterial titers (Fig. 5B). Similarly, we observed...
4- and 2-fold reductions in *Listeria* CFU titers in spleens of recipient mice transferred with NKT cells and conventional CD8+ T cells, respectively (Fig. 5B). Importantly, the reduction in bacterial burden in mice adoptively transferred with NKT cells was associated with prolonged survival compared with infected Rag2−/−gcl2−/−mice that received conventional CD8+ T cells alone (Fig. 5C). In fact, Rag2−/−gcl2−/−mice adoptively transferred with conventional CD8+ NK1.1−T cells showed no difference in survival compared with recipient mice after challenge with a lethal dose of LM-OVA (Fig. 5C). To directly confirm that the CD8+ subpopulation of NKT cells can provide early innate immunity that can protect from early lethality from *Listeria* infection, we adoptively transferred CD8+ To rC D8+ NKT cells into Rag2−/−gcl2−/−mice (Fig. 5D).

Consistent with the data in Fig. 5C, the adoptive transfer of CD8+ NKT cells delayed mortality from *Listeria* infection. Although the role of conventional CD8+ T cells in providing long-lasting protection has been well documented, our data suggest that innate responses by CD8+ NKT cells may be critical in the early defense against *L. monocytogenes* infection.

**Discussion**

With the recent development of both the NK and NKT cell fields, it has become increasingly recognized that T cells expressing the NK1.1 marker form a distinct T cell compartment comprising several subsets as diverse as the heterogeneity of the conventional T cell compartment. In this study, we distinguished the conventional T cell compartment lacking NK1.1 from the NKT cell compartment expressing NK1.1. Although the pool size of the conventional T cell compartment is ∼50 times larger than the NKT cell compartment, both cell compartments harbor CD4+ and CD8+ T cell subsets. A phenotypic comparison of surface marker expression between the conventional CD8+ T cell compartment and the CD8+ NKT cell compartment showed distinct profiles that may reflect differential roles in immunity (Supplemental Fig. 2). In this study, we showed that this minor population of CD8+ NKT cells (that is easily overlooked) can expand rapidly during the innate phase of the immune response against *Listeria* infection, produces copious amounts of IFN-γ and GzmB, and contributes to improved early survival. Using LM-OVA tetramer, we...
confirmed that the early response mediated by CD8\(^+\) NKT cells is Ag independent. Interestingly, similar to the conventional CD8\(^+\) T cells, this cell population was able to mount adaptive immunity late during infection and generate memory response after re-exposure to \textit{Listeria}.

The question of whether these CD8\(^+\) NK1.1\(^+\) T cells that have innate properties represent a distinct T cell lineage or arise from conventional CD8\(^+\) T cells that have acquired NK1.1 expression remained largely controversial (16–20, 22). On one hand, results showing the upregulation of NK1.1 on CD8\(^+\) T cells upon in vitro and in vivo stimulation suggest a conventional T cell origin (17–20, 22). On the other hand, the observation that CD8\(^+\) NK1.1\(^+\) T cells can develop in thymectomized mice supports a distinct lineage for these CD8\(^+\) NKT cells (16). Recent reports suggested an innate component within memory CD8\(^+\) T cells that express NK1.1; however, these cells were elicited only after transfer of memory \textit{Listeria}-primed CD8\(^+\) T cells and, therefore, are functionally significant in the context of pathogen re-infection (10, 13). In contrast, in the current study, we examined CD8\(^+\) NK1.1\(^+\) T cells from naive specific pathogen–free mice and demonstrated that these cells are endowed with both innate and adaptive responses after \textit{Listeria} infection. Although we cannot rule out the possibility that some CD8\(^+\) NK1.1\(^+\) T cells in the current study arise from a pool of memory T cells, the distinct phenotypic profile and the specialized innate function of NK1.1\(^+\) CD8\(^+\) T cells support a different lineage that warrants further study.

The notion that T cells can perform as innate cells was proposed previously and debated in a number of reports (6, 7, 14, 15, 37–39). Although the role of NK cells producing IFN-\(\gamma\) during the early phase of \textit{Listeria} infection is indisputable, Andersson et al. (37) showed evidence of early IFN-\(\gamma\) production in the absence of NK cells. Specifically, their results showed that infected \(\gamma c^-/^-\) mice, which lack NK cells, but not Rag\(^2^-/^-\) \(\gamma c^-/^-\) mice (which lack T, B, and NK cells), were able to mount early resistance to \textit{Listeria}, indicating that T cells can functionally replace NK cells for early IFN-\(\gamma\) production, which is necessary for activating the innate immune system upon \textit{Listeria} infection (37). When dissecting the source of IFN-\(\gamma\) during the early response to \textit{Listeria}, Thäle and Kiderlen (38) provided evidence that T cells can produce innate IFN-\(\gamma\). Of interest, only a small population (2–3\%) of T cells was able to produce early IFN-\(\gamma\); among those T cells, CD8\(^+\) T cells were described as the major source (38). D’Orazio et al. (39) confirmed that only few CD8\(^+\) T cells were capable of producing IFN-\(\gamma\) in the early response to \textit{Listeria} and reported that these cells expressed CD44\(^{high}\). Similar observations were made by Bou Ghanem and D’Orazio (40) in humans: a small population of CD8\(^+\) T cells (<3\%) was able to secrete IFN-\(\gamma\) in response to \textit{Listeria}. Recently, Schenkel et al. (14) provided evidence that CD8\(^+\) T cells can trigger innate responses to increase immunity against unrelated pathogens. Moreover, Suarez-Ramirez et al. (15) showed that CD8\(^+\) T cells can acquire innate functions and produce IFN-\(\gamma\) independently of TCR stimulation. Along the same lines, Berg et al. (8) showed that memory CD8\(^+\) T cells are able to reduce \textit{Listeria} burden in an Ag-independent and IFN-\(\gamma\)-dependent manner. However, naive CD8\(^+\) T cells had reduced (compared with memory CD8\(^+\) T cells) expression of IL-12R\(\beta2\), IL-18R\(\alpha\), and IL-18R\(\beta\) subunits, which negatively affect their ability to secrete IFN-\(\gamma\) and reduce LM-OVA burden at early (day 3) time points (8). In addition, memory CD8\(^+\) T cells, but not naive CD8\(^+\) T cells, were shown to rapidly colocalize with LM-OVA–infected cells during the early phase of re-infection (11). In this study, we show evidence of innate responses against the first exposure to \textit{Listeria} infection confined within the subset of CD8\(^+\) NKT cells.

Evidence of innate responses within CD8\(^+\) NKT cells is provided by the rapid expression of CD69, CD107, IFN-\(\gamma\), and GzmB, which develop in an Ag-independent manner. In the absence of any NK1.1\(^+\) CD8\(^+\) T cells, conventional CD8\(^+\) NK1.1\(^+\) T cells were inferior to NKT cells in providing protection against early lethality to \textit{Listeria} infection. It remains to be determined whether these cells are capable of rapid colocalization with LM-OVA–infected cells, as is the case for memory CD8\(^+\) T cells during re-infection. Also, the exact mechanisms by which CD8\(^+\)
NKT cells recognize Listeria-infected cells remain unclear. We show that CD8+ NKT cells express activating and inhibitory NK cell receptors at high levels (e.g., NK2G2D, NK2G2A) in contrast to conventional CD8+ T cells, which have minimal to no expression of these receptors (Supplemental Fig. 2). Thus, it is possible that CD8+ NKT cells use the NK cell receptor machinery to recognize and kill Listeria-infected cells in an Ag-independent, nonspecific manner. Interestingly, a recent study reported that vectors expressing the NK2G2D ligand RAE-1γ dramatically enhanced the effectiveness of the CD8+ T cell response, suggesting a promising approach in the development of CD8+ T cell–based vaccines (41). Accordingly, one possible candidate for the origin of innate CD8+ NKT cell function is the subset of MHC-unrestricted NKT cells (42, 43). These NKT cells were recently described as sharing many characteristics of NK cells, including expression of the killer cell lectin-like receptors, the rapid production of IFN-γ in response to cytokine stimuli (IL-12/IL-18), and the potent cytotoxic program (GzmB) in response to innate signals (44, 45), and the potent cytotoxic program (GzmB) in response to innate signals (44, 45), and the poten...


Supplemental Figure 1. Gating scheme and sorted cell subsets. (A) NK cells were sorted as CD1d-tet−CD3−NK1.1+DX5+ cells. (B) iNKT cells were sorted as CD1d-tet+CD3+ cells. (C) NKT cells were sorted as CD1d-tet−CD3+NK1.1− cells. (D) CD8+ conventional T cells were sorted as CD1d-tet−CD3+CD8+NK1.1− cells. (E) CD8+ NKT cells were sorted as CD1d-tet−CD8+CD3+NK1.1+ cells.
Supplemental Figure 2. Phenotypic comparison of CD8+ T cells from conventional T cell versus NKT cell compartments. (A) Plots show the distribution of CD4 versus CD8 among conventional T cell compartment (NK1.1-CD3+CD1d-tet-) versus NKT cell compartment (NK1.1+CD3+CD1d-tet-). (B) CD8+ T cells from the conventional T cell compartment (NK1.1+CD3+CD8+CD1d-tet-) and NKT cell compartment (NK1.1*CD3*CD8*CD1d-tet-) were compared for the expression of the indicated receptors. Shown as controls, NK (NK1.1*DX5*CD3*CD1d-tet-), iNKT (NK1.1*CD3*CD1d-tet-), and total NKT (NK1.1*CD3*CD1d-tet-) cells. Numbers indicate the frequency of positive cells as mean ± s.e.m. Data are representative of two independent experiments, n = 6.
Supplemental Figure 3. Experimental design. (A) Mice were intravenously injected with Listeria monocytogenes (expressing Ovalbumin, LM-Ova) at day 0 and day 42 (depicted as arrows in graphs). In prime/boost setting prime dose was $10^4$ CFU and boost dose was $2 \times 10^5$ CFU. For prime dose response single $10^3$ CFU and $2 \times 10^5$ CFU infections were also used. Mice were sacrificed at days 0, 3, 7, 14, 42, 45, 49, and 70 and innate (IFNγ, CD107, CD69, Granzyme B) and adaptive (Ova-tetramer, LemA-tetramer, OVA or LemA peptide ex vivo stimulation of recall responses) were studied. (B) Graphs of kinetics of absolute number for total splenocytes and liver lymphocytes in WT mice are shown in response to LM-Ova. Data are representative of three independent experiments, $n = 9$ (days 0, 7, 49), $n = 6$ (days 3, 14, 42, 45, 70), mean ± s.e.m.
Supplemental Figure 4. Tracking LemA-specific T cells among conventional CD8+ T cells and CD8+ NKT cells. Mice were infected with 10^4 CFU of LM-Ova on day 0 and re-challenged with 2x10^5 CFU on day 42, as indicated by arrows. Graphs show kinetics of absolute number of LemA-tetramer positive cells among CD8+ NKT and CD8+ T cells in spleen and liver. Data are representative of three independent experiments, n = 4 for each time point (for days 0, 7, 49) and data are representative from one of two independent experiments n = 3 for each (for days 3, 14, 42, 45, 70), mean ± s.e.m.