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Memory CD8+ T Cells Provide an Early Source of IFN-γ

Taku Kambayashi,* Erika Assarsson,† Aron E. Lukacher,* Hans-Gustaf Ljunggren,†‡ and Peter E. Jensen2*†

During the non-Ag-specific early phase of infection, IFN-γ is believed to be primarily provided by NK and NKT cells in response to pathogen-derived inflammatory mediators. To test whether other cell types were involved in early IFN-γ release, IFN-γ-producing cells were visualized in spleens and lymph nodes of LPS-injected mice. In addition to NK and NKT cells, IFN-γ was also detected in a significant fraction of CD8+ T cells. CD8+ T cells represented the second major population of IFN-γ-producing cells in the spleen (~30%) and the majority of IFN-γ+ cells in the lymph nodes (~70%). LPS-induced IFN-γ production by CD8+ T cells was MHC class I independent and was restricted to CD44high (memory phenotype) cells. Experiments performed with C3H/HeJ (LPS-nonresponder) mice suggested that CD8+ T cells responded to LPS indirectly through macrophage/dendritic cell-derived IFN-α/β, IL-12, and IL-18. IFN-γ was also detected in memory CD8+ T cells from mice injected with type I IFN or with poly(I:C), a synthetic dsRNA that mimics early activation by RNA viruses. Taken together, these results suggest that in response to bacterial and viral products, memory T cells may contribute to innate immunity by providing an early non-Ag-specific source of IFN-γ. The Journal of Immunology, 2003, 170: 2399–2408.

Interferon-γ is a type 1 cytokine that exhibits a wide array of proinflammatory activities. It is produced during most types of infections and plays a key role in amplifying innate and adaptive immune responses. In innate immunity, IFN-γ synergistically augments LPS-stimulated proinflammatory cytokine production (1), induces respiratory burst (2), and suppresses release of anti-inflammatory cytokines such as IL-10 (3, 4). IFN-γ also aids in development and shaping of adaptive immune responses. Most importantly, it enhances the function of the APC through stimulation of phagocytosis, up-regulation of MHC class I and class II molecules, and induction of the immunoproteasome (5). Consequently, T cells can respond more efficiently to IFN-γ-activated APCs that express MHC molecules bound with pathogen-derived peptides. Apart from Ag presentation, IFN-γ also affects adaptive immunity by promoting Th1 (cell-mediated) immune responses. Namely, IFN-γ promotes differentiation of CD4+ T cells into Th1 phenotype IFN-γ-producing cells, induces B cell IgG2a Ab class switching, and stimulates CD8+ T cell cytotoxicity (5). Experiments performed with mice lacking functional IFN-γ or IFN-γ receptors illustrate the overall importance of IFN-γ in protective immunity. These mice display increased susceptibility to many pathogens including viruses and intracellular bacteria such as Listeria monocytogenes and mycobacteria (6).

IFN-γ is produced mainly by T, NK, and NKT (3) cells (5–7). The contribution of each cell type to the overall IFN-γ response depends largely on the stage of infection (early innate vs late adaptive). During the non-Ag-specific early phase of infection, IFN-γ is thought to be primarily provided by NK and NKT cells in response to early danger signals derived from the invading pathogen (8–10). Although there is some evidence that certain microbial products can directly stimulate the production of IFN-γ from NK cells (11, 12), most involve indirect activation through APCs. Professional APCs such as macrophages and dendritic cells (DCs) express numerous Toll-like receptors, and are thus able to respond to microbial products by producing cytokines such as type I IFNs, IL-12, and IL-18 (13, 14). These cytokines in turn stimulate IFN-γ production from NK and NKT cells (7, 8, 15–17). Once the adaptive immune response is established, T cells are the predominant producers of IFN-γ. Ligation of TCR with specific MHC/peptide complexes results in abundant secretion of IFN-γ from CD8+ T cells and CD4+ T cells of Th1 phenotype (5).

Cells of the adaptive immune system are not as functionally distinct from innate immune cells as once believed. One example is the memory CD8+ T cell whose primary role is played in adaptive immunity by conferring rapid Ag-specific protection against pathogens that the host has previously experienced. A long-lived pool of memory T cells is established from Ag-specific T cells that have expanded during the first encounter with the pathogen (18). Unlike naive T cells, memory CD8+ T cells express several receptors that are commonly associated with cells of innate immunity. These include cytokine receptors such as IL-2Rβ (CD122) and NK cell-associated receptors such as Ly-49 and NKR-P1-C (NK1.1) (19). Expression of high affinity cytokine receptors allows memory, but not naive CD8+ T cells to proliferate in response to variety cytokines in the absence of TCR stimulation (20–24). In fact, recent evidence suggests that maintenance of memory CD8+ T cells is regulated by an MHC class I-independent mechanism involving IL-2 and IL-15 (18, 25, 26). Furthermore, it has been postulated that activation of T cells by cytokines (i.e., bystander activation) is an important factor in host defense, although this is highly controversial.

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In previous experiments, we noticed that cytokine-stimulated memory CD8+ T cells expressed IFN-γ mRNA in the absence of TCR cross-linking. Therefore, we hypothesized that activation of memory CD8+ T cells by cytokines may provide an early source of IFN-γ. To test this hypothesis, the cell types involved in IFN-γ production during the early innate phase of infection were investigated. Endotoxin (LPS) and poly(I:C), a synthetic dsRNA, were used to generalize early activating events induced by bacteria and viruses, respectively. When mice were injected with either LPS or poly(I:C), memory CD8+ T cells, but not naive CD8+ T cells, were major producers of IFN-γ. The data presented in this study support the notion that bystander activation of memory CD8+ T cells may contribute to early non-Ag-specific immune responses.

Materials and Methods

Mice and reagents

C57BL/6 (B6) mice, μMT, β2-microglobulin (β2m−/−), and IL-12p40−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). WBB6F1/KitFlk/W (W/W) female mice and their female +/+ (genetically littermates, WBB6F1/Kit+/+ (F/F+)) were obtained from The Jackson Laboratory. Both of these groups result from the cross of WB/ReJ-Kit W/° mice. C3H/HeJ and C3H/HeN mice were obtained from the National Cancer Institute (Frederick, MD). Animal care was provided according to protocols approved by the Institutional Animal Care and Use Committee. Both of these groups result from the cross of WB/ReJ-Kit W/° mice. C3H/HeJ and C3H/HeN mice were obtained from the National Cancer Institute (Frederick, MD). Animal care was provided according to protocols approved by the Institutional Animal Care and Use Committee. Both of these groups result from the cross of WB/ReJ-Kit W/° mice. C3H/HeJ and C3H/HeN mice were obtained from the National Cancer Institute (Frederick, MD). Animal care was provided according to protocols approved by the Institutional Animal Care and Use Committee. Both of these groups result from the cross of WB/ReJ-Kit W/° mice. C3H/HeJ and C3H/HeN mice were obtained from the National Cancer Institute (Frederick, MD). Animal care was provided according to protocols approved by the Institutional Animal Care and Use Committee. Both of these groups result from the cross of WB/ReJ-Kit W/° mice. C3H/HeJ and C3H/HeN mice were obtained from the National Cancer Institute (Frederick, MD). Animal care was provided according to protocols approved by the Institutional Animal Care and Use Committee. Both of these groups result from the cross of WB/ReJ-Kit W/° mice. C3H/HeJ and C3H/HeN mice were obtained from the National Cancer Institute (Frederick, MD). Animal care was provided according to protocols approved by the Institutional Animal Care and Use Committee. Both of these groups result from the cross of WB/ReJ-Kit W/° mice. C3H/HeJ and C3H/HeN mice were obtained from the National Cancer Institute (Frederick, MD). Animal care was provided according to protocols approved by the Institutional Animal Care and Use Committee.

MACS purification of CD8+ T cells

Single cell suspensions from spleens of B6, C3H/HeN, or C3H/HeJ mice were depleted of erythrocytes, and CD8+ cells were purified using the MACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s guidelines. Briefly, 1–5 x 10^6 erythrocyte-depleted spleenocytes were washed twice with MACS buffer (PBS without Ca2+/Mg2+ supplemented with 0.5% BSA and 2 mM EDTA) and resuspended in MACS buffer containing CD8a magnetic beads. After incubation for 30 min at 4°C, CD8+ cells were purified from the cell suspension using a magnetic LS+ column, washed once, and used directly for additional experiments.

Adoptive transfer of CFSE-labeled CD8+ T cells

FACSVantage-sorted (BD Biosciences) CD8+CD44high CD62L− cells from B6 mice or MACS-purified CD8+ cells from C3H/HeN or C3H/HeJ mice were washed thoroughly with PBS and incubated in PBS containing 0.5 μM of CFSE (1 μM) for 20 min at room temperature. The cells were washed once with complete medium and twice in PBS. A total of 2–5 x 10^6 cells were injected i.v. into β2m−/−, C3H/HeN, or C3H/HeJ mice, as indicated, 1 day before LPS challenge.

Infection with polyoma virus

Wild-type polyoma virus, strain A2, was molecularly cloned and plaque purified, and virus stocks were prepared on primary baby mouse kidney cells, as previously described (27). Adult mice were inoculated s.c. in hind footpads with 2 x 10^7 PFU of virus.

Generation of bone marrow chimeric mice

Bone marrow cells were harvested from the femur and tibia of donor C3H/HeN and C3H/HeJ mice by flushing the bones with a syringe. C3H/HeN or C3H/HeJ recipient mice were lethally irradiated at 1060 rad. A total of 10^7 bone marrow cells from donor C3H/HeN or C3H/HeJ mice were injected i.v. into the irradiated C3H/HeN or C3H/HeJ recipients. The mice were allowed to rest for 8 wk to establish chimerism before challenge with LPS.

Generation of macrophages and DCs

Peritoneal macrophages and bone marrow-derived CD11c-sorted DCs were prepared, as previously described (28, 29). Splenic DCs were prepared by sorting CD11c+ cells from spleens of mice injected i.p. with 40 μg of Flt3L for 10 days. The CD11c+ cells were sorted using the MACS separation system in a similar fashion to the CD8+ T cell purification described above using anti-CD11c-conjugated beads.
cells. Of note, CD8\(^+\)/NK1.1\(^-\) T cells were gated out from the CD3\(^+\)/NK1.1\(^-\) NKT cell population, because a proportion of memory CD8\(^+\)/NK1.1\(^-\) T cells has also been shown to express NK1.1 (32, 34). All cell types responded rapidly to LPS injection in a dose-dependent manner, reaching peak numbers of IFN-\(\gamma\)–producing cells at \(~3\) h (Fig. 2, A–C). By 24 h postinjection, few IFN-\(\gamma\) cells could be detected in these mice (Fig. 2, A and B). This correlated well with the kinetics of secreted IFN-\(\gamma\) found in the serum and spleen cell culture supernatants of these mice (data not shown). Interestingly, the induction of IFN-\(\gamma\) was detected even when remarkably low doses of LPS were used (50 ng/mouse) (Fig. 2, C). In addition, the response of CD8\(^+\) T cells to LPS seemed to be specific for IFN-\(\gamma\), because other cytokines such as TNF-\(\alpha\), IL-4, or IL-10 were not detected in CD8\(^+\) T cells of LPS-injected mice (data not shown).

In the spleen, NK cells were the major producer of IFN-\(\gamma\), representing 50–60% of all IFN-\(\gamma\) cells (Fig. 2, D). The second major contributor was CD44 high CD8\(^+\) T cells (\(~30\)%), followed by NKT cells (\(~15\)%), and CD44 high CD4\(^+\) T cells (\(~5\)%). Although IFN-\(\gamma\) was detected in a larger fraction of NKT cells

**FIGURE 1.** Visualization of IFN-\(\gamma\)-producing cells in the spleen following LPS challenge. Intracellular IFN-\(\gamma\) was detected in various spleen cell populations of LPS-injected (50 \(\mu\)g/mouse i.p.) B6 mice by flow cytometry 3 h post-LPS challenge. A, Cells were gated on CD8\(^+\) T cells (top plot) or CD4\(^+\) T cells (bottom plot). The number in each plot indicates the percentage of CD44\(^{high}\)IFN-\(\gamma\) cells of total CD44\(^{high}\) cells. B, The CD3/ NK1.1 density plot was gated on CD8 cells, and intracellular IFN-\(\gamma\) was detected in NK cells (CD3\(^-\) NK1.1\(^+\) cells, top histogram) and NKT cells (CD3\(^-\) NK1.1\(^+\) CD8\(^-\) cells, bottom histogram). The number in each histogram plot represents percentage of IFN-\(\gamma\) cells.

**FIGURE 2.** Kinetics, dose dependence, and relative contribution of IFN-\(\gamma\)-producing cell types in spleens of LPS-injected mice. Intracellular IFN-\(\gamma\) was detected in various spleen cell populations of LPS-injected B6 mice by flow cytometry. The proportion of CD8\(^+\) CD44\(^{high}\) (○), CD8\(^+\) CD44\(^{low}\) (●), CD4\(^+\) CD44\(^{high}\) (△), or CD4\(^+\) CD44\(^{low}\) cells (▲) (A), and NK1.1\(^+\)CD3\(^-\) (○) or NK1.1\(^+\)CD3\(^-\)CD8\(^-\) cells (●) (B) producing IFN-\(\gamma\) was plotted against time post-LPS injection (50 \(\mu\)g/mouse i.p., \(n = 4\) mice/time point). C, The proportion of CD8\(^+\) CD44\(^{high}\) (○), NK1.1\(^+\) CD3\(^-\) (●), and NK1.1\(^+\) CD3\(^-\) CD8\(^-\) cells (△) producing IFN-\(\gamma\) 3 h postchallenge was plotted against the amount of LPS injected per mouse (\(n = 4\) mice/dose). D, The relative contribution of each cell type at 3 h post-LPS challenge was determined by dividing the number of IFN-\(\gamma\)-producing CD8\(^+\) CD44\(^{high}\) T cells (lane 1), CD4\(^+\) CD44\(^{high}\) T cells (lane 2), NK cells (lane 3), and NKT cells (lane 4) by the total number of IFN-\(\gamma\) cells in the spleen (\(n = 4\) mice).
FIGURE 3. CD8^+CD44^{high} T cells are the major producer of IFN-γ in lymph nodes. Intracellular IFN-γ was detected in lymph node cells of LPS-injected (50 μg/mouse in hind footpad) B6 mice by flow cytometry 3 h post-LPS challenge. A. Cells were gated on CD8^+ T cells (top plots) or CD4^+ T cells (bottom plots) from pooled inguinal (left plots) or popliteal (right plots) lymph node cells (n = 4 mice). The number in each plot indicates the percentage of CD44^{high}IFN-γ^+ cells of total CD44^{high} cells. B. The relative contribution of each cell type at 3 h post-LPS challenge was determined by dividing the number of IFN-γ-producing CD8^+CD44^{high} T cells, CD4^+CD44^{high} T cells, NK cells, and NKT cells by the total number of IFN-γ^+ cells in pooled inguinal (left bars) or popliteal (right bars) lymph node cells (n = 4 mice).

compared with CD44^{high}CD8^+ T cells (Fig. 1), CD44^{high}CD8^+ T cells had a greater relative contribution to the IFN-γ-expressing cell population due to higher cell numbers.

We next examined whether LPS-stimulated lymphocytes produced IFN-γ in lymphoid organs other than the spleen. Thus, mice were injected with LPS in the hind footpad, and IFN-γ-producing cells in the draining lymph nodes were examined. As observed in the spleen, CD44^{high}CD8^+ T cells and to a lesser extent CD44^{high}CD4^+ T cells produced IFN-γ in the popliteal and inguinal lymph nodes of LPS-injected mice (Fig. 3A). However, only scarce numbers of NK or NKT cells are found in lymph nodes compared with the spleen. Owing to this fact, the major producer of IFN-γ in the lymph nodes was CD44^{high}CD8^+ T cells, representing up to 70% of all IFN-γ^+ cells (Fig. 3B).

IFN-γ is detected in memory, but not naive CD8^+ T cells of LPS-injected mice

Among CD4^+ and CD8^+ T cells in LPS-challenged mice, IFN-γ was detected only in cells with high expression of CD44, a marker indicating previous activation (Figs. 1, 2A, and 3A). This implied that only memory or recently activated T cells produced IFN-γ in response to LPS. However, it was uncertain whether or not CD44 expression was high on IFN-γ^+CD8^+ T cells before LPS challenge. It was also possible that CD44 was up-regulated on CD8^+ T cells after LPS stimulation, which would suggest that the IFN-γ^+CD8^+ T cells were activated naive rather than memory CD8^+ T cells. In support of the latter possibility, it has been reported that LPS can cause activation and up-regulation of CD69 on naive T cells (24). To address this issue, CD8^+ T cells were sorted into CD44^{high} and CD44^{low} subsets and adoptively transferred into β2m^-/- hosts. Upon challenge with LPS, IFN-γ was detected in CD44^{high}CD8^+ T cells, but not in CD44^{low}CD8^+ T cells (Fig. 4A). This suggested that IFN-γ^+CD8^+ T cells were already

FIGURE 4. The induction of IFN-γ from CD8^+ T cells is restricted to memory T cells and is MHC class I independent. A. FACS-sorted populations of CD8^+CD44^{high} (top plots) or CD8^+CD44^{low} (bottom plots) cells were labeled with CFSE and adoptively transferred i.v. into β2m^-/- mice. The next day, recipient mice were injected with PBS (left plots) or LPS (right plots). Intracellular IFN-γ was visualized in CFSE^+ cell populations by flow cytometry 3 h post-LPS challenge. The number in each plot represents percentage of IFN-γ^+ cells. B, C3H/HeN mice were infected with polyoma virus and challenged with LPS (50 μg/mouse i.p.) 90 days later. Intracellular IFN-γ was detected in virus-specific T cells (D^a-MT389^+CD8^+ T cells) by flow cytometry 3 h post-LPS challenge.
CD44<sup>high</sup> before LPS stimulation and that IFN-γ production was not induced in naive CD44<sup>low</sup>CD8<sup>+</sup> T cells.

Although these results demonstrated that IFN-γ production was restricted to CD8<sup>+</sup> T cells expressing high levels of CD44, it could still be argued that these cells were not conventional memory T cells, i.e., memory CD8<sup>+</sup> T cells arising from previous exposure to specific Ags. To confirm that conventional memory CD8<sup>+</sup> T cells were capable of responding in a similar fashion to CD44<sup>high</sup>CD8<sup>+</sup> T cells, LPS-induced IFN-γ production was examined in a pool of memory CD8<sup>+</sup> T cells established from a previous viral infection. LPS was injected into mice 90 days after infection with polyoma virus, and Ag-specific memory CD8<sup>+</sup> T cells were detected in the spleen by MHC class I (H-2D<sup>+</sup>) tetramers refolded with the polyoma-derived immunodominant MT<sub>390-398</sub> epitope. Like CD44<sup>high</sup> CD8<sup>+</sup> T cells in LPS-challenged uninfected mice, 15–25% of D<sup>+</sup>-MT<sub>390</sub> CD8<sup>+</sup> T cells produced IFN-γ in response to LPS (Fig. 4B). Of note, IFN-γ<sup>+</sup> D<sup>+</sup>-MT<sub>390</sub> CD8<sup>+</sup> T cells were not detected in mice that did not receive LPS (data not shown). This indicated that the response elicited by CD44<sup>high</sup>CD8<sup>+</sup> T cells in LPS-injected unchallenged mice reflects the activity of conventional memory T cells.

The induction of IFN-γ from CD8<sup>+</sup> T cells by LPS is MHC class I independent and is in part mediated by type I IFNs, IL-12, and IL-18

As mentioned previously, when LPS was injected into β<sub>2m</sub><sup>−/−</sup> (MHC class I-deficient) hosts that were adoptively transferred with CD44<sup>high</sup>CD8<sup>+</sup> T cells, IFN-γ was detected in the transferred CD8<sup>+</sup> T cells (Fig. 4A). This suggested that LPS-induced IFN-γ production by memory CD8<sup>+</sup> T cells did not involve TCR/MHC class I interactions. We also considered the possibility that LPS may directly activate CD8<sup>+</sup> T cells to produce IFN-γ. However, this possibility was ruled out by experiments performed with C3H/HeJ mice. Due to a mutation in Toll-like receptor 4, a vital component of the LPS receptor, spleens of LPS-injected C3H/HeJ mice contained only small numbers of IFN-γ<sup>+</sup> cells (Fig. 5A). Likewise, CD8<sup>+</sup> T cells from C3H/HeJ mice did not produce IFN-γ when adoptively transferred into LPS-injected C3H/HeJ mice (Fig. 5A). In contrast, a significant proportion of CD8<sup>+</sup> T cells from C3H/HeJ mice produced IFN-γ when adoptively transferred into LPS-injected C3H/HeN (LPS-responder) mice (Fig. 5A). The proportion of C3H/HeJ IFN-γ<sup>+</sup>CD8<sup>+</sup> T cells was equivalent to the proportion of host-derived HeN IFN-γ<sup>+</sup>CD8<sup>+</sup> T cells (Fig. 5A). These data suggested that LPS did not directly activate CD8<sup>+</sup> T cells and that another factor(s) was involved in the induction of IFN-γ from CD8<sup>+</sup> T cells.

We examined whether other activators of innate immunity could also induce IFN-γ production from memory CD8<sup>+</sup> T cells. Poly(I:C), a synthetic dsRNA, was used to mimic early non-Ag-specific activation by viruses. Similar to LPS, IFN-γ was detected in memory CD8<sup>+</sup> T cells after challenge with poly(I:C) (Fig. 5B). This demonstrated that the contribution of memory CD8<sup>+</sup> T cells to non-Ag-specific IFN-γ production was not restricted to bacterial stimuli such as LPS. Because poly(I:C) is a strong inducer of type I IFNs, we also tested whether type I IFNs could mimic the activity of poly(I:C). Similar to poly(I:C) and LPS, injection of type I IFNs was sufficient for inducing IFN-γ from CD8<sup>+</sup> T cells in vivo (Fig. 5C).

Previous studies have demonstrated that type I IFNs, IL-12, IL-15, and IL-18 are involved in non-Ag-specific proliferation of memory CD8<sup>+</sup> T cells in vivo (20–24). Upon stimulation of purified CD8<sup>+</sup> T cells with various combinations of these cytokines for 6 or 24 h, secreted IFN-γ was detected only when IL-18 was given in combination with type I IFN or IL-12 (Table 1). None of these cytokines by themselves had an IFN-γ-inducing effect. We also examined intracellular IFN-γ in unmanipulated spleen cells stimulated with type I IFN, IL-12, or IL-15 in combination with IL-18 to exclude the possibility that the IFN-γ detected in the culture supernatants came from contaminating cell populations rather than from CD8<sup>+</sup> T cells. Upon stimulation with these cytokines for 6 h, intracellular IFN-γ was detected in memory CD8<sup>+</sup> T cells, memory CD4<sup>+</sup> T cells, NK cells, and NKT cells (Fig. 6, A–C). This confirmed the results obtained with cytokine stimulation of purified CD8<sup>+</sup> T cells. However, in contrast to secreted IFN-γ, IL-15 + IL-18 had an IFN-γ-inducing effect on CD8<sup>+</sup> T cells that was equivalent to stimulation with IL-12 + IL-18 when IFN-γ was detected intracellularly (Table I, Fig. 6C).

We next tested whether type I IFN and IL-12 were involved in LPS-induced IFN-γ production in vivo. Compared with wild-type mice, IL-12p40<sup>−/−</sup> mice had similar numbers of IFN-γ<sup>+</sup>CD8<sup>+</sup> cells, but significantly fewer IFN-γ<sup>+</sup> NK cells (Fig. 7, A and B). In contrast, LPS-challenged mice treated with neutralizing anti-IFN-α/β Ab had significantly fewer IFN-γ<sup>+</sup>CD8<sup>+</sup> cells, but similar...
numbers of IFN-γ+ NK cells compared with LPS-challenged untreated mice (Fig. 7, A and B). Furthermore, LPS-challenged IL-12p40−/− mice treated with neutralizing anti-IFN-α/β Ab had even fewer numbers of both IFN-γ-producing memory CD8+ T cells and NK cells compared with LPS-treated mice lacking only one cytokine (Fig. 7, A and B). This was in agreement with a previous report demonstrating that IL-12 and type I IFNs play redundant roles in IFN-γ production (35). This redundancy was most noticeable with NKT cells in which a significant decrease in IFN-γ+ NKT cells was only observed when both IL-12 and IFN-α/β were absent (Fig. 7C). Because the inhibition of LPS-induced IFN-γ production was not complete, factors other than type I IFNs or IL-12 may also play a role in vivo. It is also possible that the Ab against IFN-α/β did not neutralize all of the IFN-α/β that was induced upon LPS injection. Nevertheless, these data suggest that at least in part, type I IFN and IL-12 are responsible for LPS-induced IFN-γ production in vivo.

Macrophages/DCs are involved in LPS-induced IFN-γ production by CD8+ T cells

Because LPS did not directly activate CD8+ T cells, this suggested that another cell type was involved in the induction of IFN-γ from CD8+ T cells. To determine whether hemopoietic or radio-resistant nonhemopoietic cells were responsible for the IFN-γ-inducing effects, bone marrow chimeras were generated using C3H/HeJ and

Table 1. IFN-γ release by cytokine-stimulated CD8+ T cellsa

<table>
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<tr>
<th>Cytokine</th>
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<th>24 h</th>
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<tr>
<td>None</td>
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<tr>
<td>IFN-α/d</td>
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<tr>
<td>IL-12</td>
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<td>5.7 ± 3.0</td>
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<td>IL-15</td>
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<td>N/D</td>
</tr>
<tr>
<td>IL-18</td>
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<td>N/D</td>
</tr>
<tr>
<td>IFN-α/d + IL-12 + IL-15</td>
<td>N/D</td>
<td>14 ± 1.4</td>
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<tr>
<td>IL-12p40, IL-18</td>
<td>2.1 ± 0.9</td>
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a Purified CD8+ T cells were stimulated with various combinations of IFN-α/d (1000 U/ml), IL-12 (100 ng/ml), IL-15 (100 ng/ml), or IL-18 (100 ng/ml) for 6 or 24 h. IFN-γ was detected in the cell-free supernatants by a europium-based anti-IFN-γ immunoassay. Results are expressed as mean IFN-γ (U/ml) ± SD of duplicate determinations of duplicate cultures.

b N/D = not detectable.

FIGURE 7. IFN-α/β and IL-12 are partially responsible for LPS-induced IFN-γ production in vivo. Intracellular IFN-γ was detected in various spleen cell populations of LPS-injected (50 μg/mouse i.p.) B6 mice, B6 mice treated with anti-IFN-α/β Ab (104 neutralizing U/mouse), IL-12p40−/− mice, and IL-12p40−/− mice treated with anti-IFN-α/β Ab by flow cytometry. The proportion of CD8+CD44high (A), NK1.1+CD3+ (B), or NK1.1+CD3+CD8− (C) cells producing IFN-γ was compared between groups. Results are expressed as mean ± SD of n = 5 mice/group. *, Indicates significance (p < 0.05) compared with LPS-injected untreated B6 mice (lane 1).

FIGURE 6. Production of IFN-γ by T, NKT, and NK cells after stimulation of whole spleen cultures with cytokines in vitro. A, Intracellular IFN-γ was detected in various spleen cell populations as indicated in each plot after a 6-h stimulation in vitro with IFN-α/d (104 U/ml) + IL-18 (100 ng/ml) (A), IL-12 (100 ng/ml) + IL-18 (B), or IL-15 (100 ng/ml) + IL-18 (C). The number in each histogram plot represents percentage of IFN-γ+ cells.
C3H/HeN mice. As shown in Fig. 8A, IFN-γ production by memory CD8⁺ T cells was only detected when donor bone marrow cells were of C3H/HeN origin. This suggested that the LPS-responding cell type was bone marrow derived. To further narrow down the possibilities, we tested whether B cells or mast cells, both of which are known to directly respond to LPS, were responsible for the IFN-γ-inducing effects. The fraction of IFN-γ⁺CD8⁺ T cells from B cell-deficient and mast cell-deficient mice was similar to their respective wild-type controls, ruling out B cells or mast cells as the responsible cell type (Fig. 8A and C).

Among the possibilities remaining, monocyte-derived cell types such as macrophages and DCs were likely candidates. Because knockout mice lacking macrophages or DCs do not exist and complete depletion in vivo is a difficult task, we examined whether purified CD8⁺ T cells would produce IFN-γ when cocultured with LPS-stimulated macrophages or DCs. Unexpectedly, coculture of Flt3L-elicited splenic DCs with purified CD8⁺ T cells did not result in LPS-stimulated IFN-γ production by CD8⁺ T cells (data not shown). Moreover, coculture of purified CD8⁺ T cells with LPS-stimulated thioglycollate-elicited peritoneal macrophages resulted in only a small proportion (2–3%) of IFN-γ⁺CD8⁺ T cells (Fig. 8D). However, when exogenous IL-18 was added to the cocultures, up to 19% of the CD44high CD8⁺ T cells produced IFN-γ, whereas IFN-γ was not detected in CD8⁺ T cells cultured alone under the same conditions (Table II). This suggested that an insufficient amount of IL-18 was responsible for the lack of IFN-γ-inducing activity by these cell types. Previously, bone marrow-derived myeloid DCs have been shown to produce substantial amounts of IL-18 (36). To see whether these cells could provide a source of IL-18, bone marrow-derived CD11c-purified DCs alone or mixed with macrophages were cocultured with CD8⁺ T cells. Upon stimulation with LPS, intracellular IFN-γ was detected in ~6 and ~9% of CD44⁺CD8⁺ T cells that were cocultured with

Table II. Induction of IFN-γ from CD44⁺CD8⁺ T cells by LPS-stimulated macrophages and DC

<table>
<thead>
<tr>
<th>DC/Macrophage:CD8 Ratio</th>
<th>DC (%)</th>
<th>Macrophages (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:1</td>
<td>7.5</td>
<td>1.4</td>
</tr>
<tr>
<td>1:1</td>
<td>13.8</td>
<td>15.4</td>
</tr>
<tr>
<td>1:5</td>
<td>3.2</td>
<td>18.7</td>
</tr>
<tr>
<td>1:25</td>
<td>2.2</td>
<td>9.1</td>
</tr>
<tr>
<td>No DC or macrophages</td>
<td>1.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Purified CD11c⁺ DCs from spleens of Flt3L-treated mice or thioglycollate-induced peritoneal macrophages were cocultured with purified CD8⁺ T cells at various DC/macrophage:CD8⁺ T-cell ratios with fixed numbers of CD8⁺ T cells. The cultures were treated with 500 or 100 ng/ml of LPS for macrophages and DCs, respectively, in the presence of IL-18 (100 ng/ml). Five hours later, brefeldin A was added for another 3 h, and intracellular IFN-γ was detected in CD8⁺ T cells by flow cytometry. Results are expressed as percentage of CD44⁺CD8⁺ T cells that were cocultured with total CD44⁺CD8⁺ T cells.
DCs alone and DCs mixed with macrophages, respectively, at a CD8:macrophage/DC ratio of 3:1 (Fig. 8D). Of note, ~6% of CD44hiCD8+ T cells produced IFN-γ even at a CD8:(macrophage + DC) ratio of 9:1 (data not shown). This suggested that cytokines released by one type of DC or macrophage are probably not sufficient for inducing IFN-γ production from CD8+ T cells and that factors derived from multiple types of macrophages or DCs are required.

Discussion

Macrophages/Monocytes and DCs respond rapidly to invading microorganisms by recognizing general pathogen-derived patterns through Toll-like receptors. These patterns are found on molecules that are commonly shared between related pathogens, e.g., LPS in Gram-negative bacteria or dsRNA in RNA viruses. In response to such stimuli, macrophages produce cytokines such as IL-12 and IL-18, which stimulate IFN-γ production from NK and NKT cells (7, 8, 15–17). In the present study, we have shown that in addition to NK and NKT cells, memory CD8+ T cells and to a lesser extent memory CD4+ T cells provide IFN-γ in response to innate immune stimuli. The contribution of memory CD8+ T cells to the early IFN-γ response is especially prominent in the lymph nodes, which are largely devoid of NK and NKT cells.

CD8+ T cells are conventionally activated through their TCR in response to specific MHC class I/peptide complexes. In the absence of TCR stimulation, memory CD8+ T cells can also be activated by cytokines, a process termed bystander activation. By-stander activation of T cells in vivo has been most extensively reported by Sprent and colleagues (21–24), who have shown that the administration of innate immune activators such as LPS, poly(I:C), and CpG DNA induces proliferation of memory CD8+ T cells through a mechanism involving type I IFN, IL-12, IL-15, and IL-18. Others have also demonstrated that certain functions of memory CD8+ T cells such as cytotoxicity can be enhanced by bystander activation during infection with viruses (37–39). However, the latter studies suggested that bystander activation of memory CD8+ T cells imposes little biological significance due to the relatively low numbers of specific T cells that are activated through this pathway. In other words, too few T cells specific for a given Ag are reactivated by cytokines to yield any Ag-specific impact on the immune response.

In contrast, IFN-γ production by bystander-activated T cells may have profound effects on the immune response. Because the effects of IFN-γ are independent of its source, the Ag specificity of the responding memory T cell is clearly not an issue. Unlike Ag-specific responses, IFN-γ can be provided by polyclonal memory T cells of various specificities in response to early bacterial and viral stimuli, as shown by our data. In support of our observations, a recent study identified memory CD8+ T cells as a major source of IFN-γ in a live bacterial model. Although most of their experiments were performed in vitro, stimulation of spleen cells with *Burkholderia pseudomallei* resulted in IFN-γ production by memory CD8+ T cells through a mechanism involving IL-12 and IL-18 (40). IFN-γ was also detected in a fraction of CD8+ T cells after in vivo infection with *Listeria monocytogenes*, but only when re-stimulated with a polyclonal activator in vitro (40). A similar result has also been recently reported in a mouse virus model. Three days after challenge with vaccinia virus, IFN-γ production was detected by lymphocytic choriomeningitis virus (LCMV)-specific memory CD8+ T cells without further stimulation in vitro (41). In addition, mice with LCMV-specific memory T cells had attenuated vaccinia viral titers and were protected against lethal viral challenge in an IFN-γ-dependent manner. However, the mechanism by which the LCMV-specific memory T cells were reactivated by vaccinia virus was not defined. The authors suggested that rather than by bystander activation, IFN-γ production by LCMV-specific memory T cells was induced by cross-reactive epitopes shared by LCMV and vaccinia virus. Similarly, Biron and colleagues (42) showed that LPS induced IFN-γ production by LCMV-specific CD8+ T cells at the peak of infection in LCMV-infected mice. Again, it was concluded that the induction of IFN-γ was dependent on TCR/MHC class I interactions, because IFN-γ was observed in CD8+ T cells only during the midst of viral infection. In contrast, our data demonstrate that specific Ags are clearly not required for IFN-γ induction from memory CD8+ T cells. The ability of adoptively transferred memory CD8+ T cells to produce IFN-γ in LPS-injected β2m−/− hosts suggests that not even a low avidity interaction between TCR and MHC/self peptide complexes is required. Thus, bystander activation by cytokines is sufficient to induce IFN-γ production by memory CD8+ T cells in vivo.

The source of early IFN-γ in acute inflammatory settings has been a topic of considerable interest. One study reported that CD3+ T cells represent the second major source of IFN-γ in spleens of LPS-challenged mice (8). In this particular study, the responding CD3+ T cells were presumed to be NKT cells, because the majority of the IFN-γ CD3+ T cells coexpressed the DX5 Ag. This conclusion may not be entirely correct, because the DX5 Ag is not a specific marker for NK and NKT cells. A large population of memory CD8+ T cells also expresses the DX5 Ag (19, 32, 34, 43), suggesting that many of the IFN-γ DX5+ CD3+ T cells could have been memory CD8+ T cells. In our study, similar numbers of IFN-γ CD4+ and IFN-γ CD8+ T cells were detected in LPS-injected NKT cell-deficient CD1−/− mice compared with LPS-injected wild-type mice (data not shown). Moreover, we found that virus-specific memory CD8+ T cells restricted to H-2Dd produced IFN-γ in response to LPS. Taken together, these data suggest that the IFN-γ-producing CD44hiCD8+ T cells and CD44hiCD4+ T cells are distinct from CD1-restricted NKT cells.

CD8+ T cells from C3H/HeJ (LPS-nonresponder) mice produced IFN-γ upon adoptive transfer into an LPS-challenged C3H/HeN (LPS-responder) recipient, but not upon transfer into LPS-challenged C3H/HeJ mice. In contrast, CD8+ T cells from C3H/HeN mice did not produce IFN-γ upon adoptive transfer into an LPS-challenged C3H/HeJ host (data not shown). This indicated that the induction of IFN-γ from CD8+ T cells was not caused by a direct effect of LPS on CD8+ T cells. This is consistent with the finding that T and NK cells express low to undetectable levels of Toll-like receptors 3 and 4, which are the receptors for poly(I:C) and LPS, respectively (44). Several lines of evidence suggested that the IFN-γ-inducing activity was mediated by multiple cell types, including macrophages and DCs. First, the responsible cell type was determined to be a bone marrow-derived cell that was not a B cell or a mast cell. Second, a large proportion of IFN-γ CD8+ T cells was detected only when LPS-activated macrophages or DCs were cocultured with purified CD8+ T cells in the presence of exogenous IL-18. Finally, the addition of exogenous IL-18 was not necessary for IFN-γ production when the macrophages were mixed with bone marrow-derived DCs and cocultured with CD8+ T cells. It is known that specific subsets of DCs preferentially produce certain cytokines when stimulated by LPS. For example, plasmacytoid DCs produce relatively large amounts of type I IFNs compared with other subtypes of DCs (45). Thus, it is conceivable that cytokines produced by multiple types of DCs and macrophages might work in concert to induce IFN-γ production from CD8+ T cells. Our data are in line with previous reports suggesting that adherent APC-like cells were required for LPS-induced IFN-γ production in vivo (8) and CD8+ T cell proliferation in vivo (5).
although we cannot exclude the involvement of other bone marrow-derived cell types.

Purified CD8⁺ T cells produced IFN-γ in vitro when stimulated by type I IFN or IL-12 in combination with IL-18. Activated macrophages and DCs are major producers of these cytokines (5, 46, 47). During a 6-h stimulation, IFN-α/d + IL-18 induced more IFN-γ release from CD8⁺ T cells than did IL-12 + IL-18. Likewise, a higher proportion of IFN-γ⁺CD8⁺ T cells was detected in spleen cell cultures stimulated with IFN-α/d + IL-18 compared with those stimulated with IL-12 + IL-18 for 6 h. In contrast, IFN-α/d and IL-12 had equal effects on IFN-γ production by NK and NKT cells. Interestingly, IL-12 had a stronger IFN-γ-inducing effect than IFN-α/d when the CD8⁺ T cells were stimulated for 24 h. It is possible that IL-12 may take a longer time to exert its effects on CD8⁺ T cells, because it has been previously reported that IL-12 and IL-18 act synergistically by up-regulating the expression of each other’s receptors (15, 48–50). Because IFN-γ production by CD8⁺ T cells is already substantially decreased by 24 h post-LPS injection in vivo, it is more likely that the 6-h cytokine stimulation in vitro mirrors the in vivo situation. Thus, in an acute setting, these data suggest that type I IFN has a stronger IFN-γ-inducing effect on CD8⁺ T cells than IL-12. Our in vivo experiments also favor a dominant role for type I IFNs in regulation of IFN-γ by CD8⁺ T cells, but not by NK or NKT cells. LPS-challenged IL-12p40⁻/⁻ mice contained a similar proportion of IFN-γ⁺CD8⁺ T cells compared with LPS-treated wild-type mice, whereas LPS-challenged mice treated with anti-IFN-α/β had significantly attenuated numbers of IFN-γ⁺CD8⁺ cells. Interestingly, IL-12 had a slightly greater effect than IFN-α/β on NK cells in vivo, although absence of both cytokines was required to obtain a substantial decrease in IFN-γ-producing cells. This was also true for NKT cells in which a significant decrease in IFN-γ⁺ NKT cells was only observed when both IL-12 and IFN-α/β were taken. Taken together, these data suggest that the regulation of LPS-induced IFN-γ production by T, NK, and NKT cells is distinct and involves multiple cytokines with overlapping functions.

It is noteworthy that not all memory CD8⁺ T cells produce IFN-γ in response to LPS. At most, the proportion of memory CD8⁺ T cells that produced IFN-γ was 30%, depending on the experiment. It is possible that the expression of certain cytokine receptors such as type I IFN receptor is required for IFN-γ production. Consequently, only a fraction of the memory CD8⁺ T cells can produce IFN-γ, because these cytokine receptors may be variably expressed on subsets of memory CD8⁺ T cells. Likewise, it is possible that naive CD8⁺ T cells do not produce IFN-γ in response to LPS due to the lack of expression of functional IL-12 and IL-18 receptors (51). We also tested whether IFN-γ production by memory CD8⁺ T cells was correlated to the expression of NK cell receptors. However, a correlation could not be found, as the proportion of IFN-γ⁺CD8⁺ T cells expressing NK1.1, NKG2A/C, or Ly-49G2 was equivalent to the proportion of IFN-γ⁺CD8⁺ T cells that did not express these receptors (data not shown).

The induction of IFN-γ from CD8⁺ T cells was not restricted to LPS. Injection of poly(I:C) or type I IFN mimicked the IFN-γ-inducing effect of LPS. This is relevant to viral infections in which large amounts of IFN-α/β are secreted 2–3 days after infection (35, 52, 53). In addition, in vitro evidence suggests that virus-stimulated type I IFNs can induce IFN-γ gene expression in human T cells (54). Thus, memory CD8⁺ T cells may provide an early source of IFN-γ not only during bacterial infections, but also during viral infections. Although much of systemic IFN-γ production may be attributed to NK cells (10), the contribution of memory CD8⁺ T cells to IFN-γ production may be important in local areas such as the lymph nodes, where memory CD8⁺ T cells comprise up to 70% of all IFN-γ⁺ cells. Because Ag presentation and subsequent clonal expansion of T cells take place in the lymph nodes, one may suspect that the local IFN-γ released by memory CD8⁺ T cells is important in stimulating APC function and promoting Th1-type responses. Experiments are currently underway to determine whether IFN-γ from bystander-activated memory CD8⁺ T cells is important in the development of subsequent adaptive immune responses.

In summary, we have demonstrated that bystander-activated memory T cells produce IFN-γ in vivo in response to non-Ag-specific bacterial and viral stimuli. The data provided in this study support a role for memory T cells in innate immunity. The accumulation of memory CD8⁺ T cells over time may not only help the host combat previously encountered pathogens, but also provide protection against unrelated newly encountered pathogens by producing IFN-γ.

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References


