A Unique Mechanism for Innate Cytokine Promotion of T Cell Responses to Viral Infections

Gary C. Pien, Khuong B. Nguyen, Lene Malmgaard, Abhay R. Satoskar and Christine A. Biron

*J Immunol* 2002; 169:5827-5837; doi: 10.4049/jimmunol.169.10.5827

http://www.jimmunol.org/content/169/10/5827
A Unique Mechanism for Innate Cytokine Promotion of T Cell Responses to Viral Infections

Gary C. Pien,* Khuong B. Nguyen,* Lene Malmgaard,* Abhay R. Satoskar,† and Christine A. Biron²*


CD8 T cell functions are critical for control of many infectious agents. Clearance of pathogens, however, often begins prior to maximal CD8 T cell expansion (1, 2). During lymphocytic choriomeningitis virus (LCMV) infections, peak CD8 T cell expansion occurs between days 7 and 9 and is accompanied by high levels of ex vivo CTL activity and IFN-γ production observed under culture conditions with antigenic or TCR stimulation. Measurement of the responses using these procedures demonstrates peak frequencies of CD8 T cells primed for IFN-γ production, but does not assess timing of in vivo function. Because reductions in viral burden are observed prior to peak CD8 T cell expansion and priming for function as measured in culture, in vivo CD8 T cell effector functions must be activated relatively early during infections. The actual in vivo response has not been previously characterized, nor have the early pathways responsible for induction and promotion of initial CD8 T cell functions been defined.

Type I interferons (IFN-αβ) are a family of innate cytokines exerting pleiotropic effects during viral infections. They can mediate both antiviral and immunoregulatory functions (3–7). Infections by LCMV elicit high levels of IFN-αβ by days 2–3, but not detectable, biologically active IL-12 (8–11). As a result, NK cell IFN-γ responses are low or absent during infections with this virus (10, 12). IFN-αβ can inhibit replication of certain LCMV strains, including Armstrong and WE, in vitro (13). Moreover, IFN-αβ play a modest role in promoting day 8 CD8 T cell IFN-γ responses in immunocompetent mice (14). Interestingly, IFN-αβ have been reported to synergize with another innate cytokine, IL-18, for enhancing human T cell IFN-γ expression in culture (15). Whether such interactions are important in the mouse and/or during viral infections in vivo is not known. IL-18 is an IFN-γ-inducing factor (16, 17). After proteolytic processing by caspase-1, mature IL-18 is biologically active and can induce IFN-γ production by NK and T cells, particularly in synergy with IL-12 (18–21). Thus, there are conditions in which innate cytokines might interact to promote and shape downstream adaptive responses. An intriguing possibility is that virus-elicited innate cytokines may boost antigenic signals delivered to T cells to cooperatively induce high levels of IFN-γ production only during active viral infection and/or at times when virus-specific subsets are present at low frequencies. The contributions of IL-18 to T cell responses, and whether IL-18 and IFN-αβ can synergize and promote early antigen-driven IFN-γ responses during LCMV infections, are not currently known.

The studies presented in this report evaluate the in vivo kinetics of IFN-γ production after LCMV infections and delineate the roles of innate cytokines in promoting this response. The results demonstrate an unexpectedly early day 4 peak of IFN-γ occurring prior to the dramatic expansion of CD8 T cells. The response is MHC class I/CD8 dependent and associated with IFN-αβ and IL-18 expression, but is independent of IL-12 or NK cells, MHC class II/CD4, or TCR-γδ cells. Antagonist peptide treatment impairs the production of day 4 IFN-γ in vivo, suggesting a role for viral Ag recognition in the response. IL-18 and IFN-αβ augment viral peptide-driven IFN-γ production in culture, and IL-18-deficient or IFN-αβ/IFN-αβ-deficient mice demonstrate impaired day 4 IFN-γ responses in vivo. Taken together, the data reveal a previously unappreciated mechanism for interaction between innate and adaptive immunity to promote delivery of specific adaptive immune responses to viral infections, and a unique mechanism in promoting the in vivo IFN-γ response during LCMV infections.
functions by low frequency CD8 T cell subsets at times preceding their maximal expansion. They also suggest that this mechanism might be in place to limit delivery of a T cell function to times associated with significant pathogen burden.

Materials and Methods

Mice
Male C57BL/6 and homozygous B6.129S2-Cd8a tm1Mak, B6.129P2-B2m tm1Unc, B6.129S2-Cd8a tm1Mak, B6.129P2-Tcra tm1Mom and B6.129-
H112fltm1Bn were purchased from The Jackson Laboratory (Bar Harbor, ME). In addition, male C57BL/6 and MHC class II-deficient B6.169-
Aβfltm5 N5-M with control B6.169-Aβfltm5 N6-W were purchased from Taconic Animal Services and Animals (Germantown, NY). Mice muta-
ted in the IL-18 (22) or IFN-αβR gene (14) were bred and maintained at Brown University (Providence, RI). These animals were age and sex matched, respectively, with C57BL/6 and 129/SvEve mice as appropriate. All mice were of the H-2b haplotype and were used between 6 and 12 wk of age in accordance with institutional guidelines for animal care and use.

In vivo treatments, sample preparation, and cytokine measurements
Infections were established i.p. on day 0 with 2 × 10⁵ PFU of either LCMV Armstrong strain clone E350 or the hepatotropic strain WE (12). In NK cell depletion experiments, either rabbit anti-AGM1 or anti-NK1.1 (PK136) Ab preparations, or the respective control rabbit IgG (Sigma-Aldrich, St. Louis, MO) or P3NS1 preparations, were administered i.p. on day −0.5 and day 3 (10). Efficacy of NK cell depletions were verified by lack of NK1.1 CD3ε (after anti-AGM1) or 2B4 CD3ε (after anti-NK1.1) (23) populations by flow cytometry and/or lack of YAC-1 target cell lysis in standard ⁵¹Cr release assays (24). In peptide administration studies, mice received 2 mg i.p. on day 3 postinfection of either control viral peptide NP ⁹⁶⁶–⁴⁰⁴ or gp33–41 demonstrated that splenics proportions of virus-specific CD8 T cells were not readily apparent on day 4, but were dramatically elevated by day 8 in C57BL/6 mice infected with either LCMV Armstrong or WE (Fig. 1A). Concomitantly, high levels of ex vivo production of IFN-γ in spontaneous and anti-CD3-elicited splenocyte-conditioned media were observed by day 8, but not prior to days 4–6 (Fig. 1B). Armstrong infections typically induced higher magnitude splenic CD8 T cell responses than infections with WE. However, it is not clear whether these ex vivo assays demonstrating CD8 T cell priming for IFN-γ production accurately represent their functions in vivo, particularly since clearance of LCMV is known to be underway prior to day 8 in the spleen (1, 14, 40–42).

To more precisely address the kinetics of CD8 T cell function in vivo, studies investigating the production of systemic and local IFN-γ during LCMV infections were done. Levels of IFN-γ were measured in serum samples and organ homogenates to assess in vivo expression of cytokines. These experiments demonstrated an unexpectedly early peak of IFN-γ production on day 4 in response to LCMV infections (Fig. 2). With Armstrong, levels reached 0.19 ± 0.03 ng/ml in serum and 2.8 ± 0.6 and 0.4 ± 0.1 ng/g tissue in spleen and liver, respectively; with WE, IFN-γ levels reached 0.65 ± 0.11 ng/ml in serum and 7.1 ± 0.7 and 0.6 ± 0.1 ng/g tissue in spleen and liver, respectively. Taken together, the results reveal a dichotomy between when peak responses occur for in vitro measurements of CD8 T cell priming of IFN-γ production and when IFN-γ is endogenously expressed in vivo during acute infections.

IL-12 and cellular dependencies of day 4 IFN-γ responses
To determine the requirements for the early IFN-γ response, different immune components were eliminated through the use of genetically manipulated or Ab-treated mice. Because spleen IFN-γ levels were higher than in the liver, subsequent experiments focused on systemic and splenic responses. Disruption of particular arms of innate immunity did not impair day 4 IFN-γ expression in either NK cell-depleted mice or IL-12p40-deficient mice (Table I),

In vitro stimulations
Spontaneous production of IFN-γ in culture was determined by plating 10⁶ cells/well in 96-well microtiter plates with 200 μl of 10% FCS-RPMI 1640. Anti-CD3-elicited IFN-γ production was similarly done using plates coated overnight with 150 μl of 10 μg/ml anti-CD3ε (145–2C11) (BD PharMingen). To evaluate the ability of exogenous cytokines to enhance Ag-driven IFN-γ production in culture, cells from LCMV-infected mice were plated at 10⁵ cells/well (bulk splenocytes) or 2 × 10⁵ cells/well (CD8 enriched). Indicated concentrations of NP ⁹⁶⁶–⁴⁰⁴ were added with or without recombinant murine IL-18 (R&D Systems, Minneapolis, MN) and/or universal IFN-α A/D (PBL Biomedical Laboratories, New Brunswick, NJ) to a final volume of 200 μl/well. NP ⁹⁶⁶–⁴⁰⁴ was chosen because this is the immunodominant epitope in H-2b hosts (30–34). Universal IFN-α A/D is a hybrid factor constructed from recombinant human IFN-α A and IFN-α D and exerts bioactivity on mouse cells. Cultures were incubated for 24 h at 37°C prior to harvesting of supernatants for determination of IFN-γ production by ELISA.

Statistical analyses
Data were analyzed using statistical functions and the two-tailed homoscedastic Student’s t test function from Microsoft Excel 98 (Microsoft, Redmond, WA). Unless otherwise indicated, results are given as means ± SEM.
nor was it impaired in IL-12p35-deficient mice (data not shown). The IFN-γ response was also intact in mice lacking TCR-γδ-bearing T cells. However, mice lacking MHC class I and/or CD8 T cells, as a result of genetic disruptions in H-2Kb or CD8, demonstrated significant reductions of 75–95% in systemic and splenic IFN-γ responses to day 4 Armstrong and WE infections (Table I). Thus, both strains elicited a MHC class I/CD8-dependent IFN-γ response during acute infections. Flow cytometric analyses of CD8 T cells demonstrated that, whereas only 7–8% expressed CD44 high CD62L low in uninfected mice, by day 4 postinfection 20% of CD8 cells acquired this activated phenotype. These results demonstrate that a subset of CD8 T cells is indeed activated as early as day 4. Furthermore, the data support a role for CD8 T cells in contributing to in vivo IFN-γ production at this time.

Role for Ag signaling in day 4 IFN-γ responses

Because MHC class I was critical for early CD8-dependent IFN-γ responses, the contributions of Ag signaling were investigated by interfering with Ag presentation on MHC class I to CD8 T cells. To antagonize in vivo H-2Db-restricted interactions, a synthetic blocking peptide (SMIENLEYM) known to block LCMV-specific CTL activity was used (25–27). Two different control peptides were tested in separate experiments, using an immunodominant viral peptide (NP396–404) and an irrelevant OVA peptide (OVA257–264). Since administered peptides were expected to have short half-lives in vivo, control and blocking peptides were injected 1 day before peak IFN-γ responses on day 4 after infection. The studies demonstrated that, as compared to control peptide treatment, administration of blocking peptide reduced systemic and splenic IFN-γ responses by 25–50% in C57BL/6 mice (Table II). Control peptide treatments yielded similar day 4 IFN-γ responses as compared to infected, but untreated mice. The differences between control and antagonist peptide treatments were statistically significant in most, but not all, experiments. It was not possible to ascertain whether blocking peptide treatments were sufficient to interfere with all H-2Db-TCR interactions involving viral Ag in vivo. Nevertheless, the decreases were reproducibly observed in additional independent studies, demonstrating a contribution of Ag signaling during the 24-h period preceding the day 4 IFN-γ response to LCMV infections. The results therefore support an Ag-specific stimulation of CD8 T cells as a factor contributing to the day 4 IFN-γ response.

To directly assess virus-specific CD8 T cell subsets on day 4 after LCMV infections, bulk splenocytes were analyzed by flow cytometry for intracellular expression of IFN-γ. Spontaneous expression of IFN-γ was difficult to detect ex vivo, thus cells were briefly restimulated with H-2Kb immunodominant viral peptides NP396–404 and gp33–41. Under these conditions, <0.1% of CD8 T cells from uninfected mice expressed intracellular IFN-γ. However, after 4 days of LCMV infection, ~0.7% of CD8 T cells were...
induced to express IFN-γ compared to ≤0.2% of CD4 T cells (Fig. 3A). Thus, consistent with data from genetically manipulated mice (Table I), CD8 T cells were found to be the predominant source of IFN-γ. Because these frequencies of IFN-γ-expressing CD8 T cells were low, bulk splenocytes were enriched for CD8+ cells prior to restimulation. These experiments confirmed that ~0.4% of enriched CD8 T cells were induced to express intracellular IFN-γ after infections (Fig. 3B). Populations from LCMV Armstrong-, as compared to WE, infected mice typically exhibited a greater magnitude ex vivo IFN-γ responses. To delineate the contributions of CD8 T cells to the IFN-γ response under these conditions, bulk splenocytes from Armstrong-infected mice were brieelly restimulated with viral peptides and magnetically sorted for IFN-γ-secreting cells. Phenotypic identification of the IFN-γ-secreting populations revealed that CD8 T cells comprised 47% of the isolated cells (Fig. 3C). Although some nonspecific signal from IFN-γ+ CD8+ populations was detected in both uninfected and day 4 Armstrong-infected mice, the data nevertheless established that virus-specific CD8 T cell subsets were primed for IFN-γ production by day 4 and were the predominant source of this cytokine. The collective studies thus indicate that MHC class I–TCR interactions are important for the in vivo CD8-dependent IFN-γ response and that likely this response is primarily from Ag-specific CD8 T cell subsets.

**Innate cytokine functions in driving T cell IFN-γ production**

Although IL-12 is not required for CD8 T cell responses to LCMV infections, several other innate cytokines may promote and/or augment T cell synthesis of IFN-γ. In particular, IFN-αβ and IL-18.

<table>
<thead>
<tr>
<th>Studies</th>
<th>Mouse Strain</th>
<th>Ab Treatment</th>
<th>Serum (pg/ml)</th>
<th>Spleen (ng/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12</td>
<td>C57BL/6</td>
<td>Control</td>
<td>286.2 ± 49.9</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antagonist</td>
<td>196.9 ± 89.9</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>C57BL/6</td>
<td>Control</td>
<td>149.3 ± 8.9</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-NK1.1</td>
<td>369.4 ± 57.2</td>
<td>5.3 ± 1.1</td>
</tr>
<tr>
<td>NK</td>
<td>C57BL/6</td>
<td>Control</td>
<td>119.7 ± 10.0</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-AMG1</td>
<td>528.7 ± 91.7</td>
<td>6.8 ± 1.1</td>
</tr>
<tr>
<td>TCR-γδ</td>
<td>IL-12</td>
<td>C57BL/6</td>
<td>184.5 ± 17.9</td>
<td>5.9 ± 1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCR-δ−</td>
<td>145.2 ± 21.4</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>CD4</td>
<td>C57BL/6</td>
<td>MHC class II</td>
<td>236.1 ± 38.9</td>
<td>3.0 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>C57BL/6</td>
<td>CD4+</td>
<td>221.5 ± 28.4</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>C57BL/6</td>
<td>CD4−</td>
<td>182.0 ± 23.3</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>CD8</td>
<td>β2-microglobulin−−</td>
<td>53.8 ± 4.9*</td>
<td>0.3 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C57BL/6</td>
<td>CD8α</td>
<td>115.5 ± 26.3</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>CD8α</td>
<td>CD8α</td>
<td>BLD**</td>
<td>BLD**</td>
</tr>
<tr>
<td></td>
<td>C57BL/6</td>
<td>CD8α</td>
<td>1.3 ± 0.3**</td>
<td>0.4 ± 0.1**</td>
</tr>
</tbody>
</table>

Table II. **Effects of antagonistic peptide administration on day 4 IFN-γ responses**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Infection</th>
<th>Peptide Treatment</th>
<th>Serum (pg/ml)</th>
<th>% Reduction</th>
<th>Spleen (ng/g tissue)</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arm</td>
<td>Control</td>
<td>8.4 ± 1.4</td>
<td>33</td>
<td>17.3 ± 1.7</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antagonist</td>
<td>4.0 ± 0.4*</td>
<td>252</td>
<td>13.7 ± 2.2</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Arm</td>
<td>Control</td>
<td>3.6 ± 0.4</td>
<td>30.8</td>
<td>2.0 ± 0.1*</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antagonist</td>
<td>2.0 ± 0.2*</td>
<td>42.9</td>
<td>2.6 ± 0.9</td>
<td>55</td>
</tr>
</tbody>
</table>

* Studies with genetically mutated or Ab-treated mice were done as described in Materials and Methods. Serum samples and spleen homogenates from day 4 LCMV Armstrong- or WE-infected mice were assayed for IFN-γ levels by sandwich ELISA. Results are expressed as the means ± SEM of three to four mice per group. BLD denotes levels that are below the limits of detection of 10 pg/ml in serum and 0.2 ng/g tissue in spleen.

**p ≤ 0.001 compared to control C57BL/6 mice.**

**p ≤ 0.007 compared to control C57BL/6 mice.**

**Studies with genetically mutated or Ab-treated mice were done as described in Materials and Methods. Serum samples and spleen homogenates from day 4 LCMV Armstrong- or WE-infected mice were assayed for IFN-γ levels by sandwich ELISA. Results are expressed as the means ± SEM of three to four mice per group. BLD denotes levels that are below the limits of detection of 10 pg/ml in serum and 0.2 ng/g tissue in spleen. BLD** denotes levels that are below the limits of detection of 10 pg/ml in serum and 0.2 ng/g tissue in spleen.

**Expt. 1**, control OVA257–264 peptide (Expt. 1), control OVA109–120, OVA257–264 peptide (Expt. 1), or the antagonistic peptide SMIFNLEYM (Expts. 1 and 2). Day 4 serum samples and spleen homogenates were then assayed by ELISA for IFN-γ levels. Results are expressed as the means ± SEM of three mice per group along with percent reductions induced by antagonist treatment as compared to control injections.

* *p ≤ 0.02 compared to control peptide treatment.

† *p ≤ 0.05 compared to control peptide treatment.
have been reported to synergize for human T cell IFN-γ in culture (15). To determine whether IFN-αβ and/or IL-18 were induced during LCMV infections, the kinetics of expression for these innate cytokines was assayed. Consistent with other reports (8), IFN-αβ was induced to high systemic levels following LCMV infections and peaked on days 2–3 (Fig. 4). In addition, in vivo

FIGURE 3. Intracellular IFN-γ expression by day 4 LCMV infection-primed CD8 T cells. Bulk splenocytes (A) or populations enriched by MACS for CD8 T cells (B) were obtained by pooling four C57BL/6 mice per group. Cells were cultured for 5 h in the presence of 100 ng/ml each of viral peptides NP396–404 and gp33–41. During the last 3 h of culture, brefeldin A was added. Cells were then harvested and stained for CD8 and CD4 surface expression, fixed, permeabilized, and stained for intracellular IFN-γ. Similar data were obtained in an additional experiment. C. A total of 5 x 10⁷ bulk splenocytes were pooled from three C57BL/6 mice per group; from uninfected or day 4 Armstrong (Arm)-infected mice. Cells were cultured for 6 h in the presence of 1 μg/ml each of NP396–404 and gp33–41 prior to magnetic enrichment using a commercial IFN-γ secretion assay according to the manufacturer’s directions. Enriched populations were stained for surface markers, fixed, and analyzed by flow cytometry.

FIGURE 4. Expression of innate cytokines during LCMV infections. Serum samples and spleen homogenates from LCMV-infected C57BL/6 mice were assayed for IL-18 and IFN-αβ as described in Materials and Methods. Samples for IL-18 measurements were collected from two experiments, demarcated on the x-axis by hatches. Arm, Armstrong.
production of IFN-αβ in spleen homogenates was demonstrated, and was found to have similar kinetics of induction as in serum (Fig. 4). Likewise, IL-18 was induced early in serum samples and spleen homogenates (Fig. 4). Systemic levels of IL-18 were induced from undetectable levels in uninfected mice to 0.11 ± 0.01 and 0.14 ± 0.02 ng/ml by day 6 after Armstrong and WE infections, respectively. Splenic levels of IL-18 peaked earlier on day 3, reaching 339 ± 30 and 342 ± 51 ng/g tissue after Armstrong and WE infections, respectively (Fig. 4). Splenic expression of the 18-kDa biologically active form of IL-18 was verified by Western blotting (data not shown). Taken together, these experiments demonstrate that IFN-αβ and IL-18 are induced early after infections, and thus are available for potentially supporting day 4 IFN-γ responses.

To assess whether IFN-αβ and/or IL-18 could indeed promote Ag-driven IFN-γ responses, splenocytes from day 4 and day 8 LCMV-infected mice were cultured with varying concentrations of the immunodominant LCMV peptide NP396–404 in combination with IFN-α and/or IL-18. Day 4 IFN-γ responses were modest under these culture conditions (Fig. 5), presumably due to the low frequencies of CD8 T cells specific for this viral epitope on day 4 (Fig. 1). Nevertheless, the addition of either IFN-α or IL-18 tended to enhance the modest Ag-driven IFN-γ response on day 4. Splenocytes from either day 8 Armstrong- or WE-infected mice produced high levels of IFN-γ in response to viral Ag, and production also could be augmented in the presence of either IFN-α or IL-18 (Fig. 5). Consistent with the greater accumulation of responding CD8 T cells in the spleens of Armstrong-, as compared to WE-, infected mice, measurements of in vitro production by isolated cell populations resulted in higher levels of IFN-γ from Armstrong rather than WE infections, particularly on day 8 (Figs. 5 and 6). At suboptimal concentrations, the combination of IL-18 and IFN-α synergistically enhanced viral peptide-driven IFN-γ production following either day 4 or day 8 LCMV infections (Fig. 6). These innate cytokines also synergized with Ag for driving IFN-γ production from CD8-enriched cell populations. Enriched CD8 populations pooled from day 8 Armstrong-infected mice produced 1.3 ng/ml IFN-γ after stimulation with 50 ng/ml NP396–404 peptide. Addition of either 1000 U/ml IFN-α or 0.4 ng/ml IL-18 enhanced IFN-γ production to 4.6 and 3.0 ng/ml, respectively. Combining all three stimuli augmented production of IFN-γ by enriched CD8 T cells to 10.2 ng/ml, suggesting that these factors could directly act upon CD8 T cells. Collectively, these studies demonstrate that innate cytokines act in concert with Ag to augment CD8 T cell IFN-γ production in culture.

To investigate the endogenous roles for IL-18 and/or IFN-αβ in promoting IFN-γ responses, mice lacking IL-18 or IFN-αβ functions were infected with LCMV to determine their IFN-γ responses in vitro and in vivo. These animals were genetically mutated in the genes encoding either IL-18 or the IFN-αβ receptor. Splenic leukocytes from Armstrong-infected, IL-18-deficient mice demonstrated reduced IFN-γ production when cultured with viral Ag (Fig. 7A). Control C57BL/6 mice produced 306.3 ± 16.4 pg/ml IFN-γ at the highest concentration of NP396 peptide. In contrast,
vivo responses in these immunodeficient mice. IL-18-deficient C57BL/6 mice exhibited significant reductions of 40–60% in serum and splenic IFN-γ production after infections by either Armstrong or WE (Fig. 8A). More dramatically, in both compartments the day 4 IFN-γ response to WE infections in 129 IFN-αβR-deficient mice was significantly reduced by >95% (Fig. 8B). Collectively, the results demonstrated that lack of IL-18 or IFN-αβ functions impairs the ability of Ag to stimulate virus-specific IFN-γ responses in culture. The results therefore define critical requirements for endogenous innate cytokines in promoting day 4 IFN-γ responses in vivo.

Discussion

The studies presented in this report demonstrate innate cytokine regulation of CD8 T cell IFN-γ production. We show for the first time the induction and expression of IL-18 during LCMV infections and delineate its role, in conjunction with IFN-αβ, in promoting an unexpectedly early in vivo day 4 IFN-γ response. This response occurs with either LCMV Armstrong or WE infections, is NK cell independent but CD8 dependent, and precedes the dramatic expansion of CD8 T cells seen after infection. Antagonist peptide studies and experiments restimulating with LCMV peptides demonstrate that day 4 IFN-γ is driven by Ag-specific responses. Either IFN-αβ or IL-18 can significantly augment IFN-γ responses in culture, and both factors synergize for this effect. Correspondingly, day 4 IFN-γ responses require IFN-αβ and, to a lesser extent, IL-18 for expression. Our studies delineate the induction of endogenous innate cytokines and the promotion by these factors of a heretofore unappreciated CD8 T cell-mediated IFN-γ response by low-frequency subsets early after LCMV infection. Thus, they define a unique mechanism by which innate cytokines promote T cell responses.

Although the frequencies of virus-specific CD8 T cells for any single immunodominant epitope were below the limits of effective detection, our data indicate that on day 4 postinfection ~0.3 to 0.7% of CD8 T cells were primed for intracellular IFN-γ expression following ex vivo restimulation with immunodominant viral peptides (Fig. 3). This is commensurate with early estimates of naive LCMV-specific CTL precursor frequencies of 1 in 560,000 (43) and with more recent approximations of 1 in 10,000 (44). Taking into account a 6- to 24-h division time for activated T cells, our measurements of day 4 IFN-γ-producing CD8 T cells are in close agreement with expected virus-specific precursor frequencies

Table III. CD8 T cell expansion in day 8 LCMV-infected mice lacking IL-18 or IFN-αβR

<table>
<thead>
<tr>
<th>Infection</th>
<th>No. of CD8 (×10^6)/Whole Spleen</th>
<th>No. of CD8 (×10^5)/g Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>IL-18^+</td>
<td>IL-18^-</td>
</tr>
<tr>
<td>Total CD8</td>
<td>5.7 ± 1.3</td>
<td>6.2 ± 1.0</td>
</tr>
<tr>
<td>H-2D^+/NP^396</td>
<td>≤0.1 ± 0.0</td>
<td>≤0.1 ± 0.0</td>
</tr>
<tr>
<td>H-2D^+/GP^34</td>
<td>≤0.1 ± 0.0</td>
<td>≤0.1 ± 0.0</td>
</tr>
<tr>
<td>Day 8 Armstrong</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CD8</td>
<td>38.9 ± 7.9</td>
<td>44.4 ± 7.3</td>
</tr>
<tr>
<td>H-2D^+/NP^396</td>
<td>10.8 ± 2.0</td>
<td>9.4 ± 1.8</td>
</tr>
<tr>
<td>H-2D^+/GP^34</td>
<td>3.8 ± 0.8</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>Day 8 WE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CD8</td>
<td>6.0 ± 1.3</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>H-2D^+/NP^396</td>
<td>0.7 ± 0.0</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>H-2D^+/GP^34</td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0.0</td>
</tr>
</tbody>
</table>

^a Indicated strains of mice were infected with LCMV Armstrong or WE for 8 days as per Materials and Methods. Freshly isolated splenocytes were stained with fluorochrome-conjugated anti-CD4, anti-CD8, and H-2D^b tetramers loaded with the indicated viral peptides. Samples were analyzed by flow cytometry to determine proportions of total and virus-specific CD8 (CD8^+CD4^-) T cells as per Materials and Methods. Numbers of CD8 T cell subsets were then calculated on a per whole organ basis (IL-18 studies) or on a per g of tissue basis (IFN-αβ studies).
after about 3–4 days of in vivo stimulation. It is not entirely surprising to us that a low-frequency subset of cells could produce the levels of IFN-γ observed on day 4. NK cells, which typically only comprise about 2% of splenocyte populations, produce 10-fold greater levels of IFN-γ at their peak response to murine cytomegalovirus infection (24). However, in vivo contributions to IFN-γ on day 4 of LCMV infection may be drawn from a broader range of CD8 T cell subsets than those quantitated with the LCMV peptides tested here. Although the day 4 IFN-γ response is at least partially driven by viral Ag presented on H-2Dk (Table II), it is possible that additional H-2Kβ interactions, subdominant peptides, and/or emerging low-affinity CD8 T cells also contribute to IFN-γ production (34). We cannot exclude the possibility that other cell populations may be contributing to CD8-dependent IFN-γ responses. Others have demonstrated that in the absence of a normal high-affinity CTL response, lower affinity CTL and/or alternative-epitope CTL can be generated against LCMV (45, 46). Interestingly, in this hierarchical context IFN-γ is required by the resultant low-affinity CTL response for clearance of virus (47). Although there are a number of pathways by which this effect could be mediated, IFN-γ might enhance CTL function by promoting the expression of MHC class I molecules and the resulting Ag presentation by these molecules to CD8 T cells (48). Thus, prior to the expansion of CD8 T cells specific for high-affinity immunodominant viral epitopes, the early IFN-γ response may be a composite derived from more diverse CD8 T cell subsets driven by LCMV Ags and augmented by IFN-αβ and IL-18, and the cytokine response might act to help promote defense through direct antiviral and/or immunoregulatory effects.

Given that the induction of innate cytokine responses can be linked to stimulation with microbial products, their requirement for enhancing an Ag-specific response may serve as a mechanism for ensuring the appropriateness of expressing T cell effector functions. Our results complement the observations, during infections with the intracellular pathogens Toxoplasma gondii (49) and Leishmania major (50), that continuous IL-12 exposure is required for maintaining T cell IFN-γ production and host resistance. The IL-12 requirement exists despite continued antigenic stimulation, either in culture with exogenous Ag or in vivo after infection reactivated due to loss of resistance. Using innate cytokines to promote Ag stimulation of T cells may limit induction of T cell functions to times when they are needed. On the other hand, there are examples where Ag is critical for priming of T cell functions, but once activated, the responses can occur independently of further antigenic stimulation in the presence of cytokines (39). Following transient exposure to Listeria monocytogenes Ag in culture, for example, peptide-specific CD8 T cells are capable of undergoing limited replication, after Ag deprivation, promoted by exogenous IL-2 (50). Similarly, IL-12 and IL-18 costimulation induces polarized CD4 T helper 1 cells to secrete IFN-γ in the absence of exogenous specific Ag (51, 52). Thus, once primed with Ag, T cells may be stimulated by cytokines to produce IFN-γ without further TCR-mediated signaling. Such mechanisms may make it possible to call memory CD8 T cells into early IFN-γ production by exposure to innate cytokines and might provide a mechanism for nonspecific activation during viral infections. Under the day 4 infection conditions studied here, however, the CD8 T cell IFN-γ responses occur in the context of antigenic stimulation and are partially dependent upon TCR signaling (Table II). Nevertheless, it is possible that the residual IFN-γ response, not inhibited by antagonistic peptide, is due either to other cell types being recruited to participate in the response or to virus-specific CD8 T cells, primed before day 3, but no longer requiring Ag to respond to cytokine-mediated signals for IFN-γ production. Taken together, our data and others demonstrate that cytokines may be a second signal required for accessing Ag-primed T cell responses. By requiring a second signal from innate cytokines induced during an acute infection, a safeguard is created by which T cell functions are only driven when licensed to do so in vivo.

The intracellular signals inducing T cell IFN-γ expression can arise from TCR and/or cytokine stimulation via different pathways. In T helper 1 CD4 T cells, TCR-induced IFN-γ is likely initiated by the NFAT (53–55), whereas IL-18-induced IFN-γ requires GADD45β (56). It is unclear whether CD8 T cells have a similar dichotomy in receptor-mediated pathways, but differences clearly exist between CD4 and CD8 T cells in their requirements for IFN-γ induction by TCR stimulation (57). Our results show that day 4 IFN-γ responses to LCMV infections are primarily derived from CD8 T cells rather than CD4 T cells. Both Ag and IL-18 play important roles in driving this response, which also requires IFN-αβ (Table II and Fig. 7). Although we have not defined the intracellular pathways for synergism in this report, the interplay between IFN-αβ and IL-18 can occur at several mechanistic levels. IFN-αβ can enhance IL-18-mediated signaling by inducing caspase gene expression, up-regulating IL-18R components (58), and/or increasing expression of the IL-18R signaling adaptor molecule myeloid differentiation factor 88 (59). Indeed, after LCMV infections, CD8 T cells up-regulated expression of the IL-18R component, IL-1R-related protein, indicating that these populations are likely primed to respond to this factor (data not shown). Thus, during in vivo infections a combination of TCR and cytokine signals contribute to CD8 T cell IFN-γ responses, particularly at
early times when viral titers and Ag load are high but virus-specific CD8 T cell frequencies are still relatively low. At these low in vivo E:T cell ratios, dichotomous but convergent TCR and cytokine signaling pathways are likely to contribute to the synergism observed in promoting CD8 T cell IFN-γ responses on day 4 postinfection.

IFN-αβ cytokines exert pleiotropic effects during viral infections, and it is becoming clear that during LCMV infections, these innate factors possess complex immunoregulatory roles. The type 1 IFNs are a family of structurally related factors encoded by a single IFN-β and multiple IFN-α genes. These molecules all utilize the same heterodimeric receptor complex. Although certain subtle differences have been suggested, the various IFN-αβ members generally have overlapping biological effects (60). Our laboratory has recently demonstrated that IFN-β and multiple IFN-α gene products are induced during LCMV infection (61). In the present study, we demonstrate that a universal recombinant type I IFN supports Ag-driven T cell IFN-γ production in vitro and that LCMV-induced IFN-αβ endogenously promote T cell IFN-γ responses in vivo.

These experiments define a unique mechanism for innate cytokine promotion of CD8 T cell IFN-γ production. IFN-γ has been shown to be essential for defense against a variety of infections (9, 10, 24, 62–64). During LCMV infections, its role has been more controversial, with IFN-γ reported to modestly inhibit viral replication in vitro (13) and to enhance viral clearance (65–67). We and others have shown that IFN-αβ are important for control of LCMV (13–14, 68). In contrast, IL-18 was dispensable for clearance of virus (data not shown), possibly due to incomplete abrogation of the IFN-γ response. This is consistent with the greater importance of IFN-αβ, as compared to IL-18, for promotion of IFN-γ (Fig. 8). However, it remains to be determined whether IFN-αβ plays a role in optimal host defense at specific times prior to maximal expansion of virus-specific CTL.

Our group has previously shown that IFN-αβ function to inhibit endogenous IL-12 expression during LCMV infection (11). In addition, 2 days after viral infections, IFN-αβ induce a refractory state in which NK cell IFN-γ responses to IL-12 and anti-CD3-elicted IFN-γ production from splenocytes are inhibited (69). The refractory state is temporary and responsiveness to both stimuli is restored by 4 days postinfection. Along with the studies presented here, the data demonstrate a biphasic role for IFN-αβ in the regulation of IFN-γ responses during LCMV infections. Initially, IFN-αβ function to inhibit IL-12 and day 2 IFN-γ expression. However, after the refractory period expires, IFN-αβ clearly enhance Ag-driven IFN-γ production in vitro (Figs. 5 and 6) and promote the endogenous day 4 IFN-γ response (Fig. 7). This duration of probability likely exists to temporally coordinate host immune responses for optimal efficacy. Since both IFN-γ and IFN-αβ can signal through STAT1, initial refractoriness to TCR stimulation may prevent inappropriate IFN-γ expression and competition for signal transducers at times when IFN-αβ exert critical antiviral and/or other immunoregulatory effects. Additionally, deferred activation of T cell functions may avoid premature IFN-γ-induced cell death or exhaustion prior to expansion of immunodominant virus-specific CD8 T cell subsets, and/or before the delivery of antiviral functions (70, 71). Indeed, administration of high-dose IL-12 during LCMV infection drives IFN-γ levels to greatly elevated levels, but is detrimental to the expansion of CD8 T cells and hinders control of viral replication (72). Thus, IFN-αβ are an important coordinator of host immune responses that span both innate and adaptive immunity.

The mechanisms by which IFN-αβ might act to promote IFN-γ expression in the human as compared to the mouse are controversial. The issue stems from the observation that one pathway to IFN-γ induction, STAT2-dependent activation of STAT4, can be elicited in response to IFN-αβ in human but not mouse cells (73). It should be noted that the studies reported here demonstrate a mechanism by which IFN-αβ promote IFN-γ responses in the mouse by acting with IL-18. Because these effects have also been reported in human culture systems (15), at least this pathway may be operational in both species. Particular biochemical differences between the species may have less significance in the context of the mixed cytokine and cellular responses elicited during pathogen challenge. Interestingly, Ab depletion of NK cells resulted in higher in vivo IFN-γ levels as compared to control-treated animals (Table I). Although the mechanism for this is unclear, it would be consistent with previous work from our laboratory demonstrating that NK cells can regulate the magnitude of CD4 and CD8 T cell responses to LCMV infection (74). Thus, cellular as well as cytokine cross-talk are likely to exist between innate and adaptive immunity.

In summary, our data demonstrate endogenous roles for innate cytokines in promoting early adaptive immune responses. Since we have captured the functioning of CD8 T cells in vivo, our results question the in vivo relevance of ex vivo measurements of T cell functions, as peak Ag- or anti-CD3-elicted IFN-γ responses in vitro occur between days 7 and 9. We show that the endogenous in vivo peak is much earlier. Access to CD8 T cell effector functions, and hence control of viral replication by adaptive immunity, can thus begin as early as 4 days after infection. These conditions may promote early antiviral defense at times when virus-specific cells are at low frequencies. Furthermore, the pathways defined in these studies are a mechanism for ensuring the appropriateness of accessing Ag-specific CD8 T cell functions as they require a second signal from innate cytokines induced during the acute infection.

Acknowledgments

We thank Dr. Philip Scott (University of Pennsylvania) for his gift of rabbit anti-mouse IFN-γ, Drs. Kiyoshi Takeda and Shizuo Akira (Osaka University, Osaka, Japan) for providing breeding pairs of IL-18-deficient mice, and the National Institute of Allergy and Infectious Diseases Tetramer Facility at Emory University (Atlanta, GA) for the LCMV tetramers. We are also grateful to Drs. Marc Dalod and Thais Salazar-Mather, along with Rachelle Salomon, Casey Lewis, and Wanda Montas for their insightful discussions and expert assistance.

References

10. Orange, J. S., and C. A. Biron. 1996. An absolute and restricted requirement for IL-12 in natural killer cell IFN-γ production and antiviral defense: studies of


