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Cutting Edge: Selective IL-18 Requirements for Induction of Compartmental IFN-γ Responses During Viral Infection[1]

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Optimal protective effects for defense against infection require orchestration of immune responses spanning multiple host compartments and divergent local regulation at particular sites. During murine cytomegalovirus infections known to target spleen and liver, IL-12-induced IFN-γ from NK cells is crucial for resistance. However, the roles for IL-18 and/or IL-12 in regulating hepatic IFN-γ responses, as compared with systemic or splenic responses, have not been defined. In this report, mice genetically deficient in either IL-18 or IL-12p35 exhibited up to 95% reductions in systemic and splenic IFN-γ responses. Surprisingly, IFN-γ responses were preserved in the livers of IL-18-deficient, but not IL-12p35-deficient, mice. Cytokine requirements for host survival also differed. Under conditions where mice lacking IL-12p35 exhibited 100% mortality, those lacking IL-18 survived. Taken together, our results delineate contrasting compartmental requirements for IL-18 and suggest that preservation of local, hepatic IFN-γ production is critical for host defense during murine cytomegalovirus challenge. ***The Journal of Immunology,*** **2000, 165: 4787–4791.**

Interleukin 18 possesses biochemical similarities to IL-1 and functional similarities to IL-12 (reviewed in Refs. 1 and 2). The factor is synthesized as an inactive precursor requiring proteolytic cleavage by caspase-1 (IL-1β-converting enzyme) to produce the biologically active species (1, 2). Mature bioactive IL-18 can stimulate IFN-γ production from NK cells and is potently synergistic with IL-12 for this function (3, 4). The importance of IL-18 in immunity and host defense is only beginning to be appreciated. Human macrophages have been demonstrated to secrete IL-18 upon influenza A and Sendai viral infections (5, 6), and administration of IL-18 has been shown to elicit antiviral effects in vaccinia virus-infected mice (7). Almost nothing is known about the endogenous induction and in vivo function of IL-18 during viral infections.

Murine cytomegalovirus (MCMV)[3] is a hepatotropic herpesvirus that induces endogenous IL-12 and NK cell-dependent IFN-γ responses crucial in early defense (8–12). IL-12 is required for both systemic and splenic IFN-γ responses (11–13), but little information is available regarding IL-12 functions in liver or the roles for IL-18 at any of these sites. Mice genetically deficient in the IL-1 receptor-associated kinase, a signaling molecule shared by the IL-1 and IL-18 receptors, have impaired systemic IFN-γ responses to MCMV infections (14). Recent work from our group has shown that conditions prohibiting recruitment of IFN-γ-producing NK cells to the liver compromise host defense and results in mortality even in the presence of intact systemic and splenic IFN-γ responses (15, 16). The studies presented here define the induction and function of IL-18 in defense against MCMV infection and contrasts these to those of IL-12. Collectively, our results demonstrate that IFN-γ responses are differentially regulated at particular sites and that there are selective and specific requirements for IL-18 as a cofactor in regulating IFN-γ responses in some, but not all, host compartments.

Materials and Methods

Mice

C57BL/6 mice were purchased from Taconic Laboratory Animals and Services (Germantown, NY) and The Jackson Laboratory (Bar Harbor, ME). The former were controls for mice with a targeted disruption of the IL-12p35 gene (13), originally provided by Genetics Institute (Andover, MA); the latter were controls for mice genetically deficient in IL-18 (17). All mice were used between 5 and 12 wk of age, and experiments were conducted in accordance with institutional guidelines for animal care and use.

In vivo treatments and sample collection

Infections were established on day 0 by i.p. injections of 5×10^4 PFU of MCMV v70 Smith strain (11). In experiments utilizing LPS, mice were treated i.p. with a 100-μg dose from *Escherichia coli* strain O111:B4 (Difco, Detroit, MI) for 6 h. At indicated times after injections, serum and organs were collected. In survival experiments, mice were infected with

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[3] Abbreviations used in this paper: MCMV, murine cytomegalovirus; MFI, mean fluorescence intensity.

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10^5 PFU of MCMV and assessed for mortality at least once daily for ≥21 days.

Preparation of leukocytes

Splenic and hepatic leukocytes were prepared as described previously (18). For flow cytometric analyses, hepatic populations were isolated by density centrifugation on Percoll gradients. Because cell yields were limiting with this protocol, 24% metrizamide gradients (Accurate Chemical and Scientific, Westbury, NY) were used for preparation of hepatic effector cells used in cytotoxic activity assays (19). Cell yields and viability were determined by trypan blue exclusion.

Cytokine measurements

For determination of cytokine levels, organs were weighed and homogenized (16). Supernatants were assayed for IFN-γ, IL-12p40 (detects p40 plus p70), or IL-18 (detects 24-kDa precursor plus 18-kDa bioactive species) levels by sandwich ELISA (10, 12). IL-18 ELISA reagents utilized a rat monoclonal Ab (R&D Systems, Minneapolis, MN) for capture, a goat polyclonal Ab (R&D Systems), and an anti-goat HRP-conjugated Ab for visualization with 2,2′-azino-di(3-ethyl-benzthiazoline-6-sulfonate) substrate. Standard curves were generated with recombinant mature murine IL-18 (R&D Systems).

NK cell flow cytometric analyses, intracellular IFN-γ staining, and cytotoxicity assays

Surface and intracellular IFN-γ protein staining were done as described previously (18). NK cells were phenotypically identified as $NK1.1^+$ TCR-β^- populations. For intracellular staining, >100,000 events were collected using a FACSCalibur (Becton Dickinson, San Jose, CA) with an argon laser operating at 15 mW at 488 nm and a blue diode laser operating at 635 nm. Specificity of intracellular IFN-γ staining was done by competition with unconjugated XMG1.2 Ab. NK cell cytotoxic activity against YAC-1 target cells was determined by standard ^{51}Cr release assays (10, 12).

Plaque assays

To determine MCMV viral titers, serial dilutions of organ homogenates were added to monolayers of NIH 3T3 fibroblast cells. After 1 wk, cells were fixed with 10% buffered Formalin. Plaques were visualized with 0.1% crystal violet and quantitated as log PFU per gram tissue. MCMV standards and negative controls were included in each assay.

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. Mortality studies were statistically analyzed by the nonparametric Mantel-Cox test from Statview version 4.51 (Abacus Concepts, Berkeley, CA).

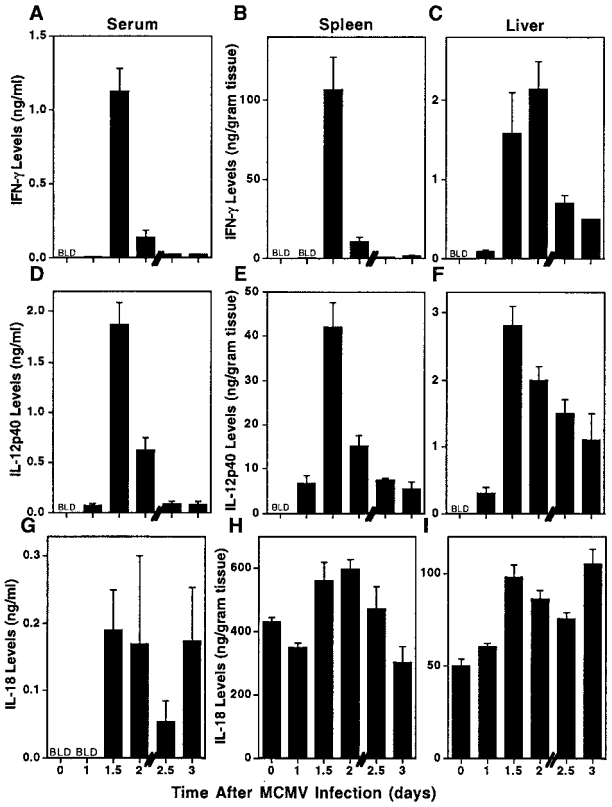

FIGURE 1. Compartmental kinetics of early total cytokine protein expression during MCMV infections. C57BL/6 mice were infected i.p. with 5×10^4 PFU of MCMV. Serum (*A*, *D*, and *G*), spleens (B, E, H), and livers (*C*, *F*, and *I*) were harvested from uninfected (day 0) or infected mice at the indicated times. IFN-γ (*A–C*), IL-12p40 (*D–F*), and total IL-18 (*G–I*) protein levels in serum and organ homogenates were determined by sandwich ELISA. Time points were collected from two separate experiments (demarcated on *x*-axes by indicated hatches) from four mice per group. Similar results were obtained in at least two other experiments. The limits of detection in serum, spleen, and liver were, respectively, 0.02 ng/ml, 0.30 and 0.05 ng/g tissue for IFN-γ and IL-12p40, and 0.04 ng/ml and 1.0 and 0.18 ng/g tissue for IL-18. Values at or below these limits are indicated as below the level of detection (BLD).

Results

Kinetics of compartmental cytokine responses

Peak systemic and splenic IFN-γ responses to early MCMV infection occur 1.5 days after challenge, with hepatic responses extended through day 2 (16, 20), and IL-12 production in serum and spleen closely mirrors the kinetics of IFN-γ induction (11–13, 20). The results presented here were consistent with these observations (Fig. 1, *A–E*). Compartmental characterization of IL-12 expression was extended to include liver, and IL-18 expression was evaluated in all three compartments for the first time. Hepatic levels of IL-12 peaked on day 1.5 but were slower to subside and were sustained through day 2 (Fig. 1*F*). As measured by ELISA, systemic levels of total IL-18 protein were below the limits of detection in uninfected mice (Fig. 1*G*). In contrast, IL-18 was expressed constitutively in both spleens and livers from uninfected mice. Modest induction was observed systemically and in both organs on day 1.5 of infection (Fig. 1, *G–I*). Western blot analyses revealed that IL-18 was primarily expressed as precursor, with moderate induction of mature factor in the spleen on days 1 through 1.5 of infection (data not shown). In contrast, although levels of precursor IL-18 were readily detectable in the liver, mature factor was nearly undetectable. These results show that total IL-18 expression is sustained more broadly than IL-12 and suggest a dichotomy in which mature IL-18 is induced to higher levels in the spleen than the liver after MCMV infection.

Requirements for compartmental IFN-γ responses

To determine the importance of endogenous IL-12 and IL-18 for regulating IFN-γ responses during infection, mice genetically deficient in either the p35 subunit of IL-12 ($IL\text{-}12^-$) or IL-18 ($IL\text{-}18^-$) were examined. Compared with $IL\text{-}12^+$ hosts, $IL\text{-}12^-$ mice exhibited up to 95% reductions in IFN-γ responses in serum, spleen, and liver on day 1.5 (Fig. 2*A*). Furthermore, the extended hepatic IFN-γ kinetics on day 2 were compromised by >85% in the absence of bioactive IL-12. Total and bioactive IL-18 proteins in spleens of $IL\text{-}12^-$ mice were induced to levels comparable to those of $IL\text{-}12^+$ mice (data not shown). Thus, IL-12 is globally required for driving peak IFN-γ production independent of effects on IL-18 protein expression.

Similarly, $IL\text{-}18^-$ mice had >90% reductions in IFN-γ responses in serum and spleen (Fig. 2*B*). However, no significant changes in liver IFN-γ responses were observed, and the sustained expression of IFN-γ on day 2 was also maintained (Fig. 2*B*). Thus,

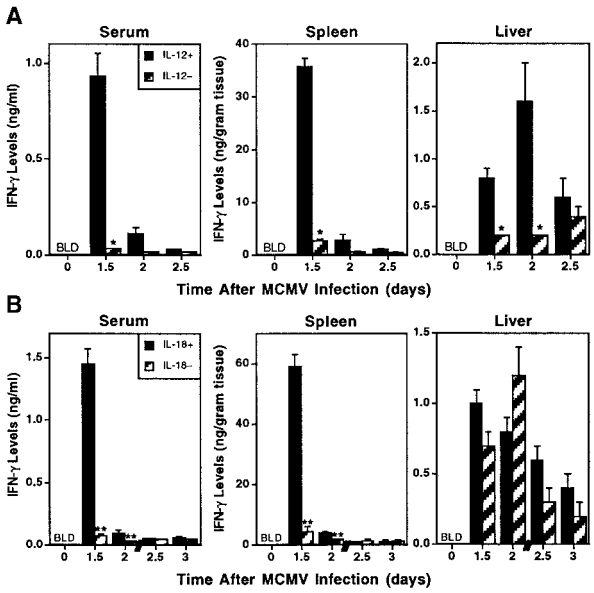

FIGURE 2. IL-12 and IL-18 requirements for induction of IFN-γ. Serum, spleens, and livers were harvested from control (denoted as IL-12$^+$ or IL-18$^+$), IL-12$^-$ (*A*), and IL-18$^-$ (*B*) mice infected with MCMV for the indicated times. Levels of IFN-γ in serum and organ homogenates were determined by sandwich ELISA. Results are presented as means ± SEM of four mice per group. The limits of detection in serum, spleen, and liver were, respectively, 0.02 ng/ml and 0.30 and 0.05 ng/g tissue. Values at or below these limits are indicated as below the level of detection (BLD). Statistically significant differences were observed between IL-12$^+$ and IL-12$^-$ mice (*, $p \leq 0.01$), and between IL-18$^+$ and IL-18$^-$ mice (**, $p \leq 0.02$).

IL-18 was selectively required for systemic and splenic IFN-γ responses, but unlike IL-12, was not required for induction of IFN-γ in the liver. Since IL-18$^+$ and IL-18$^-$ mice expressed equivalent levels of IL-12p70 (data not shown), the compartmentally selective deficits in IFN-γ responses did not appear to be due to secondary effects on IL-12 production. The lack of a role for IL-18 in the hepatic response was contextual. Consistent with other reports (21), in vivo challenge with LPS for 6 h resulted in >50% reductions in IFN-γ responses in all three sites (data not shown). In summary, IL-18 is necessary for optimal IFN-γ induction under some conditions, but is dispensable for innate hepatic IFN-γ responses during MCMV infections.

NK cell responses in IL-18$^-$ and IL-12p35-deficient mice

Because NK phenotype cells have been identified as the source of early IFN-γ during MCMV infections (10, 11, 15), NK cell proportions and numbers were evaluated in IL-18$^-$ and IL-12$^-$ mice. Flow cytometric analyses revealed no significant differences in either proportions or total numbers of NK (NK1.1$^+$TCR-β^-) cells as compared with control mice (data not shown). Because the splenic, but not hepatic, IFN-γ responses were dependent upon IL-18, intracellular staining was done to examine the effects of IL-18 deficiency on NK cell IFN-γ expression. After 1.5 days of infection, an average of 73% of NK cells from IL-18$^+$ mice were expressing IFN-γ as compared with 50% of NK cells from IL-18$^-$ mice ($p < 0.01$). Furthermore, mean fluorescence intensities (MFIs) for splenic NK cell IFN-γ staining from IL-18$^+$ and IL-18$^-$ mice were 361 and 163, respectively ($p < 0.01$). Thus, in the spleen, both the proportions of IFN-γ-producing NK cells and the levels of IFN-γ expression were reduced in the absence of IL-18. In contrast, hepatic NK cells exhibited comparable proportions and MFIs of intracellular IFN-γ expression, reaching 39 and 42%, and 80 and 78%, respectively, in IL-18$^+$ and IL-18$^-$ mice. Thus, the compartmentally selective IL-18 requirement is mediated, at least in part, at the level of NK cell IFN-γ expression.

Splenic NK cell cytotoxic activity is IL-12$^-$ independent under these conditions (11, 12). Using IL-12$^-$ mice, these observations were repeated in the spleen and extended to the liver. Cytolytic activities against YAC-1 target cells sensitive to NK cell-mediated lysis were found to be comparable between IL-12$^+$ and IL-12$^-$ mice, in both spleens and livers, after 2 days of MCMV infection (Fig. 3*A*). Likewise, average cytotoxic activity was induced to similar levels in both spleens and livers of control and IL-18$^-$ mice (Fig. 3*B*). Hence, neither IL-18 nor IL-12 is required for induction of NK cell cytotoxic activity in either site during MCMV infection.

Requirements for IL-18 in antiviral defense

Control of hepatic MCMV replication early during infection is dependent upon NK cells, IL-12, and NK cell-derived IFN-γ (9–12, 16). As the studies presented thus far have demonstrated a selective compartmental role for IL-18 in IFN-γ, but not cytolytic, responses, susceptibility to infection was evaluated in IL-18$^+$ and IL-18$^-$ mice. No differences in early hepatic viral titers were observed. On day 3 of infection, viral titers in liver reached 5.2 and 4.9 log PFU/g tissue in IL-18$^+$ and IL-18$^-$ mice, respectively. Day 3 viral titers were also comparable in the spleen, respectively, reaching 5.1 and 4.9 log PFU/g tissue. In host survival studies challenging mice with 10^5 PFU of MCMV, all immunocompetent and IL-18$^-$ mice survived beyond 21 days at this infecting dose of virus (Fig. 4). In contrast, IL-12$^-$ mice exhibited 100% mortality by day 8 of infection (Fig. 4). Taken together, these results demonstrate that under comparable conditions of MCMV infection, IL-18 is dispensable, but IL-12 is critical, for host survival.

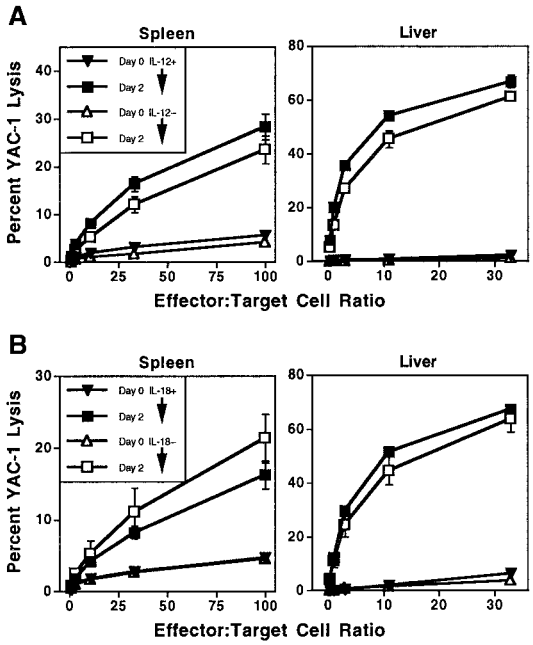

FIGURE 3. Induction of NK cell cytotoxic activity in IL-12$^-$ and IL-18$^-$ mice during MCMV infections. Splenic and hepatic populations were isolated from uninfected or day 2 MCMV-infected control, IL-12$^-$ (*A*), and IL-18$^-$ (*B*) animals. These effector cell populations were assayed for NK cell cytolytic activity against sensitive YAC-1 target cells by ^{51}Cr release assay. All data points are from three to four mice per group.

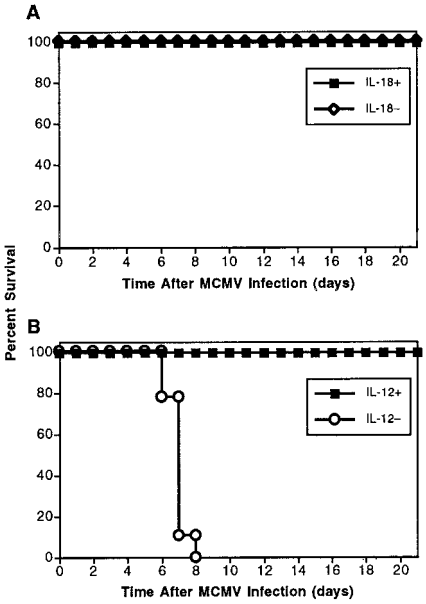

FIGURE 4. Cytokine requirements for host survival during MCMV infections. Control, IL-18$^-$ (*A*), and IL-12$^-$ (*B*) mice were infected i.p. with 10^5 PFU of MCMV on day 0. Animals were then monitored at least once daily for 21 days after infection to assess mortality. Results are from at least six mice per group. Differences in survival between IL-12$^+$ and IL-12$^-$ mice were significant ($p = 0.0001$), and results were repeated in a separate experiment.

Discussion

These studies characterize expression of IFN-γ, IL-12, and IL-18 in the sera, spleens, and livers of MCMV-infected mice. The results demonstrate that although IL-12 is required for driving IFN-γ responses in all three compartments, IL-18 is only necessary for systemic and splenic responses. This compartmentally selective deficit in IFN-γ production is observable as reductions in proportions and total numbers of IFN-γ-expressing NK cells from the spleen with concomitant maintenance of expression in hepatic NK cells. The deficit is also functionally specific, as control and IL-18$^-$ mice have comparable NK cell proportions and total numbers, as well as comparable induction of cytotoxic activity in both spleen and liver following infection. Although IL-18$^-$ mice exhibit a compartmentally selective deficit in IFN-γ responses, these mice do not succumb to infection, whereas IL-12$^-$ mice are highly susceptible. Taken together, our data conclusively define a compartmental role for IL-18 in regulating in vivo NK cell IFN-γ responses, but suggest that the role may not be critical for defense against this viral infection.

The work extends our understanding of the importance of local immune responses. Our group has recently demonstrated that in macrophage-inflammatory protein 1α-deficient mice, NK cells fail to traffick to the liver after MCMV infection (15, 16). As a result, hepatic IFN-γ responses are severely reduced. Macrophage-inflammatory protein 1α-deficient mice succumb to the infection despite the preservation of systemic and splenic IFN-γ expression (16). Conversely in the studies presented here, IL-18$^-$ mice survive despite compromised responses systemically and in the spleen (Fig. 4). Since IL-18$^-$ mice have intact responses in the liver, the studies collectively suggest that the liver may be the critical battleground for host defense against infections by hepatotropic MCMV. Although splenic IFN-γ expression, but not cytotoxic activity, was greatly diminished in IL-18$^-$ mice, viral titers were not elevated compared with control mice. This is consistent with a report in which control of viral replication in the spleen is proposed to be dependent upon perforin but not IFN-γ (22). Our results, however, also suggest that preservation of early antiviral IFN-γ responses in the liver is necessary for host survival, whereas splenic cytotoxicity is insufficient because an IL-12 deficiency results in death despite preserved cytotoxic function in both spleen and liver (Fig. 3).

The lack of requirement for IL-18 in the liver during MCMV infection is intriguing, particularly because this cytokine was originally purified from the livers of mice challenged with bacterial products (23) and has a role in LPS-induced IFN-γ responses in all three compartments. The dispensability of IL-18 may be the result of other, as yet unidentified, costimulatory interactions involved in regulating hepatic IFN-γ responses. As hepatic NK cells colocalize at sites of IFN-γ and viral Ag expression (15), NK cells may receive additional cell-matrix and/or cell-cell contact signals to promote IL-12-driven IFN-γ production locally. These signals could be delivered during the initial migration into hepatic sinusoids where integrins may supply a necessary costimulus (24) and/or within inflammatory foci through contact with cells expressing particular costimulatory molecules (25–27).

Although both IL-12 and IL-18 have been shown to be capable of augmenting NK cell cytotoxic activity in other settings (17, 23), during MCMV infections this effector function is induced independently of either cytokine (Fig. 3), but does require IFN-αβ, at least in the spleen (11, 12). Thus, there is a dichotomy between what panel of functions a specific cytokine can potentially mediate and which subsets of those functions are actually accessed in vivo during viral infections. The results also show that dichotomy of cytokine function is further regulated at the compartmental level, such that local cytokine requirements for induction of IFN-γ can vary from site to site. This schema is likely in place to afford the host a measure of fine tuning, such that potentially beneficial factors are locally regulated to allow access to antiviral pathways in some compartments while simultaneously limiting possible deleterious effects in others.

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