A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense

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Macrophage inflammatory protein 1α (MIP-1α) promotes natural killer (NK) cell inflammation in livers during murine cytomegalovirus (MCMV) infections, and NK cell–produced interferon γ (IFN-γ) contributes to defense against MCMV infections. A specific role for local NK cell IFN-γ production, however, has not been established. The importance of MIP-1α and NK cell–produced IFN-γ in shaping endogenous immune responses and defense in different compartments was examined. MIP-1α deficiency profoundly decreased resistance to MCMV and was associated with dramatically reduced NK cell accumulation and IFN-γ production in liver. MIP-1α–independent IFN-γ responses were observed in serum and spleen, and infection-induced elevations in blood NK cell populations occurred in absence of the factor, but peak liver expression of another chemokine, the monokine induced by IFN-γ (Mig), depended upon presence of MIP-1α, NK cells, and IFN-γ. The Mig response was also important for viral resistance. Thus, serum cytokine responses are insufficient; MIP-1α is critical for NK cell migration and IFN-γ delivery to mediate protection; and Mig induction in tissues is a downstream protective response resulting from the process. These results define a critical chemokine-to-cytokine-to-chemokine cascade required for defense during a viral infection establishing itself in tissues.

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Introduction

Many different host compartments must be protected against spreading of initial infections, and induced serum cytokines may promote resistance at sites distal to such infections. The relative contributions of systemically released as compared with locally produced cytokines, however, have not been established. Certain chemokines have been shown to be required for tissue inflammation. Studies of responses in the presence or absence of these may help distinguish effects mediated by cytokines released into serum as compared with those locally produced by cells migrating into tissues. In particular, the β-chemokine macrophage inflammatory protein 1α (MIP-1α) is important for inflammation induced by certain viral infections (1, 2), elicits NK cell chemotaxis in culture (3, 4), and has in vivo function in promoting NK cell migration into tissues (2, 5). During murine cytomegalovirus (MCMV) infections of mice, NK cells, characterized as non–T cells (5), migrate to sites of liver infection through an MIP-1α–dependent pathway (2). They also produce high levels of IFN-γ protein, detectable in situ in infected livers (2) and peaking in serum at 36–44 hours but subsiding by 48 hours after viral challenge (6–8). NK cell–derived IFN-γ is important for protection against MCMV (6, 7, 9), but the requirement for and effects of local NK cell IFN-γ production have not been defined. A variety of genes can be induced by IFN-γ, but many of these also can be induced by other cytokines (10–15). Among those studied to date, the α-chemokine Mig, a monokine induced by IFN-γ, appears to have a uniquely stringent requirement for IFN-γ activation (16–19). Thus, induction of Mig expression in tissues has the potential to be used to evaluate exposure to NK cell–produced IFN-γ during innate responses to MCMV. As Mig can promote migration of activated T lymphocytes (20–22), its induction may be a previously unappreciated pathway by which IFN-γ promotes resistance to infection.

The experiments presented here define roles for MIP-1α and the MIP-1α–dependent NK cell liver inflammation in the regulation of endogenous responses and protection during MCMV infections. The studies demonstrate that MCMV replication and susceptibility to virus-induced death are dramatically elevated in MIP-1α–deficient mice when compared with wild-type mice. The changes in resistance occur despite enhanced numbers of NK cells in blood, and normal NK cell IFN-γ responses in serum and spleen. However, these resistance changes are associated with profound reductions in sustained NK cell IFN-γ production in liver. The local NK cell IFN-γ expression is shown to be required for peak induction of Mig in livers, and Mig is shown to be important for antiviral defense. The results demonstrate that systemic responses are not enough, and that specific chemokines are critical contributors to antiviral defense. They provide a picture of immune responses to viral infections with systemic NK cell and IFN-γ responses in place early, but they also demon-

strate an essential need for tissue localization of these cells and the cytokines they produce in defense.

Methods

Mice. Specific pathogen-free male C57BL/6 (C57BL/6NtacfBR) and C57BL/6–SCID (C57BL/6J-*scid*/SzJ) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Homozygous MIP-1α mutants, C57BL/6-MIP-1α$^{-/-}$ (1), originally provided by M. Caligiuri (Ohio State University, Columbus, Ohio, USA) and then purchased from The Jackson Laboratory, were bred at Brown University. Breeding pairs of C57BL/6-IFN-γ–deficient (C57BL/6-IFN-γ$^{-/-}$) mice (23) from The Jackson Laboratory, and of mice deficient in both MIP-1α and IFN-γ (C57BL/6-MIP-1α$^{-/-}$, IFN-γ$^{-/-}$) generously provided by H. Virgin (Washington University, St. Louis, Missouri, USA), also were used to establish colonies. Male and female mutant mice were examined. All mice were 5 to 6 weeks old. Procedures were conducted in accordance with institutional guidelines for animal care and use.

Virus stocks, infections and titration. Stocks of Smith strain MCMV salivary gland extracts were prepared (9). Infections were initiated intraperitoneally on day 0 (uninfected) with 5×10^4 plaque-forming units (PFU) of MCMV. In survival experiments, mice were infected with either 10^4 (low dose) or 10^5 (high dose) PFU. Mouse conditions were assessed twice daily. Titers in organs were measured as PFU per gram of tissue using viral plaque assays (2, 8).

In vivo antibody treatments. NK cells were depleted with rabbit polyclonal antibody against asialo ganglio-*N*-tetraosylceramide (AGM1; Wako Chemicals, Richmond, Virginia, USA), or the mouse anti-NK1.1 IgG2a mAb, PK136, as described (9, 24). The procedures eliminated more than 95% of NK1.1$^+$CD3$^-$ cells in blood and spleen and more than 90% of NK1.1$^+$ and β chain of the T-cell receptor for antigen-(TCR-β$^-$) cells in liver. Rabbit or mouse control antibodies were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). To stimulate IFN-γ production in uninfected livers, 20 μg of anti-CD3 hamster IgG mAb 2C11 (PharMingen, San Diego, California, USA) was administered intravenously. Control treatments were injections with isotype-matched hamster antibody (PharMingen). Livers were harvested 3 hours after treatment for RNA preparation. For Mig neutralization, mice were given rabbit antiserum against Mig (22) or control normal serum (Sigma Chemical Co.) 12 hours before and on day 2 or 3 after infection.

Preparation of leukocytes, tissue homogenates, and serum. Mice were anesthetized and bled before sacrifice for organ harvest, and leukocytes were separated from macerated whole spleen and liver cell suspensions, as described (24–26). Blood cells were isolated over Histopaque-1083 (Sigma Chemical Co.) (26). Viable cell yields were enumerated by trypan blue exclusion. For preparation of tissue homogenates, weighed organs were placed into DMEM (Life Technologies Inc., Rockville, Maryland, USA) and were ground using a dounce homogenizer. Samples were centrifuged and supernatant fluids collected. Serum was prepared as described (26).

Flow cytometric analyses. Proportions and numbers of NK cells in the different populations were evaluated by flow cytometric analysis after 2-color staining for the presence of the NK cell surface marker, NK1.1, and the absence of T-cell surface markers, CD3ε or TCR-β. For spleen and blood samples, R-phycoerythrin–conjugated anti-NK1.1 mAb clone PK136 and CyChrome-conjugated anti-CD3ε mAb clone 145-2C11 (PharMingen) were used. To better distinguish classical NK cells from NK T-cell populations in liver (27), R-phycoerythrin–conjugated anti-NK1.1 mAb clone PK136 and CyChrome-conjugated anti–TCR-β chain mAb clone H57-597 (PharMingen) were used. Appropriate blocking steps were included. Control antibodies not recognizing specific determinants (PharMingen) were used to correct for background fluorescence and they set analysis gates with more than 97% of control-stained cells in negative populations. At least 20,000 events were acquired per sample using a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) with a constant argon laser output of 15 mW at a 488-nm wavelength. Data were acquired and analyzed using Cellquest software (Becton Dickinson Immunocytometry Systems).

Cytokine analyses. Serum samples or spleen and liver homogenates were tested for IFN-γ by standard sandwich ELISA (6–9). Limits of detection were 20 pg/mL for serum samples and 10 pg/mL for tissue homogenates. Colorimetric changes after addition of substrate were detected as described (26).

Preparation and analyses of RNA. Total liver RNA was prepared using UltraSpec (Biotecx Laboratory Inc., Friendswood, Texas, USA) and analyzed by Northern blot hybridization or by relative quantitative RT-PCR. Plasmid DNA, containing specific cDNA sequences for murine Mig (17, 24) or GAPDH was [^{32}P]dCTP labeled by random priming (Ambion Inc., Austin, Texas, USA) for Northern blot analysis, which was carried out as described (24). For relative quantitative RT-PCR, 1-μg total RNA samples were reverse transcribed into cDNA (2). For each sample, 5 μL cDNA was a template for PCR amplification using IFN-γ– or Mig-specific primers. As internal controls for sample preparation, gel loading, and random variations in RT-PCR, 18-S rRNA primers and 18-S rRNA Competimers (Ambion Inc.), used to modify 18-S cDNA amplification efficiency, were included in each PCR reaction with target gene–specific primers. The linear range of amplification and optimal 18-S primer/Competimer ratio was determined for each target gene following the manufacturer's recommendations (Ambion Inc.). Amplifications were carried out in a programmable thermal cycler (PTC-100; MJ Research, Waltham, Massachusetts, USA) using cycling parameters as previously described (28). Primer oligonucleotides for IFN-γ were obtained from CLONTECH Laboratories Inc., Palo Alto, California, USA. Mig primers were selected from published sequence data (29) and were synthesized by Operon (Alameda, California, USA). Products were separated on 1.8% agarose gels and examined by ethidium bromide staining. Specificity was verified by Southern blot hybridization using internal
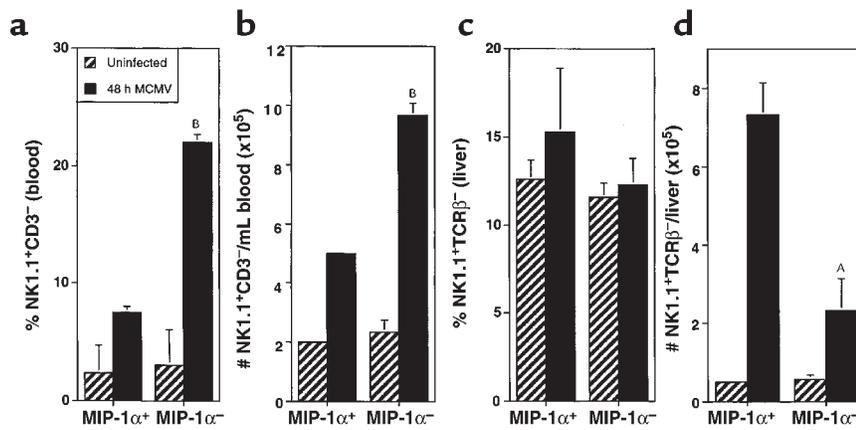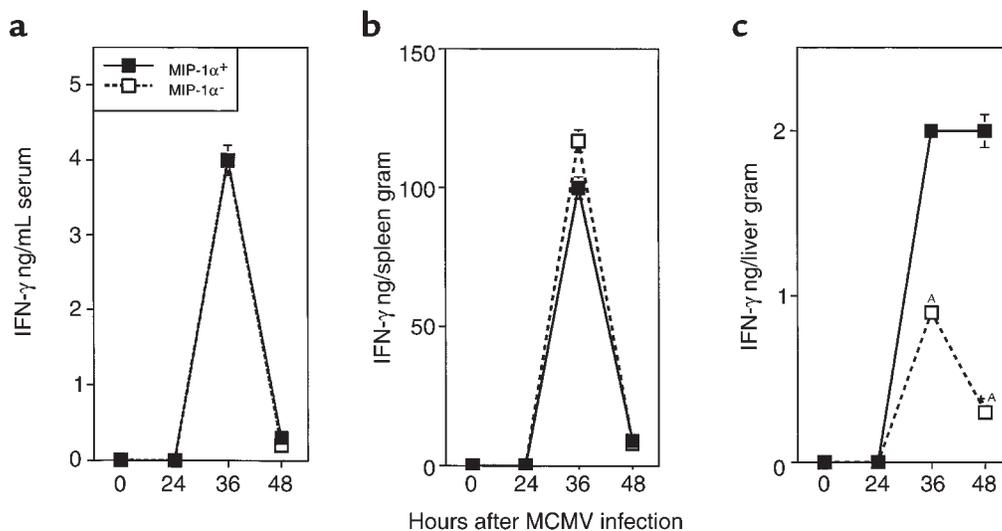
Figure 1
Compartmental changes in NK cell populations. Blood (**a** and **b**) and liver (**c** and **d**) leukocytes were prepared from C57BL/6-MIP-1α[+]/[+] (MIP-1α[+]) or C57BL/6-MIP-1α^{−/−} (MIP-1α[−]) mice either uninfected or infected with MCMV for 48 hours. Leukocytes were analyzed by flow cytometry as described in Methods. Both the percentage (**a** and **c**) and absolute number (**b** and **d**) of NK1.1^{+}CD3^{−} cells per milliliter of blood or NK1.1^{+}TCR-β^{−} cells per entire liver are shown. Data are means ± SE (*n* = 3). Compartmental changes represent results from 1 of 3 repetitive experiments. Differences between control MIP-1α[+] and MIP-1α[−] are significant, [A]$P < 0.01$, [B]$P < 0.001$.

oligonucleotide probes. For relative quantitation of generated products, stained gels were scanned with a Fotodyne Image Analysis System (Fotodyne, Hartland, Wisconsin, USA) and analyzed using Collage 3 software (Fotodyne). For each target gene, a relative corrected value was obtained by: (Σ pixel intensity of gene specific amplicon – Σ pixel intensity background) ÷ (Σ pixel intensity for the 18-S amplicon – Σ pixel intensity background). Uninfected control samples were given an arbitrary relative corrected value of 0.01, designating the lowest limit of detection. Relative intensity values represent the corrected value for each sample, of specific as compared with 18-S amplification (×100).

Immunohistochemistry. For immunohistochemical analyses, liver sections were prepared, blocked, exposed to antibodies, and detected enzymatically as described (2). Mig protein was identified with goat polyclonal IgG antibody raised against recombinant mouse Mig (R&D Systems Inc., Minneapolis, Minnesota, USA). Sections were incubated with antibodies overnight at 4°C, washed in PBS plus 0.1% Tween-20 (Sigma Chemical Co.), and then incubated for 30 minutes with biotin-SP–conjugated F(ab′)$_2$ fragment against goat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA). Equivalent concentrations of goat IgG were used as control antibodies (Sigma Chemical

Figure 2
IFN-γ production in serum, spleen, and liver. Serum samples (**a**) and spleen (**b**) or liver (**c**) homogenates were prepared from uninfected or MCMV-infected (at 24, 36, and 48 hours after infection) MIP-1α[+] or MIP-1α[−] mice. IFN-γ protein was measured by ELISA. Each spleen homogenate data point consists of 6 animals tested individually. For liver homogenates, each uninfected and 24-hour data point consists of 6 animals, and each 36- and 48-hours data point consists of 9 animals, all tested individually. The means ± SE are shown. All samples from uninfected or 24-hour MCMV-infected mice were below the level of detection. Differences between MIP-1α[+] and MIP-1α[−] are significant, [A]$P < 0.0005$.
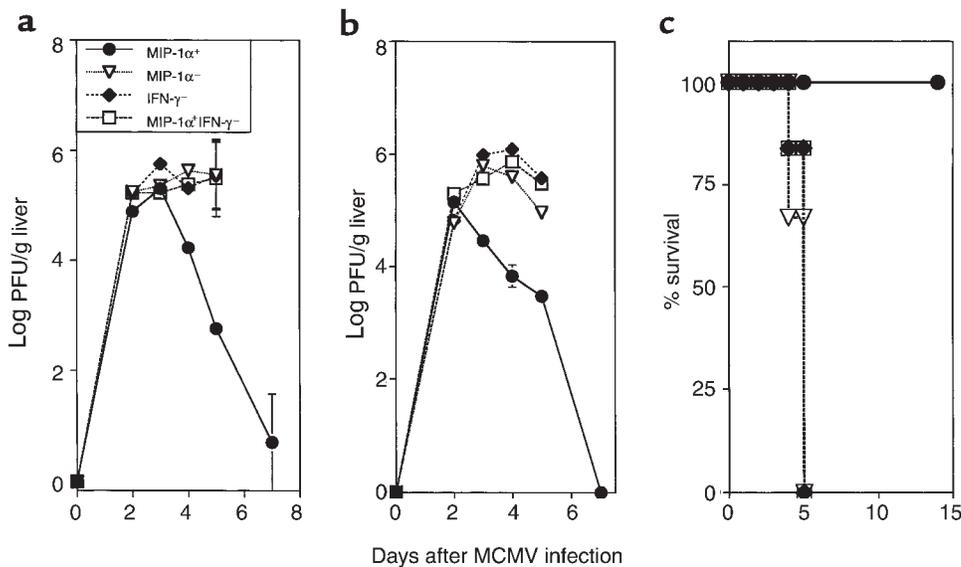
Figure 3
Effects of MIP-1α and IFN-γ on resistance to MCMV. (**a** and **b**) MIP-1α+, MIP-1α−, IFN-γ−/− (IFN-γ−), and MIP-1α− IFN-γ− mice were uninfected or infected with 5×10^4 PFU MCMV at times indicated. Spleen (**a**) and livers (**b**) were harvested and homogenized for viral titer determinations as described in Methods. Limit of detection for the assay was 2 log PFU per gram of tissue. Means ± SE ($n = 3$) are shown. (**c**) Mice were uninfected or infected with 10^5 PFU (high dose) MCMV and monitored twice daily for survival. Results represent 1 of at least 2 experiments. Data shown represent the mean ± SE ($n = 6$).

Co.). Staining specificity was documented by lack of reaction with control antibodies and in the absence of primary antibody. Sections were counterstained with methyl green (Vector Laboratories, Burlingame, California, USA). Mig-positive cell populations were quantitated by counting dark blue nucleated cells in a total of 20 randomly selected 1×1 mm^2 areas of representative liver sections at a magnification of ×400. Photographs shown were taken as described (2).

Statistical analyses. Statistical significance of experimental results were analyzed by two-tailed Student's *t*-test where indicated.

Results

MIP-1α–dependent changes in compartmental proportions and numbers of NK cells. To evaluate and quantitate NK cells in particular compartments, spleen, blood, and liver samples were isolated from uninfected or MCMV-infected mice at a peak time of inflammation (i.e., 48 hours after infection). Normal C57BL/6-MIP-1α+/+ (MIP-1α+) and C57BL/6-MIP-1α−/− (MIP-1α−) mice were examined. Leukocytes were prepared, and proportions and numbers of NK cells (i.e., NK1.1+CD3− cells in spleen and blood and NK1.1+TCR-β− cells in liver) were determined using 2-color flow cytometric and cell yield analyses. NK cell frequencies and absolute numbers were equivalent in spleen (data not shown), blood (Figure 1, a and b), and liver (Figure 1, c and d) leukocytes isolated from uninfected MIP-1α+ and MIP-1α− mice. At 48 hours after MCMV infection, they declined in a similar manner in spleens of MIP-1α+ and MIP-1α− mice (data not shown). In contrast, at 48 hours, MCMV-infected MIP-1α+ and MIP-1α− mice had 3- and 7-fold

increases in frequencies of blood NK cells (Figure 1a), respectively. Because total blood leukocyte cell yields for MIP-1α+ and MIP-1α− mice were $6.5 \times 10^6 \pm 5 \times 10^5$/mL and $5 \times 10^6 \pm 7 \times 10^5$/mL, respectively, the MIP-1α− mice had statistically significant 2-fold higher numbers of NK cells in blood (Figure 1b). Thus, infection in the presence or absence of MIP-1α induces an increase in circulating NK cells, but MIP-1α deficiency results in further accumulation of the population in blood.

Results for the liver were different from those for either spleen or blood. As total liver leukocyte numbers increased to $5 \times 10^6 \pm 10^6$ from the uninfected values of $4 \times 10^5 \pm 0$, MIP-1α+ mice had 14-fold increases in NK cell numbers at 48 hours after MCMV infection (Figure 1d). In contrast, infection-induced changes in NK cell proportions were undetectable (Figure 1c), and liver leukocyte yields only increased from uninfected values of $5 \times 10^5 \pm 4 \times 10^4$ to $2 \times 10^6 \pm 7 \times 10^5$ at 48 hours after MCMV infection of MIP-1α− mice. As a consequence, there was less than a 4-fold increase in liver NK cell numbers after infection of MIP-1α− mice (Figure 1d). Taken together, the data demonstrate a prominent role for MIP-1α in NK cell accumulation in liver and blood in the absence of MIP-1α during infection.

*Compartmentalization of IFN-γ responses.* During MCMV infections, IFN-γ production is induced in NK cells, and serum and spleen leukocyte production responses peak early but subside rapidly (6, 9). To extend characterization to liver and to examine MIP-1α effects on production, ELISA assays measuring IFN-γ protein levels were performed on serum samples and spleen and liver homogenates; the homogenates were prepared from MIP-1α+ and MIP-1α− mice uninfected or infected with

MCMV for 24, 36, and 48 hours. Peak infection-induced serum values in both MIP-1α$^+$ and MIP-1α$^-$ mice were detected at 36 hours after infection, reaching 4 ± 0.2 ng/mL in both groups (Figure 2a). IFN-γ production in spleen homogenates was also induced at 36 hours after MCMV infection, with values of 142 ± 10 ng/mL from MIP-1α$^+$ and 180 ± 15 ng/mL from MIP-1α$^-$ mice (Figure 2b). Responses with samples from both compartments in both groups subsided by 48 hours after infection (Figure 2, a and b). IFN-γ levels in liver homogenates were strikingly different in 2 ways. First, the liver response in MIP-1α$^+$ mice was sustained at 48 hours after infection, and, second, IFN-γ produced in infected MIP-1α$^-$ livers was profoundly reduced as compared with infected MIP-1α$^+$ livers (Figure 2c). At 36 hours after infection, liver IFN-γ levels were 2 ± 0.1 ng/mL in MIP-1α$^+$, but were only 0.9 ± 0.04 ng/mL in MIP-1α$^-$ mice (Figure 2c). As compared with MIP-1α$^+$, MIP-1α$^-$ mice had more than 2- to 5-fold reductions in liver IFN-γ production over time after MCMV infection. Hence, the virus-induced NK cell IFN-γ responses in serum and spleen are MIP-1α independent, but MIP-1α is absolutely required to enhance and sustain infection-induced IFN-γ production in liver.

MIP-1α deficiency and resistance to infection. Studies were carried out to characterize contributions of MIP-1α and the accompanying systemic (without localized IFN-γ) responses for establishment of antiviral states. Spleen and liver samples were isolated from MIP-1α$^+$ and MIP-1α$^-$ mice uninfected or infected with 5 × 10^4 PFU MCMV for up to 7 days. Plaque assays demonstrated that both MIP-1α$^+$ and MIP-1α$^-$ mice had similar viral titers in spleen and liver on day 2 after infection (Figure 3, a and b). After day 4, viral replication in both spleen and liver declined dramatically in MIP-1α$^+$, but remained elevated in MIP-1α$^-$, mice (Figure 3, a and b). Moreover, all of the MIP-1α$^-$,

but none of the MIP-1α$^+$ mice succumbed to the infection by day 7. At a higher dose (10^5 PFU MCMV), all of the MIP-1α$^+$ mice survived, and all of the MIP-1α$^-$ mice died by day 5 (Figure 3c). Thus, absence of MIP-1α profoundly increased susceptibility to MCMV. As the MIP-1α$^-$ mice had systemic and spleen IFN-γ responses, sensitivities resulting from the total lack of IFN-γ (i.e., IFN-γ$^-$ mice) and a double deficiency resulting from the lack of both IFN-γ and MIP-1α (i.e. MIP-1α$^-$IFN-γ$^-$ mice) were compared with that resulting from the single MIP-1α deficiency. Viral burdens in the tissue compartments were similar throughout MCMV infection in all 3 groups of deficient mice (Figure 3, a and b). Moreover, they all succumbed to lethal effects of infection with 10^5 PFU MCMV on day 5 (Figure 3c). All mice survived infection with low dose (10^4 PFU) MCMV (data not shown). These results demonstrate that MIP-1α$^-$ is critical and as important as IFN-γ for survival under these conditions. Furthermore, the results suggest that local IFN-γ production is essential, but systemic IFN-γ responses are insufficient for protection against infection.

Induction of Mig expression in liver. Mig expression was evaluated to assess downstream effects of MIP-1α, NK cells, and local versus systemic IFN-γ. Total RNA was prepared from livers of uninfected or MCMV-infected MIP-1α$^+$ and MIP-1α$^-$ mice for 36, 48, or 72 hours. Liver RNA from IFN-γ$^-$ mice infected for 48 hours was used to document the IFN-γ requirement. To evaluate NK cell contributions to liver IFN-γ and Mig expression, RNA also was prepared from uninfected or infected immunocompetent C57BL/6 and T- and B-cell–deficient C57BL/6-SCID mice; these mice had been control antibody treated or NK cell depleted by treatments with either anti-NK1.1 or anti-AGM1 antibodies. Northern blot analyses revealed a more than 200-fold induction
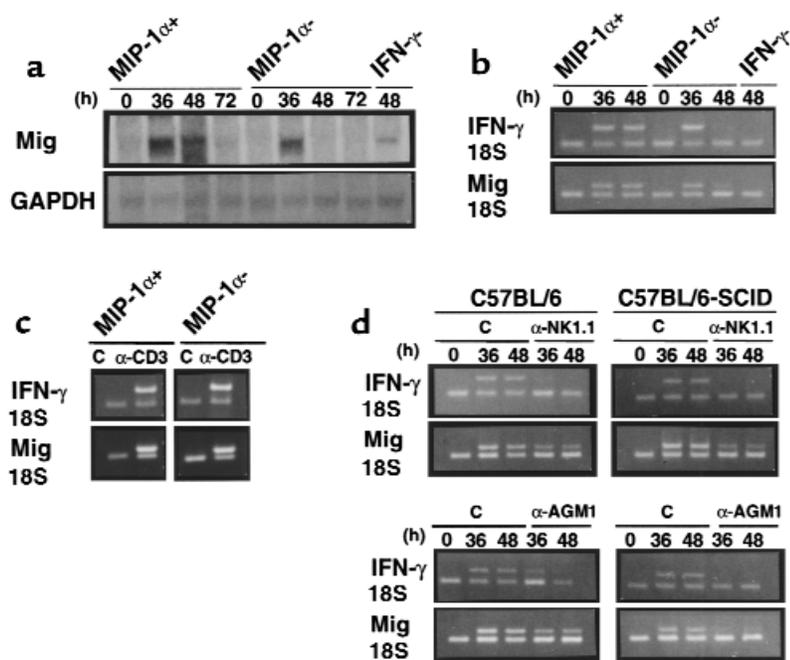
Figure 4
Requirements for IFN-γ and Mig gene expression. (**a** and **b**) Liver RNA was isolated from MIP-1α$^+$, MIP-1α-, or IFN-γ$^-$ mice that were uninfected or infected with MCMV. (**a**) Total RNA was analyzed by Northern blot hybridization. Results represent 1 of 3 experiments. (**b**) Relative quantitative RT-PCR was carried out with Competimers for 18-S rRNA as internal controls. (**c**) Total liver RNA was prepared from MIP-1α$^+$ and MIP-1α$^-$ mice administered control IgG (denoted as C) or anti-CD3 (α-CD3) at 3 hours before harvest. RNA was prepared and analyzed by relative quantitative RT-PCR as above. (**d**) Liver RNA was isolated from mice that were uninfected or infected with MCMV, with or without NK cell depletions. Mice were C57BL/6 or C57BL/6-SCID mice treated with control IgG (denoted as C), anti-NK1.1, or anti-AGM1 24 hours before infection. RNA was analyzed by relative quantitative RT-PCR as above. **b**–**d** show ethidium bromide–stained gel of amplified products, representing 1 of 3 experiments (see Table 1 for quantitation of results and Methods for details of procedures).

Table 1
Requirements for Mig mRNA expression in liver

Mouse strain	Treatment	Hours after MCMV infection	Relative intensity (×100)	
			IFN-γ	Mig
C57BL/6	None	0	<1	<1
		36	90 ± 7	80 ± 7
		48	100 ± 0	87 ± 8
C57BL/6-MIP-1α⁻	None	0	<1	<1
		36	93 ± 8	70 ± 0
		48	3 ± 2[B]	1 ± 0[B]
C57BL/6-IFN-γ⁻	None	48	<1	<1
C57BL/6	Control-IgG	0	<1	<1
		36	90 ± 7	90 ± 12
		48	97 ± 4	70 ± 21
	Anti-NK1.1	36	4 ± 4[B]	14 ± 2[B]
		48	7 ± 4[B]	12 ± 5[B]
	Control-IgG[A]	0	<1	<1
		36	80 ± 14	85 ± 21
		48	90 ± 14	70 ± 14
	Anti-AGM1[A]	36	15 ± 7[B]	15 ± 7[B]
		48	10 ± 0[C]	15 ± 7[C]
C57BL/6-SCID	Control-IgG	0	<1	<1
		36	83 ± 11	67 ± 11
		48	83 ± 11	57 ± 8
	Anti-NK1.1	36	2 ± 1[B]	10 ± 7[B]
		48	1 ± 0[B]	7 ± 7[B]
	Control-IgG[A]	0	<1	<1
		36	60 ± 14	45 ± 7
		48	55 ± 7	50 ± 14
	Anti-AGM1[A]	36	6 ± 6[C]	1 ± 0[C]
		48	1 ± 0[C]	1 ± 0[C]

Liver RNA was extracted from antibody-treated and/or MCMV-infected mice as indicated. RT-PCR was performed using IFN-γ– or Mig-specific primer sequences and 18-S ribosomal sequences as internal controls to generate cDNA products. Corrected relative intensity values for IFN-γ and Mig mRNA expression were determined as described in Methods; uninfected controls were given an arbitrary corrected value of the lowest limit of detection (0.01). Values represent corrected relative intensity values for each sample ×100. Data shown represent the means ± SE ($n = 3$, except [A] where $n = 2$). Number compared with control is significant [B]$P < 0.005$, [C]$P < 0.05$.

of Mig relative to GAPDH mRNA expression at 36 hours after infection in both MIP-1α⁺ and MIP-1α⁻ mice (Figure 4a). In contrast, at 48 hours, Mig gene expression was sustained in MIP-1α⁺, but was below the level of detection in MIP-1α⁻ mice (Figure 4a).

To increase detection sensitivity and correlate IFN-γ with Mig expression, relative RT-PCR was used for quantitation. At 36 hours after infection, IFN-γ and Mig mRNA levels were induced 70- to 93-fold relative to uninfected levels in both MIP-1α⁺ and MIP-1α⁻ livers (Figure 4b; Table 1). At 48 hours, however, IFN-γ and Mig message levels dramatically declined in MIP-1α⁻ livers to less than 3-fold induction levels; however they continued to be elevated by 87- to 100-fold in MIP-1α⁺ livers (Figure 4b; Table 1). MIP-1α was not absolutely required for elevated IFN-γ or Mig mRNA, because these were detected at 36 hours after MCMV infection (Figure 4b) and were induced equally after in vivo treatments with antibody to CD3 (Figure 4c) in both MIP-1α⁺ and MIP-1α⁻ mice.

Increased induction intensities of IFN-γ and Mig expression were observed in control-treated C57BL/6 and C57BL/6 SCID mice at both 36 and 48 hours after infection (Figure 4d; Table 1). After NK cell depletions, however, there were greater than 5-fold reductions in infection-induced IFN-γ and Mig mRNA increases in both types of mice (Figure 4d; Table 1). Thus, MIP-1α is needed for sustained (not short-term) induction of IFN-γ and Mig mRNA in MCMV-infected livers. NK cells also are required for peak levels of expression of these genes in this compartment; and classical NK cells are major contributors to the responses.

Mig protein expression was examined by immunohistochemistry. Sections were prepared from livers of the different mice. The results showed comparable low-level induction of Mig protein predominantly in both MIP-1α⁺ and MIP-1α⁻ mice at 36 hours after MCMV infection (Figure 5, a and b; Table 2). At 48 hours after MCMV infection, however, Mig protein expression had increased in MIP-1α⁺ mice (Figure 5, c and e; Table 2), but was absent in MIP-1α⁻ (Figure 5, d and f; Table 2) mice. Mig protein was predominantly observed in sinusoidal areas (Figure 5e). It also was abundant in liver sections from SCID mice, with production levels similar to those observed in immunocompetent MIP-1α⁺ mice (Figure

5h; Table 2), but it was not detected in uninfected (Figure 5g) or in MCMV-infected IFN-γ⁻ mice (Table 2). Dramatic reductions in Mig protein were evident in immunocompetent and SCID mice treated with anti-NK1.1 or anti-AGM1 (Table 2). These results demonstrate the requirements for MIP-1α, NK cells, and IFN-γ in inducing sustained production of Mig protein in liver.

Role for Mig in antiviral defense. To assess the Mig contribution to antiviral defense, spleen and liver samples were isolated from MCMV-infected C57BL/6 mice that were untreated, treated with control antiserum, or treated with antiserum-neutralizing Mig. Mice were infected with 5×10^4 PFU MCMV for 4 or 5 days, and viral burdens were determined. As compared with untreated or control serum-treated and infected mice, viral titers in both compartments were increased by 1 log on day 4 and 2 logs on day 5 after infection as a result of Mig neutralization (Table 3). Moreover, at a high-dose MCMV infection (10^5 PFU), all of the mice having Mig function neutralized, but none of the untreated or control serum-treated mice, succumbed to the lethal effects and died at day 5 after infection (Table 3). These results demonstrate that Mig is a significant contributor to protection against MCMV infection.

Discussion

These studies define roles for MIP-1α and Mig in protection against infection and evaluate relative effects of serum and tissue IFN-γ expression. They demonstrate that MIP-1α plays a significant role in defense and survival during a viral infection targeting tissue sites. The MIP-1α–dependent changes in susceptibility occur despite enhanced numbers of NK cells in blood and normal NK cell IFN-γ responses in spleen and serum, but with greatly reduced NK cell accumulation and IFN-γ production in liver. The local NK cell IFN-γ is required for peak Mig expression in this organ, and Mig is also important for defense. Taken together, the results suggest an essential defense pathway with MIP-1α required for NK cell trafficking and sustained IFN-γ production at tissue sites of viral infection, leading to local downstream induction of IFN-γ–dependent events including Mig expression. They provide a picture of immune responses to viral infections with the peak systemic NK cell IFN-γ in place early, but a critical need for tissue localization of these cells and the cytokines they produce for defense.

The observations clearly demonstrate that systemic immune responses are not enough. The presence of serum, in the absence of local liver NK cells and IFN-γ production, in MIP-1α–deficient mice is insufficient to protect against infection. There are several possible reasons for this. First, accumulation of NK cells producing the cytokine may result in the delivery of higher levels of IFN-γ. Second, cells trafficking into the liver may deliver IFN-γ to hepatic populations not able to respond to serum cytokine because they reside deep within tissues. These are both likely to occur. Moreover, there may be reciprocal interactions between infiltrating NK cells and the liver environment to promote extended NK cell IFN-γ production and enhanced IFN-γ–mediated antiviral effects. In contrast to the tightly regulated systemic NK cell responses (8; Figure 2), liver IFN-γ production proceeds for longer periods (Figure 2). Given the poten-
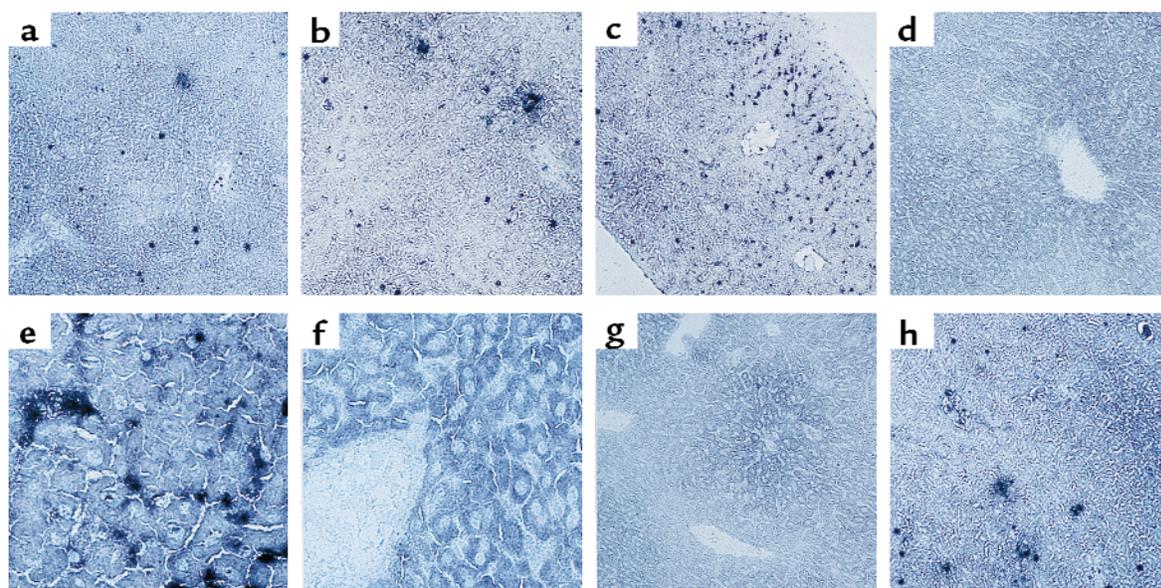
Figure 5
Induction of Mig protein expression. Livers were harvested from uninfected or 36 and 48-hour MCMV-infected MIP-1α⁺ and MIP-1α⁻ mice, or 48-hour MCMV-infected C57BL/6-SCID mice. Tissue sections were prepared and immunohistochemically stained as described in Methods. The Mig protein is identified by dark blue precipitates. Tissues are counterstained with methyl green. Results shown are from (**a**) MIP-1α⁺ mice after 36 hours of MCMV infection, (**b**) MIP-1α⁻ mice after 36 hours of MCMV infection, (**c** and **e**) MIP-1α⁺ mice after 48 hours of MCMV infection, (**d** and **f**) MIP-1α⁻ mice after 48 hours of MCMV infection, (**g**) uninfected MIP-1α⁺, and (**h**) C57BL/6-SCID mice after 48 hours of MCMV infection. Photographs were taken at magnifications of ×31.25 (**a–d**, **g**, **h**) or ×125 (**e**, **f**).

Table 2
Requirements for Mig protein production in liver

Mouse strain	Treatment	Hours after MCMV infection	No. of Mig⁺ cells per liver area
C57BL/6		0	0 ± 0
		36	134 ± 15
		48	272 ± 51
	Anti-NK1.1	48	0 ± 0[A]
	Anti-AGM1	48	0 ± 0[A]
C57BL/6-MIP-1α⁻		0	0 ± 0
		36	95 ± 33
		48	0 ± 0[A]
C57BL/6-IFN-γ⁻		48	0 ± 0
C57BL/6-SCID		0	0 ± 0
		36	133 ± 14
		48	203 ± 13
	Anti-NK1.1	48	0 ± 0[A]
	Anti-AGM1	48	0 ± 0[A]

Liver sections were prepared from uninfected mice or mice infected with MCMV for 36 or 48 hours, immunostained with anti-Mig antibody, and then counterstained with methyl green as described in Methods. The number of Mig-positive cells was enumerated using a ×40 objective lens. A total of 20 randomly selected 1 × 1 mm² areas were counted for each section. Because there were no differences between the production of Mig in untreated and control antibody–treated mice, these groups were combined. Data shown represent the means ± SE (n = 4–6 animals). Number compared with control is significant [A]P < 0.001.

tially life-threatening consequences of systemic IFN-γ (30, 31), mechanisms must be in place to regulate serum levels but allow continued local access for antiviral cytokine effects. Other work in our laboratory is demonstrating changes in spleen, but not liver, NK cell populations resulting in reduced capacity to make IFN-γ (26). Thus, NK cell accumulation in liver may not only promote the delivery of cell-produced IFN-γ to tissue cell populations, but also drive NK cells into environments promoting extended IFN-γ production.

Our studies indicate that a major role for MIP-1α is to promote migration of cells into tissues, and that this is critical to outcome. MIP-1α does not itself appear to be required to promote IFN-γ production (32, 33) because the IFN-γ responses occur in other compartments (Figure 2) and anti-CD3 antibody readily induces IFN-γ and Mig expression in the livers of MIP-1α–deficient mice (Figure 4). The experiments used conditions of infection optimizing focus on NK cell events in the liver, i.e., moderate doses of virus for infections of young mice having the C57BL/6 genetic background (2, 6–9). The effects are likely to occur in other infected solid tissues and to involve the migration of other cell types. As certain chemokines overlap in receptor use (34), it is remarkable that this particular factor is so critical to defense against MCMV infection. The results presented

demonstrate how a single chemokine can make the difference between life and death.

Recombinant vaccinia virus expression of Mig has been shown by others to increase antiviral resistance during infection (29). Our results are consistent with this report, and, further, they extend understanding to endogenous Mig function and characterization of the events needed to induce its expression in tissues. We show that MIP-1α–dependent NK cell inflammation and their sustained local IFN-γ production is required for peak induction of Mig in livers (Figure 4; Table 1), we also show that endogenous Mig is another single chemokine required for survival (Table 3).

Mig was selected for examination because its induction is reported to have a uniquely stringent and specific requirement for IFN-γ (17–19). Although it is not yet clear whether or not Mig can access multiple antiviral pathways, this chemokine is known to have potent chemotactic activity for activated T cells (20–22). Ongoing preliminary studies support the intriguing possibilities that one unique mechanism by which IFN-γ indirectly mediates antiviral effects is by promoting downstream expression of Mig to recruit T cells, and that an important function of early NK cell inflammation is to induce conditions promoting T-cell inflammation. Because T cells are required for complete protection against MCMV

Table 3
Effect of Mig on MCMV replication and susceptibility

Antibody	Day 4 viral titer log PFU/g[A]		Day 5 viral titer log PFU/g[A]		Mortality	Average survival time
Treatment	Liver	Spleen	Liver	Spleen	(Live/total)	(Days)
None	5.4 ± 0.1[B]	5.5 ± 0.2[B]	4.5 ± 0.3	5.0 ± 0.7	6/6	>21
Control serum	5.2 ± 0.1	5.6 ± 0.1	4.2 ± 0.1	4.6 ± 0.2	12/12	>21
Anti-Mig	6.4 ± 0.3[C]	6.2 ± 0.05[D]	6.4 ± 0.05[C]	6.2 ± 0.5[C]	0/9	5

Mice were untreated, treated with control antiserum, or treated with antiserum-neutralizing Mig before infection and on days 2 or 3 after infection, as described in Methods. [A]Data shown represent the means ± SE (n = 4, except [B] where n = 3). Number compared with control is significant [C]P ≤ 0.005, [D]P < 0.01.

(9), our results are defining a chemokine to cytokine to chemokine cascade for protection against viral infections where MIP-1α promotes innate responses and these responses either act sequentially or in concert to induce Mig for delivery of downstream adaptive T-cell effects.

Other genes and gene products known to be induced by IFN-γ include the class II major histocompatibility molecules (MHC class II), inducible nitric oxide synthase (iNOS or NOS2), and IFN-γ–inducible protein of 10 kDa (IP-10) (10–14). However, these all are reported to also be induced by cytokines other than IFN-γ, including type 1 IFNs (i.e., IFN-α/β and TNF). Because IFN-α/β and TNF can be made by cell types other than NK cells, and because these cytokines are induced during MCMV infections (5, 7), Mig was a better candidate for examining effects specifically requiring NK cells and NK cell–produced IFN-γ. Ongoing studies are extending our work to evaluate expression of the other molecules. We have dissected a critical chemokine to cytokine to chemokine cascade, but there are likely to be other linked accessory events modified as a result of blocking individual steps in this sequence.

In summary, the understanding of events important for defense at early stages of tissue-targeted viral infection is advanced by the work presented here. Our results prove a role for MIP-1α–dependent NK cell inflammation and sustained local IFN-γ production in mediating protection against infection. In addition, they demonstrate that systemic responses are insufficient, and suggest that this may be a consequence of their limited kinetics. Finally, our results indicate that downstream antiviral effects are mediated through the NK cell and IFN-γ–dependent induction of Mig. Taken together, they help define 1 critical cascade for protection during infection.

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