Distinct Organ-Dependent Mechanisms for the Control of Murine Cytomegalovirus Infection by Natural Killer Cells

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Antiviral mechanisms by which natural killer (NK) cells control murine cytomegalovirus (MCMV) infection in the spleens and livers of C57BL/6 mice were measured, revealing different mechanisms of control in different organs. Three days postinfection, MCMV titers in the spleens of perforin 0/0 mice were higher than in those of perforin +/+ mice, but no elevation of liver titers was found in perforin 0/0 mice. NK cell depletion in MCMV-infected perforin 0/0 mice resulted only in an increase in liver viral titers and not in spleen titers. Depletion of gamma interferon (IFN-γ) in C57BL/6 mice by injections with monoclonal antibodies to IFN-γ resulted in an increase of viral titers in the liver but not in the spleen. Analyses using IFN-γ-receptor-deficient mice, rendered chimeric with C57BL/6 bone marrow cells, indicated that in a recipient environment where IFN-γ cannot exert its effects, the depletion of NK cells caused an increase in MCMV titers in the spleens but had little effect in the liver. IFN-γ has the ability to induce a variety of cells to produce nitric oxide, and administrating the nitric oxide synthase inhibitor N^{ω}-monomethyl-L-arginine into MCMV-infected C57BL/6 mice resulted in MCMV titer increases in the liver but not in the spleen. Taken together, these data suggest that in C57BL/6 mice, there is a dichotomy in the mechanisms utilized by NK cells in the regulation of MCMV in different organs. In the spleen NK cells exert their effects in a perforin-dependent manner, suggesting a cytotoxic mechanism, while in the liver the production of IFN-γ by NK cells may be a predominant mechanism in the regulation of MCMV synthesis. These results may explain why the $Cmv-1^r$ locus, which maps closely to genes regulating NK cell cytotoxic function, confers an NK cell-dependent resistance to MCMV infection in the spleen but not in the liver.

One component of the natural immune system is the NK cell, which provides an early host response to viral and bacterial infections and plays a role in resistance to some infections, in tumor surveillance, and in the regulation of hematopoiesis (1, 31, 33, 42). NK cells have a large granular lymphocyte morphology with granules containing a group of serine proteases known as granzymes and also the membrane pore-forming molecule perforin (21). These cells can also secrete a number of cytokines, such as gamma interferon (IFN-γ), tumor necrosis factor alpha, granulocyte-macrophage colony-stimulating factors, and interleukin 1 (35). On their cell surfaces, most NK cells express a variety of NK cell-specific receptors, such as NK1.1 and Ly49 isoforms, all encoded by genes found within the distal portion of mouse chromosome 6, in a region now known as the NK gene complex (44, 46). The differential expression of NK cell receptors on NK cell surfaces allows a division of the mouse NK cell population into different subsets. The cytolytic function of these NK cell subsets depends on the signals received after the ligation of their NK cell receptors to their respective ligand, as the signals could be either activating or inhibitory (26, 45).

The importance of NK cells in the regulation of the NK-sensitive virus murine cytomegalovirus (MCMV) has been shown with several mouse strains. Depletion of NK cells in C57BL/6 mice with antisera to asialo GM_1 (aGM_1) or monoclonal antibodies (MAbs) to NK1.1 enhanced the virus growth in the spleen, lung, and liver (6, 38, 39). NK cells were also shown to be very effective in the regulation of MCMV infection in mice with severe combined immunodeficiency (SCID),

denoting the importance of these cells in an environment devoid of functional T and B cells (38). A definitive demonstration of the anti-MCMV capability of NK cells was shown by adoptive transfer experiments (5). One-week-old suckling mice have low NK cell activity and are very sensitive to MCMV. Adoptive transfer of adult splenocyte populations protected the suckling mice from a lethal MCMV infection, and NK cells were required for this protection. Furthermore, adoptive transfers of purified culture-derived NK cells protected suckling mice from MCMV. In humans, the importance of NK cells was highlighted in our institution by a patient who had complete and selective NK cell immunodeficiency (2). This patient had unusually severe cases of human cytomegalovirus infection and other herpesvirus infections.

How NK cells regulate virus infections in vivo has been a poorly understood phenomenon. NK cells possibly regulate virus infections by the secretion of antiviral cytokines, such as IFN-γ and tumor necrosis factor alpha, or alternatively, by the direct lysis of virus-infected cells prior to the release of infectious virus progeny. Most evidence to date suggests that cytokines are important (9, 14, 15, 22–25). Nude mice infected with a vaccinia virus–interleukin 2 recombinant were able to control the virus infection (14). Administration of either anti-IFN-γ Abs or anti-aGM_1 antiserum to deplete NK cells exacerbated the infection. This suggests that IFN-γ produced by NK cells regulates the infection by the vaccinia virus recombinant, but it may be an artificial system, as viruses do not encode interleukin 2, which is a strong chemoattractant for NK cells as well as an inducer of IFN-γ. Recently it has been shown that the production of IFN-γ by NK cells is important for defense against MCMV infection (23). Depletion of IFN-γ in C57BL/6 mice increased the incidence of MCMV-induced hepatitis and viral replication in the liver (23). The importance of IFN-γ was also demonstrated with CB17 SCID mice, where the adminis-
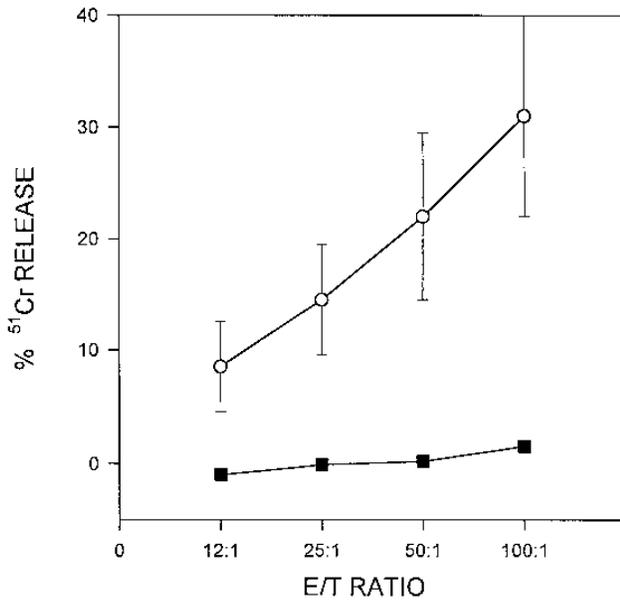
FIG. 1. NK cell activity from MCMV-infected perforin +/+ (○) and perforin 0/0 (■) mice, 3 days postinfection. The spleen cells from the mice were used as effectors against YAC-1 targets in a standard 5-h ^{51}Cr release assay.

tration of anti-IFN-γ Abs into MCMV-infected mice resulted in increases in splenic and liver MCMV titers at 7 days postinfection (9). Antibodies to IFN-γ did not affect NK cell cytolytic activity, a result consistent with the concept that IFN-α/β rather than IFN-γ activates NK cells at the early stages of infection (23). The only evidence to date suggesting that a cytotoxic mechanism may be involved in the control of MCMV comes from studies with beige mice, which control MCMV infection poorly and whose NK cells are deficient in cytotoxic function (32).

Of great interest are the findings by Scalzo et al., which showed that there is a non-major histocompatibility complex (MHC)-linked resistance gene to MCMV which maps within the NK gene complex (27, 30). Cmv-1, which maps very closely to the NK1.1 locus, confers resistance to MCMV in the spleen but not in the liver. The effects of Cmv-1 are mediated by NK1.1$^+$ cells, and mice that have the Cmv-1 gene (Cmv-1^r) have lower splenic viral titers than mice that do not have the gene (Cmv-1^s) (28). However, the liver viral titers remain similar in both strains of mice. Since Cmv-1 is found within the NK locus, it is possible that the gene may be differentially expressed in the various subsets of NK cells and that different subsets of NK cells control MCMV in different organs. Alternatively, NK cells may utilize a Cmv-1-dependent mechanism to control MCMV in the spleen but another mechanism to control the virus in the liver.

To address the latter possibility, we examined MCMV infections in the perforin-deficient mice and IFN-γ-receptor-deficient mice, as well as in normal mice treated with anti-IFN-γ Abs in an effort to delineate the mechanisms utilized by NK cells early in the infection. Here, we report that in Cmv-1^r mice, such as C57BL/6 mice, there is a dichotomy in the mechanisms utilized by NK cells in the regulation of MCMV in different organs. In the spleen, NK cells may control MCMV via a perforin-dependent cytotoxic mechanism, while in the liver IFN-γ produced by NK cells is a major mediator in the regulation of the infection.

MATERIALS AND METHODS

Cells. YAC-1, a highly NK-sensitive lymphoma line, was cultivated in suspension in RPMI 1640 (Sigma, St. Louis, Mo.), supplemented with 10% fetal bovine serum (FBS) and antibiotics. Mouse embryonic fibroblasts (MEF) from C57BL/6 mice were cultivated as monolayers in minimal essential medium (Gibco Laboratories, Grand Island, N.Y.) with 20% FBS and antibiotics.

Mice. C57BL/6 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. These mice were conventionally housed and were used at 6 to 12 weeks of age. Breeder pairs of 129 wild-type control and 129 mice homozygous for a targeted mutation disrupting the mouse interferon gamma receptor gene (IFN-γR$^{0/0}$) were originally derived and kindly supplied by M. Aguet, University of Zurich, Switzerland (10). Breeder pairs of 129 × C57BL/6 mice heterozygous for a targeted mutation disrupting the gene for perforin were originally derived and kindly provided by C. M. Walsh and W. R. Clark, University of California, Los Angeles (37). F1 offspring of the above heterozygous breeding were typed by PCR. Both perforin-deficient (perforin 0/0) and IFN-γR$^{0/0}$ mice and their respective wild-type controls were housed in a specific-pathogen-free environment and were used at 6 to 12 weeks of age.

Virus. The Smith strain of MCMV was a salivary gland-passaged virus stock prepared in BALB/c mice. MCMV was titrated by plaque assay on MEF and was given to mice intraperitoneally (i.p.) at a dose of 5 to 10 × 10^3 PFU per mouse 3 days before use.

Cytotoxicity assay. Standard 4- to 6-h ^{51}chromium (Cr) release microcytotoxicity assays were used to determine NK cell activity on YAC-1 targets (5). YAC-1 cells were used at 5 or 10 × 10^3 targets per well and at a variety of E:T ratios. All microcytotoxicity assays were performed using U-bottom 96-well plates (Falcon, Lincoln Park, N.J.).

Immune reagents. Rabbit antiserum to aGM$_1$ (Wako Labs, Dallas, Tex.) was used at a pretitrated dose that depleted lymphocytic choriomeningitis virus (LCMV)-induced NK cell activity but not cytotoxic T-lymphocyte (CTL) activity in C57BL/6 mice. Anti-aGM$_1$ antiserum was inoculated into mice i.p. 24 h before the day of infection. The anti-NK1.1 MAb, PK136 (provided by G. C. Koo, Merck Sharpe and Dohme Research Laboratories, Rahway, N.J.) (17), was produced in ascites, NH$_4$SO$_4$ cut, and affinity purified before use. PK136 was inoculated into mice intravenously (i.v.) at a dose of 200 μl of a 1:40 dilution per mouse, 24 h before the day of infection. The anti-IFN-γ MAb, R46A2, was obtained from American Type Culture Collection, Rockville, Md., and was inoculated in ascites form at 200 μl of a 1:5 dilution per mouse on the day of infection and at day 1 and day 2 after infection. By enzyme-linked immunosorbent assay, the amount of anti-IFN-γ ascites given in 3 days was enough to bind 0.9 μg/ml of recombinant mouse IFN-γ (Pharmingen, San Diego, Calif.). In vivo depletion of CD4$^+$ T cells was done by injecting i.p. 80 μl of a rat anti-mouse CD4 MAb in undiluted ascites (clone GK1.5). CD8$^+$ T cells were depleted by injecting i.p. 100 μl of a 1:40 dilution of an ascites preparation of rat anti-mouse CD8 MAb (clone 2.43). Clone 2.43 was provided by R. Fujinami, University of Utah, and clone GK1.5 was purchased from American Type Culture Collection. The anti-CD4 and anti-CD8 MAbs were routinely used in our laboratory at these concentrations to deplete CD4 and CD8 cell numbers as well as virus-induced CTL activity in vivo.

Immunofluorescence. To stain for MHC class II antigens on macrophages, 5 × 10^7 splenocytes were first treated with a rat monoclonal antibody, J11D (3, 20), plus rabbit complement (Pel Freeze Chemical Inc., Brown Deer, Wisc.) to remove the B cells and granulocytes. The residual cells were pretreated with normal mouse serum and then stained with mouse anti-mouse I-A^b-FITC (Pharmingen).

Reagents. N$^{\omega}$-monomethyl-L-arginine (L-NMA) and N$^{\omega}$-monomethyl-D-arginine (D-NMA) were obtained from Sigma Immunochemicals (St. Louis, Mo.). Both reagents, except where indicated, were given i.v. at a dose of 5 mg per mouse on the day of infection and at day 1 and day 2 of the infection.

Typing of perforin-deficient mice. The primer sequences of the perforin primers were as follows: CTCCGGCTCCTTCCCAGTGA and TTCTTCCAGTTCC TTTCTCC (Ransom Hill Bioscience Inc., Ramona, Calif.). Neomycin primer sequences (ATGATTGAACAAGATGGATTGC and GACAAAAAGAACCG GGCG) were provided by C. L. Sidman, University of Cincinnati Medical Center, Cincinnati, Ohio. The neomycin primers were made by Biosynthesis, Lewisville, Tex., and were provided by A. M. Baird, University of Massachusetts Medical Center, Worcester. PCR were performed on the tail DNA obtained from the F1 offspring of the perforin heterozygous breeding. The PCR will only amplify the 145-bp neomycin band from the DNA of perforin 0/0 and only the 414-bp perforin band from the perforin-intact mice (perforin +/+). Tail DNA from perforin heterozygous mice was used as a positive control, and two bands corresponding to the 414-bp perforin band and the 145-bp neomycin band were obtained.

Generation of bone marrow chimeras. Six- to 12-week-old 129 or IFN-γR$^{0/0}$ mice received 950 rads (1 rad = 0.01 gray) of gamma irradiation, were injected i.v. within 3 h with 5 × 10^7 bone marrow cells from 6- to 12-week-old C57BL/6 mice, and were used 6 weeks after reconstitution. The ability of anti-NK1.1 MAb to deplete the NK cell activity in the reconstituted mice was used as an indication that the bone marrow reconstitution with NK1.1$^+$ C57BL/6 cells was complete, as the 129 strain does not express the NK1.1 antigen.

TABLE 1. Regulation of MCMV infection in perforin +/+ and 0/0 mice[a]

Group	Log_{10} PFU/organ $\pm$ SD[b]							
	Expt 1[c]		Expt 2[d]		Expt 3		Expt 4	
	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver
Perforin +/+	1.5 ± 0.3	3.6 ± 0.2	2.6 ± 0.3	5.3 ± 0.04	3.4 ± 0.0	4.5 ± 0.3	1.5 ± 0.2	3.4 ± 0.1
Perforin 0/0	3.9 ± 0.1^e	4.0 ± 0.1	4.3 ± 0.3^e	5.4 ± 0.3	4.2 ± 0.4^e	3.9 ± 0.3	3.6 ± 0.1^e	3.7 ± 0.1

[a] Age-matched perforin +/+ and 0/0 mice were given i.p. 5×10^3 MCMV PFU per mouse.
[b] Spleen and liver MCMV PFU were titrated on C57BL/6 MEF 3 days after infection.
[c] Splenic NK cells were used as effectors against YAC-1 targets. The results are depicted in Fig. 1.
[d] Anti-CD4 and anti-CD8 Abs were given i.p. 1 day prior to MCMV infection.
[e] Virus titers were significantly higher than those in perforin +/+ mice ($P < 0.005$).

Statistical analyses. Data shown in Tables 1 and 3 through 6 were analyzed by Student's t test for each individual experiment. For the calculation of the average $\log_{10}$ PFU increase or decrease in MCMV titers compared to the appropriate controls, taking into consideration all of the relevant experiments, data obtained from individual mice were used, and a statistical test analyzing the variance (ANOVA) for mixed models using restricted estimation by maximum likelihood (REML) was applied. All computations were performed by using the Proc Mixed procedure in the SAS statistical software package (SAS, Cary, N.C.). The results are shown as average $\log_{10}$ PFU $\pm$ standard error (SE) in the text and in Table 2.

RESULTS

Replication of MCMV in perforin +/+ and 0/0 mice. Perforin is a key effector molecule in cell-mediated cytolysis mediated by $CD8^+$ T cells and NK cells, and the molecule has been shown to be important in the in vivo T cell-dependent regulation of virus infections and in the clearance of tumors (12, 37). To study the role of perforin in the early regulation of MCMV by NK cells, perforin +/+ and 0/0 mice were infected with MCMV for 3 days, and the NK cell activity and MCMV titers in the spleens and livers were measured. Spleen and liver viral titers were chosen because the spleen and liver are the primary organs of infection early in the infection. Figure 1 shows the NK cell activity elicited in these mice 3 days postinfection. Perforin +/+ mice had good NK cell activity, whereas the perforin 0/0 mice exhibited no NK cell activity, confirming both the genotype of these mice and the importance of perforin in cell-mediated cytolysis. Replication of MCMV in the spleens of perforin 0/0 mice was in several experiments markedly elevated compared to that in perforin +/+ mice (Table 1).

Interestingly, in both strains of mice there was no appreciable difference in the liver MCMV titers (Table 1). In four experiments, perforin 0/0 mice had an average of $1.8 \pm 0.4 \log_{10}$ PFU more MCMV in the spleen than did the perforin +/+ mice ($P = 0.0002$; Table 2). In the liver, there was a statistically insignificant $0.1 \pm 0.5 \log_{10}$ PFU MCMV increase in perforin 0/0 mice compared to perforin +/+ mice (Table 2). These results suggest that perforin plays an important role in the regulation of MCMV in the spleen but not in the liver. Although the control of MCMV infection at 3 days has been shown to be mediated by NK cells and not T cells (5, 6, 39), in one experiment mice were depleted of T cells with MAbs to CD4 and CD8. Perforin 0/0 mice treated with the anti-CD4 and anti-CD8 MAbs also exhibited similar splenic titer increases with no change in the liver MCMV titers compared to T-cell-depleted perforin +/+ mice (Table 1, expt 2).

Effect of anti-NK1.1 MAb treatment on the replication of MCMV by perforin 0/0 mice. The results from the experiments performed above suggest that NK cells utilize perforin to regulate MCMV in the spleen but not in the liver. However, these mice were of a mixed genetic background (129 × C57BL/6), and 129 mice and C57BL/6 mice have different susceptibilities to MCMV infection (30). To prove that any effects seen were indeed due to NK cells, the mice were first shown to express the NK1.1 antigen by immunofluorescence, and then they were depleted of NK cells to test for the enhancement in virus titers. Results depicted in Table 3 show that MCMV-infected, perforin 0/0 mice depleted of NK1.1$^+$ NK cells synthesized more virus in the liver than MCMV-infected, NK cell-intact perforin

TABLE 2. Summary of experiments[a]

Group + treatment[b] (n^c)	Corresponding control[b] (n^c)	Increase in $\log_{10}$ PFU MCMV/organ $\pm$ SE over controls			
		Spleen	P^d	Liver	P^d
Perforin 0/0 (11)	Perforin +/+ (11)	1.8 ± 0.4	0.0002	0.1 ± 0.5	0.6 (NS[e])
Perforin 0/0 + anti-NK1.1 (9)	Perforin 0/0 (9)	0.1 ± 0.3	0.4 (NS)	0.6 ± 0.2	0.0002
B6 + anti-IFN-γ (15)	B6 (15)	0.1 ± 0.2	1.0 (NS)	0.7 ± 0.2	0.0003
B6 + anti-NK1.1 (14)	B6 (15)	1.6 ± 0.2	0.0003	0.7 ± 0.2	0.0003
B6 + anti-IFN-γ + anti-NK1.1 (14)	B6 (15)	1.6 ± 0.2	0.0003	0.7 ± 0.2	0.0003
B6→129 + anti-NK1.1 (9)	B6→129 (10)	1.2 ± 0.2	0.0002	0.6 ± 0.3	0.0002
B6→IFN-γR$^{0/0}$ + anti-NK1.1 (15)	B6→IFN-γR$^{0/0}$ (16)	1.5 ± 0.4	0.0002	0.03 ± 0.3	1.0 (NS)
B6 + L-NMA (9)	B6 (8)	-0.2 ± 0.4	0.3 (NS)	0.6 ± 0.4	0.003
B6 + L-NMA (9)	B6 + D-NMA (6)	-0.1 ± 0.4	1.0 (NS)	0.8 ± 0.4	0.0004

[a] Analysis of data encompassing the individual experiments presented in Tables 1, 3, 4, 5, and 6. Shown is the increase in least-square mean titers ($\log_{10}$ PFU per organ $\pm$ SE) between the treatment group and the control group.
[b] Age-matched mice were infected i.p. with 10^4 PFU per mouse of MCMV. Mice were either left untreated or were treated with anti-NK1.1 Abs i.v. 1 day prior to infection. Anti-IFN-γ Abs were given i.p. at days 0, 1, and 2 of the infection. L-NMA or D-NMA was given i.v. at days 0, 1, and 2 of the infection.
[c] Total number of mice used in each group.
[d] P values were obtained by applying the statistical test analyzing the variance for mixed models using restricted estimation by maximum likelihood using the Proc Mixed procedure in the SAS statistical software package.
[e] NS, not significant.

TABLE 3. Effect of anti-NK1.1 MAb treatment on the regulation of MCMV by perforin +/+ and 0/0 mice[a]

Expt no.	Group	Log$_{10}$ PFU/organ ± SD[b]	
		Spleen	Liver
1	Perforin 0/0	4.4 ± 0.1	4.5 ± 0.1
	Perforin 0/0 + anti-NK1.1	4.5 ± 0.1	5.0 ± 0.1[d]
2	Perforin 0/0	3.9 ± 0.1	4.0 ± 0.1
	Perforin 0/0 + anti-NK1.1	3.8 ± 0.2	4.5 ± 0.1[d]
	Perforin +/+	1.5 ± 0.3	3.6 ± 0.2
	Perforin +/+ + anti-NK1.1	2.6 ± 0.2[d]	4.3 ± 0.04[c]
3	Perforin 0/0	4.6 ± 0.1	4.1 ± 0.1
	Perforin 0/0 + anti-NK1.1	4.6 ± 0.1	4.5 ± 0.2[e]

[a] Age-matched perforin +/+ and 0/0 mice were given i.p. 5 × 10^3 MCMV PFU per mouse. Anti-NK1.1 was given i.v. 1 day prior to infection.
[b] Spleen and liver MCMV titers were measured by using C57BL/6 MEF 3 days after infection.
[c] Virus titers increased significantly after NK cell depletion compared to those in infected controls ($P < 0.01$).
[d] Virus titers increased significantly after NK cell depletion compared to those in infected controls ($P < 0.005$).
[e] Virus titers increased significantly after NK cell depletion compared to those in infected controls ($P = 0.08$).

0/0 mice. However, splenic MCMV titers remained the same in both sets of mice. In three experiments, the average log$_{10}$ PFU increase in virus titers in the liver was 0.6 ± 0.2 log$_{10}$ PFU ($P = 0.0002$; Table 2), while there was only a slight increase in MCMV titers in the spleens (0.1 ± 0.3 log$_{10}$ PFU; Table 2) of anti-NK1.1-treated perforin 0/0 mice. These data suggest that at 3 days post-MCMV infection, the virus in the spleen is controlled by perforin, while in the liver, MCMV is controlled by NK cells in a perforin-independent manner.

Effect of anti-IFN-γ treatment on MCMV replication in C57BL/6 mice. One possible mechanism that NK cells can use to control MCMV in the liver is via the production of antiviral cytokines like IFN-γ. IFN-γ produced by NK cells protects the liver against MCMV infection, and this protection can be abrogated by treating mice with anti-IFN-γ Abs (23). C57BL/6 mice were infected with MCMV and simultaneously treated with anti-IFN-γ Abs to determine whether the depletion of IFN-γ would raise virus titers in both the liver and the spleen. Treatment of MCMV-infected C57BL/6 mice with anti-NK1.1 MAb or anti-aGM$_1$ antiserum depleted the NK cell activity

below the levels in the uninfected controls, while the NK cell activity in mice depleted of IFN-γ was similar to that in the infected controls (data not shown). This shows that the anti-IFN-γ treatment had no effect on the virus-induced augmentation of NK cell activity, results similar to that recently reported by Orange et al. (23). However, the effector function of NK cells in vivo was compromised, as MCMV-infected C57BL/6 mice depleted of IFN-γ grew more virus in the liver (Table 4). In contrast, the splenic titers remained the same as those in the infected untreated controls (Table 4). There was an average of 0.7 ± 0.2 log$_{10}$ PFU more MCMV in the livers ($P = 0.0003$) but only 0.1 ± 0.2 log$_{10}$ PFU more virus in the spleens of anti-IFN-γ-treated mice compared to the infected untreated controls (Table 2). The amounts of MCMV present in the livers of anti-IFN-γ-treated mice were similar to those in MCMV-infected mice that were either given anti-NK cell Abs only (anti-NK1.1 or anti-aGM$_1$) or a combination of anti-NK cell Abs and anti-IFN-γ Abs (Table 4). Although anti-IFN-γ treatment had no effect on splenic viral titers, mice that were given anti-NK1.1 Abs exhibited 1.6 ± 0.2 log$_{10}$ PFU more virus in the spleen than infected controls ($P = 0.0003$; Table 2).

To make sure that the anti-IFN-γ treatment was altering IFN-γ-induced functions in the spleen, splenocytes enriched for macrophages from uninfected, MCMV-infected, and MCMV-infected, anti-IFN-γ-treated C57BL/6 mice were stained for MHC class II expression, which MCMV-induced IFN-γ has been previously shown to upregulate (9). MCMV infection increased MHC class II expression on spleen leukocytes, but in MCMV-infected mice treated with the same dose of anti-IFN-γ Abs, MHC class II levels on the spleen leukocytes were the same as those on the uninfected control (Fig. 2). This indicated that the anti-IFN-γ treatment blocked IFN-γ-mediated functions in the spleen, yet it did not affect MCMV replication in that organ.

Replication of MCMV in B6→IFN-γR$^{0/0}$ mice. To further clarify the role of IFN-γ in the in vivo regulation of MCMV, we made use of the IFN-γR$^{0/0}$ mice. These mice lack the receptor for IFN-γ, and, even though they retain the ability to produce IFN-γ, the cells in these animals cannot respond to that cytokine (10). The 129 background of these knockout mice did not allow us to perform satisfactorily direct analyses on the NK cell control of MCMV infection, because the 129 strain has a low NK cell activity and is NK1.1$^-$ and Cmv-1^s (30). Depletion of NK cell activity in these mice with anti-aGM$_1$ antiserum led to

TABLE 4. Effects of anti-NK cell and/or anti-IFN-γ MAb treatment in the regulation of MCMV in C57BL/6 mice[a]

Group	Log$_{10}$ PFU/organ ± SD[b]					
	Expt 1		Expt 2		Expt 3	
	Spleen	Liver	Spleen	Liver	Spleen	Liver
Untreated	<2.3 ± 0.0	4.4 ± 0.4	3.2 ± 0.2	4.0 ± 0.4	2.7 ± 0.4	4.3 ± 0.1
Treated with:						
anti-IFN-γ	<2.3 ± 0.0	5.0 ± 0.2[d]	3.2 ± 0.6	4.7 ± 0.04[d]	3.0 ± 0.3	4.9 ± 0.3[d]
anti-NK1.1	4.5 ± 0.1[e]	5.1 ± 0.2[d]	4.4 ± 0.2[e]	4.6 ± 0.1	4.3 ± 0.5[e]	4.9 ± 0.2[d]
anti-NK1.1 + anti-IFN-γ	4.4 ± 0.1[e]	5.0 ± 0.02[d]	4.5 ± 0.2[e]	4.8 ± 0.3[d]	4.1 ± 0.2[e]	4.9 ± 0.3[d]
anti-aGM$_1$	4.6 ± 0.1[e]	5.3 ± 0.3[d]	ND[c]	ND	ND	ND
anti-aGM$_1$ + anti-IFN-γ	4.1 ± 0.2[e]	5.2 ± 0.2[d]	ND	ND	ND	ND

[a] Age-matched C57BL/6 mice were infected i.p. with 10^4 PFU of MCMV per mouse. Anti-NK1.1 or anti-aGM$_1$ was given i.v. or i.p., respectively, 1 day prior to infection. Anti-IFN-γ was given i.p. at days 0, 1, and 2 of the infection.
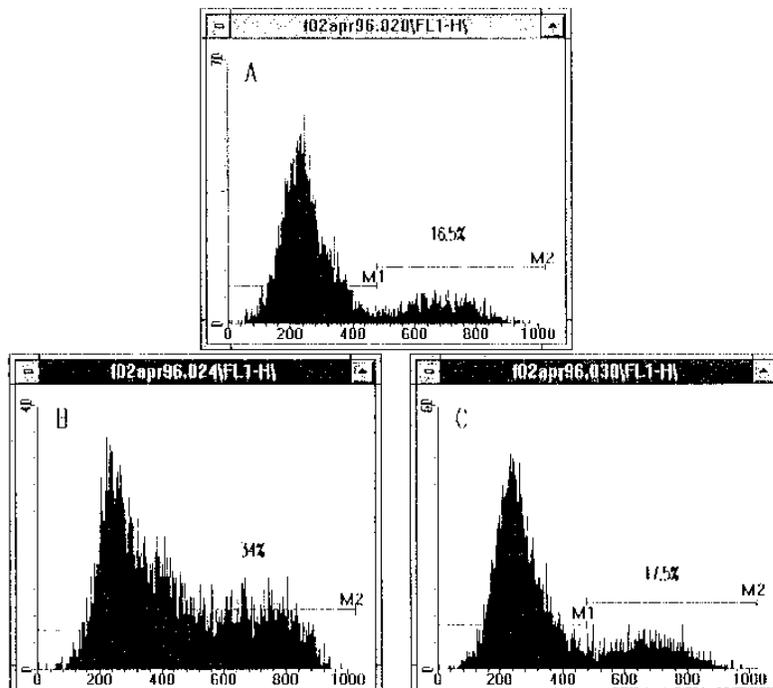[b] Spleen and liver MCMV PFU were titrated on C57BL/6 MEF 3 days after infection.
[c] ND, not done.
[d] NK cell depletion or anti-IFN-γ treatment significantly increased the virus titers compared to those in infected controls ($P < 0.05$).
[e] NK cell depletion or anti-IFN-γ treatment significantly increased the virus titers compared to those in infected controls ($P < 0.005$).

FIG. 2. MHC class II expression on splenic macrophages. Spleen cells from uninfected (A), MCMV-infected (B), and MCMV-infected, anti-IFN-γ-treated (C) mice were partially enriched for macrophages by treatment with J11D plus complement and then stained with a mouse anti-mouse I-A^b-FITC Ab. The number above the positive peak reflects the percentage of cells that stained positively for MHC class II expression.

only marginal increases in the spleen and liver MCMV titers. In five experiments each with 4 to 5 mice per group, anti-aGM$_1$-treated, MCMV-infected 129 mice had an average of a 0.5 ± 0.4 log$_{10}$ PFU increase in splenic titers and only a 0.1 ± 0.2 log$_{10}$ PFU increase in liver titers compared to infected controls. In seven experiments, anti-aGM$_1$-treated, MCMV-infected 129/IFN-γR$^{0/0}$ mice had only a 0.2 ± 0.2 log$_{10}$ PFU increase in splenic titers and a 0.1 ± 0.1 log$_{10}$ PFU MCMV decrease in liver titers compared to infected controls. Scalzo et al. had shown that, in BALB/c mice congenic for C57BL/6 *Cmv-1* and the NK gene complex, susceptibility of these mice to MCMV changed from a *Cmv-1^s* to a *Cmv-1^r* phenotype (29). Therefore, to circumvent the 129/*Cmv-1^s* background problem, we reconstituted the IFN-γR$^{0/0}$ mice with C57BL/6 bone marrow cells, thereby creating a strain of mouse (B6→IFN-γR$^{0/0}$) that has C57BL/6 NK cells, is *Cmv-1^r*, and retains its IFN-γR-deficient phenotype.

Figure 3 shows the NK cell activity from B6→129 and B6→IFN-γR$^{0/0}$ chimeras 3 days after MCMV infection, with or without anti-NK1.1 MAb treatment. Both strains of mice exhibited robust NK cell activity, which was depleted with the anti-NK1.1 MAb. B6→129 mice depleted of NK1.1$^+$ cells had higher virus titers in both their spleens and livers than infected controls, further showing the efficacy of the bone marrow reconstitution (Table 5). In two experiments, NK cell-depleted B6→129 mice had 1.2 ± 0.2 log$_{10}$ PFU more virus in the spleen ($P = 0.0002$) and 0.6 ± 0.3 log$_{10}$ PFU more virus in the liver ($P = 0.0002$) than NK cell-intact, MCMV-infected controls (Table 2). However, NK cell-depleted B6→IFN-γR$^{0/0}$ mice showed only an increase in splenic titers with no appreciable
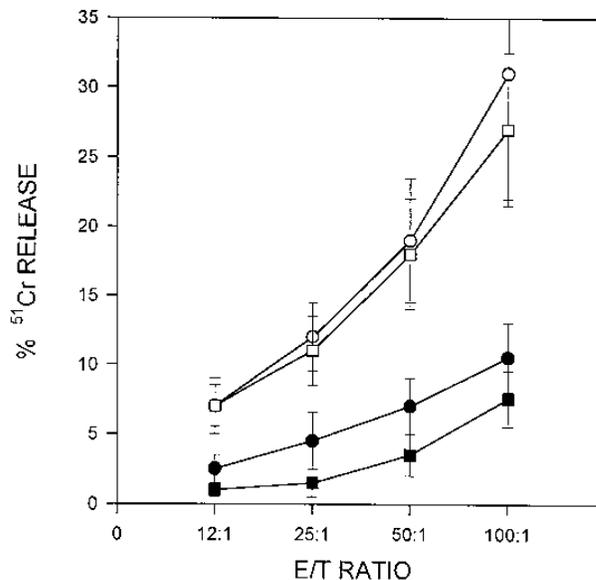

FIG. 3. NK cells from MCMV-infected B6→IFN-γR$^{0/0}$ control (○), MCMV-infected, anti-NK1.1-treated B6→IFN-γR$^{0/0}$ (●), MCMV-infected B6→129 control (□), and MCMV-infected, anti-NK1.1-treated B6→129 (■) mice were used 3 days postinfection as effectors against YAC-1 targets in a standard 5-h ^{51}Cr release assay.

TABLE 5. Effects of anti-NK1.1 MAb treatment on the regulation of MCMV in B6→IFN-γR$^{0/0}$ and B6→129 mice[a]

Expt no.	Group	Log$_{10}$ PFU/organ ± SD[b]	
		Spleen	Liver
1	B6→IFN-γR$^{0/0}$	<1.4 ± 0.2	4.8 ± 0.2
	B6→IFN-γR$^{0/0}$ + anti-NK1.1	3.8 ± 0.3[e]	4.6 ± 0.5
2	B6→IFN-γR$^{0/0}$	<1.1 ± 0.2	4.0 ± 0.2
	B6→IFN-γR$^{0/0}$ + anti-NK1.1	2.6 ± 0.5[e]	4.3 ± 0.4
3	B6→IFN-γR$^{0/0}$	<2.5 ± 0.3	4.4 ± 0.4
	B6→IFN-γR$^{0/0}$ + anti-NK1.1	3.6 ± 0.5[e]	4.8 ± 0.1
4[c]	B6→IFN-γR$^{0/0}$	<1.4 ± 0.2	3.8 ± 0.6
	B6→IFN-γR$^{0/0}$ + anti-NK1.1	2.6 ± 0.3[e]	3.9 ± 0.2
	B6→129	1.3 ± 0.0	3.4 ± 0.3
	B6→129 + anti-NK1.1	2.8 ± 0.4[e]	4.1 ± 0.1[d]
5	B6→129	<1.9 ± 0.5	3.9 ± 0.1
	B6→129 + anti-NK1.1	2.9 ± 0.3[e]	4.4 ± 0.2[e]

[a] Age-matched B6→IFN-γR$^{0/0}$ mice or B6→129 mice were infected i.p. with 10^4 MCMV PFU per mouse. Anti-NK1.1 was given i.v. 1 day prior to infection.
[b] Spleen and liver MCMV PFU were titrated on C57BL/6 MEF 3 days postinfection.
[c] Splenic NK cells were used as effectors against YAC-1 targets. The results are shown in Fig. 3.
[d] NK cell depletion caused a significant increase in virus titers compared to those in the appropriate controls ($P < 0.01$).
[e] NK cell depletion caused a significant increase in virus titers compared to those in the appropriate controls ($P < 0.005$).

increase of MCMV in the liver (Table 5). In four experiments performed, there was an average 1.5 ± 0.4 log$_{10}$ PFU increase in splenic titers ($P = 0.0002$) but only a 0.03 ± 0.3 log$_{10}$ PFU increase in the livers of NK cell-depleted B6→IFN-γR$^{0/0}$ mice over the infected untreated controls (Table 2). These results are compatible with the concept that IFN-γ is the NK cell-dependent mechanism that regulates MCMV synthesis in the liver but not in the spleen. To be certain that the effect seen in the spleens of NK1.1-depleted B6→IFN-γR$^{0/0}$ mice was not due to the donor cells' ability to respond to IFN-γ, perforin 0/0 bone marrow cells were used to reconstitute IFN-γR$^{0/0}$ mice, and the effects of depleting NK1.1$^+$ cells in MCMV-infected, perforin 0/0→IFN-γR$^{0/0}$ chimeric mice were measured. Three days post-MCMV infection, NK cell-depleted perforin 0/0→IFN-γR$^{0/0}$ mice (spleen, 3.2 ± 0.2 log$_{10}$ PFU; liver, 4.8 ± 0.2 log$_{10}$ PFU) had the same amount of virus in the spleen and liver as the NK cell-intact, untreated control (spleen, 3.4 ± 0.1 log$_{10}$ PFU; liver, 4.9 ± 0.2 log$_{10}$ PFU). This result strongly suggests that the early regulation of MCMV in the spleen is controlled by perforin.

Effect of L-NMA on the in vivo replication of MCMV by C57BL/6 mice. One of the ways IFN-γ can inhibit virus replication is by inducing the expression of the gene *iNOS*, which encodes nitric oxide synthase (NOS), which in turn catalyzes the guanidino nitrogen of L-arginine into a free radical gas, nitric oxide (NO) (19). NO production by NOS has been shown both in vivo and in vitro to inhibit ectromelia virus, vaccinia virus, and herpes simplex virus type 1 replication (8, 16). To see whether MCMV is regulated by IFN-γ via the production of NO and to determine whether this regulation is organ dependent, we treated MCMV-infected mice with a competitive inhibitor of NOS, L-NMA, and measured the NK cell activity as well as the viral titers in both spleens and livers of these mice. Three days post-MCMV infection, L-NMA or its stereoisomer D-NMA had no effect on the NK cell-medi-
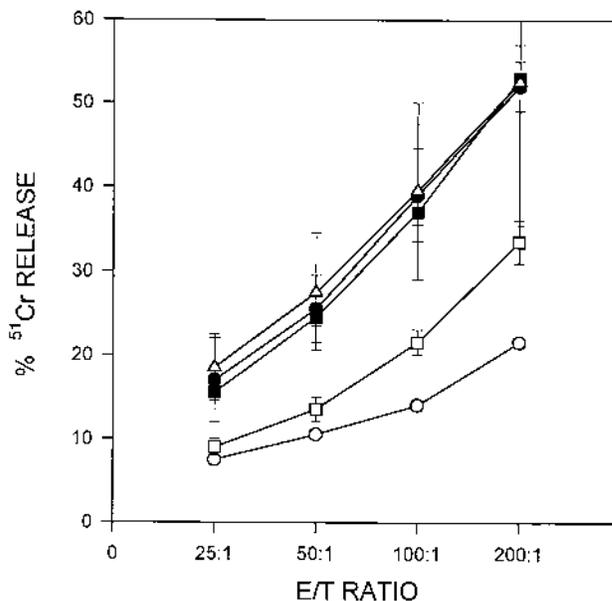

FIG. 4. NK cell activity from uninfected (○), MCMV-infected (●), MCMV-infected, anti-NK1.1-treated (□), MCMV-infected, L-NMA-treated (■), and MCMV-infected, D-NMA-treated (△) C57BL/6 mice, 3 days postinfection. Spleen cells from the mice were used as effectors against YAC-1 targets in a standard 5-h ^{51}Cr release assay.

ated lysis (Fig. 4). L-NMA-treated mice had more virus in the liver than the infected controls, but the splenic titers remained the same in these mice (Table 6). The enantiomeric analog of L-NMA, D-NMA, had no effect on the virus titers in either the spleen or the liver (Table 6). In three experiments, L-NMA treatment induced an average of a 0.6 ± 0.4 log$_{10}$ PFU increase in liver virus titers ($P = 0.003$) but caused a 0.2 ± 0.4 log$_{10}$ PFU decrease in the spleen compared to untreated controls (Table 2). Compared to the D-NMA control, L-NMA-treated mice had 0.8 ± 0.4 log$_{10}$ PFU more virus in the liver ($P = 0.0004$), but there was no appreciable difference in splenic MCMV titers (Table 2). This suggests that MCMV synthesis in the liver but not in the spleen is regulated by IFN-γ-induced nitric oxide and adds further evidence to support the concept that the control of MCMV infection is mediated by different mechanisms in the spleen and liver.

DISCUSSION

This is the first report of NK cells using different mechanisms to regulate viral infections in different organs. In a 3-day MCMV infection, we were surprised to see that the perforin 0/0 mice had more virus in the spleen than perforin +/+ mice, while the liver titers in both groups of mice remained similar. This suggests that the perforin-dependent mechanism is of greater importance in the spleen than in the liver. Because perforin is related to the cytotoxic function of the NK cells, this may indicate that the cytotoxic function of NK cells is important for the control of MCMV in the spleen but not in the liver. Guidotti et al. has recently reported that in hepatitis B virus transgenic mice, the regulation of the virus in the liver is carried out by CTLs via the production of IFN-γ and TNF-α (7). The cytokines do not kill the virus-infected hepatocytes but rather selectively degrade the hepatitis B virus nucleocapsid particles and their replicating genomes and destabilize the viral RNA. Direct lysis of the hepatocytes by the CTLs was minimal.

TABLE 6. Effects of L-NMA and D-NMA treatment on the regulation of MCMV in C57BL/6 mice[a]

| Group | Log$_{10}$ PFU/organ $\pm$ SD[b] | | | | | |
| | Expt 1[c] | | Expt 2[c,d] | | Expt 3[e] | |
	Spleen	Liver	Spleen	Liver	Spleen	Liver
Untreated	2.5 ± 0.2	4.2 ± 0.04	1.8 ± 0.2	3.0 ± 0.4	1.9 ± 0.2	3.6 ± 0.5
Treatment with:						
anti-NK1.1	3.5 ± 0.2^f	4.9 ± 0.2^f	2.6 ± 0.2^g	4.1 ± 0.2^f	2.9 ± 0.1^g	4.5 ± 0.2^f
L-NMA	2.5 ± 0.2	4.7 ± 0.1^f	1.4 ± 0.2	3.7 ± 0.03^f	1.8 ± 0.0	4.2 ± 0.2^h
D-NMA	ND	ND	1.4 ± 0.2	2.6 ± 0.6	2.0 ± 0.2	3.7 ± 0.3

[a] Age-matched C57BL/6 mice were infected i.p. with 10^4 MCMV PFU of MCMV per mouse. Anti-NK1.1 was given i.v. 1 day prior to infection. L-NMA and D-NMA were given i.v. at day 0, day 1, and day 2 of the infection.
[b] Spleen and liver MCMV PFU were titrated on C57BL/6 MEF 3 days postinfection. ND, not determined.
[c] L-NMA and D-NMA were used at a dose of 5 mg per mouse per day.
[d] Splenic NK cells were used as effectors against YAC-1 targets. The results are shown in Fig. 4.
[e] L-NMA was used at a dose of 10 mg per mouse per day, while D-NMA was used at a dose of 5 mg per mouse per day.
[f] NK cell depletion or treatment with L-NMA resulted in increases of virus titers compared to those in infected controls ($P < 0.05$).
[g] NK cell depletion or treatment with L-NMA resulted in increases of virus titers compared to those in infected controls ($P < 0.005$).
[h] Treatment with L-NMA resulted in increases of virus titers compared to those in the infected controls ($P = 0.1$).

This lends credence to our experiments, as the regulation of MCMV in the liver seems to be by a perforin-independent mechanism. Furthermore, Kagi et al. have shown in a T cell-dependent system, the amount of bacteria in the spleens of 5-day *Listeria monocytogenes*-infected perforin 0/0 mice was significantly higher than that in perforin +/+ mice, but the bacterial loads in the livers of both strains of mice were similar. This suggests that the T cell-mediated regulation of *Listeria* species in the spleen but not in the liver was perforin dependent (13).

Depletion of NK cells with either anti-aGM$_1$ antiserum or anti-NK1.1 MAb has always resulted in increases in both splenic and liver MCMV titers in BALB/c, BALB/c SCID, C57BL/6, or C57BL/6 SCID mice (6, 38, 39, 41). However, depletion of NK cells in perforin 0/0 mice with anti-NK1.1 MAb resulted only in the increase of liver MCMV titers (Table 3). Such a result when taken in context with the above-mentioned experiments is consistent with the regulation of MCMV in the spleen being mediated via a perforin-dependent mechanism, and the removal of NK cells in the perforin 0/0 mice should not affect the splenic titers. However, MCMV in the livers of perforin 0/0 mice is controlled by NK cells via a perforin-independent mechanism, as the removal of NK cells in the mice abrogated the resistance in that organ.

Orange et al. have shown that NK cells in C57BL/6 mice synthesize IFN-γ and regulate MCMV in the liver, but they did not report data for the spleen (23). Our results confirm their work with the liver but show that this cytokine has a negligible antiviral role in the spleen under these conditions of infection. Our analyses are focused on 3 days post-MCMV infection of C57BL/6 mice, at a period when NK1.1$^+$ NK cells are exhibiting their peak NK cell activity. However, Heise et al. have shown that in a 7-day MCMV infection of CB17 SCID mice, IFN-γ depletion increased MCMV titers in both the spleen and liver (9). The CB17 SCID mice used in their analyses were NK1.1$^-$ and *Cmv-1^s*, and this might not allow for the dichotomy in mechanisms used in different organs in the regulation of MCMV by NK1.1$^+$, *Cmv-1^r* mice. It is also likely that in this lengthier time of infection, the enhanced titers of virus in the liver would seed the spleen with more virus, eventually leading to higher titers in that organ. Interestingly, it was also reported in the same study that the depletion of TNF-α resulted in increases in splenic but not liver MCMV titers (9).

The B6→IFN-γR$^{0/0}$ mice experiments further reinforced the results obtained with mice treated with anti-IFN-γ Abs. The liver cells of B6→IFN-γR$^{0/0}$ mice do not have the ability to respond to IFN-γ, and the depletion of NK cells from day 3 MCMV-infected B6→IFN-γR$^{0/0}$ mice increased only the splenic but not the liver titers. It is possible that the increase in titers in the spleens of B6→IFN-γR$^{0/0}$ mice after NK cell depletion could be explained by an IFN-γ-mediated mechanism, as the spleens of these mice became repopulated by the donor immune cells that would have the ability to respond to IFN-γ. However, IFN-γR$^{0/0}$ mice reconstituted with perforin 0/0 bone marrow cells showed no increase in splenic MCMV titers after NK cell depletion, arguing against that interpretation. Also, spleen cells from normal unreconstituted perforin 0/0 mice have the ability to respond to IFN-γ, but after NK cell depletion, titers of MCMV in the spleen are not enhanced (Table 3). It is interesting to note that the depletion of NK cells in B6→129 chimeric mice resulted in increases in both splenic and liver MCMV titers (Table 5), suggesting that NK cell-dependent factors other than *Cmv-1* are missing or nonfunctional in the 129 strain, factors which may play important roles in the in vivo regulation of the virus.

Treating mice with L-NMA, a competitive inhibitor of NOS, enhanced MCMV titers in the liver but not in the spleen. This set of data adds further evidence that IFN-γ is more important in the liver, as one of the ways IFN-γ can exert its antiviral actions is through the induction of NOS to produce NO. Interestingly, the expression of NO in the spleen but not in the liver correlated with the resistance to blood-stage malaria in C57BL/6 mice (11). However, in a 5-day ectromelia virus infection, the control of the virus by NO was reported in both the spleen and the liver (16), suggesting that, depending on the pathogen, the productive function of NO may be an organ-dependent phenomenon or a general occurrence.

A selective lysis of virus-infected cells would require the NK cell to bind to the target cell, but regulation of the infection via the production of antiviral cytokines may just entail the cytokine-activated NK cell to produce the cytokines without contact between the NK cell and the virus-modified target cell. Target cells in the spleen may be more sensitive to NK cell-mediated lysis than target cells in the liver. Reports on the natural targets of NK cell cytotoxicity in vivo have been limited to lymphocytes, lymphoma cells, and cells of hematopoietic origins, and most demonstrations of selectivities in NK cell recognition and lysis have used lymphocyte targets (4). Early

work by Wu et al. showed that, 3 days post-MCMV infection, T and B cells isolated from blood constituted the major fraction of cells infected with MCMV (43). Furthermore, normal mice by 3 days post-MCMV infection have 100-fold more virus in the spleen than do SCID mice (38). These results suggest that lymphocytes are targets of MCMV infection in the spleens, and such targets may be good candidates for NK cell killing. On the other hand, in the liver hepatocytes constitute the major fraction of the infected cells. Taking into consideration the constraints on NK cell movement into a solid organ like the liver, the production of antiviral cytokines may be the most efficient way to regulate MCMV therein.

Scalzo et al. have shown the importance of $Cmv-1^r$ in the regulation of MCMV by NK cells in the spleen (27, 28). In all the experiments performed here, the presence of the $Cmv-1^r$ gene is a prerequisite. Finding the $Cmv-1$ gene within the NK gene complex might suggest that the yet identified $Cmv-1$ gene product may be a receptor molecule associated with the cytotoxic function of NK cells. Generally, NK cells tend to lyse target cells that are expressing low levels of MHC class I antigens on their cell surfaces instead of cells that are expressing higher levels (18, 36). A virus may alter MHC class I expression in the virus-infected cells via global or selective inhibition of protein synthesis, by encoding proteins that interfere with the transport of the MHC to the cell surface, by qualitatively altering MHC class I antigens by changing the peptides in the binding groove, or by preventing IFN from upregulating class I expression (4, 40). However, NK cells in β_2-microglobulin-deficient mice, which do not express MHC class I molecules in the correct conformation, control MCMV infection normally in the spleen (34). This may suggest that the regulation of MCMV may not require the recognition of MHC class I molecules by NK cells.

It is possible that $Cmv-1$ might encode a receptor that recognizes MCMV-infected cells or even MCMV itself. If all NK cells from C57BL/6 mice express $Cmv-1$, it would suggest that all the NK cells have the ability to utilize different mechanisms to control MCMV in different organs. On the other hand, if only a certain subset of NK cells express $Cmv-1$, this would suggest that the particular subset of NK cells are very important in the spleen, while in the liver, other NK cell subsets may suffice. One can postulate that in MCMV infection of C57BL/6 mice, a subset of NK cells may traffic to the liver and control the infection there. The cloning of the $Cmv-1$ gene and the identification of the NK cell populations in the different peripheral organs may shed more light on the dichotomy in mechanisms used by NK cells in different organs to regulate MCMV.

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