Requirement For Natural Killer Cell–produced Interferon γ in Defense against Murine Cytomegalovirus Infection and Enhancement of This Defense Pathway by Interleukin 12 Administration

By Jordan S. Orange,* Baoping Wang,‡ Cox Terhorst,‡ and Christine A. Biron*

*From the *Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912; and the ‡Division of Immunology, Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts 02115*

Summary

The presence of natural killer (NK) cells contributes to early defense against murine cytomegalovirus (MCMV) infection. Although NK cells can mediate in vivo protection against MCMV, the mechanism by which they do so has not been defined. The studies presented here evaluate cytokine production by NK cells activated during MCMV infection and the role of NK cell–produced cytokines in early in vivo antiviral defenses. Experiments with normal C57BL/6, T cell–deficient C57BL/6 nude, and severe combined immunodeficient mice lacking T and B cells demonstrated that both interferon γ (IFN-γ) and tumor necrosis factor (TNF) production were induced at early times after infection with MCMV. Conditioned media samples prepared with cells from these mice, on day 2 after infection, produced 11–43 pg/million cells of IFN-γ and 12–19 pg/million cells of TNF as evaluated by specific protein enzyme-linked immunosorbent assays. Studies in the NK- and T cell–deficient mouse line, E26, in mice that had been depleted in vivo of NK cells by treatment with antibodies eliminating NK cells, anti–asialo ganglio-*N*-tetraosylceramide or anti-NK1.1, and with populations of cells that had been depleted of NK cells by complement treatment with the anti–NK cell antibody, SW3A4, demonstrated that NK cells were solely responsible for the IFN-γ but were not required for TNF production. The in vivo absence of NK cells was accompanied by increased viral hepatitis and viral replication in both immunocompetent and immunodeficient mice, as well as decreased survival time of immunodeficient mice. In vivo treatments with antibodies neutralizing IFN-γ demonstrated that this factor contributed to the NK cell–mediated antiviral defense and reduced the measured parameters of viral defense to levels indistinguishable from those observed in NK cell–deficient mice. These effects appeared to be independent of cytolytic activity, as NK cells isolated from anti–IFN-γ–treated mice mediated killing at levels comparable to those observed in control-treated mice. The consequences of interleukin 12 (IL-12) administration, a known potent inducer of IFN-γ production by NK cells, were evaluated in MCMV-infected mice. Low IL-12 doses, i.e., 1 ng/d, increased NK cell cytotoxicity and IFN-γ production up to twofold and resulted in improved antiviral status; virus-induced hepatitis was decreased as much as fivefold, and viral burdens were decreased to levels below detection. The beneficial effects of IL-12 treatment were prevented by depleting either NK cells or IFN-γ. As infection of severe combined immunodeficiency mice was exacerbated by anti–IFN-γ administration and benefited from IL-12, the antiviral effects of both administered IL-12 and NK cell–produced IFN-γ in vivo could be independent of T and B cells. The results elucidate a cytokine-mediated mechanism for antiviral function of NK cells in vivo. Furthermore, they demonstrate the therapeutic efficacy of IL-12 administration in the context of an acute viral infection.

Murine cytomegalovirus (MCMV)[1] is a herpesvirus with considerable homology to human CMV on both biological and molecular levels (1). Acute infection of immunodeficient or susceptible mice with MCMV can lead to profound hepatitis (2), pneumonia (3), bone marrow toxicity (4), and multiorgan failure (5). The immune response in mice acutely infected with MCMV is characterized by an early activation of NK cell cytotoxicity (6) and a later acti-

[1]*Abbreviations used in this paper:* AGM1, asialo ganglio-*N*-tetraosylceramide; LCMV, lymphocytic choriomeningitis virus; MCMV, murine cytomegalovirus; mrIL-12, murine rIL-12.

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vation of T cells (7), peaking on days 3–5 and 7–10 after infection, respectively. NK (8–10), $CD4^+$ T (11, 12), and $CD8^+$ (13) T cell responses have all been shown to play important roles in defense against MCMV infection. $CD8^+$ T cells mediate MHC class I–restricted lytic activity and are responsible for later peripheral clearance of virus (13, 14). $CD4^+$ T cells produce IFN-γ and TNF, which are responsible for clearance of MCMV from salivary glands (15, 16). NK cells mediate protection by regulating viral load at early times after MCMV infection (9).

Although it is clear that NK cells can control MCMV replication, the mechanisms by which they do so are not well understood. NK cells may contribute to the in vivo regulation of virus replication by killing infected cells (17). They may alternatively or additionally make cytokines to inhibit viral replication. Both IFN-γ and TNF have direct antiviral effects on MCMV replication (18). Under appropriate conditions, NK cells can be activated to make IFN-γ and/or TNF (19). However, cytokine production by NK cells and a resulting role of any produced cytokines on antiviral states have not yet been examined.

The studies presented here were undertaken to evaluate cytokine production by NK cells activated during MCMV infection and to determine the contribution of endogenous NK cell cytokine production to regulation of viral replication and virus-induced disease. In addition to evaluating the basal expression and function of NK cell–produced cytokines, the therapeutic effects of IL-12 administration were examined. This factor was evaluated because it has been shown to induce IFN-γ and TNF production in vivo (20–22), to activate NK cell killing in vitro (23) and in vivo (22), and to stimulate NK cell production of IFN-γ and TNF in vitro (24, 25) (for review see reference 26). The results of our studies conclusively demonstrate that NK cells are induced to express IFN-γ during MCMV infection, and that this factor is a major contributor to NK cell–mediated protection against MCMV infection. They also show that low-dose IL-12 administration, primarily as a result of inducing increased IFN-γ production by NK cells, can enhance NK cell–mediated defense against MCMV infection. Taken together, the studies define a mechanism for antiviral protection mediated by NK cells and demonstrate beneficial consequences of IL-12 administration in defense against viral infections.

Materials and Methods

Mice. Specific pathogen-free male C57BL/6 (C57BL/6NTacfBR), Swiss athymic nude (Tac:N:NIHS-nufDF), C.B-17-SCID (C.B-17/IcrTac-scidfDF), and C57BL/6-NUDE (C57BL/6NTac-nufDF N9) mice were obtained from Taconic Inc. (Germantown, NY). Male C57BL/6J mice homozygous or heterozygous for the nude gene were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were 5–10 wk old when used in experiments. The NK- and T cell–deficient E26 mouse line, transgenic for the human CD3ε gene, was established as described (27) and bred homozygous for the transgene (+/+) under strict isolation in our pathogen-free breeding facility at Brown University (Providence, RI). The facility is monitored serologically by evaluation of control mice. Male and female E26 mice were between 6 and 20 wk of age when used in experiments. Virus- and antibody-free CD-1 (Crl:CD-1[ICR]BR) mice were obtained from Charles River Laboratories (Wilmington, MA) to prepare viral stocks.

Virus and Virus Titration. MCMV V70 CD1 salivary gland extract was kindly provided by Dr. Mary Jane Selgrade (Environmental Toxicology Division, U.S. Environmental Protection Agency, Triangle Park, NC). Subsequent stocks of virus were prepared as a 10% (wt/vol) homogenate of salivary glands from infected CD-1 or C57BL/6 mice (28). MCMV was quantitated by plaque assay on monolayers of mouse embryo fibroblasts. MCMV titers were assessed by weighing and homogenizing left lateral liver lobes. Duplicate log dilution samples of organ supernatants were used to infect mouse embryo fibroblast monolayers, which were then overlayed with 1× medium 199 containing 5% heat-inactivated fetal bovine serum, 10,000 U nystatin (GIBCO BRL, Gaithersburg, MD), 100 U penicillin/100 μg streptomycin, 2 mM glutamine, and 0.6% low–gelling temperature agarose (FMC Corp. Bioproducts, Rockland, ME). Cell monolayers were incubated for 4.5 d at 37°C in 5% CO_2 and stained with 0.1% crystal violet in 1% formaldehyde. Virulence of MCMV was tested by analyzing hepatic pathology at 2 d after infection of mice.

Antibodies and Cytokine ELISAs. Rabbit polyclonal antibody against asialo ganglio-*N*-tetraosylceramide (AGM1) was obtained from Wako Chemicals (Richmond, VA). Rabbit IgG (Sigma Chemical Co., St. Louis, MO) was used as a control. PK136 and SW3A4 hybridomas, producing a mouse IgG2b specific for NK1.1 (29) and IgM specific for the SW3A4 determinant of NK1.1 (30), respectively, were kindly given by Dr. Vinay Kumar (Southwestern Medical School, Dallas, TX). Anti–IFN-γ XMG1.2 and isotype control GL113.1 hybridomas were generously provided by Dr. Robert Coffman (DNAX Research Institute, Palo Alto, CA). Filter-sterilized ascites from PK136, control P3NS1 (31), XMG1.2, and GL113.1 as well as cell culture supernatants from SW3A4 were used. Antibodies were quantitated by specific IgG subclass ELISA and, when appropriate, were additionally quantified by protein assay (Pierce, Rockford, IL), flow cytometric analysis of binding, and in vivo titration for elimination of cells or functions.

TNF was quantitated in biological samples by specific sandwich ELISA as described (21). IFN-γ was detected by sandwich ELISA using rat anti–IFN-γ XMG1.2 as capture reagent and rabbit anti–mouse IFN-γ polyclonal serum (the kind gift of Dr. Philip Scott, University of Pennsylvania, Philadelphia, PA) as detecting reagent. TNF and IFN-γ standards were from R&D Systems (Minneapolis, MN) or PharMingen (San Diego, CA). Sensitivity of the IFN-γ and TNF ELISAs were 39 pg/ml and 19 pg/ml, respectively.

In Vivo Treatment Protocol. Murine recombinant IL-12 (mrIL-12), batch number MRB 630717292-A, specific activity 8.4×10^6 U/mg with endotoxin contamination <1.8 EU/ml (as measured by *Limulus* amebocyte assay), was produced by the Mammalian and Microbial Cell Sciences and Process Biochemistry groups of Genetics Institute, Inc. (Cambridge, MA) and was kindly provided by Dr. Stanley Wolf. Frozen microgram aliquots of IL-12 stock were diluted to 1 ml with 0.45-μm filter-sterilized 10% C57BL/6 male serum in PBS at the start of an experiment and stored at 4°C for individual experiment durations. Dilutions of mrIL-12 used for injections were made from this stock into sterile 5% C57BL/6 male serum in PBS. Animals were given either vehicle or 1 ng mrIL-12 in vehicle the day before infection

and on the day of infection (day 0) 3 h before injection of 2×10^5 or 4×10^5 PFU MCMV and again on each day after infection until the day before killing. Selection of the 1-ng/d dose of IL-12 was based upon dose response experiments in which 0.1, 1, 10, 100, and 1,000 ng/d were administered, and results were analyzed on days 1, 2, 3, and 5 after infection. In survival experiments, animals were treated with 1 ng mrIL-12 daily until day 5, and then every 3 d until death.

Analyses of the role of NK cells or IFN-γ were performed by administration of cell-depleting or neutralizing antibodies the day before the first mrIL-12 injection. In day 2 and 3 experiments, an additional injection of antibody was given on day 1 after infection. To deplete NK cells, 0.5 mg anti–AGM1 or 1 mg of PK136 was given 1 d before IL-12 treatment and again before cytokine injection on day 1 after infection. In survival experiments, 0.25 mg of anti-AGM1 was given on the day before beginning treatment and again on day 3 after infection. These protocols were shown to deplete >95% of NK cell subsets. IFN-γ was neutralized by administration of 1 mg XMG1.2 the day before first IL-12 treatment and 0.25 mg on day 1 after infection. In survival experiments, 1 mg XMG1.2 was given the day before treatment and 0.5 mg was given every 7 d. All experiments contained parallel control groups that used rabbit IgG for anti-AGM1, P3NS1 for PK136, and GL113.1 for XMG1.2.

Preparation and Evaluation of Biologic Samples. Splenic leukocytes were prepared and cytotoxicity against YAC-1 target cells was measured in ^{51}Cr release assay as previously described (32). NK cell populations were measured by flow cytometry based on staining of $NK1.1^+$ $CD3^-$ cells (33). Conditioned media were prepared by 24 h incubation of 5×10^6 splenic leukocytes/ml, in RPMI 1640 medium (GIBCO BRL), supplemented with 2 mM glutamine, 100 U penicillin/100 μg streptomycin, and 1% heat-inactivated fetal bovine serum, at 37°C in 5% $C0_2$. Where necessary, supernatants were concentrated 5- to 10-fold using Centricon or Centriprep 10 concentrators (Amicon Inc., Beverly, MA). Samples were tested in ELISA for presence of IFN-γ and TNF. Results are reported as picograms of cytokine per million cells. Limits of detection were ~0.8 pg/million cells and 0.4 pg/million cells for IFN-γ and TNF production, respectively. In some experiments, NK cells were removed from total splenic leukocytes before preparation of conditioned medium by treatment with SW3A4 antibody and rabbit complement (Pel-Freez Biologicals, Rogers, AR).

Whole livers were removed intact from mice and examined for the presence of macroscopic necrotic foci. A necrotic focus was defined as an isolated region of necrotic liver tissue, ≥1 mm^2, that appeared white upon gross examination. Left and right median lobes of the liver were prepared for histology by fixation in

Table 1. *NK Cell Cytotoxicity During MCMV Infection*

Mouse strain	Day after infection	Treatment*	Number of mice	Splenic leukocyte yield‡	% YAC-1 lysis§	
					100 E/T	33 E/T
C57BL/6	0	None	7	56 ± 1	6.8 ± 1.9	3.7 ± 1.5
	2	Vehicle	12	64 ± 4	23.6 ± 3.4	13.1 ± 2.0
		IL-12	12	65 ± 3	31.8 ± 3.7‖	20.6 ± 2.0‖
	3	Vehicle	5	68 ± 14	20.2 ± 5.2	11.4 ± 3.6
		IL-12	5	91 ± 8	19.2 ± 3.2	11.0 ± 2.2
C.B-17 SCID	0	None	4	12 ± 2	0.4 ± 0.2	0.8 ± 0.3
	2	Vehicle	3	15 ± 4	24.0 ± 4.9	13.0 ± 4.7
		IL-12	3	17 ± 1	39.5 ± 1.6‖	24.6 ± 2.2‖
	3	Vehicle	3	17 ± 4	42.5 ± 1.1	28.1 ± 4.9
		IL-12	3	11 ± 1	37.0 ± 2.8	26.6 ± 1.0
C57BL/6 nude	0	None	2	65 ± 4	11.0 ± 1.8	3.1 ± 1.6
	2	Vehicle	3	66 ± 2	42.7 ± 12.2	29.7 ± 8.9
		IL-12	2	44 ± 1	61.6 ± 3.2‖	48.4 ± 4.9‖
	3	Vehicle	2	71 ± 1	47.0 ± 0.1	34.0 ± 1.1
		IL-12	2	78 ± 6	42.5 ± 5.6	29.8 ± 7.6
E26	0	None	4	76 ± 1	2.4 ± 0.7	0.9 ± 0.7
	2	Vehicle	4	41 ± 8	0.4 ± 0.4	0.6 ± 0.3
		IL-12	5	46 ± 6	1.4 ± 0.2	0.8 ± 0.4
	3	Vehicle	4	49 ± 9	1.0 ± 0.5	1.0 ± 0.1
		IL-12	6	43 ± 4	0.2 ± 0.3	1.0 ± 0.3

*Mice were treated with 1 ng/d mrIL-12 or vehicle as described in Materials and Methods.
‡Splenic leukocyte yield in millions of cells per spleen ± SE.
§YAC-1 lysis as measured in ^{51}Cr release assay ± SE. Data shown were obtained at E/T cell ratios of 100 or 33 to 1.
‖IL-12 treatment mediated significant enhancement conmpared with vehicle control, $P < 0.05$.

Table 2. *IFN-γ and TNF Production in Picograms/Million Cells During MCMV Infection*

Mouse Strain	Day after infection	Control-treated*		IL-12–treated*	
		IFN-γ	TNF	IFN-γ	TNF
C57BL/6‡	0	<0.9 ± 0.0 (4)	5.8 ± 1.5 (4)	<0.9§ (1)	5.0§ (1)
	2	11.2 ± 2.9 (5)	12.3 ± 1.8 (5)	22.4 ± 3.7‖ (5)	14.2 ± 3.4 (5)
	3	1.1 ± 0.1 (2)	13.9 ± 3.5 (2)	6.9 ± 0.1 (2)	19.3 ± 5.2 (2)
C.B-17 SCID¶	0	<0.9	4.9	nd	nd
	2	42.6	18.6	100.0	23.4
	3	<0.9	3.5	<0.9	4.1
E26**	0	<0.9 ± 0.0 (4)	4.1 ± 0.4 (4)	nd	nd
	2	<0.9 ± 0.1 (3)	10.6 ± 0.9 (3)	<0.9 ± 0.1 (3)	14.1 ± 0.9 (3)
	3	<0.9 ± 0.0 (4)	8.6 ± 2.5 (4)	<0.9 ± 0.0 (4)	6.0 ± 1.2 (4)

*Values for cytokine production are given in picograms/million cells in culture as explained in Materials and Methods. Where appropriate, values are ± SE. Parentheses identify the number of independent samples tested. All tests were carried out in duplicate.
‡Conditioned media from C57BL/6 mice were prepared either from individual animals or from two or three pooled animals, such that results shown were obtained from 5–12 animals per point.
§Point consists of three pooled animals.
‖IL-12 treatment mediated significant enhancement compared with vehicle control, $P < 0.05$.
¶Conditioned media samples from C.B-17 SCID mice were prepared with cells pooled from three to four animals.
**Conditioned media samples from E26 +/+ mice were prepared either from individual animals or from two or three pooled animals such that results shown were obtained from four to eight animals per point.

10% buffered formalin. Paraffin-embedded sections (5 μM) were prepared, hematoxylin and eosin stained, and analyzed microscopically. Inflammatory foci were defined as discrete clusters of cells containing between 6 and 60 individual cells. Inflammatory foci were quantitated by counting clusters in 8 mm^2 of representative liver tissue.

Statistical Analysis. Data were analyzed by Student's *t* test in all experiments except survival curves, which were analyzed by Wilcoxon signed rank correlation.

Results

Induction of NK Cell–mediated Cytotoxicity during MCMV Infection and Effects of IL-12 Treatment on This Response. NK cell activity during MCMV infection of immunocompetent C57BL/6 mice, as measured by cytotoxicity against YAC-1 target cells in ^{51}Cr release assay, became apparent at day 1 after infection, peaked on days 3–5 after infection, and returned to almost baseline levels by day 7 after infection (data not shown). The percentage of NK cells in the spleen, as measured by flow cytometry, did not change significantly over the course of infection. Administration of 1 ng/d IL-12 starting the day before infection did not modify the numbers of NK cells or extend the longevity of the NK cell response; however, NK cell–mediated cytotoxicity was accelerated such that the peak occurred on day 2, instead of days 3–5 after infection (Table 1, C57BL/6). Compared with vehicle-treated controls, IL-12–treated mice had ~35–55% greater NK activity on day 2 after infection.

To isolate the NK cell response from other lymphocyte responses, T and B cell–deficient SCID, T cell–deficient nude, and NK- and T cell–deficient E26 (27) mice were examined. As in the immunocompetent animals, NK cell cytotoxic activity peaked on day 3 after infection in control-treated SCID and nude mice, and IL-12 treatment accelerated NK cell responses in these strains such that peak killing was present on day 2 after infection (Table 1, SCID and nude). There was an average of ~1.5 fold more cytotoxicity in IL-12–treated nude and SCID mice on day 2 than in vehicle-treated controls. In E26 mice deficient for both NK and T cells, NK cell activity was absent on days 2 and 3 after infection with MCMV, and IL-12 treatment failed to induce significant cytotoxic activity (Table 1, E26). These observations demonstrate that administration of 1 ng/d IL-12 is capable of accelerating NK cell cytotoxic responses to infection in immunocompetent or immunodeficient, but not NK cell–deficient mice.

Induction of IFN-γ and TNF during MCMV Infection and Effects of IL-12 Treatment. To measure early IFN-γ and TNF production during MCMV infection and to characterize the effects of IL-12 treatment on these cytokine responses, ex vivo splenic leukocytes were used to prepare conditioned media for testing in ELISAs. In C57BL/6 mice, MCMV infection induced production of both IFN-γ and TNF on day 2 after infection (Table 2, C57BL/6). By day 3 after infection, IFN-γ production decreased to marginally detectable levels, while TNF production was still at induced levels. As increased TNF production, but not IFN-γ production, was present on day 1 after infection, induction of TNF appeared to precede IFN-γ (data not shown). Administration of 1 ng/d IL-12 significantly enhanced the IFN-γ response at day 2 after infection; there was a twofold increase in IFN-γ production on a per-cell

basis (Table 2). IL-12 did not significantly increase TNF levels. IL-12 enhancement of the day 2 IFN-γ response was also evident in T and B cell deficient SCID mice. The IFN-γ levels in SCID mice appeared higher than in C57BL/6 mice when measured on a per-cell basis; however, there were approximately three- to fourfold fewer cells in the SCID spleens. Thus, levels of IFN-γ production per spleen were similar. These data demonstrate that IFN-γ and TNF are produced at early times after infection with MCMV. Furthermore, they show that the IFN-γ response can be significantly increased by administration of IL-12.

Role of NK Cells in Cytokine Production. A variety of approaches were used to determine the NK cell contribution to IFN-γ and TNF production. First, responses in NK- and T cell–deficient E26 mice were examined. In contrast to mice with NK cells, cells from E26 mice did not produce IFN-γ during MCMV infection or MCMV infection with IL-12 treatment (Table 2, E26). E26 mouse TNF responses were comparable to those in NK cell–containing mice. Second, normal C57BL/6 mice or nude mice were NK cell depleted in vitro or in vivo. For in vitro depletion, NK cells were eliminated from isolated splenic leukocyte populations using SW3A4 and rabbit complement. In C57BL/6 mice either heterozygous or homozygous for the nude mutation, depletion of NK cells reduced production of IFN-γ (Fig. 1 *A*). Production of IFN-γ after IL-12 administration was also dramatically decreased after NK cell depletion. In contrast, elimination of NK cells did not affect TNF production (Fig. 1 *B*). In vivo depletion of NK cells gave equivalent results. Cells isolated from MCMV-infected C57BL/6 and C57BL/6 nude mice treated with anti-AGM1 or anti-NK1.1, to eliminate NK cells, were blocked in IFN-γ production (Fig. 1 *C*). Likewise, IFN-γ production by cells from IL-12–treated, MCMV-infected animals was greatly reduced as a result of in vivo NK cell depletion. Induced TNF production, however, was observed regardless of the presence or absence of NK cells (Fig. 1 *D*). These results demonstrate that the IFN-γ produced at early times after infection with MCMV and/or after IL-12 administration at these times is made by NK cells.

Effects of IL-12 on MCMV-induced Liver Pathology and Viral Replication. MCMV replicates to high levels in the liver, and antiviral effects can be measured both by viral load and virus-induced pathology at this site (Tables 3 and 4). Pathology induced by MCMV infection is characterized by a mononuclear cell infiltrate and large areas of hepatic necrosis. Histologically, the cellular inflammation has the form of discrete foci or clusters of cells present throughout the liver. Necrosis is visualized grossly as small white foci on freshly isolated livers, and histologically as large, discrete subcapsular regions of eosinophilic staining.

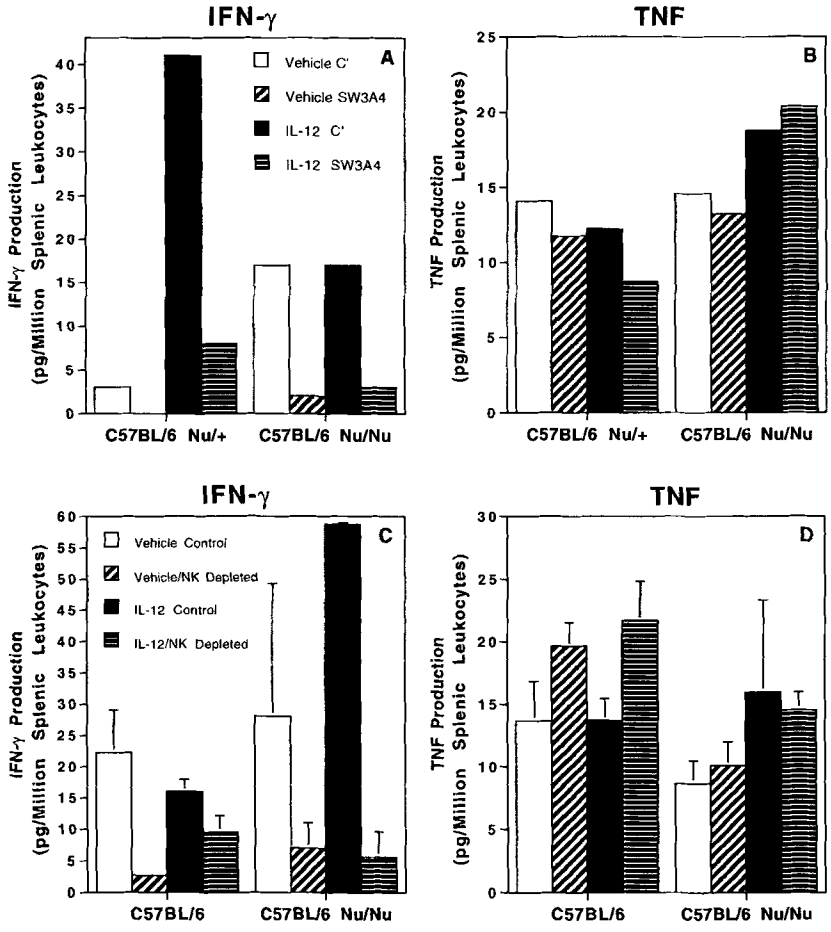

Figure 1. Effects of NK cell depletion on IFN-γ and TNF production in response to MCMV infection. Mice were depleted of NK cells either in vitro (*A* and *B*) or in vivo (*C* and *D*). In vitro depletions were performed with pooled splenic leukocytes from groups of two C57BL/6 nude/+ or C57BL/6 nude/nude mice on day 3 after infection using SW3A4 and rabbit complement (SW3A4). Rabbit complement was used alone as a control (C'). In vivo NK cell depletions were performed by administration of antibodies against AGM1 or NK1.1 to groups of two or three C57BL/6 and C57BL/6 nude/nude mice, respectively. Samples were evaluated for IFN-γ (*A* and *C*) or TNF (*B* and *D*) by ELISA. Error bars shown are ± SE.

Table 3. *Effects of IL-12 on Antiviral State in Immunodeficient Mice*

Mouse strain	Day after infection	Treatment	Number of mice	Hepatic inflammatory foci*	Hepatic necrotic foci per liver‡	Log viral titer PFU/g liver
C.B-17 SCID	2	Vehicle	4	25 ± 3	BLD	3.50 ± 0.10
		IL-12	5	8 ± 1§	BLD	<2.07 ± 0.09§
	3	Vehicle	2	21 ± 1	BLD	3.44 ± 0.30
		IL-12	2	4 ± 1§	BLD	<2.18 ± 0.18§
E26	2	Vehicle	3	14 ± 4	45 ± 10	4.88 ± 0.12
		IL-12	3	17 ± 3	60 ± 13	4.87 ± 0.03
	3	Vehicle	3	15 ± 6	19 ± 10	5.06 ± 0.45
		IL-12	3	22 ± 1	31 ± 1	5.31 ± 0.02

Values are shown ± SE.
*Microscopic inflammatory foci are shown per area liver as described in Materials and Methods.
‡Macroscopic necrotic foci are shown per whole liver as described in Materials and Methods.
§IL-12 treatment mediated significant decrease compared to vehicle control $P < 0.05$.
BLD, below level of detection.

Table 4. *Effects of IL-12 on NK Cell–dependent Antiviral State In Vivo*

Mouse strain	Day after infection	Cytokine treatment	Antibody treatment	Number of mice	Hepatic inflammatory foci	Hepatic necrotic foci per liver	Log viral titer PFU/g liver
C57BL/6	0	None	None	8	0 ± 0	0 ± 0	0.00 ± 0.00
	2	Vehicle	Control	3	69 ± 12	8 ± 5	4.78 ± 0.13
		IL-12	Control	3	22 ± 9*	0 ± 0*	4.26 ± 0.13
		Vehicle	Anti-AGM1	3	33 ± 3	49 ± 21	5.24 ± 0.02‡
		IL-12	Anti-AGM1	3	37 ± 5	51 ± 29	5.00 ± 0.31‡
	2	Vehicle	Control	3	31 ± 5	33 ± 6	4.63 ± 0.16
		IL-12	Control	3	13 ± 1*	1 ± 1*	3.78 ± 0.02*
		Vehicle	PK136	3	18 ± 5	46 ± 17	5.32 ± 0.10‡
		IL-12	PK136	3	8 ± 1	14 ± 10	4.79 ± 0.06‡
	3	Vehicle		5	56 ± 9	7 ± 4	4.30 ± 0.13
		IL-12		5	14 ± 4*	1 ± 4	BLD <2.00*
C57BL Nu/Nu	2	Vehicle	Control	3	43 ± 21	BLD	4.99 ± 0.09
		IL-12	Control	2	26 ± 6	BLD	4.23 ± 0.04*
		Vehicle	PK136	2	21 ± 1	BLD	5.27 ± 0.20‡
		IL-12	PK136	3	24 ± 15	BLD	4.84 ± 0.12
	3	Vehicle		2	37 ± 1	BLD	3.95 ± 0.44
		IL-12		2	14 ± 4*	BLD	2.34 ± 0.18*

Values are shown ± SE.
*IL-12 treatment mediates significant decrease compared with vehicle control, $P < 0.05$.
‡NK cell depletion causes significant increase relative to appropriate control, $P < 0.05$.
BLD, below level of detection.

IL-12 treatment significantly inhibited the development of both pathology and viral replication in the liver. On days 2 and 3 after infection, IL-12–treated SCID mice had >80% reductions in numbers of inflammatory foci per area liver and >1 log decreases in hepatic viral replication to levels below detection (Table 3, SCID). Because gross necrotic foci were not detectable in MCMV-infected SCID mice, there appeared to be a strain-dependent genetic component to the development of this pathological manifestation. IL-12 treatment of immunocompetent C57BL/6 and T cell–deficient C57BL/6 nude mice also resulted in decreased hepatitis and viral replication (Table 4). By day 3 after infection in these mice, numbers of inflammatory foci were decreased by >60% and viral replication was reduced >1.5 logs. In C57BL/6 mice, necrotic foci were present during MCMV infection and decreased by >75% after IL-12 treatment. These data demonstrate that, at early times after infection, IL-12 treatment of mice possessing NK cells enhances the antiviral state.

Requirement for NK Cells in IL-12 Enhancement of the Antiviral State. To examine the role of NK cells in IL-12–mediated enhancement of antiviral responses, NK cell–deficient mice were evaluated. NK- and T cell–deficient E26 mice displayed both inflammatory and necrotic foci on days 2 and 3 after infection (Table 3, E26). The development of hepatitis in these mice correlated with high level viral replication in the liver (Table 3, E26). Treatment of E26 mice with 1 ng/d IL-12 did not alter either the pathology or viral replication in the liver (Table 3, E26). In immunocompetent C57BL/6 or C57BL/6 nude mice, depletion of NK cells with anti-AGM1 or anti-NK1.1 antibodies abolished the beneficial effects of IL-12 administration (Table 4). These data demonstrate that IL-12–mediated enhancement of early antiviral states in immunocompetent or immunodeficient mice requires NK cells.

To determine if the IL-12–mediated enhancement of the antiviral state had long-term benefits in immunodeficient mice, survival experiments were performed. In these experiments, animals were treated with 1 ng/d IL-12 on the day before infection, through day 5 after infection, and every 3 d thereafter. In NK cell–possessing SCID and nude mice, IL-12 significantly extended life span by ~20% (Fig. 2, *A* and *B*). In contrast, IL-12 administration to NK cell–deficient E26 mice had no effect on their survival (Fig. 2 *C*). IL-12 treatment also had no beneficial effect in nude mice depleted of NK cells by anti-AGM1 treatment (Fig. 2 *B*). These observations demonstrate that IL-12 administration is able to prolong survival in immunodeficient mice and that this process is NK cell dependent.

Requirement for NK Cell–produced IFN-γ in Protective Antiviral Responses. To determine the importance of NK cell–produced IFN-γ in defense against MCMV infection, effects of administration of the specific neutralizing anti–IFN-γ mAb XMG1.2 were contrasted with those of the isotype-matched nonspecific control antibody GL113.1. Anti–IFN-γ did not significantly alter the induced NK cell cytotoxicity against YAC-1 target cells (Fig. 3). Anti–IFN-γ also did not affect TNF production nor the ability of cells to produce IFN-γ when production was measured ex vivo (data not shown). Treatment with anti–IFN-γ did significantly inhibit the antiviral state. C57BL/6 mice given anti–IFN-γ had exacerbated parameters of virus-induced pathology; there were sevenfold more necrotic foci and twofold more inflammatory foci in anti–IFN-γ–treated as compared with control–treated mice on day 3 after infection (Table 5). Anti–IFN-γ treatment also resulted in a log increase in viral titers at day 3 after infection. The role of NK cell–produced IFN-γ was specifically examined using SCID mice. As in immunocompetent mice, anti–IFN-γ decreased the antiviral state in SCID mice, evidenced by 1 log increased viral replication on day 3 after infection (Table 5). Interestingly, viral replication in anti–IFN-γ–treated mice was indistinguishable from that in NK cell–depleted animals (Table 4). These data show that IFN-γ is a major contributor to antiviral effects mediated by NK cells during MCMV infection.

To evaluate the importance of IFN-γ production in enhanced NK cell–mediated defense after IL-12 treatment, anti–IFN-γ was given to mice that were cytokine treated and MCMV infected. Similar to depletion of NK cells (Ta-

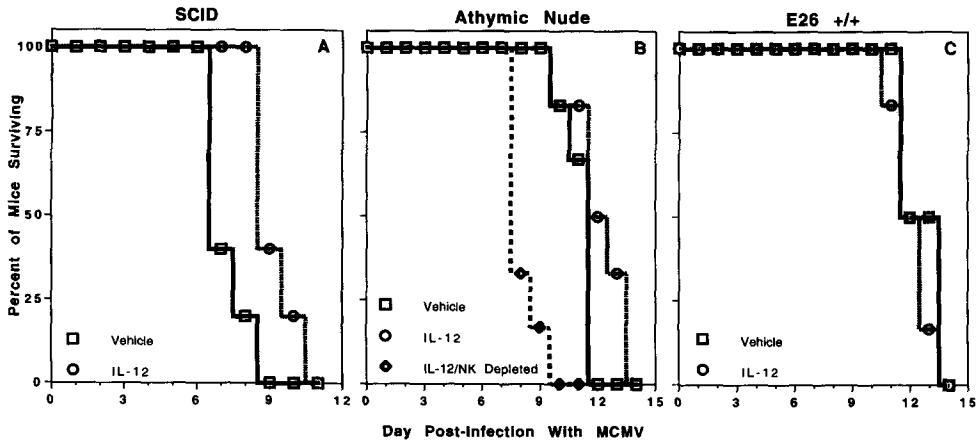

Figure 2. Effects of IL-12 on survival of MCMV-infected immunodeficient mice. (*A*) C.B-17 SCID, (*B*) athymic nude, and (*C*) E26 mice were treated with 1 ng/d IL-12 (—○—) or vehicle (—□—) as described in Materials and Methods. Nude mice were given either antibody against AGM1 (-◇-) or control rabbit IgG in addition to cytokine on the day before infection and on day 3 after infection (*B*). All experimental groups contained between five and seven animals. Prolongation of life span mediated by IL-12 administration was significant compared with vehicle-treated controls in both SCID and athymic nude mice ($P = 0.03$ and 0.06, respectively).

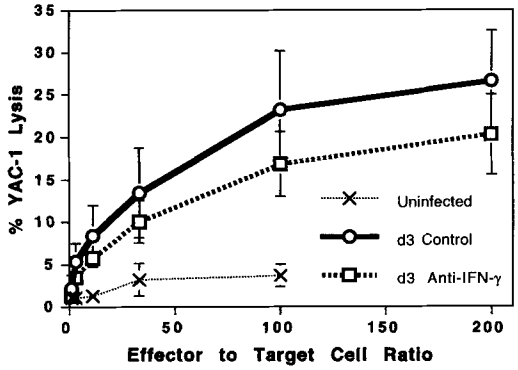

Figure 3. Effects of anti–IFN-γ treatment on NK cell cytotoxic activity. Mice were antibody treated with either XMG1.2 anti–IFN-γ or GL113.1 isotype control on the day before infection and on day 1 after infection. Cytotoxic activity of splenic leukocytes against YAC-1 target cells was evaluated on day 3 after infection in ^{51}Cr release assays. Groups contained three animals each, and error bars shown are ± SE. The difference between the XMG1.2 or GL113.1 data points at respective E:T ratios is not of statistical significance.

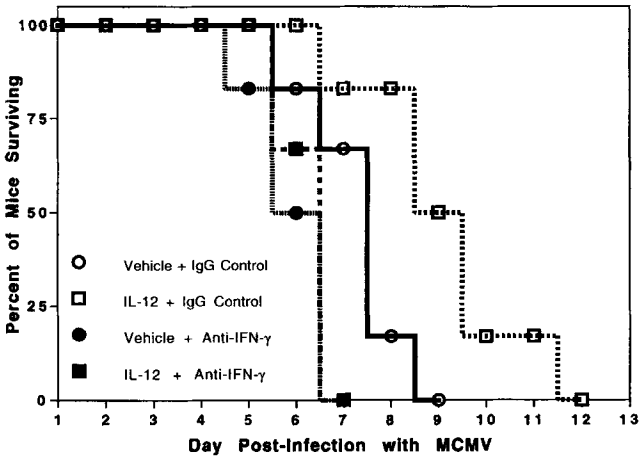

Figure 4. Effects of anti–IFN-γ administration on survival of MCMV-infected, IL-12–treated nude mice. Athymic nude mice were treated with 1 ng/d IL-12 or vehicle as described in Materials and Methods. The anti–IFN-γ antibody, XMG1.2, or isotype control antibody, GL113.1, was administered on the day before treatments began and on day 5 after infection. Groups contained six animals each. Prolongation of life span mediated by IL-12 treatment was significant ($P = 0.02$) compared with vehicle-treated controls. Decrease of life span mediated by anti–IFN-γ treatment was significant in vehicle-treated ($P = 0.06$) and IL-12–treated mice ($P = 0.03$).

ble 4), the beneficial effects of IL-12 treatment were ablated by in vivo neutralization of IFN-γ. In IL-12–treated C57BL/6 mice given anti–IFN-γ, both hepatitis and viral replication were increased; there were threefold more inflammatory foci, 10-fold more necrotic foci, and almost a 2-log increase in viral titer in IL-12–treated mice receiving anti–IFN-γ as compared with IL-12–treated controls (Table 5, C57BL/6). The antiviral effects of IL-12 in SCID mice were also prevented by anti–IFN-γ treatment (Table 5, SCID). Viral titers in all of the anti–IFN-γ–treated animals were similar whether or not they received IL-12. These results demonstrate that the IL-12–induced increase in NK cell–mediated antiviral function is dependent upon IFN-γ.

Experiments were carried out to analyze the importance of NK cell–produced IFN-γ in survival after MCMV infection of immunodeficient nude mice. Similar to NK cell depletion (Fig. 2), anti–IFN-γ treatment of nude mice resulted in >20% reduction of life span (Fig. 4). Prolongation of life induced by IL-12 treatment was prevented by administration of anti–IFN-γ antibodies, and anti–IFN-γ–treated mice died at similar days after infection regardless of

Table 5. *Interferon-γ-mediated Antiviral State During MCMV Infection: Mechanism for Endogenous and IL-12–Induced Protection*

Mouse strain	Day after infection	Treatment	Hepatic inflammatory foci		Hepatic necrotic foci per liver		Log viral titer PFU/g liver	
			Control	Anti–IFN-γ	Control	Anti–IFN-γ	Control	Anti–IFN-γ
C57BL/6	2	Vehicle	61 ± 13	74 ± 13	35 ± 14	98 ± 34*	5.18 ± 0.06	5.43 ± 0.02*
		IL-12	33 ± 7	73 ± 3*	7 ± 1‡	59 ± 11*	4.33 ± 0.10‡	5.47 ± 0.10*
	3	Vehicle	77 ± 1	140 ± 8*	9 ± 7	69 ± 25*	5.05 ± 0.06	5.90 ± 0.16*
		IL-12	34 ± 3‡	110 ± 16*	3 ± 1‡	38 ± 4*	4.03 ± 0.09‡	5.73 ± 0.15*
C.B-17 SCID	2	Vehicle	37 ± 5	50 ± 2*	BLD	BLD	4.87 ± 0.15	5.23 ± 0.08*
		IL-12	23 ± 2‡	46 ± 5*	BLD	BLD	4.24 ± 0.09‡	5.14 ± 0.05*
	3	Vehicle	71 ± 4	99 ± 8	BLD	BLD	4.65 ± 0.04	5.64 ± 0.09*
		IL-12	30 ± 2	88 ± 5	BLD	BLD	3.89 ± 0.14‡	5.52 ± 0.05*

Values shown represent three mice per group ± SE.
*Administration of anti–IFN-γ neutralizing antibodies causes significant increase relative to appropriate control, $P < 0.05$.
‡IL-12 treatment mediates significant enhancement compared with vehicle control, $P < 0.05$.

IL-12 treatment (Fig. 4). These results demonstrate that NK cell–produced IFN-γ is important to the longevity of immunodeficient animals and that beneficial effects of IL-12 require IFN-γ. Taken together with the studies on early viral load and virus-induced pathology, they show that IFN-γ regulation of MCMV replication can be enhanced by IL-12 administration.

Discussion

The studies presented here prove the biological importance of NK cell–produced IFN-γ in protective antiviral responses, demonstrate conditions under which IL-12 can be used therapeutically during a viral infection, and identify NK cell–dependent production of IFN-γ as a mechanism for IL-12–induced antiviral effects. To our knowledge, this is the first demonstration of the importance of cytokine production exclusively by NK cells in protection against viral infection and the therapeutic efficacy of IL-12 during a viral infection.

Demonstration of a role for NK cell–produced IFN-γ in defense against MCMV infection complements and extends work from others demonstrating the following: (*a*) that IFN-γ can directly inhibit MCMV replication in cell culture (15, 18, 34), and (*b*) that this factor is important for late protective T cell responses to MCMV infection (15, 35). Our experiments show that NK cell–produced IFN-γ regulates MCMV replication and MCMV-induced pathology in both immunocompetent and immunodeficient mice (Table 5), and it contributes to the establishment of mean survival times in T cell–deficient mice (Fig. 4). Thus, our studies extend the characterization of the role of IFN-γ in protection against MCMV infection to early times after infection preceding T cell responses or in the absence of T cells.

Taken together with the earlier culture studies (18, 34), our results are consistent with a model whereby IFN-γ mediates protection by directly inhibiting MCMV replication in infected cells. Furthermore, although the studies presented here do not directly examine a role for TNF in the antiviral effects, they do demonstrate that TNF is being produced at times of IFN-γ production (Table 2 and Fig. 1). As others have shown that TNF can also act alone and/or in synergism with IFN-γ to inhibit MCMV replication in culture (18), IFN-γ may synergize with this factor at early times during infection to directly regulate MCMV replication in vivo. IFN-γ can also activate macrophages (36), and activated macrophages have increased TNF production (37). Although it is possible that IFN-γ also may be amplifying TNF production in vivo, our data on ex vivo production of TNF by cells isolated from anti–IFN-γ–treated mice suggest that this does not explain the induced levels of TNF detected (see Results). However, as IFN-γ has also been shown to increase sensitivity to TNF (38), this factor might alternatively enhance antiviral defenses by increasing endogenous cell sensitivity to TNF. Thus, the antiviral effects of NK cell–produced IFN-γ may be a result of in vivo synergism between TNF and IFN-γ.

The role of NK cells in defense against MCMV has been previously established through a variety of independent approaches. First, endogenous NK cell cytotoxic activity correlates with resistance to MCMV infection (6). Second, beige mutant mice with defective NK cells are more susceptible to lethal MCMV infection (8). Third, in vivo depletion of NK cells by antibody treatment results in increased sensitivity to MCMV infection (9, 10). Thus, the evidence supporting the role of NK cells in defense against MCMV infection is persuasive. It has long been assumed that the mechanism for NK cell–mediated defense is lysis of infected cells. However, there are limited studies testing this hypothesis and no clear in vivo data to support it. In vitro experiments testing sensitivity of MCMV-infected target cells to NK cell–mediated lysis have resulted in conflicting results. In contrast with normal cells, MCMV-infected fibroblasts have only moderate to low sensitivity to NK cell–mediated cytotoxicity, but they do not acquire resistance to NK cell–mediated lysis upon exposure to IFN-β (17). These results have been interpreted to suggest that NK cells can specifically kill MCMV-infected cells in the presence of in vivo IFN-α/β, but the pathway has not been conclusively demonstrated in vivo. Although chemical induction of NK cell activity has been associated with enhanced survival after MCMV infection (39), it is not clear if any other parameters of NK cell or cytokine responses are altered by this treatment. Even if some of the NK cell–mediated effects against MCMV infection are a consequence of infected cell lysis, the studies presented here definitively show that IFN-γ is a major contributor to NK cell–mediated defense against MCMV. The IFN-γ–mediated effect does not appear to be a result of induction of NK cell–mediated lysis (Fig. 3). Although it is not possible to rule out IFN-γ–dependent changes in target cell range or in vivo NK cell trafficking, our results are consistent with the earlier observation that administration of IFN-γ can substitute for NK cells in defense against mortality (40). They suggest that NK cell–produced IFN-γ mediates protection through mechanisms independent of target cell lysis.

Activation of NK cell cytotoxic activity and IFN-γ production correlated during MCMV infection, but this may not be the NK cell response to all viral infections. Although we have conclusively demonstrated IFN-γ mRNA expression by a proportion of NK cells activated to mediate elevated killing at early times after infection with lymphocytic choriomeningitis virus (LCMV) (41, and Salazar-Mather, T., R. Ishikawa, and C. A. Biron, manuscript submitted for publication), we have been unable, using the methods presented in this report, to demonstrate detectable IFN-γ protein production by LCMV-induced NK cells (Orange, J. S., and C. A. Biron, manuscript submitted for publication). It is interesting to note that, although NK cells are important in defense against MCMV, they have no demonstrable role in controlling early LCMV infection (42, 43). Thus, the induction of NK cell lytic activity and NK cell–mediated antiviral effects do not correlate. Our studies in LCMV as compared with the MCMV infection

suggest that there is an association between IFN-γ production by NK cells and NK cell–mediated defense against a virus.

The question of why NK cells are activated to mediate killing and produce IFN-γ in response to MCMV infection arises from our results. This could be the result of either the presence of additional activation signals (i.e., and/or the absence of negative signals). We are currently exploring this question in depth. The studies presented here show NK cell–independent production of TNF (Table 2 and Fig. 1). A distinguishing feature of MCMV infection is that macrophages, in particular, are targeted by the virus (44). Direct infection of macrophages most likely results in their early activation and accounts for the rapid TNF induction. MCMV infection of macrophages has also been shown to result in IFN-α/β production (45), and these factors are known to activate NK cell killing (46, 47) but are poor inducers of cytokine production by NK cells (Biron, C. A., unpublished results). As macrophages can also be an excellent source of IL-12 (48) and this factor can activate IFN-γ production by NK cells (26, 49–50), MCMV infection of macrophages may elicit an IL-12 response to induce NK cell production of IFN-γ. Recent evidence from our laboratory shows that IL-12 is produced in response to MCMV infection and proves that this factor is necessary for the day 2 IFN-γ production during MCMV infection (Orange, J. S., and C. A. Biron, manuscript submitted for publication). Thus, there appears to be cooperation between NK cells and macrophages at early times during MCMV infection to create a protective antiviral state.

Administration of 1 ng/d IL-12 enhanced antiviral states (Tables 3 and 4, and Fig. 2). The enhanced antiviral states were shown to be dependent upon NK cell production of IFN-γ (Table 5 and Fig. 4). The dose of IL-12 used was critical for achieving enhanced protection. Higher doses of IL-12 either had no effect (10–100 ng/d) or had detrimental effects (>100 ng/d) (Materials and Methods, and data not shown). We have previously shown that, during LCMV infection, doses of IL-12 in excess of 100 ng/d synergize with the endogenous immune response to induce systemic TNF (20, 21). A consequence of the systemic TNF production is the elimination of responding $CD8^+$ T cells (21). As this population is responsible for viral clearance, there is also a resulting increase in viral burden. In contrast, low doses (1 ng/d) significantly enhance $CD8^+$ T cell responses to either LCMV or MCMV (20). The studies presented here demonstrate that low doses can be beneficial in enhancing early responses to MCMV infection. They underscore the importance of evaluating administration dose in the context of an ongoing endogenous immune response.

Our studies have used the genetically altered E26 mouse line. These mice are stably deficient in both NK and T cells (27). The experiments presented here are the first evaluating the use of these mice as a vehicle for examining NK cell–mediated effects in vivo. The results demonstrate that, during MCMV infection, the NK cell–mediated functions of cytotoxicity (Table 1) and IFN-γ production (Table 2) are missing in these animals. Because the E26 mice are induced to express TNF (Table 2), however, they are clearly responding to the viral challenge. IL-12 treatment of E26 mice did not induce NK cell–mediated functions (Tables 1 and 2), nor did it induce an antiviral state in these mice (Table 3 and Fig. 2). In all parameters evaluated, the E26 mice responses were comparable to those of nude mice rendered NK cell deficient by in vivo antibody treatment (Table 4 and Fig. 2). E26 mice have an advantage over the antibody-treated mice because they are rendered NK cell deficient in a complete and long-term manner as a result of their genetic manipulation.

In conclusion, by using NK cell–deficient mice and antibody-mediated neutralization of IFN-γ in vivo, the studies here demonstrate a cytokine-mediated mechanism for NK cell defense against viral infection. In addition, they show that IL-12 can be effectively used as a therapeutic agent to enhance this antiviral pathway. The results suggest that the contribution of an NK cell–mediated component of defense against viral infection may require the induction of IFN-γ production by NK cells in addition to, or as an alternative to, the activation of NK cell–mediated cytotoxicity.

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Address correspondence to Dr. Christine A. Biron, Division of Biology and Medicine, Box G-B618, Brown University, Providence, RI 02912.

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