Exacerbated Viral Hepatitis in IFN-γ Receptor-Deficient Mice Is Not Suppressed by IL-12

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Both IL-12 and IFN-γ have been implicated as principal inducers of type 1 immune responses required for the elimination of intracellular pathogens, such as viruses. We examined the in vivo antiviral role of both cytokines during coronavirus-induced hepatitis in a mouse hepatitis virus (MHV) model. The absence of IFN-γ function in mice with a targeted disruption of the IFN-γR α-chain gene (IFN-γR −/−) resulted in increased susceptibility to coronaviral hepatitis associated with augmented viral replication and increased hepatocellular injury. The mutant mice showed a type 1 lymphokine response characterized by the normal high IFN-γ and low IL-4 production. Unlike MHV-infected wild-type mice, however, the mutant IFN-γR −/− mice showed no increase in IL-12 *p40* gene expression, similar to that in naive animals. IL-12 treatment failed to restore host resistance in IFN-γR −/− mice, but significantly protected MHV-susceptible C57BL/6 mice against lethal infection, although less than IFN-γ treatment. Mice protected by IL-12 or IFN-γ showed resistance against an otherwise lethal second MHV infection. Our data demonstrate that despite reduced IL-12 gene expression and defective IFN-γR function, virus-induced IFN-γ production can occur. Furthermore, they emphasize the pivotal antiviral role of IFN-γ in protection against acute coronavirus-induced hepatitis. *The Journal of Immunology,* **1996, 157: 815–821.**

Immunity to viruses and other intracellular pathogens is ascribed to type 1 immune responses that are characterized by IFN-γ production (1). Especially early IL-12 production is implicated in the promotion of type 1 cytokine responses, since it augments the proliferative and cytolytic activities of NK and T cells and their capacity to produce IFN-γ. IL-12 is, therefore, considered to be a therapeutic agent against intracellular pathogens, including HIV and other viruses, and is currently being tested in phase I trials (2), although little is known about its antiviral activity in vivo. No beneficial effect of IL-12 was demonstrated in mice infected with influenza virus, encephalomyocarditis virus, or respiratory syncytial virus (3). During experimental LCMV[2] infection, administration of low doses of IL-12 enhanced $CD8^+$ T cell activity and reduced viral replication, while high doses were detrimental to LCMV resistance due to reduction of CTL activity and high TNF-mediated toxicity (4, 5).

A variety of viruses, including lactate dehydrogenase-elevating virus, mouse adenovirus, and mouse hepatitis virus, when inoculated into mice induced transient in vivo IL-12 *p40* gene expression, predominantly in macrophages (6). According to the prevailing view, IL-12 synthesis precedes IFN-γ production; recently, however, it has been suggested that IFN-γ is required for IL-12 production, since macrophages of IFN-γR-deficient mice fail to produce IL-12 after infection with *Mycobacterium* (7).

In the present study, we examined the in vivo antiviral activity and immunomodulatory function of IL-12 and IFN-γ in an experimental mouse model of coronavirus-induced acute hepatitis. Mouse hepatitis virus strain A-59 (MHV-A59) when injected i.p. is mainly hepatotropic and causes lytic infections of liver cells. Immunity to hepatotropic MHV-A59 infection is associated with MHC class II-restricted, IFN-γ-producing CTLs that can confer protection to acutely infected mice upon adoptive transfer; as a result, viral titers in the liver are reduced (8). No evidence was found for activation of class I-restricted CTL (8).

We demonstrate that mutant mice with blocked IFN-γR signaling due to a targeted IFN-γR gene exhibit increased susceptibility to acute coronavirus-induced hepatitis associated with impaired IL-12 *p40* gene expression. Remarkably, these mice produce a normal type 1 lymphokine profile. Treatment with rIL-12 fails to compensate for the IFN-γR gene deletion, but significantly stimulates protective immunity in normal C57BL/6 mice.

Materials and Methods

Mice

The mutant (129/Sv/Ev) mouse strain deficient in IFN-γR α-chain (IFN-γR −/−), generated by gene targeting in murine embryonic stem cells (9), was kindly provided by Dr. M. Aguet (Genentech, Inc., South San Francisco, CA). These mice develop a normal immune response, possess IFN-γ-independent macrophage and NK cell activities, and constitutively express MHC Ag. However, they lack a functional IFN-γ system (10). In these experiments we used IFN-γR −/− mice, wild-type 129/Sv/Ev mice, or C57BL/6 mice of both sexes (bred at the breeding facilities of the Central Animal Laboratory, Utrecht). For each experiment the groups of mice were matched for sex and age as much as possible. All animals were housed in filter-top cages. The animal experiments had been approved by the institutional animal welfare committee.

Virus

MHV (strain A-59), a virulent hepatotropic strain, was propagated on Sac(−) cells and virus stocks were prepared as described previously (11). When the virus is injected i.p., the disease susceptibility of inbred mouse strains varies; preliminary experiments revealed that a dose of 10^5 PFU causes mortality among 80% of BALB/c mice, while C57BL/6 mice are unaffected. A dose of 10^6 PFU kills all BALB/c mice and 80 to 90% of C57BL/6 mice. A dose of 10^7 induces disease and mortality in only 15%

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[2] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; IFN-γR −/−, IFN-γ receptor α-chain deficient; MHV, mouse hepatitis virus; PFU, plaque forming unit; AST, aspartate aminotransferase; ALT, alanine aminotransferase; RT-PCR, reverse transcriptase-polymerase chain reaction.

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of 129/Sv/Ev mice. Inactivated virus was prepared by UV irradiation for 15 min.

Cytokines

Murine rIL-12 (lot MRB 021893–3.2) was kindly provided by Dr. S. Wolf (Genetics Institute, Inc., Cambridge, MA). It was expressed from cloned cDNAs and had a sp. act. of 5.5×10^6 U/mg. Endotoxin contamination, as measured in the *Limulus* amebocyte assay, ranged from 11.4 to 22.7 endotoxin units/mg IL-12. Rat rIFN-γ (4×10^6 U/mg), which displays activity in both rats and mice (*12*), was kindly provided by Dr. P. H. Van der Meide (ITRI-TNO, Rijswijk, The Netherlands). Either 0.1 to 1 μg (i.e., $0.55–5.5 \times 10^3$ U) IL-12 or 0.25 to 2.5 μg (i.e., 10^3-10^4 U) IFN-γ/mouse as a dilution in 500 μl of PBS was injected at the indicated time points. The choice of these cytokine doses and regimens was based on previous studies reporting in vivo antiviral activity of IL-12 (4) and IFN-γ (13) in other model systems.

Experimental protocol

On day 0, groups of 6 to 10 mice were infected i.p. with virulent MHV-A59. In susceptible mice, this infection causes a rapidly progressing acute hepatitis leading to illness (hypomotility, hunchback, and ruffled fur) after 4 to 5 days, followed by death of the animal generally within 7 to 10 days. The severity of the disease varies with the virus inoculum dose, genetic background, age, and immune status of the host. On day 1 or 4 postinfection, before the onset of symptoms, the spleens were removed, and splenocyte single cell suspensions were tested for cytokine and nitrite production. On days 1 and 5 postinfection, the livers of infected mice were removed and homogenized (10%, w/v) in DMEM containing 10% FCS; supernatants were assayed for infectious virus after low speed centrifugation. At designated time points after infection, the livers and spleens were removed, flash-frozen in liquid nitrogen or embedded in paraffin, and analyzed immunohistochemically for viral Ag expression. Other groups of mice were monitored daily for clinical symptoms and mortality. They were bled from the metro-orbital plexus on day 4. Their sera were analyzed biochemically for determination of hepatocellular injury by examining serum aspartate aminotransferase (AST) or serum alanine aminotransferase (ALT) activity.

Infectivity assay

MHV infectivity titers in liver homogenates were determined on monolayers of L cells using an end-point titration of 10-fold diluted tissue homogenate (10%, w/v) supernatants. After 24-h incubation, the cell layers were stained with 0.1% crystal violet in 1% formaldehyde. Titers are expressed as PFU per gram of tissue.

Immunohistology

To block endogenous peroxidase activity, cryostat sections (5 μm) were fixed for 10 min in acetone containing 0.5% H_2O_2, while paraffin section were incubated in methanol containing 0.5% H_2O_2. Sections were examined for the presence of viral Ag. The slides were washed and incubated for 2 h with polyclonal rabbit anti-MHV (K134) (14) diluted in PBS containing 0.1% BSA. After washing, they were incubated for 1 h with secondary anti-rabbit peroxidase conjugate diluted in PBS containing 0.1% BSA. All washes were performed with PBS. Peroxidase activity was visualized by using 0.003% H_2O_2 and 0.5% 3,3′-diaminobenzidine in 0.05 M Tris-HCl buffer, pH 8.3. All incubations were conducted at room temperature. Other sections were counterstained with hematoxylin-eosin. The preparations were rinsed in tap water and mounted in Aquamount, BDH Laboratory Supplies, Poole, England.

In vitro analysis of lymphokine and NO_2^- production

For the analysis of cytokine production, splenocytes (1.5×10^6 cells/ml) were cultured in 24-well plates (Nunc, Breda, The Netherlands) and stimulated with MHV Ag (antigenic mass of 5×10^5 PFU MHV, UV inactivated) or Con A (5 μg/ml). Supernatants of stimulated cultures were harvested at 48 h and stored at −20°C until use. IFN-γ and IL-4 were measured by two-site ELISA (Holland Biotechnology, Leiden, The Netherlands; and PharMingen, San Diego, CA) using standard curves established with known amounts of murine rIFN-γ (kindly provided by Dr. H. Heremans, Leuven, Belgium) and rIL-4 (Genzyme Corp., Cambridge, MA). NO_2^- was measured as previously described (15).

Semiquantitative RT-PCR analysis of cytokine mRNAs

Total cellular RNA was extracted from 2×10^6 splenocytes obtained 24 h after infection. The mRNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase using oligo(dt) primers. To calibrate the input cDNA of each sample, the cDNAs were first amplified by PCR with primers specific for β-actin: sense, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′; and antisense, 5′-TAAAACGCAGTCAGTAACAGTCCG-3′. For subsequent amplification with cytokine-specific primers, we used the following primers specific for IL-12 p40: sense, 5′-TCACCTGTGACACGCCTGAAG-3′; and antisense, 5′-GATGTCCCTGATGAAGAAGC-3′; for IL-12 p35: sense, 5′-CAGTGCCGGCTCAGCATGTG-3′; and antisense, 5′-ATGAAGAAGTATGCAGAGCTT-3′; for IFN-γ: sense, 5′-AGCGGCTGACTGAACTCAGATTGTAG-3′; and antisense, 5′-GTCACAGTTTTCAGCTGTATAGGG-3′; or for IL-4: sense, 5′-CGAAGAACACCACAGAGAGTGAGCT-3′; and antisense, 5′-GACTCATTCATGGTGCAGCTTATCG-3′. In most cases, 30 cycles of denaturation (95°C), annealing (60°C), and elongation (72°C) were performed in a thermocycler (Perkin-Elmer, Norwalk, CT). The products were separated and visualized by electrophoresis in ethidium bromide-stained 1.5% agarose gels. The identity of the PCR products of appropriate length was confirmed by cycle sequencing using dye-labeled terminators (Amersham International, Buckinghamshire, U.K.).

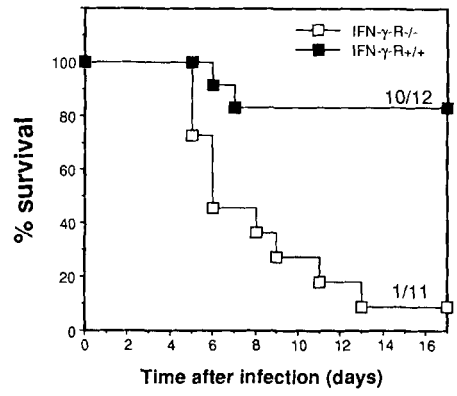

FIGURE 1. Increased susceptibility of IFN-γR −/− mice to MHV infection. Ten-week-old IFN-γR −/− mice (open squares; five female and six male animals) and age-matched wild-type 129/Sv/Ev mice (closed squares; eight female and four male animals) were i.p. infected with 10^7 PFU MHV/mouse. Survival was monitored daily.

Analysis of hepatocellular injury

For biochemical assessment of liver injury, the sera of infected animals were analyzed by an enzymatic rate method to quantitatively determine AST activity (Synchron CX system, Beckman Instruments, Brea, CA) or ALT activity (Sopar-Biochem, Brussels, Belgium); both are enzyme markers for hepatocellular necrosis.

Statistical analysis

Evaluation of statistical differences between data obtained from mutant and wild-type mice and from differently treated groups of mice was performed using the Wilcoxon-Mann-Whitney test.

Results

Pathogenesis of acute MHV infection in IFN-γR −/− mice

To assess the importance of IFN-γ function for the susceptibility to acute MHV infection, wild-type and IFN-γR −/− mice were inoculated i.p. with 10^7 PFU. In the mutant mice, host resistance was strongly impaired, with only 1 of the 11 IFN-γR −/− mice surviving (Fig. 1). In contrast, wild-type 129/Sv/Ev mice were relatively resistant, exhibiting symptoms and mortality in only 2 of 12 infected animals.

In a subsequent experiment, four IFN-γR −/− and four 129/Sv/Ev mice were infected, and after 4 days, liver and spleens were removed and examined histologically. At this point in time, one of the mutant animals already exhibited clinical symptoms. Immunohistologic examination revealed a marked increase in the number and size of foci of virus Ag-positive cells in the livers of IFN-γR −/− mice (Fig. 2). Similarly, increased numbers of viral Ag-positive cells were noted in

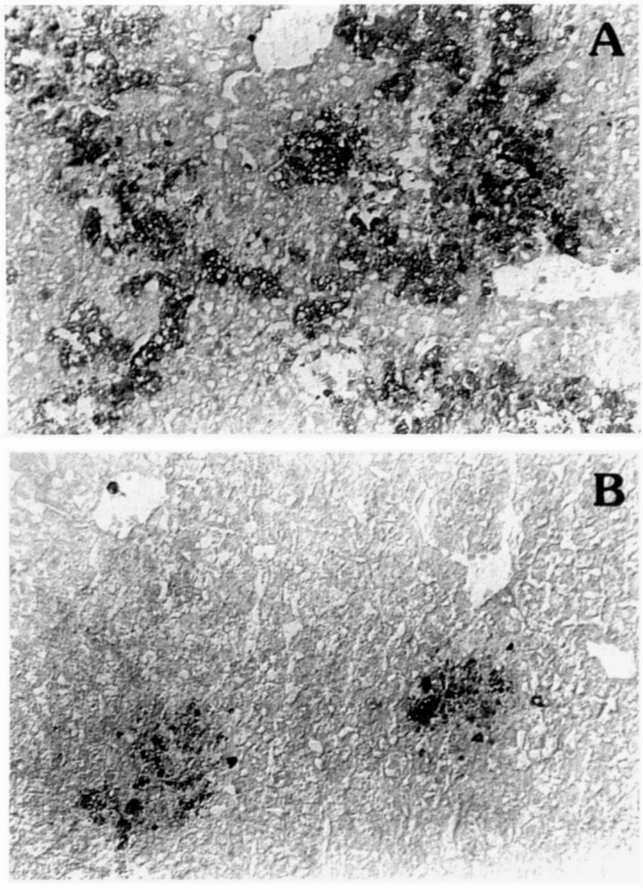

FIGURE 2. Immunohistologic evidence for increased MHV replication in IFN-γR −/− mice. Shown are representative cryostat sections of livers isolated from 11-wk-old IFN-γR −/− mice (*A*) and age-matched wild-type mice (*B*) on day 4 post-MHV infection, stained with MHV specific Ab k134. Sections from noninfected mice showed no staining (not shown). Magnification, ×235.

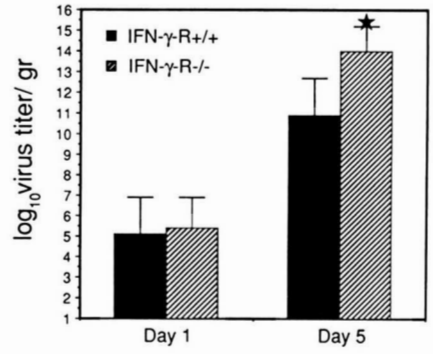

FIGURE 3. Increased replication of MHV in IFN-γR −/− mice. Eleven-week-old IFN-γR −/− mice (hatched bars; eight male animals) and age-matched wild-type 129/Sv/Ev mice (closed bars; three female and six male animals) were i.p. infected with 10^7 PFU MHV/mouse. Groups of four animals were killed on day 1 or 4 after infection. Mean infectivity titers of the livers are given as PFU per gram of tissue ± SEM. An asterisk indicates significant difference vs the control group ($p = 0.04$).

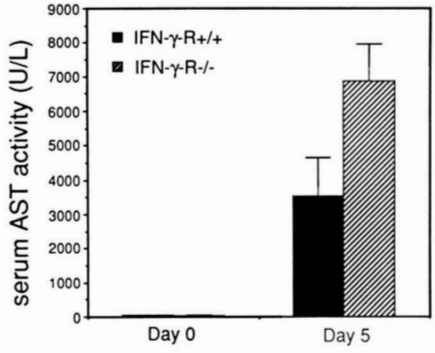

FIGURE 4. Increased hepatocellular injury in MHV-infected IFN-γR −/− mice. Ten-week-old IFN-γR −/− mice (hatched bars; three female and two male animals) and age-matched wild-type 129/Sv/Ev mice (closed bars; two female and three male animals) were i.p. infected with 10^7 PFU MHV/mouse. Groups of five animals were bled from the retro-orbital plexus on day 0 and 5 after infection, and their sera were analyzed for AST activity. Mean values are given as units per liter ± SEM.

the spleens of the mutant mice (not shown). On day 4, virus titers in the liver were also significantly increased ($p = 0.047$), approximately 1000-fold in the mutant mice (Fig. 3). Biochemical assessment of liver disease revealed increased serum AST activities in the mutant mice (Fig. 4).

These data indicate that physiologic IFN-γ is essential for the control of an acute MHV infection.

MHV-infected IFN-γR −/− mice generate an antiviral type 1 lymphokine response

The necessity for IFN-γ to generate Th1-like responses is controversial. We and others have recently shown that IFN-γR −/− mice infected with pseudorabies virus (16) or *Leishmania major* (17) produce an Ag-specific type 1 cytokine response. We, therefore, determined whether the exacerbated viral hepatitis associated with defective IFN-γR function had resulted from alterations in lymphokine profiles. IFN-γR −/− mice showed no differences in IFN-γ or IL-4 gene expression in splenocytes isolated 24 h after infection when examined by semiquantitative RT-PCR (not shown). Similarly, ex vivo cultured splenocytes of both normal and IFN-γR −/− mice isolated 4 days after infection and restimulated with MHV Ag or Con A produced high levels of IFN-γ and low amounts of IL-4 (Fig. 5). These data show that the increased susceptibility of IFN-γR −/− mice cannot be explained by an altered lymphokine profile.

Lack of IL-12 production in MHV-infected IFN-γR −/− mice

Time-course analyses revealed that MHV-A59 induces transient IL-12 *p40* gene expression in normal 129/Sv/Ev mice only 18 to 24 h after infection, while p35 mRNA levels remain unaltered (6). Remarkably, macrophages from IFN-γ-receptor deficient mice fail to produce IL-12 after mycobacterial stimulation (7). We hypothesized that the increased susceptibility to MHV infection in the absence of IFN-γ function resulted from the impaired production of IL-12. To examine in vivo IL-12 induction by MHV, we isolated splenocytes 24 h postinfection and analyzed mRNA encoding the IL-12 p40 chain by semiquantitative RT-PCR. Indeed, while IL-12 p40 mRNA was readily detected in MHV-infected wild-type mice 24 h after infection, minimal IL-12 gene expression was noted in splenocytes isolated from infected IFN-γR −/− mice, similar to that in uninfected mice (Fig. 6). This finding indicates reduced IL-12 synthesis in MHV-infected IFN-γR −/− mice.

Effect of IL-12 on MHV infection

IL-12 is a powerful inducer of IFN-γ and stimulates NK and T cells, both antiviral effector cells. We, therefore, investigated the

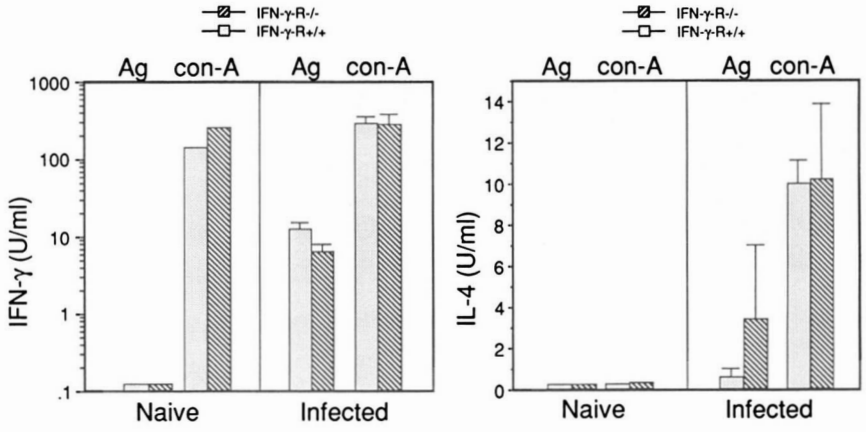

FIGURE 5. In vitro cytokine production by wild-type (stippled bars) and IFN-γR −/− splenocytes (hatched bars) after stimulation with MHV Ag (an antigenic mass of 5×10^5 PFU MHV, UV inactivated) or Con A (5 μg/ml). Splenocytes isolated from 10-wk-old female naive and MHV-infected mice inoculated 4 days previously with 10^7 PFU MHV-A59 were cultured, and their supernatants were harvested at 48 h for determination of IFN-γ and IL-4 by ELISA. Mean cytokine concentrations ± SEM in cultures of two individual mice are shown.

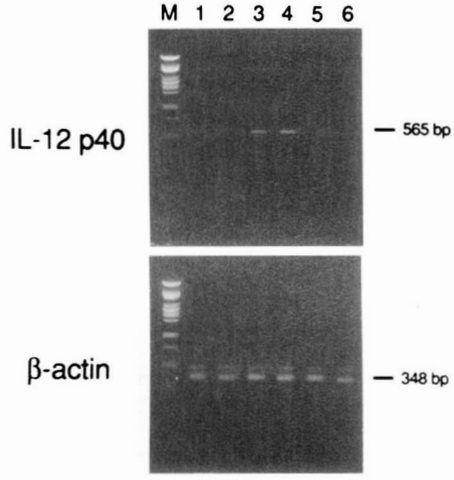

FIGURE 6. IL-12 p40 mRNA expression in splenocytes of individual MHV-infected wild-type 8-wk-old female 129/Sv/Ev mice (*lanes 1* and *2*), age-matched female IFN-γR −/− mice (*lanes 3* and *4*), and noninfected female 8-wk-old C57BL/6 mice (*lanes 5* and *6*). Total cellular RNA was extracted from 2×10^6 splenocytes/mouse isolated 24 h after infection. The RNA was reverse transcribed and amplified by PCR with primers specific for IL-12 p40 and β-actin. *Lane M*, DNA m.w. marker (λ *Pst*I digest, 15–14,057 bp). Note the presence of IL-12 p40 mRNA in infected wild-type mice only (*lanes 3* and *4*). IL-12 p35 mRNA expression was similar in MHV-infected wild-type and mutant mice (not shown).

possibility of compensating for the reduced IL-12 synthesis in IFN-γR −/− mice by exogenous IL-12, eventually resulting in restoration of IFN-γ-independent antiviral defense functions. First we examined the in vivo antiviral activity of IL-12 in the MHV-susceptible C57BL/6 mouse strain. The mice were treated twice with 1 μg of IL-12 1 day and 1 h before inoculation with 10^6 PFU MHV. This treatment failed to fully reconstitute host resistance and did not prevent mortality (Fig. 7*A*). Nonetheless, IL-12-treated animals showed an extension of survival time; their mean time to death ± SEM was 8.5 ± 0.56 vs 6.42 ± 0.29 days in the control group. This IL-12 treatment inhibited liver disease, as reflected by reduced serum AST and ALT activities, although the effect was not statistically significant ($p = 0.46$ for both parameters; not shown). Daily treatment with 100 ng IL-12/mouse for 8 days starting 1 day before infection, however, protected five of nine mice against lethal MHV infection (Fig. 7*A*) and reduced virus titers in the liver (Table I). These surviving animals initially showed clinical signs such as a ruffled fur and hypomotility, but recovered completely. The IL-12-induced protection was reflected by a reduction of macroscopic liver lesions (not shown), by less viral Ag in the liver (Fig. 8) and spleen (not shown), and by less hepatocellular necrosis with significantly reduced serum AST and serum ALT activities ($p = 0.04$ for both parameters; Table I).

When the surviving animals were rechallenged 4 wk after the first infection, all survived and showed no signs of illness. In contrast, a similar IL-12 regimen failed to induce protection or extension of survival time in IFN-γR −/− mice (Fig. 7*B*), indicating that physiologic IFN-γ is required for IL-12 to mediate its in vivo antiviral activity.

Effect of rIFN-γ on MHV infection

To assess the effect of exogenous IFN-γ on MHV replication, we first determined the in vitro antiviral activity of IFN-γ in cultures of MHV-permissive L cells. Monolayers of L cells were incubated with different concentrations of IFN-γ for 24 h before infection with approximately 10^3 PFU MHV and overlayering with agar. Plaques were counted 2 days postinfection after fixation of the monolayers with 1% formaldehyde and staining with 0.1% crystal violet. Figure 9*A* shows that pretreatment with IFN-γ inhibits MHV plaque formation in a dose-dependent fashion.

Due to the low susceptibility of wild-type 129/Sv/Ev mice to MHV infection, the in vivo antiviral activity of rIFN-γ was determined in the more susceptible C57BL/6 strain. One dose of 10^4 U rIFN-γ/animal was administered 1 day before infection; a second dose was given 1 h before inoculation with 10^6 PFU MHV. IFN-γ completely protected a group of eight animals that remained without signs of illness, whereas seven of nine mice in the untreated control group succumbed (Fig. 9*B*). A regimen with a 10-fold lower dose of 10^3 U IFN-γ had a similar protective effect.

The IFN-γ-induced protection was reflected by reduced liver disease, as revealed by obvious reductions of macroscopic liver lesions (not shown), by less viral Ag in the liver (Fig. 8) and spleen (not shown), and by significant reductions in serum AST and ALT activities when measured 4 days after infection ($p = 0.014–0.028$, respectively; Table I). Also, the IFN-γ-treated mice had reduced infectivity titers in the liver (Table I).

Mice protected by IFN-γ were challenged 4 wk after the first infection with the same lethal dose of virus. They all showed resistance to the second challenge, whereas only one of eight animals survived in the age-matched control group (not shown).

These data indicate that rIFN-γ exerts direct antiviral activity against MHV in vitro, and that in mice, exogenous IFN-γ can prevent the virus-induced pathology and mortality, leading to immunity against a second challenge infection.

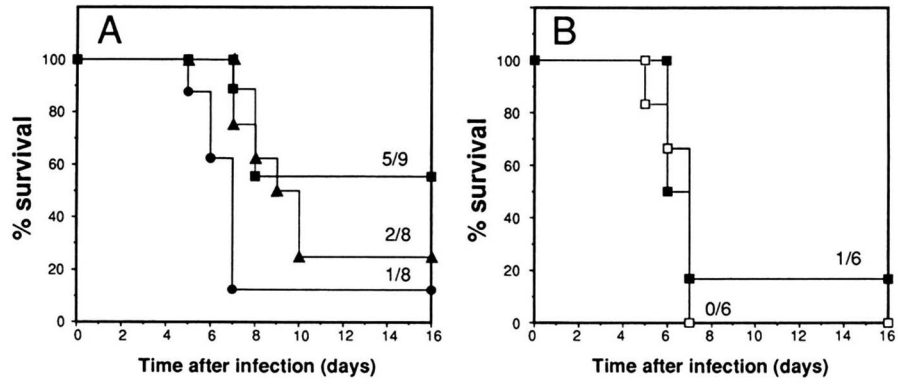

FIGURE 7. In vivo antiviral activity of IL-12 against MHV infection in C57BL/6 mice (*A*) and IFN-γR −/− mice (*B*). *A*, Groups of 6-wk-old male C57BL/6 mice were infected with 10^6 PFU MHV/mouse. IL-12 (0.1 μg/mouse, i.e., 0.55×10^3 U) was given on 8 consecutive days starting 1 day before infection (squares; $n = 9$). Another group of mice (triangles; $n = 8$) received an injection of 1 μg of IL-12 (i.e., 5.5×10^3 U) as a dilution in 500 μl of PBS on days −1 and 0. PBS-treated mice (circles) served as controls. *B*, Two groups of six female 12-wk-old IFN-γR −/− mice were infected with 10^7 PFU MHV/mouse. One group received 0.1 μg of IL-12/mouse (i.e., 0.55×10^3 U) on 8 consecutive days starting 1 day before infection (open symbols). Another group received no IL-12 (closed symbols).

Table I. *Effect of IL-12 and IFN-γ on hepatocellular injury and hepatic virus load*[a]

Treatment	AST[b] (U/L ± SEM)	ALT (U/L ± SEM)	Log Virus Titer in Liver (PFU/g ± SEM)
None	2150 ± 274	2585 ± 85	10.9 ± 0.22
IL-12	713 ± 90*	1047 ± 67*	9.8 ± 0.16*
IFN-γ	802 ± 325*	997 ± 483*	9.2 ± 0.14*

[a] Male 6-wk-old C57BL/6 mice ($n = 3$–5) were either untreated, treated with IL-12 (0.1 μg = 0.55×10^3 U/animal daily starting on day −1 until day 6 after infection), or injected twice with IFN-γ (0.25 μg = 10^3 U/animal at 24 and 1 h before infection). All animals were infected with 10^6 PFU MHV per mouse on day 0.

[b] The animals were bled from the reto-orbital plexus at day 4 after infection, and their sera were analyzed for AST and ALT activity. Uninfected animals treated with IL-12 only had mean AST and ALT values of 56 ± 5 and 42 ± 9, respectively, which was similar to preserum values.

*A significant difference ($p < 0.05$) when compared with the mean value obtained from untreated MHV-infected mice.

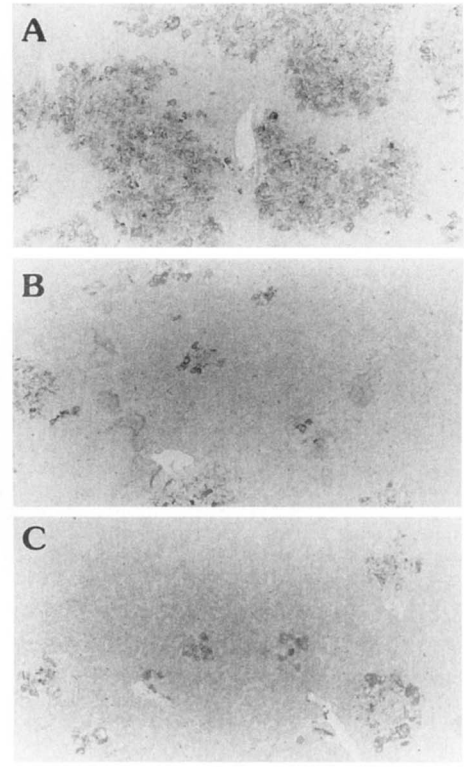

FIGURE 8. Immunohistologic evidence for suppressed MHV replication in IL-12- and IFN-γ-treated 6-wk-old male C57BL/6 mice. Shown are representative paraffin sections of livers isolated from untreated (*A*), IL-12-treated (*B*), and IFN-γ-treated (*C*) mice on day 4 post-MHV infection, stained with MHV-specific Ab k134. Sections from noninfected mice showed no viral Ag staining (not shown). The animals were treated as described in Table I. Magnification, ×148.

Discussion

IL-12 and IFN-γ are currently viewed as the principal inducers of Th1-like immune responses necessary for the elimination of intracellular pathogens. In the present study we examined the in vivo antiviral activity and immunoregulatory function of both cytokines during coronavirus MHV-induced acute hepatitis in mice.

IL-12 is regarded as the cytokine initiating cell-mediated immune responses (18), and its synthesis is therefore considered to precede IFN-γ production (18). IFN-γ, on the other hand, has been shown to stimulate IL-12 synthesis in cultured macrophages (19, 20), suggesting a positive feedback. In line with data from Flesh and co-workers (7), we observed an impaired IL-12 induction in IFN-γR −/− mice, suggesting that endogenous IFN-γ is required for its normal synthesis. The discrepancies between different model systems concerning the IFN-γ dependence of IL-12 synthesis may be explained by the cellular tropism of the particular pathogen and its intrinsic capacity to stimulate IL-12 production in APC. It would be of interest to determine the cellular source of both cytokines during MHV infection and whether IL-12-deficient or -depleted 129/Sv/Ev mice are able to produce IFN-γ.

The observed lack of endogenous IL-12 in MHV-infected IFN-γR −/− mice surprisingly resulted in a normal type 1 lymphokine response, characterized by high IFN-γ and low IL-4 levels. This is unexpected, since both IL-12 and IFN-γ are considered to be principal inducers of type 1 characteristic IFN-γ production. Previously, we demonstrated that IFN-γR ablation does not affect antiviral Th1 characteristic cytokine production during pseudorabies virus infection (16). Similarly, *L. major*-infected IFN-γR −/− mice exhibited a Th1 cytokine profile despite increased susceptibility (17). In contrast, other in vivo studies reported that anti-IFN-γ Abs promote Th2 and impair Th1 activities (21, 22). Also, mice with a disruption in the IFN-γ gene show increased influenza

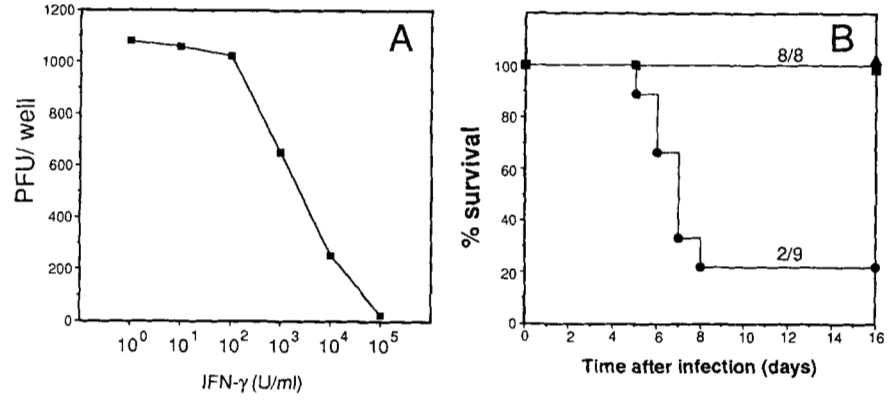

FIGURE 9. Antiviral activity of IFN-γ against MHV in vitro (*A*) and in vivo (*B*). *A*, Monolayers of L cells were cultured in different concentrations of IFN-γ for 24 h, subsequently infected with $\pm 10^3$ PFU per well, and overlayered with agar. After a 48-h incubation, the cell layers were fixed and stained with 0.1% crystal violet with 1% formaldehyde. The mean PFU per well are shown from one of three experiments that gave similar results. *B*, Groups of 6-wk-old male C57BL/6 mice (n = 9) were injected twice with IFN-γ at 24 and 1 h before infection with 10^6 PFU MHV/mouse. Either 0.25 μg (i.e., 10^3 U) of IFN-γ (squares) or 10^4 U of IFN-γ (triangles) per mouse as a dilution in 500 μl of PBS was injected. PBS-treated mice (circles) served as controls.

virus and *L. major* Ag-specific IL-4 and IL-5 production (23, 24). The discrepancies with our data may be explained by genetic background differences. There is no evidence for a compensation of the IFN-γR function by other (cytokine) receptors. Moreover, treatment with IFN-γ-neutralizing Ab did not alter the Th1 characteristic cytokine responses in IFN-γR −/− and IFN-γR +/+ mice after *L. major* infection (17). In agreement with our data is the observation of Flynn and co-workers that splenocytes of *Mycobacterium tuberculosis*-infected mice lacking the IFN-γ gene do not produce increased amounts of IL-4 after antigenic stimulation (25). Seder et al. showed that anti-IFN-γ Ab did not reduce IL-12-induced priming for IFN-γ production in an accessory cell-dependent system (26). Also, IL-12-induced Th1 development in C57BL/6 mice is not prevented by IFN-γ-neutralizing Ab treatment (27). We recently reported that co-injection of IL-12 during immunization strongly increases Ag-specific IFN-γ production in IFN-γR −/− mice, as it does in normal mice (28).

Our observations indicate that other cytokines or host factors can apparently replace or dominate IFN-γ and IL-12 to induce Th1-type responses. It is possible that the maintenance of IFN-γ production in IFN-γR −/− mice results from a lack of endogenous IL-4 induction, as previously suggested in other antigenic systems (17, 26). Indeed, we could confirm that Con A-stimulated splenocytes of 129/Sv/Ev mice, in contrast to those of BALB/c mice, failed to produce IL-4 even when precultured in the presence of rIL-4 (16). Yet another possibility includes the production of the recently identified cytokine IFN-γ-inducing factor, which strongly induces IFN-γ production independently of IL-12 (29).

In terms of in vivo antiviral protection, exogenous IL-12 could not compensate for the reduced endogenous IL-12 synthesis observed in IFN-γR-deficient mice. Endogenous IFN-γ is obviously necessary for the antiviral effect of exogenous IL-12, as has recently been demonstrated in several models (30–34).

In vivo augmentation of antiviral resistance by exogenous IL-12 has been demonstrated in experimental LCMV and murine CMV infections using daily doses of $<10^3$ ng/animal (4, 35). However, doses of 1 μg IL-12/day impaired antiviral defense against LCMV infection (5). No beneficial effect of IL-12 was demonstrated in mice infected with influenza virus, encephalomyocarditis virus, or respiratory syncytial virus (3). We demonstrated that the in vivo anti-MHV activity of exogenous IL-12 is less pronounced compared with that of IFN-γ. Since two injections of only 10^3 U IFN-γ are fully protective, the IL-12 regimens tested may have induced insufficient amounts of endogenous IFN-γ. However, as suggested by two recent studies, the exogenous IL-12 may have induced additional factors such as TNF-α that, depending on the endogenous cytokine environment, can be harmful to the host (5, 33).

We demonstrated that mice lacking the ligand-binding chain of the IFN-γR are less able to control the cytolytic virus infection. Several mechanisms can be proposed by which physiologic IFN-γ contributes to defense against acute MHV infection. One possibility includes the activation of innate and/or specific immune reactions. Indeed, enhancement of MHV infection has been observed in mice treated with anti-asialo-GM_1 (34), although not reproducibly (36). During MHV-induced hepatitis, MHC class II-restricted $CD4^+$ CTL encounter the liver (8). These $CD4^+$ T cells secrete IFN-γ, but not IL-4, upon recognition of the immunodominant MHV *S*-glycoprotein peptide (37), and when adoptively transferred, they confer protection against lethal MHV infection and reduce infectivity titers in the liver (8). No functional or immunohistochemical evidence was found for activation of classical $CD8^+$ class I-restricted CTL. We demonstrated that MHV-infected IFN-γR −/− mice generate a normal type 1 lymphokine profile, with high IFN-γ and low IL-4 levels. Their proliferative splenocyte responses were even increased (not shown), as in other IFN-γ-deficient model systems (16, 38, 39). This suggests that the generation of antiviral T cell functions in the mutant mice is not impaired and that endogenous IFN-γ has antiproliferative activity (38).

Another or additional scenario explaining the in vivo IFN-γ function includes the induction of an antiviral state in cells adjacent to foci of infected cells, thereby preventing virus spread. The pronounced in vitro and in vivo antiviral activities of exogenous IFN-γ indicate that this assumption is feasible. The exact cellular source of IFN-γ still needs to be identified, as well as whether its in vivo antiviral activity is based on immune-mediated or direct mechanisms. This could be revealed in immunodeficient or selectively immunodepleted mice (13).

Even at high doses, IFN-αβ cannot fully replace IFN-γ in stimulating the generation of NO_2^- by macrophages (9); therefore, IFN-γ-dependent nitric oxide production may also contribute to antiviral resistance. IFN-γR −/− mice inoculated with *Plasmodium sporozoites* produce minimal amounts of inducible nitric oxide synthetase mRNA in the liver (40). Also, in vitro-stimulated IFN-γR −/− macrophages failed to release nitrites (9, 10). Indeed, we observed in preliminary experiments that Con A-stimulated IFN-γR −/− splenocyte cultures of MHV-infected mice produced low amounts of NO_2^-, in contrast to cultures of MHV-infected wild-type mice (not shown). Interestingly, mice protected by IFN-γ or IL-12 were also immune to a second challenge infection,

indicating that viral epitopes are recognized by specific immune cells during inhibition of viral replication.

In conclusion, MHV infection of mice with an inactivated IFN-γR showed that 1) physiologic IFN-γ is important in the defense against coronavirus-induced acute hepatitis; 2) IFN-γ is not essential for the development of a type 1 lymphokine profile in a virus infection; 3) IFN-γ contributes to virus-induced in vivo IL-12 synthesis; and 4) in vivo antiviral protection incited by exogenous IL-12 depends upon IFN-γ. Furthermore, this study demonstrates that in vivo protection against acute viral hepatitis mediated by rIFN-γ is superior to that of rIL-12 and leads to protective immunity against a second challenge. The data suggest that during particular virus infections, immunotherapy using rIFN-γ treatment would be more promising than interventions with rIL-12.

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References

1. Scott, P., and S. H. E. Kaufmann. 1991. The role of T-cell subset and cytokines in the regulation of infection. *Immunol. Today 12:346.*
2. Trinchieri, G., and P. Scott. 1994. The role of interleukin 12 in the immune response, disease and therapy. *Immunol. Today 15:460.*
3. Gladue, R. P., A. M. Laquerre, H. A. Magna, L. A. Carroll, M. O'Donell, P. S. Changelian, and A. E. Franke. 1994. In vivo augmentation of IFN-γ with a rIL-12 human/mouse chimera: pleiotropic effects against infectious agents in mice and rats. *Cytokine 6:318.*
4. Orange, J. S., S. F. Wolf, and C. A. Biron. 1994. Effects of IL-12 on the response and susceptibility to experimental viral infections. *J. Immunol. 152:1253.*
5. Orange, J. S., T. P. Salazar-Mather, S. M. Opal, R. L. Spencer, A. H. Miller, B. S. McEwen, and C. A. Biron. 1995. Mechanism of interleukin-12-mediated toxicities during experimental viral infections: role of tumor necrosis factor and glucocorticoids. *J. Exp. Med. 181:901.*
6. Coutelier, J.-P., J. van Broeck, and S. F. Wolf. 1995. Interleukin-12 gene expression after viral infection in the mouse. *J. Virol. 69:1955.*
7. Flesh, I. E. A., J. H. Hess, S. Huang, M. Aguet, J. Rothe, H. Bluethmann, and S. H. E. Kaufmann. 1995. Early interleukin 12 production by macrophages in response to mycobacterial infection depends on interferon γ and tumor necrosis factor α. *J. Exp. Med. 181:1615.*
8. Heemskerk, M. H. M., H. Schoenmaker, W. J. M. Spaan, and C. J. P. Boog. 1995. Predominance of MHC class II-restricted $CD4^+$ cytotoxic T cells against mouse hepatitis virus A59. *Immunology 84:521.*
9. Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilček, R. M. Zinkernagel, and M. Aguet. 1993. Immune response in mice lacking the interferon-γ receptor. *Science 259:1742.*
10. Kamijo, R., D. Shapiro, J. Le, S. Huang, M. Aguet, and J. Vilcek. 1993. Generation of nitric oxide and induction of major histocompatibility complex class II antigen in macrophages from mice lacking the interferon γ receptor. *Proc. Natl. Acad. Sci. USA 90:6626.*
11. Spaan, W. J. M., P. J. M. Rottier, M. C. Horzinek, and B. A. M. van der Zeist. 1981. Isolation and identification of virus-specific mRNAs in cells infected with mouse hepatitis virus (MHV-A59). *Virology 108:424.*
12. Van der Meide, P. H., M. Dubbeld, K. Vijverberg, T. Kos, and H. Schellekens. 1986. The purification and characterization of rat gamma interferon by use of two monoclonal antibodies. *J. Gen. Virol. 67:1059.*
13. Schijns, V. E. C. J., T. H. Borman, H. Schellekens, and M. C. Horzinek. 1988. Antiviral activity of recombinant rat interferon gamma in immunologically impaired and immunosuppressed rats. *J. Gen. Virol. 69:1979.*
14. Rottier, P. M. J., W. J. M. Spaan, M. C. Horzinek, and B. A. M. Van der Zeist. 1981. Translation of the three mouse hepatitis virus strain A59 subgenomic RNAs in *Xenopus laevis* oocytes. *J. Virol. 38:20.*
15. Green, L. C., D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum. 1982. Analysis of nitrate, nitrite, and (15N) nitrate in biological fluids. *Anal. Biochem. 126:131.*
16. Schijns, V. E. C. J., B. L. Haagmans, E. O. Rijke, S. Huang, M. Aguet, and M. C. Horzinek. 1994. Interferon-γ receptor-deficient mice generate antiviral Th1-characteristic cytokine profiles but altered antibody responses. *J. Immunol. 153:2029.*
17. Swihart, K., U. Fruth, N. Messmer, K. Hug, R. Behin, S. Huang, G. Del Giudice, M. Aguet, and J. A. Louis. 1995. Mice from genetically resistant background lacking the interferon γ receptor are susceptible to infection with *Leishmania major* but mount a polarized T helper cell 1-type $CD4^+$ T cell response. *J. Exp. Med. 181:961.*
18. Scott, P. 1993. IL-12: initiation cytokine for cell-mediated immunity. *Science 260:496.*
19. Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf, and A. Sher. 1993. Interleukin-12 is required for the T-lymphocyte-independent induction of interferon-γ by an intracellular parasite and induces resistance in T cell-deficient hosts. *Proc. Natl. Acad. Sci. USA 90:6115.*
20. Chensue, S. W., J. H. Ruth, K. Warmington, P. Lincoln, and S. L. Kunkel. 1995. In vivo regulation of macrophage IL-12 production during type 1 and type 2 cytokine-mediated granuloma formation. *J. Immunol. 155:3546.*
21. Scott, P. 1991. IFN-γ modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *J. Immunol. 147:3149.*
22. Murray, J. S., J. Madri, T. Pasqualini, and K. Bottomly. 1993. Functional CD4 T cell subset interplay in an intact immune system. *J. Immunol. 150:4270.*
23. Graham, M. B., D. Dalton, D. Giltinan, V. L. Braciale, T. A. Stewart, and T. J. Braciale. 1993. Response to influenza infection in mice with a targeted disruption in the interferon γ gene. *J. Exp. Med. 178:1725.*
24. Wang, Z.-E., S. L. Reiner, S. Zheng, D. K. Dalton, and R. M. Locksley. 1994. $CD4^+$ effector cells default to the Th2 pathway in interferon-γ-deficient mice infected with *Leishmania major*. *J. Exp. Med. 179:1367.*
25. Flynn, J. A., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom. 1993. An essential role for interferon γ in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med. 178:2249.*
26. Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul. 1993. Interleukin 12 acts directly on $CD4^+$ T cells to enhance priming for interferon γ production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA 90:10188.*
27. McKnight, A. J., G. J. Zimmer, I. Fogelman, S. F. Wolf, and A. K. Abbas. 1994. Effects of IL-12 on helper T cell-dependent immune responses in vivo. *J. Immunol. 152:2172.*
28. Schijns, V. E. C. J., B. L. Haagmans, and M. C. Horzinek. 1995. IL-12 stimulates an antiviral type 1 cytokine response but lacks adjuvant activity in IFN-γ-receptor deficient mice. *J. Immunol. 155:2525.*
29. Okamura, H., H. Tsutsui, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, K. Akita, M. Namba, F. Tanabe, K. Konishi, S. Fukuda, and M. Kurimoto. 1995. Cloning of a new cytokine that induces IFN-γ production by T cells. *Nature 378:88.*
30. Morris, S. C., K. B. Madden, J. J. Adamovicz, W. C. Gause, B. R. Hubbard, M. K. Gately, and F. D. Finkelman. 1994. Effects of IL-12 on in vivo cytokine gene expression and Ig isotype selection. *J. Immunol. 152:1047.*
31. Flynn, J. L., M. M. Goldstein, K. J. Triebold, J. Sypek, S. Wolf, and B. R. Bloom. 1995. IL-12 increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection. *J. Immunol. 155:2515.*
32. Zhou, P., M. C. Sieve, J. Bennett, K. J. Kwon-Chung, R. P. Tewari, R. T. Gazzinelli, A. Dher, and R. A. Seder. 1995. IL-12 prevents mortality in mice infected with *Histoplasma capsulatum* trough induction of IFN-γ. *J. Immunol. 155:785.*
33. Stevenson, M. M., M. F. Tam, S. F. Wolf, and A. Sher,. 1995. IL-12 induced protection against blood-stage *Plasmodium chabaudi AS* requires IFN-γ and TNF-α and occurs via a nitric oxide-dependent mechanism. *J. Immunol. 155: 2545.*
34. Bukowski, J. F., B. A. Woda, S. Habu, K. Okumura, and R. M. Welsh. 1983. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. *J. Immunol. 131:1531.*
35. Orange, J. S., B. Wang, C. Terhorst, and C. A. Biron. 1995. Requirement for natural killer cell-produced interferon γ in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J. Exp. Med. 182:1045.*
36. Welsh, R. M., and M. Vargas-Cortes. 1992. Natural killer cells in viral infection. In *The Natural Killer Cell.* C. E. Lewis and J. O'D. McGee, eds. IRL Press, Oxford University Press, New York, pp. 108–134.
37. Heemskerk, M. H. M., H. Schoenmaker, I. De Jong, V. E. C. J. Schijns, W. J. M. Spaan, and C. J. P. Boog. 1995. Differential activation of mouse hepatitis virus-specific $CD4^+$ cytotoxic T cells is defined by the peptide length. *Immunology 85:517.*
38. Gajewski, T. F., and F. W. Fitch. 1988. Anti-proliferative effect of IFN-γ in immune regulation. I. IFN-γ inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J. Immunol. 140:4245.*
39. Dalton, D. K., S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart. 1993. Multiple defects of immune function in mice with disrupted interferon-γ genes. *Science 259:1739.*
40. Tsuji, M., Y. Miyahira, R. S. Nussenzweig, M. Aguet, M. Reichel, and F. Zavala. 1995. Development of antimalaria immunity in mice lacking IFN-γ receptor. *J. Immunol. 154:5338.*