Interferon γ Regulates Acute and Latent Murine Cytomegalovirus Infection and Chronic Disease of the Great Vessels

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Summary
To define immune mechanisms that regulate chronic and latent herpesvirus infection, we analyzed the role of interferon γ (IFN-γ) during murine cytomegalovirus (MCMV) infection. Lethality studies demonstrated a net protective role for IFN-γ, independent of IFN-α/β, during acute MCMV infection. Mice lacking the IFN-γ receptor (IFN-γR-/-) developed and maintained striking chronic aortic inflammation. Arteritis was associated with inclusion bodies and MCMV antigen in the aortic media. To understand how lack of IFN-γ responses could lead to chronic vascular disease, we evaluated the role of IFN-γ in MCMV latency. MCMV-infected IFN-γR-/- mice shed preformed infectious MCMV in spleen, peritoneal exudate cells, and salivary gland for up to 6 mo after infection, whereas the majority of congenic control animals cleared chronic productive infection. However, the IFN-γR was not required for establishment of latency. Using an in vitro explant reactivation model, we showed that IFN-γ reversibly inhibited MCMV reactivation from latency. This was at least partly explained by IFN-γ-mediated blockade of growth of low levels of MCMV in tissue explants. These in vivo and in vitro data suggest that IFN-γ regulation of reactivation from latency contributes to control of chronic vascular disease caused by MCMV. These studies are the first to demonstrate that a component of the immune system (IFN-γ) is necessary to regulate MCMV-associated elastic arteritis and latency in vivo and reactivation of a herpesvirus from latency in vitro. This provides a new model for analysis of the interrelationships among herpesvirus latency, the immune system, and chronic disease of the great vessels.

Key words: interferon γ • latency • reactivation • cytomegalovirus • vasculitis

Human cytomegalovirus (HCMV) establishes a chronic infection in normal hosts, characterized by latency and intermittent shedding of infectious virus. HCMV causes severe reactivation disease in situations of immunocompromise, and is a frequent cause of morbidity and mortality in transplant and AIDS patients. The specific components of the immune system that are responsible for preventing reactivation disease have not been identified. Reactivation disease in humans involves multiple tissues, including the retina, liver, lung, and gastrointestinal tract. In apparently immunocompetent hosts, CMV has been implicated in the genesis of atherosclerosis (1–3), rapidly progressive coronary artery disease and endothelialitis in cardiac transplant patients (4–7), coronary restenosis after angioplasty (8, 9), and inflammatory aortic disease (10, 11). Although these studies suggest a role for HCMV in human vascular disease, this is an area of considerable controversy (12–15). Since MCMV can cause aortic inflammation in weanling mice (16), we focused on the great vessels as possible targets for chronic MCMV disease.

The role of the innate and adaptive immune systems in controlling acute MCMV infection has been well characterized. CD8 T cells are primary effector cells in clearance of MCMV during acute infection (17, 18). However, MCMV replication can be controlled by CD4 T cells when CD8 T cells are depleted (19), and CD4 T cells are required to clear virus from the salivary gland (18). NK cells play a significant role in the control of acute infection with MCMV, and at least one mechanism for NK cell pro-
tection is secretion of IFN-γ (20–22). IFN-γ has a number of functions that probably play a role in controlling acute MCMV infection. These include activation of macrophages during MCMV infection (21), enhancement of MHC class I-dependent antigen presentation by infected cells to CD8 T cells (23), and inhibition of lytic MCMV replication and gene expression (21, 24, 25). Administration of recombinant IFN-γ can protect against lethal infection (26), and administration of anti-IFN-γ can both decrease the effectiveness of anti-MCMV CD8 and CD4 T cells (23, 27) and increase viral titers in visceral organs (21, 27). CD4 T cells specific for the HCMV immediate early 1 protein produce cytokines including IFN-γ and TNF-α on antigenic stimulation, which inhibit viral replication in MRC5 cells (28). Although these studies all argue for a protective role for IFN-γ, other studies in both rat CMV and HCMV have argued that IFN-γ is required to generate productive infection of macrophages and efficient replication in vivo (29, 30). These results raise the question of whether the net effect of endogenous IFN-γ is protective.

A period of persistent productive infection in the salivary gland, MCMV establishes a predominantly latent infection in immunocompetent mice (31, 32). Latency is characterized by the presence of viral genome in the absence of preformed infectious virus combined with the capacity to reactivate either in vivo or in vitro from tissue explants. The extent to which the immune system regulates and controls latency is not fully understood. It is thought that the immune system regulates latency because immunosuppression results in reactivation of lytic MCMV (33–35). Reactivation is a multistep process that may be divided into two phases: cellular reactivation and subsequent growth of virus. Cellular reactivation probably results from a shift from latent to lytic gene expression; nothing is known about whether or how the immune system alters this process. Cellular reactivation is followed by growth of reactivated virus to levels that cause disease or are experimentally detectable. The mechanisms responsible for controlling viral replication after cellular reactivation are also incompletely defined, although the immune system is likely to play a role at this step in reactivation. For example, antibody has been shown to limit dissemination of MCMV after reactivation (36). IFN-γ is a good candidate in regulating growth of reactivated MCMV given its importance for clearing persistent productive infection of the salivary gland (27).

In this study we first demonstrate that the net effect of endogenous IFN-γ is protective during acute infection in vivo. We then show that IFN-γ plays an important role during chronic MCMV infection in vivo and during reactivation in vitro. In the course of these studies we have defined a novel role for IFN-γ in regulating chronic vascular pathology due to MCMV.

**Materials and Methods**

Viruses and Viral Antigens. MCMV Smith strain was obtained from the American Type Culture Collection (no. VR-194, Lot 10; Rockville, MD). Tissue culture–passaged MCMV (tc-MCMV) and salivary gland MCMV (sgMCMV) were generated as described previously (21, 31). All media were prepared as described previously (37). Mice embryonic fibroblasts (MEFs) were prepared and used during initial passaging or thawed from frozen stocks (31). All MEFs were used before passage 4. To detect and quantitate MCMV genome, nested PCR detecting sequences of the MCMV immediate early 1 gene were performed on DNA from bone marrow cells as described previously (31). This nested PCR assay reproducibly detects one copy of target plasmid (31, 37). MCMV sequences are quantitatively recovered in tissue DNA preparations (31). Herpes simplex virus (HSV) was grown and titered as described previously (21). The myocarditic reovirus serotype 3 BB was grown and titered as described previously (38).

**Mice, Mouse Infections, and Tissue Harvests.** 129/Sv mice and mice with null mutations in the IFN-γ receptor (IFN-γR−/−), the IFN-α/β receptor (IFN-α/βR−/−), and both the IFN-γ and IFN-α/β receptors (IFN-α/βR/γR−/−), were obtained from Dr. Michel Aguet (39) and bred at Washington University (St. Louis, MO). These mice were infected intraperitoneally with doses of sgMCMV described in the text. BALB/c (National Cancer Institute, Frederick, MD) and C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) mice were infected with 2 × 105 or 1 × 106 PFU sgMCMV intraperitoneally and rested for 4–10 mo to establish a latent infection. To assess mice for the presence of preformed infectious MCMV, spleens and salivary glands were harvested aseptically and handled individually. Bone marrow cells and peritoneal exudate cells (PECs) were harvested as described previously (37). Low levels of preformed infectious MCMV were detected using coculture of sonicated cells or tissues with MEFs in an assay that detects 1–10 PFU per organ as described (31, 37). In brief, solid organs were minced, then sonicated twice, and three T75 flasks (Sarstedt, Newton, NC) of confluent MEFs were inoculated with the sonicated tissue. Dilution of tissue sonicates into large volume cultures (total vol 30 ml/T75 flask) is essential to generate the high sensitivity of this assay (31). One T75 flask of confluent MEFs was inoculated per sample of sonicated PECs or bone marrow cells (generally between 2 and 3 × 105 PECs or 2 and 3 × 107 bone marrow cells). Flasks were observed for 3 wk to detect MCMV-associated cytopathic effect. When necessary, results were confirmed by transfer of supernatant onto fresh MEF monolayers. Positive controls consisted of adding 5 PFU of MCMV to flasks and were consistently positive.

Pathology and Immunohistochemistry. To analyze aortic pathology, the heart, lungs and great vessels were resected en bloc after death and processed as described elsewhere (40). In brief, serial sections from the posterior of the heart through the thymus were prepared and stained with hematoxylin and eosin and then read blindly for aortic pathology by either H.W. Virgin or A.J. Dal Canto. Samples were scored as positive if evidence of arteritis in the aorta or pulmonary artery was observed. Since arteritis most commonly involved the base of the aorta, samples were called positive only if a clear view of the base of the aorta was available; samples were excluded if the base of the aorta was not visible. Mice monoclonal antibody to MCMV was prepared as follows. BALB/c mice were infected with 105 PFU tcMCMV. Salivary glands were harvested at 20 d after infection, homogenized in medium lacking FCS, and titered by plaque assay. BALB/c mice were infected with 104 PFU of this stock. Mice were bled through the retroorbital plexus at 1, 2, and 3 mo after additional boosts with 1.2 × 105 PFU of sgMCMV. This approach was taken to minimize immunization with FCS in tissue culture preparations of virus. Immunization with murine cellular antigens was
avoided by selection of BALB background mice for both preparation of virus and serum generation. Immunohistochemical staining was performed as described previously (40) except that the murine polyclonal antibody to MCMV was diluted 1:1000. Preimmune serum was also used at a 1:1000 dilution as a negative control. Horseradish peroxidase (HRP)-conjugated donkey anti–mouse secondary antibody (Jackson Immunoresearch Labs, West Grove, PA) was used at 1:1000. Tyramide signal amplification (NEN Life Science Products, Boston, MA) was performed and HRP activity was localized as previously described (40).

MCMV reactivation from Explanted Organs and Bone Marrow. Spleens or lungs from 8–13 latently infected BALB/c or C57BL/6 mice were pooled, mixed, homogenized using a Tenbroeck Tissue Homogenizer (Belco, Vineland, NJ), and plated evenly into 12 six-well plates (Falcon, Franklin Lakes, NJ). An aliquot (0.3–0.5 spleen or lung equivalents) was diluted, sonicated, and cocultured with MEFs to rule out the presence of preformed infectious virus in pooled spleen and lung explants (see above). Explants were cultured in the presence of mock IFN (prepared by Lee Biomolecular, San Diego, CA, in the same manner as their IFN preparations, except that it is mock induced), 100 U/ml IFN-α (Lee Biomolecular), IFN-β (Lee Biomolecular), or rIFN-γ (gift of Genentech, San Francisco, CA, or purchased from Genzyme Corp., Cambridge, MA). Explants were maintained with media changes with every 3–4 d for 64–116 d. IFN was replaced with these media changes for the number of days indicated in Figs. 5 and 6. Reactivation was detected by transfer of 100 μl culture medium to fresh MEF monolayers which were observed for cytopathic effect for 12 d (31). For determination of the effects of IFNs on growth of low levels of MCMV in explant cultures, spleen and lung explants from naive mice were prepared as described above. 12 d after explantation, MCMV was added to the cultures at doses described in the text and the plates were incubated as described above. To determine if IFN or other factors from explant wells could prevent detection of MCMV in secondary MEF cultures, supernatant was harvested from wells before reactivation and cultured with MEFs to which 5 PFU of MCMV were added. To assess reactivation of MCMV from bone marrow, bone marrow was harvested, treated with a hypotonic solution to lyse red blood cells, and cultured with L cell–conditioned medium as described previously (41). After 19 d, media was changed to DMEM with 10% FCS (31) and MEFs were added as an indicator monolayer for MCMV–induced cytopathic effect.

Statistical Methods. Statistical analysis was carried out through the Division of Biostatistics at Washington University School of Medicine. The data were analyzed using the SAS procedure PHREG, which performs regression analysis of survival data based on the Cox proportional hazards model. This model was particularly relevant to these data as it takes into account the censoring of the day of death or killing. The dependent variables used to analyze lethality data (Fig. 1) were day of death censored by whether or not the animal died on its own. Cox proportional hazards models were developed to first test all of the strains against the control (129 or B6) with dose and sex as covariates. Then models were developed to pairwise compare each experimental strain against the others, still using dose and sex as covariates. Mice strain was a significant predictor of outcome independent of viral dose or sex (see text). The arteritis data (Table 1) were also analyzed using a Cox proportional hazards model, comparing the IFN-γR−/− strain to the 129 control with dose and sex as covariates. The dependent variables were day of sacrifice censored by whether or not the animal displayed evidence of arteritis. For the persistence data (Table 2), stepwise proportional hazards regression techniques were used to determine the best fitting variables among the different experiments. The final analyses compared IFN-αR−/− to the 129 controls using as dependent variables day of killing censored by whether or not the animal displayed evidence of shedding virus in any organ, or in the salivary gland, spleen, or PECs. The bone marrow results were not statistically analyzed as there were no mice that displayed evidence of the shedding virus in their bone marrow.

Results

Protective Role of IFN-γ in Acute Infection. Control congenic mice and mice with various null mutations influencing IFN responsiveness were challenged with varying doses of MCMV and followed for mortality (Fig. 1). Sharp changes in mortality observed with small changes in viral dose have been previously reported and necessitated the use of large numbers of age-matched mice across many experiments to obtain clear data (42, 43). We have evaluated 416 mice in the course of these experiments. We aged mice at least 2 mo before challenge to minimize the impact of large changes in susceptibility to lethal MCMV infection that occur over the first weeks of life (our unpublished data). The LD50's for IFN-γR−/− and IFN-γ/− mice were approximately five- and fourfold lower, respectively, than for congenic (129 and C57BL/6) control mice (P < 0.02; P < 0.005). IFN-α/β might compensate for some effects of IFN-γ since both can inhibit MCMV replication in vivo and in vitro (21, 24, 25). We therefore evaluated IFN-α/βR−/− mice and found that the LD50 for IFN-α/βR−/− mice was 800-fold lower than for control 129 mice (P < 0.0002). To determine the importance of IFN-γ in the absence of IFN-α/β responses, we compared IFN-α/βR−/− and IFN-α/β/γR−/− mice. As few as 10 PFU of MCMV killed 100% of IFN-α/β/γR−/− mice, and thus an LD50 could not be determined. However, the LD50 for IFN-α/β/γR−/− mice was 109-fold lower than for IFN-α/βR−/− mice. The difference in LD50 for IFN-α/βR−/− versus IFN-α/β/γR−/− mice was statistically significant (P < 0.0002).

Figure 1. Lethality studies in IFN unresponsive mice. Mice with targeted mutations in IFN responsiveness were infected with between 10 and 5 × 103 PFU of sgMCMV intraperitoneally and followed for mortality for 21 d or for 5 d after the last death observed in an experimental group. Data is shown as percentage of mortality. Data was derived from the following numbers of mice and experiments: 129: 84 mice in 7 experiments; C57BL/6: 69 mice in 3 experiments; IFN-γR−/−: 85 mice in 6 experiments; IFN-α/βR−/−: 93 mice in 5 experiments; IFN-α/β/γR−/− mice: 44 mice in 4 experiments; IFN-γ−/−: 41 mice in 3 experiments.

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Because MCMV can cause aortic disease in newborn immunocompetent mice (16), we examined the aorta for signs of pathology over the first 154 d of infection (Table 1). Early after infection (days 28–56), lesions of the aorta were seen in 4 out of 10 normal 129 and 5 out of 6 IFN-γR2/2 mice. Interestingly, after day 84 aortic lesions were not seen in 129 mice (0 out of 24 mice; Table 1). In contrast, most IFN-γR2/2 mice had significant lesions as late as 154 d after infection (15 out of 27 mice; Table 1 and Fig. 2). This difference is statistically significant, with P, 0.0033. These lesions involved all layers (intima, media, and adventitia) of the vessels (Figs. 2, C and D, and 3 A). A prominent inflammatory infiltrate consisting primarily of mononuclear cells was observed (Figs. 2 D and 3 A). Cytomegalic inclusion bodies were seen within cells in the aortic media (Fig. 3 B). Immunohistochemistry revealed MCMV antigens in the media of affected aortas (Fig. 3, C and D). Cells in the aortic media containing inclusions and MCMV antigen were morphologically consistent with smooth muscle cells. We have previously published that arteritis is not seen in mock infected IFN-γR2/2 mice observed for 6 to 15 wk (40). Furthermore mice infected with either a myocarditic reovirus reassortant (8B) or HSV failed to show lesions 84 d after infection (Table 1). At this time IFN-γR2/2 mice infected with MCMV showed aortic pathology. These experiments demonstrate that MCMV causes chronic vascular pathology in IFN-γR2/2 mice, and illustrate a tropism for cells of the aortic media during chronic infection.

MCMV-infected IFN-γR2/2 Mice Develop Chronic Aortitis. Given the importance of IFN-γ to regulating acute MCMV infection, we examined the course of chronic MCMV infection in IFN-γR2/2 mice given a dose that most would survive for at least 21 d of infection (Fig. 1; 1–5 x 10^4 PFU). Because MCMV can cause aortic disease in newborn immunocompetent mice, we examined the aorta for signs of pathology over the first 154 d of infection (Table 1). Early after infection (days 28–56), lesions of the aorta were seen in 4 out of 10 normal 129 and 5 out of 6 IFN-γR2/2 mice. Interestingly, after day 84 aortic lesions were not seen in 129 mice (0 out of 24 mice; Table 1). In contrast, most IFN-γR2/2 mice had significant lesions as late as 154 d after infection (15 out of 27 mice; Table 1 and Fig. 2). This difference is statistically significant, with P, 0.0033. These lesions involved all layers (intima, media, and adventitia) of the vessels (Figs. 2, C and D, and 3 A). A prominent inflammatory infiltrate consisting primarily of mononuclear cells was observed (Figs. 2 D and 3 A). Cytomegalic inclusion bodies were seen within cells in the aortic media (Fig. 3 B). Immunohistochemistry revealed MCMV antigens in the media of affected aortas (Fig. 3, C and D). Cells in the aortic media containing inclusions and MCMV antigen were morphologically consistent with smooth muscle cells. We have previously published that arteritis is not seen in mock infected IFN-γR2/2 mice observed for 6 to 15 wk (40). Furthermore mice infected with either a myocarditic reovirus reassortant (8B) or HSV failed to show lesions 84 d after infection (Table 1). At this time IFN-γR2/2 mice infected with MCMV showed aortic pathology. These experiments demonstrate that MCMV causes chronic vascular pathology in IFN-γR2/2 mice, and illustrate a tropism for cells of the aortic media during chronic infection.

MCMV-infected IFN-γR2/2 Mice Maintain Chronic Productive MCMV Infection for 180 d after Infection. Aortic pathology in IFN-γR2/2 mice seemed likely to be due to an important role of IFN-γ in controlling chronic MCMV infection. 29 IFN-γR2/2 mice and 28 wild-type 129 mice were therefore evaluated for the presence of preformed infectious virus at times during which MCMV transitions from a

### Table 1. Arteritis in 129 and IFN-γR−/− Mice

| Mouse strain | Virus | Dose (× 10^4 PFU) | Day killed | Elastic arteritis (positive/total) |
|--------------|-------|-------------------|------------|----------------------------------|
| 129          | MCMV  | 5                 | 28         | 2/5                              |
|              | MCMV  | 5                 | 56         | 2/5                              |
|              | MCMV  | 5                 | 84         | 0/5                              |
|              | MCMV  | 1                 | 120        | 0/5                              |
|              | MCMV  | 1–1.25            | 154        | 0/14                             |
| IFN-γR−/−    | MCMV  | 5                 | 28         | 2/3                              |
|              | MCMV  | 5                 | 56         | 3/3                              |
|              | MCMV  | 5                 | 84         | 3/4                              |
|              | MCMV  | 1                 | 120        | 5/8                              |
|              | MCMV  | 1–1.25            | 154        | 7/15*                            |
| IFN-γR−/−    | HSV   | 1                 | 84         | 0/9                              |
|              | Reovirus | 200             | 84         | 0/10                             |

*In one experiment, 5 out of 5 mice had arteritis, and in a second experiment, 2 out of 10 mice had arteritis.

### Table 2. Shedding of Preformed Infectious MCMV in Tissues of Control and IFN-γ−/− Mice over Time

| Mouse strain | Days after infection | Preformed infectious MCMV (number shedding MCMV/total evaluated) |
|--------------|----------------------|---------------------------------------------------------------|
|              |                      | Salivary gland | Spleen | Peritoneal cells | Bone marrow | Total |
| 129          | 72                   | 4/4           | 1/4    | 0/4             | 0/4         | 4/4   |
|              | 90                   | 1/3           | 0/3    | 0/3             | 0/3         | 1/3   |
|              | 120                  | 1/8           | 0/8    | 0/3             | 0/3         | 1/8   |
|              | 154                  | 1/8           | 1/3    | 0/8             | 0/8         | 2/8   |
|              | 166–180              | 3/5           | 0/5    | 0/4             | 0/4         | 3/5   |
| IFN-γR−/−    | 72                   | 3/3           | 1/3    | 0/3             | 0/3         | 3/3   |
|              | 90                   | 3/3           | 3/3    | 3/3             | 0/3         | 3/3   |
|              | 120                  | 8/9           | 8/9    | 1/3             | 0/3         | 9/9   |
|              | 154                  | 8/8           | 3/8    | 2/8             | 0/8         | 8/8   |
|              | 166–180              | 6/6           | 1/6    | 0/5             | 0/5         | 6/6   |

*Number of mice shedding preformed infectious MCMV in at least one tissue.
chronic productive salivary gland infection to a latent infection in a majority of immunocompetent mice (Table 2). Spleen, salivary gland, PECs, and bone marrow cells were sonicated and cultured on MEFs to detect low levels of preformed infectious virus. IFN-\(\gamma\)R\(^2/2\) mice were productively infected in at least one of the tissues tested out to 180 d after infection. Only one of four IFN-\(\gamma\)R\(^2/2\) mice survived to the 180 d time point. Although preformed infectious virus was detected in some congenic control 129 mice, the majority established a latent infection in the absence of persistent productive infection as has been shown for BALB/c mice (31, 32). Differences between IFN-\(\gamma\)R\(^-/-\) and 129 mice are statistically significant for total mice shedding (\(P<0.009\)), mice shedding in the spleen (\(P<0.001\)) and salivary gland (\(P<0.009\)). The presence of low levels of preformed MCMV in IFN-\(\gamma\)R\(^-/-\) mice in spleen and PECs demonstrates that IFN-\(\gamma\) regulates chronic infection with MCMV in several locations in addition to salivary gland (27).

Establishment of Latent Infection in IFN-\(\gamma\)R\(^-/-\) Mice. Since we detected chronic productive infection in several tissues in IFN-\(\gamma\)R\(^-/-\) mice, we were interested in assessing whether a latent infection could be established in the absence of IFN-\(\gamma\) responsiveness. Of the tissues tested for chronic productive MCMV infection in IFN-\(\gamma\)R\(^-/-\) mice, only bone marrow consistently lacked detectable preformed infectious MCMV. Bone marrow cells (between 10 and \(25 \times 10^6\) per mouse) were tested from 22 IFN-\(\gamma\)R\(^-/-\) mice and contained no preformed infectious virus as detected by coculture with MEFs (six independent experiments). Since coculture with MEFs regularly detects 5 PFU of MCMV, these experiments showed that there was \(<1\) PFU of MCMV per 2-5 \(\times 10^6\) bone marrow cells from IFN-\(\gamma\)R\(^-/-\) mice. To determine if the lack of infectious MCMV in bone marrow cells reflected a lack of MCMV infection in the bone marrow of IFN-\(\gamma\)R\(^-/-\) mice, DNA was isolated from bone marrow cells and PCR was used to

Figure 2. MCMV induced vascular pathology in IFN-\(\gamma\)R\(^-/-\) mice. Mice were killed 154 d after infection with 10⁴ PFU MCMV. All sections were stained with hematoxylin and eosin. L, lumen; I, intima; M, media; Adv, adventitia; V, aortic valves. (A) Normal aorta in an MCMV-infected 129 mouse. (B) High power view from the boxed region shown in A. Uninfected IFN-\(\gamma\)R\(^-/-\) mice show the same histology. (C) Aorta from an MCMV-infected IFN-\(\gamma\)R\(^-/-\) mouse. (D) High power view of the boxed region shown in C.
quantitate the level of viral genome (31). Dilutions of DNA from 129 and IFN-γR−/− mice revealed that the amount of viral genome in DNA samples was similar (Fig. 4). The presence of viral genome in the absence of productive infection suggests that MCMV establishes either a latent or abortive infection in the bone marrow of IFN-γR−/− mice. To demonstrate that the viral genome that we detected was due to latent infection, bone marrow cells were cultured in vitro to induce reactivation. 8 out of 19 cultures of IFN-γR−/− bone marrow reactivated MCMV. 2 out of 18 cultures of wild-type 129 bone marrow reactivated MCMV. The presence of viral genome in bone marrow cells, and the capacity to reactivate MCMV from bone marrow cultures demonstrates that IFN-γ is not required to establish latency in the bone marrow.

IFN-γ Directly Inhibits MCMV Reactivation from Latency in Spleen and Lung Explants. Since latency can be established in the absence of IFN-γ responsiveness, we examined the hypothesis that IFN-γ controls reactivation from MCMV latency. We took advantage of an in vitro assay in which latently infected spleen (31, 33, 44, 45) or lung explants reactivate at high frequency upon culture in vitro. Explants were cultured in the presence of media alone, media plus mock IFN, or media with 100 U/ml IFN-α, IFN-β, or IFN-γ. Explant supernatant was sampled every 3–4 d for 64–116 d and tested for infectious virus by culture on MEFs (Fig. 5, A and B). Explants were tested for chronic productive MCMV infection present at the time of explantation by culturing a sonicated aliquot with MEFs (see Materials and Methods). These control cultures were consistently negative. Although 100% of lung explants that were treated with media alone or with mock IFN reactivated by day 35, only 12.5–15% of explant cultures treated with IFN-γ reactivated (Fig. 5 A). Explants treated with IFN-α

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or IFN-γ or IFN-β showed an intermediate block to reactivation. Similar results were seen with spleen explants, although the frequency of reactivation is lower (Fig. 5B). We considered several explanations for the lack of detection of reactivated MCMV in explants treated with IFN-γ. IFN treatment might prevent reactivation of MCMV by killing latently infected cells. To assess this possibility, IFN treatments were withdrawn on day 35 for lung explants and day 38 for spleen explants. In some explants, IFN-γ treatment was continued. After cytokine removal, an additional 9 out of 21 lung explant cultures previously treated with IFN-γ reactivated, suggesting that the blockade of reactivation induced by IFN-γ is at least partially reversible (Fig. 5A). Cultures that continued to receive IFN-γ during this time did not reactivate. Similar results were seen in spleen explant cultures (Fig. 5B). This reversibility of IFN-γ effect argues against IFN-γ-induced killing of cells containing latent virus as a mechanism of reactivation blockade. Another explanation for our lack of detection of MCMV reactivation in these treated explants may be that the IFNs carried over from explant cultures decrease our ability to detect infectious MCMV in MEF cultures. However, 5 PFU of MCMV added to media from IFN-γ-treated explants was detected on MEF monolayers in 18 out of 18 times. These data were consistent with a direct blockade of MCMV reactivation by IFN-γ treatment.

Mechanism of Action of IFN-γ in Explant Reactivation. IFN-γ could inhibit reactivation by blocking either cellular reactivation or growth of reactivated virus. Since the molecular nature of MCMV latency is unknown, we could not examine an effect of IFN-γ on cellular reactivation directly. However, we hypothesized that IFN-γ could block

Figure 4. IFN-γR−/− mice are capable of establishing latency in the bone marrow. DNA was prepared from bone marrow from IFN-γR−/− and 129 mice between 72 and 154 d after infection. DNA was adjusted to 0.1 μg/μl in TE and then serially diluted in trNA (0.1 μg/μl). Each sample was tested in a nested PCR assay for the presence of MCMV immediate early 1 DNA (sensitivity 1 copy). Data is expressed as the percentage of PCR reactions for a given sample which were positive. 14–26 reactions were performed for each dilution.

Figure 5. Effects of IFN-γ on reactivation in vitro. (A) Lungs from 8–10 latently infected BALB/c mice were pooled, minced, homogenized, and divided evenly for culture in six 6-well plates (12 wells per condition in each of two experiments) in the presence of media alone (no IFN), mock IFN, or 100 U/ml IFN-α, IFN-β, or IFN-γ. Supernatant was tested for the presence of infectious virus, and media was changed every 3–4 d. On day 35 cytokine treatments were removed from all cultures except for two 6-well plates per experiment, which contained 100 U/ml IFN-γ, until day 116. Data shown is the average of two independent experiments. (B) Spleen explants prepared from 8–13 latently infected BALB/c or C57BL/6 mice treated in the same manner as the lung explants (two independent experiments, results from BALB/c and C57BL/6 mice showed similar trends).
reactivation in explant cultures by blocking the growth of small amounts of virus released during cellular reactivation. We therefore made lung and spleen explant cultures using organs from uninfected mice and treated them with IFNs as for explants from latently infected organs. To mimic a reactivation event, either 10 or 50 PFU of MCMV was added on day 12, and the explants were observed for production of MCMV for the ensuing 39 d (Fig. 6). The number of wells productively infected with MCMV was decreased by IFN-γ treatment in both spleen and lung explants given either 10 or 50 PFU (Fig. 6). This shows that IFN-γ can block productive infection with low levels of MCMV. IFN-α had a similar effect when an inoculum of 10 PFU was used, but was less effective than IFN-γ when an inoculum of 50 PFU was used. These data demonstrate that under certain conditions of infection, IFN-γ can effectively prevent growth of low levels of MCMV in explant cultures. This blockade could account for the inhibition of reactivation we observed in explant cultures from latently infected mice (Fig. 5). The lack of IFN-γ-mediated inhibition of outgrowth of reactivated virus could contribute to the presence of preformed infectious MCMV in IFN-γR−/− mice detected during chronic infection (Table 2).

**Discussion**

The experiments reported here demonstrate the following important points: (a) IFN-γ regulates chronic infection with MCMV in multiple tissues in addition to the salivary gland (27); (b) IFN-γ is not required for establishment of MCMV latency; (c) IFN-γ can reversibly block reactivation from latency in an in vitro model; (d) one mechanism by which IFN-γ might regulate chronic MCMV infection is blockade of the growth of low levels of infectious virus; and (e) one consequence of the absence of IFN-γ responsiveness is chronic inflammatory disease in the large elastic arteries. Previous work has shown that IFN-γ is present for prolonged times in trigeminal ganglia latently infected with the α-herpesvirus HSV (46, 47), suggesting IFN-γ as a candidate for regulating chronic HSV infection. IFN-γ plays a role in chronic infection with γ-herpesviruses, as is illustrated by the severe chronic elastic arteritis seen in IFN-γ nonresponsive mice infected by the murine γ-herpesvirus 68 (γHV68; reference 40). Experiments presented here provide the first evidence that IFN-γ regulates chronic infection with a β-herpesvirus, MCMV, potentially by controlling reactivation from latency. In addition, lack of IFN-γ regulation of MCMV infection has significant pathologic consequences for the host. The fact
that lack of response to a single cytokine results in severe MCMV-associated pathology suggests that penetrance and severity of disease during chronic herpesvirus infection is regulated by relatively subtle changes in host defense.

IFN-γ and Chronic MCMV Infection. We found that IFN-γ is important for controlling chronic infection with MCMV, but was not essential for the establishment of latency in bone marrow cells. It was possible that events during early stages of infection set the stage for chronic productive infection (e.g., by altering the cells infected or the viral load in specific cells or tissues). However, IFN-γ was effective at preventing the reactivation of latent MCMV from tissue explants, suggesting that IFN-γ has significant effects during chronic infection in addition to any effects of IFN-γ during acute infection. Notably, the effect of IFN-γ lasted for up to 116 d, and was reversible after 30–40 d of culture. These results argue that IFN-γ is very effective in this role, and that IFN-γ does not act by killing all latent cells. IFN-α and IFN-β were less effective. This, combined with the fact that IFN-γ has a role during acute infection that is not redundant to the effects of IFN-α and IFN-β (Fig. 1), argues strongly that IFN-γ is a key regulator of all phases of MCMV infection.

The effect of IFN-γ on reactivation from tissue explants could be due to effects on either cellular reactivation or growth of virus in explant cultures after cellular reactivation has occurred. We found that IFN-γ can completely block the growth of low levels of MCMV in explant cultures. This effect was significant enough that it could account entirely for the effects of IFN-γ on reactivation from latent tissue explants. This being the case, we cannot address the possibility that IFN-γ might have a role in regulating cellular reactivation. The mechanism by which IFN-γ works to prevent MCMV growth is controversial. Several studies have shown that IFN-γ can decrease viral yields (21, 24, 25), but studies demonstrating an effective block on replication of low levels of infectious MCMV have not previously been reported. Some studies have suggested a role for IFN-γ in regulating transcription of the MCMV immediate early genes, whereas others have suggested a role for IFN-γ in the lytic replication cycle (24, 25). Further studies will be required to define these mechanism(s) at the molecular level, and to understand how they might contribute to prolonged blockade of viral growth.

It is interesting that MCMV and HCMV both have mechanisms to block the signaling of IFN-γ in infected cells. MCMV inhibits IFN-γ induction of HCMV gene expression at a step downstream from STAT (signal transducer and activator of transcription)-1 phosphorylation and nuclear translocation (48). HCMV inhibits IFN-γ signaling in infected cells by altering the turnover of Janus (JAK) family kinases required for IFN-γ action (49). Thus, both the viruses have developed countermeasures that combat the effects of IFN-γ in infected cells. This creates an interesting balance between cytokine secretion and effectiveness in vivo, and virus-encoded anticytokine actions. It is likely that this critical balance defines the outcome of chronic CMV infection. The capacity of HCMV to inhibit IFN-γ signaling may explain the lack of effectiveness of IFN-γ in macrophages treated with IFN-γ after infection (30).

The source of IFN-γ during chronic infection has not been determined. Since IFN-γ is important in both the adaptive (both CD4- and CD8-mediated) and the innate (NK cell–mediated) responses to MCMV infection, future studies will have to assess the role of all three of these lymphocyte subsets as IFN-γ producers during chronic infection. Since CD8 T cell–mediated secondary vaccine responses do not require IFN-γ (42), we favor the hypothesis that CD4 T cells or NK cells are important during chronic infection. This would be consistent with the proven role for CD4 T cells and IFN-γ in controlling persistent productive infection in the salivary gland (18, 27).

MCMV and Vascular Disease. In this study, we confirmed that MCMV infects the great elastic arteries (16), and demonstrated for the first time that in the absence of the IFN-γ receptor MCMV causes chronic vascular pathology even in adult mice. It was notable that lack of IFN-γ responsiveness was not a prerequisite for MCMV induction of vascular pathology. Thus, normal mice showed signs of disease early after infection. However, IFN-γR−/− mice maintained lesions for much longer than normal mice, suggesting that IFN-γ is critical for controlling infection in the large elastic arteries. This is remarkably parallel to our studies in mice infected with γHV68. γHV68 causes severe elastic arteritis in IFN-γ−/− mice (40). The fact that MCMV and γHV68 are quite distinct in primary sequence (50, 51) but cause disease of the elastic arteries in similar immunodeficient mice argues for the nature of the interaction between the tissue (elastic arteries) and IFN-γ as the critical determinant of virus induced vasculitis rather than specific receptors or other proteins shared between MCMV and γHV68. Alternatively it is possible that both MCMV and γHV68 share specific IFN-γ–sensitive interactions with smooth muscle cells or other cells of the elastic arteries.

Latent MCMV has been identified in endothelial cells by PCR in situ hybridization (52). It is tempting to speculate that the lack of IFN-γ results in chronic vascular disease because of a failure to control reactivation in endothelial cells. An alternative hypothesis is that latently infected macrophages (37, 52) are the source of reactivating MCMV that causes arteritis. IFN-γ may also be essential for regulating vascular pathology via effects on the proliferation of smooth muscle cells (53) or the development of foam cells, which characterize atherosclerotic lesions (54).

In this report, we provide evidence that IFN-γ is a critical component in the regulation of chronic MCMV infection, without which severe pathologic consequences develop. This may have implications for the role of herpesviruses in vascular pathology. Explant studies suggest that IFN-γ regulates MCMV latency by inhibiting reactivation from latency. One mechanism for this inhibition is the prevention of outgrowth of low levels of virus. Further studies are needed to evaluate the mechanism of this block of reactivation, and to define the role of reactivation in the induction of vascular pathology.
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