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Gamma Interferon Is Not Essential for Recovery from Acute Infection with Murine Gammaherpesvirus 68

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Murine gammaherpesvirus 68 (MHV-68) when administered intranasally induces high levels of gamma interferon (IFN-γ) in the lymphoid tissues of infected mice. In order to investigate the role of this cytokine in the immune response to MHV-68, mice which were congenitally deficient in the IFN-γ gene (IFN-γ knockout mice) were infected with the virus. Comparison of the courses of the disease in wild-type control and IFN-γ knockout mice revealed surprisingly little difference. Both groups of mice had cleared infectious virus from the lungs 15 days after infection, although there did appear to be a slight delay in viral clearance in the IFN-γ knockout mice. In addition, after the initial phase of viral clearance, the lungs of both groups remained clear of replicating virus throughout the course of the experiment, which concluded 34 days after infection. Consistent with these observations, cytotoxic T-cell activities were similar in the two groups of mice. Levels of latent virus were comparable in wild-type and knockout mice over the time course studied. Furthermore, analysis of the numbers, types, and activation status of cells in the lungs, lymph nodes, and spleens of control and knockout mice revealed no striking difference. This suggests that IFN-γ is not essential for regulating the cell recruitment or proliferation that normally occurs during this viral infection. Apart from the expected lack of IFN-γ, cytokine profiles were not dramatically altered in IFN-γ knockout mice, demonstrating that IFN-γ did not suppress the proliferation or differentiation of Th2 cells during MHV-68 infection. These observations indicate that IFN-γ plays a nonessential or redundant role in the control of acute infection with MHV-68.

Murine gammaherpesvirus 68 (MHV-68) is a naturally occurring rodent pathogen (2) which is closely related to Epstein-Barr virus, human herpesvirus 8, and herpesvirus saimiri (6). Intranasal (i.n.) administration of MHV-68 results in acute productive infection of lung alveolar epithelial cells and a latent infection in B lymphocytes (4, 17, 18, 19). The virus induces an inflammatory infiltrate in the lungs and enlargement of the lymph nodes and spleen. Infectious virus is cleared from the lungs 10 to 13 days after infection by a mechanism which is dependent on immune CD8, but not CD4, T cells (4, 7). Previous studies have shown that MHV-68 induces high levels of gamma interferon (IFN-γ) production in the spleens and lymph nodes of mice infected i.n. with the virus (12). Levels of IFN-γ peak just prior to viral clearance from the lungs, indicating a potential role in this process (12). Class II knockout mice, which lack CD4 T cells, lose the ability to mount a recall IFN-γ response to MHV-68, whereas this response is maintained in wild-type mice. This coincides with recrudescence of MHV-68 in the lungs of class II knockout, but not wild-type, mice (4). Furthermore, IFN-γ is known to play a role in the clearance of several other herpesviruses (3, 11, 16). Taken together, these results suggested that IFN-γ might be an important immunomodulatory factor in MHV-68 infection. To test this hypothesis, we examined various aspects of the immune response to MHV-68 in mice with a targeted disruption of the IFN-γ gene.

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MATERIALS AND METHODS

Mice. Breeding pairs of BALB/c mice which were heterozygous for the disruption of the IFN-γ gene were originally obtained from Genentech Inc. (San Francisco, Calif.). The mice were bred and housed under specific-pathogen-free conditions in the animal facility at St. Jude Children's Research Hospital. The genotypes of subsequent littermates were screened by PCR on tail DNA according to standard protocols obtained from Genentech. Tail DNA was isolated by using a kit from Qiagen (Chatsworth, Calif.) according to the manufacturer's instructions. The genotypes of IFN-γ knockout and wild-type mice were verified by an enzyme-linked immunosorbent assay (ELISA) for IFN-γ, using culture supernatants from cells restimulated in vitro with MHV-68 as described below. In some experiments, culture supernatants from spleen cells restimulated with 2 μg of concanavalin A (Sigma) for 48 to 72 h were also tested. Male and female IFN-γ knockout and wild-type mice, aged 6 to 15 weeks, were used. In some cases, BALB/c mice purchased from Jackson Laboratory (Bar Harbor, Maine) were substituted for the wild-type controls. Wild-type and IFN-γ knockout mice were age- and sex-matched in all experiments.

Viral infection and sampling. MHV-68 virus (clone G2.4) was obtained from A. A. Nash, Edinburgh, United Kingdom, and stocks were grown in owl monkey kidney (OMK) cells (ATCC CRL 1556). Mice were anesthetized with 2,2,2 tribromoethanol and infected i.n. with 4,000 PFU of the virus in phosphate-buffered saline. At various times after infection, the mice were terminally anesthetized with 2,2,2 tribromoethanol and bled from the right axilla or vena cava. The infiltrating inflammatory cells were harvested by bronchoalveolar lavage (BAL) via the trachea, and single-cell suspensions were prepared from the spleen and mediastinal and cervical lymph nodes (MLN and CLN, respectively) as previously described (1). Live cell counts were done by trypan blue exclusion. Following lavage, lungs were removed and sonicated in medium on ice prior to virus titration.

Treatment with antibodies against IFN-γ or CD4 in vivo. In one set of experiments, wild-type mice were infected with MHV-68 and, after a 10-week interval, were treated with either anti-IFN-γ antibody XMG1.2 (0.5 ml [per mouse] of a 1/10 dilution of ascites fluid containing 20 mg of immunoglobulin G (IgG) per ml intraperitoneally at 2- to 3-day intervals) or 0.5 ml of rat IgG (2 mg/ml) as a control for 14 days. Lungs were then harvested for virus titration as described above. The effect of depletion of CD4 cells in vivo in IFN-γ knockout and wild-type mice was also assessed by treatment of groups of mice with either anti-CD4 antibody (GK1.5) or control rat IgG as described above, commencing 1 day prior to infection. Lungs were harvested for virus titration 22 days after infection.

Virus titration and infectious centers assay. Virus titers were determined by plaque assay on NIH 3T3 cells (ATCC CRL 1658) as described previously (4). Briefly, dilutions of stock virus, sonicated mouse tissues, or sonicates of lympho-

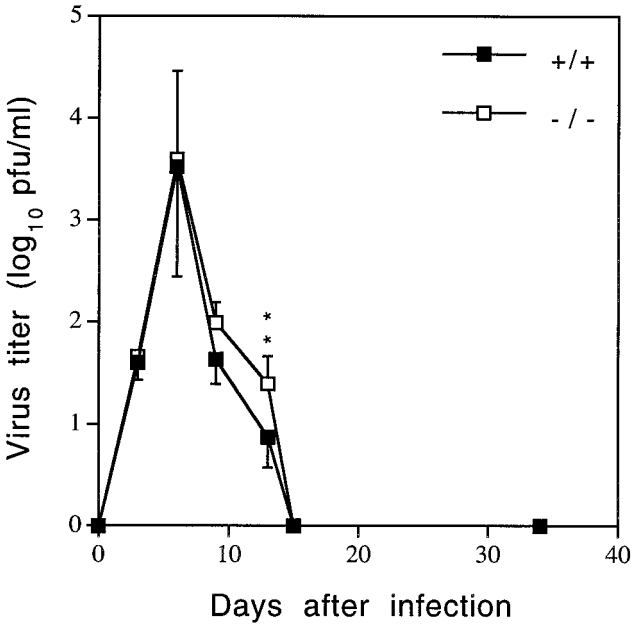

FIG. 1. Virus titers in the lungs of MHV-68-infected IFN-γ knockout (−/−) and wild-type (+/+) mice. Lung virus titers were determined at specified times after infection of mice as described in Materials and Methods. Results are expressed as mean log_{10} PFU/ml of a 10% tissue sonicate ± standard deviation for three separate experiments at day 6; two experiments each at days 13, 15, and 34; and one experiment at day 3. Tissues from three to four mice were tested at each time point in each experiment. The limit of detection was 3 PFU. The double asterisk indicates a significant difference in virus titers between the two groups at day 13 after infection ($P < 0.01$).

cytes were adsorbed onto NIH 3T3 monolayers for 1 h at 37°C and overlaid with carboxymethyl cellulose (CMC). After 5 days, the CMC overlay was removed, and the monolayers were fixed with methanol and stained with Giemsa to facilitate determination of the number of plaques.

The frequency of latently infected lymphocytes was determined with an infectious centers assay. Cell suspensions prepared from lymph nodes or spleen were plated at various cell densities on monolayers of NIH 3T3 cells, incubated overnight, and then overlaid with CMC. The cells were cocultured for 5 to 6 days, after which the overlay was removed and the number of plaques was determined as described above.

Cytotoxicity assays. Cytotoxic T-cell activity was determined with a redirected assay on suspensions of lymph node, spleen, or BAL cells. The BAL cells were incubated in petri dishes for 1 h at 37°C to remove macrophages by plastic adhesion. Cell suspensions were incubated with ^{51}Cr-labeled P815 cells in the presence of the 2C11 monoclonal antibody (MAb) to CD3ε for 6 h at 37°C as previously described (4). The level of specific ^{51}Cr release is a measure of the total (virus-specific and nonspecific) cytotoxicity.

Flow cytometric analysis. Cells were stained with phycoerythrin or fluorescein-conjugated MAbs as previously described (13), except that the cells were fixed in 1% paraformaldehyde (Ted Pella Inc., Redding, Calif.) after staining. All antibodies were purchased from PharMingen (San Diego, Calif.). Isotype controls were included in each assay.

Cytokine ELISAs. Cytokine levels in culture supernatants from cells that had been restimulated in vitro with virus-infected, irradiated, antigen-presenting cells were assayed by sandwich ELISA as described previously (13). All reagents were obtained from PharMingen.

Statistical analysis. Data was analyzed with the Student *t* test.

RESULTS

IFN-γ is not essential for clearance of infectious virus from the lungs. The absence of IFN-γ did not prevent mice from clearing infectious MHV-68 from their lungs. Both wild-type and IFN-γ knockout mice had cleared virus 15 days after infection with MHV-68 (Fig. 1), in agreement with previous reports on wild-type BALB/c (7) and C57/BL6 mice (4). Peak lung virus titers were detected at day 6 after infection in both knockout and wild-type mice (Fig. 1). There was no significant difference between the lung virus titers for wild-type and IFN-γ knockout mice at this time point. However, there did appear to be a slight delay in viral clearance in the IFN-γ knockout mice, as they had higher lung virus titers than the wild-type mice ($P = 0.003$) at day 13 after infection. The lungs of both groups of mice remained clear of virus for at least 34 days after infection. Mice in both groups appeared healthy, at the latter time point, and the lungs appeared normal upon macroscopic examination.

To further investigate the role of IFN-γ in the control of latent virus, wild-type mice were infected with MHV-68 and left for 8 weeks. Groups of three mice were then treated for 14 days with neutralizing antibodies to IFN-γ or with control rat Ig. Neither group showed evidence of significant viral reactivation (lung virus titers for both groups were 0.1 ± 0.2 log PFU/ml).

Failure to control viral replication in mice lacking CD4 T cells is independent of IFN-γ. It had been noted previously that recrudescence of MHV-68 in the lungs of CD4 T-cell-deficient class II knockout mice at 20 to 30 days after infection coincided with loss of IFN-γ production (4). It therefore seemed possible that the combined lack of these factors, during the course of infection, might lead to uncontrolled viral proliferation. To test this hypothesis, groups of IFN-γ knockout and wild-type mice were treated with either anti-CD4 antibody or control rat Ig commencing 1 day prior to infection with MHV-68. Lungs were harvested for determination of virus titer 22 days after infection. Two separate experiments were done, with a total of four lungs for each group tested. However, the degree of viral replication appeared to be independent of IFN-γ, regardless of whether CD4 cells were present. Thus, at day 22 after infection, there was no significant difference in lung virus titers between CD4-depleted IFN-γ knockout and wild-type mice ($P = 0.10$). Consistent with previous observations of class II knockout mice (4), both CD4-depleted groups showed low levels of virus in the lungs (mean lung virus titers were 0.8 ± 0.34 and 1.34 ± 0.5 log_{10} PFU/ml for the IFN-γ knockout and the wild-type mice, respectively), whereas neither of the mock-depleted groups had detectable lung virus titers. The efficacy of CD4 depletion was assessed by fluorescence-activated cell sorter staining of spleen cells, as previously described (1). Less than 2% of the cells were $CD4^+$, indicating efficient depletion. The data show that, even in the absence of CD4 T cells, IFN-γ does not contribute significantly to the control of MHV-68 replication in the lungs.

IFN-γ does not regulate the frequency of latently infected cells during acute infection. The effect of IFN-γ on the frequency of latently infected cells was examined in the spleens and lymph nodes of MHV-68-infected IFN-γ knockout and wild-type mice. Although there were significantly more latently infected cells at day 6 in the MLN of IFN-γ knockout mice (Table 1), the difference was not statistically significant by day 13, as the frequency of latently infected cells varied considerably between animals. Moreover, there was no significant difference in the frequency of latently infected cells in the CLN or spleens of IFN-γ knockout and wild-type mice. Thus, the absence of IFN-γ did not lead to a general increase in latently infected cells.

IFN-γ is not essential for the generation of cytotoxic T cells. Following infection with MHV-68, significant levels of cytotoxic T-cell activity were detected in the spleens, MLN, CLN, and BAL populations of both IFN-γ knockout and wild-type mice (Fig. 2). A redirected cytotoxicity assay, which measures total activity (both virus-specific and nonspecific T-cell cytotoxicity), was used. Cytotoxic T-cell activity in uninfected mice was low or undetectable, indicating that the majority of this

TABLE 1. Frequencies of latently infected cells in the spleens and lymph nodes of mice infected with MHV-68[a]

Time after infection (days)	Mouse type[b]	Infectious centers/10^7 lymphocytes		
		Spleen	MLN	CLN
3	IFN-γ +/+	68	60	17
	IFN-γ −/−	72	40	20
6	IFN-γ +/+	36 ± 36	45 ± 21	133 ± 55
	IFN-γ −/−	30 ± 10	102 ± 2	220 ± 165
13	IFN-γ +/+	924 ± 1,281	229 ± 195	503 ± 631
	IFN-γ −/−	675 ± 926	688 ± 849	390 ± 480
15	IFN-γ +/+	1,140 ± 127	665 ± 191	1,981 ± 1,429
	IFN-γ −/−	1,540 ± 622	1,515 ± 969	1,820
34	IFN-γ +/+	57 ± 14	ND	67
	IFN-γ −/−	72 ± 16	ND	17

[a] Numbers of latently infected cells in lymphocyte suspensions were determined by using an infectious centers assay as described in Materials and Methods. Results are mean infectious centers per 10^7 lymphocytes ± standard deviation for three separate experiments at day 6; two experiments each at days 13, 15, and 34 (except for the CLN data at day 15, which was from a single experiment); and one experiment at day 3. Tissues from three mice were tested at each time point in each experiment. The limit of detection was three infectious centers. ND, not determined.

[b] +/+, wild type; −/−, IFN-γ knockout.

activity was induced by the virus (Fig. 2). In each site tested, the activity peaked at around day 13 to 15 after infection, coinciding with virus clearance. The maximum percentage of P815 target cells which were lysed peaked at 30 to 40% in lymph nodes, 25 to 35% in spleen, and 40 to 45% in BAL cells (Fig. 2). Generally, levels of activity in IFN-γ knockout mice were comparable or slightly higher than those in wild-type mice. These results indicate that IFN-γ was not essential for the generation of cytotoxic cells.

IFN-γ does not regulate cell recruitment or proliferation during MHV-68 infection. Previous studies indicated that IFN-γ might play a role in the activation and recruitment of cells into inflammatory sites or in the regulation of cell proliferation. Therefore, the numbers and types of cells in the lungs, lymph nodes, and spleens of IFN-γ knockout and wild-type mice were assessed. The increase in cellularity in the lymph nodes and spleens and the development of an inflammatory infiltrate in the lungs followed similar kinetics in IFN-γ knockout and wild-type mice (Fig. 3). As previously reported (4, 7, 12), 5- to 10-fold increases in the numbers of cells in spleens and lymph nodes were observed after infection of the mice with MHV-68. Very few cells were detected in BAL fluids from uninfected mice (data not shown). In the lymph nodes, spleens, and BAL fluids of both IFN-γ knockout and wild-type mice, cell numbers were maximal at around day 15 after infection and had declined by day 34. Thus, IFN-γ does not appear to be essential for the normal regulation of cell proliferation or recruitment during MHV-68 infection.

The cell subsets contributing to the increased cellularity in the MLN, spleen, and BAL populations were analyzed at day 15 after infection (the time point at which cell numbers were maximal (Fig. 3) and at day 34 after infection, when cell numbers had declined considerably (Fig. 3). The relative proportions of B cells and αβ T-cell-receptor-positive, CD4, and CD8 T-cells were very similar in IFN-γ knockout and wild-type mice (Fig. 4). Only small or inconsistent differences were seen. Only a small proportion of the cells in any of the tissues analyzed were γδ T-cell-receptor-positive T cells (Fig. 4). The level of expression of CD62L was used to subdivide CD4 or CD8 T cells into activated or memory and resting-cell subsets. The relative proportions of each subset (Fig. 4) were comparable in IFN-γ knockout and wild-type mice. In both cases, the majority of CD4 and CD8 cells in the BAL fluid exhibited low levels of expression of CD62L (indicating an activated or memory state) at both day 15 and day 34 after infection. A smaller but substantial proportion of the CD4 and CD8 T cells in the spleen

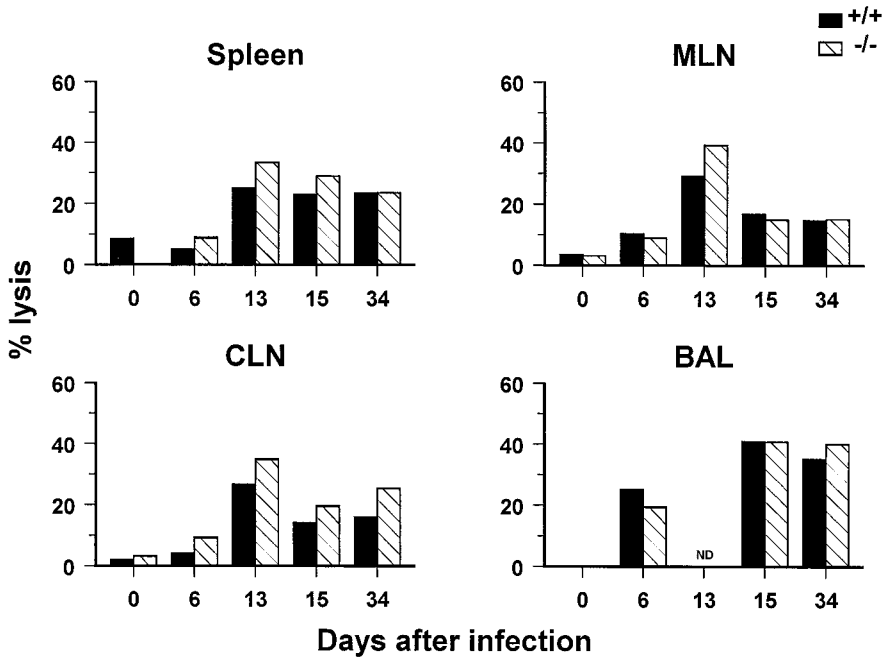

FIG. 2. Effector cytotoxic T-lymphocyte activity in the spleen, MLN, CLN, and BAL populations. Single-cell suspensions were prepared from groups of three IFN-γ knockout (−/−) or wild-type (+/+) mice, and cytotoxic T-lymphocyte activity was determined in a redirected 6-h ^{51}Cr release assay using P815 cells as targets. Data for an effector-to-target ratio of 40:1 is shown. Means for two separate experiments at each time point are shown. ND, not determined.

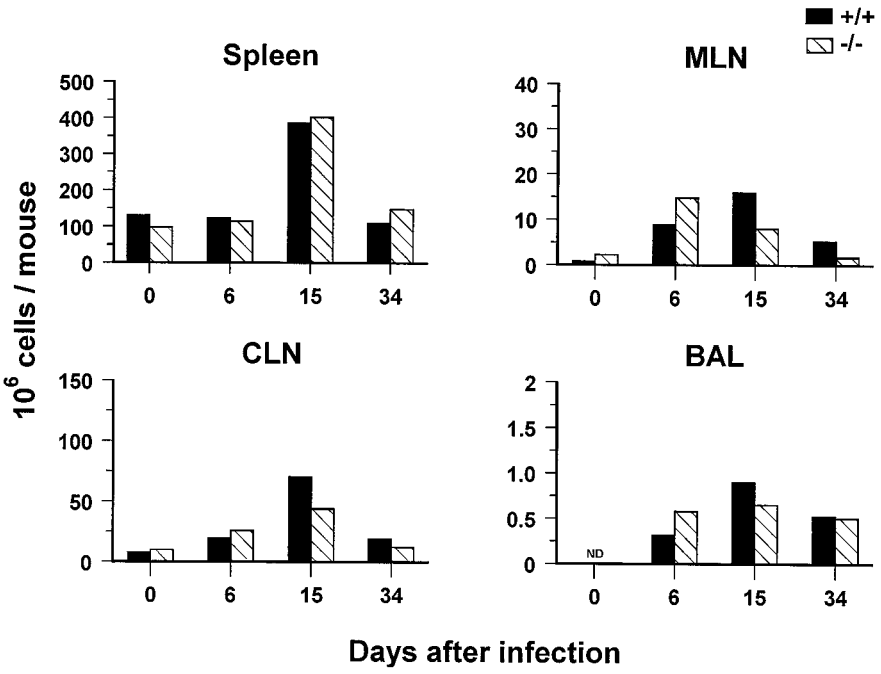

FIG. 3. Cell numbers in the BAL population, MLN, CLN, and spleen were determined at intervals following i.n. infection of IFN-γ knockout (−/−) and wild-type (+/+) mice with MHV-68. Single-cell suspensions were prepared from lymphoid tissue from groups of three mice, and viable cell counts were determined by trypan blue exclusion. Means of two separate experiments at each time point are shown. ND, not determined.

had low levels of expression of CD62L. This was >50% at day 15 and a little lower at day 34 and presumably reflects the massive splenomegaly during this viral infection. The situation was similar in the MLN: the proportions of CD4 and CD8 T cells which exhibited low levels of expression of CD62L were comparable in the IFN-γ knockout and wild-type mice. Again, these results suggest that IFN-γ is not essential for the activation of T cells during MHV-68 infection.

Lack of IFN-γ during MHV-68 infection does not induce a switch in the cytokine profile. Cytokines were assayed in culture supernatants following in vitro restimulation of splenocytes from virus-infected or control (day 0) mice. Supernatants were harvested at 24, 72, and 96 h after restimulation. Peak values are shown in Fig. 5 and were found at 72 h for all cytokines detected. The detection limits were 0.4 U/ml for interleukin 4 (IL-4) and IL-5, 0.4 ng/ml for IL-10, and 0.8 U/ml for all other cytokines. The results confirmed that the gene for IFN-γ had been inactivated in the IFN-γ knockout mice, as lymph nodes or spleen cells from the latter failed to produce IFN-γ, whereas those from wild-type mice produced substantial amounts of this cytokine (Fig. 5). Similar results were obtained with culture supernatants from concanavalin A-stimulated splenocytes (data not shown). No other major change in the cytokine profile was seen after restimulation with MHV-68-infected splenocytes. Neither wild-type nor knockout splenocytes produced detectable IL-2 or IL-4, although both produced IL-6 and IL-10 (Fig. 5). As reported previously, IL-6 and IL-10 were produced by naive (day 0) cells on restimulation with the virus. The relative levels of these two cytokines in IFN-γ knockout and wild-type mice varied, depending on the time point and tissue examined. However, the levels were not consistently higher in either the knockout or wild-type mice, and both groups produced significant levels of each cytokine in response to MHV-68 infection. Cells from the IFN-γ knockout mice did secrete very low levels of IL-5, whereas those from the wild-type mice did not. However, the amounts of IL-5 detected in the cultures from IFN-γ knockout mice were close to the detection limit of 0.4 U/ml. Taken together, these data do not indicate a major role for IFN-γ in controlling the cytokine profile.

DISCUSSION

Infection of mice with murine gammaherpesvirus and many other viruses induces T cells (and possibly other cells such as NK cells) to produce high levels of IFN-γ. In the case of MHV-68, this is a specific immune response which requires prior in vivo priming with the virus and is predominantly a function of αβ T cells (12).

Despite the high levels of IFN-γ induced during MHV-68 infection, this cytokine does not appear to be essential for clearance of infectious virus from the lungs (Fig. 1) or for controlling the numbers of latently infected cells, at least over a period of 35 days (Table 1). Only a slight delay in viral clearance was seen in the IFN-γ knockout mice. In a previous study on MHV-68-infected class II knockout mice, infectious virus reemerged in the lungs at around 22 days after infection (4). This correlated with a loss in the recall IFN-γ response, leading us to postulate that IFN-γ might be involved in immune surveillance. This does not appear to be the case, as viral reactivation was not observed in IFN-γ knockout mice or in wild-type mice treated with neutralizing antibodies to IFN-γ. It was possible that the combined lack of CD4 T cells and IFN-γ, rather than a lack of IFN-γ alone, led to a failure in immune surveillance. To address this possibility, we depleted CD4 T cells in vivo from either IFN-γ knockout or wild-type mice. There was no difference in lung virus titers between the CD4-depleted IFN-γ knockout and wild-type mice. It seems more likely that the inability to produce IFN-γ signals a more gen-

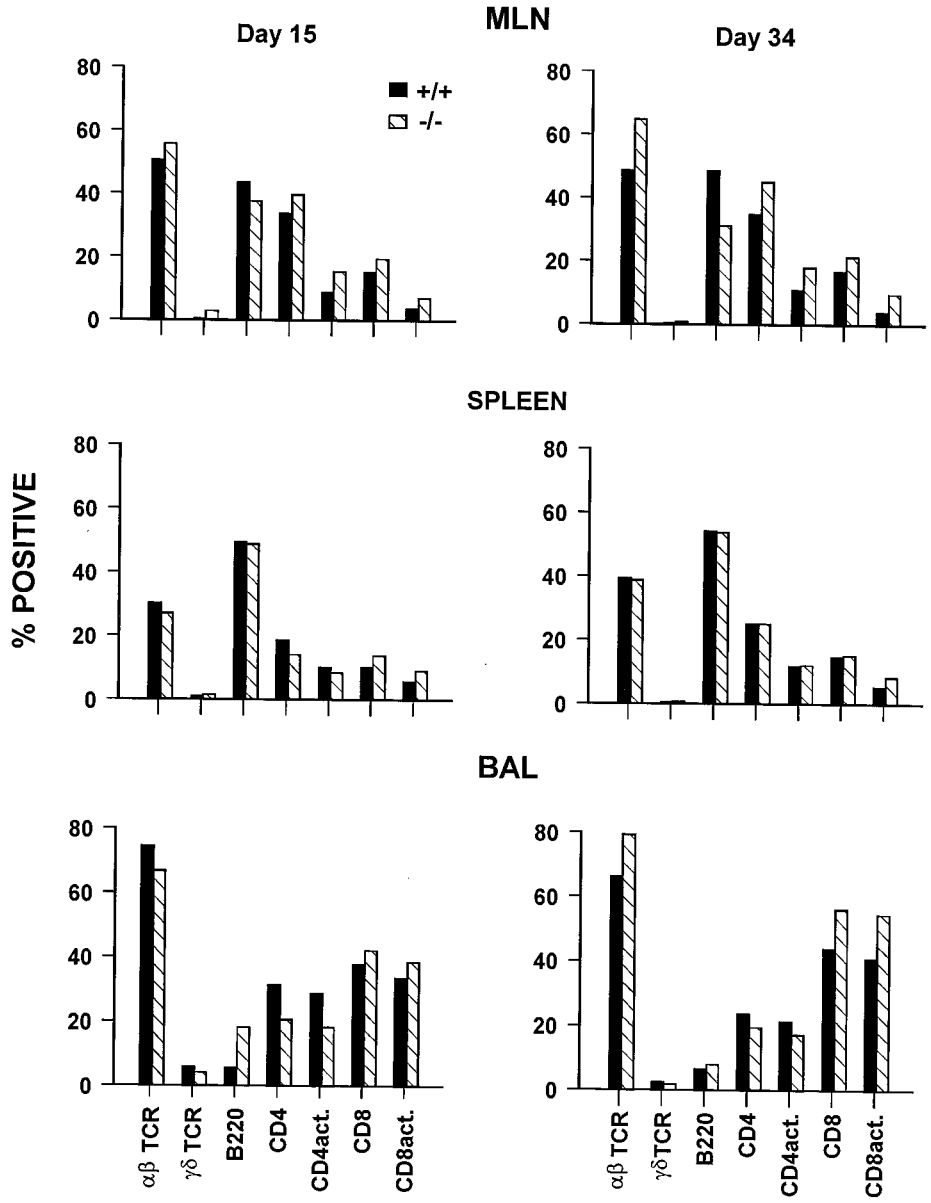

FIG. 4. Lymphocyte subsets in the MLN, spleens, and BAL populations of IFN-γ knockout (−/−) and wild-type (+/+) mice infected with MHV-68. Cells suspensions were prepared and stained with phycoerythrin or fluorescein-conjugated MAbs as previously described (13), except that cells were fixed in 1% paraformaldehyde (Ted Pella Inc.) after staining. The detection limit was less than 1% on the basis of staining with isotype-matched controls. CD4act. and CD8act. refer to CD4 and CD8 cells, respectively, which were classified as activated-memory cells based on their low expression of CD62L. TCR, T-cell receptor. The results are expressed as mean percentages of positive cells (determined by flow cytometry) for two separate experiments at each time point.

eral failure in the immune response which is dependent on CD4 T cells.

In two earlier studies on IFN-γ and IFN-γ receptor knockout mice, increased proliferative responses were observed in vitro (5, 15). However, in the present study, the increases in cellularity in the lymph nodes, spleen, and BAL fluid were similar in knockout and wild-type mice, suggesting a lack of effect in vivo. Furthermore, the rates of decrease in cellularity in these tissues after viral clearance were comparable in knockout and wild-type mice, suggesting that IFN-γ did not limit cell proliferation or accumulation.

Dalton et al. (5) showed increased cytolytic activity in IFN-γ knockout mice, while Wille et al. (20) showed diminished cytolytic activity in the absence of this cytokine and other studies showed no change in cytolytic activity (9–11). This probably reflects the use of different experimental models. In agreement with the latter results, we saw no change in cytolytic activity in MHV-68-infected mice (Fig. 2). CD8 T cells have been reported to play an essential role in the clearance of infectious MHV-68 and in maintaining immune surveillance (4, 7). It seems likely that this activity is mediated through cytotoxicity, and therefore, our data showing no change in T-cell cytotoxicity in IFN-γ knockout mice is consistent with the lack of any substantial effect on viral clearance.

Although IFN-γ has been reported to suppress the generation of Th2 cells (8), we saw little increase in IL-4 or IL-5 production in the IFN-γ knockout mice infected with MHV-68. Thus, consistent with other studies on IFN-γ or IFN-γ

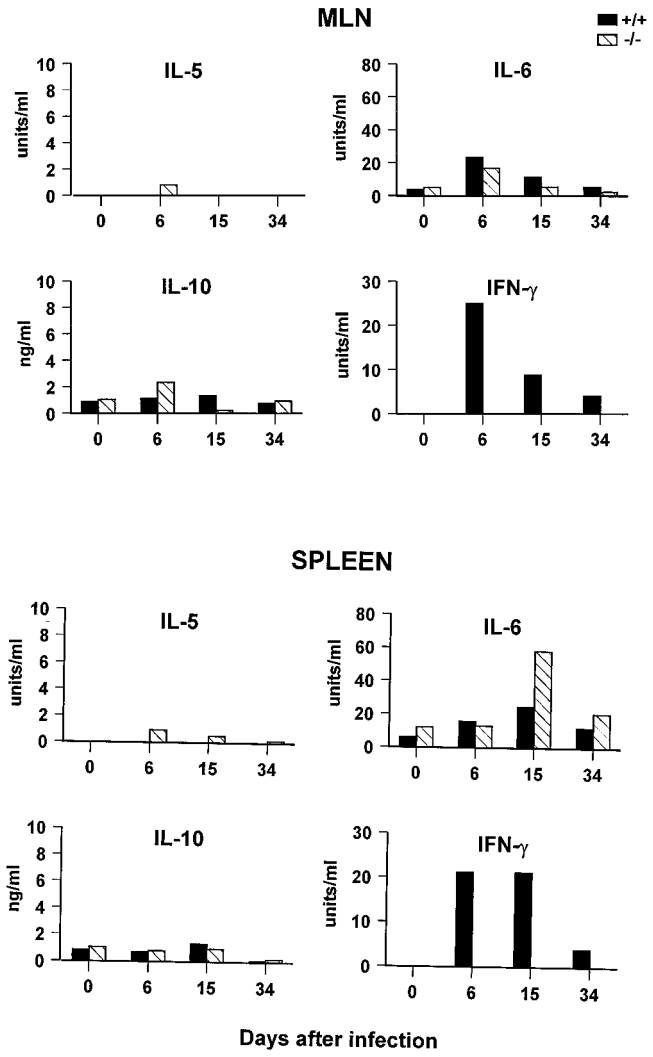

FIG. 5. Cytokine production during MHV-68 infection of IFN-γ knockout (−/−) and wild-type (+/+) mice. MLN or spleen cell suspensions were prepared and restimulated in vitro with virus-infected irradiated spleen cells. Cytokines were assayed in the culture supernatants by sandwich ELISA as described previously (13). The detection limit was below 0.4 U/ml for IL-4 and IL-5, below 0.4 ng/ml for IL-10, and below 0.8 U/ml for the other cytokines. Means of two separate experiments at each time point are shown. No IL-2 or IL-4 was detected.

receptor-deficient mice (14, 15), it does not appear that IFN-γ always mediates this kind of suppression. Other cytokines or costimulatory factors and the nature of the epitopes recognized may also play a part in determining the cytokine profile.

IFN-γ has been reported to play an important role in the clearance of several acute viral infections, including those of various strains of *Herpesviridae* (3, 11, 16). However, in murine cytomegalovirus infection, CD4-mediated clearance of virus from the salivary gland was found to be IFN-γ dependent, whereas clearance from other sites was not (11). Moreover, IFN-γ did not appear to play a major role in controlling the replication of pseudorabies virus (15). This suggests that IFN-γ may be produced as a general response to viral infection and is an effective defense mechanism against some, but not other, strains of virus (thus conferring a survival advantage).

To summarize, IFN-γ does not play an essential role in the clearance of infectious MHV-68. This may be due to redundancy of its actions with those of other cytokines, possibly via common signal transduction pathways. Alternatively, the lack of effect of IFN-γ may point to the importance of other immune mechanisms in the control of this virus.

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