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Mouse Strain Differences in the Chemokine Response to Acute Lung Infection with a Murine Gammaherpesvirus

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ABSTRACT

Numerous mouse strain–based differences in the immune response and in susceptibility to numerous pathogens have been described, but it is not known if these differences extend to chemokine responses to viral infection of the lungs. To define mouse strain–based differences in the host chemokine response and susceptibility to infection with murine gammaherpesvirus–68 (MHV-68), we compared the induced chemokine response to MHV-68 infection in the lungs of BALB/c and C57BL/6 mice at 1–15 days post-infection. CC and CXC chemokines were induced in both BALB/c and C57BL/6 following infection but the level of chemokine induction was significantly higher in the BALB/c mice for all chemokines measured. In addition, interferon-γ (IFN-γ) was also induced to a significantly higher level in the lungs of BALB/c infected mice compared to C57BL/6 mice. Interestingly, viral gene expression was lower in the lungs of C57BL/6 mice during the acute phase of replication. Titers of infectious virus were also greater in BALB/c lungs, although they did not achieve statistical significance. In contrast, latent viral load in the spleen, as measured by quantitative real-time PCR, did not significantly differ between mouse strains, suggesting that the establishment of latency is not affected by the amount of virus present during acute infection. This data suggests that robust chemokine response and expression of IFN-γ in the lungs of infected BALB/c mice does not correlate with increased resistance to infection. In addition, the significant differences in chemokine responses observed will be important factors to consider in future studies of viral pathogenesis using mouse models.

INTRODUCTION

THE LUNG is the site of entry for many viruses into their host and the dynamic balance of host/virus interactions in this important organ system is critical for the subsequent development of virally induced pathology and disease. Infection of mice with a variety of respiratory pathogens has proven to be a useful model system to elucidate many of the host factors. Of these host factors, chemokines are increasingly be-

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ing recognized as key mediators of inflammation and leukocyte recruitment in response to inflammatory stimuli including viral infections (16,22,25).

Infection of mice with murine gammaherpesvirus (MHV)–68 is an amenable model to study the pathogenesis of the gammaherpesviruses of which the human pathogens, Epstein-Barr virus and Kaposi's sarcoma herpesviruses, are members (31,40). MHV-68 replicates in the lungs following intranasal (i.n.) inoculation and is cleared by 12 dpi (35). The virus establishes latency early in infection in both mediastinal lymph nodes and spleen suggesting that trafficking from the site of acute replication in the lungs to the lymphoid compartments is a critical event in the viral life cycle. We have found that there is a biphasic induction of chemokines in the lungs of BALB/c mice infected with MHV-68 (42); others have shown a pronounced inflammatory infiltrate in lungs of MHV-68 infected mice subsequent to the resolution of acute infection (19).

Numerous mouse strain differences in immune responses have been described following infection with various viruses (1,29,37,43), bacteria (7,11), and parasites (9,12,20,23,30). These differences have been described primarily in the T_H1 or T_H2 pattern of cytokine production following *ex vivo* culture of lymphocytes isolated from a given mouse strain (18). In general, C57BL/6 mice tend to mount a predominantly T_H1 response characterized by IFN-γ production, while BALB/c mice tend to mount a predominantly T_H2 response characterized by interleukin (IL)–4 (34). Differing patterns of chemokine production and response to chemokine stimulation have also been correlated with T_H1 or T_H2 polarization *in vitro* (26,33,45), while *in vivo* mouse strain–based differences in chemokine or chemokine receptor expression have only been described at baseline in the spleen (4), and following infection or other antigenic stimulation in the cornea (44) or genital tract (7,8). Similar mouse strain–based differences in chemokine response have not been described in the lungs.

In this report, we determined whether there was a difference in the pattern or degree of chemokines induced in the lungs of C57BL/6 or BALB/c mice following i.n. inoculation with MHV-68. We found that there were significant differences between mouse strains in the chemokine response to acute infection with a markedly depressed chemokine response in C57BL/6 mice. In addition, little IFN-γ was produced in lung tissue of acutely infected C57BL/6 mice. These data suggest that mouse strains also exhibit polarization in their chemokine responses in infected lungs but this polarization does not reflect a classic T_H1 or T_H2 bias. Finally, differences in levels of viral gene expression between the two mouse strains were also observed, suggesting that C57BL/6 mouse was more resistant to MHV-68 infection than the BALB/c mouse.

MATERIALS AND METHODS

Cell lines, viruses, and mice. Owl monkey kidney (OMK) cells (ATCC #CRL-1556, Manassas, VA) and NIH 3T3 cells (ATCC #CRL-1658, Manassas, VA) were maintained in culture as described (24). The MHV-68 sequence clone G2.4 was used for wildtype infections. Generation of virus stocks and determination of viral titer by plaque assay was performed as described (3).

Four to six week old male BALB/c and C57BL/6 mice were purchased from Harlan Sprague (Indianapolis, IN) and maintained in specific-pathogen-free housing. Mice were inoculated i.n. under light anesthesia with 4×10^4 plaque-forming units (PFU) of virus diluted in 20 μL of sterile phosphate buffered saline (PBS; GibcoBRL, Grand Island, NY). Control mice were inoculated with 20 μL of supernatant from mock-infected OMK cells at an equivalent dilution in PBS. Organs were harvested at indicated times post-infection, snap frozen in a dry ice and ethanol bath, and stored at -80°C until processed. Between three and eight mice were used per time point, with the exception of two mice at 0 and 4 dpi.

RNA, DNA, and protein isolation. RNA was extracted from the left lung as described (5,24). DNA was extracted from the spleen using the DNeasy® Tissue Kit (Quiagen Inc., Valencia, CA) according to the manufacturer's instructions. Using a Tissue Tearor (BioSpec Products, Bartlesville, OK), the right lung was homogenized in sterile filtered Dubelco's MEM (BioWhittaker, Walkersville, MD) supplemented with glucose (4.5 g/L), 2 mM L-glutamine (Gibco-BRL, Gaithersburg, MD), penicillin-streptomycin (Gibco-BRL), 10% fetal bovine serum (Gemini BioProducts, Calabasas, CA), one Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Manheim, Germany) per 7 mL and Triton-X-100 (Fisher Scientific, Pitts-

burgh, PA; final concentration 0.01%). The homogenate was centrifuged in a Sorvall RT7 tabletop centrifuge (Sorvall Products, L.P., Newton, CT) at 468 RCF for 5 min at 4°C to pellet debris and the remaining supernatant was used for ELISA.

Measurement of chemokine protein by ELISA. The murine chemokines RANTES, TCA-3, MIP-1α, MIP-2, and IP-10, as well as the cytokine IFN-γ, were quantified using a standard method of sandwich ELISA as previously described (14,41). Samples were used at 1:4 dilution for RANTES and 1:2 dilution for all other chemokines and IFN-γ.

Measurement of viral gene expression. MHV-68 gene expression was measured using the γ-3 riboprobe template (24). All riboprobe syntheses were driven by T7 bacteriophage RNA polymerase with [α-^{32}P]UTP (Amersham, Arlington Heights, IL) as the labeling nucleotide as described (13). Probe bands were visualized by autoradiography (XAR film, Kodak, Rochester, NY) and were quantified by using the Storm PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For the latter, volume measurement with rectangular objects was used to generate PhosphorImager (PI) counts, which are presented as a percentage of the internal housekeeping signal (i.e., L32) present in each lane.

Measurement of viral titers. Viral titers in the lungs of infected mice were measured using standard plaque assay. Lung homogenates were thawed and filtered through a 0.2-μm syringe filter. Serial 10-fold dilutions of the filtered homogenate were then used in plaque assays as previously described (3).

Measurement of latent viral load. Latent viral load was quantified in the spleens of infected mice by real-time PCR using the ABI Prism 7700 Sequence Detector (PE Biosystems, Foster City, CA). We designed primers and probes using Primer Express® Software (PE Applied Biosystems) as follows to detect a 70-bp region of the MHV-68 gB gene: forward 5′-GGCCCAAATTCAATTTGCCT-3′; reverse 5′-CCCTGGACAACTCCTCAAGC-3′; probe (5′) 6-(FAM)-ACAAGCTGACCACCAGCGTCAACAAC-(TAMRA) (3′) where FAM is a reporter dye and TAMRA a quencher dye. Standard curves were generated using known amounts of plasmid containing the gB PCR fragment. The DNA copy number was calculated from the molecular weight of the DNA construct and Avogadro's number. One hundred nanograms of DNA extracted from each mouse spleen was used per reaction. For each mouse strain, data were combined from spleens harvested at 14 and 15 dpi for a total of six mice per strain.

Statistical analysis. Microsoft Excel (Microsoft Corporation, Redmond, WA) was used to analyze results for statistical significance. Comparison of RPA and ELISA results between BALB/c and C57BL/6 mice were made using two-tailed Student's *t*-test. A p less than 0.05 was considered statistically significant.

RESULTS

Chemokine production during acute infection in the lungs. To determine if there were differences in the chemokine response to MHV-68 infection following infection of BALB/c and C57BL/6 mice, 4–6-week-old BALB/c and C57BL/6 mice were infected i.n. with 4×10^4 PFU of MHV-68. Lungs were harvested at 1, 4–8, 10, 14, and 15 days post-infection (dpi). RNA was extracted from the left lobe of the lung and tissue homogenate was generated from the remainder of the lung as previously described (5,24,42). We measured levels of representative CC (TCA-3, RANTES, MIP-1α) and CXC (MIP-2, IP-10) chemokines in lung homogenates from both strains using ELISA (Fig. 1). Chemokine protein levels were negligible in control mice of both strains inoculated i.n. with supernatant from mock-infected owl-monkey kidney cells at an equivalent dilution in PBS (data not shown). Striking differences in elicited chemokine responses were observed between the two mouse strains. Whereas the BALB/c mice exhibited a robust chemokine response overall, a significantly dampened chemokine induction was observed in the lungs of C57BL/6 mice at all times post-infection and for all chemokines measured. A peak in RANTES occurred at 10 dpi in C57BL/6 mice. This peak was smaller than that measured in BALB/c mice, although this difference was not statistically significant. BALB/c mice also exhibited induction of the CC chemokines TCA-3 and MIP-1α and the CXC chemokines MIP-2 and IP-10 that peaked between 6 and 10 dpi, similar to our previously reported findings (42). Very little induction of these chemokines was measured in C57BL/6 mice.

Since IP-10 is an IFN-γ inducible cytokine (25) and we observed only low levels of IP-10 in C57BL/6

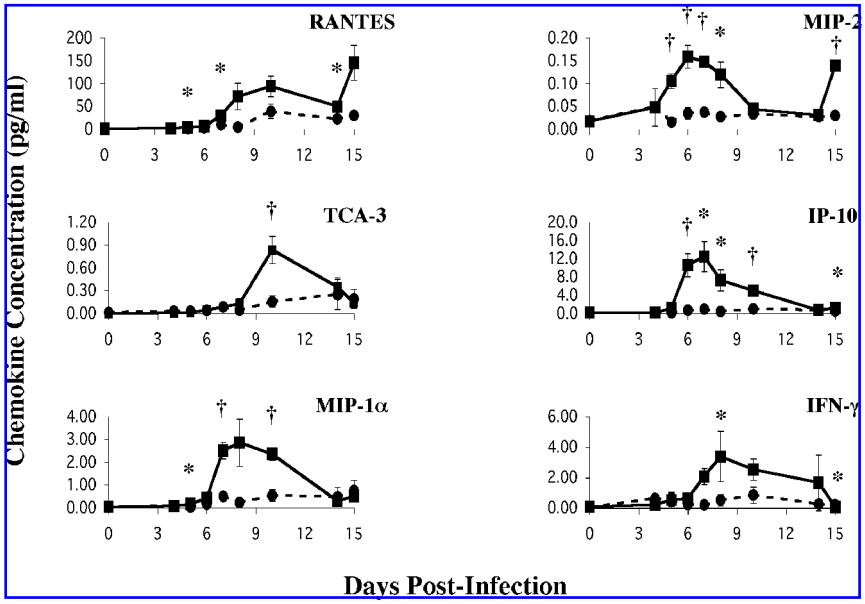

FIG. 1. Chemokine production during acute infection in the lungs. ELISA was performed on lung homogenate from mock-infected mice or from mice infected with 4×10^4 pfu of MHV-68 for representative chemokines and IFN-γ. Means ± SEM are shown for BALB/c (squares) and C57BL/6 (circles) mice at each time point. Comparison with Student's *t* test shows statistically significant differences between BALB/c and C57CL/6 mice (*$p < 0.05$; †$p < 0.01$).

mice, we next determined if IFN-γ levels were also reduced in C57BL/6 mice relative to BALB/c mice (Fig. 1). Interestingly, we observed significantly reduced levels of IFN-γ in the lungs of infected C57BL/6 mice.

Viral replication during acute infection in the lungs. To determine if there were differences in the ability of MHV-68 to replicate in BALB/c versus C57BL/6 mice, RNA extracted from lungs of mice infected with MHV-68 at various time-points was analyzed for MHV-68 gene expression by RPA as previously described (24). A representative RPA is shown in Figure 2A, and the quantitation of the immediate-early (rta), early (DNApol), and late (gB) transcripts is shown in Figure 2B. By 4 dpi, we observed significantly lower levels of viral transcripts in the lungs of MHV-68–infected C57BL/6 mice. The low level of viral gene expression was observed throughout the acute phase of replication in the lungs suggesting that C57BL/6 mice are more resistant to MHV-68 infection.

To determine if the reduced levels of viral gene expression also correlated with reduction in infectious virus, plaque assays were performed on lungs of MHV-68–infected BALB/c and C57BL/6 mice at selected time-points post-infection (Fig. 3). At 5 dpi, we observed a lower overall titer of infectious virus in the lungs of C57BL/6 mice (mean 4.6 $\log_{10}$ pfu/mL/g) compared to BALB/c mice (mean 5.9 $\log_{10}$ pfu/mL/g), although this difference did not reach statistical significance ($p = 0.15$). At 10 dpi, a very low titer of virus was present in the lung of one C57BL/6 mouse. Otherwise no virus was detected in the lungs of the other C57BL/6 mice or BALB/c mice at 10 dpi. Infectious virus was therefore present to a smaller degree in the lungs of C57BL/6 mice following inoculation with equivalent input titers. Despite the differences in viral titers in the lungs of the two mouse strains, virus was cleared from the lungs at the same time.

Latent viral load in spleens of infected mice. Following clearance of infectious virus from the lungs of infected mice, latency is established in the spleen (3,24,32,36). To determine whether the observed strain-based differences in viral replication in the lungs were associated with corresponding strain-based differences in latent viral load in the spleen, we measured levels of MHV-68 DNA by analyzing DNA extracted from the spleens of infected mice using quantitative real-time PCR. Spleens were harvested from infected mice at 14–15 dpi, a time when viral latency is established (36). The lower limit of detection using this

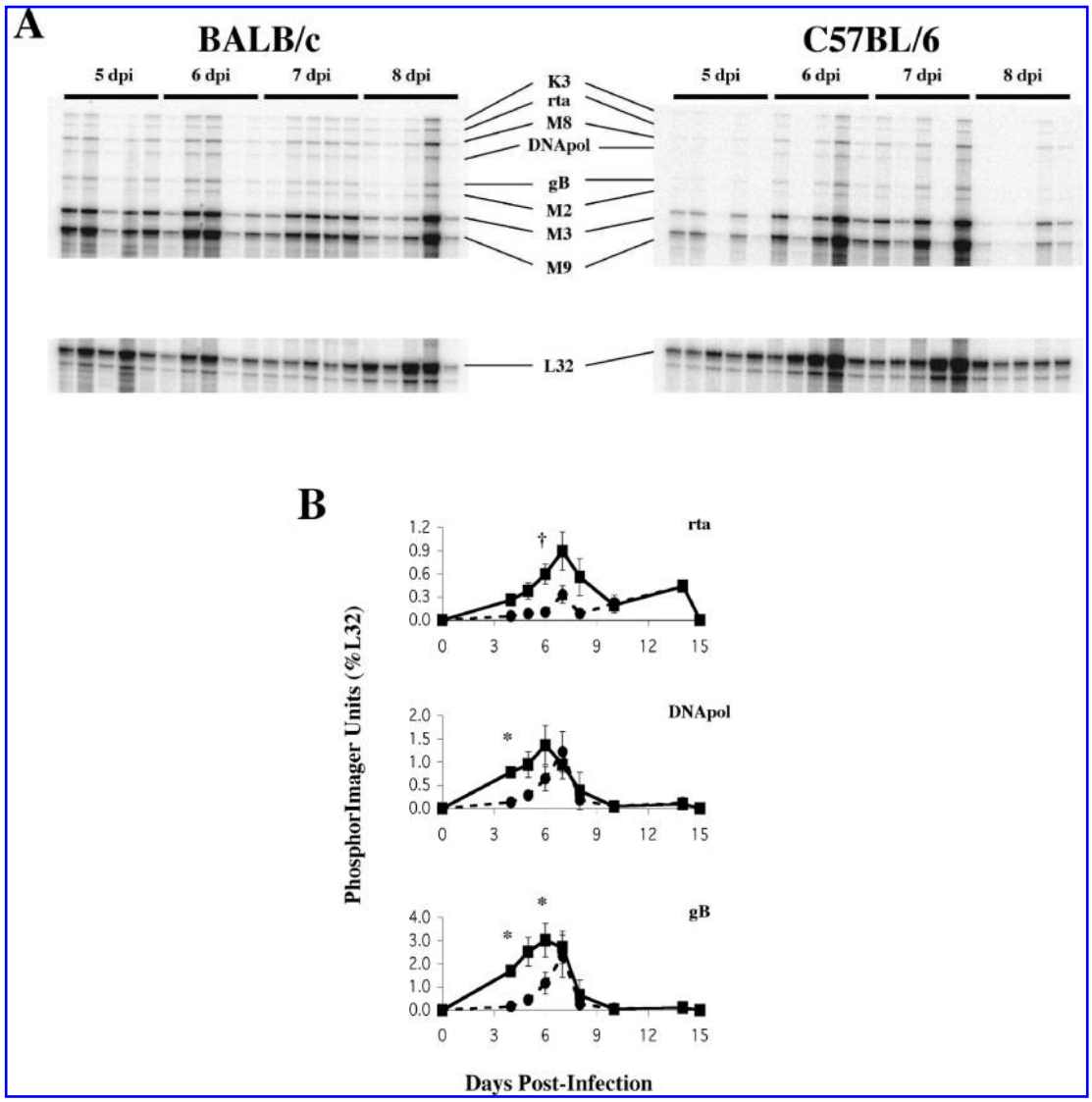

FIG. 2. Viral gene expression in the lungs of infected mice. (**A**) Representative ribonuclease protection assay (RPA) detecting viral gene expression in BALB/c lung. RNA extracted from the lungs of MHV-68–infected mice was analyzed by multi-probe ribonuclease protection assay. Each lane represents RNA extracted from the lungs of an individual mouse; five mice per time point were analyzed. Bands corresponding to protected probe fragments of specific viral genes are visible for five mice per timepoint. Labels indicate representative viral genes and the internal housekeeping gene, L32. (**B**) PhosphorImager counts were obtained for protected probe fragments, and data for representative immediate-early (rta), early (DNApol) and late (gB) viral genes are presented as a percentage of the internal housekeeping signal L32. Means ± SEM are shown for each time point for BALB/c (squares) and C57BL/6 (circles) mice. Comparison with Student's *t* test shows statistically significant differences between BALB/c and C57CL/6 mice (*$p < 0.05$; †$p < 0.01$).

method was 1.2 log copies of the target DNA sequence. Calculated values below this threshold were assigned the value of 1.2 log copies for statistical analysis. The analysis did not substantially differ when values below this threshold were assigned the value zero. Viral DNA was detectable in spleens of both mouse strains (Fig. 4), and no differences were observed between strains in the levels of MHV-68 DNA ($p = 0.53$).

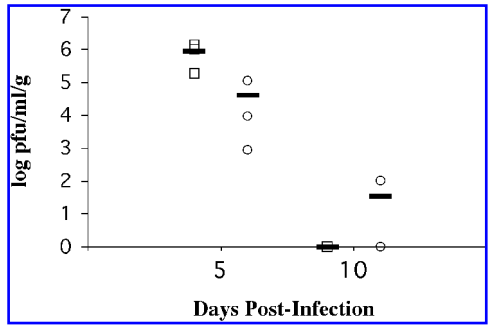

FIG. 3. Titers of infectious virus in lungs during acute infection. Plaque assays were performed on 3T3 cell monolayers using lung homogenates obtained from BALB/c (squares) and C57BL/6 (circles) mice at 5 and 10 dpi ($n = 3$ mice per strain for each time point). Log viral titers are shown for individual mice (open symbols) and for means of each strain at each time point (bars).

DISCUSSION

Induction of chemokines is associated with a variety of inflammatory stimuli including infection with viruses, bacteria, and other agents. Chemokines play a role in host defense by recruiting leukocytes to sites of inflammation so that they may act to control or clear the infection. In this report, we describe mouse strain-based differences in levels of chemokines induced by i.n. infection with MHV-68. BALB/c mice exhibited a robust chemokine response overall, while a significantly lower chemokine response was observed in the lungs of C57BL/6 mice at all times post-infection and for all chemokines measured. The greater chemokine induction in the lungs of BALB/c mice did not result in improved control of acute MHV-68 infection. In contrast, we demonstrate increased levels of MHV-68 gene expression and viral titers in the lungs of BALB/c compared to C57BL/6 mice. Finally, these differences in acute viral replication did not correlate with mouse strain–based differences in latent viral load in the spleen, as similar amounts of viral DNA were measured in spleens of BALB/c and C57BL/6 mice.

It is possible that the lower level of viral replication in the lungs of C57BL/6 mice served as a less potent inflammatory stimulus for chemokine production. The observed differences in acute viral replication in the lungs would therefore presumably be due to other underlying genetic differences in the immune response of BALB/c and C57BL/6 mice not assessed in this study. Alternatively, differential chemokine production between strains may reflect inherent differences in their chemokine response to infection. Augmented leukocyte recruitment to the lungs of BALB/c mice secondary to increased levels of chemokine production may actually serve to augment viral replication by increasing numbers of target cells for MHV-68 infection in the lungs. Further work using chemokine and chemokine receptor knockout mice will aid in distinguishing these possibilities.

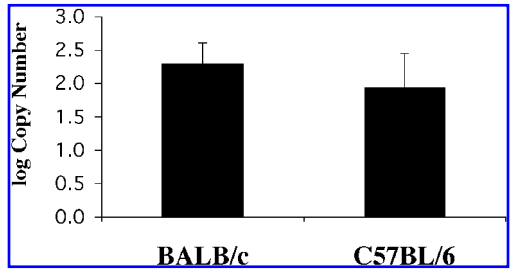

FIG. 4. Latent viral load in spleens of infected mice. MHV-68 DNA was detected in the spleens of BALB/c and C57BL/6 mice at 14–15 dpi using quantitative real-time PCR to detect a 70-bp region of the MHV-68 gB gene. Results are expressed as log copy number per 100 ng input DNA. Means ± SEM are shown for each strain ($n = 6$ for each strain).

The less pronounced IFN-γ response observed in T_H1-predominant C57BL/6 mice compared to T_H2-predominant BALB/c mice is inconsistent with the classic T_H1/T_H2 paradigm, in which IFN-γ is thought to be a predominant T_H1-type cytokine. However, organ-specific differences are likely to exist, with the chemokine and IFN-γ responses in the lung differing from those in the spleen, nodes or other organs based on differing cytokine profiles in localized microenvironments. Likewise, responses measured in vivo may be dramatically different than those measured in vitro, potentially accounting for the observations in this study which differ from the T_H1/T_H2 paradigm. Sarawar and colleagues (27), demonstrated that MHV-68 is cleared following infection of IFN-γ knockout mice, suggesting that IFN-γ does not play an important role in control of acute infection. Our data indirectly supports that conclusion.

We detected very little IP-10 protein in the lungs of C57BL/6 mice. This is in contrast to the report by Sarawar et al. (28) who observed IP-10 mRNA expression in the lungs of MHV-68–infected C57BL/6 mice. In their system, IP-10 mRNA was measured using a commercially available ribonuclease protection assay. However, Hallensleben et al. (10) has suggested that polymorphisms in IP-10 gene result in aberrant signal patterns using this ribonuclease protection assay, which could potentially explain the discrepant results. What is surprising is the differences in kinetics of IP-10 induction relative to IFN-γ, as IP-10 is an IFN-γ–inducible gene. However, it is possible that very little IFN-γ is necessary for induction of IP-10 or that other cytokines such as tumor necrosis factor–α are important in this system.

MHV-68 encodes for M3, a highly secreted chemokine binding protein that binds to both CC and CXC chemokines (21,38). Studies of MHV-68 pathogenesis have used either BALB/c (2,24,35,42) or C57BL/6 (3,6,15,17,28,40) mouse strains. Interestingly, infection of BALB/c mice with a recombinant virus deleted of M3 did not result in the establishment of latency (2). In contrast, when C57BL/6 mice were infected with a different recombinant virus that did not express M3, establishment of latency was maintained (39). Although differences in construction of the recombinant viruses have been postulated to account for these discordant results, an additional explanation could be differences in chemokine responses between the two mouse strains. In a more general sense, given the findings of our study it will be important in future studies to address potential mouse strain-based differences in chemokine responses to inflammatory stimuli in the lungs.

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