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MHV68 Latency Modulates the Host Immune Response to Influenza A Virus

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Abstract—Murine gammaherpesvirus 68 (MHV68) is a natural rodent pathogen that has been used as a model to study the pathogenesis of human gammaherpesviruses. Like other herpesviruses, MHV68 causes acute infection and establishes life-long latency in the host. Recently, it has been shown that mice latently infected with MHV68 have resistance to unrelated pathogens in secondary infection models. We therefore hypothesized that latent MHV68 infection could modulate the host response to influenza A virus. To test this hypothesis, mice were infected intranasally with influenza virus following the establishment of MHV68 latency. Mice latently infected with MHV68 showed significantly higher survival to influenza A virus infection than did PBS mock-infected mice. Latent MHV68 infection led to lower influenza viral loads and decreased inflammatory pathology in the lungs. Alveolar macrophages of mice latently infected with MHV68 showed activated status, and adoptive transfer of those activated macrophages into mice followed the infection with influenza A virus had significantly greater survival rates than control mice, suggesting that activated alveolar macrophages are a key mechanistic component in protection from secondary infections.

KEY WORDS: gammaherpesvirus; influenza A virus; alveolar macrophages; neutrophils.

INTRODUCTION

Humans in a natural environment are exposed to multiple pathogens, and face a constant risk of infection. It has been demonstrated that in certain cases infection with one pathogen can affect the immune response to subsequent infection with non-related pathogens, leading to "cross-protective immunity" or "heterologous immunity" [1–5]. This protective effect has been shown between diverse pathogens, including viruses, bacteria, and fungi.

Gammaherpesviruses such as Epstein-Barr virus (EBV) are double-stranded DNA viruses that are important pathogens in humans and animals. Most of the world's population is latently infected with multiple herpesviruses; however, these infections are usually asymptomatic in immunocompetent persons. Murine gammaherpesvirus 68 (MHV68) is a natural pathogen of rodents that is closely related to the human gammaherpesviruses and has been used as a model to study the pathogenesis of human gammaherpesviruses [6, 7]. Also, MHV68 has been shown to establish life-long latency in B cells, macrophages, and dendritic cells in Balb/c mice [8, 9].

Recently, it has been shown that mice latently infected with MHV68 are protected against two non-related pathogens, *Listeria monocytogenes*, and *Yersinia pestis*, and this protective effect is due in part to the activation of innate immunity and constitutive expression of interferon (IFN)-gamma (IFN-γ) that suppresses viral reactivation [10]. These data suggested that "cross-protective immunity" may be a common phenomena, but to date only a few pathogens have been studied.

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In the present study, we investigated whether MHV68 latency protects mice from lethal infection with influenza A virus, a single-stranded RNA virus that causes acute respiratory infections that result in significant morbidity and mortality in humans and animals. We evaluated the immunological response to influenza virus in PBS mock-infected and MHV68-infected mice and further, assessed the mechanisms underlying the increased survival seen in mice latently infected with MHV68.

MATERIALS AND METHODS

Mice

Female Balb/c mice (6–8 weeks age; The Jackson Laboratory) were housed under specific pathogen-free conditions at the Unit for Laboratory Animal Medicine of the University of Michigan and treated in accordance with the guidelines of the animal ethical committee. All experiments were done with the approval of the University of Michigan Committee for Use and Care of Animals (UCUCA).

Virus infection and sampling

MHV68 (strain WUMS) and influenza A virus (strain A/PR8/34; H1N1 isotype) were obtained from the American Type Culture Collection. To establish MHV68 latency, mice were anesthetized and challenged intranasally with 4×10^4 PFU of MHV68 (MHV68-infected mice) or with control PBS (mock-infected mice). Twenty-eight, 60, or 120 days following MHV68 or PBS challenge, mice were infected intranasally with 1×10^4 PFU of influenza virus. Whole lungs were harvested at the indicated times after influenza infection.

Histological examination

Individual excised lung lobes were inflated and fixed with 10 % buffered formalin for morphometric analysis. Sections of lung tissue were fixed in 4 % neutral buffered paraformaldehyde, embedded in paraffin and sectioned at 5-micron thickness. Sections were stained with hematoxylin and eosin.

Reverse transcription and real-time quantitative PCR analysis

Total RNA was isolated from whole lungs using TRIzol (Invitrogen Corp.) according to manufacturer's instructions. Real-time quantitative PCR analysis was performed using the ABI 7700 sequence detector system (PE Applied Biosystems). For virus quantification, cDNA was synthesized using MultiScribe reverse transcriptase and random hexamers (PE Applied Biosystems) as previously described [11]. For real-time quantitative PCR, the following primers were used: for the MP-1: forward 5′-CATCCCGTCAGGCCCCCTCA-3′, reverse 5′-GGGCACGGTGAGCGTGAACA-3′; for the NS: forward 5′-GGGGCAGCACTCTTGGTCTGG-3′, reverse 5′-CGCGACGCAGGTACAGAGGC-3′.

Protein analysis of cytokine and chemokine levels

Murine cytokine and chemokine levels were measured in 50 μl samples from whole lung homogenates using a Bio-plex bead-based cytokine assay purchased from Bio-Rad Laboratories. The cytokine and chemokine levels in supernatants from lung homogenates were normalized to the total protein in supernatants of each lung sample measured by the Bradford assay [12]. Albumin levels in bronchoalveolar lavage (BAL) fluid were quantified using the Albumin Quantification Kit (Bethyl Laboratories, Montgomery, TX).

Antibodies and flow cytometry

Flow cytometric analyses of lung cells were performed as previously described [12]. In brief, whole lungs were dispersed in 0.1 % collagenase (Sigma-Aldrich) in RPMI 1640 (MediaTek) and 5 % FBS (Atlas Biologicals) at 37 °C for 45 min to obtain a single-cell suspension. The cells were stained with the indicated Abs after 10 min of pre-incubation with CD16/CD32 Abs (Fc block) and fixed overnight with 4 % formalin. Cells were analyzed using a BD LSR II Flow Cytometer (BD Biosciences), and data generated were analyzed by FlowJo software (Tree Star Inc.). Flow cytometric analyses of BAL cells were performed in the same way.

Bronchoalveolar lavage

BAL was performed for assessment of leukocyte recruitment. The trachea was exposed and intubated using a 21-gauge catheter. One millimeter of PBS was injected into the airway and then collected. Lavaged fluid was centrifuged to pellet cells, and the supernatant was stored for albumin analysis. BAL cells were counted and used for flow cytometry analysis or adoptive transfer experiments.

Alveolar macrophages and CD8+ T cells purification, and adoptive transfer

Alveolar macrophages were isolated from BAL cells of PBS mock-infected or MHV68-infected mice at 28 days post-infection using adherence purification. For adoptive transfer experiments, 2×10^5 alveolar macrophages were transferred intranasally into wild-type Balb/c mice. For T cell isolation from mice injected with MHV68 or PBS control, spleens were harvested 28 days post-infection. The spleens were mechanically dispersed into a single cell suspension using a 40-μm filter, and ammonium chloride lysis buffer was used to lyse erythrocytes. For purification of CD8+ T cells, ferromagnetic beads were used (CD8+ T cell isolation kit, Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. For T cell transfer experiments, 1.5×10^6 CD8+ T cells were transferred via intravenous injection (tail vein) into wild-type Balb/c mice. A day following adoptive transfer of either macrophages of T cells, recipient mice were intranasally challenged with 1×10^4 PFU of influenza virus. Four days following challenge, mice were sacrificed for analysis, as this timepoint represents the peak of viral RNA expression in lungs.

Statistical analysis

Two-tailed Student's *t* test was performed using GraphPad Prism version 5.00 for Mac OS (GraphPad Software, San Diego, CA) in all cases.

RESULTS

MHV68 latency increased survival after influenza virus infection

For these studies we hypothesized that MHV68 latency would provide the host with protective immunity against influenza virus infection. To address this, we infected mice with MHV68 by intranasal inoculation, and then challenged these mice with influenza virus also administered intranasally, 28, 60, or 120 days later and monitored survival. Control mice did not receive MHV68 but rather were mock infected with PBS.

While 84 % of mock-infected mice died 8–12 days after influenza virus infection, mice that were infected with MHV68 and challenged with influenza virus 28 days later demonstrated 92 % survival, and thus a significantly lower mortality rate ($p<0.0001$, Fig. 1a). When influenza virus was injected 60 days after MHV68 a significant protective effect was also observed (80 versus 20 % survival, $p<0.05$, Fig. 1b). At 120 days after MHV68 infection mice infected with influenza virus still had increased survival (33 %) compared to mock-infected mice (16.7 %), but this difference was not significant (Fig. 1c). Using limited dilution qPCR at 60 days post infection we confirmed the presence of MHV68 viral specific RNA in Balb/c splenocytes (data not shown).

This data shows that MHV68 infection protects mice from influenza virus mortality, and in agreement with other studies, this survival effect decreases as the time between the primary and secondary infection increases and loses significance by day 120 [5, 8].

Effect of MHV68 latency on influenza virus burden and lung inflammation

To understand the mechanisms underlying the immunity to influenza virus caused by prior MHV68 infection, we first assessed the influenza viral burden by measuring the expression of influenza virus-producing proteins, matrix protein-1 (MP-1), and non-structural protein (NS), as well as RNA levels of these from lungs of mice injected with influenza virus 28 days after MHV68 injection or from PBS mock injection.

mRNA levels for both of these proteins were significantly lower 4 days after influenza virus challenge when MHV68 primary injected mice were compared to mock-infected control mice. Eight days after influenza virus challenge, mRNA expression levels were significantly reduced compared to 4-day expression levels but there was no significant difference between MHV68 injected and PBS mock-injected mice (Fig. 2a). These results suggest that MHV68 latency results in accelerated influenza viral clearance at early time points.

We next evaluated the histopathological changes in hematoxylin and eosin-stained lung sections during influenza virus infection. In agreement with previous reports [13, 14], lungs latently infected with MHV68 were histologically similar to uninfected lungs (data not shown). By day 8 after influenza virus challenge, lungs of both mock-infected and MHV68-infected mice exhibited significant inflammation, which included infiltrates of inflammatory cells. Further, influenza virus infection in mice previously PBS mock injected showed increased inflammatory pathology including

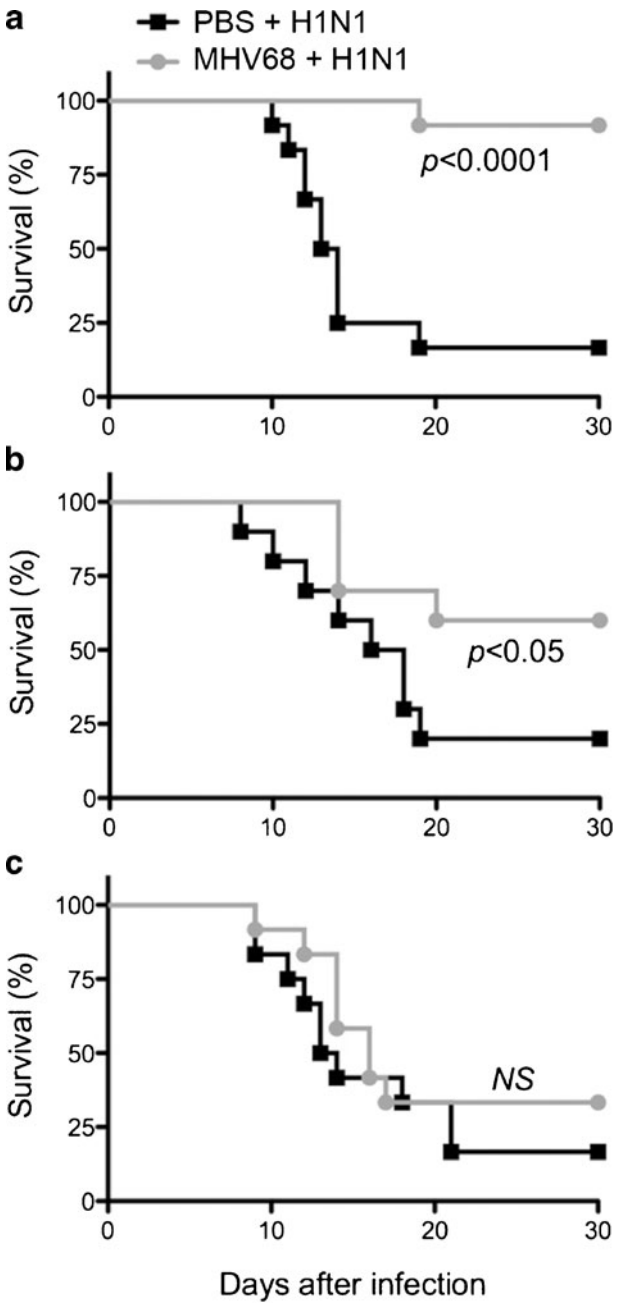

Fig. 1. MHV68 latency increased survival of mice after influenza challenge. Mice were infected intranasally with 4×10^4 PFU of MHV68 and control mice received PBS. **a** 28, **b** 60, or **c** 120 days after MHV68 infection or after PBS mock-infection, mice were inoculated with 1×10^4 PFU of influenza virus. $n=10$–12 mice. *Black squares* control PBS-infected mice, *gray circles* MHV68-infected mice.

more infiltrates and greater destruction of alveolar architecture compared to mice previously injected with MHV68 (Fig. 2b).

To quantitate the magnitude of alveolar injury, the permeability of the alveolar-capillary membrane was assessed by measurement of albumin concentrations in BAL fluid. At day 8, BAL fluid albumin levels were significantly decreased in mice latently infected with MHV68 compared with PBS mock-infected mice ($p<0.01$, Fig. 2c), suggesting that MHV68 had a protective effect regarding alveolar injury.

Lung leukocyte populations in mock-infected and MHV68-infected mice after influenza virus infection

To further characterize the cellular profile, we next investigated differences in lung inflammatory cell accumulation and activation in PBS mock-infected and MHV68-infected mice during the development of influenza viral infection (Fig. 3). The numbers of both CD4+ and CD8+ T cells in the lungs were increased after influenza virus challenge, and were significantly greater in MHV68-infected mice compared to PBS mock-infected mice. Increased numbers of CD69 expressing activated CD4+ and CD8+ T cells were also observed during influenza infection, with significantly greater numbers seen in MHV68-infected mice than in PBS mock-infected mice. More than 50 % of the lung CD8+ T cells were activated while approximately 20 % of the lung CD4+ T cells were activated, suggesting the importance of CD8+ T cells in antiviral immunity.

Next we examined the composition of BAL cells (Fig. 3b). No significant differences in the number of total cells or macrophages were seen. The number of neutrophils in BAL fluid increased remarkably after influenza virus challenge, but the increase in MHV68-infected mice was significantly less than in PBS mock-infected mice at day 8 after influenza virus challenge.

We next evaluated the levels of chemokines CXCL1 (KC) and CXCL2 (MIP-2a) that attract neutrophils to the lungs during influenza virus infection. The levels of these chemokines were both increased after influenza virus challenge, especially at day 4 and in agreement with the above results the levels of these chemokines were significantly lower in MHV68-infected than in mock-infected mice (Fig. 3c).

MHV68 latency led to altered cytokine production and an altered cellular profile in the lungs of mice

To determine the mechanisms responsible for the rapid influenza viral clearance and the relatively mild

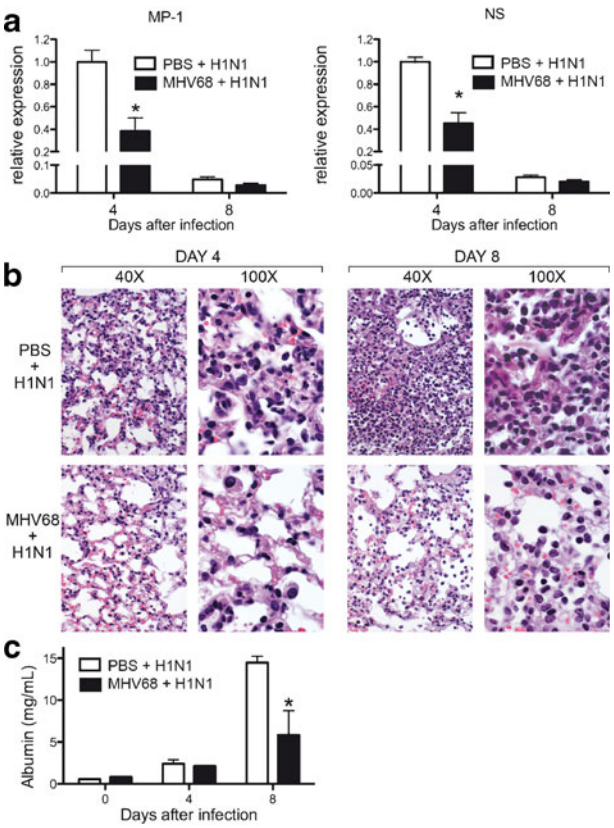

Fig. 2. Viral burden, histopathological changes, and lung permeability of MHV-68 latently infected mice after influenza challenge. **a** Viral burden (n=8–10). Lung homogenates were collected 4 and 8 days after influenza challenge and transcripts for influenza viral proteins, MP-1 and NS, were measured. **b** H&E-stained histopathological analysis of lungs collected 4 and 8 days after influenza virus that was administered 28 days post-MHV68 infection or post PBS-mock infection. **c** Lung permeability of MHV68-infected and PBS control mice infected with influenza virus (n=4) assessed by quantitation of albumin concentrations in BALF from mice 8 days after influenza challenge. Means and SEM are shown. *p<0.001.

inflammation in MHV68-infected mice, we assessed the production of proinflammatory cytokines in the lungs of PBS control or MHV68-infected mice by qPCR. The expression levels of IFN-γ in the lung were upregulated in MHV68-infected mice compared to PBS mock-infected mice. Upregulation of IFN-γ was greater when influenza virus was administered 28 days versus 60 days after MHV68 infection, but at both time points the increases were significant compared to mock-infected control IFN-γ levels. In contrast, there was no significant difference in IFN-γ levels in lungs from PBS mock- or MHV68-infected mice when influenza virus was given at the 120-day timepoint (Fig. 4a). Two other proinflammatory cytokines, TNF-α and IL-12p40, were also significantly upregulated compared to PBS controls when infected with influenza virus either 28 or 60 days after MHV68 infection (Fig. 4a).

Since these cytokines are all related to the activation of macrophages, we analyzed alveolar macrophages collected from BAL fluid. As in our above results (Fig. 3b), the numbers of alveolar macrophages in BAL fluid of MHV68-infected mice were not different from mock-infected mice before influenza virus challenge. But using flow cytometry, we found that alveolar macrophages of MHV68-infected mice showed upregulation of surface major histocompatibility complex (MHC) class II molecules, indicating they were activated (Fig. 4b).

Protective effects were dependent on activated macrophages

To determine whether activated alveolar macrophages contributed to the observed protective immunity, we performed adoptive macrophage transfer experiments. Alveolar macrophages collected from MHV68-infected or PBS mock-infected mice were transferred intranasally to wild-type mice following primary influenza virus infection and survival was monitored. Mice receiving macrophages from MHV68-infected animals showed significantly increased survival compared to mice receiving macrophages from mock-infected mice (60 versus 10 %, respectively; Fig. 5a). Further, at day 4 after influenza virus challenge, mRNA expression of influenza virus proteins MR-1 and NS were significantly decreased in the lungs of mice receiving alveolar macrophages from MHV68-infected mice compared to mice receiving alveolar macrophages from mock-infected mice(Fig. 5b). The levels of neutrophil attracting chemokines CXCL1 (KC) and CXCL2 (MIP-2a) were decreased in mice that received alveolar macrophages from MHV68-infected mice (Fig. 5c). These results suggest that activated alveolar macrophages are critical for the observed protective effect afforded by prior MHV68 infection. Lastly, we performed an adoptive CD8+ T cell transfer experiment (Fig. 5d) that showed no difference in expression levels of viral proteins in lungs from mice receiving CD8+ T cells from MHV68-infected mice or from PBS mock-infected mice.

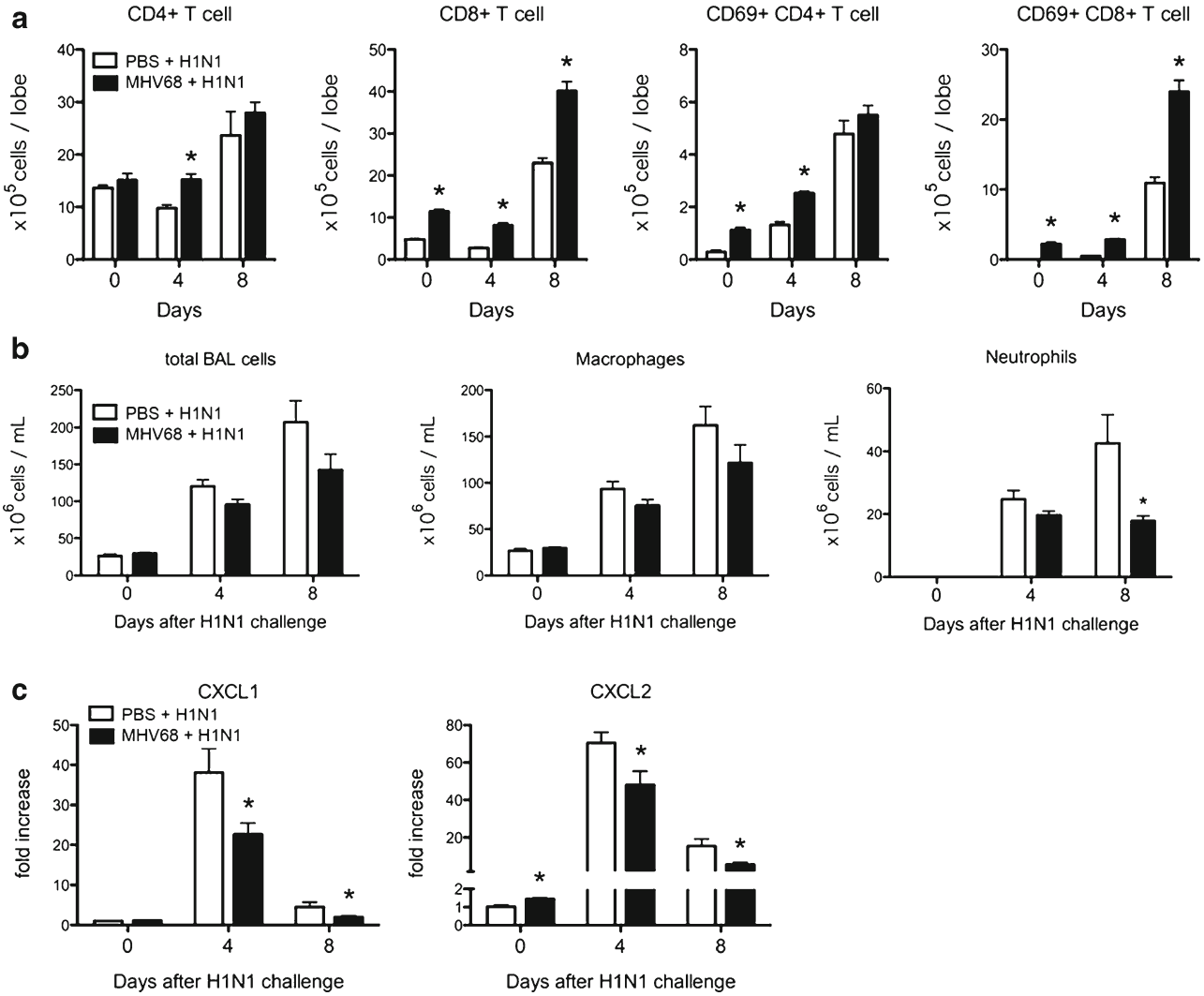

Fig. 3. Cellular profile of Influenza infected lungs. **a** The cellular profile of lungs collected from mice at the indicated times after influenza virus challenge were determined using flow cytometry (n=5–6). **b** Differential cellular composition in BAL fluid of infected lungs (n=4). BAL fluid was collected from mice at days 4 and 8 after infection with influenza virus. **c** Neutrophil-attractant chemokine levels were measured by qPCR in whole lungs collected from mice on the indicated days after influenza virus challenge. n=5–6; *p<0.05.

DISCUSSION

In the present study, we report that MHV68 latency alters the host response to lethal respiratory infection with influenza virus. Mice latently infected with MHV68 showed higher survival rates after influenza viral challenge, lower viral loads, and less inflammation in the lungs than did mock-PBS-infected mice. Further, we demonstrated that this protective effect lasted for at least 2 months, a length of time that is a significant portion of a mouse's lifespan. Cross-protection is the term describing the protective effect against a secondary pathogen due to a prior infection with another, heterologous pathogen. Several groups have shown that latent infection with MHV68 provides the host with cross-protection from subsequent infection, including bacterial pathogens *L. monocytogenes* [10, 15], *Y. pestis* [10], murine adenovirus type 1 [16], and *Plasmodium yoelii* [17]. We have now added lethal respiratory influenza virus to this list.

The main route of infection with MHV68 is unknown, but is presumed to be through the respiratory tract. Similar to infection with EBV, after intranasal inhalation of MHV68, lytic infection occurred in the

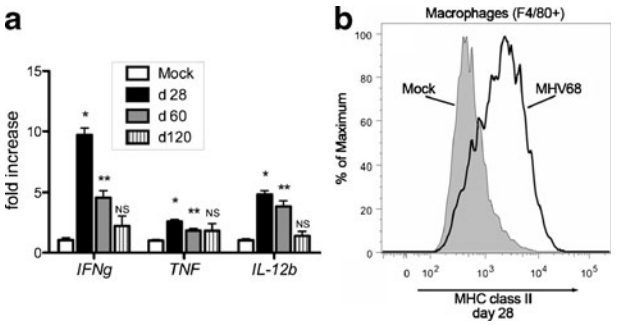

Fig. 4. Cytokine production and cellular profile in the lungs of MHV68-infected and PBS mock-infected mice. These mice were not infected with influenza virus. **a** Cytokine levels ($n=5$) were measured by qPCR in whole lungs collected from mice at the indicated days after MHV68 infection. Means and SEM are shown. *$p<0.0001$. **$p<0.01$. **b** Surface class II MHC levels on alveolar macrophages were analyzed by flow cytometry in F4/80+ cell population in BAL cells harvested 28 days post infection. Bal cells from MHV68-infected mice were compared to those from mock-infected mice.

lung epithelial cells followed by viral clearance in 10 to 14 days, followed by life-long and systemic latency. Inflammatory cytokines including IFN-γ are important in preventing latent virus reactivation [18, 19]. Since our studies showed an upregulation of IFN-γ, TNF-α, and IL-12b in the lungs of MHV68-infected mice (compared to mock-infected mice), it is unlikely that acute influenza virus infection reactivated MHV68, at least out to the 60-day timepoint. Another study showed that intranasal infection with MHV68 caused upregulation of inflammatory cytokines in the serum and activation of peritoneal macrophages, which was essential for the protective effect against bacterial infection [8]. Similarly, we demonstrated that proinflammatory cytokines were upregulated in the lungs of MHV68-infected mice compared to PBS mock-infected mice and further, that alveolar macrophages in MHV68-infected mice were activated.

Alveolar macrophages are one of the first cells of the innate immune system to encounter inhaled pathogens and antigens. After the invasion of pathogens or antigens, alveolar macrophages become highly phagocytic and produce robust amounts of inflammatory cytokines and induce the adaptive immune system [20]. Depletion of alveolar macrophages before influenza virus infection results in higher mortality and greater virus load [21–23], but depletion 3 or 5 days following infection does not [22], which suggests alveolar macrophages are critical for managing influenza viral infection, especially in its very early stage. Our adoptive transfer of alveolar macrophages from MHV68-infected mice resulted in significantly higher survival rates and lower influenza viral titers compared to mice receiving macrophages from mock-infected mice. This result confirmed the mechanistic importance of activated alveolar macrophages in protection from secondary infection with influenza virus. Even if virus is latent and undetectable, it can have a major effect on immune cellular components in relevant organ compartments as we here saw with the lung macrophages. However, we also examined the role of NK cells in the innate immune response to viral infection. Recently, it was demonstrated that latent herpesvirus infection causes NK cells to produce more granzyme B and IFN-γ and to display increased cytotoxity [24]. Our NK cell depletion studies (>90 % of NK cells were depleted in the lungs) resulted in no significant difference in viral titers (data not shown) suggesting that NK cells are dispensable in the innate response to influenza virus. However, IFN-γ made by NK cells may contribute to the activation of macrophages.

In addition to macrophages and NK cells, neutrophils are also recruited into the lungs during influenza virus infection and play an important role in the immunopathology [25, 26]. However, the exact role of neutrophils in influenza virus infection is controversial. Two reports showed that depletion of neutrophils before influenza virus infection resulted in higher mortality

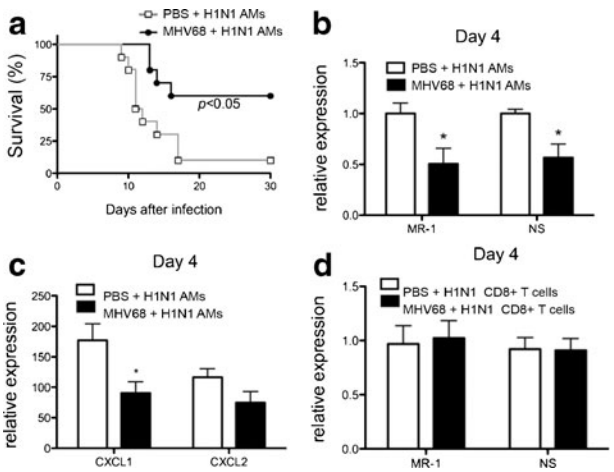

Fig. 5. Adoptive transfer of alveolar macrophages (AMs) from MHV68-infected or PBS mock-infected mice. AMs were transferred intranasally 1 day before infection with 1×10^4 PFU of influenza virus. **a** Survival curves. **b** Viral load assayed by measuring amounts of viral MR-1 and NS RNA by qPCR. **c** Measurement of neutrophil attracting chemokines after influenza infection. **d** Adoptive transfer of splenic 1.5×10^6 CD8+ T cells through tail vein from MHV68-infected or PBS mock-infected mice. Means and SEM are shown. All measurements were performed 4 days after viral infection as this timepoint is the peak of viral load. $n=5$–10; *$p<0.05$.

rates in mice [21, 27], while three other reports showed that increased numbers of neutrophils in the lungs during influenza infection were associated with severe pneumonia and high mortality [22, 28]. In our study, lower numbers of neutrophils in BAL were observed in MHV68-infected mice after influenza virus challenge compared to PBS mock-infected mice. We also demonstrated that the levels of neutrophil-attractant chemokines, KC and MIP-2a, were lower in MHV68-infected mice than in mock-infected mice during influenza virus infection. These results suggest that activated macrophages regulate the influx of neutrophils into the lungs directly or indirectly and this regulation is important for the observed cross-protection in MHV68-infected mice.

We also demonstrated that the numbers of both CD4+ and CD8+ T cells after influenza virus challenge were increased in MHV68-infected mice compared to mock-infected mice. Furthermore, the numbers of activated T cells indicated by expression of CD69 were greater in MHV68-infected mice. Thus it appears that activated macrophages in MHV68-infected mice enhance the adaptive immune system response to counter the influenza virus challenge. On the other hand, the mechanism of cross-protection between viruses, which is T cell dependent, has been termed "heterologous immunity" [3, 29]. Heterologous immunity protection is believed to be due to cross-reactive antigenic epitopes shared by the primary and secondary viruses, so during secondary infection memory T cells induced by the primary virus are reactivated [3]. Some examples of this T cell cross-reactivity between viruses in both mouse and human were observed [3]. To test this mechanism in our model we adoptively transferred CD8+ T cells from the spleen of MHV68-infected or PBS mock-infected mice to wild-type mice, following challenge with influenza virus. The influenza viral loads were not significantly different between these groups, which suggests a lack of cross-reactivity of antigenic epitopes between MHV68 and influenza.

Our results are another example showing that persistent latent infection with one virus can modulate a secondary pathogen assault. If this proves to translate to the clinic, latent infection with human herpesviruses, including EBV and Kaposi sarcoma-associated herpesvirus, even when undetectable may play a heretofore unrecognizable role in modulating the immune response to life-threatening respiratory pathogens like influenza virus. Although the effects that we observed in mice appeared to be active during a limited but significant time frame in mice, how exactly this window of time will translate to human life span is unknown, but may well extend into adulthood.

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