



γ -herpesvirus latency attenuates *Mycobacterium tuberculosis* infection in mice

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ABSTRACT

Tuberculosis is caused by *Mycobacterium tuberculosis* (Mtb), a bacterial pathogen which is transmitted via aerosol and establishes a chronic lung infection. In naïve hosts, Mtb grows for several weeks without being restricted by IFN γ -producing T cells, which eventually accumulate and limit Mtb dissemination. In this study, we used a mouse model of Mtb/ γ -herpesvirus (γ HV) coinfection to test the hypothesis that latent γ HV infection alters host resistance to Mtb. γ HVs are DNA viruses which elicit a polyclonal T cell response and attenuate some acute bacterial pathogens in mice; whether γ HVs modulate infection with Mtb is unknown. Here, mice harboring latent mouse gammaherpesvirus 68 (MHV68)—a γ HV genetically and biologically related to human Epstein Barr virus (EBV)—were infected via aerosol with a low dose of virulent Mtb. Mtb burdens and IFN γ ⁺ T cell frequencies in mice with latent MHV68 (MHV68^{POS} mice) were subsequently measured and compared to control mice that did not harbor latent MHV68 (MHV68^{NEG} mice). Relative to MHV68^{NEG} controls, MHV68^{POS} mice more effectively limited Mtb growth and dissemination, and had higher frequencies of CD4⁺ IFN γ ⁺ cells in lung-draining lymph nodes. Collectively, our results support a model wherein latent γ HV confers moderate protection against subsequent Mtb infection.

1. Introduction

Tuberculosis (TB) is the leading cause of death from an infectious disease [1]. TB is caused by aerogenic transmission of *Mycobacterium tuberculosis* (Mtb), a bacterial species which infects alveolar macrophages and can disseminate to extrapulmonary tissues via lymphatic or hematogenous spread [2]. TB has a spectrum of clinical manifestations that vary depending on the host response, including severe, active, chronic, subclinical and latent forms [3]. Improved socioeconomic conditions, public health practices and the use of effective drug treatment have reduced global TB rates; however, these rates fell short of the World Health Organization (WHO) goal of reversing TB incidence by 2015 [4], and are not on target to achieve WHO's goal of ending the TB epidemic by 2030 [5]. For these reasons, it is important to continue research into understanding host responses which restrict Mtb growth and dissemination.

T cells have been recognized for ~50 years as being essential for restricting Mtb growth and dissemination [6]. In the mouse model of TB, Mtb growth in the lung is near-logarithmic for the first 4 weeks post-infection [7]; after this period, CD4⁺ and CD8⁺ T cells begin to accumulate in the lung and secrete IFN γ , a cytokine which enhances the bactericidal activities of macrophages and other innate lineages. Relative to many lung infection models, the kinetics of T cell accumulation during TB are delayed [8]; this delay is important, as it provides Mtb a window of opportunity to express genes that are necessary for

establishing chronic infection [9]. The only known way of shortening this window is to expedite T cells' response to Mtb, as achieved through either BCG vaccination or, in animal models, the adoptive transfer of T cells from a previously-infected host [7]. The T cell IFN γ response is accompanied by reductions in lung Mtb burden and the delayed appearance of Mtb in extrapulmonary tissues [10,11].

γ -Herpesviruses (γ HVs) elicit a prolonged T cell IFN γ response which may affect Mtb pathogenesis. γ HVs are a large family of double-stranded DNA viruses that establish life-long, latent infections in mammals. Epstein-Barr virus (EBV), one of two known human γ HVs, infects ~50% of children by age 8, and ~90% of adults after age 18^{12,13}. Related mouse gammaherpesvirus 68 (MHV68) represents a tractable model of chronic gammaherpesvirus infection and overcomes limitations associated with exquisite species specificity of gamma-herpesviruses [14–16]. Latent γ HV infection, which occurs during the time of chronic infection (after day 12 of MHV68 infection), causes a polyclonal, antigen-independent activation of B and T cells: infection with MHV68 stimulates a significant increases in the number of splenic B cells, CD4⁺ and CD8⁺ T cells which are not specific for MHV68 epitopes [14–16]. The increase in the number of immune cells occurs concurrently with activation, as ~40% of splenic CD4⁺ T cells are CD62L^{Lo} by day 16 post-MHV68 infection, and remain elevated as late as day 90 post-MHV68 infection [17]. In humans, the polyclonal nature of γ HV infection is best highlighted by the fact that a clinical diagnosis for a recent EBV infection involves testing for antibodies that are

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reactive with horse red blood cells (heterophile antibodies test). In addition to polyclonal stimulation of adaptive immunity, γ HV infection is also associated with a substantial increase in systemic and organ-specific type I and type II interferon levels, an increase that is sustained throughout latent infection [18–20]. In mice, γ HV latency provides IFN γ -dependent protection against subsequent *Listeria monocytogenes* and *Yersinia pestis* infection [18]. Whether γ HV latency influences TB has not been reported.

Given the variety of innate and adaptive immune responses elicited by γ HV [21], we hypothesized that latent γ HV infection alters the immune response to Mtb to confer protection. To test this hypothesis, we infected two groups of mice—an experimental group harboring latent MHV68 (i.e. MHV68^{POS} mice), and a control group that was never exposed to MHV68 (i.e. MHV68^{NEG} mice)—with Mtb via aerosol, and subsequently measured Mtb burdens in the lung and spleen on post-infection days 17 and 38. The frequencies of IFN γ -producing CD4⁺ and CD8⁺ T cells in the mediastinal lymph node were also determined. Our results support a model wherein γ HV latency confers a moderate degree of protection against TB, which has important implications for our understanding of human TB resistance.

2. Methods

2.1. Mice

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were bred and housed in a specific-pathogen-free barrier facility in accordance with institutional and federal guidelines. All mouse experiments were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

2.2. MHV68 inoculation

Intranasal inoculation of 6–8 week old mice with 10⁴ PFU of wild type murine gammaherpesvirus 68 (MHV68; WUMS strain) or 15 μ l of phosphate buffered saline (PBS) was performed under light anesthesia, as previously reported [22]. MHV68-inoculated mice were housed separately from carrier-inoculated mice for the duration of the study.

2.3. Mtb infection and burden assessment

Eighteen days post-MHV68 (or carrier) inoculation, mice were aerosol infected with virulent Mtb H37Rv per our reported protocols [11,23]. For bacterial load determinations, lungs and spleen were removed from euthanized mice and individually homogenized in normal sterile saline; serial dilutions of each homogenate were then plated on 7H11 and colonies counted after ≥ 2 weeks incubation at 37 °C and 5% CO₂. Lungs from control mice were plated on day 1 post-infection to confirm the delivery of ~ 80 Mtb CFU per lung.

2.4. Flow cytometry

Mediastinal lymph node cells were prepared and stained for surface and intracellular markers per our previous protocols [11,23], using antibodies specific for mouse CD4, CD8 and IFN γ (BD Biosciences). Flow cytometry data files were collected on a Guava easyCyte 8HT cytometer, and analyzed using FlowJo software to determine the frequency of CD4⁺IFN γ ⁺ and CD8⁺IFN γ ⁺ cells.

2.5. Statistics

GraphPad Prism software was used for graph preparations and statistical analyses. Bars in the figures show means plus standard deviations (SD). Student's *t*-test was used for all comparisons of MHV68^{POS} and MHV68^{NEG} values. The asterisks shown between MHV68^{POS} and MHV68^{NEG} groups indicate the significance of a comparison, where

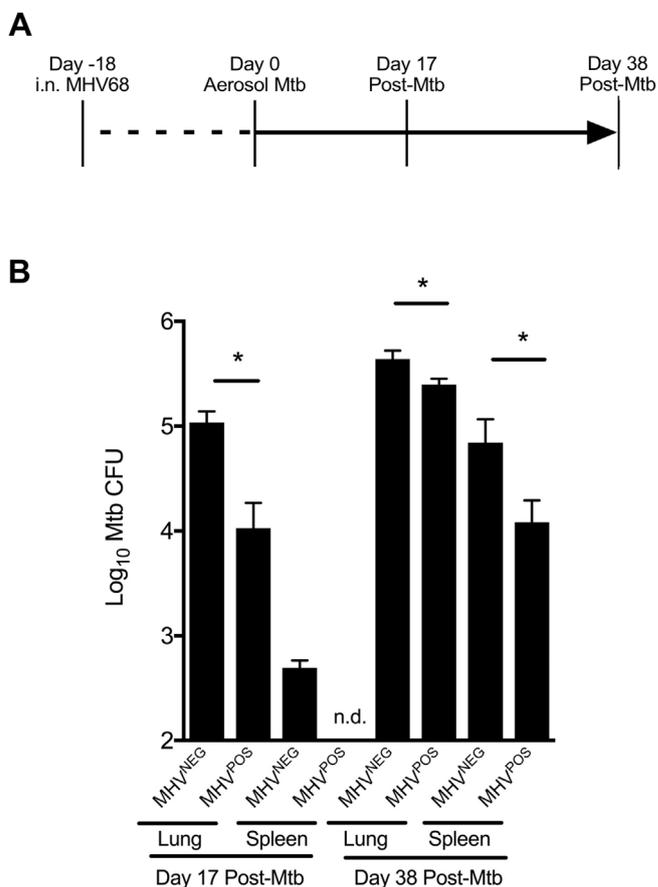


Fig. 1. MHV68^{POS} mice are more resistant to Mtb growth and dissemination. (A) For this study, adult C57BL/6 mice were intranasally infected with 10⁴ PFU of MHV68, or vehicle alone. Eighteen days later, all mice were aerosolically infected with ~ 80 CFU of virulent Mtb (H37Rv). On post-Mtb infection days 17 and 38, mice from each group were euthanized and the lungs, spleen and MLNs were removed for either CFU burden assessment or measuring the frequency of IFN γ -producing T cells. (B) Mtb burdens in the lungs and spleen from indicated groups were assessed by plating serial dilutions of homogenized organs on 7H11. Shown for each time is the mean number of CFU (log₁₀) present in 3–4 mice per group per time point. Error bars represent \pm SD; asterisks indicate that a significant difference between MHV68^{POS} and MHV68^{NEG} mice was observed at the indicated time point (i.e., $P \leq 0.05$ as determined using Student's *t*-test). Results are representative of three independent experiments.

$P \leq 0.05$.

3. Results

3.1. MHV68^{POS} mice are more resistant to Mtb growth and dissemination

To determine if MHV68 latency affects Mtb growth and dissemination, we inoculated groups of C57BL/6 mice with either MHV68 (10⁴ PFU, MHV68^{POS} mice) or carrier solution (MHV68^{NEG} mice); eighteen days later—a point when viral latency is established in MHV68^{POS} mice [24,25]—all mice were infected via aerosol with ~ 80 CFU of virulent Mtb (Fig. 1A). MHV68^{POS} and MHV68^{NEG} mice were maintained in separate cages for duration of the experiment. On post-infection days 17 and 38, groups of mice were euthanized and the lungs, spleen and mediastinal lymph nodes (MLNs) were removed and assayed for Mtb CFU burdens or the frequency of IFN γ ⁺ T cells. The results of our Mtb CFU burden assessment are shown in Fig. 1B, and demonstrate that post-infection day 17 lung Mtb burdens are significantly lower in MHV68^{POS} mice compared to MHV68^{NEG} controls.

Whereas we detected Mtb CFUs in MHV68^{NEG} spleens on post-infection day 17—a result of lymphatic drainage and hematogenous spread [26]—spleen Mtb CFUs were not detectable in MHV68^{POS} mice at that time. On post-infection day 38, MHV68^{POS} lung and spleen Mtb CFU burdens continued to be significantly lower than MHV68^{NEG} controls (Fig. 1B). Collectively, these data demonstrate that mice that are latently infected with MHV68 are more resistant to Mtb growth and dissemination.

3.2. Mtb-infected MHV68^{POS} mice have a transient increase in the frequency of IFN γ -producing CD4⁺ cells

MHV68 elicits an IFN γ ⁺ T cell response that is sustained during viral latency [18–20]. Since IFN γ -producing T cells promote TB resistance [10,11], we next determined if Mtb-infected MHV68^{POS} mice—which have lower CFU burdens than MHV68^{NEG} mice on post-infection day 17—correspondingly have higher frequencies of IFN γ -producing T cells. MLNs were removed from the same mice used for CFU burden assessments (Fig. 1), processed into single cell suspensions, stimulated with PMA/ionomycin in the presence of brefeldin A, and stained for surface CD4, surface CD8 and intracellular IFN γ . The results of our flow cytometry analysis are shown in Fig. 2, and demonstrate that on post-infection day 17 the MLNs of Mtb-infected MHV68^{POS} mice have significantly higher frequencies of CD4⁺IFN γ ⁺ cells, relative to MHV68^{NEG} controls. There was no significant difference in the frequencies of CD8⁺IFN γ ⁺ cells between MHV68^{POS} and MHV68^{NEG} mice. By post-infection day 38, no differences in CD4⁺IFN γ ⁺ frequencies were observed between Mtb-infected MHV68^{POS} and MHV68^{NEG} mice. Collectively, these data demonstrate that Mtb-infected mice with latent MHV68 have transiently higher frequencies of CD4⁺IFN γ ⁺ cells.

4. Discussion

Animal models have been used for over a century to identify host mechanisms which govern TB resistance [27]. Following aerosol infection with Mtb, large outbred animals develop a range of pulmonary disease forms which model the diversity of human TB forms [3]; however, the uniformity and reproducible nature of TB in mice has

enabled the discovery of multiple immune mechanisms that regulate TB resistance in mice and humans alike [28]. Regardless of the species used, a difference between animal models and humans is that human immune responses are modified by ubiquitous, life-long viral infections that are absent from most animal research. In the absence of these viruses, animals' immune responses do not fully reflect the human situation.

γ -herpesviruses (γ HV) are one such group of immune-altering viruses, which establish a life-long infection in immunocompetent individuals [21]. In most immunocompetent individuals, the two human γ HVs—Kaposi's sarcoma herpesvirus (KSHV), and Epstein-Barr virus (EBV)—infect B cells and establish a latent infection wherein they maintain their genome as a circular episome in the host nucleus and only produce few if any viral proteins that promote B cell proliferation (thus maintaining their cellular reservoir), and occasionally reactivate from latency to lytic cycle (thus maintaining their ability to infect a new host). Although most KSHV^{POS} and EBV^{POS} individuals experience mild-to-no clinical disease, a subset develops lymphoproliferative disorders and other malignancies [29]. In lymphopenic individuals, such as those who have HIV/AIDS or transplant-associated immunosuppression, KSHV infection causes malignant diseases of the skin and mucosal surfaces. Communities in sub-Saharan Africa have the highest prevalence of KSHV infection, (seropositivity rates of > 50%), followed by those in the Mediterranean (20–30% seropositivity rates), Europe, Asia and United States (< 10% seropositivity rates) [30]. In contrast to the wide regional variation in KSHV prevalence, EBV infects ~90% of adults worldwide [12,13]. EBV is best known for being the cause of non-malignant, infectious mononucleosis; however, it is also associated with malignant lymphoproliferative diseases such as Burkitt lymphoma and Hodgkin's lymphoma. Importantly, the parameters of γ HV infection are further modified by the developmental stage of the host: γ HV-driven polyclonal activation and expansion of B and T cells is more subtle in EBV-infected children and MHV68-infected neonatal mice [31]. Unlike adults that clear persistently replicating MHV68, MHV68-infected neonatal mice are also more likely to maintain low levels of persistent MHV68 replication in the lungs [31].

During their initial infection of host cells, KSHV and EBV elicit adaptive, polyclonal immune responses that persists after γ HV enters a non-replicative state (i.e. latency). Barton et al. [18] identified a benefit of this response, using the MHV68-infected mouse model: latent γ HV-infected mice were resistant to subsequent infection with the bacterial pathogens *L. monocytogenes* and *Y. pestis*. Both *L. monocytogenes* and *Y. pestis* cause acute diseases (i.e. listeriosis and plague), resistance to which depends on the cytokine IFN γ [32–34]. γ HV-driven resistance to *L. monocytogenes* and *Y. pestis* is likewise dependent of IFN γ , which is produced by CD4⁺ and CD8⁺ T cells during γ HV latency [35,36]. The results of Barton et al. were independently reproduced [37], and drove our curiosity whether γ HV-driven resistance also applied to TB, which—unlike listeriosis and plague—has both active and latent stages, the latter of which could be considered a chronic bacterial disease. Specifically, we hypothesized that the course of experimental TB would be improved by latent MHV68 infection, since γ HVs elicit a polyclonal T cell response that persists in the lung after viral latency is established (12 days in the MHV68 model). Indeed, we observed that latent MHV68 infection reduced the lung and spleen Mtb burdens of mice, and this reduction was associated with elevated numbers of CD4⁺IFN γ ⁺ cells in the MLN. Mtb infection elicits the clonal expansion of MLN T cells that are specific to Mtb-proteins EsxH, ESAT6 and Ag85, and can transfer immunological memory with varying efficacy [38]. Since Mtb benefits from a suboptimal T cell response *in vivo* [39], we predict that MHV68 infection expands the pool of Mtb-specific CD4⁺ T cells prior to Mtb-infection, thus increasing the probability that these cells will encounter Mtb-infected macrophages in the lung and spleen. Additional experiments are needed to test this hypothesis, using Mtb-peptide conjugated MHC tetramers to track the frequency of Mtb-specific T cells in the presence or absence of MHV68.

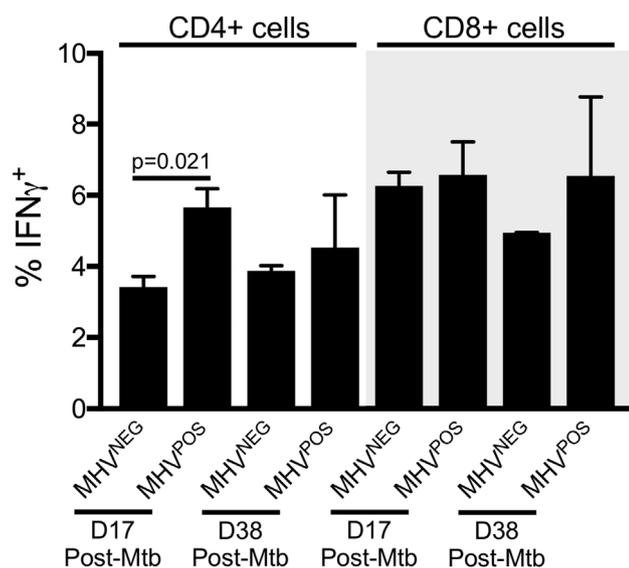


Fig. 2. Mtb-infected MHV68^{POS} mice have a transient increase in the frequency of IFN γ -producing CD4⁺ cells. C57BL/6 mice were infected with MHV68 and Mtb H37Rv as depicted in Fig. 1A. On post-Mtb infection days 17 and 38, we determined the frequency of MLN CD4⁺ and CD8⁺ cells capable of producing IFN γ following PMA/Ionomycin stimulation. Results are representative of three independent experiments; significance values are indicated above the data, as determined using Student's t-test.

Finally, our results suggest human γ HV-infection may be an important but unrecognized factor which modifies TB outcome, particularly in high TB burden countries where most children acquire EBV by 3 years of age [40,41] (in comparison, only 70% of 12-year-old children are seropositive for EBV in developed countries [42]). Whereas Mtb infection of adults almost uniformly occurs in the setting of chronic γ HV-infection, Mtb infection of children more likely occurs in both γ HV-naïve and γ HV-infected settings. Regarding the TB incidence data of McShane and colleagues [43], who were comparing the relative efficacy of BCG \pm MV85A or placebo in children aged 0–3 in South Africa (i.e. the MV85A efficacy trial): in light of our data, it will be important to determine if these two groups also had equivalent numbers of children that were exposed to EBV, as evidenced by the presence of circulating EBV-specific antibodies. The MV85A efficacy trial was important and led to follow-up studies to identify immune correlates of risk in vaccinated children [44]; however, to our knowledge, it has not yet been reported whether there is a correlation between serum anti-EBV titers and TB incidence in these same children. Based on our animal model data, we would predict TB incidence is lower in EBV-infected children. Differences in γ HV-infection prevalence may underlie an important clinical difference between adult and pediatric TB: whereas adult TB generally presents as pulmonary disease, widespread dissemination of Mtb is more common in children, resulting in extrapulmonary disease and infection of the brain, bones and other organs. Despite treatment regimens, mortality rates for children with disseminated forms of the disease are 10–20% and over 50% of survivors suffer long term neurological deficits [45]. We hope the results of our study will prompt the design of retrospective or prospective studies to assess whether γ HV-infection status associates with TB outcome in adults and children.

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