LETTERS

Herpesvirus latency confers symbiotic protection from bacterial infection

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All humans become infected with multiple herpesviruses during childhood. After clearance of acute infection, herpesviruses enter a dormant state known as latency. Latency persists for the life of the host and is presumed to be parasitic, as it leaves the individual at risk for subsequent viral reactivation and disease[1]. Here we show that herpesvirus latency also confers a surprising benefit to the host. Mice latently infected with either murine gammaherpesvirus 68 or murine cytomegalovirus, which are genetically highly similar to the human pathogens Epstein–Barr virus and human cytomegalovirus[2], respectively, are resistant to infection with the bacterial pathogens *Listeria monocytogenes* and *Yersinia pestis*. Latency-induced protection is not antigen specific but involves prolonged production of the antiviral cytokine interferon-γ and systemic activation of macrophages. Latency thereby upregulates the basal activation state of innate immunity against subsequent infections. We speculate that herpesvirus latency may also sculpt the immune response to self and environmental antigens through establishment of a polarized cytokine environment. Thus, whereas the immune evasion capabilities and lifelong persistence of herpesviruses are commonly viewed as solely pathogenic, our data suggest that latency is a symbiotic relationship with immune benefits for the host.

Murine gammaherpesvirus 68 (γHV68) undergoes brief lytic replication in a number of cell types, and establishes lifelong latency in memory B cells, macrophages and dendritic cells *in vivo*[3]. While investigating the role of interferons in controlling γHV68 reactivation after intranasal infection, we noted that peritoneal macrophages from latently infected mice were uniformly activated, displaying cytoplasmic vacuolization, membrane ruffling, increased size and upregulation of surface major histocompatibility complex (MHC) class II molecules (Fig. 1a–d; Supplementary Fig. 1). Peritoneal macrophage activation was prominent for at least two months after mucosal inoculation of γHV68 and clearance of lytic infection (data not shown). As fewer than 1 in 500 peritoneal macrophages are latently infected[4], macrophage activation was not a direct consequence of infection. In contrast to macrophages explanted from mock-infected mice (Fig. 1e), macrophages from latently infected mice were bactericidal, killing *L. monocytogenes* rapidly after uptake (Fig. 1f, 6 h after infection).

Because activated macrophages are an important innate defence against bacterial pathogens, we considered the hypothesis that herpesvirus latency might be associated with host resistance to infection. To test this hypothesis, we challenged mice with *L. monocytogenes*, a Gram-positive intracellular pathogen controlled *in vivo* by activated macrophages[5]. Acute γHV68 infection did not alter susceptibility to this pathogen (Fig. 2a). In contrast, latently infected mice were highly resistant to *L. monocytogenes* (Fig. 2b). Significant protection from *L. monocytogenes* was observed up to 3 months after γHV68 infection (Fig. 2c), the latest time point assessed. Protection correlated with ~100-fold reduction in bacterial burden in the spleen and liver (Fig. 2d, e). A point mutant of γHV68 that is capable of lytic infection but severely defective in establishment of latency (ORF73.stop)[6] did not protect mice from *L. monocytogenes* infection and did not stimulate chronic macrophage activation (Fig. 2d, e; Supplementary Fig. 1), demonstrating the importance of latent infection in this

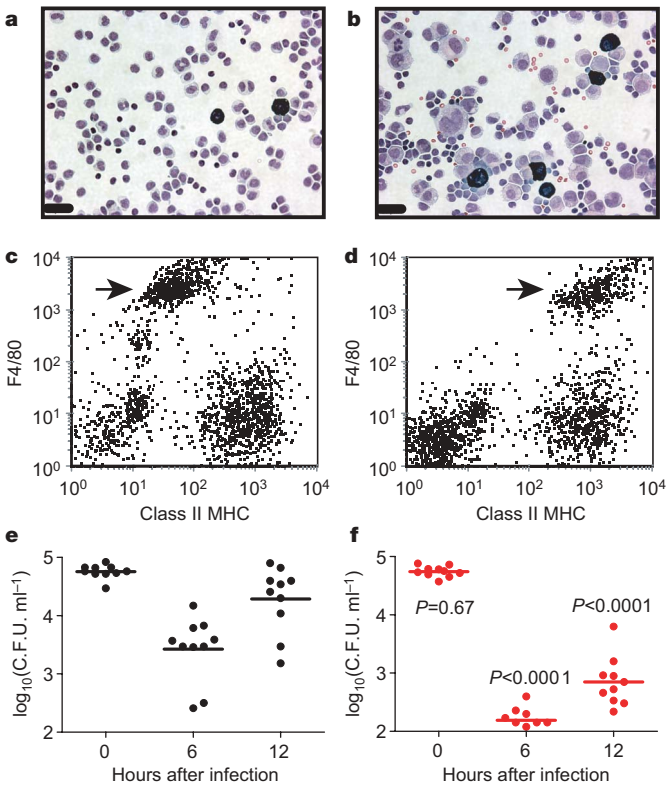

Figure 1 | Macrophages are activated during γHV68 latency. **a–f,** Peritoneal macrophages from mock (**a**, **c**, **e**) or latently infected (**b**, **d**, **f**) mice. **a**, **b**, Wright's Giemsa stain. Scale bar, 25 μm. **c**, **d**, Surface class II MHC levels on F4/80$^+$ peritoneal macrophages (arrow) in mock-infected mice (57 ± 14 (mean fluorescence intensity ± s.e.m.); $n = 23$) compared with latently infected mice (1,363 ± 144; $n = 18$). **e**, **f**, *Ex vivo* bactericidal activity of peritoneal macrophages at the indicated times. *P* values, calculated using the Mann–Whitney rank sum test, compare latently infected and mock-infected mice at the same time points. Data are representative of two to five independent experiments. c.f.u, colony-forming units. Horizontal bars indicate the arithmetic mean of log-transformed data.

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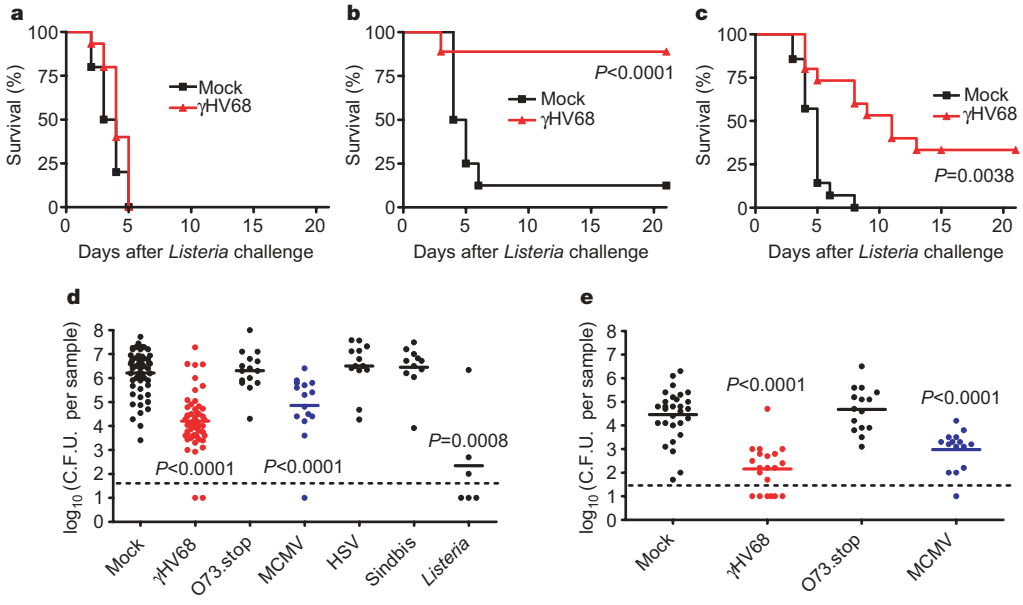

Figure 2 | Latent herpesvirus infection renders mice resistant to *L. monocytogenes*. **a**–**c**, Survival of mice infected with γHV68 1 (**a**), 4 (**b**) or 12 (**c**) weeks before challenge with *L. monocytogenes*. *P* values were calculated using logrank analysis (two to three experiments, $n = 8$–15 mice per condition). **d**, **e**, Bacterial titres in the spleen (**d**) and liver (**e**) 3 days after challenge in mice infected 28–42 days previously with the indicated virus or a sublethal dose of *L. monocytogenes*. *P* values were calculated using Mann–Whitney rank sum test compared to mock (three to five independent experiments). Horizontal bars indicate the arithmetic mean of log-transformed data. The dashed line represents the limit of detection. Where not indicated, $P > 0.3$.

protection. Latency-induced protection and macrophage activation were also observed in mice infected with murine cytomegalovirus (MCMV), a betaherpesvirus (Fig. 2d, e; Supplementary Fig. 1). Previous infection with human herpes simplex virus type-1 (HSV-1) or Sindbis virus (an RNA virus incapable of latent infection) triggered neither protection from bacterial infection nor chronic macrophage activation (Fig. 2d; Supplementary Fig. 1). Thus, latent infection with two divergent herpesviruses results in protection of the host from *L. monocytogenes*, indicating that this phenotype is a general and previously unappreciated consequence of herpesvirus latency.

To explore the possibility that herpesvirus latency enhanced antibacterial resistance more generally, we challenged γHV68 latently infected mice with the human and rodent pathogen *Y. pestis*, a Gram-negative extracellular bacterium and the causative agent of plague[7]. Using a pneumonic plague model, mock-infected mice displayed rapid *Y. pestis* replication in the lung, with spread to the spleen at later time points (Fig. 3a, b). In contrast, latently infected mice were resistant, exhibiting decreased *Y. pestis* replication in the lung (Fig. 3a) and decreased systemic spread (Fig. 3b). Latently infected mice also exhibited resistance in a bubonic plague model, displaying 100- to 1,000-fold decreases in systemic *Y. pestis* replication (Fig. 3c, d). The protection afforded by latency was not universal, as latently infected mice succumbed to West Nile virus (WNV) infection with kinetics indistinguishable from mock-infected mice (Fig. 3e).

To define the mechanism whereby herpesvirus latency protects against two distinct bacterial pathogens, we measured cytokine levels in the serum of latently infected mice. We found that latent infection triggered elevated levels of interferon-γ (IFN-γ) and tumour-necrosis factor-α (TNF-α), soluble mediators of macrophage activation and resistance to bacterial infection (Fig. 4a). IFN-γ was required for latency-induced protection from bacterial challenge (Fig. 4b), and consistent with a critical role for latent infection in this process, we did not observe IFN-γ upregulation in mice infected with the latency-defective ORF73.stop mutant (data not shown). Infection of $H\text{–}2K^{b-/-}$ $H\text{–}2D^{b-/-}$ $B_2M^{-/-}$ mice indicated that classical class I MHC-restricted $CD8^+$ T-cells, an important source of IFN-γ during γHV68 infection[8], were completely dispensable for protection (Fig. 4c). Notably, both $Ifng^{-/-}$ and $H\text{–}2K^{b-/-}$ $H\text{–}2D^{b-/-}$ $B_2M^{-/-}$ mice clear acute infection with γHV68 and

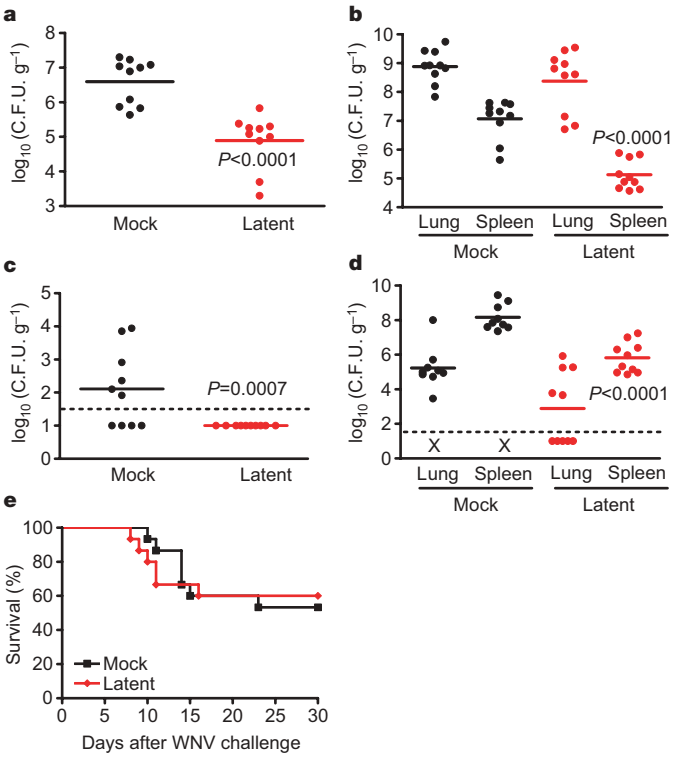

Figure 3 | Latently infected mice are resistant to *Y. pestis* but not WNV. **a**–**d**, Bacterial titres in mock (black symbols) or latently infected (γHV68, red symbols) mice challenged intranasally (**a**, 24 hours past infection (h.p.i.), lung, **b**, 48 h.p.i.) or subcutaneously (**c**, 36 h.p.i., spleen, **d**, 60 h.p.i.) with *Y. pestis*. *P* values, calculated using the Mann–Whitney rank sum test, compare latently infected and mock-infected mice (two independent experiments). X indicates death before harvest. Horizontal bars indicate the arithmetic mean of log-transformed data. The dashed line represents the limit of detection. Where not indicated, $P > 0.08$. **e**, Survival of mock or latently infected mice challenged by footpad inoculation with WNV. The *P* value was calculated using logrank analysis with ten mice per group.

establish latent infection, which is associated with persistent replication below the level of detection of standard plaque assays[8–10]. This indicates that latency-induced protection does not require classical $CD8^+$ T-cell-derived IFN-γ, and may be mediated by other cell types that have been demonstrated to produce IFN-γ during gammaherpesvirus latency, including activated $CD4^+$ T cells[3,11–13].

Infection-induced 'cross protection' between unrelated bacterial pathogens was the foundational observation defining cell-mediated immunity[14]. Classical cross protection occurs when effector lymphocytes responding to the initial infection secrete IFN-γ, thereby activating bystander macrophages and generating a heightened state of innate immunity to secondary infection. This classical form of cross protection wanes rapidly once the primary pathogen is cleared and responding lymphocytes differentiate from effector to memory phenotype[15], and has not previously been observed during latent virus infection.

In contrast, two mechanisms of cross protection that are mediated by memory lymphocytes have been recently described. In the first, termed heterologous immunity, cross-reactive antigenic epitopes between the primary and secondary pathogens result in antigen-specific memory lymphocyte activation after secondary infection[16]. In the second mechanism, $CD8^+$ memory T cells generated after primary infection can become activated in an antigen-independent manner by cytokines (including interleukin (IL)-12 and IL-18) expressed during the early stages of subsequent infections. In both cases, memory T-cell activation leads to secretion of IFN-γ and an enhanced response to the secondary infection[17]. In contrast to classical cross protection, memory-mediated cross protection does not involve heightened innate immunity before secondary challenge.

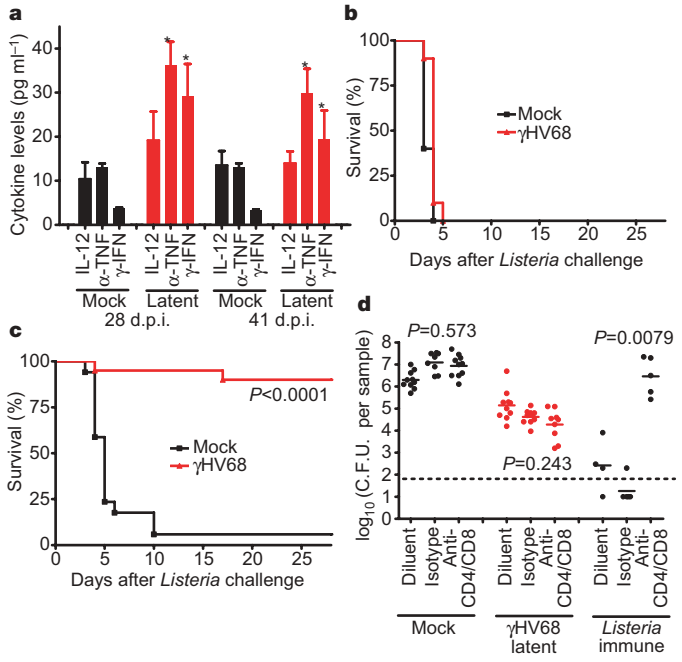

Figure 4 | Mechanism of enhanced innate immunity in latently infected mice. **a**, Cytokine levels (mean ± s.e.m.) following infection with γHV68. Asterisk, $P \leq 0.0002$, relative to mock, Mann–Whitney rank sum. Where not indicated, $P > 0.4$. **b**, **c**, Survival of mock or latently infected *Ifng*$^{-/-}$ (**b**) or *H–2K*$^{b-/-}$ *H–2D*$^{b-/-}$ *B*$_2$*M*$^{-/-}$ (**c**) mice, challenged with *L. monocytogenes*. The *P* value is relative to mock, logrank analysis (two to three experiments, $n = 10$–20 mice per condition). **d**, Bacterial titres in spleen 3 days after challenge with *L. monocytogenes* in mock, latently infected or *L. monocytogenes*-immune mice. $CD4^+$ and $CD8^+$ T cells were depleted 1 day before challenge. Horizontal bars indicate the arithmetic mean of log-transformed data. *P*-values, calculated using the Mann–Whitney rank sum test, compare depleted to isotype controls (two experiments).

Rather, the second pathogen activates an innate cytokine response that triggers bystander memory T-cell activation.

The chronic IFN-γ secretion and macrophage activation we observe in latently infected mice suggest that latency induces a prolonged state of classical cross protection. However, these observations do not exclude a role for the T-cell-dependent mechanisms described above. To determine whether latency-induced cross protection requires activation of memory T cells at the time of bacterial challenge, we depleted both $CD4^+$ and $CD8^+$ lymphocytes one day before *L. monocytogenes* challenge (Fig. 4d). T-cell depletion was effective, as indicated by elimination of *L. monocytogenes*-specific memory in depleted *L. monocytogenes*-vaccinated mice (Fig. 4d). In contrast, the resistance of latently infected mice to *L. monocytogenes* remained when $CD4^+$ and $CD8^+$ T cells were depleted. We conclude that viral latency generates a state of broadly effective innate cross protection that does not require the effector functions of lymphocytes at the time of secondary challenge.

Latency-induced cross protection differs from classical cross protection in at least two aspects. First, IFN-γ-dependent cross protection can occur between latent viral infection and acute bacterial infections. Second, this protection is quite prolonged, lasting months after clearance of acute infection. The durability of cross protection that we observe during gammaherpesvirus latency leads us to propose that latent infection represents a true symbiotic relationship, protecting the host from subsequent infection by means of heightened innate immune activation. Although our data suggest that IFN-γ-activated macrophages mediate latency-induced cross protection during both beta- and gammaherpesvirus infection, other cell types may contribute to the protective effects observed.

Are our findings with murine herpesviruses relevant for other latently infected hosts? We are unaware of studies that directly address the role of herpesvirus infection in human susceptibility to secondary bacterial infection. However, the observation of latency-induced cross protection in two subfamilies of the Herpesviridae suggests that this is an evolutionarily conserved aspect of the virus–host relationship. Furthermore, accumulating evidence indicates that latency with all three herpesvirus subfamilies in humans involves chronic, low-level immune activation accompanied by IFN-γ and TNF-α secretion in response to frequent but subclinical viral reactivation[18–22]. Although we do not observe latency-induced cross protection in mice infected with the alpha-herpesvirus HSV-1, this may reflect the tight anatomical restriction of HSV-1 latency in neurons, in contrast to latent infection of circulating haematopoietic cells by beta- and gammaherpesviruses. A plausible hypothesis for the mechanism of latency-induced cross protection involves the chronic, low-level presentation of viral antigens as a result of viral reactivation, resulting in prolonged T-cell activation and secretion of IFN-γ. This effect may be particularly important at mucosal and epithelial sites, where herpesvirus reactivation occurs and most pathogenic challenges initiate. Our data clearly indicate that IFN-γ expression during latency can have beneficial effects beyond control of viral reactivation, and suggest the need for further epidemiological studies of the immune consequences of herpesvirus infection in humans. These studies should also take into account the possible deleterious consequences of chronic inflammation elicited during latency, such as the development of autoimmune disease or cancer[23].

Beneficial immune modulation during herpesvirus latency may extend beyond protection from pathogens. Substantial epidemiological data support the 'hygiene' hypothesis, in which prior infection protects against development of allergy[24]. Few studies have addressed the role of herpesvirus latency in skewing immune responses, despite the increasing age of seroconversion to herpesvirus infection in populations with increased atopy and autoimmunity[25]. One study reports a protective effect of Epstein–Barr virus infection in the reduction of IgE sensitization to environmental allergens, an effect that was more pronounced on co-infection with human cytomegalovirus and Epstein–Barr virus[26]. Our findings suggest a mechanistic

explanation for this observation, whereby prolonged secretion of T-helper-1-type cytokines during latency may inhibit the development of T-helper-2-driven immune pathology. Accordingly, potential benefits of herpesvirus latency should be considered when evaluating vaccines against herpesvirus infection, as decreased infection rates may be associated with unintended negative consequences for vaccinated individuals.

Our current understanding of cellular and molecular immunity is founded largely on studies in specific-pathogen-free mice that have no known herpesvirus infections. We now demonstrate that herpesvirus infection triggers systemic, profound immune modulation, with the potential to alter significantly the kinetics and nature of host response to foreign antigens. As there are few herpesvirus-free humans, we speculate that an accurate understanding of 'normal' immunity may require a re-evaluation of immune function in the presence of these symbionts, which have been exerting selective pressures on the mammalian immune system since before the vertebrate radiation[27].

METHODS SUMMARY

Mice, viruses and infections. C57BL/6J mice were infected with virus between 8 and 20 weeks of age. *Ifng*$^{-/-}$ or *H–2K*$^{b-/-}$ *H–2D*$^{b-/-}$ *B*$_2$*M*$^{-/-}$ mice are described elsewhere[8,10]. Mice received wild-type or ORF73.stop γHV68 (WUMS strain, intranasal), HSV-1 (strain 17, intraperitoneal), MCMV (strain Smith, intraperitoneal), or Sindbis virus (AR339, intraperitoneal). Mice were considered latently infected at 28 days after infection. *Listeria monocytogenes* was administered intraperitoneally. *Yersinia pestis* was administered intranasally[7] or subcutaneously. WNV was administered by footpad inoculation[28]. Lymphocytes were depleted by administration of anti-CD4 (YTS-191.1), anti-CD8 (H35), or isotype control SFR-DR5 rat monoclonal antibodies intraperitoneally[29].

Flow cytometric analysis. Peritoneal cells were obtained by lavage, blocked in 10% normal mouse serum, and stained for cell-surface MHC class II (I–Ab/I–Eb; Phyroerythrin-conjugated, Pharmingen) and F4/80 (allophycocyanin-conjugated, Caltag) and analysed on a Beckton-Dickinson FACScalibur.

***L. monocytogenes* growth in macrophages.** Adherent peritoneal macrophages were infected with *L. monocytogenes*; unbound bacteria were removed. At 30 min after-adsorption, gentamicin (5 μg ml^{-1}) was added to kill extracellular bacteria, permitting the direct assessment of macrophage capacity to kill phagocytosed *L. monocytogenes*. Viable intracellular bacteria were quantified by hypotonic lysis of peritoneal cells and plating onto BHI agar plates[30].

Quantification of bacterial growth *in vivo*. Spleens, livers and lungs were removed from *L. monocytogenes*- or *Y. pestis*-infected mice at the indicated times after bacterial challenge. For quantification of *L. monocytogenes*, spleens were homogenized in 10 ml PBS/0.05% Triton X-100 and plated on BHI-agar plates. Organs from *Y. pestis*-infected mice were homogenized in sterile PBS and plated onto BHI agar plates.

Quantification of cytokine expression. Cytokines were detected in whole serum using the mouse inflammation cytometric bead array (BD Pharmingen) according to the manufacturer's instructions.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions The original hypothesis of the article was formulated by E.S.B. and H.W.V. E.S.B. and D.W.W performed all experiments except those characterizing *Y. pestis* infection (J.S.C. and V.L.M.) and WNV infection (M.E. and M.S.D.). K.A.B.-M. made the initial observation of chronic IFNγ secretion during latency. The manuscript was written by E.S.B., D.W.W., and H.W.V. and all authors commented on data and conclusions prior to submission.

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METHODS

Mice and infections. C57BL/6J mice (Jackson and internal breeding colonies) were infected with virus between 8 and 20 weeks of age. *Ifng*$^{-/-}$ or *H–2K*$^{b-/-}$ *H–2D*$^{b-/-}$ $B_2M^{-/-}$ mice are described elsewhere[8,10] and were from internal breeding colonies. γHV68 infections (10^4 plaque-forming units (p.f.u.)) were performed intranasally in a volume of 40 μl DMEM/10% FBS (DMEM/10). Lytic infection of ORF73.stop virus after intranasal inoculation was confirmed by enzyme-linked immunosorbent assay (ELISA) against total γHV68 antigen, which demonstrated equivalent serum levels of γHV68-specific antibody after wild-type γHV68 or ORF73.stop infection (data not shown). HSV1 (10^4 p.f.u.), MCMV (10^5 p.f.u.), and Sindbis virus (10^4 p.f.u.) were administered intraperitoneally in 0.5 ml DMEM/10. *Listeria monocytogenes* (nonlethal dose, 10^3 c.f.u; lethal dose 10^6 c.f.u) was administered intraperitoneally in 0.5 ml saline. MCMV infected mice displayed no serological evidence of γHV68 infection by ELISA (data not shown). *Yersinia pestis* used for subcutaneous infections were cultured at 26 °C in BHI for 16–18 h, and for intranasal infections bacteria were cultured at 37 °C in BHI supplemented with 2.5 mM $CaCl_2$ for 16–18 h. For analysis of *Y. pestis* colonization and dissemination mice were infected intranasally with 1×10^4 CFU essentially as described[7] or subcutaneously with 1×10^2 c.f.u. in 100 μl at the base of the tail. WNV (100 p.f.u.) was administered by footpad inoculation as described[28]. In some experiments, lymphocytes were depleted by administration of a single 1-mg dose of anti-CD4 (YTS-191.1), anti-CD8 (H35), or isotype control SFR-DR5 (American Type Culture Collection) rat monoclonal antibody intraperitoneally[29].

Viral and bacterial stocks. γHV68 (strain WUMS) stocks were prepared and titrated in murine 3T12 fibroblasts. The latency-deficient γHV68 mutant ORF73.stop was obtained from the laboratory of S. H. Speck[6]. MCMV strain Smith was obtained from the laboratory of M. Colonna. HSV-1 strain 17 and Sindbis virus strain AR339 are laboratory stocks. *Listeria monocytogenes* strain EGD stocks were amplified from infected splenic lysate and were obtained from the laboratory of E. R. Unanue. The virulent wild-type *Y. pestis* strain CO92[7] was obtained from the US Army, Ft. Detrick, Maryland. WNV (strain 3000.0259) was isolated in New York in 2000 and passaged once in C6/36 *Aedes albopictus* cells[28].

Quantification of *L. monocytogenes* growth in cultured peritoneal macrophages. This assay has been described in detail previously[30]. Briefly, peritoneal cells were removed by lavage with 10 ml ice-cold DMEM/10 and allowed to adhere to polystyrene 24-well plates overnight. Non-adherent cells were removed by washing with agitation in warm DMEM/10. Monolayers were infected with 1×10^5 c.f.u. of fresh-standing overnight culture of *L. monocytogenes* by centrifugal adsorption. Unbound bacteria were removed by rinsing with warm PBS and then cultured in the presence of gentamicin (5 μg ml^{-1}, added at 30 min after bacterial adsorption) to kill extracellular bacteria that have not been phagocytosed, thereby permitting the direct assessment of macrophage capacity to kill phagocytosed *L. monocytogenes* before bacterial escape from the endosome. At various times after adsorption, viable intracellular bacteria were quantified by hypotonic lysis of macrophage monolayers in water and inoculation of serial dilutions onto BHI agar plates.

Figure S1

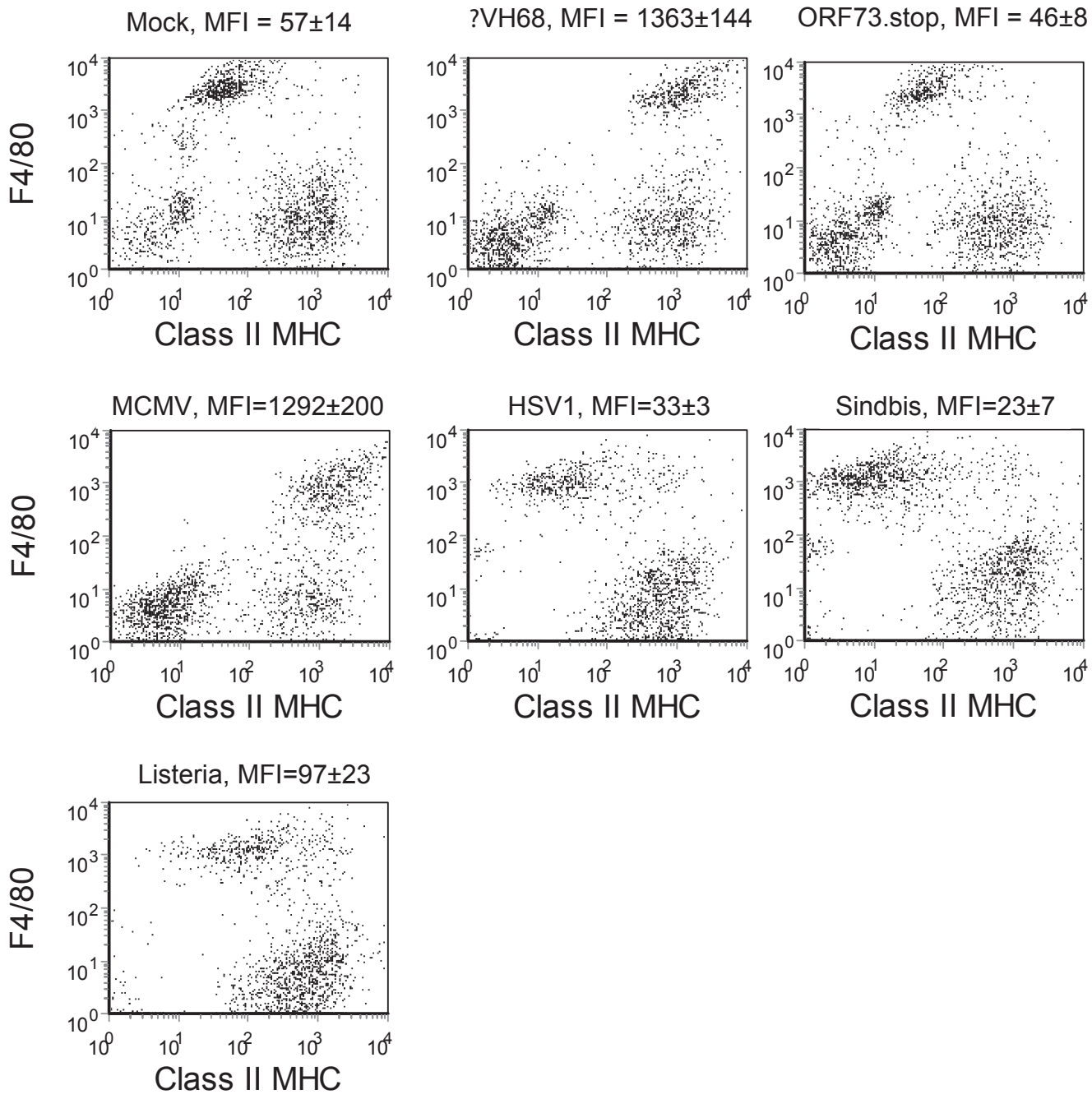

Figure S1. Latent infection with γHV68 or MCMV results in chronic, systemic macrophage activation. Representative scatter plots showing surface F4/80 and class II MHC levels on peritoneal macrophages from mice infected 28 days previously with the indicated virus or a sublethal dose of *L. monocytogenes.* Mean fluorescence intensity (MFI, +/- s.e.m.) of class II MHC on ungated F4/80+ cells from all experiments is indicated (mock, n=23; gHV68, n=18; ORF73.stop, n=10; MCMV, n=12; HSV-1, n=4; Sindbis virus, n=4; *L. monocytogenes*, n=4).