Interferon-induced transmembrane protein 3 prevents cytokine-driven cytomegalovirus pathogenesis

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Abstract

The antiviral restriction factor Interferon-induced transmembrane protein 3 (IFITM3) inhibits cell entry of a number of viruses and genetic diversity within IFITM3 determines susceptibility to viral disease in humans. Using the murine cytomegalovirus (MCMV) model of infection, we discovered that Ifitm3 limits herpesvirus-associated pathogenesis without directly preventing virus replication. Instead, Ifitm3 promoted antiviral cellular immunity through the restriction of virus-induced lymphopenia, apoptosis-independent NK cell death and loss of T cells. Viral disease in Ifitm3/-/ mice was accompanied by elevated production of cytokines, most notably interleukin-6 (IL-6). Ifitm3 inhibited IL-6 production by myeloid cells in response to replicating and non-replicating virus, and also following stimulation with TLR ligands Poly(I:C) and CpG. Although IL-6 promoted virus-specific T cell responses, uncontrolled IL-6 expression in Ifitm3/-/ mice triggered the loss of NK cells, and subsequently impaired control of MCMV replication. Thus, Ifitm3 represents a checkpoint regulator of anti-viral immunity, which controls cytokine production to restrict viral pathogenesis. These data suggest the utility of cytokine-targeting strategies in the treatment of virus-infected individuals with impaired IFITM3 activity.
Introduction

Antiviral immune responses elicited following acute viral infections are tightly regulated to limit uncontrolled immune pathology whilst ensuring adequate control of the primary infection. Herpesvirus infections are typically controlled by asymptomatic resolution of the primary infection and establishment of virus latency, where the adaptive immune response controls replication of reactivating virus. Thus, host innate and adaptive immune mechanisms work to hold herpesvirus replication in check. In some settings, failure in anti-viral defense during the primary infection leads to elevated virus replication and virus-induced disease pathology (1-3).

Herpesvirus restriction of immune activation may contribute to limited pathology during acute infection. Indeed, a clear evolutionary advantage for herpesviruses exists to modulate antiviral immunity to maintain host fitness during acute infection, but also to facilitate persistence and the establishment of latency. The β−herpesvirus human cytomegalovirus (HCMV) represents a paradigm for viral immune evasion. It encodes numerous proteins with putative immune modulatory actions (4, 5), and HCMV infection profoundly influences the expression of host immune-related proteins (6). Studies in the murine cytomegalovirus (MCMV) model of β−herpesvirus infection have highlighted that CMV also exploits host immune inhibitory mechanisms, including the immune regulatory cytokine interleukin-10 (IL-10), to facilitate virus persistence (7-9). Paradoxically, both cellular (10) and viral (11) IL-10 restrict acute pathologies in experimental models of CMV infection. Consequently, a delicate and important balance exists between control of acute replication, virus-induced inflammation and virus persistence. The factors governing this balance and
the potential influence that host and virus genetic variation exerts on this process requires a better understanding.

Interferon-induced transmembrane protein 3 (IFITM3) is an IFN-inducible antiviral restriction factor that is enriched in endosomal compartments (12). IFITM3 restricts endocytosis-dependent entry of diverse viruses, most notably influenza, Dengue Virus, West Nile Virus and HIV (13, 14). Importantly, genetic studies emphasize the pivotal role that IFITM3 plays in governing viral disease in humans. A number of polymorphisms within human IFITM3 have been identified that may potentially influence IFITM3 function (15). Notably, the minor rs12252-C allele in IFITM3, that has an allele frequency of 0.03 and 0.5 in European Caucasian and Han Chinese populations respectively (16, 17), is associated with impaired restriction of influenza replication (15, 16, 18), increased susceptibility to severe influenza-associated disease (16, 17) and HIV progression (19).

Studies in murine infection models have highlighted a critical role for Ifitm3 in restricting viral pathogenesis in vivo. Ifitm3<sup>−−</sup> mice exhibit increased susceptibility to infection with influenza (16, 20), arthritogenic and encephalitic alphaviruses (21), respiratory syncytial virus (22) and West Nile Virus (23). Susceptibility is associated with the significant impairment of direct control of viral replication, in accordance with the established role for IFITM3 as an antiviral restriction factor. Interestingly, however, alterations in immune responses have also been described in these models (16, 21-23). Whilst these observations suggest a possible link between Ifitm3 and the regulation of antiviral immunity, the direct impact of Ifitm3 on virus replication has not been disentangled from any immune regulatory functions of Ifitm3. Furthermore, studies in influenza infection have revealed that impaired antiviral immune responses
in Ifitm3−/− mice can occur as a consequence of unregulated infection of immune cells (24, 25).

Ifitm3 does not directly impinge on HCMV replication in vitro (26, 27). Consequently, we were intrigued to establish whether Ifitm3 influences herpesvirus pathogenesis in vivo. Using the MCMV model of infection, we identified that murine Ifitm3 is a critical checkpoint regulator of herpesvirus-induced immune pathology during acute and chronic infection in vivo. Consistent with observations in HCMV infection, Ifitm3 did not directly restrict MCMV replication, but instead acted to limit over-exuberant production of cytokines, in particular interleukin-6 (IL-6). Thus, Ifitm3 activity acts as a rheostat of antiviral immunity that determines the pathological outcome of acute MCMV infection.
Results

Ifitm3 determines the primary outcome of MCMV infection

To assess the impact of Ifitm3 on herpesvirus pathogenesis, we first infected control wild type (WT) and ifitm3-deficient mice with MCMV. Infection of WT mice with $3 \times 10^4$ PFU salivary gland-propagated Smith strain MCMV resulted in a non-lethal infection (Figure 1A) and moderate (~10%) weight loss (Figure 1B). However, Ifitm3−/− mice exhibited only a 60% survival, with mice succumbing to infection or being culled in accordance with UK Home Office guidelines regarding disease severity/weight loss, between 6-8 days post-infection (pi) (Figure 1A&B). Surviving Ifitm3−/− mice also exhibited delayed weight loss recovery still evident at 12 days post-infection (Figure 1B). Furthermore, sub-clinical infection with an inoculum of $5 \times 10^3$ PFU/mouse also induced weight loss in Ifitm3−/− mice but not in WT mice (Figure S1A).

Exacerbated MCMV-induced weight loss in Ifitm3−/− mice was accompanied by statistically significant higher virus load from days 4 pi in spleen (Figure 1C) and lung (Figure 1D) but not the liver (Figure 1E) after infection with standard inoculum ($3 \times 10^4$ PFU). Weight loss in Ifitm3−/− mice induced following low dose inoculum ($5 \times 10^3$ PFU) also resulted in significantly increased virus load in the spleen 4 days pi (Figure S1B). Furthermore, 14 days after infection with standard inoculum, viral persistence in the salivary gland was evident in WT and Ifitm3−/− mice, but Ifitm3−/− mice harbored elevated virus load in this established site of persistent MCMV replication (Figure 1F). Moreover, extensive pathology in spleens of Ifitm3−/− mice was observed 14 days pi (Figure 1G) with severe disruption of follicular structures evident (Figure 1H). Spleens were not recoverable from Ifitm3−/− mice 3 months post-infection (data not
shown), demonstrating the irreversible nature of organ damage. Thus, Ifitm3 promoted host survival and control of virus replication during CMV infection.

**Ifitm3 does not influence MCMV cell entry or infectious virion production**

IFITM3 restricts entry of a number of RNA viruses that utilize the endocytic pathway during cell entry (13). However, IFITM3 does not restrict HCMV entry into fibroblasts and epithelial cells (26, 27), the latter of which requires endocytosis (28). Bone marrow chimera experiments revealed that Ifitm3 deficiency within the hematopoietic cell compartment was sufficient to increase virus load 4 days pi in vivo (Figure 2A&B). Cytomegalovirus does not productively infect lymphocytes (29), and we detected no MCMV infection of WT or *Ifitm3*−/− T cells or NK cells, as determined by the absence of detection of the MCMV m06 early protein in vitro (data not shown). Interestingly, MCMV infection of M-CSF, GM-CSF and Flt3L differentiated bone marrow-derived myeloid cells was prevented by pretreatment with the endocytosis inhibitor EIPA (Figure S1C-E). Thus, we investigated whether Ifitm3 influenced endocytosis-dependent MCMV replication within myeloid cells. MCMV entry was unaffected by *Ifitm3* deficiency when cells were infected at multiplicities of infection (MOI) that result in non-saturating infection in WT cells (MOI of 0.1 or 1, Figure 2C-H) or following infection with a higher MOI (MOI = 10, Figure S1F). Furthermore, Ifitm3 had no impact on type I IFN-mediated endocytosis-dependent control of MCMV infection (Fig. S1G-I). Moreover, MCMV infection of primary mouse embryo fibroblasts (MEFs) was also unaffected by the absence of ifitm3 (Figure 2I&J). Thus, in contrast to influenza infection (Figure S1J), Ifitm3 did not influence infection efficiency of MCMV in our assays. Furthermore, productive virus replication following infection of all cell types examined was also unaffected by Ifitm3 deficiency (Figure
Thus, as observed in HCMV infection (26, 27), our data suggest that Ifitm3 does not directly restrict MCMV cell entry and subsequent replication.

Iftm3<sup>−/−</sup> mice exhibit exacerbated lymphopenia and increased leukocyte death during MCMV infection

We investigated the mechanisms underpinning MCMV-induced pathogenesis in Iftm3<sup>−/−</sup> mice. Lymphopenia is a hallmark of severe viral disease (30). MCMV-infected Iftm3<sup>−/−</sup> mice exhibited exacerbated systemic lymphopenia and a concomitant elevation in circulating granulocytes during MCMV infection (Figure 3A). This was accompanied by a large reduction in splenocyte numbers at 4 pi (Figure 3B). Circulating blood platelet and red blood cells were not reduced in Iftm3<sup>−/−</sup> mice, suggesting that lymphopenia was not a consequence of generalized bone marrow suppression (Figure S2A&B). Furthermore, concentrations of chemokines including lymphocyte-attracting chemokines CCL5 and CXCL10 within the spleens of Iftm3<sup>−/−</sup> mice were elevated as compared with WT controls (Figure S2C), highlighting that tissue lymphopenia was likely not due to impaired chemokine-mediated cellular recruitment. Instead, cell death analysis revealed enrichment of late apoptotic/necrotic (Annexin V<sup>+</sup>7AAD<sup>+</sup>) NK cells (Figure 3C) and CD3<sup>+</sup> cells (Figure 3D) day 4 pi in Iftm3<sup>−/−</sup> mice. In contrast, we observed no significant enrichment of early apoptotic NK1.1<sup>+</sup> or CD3<sup>+</sup> cells, as detected by either caspase-3 expression or Annexin V<sup>−/−</sup>7AAD<sup>−</sup>staining either 2 or 4 days post-infection (Maria Stacey and Ian Humphreys, data not shown). Collectively these data implied that apoptosis-independent cell death was a significant factor underpinning lymphopenia in MCMV-infected Iftm3<sup>−/−</sup> mice.
Impaired cellular immune responses in MCMV-infected Ifitm3−/− mice

We examined the influence of cellular immunity on the outcome of MCMV infection in Ifitm3−/− mice. Neutrophil depletion exacerbated MCMV-induced weight loss in both WT and Ifitm3−/− mice (Figure S2D), consistent with their antiviral function (31) and the conclusion that, as suggested by elevated granulocyte responses in Ifitm3−/− mice (Figure 3A), impaired neutrophil responses were not responsible for increased susceptibility of Ifitm3−/− mice to viral pathogenesis.

NK cells afford critical protection from HCMV (32) and MCMV (2) infections. Increased MCMV replication in Ifitm3−/− mice was accompanied by a 5-fold reduction in NK cell numbers (Figure 3E) similar to the reduction in splenocytes observed in Ifitm3−/− mice (Figure 3B). This equated to a comparable defect in the accumulation of degranulating (CD107a+) and IFN-γ+ cells (Figure 3F) NK cells, although frequencies of NK cells spontaneously expressing IFN-γ+ were low in both groups consistent with the dominant role of perforin-mediated control of MCMV replication in the spleen (33). This is consistent with the conclusion that Ifitm3 deficiency impinged on antiviral NK cell responses by influencing NK cell accumulation and survival rather than by directly influencing NK cell function. Notably, NK cell depletion using established methodology (34) resulted in comparable virus load in WT and Ifitm3−/− mice 4 days pi (Figure 3G), suggesting that the NK cell defect significantly contributed to acute MCMV replication and pathology in the Ifitm3−/− mice. In accordance, Ifitm3−/− mice did not exhibit impaired control of a strain of MCMV lacking the m157 protein (Figure S2E) that specifically induces NK cell activation (35).

During the latter stages of MCMV infection (day 7 pi), in accordance with substantial CD3+ cell death at early time-points (Figure 3D), accumulation of CD4+ and CD8+ T cell numbers (Figure 3H) and virus-specific CD8+ T cells (Figure 3I) were also
drastically reduced in Ifitm3<sup>−/−</sup> mice, in addition to NK cells (Figure 3H). Therefore, the broad protection afforded by Ifitm3 on T cell survival in vivo promoted the generation of virus-specific T cell immunity.

**Ifitm3 regulates MCMV-induced cytokine production**

Over-exuberant cytokine production is associated with virus-induced lymphopenia (30) and with reduced NK cell accumulation during acute MCMV infection (34). We therefore hypothesized that unregulated cytokine production was driving loss of cellular antiviral immunity in this model. Small but significant elevations of IL-12p70, GM-CSF and IFN-α were detected in spleen homogenates from Ifitm3<sup>−/−</sup> mice (Figure 4A). We also observed a moderate increase in TNF-α expression d4 pi (Figure 4A), a result reproduced in 3 of 4 experiments (data not shown). Importantly, however, dramatic increases in IL-6 protein concentrations were routinely observed in Ifitm3<sup>−/−</sup> mice at multiple time-points following acute infection (Figure 4A&B).

IL-6 is implicated in numerous inflammatory pathologies (reviewed in (36)). Therefore we investigated the role that IL-6 played in MCMV-associated pathogenesis. Myeloid cells (conventional (c)DCs, macrophages and plasmacytoid (p)DCs) were identified as primary sources of IL-6 expression during MCMV infection in vivo, and the percentage of myeloid cells expressing IL-6 within these populations was substantially elevated in Ifitm3<sup>−/−</sup> mice (Figure 4C). Importantly, MCMV infection of chimeric mice expressing Ifitm3 in hematopoietic and/or non-hematopoietic cells revealed that hematopoietic cell expression of Ifitm3 was essential for controlled IL-6 production (Figure 4D). Macrophages, cDCs and pDCs all expressed Ifitm3 during MCMV infection (Figure 4E). We therefore hypothesized that Ifitm3 directly inhibited virus-induced IL-6 production by myeloid cells. To test this, we generated myeloid
cell cultures from WT and Ifitm3−/− bone marrow stem cells. Although Ifitm3 deficiency did not impact on low levels of MCMV-induced IL-6 production by M-CSF-differentiated cells or by MEFs, both GM-CSF- and Flt3L-differentiated Ifitm3−/− cells produced significantly more IL-6 than WT cells in response to MCMV (Figure 4F). Importantly, these data were derived from the same experiments in which we confirmed that Ifitm3 did not restrict MCMV entry (Figure 2).

To further confirm that increased MCMV-induced IL-6 production in Ifitm3−/− myeloid cells was not a consequence of enhanced viral replication and/or cell entry, we incubated GM-CSF- and Flt3L-differentiated myeloid cells with irradiated MCMV and detected increased IL-6 production by Ifitm3−/− cells (Figure 5A&B). Toll like receptors (TLRs) 3 and 9 are stimulated by MCMV (37, 38), and Ifitm3−/− myeloid cells also produced more IL-6 in response to the TLR9 ligand CpG and, in the case of Flt3L-generated cells, the TLR3 ligand Poly(I:C) (Figure 5C&D). In contrast, IL-6 induction in response to cell surface expressed TLR4 and cytoplasmic DNA sensor STING was not significantly altered by Ifitm3 deficiency, although we observed a trend in increased IL-6 production following stimulation of both innate immune sensors (Fig. S3). Thus, Ifitm3 significantly suppresses myeloid cell regulation of IL-6 production in response to endosomal TLR ligands, and replicating and non-replicating MCMV.

**IL-6R signaling mediates MCMV-induced pathogenesis**

We assessed whether enhanced cytokine production was responsible for the pathogenesis observed in Ifitm3−/− mice. Administration of an antagonist anti-IL-6 receptor (anti-IL-6R) monoclonal antibody that blocks both classical IL-6 receptor signaling and IL-6 trans-signaling in vivo (39) significantly alleviated virus-induced weight loss in WT and Ifitm3−/− mice. Here, IL-6R blockade halved the loss of weight
in *Ifitm3<sup>−/−</sup>* mice seen during the first 4 days of infection (Figure 6A). In contrast, neutralization of TNF-α had no impact on MCMV-driven weight loss in *Ifitm3<sup>−/−</sup>* mice (Figure S4). These data suggested a dominant role for the Ifitm3 regulation of IL-6 production in determining the pathological outcome of MCMV infection.

Improved outcome of acute infection after anti-IL-6R treatment of *Ifitm3<sup>−/−</sup>* mice was accompanied by a dramatic reduction in virus replication (4 days pi). The virus load in these antibody-treated animals resembled levels observed in infected WT mice (Figure 6B). This was associated with a recovery of leukocyte (Figure 6C) and NK cell accumulation at this time (Figure 6D&E). Unregulated cytokine production during acute MCMV infection promotes NK cell death (34). Interestingly, IL-6R blockade completely reversed the accumulation of AnnV<sup>+</sup>7AAD<sup>+</sup> NK cells (Figure 6F).

Correlation analysis of NK cell accumulation versus PFU of all treated and untreated WT and *Ifitm3<sup>−/−</sup>* mice revealed a significant inverse correlation between NK cell numbers and virus control (Figure 6G), whereas virus load and CD3<sup>+</sup> T cells accumulation showed no statistically significant correlation (Figure S5A). Thus, inhibition of IL-6 receptor signaling alleviates early virus-induced disease in *Ifitm3<sup>−/−</sup>* hosts. These findings support a role for IL-6 in control of viral replication and NK cell survival.

**IL-6R signaling is required for the development of antiviral cellular immunity**

The beneficial impact of anti-IL-6R treatment was reduced at later time-points of infection in both WT and *Ifitm3<sup>−/−</sup>* mice (Figure 6A). IL-6R blockade failed to reverse the dramatic loss of T cell accumulation in the spleens of *Ifitm3<sup>−/−</sup>* mice 7 days pi (Figure 6H) without selectively antagonizing the accumulation of particular lymphocyte subsets (Figure S5B). The failure of IL-6R blockade to rescue T cell
responses did not reflect a cell-intrinsic role for Ifitm3 in maintaining T cell responses in vivo, as demonstrated by comparable recovery of WT and Ifitm3<sup>−/−</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells following MCMV infection (Figure S5C). Instead, in accordance with data derived from influenza infection models (40), IL-6 was required for the development of virus-specific T cell responses in MCMV (Figure S5D). Furthermore, NK cell accumulation at this time was also dependent upon IL-6, and anti-IL-6R treatment also failed to rescue the loss of NK cells in Ifitm3<sup>−/−</sup> mice (Figures 6H and S5B). Consequently, the early control of virus replication observed following IL-6R blockade in Ifitm3<sup>−/−</sup> mice was not sustained at 7 days pi (Figure 6I). These data highlight IL-6 as an important mediator of viral pathogenesis and suggest a critical role for Ifitm3 in the appropriate temporal regulation of production of this cytokine in response to herpesvirus infection.
Discussion

Experimental evidence to date has suggested that exacerbated viral pathogenesis in hosts with deficient or impaired IFITM3 activity is a consequence of impaired restriction of virus entry and subsequent replication. Herein, we provide evidence that direct regulation of virus-induced cytokine production is an important additional in vivo function of Ifitm3 and, in the context of CMV infection, represent the dominant mechanism through which Ifitm3 controls virus-induced disease.

We demonstrated that the production of multiple cytokines were restricted by Ifitm3. However, our data define Ifitm3 regulation of IL-6 production as a central determinant of pathogenesis associated with MCMV infection. In the early stages of infection, IL-6 was the key driver of virus-induced weight loss in WT and Ifitm3−/− mice and impinged on cellular antiviral immunity. These findings are consistent with IL-6 driving lymphopenia (41), inhibiting lymphopoiesis (42) and inducing glucocorticoid expression (43). Further, our data suggests that IL-6-induced apoptosis-independent lymphocyte death was associated with lymphopenia in MCMV-infected Ifitm3−/− mice. Although it is not possible to discriminate directly ex-vivo between different forms of apoptosis-independent cell death, IL-6 may promote cell death directly via induction of autophagy (44) or STAT3-mediated necrosis (45). Alternatively, IL-6 may indirectly trigger cell death via induction of multiple cytokines implicated in activation-induced death of T cells and NK cells (46, 47). Given that IL-6 was also required for the latter accumulation of NK cells and virus-specific T cells during MCMV infection, IL-6 likely exerts differential context-dependent pro-death or pro-survival signals in leukocytes during infection (reviewed in (36)).
NK cells exert critical control of HCMV (32) and MCMV (2) infections. Although we cannot formally exclude the possibility that Ifitm3 may influence virus replication in an unidentified cell type in vivo, the observation that virus load after 4 days of infection was comparable in WT and \textit{Ifitm3}\textsuperscript{-/-} mice following NK cell depletion or after challenge with delta-m157 MCMV, strongly suggests that impaired NK cell responses was primarily responsible for elevated virus replication in \textit{Ifitm3}\textsuperscript{-/-} mice. In this regard, IL-6R blockade was also found to rescue both NK cell responses and virus control in \textit{Ifitm3}\textsuperscript{-/-} mice. Interestingly however, although IL-6 has recently been implicated in direct control of HCMV replication (48), inhibiting the action of IL-6 restricts HCMV reactivation from latency in dendritic cells (49). Thus, it is possible that the beneficial impact of anti-IL-6R treatment on control of virus replication in our experiments may extend beyond restriction of lymphopenia and NK cell death.

\textit{Ifitm3} restricted myeloid cell production of IL-6 upon exposure to MCMV in vitro. \textit{Ifitm3} expression by hematopoietic cells was critical for controlled production of IL-6 in vivo, and frequencies of IL-6-secreting myeloid cells were elevated in MCMV-infected \textit{Ifitm3}\textsuperscript{-/-} mice. The latter observation may reflect in part a feedback mechanism through which elevated virus load in \textit{Ifitm3}\textsuperscript{-/-} mice further stimulated myeloid cell production of IL-6 and hence increases the frequency of myeloid cells capable of producing IL-6 ex-vivo. Critically, however, we demonstrate that \textit{Ifitm3}\textsuperscript{-/-} myeloid cells produced more IL-6 in response to non-replicating virus, and IL-6R blockade is sufficient to restore control of MCMV replication in \textit{Ifitm3}\textsuperscript{-/-} mice. Thus, \textit{Ifitm3} acts as an immune regulator that restricts virus-induced IL-6 production by myeloid cells independently of controlling virus entry and replication. The \textit{Ifitm3}-IL-6 axis is therefore responsible for determining viral pathogenesis in vivo.
TLRs located in endosomes are triggered by MCMV (37, 38) and induce expression of cytokines including IL-6 upon stimulation (50). Critically, our data reveal that Ifitm3 suppresses TLR3 and TLR9-induced cytokine production by myeloid cells in response to non-replicating ligands. In contrast, IL-6 production by Ifitm3−/− cells was only moderately elevated following stimulation of cell membrane-expressed TLR4 or the cytoplasmic DNA sensor STING. These data suggest that Ifitm3 may preferentially regulate the activation and/or down-stream signaling triggered by endosomal TLRs, and that this represents a broad regulatory function not restricted to antiviral responses. IFITM3 localization within endosomes may be critical for its regulatory function either directly or by influencing the entry of virus, TLR ligands or TLR receptors into the endosomal pathway. Importantly, however, a trend of increased cytokine expression in response to STING and TLR4 ligands also suggests that the regulatory function(s) of IFITM3 extend beyond modulation of endosomal TLR activation. A role for IFITM3 in membrane trafficking has been reported (51) and thus IFITM3 may influence downstream events induced by innate pathogen recognition such as expression or secretion of cytokines and/or the activity of cytokine receptors. Understanding the exact function of IFITM3 within virus-exposed, virus-infected and uninfected cells clearly represents an important future area of study.

Overt cytokine production observed in MCMV-infected Ifitm3−/− mice displayed some similarities to the inflammatory disease hemophagocytic lymphohistiocytosis (HLH). Herpesvirus infections, particularly Epstein Barr virus (EBV), are common triggers of HLH, and EBV-associated HLH is highly prevalent in Asia suggesting an influence of host genetics on disease (52). Our data imply that genetic variation in genes encoding proteins such as IFITM3 that exhibit immune modulatory functions may
influence the occurrence and/or severity of herpesvirus-triggered HLH. The data derived from our in vivo model also imply that individuals with the minor IFITM3 rs12252-C SNP may also have altered susceptibility to HCMV disease.

An important implication of our data is that individuals with reduced IFITM3 activity suffer from virus-induced pathogenesis that is driven, at least part, by unregulated cytokine production. In support of this hypothesis, heightened early production of inflammatory-associated cytokines including IL-6 is associated with the fatal outcome of influenza H7N9 infection (53), consistent with a role for inflammation in driving influenza-associated diseases (54-56). Critically, Wang et al demonstrated the rs12252-C SNP as a concurrent risk factor of fatal influenza infection (53). We now provide direct evidence of a link between excessive cytokine production as a consequence of impaired IFITM3 function and fatal viral infection.

Overall, our data demonstrate that restriction of virus-induced cytokine production is an important and previously unexplored mechanism through which IFITM3 regulates both virus-induced pathogenesis, and that this process exerts a critical influence on the outcome of cytomegalovirus infection. Although the long-term benefits of IL-6R blockade were limited due to the latter requirement of this cytokine pathway in cellular immune responses including the development of virus-specific T cell immunity, these results highlight that reducing overt cytokine production, perhaps using more subtle and/or broader approaches, may represent an effective strategy for the treatment of virus-infected individuals with impaired IFITM3 activity. Finally, the discovery that IFITM3 inhibits TLR-mediated cytokine production may provide insight into why the rs12252-C SNP has been conserved in human population despite its potential deleterious effect associated with reduced IFITM3 function.
Methods

Mice, viral infections and treatments

Ifitm3-deficient (Ifitm3<sup>−/−</sup>) and WT controls (95% C57BL/6, 5% 129) have been previously described (16). Age- and sex-matched mice between 7-12 weeks were used in experiments. MCMV Smith strain (ATCC) for in vivo experimentation was prepared in salivary glands of 3-4 week old BALB/c mice. Salivary glands were homogenized and virus from supernatant purified over a sorbital gradient. Virus was passaged no less than 3 and no more than 5 times in vivo. Virus from homogenized organs and tissue culture supernatants were titered for 6 days on 3T3 cells with a carboxymethylcellulose overlay. Mice were infected intraperitoneally (i.p.) with 3 x 10<sup>4</sup> PFU MCMV. For NK cell depletion, mice were injected i.p. with 200μg αNK1.1 (clone PK136, BioXCell) or IgG control on days −2, 0, and +2 pi, or in repeat experiments were injected i.p with 250μg αAsialo-GM1 (polyclonal antibody, eBioscience) or PBS control on days -3 and 0 pi. For IL-6R blockade, mice were injected i.p with 300μg αIL-6R (clone 2B10 or IgG control on day 0, and for 7-day experiments, on day 4 pi. For WT/Ifitm3<sup>−/−</sup> bone marrow chimeras, mice were irradiated at 2 x 4.5 Gy and transfused intra-venous (i.v) with 1 x 10<sup>6</sup> bone marrow cells 24 hours later. Mice were then treated for 2 weeks with antibiotic supplemented water. Mice were infected with MCMV 8 weeks after irradiation.

Leukocyte isolation, intracellular cytokine staining, and flow cytometry

Leukocytes (1 x 10<sup>6</sup>) were stained, in most experiments, with Zombie Aqua dye, incubated with Fc block (both from Biolegend) and stained for surface markers with a combination of the following antibodies (all from Biolegend, eBioscience or BD Biosciences): αNK1.1 (clone PK136), αCD3ε (Clone 145-2C11), αCD25 (Clone 3C7) αCD4 (Clone GK1.5), αCD8 (Clone 53-6.7), αCD11b (clone M1/70), αCD11c (clone
N418), αCD45R/B220 (clone RA3-6B2), αF4/80 (clone BM8), αI-A/I-E (clone M5/114.15.2), αLy6G (clone 1A8), and αSiglec H (clone 551). Following surface staining, some cells were fixed and permeabilized with saponin buffer (PBS, 2% FCS, 0.05% sodium azide, and 0.5% saponin) and stained with αIL-6 (clone MP5-20F3, Biolegend), or rabbit polyclonal αIFITM3 (Abcam) followed by αrabbit-PE (Sigma-Aldrich). For analysis of NK cell function, cells were incubated for 5 hours in monensin (BD Pharmingen) and αCD107a (clone 1D4B, Biolegend), stained with αNK1.1 and αCD3ε, permeabilized and then stained with αIFN-γ (clone XMG1.2, Biolegend). Other unfixed cell preparations were stained with 7AAD and Annexin V (both Biolegend). M45-specific CD8 T cell responses (57) and functional NK cell responses were assessed as described previously (34).

At least 20,000 leukocytes were analyzed using a BD FACSCanto II flow cytometer (BD Biosciences). Electronic compensation was performed with Ab capture beads stained separately with individual mAbs used in the experimental panel. Data were analyzed using FlowJo software version 8.5.3 (Tree Star). Total numbers of different cell populations were calculated by multiplying the total number of viable leukocytes (assessed by trypan blue exclusion) by percent positive cells, as detected by flow cytometry.

**Peripheral leukocytes, platelet and red blood cell assessment**

Small (50 μl) blood samples were collected from the lateral tail vein into K2 EDTA-coated 0.1 ml paediatric tubes with an integrated capillary (Kabe Labortechnik GmbH) for complete blood counts determination using a Scil Vetabc system.
**In vitro infections**

Bone marrow cells were incubated at $2 \times 10^5$ cells/ml or $4 \times 10^5$ cells/ml in media supplemented with 20ng/ml of M-CSF (Biolegend) or with 50µM 2-Mercaptoethanol (Gibco) and 20ng/ml of GM-CSF (Biolegend) for 7 days, respectively. Media was replenished after 3 days. For bone-marrow derived Flt3-Ligand (Flt3L) induced myeloid cells, cells were incubated a $4 \times 10^5$ cells/ml in R10 media supplemented with 50µM 2-Mercaptoethanol and 100ng/ml of Flt3L (Biolegend) for 9 days, replenishing media on days 4 and day 8. Myeloid cells were then mock infected or infected with MCMV (pSM3fr-MCK-2fl, a kind gift from Chris Benedict and Barbara Adler) at an MOI of 1 or 0.1. Some cells were incubated with pSM3fr-MCK-2fl that was irradiated (2520Gy) and confirmed as replication deficient by failed infection of 3T3 cells. Some cells were treated with 80µM EIPA (Sigma-Aldrich) 30 minutes prior to infection whereas others were treated with 10µg/ml Poly(I:C) (Sigma-Aldrich) or CpG ODN2395 or ODN2395 control (ODN5328) (Miltenyi). After 6 or 24 hours, supernatants were collected for IL-6 protein analysis.

To quantify virus infection, M-CSF and GM-CSF generated myeloid cells were infected with pSM3fr-MCK-2fl MCMV (MOI of 1 or 0.1), and 24hrs later cells stained with Zombie Aqua dye and FC block, stained for surface markers as described above prior to fixation and permeabilization and intracellular staining with anti-m06 antibody (CapRi) conjugated with APC (Innova Biosciences). Some cells were incubated for 4 days and supernatant analyzed for infectious virus by plaque assay.

To generate primary fibroblasts, adult *Ifitm3* knockout mice were intercrossed and MEFs were derived from embryos at day 13.5 of gestation, as described previously (13). MEFs were cultured in DMEM, containing 10% FBS, 1X MEM essential amino acids, 1X 2-
Mercaptoethanol (Gibco). MEFs were then infected with MCMV as above and replicating virus in the supernatant quantified by plaque assay 4 days later.

**Cytokine and chemokine analysis**

IL-6 protein was measured by ELISA (Biolegend). Pro-inflammatory cytokines were measured using a ProcartaPlex Multiplex Immunoassay kit (eBioscience) and run on a Bio-Plex 200 luminex machine (Biorad).

**Histology**

For histological examination tissues of MCMV-infected tissues, organs were fixed in 4% formaldehyde and then processed to paraffin blocks. 5μm sections of paraffin-embedded tissue were stained with haematoxylin and eosin (Sigma-Aldrich).

**Statistics**

For viral load analysis, statistical significance was determined using the Mann-Whitney U test for comparing WT with Ifitm3−/− groups. To analyze viral load data from multiple groups given different treatments, data were first subjected to logarithmic transformation and subsequent two-way ANOVA analysis was performed. For paired analysis of flow cytometry data and ELISA data, the two-tailed Student’s t test was used for paired data sets, and 1-way ANOVA adopted for analysis of data derived from >2 groups of mice, including grouped weight loss data. *p<0.05, **p<0.01, ***p<0.001.

**Study Approval**

All animal studies were performed at the Wellcome Trust Sanger Institute research support facility under UK Home Office Project License number 80/2596.
**Author Contributions**


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References


variant rs12252-C is associated with severe influenza in Chinese individuals. 


**Figure Legends**

**Figure 1. Ifitm3 affords critical protection from MCMV infection.** WT and *Ifitm3<sup>−/−</sup>* mice were infected with 3x10<sup>4</sup> PFU MCMV, and survival (A) and weight loss (B) were assessed over time. Survival data includes mice culled according to UK Home Office restrictions of virus-induced weight loss. Data from 14 (WT) and 21 (*Ifitm3<sup>−/−</sup>*) mice/group merged from 3 experiments is shown. Replicating virus in spleens (C), lung (D), liver (E) and salivary glands (F) day 4 (C-E) or 14 (F) post-infection was quantified by plaque assay. (G) Spleen morphology in WT and *Ifitm3<sup>−/−</sup>* mice 14 days pi. (H) Spleens were taken 14 days pi and sections stained with H&E. Magnification = 20x, scale bars = 10mm. Data is representative of at least 3 separate experiments.

**Figure 2. Ifitm3 does not restrict MCMV replication.** Mixed WT/*Ifitm3<sup>−/−</sup>* bone marrow chimeras were generated and infected with MCMV, and after 4 days PFU in spleen (A) and lung (B) were measured. Individual mice + median are shown from one of two experiments. M-CSF (C&D), GM-CSF (E&F) and Flt3L (G&H) differentiated myeloid cells derived from WT and *Ifitm3<sup>−/−</sup>* bone marrow, and WT and *Ifitm3<sup>−/−</sup>* MEFs (I&J) were infected with MCMV (pSM3fr-MCK-2fl) at different MOIs and MCMV m06 protein was detected 24hrs later by flow cytometry. Data represents 2-3 experiments and panels B, D, F & H show mean + SEM of quadruplet wells. (K) WT and *Ifitm3<sup>−/−</sup>* MEFs, M-CSF, GM-CSF and Flt3L differentiated myeloid cells were infected with pSM3fr-MCK-2fl MCMV at an MOI=1 and supernatant taken 4 days later and replicating virus quantified by plaque assay. Results are representative of 2-4 experiments.

**Figure 3. Ifitm3 deficiency leads to an impairment of cellular immunity.** WT and *Ifitm3<sup>−/−</sup>* mice were infected with MCMV. (A) On days 0, 2 and 4, frequencies of
circulating leukocyte populations in blood were quantified. Mean + SEM of 3 mice/group is shown and represents 3 experiments. (B) Viable splenocytes were quantified d4 pi. Mean + SEM of 3-9 mice per group is shown and represents at least 5 experiments. NK1.1^CD3^- cells (C) and CD3^+ cells (D) were stained with 7AAD and Annexin V. Mean + SEM of 4-6 mice per group is shown and represents at least 3 experiments. (E) Representative bivariate flow plots of NK1.1 versus CD3, gated on viable cells (left), and total viable NK cells (right) D4 pi. Mean + SEM of 3-9 mice per group is shown and represents at least 5 experiments. (F) Total numbers of NK cells positive for CD107a or intracellular IFNg were quantified by flow cytometry at d4 pi. Mean + SEM of 8-9 mice/group is shown and represents 3 experiments. (G) WT and Ifitm3^−/− mice were depleted of NK cells and splenic virus load assessed by plaque assay 4 days later. Individual mice + median is shown and represents 3 experiments (2 using aNK1.1 and 1 using aASGM1 treatment). (H) Numbers of CD4^+ cells, CD8^+ cells and NK1.1^+ cells were quantified in the spleen d7 pi. Individual mice + mean are shown and represent 3 similar experiments. (I) % and total M45-specific CD8^+ T cells 7 pi. All results represent at least 3 experiments.

Figure 4. Ifitm3 suppresses MCMV-induced IL-6 production. (A-C) WT and Ifitm3^−/− mice were infected or not with MCMV. (A) Pro-inflammatory cytokines were measured by multiplex immunoassay in spleen homogenates 4 days pi. Mean values + SEM of 8-9 mice/group are shown. (B) Spleens were taken on days 0, 2 and 4 days pi and IL-6 measured by ELISA in tissue homogenates. Individual mice + mean values are depicted and data represent at least 2 experiments at each time point. (C) IL-6 expression by (unstimulated ex-vivo) CD11c^{hi}MHCII^+ (cDCs), CD11b^-CD11c^-B220^-SiglecH^+ (pDCs) and F4/80^-CD11b^+ (macs) was detected by flow cytometry. Mean + SEM of expression values from 4-5 mice/group is shown and represents 2 experiments. (D) Mixed WT/Ifitm3^−/− bone marrow chimeras were generated and infected with MCMV. After 4 days, IL-6 in spleen supernatants was
quantified by ELISA. Data from one of two experiments is shown. (E) Ifitm3 expression by splenic macrophages (macs), cDCs and pDCs was assessed by flow cytometry (blue line = WT at day 0, green line = WT at day 2 pi, red line = Ifitm3\(^{-/-}\) at day 2 pi). (F) WT and Ifitm3\(^{-/-}\) Flt3L-, GM-CSF- and M-CSF-generated myeloid cells and primary MEFs were infected with pSM3fr-MCK-2fl MCMV (MOI=1) and IL-6 protein measured 6 hours later. Mean + SEM of 4 quadruplet wells are shown. Data represents at least 3 experiments.

Figure 5. Ifitm3\(^{-/-}\) myeloid cells are hyper-responsive to replication deficient virus and endosomal TLR ligand stimulation. (A&B) GM-CSF and Flt3L differentiated myeloid cells were infected or not with irradiated MCMV and IL-6 protein in the supernatants was analyzed by ELISA 24 hours later. (C&D) GM-CSF and Flt3L differentiated myeloid cells were stimulated or not with a control CPG or CPG (both 0.5mg/ml) or Poly(I:C) (10mg/ml) for 24 hours and IL-6 protein in the supernatants was then analyzed by ELISA. Mean + SEM of quadruplet wells are shown and represent 2 (C&D) or 3 (A&B) experiments.

Figure 6. IL-6 is a critical regulator of MCMV-induced pathology in Ifitm3\(^{-/-}\) mice. WT and Ifitm3\(^{-/-}\) mice were infected with 3x10\(^4\) PFU MCMV and treated with IgG or anti-IL-6R (2B10) on days 0 and 4 pi. (A) Weight loss was measured over a 7-day time course. Mean + SEM of 4-11 mice/group is shown. (B) Virus load in the spleen was quantified by plaque assay at 4 days pi. Individual mice + median are shown and represents 2 experiments. (C) Viable splenocytes were counted on day 4 pi. Mean + SEM are shown of 2 merged experiments containing 9-11 mice/group. (D) Representative bivariate plots of NK1.1 versus CD3 expression in WT (left) and Ifitm3\(^{-/-}\) (right) mice 4 days pi after IgG (top) or anti-IL-6R (bottom) treatment. Viable NK cells (E) and Annexin V\(^+\)7AAD\(^+\) NK cells (F) were quantified at 4 days pi. (G) Correlation between virus load and NK1.1\(^+\)CD3\(^-\) cells in WT/Ifitm3\(^{-/-}\) mice treated with
IgG or anti-IL-6R. (H&I) After 7 days, viable splenic T cell and NK1.1\(^+\) cells were quantified and expressed as mean \(\pm\) SEM from 4-6 mice/group, (H) and virus load in the spleen was measured (I). All results represent 2-3 experiments.
Figure 1
Figure 2
Figure 3
Figure 5
Figure 6