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Mouse gammaherpesvirus-68 infection acts as a rheostat to set the level of type I interferon signaling in primary macrophages

Brittani M. Wood, Wadzanai P. Mboko, Bryan C. Mounce, Vera L. Tarakanova *

Department of Microbiology and Molecular Genetics, Cancer Center, Medical College of Wisconsin, USA

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ABSTRACT

Type I interferon (IFN) is a critical antiviral response of the host. We found that Interferon Regulatory Factor 3 (IRF-3) was responsible for induction of type I IFN following mouse gammaherpesvirus-68 (MHV68) infection of primary macrophages. Intriguingly, type I IFN signaling was maintained throughout the entire MHV68 replication cycle, in spite of several known viral IFN antagonists. However, MHV68-infected primary macrophages displayed attenuated responses to exogenous type I IFN, suggesting that MHV68 controls the level of type I IFN signaling that is allowed to occur during replication. Type I IFN receptor and IRF-3 were necessary to attenuate transcription of MHV68 RTA, an immediate early gene critical for replication. Furthermore, higher constitutive activity of RTA promoters was observed in the absence of type I IFN signaling. Our study suggests that MHV68 has preserved the ability to sense type I IFN status of the host in order to limit lytic replication.

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Introduction

Type I interferon (IFN) signaling network is an innate defense system of the host with potent antiviral activity. In a canonical signaling pathway, virus products, such as double stranded RNA, are recognized by a combination of cytoplasmic and membrane-associated pattern recognition receptors. These pattern recognition receptors initiate signaling that activates interferon regulatory factors (IRF) 3 and 7. Upon activation, IRF-3 and IRF-7 stimulate transcription of several type I IFN genes, including IFNβ and IFNα. All members of type I IFN family interact with a single receptor (IFNAR) that is critical for signal propagation [reviewed in Platanias (2005)]. Engagement of the type I IFN receptor leads to activation of receptor-associated kinases Jak1 and Tyk2 that phosphorylate specific residues of Stat1 and Stat2. A complex of phosphorylated Stat1, Stat2, and IRF9 (ISGF3) subsequently mediates transcription of hundreds of cellular genes that collectively create an antiviral state. Importantly, only a handful of IFN-stimulated genes (ISG) have proven antiviral functions in the context of a particular virus infection; furthermore, the mechanism by which these antiviral functions are conferred is generally poorly understood.

Gammaherpesviruses, such as Epstein-Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV), establish a life-long infection in a majority of human population and are associated with several malignancies. Similar to a plethora of other viruses, gammaherpesviruses encode a number of proteins that interfere with type I IFN induction and signaling [reviewed in Versteeg and Garcia-Sastre (2010); Sathish and Yuan (2011)]. Induction of type I IFN in gammaherpesvirus-infected cells is in part controlled by KSHV tegument protein orf45 that interacts with and inhibits phosphorylation of IRF-7 (Zhu et al., 2002). Virion-associated KHSV orf64 reduces activation of RIG-I, a cytosolic sensor that initiates type I IFN cascade (Inn et al., 2011). Another tegument protein, a conserved protein kinase encoded by EBV (BGLF4) and mouse gammaherpesvirus-68 (MHV68, orf36), attenuates type I IFN induction by binding to IRF-3 and inhibiting activity of IRF-3-driven promoters, including IFNβ (Hwang et al., 2009;Wang et al., 2009). RTA is an immediate early gammaherpesvirus transactivator that is essential for MHV68 and KSHV replication and reactivation from latency (Liu et al., 2000;Lukac et al., 1998;Sun et al., 1998;Wu et al., 2000, 2001). KSHV RTA targets both IRF-3 and IRF-7 for degradation (Yu et al., 2005;Yu and Hayward, 2010). Importantly, the relative contributions of IRF-3 and IRF-7 to the induction of type I IFN response in gammaherpesvirus-infected cells have not been defined.

Type I IFN signaling is further inhibited by another cohort of gammaherpesvirus proteins. KSHV RIF (encoded by *orf10*) forms complexes with type I IFN receptor and receptor-associated kinases Jak1 and Tyk2 to attenuate type I IFN signaling (Bisson et al., 2009). MHV68-encoded M2 protein interferes with type I IFN signaling concurrent with downregulation of STAT2 expression (Liang et al., 2004). Another MHV68 protein encoded by *orf54* targets type I IFN receptor for degradation (Leang et al., 2011).

Paradoxically, some of gammaherpesvirus proteins appear to have a positive effect on type I IFN signaling. Specifically, EBV-encoded lytic Sm protein induces Stat1 phosphorylation

* Correspondence to: Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, United States. Fax: +1 414 955 6535.
E-mail addresses: vera@mcw.edu, vtarakan@mcw.edu (V.L. Tarakanova).

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(Ruvolo et al., 2003). Intriguingly, LMP1, a latent EBV protein, has been shown to both activate and suppress type I IFN signaling (Zhang et al., 2004; Xu et al., 2006; Geiger and Martin, 2006). A majority of studies examining the regulation of type I IFN signaling by EBV- or KSHV-encoded proteins have been done in the context of protein overexpression and/or using transformed cell lines. Because type I IFN responses are altered upon transformation (Tomic et al., 2011; Clifford et al., 2002; Klampfer et al., 2003), it is not clear whether the same regulation of type I IFN signaling is preserved during infection of primary, physiologically relevant cell types. Significantly, infection of primary human foreskin fibroblasts and endothelial cells with KSHV leads to a significant induction of ISG at 2 and 4 h post infection (Naranatt et al., 2004). Furthermore, live, but not UV-inactivated KSHV infection increased activation and type I IFN production in plasmacytoid dendritic cells (West et al., 2011); however, it is not clear whether KSHV can adequately express all of its lytic genes to undergo productive replication in this cell type. Thus, gammaherpesvirus infection of primary cells is likely to induce type I IFN signaling, in spite of several type I IFN inhibitors (including tegument proteins) encoded by the virus.

MHV68 is genetically and biologically related to EBV and KSHV (Efstathiou et al., 1990; Virgin et al., 1997) and offers a powerful experimental system to dissect virus–host interactions in the context of primary cell types and *in vivo*. Similar to that observed for human gammaherpesviruses *in vitro* (Chang et al., 2000; Krug et al., 2004; Monini et al., 1999; Perry and Compton, 2006), type I IFN plays an important role in controlling MHV68 replication *in vitro* and chronic infection *in vivo* (Barton et al., 2005; Dutia et al., 1999; Hwang et al., 2009; Mandal et al., 2011). Overexpression of several ISG prior to MHV68 infection restricts MHV68 replication (Liu et al., 2012); however, the mechanism by which type I IFN attenuates MHV68 lytic infection is poorly understood. We have recently reported that activation of the DNA damage response, a major tumor suppressor system of the host, stimulates type I IFN and ISG expression in primary macrophages (Mboko et al., 2012). MHV68-infected primary macrophages were resistant to further increase in ISG expression upon irradiation, suggesting that the virus uncouples the connection between the DNA damage response and type I IFN signaling. Intriguingly, we observed elevated baseline ISG expression in MHV68-infected macrophages at an advanced stage of infection [36 h post infection (Mboko et al., 2012)], suggesting that type I IFN signaling is active in infected cells. In this study we show that, following *de novo* infection of primary macrophages, type I IFN signaling was induced as early as 4 h post infection and was maintained throughout the entire viral replication cycle. IRF-3 was critical for type I IFN induction during the early stage of lytic MHV68 infection. In spite of active IFN signaling during infection, response to increased levels of exogenous type I IFN was attenuated in infected macrophages, suggesting that the MHV68 infection functions as a rheostat that sets a defined level of type I IFN signaling in infected cells. Finally, expression of RTA, an immediate early viral transactivator, and activity of RTA promoters were elevated in macrophages lacking type I IFN receptor, suggesting that MHV68 has evolved to sense the innate immune status of the host in order to control its lytic replication.

Results

MHV68 infection of primary macrophages is associated with increased phospho- and total Stat1 levels

We have recently shown that activation of the DNA damage response induces type I IFN signaling (Mboko et al., 2012). Irradiation failed to further increase ISG expression in MHV68-infected macrophages, suggesting that the virus blocked DNA damage-induced transcription of ISG. Intriguingly, even in the absence of irradiation, a significant increase in transcription of two antiviral ISG (viperin and Mx1) was observed in MHV68-infected macrophages at an advanced stage in viral replication [36 h post infection (Mboko et al., 2012)], suggesting that IFN signaling may already be active in infected cells. To further dissect the status of type I IFN signaling during infection, primary bone marrow derived macrophages were mock-treated or infected with wt MHV68, N36S viral mutant that lacks expression of orf36, a viral kinase responsible for inhibition of IRF-3 in fibroblasts (Hwang et al., 2009), or UV-inactivated wt MHV68 to control for the effects of the virus particle and tegument proteins.

Phosphorylation of Stat1 Y701 (α and β isoforms) was induced as early as 4 h post infection with wt MHV68, N36S, or UV-inactivated wt virus (Fig. 1A). Levels of phosphorylated Stat1 peaked at 4 h post infection and decreased thereafter. Importantly, phosphorylated Stat1 levels remained above background in wt MHV68- and N36S-infected macrophages for the duration of the replication cycle (48 h, Fig. 1A), suggesting that some level of type I IFN signaling was maintained throughout the infection. Maintenance of phosphorylated Stat1 required viral gene expression and/or replication, as phosphorylated Stat1 was no longer detected in macrophages infected with UV-inactivated MHV68 by 24 h post infection. Consistent with a positive feedback loop mediated by Stat1 (Cheon and Stark, 2009), total Stat1 levels increased in

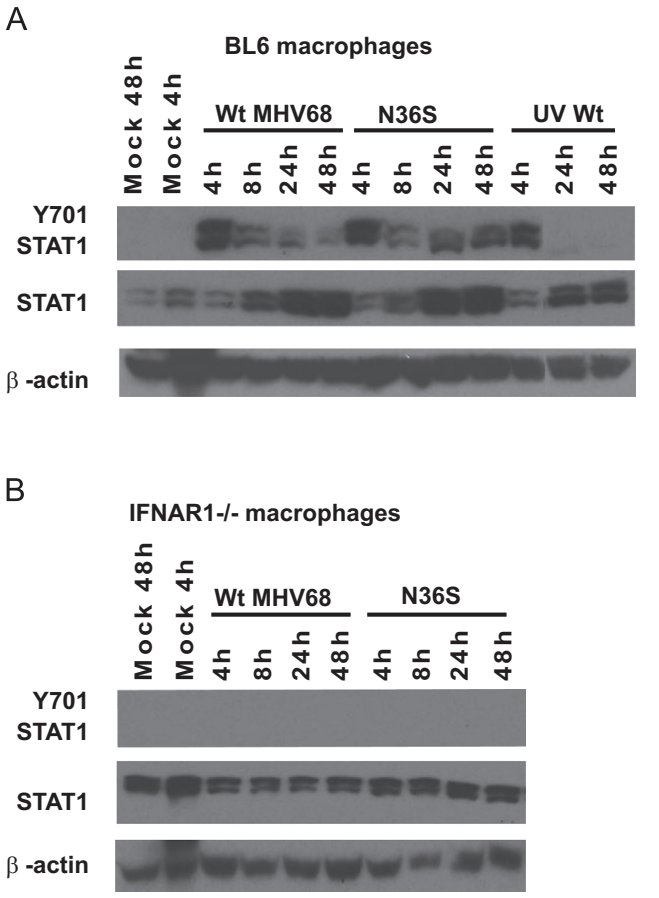

Fig. 1. MHV68 infection of primary macrophages is associated with increased phospho- and total Stat1 levels. Primary macrophages were derived from bone marrow isolated from BL6 (wt) (A) or IFNAR1-/- (B) mice. Macrophages were mock-treated or infected with live wt MHV68, N36S mutant, or UV-inactivated wt MHV68 at an MOI of 10. Cell lysates were collected at indicated times post infection and levels of total Stat1, tyrosine 701-phosphorylated Stat1, and β-actin measured by western analysis. Images representative of 3–5 independent experiments are shown. Western blots measuring levels of tyrosine 701-phosphorylated Stat1 in A and B were performed and developed concurrently and represent the same exposure time.

infected macrophages by 8 h post infection and remain elevated for the duration of the replication cycle (Fig. 1A). Importantly, infection of IFNAR1-/- macrophages failed to upregulate either total or phosphorylated Stat1 levels (Fig. 1B). Thus, MHV68 infection of primary macrophages induced an early Stat1 phosphorylation that was maintained for the duration of the replicative cycle and required active viral replication and expression of type I IFN receptor.

Expression of type I IFN receptor is required for induction of interferon stimulated genes (ISG) in MHV68-infected primary macrophages

In a canonical type I IFN signaling pathway, phosphorylation of Stat1 triggers the assembly of the ISGF3 transcriptional complex that culminates in increased transcription of a large number of ISG, including those with known antiviral functions. Having observed increased levels of total and phospho-Stat1 in infected macrophages, we wanted to determine whether transcription of antiviral ISG was increased in MHV68-infected cells concurrent with increase in phospho-Stat1.

Indeed, transcription of Mx1 and expression of viperin, ISGs with known antiviral function (Pavlovic et al., 1992; Wang et al., 2007), peaked at 8 h post infection and was sustained for the duration of replicative cycle in wt MHV68- and N36S-infected macrophages (Fig. 2A–C). Importantly, peak expression of Mx1 and viperin was lower in macrophages infected with UV-inactivated MHV68 (Fig. 2A and B). Mx1 and viperin mRNA levels further declined to baseline by 24 h post infection with UV-inactivated virus (Fig. 2A and B), suggesting that a UV-sensitive virion component and active viral gene expression were necessary for peak induction and sustained transcription of ISG in MHV68-infected macrophages. Type I IFN signaling was required for ISG transcription throughout the infection, as ISG mRNA levels remained at baseline in IFNAR1-/- macrophages under all experimental conditions (Fig. 2D and E, data not shown). Consistent with the phenotype of the N36S virus mutant in fibroblasts (Hwang et al., 2009), increased Mx1 and viperin mRNA levels

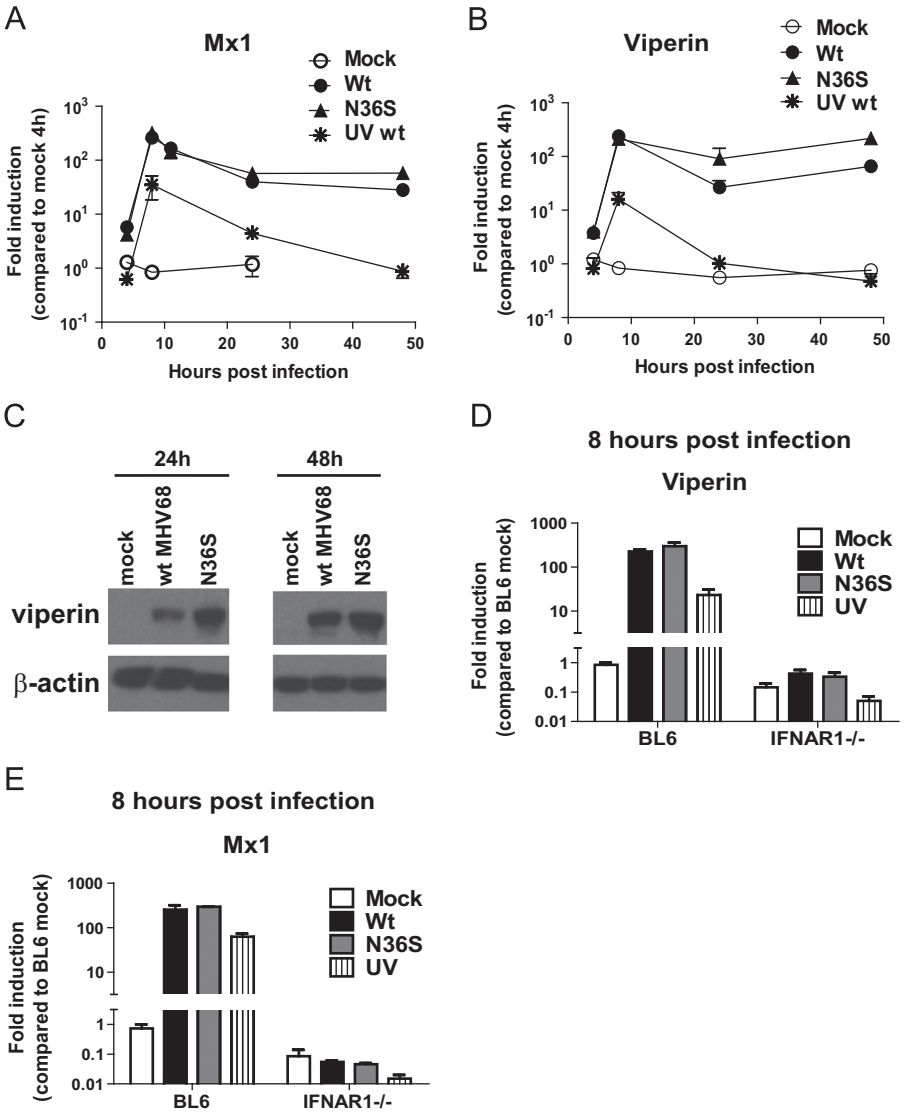

Fig. 2. Expression of type I IFN receptor is required for induction of interferon stimulated genes in MHV68-infected primary macrophages. A, B, D, E. Primary bone marrow derived macrophages were generated from BL6 or IFNAR1-/- mice. Macrophages were infected as described in Fig. 1 and total RNA harvested at indicated times post infection. Mx1 and viperin mRNA levels were measured by qRT-PCR. Relative levels of mRNA present in BL6 mock-infected cells were set to 1 and the rest of the data expressed as fold induction. Data were pooled from 3 to 5 independent experiments, with each experimental condition assessed in duplicate within each experiment. Pooled data are shown as average with standard error of measurement. C. Primary BL6 derived macrophages were infected as described above and viperin and β-actin protein levels measured at 24 and 48 h post infection. Data is representative of 3 independent experiments.

were observed in N36S-infected cells as compared to wt MHV68-infected cells at 48 h post infection (Fig. 2A, 2.1-fold difference; Fig. 2B, 3.3-fold difference). In summary, transcription of ISG was induced early in MHV68 infection and was sustained for the duration of the replication cycle. Both peak and sustained ISG expression required live virus and expression of host IFNAR1.

IRF-3 plays a critical role in the induction of type I IFN responses during early MHV68 infection

IRF-3 was initially shown to play an important role in the induction of type I IFN expression following Newcastle Disease virus infection (Yoneyama et al., 1998) and was later found to have a more global role in type I IFN induction by several viruses. Importantly, IRF-3 is dispensable for induction of type I IFN in response to myxoma virus infection of dendritic cells *in vitro* and infection with West Nile virus virus-like particles *in vivo* (Bourne et al., 2007; Dai et al., 2011). Because IRF-3 is targeted by KSHV RTA and by orf36 in MHV68-infected fibroblasts (Hwang et al., 2009; Yu and Hayward, 2010), we expected that IRF-3 would only partially contribute to the robust induction of type I IFN responses in MHV68-infected macrophages during early infection. Surprisingly, at 8 h post infection, when peak levels of Mx1 and viperin mRNA are observed in MHV68-infected macrophages, transcription of Mx1 and viperin was abolished in IRF-3 deficient macrophages under all experimental conditions (Fig. 3A, data not shown), suggesting that IRF-3 plays a critical and unique role in increased ISG transcription during early MHV68 infection.

Because IFNAR1 and IRF-3 expression were required for increased transcription of Mx1 and viperin at 8 h post infection (Figs. 2D, E and 3A), it was likely that IRF-3 was driving type I IFN induction shortly following virus entry. Over a dozen of type I IFN family members interact with type I IFN receptor to induce signaling [reviewed in (Platanias, 2005)]. Therefore, we employed EMCV bioassay to capture production of any type I IFN by infected macrophages. Primary macrophages isolated from control (BL6), IFNAR1-/-, or IRF-3-/- mice were mock-treated, infected with live MHV68 or UV-inactivated virus, and antiviral activity of culture supernatants measured during the first four hours of infection. Very little antiviral activity was observed under all experimental conditions during the first two hours of infection (Fig. 3B). However, increased antiviral activity was seen in supernatants collected at 3 and 4 h post infection from BL6 and IFNAR1-/- macrophages infected with live MHV68 (Fig. 3B and C). EMCV antiviral activity was also present in the supernatants collected at 4 h post infection from UV virus-infected BL6 and IFNAR1 -/- macrophages, although this antiviral activity remained significantly lower than that induced by live MHV68 (Fig. 3B and C). In contrast, antiviral activity was not detected in any of the supernatants collected from IRF-3-/- cultures (Fig. 3D). Consistent with the bioassay results, mRNA levels of IFNα and IFNβ increased in virus-infected BL6 macrophages, but not in IRF-3 deficient cultures at 4 h post infection (Fig. 3E). Thus, IRF-3 functioned upstream of IFNAR1 to induce secreted antiviral activity and type I IFN transcription in infected macrophages.

To determine the relative importance of IRF-3 in type I IFN responses later in MHV68 infection, levels of Stat1 and ISG15 were measured in wild type (BL6) and IRF-3-/- macrophages infected with either wt MHV68 or the N36S mutant. Both free and conjugated forms of ISG15 were increased in infected BL6 macrophages at 24 and 48 h post infection (Fig. 3F); this increase was similar in wt- and N36S-infected cultures. In contrast, minimal ISG15 induction was seen in IRF3-/- macrophages infected with wt MHV68 at both 24 and 48 h post infection. Surprisingly, some induction of ISG15 was observed in IRF-3-/- macrophages infected with the N36S mutant at 24 and 48 h post infection (Fig. 3F). Total Stat1 levels were minimally induced in IRF3-/- macrophages at 24 h post infection. However, appreciable induction of total Stat1 was observed at 48 h post infection in the absence of IRF-3. Because increased expression of total Stat1 is dependent on type I IFN signaling (Fig. 1B), these results suggest that another mechanism of type I IFN induction compensated for IRF-3 deficiency at a late stage of MHV68 infection. While total Stat1 levels were similarly induced in wt MHV68- and N36S-infected BL6 macrophages (Fig. 1A), Stat1 levels were higher in N36S-infected IRF-3-/- macrophages at 48 h post infection (Fig. 3F), suggesting that, in addition to IRF-3 (Hwang et al., 2009), orf36 is likely to inhibit another component of type I IFN induction/signaling network. Consistent with the proposed role of orf36 in counteracting IRF-3-independent IFN induction in infected macrophages, phospho-Stat1 levels were increased in N36S-infected, but not wt MHV68-infected IRF-3 deficient macrophages at 24 h post infection (Fig. 3G).

MHV68 infection attenuates further Stat1 phosphorylation upon exogenous IFNβ treatment

Although phospho-Stat1 and ISG transcription were detected in MHV68-infected macrophages, the extent to which MHV68 controlled type I IFN signaling was not clear. Thus, exogenous IFNβ treatment was used to test the ability of infected cells to respond to further stimulation of type I IFN signaling. At 24 h post infection, mock, wt-, and N36S-infected macrophages were treated with recombinant mouse IFNβ and total and phospho-Stat1 levels measured at the end of cytokine or mock treatment. Low concentration of IFNβ (10 U/ml) increased phospho-Stat1 levels in mock-infected cells between 15 and 60 min of treatment (Fig. 4). Similar IFNβ treatment did not induce the same increase in Stat1 phosphorylation in wt MHV68- or N36S-infected macrophages. As expected, high levels of phospho-Stat1 were induced in mock-infected cells treated with 100 U/ml of IFNβ. Importantly, wt MHV68- and N36S-infected macrophages showed attenuated phospho-Stat1 levels even following treatment with high dose of exogenous IFNβ (Fig. 4, 100 U/ml). This attenuated Stat1 phosphorylation in response to exogenous cytokine occurred in spite of an increase in total Stat1 levels observed in infected cells at 24 h post infection (Figs. 4 and 1A). Thus, MHV68 infection controlled the extent of Stat1 phosphorylation within infected macrophages.

Type I IFN signaling represses RTA expression

Live MHV68 triggered type I IFN signaling within the first 4 h of infection, and this signaling persisted throughout the entire MHV68 replication cycle (Figs. 1–3). Virus-induced type I IFN signaling was inhibitory, as MHV68 replication was enhanced in macrophages derived from IFNAR1-/- mice (Fig. 5A). As expected, attenuated replication of the N36S viral mutant was partially rescued in IFNAR1-/- macrophages (Fig. 5A). Consistent with the role of IRF-3 in the induction of type I IFN, replication of wt MHV68 was also increased in IRF-3 deficient macrophages (Fig. 5B). Enhanced viral replication in IRF-3-/- macrophages was most obvious at 24 h post infection and never reached the same magnitude as compared to wt MHV68 replication in IFNAR1-/- macrophages, consistent with induction of type I IFN responses at a later stage of MHV68 infection in IRF-3-/- macrophages (Fig. 3F). As expected (Hwang et al., 2009), replication of the orf36 null N36S virus mutant was partially rescued in IRF-3-/- macrophages (Fig. 5B).

To further define the step of MHV68 replication attenuated by type I IFN, immediate early viral gene transcription was measured in BL6 and IFNAR1-/- macrophages. RTA is an immediate early gammaherpesvirus gene that is critical for viral replication and

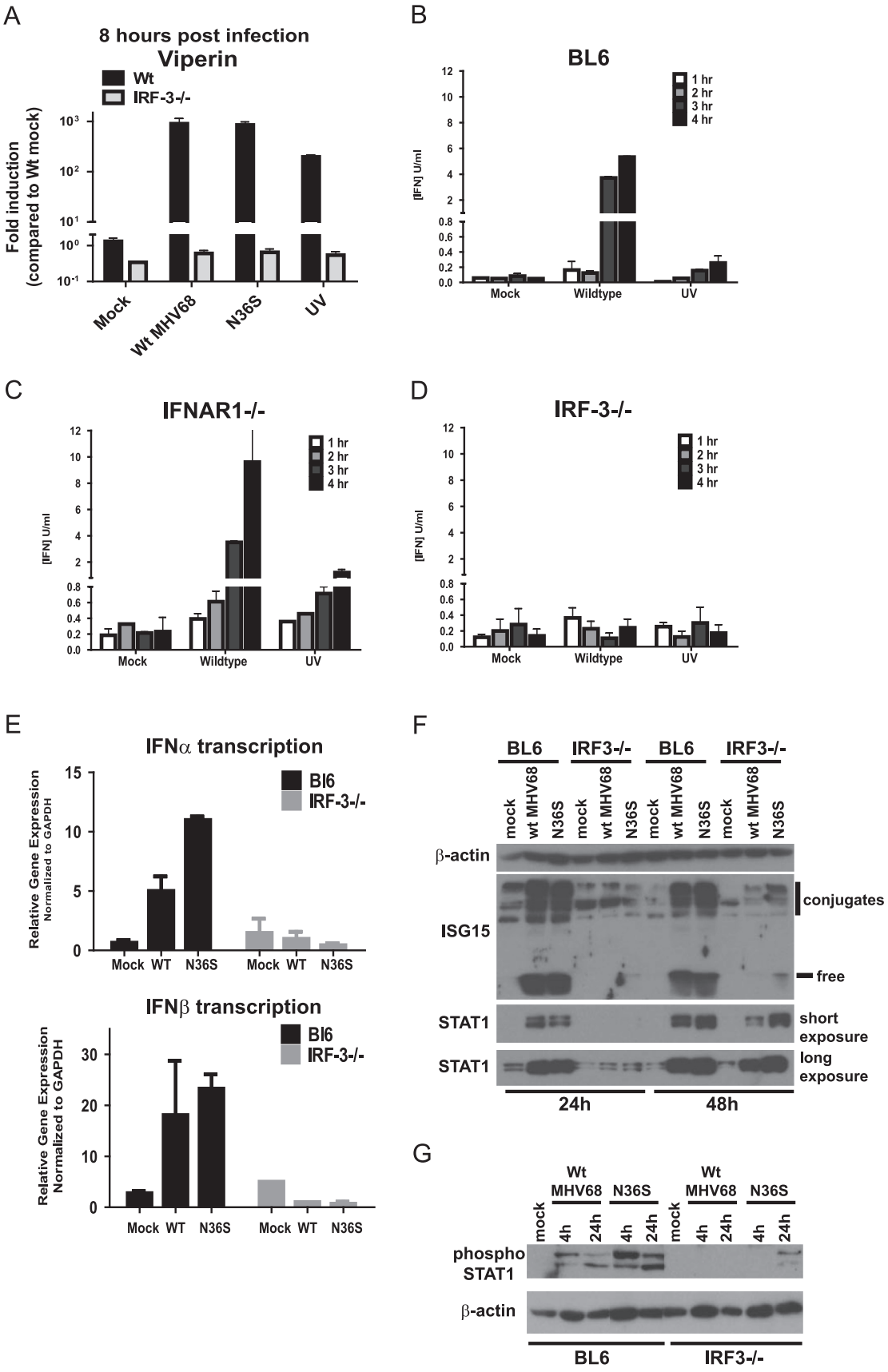

Fig. 3. IRF-3 is required for induction of type I IFN responses during early MHV68 infection. Primary bone marrow derived macrophages were generated from BL6 (Wt), IFNAR1-/-, or IRF-3-/- mice. Macrophages were infected as described in Fig. 1. A. Total RNA was harvested at 8 h post infection and subjected to qRT-PCR to measure viperin mRNA levels. B–D. EMCV bioassay was used to measure antiviral activity of conditioned tissue culture medium collected at indicated times post infection. Conditioned tissue culture medium was UV-irradiated prior to the bioassay. E. mRNA levels were measured at 4 h post infection using universal primers against IFNα or IFNβ sequences, respectively. F, E. Protein levels of β-actin, ISG15, total STAT1, and phospho-Stat1 were measured by western analysis at 4, 24 and 48 h post infection as indicated. Both free and conjugated forms of ISG15 are indicated. Data are representative of 2–3 independent experiments. Within an experiment, each experimental condition was done in duplicate, with average and standard error shown.

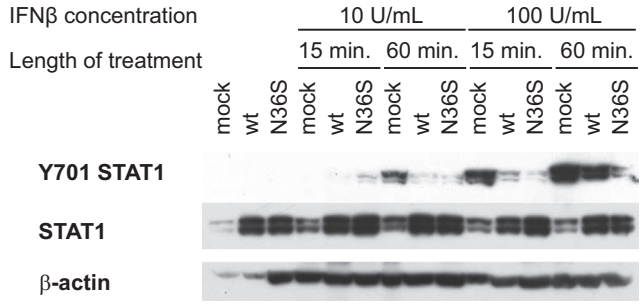

Fig. 4. MHV68 infection prevents further Stat1 phosphorylation upon exogenous IFNβ treatment. Primary bone marrow derived macrophages (BL6) were mock-infected or infected with live MHV68 or N36S virus mutant at an MOI of 10. At 23 h post infection, cultures were mock-treated or treated with indicated amounts of exogenous IFNβ, and cell lysates collected at indicated times post treatment or at 24 h post infection. Levels of Y701-phosphorylated Stat1, total Stat1, and β-actin were measured by western analysis. Presented data is representative of two independent experiments.

reactivation from latency (Liu et al., 2000; Lukac et al., 1998; Sun et al., 1998; Wu et al., 2000, 2001). RTA mRNA levels were significantly increased in IFNAR1-/- macrophages at 8 h post infection (Fig. 5C). The difference in RTA mRNA levels observed at 8 h post infection in BL6 and IFNAR1-/- macrophages was no longer seen at 24 h post infection (Fig. 5C), suggesting that type I IFN-mediated attenuation of RTA transcription was counteracted later in infection by an unknown viral mechanism. A similar increase in RTA transcription was seen at 8 h post infection in the absence of IRF-3 (Fig. 5D), consistent with the critical role of IRF-3 in the induction of type I IFN response during early stages of MHV68 infection (Fig. 3).

Increased expression of RTA in the absence of IFNAR1 or IRF-3 led to increased expression of MHV68 early genes. Specifically, protein levels of MHV68 single stranded DNA binding protein (ssDBP), a critical component of viral DNA replication machinery, were increased in IFNAR1-/- and IRF-3-/- macrophages at 24 h post infection (Fig. 5E). Later in infection (48 h) ssDBP levels remained elevated in the absence of IRF-3 or IFNAR1 (Fig. 5F, data not shown).

Consistent with an elevated expression of MHV68 ssDBP, MHV68 DNA accumulation was increased in IRF-3-/- and IFNAR1-/- macrophages (Fig. 5G and H). Furthermore, attenuated viral DNA synthesis in the absence of orf36, was partially rescued in the absence of IRF-3 or IFNAR1. Thus, type I IFN signaling attenuated early stages of MHV68 replication and restricted progression through the rest of the replication cycle.

MHV68 RTA transcription is inhibited by type II IFN in a Stat1-dependent manner (Goodwin et al., 2010). Because RTA mRNA levels were significantly increased in IFNAR1-/- and IRF-3-/- macrophages at 8 h post infection, we wanted to further explore the effects of type I IFN signaling on RTA expression. RTA transcription is driven by two promoters, termed core and distal (Gray et al., 2008;Liu et al., 2000), and is further amplified by RTA. Type II IFN represses both RTA promoters (Goodwin et al., 2010); however, the effect of type I IFN on the activity of RTA promoters has not been defined. To determine the extent to which type I IFN signaling affects the baseline activity of RTA promoters, reporter plasmids containing either RTA promoter or a control promoter-less vector (pGL4.10) were nucleofected into primary macrophages and promoter activity assessed at 16 h post nucleofection. Both core and distal RTA promoters displayed low activity in nucleofected primary macrophages derived from wt mice (Fig. 6A). Interestingly, a significantly higher activity of core and distal RTA promoters, but not control vector, was observed in IFNAR1-/- macrophages as compared to wild type cells (3.5-fold increase for the core promoter, 1.8-fold increase for the distal promoter, Fig. 6A).

Pretreatment of macrophages with type II IFN suppresses RTA transcription (Goodwin et al., 2010). To directly compare the effects of type I and type II IFN, primary macrophages were mock-treated or pretreated with 10U/ml of recombinant IFNβ or IFNγ for 16 h, infected with wt MHV68, treated with the same amount of IFNβ or IFNγ following virus absorption, and RTA mRNA levels measured at 8 or 24 h post infection. Consistent with the published study (Goodwin et al., 2010), IFNγ significantly decreased RTA transcription at 8 and 24 h post infection (Fig. 6B). A similar magnitude of decrease was seen in infected macrophages that were treated with IFNβ. Thus, type I IFN signaling suppressed the constitutive activity of both RTA promoters independent of virus infection, and inhibited RTA transcription in MHV68-infected primary macrophages.

Discussion

In this manuscript we show that MHV68 infection of primary macrophages induces type I IFN response that proceeds through the entire signaling pathway culminating in increased transcription of antiviral ISG. In spite of many type I IFN inhibitors encoded by herpesviruses, activation of type I IFN signaling by individual herpesvirus proteins or in the context of herpesvirus entry is not without a precedent. Entry of human cytomegalovirus (HCMV) initiates type I IFN signaling, including increased expression of viperin, an ISG also examined in the present study (Juckem et al., 2008; Chin and Cresswell, 2001). ISG induction is observed as early as 2 h following KSHV infection of primary cells (Naranatt et al., 2004) and both type I and type II IFN are constitutively secreted into the supernatants of cultured lymphoblastoid cell lines latently infected with EBV (Xu et al., 2006; Zhang et al., 2004). Intriguingly, activation of type I IFN signaling was not limited to early stages of MHV68 infection, but was, instead, maintained throughout the entire replication cycle, in spite of expression of viral type I IFN antagonists.

Triggering of pattern recognition receptors by viral constituents is a classical mechanism that would be expected to induce type I IFN signaling in infected cells. Consistent with this mechanism, infection of primary macrophages with UV-inactivated MHV68 induced type I IFN signaling and both IRF-3 and IFNAR1 were required for this induction (Figs. 1–3). Surprisingly, induction of type I IFN signaling was stronger and persisted throughout the entire replication cycle in macrophages infected with live MHV68, as compared to UV-inactivated virus. This suggests that UV-sensitive virion components contribute to induction of type I IFN signaling in infected primary macrophages. It was previously shown that repeat regions of MHV68 and KSHV DNA are sufficient to induce type I IFN (Sanchez et al., 2008); however, it is not clear whether UV treatment alters the ability of repeat regions to engage pattern recognition receptors. Furthermore, all virus stocks used for this study were treated with DNase to eliminate stimulation of pattern recognition receptors by unpackaged MHV68 DNA. Induction of Mx1 and viperin transcription occurred as early as 4 h post infection in macrophages infected with live MHV68, but not UV-inactivated virus (Fig. 2A and B). Expression of RTA, an immediate early gene, remains below the detection level for the first 5 h of infection under high MOI conditions (Mounce et al., 2011b), suggesting that *de novo* viral gene expression is unlikely to contribute to induction of ISG expression seen at 4 h post infection in live MHV68-infected macrophages. EBV and MHV68 encode a number of small viral RNAs that are packaged into the virion and have recently been shown to regulate several cellular processes, including cytokine expression, immediately following EBV infection of primary B cells (Jochum et al., 2012). It is intriguing to speculate that virion-associated RNA also contributes to early

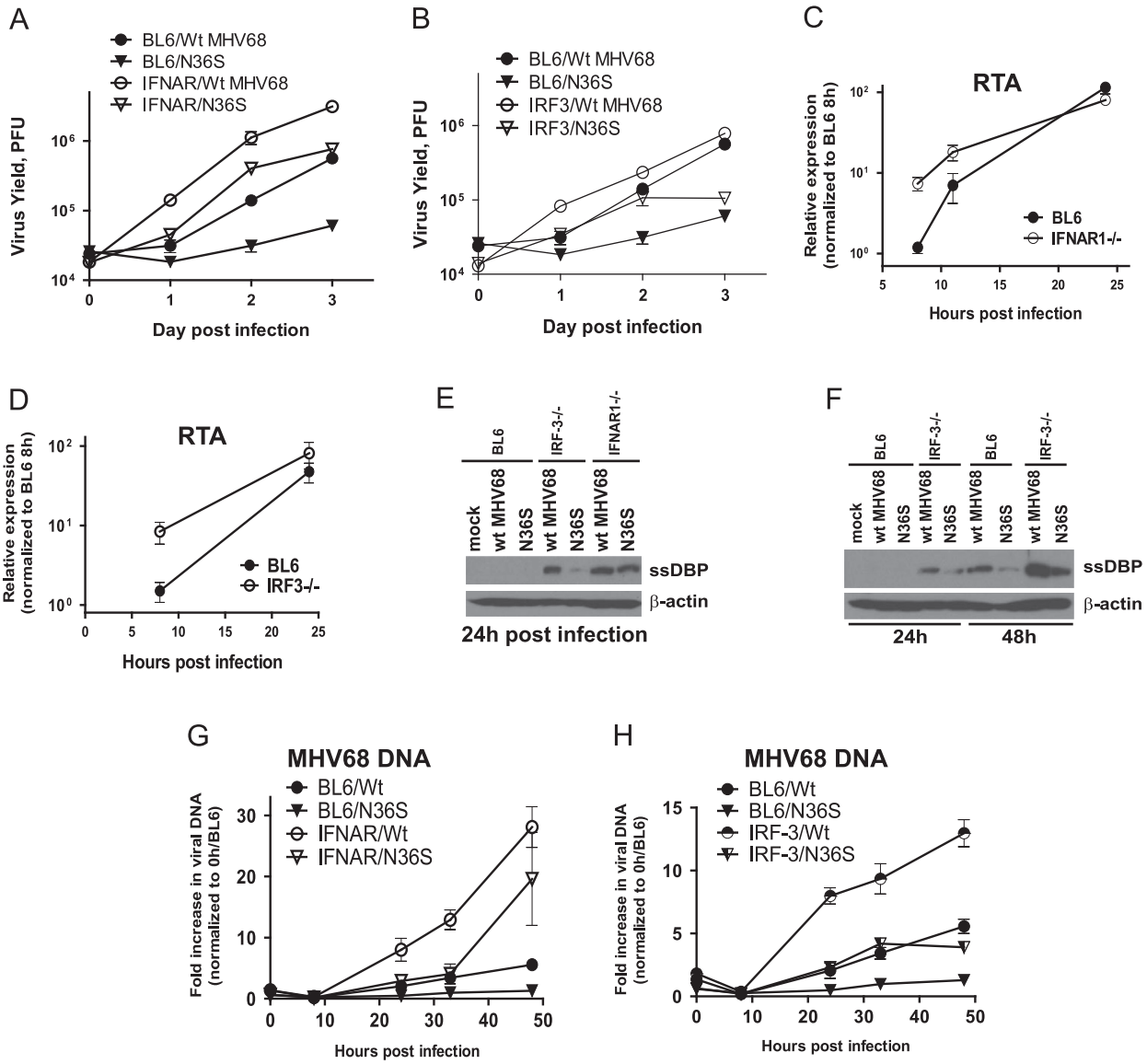

Fig. 5. Type I IFN signaling attenuates MHV68 replication in primary macrophages. Bone marrow derived macrophages were isolated from BL6 (Wt), IFNAR1-/-, or IRF-3-/- mice. Macrophages were infected with wt MHV68 or the N36S mutant at an MOI of 10. A, B. Virus yield was measured at indicated times post infection. Data are representative of three independent experiments. C, D. Total RNA was collected at indicated time post infection and RTA mRNA levels measured using qRT-PCR. Relative levels of RTA mRNA in BL6 cells at 8 h post infection were set to 1, with the rest of the conditions normalized to BL6 8 h levels within each experiment. Data were pooled from 2 to 3 independent experiments, with each experimental condition performed in duplicate within each experiment. E, F. Primary macrophages of indicated genotypes were mock-infected or infected with wt MHV68 or the N36S mutant at an MOI of 10. MHV68 ssDBP and β-actin protein levels were measured by western analysis in cell lysates collected at 24 and 48 h post infection. Data are representative of two independent experiments. G, H. Total DNA was isolated from BL6, IFNAR1-/-, or IRF-3-/- macrophages infected with wt MHV68 or the N36S viral mutant. The relative amount of viral DNA was measured by real time PCR with subsequent normalization to cellular DNA levels. Data are representative of 2–3 independent experiments, each data point derived from a biological triplicate with mean and standard error of measurement shown.

induction of type I IFN in MHV68-infected macrophages. Alternatively, a UV-sensitive virion protein may also function to induce ISG transcription at 4 h post infection.

Once viral gene expression ensues, type I IFN signaling may also be directly driven by viral proteins expressed in infected macrophages. Consistent with this hypothesis, expression of EBV-encoded lytic Sm protein is sufficient to induce Stat1 phosphorylation (Ruvolo et al., 2003). Furthermore, expression of HCMV immediate early IE1 induces ISG transcription that is dependent on Stat1 expression and phosphorylation (Knoblach et al., 2011). Alternatively, ongoing viral gene expression and replication may create nucleic acid products that engage pattern recognition receptors, contributing to sustained type I IFN signaling in MHV68-infected macrophages.

We have observed increasing levels of unphosphorylated Stat1 (uStat1) that accumulated in infected macrophages by 24 h post infection (Fig. 1A). uStat1 is an active transcription factor with an altered DNA binding preference (as compared to phosphorylated Stat1) that mediates expression of immune and non-immune genes in a cell type-specific manner (Cheon and Stark, 2009; Yang and Stark, 2008). In the future it will be important to define whether the accumulation of uStat1 in MHV68-infected macrophages has the capacity to regulate gammaherpesvirus infection.

While several gammaherpesvirus proteins are known to counteract IRF-3, the role of IRF-3 in generating antiviral response in EBV- or KSHV-infected cells has not been defined. Here we show that IRF-3 expression was critical for induction of type I IFN signaling at 8 h post MHV68 infection, including in macrophages

infected with the orf36 null MHV68 mutant (N36S, Fig. 3A). The fact that MHV68-mediated type I IFN induction was brought to baseline in the absence of IRF-3 (Fig. 3D) was particularly surprising in light of the well-established roles of MHV68 orf36 and its closely related EBV protein kinase in antagonizing IRF-3 (Hwang et al., 2009; Wang et al., 2009) and targeting of both IRF-3 and IRF-7 by KSHV RTA (Yu et al., 2005; Yu and Hayward, 2010). The viral kinase-IRF-3 interaction was demonstrated exclusively in fibroblasts or epithelial cells. Because stromal cell types and professional immune cells differ in their capacity to induce and respond to type I IFN (Fang et al., 2012), it is possible that the function of viral type I IFN antagonists may be modified in a cell type-specific fashion. Gammaherpesvirus infection of other relevant cell types, such as dendritic cells and B cells, may activate additional IRF family members to trigger type I IFN expression. Identifying cell type-specific signaling pathways that induce type I IFN in response to gammaherpesvirus infection is an important future endeavor. Intriguingly, our findings also suggest that, in addition to IRF-3, orf36 regulates another aspect of type I IFN induction and/or signaling, as expression of type I IFN-dependent ISG15 and Stat1 and Stat1 phosphorylation was increased in IRF-3-/- macrophages infected with the N36S mutant as compared to wt virus, especially at later time points of replication (Fig. 3F, G). This finding suggests that gammaherpesvirus infection of macrophages is likely to trigger multiple, IRF-3 dependent and independent mechanisms of type I IFN induction that operate at different times of the replication cycle.

Consistent with multiple type I IFN antagonists encoded by gammaherpesviruses, we also show that MHV68 attenuates type I IFN signaling induced by exogenous type I IFN (Fig. 4). It is intriguing that MHV68 allows some degree of type I IFN signaling to be maintained throughout the entire replication cycle, in spite of a significant detriment to viral replication (Fig. 5). Because gammaherpesviruses have evolved to maintain a lifelong infection, it is possible that these viruses have developed mechanisms to limit viral replication and pathogenesis in an immunocompetent individual. In support of this idea, our study shows that constitutive type I IFN signaling in primary macrophages limits the activity of the RTA promoters (Fig. 6A). The mechanism by which type I IFN suppresses RTA transcription remains unclear. Several putative interferon gamma activated sequences were identified in both core and distal RTA promoters, however, these sequences are dispensable for Stat1-mediated repression of RTA promoter activity in response to IFNγ (Goodwin et al., 2010). MHV68 has evolved an interferon stimulated response element in its *M2* promoter that acts to repress M2 expression and, thus, limit viral lytic replication and reactivation *in vivo* (Mandal et al., 2011). It is also possible that MHV68 may usurp certain ISGs to facilitate its lytic or latent life cycle, similar to the HCMV-driven induction of viperin that this virus coopts to enhance its replication (Seo et al., 2011). Thus, it is prudent to explore virus-type I IFN interactions not only from the purely antagonistic perspective, but to also consider the benefits of type I IFN signaling for viral processes, especially for viruses that establish chronic asymptomatic infection in most infected hosts.

Materials and methods

Animals and primary cell cultures

C57BL/6J(BL6) mice were obtained from Jackson Laboratories (Bar Harbor, ME). IFNAR1-deficient mice (Sun et al., 1998) were obtained from Dr. Mitchell Grayson. IRF-3 deficient mice were obtained from Dr. Michael Diamond (Sato et al., 2000). Mice were bred and housed in a specific-pathogen-free barrier facility in accordance with institutional and federal guidelines. All experimental manipulations of mice were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin. Bone marrow was harvested from mice between 3 and 10 weeks of age. Primary bone marrow derived macrophages were generated as previously described (Tarakanova et al., 2007).

Viral DNA quantitation

Infected cells were washed with PBS and lysed in a buffer containing 10 mM Tris–Cl, 1 mM EDTA, 0.8% SDS, and 20 μg/ml of proteinase K (Sigma-Aldrich, St. Louis, MO). Following overnight protein digestion at 56 °C, DNA was extracted with phenol/chloroform and precipitated using standard sodium acetate/ethanol treatment. DNA pellet was resuspended in TE (10 mM Tris–Cl, pH 8, 1 mM EDTA), viral DNA was measured by real time PCR using core

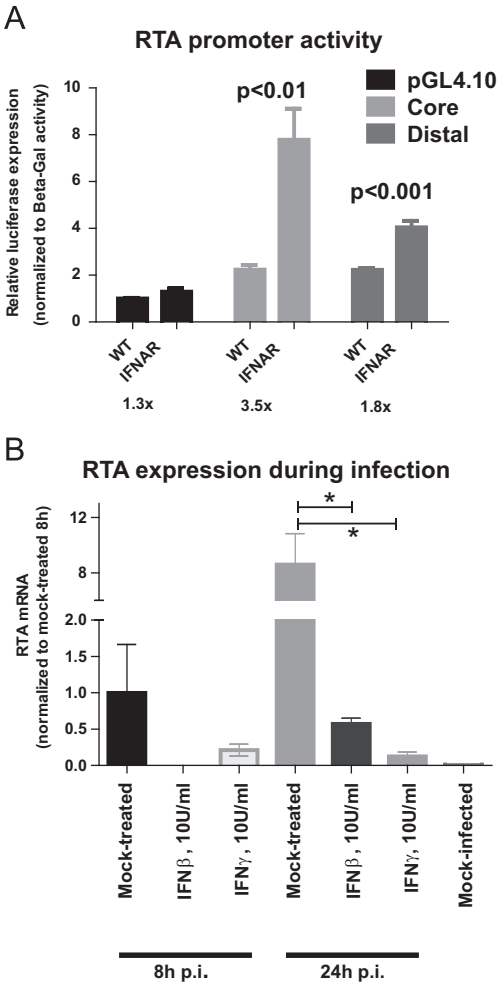

Fig. 6. Type I IFN signaling represses MHV68 RTA expression. A. Primary bone marrow derived macrophages isolated from BL6 (Wt) or IFNAR1-/- mice were nucleofected with indicated reporter constructs or a promoterless vector in combination with a plasmid encoding constitutively expressed β-gal. Cell lysates were collected at 16 h post nucleofection, luciferase activity measured, and normalized to the corresponding β-gal activity. Normalized luciferase data were pooled from two independent experiments, each experimental condition done in duplicate within each experiment. Average and standard error of measurement are shown. B. Primary bone marrow derived BL6 macrophages were mock-treated or treated with 10U/ml of recombinant IFNβ or IFNγ for 16 h. Following treatment, macrophages were infected with wt MHV68 at an MOI of 10, and corresponding treatment (mock, IFNβ, or IFNγ at 10 U/ml) was continued following virus absorption. Total RNA was harvested at 8 and 24 h post infection and RTA mRNA levels measured by qRT-PCR. RNA from mock-infected macrophages was used as a specificity control. Data is displayed as average with standard error of measurement. $^{*}p < 0.05$.

gene 50 promoter primers (Mounce et al., 2011b), and normalized to corresponding GAPDH levels as previously described (Mounce et al., 2011b).

Quantitative real-time PCR

Total RNA was harvested from cells, DNase treated, and reverse transcribed as previously described (Mboko et al., 2012). cDNA was assessed in triplicate, along with corresponding negative RT reactions by real time PCR using iCycler (Bio-Rad, Hercules, CA) (Mboko et al., 2012; Mounce et al., 2011a). Sequences of primers used for this study were previously published (Mboko et al., 2012; Tarakanova et al., 2010). Universal IFNα primers were as follows: forward 5′–ATG GCT AGR CTC GTG CTT TCC T–3′ (R is a wobble of A or G); reverse 5′–AGG GCT CTC CAG AYT TCT GCT CTG–3′ (Y is a wobble of C or T). Relative abundance of each cDNA was normalized to corresponding GAPDH levels and quantified using the ΔC_T method.

Western analysis

Cells were washed with PBS, directly lysed in the Laemmli buffer, and analyzed as previously described (Mboko et al., 2012; Mounce et al., 2011a). The antibodies used were anti-β actin (1:20,000; Novus Biological, Littleton, CO), anti-Y701 phospho-Stat1 (1:1,000; Millipore, Billerica, MA), anti-total Stat1 (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-ssDBP [1:1000; (Mounce et al., 2011a)], rabbit anti-mouse ISG15 (1:3000, a generous gift of Dr. Lenschow), and a secondary goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:25,000; Jackson Immunoresearch, Westgrove, PA).

Interferon treatment

Macrophages were treated with recombinant mouse IFNβ (Biolegend, San Diego, CA) diluted in tissue culture medium at concentrations of 10 U/mL or 100 U/mL. Cells were treated either 15 or 60 min prior to lysis. To measure the effect of IFN signaling on RTA transcription during infection (Fig. 6B), macrophages derived from bone marrow of BL6 mice were mock-treated or pretreated with 10 U/ml of recombinant mouse IFNβ or IFNγ for 16 h, infected with wild type MHV68 at an MOI of 10, and subjected to the same IFN treatment for additional 8 or 24 h.

Viral stock preparation and infections

N36S virus mutant (Hwang et al., 2009; Tarakanova et al., 2007) and wt MHV68 viral stocks were prepared and titered on NIH 3T12 cells as previously described (Mounce et al., 2011b). Wt MHV68 was inactivated by UV crosslinking (0.75 J/cm^2; Stratagene UV Stratalinker 1800, Agilent Technologies, Santa Clara, CA) prior to infection. Bone marrow derived macrophages were infected with either wt, N36S, or UV-inactivated MHV68 at a multiplicity of infection (MOI) of 10 PFU/cell for 1 h at 37 °C and 5% CO_2 to allow for adsorption, and washed twice with PBS prior to medium replenishment.

Luciferase reporter constructs

Sequences containing the core and distal RTA promoters were amplified using the following primers: core, 5′–TGG-CGG-GTA-CCG-AAT-CAT-AGA-TTT-TTT-TAG-C–3′ (forward), 5′–GAT-CGC-TAG-CAG-GTG-GTG-GTT-GCC-AGC-AGG–3′ (reverse); distal, 5′–TGG-CGG-GTA-CCT-TAA-TCC-TAT-ATG-GAG-AT–3′ (forward), 5′–GAT-CGC-TAG-CGT-GCT-GGG-TTG-TGA-A–3′ (reverse). Amplified and purified DNA was cloned into the pGL4.10[luc2] vector (Promega, Madison, WI) using KpnI and NheI restriction enzyme sites. Constructs were verified by sequencing and purified on cesium chloride gradients prior to nucleofection.

Nucleofection of primary bone marrow macrophages

Macrophages were cultured as described previously (Tarakanova et al., 2007), cells were lifted on day 10 of culture, combined with plasmid DNA, and resuspended in Buffer T according to manufacturer's instructions (Lonza, Basel, Switzerland). Electroporation was performed using program T-20 on Amaxa Nucleofector (Lonza, Basel, Switzerland). At 16 h post transfection, cells were collected in 1X rapid lysis buffer (Promega, Madison, WI) and subjected to luciferase assays with subsequent normalization against beta-galactosidase activity.

Luciferase assay

Nucleofected cells were collected in 200 μL 1X rapid lysis buffer (Promega, Madison, WI) and freeze-thawed twice. 20 μL of lysate was then subjected to luciferase assay according to the manufacturer's recommendations (Promega, Madison, WI) using Victor V3 (PerkinElmer, Waltham, MA).

Beta-galactosidase assay

Nucleofected cell lysate was prepared as described above, combined with 4 mg/mL ONPG (Sigma Aldrich, St. Louis, MO) and incubated at 37 °C for 30 m. Absorbance measures were taken at 420 nm and used to normalize luciferase readings.

IFN bioassay

Conditioned medium (CM) was collected from bone marrow derived macrophages which were mock-infected or infected with either live or UV-inactivated wild type MHV68. CM and serially diluted IFNβ standards were UV irradiated to inactivate live MHV68 (0.25 J/cm^2; Fisher Biotech UV Crosslinker FB-UV XL-1000, Fischer Scientific, Pittsburgh, PA). Monolayers of L929 cells were overlaid with CM or diluted IFNβ standards and incubated overnight at 37 °C, 5% CO_2. After 24 h, CM was removed, monolayers were infected with EMCV (B strain) at MOI 5, and incubated for additional 24 h. CellTiter 96 AQ_{eous} One Solution Reagent (Promega, Madison, WI) was added to each well 24 h post infection, according to manufacturer's instructions, and incubated at 37 °C, 5% CO_2 for 45 min. Absorbance (OD) was measured at 450 nm (1420 Multilabel counter Victor^{3}V, PerkinElmer, Waltham, MA). A Non-linear fit regression was performed (GraphPad Prism, GraphPad, La Jolla, CA) to determine the values defining the maximum and minimum plateau, and the EC50 for the standard curve. Concentration of IFN in CM was determined using the OD450 as follows: Log IU/mL=logEC50+Log [(OD450−minimum)/(maximum−OD450)].

Statistical analysis

A Student's *T*-test (GraphPad Prism, GraphPad, La Jolla, CA) was used to measure statistical significance with an α-value of 0.05.

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