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Synergistic control of herpes simplex virus pathogenesis by IRF-3, and IRF-7 revealed through non-invasive bioluminescence imaging

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Abstract

Interferon regulatory factors IRF-3 and IRF-7 are central to the establishment of the innate antiviral response. This study examines HSV-1 pathogenesis in IRF-3$^{-/-}$, IRF-7$^{-/-}$ and double-deleted IRF3/7$^{-/-}$ (DKO) mice. Bioluminescence imaging of infection revealed that DKO mice developed visceral infection following corneal inoculation, along with increased viral burdens in all tissues relative to single knockout mice. While all DKO mice synchronously reached endpoint criteria 5 days post infection, the IRF-7$^{-/-}$ mice survived longer, indicating that although IRF-7 is dominant, IRF-3 also plays a role in controlling disease. Higher levels of systemic proinflammatory cytokines were found in IRF7$^{-/-}$ and DKO mice relative to wild-type and IRF-3$^{-/-}$ mice, and IL-6 and G-CSF, indicative of sepsis, were increased in the DKO mice relative to wild-type or single-knockout mice. In addition to controlling viral replication, IRF-3 and −7 therefore play coordinating roles in modulation of inflammation during HSV infection.

Keywords

Herpes simplex virus; IRF-3; IRF-7; Innate immunity

INTRODUCTION

Herpes simplex virus 1 (HSV-1) is highly sero-prevalent in the human population (D. E. Alexander et al., 2007). Its ability to establish latency renders it refractory to clearance by the immune system, allowing it to persist for the lifetime of the host (Whitley et al., 1998). HSV-1 infection most commonly manifests as orofacial lesions (Spruance, 1992), but also causes more serious diseases such as stromal keratitis (Behrens-Baumann, 2010; Hsiao et al., 2009; Inoue, 2008), meningitis, encephalitis (Baringer, 2008; Brochet et al., 1990; McGrath et al., 1997; Sköldenberg, 1996; Kenneth L Tyler, 2004) and hepatitis (Al Midani et al., 2011; Riediger et al., 2009). These can occur during primary infection or reactivation events, particularly in immune-deficient hosts (Guo et al., 2011; Sancho-Shimizu et al., 2011, 2007; S.–Y. Zhang et al., 2007).

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Type I interferons (IFNα and IFNβ) play major roles in the host innate immune response to HSV-1 infection, and both humans and mice lacking components of the IFN pathway show increased susceptibility and mortality during infection with HSV-1 (Guo et al., 2011; Ishikawa et al., 2009; Lafaille et al., 2012; Luker et al., 2003; Pasieka et al., 2011, 2008; Sancho-Shimizu et al., 2011; S.-Y. Zhang et al., 2007). The importance of IFN signaling in the containment of HSV-1 infection is also reflected by the array of proteins encoded by HSV-1 that function to subvert establishment of the anti-viral state and facilitate viral replication (Chou et al., 1995; He et al., 1997; Johnson et al., 2008; Rongtuan Lin et al., 2004; Melroe et al., 2004; Mulvey et al., 2004; Orvedahl et al., 2007; Pasieka et al., 2012; Sànchez and Mohr, 2007; Xing et al., 2012). Pattern recognition receptors (PRR) such as toll-like receptors (TLRs) (Lemaitre et al., 1996), melanoma differentiation factor (MDA5) (Kang et al., 2002), retinoic acid inducible gene (RIG-I) (Yoneyama et al., 2004), interferon inducible protein-16 (IFI16) (Unterholzner et al., 2010), absent in melanoma 2 (AIM2) (Bürckstümmer et al., 2009), and DNA dependent activator of IRFs (DAI) (Takaoka et al., 2007) recognize pathogen-associated molecular patterns (PAMPs) such as dsRNA and viral DNA during infection. Once activated, these PRRs initiate signaling pathways that culminate in the activation of the transcription factors IRF-3 and IRF-7, leading to production of type 1 IFN.

IRF-3 is a constitutively expressed protein in most cells, existing as an inactive monomer in the cytoplasm. Upon activation, serine residues at the C terminus of IRF-3 are phosphorylated (J Hiscott et al., 1999; R Lin et al., 1998; Sonia Sharma et al., 2003), IRF-3 assembles into dimers and translocates to the nucleus (M Sato et al., 1998), facilitating transcription of interferon β. Newly-produced IFNβ secreted from virus infected cells interacts with cell surface IFNα/β receptors (IFNARs) on infected and uninfected neighboring cells (Mogensen et al., 1999), activating the JAK-STAT signaling pathway (Levy and García-Sastre, 2001), inducing transcription of IFN responsive genes including IRF-7 (Fu et al., 1992; Reich, 2007; Zimmerer et al., 2007). The C-terminus of newly synthesized IRF-7 is phosphorylated, promoting dimerization and translocation to the nucleus (Mitsuharu Sato et al., 2000), where it induces production of large amounts of IFNα, IFNβ and IFN stimulated genes, thus establishing the antiviral state. IRF-7 has long been recognized as the “master regulator” of interferon signaling (Honda et al., 2005), but it is also accepted that IRF-3 plays an important role in controlling HSV-1 replication - particularly within tissues of the central nervous system (Menachery et al., 2010). In addition to its role in inducing anti-viral responses, recent reports (Tarassishin et al., 2011a, 2011b) have indicated that IRF-3 also plays a role in minimizing immune mediated pathology through modulation of pro-inflammatory cytokine expression. Given the non-regenerative nature of certain cell types within the CNS, this ability to switch from a tissue-damaging pro-inflammatory state to a tissue recovery-promoting, anti-inflammatory state is likely to play a role in successful resolution of infection and recovery of the host. The study herein utilizes single and double knockout mouse models of IRF-3 and IRF-7 to examine in detail the roles played by IRF-3 and IRF-7, both singly and in combination, in controlling HSV-1 replication, and disease pathology.

MATERIALS AND METHODS

Cell culture and viruses

Vero cells, maintained in Dulbecco’s Modification of Eagle Medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS) and 1% penicillin/streptomycin were used to propagate and titer HSV-1 preparations. Bone marrow derived dendritic cells (BMDCs) were generated from 6–8 week old wild-type, IRF-$3^{-/-}$, IRF-$7^{-/-}$ and IRF-$3/7^{-/-}$ mice. Briefly, bone marrow was flushed from femurs and cultured in RPMI supplemented with 10 % FBS, glutamax, sodium pyruvate, non-essential amino acids, 1% penicillin/

streptomycin and 2% granulocyte macrophage colony stimulating factor (GM-CSF) for 7 days. BMDCs were harvested and divided into aliquots for infection. Virus was added in a minimal volume of medium for 1 h at 37°C. After 1 h adsorption, cells were centrifuged at low speed, washed and re-suspended in fresh medium in 6 well plates for the duration of the experiment.

HSV-1 strain 17 syn+ (S. M. Brown et al., 1973) and HSV-1 McKrae/Dlux were used in this study. McKrae/Dlux was generated through homologous recombination of HSV-1 McKrae strain infectious DNA with a pUIC plasmid containing a pDlux cassette, as previously described (Summers et al., 2001). Recombinant viruses were screened for luciferase expression, plaque purified three times and verified by Southern blot (data not shown). All viral stocks were titered by plaque assay on vero cell monolayers as described previously (D A Leib et al., 1989). Briefly, serial 10-fold dilutions of virus were applied to cell monolayers, adsorbed for 1 h and overlaid with a semi-solid methylcellulose overlay. After 4 days monolayers were stained with neutral red and plaques were counted.

Migration assay

Chemotaxis in response to CCL19 was analyzed by measuring the number of cells migrating through a polycarbonate filter in transwell chambers (2 μm pore size; Corning). 3×10^5 wild-type, IRF-$3^{-/-}$, IRF-$7^{-/-}$ or DKO BMDCs were seeded into the top chambers of the transwells in culture medium. Culture medium supplemented with 20 ng/ml of CCL19 was added to the bottom chambers. Cultures were incubated for 3 h at 37°C and cells that migrated from the top to the bottom chamber were counted using an automated cell counter.

Mice

Mouse strains used in this study included wild-type C57BL6 mice and IRF-$3^{-/-}$ (Mitsuharu Sato et al., 2000), IRF-$7^{-/-}$ (Honda et al., 2005) and IRF-3/$7^{-/-}$ (generated by crossing IRF-$3^{-/-}$ X *IRF-*$7^{-/-}$ single knockout mice) knockout, referred to hereafter as DKO (double knockout) strains, all on the C57BL6 background. Mice were genotyped by PCR and housed in the barrier facility in the Center for Comparative Medicine and Research at The Geisel School of Medicine at Dartmouth and were infected in the biohazard facility between the ages of 6–8 weeks. Mice were housed, treated, and euthanized when necessary in accordance with all Federal and University policies.

Animal infection, disease scoring and organ harvest

Mice were anesthetized intraperitoneally with ketamine (87 mg/kg body weight) and xyalazine (13mg/kg). Corneas were bilaterally scarified and mice were inoculated by adding 2×10^6 p.f.u per eye in a 5μl volume (Rader et al., 1993). At indicated times post infection, mice were weighed and scored for disease pathology using the following disease scores: 0 – no visible pathology, 0.5 – minor eyelid swelling, 1.0 – minor eyelid and nasal swelling, 1.5 – moderate eyelid and nasal swelling, 2.0 – severe eyelid swelling, minor periocular hair loss and skin lesions, 2.5 – severe eyelid swelling, moderate periocular hair loss and skin lesions, 3.0 – neurological symptoms. Endpoint criteria for sacrificing mice for mortality include loss of ≥15% of starting body weight, altered locomotion, and labored breathing. Eye swab material was collected for analysis by plaque assay at selected times post infection, as previously described (D A Leib et al., 1989). Mice were sacrificed and trigeminal ganglia, brain stems, brains, livers and spleens were harvested into an appropriate volume of media, mechanically disrupted with either 1 mm or 2.5 mm glass beads, sonicated and titered via standard plaque assay (Menachery et al., 2010).

Bioluminescence imaging

Mice infected with McKrae/Dlux were injected intraperitoneally with 150 μg/g body weight of D-Luciferin potassium salt (GoldBio) in PBS, anaesthetized with 2.5 % isoflurane and imaged (exposure time of 1–60 s, f-stop 1 or 2, field of view 15 or 19.6) with a cooled charge coupled device (CCD) camera based bioluminescence *in vitro* imaging system (IVIS 100, Caliper Life Sciences, Hopkinton, MA) (Pasieka et al., 2011). For analysis, regions of interest (ROI) were defined manually around eyes, lymph nodes and liver. Background light emission was subtracted from ROIs and photon flux was calculated using Living Image and Igor pro software (Version 2.6).

Cytokine analysis

Blood was harvested from mice at indicated times post infection and plasma was separated by centrifugation. For this experiment, a mouse 32-plex kit (Milipore) was utilized. Samples were diluted 1:1 with assay buffer, and processed as per manufacturers instructions. Cytokines concentrations were determined by comparison to recombinant cytokine standard curves.

Real time reverse transcription PCR of tissue

At the indicated time post infection tissues were harvested into Trizol (Invitrogen). Tissue was homogenized using a tissue blender, RNA was extracted as per manufacturers instructions and DNAse treated with DNA-free (Ambion). cDNA was generated with superscript III reverse transcriptase (Invitrogen) using random hexamers to prime the reaction. Efficiency curves were generated for all primer sets to determine PCR efficiencies of target and reference genes, as described by Pfaffl (Pfaffl, 2001). PCR mixtures were prepared with iQ SYBR green supermix (Biorad), 0.2 – 0.5 μM primers, and 2 μl cDNA. For each amplified PCR product, a single peak was obtained by melting curve analysis, and only a single band of the predicted size was observed by agarose gel electrophoresis. The Pfaffl method (Pfaffl, 2001), which takes PCR efficiencies into account, was used for data analysis.

RESULTS

Bioluminescence imaging of mice infected with HSV-1 McKrae/Dlux

Bioluminescence imaging (BLI) has proved useful in several previous studies (Luker et al., 2003; Pasieka et al., 2011) when attempting to gain an overview of viral pathogenesis in a variety of knockout mice. One issue, however, has been that existing bioluminescent strains in the HSV-1 KOS background are of insufficient virulence to robustly infect mice in the C57BL/6 background. We had, however, observed that strain McKrae had a high rate of lethal infection following corneal challenge of C57BL/6 wild-type and IRF-3$^{-/-}$ mice (Menachery et al., 2010). We wanted to determine the roles played by IRF-3 and IRF-7 when faced with a virulent HSV-1 infection so we created a new luciferase-expressing virus in the McKrae background (McKraeDlux) and performed BLI (Fig. 1A and 1B). Robust photon flux was observed for all mouse strains at 3, 4, and 5 days post-infection, underscoring the utility of McKraeDlux. When taken as a whole, BLI signals were not significantly different between C57BL/6 and the single knockout mice (IRF3$^{-/-}$, and IRF7$^{-/-}$), although there were trends towards higher abdominal signals in the IRF-3$^{-/-}$ mice and higher head/sub-mandibular lymph node signals in the IRF-7$^{-/-}$ mice. In contrast, imaging of the DKO mice revealed significantly higher photon fluxes in lymph node, eye and abdominal regions relative to wild-type and single knockout mice at days 3, 4 and 5 post-infection. Having shown a pattern of increased BLI activity and generalized spread in the DKO we next wished to assess viremia during acute infection (Fig. 1C). All mice

showed viremia on day 2, although the DKO mice had almost 10-fold more virus in their serum than wild-type or single knockout mice. Wild-type and IRF-3$^{-/-}$ mice were, however, capable of clearing virus from the bloodstream by day 4. In contrast, the DKO mice were unable to clear virus from the bloodstream with titers remaining relatively constant between days 2 and 4. In this time frame, titers decreased in the blood of IRF-7$^{-/-}$ mice although there was still significant viremia on day 4 post-infection. The widespread viral dissemination observed on day 5 in the DKO mice by BLI, with especially strong signals in the liver, is therefore consistent with their inability to clear virus from the bloodstream.

Combined IRF-3 and IRF-7 loss leads to enhanced HSV-1 replication in peripheral tissues

Having established the general patterns of pathogenesis in the DKO mice using BLI, we wished to examine virus replication more specifically in corneas and trigeminal ganglia. For studies which did not require BLI we switched to infection with wild-type McKrae virus, but this led to very rapid death (≤4 days) of the DKO mice (data not shown). We therefore used the less virulent HSV-1 strain 17 for these experiments to allow the study of HSV-1 replication in these tissues over a longer time frame. Analysis of corneal swabs (Fig. 2A) revealed no defect in IRF-3$^{-/-}$ mice for control of HSV-1 replication on either day 2 (data not shown) or day 4 post infection relative to wild-type mice, consistent with previously published data (Menachery et al., 2010). In contrast, HSV replication in corneas of IRF-7$^{-/-}$ mice was significantly increased ($p \leq 0.05$) relative to wild-type, and even greater viral loads ($p \leq 0.001$) were observed in the corneas of DKO mice. Consistent with this, we found the trigeminal ganglia of DKO infected mice had significantly higher viral loads than wild-type or single knockout mice (Fig. 2B). In contrast to the cornea, IRF-7$^{-/-}$ mice showed no defect in controlling virus replication in the trigeminal ganglia. These data show, consistent with the BLI data, that there is an increase in productive virus infection in corneas and trigeminal ganglia of DKO mice relative to wild-type or single IRF3/7 knock-out mice.

IRF-3 and IRF-7 are both required for control of disease pathology and lethality

Following infection with HSV-1 strain 17, we monitored mice for development of disease, and for timing to reach endpoint disease criteria (Fig. 3*A*, 3*B*). Wild-type and IRF-3$^{-/-}$ mice displayed a similar degree and rate of disease development, with symptoms such as eyelid and nasal swelling reaching maximum levels between 5 and 6 days post infection, and remaining steady, or declining thereafter. IRF-7$^{-/-}$ mice developed symptoms at a similar rate to both the wild-type and IRF-3$^{-/-}$ mice up until 5 days postinfection (Fig 3A). There was, however, a continued increase in disease severity, with all IRF-7$^{-/-}$ mice reaching end-point criteria 6–8 days post-infection (Fig 3B). DKO mice showed an increase in both rate of onset and severity of disease symptoms relative to wild-type or single knockout mice (Fig 3A). Symptoms progressed rapidly and all DKO mice reached end point criteria synchronously on the fifth day postinfection (Fig. 3B). This was 2–3 days earlier than IRF-7 $^{-/-}$ mice, indicating that while IRF-7 may be the critical regulator of IFN responses, IRF-3 becomes critical for controlling HSV-1 disease progression in the absence of IRF-7.

We reasoned that the increased mortality in the 1RF7$^{-/-}$ and DKO mice might be due to viral neuroinvasion and encephalitis, as neurological symptoms were present prior to euthanasia. To examine whether death correlated with viral titers within the CNS, we compared viral loads in brain stems and brains of infected mice (Figs. 3C and 3D). Interestingly, we observed small but significant increases in viral loads in the brainstems of IRF-3$^{-/-}$ and DKO mice, relative to wild-type and IRF-7$^{-/-}$ mice. Higher constitutive expression levels of IRF-3 than IRF-7 within CNS tissue (J. E. Christensen et al., 2012; Delhaye et al., 2006; Ousman et al., 2005), supports IRF-3 having a more important role during the early stages of HSV-1 replication within the CNS, prior to induction of IRF-7. Comparison of viral loads within the whole brain revealed that only brains from the DKO

mice had significantly higher viral loads than wild-type mice (Fig 3D). These data are consistent with the hypothesis that while IRF-3 may be important in controlling initial entry into the brain stem, IRF-3 and IRF-7 act in concert to limit further virus spread within the brain. That said, it seemed unlikely that loss of IRF-3/7 leading to increased virus replication in the CNS could be the sole explanation for the drastic changes in disease and survival seen in IRF7$^{-/-}$ and DKO mice (Fig 3A, 3B).

Role of IRF-3 and IRF-7 in migration of immune derived cells *in vitro* and production of cytokines *in vivo*

Previous work has demonstrated that while IRF-3 is important for control of HSV-1 replication in immune cells, IRF-7 is dispensable (Menachery and David A. Leib, 2009). Consistent with this, virus replication was increased in IRF-3$^{-/-}$ relative to wild-type and IRF-7$^{-/-}$ BMDCs, and no additional increases in replication were observed in the DKO BMDCs (Fig.4A). In addition to impairments in controlling viral replication, it has been shown that dendritic cells of IRF-3$^{-/-}$ mice are impaired in their ability to migrate to lymph nodes relative to wild-type DCs, possibly leading to sub-optimal priming of the adaptive immune response (Marichal et al., 2010). Using an *in vitro* transwell system, we investigated the ability of wild-type, IRF-3$^{-/-}$, IRF-7$^{-/-}$ and DKO BMDCs to migrate towards CCL19, a chemokine expressed abundantly in lymph nodes. As expected, IRF-3$^{-/-}$ BMDCs showed defective migration (Fig. 4B). IRF-7$^{-/-}$ BMDCs exhibited no defect in migration relative to wild-type, and DKO BMDCs had no additional migratory defects above those observed in IRF-3$^{-/-}$ BMDCs, indicating that any defects in migration are due solely to loss of IRF-3.

To examine whether increased disease pathology in the DKO mice correlated with altered cytokine levels, we examined plasma of mock and HSV-1 infected wild-type, IRF-3$^{-/-}$, IRF-7$^{-/-}$ and DKO mice with a BioRad Bio-Plex multiple cytokine assay (Fig. 5). While many cytokines showed elevated expression during infection with HSV-1 we focused on those which were elevated in the IRF-7$^{-/-}$ and DKO populations, compared to control and IRF-3$^{-/-}$ mice. Disruption of interferon signaling can inhibit the IFNγ negative feedback loop, leading to increased expression of the pro-inflammatory cytokine IFNγ (Ben-Asouli et al., 2002; Kaempfer, 2006). In agreement with this, IFNγ was only detectable in IRF-7$^{-/-}$ and DKO mice, and expression of MIG (Farber, 1997), a biomarker for IFNγ activity was also higher in IRF-7$^{-/-}$ and DKO mice (Fig 5A). TNFα and IL-1α, pro-inflammatory cytokines which play roles in development of stromal keratitis (Keadle et al., 2000), were both elevated in IRF-7$^{-/-}$ and DKO relative to wild-type and IRF-3$^{-/-}$ mice. Interestingly, some anti-inflammatory cytokines, IL-10 and IL-13, were also expressed at higher levels in IRF7$^{-/-}$ and DKO mice than wild-type and IRF3$^{-/-}$ mice, although the significance of this is unclear (Fig 5A).

While the elevated levels of the above-mentioned cytokines likely contribute to the altered pathology observed in infected mice, their levels were largely equivalent in IRF-7$^{-/-}$ and DKO mice, suggesting that other factors likely contribute to the especially rapid lethality observed in the DKO mice. Leukemia inhibitory factor (LIF), a member of the IL-6 family of proteins with pro- and anti-inflammatory properties (Mathieu et al., 2012), and two pro-inflammatory cytokines, G-CSF and IL-6, had notably high expression levels in DKO mice relative to IRF-7$^{-/-}$ mice: (Fig 5A). Elevated levels of G-CSF mobilize cells from bone marrow and stimulate prolonged survival, leading to increased numbers of circulating neutrophils (Lawlor et al., 2004; Xu et al., 1996), while increased IL-6 production in IFN signaling deficient mice has previously been associated with increased neutrophil infiltration and corneal inflammation during HSV-1 infection (Pasieka et al., 2009). Corneas from DKO mice showed increased opacity post infection with HSV-1, and real time PCR of corneal tissues confirmed that transcript levels of IL-6 mRNA were 12 fold higher in DKO mice relative to wild-type infected mice, while they were only 4 fold higher in IRF-7$^{-/-}$ mice

relative to wild-type mice (Fig 5B). IL-6 transcript levels were also higher in trigeminal ganglia of DKO mice relative to wild-type mice, than in trigeminal ganglia of IRF-7$^{-/-}$ mice (Fig. 5B), confirming an elevated IL-6 signaling response in the peripheral tissues of DKO mice.

DISCUSSION

The importance of a timely and measured type 1 IFN response in the control of HSV-1 infection is well-established (C D Conrady et al., 2012; Christopher D Conrady et al., 2011; Ishikawa and Barber, 2008; Lundberg et al., 2008; Pasieka et al., 2009, 2008), and defects in the IFN signaling pathway can lead to life-threatening conditions such as encephalitis (Lafaille et al., 2012; McGrath et al., 1997; Kenneth L Tyler, 2004). Interferon regulatory factors 3 and 7 are central to the establishment of this response (Daffis et al., 2008; Honda et al., 2005; Juang et al., 1998; Marié et al., 1998; M Sato et al., 1998), and this study demonstrates that a combined deficiency of IRF-3 and IRF-7 results in a rapid onset, severity and progression of disease pathology, along with increased mortality following infection with HSV-1. That said, the deletion of IRF-7 alone had a significant impact on pathogenesis, especially on viremia and mortality, and the impact of IRF-7 loss was greater than the impact of loss of IRF-3 alone. This non-redundancy of IRF-3 and 7 is similar to the pattern observed for West Nile virus (Daffis et al., 2008), but distinct from observations with Chikungunya and murine norovirus (Schilte et al., 2012; Thackray et al., 2012) in which significant changes in susceptibility were observed only after deletion of both IRF-3 and −7.

Despite evidence for a dominant role for IRF-7, this study has revealed that IRF-3 and IRF-7 act synergistically to exert control over viral replication, with significantly elevated viral burdens in all tissues examined in DKO relative to single knockout mice. Altered tissue tropism and elevated levels of viremia were also observed, correlating with a previous study characterizing WNV infection of DKO mice (Daffis et al., 2009) . Although 100% mortality was observed in DKO mice in both studies, the earlier invasion of virus into the CNS and the lethal uncontrolled viral replication that was observed during WNV infection was not recapitulated during HSV infection. Based on the BLI study it appears more likely that the susceptibility of the DKO mice is largely due to the profound viscerotropism of HSV-1 in the absence of IRF-3 and −7, consistent with the pattern of HSV infection in IFNR$^{-/-}$ mice (Luker et al., 2003). Such patterns of infection are accompanied with alterations in AST/ALT levels within the livers of both experimental mice and in humans resulting in herpetic hepatitis and fatal liver failure (Minuk and Nicolle, 1986; Norvell et al., 2007) .

Previous studies have demonstrated that loss of critical components of the IFN signaling pathway, such as STAT1, can lead to enhanced inflammation and pathology during infection with HSV-1, and such disease can be independent of changes in viral titers (Lundberg et al., 2008; Pasieka et al., 2009, 2008). Several studies have also reported that IRF-3 has anti-inflammatory properties which may help control immune-mediated pathology (Hua et al., 2002; Suh et al., 2009; Tarassishin et al., 2011a, 2011b). These studies therefore prompted us to investigate in this study whether a dysfunctional immune response was contributing to the observed pathology and mortality. Consistent with this idea, cytokine profiling revealed higher levels of systemic pro-inflammatory cytokines in 1RF7$^{-/-}$ and DKO mice relative to wild-type and IRF-3$^{-/-}$ mice. IL-6 and G-CSF, which lead to increased infiltration of immune cells into tissues and increased pathology (Fielding et al., 2008; Lawlor et al., 2004; Xu et al., 1996; Yong, 1996) have previously been implicated in the development of herpes stromal keratitis (Divito and Hendricks, 2008; Fenton et al., 2002; Inoue, 2008). Consistent with this, we observed high levels of IL-6 transcripts in the corneas of DKO mice, indicating that it likely contributes to the ocular and periocular pathology that occurs during infection in these mice.

Studies have also shown a negative correlation between IL-6 levels and survival of herpes simplex encephalitis (Bociaga-Jasik et al., 2011). Additionally, elevated levels of LIF (Auernhammer and Melmed, 2000; Villers et al., 1995), IL-6 (Damas et al., 1997; Ebong et al., 1999) and G-CSF have all been reported during the development of systemic inflammatory response syndrome, or sepsis. Sepsis is characterized by an initial hyper-inflammatory response to infection, which can be followed by a period of immune suppression (Hotchkiss et al., 2009), and is associated with development of organ dysfunction, hypoperfusion or hypotension and intravascular coagulation (Sriskandan and Altmann, 2008). Anti-inflammatory cytokines are also produced during sepsis as the host attempts to modulate the inflammatory response (Marchant et al., 1994), perhaps explaining our observation of elevated levels of IL-10 and IL-13. Interestingly, the pathophysiology of neonatal HSV infection is typical of a systemic inflammatory response, and it has been reported that neonates, whose immune systems are immature in comparison to the adult immune system, produce higher quantities of pro-inflammatory cytokines such as IL-6 in response to HSV challenge. This correlates well with our observations that deficiencies in the innate immune response lead to uncontrolled inflammation and sepsis, and indicate that this may be a useful model for further study of neonatal HSV pathology.

While our data supports the development of a hyper-inflammatory state in IRF-3/7$^{-/-}$ deficient mice, it contrasts with a previous study which reported increased expression of transcripts encoding multiple inflammatory markers within the CNS of STAT1$^{-/-}$ mice with an accompanying breach of the blood brain barrier (Pasieka et al., 2009). In this study we analyzed expression of cytokines and markers of inflammation in brain stem tissues. Surprisingly, we found no evidence of further elevation of proinflammatory cytokines in the brains of the DKO mice relative to the other mice (data not shown). We also found no evidence of increased cellular infiltrates or breach of the blood-brain barrier (data not shown). These data suggest that the elevated cytokines observed in the DKO mice are causing their damage outside of the CNS, most likely coupled with the significant generalized infection visualized through the BLI experiments. In the absence of IRF-3 and IRF-7, it is possible that the initial hyperinflammatory phase of sepsis is so rapid that death ensues before the development of breach of the BBB.

In summary, our study has revealed that while IRF-7 is the dominant factor in the control of HSV-induced disease and mortality, IRF-3 and IRF-7 act synergistically to control viral replication, with loss of both leading to higher viral loads. We also demonstrate that they play an essential role in modulation of inflammatory responses during infection with HSV, preventing the development of severe systemic inflammation. It is likely that a combination of increased viral replication and markedly increased levels of pro-inflammatory cytokines such as G-CSF and IL-6 in the DKO mice are responsible for the increased disease and mortality observed. These mice will therefore likely prove a useful model for future studies of sepsis and immune pathology.

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Highlights

- We compare HSV-1 infection of IRF-3, IRF-7, and IRF-3/7 (DKO)-deficient mice.
- DKO develop visceral disease and succumb rapidly to infection.
- IRF-7 was more critical than IRF-3 for protection of mice from fatal infection.
- Both factors are critical for modulating the inflammatory responses to infection.

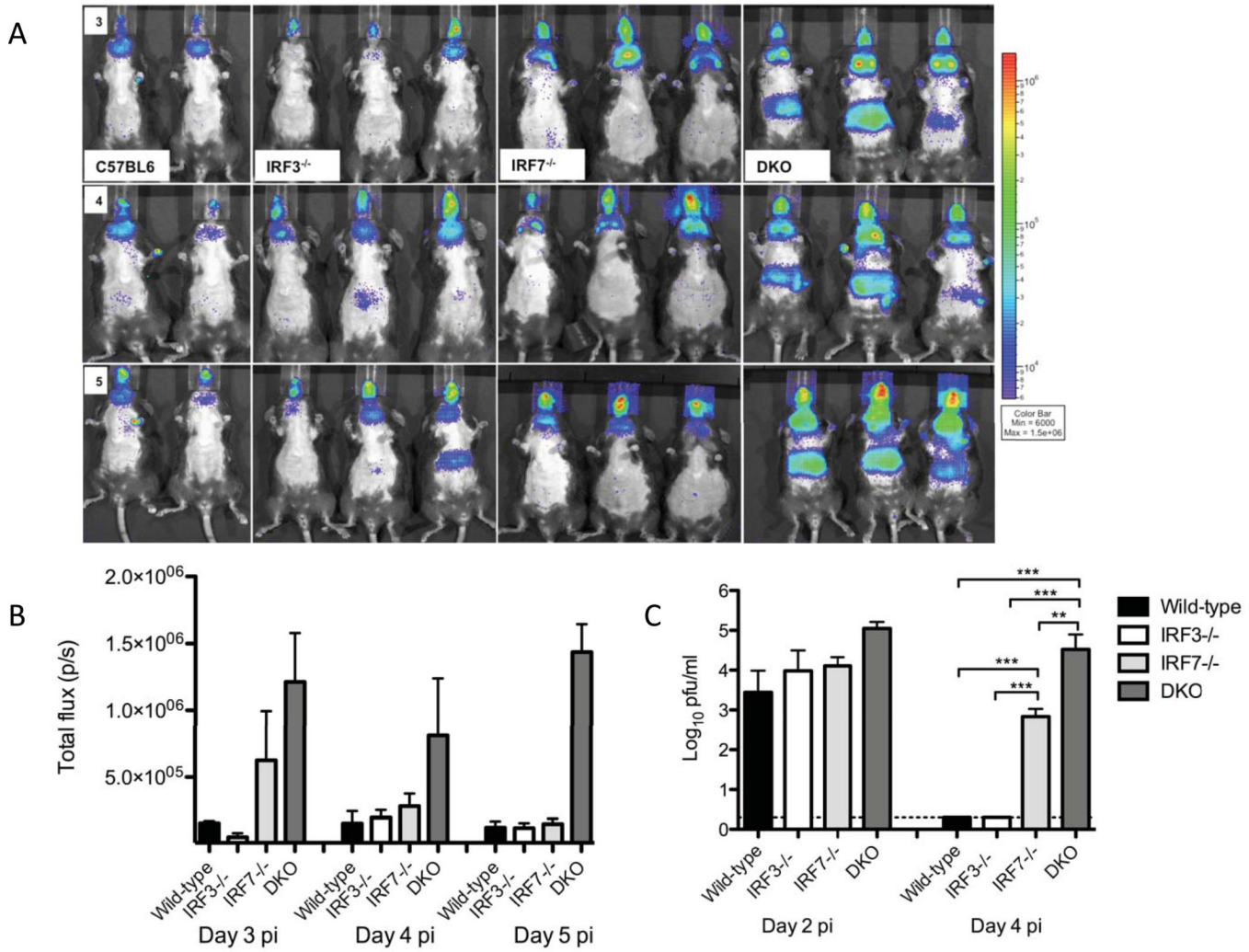

Figure 1. Pattern of viral spread in wild-type, IRF-3$^{-/-}$, IRF-7$^{-/-}$ and DKO mice

(A) In vivo bioluminescent imaging analysis was performed on days 2 through 5 post corneal infection with McKrae/DLux. Daily images for the same mice are shown on identical photon flux scales and are representative of 2 independent experiments. (B) Region of interest analysis of lymph node bioluminescence. ROIs were drawn around the lymph node area using the Living Image and IgorPro software and bioluminescent signal was reported in photons/sec. (C) Viral titers in the blood stream. Serum was isolated from mice two and four days post infection with McKrae/DLux and assayed on vero cell monolayers (n=4 mice per group, 2 independent experiments).

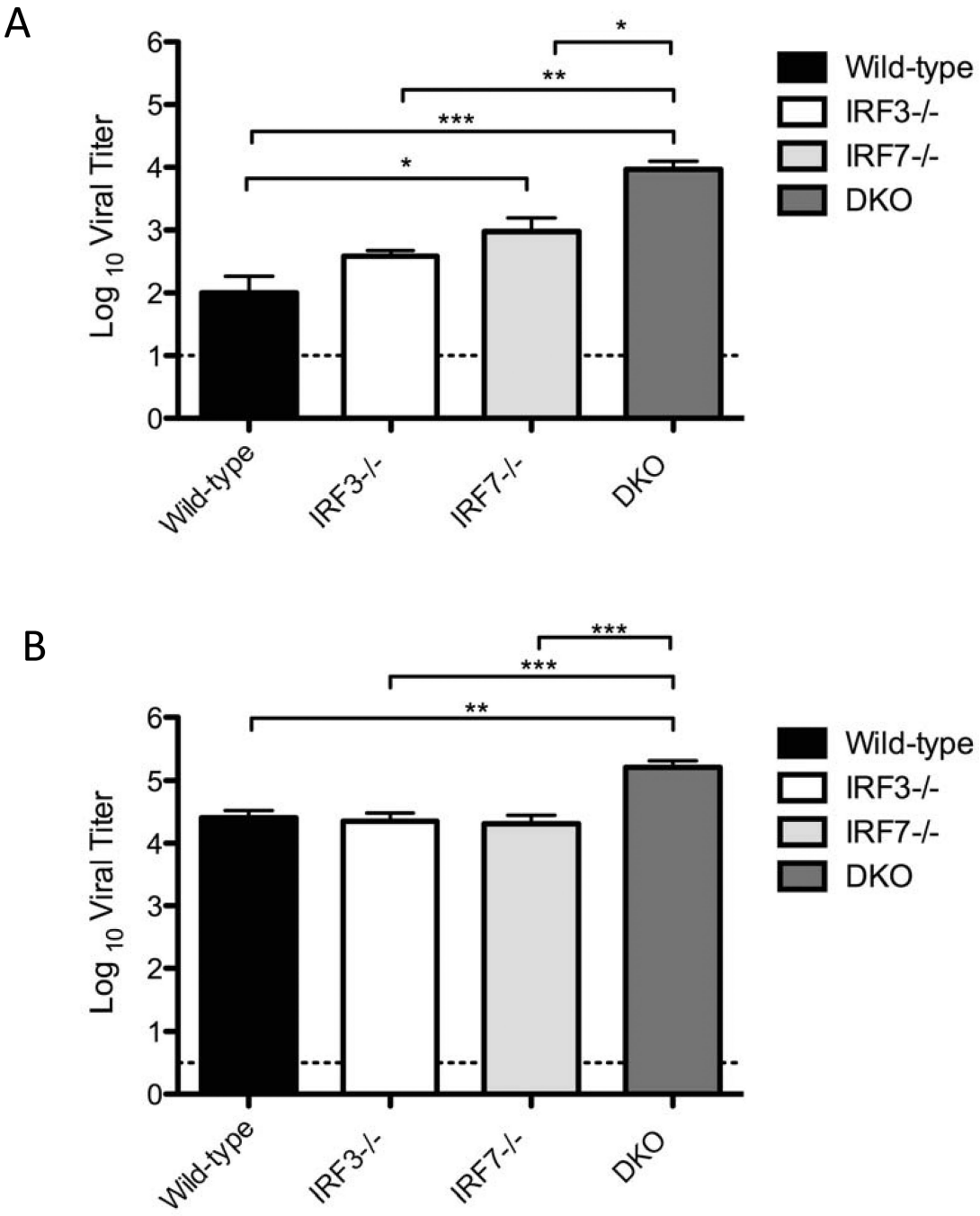

Figure 2. Virus replication in peripheral tissues of wild-type and IRF-3$^{-/-}$, IRF-7$^{-/-}$, and IRF3/7$^{-/-}$ (DKO) mice

Viral titers in (A) cornea, (B) trigeminal ganglia, 4 days following corneal infection with 2 × 10^6 pfu/eye. Graphs represent averages and standard errors of the mean for several mice from two independent experiments (n ≥ 5 mice per group). The dotted line indicates the threshold of detection. Statistical analysis (ANOVA) was performed using GraphPad Prism 5.0. Asterisks indicate ranges of significance (* , P ≤ 0.05; ** , P ≤0.01; ***, P ≤0.001).

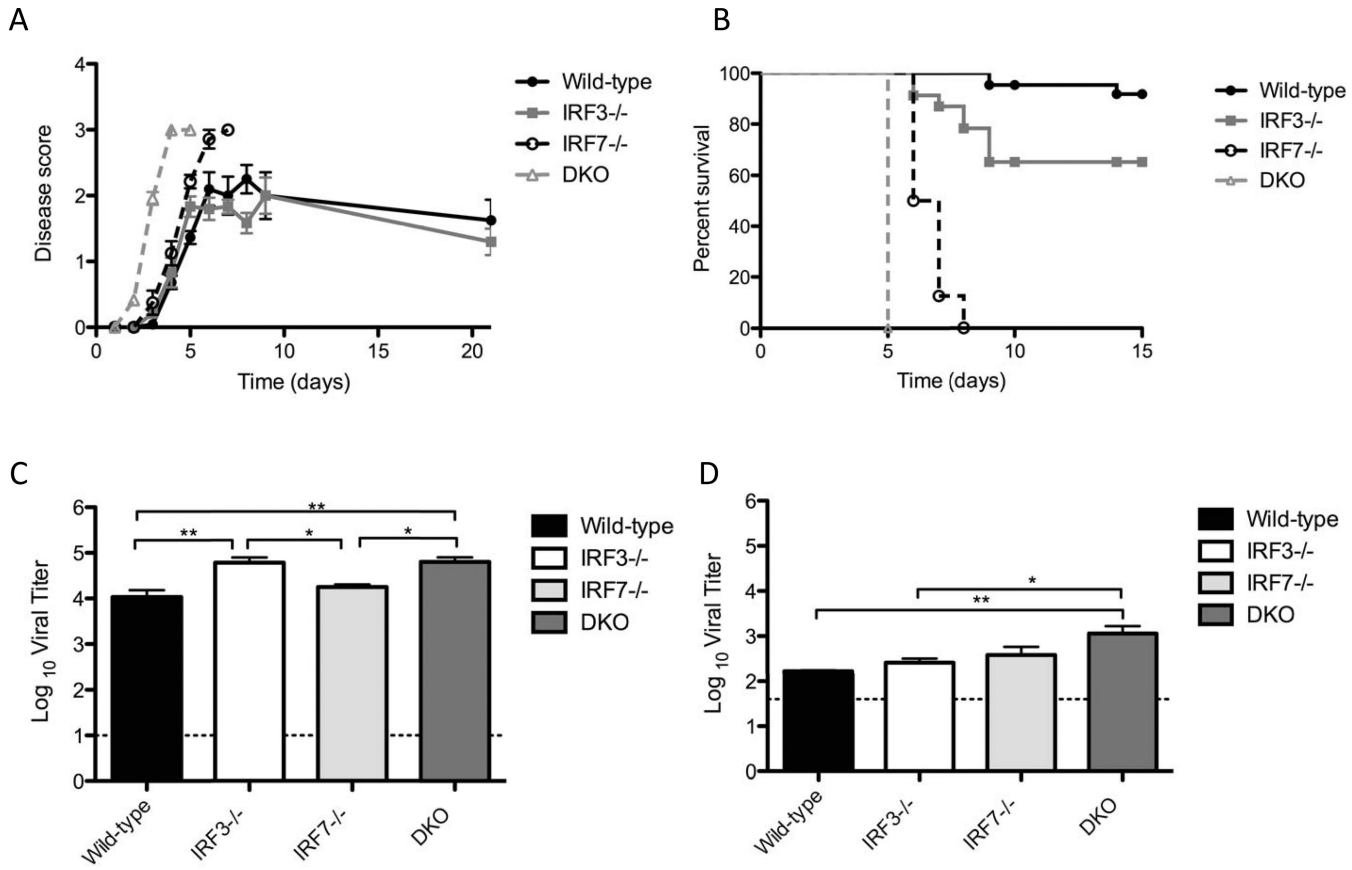

Figure 3. Disease, mortality and CNS replication following HSV-1 replication in C57BL6, IRF-3$^{-/-}$, IRF-7$^{-/-}$ or IRF-3/7$^{-/-}$ (DKO) mice

(A) Pathology following HSV-1 infection. Corneas of C57BL6, IRF-3$^{-/-}$, IRF-7$^{-/-}$ and DKO mice were scarified and inoculated with 2×10^6 pfu/eye. Disease scores were recorded in accordance with parameters detailed in the materials and methods. Data represents disease scores from three independent experiments (n ≥ 12 mice per group). (B) Survival of wild-type, IRF-3$^{-/-}$, IRF-7$^{-/-}$ and DKO mice following corneal infection with 2×10^6 p.f.u HSV-1 strain 17 per eye. Survival curves were carried out independent of other studies and represent data from three individual experiments. (n ≥ 12 mice per group). Brain stems (C) and brains (D) were harvested at specified days following infection of wild-type, IRF-3$^{-/-}$, IRF-7$^{-/-}$ and DKO mice with 2 × 10 p.f.u. HSV-1 strain 17 per eye. Graphs represent mean +/− SEM of two independent experiments, (n ≥ 5 mice per group)

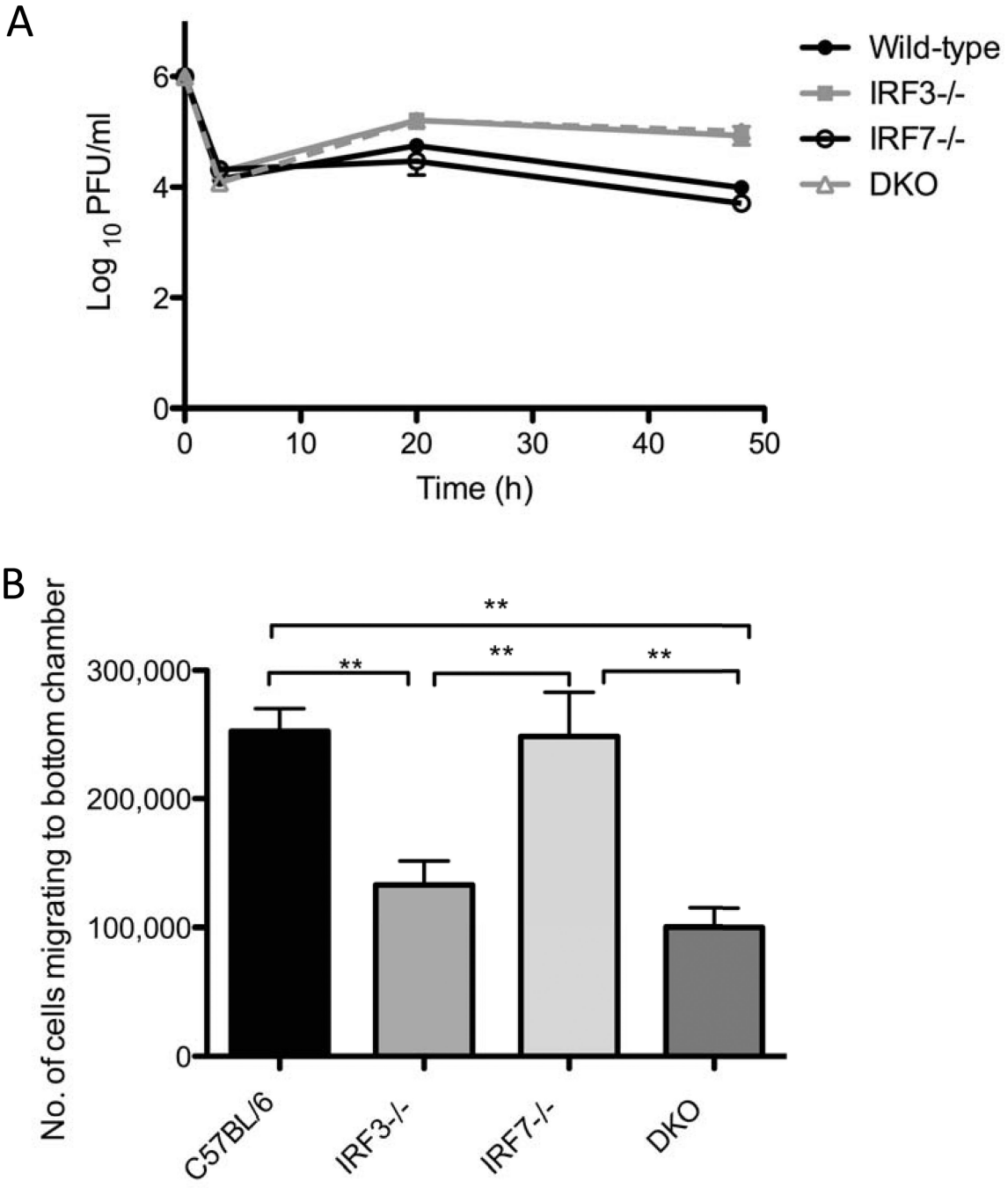

Figure 4. HSV replication in wild-type and IRF-3$^{-/-}$IRF-7$^{-/-}$and IRF3/7$^{-/-}$(DKO) BMDCs and impact on DC migration

(A) In vitro replication in bone marrow derived dendritic cells. Primary BMDCs were infected with HSV-1 strain 17 at MOI 1. At indicated times post infection, cell supernatants were harvested and viral titers assayed on vero cell monolayers. Results shown represent the mean +/− SEM of two independent experiments, each carried out in triplicate. (B) Migratory ability of primary BMDCs. The ability of wild-type, single and DKO BMDCs to migrate towards the chemokine CCL19 was determined by counting the number of cells which migrated through a 2 μm pore size polycarbonate filter of a transwell plate. Results shown represent one experiment carried out in duplicate. The experiment was repeated twice with

identical results. Statistical analysis (ANOVA) was performed using GraphPad Prism 5.0. (* , $P \leq 0.05$; ** , $P \leq 0.01$; ***, $P \leq 0.001$).

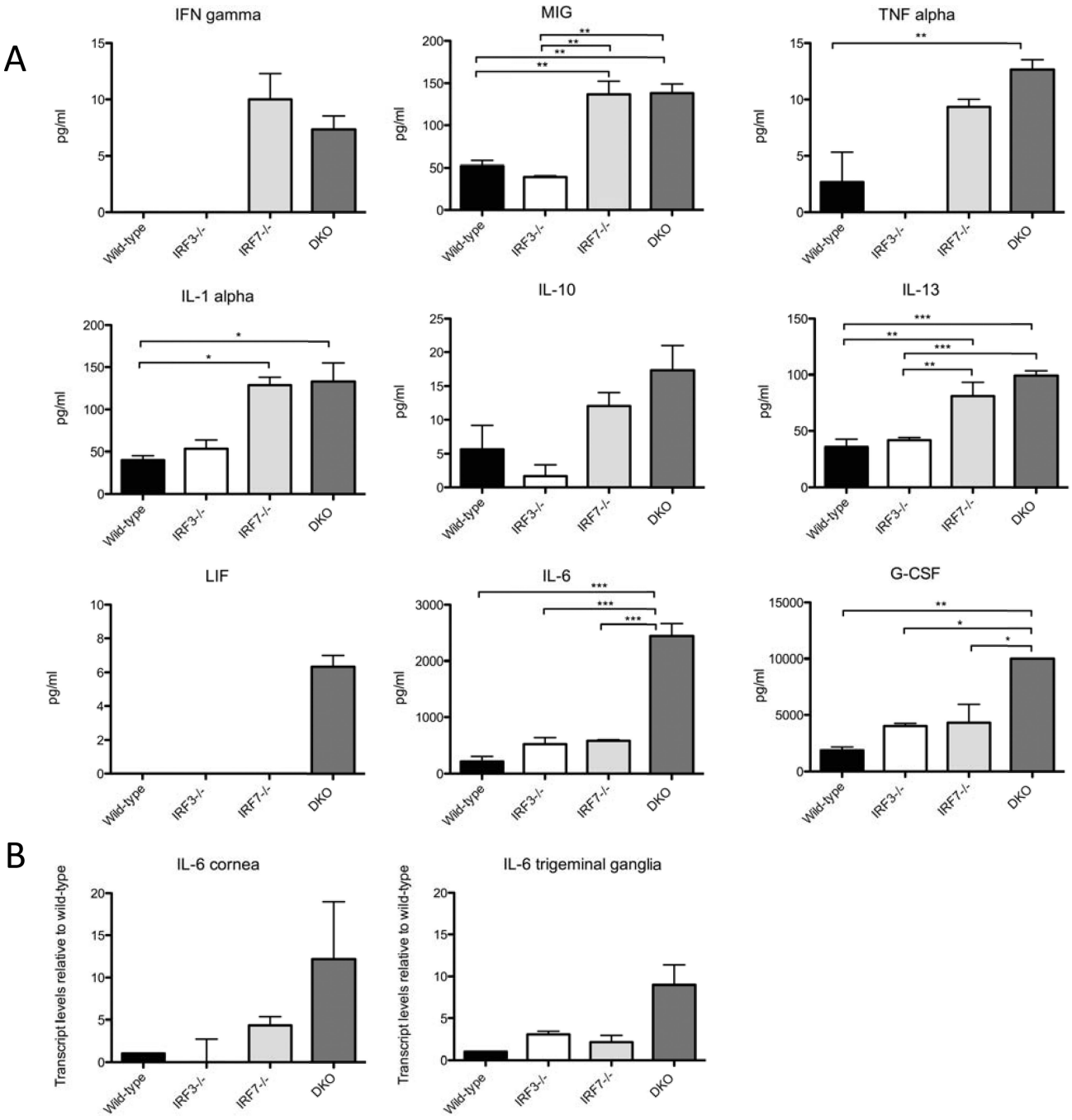

Figure 5. Cytokine analysis from sera of wild-type, IRF-3$^{-/-}$, IRF-7$^{-/-}$, and IRF3/7$^{-/-}$ mice following HSV infection

(A) Multiplex cytokine analysis of plasma from HSV-1 infected wild- type, IRF3$^{-/-}$*IRF7*$^{-/-}$ and DKO mice. Mice were infected by corneal scarification and blood was harvested 4 days pi. Average concentrations from groups of 3 mice are reported as pg/ml. Mock samples were omitted from the graphs for clarity. (B) Transcript levels of IL-6 within corneal and trigeminal tissue. RNA was extracted from corneas and trigeminal ganglia of wild-type, IRF-3$^{-/-}$, IRF-7$^{-/-}$ and DKO mice 4 days post infection with HSV-1 strain 17. After conversion to cDNA, transcript levels of IL-6 in knockout mice were compared to levels in

wild-type mice. Graphs represent means (+/− SEM) from three biological replicates. Each biological replicate was subjected to three technical replicates.