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Article

Crosstalk between the Type 1 Interferon and Nuclear Factor Kappa B Pathways Confers Resistance to a Lethal Virus Infection

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SUMMARY

Nuclear factor kappa B (NF-κB) and type 1 interferon (T1-IFN) signaling are innate immune mechanisms activated upon viral infection. However, the role of NF-κB and its interplay with T1-IFN in antiviral immunity is poorly understood. We show that NF-κB is essential for resistance to ectromelia virus (ECTV), a mouse orthopoxvirus related to the virus causing human smallpox. Additionally, an ECTV mutant lacking an NF-κB inhibitor activates NF-κB more effectively in vivo, resulting in increased proinflammatory molecule transcription in uninfected cells and organs and decreased viral replication. Unexpectedly, NF-κB activation compensates for genetic defects in the T1-IFN pathway, such as a deficiency in the IRF7 transcription factor, resulting in virus control. Thus, overlap between the T1-IFN and NF-κB pathways allows the host to overcome genetic or pathogen-induced deficiencies in T1-IFN and survive an otherwise lethal poxvirus infection. These findings may also explain why some pathogens target both pathways to cause disease.

INTRODUCTION

Upon infection, pathogen-associated molecular patterns (PAMPS) are sensed by pattern recognition receptors (PRRs), resulting in the activation of various transcription factors, prominently canonical nuclear factor kappa B (NF-κB) and the interferon regulatory factors 3 and 7 (IRF3 and IRF7) (Barnes et al., 2002; Brennan and Bowie, 2010; O'Neill, 2006).

Under resting conditions, the p105 and p65 (RelA) subunits of canonical NF-κB form a complex with the inhibitor of κB (IκB). After stimulation through various receptors, p105 is phosphorylated by activated IκB kinases (ikks) and cleaved to generate the mature p50 subunit. IκB is also phosphorylated by ikks and degraded by the proteasome. This results in the release of p50-p65, which translocates to the nucleus and binds to the promoters of many genes, including proinflammatory cytokines such as TNF-α, IL-1α, and IL-1β and various chemokines that further induce the activation of NF-κB in a positive feedback loop (Liu, 2005).

In the mouse, the type 1 interferons (T1-IFNs) are represented by one IFN-β and 12 IFN-αs (IFN-α1, IFN-α2, IFN-α4–IFN-α7, and IFN-α9–IFN-α14). The transcription factors IRF3 and IRF7 play an important role in the T1-IFN expression. IRF3 is constitutively expressed in most cells. Activation of IRF3 through most PRRs induces the transcription of IFN-α4 and IFN-β, which are known as "early" T1-IFNs. IRF7 is expressed constitutively only in some cells, such as plasmacytoid dendritic cells (pDCs) (Honda et al., 2005), but can be induced in all cells by T1-IFN (Lu et al., 2000) and possibly by other stimuli as its promoter contains an NF-κB response element (Lu et al., 2002). Activation of IRF7 through the PRR TLR9 and its adaptor MyD88 results in the rapid production of IFN-β, IFN-α4, and also non-4 IFN-α which are known as "late" TI-IFNs (Fitzgerald et al., 2003; Sharma et al., 2003).

T1-IFN signaling through the IFN-α receptor (IFNAR) results in the expression of a large number of IFN-stimulated genes (ISGs) with antiviral function. While in vitro some ISGs can be induced independently of T1-IFN and dependently (Basagoudanavar et al., 2011) or not (Hasan et al., 2013) on NF-κB, whether ISGs can be induced in vivo by stimuli other than T1-IFN is not known. Because IRF7 is itself an ISG, the T1-IFN pathway can also be regulated by a positive feedback loop in an autocrine and paracrine manner (Honda et al., 2005; Sato et al., 1998).

While T1-IFNs are known to be critical for the clearance of many viruses in vivo (Bogdan, 2000), the role of NF-κB is less clear due to the variety of defects in mice deficient in this pathway (Gerondakis et al., 2006; Weih and Caamaño, 2003). However, many viruses developed mechanisms to evade NF-κB signaling (Bowie and Unterholzner, 2008; Seet et al., 2003), suggesting that it is required for innate virus control in vivo. Importantly, the promoters of the early T1-IFNs have NF-κB response elements, which are essential for constitutive as well as early expression of IFN-β after viral infection. In contrast, they are dispensable for late expression of IFN-β in cells infected with RNA viruses (Balachandran and Beg, 2011; Basagoudanavar et al., 2011; Wang et al., 2010; Wang et al., 2007).

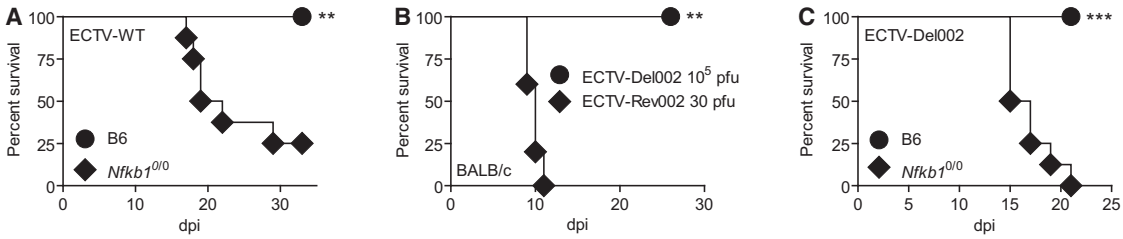

Figure 1. Role of p105 in Resistance to Mousepox and of p105bp in ECTV Virulence

Survival of B6 (n = 8, circles) and *Nfkb1*$^{0/0}$ mice (n = 8, diamonds) infected with 3,000 pfu ECTV-WT (A), BALB/c mice (n = 5) infected with 30 pfu ECTV-Rev002 (diamonds) or 100,000 pfu ECTV-Del002 (circles) (B), and B6 (n = 8, circles) and *Nfkb1*$^{0/0}$ mice (n = 8, diamonds) infected with 3,000 pfu ECTV-Del002 (C). Data in (A) and (C) are aggregates of two experiments. See also Figure S1.

Thus, rather than being separate, the NF-κB and T1-IFN pathways appear to be part of a complex antiviral network. How this networking improves virus control in vivo and why some viruses must subvert several of its components remains relatively unexplored.

The genus Orthopoxvirus (OPV) comprises many species, including variola virus, zoonotic monkeypoxvirus, the vaccine species vaccinia virus, and ectromelia virus (ECTV), a natural mouse pathogen that penetrates the body through microabrasions in the footpad and spreads through the lymphohematogenous (LH) route (Esteban and Buller, 2005; Fenner, 1949). Mousepox-susceptible mouse strains such as BALB/c cannot control virus replication and LH spread, dying 7–14 days postinfection (dpi) with high virus loads and extensive damage to the liver and spleen. Resistant mouse strains such as C57BL/6 (B6) control virus replication and LH spread through combined innate and adaptive immune mechanisms (Wallace and Buller, 1985).

OPVs encode modulators of both the NF-κB and the T1-IFN pathways (Seet et al., 2003). Recently, a protein that binds to the NF-κB subunit p105 (herein p105 binding protein, p105bp) was identified in a proteomic screening of variola virus. In transfected cells, p105bp from variola, monkeypox, cowpox, and ECTV inhibited the proteolytic processing of p105 to p50 and blocked NF-κB activation (Mohamed et al., 2009a). Moreover, a CPXV deficient in p105bp was attenuated in mice and recruited more inflammatory cells to the infected lung than the wild-type (WT) virus (Mohamed et al., 2009b). However, the effect of p105bp deficiency on the activation of NF-κB and downstream effects in vivo has not studied.

RESULTS

p105 Is Required for Resistance to Mousepox and Its Inhibition Is Essential for ECTV Virulence

Different than wild-type B6 mice, B6.Cg-*Nfkb1*^{tm1Bal}/J mice (Sha et al., 1995) in a mousepox-resistant B6 background (*Nfkb1*$^{0/0}$) succumbed to wild-type ECTV (ECTV-WT) infection (Figure 1A), and the few that survived became very ill, indicating a crucial role for canonical NF-κB in resistance to mousepox. However, *Nfkb1*$^{0/0}$ mice have severe immune defects that make them useless for in-depth studies of viral pathogenesis (Gerondakis et al., 2006). Indeed, it has been shown that most *Nfkb1*$^{0/0}$ mice lack inguinal lymph nodes (LNs) (Lo et al., 2006), and we found that all of them lack popliteal LNs (data not shown). Because the popliteal LN participates in the dissemination and the control of ECTV (Fang et al., 2008; Fang et al., 2011; Fang et al., 2010; Fenner, 1949), this finding makes *Nfkb1*$^{0/0}$ mice unsuitable for in-depth studies of NF-κB in the control of ECTV.

In ECTV, p105bp is encoded by *Evm002*, and its ability to inhibit NF-κB in cultured cells has already been demonstrated by the McFadden group using cells transfected with *Evm002* (Mohamed et al., 2009a). To elude the inherent problems of NF-κB-deficient mice, we generated an ECTV deficient in *Evm002* (ECTV-Del002; see Figure S1A available online) with the purpose of using it to study the role of NF-κB in antiviral defense in vivo. In BS-C-1 cells, ECTV-Del002 and its revertant (ECTV-Rev002; Figure S1B) replicated similarly (Figures S1C and S1D). In mousepox-susceptible BALB/c mice, ECTV-Rev002 was 100% lethal at a dose of 30 plaque-forming units (pfu), while ECTV-Del002 was nonlethal at 10^5 pfu (Figure 1B). However, 3,000 pfu ECTV-Del002 killed all *Nfkb1*$^{0/0}$ mice (Figure 1C), indicating that NF-κB activation is essential for resistance to ECTV and that p105bp is required for ECTV virulence.

Inhibition of p105/p50 Reduces the Speed and Efficiency of NF-κB Activation in the Draining LN and Liver

It has been shown that ECTV p105bp blocks NF-κB activation in transfected cells (Mohamed et al., 2009a). To compare NF-κB activation by ECTV in the presence or absence of p105bp in vivo, we transfected the livers of BALB/c mice with an NF-κB luciferase reporter plasmid using hydrodynamic injection (Liu et al., 1999). In these mice, ECTV-Del002 activated NF-κB in the liver earlier than ECTV-WT as determined by whole-body luciferase imaging (Figures 2A and 2B). Further, we could observe cells with nuclear p65 in areas of infection in the draining LN (D-LN) at 2 dpi (Figure S2A) and in the liver at 5 dpi (Figure S2B) in mice infected with ECTV-Del-002 but not with ECTV-WT. Thus, p105bp slows and decreases the efficiency of NF-κB activation in vivo.

Inhibition of p105/p50 by p105bp Accelerates ECTV LH Spread

At 3 dpi, virus titers in the D-LN, liver, and spleen were significantly lower in BALB/c mice infected with ECTV-Del002 than with ECTV-WT, suggesting that ECTV-Del002 replicated and/or disseminated less efficiently. From 5 to 7 dpi, the titers of ECTV-WT in the liver and spleen increased but those of ECTV-Del002 decreased (Figure 2C). Similar results were

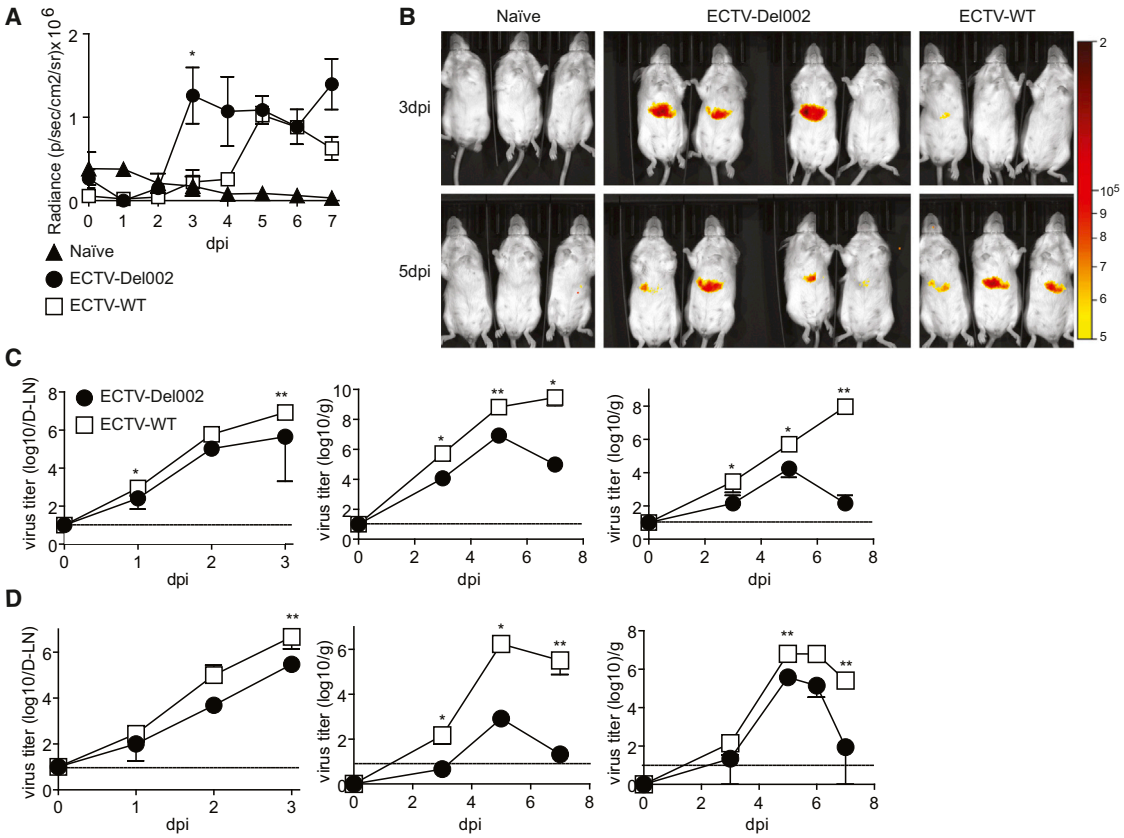

Figure 2. Role of p105bp in NF-κB Activation and Virus Spread

(A and B) In vivo NF-κB activation. The livers of BALB/c mice were transfected in vivo with an NF-κB reporter plasmid expressing firefly luciferase. Twelve days after transfection, the mice were infected with 3,000 pfu ECTV-WT in the footpad and imaged daily for 7 days 5 min after luciferin injection.

(A) Radiance (p/sec/cm^2/sr) in the liver area at the indicated dpi was determined in naive (n = 3), ECTV-Del002-infected (n = 4), and ECTV-WT-infected (n = 3) mice and plotted as mean ± SEM with significant difference indicated at 3 dpi.

(B) Radiance images of the mice in (A) at 3 and 5 dpi.

(C and D) Virus titers. BALB/c (C) or B6 (D) mice were infected in the footpad with 3,000 pfu ECTV-WT (empty squares) or ECTV-Del002 (filled circles) viruses. Virus loads were monitored in D-LN (left panels), spleen (middle panels), and liver (right panels). n = 5 mice per time point.

Data are displayed as mean ± SEM. Horizontal lines indicate limit of detection. See also Figure S2.

obtained in mousepox-resistant B6 mice, except that at 7 dpi the titers of ECTV-WT were lower than at 5 dpi while ECTV-Del002 had almost been cleared (Figure 2D). Hence, inhibition of p105/p50 by p105bp promotes LH spread and slows liver clearance in mousepox-sensitive and also in mousepox-resistant mice. While natural killer (NK) cell recruitment to the D-LN at 2–3 dpi is important to control early ECTV spread (Fang et al., 2008), the decreased LH spread of ECTV-Del002 was not due to enhanced NK cell recruitment (Figure S2C).

Inhibition of p105/p50 Decreases Transcription of Several Inflammatory Genes in the D-LN of BALB/c and B6 Mice

At 2.5 dpi, transcription of the ECTV gene *Evm166* was higher in the D-LN of ECTV-WT- than in ECTV-Del002-infected BALB/c mice indicating higher virus loads in ECTV-WT-infected mice (Figure 3A). ECTV-WT induced significantly higher transcription of early and late T1-IFNs (Figure 3B). However, ECTV-WT and ECTV-Del002 induced similar levels of the ISGs *Mx1*, *Irf7*, *Isg15*, and *Ifit3* (Figure 3C). Transcripts of proinflammatory *Il1a*, *Il1b*, *Ccl2*, *Irg1*, and *Rsad2* but not *Il6* were induced significantly more by ECTV-Del002 (Figure 3D). Results in B6 mice were similar (Figure S3) except that only the proinflammatory *Il1a* and *Irg1* were expressed higher with ECTV-Del002 (Figure S3D). Hence, while ECTV-Del002 replicated less efficiently in vivo, it induces several proinflammatory genes more effectively than ECTV-WT indicating that p105bp dampens the expression of proinflammatory genes in the D-LN. Moreover, even though ECTV-WT replicated better and induced more T1-IFN, genes traditionally considered ISGs were equally induced by both viruses suggesting that p105bp also reduces ISG expression.

Inhibition of p105/p50 In Vivo Decreases the Expression of Proinflammatory Mediators in Uninfected Cells

During in vivo infection, cytokines could be produced by the infected cells themselves and/or by uninfected cells receiving cues from those infected. However, to what extent infected and uninfected cells contribute to the overall cytokine response in vivo is unknown. ECTV-Del002 expresses EGFP and we have previously generated a fully virulent recombinant ECTV

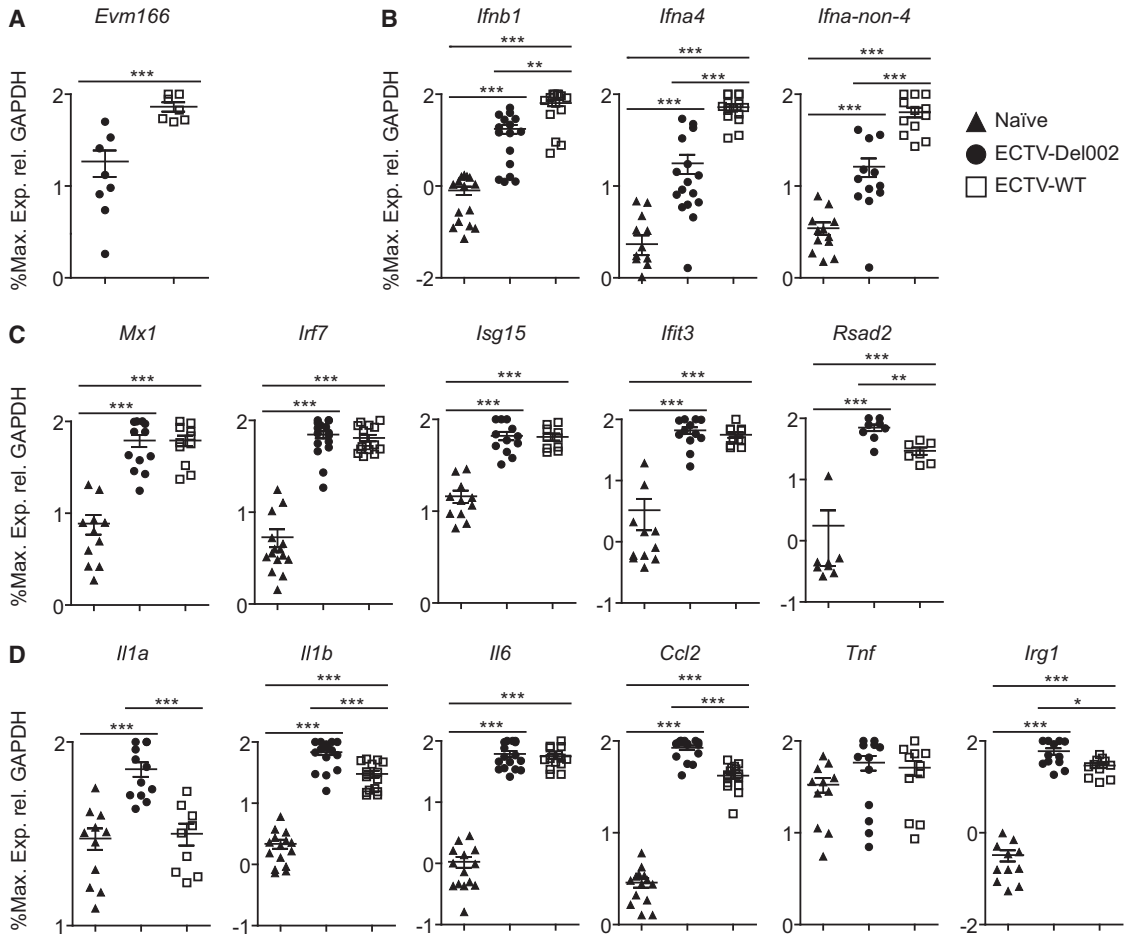

Figure 3. ECTV Inhibition of p105/p50 Results in Decreased Transcription of Inflammatory Genes in Susceptible BALB/c Mice

BALB/c mice were infected with ECTV-Del002 or ECTV-WT. At 2.5 dpi, the indicated transcripts were quantified by quantitative RT-PCR (qRT-PCR). Data correspond to four independent experiments combined. Virus load (A), T1-IFNs (B), ISGs (C), and inflammatory genes (D) are shown. Triangles, naive; circles, ECTV-Del002; squares, ECTV-WT. Data are expressed as percentage of maximum as calculated for each individual experiment and shown in a log10 scale as mean ± SEM. See also Figure S3.

expressing EGFP (ECTV-EGFP) (Fang et al., 2008). Given that p105bp is a nonstructural intracellular protein and should therefore only directly affect NF-κB in infected cells, we investigated whether in vivo, ECTV inhibition of NF-κB mainly affects the ability of EGFP$^+$ infected cells to produce cytokines and/or indirectly affects EGFP$^-$ uninfected cells to generate those mediators. To gain easy access to infected and uninfected cells, we infected mice with ECTV-Del002 or ECTV-EGFP intraperitoneally. At 2 dpi, the cells in the peritoneal cavity were obtained by lavage and stained with antibodies to various hematopoietic lineage markers. We found that for both viruses, most infected cells were CD3$^-$ DX5$^-$ B220$^-$ CD11b$^+$ as determined by EGFP expression (data not shown). Hence, we used fluorescence-activated cells sorting (FACS) to sort EGFP$^+$ CD3$^-$ DX5$^-$ B220$^-$ CD11b$^+$ and EGFP$^-$ CD3$^-$ DX5$^-$ B220$^-$ CD11b$^+$ and gene expression was determined by qRT-PCR in the two populations. Transcription of *Evm166* was strong in EGFP$^+$ cells but not in EGFP$^-$ cells independently of the virus used for the infection (Figure 4A). This confirmed that sorting based on EGFP expression distinguished infected from uninfected cells. Independently of the virus type, early as well as late T1-IFNs were expressed almost exclusively by infected cells. Unexpectedly, the proinflammatory cytokines *Il1b* and *Ccl2* were only expressed in uninfected cells. More strikingly, *Ccl2* was expressed at significantly higher levels in uninfected cells from mice infected with ECTV-Del002 (Figure 4A). This was confirmed at the protein level by flow cytometry following intracellular staining with anti-CCL2 Ab. (Figure 4B).

Inhibition of p105/p50 bp Is Required for Lethality in *Irf7*$^{0/0}$ but Not in *Tlr9*$^{0/0}$ or *Myd88*$^{0/0}$ Mice

As reported by others (Samuelsson et al., 2008; Sutherland et al., 2011), *Tlr9*$^{0/0}$ and *Myd88*$^{0/0}$ mice succumbed to ECTV-WT. These mice were also highly sensitive to ECTV-Del002, suggesting that the TLR9/MyD88 activation of NF-κB is essential for resistance to ECTV-Del002. Remarkably, mice deficient in IRF7 (*Irf7*$^{0/0}$), which is also activated by TLR9/MyD88, succumbed to ECTV-WT but not to ECTV-Del002 (Figure 5A). This indicates that a deficiency in T1-IFN production can be compensated by increased NF-κB activation through TLR9/MyD88.

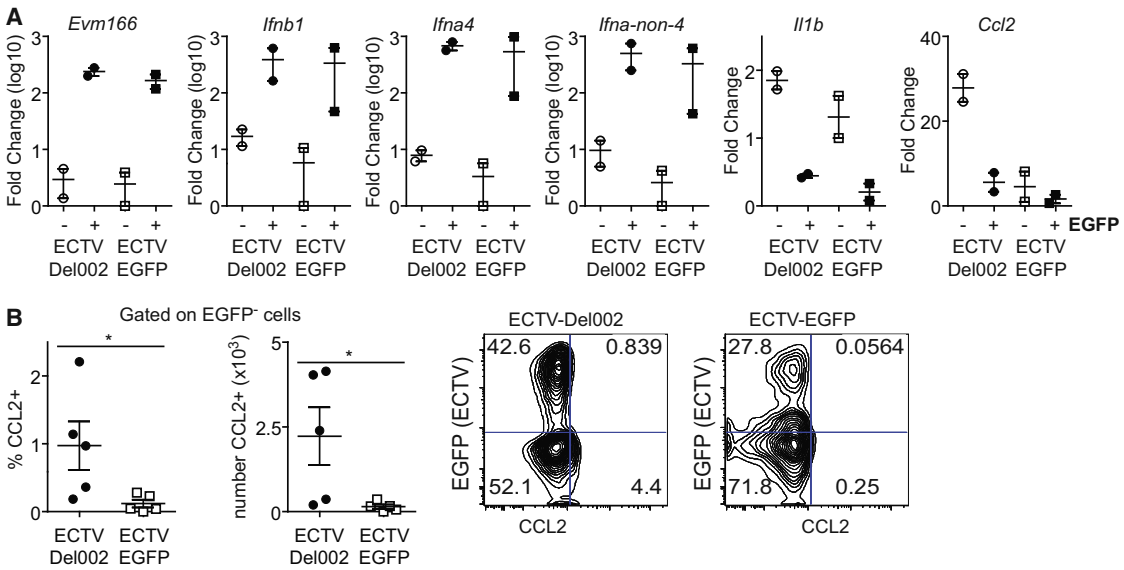

Figure 4. Inhibition of p105/p50 In Vivo Decreases Expression of Proinflammatory Mediators in Uninfected Cells

(A) Groups of six mice were infected intraperitoneally with 10^4 pfu ECTV-EGFP or ECTV-Del002. At 2 dpi, $CD3^-$ $DX5^-$ $B220^-$ $CD11b^+$ $EGFP^+$ and $CD3^-$ $DX5^-$ $B220^-$ $CD11b^+$ $EGFP^-$ peritoneal cells from pools of three mice were purified by FACS, and the expression of the indicated genes was determined by qRT-PCR. Each data point corresponds to a pool of three mice. The experiment was repeated with similar results.

(B) Left: Frequency and calculated absolute number of $CCL2^+$ cells in the $CD3^-$ $DX5^-$ $B220^-$ $CD11b^+$ F4/80$^+$ $EGFP^-$ gate as determined by flow cytometry at 2 dpi in peritoneal cells from mice infected with the indicated viruses. Each dot represents an individual mouse (n = 5). Mean ± SEM are shown. Right: Representative flow cytometry plots. Plots are gated on $CD3^-$ $DX5^-$ $B220^-$ $CD11b^+$ F4/80$^+$ cells.

Inhibition of p105/p50 Reduces Transcription of Several Inflammatory Genes in *Irf7*$^{0/0}$ Mice

At 2.5 dpi, transcription of *Evm166* was higher in the D-LN of ECTV-WT than in ECTV-Del002-infected *Irf7*$^{0/0}$ mice, indicating higher virus loads in the ECTV-WT-infected animals (Figure 5B). ECTV-WT induced significantly more early T1-IFNs, indicating that increased virus loads induce early T1-IFN independently of IRF7. In vitro, IRF7 is essential for the transcription of late T1-IFNs (Honda et al., 2005). Accordingly, neither virus induced the transcription of late T1-IFNs (Figure 5C). Both viruses induced ISGs to similar levels (Figure 5D), but proinflammatory *Il1a*, *Il6*, and *Irg1* were induced significantly more by ECTV-Del002 (Figure 5E). Thus, the enhanced transcription of proinflammatory genes in the absence of p105bp compensates for the deficient production of late T1-IFN in *Irf7*$^{0/0}$ mice promoting their resistance to ECTV-Del002.

Inhibition of Proinflammatory Gene Transcription by p105bp in the D-LN Is Independent of Virus Loads and T1-IFN Signaling

Ifnar1-deficient (*Ifnar1*$^{0/0}$) mice are known to be highly susceptible to ECTV-WT infection (Panchanathan et al., 2005; Xu et al., 2008). *Ifnar1*$^{0/0}$ mice also succumbed to ECTV-Del002 but significantly later than to ECTV-WT (Figure 5A), suggesting that the enhanced activation of NF-κB that occurs in the absence of p105bp can moderately compensate for the absence of T1-IFN signaling. Still, at 2 dpi, transcription of *Evm166* in the D-LN of *Ifnar1*$^{0/0}$ mice infected with ECTV-WT or ECTV-Del002 were indistinguishable, indicating similar virus loads (Figure 6A). Thus, we took advantage of this to determine host gene transcription without any confounding differences in virus loads or T1-IFN signaling. Both viruses induced all T1-IFNs (Figure 6B), indicating that the induction of early and late T1-IFN transcription by ECTV does not require positive feedback through IFNAR. Also, the two viruses induced the ISGs *Isg15*, *Ifit3*, and *Rsad2* to similar levels, indicating that many ISGs can be induced independently of IFNAR and probably also independently of NF-κB in vivo. *Irf7* was induced by both viruses, but expression was significantly higher in ECTV-Del002-infected mice, suggesting that the NF-κB site in *Irf7* (Lu et al., 2000) plays an important role in its induction in vivo. Expression of *Mx1* was not induced by either virus, indicating that *Mx1* is a strict ISG in the D-LN (Figure 6C). Now that virus loads were equal and T1-IFN signaling was abolished, all of the inflammatory genes tested (*Il1a*, *Il1b*, *Il6*, *Ccl2*, *Tnf*, and *Irg1*) were induced significantly higher by ECTV-Del002 (Figure 6D). Thus, NF-κB stimulates the expression of proinflammatory genes, but it is not the only transcription factor that can induce them, and its effects can be obscured by high virus loads and T1-IFN signaling after infection with ECTV-WT. We also compared gene expression at 4 dpi in the livers of *Ifnar1*$^{0/0}$ mice infected with ECTV-WT and ECTV-Del002 (Figure S4). Transcription of *Evm166* was similar, indicating similar virus loads (Figure S4A). The induction of T1-IFN was inconsistent and therefore difficult to evaluate (Figure S4B). However, transcripts for most of the ISGs (Figure S4C) and proinflammatory genes (Figure S4D) were induced significantly higher by ECTV-Del002- as compared to ECTV-WT-infected mice. Thus, when virus loads are similar and IFNAR signaling is absent, increased NF-κB activation in the liver results in stronger induction not only of proinflammatory genes, but also of many ISGs, including *Mx1*.

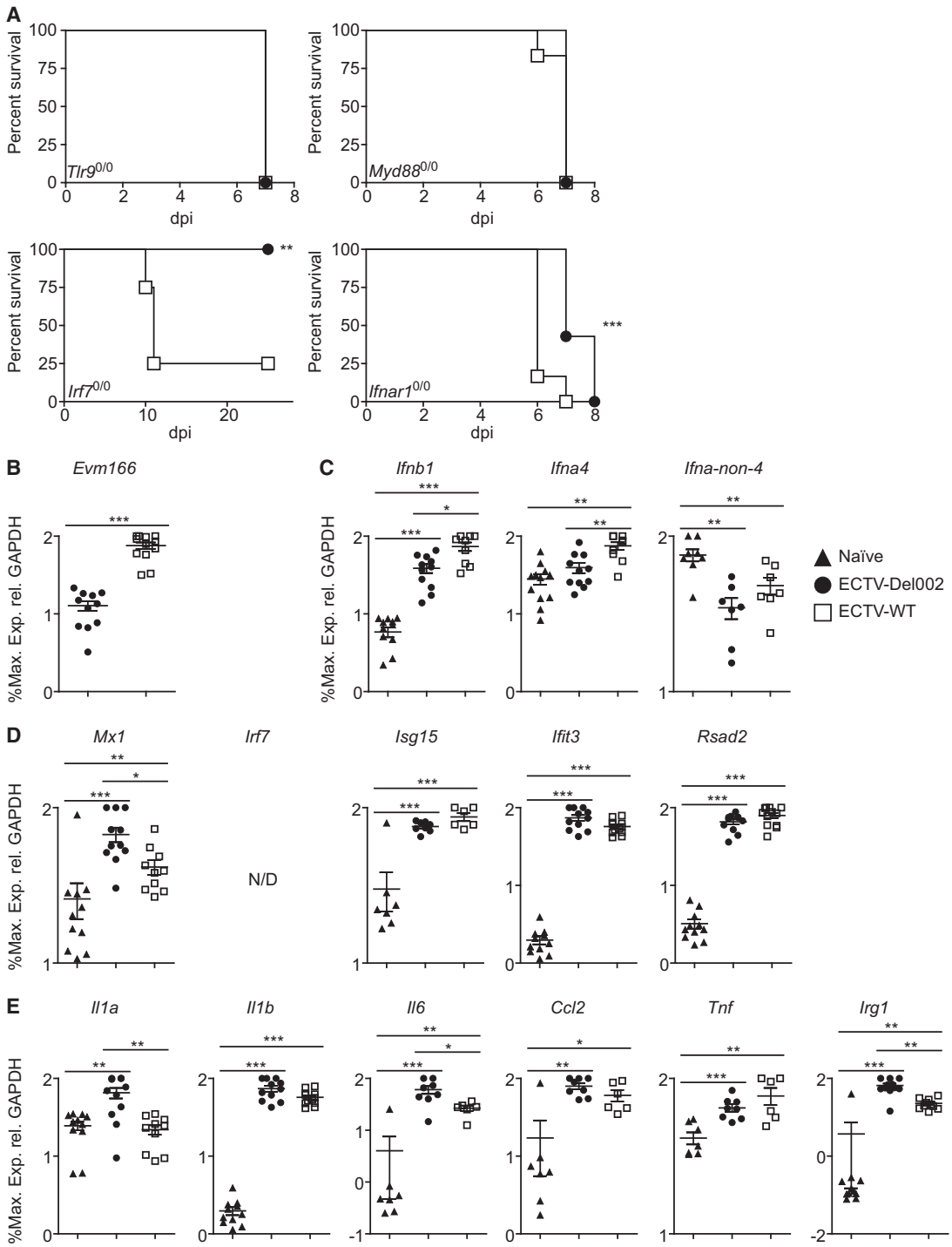

Figure 5. ECTV-Del002 Is Lethal to *Myd88*$^{0/0}$ and *Tlr9*$^{0/0}$ Mice but Not to *Irf7*$^{0/0}$ Mice, in which It Induces Higher Inflammatory Gene Expression than Lethal ECTV-WT

(A) Survival of the indicated mice. Mice (n = 5/group) infected with ECTV-Del002 (filled circles) or ECTV-WT (empty squares).

(B–E) Gene expression in *Irf7*$^{0/0}$ mice. At 2.5 dpi, gene transcription was determined as in Figure 2. The results are from three independent experiments combined. Virus (B), T1-IFNs (C), ISGs (D), and inflammatory genes (E) are shown. Triangles, naive; circles, ECTV-Del002; squares, ECTV-WT.; N/D, not detected. Data are expressed as percentage of maximum as calculated for each individual experiment and shown in a log10 scale as mean ± SEM.

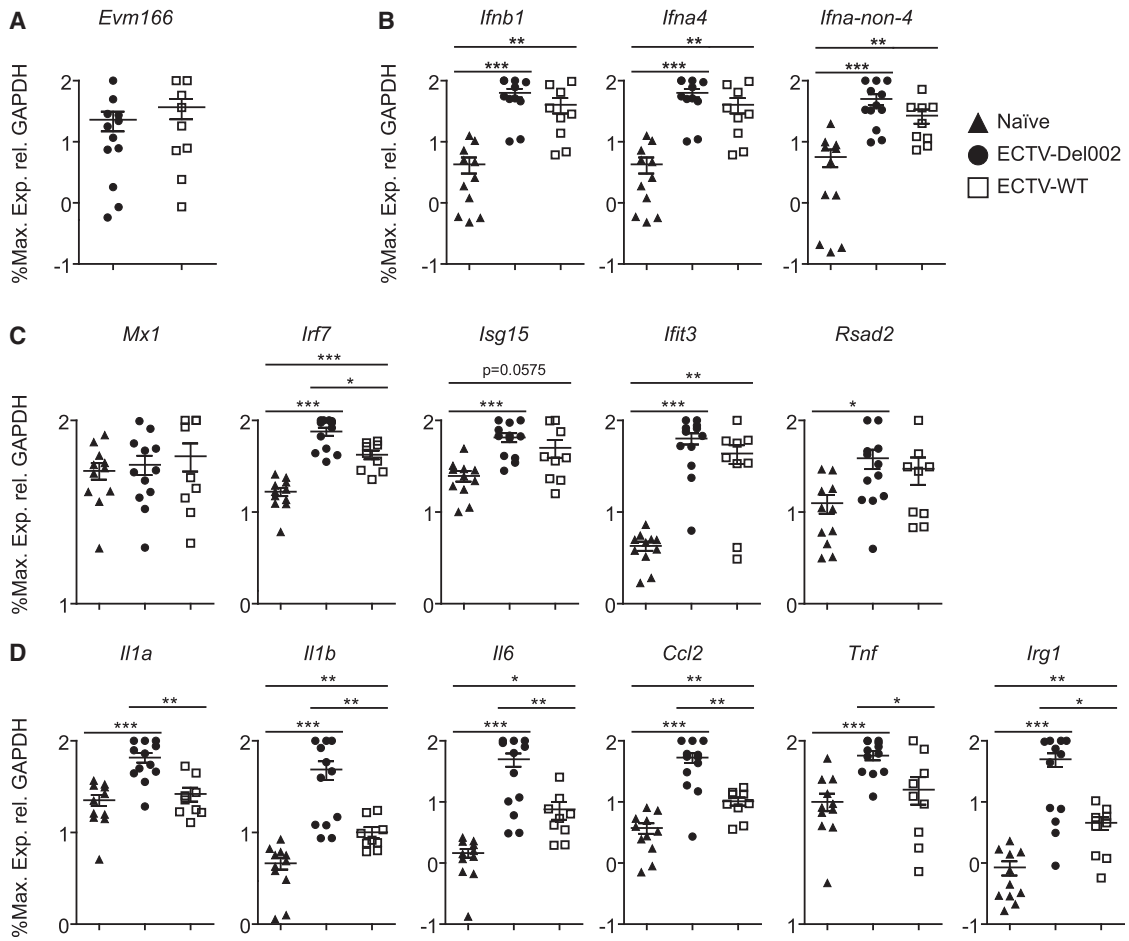

Figure 6. Reduction of Inflammatory Gene Transcription by p105bp Is Independent of Virus Loads and T1-IFN Signaling in the D-LN

Gene transcription in *Ifnar1*$^{0/0}$ mice. At 2 dpi, gene transcription determined as in Figure 2. Virus (A), T1-IFNs (CB), ISGs (C), and inflammatory genes (D) are shown. Triangles, naive; circles, ECTV-Del002; squares, ECTV-WT.; N/D, not detected. Data are expressed as percentage of maximum as calculated for each individual experiment and shown in a log10 scale as mean ± SEM. See also Figure S4.

DISCUSSION

It is well established that NF-κB helps control viral replication in cultured cells (Balachandran and Beg, 2011; Wang et al., 2010). However, while it is known that NF-κB plays a role in the control of reovirus replication in the heart (O'Donnell et al., 2005) and that *Nfkb1*$^{0/0}$ mice are more resistant than WT mice to encephalomyocarditis virus (Sha et al., 1995), to what extent the induction of proinflammatory genes by the transcription factor NF-κB contributes to virus control in vivo is less clear (Gerondakis et al., 2006). Also, whether the T1-IFN and NF-κB pathways cooperate or overlap to control an infection in vivo is unknown. Studies to understand the role of canonical NF-κB activation in the innate control of viruses using mutant mice have been hampered by the embryonic lethality of *Rela*$^{0/0}$ mice and the multifocal innate and adaptive immune defects of *Nfkb1*$^{0/0}$ mice (Gerondakis et al., 2006). This problem became evident in our own studies where we found that *Nfkb1*$^{0/0}$ mice lack popliteal LNs, which play an important role in the normal pathogenesis of ECTV. Thus, while our experiments with *Nfkb1*$^{0/0}$ demonstrate that canonical NF-κB is essential to control ECTV, in-depth studies of the role of the pathway in vivo were not possible with these mice. Of note, *Nfkb1*$^{0/0}$ mice succumbed to WT and ECTV-Del002, but slower than other susceptible strains. This suggests that in the absence of popliteal D-LN, viral spread occurred by a slower alternative route, such as direct hematogenous spread from the footpad. Therefore, we took the alternative approach of deleting the canonical NF-κB inhibitor p105bp from ECTV and found that ECTV-Del002 was pathogenic in *Nfkb1*$^{0/0}$ mice but was severely attenuated in mousepox susceptible BALB/c mice. Hence, we used ECTV-Del002 and ECTV-WT as probes to help unravel how the NF-κB and T1-IFN pathway interact in vivo.

We found that p105bp inhibition of NF-κB accelerated the spread of the virus from the D-LN to the spleen and liver and also enhanced virus replication in the D-LN and liver. Also, p105bp decreased translocation of the NF-κB p65 subunit to the nucleus of cells in the D-LN and liver and delayed the activation of NF-κB in the liver of BALB/c mice. Thus, despite its lower loads, ECTV-Del002 induces NF-κB more efficiently at the organ level than ECTV-WT, suggesting that the early activation NF-κB that occurs in the absence of p105bp takes place in the infected

cell, which then releases fewer virions, and/or that in the absence of p105bp, infected cells increase the production of inflammatory factors to further enhance NF-κB activation and the antiviral state in other cells.

We have previously shown that the recruitment of NK cells to the D-LN at 2 dpi is essential for the ability of B6 mice to resist mousepox (Fang et al., 2008; Fang et al., 2011; Fang et al., 2010). Further, we showed that a severely attenuated ECTV lacking a T1-IFN decoy receptor recruited more NK cells to the D-LN than ECTV-WT (Xu et al., 2008). However, this did not occur with ECTV-Del002. Thus, while both T1-IFN and NF-κB activation are essential to clear ECTV, their individual roles in the D-LN appear to be different.

We analyzed the transcriptional activity induced by the two viruses in mousepox-susceptible and -resistant mice and found that the increased virus loads in the presence of p105bp correlated with increased T1-IFN, but this did not translate into a difference in the transcription levels of ISGs. Consequently, effective ISG induction in vivo can be mediated by NF-κB independently of T1-IFN. This is consistent with previous reports showing that NF-κB induces a subset of ISGs in mouse embryo fibroblasts infected with RNA virus (Basagoudanavar et al., 2011). Regardless of the lower virus loads, absence of p105bp in ECTV-Del002 resulted in stronger stimulation of inflammatory genes. Given that p105bp is intracellular (Mohamed et al., 2009a, 2009b), this finding could indicate that enhanced NF-κB activation and increased cytokine production and virus control occurred in the infected cells themselves in the absence of p105bp. Alternatively, slight changes in NF-κB activation and cytokine expression by the infected cells could provide cues to uninfected cells that amplified the response by producing proinflammatory cytokines. However, we found that only infected cells produced early and late T1-IFNs. This was remarkable because early T1-IFNs in infected cells are thought to stimulate the autocrine and paracrine production of late T1-IFN. The absence of a paracrine T1-IFN positive feedback loop could be due to the secreted T1-IFN decoy receptor expressed by ECTV (Smith and Alcami, 2002; Xu et al., 2008). Interestingly, there were no differences in T1-IFN production between ECTV-WT and ECTV-Del002 in infected cells, suggesting that the activation of NF-κB does not play a major direct role in inducing T1-IFN expression in OPV-infected cells in vivo. This is consistent with previous findings that in cultured cells, NF-κB is important for the constitutive expression of IFN-β or for its upregulation soon after infection but not for its long term expression (Balachandran and Beg, 2011; Basagoudanavar et al., 2011; Wang et al., 2010; Wang et al., 2007). More remarkable, only uninfected cells produced detectable amounts of the proinflammatory cytokines that we tested (we could not test more due to limitations in the amount of RNA we could obtain). Notably, *Ccl2* was induced significantly more in uninfected cells from ECTV-Del002-infected mice than those of ECTV-WT-infected mice. These data strongly suggests that uninfected cells have a major role in producing at least some proinflammatory mediators upon receiving signals induced by NF-κB activation in the infected cells. While these signals remain unidentified, they are unlikely to be T1-IFNs as cells infected with both viruses expressed similar levels of T1-IFN. Of note, in addition to p105bp, poxviruses encode multiple proteins that interfere with NF-κB activation (Mohamed and McFadden, 2009). Thus, ECTV-Del002 may still not fully activate NF-κB in infected cells, and this may be the reason why cells infected with either virus do not produce detectable amounts of cytokines. Nevertheless, the amount of NF-κB induced by ECTV-Del002 appears to be sufficient to generate enough signals to stimulate paracrine cytokine production.

Despite the lower virus loads and T1-IFN transcription in the D-LN, enhanced NF-κB activation in the absence of p105bp resulted in efficient induction of ISGs and resistance of BALB/c mice to mousepox. Thus, we hypothesized that stronger NF-κB activation could overcome a defect in T1-IFN production. We compared resistance to ECTV-WT and ECTV-Del002 in mice with deficiencies in different genes involved in T1-IFN induction. As previously reported, *MyD88*$^{0/0}$- and *TLR9*$^{0/0}$-deficient mice were highly susceptible to ECTV-WT (Samuelsson et al., 2008; Sutherland et al., 2011). These mice were also susceptible to ECTV-Del002. We also found that IRF7, the transcription factor responsible of T1-IFN induction downstream of TLR9/MyD88, was essential for resistance to ECTV-WT. Strikingly, *Irf7*$^{0/0}$ mice were fully resistant to ECTV-Del002, indicating that enhanced NF-κB activation can overcome suboptimal T1-IFN production. Of note, *Irf3*$^{0/0}$ mice were resistant to ECTV-WT (data not shown), suggesting that endosomal TLR9 but not cytosolic PRRs are essential for innate recognition and T1-IFN production during ECTV infection. Of interest, *Irf7*$^{0/0}$ but not *Irf3*$^{0/0}$ mice also succumb to herpes simplex virus 1 (HSV-1) infection (Honda et al., 2005), suggesting that IRF7 but not IRF3 may be generally required for resistance to DNA virus infections. Virus loads in the D-LN at 2.5 dpi were higher in *Irf7*$^{0/0}$ mice infected with ECTV-WT than with ECTV-Del002, correlating with early *Ifna4* and *Ifnb1* induction. Late T1-IFNs were not induced indicating that early T1-IFNs are sufficient to provide protection from mousepox when NF-κB is efficiently activated. As in BALB/c and B6 mice, most ISGs were induced to similar levels by both viruses, and several proinflammatory genes were induced stronger with ECTV-Del002. Therefore, IRF7 is dispensable to induce many ISGs in vivo.

A complication when comparing transcription levels induced by virulent and attenuated viruses is that the differences in virus loads and T1-IFN levels could affect transcription. Thus, we analyzed gene expression in *Ifnar1*$^{0/0}$ mice, which are susceptible to both viruses and do not signal T1-IFN. Analysis of virus loads in the D-LN at 2 dpi by qRT-PCR indicated that both viruses replicated similarly. In the absence of T1-IFN signaling and with similar virus loads, the ISGs *Isg15*, *Ifit3*, and *Rsad2*, traditionally considered to be T1-IFN dependent (Chin and Cresswell, 2001; Fensterl and Sen, 2011; Okumura et al., 2008), were induced to similar levels by both viruses. Hence, in vivo, most ISGs can be induced by viral infection independently of both T1-IFN and NF-κB signaling. This finding is consistent with a recent report showing IFNAR1- and NF-κB-independent induction of ISGs in culture cells (Hasan et al., 2013). On the other hand, *Mx1* in the D-LN was fully dependent on T1-IFN signaling. Consistent with an NF-κB site on its promoter (Lu et al., 2002), *Irf7* was induced more potently in the absence of NF-κB inhibition. Moreover, all proinflammatory genes were induced more potently by ECTV-Del002. Analysis of the liver at 4 dpi showed similar virus loads with both viruses. However, while both viruses induced all tested ISGs (including *Mx1*),

transcription was generally stronger with ECTV-Del002. Therefore, in the liver, ISGs can be induced independently of T1-IFN signaling but, different to the D-LN, NF-κB activation can increase their transcription. ECTV-Del002 also induced all proinflammatory genes more potently. Of note, *Tnf* was potently induced in the liver. This finding contrast with the low level of induction of this cytokine in the D-LN, indicating that the induction of *Tnf* by virus infection is highly tissue specific.

In summary, our results show that NF-κB induction of proinflammatory genes helps to control ECTV in the D-LN and reduce LH spread even when the induction of T1-IFN is defective. Furthermore, we found tissue-specific effects of NF-κB activation in the liver, because absence of p105bp resulted not only in a more potent proinflammatory response, but also in increased T1-IFN-independent induction of ISGs. Our data also demonstrate that both T1-IFN and NF-κB are essential to survive ECTV infection. However, because T1-IFN and NF-κB have overlapping functions, enhanced NF-κB activation can compensate a partial deficiency in T1-IFN production to control the virus. Our work also shows that, as previously demonstrated in cultured cells (Balachandran and Beg, 2011; Basagoudanavar et al., 2011), NF-κB can induce ISGs independently of T1-IFN in vivo. Moreover, work with cultured cells has also demonstrated T1-IFN/NF-κB independent induction of ISGs (Dixit et al., 2010; Hasan et al., 2013). Our work shows that this also occurs upon viral infection in vivo. This suggests an even more complex network of gene activation during viral infection and hints that this is the reason why pathogenic viruses must target multiple components of the T1-IFN and NF-κB pathways to cause disease.

EXPERIMENTAL PROCEDURES

Viruses

Virus stocks were made in BS-C-1 cells as before (Xu et al., 2012). Infections were performed with lysates clarified by centrifugation. ECTV-Del002 was generated by homologous recombination replacing *Evm002* for enhanced green fluorescent protein driven by the 7.5 promoter (Figure S1A) as previously described for *Evm166* (Xu et al., 2008). ECTV-Rev002 was generated with a modified transient dominant selection protocol (Falkner and Moss, 1988). In brief, we reintroduced *Evm002* to its original location by homologous recombination using a plasmid encoding *E. Coli* Guanine-xanthine phosphoribosyltransferase phosphotransferase (GPT) and mCherry outside the recombination site (Figure S1B). More details can be found in the legend of Figure S1.

Mice and Animal Experiments

All protocols involving mice were approved by the FCCC Institutional Animal Care and Use Committee. BALB/c (BALB/cAnNTac) and B6 (C57BL/6NTac) mice were purchased from Taconic. All other mice were bred at the FCCC from original breeders obtained from various sources. B6.Cg-Nfkb1tm1Bal/J ($Nfkb1^{0/0}$) mice were from Jackson Laboratories, Ifnar1-deficient mice in a 129S2/SvPas background (Müller et al., 1994) were from Dr. R. Schreiber (St Louis, MO), B6;129P2-Irf7tm1Ttg/TtgRbrc ($Irf7^{0/0}$) mice were from Dr. T. Taniguchi (Honda et al., 2005), and B6.129-Tlr9tm1Aki/Obs ($TLR9^{0/0}$) and B6.129-Myd88tm1Aki/Obs ($Myd88^{0/0}$) mice were originally produced by Dr. S. Akira (Adachi et al., 1998; Hemmi et al., 2000) and were generously provided by Dr. R. Finberg (Worcester, MA). All mice used in experiments were 5–12 weeks old. Infections were performed in the footpad with 3,000 pfu unless otherwise indicated. For in vivo bioluminescence, the livers of BALB/c were hydrodynamically transfected (Chang et al., 2001; Liu et al., 1999) with a plasmid containing the firefly luciferase gene driven by the NF-κB promoter as described (Gross and Piwnica-Worms, 2005). Transfected mice were rested 12 days and then infected with ECTV-WT or ECTV-Del002. Mice were imaged daily for the presence of Firefly luciferase activity with an IVIS LXR system (Caliper Life Sciences) 5 min after injection of luciferase substrate as indicated by the manufacturer (Caliper Life Sciences). Cell sorting was performed with a BD Vantage sorter. qPCR of transcripts in organs was performed as before (Xu et al., 2012) except that we used transcript specific oligonucleotides and Probelibrary probes (Roche) (Table S1) and a 2X FastStart Universal Probe Master with ROX (Roche). To combine independent experiments in order to increase the statistical power of the analysis, we normalized the values for each sample by GAPDH expression. The sample with the highest numerical value was considered 100%, and the remaining data were calculated accordingly. Data were analyzed with Prism 5 software (GraphPad Software). We analyzed the survival curves with a log rank (Mantel-Cox) test and the qPCR experiments with a non parametric t test. In all figures, $^{*}p < 0.05$, $^{**}p < 0.01$, and$^{***}p < 0.001$. All experiments were repeated a minimum of two times, and most were repeated at least three times.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.04.015.

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