The I κ B Kinases Restrict Human Cytomegalovirus Infection

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ABSTRACT Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus that causes disease in immunosuppressed populations. HCMV has a complex relationship with innate immune signaling pathways. Specifically, HCMV has been found to block some aspects of inflammatory signaling while benefiting from others. Through analysis of knockout cell lines targeting the NF- κ B regulatory kinases I κ B kinase α (IKK α) and IKK β , we find that the IKKs are host restriction factors that contribute to cytokine-mediated resistance to viral infection, limit the initiation of HCMV infection, and attenuate viral cell-to-cell spread. The HCMV U_L26 protein is a viral immune modulator important for HCMV infection that has been shown to inhibit host cell NF- κ B signaling, yet it has remained unclear how U_L26-mediated NF- κ B modulation contributes to infection. Here, we find that U_L26 modulation of NF- κ B signaling is separable from its contribution to high-titer viral replication. However, we find that IKK β is required for the induction of cytokine expression associated with Δ U_L26 infection. Collectively, our data indicate that the IKKs restrict infection but HCMV targets their signaling to modulate the cellular inflammatory environment.

IMPORTANCE Innate immune signaling is a critical defense against viral infection and represents a central host-virus interaction that frequently determines the outcomes of infections. NF- κ B signaling is an essential component of innate immunity that is extensively modulated by HCMV, a significant cause of morbidity in neonates and immunosuppressed individuals. However, the roles that various facets of NF- κ B signaling play during HCMV infection have remained elusive. We find that the two major regulatory kinases in this pathway, IKK α and IKK β , limit the initiation of infection, viral replication, and cell-to-cell spread. In addition, our results indicate that these kinases contribute differently to the host cell response to infection in the absence of a virally encoded NF- κ B inhibitor, U_L26. Given the importance of NF- κ B in viral infection, elucidating the contributions of various NF- κ B constituents to infection is an essential first step toward the possibility of targeting this pathway therapeutically.

KEYWORDS IKK, NF- κ B, cytomegalovirus, innate immunity

Cells express a variety of sensors that are capable of triggering a diverse array of innate immune responses during viral infection. These immune mechanisms stimulate the production of antiviral effectors and induce inflammatory responses that can attenuate productive viral replication. Consequently, viruses have evolved viral effectors that inhibit, co-opt, or otherwise evade host cell immune responses to facilitate their proliferation and transmission. The various interactions between host cell immune signaling pathways and viral immune modulators are key determinants that can dictate the outcomes of infections. One of the viruses known to have a complex relationship with the host innate immune response is human cytomegalovirus (HCMV), a prevalent opportunistic betaherpesvirus that establishes latent infection and persists in the host indefinitely (1, 2). HCMV, the largest human herpesvirus, has an ~235-kb genome encoding over 200 viral genes (3). A significant portion of this viral coding potential is

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FIG 1 CRISPR-mediated inactivation of IKK α and IKK β . (A) The schematic demonstrates canonical and noncanonical NF- κ B signaling. (B) BJ/hTert cells were transduced with either an untargeted CRISPR control plasmid or a CRISPR construct targeting either IKK α (encoded by the CHUK gene) or IKK β (encoded by the IKKB gene). Monoclonal cell lines with frameshift mutations in both IKK α alleles (position -1 and -37 deletions) or both IKK β alleles (position +1 and +1 insertions) were isolated via clonal selection. (C) Total cell lysates from CRISPR control, IKK α ^{-/-}, and IKK β ^{-/-} cells were Western blotted with the indicated antibodies. (D) A known number of CRISPR control, IKK α ^{-/-}, and IKK β ^{-/-} cells were seeded and allowed to grow for 3 days, at which point viable cell counts were assessed by trypan blue exclusion staining ($n \geq 3$). *, $P < 0.05$; ns, not significant.

dedicated to targeted manipulation of the host cell innate immune response to support infection (reviewed in references 4 and 5).

One of the most prominent immune pathways targeted by HCMV during infection is NF- κ B signaling. The NF- κ B proinflammatory pathway is a broad signaling architecture that can be induced by various stimuli, including irradiation, infection, and cytokine exposure. The canonical form of NF- κ B signaling is induced by the cytokine tumor necrosis factor alpha (TNF- α) and results in the phosphorylation and activation of the kinase I κ B kinase β (IKK β) (Fig. 1A), which targets the I κ B repressor protein for proteasomal degradation and frees the NF- κ B transcription factors RelA and p50 to translocate to the nucleus and to alter cellular gene transcription (6). Noncanonical NF- κ B signaling is controlled by IKK α activation and relies on the RelB and p52 transcription factors to alter transcription (Fig. 1A) (7). HCMV has a nuanced relationship with the NF- κ B pathway, and multiple reports indicate that HCMV can both induce and inhibit NF- κ B activity in various contexts and at different times during infection. Early HCMV infection is characterized by activated NF- κ B signaling (8, 9). Furthermore, pharmacological inhibition or expression of dominant negative forms of IKK β restrict productive replication (10–12). Conversely, HCMV strongly inhibits canonical NF- κ B signaling at later stages of infection via multiple mechanisms, including the inhibition of IKK phosphorylation and receptor-interacting protein kinase 1 (RIP1) deubiquitination (13–15). The contributions that various aspects of NF- κ B signaling make to early and late stages of viral infection remain poorly understood.

The U_L26 protein was found previously to be a viral inhibitor of NF- κ B signaling (13). The U_L26 open reading frame has two in-frame start codons, resulting in both large

(27-kDa) and small (21-kDa) isoforms, which are both delivered with the tegument upon initial infection and expressed *de novo* during the viral life cycle, with early expression kinetics (16, 17). Early during infection, U_L26 is required for maximal transcriptional activation of the viral major immediate early promoter and localizes to the nucleus of the host cell (16, 18). As infection progresses, U_L26 exits the nucleus and is recruited to cytoplasmic virion assembly centers, where it has been shown to be required for the formation of stable virions with properly phosphorylated tegument constituents (18). Studies utilizing HCMV mutant strains lacking the U_L26 open reading frame have shown that loss of U_L26 during infection results in growth defects, including an ~90% reduction in productive viral replication and significantly reduced cell-to-cell spread (18, 19). U_L26 also plays a crucial role in attenuating host cell innate immune pathways. Previous work in our laboratory showed that U_L26 is necessary and sufficient for the inhibition of TNF- α -induced NF- κ B signaling during infection and the absence of U_L26 during viral infection is linked to multiple NF- κ B phenotypes, including inhibition of IKK phosphorylation, stabilization of the I κ B repressor, and increased nuclear accumulation of the noncanonical transcription factor RelB (13).

Here, to assess the contributions of canonical and noncanonical NF- κ B signaling to HCMV infection, we employed clustered regularly interspaced short palindromic repeat (CRISPR) methodology to generate cell lines with targeted knockouts of the key NF- κ B kinases IKK α and IKK β . We report that the IKKs act as antiviral restriction factors that limit HCMV infection. In addition, we explored the contributions that IKK α and IKK β make to the growth defect associated with a U_L26 deletion mutant. Analysis of various U_L26 mutants suggests that U_L26 inhibition of NF- κ B signaling is insufficient for wild-type (WT) levels of viral replication, indicating that U_L26 possesses distinct functions that contribute to productive infection. With respect to NF- κ B signaling, we find that IKK β mediates the induction of NF- κ B target genes during Δ U_L26 infection. Taken together, these results highlight antiviral roles for the kinases IKK α and IKK β that limit HCMV infection, and we identify IKK β as a substantial contributor to the inflammatory transcriptional state inhibited by U_L26.

RESULTS

Loss of IKK α and IKK β attenuates HCMV sensitivity to TNF- α treatment. The IKKs play central roles in the signal transduction cascades of canonical and noncanonical NF- κ B signaling (Fig. 1A). We employed CRISPR to generate monoclonal cell lines with knockouts of either IKK α or IKK β , to determine the contributions that the IKKs make to HCMV infection. Frameshift mutations were introduced into both alleles of either the conserved helix-loop-helix ubiquitous kinase (CHUK) gene encoding IKK α (position -1 and -37 deletions) or the IKBKB gene encoding IKK β (position +1 and +1 insertions) (Fig. 1B). In both cases, we observed total loss of the targeted IKK protein in each cell line. There was an observable decrease in the accumulation of the nontargeted IKK protein (e.g., reduced IKK α was evident upon knockout of IKK β) (Fig. 1C). Cell viability of the IKK α and IKK β knockout cell lines was assessed via the trypan blue exclusion cell viability assay, and we observed ~2-fold growth reduction in cells lacking IKK α expression, whereas no discernible defect was observed in IKK β ^{-/-} cells.

RelA and RelB are prominent transcription factors that are strongly associated with canonical and noncanonical NF- κ B signaling, respectively. To analyze how loss of the IKKs perturbs the expression of downstream NF- κ B pathway constituents, we assessed the accumulation of these transcription factors in our CRISPR control and IKK knockout cell lines. We found that loss of IKK α strongly limited the accumulation of RelA and RelB in both mock-infected and HCMV-infected cells, whereas both RelA and RelB still accumulated to WT levels in the absence of IKK β (Fig. 2A). These observations suggest that IKK α is required for optimal expression of the RelA and RelB NF- κ B transcription factors.

TNF- α pretreatment of cells has been shown to negatively influence the ability of HCMV to establish initial lytic infection and to form plaques in human fibroblasts (20). To determine the extent to which the IKKs contribute to this viral sensitivity to TNF- α

FIG 2 IKK inactivation alters the accumulation of NF- κ B transcription factors and reduces viral sensitivity to TNF- α . (A) Serum-starved CRISPR control, IKK α ^{-/-}, and IKK β ^{-/-} cells were mock infected or infected with WT HCMV at a MOI of 3.0. Cell lysates were collected at 48 hpi and Western blotted with the indicated antibodies. (B and C) Confluent, serum-starved CRISPR control, IKK α ^{-/-}, or IKK β ^{-/-} cell lines were mock treated or treated with either 10 ng/ml TNF- α (B) or 5 U/ml IFN- γ (C) for 4 h prior to infection with 40 PFU of WT-GFP HCMV. Relative plaque formation was measured by dividing the cytokine-treated plaque number for each virus by the number of plaques for that virus on the mock-treated cell line (CRISPR control, IKK α ^{-/-}, or IKK β ^{-/-}) and plotted as a percentage. Error bars indicate standard errors ($n \geq 4$). **, $P < 0.01$; ns, not significant.

treatment, we assessed the impact of TNF- α treatment on viral plaque formation efficiencies in our CRISPR control and IKK knockout cell lines. We observed that treatment of CRISPR control cells with 10 ng/ml TNF- α reduced viral plaque formation to 40% of the rate seen in nontreated cells (Fig. 2B). In IKK α ^{-/-} cells, the rate of plaque formation increased to 60% of that in nontreated controls. Further, this ratio increased to 80% in IKK β ^{-/-} cells (Fig. 2B). These results suggest that both IKK α and IKK β contribute to the antiviral effects of TNF- α treatment. However, the plaque formation efficiency in both IKK knockout cell lines after TNF- α challenge was still reduced, relative to non-TNF- α -treated cells (Fig. 2B), suggesting that, in each case, the presence of the reciprocal IKK could contribute to the TNF- α -induced antiviral state. Interferon gamma (IFN- γ) has also been shown to inhibit the establishment of HCMV lytic infection in human fibroblasts, putatively through JAK/STAT signaling (21). In contrast to the findings for TNF- α challenge, we found that knockout of each IKK had little impact on the plaque formation efficiency of HCMV on cells pretreated with 5 U/ml IFN- γ (Fig. 2C). Collectively, these data indicate that the IKKs are important for HCMV-associated sensitivity to TNF- α treatment but are largely dispensable for the antiviral signaling associated with IFN- γ treatment.

IKK α and IKK β restrict HCMV viral replication. The relationship between NF- κ B signaling and the HCMV life cycle is complex. HCMV induces IKK activity, which is important for viral replication in certain contexts (11, 12, 22). However, NF- κ B activation has been found to inhibit HCMV infection in other contexts (21, 23, 24). Therefore, we wanted to explore how IKK α and IKK β contribute to HCMV viral growth. We found that knockout of either IKK increased low-multiplicity of infection (MOI) viral replication by ~1 log unit, compared to growth in CRISPR control cells, as assessed by 50% tissue culture infective dose (TCID₅₀) analysis (Fig. 3A). Analysis of viral DNA accumulation during low-MOI infection showed an ~4-fold increase in viral DNA replication in the absence of IKK α (Fig. 3B). Upon inactivation of IKK β , a much smaller but still statistically significant increase in viral DNA abundance was also evident (Fig. 3B). The increased viral DNA abundance observed in the absence of IKK α correlated with an increase in the accumulation of viral gene products observed in these cells (Fig. 3C), most notably in the true late protein pp28. In contrast, IKK β -deficient cells did not display a substantial change in the accumulation of viral immediate early or late proteins (Fig. 3D). Notably, this difference in viral gene expression did not affect the production of infectious viral progeny, as the loss of either IKK β or IKK α increased production of infectious virions to similar extents, relative to CRISPR control cells. These results demonstrate that IKK α and IKK β each play a role in restricting HCMV infection and reducing the viable progeny produced during viral replication but each kinase affects viral gene expression differently.

FIG 3 IKK α and IKK β restrict WT HCMV infection. (A) Confluent, serum-starved CRISPR control, IKK α ^{-/-}, and IKK β ^{-/-} fibroblasts were infected with WT-GFP HCMV at a MOI of 0.1. Viral supernatants were harvested at 2, 6, and 8 dpi, and infectious viral progeny were quantified via TCID₅₀ assay. The indicated statistical comparisons were made using Student's *t* test, and results are color coded for each cell line relative to the control cells. *, *P* < 0.05; **, *P* < 0.01; #, *P* = 0.056. (B) Viral DNA replication of WT HCMV was measured at 6 dpi in CRISPR control, IKK α ^{-/-}, and IKK β ^{-/-} fibroblasts infected as in panel A (*n* ≥ 3). *, *P* < 0.05; **, *P* < 0.01. (C and D) Confluent CRISPR control and IKK α ^{-/-} fibroblasts (C) or IKK β ^{-/-} fibroblasts (D) were infected with WT HCMV as in panel A. Cells were harvested at the indicated time points and Western blotted with antibodies directed against IE2, pp28, and GAPDH. IE2 and pp28 blots are displayed with both light and dark exposures.

Previously, we found that the U_L26 protein is necessary and sufficient to attenuate TNF- α -induced NF- κ B activity and that U_L26-deficient HCMV (Δ U_L26) is more susceptible to challenge with TNF- α treatment (13). To determine whether either of the IKKs was functioning in a U_L26-specific manner during viral infection, we assessed viral growth of a U_L26-deficient green fluorescent protein (GFP)-positive HCMV mutant in our CRISPR IKK knockout cell lines (Fig. 4A). We found that loss of either IKK increased Δ U_L26 infectious virion production by approximately 1 log unit at 6 days postinfection (dpi) (Fig. 4A), which was similar to the effect observed for GFP-positive WT (WT-GFP) HCMV (Fig. 3A). The difference in infectious virion production between IKK-deficient and control cells was reduced by day 8 (Fig. 4A). Viral DNA accumulation was also affected somewhat similarly by the loss of the IKKs, with an ~9-fold increase in the amount of viral DNA present in IKK α ^{-/-} cells and an almost 2-fold increase in IKK β ^{-/-} cells. Concomitantly, during Δ U_L26 infection of the IKK α ^{-/-} cells, we observed significantly more production of the late viral protein pp28 (Fig. 4C). Levels of the pp28 protein were also elevated in IKK β knockout cells during Δ U_L26 infection (Fig. 4D), albeit to a lesser extent. The accumulation of the immediate early viral protein IE2 is enhanced in IKK α ^{-/-} cells but is unaffected in IKK β -deficient cells (Fig. 4C and D). The elevated expression of pp28 at later time points during Δ U_L26 infection in the IKK β ^{-/-} cells differs from WT infection of the same cells (Fig. 3D), i.e., IKK β inactivation did not substantially increase pp28 accumulation. This suggests the possibility that, in the absence of U_L26, IKK β may be limiting DNA replication and the expression of viral late genes. However, the observation that the loss of the IKKs increased WT and Δ U_L26 viral

FIG 4 IKK α and IKK β restrict Δ U_L26 infection. (A) Confluent, serum-starved CRISPR control, IKK α ^{-/-}, and IKK β ^{-/-} fibroblasts were infected with Δ U_L26-GFP HCMV at a MOI of 0.1. Viral supernatants were harvested at 2, 6, and 8 dpi, and infectious viral progeny were quantified via TCID₅₀ assay. The indicated statistical comparisons were made using Student's *t* test, and results are color coded for each cell line. *, *P* < 0.05. (B) Viral DNA replication of Δ U_L26-GFP HCMV was measured at 6 dpi in CRISPR control, IKK α ^{-/-}, and IKK β ^{-/-} fibroblasts infected as in panel A. Values are means $\pm$ standard errors (*n* $\geq$ 3). **, *P* < 0.01. (C and D) Confluent CRISPR control and IKK α ^{-/-} fibroblasts (C) or IKK β ^{-/-} fibroblasts (D) were serum starved and infected with Δ U_L26 HCMV as in panel A. Cells were harvested at the indicated time points and Western blotted with antibodies directed against IE2, pp28, and GAPDH. IE2 and pp28 blots are displayed with both light and dark exposures.

replication to similar extents ($\sim$ 10-fold at 6 dpi) suggests that the IKKs influence overall viral replication in a largely U_L26-independent manner.

The U_L26 domains required for high-titer viral replication are dispensable for inhibition of TNF- α -induced NF- κ B activation. Previously, we generated a panel of HCMV mutant strains encoding truncated forms of the U_L26 protein and examined the contributions of the various U_L26 domains to infection (Fig. 5A) (19). To determine whether specific U_L26 domains are required for both NF- κ B modulation and high-titer viral replication, we analyzed these mutants with respect to their ability to inhibit NF- κ B signaling. In contrast to Δ U_L26-infected cells, cells infected with recombinant viruses encoding N-terminal- and C-terminal-truncated U_L26 isoforms were sufficient to inhibit TNF- α -induced phosphorylation of the IKK complex during infection (Fig. 5B). Similarly, in the absence of infection, transient expression of either the N-terminal or the C-terminal U_L26 truncation mutant was sufficient to inhibit TNF- α -induced NF- κ B-dependent luciferase activity (Fig. 5C and D). Collectively, these data suggest that the N- and C-terminal domains of U_L26 are not necessary for attenuation of TNF- α -induced NF- κ B activation. Further, given that the U_L26 Δ C mutant inhibits NF- κ B signaling but has a replication defect similar in magnitude to that of the Δ U_L26 null mutant (19), these data indicate that the U_L26 sequences necessary for high-titer replication are separable from those necessary for prevention of TNF- α -induced NF- κ B activation.

IKK α and IKK β restrict initiation of infection and cell-to-cell spread. To further explore the roles the IKKs play during viral infection, we analyzed how the loss of IKK α or IKK β activity affected various plaque phenotypes. We found that knockout of either

FIG 5 The U_L26 domains required for viral growth are separable from those required for TNF- α -induced NF- κ B activation. (A) The diagram shows the truncations made to the U_L26 open reading frame to generate the U_L26 Δ N and U_L26 Δ C mutants. (B) Serum-starved, confluent fibroblasts were infected with WT, U_L26 Δ N, U_L26 Δ C, or Δ U_L26 HCMV at a MOI of 3.0. At 48 hpi, cells were subjected to mock treatment or 10 ng/ml TNF- α treatment for 10 min. Cell protein was then harvested and analyzed via Western blotting with the indicated antibodies. (C) HEK 293T cells were transiently transfected with an NF- κ B-dependent luciferase expression construct, a *Renilla* luciferase expression construct, and either an empty vector (EV) or an expression vector encoding the U_L26WT, U_L26 Δ N, or U_L26 Δ C isoform. At 24 hpi, cells were either mock treated or treated with 10 ng/ml TNF- α for 24 h prior to being harvested and subjected to a luciferase assay. Values are means $\pm$ standard errors ($n = 3$). Firefly luciferase activity was normalized to *Renilla* luciferase activity. *, $P < 0.05$; **, $P < 0.01$. (D) HEK 293T cells transfected with U_L26 mutant expression constructs as in panel C were harvested at 48 hpi and Western blotted with antibodies specific to U_L26 or tubulin.

IKK α or IKK β resulted in an $\sim$ 2-fold increase in the incidence of WT plaque formation (Fig. 6A). Similarly, treatment with 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1), a pan-IKK inhibitor at the concentrations employed (25), also induced WT plaque formation by $\sim$ 2-fold (Fig. 6B). Plaque formation of the Δ U_L26 virus was affected differentially in response to individual IKK deletions. First, knockout of IKK β induced a 3-fold increase in Δ U_L26 plaque formation efficiency, relative to control cells; this was higher than the level observed for the WT virus (Fig. 6A and C), suggesting that, in the absence of U_L26, IKK β restricts plaque formation to a greater extent. A more notable U_L26-specific difference was that the knockout of IKK α did not increase the plaque formation of the Δ U_L26 virus (Fig. 6C), which suggests that, in this context, U_L26 is necessary for HCMV to benefit from the absence of IKK α . When both IKKs were inhibited via TPCA-1 treatment, the plaque formation efficiency of the Δ U_L26 virus increased 2-fold (Fig. 6D), similar to WT infection.

In addition to measuring the impact on plaque formation efficiency, we measured the role that the IKKs play in cell-to-cell spread via an analysis of plaque size. WT HCMV plaques increased in size in the absence of the IKKs but to differing degrees. The size of WT viral plaques was enhanced $\sim$ 2-fold in the IKK α knockout cell line, while the IKK β knockout cell line exhibited a plaque size increase of almost 5-fold (Fig. 6E). Similar increases in Δ U_L26 plaque size were observed in both IKK α and IKK β knockout cells, which exhibited $\sim$ 2-fold and $\sim$ 5-fold increases, respectively (Fig. 6F). These results suggest that, regardless of the presence of U_L26, IKK β restricts HCMV cell-to-cell spread to a greater extent than does IKK α and their individual contributions to plaque initiation can largely be separated from their contributions to cell-to-cell spread. In contrast to the knockout of either IKK alone, treatment with TPCA-1, which inhibits both IKKs, did not affect WT cell-to-cell spread, as evidenced by equal plaque sizes (Fig. 6G). Notably, Δ U_L26 plaque sizes increased by $\sim$ 2.5-fold in the presence of TPCA-1 (Fig. 6H). These results suggest the possibility that, during WT infection, the potential benefits

FIG 6 IKKα and IKKβ limit the initiation and cell-to-cell spread of HCMV infection. (A) CRISPR control, IKKα^{-/-}, and IKKβ^{-/-} fibroblasts were grown to confluence, serum starved, and infected with 40 PFU of WT-GFP HCMV for analysis of plaque phenotypes. The average efficiencies of plaque formation in IKK knockout fibroblasts were normalized to CRISPR control cell values ($n \geq 4$). (B) Confluent, serum-starved MRC5 fibroblasts were either mock treated or treated with 10 μ M TPCA-1 for 2 h before being infected with 25 PFU of WT-GFP HCMV. The average plaque formation efficiency under each condition was normalized to the number of plaques in mock-treated cells ($n \geq 4$). (C) CRISPR control, IKKα^{-/-}, and IKKβ^{-/-} fibroblasts were infected as in panel A with ΔU_L26-GFP HCMV. The average efficiencies of plaque formation in IKK knockout fibroblasts were normalized to CRISPR control cell values ($n \geq 4$). (D) MRC5 fibroblasts were treated with TPCA-1 and infected as in panel B with ΔU_L26-GFP HCMV. Values were normalized as in panel B ($n \geq 4$). (E and F) CRISPR control, IKKα^{-/-}, and IKKβ^{-/-} fibroblasts were infected as in panel A with either WT-GFP (E) or ΔU_L26-GFP (F) HCMV. Individual plaque sizes were quantified using ImageJ software, averaged, and normalized to the average plaque size in CRISPR control cells ($n = 40$). (G and H) MRC5 fibroblasts were infected as in panel B with WT-GFP (G) or ΔU_L26-GFP (H) HCMV. Plaques for each condition were quantified using ImageJ software, averaged, and normalized to the average plaque size in untreated cells ($n \geq 19$). (I, J, K, and L) The CRISPR control and a heterogeneous pool composed of multiple CRISPR IKKβ^{-/-} cell lines were serum starved and infected as in panel A for plaque formation (I and J) and plaque size (K and L) analyses. Plaque formation efficiencies were normalized as in panel A ($n \geq 4$). Plaque size was measured and normalized as in panel E ($n \geq 20$). All values are plotted as means $\pm$ standard errors, and all statistical comparisons were made using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

associated with losing one IKK are masked by the simultaneous loss of the other IKK. This is not seen in the context of ΔU_L26 infection, however, where dual inhibition of the IKKs in the absence of the NF- κ B regulator U_L26 still increases the extent of cell-to-cell spread.

To rule out the possibility that the increased plaque formation and size phenotypes observed in the original $IKK\beta^{-/-}$ cell line were a result of clonal artifacts, we assessed these phenotypes in a polyclonal pool of newly generated $IKK\beta$ clones in which we combined six individual monoclonal $IKK\beta^{-/-}$ clones in equal numbers. Upon assessment of WT and ΔU_L26 plaque formation (Fig. 6I and J), we observed similar increases in plaque formation efficiency, with plaque formation increases of 1.5-fold and 2.5-fold for HCMV WT and ΔU_L26 viruses, respectively (Fig. 6I and J). Similarly, cell-to-cell spread for both WT and ΔU_L26 viruses was also increased ~ 3 - to 4-fold in this pooled population, only a slight reduction in comparison with the ~ 4 - to 5-fold increases observed in the monoclonal $IKK\beta^{-/-}$ cell line (Fig. 6K and L). These findings imply that clonal selection effects are not a likely explanation for the $IKK\beta$ -related plaque formation phenotypes we observe. Collectively, our results indicate that the IKKs restrict the initiation of infection and restrict cell-to-cell spread.

Contributions of $IKK\alpha$ and $IKK\beta$ to ΔU_L26 innate immune phenotypes. The ΔU_L26 virus is more vulnerable to TNF- α challenge than WT HCMV (13). To assess the extent to which the IKKs contribute to viral TNF- α sensitivity in a U_L26 -dependent manner, we assessed viral plaque formation in our IKK knockout cell lines in the face of TNF- α or IFN- γ challenge (Fig. 7A and B). We found that loss of either of the IKKs modulated ΔU_L26 sensitivity to TNF- α treatment, with the $IKK\beta$ knockout having a larger effect than the $IKK\alpha$ knockout and exhibiting approximately twice the number of plaques formed upon TNF- α treatment, relative to the control cells (Fig. 7A). In contrast, knockout of either $IKK\alpha$ or $IKK\beta$ had little effect on the plaque formation efficiency after treatment with IFN- γ (Fig. 7B). The increase in plaque formation efficiency observed in the $IKK\alpha$ or $IKK\beta$ knockout cells, relative to control cells, was slightly higher in the context of ΔU_L26 infection, compared to WT HCMV infection (Fig. 7C). However, the effect was very modest and not statistically significant, suggesting that IKK activity does not have a substantially larger inhibitory effect on plaque formation efficiency in the absence of U_L26 .

Previously, we found that ΔU_L26 infection induces noncanonical NF- κ B activation, including inducing the nuclear translocation of RelB, which is typically mediated by $IKK\alpha$ (Fig. 1A) (reviewed in reference 26). In addition, expression and secretion of the antiviral cytokine interleukin 6 (IL-6), a canonical NF- κ B target gene, are induced during ΔU_L26 infection (Fig. 7D) (13). To elucidate how $IKK\alpha$ and $IKK\beta$ contribute to ΔU_L26 -mediated induction of IL-6, we measured the accumulation of IL-6 mRNA during WT and ΔU_L26 infection. $IKK\alpha$ knockout cells showed notably higher levels of IL-6 transcription during mock infection. This large induction of IL-6 transcription was preserved during infection in the absence of U_L26 but not WT infection. Loss of $IKK\beta$, however, entirely ablated the transcription of IL-6 across all mock infection and infection conditions (Fig. 7D). To corroborate these findings, we utilized the IKK inhibitor TPCA-1 to impede IKK signaling during infection with either WT or ΔU_L26 HCMV (MOI of 3.0), and we measured the IL-6 mRNA abundance at 48 h postinfection (hpi). We observed that TPCA-1 inhibition had little effect on the transcription of IL-6 during WT infection but significantly reduced IL-6 mRNA abundance, almost 2-fold, during ΔU_L26 infection (Fig. 7E). These results indicate that the IKKs both regulate the accumulation of the downstream NF- κ B target gene IL-6 and that $IKK\beta$ in particular is required for the upregulation of IL-6 during infection in the absence of U_L26 .

DISCUSSION

The NF- κ B pathway has a complex relationship with HCMV infection. Studies have shown that HCMV can induce NF- κ B activation (9, 27, 28), and viral replication in certain cellular contexts is reduced by pharmaceutical inhibition or expression of dominant negative IKK constructs (11, 12, 22). Conversely, aspects of the NF- κ B response are

FIG 7 IKK β is required for ΔU_L26 -mediated induction of IL-6 expression. (A and B) Confluent, serum-starved CRISPR control, IKK $\alpha^{-/-}$, or IKK $\beta^{-/-}$ cell lines were mock treated or treated with either 10 ng/ml TNF- α (A) or 5 U/ml IFN- γ (B) for 4 h prior to infection with 40 PFU of ΔU_L26 -GFP HCMV. Relative plaque formation was measured by dividing the cytokine-treated plaque number for each virus by the number of plaques for that virus on the mock-treated cell line (CRISPR control, IKK α , or IKK β) and plotted as percentage $\pm$ standard error ($n \geq 4$). (C) The plaque formation efficiencies of TNF- α -challenged WT-GFP (Fig. 2B) and ΔU_L26 -GFP (Fig. 6A) HCMV in IKK $\alpha^{-/-}$ and IKK $\beta^{-/-}$ cell lines were divided by the plaque formation efficiency of each virus in TNF- α -challenged CRISPR control cells to determine the ratio representing the extent to which loss of each IKK rescued viral sensitivity to TNF- α . Error bars indicate standard errors ($n \geq 4$). (D) CRISPR control, IKK $\alpha^{-/-}$, and IKK $\beta^{-/-}$ fibroblasts were grown to confluence, serum starved, and either mock infected (M) or infected with WT or ΔU_L26 HCMV at a MOI of 3. Cellular RNA was harvested at 48 hpi, and the abundance of IL-6 transcript was measured by real-time PCR and normalized to that of GAPDH. Values are plotted as means $\pm$ standard errors ($n \geq 3$). (E) Confluent, serum-starved MRC5 fibroblasts were either mock treated or treated with 10 μ M TPCA-1 for 2 h before being infected with WT or ΔU_L26 HCMV at a MOI of 3. Cellular RNA was harvested and assayed for IL-6 expression at 48 hpi as in panel D ($n \geq 4$). All statistical comparisons were made using Student's t test. *, $P < 0.05$; **, $P < 0.005$; ns, not significant.

downregulated by HCMV infection (15, 29), and various gene products, such as U_L26 , have been found to inhibit NF- κ B activation (13, 30, 31). Further, stimulation of NF- κ B signaling with cytokines such as TNF- α decreases viral replicative success (20). Collectively, these data suggest that monolithic declarations about the impact of NF- κ B signaling and HCMV infection are insufficient. This insufficiency arises in part from the associated complexity of the NF- κ B signaling architecture, which includes various

related but distinct pathways that can result in substantially different transcriptional responses (32). Another contributing factor is the complexity of HCMV biology. In all likelihood, the virus tailors its interactions with the NF- κ B pathway to favor a lytic or latent infection, depending on the cellular context and both external and internal cues.

To begin to deconvolute some of the issues raised above, we utilized CRISPR-mediated mutagenesis to assess how the key NF- κ B regulatory kinases IKK α and IKK β contribute to HCMV replication in fibroblasts. In total, our results indicate that both IKKs restrict lytic HCMV infection, although they functionally diverge with respect to several viral phenotypes. Our results indicate that inactivation of either IKK α or IKK β increases low-MOI HCMV replication by ~ 10 -fold (Fig. 3A). In addition to viral replication, inactivation of either IKK increased the ability of HCMV to initiate infection and to form a plaque, increasing plaque formation efficiency to a similar extent (Fig. 6A). In these contexts, our results indicate that both IKK α and IKK β restrict HCMV infection. Notably, the accumulation of viral progeny during high-MOI infection (MOI of 3.0) in IKK $\alpha^{-/-}$ or IKK $\beta^{-/-}$ fibroblasts was not increased, relative to CRISPR control cells (data not shown), suggesting that the antiviral contributions of the IKKs are MOI dependent.

Although the increases in viral replication and plaque formation efficiency in CRISPR IKK $\alpha^{-/-}$ or IKK $\beta^{-/-}$ cells are similar in magnitude, the IKKs have divergent effects on viral protein accumulation during infection. Loss of IKK α increased the accumulation of viral immediate early and late proteins, relative to control cells, which contrasts with the IKK β knockout cells, in which there was no detected difference in viral protein accumulation (Fig. 3C and D). The similar magnitudes of increased replication between the IKK β and IKK α knockout cells suggest that this increase in viral protein expression in the IKK α knockout cells does not functionally contribute to the production of viral progeny in this context. In addition to differentially affecting viral gene expression, the knockout of IKK α and IKK β appears to disparately influence viral spread between cellular neighbors, as plaques in IKK $\beta^{-/-}$ cells were substantially larger than those observed in IKK $\alpha^{-/-}$ cells (Fig. 6E). These differential effects on viral protein accumulation and cell-to-cell spread point to IKK α and IKK β contributing to aspects of viral infection via different mechanisms. It is well established that IKK α and IKK β can direct different NF- κ B transcriptional programs via differential activation of the RelA and RelB transcription factors (Fig. 1A) (reviewed in reference 32), raising the possibility that these unequal contributions to HCMV infection could be mediated through divergent NF- κ B transcriptional programs regulated by IKK α or IKK β .

Deletion of the U_L26 reading frame is associated with an ~ 10 -fold defect in viral replication (18). More recently, we found that the U_L26 protein inhibits NF- κ B signaling and that the Δ U_L26 virus induces the nuclear translocation of RelB and is sensitive to treatment with TNF- α (13). Here, we explore the contributions of IKK α and IKK β to the Δ U_L26 replication defect. We find that inactivation of either IKK α or IKK β increases Δ U_L26 viral replication, although the extent of the increase is similar to that observed for WT HCMV, i.e., ~ 10 -fold (Fig. 3A and Fig. 4A). This similarity suggests that inactivation of IKK α or IKK β is not sufficient to rescue the replication defect specifically associated with the absence of U_L26. Our current analysis of various U_L26 mutants also provides evidence that the modulatory role of U_L26 in NF- κ B signaling is independent of its contributions to viral replication. Specifically, we find that a C-terminal U_L26 deletion mutant that replicates with the same kinetics as a Δ U_L26 virus (19) is still capable of blocking TNF- α -induced IKK phosphorylation and activation of NF- κ B (Fig. 5B and C). Collectively, these data suggest that U_L26-mediated modulation of NF- κ B activity is likely separable from its contribution to high-titer viral replication.

Despite the likely independence of the NF- κ B modulatory activities of U_L26 from its contributions to *in vitro* viral replication, the interplay between U_L26 and NF- κ B is still of particular interest, given the critical roles that NF- κ B and innate immunity play in determining the outcome of infection in a natural host. In this regard, the impact of IKK knockout on U_L26-associated NF- κ B phenotypes can provide insight into this interaction. Previously, we found that the Δ U_L26 mutant induces the expression and secretion of IL-6, a well-described NF- κ B target (33). Here, we find that the inactivation of IKK β ,

but not IKK α , blocks the induction of IL-6 expression that is associated with ΔU_L26 infection (Fig. 7D). These data suggest that IKK β and, by extension, canonical NF- κ B signaling are critical mediators of the aberrant NF- κ B transcriptional environment induced by ΔU_L26 infection, and they provide further evidence that U_L26 targets canonical NF- κ B signaling for inhibition during infection. Further, the accumulation of pp28 was significantly increased in IKK $\beta^{-/-}$ cells (Fig. 4D), a phenomenon not observed during WT infection of the same cells. This suggests that an interaction between IKK β signaling and U_L26 can alter the kinetics of late gene expression during infection.

We found previously that, in contrast to WT infection, ΔU_L26 infection induces the nuclear accumulation of RelB (13), consistent with activation of noncanonical NF- κ B signaling. In combination with the ability of U_L26 to inhibit TNF- α -induced NF- κ B activity, these results suggest that U_L26 can inhibit both canonical and noncanonical NF- κ B signaling. It remains to be determined whether U_L26 -mediated modulation of both canonical and noncanonical NF- κ B signaling occurs via targeting of an NF- κ B component capable of modulating both pathways or whether U_L26 affects the activity of more than one NF- κ B-associated signaling factor. Our current results indicate that inactivation of IKK α increases the efficiency with which WT HCMV forms plaques but U_L26 is necessary for this increase, i.e., the ΔU_L26 mutant does not display this increased plaque formation efficiency in IKK $\alpha^{-/-}$ cells (Fig. 6C). Given our results linking U_L26 -mediated NF- κ B gene expression with IKK β , the simplest explanation for this could be that IKK β -activated NF- κ B signaling in the absence of U_L26 , e.g., leading to increased IL-6 expression, compensates for the absence of IKK α during ΔU_L26 infection in this context. Generally speaking, potential compensation by one IKK for the loss of the other must be considered when making conclusions about the role that the IKKs play during HCMV infection.

In summary, our results indicate that IKK α and IKK β are important host determinants of viral growth, infection initiation, and cell-to-cell spread. We report that the IKKs contribute differently to WT and ΔU_L26 infection, with IKK β playing a specific role in shaping the transcriptional response to ΔU_L26 viral infection, whereas IKK α has an independent role in downregulating the accumulation of viral immediate early and late gene products. Moving forward, it will be important to elucidate the role that the HCMV-NF- κ B interaction plays in other infectious contexts, including other cell types important to pathogenesis, as well as during latency and reactivation. Viral targeting of innate immune pathways is a critical contributor to successful viral infection. Elucidating the mechanisms through which innate immune signaling limits viral replication, as well as the means by which HCMV subverts these mechanisms, will likely continue to provide novel avenues for antiviral therapeutic intervention.

MATERIALS AND METHODS

Cell culture, viruses, and viral infection. MRC5 fibroblasts (ATCC CCL-171), human embryonic kidney (HEK) 293T cells, and telomerase-expressing BJ fibroblasts (generated via lentiviral transduction, as described previously [34]) were cultured at 37°C in Dulbecco's modified Eagle serum (DMEM) (Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 4.5 g/liter glucose, and 1% penicillin-streptomycin (Life Technologies), in a 5% (vol/vol) CO₂ atmosphere. For seeding and cell growth assays, cell viability was determined via the trypan blue exclusion method, with a TC10 automated cell counter (Bio-Rad). The WT strain of HCMV used in this work was BADwt, a bacterial artificial chromosome (BAC) clone of AD169 (35). The ΔU_L26 -DBmet Δ (referred to here as ΔU_L26), $U_L26\Delta N$, and $U_L26\Delta C$ recombinant viruses were previously generated from this BAC as described (19). GFP-expressing AD169 (BADsubUL21.5, referred to as WT-GFP) and ΔU_L26 with a transposon insertion (ΔU_L26 -TI, referred to as ΔU_L26 -GFP) were used for plaque formation efficiency and plaque size experiments and have been described previously (19, 36). Unless otherwise indicated, viral infection was performed as follows: cells were grown to a confluence of $\sim 3.2 \times 10^4$ cells per cm² in a 6-well plate and were serum starved for 24 h prior to infection, after which viral inocula were added for an adsorption period of 1.5 h and then replaced with fresh serum-free medium. Cells were infected at a MOI of 3.0 unless otherwise indicated. Culturing in serum-free medium prior to infection was employed to minimize the effects of serum on cellular signaling, as well as to synchronize and to control for cell cycle progression in the cell population. Viral stocks were propagated in MRC5 fibroblasts. For titrating of viral stocks, as well as experiments involving the assessment of viral replication in CRISPR cell lines, viral titers were determined via TCID₅₀ analysis.

Lentiviral plasmids. CRISPR-targeted IKK knockout in BJ/hTert cells was mediated by the following guide RNAs: IKK α , 5'-ACAGACGTTCCTCCGAAGCCGC-3'; IKK β , 5'-ACCACCGCTCTCGGTCCGG-3'. These sequences were determined by inserting early segments of the CHUK (IKK α) and IKBKB (IKK β) open reading frames into the Zhang Lab online generator (<http://crispr.mit.edu>). Guide RNAs were appended with flanking homology arms and cloned into the LentiCRISPRv2 Cas9 expression platform according to the Zhang Lab single guide RNA protocol (37, 38). CRISPR control cells were generated via lentiviral transduction of the unmodified LentiCRISPRv2 plasmid.

Lentiviral transduction and cell line generation. Pseudotyped lentivirus was produced in 293T cells seeded in 10-cm dishes at a density of 2×10^6 cells per cm² and grown for 24 h prior to transfection with 2.6 μ g lentiviral vector, 2.4 μ g PAX2, and 0.25 μ g vesicular stomatitis virus G glycoprotein expression plasmid, using Fugene 6 (Promega). The medium was aspirated after 24 h, and 4 ml fresh medium was added to the plate. After another 24 h, the supernatant was filtered through a 0.45- μ m filter and applied to fibroblast cells in the presence of 5 μ g/ml Polybrene (Millipore), and the cells were incubated for 24 h. The medium was refreshed and the cells were allowed to recover for 72 h prior to selection with 1 μ g/ml puromycin (VWR). Following transduction with guide RNA-expressing lentivirions and subsequent puromycin selection, BJ/hTert fibroblasts were clonally selected by seeding individual cells in 96-well plates. CRISPR control cells transduced with non-guide RNA-containing LentiCRISPRv2 were retained as heterogeneous polyclonal populations. Genomic DNA was harvested from monoclonal lines, and the guide RNA-targeted region of each IKK was amplified by PCR using the following primers: IKK α forward primer, 5'-GGGGACTTAAAGAGCGGACC-3'; IKK α reverse primer, 5'-CCCCACTGATATCATATGGCCT-3'; IKK β forward primer, 5'-TGGATGGATAGAAATGAGGTGGG-3'; IKK β reverse primer, 5'-TAGAAGCAGCAGAGTCACCGT-3'. To verify the presence of indels in the IKK α /IKK β reading frames of both alleles in clonally isolated cell lines, the amplified PCR product was gel purified, ligated into the pCR2.1-TOPO TA vector using a TA cloning kit (Invitrogen), according to manufacturer's instructions, and transformed into DH10B competent cells. Bacterial colonies were grown and color selected on LB plates containing 50 μ g/ml kanamycin and supplemented with 40 μ l each of 100 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and 40 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), spread over the surface of the plate. Plasmids were isolated from colorless colonies containing the PCR inserts and sequenced using M13 forward (position -20) and M13 reverse primers. CRISPR-mediated indel generation in the genomic IKK α and IKK β reading frames was also confirmed via TIDE analysis (39). In addition to the monoclonal IKK α and IKK β cell lines, an IKK β ^{-/-} pooled cell line was created by combining equivalent cell numbers of multiple IKK β ^{-/-} clones with the following frameshift deletions: positions -7 and +1, positions +1 and +10, and positions +1 and +1.

Immunoblotting. Cells were harvested in disruption buffer (50 mM Tris [pH 7.0], 2% SDS, 5% 2-mercaptoethanol, and 2.75% sucrose), and the proteins were solubilized via sonication and boiling and separated by 10% SDS-PAGE. Samples were transferred to a nitrocellulose membrane in Tris-glycine transfer buffer and stained with Ponceau S to ensure uniform protein loading, and blots were blocked with 5% milk solution in Tris-buffered saline-Tween 20 (TBST). Primary and secondary antibody incubations were followed by development with an enhanced chemiluminescence (ECL) kit (Bio-Rad) and imaging using a Molecular Imager Gel Doc XR+ system (Bio-Rad). Primary antibodies were specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (D16H11; Cell Signaling), phospho-IKK α (Ser176)/IKK β (Ser177) (16A6; Cell Signaling), IKK α (2682; Cell Signaling), IKK β (2684; Cell Signaling), RelA (C-20; Santa Cruz Biotechnology), RelB (C-19; Santa Cruz Biotechnology), α -tubulin (11H10; Cell Signaling), IKK α /IKK β (H-470; Santa Cruz Biotechnology), pp28 (40), IE2 (41), and U_L26 (7H19) (18, 40). Secondary antibodies were rabbit polyclonal (Santa Cruz Biotechnology) and mouse monoclonal (Abcam) anti-IgG antibodies.

Analysis of DNA and transcripts during infection. Viral DNA accumulation was assessed by real-time quantitative PCR, as described previously (42). Briefly, CRISPR control, IKK α ^{-/-}, and IKK β ^{-/-} cells were infected with WT or Δ U_L26 virus at a MOI of 0.1 and then, at various time points, scraped in medium, washed with phosphate-buffered saline, and resuspended in lysis buffer (100 mM NaCl, 100 mM Tris [pH 8.0], 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K, and 40 μ g/ml RNase A). Cells were lysed overnight at 55°C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), extracted again with chloroform/isoamyl alcohol (24:1), precipitated with ethanol, and resuspended in water. The DNA abundance was assessed via quantitative PCR using Fast SYBR green master mix (Applied Biosystems), a model 7500 Fast real-time PCR system (Applied Biosystems), and Fast 7500 software (Applied Biosystems), according to the manufacturer's instructions. Viral DNA abundance was determined with a primer pair targeting the viral IE1 gene, i.e., 5'-CCATGTCCACTCGAACCTTAAT-3' (forward) and 5'-TGAACAAGTGACCGAGGATTG-3' (reverse). Host cell DNA was assessed using the following primers targeting GAPDH: 5'-CATGTCGTCATGGGTGTGAACCA-3' (forward) and 5'-ATGGCATGGACTGTGGTCATGAGT-3' (reverse). IE1 and GAPDH gene equivalent values were determined using the 2^{- $\Delta\Delta$ CT} method, and normalized viral DNA accumulation values were obtained by dividing the abundance of IE1 gene equivalents by the abundance of GAPDH gene equivalents. To measure IL-6 transcription during infection, RNA was isolated from infected cells via TRIzol (Invitrogen) extraction, according to manufacturer's instructions, and used to synthesize cDNA with random hexamer primers and the Superscript II reverse transcriptase system (Invitrogen). Relative quantities of gene expression were measured and normalized to GAPDH levels via the 2^{- $\Delta\Delta$ CT} method, using the following primers: IL-6, 5'-AAATTCGGTACATCCTCGACGGCA-3' (forward) and 5'-AGTGCCTCTTTGCTGCTTTCACAC-3' (reverse); GAPDH, 5'-CATGTCGTCATGGGTGTGAACCA-3' (forward) and 5'-ATGGCATGGACTGTGGTCATGAGT-3' (reverse).

NF- κ B luciferase assay. The NF- κ B reporter construct pNF- κ B-FF was induced by TNF- α treatment, as described previously (43). Briefly, calcium phosphate (Stratagene) was used to cotransfect 50%

confluent 293T cells in 96-well plates with 0.25 μ g pNF κ B-FF, 0.25 μ g of a *Renilla* luciferase expression plasmid with an SV40 promoter (pRL-SV40), and 0.25 μ g of either pCDNA3.1(+) empty vector (Invitrogen) or one of a panel of pCDNA vectors encoding U_L26 mutants (pCDNA-U_L26WT, pCDNA-U_L26 Δ N, and pCDNA-U_L26 Δ C). The following oligonucleotides were used to amplify portions of the U_L26 open reading frame and to insert the sequences into the EcoRI-NotI region of the pCDNA3.1(+) vector via Gibson assembly: pCDNAU_L26 short isoform, 5'-CGGATCCACTAGTCCAGTGTGGTGAATTATGACGAGTAGGCGCGCACCCGACGGCGG-3' (forward) and 5'-GTTTAAACGGGCCCTCTAGACTCGAGCGGCCTTACGGCAACAGCGCTGATGGCAGTTGC-3' (reverse); pCDNAU_L26 Δ C isoform, 5'-CGGATCCACTAGTCCAGTGTGGTGAATTATGTACGCCGTTTTCGGCCTCACGAGGTCG-3' (forward) and 5'-GTTTAAACGGGCCCTCTAGACTCGAGCGGCCTTACGCGATGACCTGGCGTCGCGTCGC-3' (reverse). The U_L26 WT insert was amplified using the pCDNAU_L26 Δ C isoform forward primer and the pCDNAU_L26 short isoform reverse primer. Cells were allowed to recover from transfection for 24 h and then exposed to 10 ng/ml TNF- α treatment for 24 h prior to analysis. Luciferase activity was measured using the Dual-Glo luciferase assay system (Promega), according to the manufacturer's instructions, and a Synergy 2 plate reader (BioTek). NF- κ B reporter activity was normalized to *Renilla* luciferase activity as a transfection efficiency control.

Plaque formation and size assays. Serum-starved confluent cells were infected for 1.5 h with a known number of PFU from freshly thawed stocks of indicated GFP-positive viruses. A standard agarose gel overlay was placed over the cells, and plaques were allowed to develop for 10 days before the number of plaques was counted and plaque sizes were measured using ImageJ software. The criterion for identification of a plaque was a locus of multiple GFP-positive cells displaying a cytopathic effect. For experiments involving plaque formation during cytokine treatment, cells were exposed to 10 ng/ml TNF- α (Sigma) or 5 U/ml IFN- γ (PBL Assay Science) for 4 h prior to infection. For plaque formation and size experiments using the IKK α /IKK β inhibitor TPCA-1, cells were pretreated with 10 μ M TPCA-1 (Sigma) for 2 h prior to infection.

Statistical analysis. All Western blot analyses are representative blots from at least two independent experiments. Statistical significance was determined using a nonpaired one-tailed homoscedastic Student's *t* test unless otherwise indicated. A probability value of <0.05 was considered statistically significant. For the growth curves in Fig. 3 and Fig. 4, the values produced for a given biological replicate experiment were normalized to the average amount of infectious virus produced in the control cells for the biological replicate in the same experiment before statistical comparisons between the infectious virions produced by control and IKK knockout cells were performed. This normalization serves to reduce the noise associated with the variability in the kinetics of peak viral titers between experiments, which are independent of any potential IKK α /IKK β contributions to infectious virus production.

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