An Herpesvirus Virulence Factor Inhibits Dendritic Cell Activation Through Protein Phosphatase 1 and IκB Kinase

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Abstract

Dendritic cells are sentinels in innate and adaptive immunity. Upon virus infection, a complex program is in operation which activates IκB kinase, a key regulator of inflammatory cytokines and co-stimulatory molecules. Here we show that the $\gamma_1$34.5 protein, a virulence factor of herpes simplex viruses, blocks Toll-like receptor-mediated dendritic cell activation. While wild type virus inhibits the induction of MHC class II, CD86, IL-6 and IL-12 the $\gamma_1$34.5 null mutant does not. Notably, $\gamma_1$34.5 works in the absence of any other viral proteins. When expressed in mammalian cells, $\gamma_1$34.5 associates with IKKα/β and inhibits NF-κB activation. This is mirrored by the inhibition of IKKα/β phosphorylation, p65/RelA phosphorylation and nuclear translocation in response to lipopolysaccharide or ploy (I:C) stimulation. Importantly, $\gamma_1$34.5 recruits both IKKα/β and protein phosphatase 1, forming a complex that dephosphorylates two serine residues within the catalytic domains of IκB kinase. The amino terminal domain of $\gamma_1$34.5 interacts with IKKα/β whereas the carboxyl terminal domain binds to protein phosphatase 1. Deletions or mutations in either domain abolish the activity of $\gamma_1$34.5. These results suggest that control of IκB kinase dephosphorylation by $\gamma_1$34.5 represents a critical viral mechanism to disrupt dendritic cell functions.

INTRODUCTION

Herpes simplex virus 1 (HSV-1), a member of *herpevirdae*, establishes both latent and lytic infection (41). Upon infection of the mucosal tissues HSV encounters a variety of cells, including dendritic cells (DCs) that bridge innate and adaptive immunity (7). Immature DCs are able to capture and process viral antigens. When activated DCs express a high level of co-stimulatory molecules. Moreover, DCs release inflammatory cytokines to promote DC maturation. A prominent feature of DCs is to activate naive T cells, where myeloid mucosal and lymph node resident-DCs are responsible for HSV-specific T cell activation (1, 37, 43).

A complex program is in operation upon DC activation, which is coupled to Toll-like receptor (TLR) related pathways (32). For example, when exposed to lipopolysacharride (LPS) or viral proteins (3, 23, 38), TLR4 activates the two arms of downstream signaling. In this process, TLR4 recruits TRIF via an adaptor TRAM and migrates to the endosomal membrane, where it activates TANK-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3). In parallel, TLR4 engages with MyD88 through adaptor TIRAP (also called Mal) at the plasma membrane, which facilitates the formation of a complex consisting of TRAF6, TAK1, TAB2 and IRAK. This relays signals to IκB kinase (IKK) containing IKKα, IKKβ and IKKγ subunits. Activated IκB kinase phosphorylates IκB proteins to trigger their ubiquitination and proteosome-mediated degradation, leading to nuclear translocation of NF-κB that usually exists as a p65(RelA)/p50 heterodimer (12). Accordingly, TLR activation up-regulates the expression of inflammatory cytokines and co-stimulatory molecules.

While DCs can recognize HSV through TLR-related mechanisms (15, 21, 25, 31), viral replication compromises DC functions. Several lines of evidence demonstrate that HSV interactions with immature DCs result in down-regulation of co-stimulatory molecules and

inflammatory cytokines (18, 27, 30, 34). Recently, we reported that the $\gamma_1$34.5 protein of HSV-1 is required to suppress DC maturation, resulting impaired T cell activation (17). This phenotype fits with efficient viral replication (17). HSV $\gamma_1$34.5 is a virulence factor that promotes viral pathogenesis (4, 40). In infected cells, it precludes translational arrest by double-stranded RNA dependent protein kinase PKR(5). In doing so, HSV $\gamma_1$34.5 redirects protein phosphatase 1 (PP1) to dephosphorylate the α subunit of the translation initiation factor 2 (13, 14). Additionally, $\gamma_1$34.5 inhibits TBK1-mediated type I interferon induction early in infection (39). HSV $\gamma_1$34.5 also interacts with Beclin 1 and blocks autophagy (28). Nevertheless, the mechanism through which $\gamma_1$34.5 impairs DC activation remains largely unknown. Here, we demonstrate that HSV $\gamma_1$34.5 abrogates the induction of inflammatory cytokines and costimulatory molecules by TLR in infection. Remarkably, HSV $\gamma_1$34.5 recruits both PP1 and IκB kinase, forming a complex that dephosphorylates the α/β subunit of IκB kinase and inactivates NF-κB. These results highlight a novel HSV-mediated mechanism.

MATERIALS AND METHODS

Mice. BALB/c mice were purchased from Harlan Sprague Dawley Inc. and housed under specific pathogen free conditions in biosafety level 2 containment. Groups of five week-old mice were selected for this study. Experiments were performed in accordance with the guideline of the University of Illinois at Chicago.

Cells and viruses. HeLa, and 293T cells were obtained from the American Type Culture Collection and propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Myeloid DCs were generated as previously described (16). Briefly, bone marrow cells were removed from the tibias and femurs of BALB/c mice. Following red

blood cell lysis and washing, progenitor cells were plated in RPMI-1640 medium (Invitrogen, Auckland, NZ) supplemented with 10% FBS, 0.1 mM nonessential amino acids, 1mM sodium pyruvate and 20 ng/ml granulocyte-machrophage colony stimulating factor (GM-CSF, Biosource, Camarillo, CA) in 6-well plates at 4×10^6/well. Cells were supplemented with fresh medium every other day. On day 8, DCs were positively selected for surface CD11c expression using magnetic beads (Miltenyi Biotech, Auburn, CA) to give >97% pure population of $CD11c^+$ $MHCII^+$ cells. DCs displayed low levels of CD40, CD80, CD86, and major histocompability complex class II (MHC class II) molecules, which is characteristic of immature DCs. Purified $CD11c^+$DCs were cultured in fresh medium with FBS and GM-CSF and used in subsequent experiments. HSV-1(F) is a prototype HSV-1 strain used in this study (9). In recombinant virus R3616, a 1 kb fragment from the coding region of the $\gamma_1$34.5 gene was deleted (4).

Viral infection. HeLa cells were infected with viruses at an indicated multiplicity of infection. Purified $CD11c^+$ DCs were plated in 12-well plates ($5x10^5$ cells/well) or in 96-well round bottom plates ($5x10^4$ cells/well) and infected with viruses. After 2 h incubation, cells were washed with phosphate-buffer saline (PBS) and resuspended in RPMI1640 supplemented with 10% FBS and 20 ng/ml GM-CSF. At different time points after infection, cells were harvested for analysis.

Plasmids. Plasmids pcDNA3, pTK-luc, dN200, FLAG-$\gamma_1$34.5, and N159 have been described elsewhere (10, 39). Plasmids HA-MyD88, HA-TRAF6, and FLAG-PP1 were constructed by cloning of PCR fragments into pcDNA3. To construct FLAG-γ_1MT, a DNA fragment encoding a mutant $\gamma_1$34.5 with $V^{193}E$ and $F^{195}L$ substitutions in the PP1 binding site was cloned into pcDNA3. To construct ΔN146, a DNA fragment encoding amino acids 146-263 of $\gamma_1$34.5 was cloned into pcDNA3. For retrovirus transduction, DNA fragments of wild type

$\gamma_1$34.5, γ_1MT, ΔN146 and N159 were cloned into pSIN-Ova_GFP, a dual-promoter human immunodeficiency virus type 1 vector from Mary Collins (University College London, UK) (33). Plasmid FLAG-IKKβ, pNFκB-Luc, IKKα, FLAG-TRIF were gifts from Warner Greene (University of California, San Francisco), Zuoming Sun (Beckman Research Institute of City of Hope, Duarte, CA) and Jurg Tschopp (University of Lausanne, Switzerland).

Western blot and Immunoprecipitation analyses. To analyze protein expression, cell lysates were subjected to electrophoresis, transferred to nitrocellulose membranes, and reacted with primary antibodies (10). The membranes were rinsed in phosphate-buffered saline and reacted with either goat anti-rabbit or goat anti-mouse antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Inc).

To examine protein interactions, cells were transfected with the indicated plasmids. At 36 h after transfection, cells were harvested and lysed in 50 mM Tris-HCL (pH7.4) buffer containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 1mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1μg/ml aprotinin/leupeptin/pepstatin, 1 mM Na_3VO_4, 1mM NaF. Lysates were incubated overnight at 4^{o}C with anti-FLAG M2 affinity gel (Sigma) or anti-HA antibody (Applied Biological Materials Inc) plus protein A/G-agarose beads (Santa Cruz Biotechnology). Immunocomplexes captured on the affinity gel or protein A/G-agarose beads were subjected to electrophoresis and immunoblotting analysis.

Reporter assays. Luciferase reporter assays were performed as described previously (10). Briefly, 293T cells grown on 12-well plates were transfected with a control plasmid or plasmid vector expressing TRIF, TRAF6, MyD88, IKKβ, IKKα and $\gamma_1$34.5 variants, along with an NFκB reporter plasmid expressing firefly luciferase using lipofectamine 2000 (Invitrogen). Total levels of transfected DNA were kept constant with empty vector plasmid. As a control for

transfection efficiency, a plasmid containing the *Renilla* luciferase gene driven by the HSV-1 TK promoter was included. At 36 h after transfection, cells were harvested, and luciferase activities were measured using the dual luciferase assay system from Promega.

Flow cytometry. Flow cytometry of cell surface markers, CD11c, MHCII, CD80 and CD86 on DCs were performed according to a standard protocol, with some modifications (17). Cells were blocked with 1 μl of Fcγ mAb (0.5 μg/ml) for 30 min at 4^{o}C. After washing with PBS, cells were stained with isotype-matched antibodies, anti-CD11c-PE, anti-MHCII-FITC, anti-CD80-FITC, and anti-CD86-FITC antibodies (eBioscience) for 30 min on ice with gentle shaking. Samples were processed and screened using FACSCalibur and data were analyzed with Cell Questpro software (BD). To determine viral infectivity, DCs mock infected or infected with viruses were fixed in 4% paraformaldehyde (Sigma) and permeabilized in permeabilizing buffer (eBioscience, San Diego, CA). Cells were blocked with 5% normal mouse serum (Sigma), incubated with a mAb against HSV-1 ICP27 (Virusys, Sykesville, MD) and allowed to react with a goat anti-mouse FITC-conjugated antibody (Santa Cruz Biotech, CA). ICP27 expression was evaluated by flow cytometry.

Transduction and ELISA. Plasmids were co-transfected along with HIVtrans and VSV-G into 293T cells using Lipofectamine 2000 (Invitrogen) as described previously (10). At 48 h after transfection, supernatant was collected and the titers were determined by GFP expression. Immature DCs were transduced with retroviral constructs and cells were grown with fresh RPMI 1640 medium containing GM-CSF (20 ng/ml) every 2 days. On day 5, GFP-positive DCs were sorted by FACS. Levels of IL-6 and IL-12 in supernatants of cell culture were quantified by ELISA using kits from R&D systems according to the manufacturer's instruction.

Cell Fractionation Assays. Cells were lysed in phosphate-buffed saline containing 0.4% Nonidet P-40 and protease inhibitor mixture (Sigma) and kept on ice with gentle inversion. After brief centrifugation, the nuclei were pelleted and supernatants were collected. After washing, the nuclei were resuspended in phosphate-buffed saline containing 0.4% Nonidet P-40 and frozen at -80^{o}C for 30 min. The cytoplasmic and nuclear fractions were then solubilized in disruption buffer. Samples were subjected to electrophoresis and Western blot analysis with antibodies against p65 (Santa Cruz Biotechnology), GRP78 (glucose-regulated protein 78) (BD Transduction Laboratories), and histone H3 (Cell Signaling), respectively.

Quantitative real-time PCR. Total RNAs from mock-infected or virus-infected DCs were extracted using the Reasy kit (Qiagen Inc.) Equal amounts of RNA from each sample were used to synthesize cDNA as suggested by the manufacturer (Invitrogen). cDNAs were then subjected to real-time PCR analysis for ICP27, UL30 and UL44 with specific primers (19). Real-time PCR analysis was performed with the SYBR Green system and all data are presented as relative expression units after normalization to 18s rRNA.

RESULTS

HSV $\gamma_1$34.5 suppresses dendritic cell activation by lipopolysaccharide. We sought to investigate the function of HSV $\gamma_1$34.5 in DC activation by TLR agonist. Immature CD11c^{+} DCs, mocked-infected or infected with viruses, were treated with lipopolysaccharide (LPS), a prototype stimulator of DC maturation. Cells were then subjected to flow cytometry analysis. As illustrated in Fig. 1A and B, mock-infected immature DCs exhibited basal levels of major histocompatibility complex (MHC) class II and CD86. Infection with the $\gamma_1$34.5 null mutant R3616, but not wild type HSV-1(F), stimulated the expression of MHCII and CD86. LPS increased levels of MHCII and CD86 in mock or R3616-infected DCs. However, this was

drastically suppressed in DCs infected with HSV-1(F). A similar response pattern was seen for the production IL-6 and IL-12 as determined by ELISA (Fig. 1C and D). Under these conditions, both HSV-1(F) and R3616 infected DCs equally well (Fig. 1E). Quantitative real-time PCR analysis revealed no significant difference in the expression of immediately early (ICP27), early (UL30) and late (UL44) genes for HSV-1(F) and R3616 (Fig. 1F). Further, treatment with PAA, an inhibitor of HSV DNA replication, did not alter the effects of HSV-1(F) and R3616 on DCs (Fig. 1G and D). These results suggest that $\gamma_1$34.5 may play a direct role in blocking DC activation.

To test this hypothesis, immature DCs transduced with a retrovirus vector or $\gamma_1$34.5 were examined in response to LPS stimulation. As shown in Fig. 2A and B, when left untreated, cells displayed low levels of MHCII and CD86 expression. Upon LPS stimulation, mock or vector-transduced cells expressed significantly higher levels of costimulatory molecules as compared to wild-type $\gamma_1$34.5 transduced cells. Similarly, LPS induced IL-6 and IL-12 expression in mock or vector transduced cells. These phenotypes were suppressed in cells transduced with wild-type $\gamma_1$34.5, which correlated with $\gamma_1$34.5 expression (Fig. 2C, D and E). Therefore, $\gamma_1$34.5 functions in the absence of any other HSV proteins.

HSV $\gamma_1$34.5 inhibits p65/RelA phosphorylation and nuclear translocation in dendritic cells. DC maturation is linked to type I IFN production (32). Because $\gamma_1$34.5 inhibits type I IFN induction (17, 39), we asked whether exogenous type I IFN was able to relieve the inhibitory effect of $\gamma_1$34.5. Data presented in Fig.3A, B, C and D show that $\gamma_1$34.5 suppressed LPS-stimulated expression of MHC class II, CD86, IL-6, and IL-12 as compared to control cells. Addition of IFN-β moderately or marginally relieved the inhibitory effect by $\gamma_1$34.5. This was more evident with respect to IL-6 and IL-12 expression. We reasoned that $\gamma_1$34.5 might regulate

209 the expression of MHC class II, CD86, IL-6, and IL-12 through a component independently of
210 type I IFN.

211 In addition to IRF3 which induces type I IFN, NF-κB is critical in DC maturation (32),
212 where it positively regulates the expression of costimulatory molecules and cytokines.
213 Accordingly, we evaluated the impact $\gamma_1$34.5 on NF-κB activation. As illustrated in Figure 4A,
214 p65/RelA was primarily located in the cytoplasm fraction in unstimulated cells. Upon LPS
215 stimulation, p65/RelA appeared in the nuclear fraction in mock or vector transduced DCs. In
216 stark contrast, its nuclear translocation was blocked in DCs transduced with $\gamma_1$34.5. In
217 correlation, p65/RelA phosphorylation was inhibited by $\gamma_1$34.5 although p65 expression was
218 comparable in all cells (Fig. 4B). p65/RelA phosphorylation was further confirmed with ELISA
219 (Fig. 4C). Thus, when expressed in DCs HSV $\gamma_1$34.5 is able to suppress NFκB activation by the
220 TLR4 pathway.

221 **HSV $\gamma_1$34.5 blocks NF-κB activation by associating with Iκ-B kinase.** In response to
222 LPS, TLR4 relays the signal to several adaptors and kinases, leading to NF-κB activation (38).
223 To assess at which step $\gamma_1$34.5 exerted its activity, we carried out luciferase reporter assays. As
224 indicated in Fig. 5, when expressed in 293T cells, TRIF, TRAF6, MyD88, IKKβ, and IKKα
225 activated the NFκB reporter. Co-expression of $\gamma_1$34.5 inhibited NFκB activation by all these
226 adaptors or kinases in a dose-dependent manner. To test the potential interaction of $\gamma_1$34.5 with
227 a cellular component, we performed co-immunoprecipitation assays. As shown in Fig. 6A, when
228 expressed in HeLa cells, HSV $\gamma_1$34.5 associated with endogenous IKKα and IKKβ. Under this
229 condition, it exhibited no interaction with TRIF, TRAF6, MyD88 or p65/RelA, indicating that
230 $\gamma_1$34.5 specifically targets the Iκ-B kinase complex. IKKα and IKKβ share significant amino
231 acid identity, and their domain structure is similar with an amino-terminal kinase, leucine zipper,

and helix-loop-helix motifs (12). Because of its essential role in the classical NF-κB pathway that regulates inflammatory cytokines and co-stimulatory molecules, we further analyzed IKKβ. As illustrated in Fig. 6B, when ectopically expressed in 293T cells, $\gamma_1$34.5 specifically associated with IKKβ but not with a control protein dN200 (a mutant Ebola VP35). Further, $\gamma_1$34.5 associated with IKKβ in virus infected cells (Fig. 6C). We conclude from these experiments that HSV $\gamma_1$34.5 inhibits NF-κB promoter activation by targeting Iκ-B kinase.

HSV $\gamma_1$34.5 precludes IKKβ phosphorylation via protein phoshatase 1. Association of $\gamma_1$34.5 with IKKα/β raised the possibility that it may inhibit Iκ-B kinase activation. To address this, we examined the phosphorylation status of IKKβ in 293T cells over-expressing IKKβ, PP1, and wild type $\gamma_1$34.5 (WT). Since $\gamma_1$34.5 is a virus-encoded protein phosphatase 1 regulator (13, 14), we also hypothesized that its PP1 site may be required to modulate Iκ-B kinase. For this purpose, we included a $\gamma_1$34.5 mutant (γ_1MT) with $V^{193}E$ and $F^{195}L$ substitutions which disrupt its binding to PP1 (42). As shown by Western blot analysis (Fig. 7A), these proteins were expressed at comparable levels after transfection. When ectopically expressed alone, IKKβ remained phosphorylated (lane 2). Addition of wild type $\gamma_1$34.5 or PP1 reduced IKKβ phosphorylation slightly (lanes 3 and 4). When co-expressed with PP1 and wild type $\gamma_1$34.5, IKKβ became completely unphosphorylated (lane 5). Nonetheless, IKKβ remained phosphorylated in cells expressing the $\gamma_1$34.5 mutant (γ_1MT) (lanes 6-7). Without wild type $\gamma_1$34.5, PP1 had little effect on IKKβ phosphorylation. Next we examined endogenous IKKβ phosphorylation in HeLa cells which express TLR3. As illustrated in Fig. 7B, IKKβ was un-phosphorylated in unstimulated cells (lanes 1-3). Poly(I:C) stimulation induced IKKβ phosphorylation in mock transfected cells (lanes 4 and 7). Expression of wild type $\gamma_1$34.5

precluded poly (I:C)-induced IKKβ phosphorylation (lanes 5 and 8). In contrast, expression of the mutant $\gamma_1$34.5 (γ_1MT) was unable to prevent IKKβ phosphorylation (lanes 6 and 9). These data suggest that the interaction of $\gamma_1$34.5 with protein phosphatase 1 inhibits IKKβ phosphorylation.

HSV $\gamma_1$34.5 forms a multi-protein complex which blocks NF-κB activation. Based on the above analysis, we further examined the nature of $\gamma_1$34.5, IKKβ, and PP1 interactions in a series of experiments. As indicated in the immunoprecipitation assay (Fig.8A), IKKβ coimmunoprecipiated with both wild type $\gamma_1$34.5 and PP1 in transfected-293T cells (lane 2) where IKKβ, PP1 and $\gamma_1$34.5 variants expressed at comparable levels (lanes 1-3). This indicates that IKKβ, PP1 and $\gamma_1$34.5 indeed formed a complex. Intriguingly, in the absence of $\gamma_1$34.5, IKKβ did not associate with PP1 (lane 1). Hence, HSV $\gamma_1$34.5 serves as a bridge for IKKβ and PP1. Interestingly, the mutant $\gamma_1$34.5 (γ_1MT), which cannot bind to PP1, retained its ability to interact with IKKβ, suggesting that the interaction of $\gamma_1$34.5 with IKKβ is independent of PP1 (lane 3). To define the domain required to interact with IKKβ, we tested additional $\gamma_1$34.5 mutants. As shown in Fig. 8B, like wild type $\gamma_1$34.5, γ_1MT associated with IKKβ (lanes 2 and 3). Moreover, the $\gamma_1$34.5 mutant N159, which lacked amino acids 160 to 263, was able to interact with IKKβ (lane 5). In contrast, the $\gamma_1$34.5 mutant ΔN146 which lacked amino acids 1 to 145 was unable to interact with IKKβ (lane 4). Thus, the amino-terminus of $\gamma_1$34.5 is sufficient to associate with IKKβ. To establish a functional link between $\gamma_1$34.5 and IKKβ, and PP1, we examined $\gamma_1$34.5 variants in report assays (Fig. 8C). When expressed, IKKβ induced NFκB promoter activation by 40-fold. Expression of wild type $\gamma_1$34.5 drastically inhibited its activation. However, co-expression of N159, ΔN146 or γ_1MT failed to suppress NF-κB

promoter activation by IKKβ. Hence, both the amino- and carboxyl-terminal domains are required to inhibit NF-κB activation. These results suggest that $\gamma_1$34.5 forms a complex with IKKβ and PP1, where $\gamma_1$34.5 mediates IKKβ dephosporylation by recruting PP1.

IKKβ dephosphorylation by $\gamma_1$34.5 impairs dendritic cell activation. To determine whether IKKβ dephosphorylation by $\gamma_1$34.5 is linked to impaired DC activation, we tested $\gamma_1$34.5 variants in immature DCs. As shown in Fig. 9A and B, all cells exhibited basal levels of CD80 and CD86 expression. Addition of LPS greatly stimulated the expression of these co-stimulatory molecules in cells transduced with a retroviral vector, N159, ΔN146 or γ_1MT. This stimulation was suppressed in cells transduced with wild type $\gamma_1$34.5. A similar phenotype was noted for IL-6 and IL-12 expression (Fig. 9C and D). Western blot analysis showed that $\gamma_1$34.5 variants were expressed at a comparable level (Fig. 9E). To assess whether $\gamma_1$34.5 variants inhibited NF-κB activation in DCs, we measured phosphorylation of p65 and IKKβ in dendritic cells. As indicated in Fig. 9F, wild type $\gamma_1$34.5 precluded phosphorylation of p65/RelA and IKKβ stimulated by LPS (lane 3) whereas N159, ΔN146 and γ_1MT failed to do so (lanes 4-6). These phenotypes correlated with the ability of $\gamma_1$34.5 variants to suppress DC maturation. Thus, both IKKβ and PP1 binding domains of $\gamma_1$34.5 are indispensible. Taken together, these data demonstrate that dephosphorylation of Iκ-B kinase by $\gamma_1$34.5 impairs DC maturation.

DISCUSSION

HSV replicates in DCs and perturbs DC maturation, resulting in impaired T cell activation (18, 27, 30, 34). In this study, we provide evidence that HSV $\gamma_1$34.5 suppressed the induction of co-stimulatory molecules and inflammatory cytokines by TLR stimulation. When expressed, HSV $\gamma_1$34.5 recruited both cellular protein phosphatase 1 and IKK kinase.

298 Accordingly, these proteins formed a complex that precluded phosphorylation of IKKα/β and
299 subsequent activation of NF-κB. In this context, it is noteworthy that HSV interaction with DCs
300 determines the outcome of infection (1, 19, 37). Given that HSV $\gamma_1$34.5 is a virulence factor (4,
301 40), these results support the view that interference of TLR or virus-mediated DC activation by
302 $\gamma_1$34.5 contributes to viral pathogenesis.

303 HSV $\gamma_1$34.5 plays a direct role in abrogation of TLR-mediated DC activation. We
304 previously noted that $\gamma_1$34.5 is required to inhibit DC maturation both in vivo and in vitro (17).
305 In agreement with this observation, we found that $\gamma_1$34.5 also blocked DC activation by TLR
306 stimulation. Further, when expressed alone this viral factor was capable of suppressing the
307 induction of MHC class II, CD86, IL-6 and IL-12 by LPS. Herein, these phenotypes are not due
308 to a secondary effect by other HSV gene products. HSV $\gamma_1$34.5 associates with and inhibits
309 TANK1 binding kinase 1 (13), an essential factor that activates interferon regulatory factor 3 and
310 subsequent type I IFN expression. One possible reason that $\gamma_1$34.5 perturbs DC activation is the
311 block of type I IFN production. Our experimental data suggest that this model is not sufficient to
312 reconcile the effect of $\gamma_1$34.5 on DCs. First, exogenous type I IFN marginally or moderately
313 relieved the inhibitory effect by $\gamma_1$34.5. Second, when expressed in immature DCs, HSV $\gamma_1$34.5
314 blocked phosphorylation and nuclear migration of p65/RelA in response to TLR4 activation. A
315 logical explanation is that $\gamma_1$34.5 may work via an additional mechanism.

316 The Iκ-B kinase complex sits at the center of innate immune pathways leading to the
317 expression of cytokines and co-stimulatory molecules (12, 38). Our work reveals Iκ-B kinase as
318 a novel cellular target of HSV $\gamma_1$34.5. When expressed in mammalian cells, $\gamma_1$34.5 inhibited
319 NF-κB activation mediated by IKKα, IKKβ, TRIF, TRAF6, and MyD88 effectively. Although

320 associated with IKKα or IKKβ, $\gamma_1$34.5 failed to interact with other components including TRIF,
321 TRAF6, MyD88 and p65/RelA. A specific interaction between $\gamma_1$34.5, IKKα and IKKβ seems
322 required to modulate NF-κB activation. The Iκ-B kinase complex is activated in response to a
323 variety of stimuli including TLR signaling (12, 38). This requires phosphorylation of either
324 IKKα or IKKβ on two specific serine residues within the catalytic domain of each subunit (12).
325 Importantly, $\gamma_1$34.5 inhibited phosphorylation of IKKα/β mediated by TLR3 or TLR4
326 stimulation. Because IKKα/β sits at a converging point (12, 38), it is possible that $\gamma_1$34.5 may
327 exert a broad impact on multiple pathways leading to NF-κB activation. Additional work is
328 needed to test this hypothesis.

329 HSV $\gamma_1$34.5 consists of 263 amino acids with a large amino-terminal and carboxyl-
330 terminal domains which are linked by triplet repeats of three amino acids (ATP) (4). The fact
331 that it interacts with IKKα/β and PP1 raises the question of how $\gamma_1$34.5 works. In principle,
332 $\gamma_1$34.5 may bind to the Iκ-B kinase complex and impose a physical block to prevent its
333 phosphorylation. Alternatively, $\gamma_1$34.5 may serve as a link to bridge Iκ-B kinase and PP1 that
334 dephosphorylates Iκ-B kinase. Our data favor the latter possibility. We noted when expressed
335 alone, the amino terminus of $\gamma_1$34.5 interacted with IKKα/β but had no effect on IKKα/β
336 phoporylation. Although binding to PP1 (14), the carboxyl terminus exhibited no activity on
337 IKKα/β phoporylation. Moreover, $V^{193}E$ and $F^{195}L$ substitutions abolished the interaction of
338 $\gamma_1$34.5 with PP1 but not with IKKβ. Interestingly, such mutations disrupted the ability of $\gamma_1$34.5
339 to preclude IKKβ phosphorylation. We postulate that upon HSV infection $\gamma_1$34.5 recruits
340 IKKα/β by the amino-terminus and PP1 by its carboxyl-terminus, resulting in a multi-protein

341 complex that prevents activation of IKKβ, NF-κB and DCs. Work is in progress to investigate
342 details of the underlying mechanism.

343 HSV is a large DNA virus that replicates in DCs and perturbs their functions (18, 27, 30,
344 34). Previous studies demonstrated that the virion host shutoff protein functions to inhibit DC
345 maturation (35). This is believed to result from its intrinsic RNase activity. Notably, the virion
346 host shutoff protein blocks DC activation by TLR-independent pathways of viral recognition (6).
347 On the other hand, an immediate early protein ICP0 perturbs the function of mature DCs (22),
348 where it mediates CD83 degradation via cellular proteasomes. Additionally, ICP0 as well as
349 ICP27 inhibits NF-κB activation (8, 20, 26). Our work reveals that $\gamma_1$34.5 functions to impair
350 DC activation by TLR. These viral proteins possibly work coordinately, which creates a
351 favorable environment for HSV infection. In this context, it is noteworthy that $\gamma_1$34.5 mediates
352 IKKα/β dephosphorylation rather than a physical block of IKKα/β phosphorylation. This is
353 probably related to the fact that HSV both activates and inhibits NF-κB (2, 8, 24, 26, 36). While
354 NF-κB activity is required for optimal HSV replication (11, 29), its activation is also antiviral in
355 nature (8, 26). Thus, NF-κB activity must be tightly regulated during the complex HSV life
356 cycle. We speculate that HSV has evolved sophisticated mechanisms to control NF-κB activity
357 during evolution. In this regard, $\gamma_1$34.5 acts to regulate the level of NF-κB activation by a cycle
358 on-off mechanism via dephosphorylation of IKKα/β. This may serve as a strategy to down-
359 regulate host antiviral immunity.

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FIGURE LEDENDS

Figure 1. HSV $\gamma_1$34.5 is required to suppress LPS-induced dendritic cell activation in virus infection. $CD11c^+$ dendritic cells were mock infected or infected with viruses (2 PFU/cell). At 12 h after infection, the cells were stimulated with LPS (500ng/ml, Sigma) for an additional 12 h. The cells were stained with PE-labeled antibodies against with MHCII (A) and CD86 (B) and subjected to FACS analysis. In parallel, cell supernatants were collected to measure the production of IL-6 (C) and IL-12 (D) by ELISA. (E) Viral infectivity of DCs was determined by examing ICP27 expression as described in MATERIALS AND METHODS. (F) Viral gene expression in DCs was determined 20 h after infection by quantitative real-time RT-PCR. Data are presented as relative expression percentage after normalization to 18srRNA. (G) and (H) The effect of viral DNA replication inhibitor on DC maturation. Immature DCs were infected

518 with HSV-1(F) or R3616 in the absence or presence of PAA (400μg/ml, Sigma). Cells were
519 stained for the expression of MHCII and CD86. The data are a representative of three
520 independent experiments with standard deviations. Asterisks denote statistical differences
521 ($p<0.05$) between different treatment groups.

522 Figure 2. HSV $\gamma_1$34.5 inhibits LPS-induced dendritic cell activation. Immature $CD11c^+$
523 dendritic cells were transduced with a retroviral vector or $\gamma_1$34.5. At 5 days after transduction,
524 GFP positive DCs were isolated by FACS and treated with or without LPS (500ng/ml) for 12h.
525 Cells were stained with PE-labeled antibodies against with MHCII (A) and CD86 (B) and
526 subjected to FACS analysis. Cell supernatants were collected to measure the production of IL-6
527 (C) and IL-12 (D) by ELISA. (E) Protein expression. Lysates of cells were subjected to
528 Western blot analysis with antibodies against β–actin (Sigma) and $\gamma_1$34.5 (39), respectively. The
529 data are a representative of three independent experiments with standard deviations. Asterisks
530 denote statistical differences ($p<0.05$) between different treatment groups.

531 Figure 3. Effects of exogenous IFN-β on the $\gamma_1$34.5 activity. Immature $CD11c^+$ dendritic cells
532 were mock treated or transduced with a retroviral vector or $\gamma_1$34.5. GFP positive cells were
533 isolated and treated with IFN-β (800 U/ml, PBL laboratories) overnight. Cells were then
534 stimulated with LPS (500ng/ml, Sigma) for 12 h and stained with PE-labeled antibodies against
535 MHC class II (A) and CD86 (B). Cell supernatants were used to determine the production of IL-
536 6 (C) and IL-12 (D) by ELISA. The data are a representative of three independent experiments
537 with standard deviations. Asterisks denote statistical differences ($p<0.05$) between different
538 treatment groups.

Figure 4. The $\gamma_1$34.5 protein blocks NFκB activation in dendritic cells. (A) Cell fractionation analysis. Immature $CD11c^+$ dendritic cells, transduced with a retroviral vector or wild type $\gamma_1$34.5, were stimulated with LPS (500ng/ml). The cytoplasmic and nuclear fractions were then subjected to Western blot analysis with antibodies against p65/RelA (Santa Cruz Biotech), GRP78 (BD Transduction Laboratories), and histone H3 (Cell Signaling), respectively. (B) Cells were treated as in (A) and cells lysates were processed for Western blot analysis with antibodies against phosphorylated p65/RelA and p65/RelA (Santa Cruz Biotech), respectively. (C) Lysates of cells were also collected to quantify p65 phosphorylation by ELISA (Cell signaling Tech.). Results were expressed as fold of activation with standard deviations among triplicate samples. The data are a representative of three independent experiments.

Figure 5. HSV $\gamma_1$34.5 inhibits NFκB reporter activation. 293T cells were transfected with an empty vector or plasmids expressing TRIF (A), TRAF6 (B), MyD88 (C), IKKβ (D), IKKα (E) and $\gamma_1$34.5 along with an NFκB reporter gene expressing firefly luciferase using lipofectamine 2000 (Invitrogen). At 36 h post-transfection, cells were harvested for luciferase assays. Results were expressed as fold of activation with standard deviations among triplicate samples. The data are a representative of three independent experiments.

Figure 6. (A) The $\gamma_1$34.5 protein associates with endogenous IKKα/β. HeLa cells were transfected with FLAG-$\gamma_1$34.5 or an empty vector. At 36 h after transfection, lysates of cells were immunoprecipated with anti-FLAG antibody. Samples from both cell lysates and immunoprecipitates were probed with antibodies against FLAG, TRIF (Cell signaling Tech.), TRAF6 (Santa Cruz Biotech), MyD88 (Santa Cruz Biotech), IKKβ (Cell signaling Tech.), IKKα (Cell signaling Tech.) and p65/RelA (Santa Cruz Biotech), respectively. (B) The

interaction of $\gamma_1$34.5 and IKKβ. 293T cells were co-transfected with FLAG-$\gamma_1$34.5 along with an empty vector, HA-IKKβ, or FLAG-dN200 (a mutant of Ebola VP35). At 36 h after transfection, lysates of cells were immunoprecipated (IP) with anti- FLAG antibody. Samples were probed with antibodies against FLAG, HA, and β-actin, respectively. (C) HeLa cells were transfected with HA-IKKβ. 24 h after transfection, cells were mock infected or infected with viruses (5 PFU/cell). At 10 h after infection, lysates of cells were immunoprecipated with anti-HA antibody (Santa Cruz Biotech). Samples from both cell lysates and immunoprecipitates were analyzed with antibodies against HA, $\gamma_1$34.5 and β-actin, respectively. The data are a representative of three independent experiments.

Figure 7. The $\gamma_1$34.5 protein mediates dephosphorylation of IKKβ. (A) 293T cells were transfected with an empty vector, HA-IKKβ, or FLAG-PP1 along with FLAG-$\gamma_1$34.5 or FLAG-γ_1MT which bears V^{193E} and $F^{195}L$ substitutions in the PP1 binding motif. At 36 h after transfection, lysates of cells probed with antibodies against phosphorylated IKK-β, IKK-β, $\gamma_1$34.5, PP1, and β-actin, respectively. (B) The $\gamma_1$34.5 protein suppresses phosphorylation of endogenous IKKβ. HeLa cells were transfected with FLAG-$\gamma_1$34.5 or FLAG-γ_1MT. At 36 h after transfection, cells were left untreated or treated with poly I:C (20μg/ml). Lysates of cells were then processed for Western blot analysis with antibodies against phosphorylated IKK-β, IKK-β, $\gamma_1$34.5 and β-actin, respectively. The data are a representative of three independent experiments.

Figure 8. (A) HSV $\gamma_1$34.5 mediates the formation of an IKK-$\gamma_1$34.5-PP1 complex. 293T cells were co-transfected with FLAG-$\gamma_1$34.5 or FLAG-γ_1MT along with an empty vector, HA-IKKβ, or FLAG-PP1. At 36 h after transfection, lysates of cells were immunoprecipated with anti- HA

583 antibody. Samples were probed with antibodies against PP1, HA, or $\gamma_1$34.5 and β-actin,
584 respectively. (B) Interaction of $\gamma_1$34.5 variants with IKKβ. 293T cells were cotransfected with
585 HA-IKKβ and FLAG-$\gamma_1$34.5 variants as indicated. At 36 h after transfection, lysates were
586 immunopreciated with anti-HA antibody. Proteins in the lysates and precipitates were analyzed
587 by immunoblotting with anti-HA and anti-FLAG antibodies. (C) Effect of $\gamma_1$34.5 variants on
588 NFκB promoter activation. 293T cells were cotransfected with an empty vector, HA-IKKβ
589 (50ng), Flag-$\gamma_1$34.5 variants (800ng), and an NFκB luciferase reporter. At 36 h post-
590 transfection, cells were harvested for luciferase assays. Results were expressed as fold of
591 activation with standard deviations among triplicate samples. The data are a representative of
592 three independent experiments.

593 Figure 9. Effects of $\gamma_1$34.5 variants on DC maturation. Immature $CD11c^+$ dendritic cells,
594 transduced with a retroviral vector or $\gamma_1$34.5 variants, were stimulated with LPS (500ng/ml). At
595 12 h after stimulation, cells were stained with PE-labeled antibodies against MHCII (A) and
596 CD86 (B) and subjected to FACS analysis. Cell media were collected to determine the
597 production of IL-6 (C) and IL-12 (D by ELISA. (E) Expression of $\gamma_1$34.5 variants. Lysates of
598 cells were subjected to Western analysis with anti-FLAG and anti-β-actin. (F) Effects of $\gamma_1$34.5
599 variants on LPS-induced phosphorylation of IKKβ and p65/RelA. DCs transduced with a
600 retroviral vector or $\gamma_1$34.5 variants were stimulated with LPS (500ng/ml). At 20 min after
601 stimulation, cells lysates were processed and probed with antibodies against phosphorylated
602 p65/RelA, p65/RelA, phosphorylated IKKβ, IKKβ, and β-actin, respectively. The data are a
603 representative of three independent experiments with standard deviations. Asterisks denote
604 statistical differences ($p<0.05$) between different treatment groups.

Figure 1

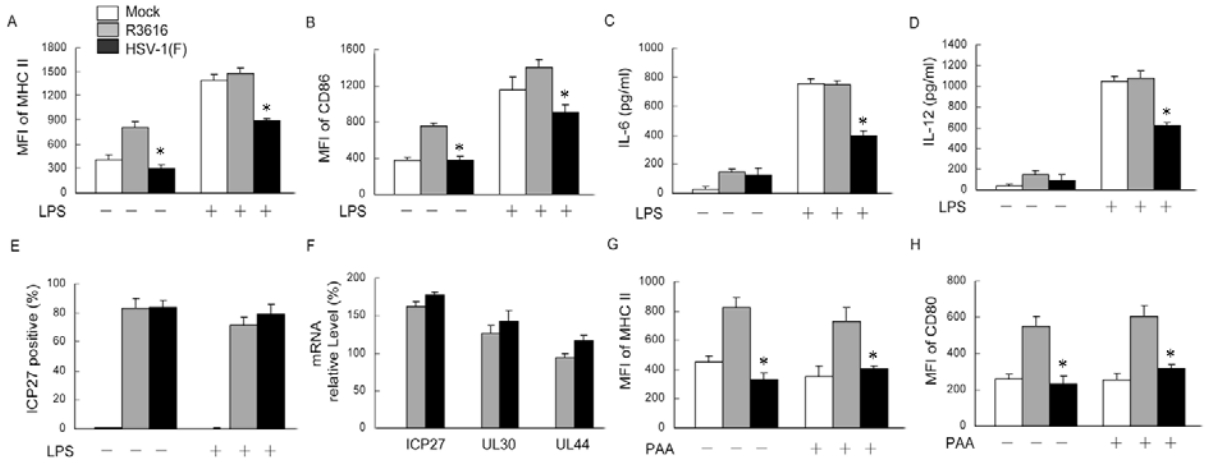
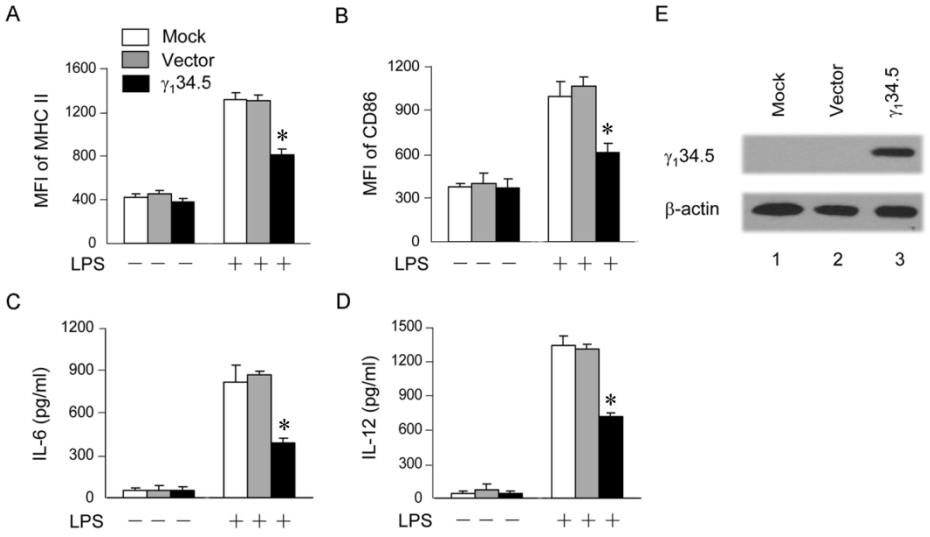


Figure 2



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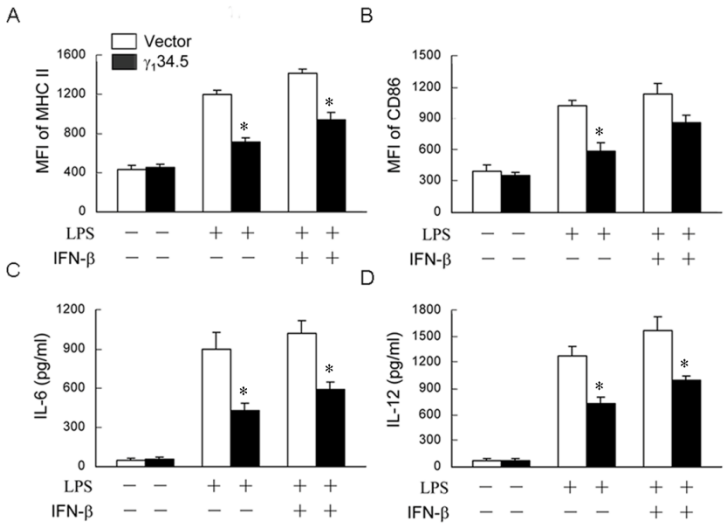

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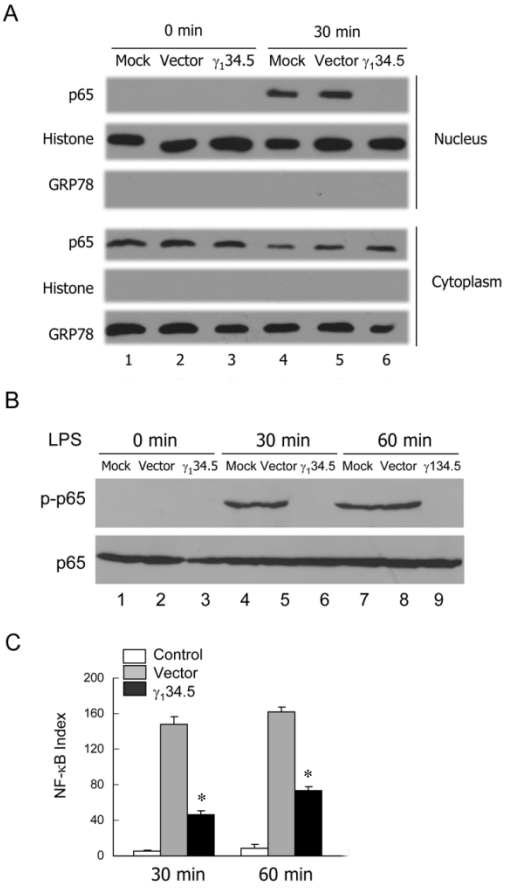
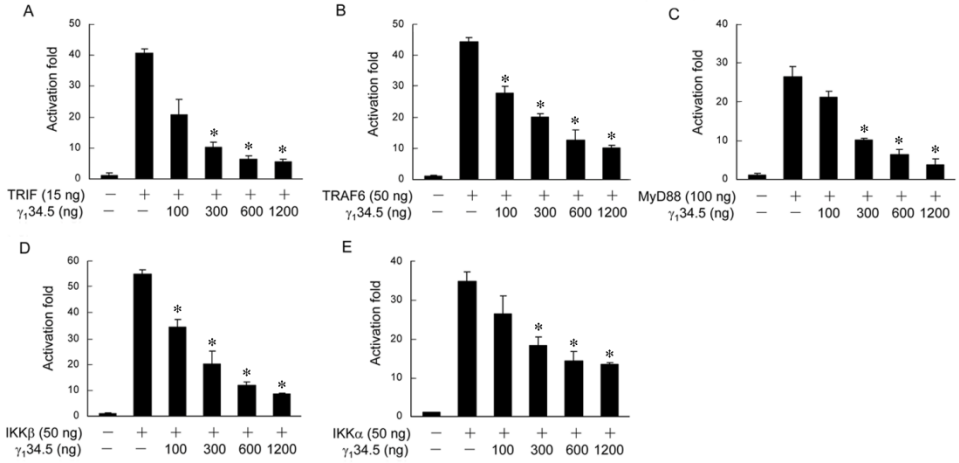


Figure 5



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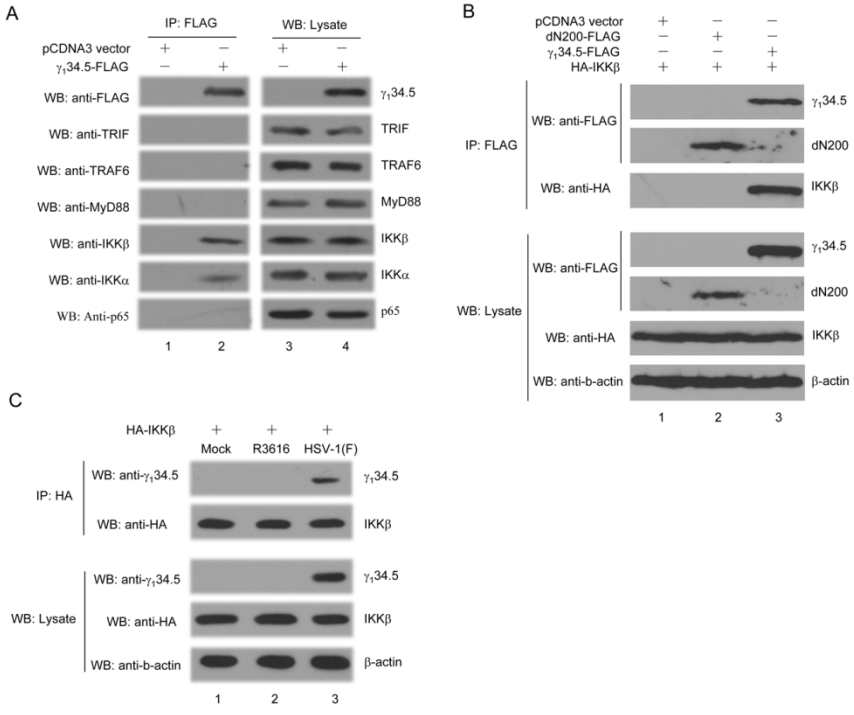


Figure 7

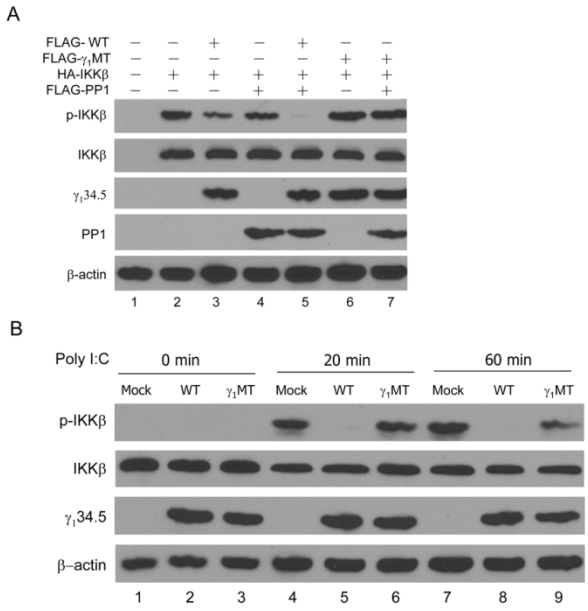


Figure 8

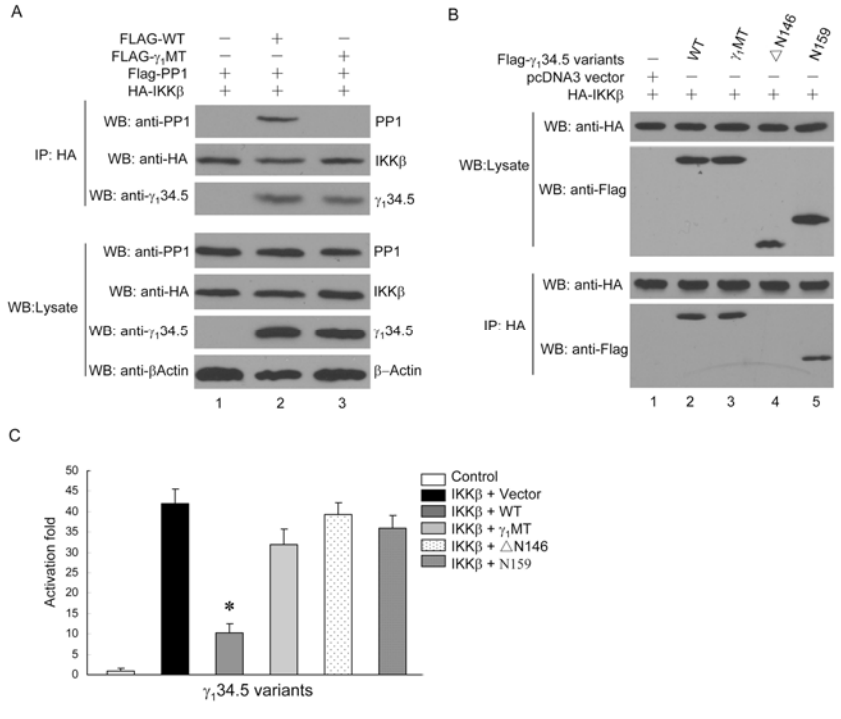
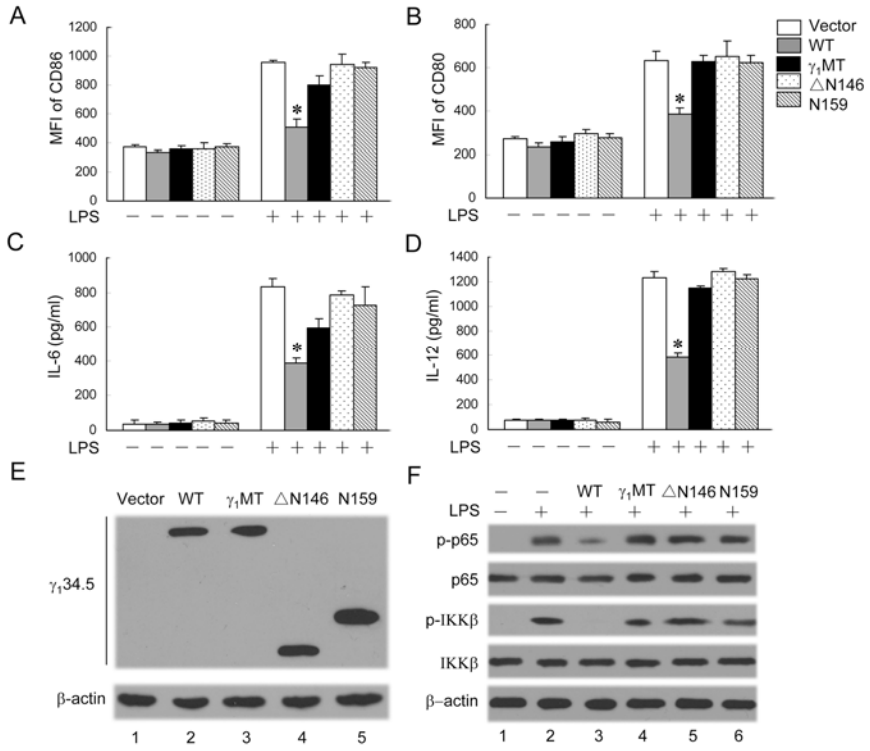


Figure 9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