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Poxvirus Protein N1L Targets the I-κB Kinase Complex, Inhibits Signaling to NF-κB by the Tumor Necrosis Factor Superfamily of Receptors, and Inhibits NF-κB and IRF3 Signaling by Toll-like Receptors*

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Poxviruses encode proteins that suppress host immune responses, including secreted decoy receptors for pro-inflammatory cytokines such as interleukin-1 (IL-1) and the vaccinia virus proteins A46R and A52R that inhibit intracellular signaling by members of the IL-1 receptor (IL-1R) and Toll-like receptor (TLR) family. *In vivo*, the TLRs mediate the innate immune response by serving as pathogen recognition receptors, whose oligomerized intracellular Toll/IL-1 receptor (TIR) domains can initiate innate immune signaling. A family of TIR domain-containing adapter molecules transduces signals from engaged receptors that ultimately activate NF-κB and/or interferon regulatory factor 3 (IRF3) to induce pro-inflammatory cytokines. Data base searches detected a significant similarity between the N1L protein of vaccinia virus and A52R, a poxvirus inhibitor of TIR signaling. Compared with other poxvirus virulence factors, the poxvirus N1L protein strongly affects virulence *in vivo*; however, the precise target of N1L was previously unknown. Here we show that N1L suppresses NF-κB activation following engagement of Toll/IL-1 receptors, tumor necrosis factor receptors, and lymphotoxin receptors. N1L inhibited receptor-, adapter-, TRAF-, and IKK-α and IKK-β-dependent signaling to NF-κB. N1L associated with several components of the multisubunit I-κB kinase complex, most strongly associating with the kinase, TANK-binding kinase 1 (TBK1). Together these findings are consistent with the hypothesis that N1L disrupts signaling to NF-κB by Toll/IL-1Rs and TNF superfamily receptors by targeting the IKK complex for inhibition. Furthermore, N1L inhibited IRF3 signaling, which is also regulated by TBK1. These studies define a role for N1L as an immunomodulator of innate immunity by targeting components of NF-κB and IRF3 signaling pathways.

Vaccinia virus is a poxvirus that was used over 20 years ago to eradicate variola virus infection (smallpox) (1). The variola and vaccinia orthopoxviruses express homologs of proteins encoded by the immune response genes of their hosts (1). These homologs include secreted decoy receptors for interleukins 1 and 18 (IL-1 and IL-18)[1] (1). Vaccinia also encodes proteins that inhibit intracellular signaling by the toll-like receptor family (TLRs) (2). *In vivo*, the TLRs mediate the innate immune response by serving as pathogen-associated pattern recognition receptors (3), whose oligomerized intracellular regions can cluster intracellular Toll/IL-1 receptor (TIR) domains. Clustering of homotypically associated TIR domain-containing adapters transduces signals that ultimately activate NF-κB and cytokine response pathways (4). Vaccinia virus encodes the A46R and A52R proteins, which have been shown to act as dominant negative inhibitors of TIR signaling. A46R has been shown to inhibit IL-1 signaling, whereas A52R interferes with IL-1, IL-18, and TLR signaling, thereby inhibiting both the innate and ultimately the adaptive immune response (2).

Signals initiated by several pro-inflammatory cytokines, or by Toll receptor engagement, lead to TIR clustering that recruits one or more TIR adapter proteins: Mal/TIRAP, TRIF/TICAM-1, MyD88, and TRAM/TIRP/TICAM-2 (5–12). Recruitment of these TIR domain-containing adapter proteins leads to the recruitment of IRAK family members, which subsequently activate TRAF6 (13). Activated TRAF6 is thought to autoubiquitinate and then bind and activate a transforming growth factor-β activated kinase 1-containing complex. Transforming growth factor-β activated kinase 1 then likely phosphorylates and activates the I-κB kinase (IKK) complex (14). The IKK complex is composed of two structurally similar kinases, IKK-α and IKK-β, as well as the noncatalytic regulatory subunit IKK-γ (also called NEMO). The activated IKK complex phosphorylates I-κBα on serines 32 and 36 leading to its polyubiq-

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[1] The abbreviations used are: IL-1, IL-18, interleukins 1 and 18; TLR, Toll-like receptor; TIR, Toll/IL-1 receptor; IKK, I-κB kinase; TNF, tumor necrosis factor; IRF3, interferon regulatory factor 3; HN, C-terminal 6× histidine-arginine tag; HA, hemagglutinin; LPS, lipopolysaccharide; PVDF, polyvinylidene difluoride; dsRNA, double strand RNA; TBK1, TANK-binding kinase 1; tK, thymidine kinase; TFN, tumor necrosis factor; IRAK, interleukin-1 receptor-associated kinase; TRAF, tumor necrosis factor receptor-associated factor; ISRE, interferon-sensitive response element; TRAM, Toll-IL-1-resistance domain-containing adaptor-inducing IFN-β-related adaptor molecule.

uitination and degradation by the proteasome. Degradation of I-κB permits nuclear translocation of NF-κB, ultimately driving transcription of NF-κB-responsive genes (reviewed in Refs. 15 and 16). Current data indicate that the IKK complex is critical for IκB degradation and NF-κB activation in response to pro-inflammatory signals.

Vaccinia virus was known to encode two proteins that inhibit the components of the Toll and IL-1 signaling pathways (2). Other poxviruses encode several uncharacterized homologs of these Toll/IL-1R signaling inhibitors (17). We conducted data base searches that detected a significant similarity among previously identified poxvirus inhibitors of Toll/IL-1R signaling (2) and the N1L protein of vaccinia virus. N1L is among the strongest determinants of vaccinia virus virulence (18, 19). Whereas N1L has modest effects on adaptive immunity, N1L suppresses initial host defenses against vaccinia virus challenge by a factor of 10^4 (18), suggesting that N1L inhibits innate immunity. However, the mechanism whereby the N1L virulence factor functions was previously unknown (18, 19).

Here it is shown that signaling via TNF-α, lymphotoxins, and Toll/IL-1R family members are fundamental immune response pathways inhibited by N1L. Signaling by inflammatory cytokines, TIR adapter molecules, and several downstream components of the NF-κB signaling pathway is inhibited by N1L. N1L co-immunoprecipitates along with members of the IKK complex. N1L also inhibited the activation of interferon regulatory factor 3 (IRF3) in response to double-stranded RNA stimulation of TLR3 or transfection of a TIR adapter that mediates signaling to IRF3. Taken together, these findings are consistent with the hypothesis that N1L disrupts the IRF3/NF-κB signaling pathways by targeting the IKK kinase complex, thereby establishing the role of N1L as a viral immunomodulator of innate immunity.

MATERIALS AND METHODS

Cell Culture and Cell Lines—Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. HEK 293 cell-stable transfectants were maintained in Dulbecco's modified Eagle's medium in the presence of 400 μg/ml Geneticin sulfate, 10% heat-inactivated bovine calf serum, 600 μg/ml L-glutamine, penicillin-streptomycin (Invitrogen), and 20 μg/ml ciprofloxacin. Wild type HEK cells were maintained in the identical medium without Geneticin. The HEK cells stably expressing CD14, MD2, and either TLR2, TLR3, or TLR4 have been described previously (6, 20, 21). For use in transfection assays, HEK 293 cells were typically seeded at 2×10^4 cells per milliliter in 96-well plates 24 h prior to transfection.

Vector Construction and Plasmids—An N1L construct was created by PCR amplification of the N1L open reading frame of vaccinia virus (Copenhagen strain) DNA donated by Francis Ennis and Masanori Terajima (University of Massachusetts Medical School, Worcester, MA). To create N1L-encoding vectors, N1L was amplified using the primers: 5′-ATGAGGACTCTACTTATTAGA-3′ and 5′-TTATTTTTCACCATATAGATC-3′. The PCR product was TA-cloned into pCR3.1 (Invitrogen). The resulting construct, pCR3.1N1L, was sequenced in both directions using bGH reverse and T7 forward primers. N1L cDNA was subcloned into the pDNR-dual vector, which encodes a C-terminal 6× histidine-arginine (HN) tag (BD Biosciences). pDNRN1L-6×HN underwent recombination with a hemagglutinin (HA)-acceptor vector yielding an HA-N1L-HN-tagged version of N1L. The resulting construct was also sequenced. pcDNA3.1N1L was constructed by ligating BamHI-NotI-digested pCR3.1N1L into pcDNA3.1(+) that had been identically digested. FLAG-tagged Mal, TRIF, IKKε, TBK1, and the pGL3–5XκB-luc plasmid were previously described (6). The ISG54 ISRE luciferase vector was purchased from Stratagene. (FLAG-tagged TRAM was also previously described (6).) N-terminally FLAG-tagged IKK-α (22), a C-terminally FLAG-tagged modification of IKK-β (23), FLAG-TRAF6, and FLAG-TRAF2 (24) were gifts of D. Goeddel (Tularik, Inc., San Francisco, CA). The plasmid encoding BZLF1 was a gift of Joan Mannick (University of Massachusetts Medical School). The plasmid thymidine kinase *Renilla* luciferase (pTk-*Renilla*) was purchased from Promega Inc. (Madison, WI). The plasmid encoding MyD88 was from M. Muzio (Mario Negri Institute, Milan, Italy). Chimeric TLR receptors CD4-TLR1, CD4-TLR2, CD4-TLR4, and CD4-TLR6, composed of the extracellular domain of CD4 fused to the transmembrane domain and cytosolic tail of the TLR, were gifts from R. Medzhitov (Yale University, New Haven, CT).

Reagents—Poly(dI·dC) was purchased from Amersham Biosciences. The *Mycoplasma fermentans*-derived membrane macrophage-activating lipopeptide of 2 kDa was generated as previously described (25) and was obtained both from P. F. Mühlradt (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany) and from G. Rawadi (Institute Pasteur, Paris, France). Bacterial lipopolysaccharide (LPS) derived from *Escherichia coli* strain 011:B4 was purchased from Sigma-Aldrich and either used directly or purified as described before (26). Human IL-1-β and TNF-α were purchased from Cell Sciences (Woburn, MA).

Transient Transfection and NF-κB or IRF3 Reporter Assays—HEK 293 cells were transfected with vectors encoding components of either the TIR or the lymphotoxin signal transduction pathways. HEK cells were co-transfected with ISRE luciferase vector or NF-κB luciferase vector, Tk-*Renilla* luciferase vector, N1L or empty vector, and in certain cases with a component of the NF-κB signaling pathway. All HEK 293 cell transfections were performed using GeneJuice (Novagen, Madison, WI) according to the manufacturer's recommendations. Transfection efficiency was normalized by transfection of cells with a plasmid encoding *Renilla* luciferase. The amount of DNA transfected was equalized among experiments by the addition of various amounts of the appropriate empty vector plasmid. To assay firefly and *Renilla* luciferase activity, cells were lysed using passive lysis buffer (Promega), and luciferase activity was determined by standard protocols, using methods as described (7). Results are presented as mean ± S.E. and are consistent with the results of at least three separate experiments. Statistical analyses were via an unpaired, two-tailed *t* test performed using GraphPad Prism software.

Co-immunoprecipitation—HEK cells were co-transfected with HA/HN-tagged N1L and a FLAG-tagged component of the NF-κB signal transduction system, as indicated in the figure legends. For each transfection, a confluent T25 flask of HEK 293 cells was trypsinized and washed. Cells were then lysed on ice for 1 hour in 100 μl of a lysis buffer comprising 0.5% Nonidet P-40, 300 mM NaCl, 50 mM Tris, pH 7.6, and Complete™ protease inhibitor cocktail (Roche Applied Science). Lysates were centrifuged at 4 °C for 20 min at 14,000 × *g*, and lysates were immunoprecipitated with anti-HN antibody (BD Biosciences) bound to Protein A-Sepharose beads (Amersham Biosciences). Immunoprecipitated proteins were boiled in Laemmli buffer for 5 min and electrophoresed on a 15% SDS-PAGE gel. Transfer to a 0.45-μm PVDF membrane was followed by blocking, and then the blot was probed with anti-FLAG monoclonal antibodies (M2, Sigma-Aldrich) and later probed with a donkey anti-mouse horseradish peroxidase (Amersham Biosciences) for 30 min essentially as described (27). In certain experiments the primary antibody step was followed by incubation with a horseradish peroxidase-conjugated antibody that recognizes native rabbit IgG (TrueBlot™, eBioscience, San Diego, CA). Between the blocking and antibody incubation steps the PVDF membrane was washed repeatedly with phosphate-buffered saline/Tween 20. Resulting bands on the immunoblot were developed with the ECL developer system (Amersham Biosciences) and visualized on a Fuji Imager. Immunoprecipitation with anti-HN-coated Protein A-Sepharose beads followed by immunoblot with anti-FLAG antibody was used to confirm the specificity of the results.

In other experiments, anti-HN antibodies (Clontech) were used to co-immunoprecipitate HA-TANK and HA-IKKγ alongside HA-N1L-HN. Subsequently, an immunoblot with anti-HA antibody (Clontech) was performed as above. Finally, anti-IKKγ antibody (Cell Sciences) was used to immunoblot lysates from the indicated transfectants that were immunoprecipitated with anti-FLAG antibody. Presence of IKKγ in lysates was also confirmed.

In Vitro Transcription and Translation and Protein-Protein Interaction—In vitro transcription and translation were performed essentially according to the manufacturer's instructions as outlined in the TnT Quick kit from Promega (Madison, WI) using 0.5 μg of the indicated DNA template, a biotinylated lysine label (Pathdetect, Promega). After confirmation of protein expression with an aliquot of protein, aliquots of N1L and various IKK components were incubated in 20 mM HEPES, pH 7.9, 60 mM NaCl, 1 mM dithiothreitol, 6 mM $MgCl_2$, 8.2% glycerol, and 0.1 mM EDTA and Complete™ protease inhibitor (Roche Applied Science) at 4 °C for 1 h. FLAG-tagged proteins were immunoprecipitated with Protein G-Sepharose-coupled anti-FLAG antibody, or HA-tagged proteins were immunoprecipitated with Protein A-Sepharose-coupled anti-HA antibody and then washed four times in hypotonic lysis buffer (see above). Samples were then analyzed by SDS-PAGE and blotted

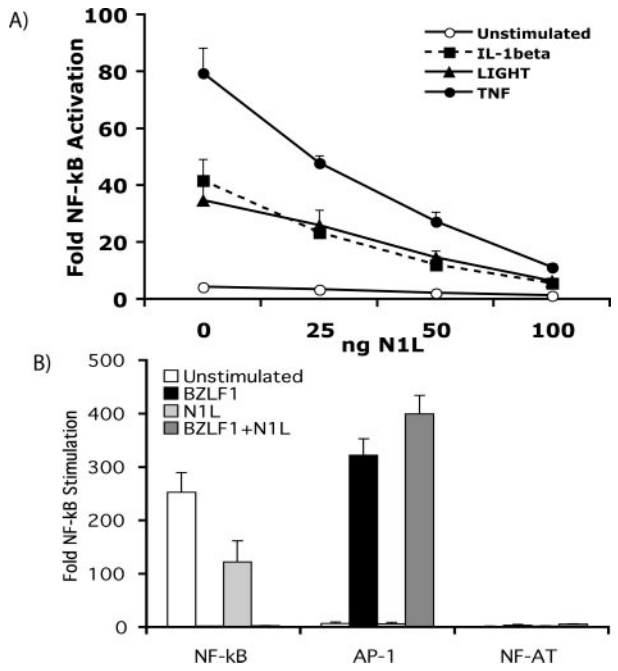

FIG. 1. **N1L inhibits signaling by IL-1, TNF, and the lymphotoxin LIGHT.** *A*, HEK cells were co-transfected with NF-κB-luciferase and *Renilla*-luciferase reporters and 0, 50, or 100 ng/well N1L. At 24 h following transfection, cells were stimulated with 10 ng/ml IL-1β, LIGHT, or TNF-α for 4–6 h. Luciferase activity was measured as described before (2) in triplicate wells in three separate experiments. Results were normalized to *Renilla* luciferase intensity in the same well and are expressed as a ratio to the lowest relative luminescence unit value. These results are representative of at least four individual experiments. *B*, N1L does not inhibit AP-1 luciferase activity. HEK 293 cells were transfected with one of three reporter constructs: NF-κB luciferase (*NF-κB*), AP-1 luciferase (*AP-1*), or NFAT luciferase (*NFAT*). Constructs were co-transfected with *Renilla* luciferase and either empty vector or BZLF1 and analyzed as above. These results are representative of three individual experiments.

onto PVDF membranes, which were blocked in phosphate-buffered saline, 0.05% Tween 20 and subsequently incubated with streptavidin horseradish peroxidase. Labeled proteins on the blot were detected by chemiluminescence (Promega), and images were captured on a Fuji Imager.

RESULTS

The N1L Proteins from Vaccinia and Other Poxviruses Have Sequence Similarity to Inhibitors of NF-κB Signaling—The amino acid sequence of N1L is relatively conserved from sheeppox virus to vaccinia, and N1L amino acid sequence is almost identical among most vaccinia viruses (19). Vaccinia virus encodes two known inhibitors of IL-1 and TLR signaling that are similar in sequence (2), albeit functionally distinct inhibitors of NF-κB signaling (27). Several poxvirus proteins have been noted as potential inhibitors of NF-κB signaling based upon their sequence similarity (17, 28) to known poxvirus inhibitors of NF-κB stimulation (2, 27). Our data base searches demonstrate that N1L is very similar (PHI-BLAST e = 3×10^{-12}) to A52R, which has been shown to inhibit Toll, IL-1β, and IL-18 signaling to NF-κB (2). Because of this sequence similarity, we hypothesized that N1L inhibited signaling to NF-κB.

N1L Inhibits Signaling to NF-κB by IL-1β, TNF-α, and Lymphotoxins—Because N1L has sequence similarity to poxvirus inhibitors of signaling by IL-1 and TLRs (2), we first tested the hypothesis that N1L inhibits IL-1 function. Stimulation of NF-κB activity by IL-1β was significantly suppressed by N1L (Fig. 1*A*, $p = 0.0084$). N1L also inhibited stimulation of NF-κB by TNF-α signaling (Fig. 1*A*, $p = 0.015$). Next, the effect of N1L on stimulation by the lymphotoxin family member, LIGHT (29), was examined. In a simultaneous experiment (Fig. 1*A*), N1L inhibited LIGHT-mediated NF-κB signaling 5-fold compared with vector-transfected cells, which was significant ($p = 0.02$). The finding that N1L also inhibited signaling by TNF-α and LIGHT (Fig. 1*A*), whereas A52R does not block TNF-α (2), indicated that N1L was a functionally distinct vaccinia virus inhibitor of signaling to NF-κB.

To determine the specificity of the inhibition of NF-κB activity by N1L, the activity of N1L on reporter constructs other than NF-κB was tested. N1L was transfected with one of three reporter constructs: NF-κB luciferase, AP-1 luciferase, or NFAT luciferase. Reporter constructs were co-transfected with the Epstein-Barr virus transactivator BZLF1, which both binds to and stimulates transcription from AP-1 sites (30) while inhibiting NF-κB activity in lymphocytes (31). Despite 2-fold inhibition of basal NF-κB activity, N1L did not inhibit AP-1 luciferase activity (Fig. 1*B*). BZLF1 suppressed NF-κB activity, consistent with results in lymphocytes (31). NFAT luciferase activity was not observed. Thus, the inhibitory effect of N1L is specific to the NF-κB and not the AP-1 pathway. In separate experiments, no cytotoxicity was evident in N1L-transfected cells based upon Annexin V staining or flow cytometric analysis (data not shown). Overall, these data indicate that N1L specifically down-modulates signaling by NF-κB that occurs in response to several inflammatory cytokines.

N1L Inhibits Signaling to NF-κB by Toll Receptors TLR2, TLR3, and TLR4—The hypothesis that N1L inhibits Toll receptor signaling was considered for several reasons. First, N1L possesses sequence similarity to vaccinia virus inhibitors of toll-like receptor (TLR) signaling (2). Second, N1L inhibits IL-1β signaling (Fig. 1*A*), suggesting that N1L may interact with a component of the Toll/IL-1 signaling pathway. Finally, whereas N1L does not appreciably inhibit adaptive immunity, such as antibody response and cytotoxic T lymphocyte activity, N1L significantly increases the ability of vaccinia virus to overcome host defenses to initial viral challenge (18), suggesting that N1L inhibits innate immunity. Therefore, we hypothesized that N1L is involved in inhibition of the Toll receptors that mediate an antiviral innate immune response.

To test whether N1L inhibited NF-κB activation by TLR2, TLR3, or TLR4, we used two different approaches: a ligand-dependent system utilizing stable transfection of HEK 293 cells and a transient, constitutively active TLR expression system. HEK 293 cells stably expressing either TLR2, TLR3, or TLR4 were treated with their respective ligands: lipopeptide, the dsRNA mimetic, poly(dI·dC), or bacterial lipopolysaccharide (LPS) (8, 32, 33) and analyzed as in Fig. 1. Chimeric versions of the TLRs, comprising the murine CD4 extracellular domain fused to the cytoplasmic domain of a given human TLR, have proved useful in probing TLR signaling pathways (34). Some TLR cytoplasmic domains can induce gene expression as homodimers (TLR4 and TLR5), whereas others signal as heterodimers (TLR1, TLR2, and TLR6) (35). The extracellular domain of CD4 renders the TLR chimera constructs constitutively active. Using these chimeras, TLR signaling can be examined in the absence of an exogenous activator.

Signaling by TLR2 was first analyzed in stable HEK 293 transfectants. N1L inhibits TLR2-dependent signaling in response to lipopeptide agonists (Fig. 2*A*, *top panel*, $p = 0.0014$ for unpurified LPS, $p = 0.044$ for membrane macrophage-activating lipopeptide of 2 kDa). Use of transient co-transfection of N1L and constitutively active chimeric CD4-TLR1, CD4-TLR2, CD4-TLR4, and CD4-TLR 6 demonstrates N1L-mediated inhibition of TLR2 signaling in conjunction with TLR1 (Fig. 2*A*, *middle panel*, $p = 0.0246$) or TLR6 (Fig. 2*A*, *bottom panel*, $p = 0.0017$).

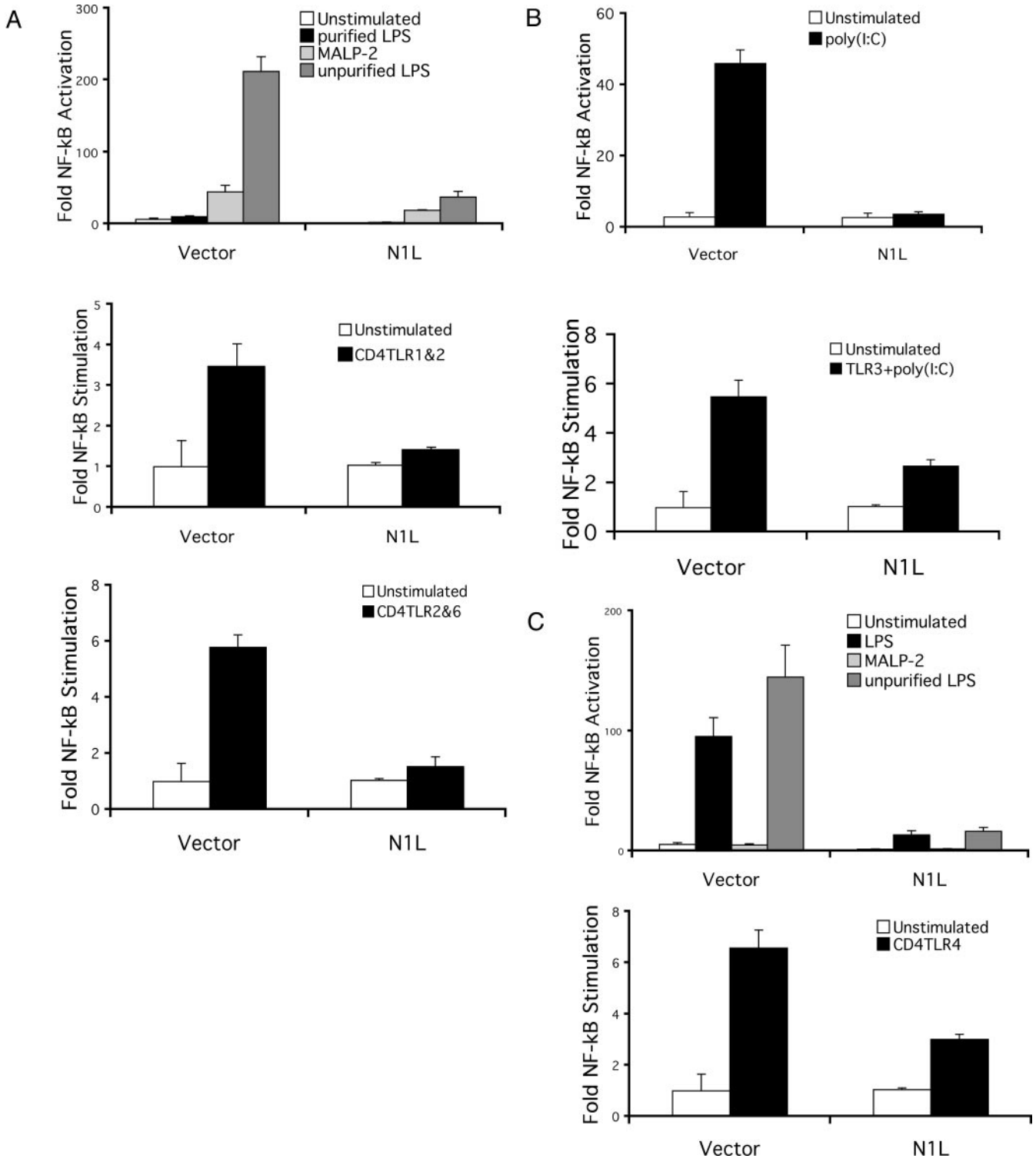

FIG. 2. **N1L inhibits signaling by several Toll receptors.** *A*, N1L inhibits TLR2-dependent signaling in response to lipopeptide agonists. HEK 293 cells transfected with TLR2 were transiently co-transfected with luciferase reporters as above, and N1L or control vector. At 24 h following transfection, cells were stimulated with the indicated ligands. 6 h later luciferase activity was assayed as above. *Middle panel*, N1L inhibits signaling by transiently co-transfected, constitutively active chimeric CD4-TLR1 and CD4-TLR2. *Bottom panel*, N1L inhibits signaling by transiently co-transfected CD4-TLR1 and CD4-TLR6. These results are representative of three independent experiments. *B*, N1L inhibits stimulation of stable TLR3 transfectants with poly(dI·dC). In five independent experiments, HEK 293 cells stably transfected with TLR3 were transfected with N1L and luciferase reporters, stimulated with the indicated ligand, and analyzed 18 h later as above. *Bottom panel*, N1L inhibited signaling induced by poly(dI·dC) treatment of HEK cells transiently transfected with TLR3. These results are representative of three independent experiments. *C*, N1L inhibits the response of TLR4 transfectants to LPS. *Top panel*, HEK 293 cells transfected with TLR4/MD2 were transfected with N1L and luciferase reporters, stimulated with the indicated ligand, and analyzed 18 h later as above. *Bottom panel*, signaling by transiently co-transfected CD4-TLR4 was inhibited by N1L. These results are representative of three independent experiments.

TLR3, which is known to interact with viral dsRNA, was analyzed next. The TLR3 ligand poly(dI·dC) was used to stimulate stable TLR3 transfectants. 12-fold inhibition by N1L of HEK 293 cells stably expressing TLR3 was observed (Fig. 2*B*, *top panel*, $p = 0.0004$). N1L inhibits signaling induced by poly(dI·dC) treatment of HEK cells transiently transfected with

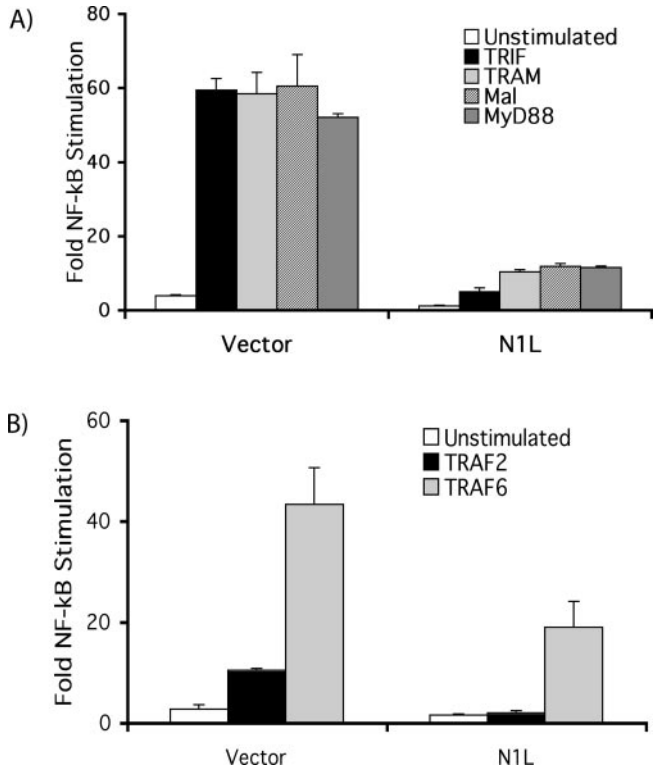

FIG. 3. **TIR adapter and TRAF signaling are inhibited by N1L.** *A*, NF-κB signaling of the TIR adapters MyD88, TRIF, TRAM, and Mal is inhibited by N1L. Several TIR adapters, TRIF, TRAM, MyD88, and Mal/TIRAP, were separately co-transfected with NF-κB luciferase, Tk-*Renilla* luciferase reporters, and N1L or control empty vector (*EV*) into HEK 293 cells. At 24 h following transfection, luciferase activity was assayed in the cell lysates (2). Results with MyD88 and Mal are representative of at least five independent experiments, whereas results with TRIF and TRAM are representative of at least three independent experiments. *B*, induction of NF-κB signaling by TRAF2 and TRAF6 is inhibited by N1L. TRAF2 and TRAF6 cDNAs were separately co-transfected with luciferase reporters as above. NF-κB activation was measured as above in the presence or absence of co-transfected N1L. N1L inhibited NF-κB activation induced by TRAF2 and TRAF6 in three independent experiments.

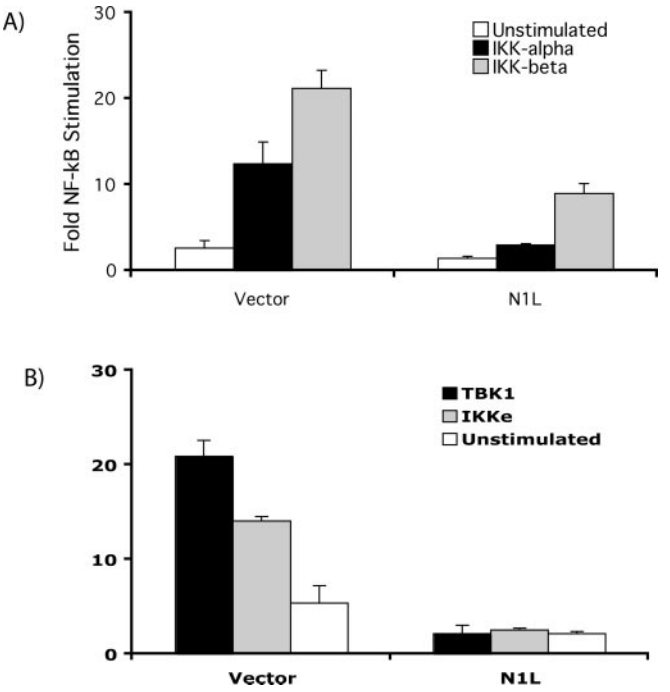

FIG. 4. **N1L inhibits NF-κB activation by TBK1, IKK-α, and IKK-β.** *A*, cDNAs encoding IKK-α and IKK-β were separately co-transfected with N1L into HEK 293 cells with luciferase reporters and analyzed as above. N1L inhibited NF-κB activation induced by ectopic expression of IKK-α and IKK-β in at least five independent experiments. *B*, cDNAs encoding IKK-ε and TBK1 were transfected with N1L or vector control and analyzed as above. N1L inhibited NF-κB activation induced by ectopic expression of TBK1 and IKK-ε in three independent experiments.

TLR3 ($p = 0.0174$) (Fig. 2*B*, *bottom panel*). TLR4 function was next examined and is inhibited by N1L (Fig. 2*C*, *top panel*, $p = 0.011$). CD4-TLR4 signaling is inhibited by N1L ($p = 0.0146$) (Fig. 2*C*, *bottom panel*). Given that N1L inhibits signaling by all Toll receptors tested, one logical candidate for inhibition by N1L was the TIR adapters that transduce signals from Toll, IL-1, and IL-18 receptors (4, 36).

Signaling to NF-κB by TIR Adapter Molecules Is Inhibited by N1L—To identify the mechanism whereby Toll, IL-1, TNF-α and lymphotoxin signal transduction pathways are inhibited by N1L (Fig. 1), we studied the effect of N1L upon membrane-proximal components of the Toll/IL-1R signaling pathways such as Mal/TIRAP, TRIF/TICAM-1, MyD88 (7–11) and TRAM (5, 6) (Fig. 3*A*). Signaling via TRIF/TICAM-1 is especially relevant to innate immunity against vaccinia virus since mutations in TRIF are associated with a 20-fold rise in vaccinia virus production in infected macrophages (37). N1L inhibits TRAM and MyD88 activation of NF-κB 4-fold, which is significant ($p < 0.002$). N1L inhibits Mal/TIRAP and TRIF activation of NF-κB by 6-fold (Fig. 3*A*, $p < 0.005$). In general, the N1L-induced inhibition of the TIR adapters is comparable, suggesting that a common signaling component downstream in the Toll/IL-1R signaling pathway might be inhibited. This suggested the hypothesis that TRAF family members or components of the IKK complex might be targeted by N1L. This hypothesis that the IKK complex is the target of N1L is consistent with the fact that TIR-independent signaling, such as signaling induced by TNF and lymphotoxins, is inhibited by N1L (Fig. 1*A*).

NF-κB Signaling by TRAF2 and TRAF6 Is Inhibited by N1L—Signals initiated by pro-inflammatory cytokines or Toll receptor engagement lead to the activation of a family of serine/threonine kinases, the IRAK family, and TRAF6, ultimately activating the I-κB kinase (IKK) complex and leading to NF-κB translocation (15). Furthermore, because of the known interaction between A52R and TRAF6 (27) and because N1L has sequence similarity to vaccinia A52R, functional interactions between N1L and TRAF6 were examined next.

Fig. 3*B* demonstrates that signaling by ectopic overexpression of TRAF2 or TRAF6 is inhibited by N1L. In Fig. 3*B*, 4-fold inhibition of TRAF6 signaling and a 5-fold inhibition of TRAF2 signaling were observed, which is significant ($p = 0.0002$ and $p = 0.05$, respectively). Although it is formally possible that N1L individually targets both TRAF2 and TRAF6 for inhibition, the data of Fig. 3 suggest that inhibition by N1L may occur downstream of these TRAF proteins.

NF-κB Signaling by TBK1, IKK-α, and IKK-β Is Inhibited by N1L—Several lines of evidence pointed to the IKK complex as being central to the function of N1L. Signaling by IL-1β, TNF-α, Toll, and LIGHT converged at the IKK complex and was inhibited by N1L (Figs. 1 and 2). Furthermore, both IKK-α and IKK-β signal downstream of the distinct TRAF2 and TRAF6 signaling pathways, which are inhibited by N1L (Fig. 3). Finally, viral modulation of signaling by the IKK complex occurs via the adenovirus 14.7-kDa protein (38), which was reported to be similar to N1L (18).

To test the hypothesis that N1L-mediated inhibition of NF-κB signaling occurs at (or distal to) the IKK complex itself, ectopic overexpression of IKK-α and IKK-β was performed in

cells transfected with N1L or empty vector (Fig. 4*A*). The function of IKK-α and IKK-β is significantly inhibited by N1L (Fig. 4*A*, $p < 0.02$ for IKK-α and $p < 0.0066$ for IKK-β). Two related kinases, IKKε (also designated IKK-i) and TANK-binding kinase 1 (TBK1) (alternatively designated NAK and T2K), have been found to be part of a multiprotein complex that also contains IKK-α, IKK-β, IKK-γ, and the TRAF family member-associated NF-κB activator, TANK (39). The ability of N1L to inhibit these kinases was tested in Fig. 4*B*. N1L inhibits TBK1 stimulation of NF-κB ($p = 0.003$) and IKKε ($p = 0.01$). Thus, N1L inhibits the NF-κB stimulatory function of four kinases in the IκB kinase complex.

Co-immunoprecipitation of N1L and Certain NF-κB Signaling Components of the IKK Complex—Poxvirus protein A52R physically associates with IRAK2 and TRAF6, thereby inhibiting Toll and IL-1 signaling (27). Many other inhibitors of the TLR and IL-1R pathway physically associate with the components that they inhibit (4, 32). Because N1L inhibited NF-κB signaling components at and/or upstream of the IKK complex (Figs. 2–4), we tested for a physical association between N1L and components of the IKK complex such as IKK-α and IKK-β (Fig. 5*A*). Two related kinases, IKKε and TBK1, have also been shown to be part of the IKK complex (39). Therefore, we also examined the interactions of N1L with IKKε and TBK1 (Fig. 5, *B* and *C*).

To identify which kinases of the IKK complex associated with N1L, a 6×HN-tagged N1L fusion protein was co-expressed in HEK cells with several FLAG-tagged components of the NF-κB signal transduction system. Immunoprecipitation by anti-6×HN antibody and immunoblot with anti-FLAG antibody was performed, which revealed an association between N1L and IKK-α and IKK-β (Fig. 5*A*, *top panel*). The *bottom panel* is an anti-HN blot of the lysates used in the immunoprecipitation, and it demonstrates approximately equal loading of HA-N1LHN-tagged protein. Thus, Fig. 5*A* indicates that N1L can co-immunoprecipitate IKK-α and IKK-β. When the same lysate was immunoprecipitated with anti-FLAG antibody, only a minimal amount of N1L fusion protein was detected, suggesting only a minimal association between IKK-α and IKK-β on the reverse immunoprecipitation (data not shown).

TBK1 and IKKε co-immunoprecipitate with N1L (Fig. 5, *B* and *C*). Because the association between N1L and TBK1 occurs whether either N1L or TBK1 is the protein being immunoprecipitated, these interactions in the immunoprecipitations provide further evidence that N1L and TBK1 are specifically associated. Fig. 5 suggests an association between N1L and components of the IKK complex. IKK-α and IKK-β have been shown to form a large complex with IKKε, TBK1, TANK, and members of the TRAF family. IKK-γ forms an oligomeric structure with IKK-α and IKK-β (40), associating with TANK, and thereby, TBK1 and IKKε (39). Furthermore, several other viral proteins that modulate signaling to NF-κB specifically target IKK-γ. Therefore, we examined interactions between N1L and IKK-γ (Fig. 6*A*). Fig. 6A indicates that HA-IKK-γ and HA-TANK co-immunoprecipitate with N1L.

Because HA-IKK-γ co-immunoprecipitates with N1L, we considered whether N1L might disrupt the association of IKK-γ with IKK-α/β by binding to sites common to their point of physical association. Peptides containing a C-terminal motif of IKK-α/β, which is required for IKK-γ binding, have been shown to disrupt formation of IKK-γ·IKK-α/β complexes (41). Thus, another hypothetical mechanism of action of N1L would be to disrupt the association of IKK-γ with IKK-α/β. If N1L bound IKK-α/β directly at the IKK-γ·IKK-α/β binding site, formation of IKKγ·IKK-α/β complexes would be inhibited. This is not seen in Fig. 6*B*, where the association between IKKβ·IKKγ

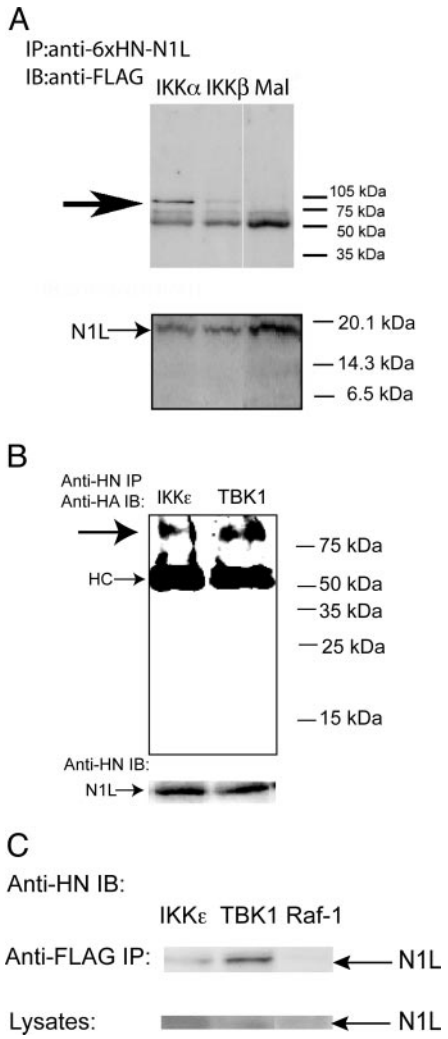

FIG. 5. **N1L associates with components of the IKK signaling complex.** *A*, IKK-α and IKK-β associate with N1L. *Top panel*, an N1L-HN fusion protein was co-expressed in HEK cells with FLAG-tagged: IKK-α (*lane 1*), IKK-β (*lane 2*), and Mal (*lane 3*). Proteins immunoprecipitated with anti-HN were detected on the blot with anti-FLAG-antibody. The *arrow* at the *left* denotes the approximate size of IKK-α and IKK-β. Control, FLAG-tagged Mal does not co-immunoprecipitate with N1L-6×HN. The *bottom panel* denotes loading of the N1L-6×HN fusion protein. *B*, TBK1 associates with N1L. *Top panel*, an N1L-HN fusion protein was co-expressed in HEK cells with FLAG-tagged: IKKε (*lane 1*) and TBK1 (*lane 2*). Proteins immunoprecipitated with anti-HN were detected on the blot with anti-FLAG-antibody. The *arrow* at the *left* denotes the approximate size of IKK-ε and TBK1. Control, FLAG-tagged TRAF6 does not co-immunoprecipitate with N1L-6×HN. The *bottom panel* denotes loading of the N1L-6×HN fusion protein. *C*, N1L associates with TBK1. *Top panel*, FLAG-tagged IKK-ε (*lane 1*), FLAG-tagged TBK1 (*lane 2*), and endogenous raf-1 (*lane 3*) were immunoprecipitated and probed on the blot with anti-HN antibody. The *arrow* at the *left* denotes the size of HAN1L-6×HN. The *bottom panel* demonstrates the amount of N1L-6×HN fusion protein in 5% of the lysate from each sample. *IP*, immunoprecipitation; *IB*, immunoblot.

persists in the presence of N1L. N1L also associated with TANK (Fig. 6*A*), which suggested that TANK, as well as TBK1, might together be direct targets of N1L. Because other poxvirus inhibitors target tandem members of the NF-κB signaling pathway (27), we sought to test whether N1L directly associated with TANK, TBK1, and IKK-γ.

In Fig. 7, protein-protein interactions are compared between *in vitro* transcribed and translated N1L and either TBK1, IKK-γ, and TANK. Aliquots of N1L and various IKK complex components were incubated, subsequently immunoprecipi-

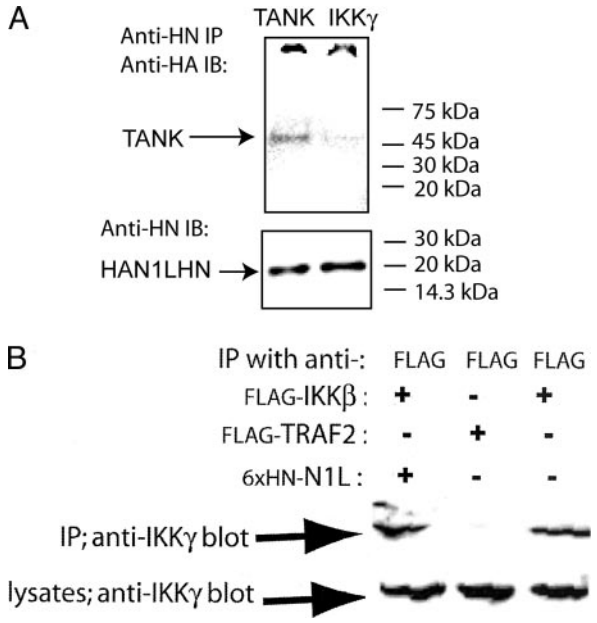

FIG. 6. **TANK and IKKγ associate with N1L.** *A*, HEK cells were co-transfected with either HA-TANK or HA-IKKγ and N1L-6×HN. Lysates were immunoprecipitated as denoted in the figure, and proteins were detected with anti-HA antibody. *B*, IKK-β·IKKγ associations persist in the presence of N1L. A N1L-HN fusion protein was co-expressed in HEK cells with FLAG-IKK-β or FLAG-TRAF2. Lysates were immunoprecipitated as denoted in the figure, and proteins were detected with anti-IKKγ antibody.

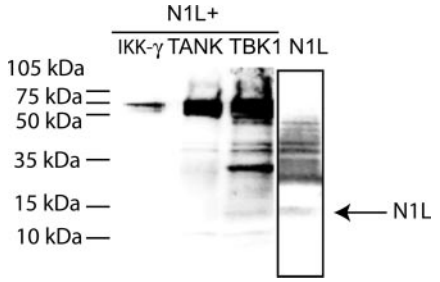

FIG. 7. **N1L interacts with TBK1 *in vitro*.** Protein-protein interactions were assessed using *in vitro* transcribed and translated N1L (*lane 4*) and either IKK-γ (*lane 1*), TANK (*lane 2*), or TBK1 (*lane 3*). Following binding *in vitro* the proteins were immunoprecipitated and then analyzed by SDS-PAGE. The *arrow* at the *right* denotes a 15-kDa band in the *N1L* and *TBK1 lanes*. A band of ~50 kDa is present in the *TANK* and *IKK-γ lanes*, consistent with their molecular sizes.

tated, and then analyzed by SDS-PAGE. The results in Fig. 7 demonstrate a band in the N1L and TBK1 lanes at ~15 kDa, which corresponds to the anticipated size of N1L (18). This N1L band is not present in the lanes containing *in vitro* translated IKK-γ and TANK. *In vitro* translated/transcribed IKK-γ and TANK are each present in the lanes as indicated by a band of ~50 kDa. These data are consistent with the finding in Fig. 5 that TBK1 immunoprecipitates with N1L and N1L immunoprecipitates with TBK1. Furthermore, signaling to NF-κB is inhibited by N1L (Figs. 1–4), and N1L inhibits NF-κB signaling by overexpression of TBK1 (Fig. 4*B*). Overall, the results of Fig. 7 suggest that N1L directly targets TBK1 to inhibit activation of NF-κB.

N1L Inhibits Signaling by the Interferon Response Pathway—The host innate immune system responds to pathogens via the interferon response pathway, which can be induced by TLR3 and TLR4 signaling in response to viruses, double-stranded RNA, and LPS (43). The TIR adapters TRIF and TRAM have been shown to mediate signaling via the IRF3

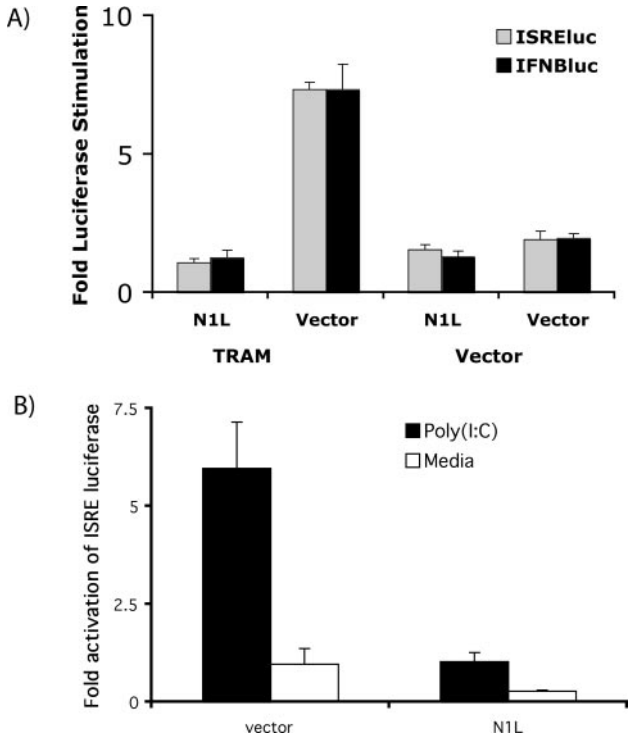

FIG. 8. **N1L inhibits IRF3 signaling.** *A*, N1L inhibits activation of the ISRE and the IFN-β promoter induced by transfection the TIR adapter, TRAM. HEK cells were co-transfected with *Renilla*-luciferase and either ISRE- or IFN-β-promoter luciferase, either 25 ng/well vector or TRAM, and either 100 ng/well N1L-encoding plasmid, or empty vector to a total of 200 ng/well. Luciferase activity was measured at 24 h as described (2). Results are consistent with three experiments. *B*, N1L inhibits IRF3 signaling induced by double-stranded RNA. HEK cells stably expressing TLR3 were co-transfected with *Renilla*-luciferase, ISRE-luciferase, and either 100 ng/well N1L-encoding plasmid or empty vector to a total of 200 ng/well. Luciferase activity was measured at 24 h as described (2). Results are consistent with three experiments.

pathway in response to dsRNA (poly(dI·dC)) or LPS stimulation of TLR3 and -4, respectively (44). The downstream kinases involved in signaling include TBK1 (42) and IKKε (6), which phosphorylate the IRF3 transcription factor. Phosphorylation permits IRF3 activation and translocation to the nucleus where IRF3 binds co-ordinately with NF-κB to promoters such as the IFN-β promoter driving the production of cytokines such as IFN-β (6).

The observation that N1L physically targets TBK1 and IKKε (Fig. 5) suggested the hypothesis that N1L would inhibit TIR adapter stimulation driving the ISRE promoter. Because N1L inhibits signaling to NF-κB, we hypothesized N1L would inhibit the activity of promoters containing both NF-κB and ISRE elements, such as the interferon β (IFN-β) promoter. To test this hypothesis, N1L was co-transfected with plasmids encoding ISRE and IFN-β promoter elements driving a luciferase construct. The *top panel* in Fig. 8 demonstrates N1L-mediated inhibition of ISRE ($p = 0.0002$) and IFN-β ($p = 0.0046$) luciferase activity. The *bottom panel* in Fig. 8 demonstrates that N1L inhibits IRF3 signaling induced by the dsRNA ligand, poly(dI·dC) in TLR3 expressing HEK cells ($p = 0.01$). Taken together these data indicate that N1L associates with the IKK complex and that N1L inhibits NF-κB and IRF3 signaling.

DISCUSSION

The virulence factor N1L down-modulates host cytokine responses and dysregulates innate immune signaling by IL-1R, TLRs, and members of the TNF receptor superfamily. The data presented here demonstrate that N1L functions, at least part, by associating with, and inhibiting, the IKK complex. The

demonstration that N1L associated with several IKK complex kinases provides a mechanistic explanation for the N1L-mediated inhibition of NF-κB-activating stimuli such as cytokines (LIGHT, TNF-α, and IL-1 in Fig. 2) or overexpression of intracellular signaling components such as TRAF2, TRAF6, and the TIR adaptors (Figs. 2 and 3). All these pathways converge on the IKK signaling complex (23, 45, 46), which is inhibited by N1L (Fig. 4). Because IKK-α and IKK-β are associated with the large IKK multiprotein complex via their regulatory subunit IKK-γ (39), IKK-α and IKK-β may be indirectly targeted because both kinases associate with IKK-γ, which physically associates with N1L (Fig. 6*A*). Other viral proteins have been described that physically associate with IKK-γ, thereby altering signaling to NF-κB (38, 47, 48).

However, several lines of evidence suggest that N1L does not function by inhibiting the association of IKK-α or IKK-β with IKK-γ. First, indirect evidence against N1L functioning by association with IKK-γ is provided by the association of N1L with TBK1 and IKKε (Fig. 5*B*) and *vice versa* (Fig. 5*C*). TBK1 and IKKε are linked to IKK-α/β by IKKγ and TANK, suggesting that a proportion of the IKK-γ remains associated with IKK-α/β in the presence of N1L. Second, N1L does not interfere with the IKK-γIKK-β association (Fig. 6*B*), which further suggests that N1L does not target IKK-γ directly. The limited amount of HA-IKKγ associated with N1L (Fig. 6*A*) suggests that N1L may target another member of the IKK-containing multiprotein complex (39). The direct interaction of N1L with TBK1 (Fig. 7) suggests that a principal target of N1L is TBK1. Overall, these observations are consistent with the hypothesis that N1L targets the IKK complex by a mechanism that may be distinct from other viruses (38, 47, 48).

TBK1 is the IKK component that is most strongly implicated as a target of N1L (Fig. 8). TBK1 has been shown to be involved in activation of NF-κB-dependent transcription (49–51). Most recently a novel, TBK1-interacting IKK component, NAP-1, has been shown to mediate NF-κB activation by TBK1 via phosphorylation of the p65 subunit of NF-κB (52). However, the role of TBK1 in NF-κB activation is still unclear, because some of the effects attributed to TBK1 deficiency cannot reproducibly be linked to NF-κB and are instead hypothesized to relate primarily to effects on signaling by interferon regulatory factor 3 (IRF3) (42). TBK1 and IKKε mediate the type I interferon antiviral response (6, 42). The data presented here indicate that TBK1 associates with N1L (Figs. 5*B*, 5*C*, and 7), and IKKε associates with N1L less strongly (Figs. 5*B*, 5*C*, and 7). These findings suggested the hypothesis that, in addition to inhibiting NF-κB activation, N1L might also inhibit IRF3 signaling that is mediated by TBK1 and IKKε. In Fig. 8, N1L inhibits IRF3 signaling. Future studies will be needed to further test the hypothesis that the association of N1L with TBK1 interrupts the type I interferon antiviral response that is mediated in part by IRF3.

N1L-mediated inhibition of signaling by TNF superfamily members is a distinct, and perhaps vital, viral immune evasion mechanism. The lymphotoxins (LTs), LT-α, LIGHT, and LT $\alpha 1\beta 2$ (LT-β), are part of the TNF superfamily (53). The activation of lymphotoxin receptors stimulates antiviral responses (54–56), NF-κB translocation (57), and lymphoid organogenesis. Furthermore, TNF-α itself has potent antiviral effects and can induce programmed cell death in certain virus-infected cells (reviewed in Refs. 54 and 58). Several viral mechanisms for avoiding immune responses mediated by TNF-α have been described (54, 58). Although certain poxviruses encode decoy TNF-α receptors, only certain decoy receptors neutralize LT-α, and no other poxviral lymphotoxin antagonist has been identified to date (59). Thus, N1L-mediated inhibition of the lymphotoxin and TNF-α antiviral pathways may influence the outcome of poxviral infections.

Poxviral inhibitors of signaling inhibit a wider array of cellular anti-viral genes than is possible with a single, virus-encoded soluble decoy receptor. For example, N1L targets signaling by IL-1β, TNF-α, lymphotoxins, and Toll receptors (Figs. 1 and 2). Thus, targeting of the IKK complex identifies N1L as a unique vaccinia virus inhibitor of certain antiviral signaling pathways. Simultaneously, N1L is one of several poxvirus proteins that target distinct points in the innate antiviral signaling pathway (17, 28), all of which converge at the IKK complex. Furthermore, N1L inhibits signaling by the innate immune pathways that simultaneously suppress vaccinia virus replication (37) and activate the adaptive immune response (3, 4, 60). We hypothesize that the much less virulent, yet almost equally immunogenic, phenotype of N1L-deficient vaccinia virus (18) reflects suppression of innate immune signaling by N1L (Figs. 1–4, and 8), resulting in increased virulence (18, 19) and an inhibited adaptive immune response during wild type vaccinia virus infection *in vivo* (18). Overall, identification of NF-κB and IRF3 signaling pathways as a target of N1L identifies the role of N1L as a viral protein with novel immunomodulatory properties.

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