Epstein–Barr latent membrane protein 1 transformation site 2 activates NF-κB in the absence of NF-κB essential modifier residues 133–224 or 373–419

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Epstein Barr virus latent membrane protein 1 (LMP1) induces NF-κB activation through transformation effector sites (TES) 1 and 2, both of which are critical for B-lymphocyte transformation. TES2 principally activates canonical NF-κB, which we confirm is NF-κB essential modifier (NEMO)-dependent and requires an intact ubiquitin binding in A20 binding inhibitor of NF-κB and NEMO (UBAN) domain. LMP1 TES2 activated NF-κB in Jurkat cell lines harboring NEMO truncated at 372 (A45) or NEMO with an in-frame deletion of 133–224 (2C), whereas TNFα, 12-O-Tetradecanoylphorbol-13-acetate, human T-cell leukemia virus 1 Tax, and CD40 did not. In both A45 and 2C Jurkat cell lines, LMP1 TES2-mediated NF-κB activation was blocked by siRNAs to TNFα receptor-associated factor 6 and NEMO, by IκB kinase inhibitors, and by the IκBα superrepressor, indicating that the NEMO mutants function to support canonical NF-κB activation. Expression of A45 or 2C mutants in NEMO-deficient murine embryonic fibroblasts reproduced the Jurkat phenotypes: LMP1 TES2 activated NF-κB in fibroblasts lacking NEMO amino acids 133–224 or 373–419, but TNFα and Tax did not. Further analysis indicated that TES2 did not activate NF-κB in cells expressing the double deletion mutant Δ133–224/Δ372–419. These data provide further evidence of the essential role for NEMO in LMP1 TES2 NF-κB activation and highlight the importance of unique domains within NEMO for sensing distinct NF-κB stimuli.

IκB kinase γ | tumor necrosis factor alpha | human T-cell leukemia virus 1 Tax | CD40

Epstein-Barr virus (EBV) transforms resting B lymphocytes into continuously proliferating lymphoblastoid cell lines (LCLs) (1). Latent membrane protein 1 (LMP1)-mediated NF-κB activation is essential for the initial establishment and long-term survival of LCLs (2). LMP1 constitutively activates NF-κB through two domains in the cytoplasmic tail, transformation effector sites (TES) 1 and 2, which interact with TNFα receptor (TNFR)-associated factors (TRAFs) and death domain proteins including TNFR-associated death domain protein (TRADD) and receptor interacting protein (RIP) (3–8). Viruses that harbor LMP1 mutations that cannot bind TRAFs or TRADD are deficient in NF-κB activation and transformation of B lymphocytes (5, 6, 9). NF-κB inhibitors cause LCLs death (10, 11). Thus, understanding the mechanisms by which LMP1 mediates NF-κB activation is important for potential interventional therapies.

NF-κB transcription factors are held inactive in the cytoplasm by inhibitors, IκBs. NF-κB complexes translocate to the nucleus after the IκBs are phosphorylated, ubiquitinated, and degraded. LMP1 TES1 primarily activates the noncanonical pathway in which p100 is cleaved to p52, eliminating the IκB activity of the C terminus and allowing p52 complexes to move into the nucleus (12–15). In contrast, LMP1 TES2 activates the canonical NF-κB pathway in which IκB activity is encoded in a separate protein IκBα, IκBβ, or IκBε. IκB degradation releases p50/Rel heterodimers or Rel homodimers that translocate to the nucleus (reviewed in ref. 16).

The IκB kinase (IKK) complex phosphorylates IκBs and p100. The IKK complex consists of two catalytic subunits, IKKα and IKKβ, and dimer of a noncatalytic subunit, NF-κB essential modifier (NEMO, also IKKγ reviewed in ref. 16). Generally, NEMO is not required for noncanonical NF-κB activation but is essential for the canonical pathway. NEMO is a 419 amino acid protein that consists of two coiled-coil domains (CC1 and CC2), a ubiquitin (Ub) binding in A20 binding inhibitor of NF-κB (ABIN) and NEMO (UBAN) domain, a leucine zipper domain (LZ), and a zinc finger domain (ZNF) (17) (Fig. 1*A*). IKKα/β are each constitutively bound to a NEMO dimer through NEMO residues 50–86 (18). The IKK complex is activated after NEMO is recruited to Ub-modified receptor-associated proteins where kinases and Ub ligases further modify the IKKs and NEMO, respectively. This is best characterized in TNFR1 signaling, where TNFR1-associated proteins such as RIP are modified with K63-linked Ub by the combined action of TRAFs and Cellular Inhibitor of Apoptosis Proteins (cIAPs) (19–21). K63-linked Ub chains recruit the transforming growth factor beta activated kinase 1 (TAK1)/TAK1 binding (TAB) protein complex and the Linear Ubiquitin Chain Assembly Complex (LUBAC) (22). The IKK complex is also recruited to the receptor through the NEMO UBAN domain. Subsequently, the TAB/TAK1 complex phosphorylates IKKβ in the activation loop, and LUBAC introduces head-to-tail linear Ub modifications onto NEMO (and perhaps, also other NF-κB signal transduction components) (23). It is unclear if the recruitment of the IKK complex occurs through NEMO binding to K63-linked or linear Ub chains. The isolated NEMO UBAN domain binds to linear Ub chains with 100-fold higher affinity than K63-linked Ub chains (24–26). However, the NEMO UBAN and ZNF domains together bind equally well to K63 and linear Ub (27). Recent data suggest that both K63 and linear Ub may be required for IKK activation. Initial binding of the IKK complex to TNFR may result from the NEMO UBAN–ZNF interaction with K63-modified RIP. However, NF-κB activation occurs only after LUBAC modifies NEMO with linear Ub, creating a high-affinity IKK binding site (22).

The mechanisms by which IKK complex activation occurs may differ with distinct stimuli. This is most clearly evident in some human NEMO mutations. NEMO is encoded on the X chromosome, and deletion is lethal in males. However, hypomorphic mutations have been identified in boys with ectodermal dysplasia and immunodeficiency (EDA-ID) (28–30). Where tested, each mutation has distinct effects on CD40, TNFα, Toll-like receptors (TLRs), and antigen receptor signaling. Mutations in the ZNF are most common and are usually defective for TNFα and IL-1 NF-κB activation (30).

Because NEMO is essential for canonical NF-κB activation, we were surprised to observe LMP1 TES2-mediated NF-κB activation in cell lines where NEMO was reported absent (14, 31). We, therefore, examined the role of NEMO in LMP1 TES2-mediated

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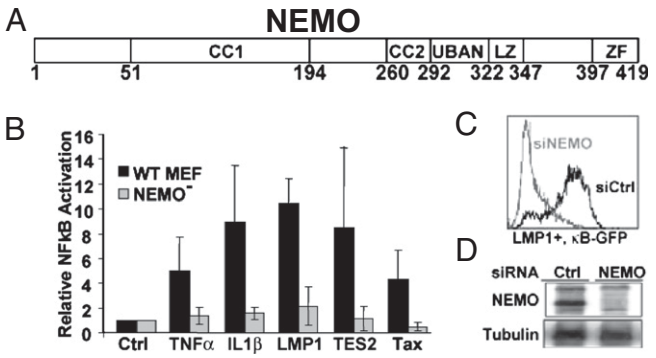

Fig. 1. NEMO is required for LMP1 TES2-mediated NF-κB activation in MEFs and 293 cells. (*A*) Schematic of NEMO: coiled-coil (CC) 1, CC2, ubiquitin binding in ABIN and NEMO (UBAN) domain, leucine zipper (LZ), zinc finger domain (ZF), and residues as indicated. (*B*) WT or NEMO$^-$ murine embryonic fibroblasts were transfected with an NF-κB–luciferase reporter, PGKβ-gal, and cotransfected with PGK2 control vector (ctrl), flag LMP1 (LMP1), flag-LMP1 TES2 (P204A/Q206A TES1 mutation; TES2), or HTLV1 TAX (Tax) as indicated. Vector control cells were either untreated or treated with TNFα (10 ng/mL) or IL-1β (10 ng/mL) for 18 or 6 h posttransfection. Luciferase and β-gal activity was determined at 24 h. (*C*) 293 cells with regulated expression of LMP1 TES2 and an integrated NF-κB–EGFP reporter plasmid were transfected with nontargeting siRNA (siCtrl) or a pool of four siRNAs to NEMO (siNEMO); 3 d later, LMP1 TES2 expression was induced for 24 h, and GFP was determined by FACS. (*D*) Western blot analysis of NEMO and tubulin of lysates from *C*.

NF-κB activation and confirmed that NEMO is essential for IKKβ activation. However, the requirements for domains within NEMO differ for LMP1 TES2-mediated NF-κB activation from those required for TNFα, human T-cell leukemia virus 1 (HTLV1) Tax, 12-O-Tetradecanoylphorbol-13-acetate (TPA), or CD40 activation.

Results

LMP1 TES2-Mediated NF-κB Activation Requires NEMO. To determine whether NEMO is required for LMP1 TES2-mediated NF-κB activation, we performed NF-κB luciferase reporter assays in wild-type (WT) and NEMO-deficient murine embryonic fibroblasts (MEFs). Although LMP1 and LMP1 TES2 induced NF-κB activity 8- to 10-fold in WT MEFs, both induced less than twofold activity in NEMO$^-$ MEFs (Fig. 1*B*). Likewise, TNFα, IL-1β, and HTLV1 Tax activated NF-κB in WT but not NEMO$^-$ MEFs (Fig. 1*B*). We also examined the effect of siRNA targeting NEMO in 293 cells. NEMO knockdown substantially diminished both NEMO expression and LMP1 TES2-mediated NF-κB activation of an integrated NF-κB–EGFP reporter (Fig. 1 *C* and *D*). Similar results were obtained with two other siRNA pools targeting distinct portions of the NEMO mRNA. TES2 NF-κB activation could be rescued by introduction of a siRNA-resistant NEMO cDNA. These data indicate that NEMO is essential for LMP1 TES2-mediated NF-κB activation in fibroblasts and epithelial cells and extend the observations made in Rat 1 fibroblasts expressing NEMO 1–151 (13). These data also suggest that the reported LMP1 induced p65 nuclear translocation in NEMO$^-$ MEFs may have been TES1-mediated (14, 32).

LMP1 and LMP1 TES2 Activate NF-κB in Mutant Jurkat Cells, Whereas TNFα, Tax, TPA, and CD40 Do Not. Because NEMO was required for LMP1 TES2-mediated NF-κB activation in MEFs and 293 cells, we were surprised to find that LMP1 TES2 induced robust NF-κB reporter activity in two Jurkat cell lines reported to be NEMO-deficient. Both Jurkat mutants, A45 and 2C, were generated by chemical mutagenesis and selected for deficiencies in either TNFα- or Tax-mediated NF-κB activation (14, 31). In 2C cells, LMP1 and LMP1 TES2 activated NF-κB 20- to 30-fold compared with >60-fold in the 2C reconstituted cell line 2Cγ (Fig. 2*A*). LMP1 effects contrasted with TNFα, TPA, and Tax, all of which failed to induce any NF-κB activity in 2C. Likewise, LMP1 and LMP1 TES2 activated NF-κB in A45 cells as well or better than in parental NEMO+ Jurkat cell line. TNFα, Tax, and TPA differed from LMP1 in that NF-κB activation was substantially diminished in A45. CD40-mediated NF-κB activation by an LMP1/CD40 chimera (33, 34) was impaired in both 2C and A45 cells, with the residual NF-κB activity likely mediated through the noncanonical pathway (Fig. 2 *A* and *B*).

Because NEMO expression was essential for LMP1 TES2-mediated NF-κB activation in epithelial cells but seemed dispensable in Jurkat cells, we further characterized the A45 and 2C cell lines. Western blots using a polyclonal antibody raised against full-length NEMO showed weak immunoreactive proteins present at ~42 kD in A45 and 2C (Fig. 2*C*). The NEMO mutant expressed in A45 cells was incorporated into IKK complexes as shown by coimmunoprecipitation with IKKα (Fig. S1*A*).

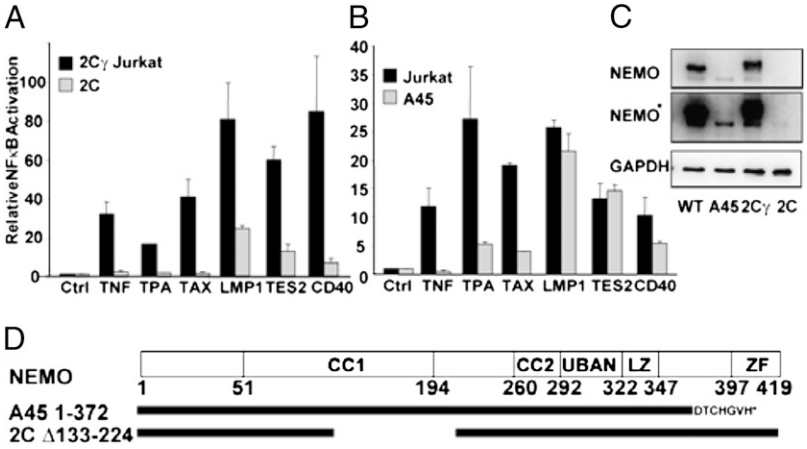

Fig. 2. LMP1 and LMP1 TES2 induce NF-κB in NEMO mutant Jurkat cells. NF-κB reporter assays were performed in (*A*) 2C (NEMO mutant) and reconstituted 2Cγ or (*B*) A45 (NEMO mutant) and parental Jurkat cells. As in Fig. 1, cells were cotransfected with reporter constructs and either PGK2 (Ctrl), HTLV1 TAX (TAX), Flag-LMP1 (LMP1), Flag LMP1 TES2 (TES2), or a ligand-independent FLAG-LMP1 CD40 chimera (CD40). Duplicate PGK2 transfected cells were treated with TNFα (10 ng/mL) or TPA (2.5 ng/mL) for 18 or 30 h posttransfection. Luciferase and β-gal were measured 48 h posttransfection. (*C*) Western blot analysis of Jurkat cell lines with polyclonal antibody to NEMO detects hypomorphic proteins on long exposure (NEMO*). (*D*) Schematic of encoded NEMO mutants in A45 and 2C Jurkat cells in relation to WT NEMO.

Because A45 and 2C cells expressed truncated NEMO mutants, we cloned and sequenced the NEMO cDNA from both cell lines. The NEMO cDNA expressed in A45 results from aberrant splicing of exon 9 into the middle of exon 10, with deletion of bases 1,376–1,491 (WT RefSeq NM_003639.3). The resulting protein enters an alternate reading frame after amino acid 372 and terminates with the sequence DTCHGVH* (Fig. 2*D*). The mutant no longer encodes for the polyproline sequence that mediates cylindromatosis protein (CYLD) binding (35) or the C-terminal ZNF that cooperates with the UBAN domain to confer low micromolar binding to K63-linked Ub (27). The cDNA from 2C differs from WT by an internal deletion of base pairs 655–930, caused by aberrant use of a splice donor site 4 bp from the end of exon 3 into the splice acceptor site for exon 6. The resulting protein has an internal in-frame deletion of NEMO residues 133–224 (Fig. 2*D*). This region of NEMO includes the intermediate domain between CC1 and CC2 and part of CC2. Both NEMO mutants have intact IKK binding sites at the amino terminus and when expressed in 293 cells, coimmunoprecipitate with IKKα (Fig. S1*B*). Northern blot analysis indicated that the mRNAs in A45 and 2C were far less abundant than those in WT Jurkat cells (Fig. S1*C*).

To show that the A45 and 2C NEMO were functional for LMP1-mediated NF-κB activation, we carried out siRNA silencing of NEMO in both cell lines. A pool of four siRNAs targeting NEMO or control siRNAs were transfected into each cell line. LMP1 and LMP1 TES2-mediated NF-κB activation in both 2C and A45 was abrogated after NEMO knockdown (Fig. 3). Thus, LMP1 TES2-mediated NF-κB activation is NEMO-dependent in Jurkat cells, and NEMO 1–372 and Δ133–224 uniquely support LMP1-mediated NF-κB activation.

LMP1 TES2-Mediated NF-κB Activation in Mutant NEMO Jurkat Cell Lines Is TRAF6- and IKKβ-Dependent. We sought to determine if LMP1 TES2 used the equivalent signaling cascade in NEMO mutant Jurkat lines as expected for canonical NF-κB activation in WT cells. TRAF6 is an essential E3 ligase needed for LMP1 TES2-mediated NF-κB activation in multiple cell lines (33–38). siRNA-mediated silencing of TRAF6 interfered with LMP1 TES2-induced NF-κB activation in both mutants of the A45 and 2C cell lines (Fig. 4). These data indicate that the requirement for TRAF6 is not changed in NEMO 1–372*- and Δ133–224-expressing cells.

Next, we determined if NEMO mutants expressed in A45 and 2C supported LMP1 TES2-mediated NF-κB activation through IKKβ using multiple assays of IKKβ activation. IKKβ has higher specific activity to IκBα than IKKα (39–40), and thus, IκBα phosphorylation is a putative read out of IKKβ activity. In A45 Jurkat cells, LMP1 induced phosphorylation of IκBα (Fig. S2*A*), suggesting that IKKβ was activated. LMP1-mediated NF-κB activation was inhibited by mutant IκBα proteins that cannot be phosphorylated and degraded (ΔN-IκBα and IκBα S32AS36A) (41, 42), indicating that IκBα phosphorylation is required for LMP1 TES2-mediated NF-κB activation (Fig. S2 *B* and *C*). Furthermore, LMP1 TES2-mediated IκBα phosphorylation and NF-κB activity were inhibited >90% by an IKKβ-selective chemical inhibitor (Fig. 5 *A* and *B* and Fig. S2*A*). The inhibitor was indeed specific for IKKβ, because LMP1 and LMP1 TES1 induced similar levels of IKKα-mediated p100 processing to p52 in both control and IKKβ inhibitor-treated A45 and 2C cells (Fig. 5*C*). The modest threefold LMP1 TES1-mediated NF-κB reporter activity was similarly unaffected by the IKKβ inhibitor (Fig. 5 *A* and *B*). Consistent with these findings, LMP1 TES2 predominately induced p50/p65 NF-κB complexes, as assessed by electrophoretic mobility shift assays (EMSA) in both mutant NEMO cell lines (Fig. S3). These data indicate that LMP1 and LMP1 TES2 functioned normally in promoting the activation of IKKβ in A45 and 2C cells.

NEMO Zn Finger and Residues 133–224 Are Individually Dispensable for LMP1 TES2-Mediated NF-κB Activation, but Residues 303–372, Encompassing the UBAN Domain, Are Essential. To delineate which domains of NEMO are required for LMP1 TES2-mediated NF-κB activation, NEMO$^-$ MEFs were stably transfected with FLAG-HA–tagged NEMO or one of six deletion mutants, including the two isolated from the A45 and 2C Jurkat cell lines (Fig. 6*A*). All mutants were expressed at levels similar to each other and endogenous NEMO (Fig. 6*B*), confirming that the diminished expression in Jurkat cells is because of low mRNA levels and not reduced protein stability. All of the NEMO proteins associated with IKKα in 293T, indicating that they fold properly (Fig. S1*B*). The subcellular localization of each mutant was predominately cytoplasmic, with a small but reproducible fraction in the nucleus. The 2C NEMO mutant Δ133–224 showed an increased association with cytoplasmic vesicles (Fig. S4).

LMP1 TES2-, TNFα–, and Tax-mediated NF-κB activation was restored by reconstitution of NEMO-deficient MEFS with full-length 1–419 NEMO expression (Fig. 6*C*). NEMO 51–419 similarly restored NF-κB activation to all three stimuli. Truncating NEMO at residue 372 had no detrimental effect on LMP1 TES2-mediated NF-κB activation and confirmed the NEMO ZNF is dispensable for LMP1 TES2-mediated NF-κB activation. Neither TNFα nor Tax could activate NF-κB in 1–372*-expressing cells. LMP1 TES2 activated NF-κB in NEMO Δ133–224-expressing cells with no significant difference from 1–419 expressing cells ($P > 0.05$). Therefore, the reduced level of TES2 signaling in the 2C Jurkat cells compared with 2Cγ is associated with the decreased mRNA and protein levels. The double deletion mutant Δ133–224/372* could not restore LMP1 TES2-mediated NF-κB activation (Fig. 6*C*). These data indicate that, in the context of the rest of NEMO, 373–419 or 133–224 suffice for LMP1 TES2 signaling and suggest that these regions have a redundant function.

LMP1 TES2 did not activate NF-κB in cells expressing NEMO 1–303. Thus, amino acids 303–372 encompassing the UBAN/LZ

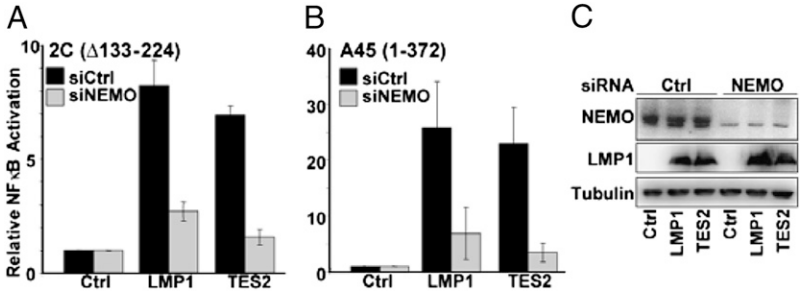

Fig. 3. NEMO Δ133–224 and 1–372 function to support LMP1- and LMP1 TES2-mediated NF-κB activation. Control or NEMO-specific siRNA was transfected into (*A*) 2C (Δ133–224) or (*B*) A45 (1–372) Jurkat cells simultaneously with reporter plasmids and either PGK2 (Ctrl), Flag-LMP1 (LMP1), or Flag LMP1 TES2 (TES2). Luciferase and β-gal were measured 72 h posttransfection. (*C*) Western blots for NEMO, LMP1, and tubulin from *B*.

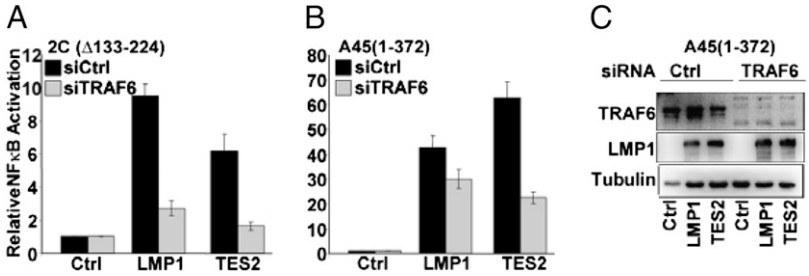

Fig. 4. TRAF6 is essential for LMP1 TES2-mediated NF-κB activation in NEMO 2C (Δ133–224)- and A45 (1–372)-expressing cells. Control or TRAF6-specific siRNA was transfected into (*A*) 2C (Δ133–224) or (*B*) A45 (1–372) Jurkat cells simultaneously with reporter plasmids and PGK2 (Ctrl), Flag-LMP1 (LMP1), or Flag LMP1 TES2 (TES2). Luciferase and β-gal were measured 72 h posttransfection. (*C*) Western blots for TRAF6, LMP1, and tubulin from *B*.

domain are required for LMP1 TES2-mediated NF-κB activation (Fig. 6*C*).

Discussion

Our data indicate that LMP1 TES2 stimulates IKK activation in ways that are overlapping but yet distinct from many NF-κB stimuli. The NEMO region 303–372 encompassing the UBAN domain is required for TNFα, Tax, and LMP1 signaling and for all other canonical NF-κB stimuli examined to date (20, 26, 43, 44). However, LMP1 is unique in that NEMO lacking its ZNF or the C-terminal portion of CC1 supports LMP1 TES2-mediated NF-κB activation but not TNFα, Tax, TPA, or CD40 signaling. This may be related to LMP1 itself. LMP1 constitutively forms large patches in the membrane and is directly modified at the amino terminus by Ub (45, 46). Thus, the focused concentration of Ub-modified LMP1 may be sufficient to overcome an abnormally high threshold necessary for NF-κB activation caused by NEMO mutation. However, the chimera of the LMP1 amino terminus and transmembrane domains with the CD40 cytoplasmic domain was largely defective for NF-κB activation. Therefore, the amino and transmembrane domains of LMP1 are not sufficient to initiate signaling through CD40 in A45 and 2C cells.

Another difference between LMP1 and TNFα or Tax may arise from the role of negative regulators of NF-κB signaling. LMP1 signaling in LCLs is continuous and seemingly resistant to the negative feedback loops that regulate TNFR signaling. LMP1 may uniquely activate independent of the NEMO ZNF and CC1 domains, because negative regulators of the pathway do not function in the same manner. For example, LMP1 induces A20 expression. A20 inhibits TNFα signaling by catalyzing Ub modification of NEMO and thereby, targeting it for degradation (47). A20 inhibits LMP1-induced NF-κB activity in overexpression studies (48). However, A20 is expressed at very high levels in LCLs, and NF-κB activation persists. These data suggest a fundamental difference between the roles of A20 in TNFα and LMP1 signaling.

Recruitment and activation of the IKK complex may differ with LMP1 compared with other canonical stimuli. NEMO binding to linear Ub linkages but not K63-linked chains may be required. This model is consistent with the finding that the NEMO ZNF, which is necessary for high-affinity binding to K63 linkages, is not required for LMP1 TES2 NF-κB activation. Alternatively, residues 133–224 and the ZNF may serve a redundant function to support binding to K-63 Ub linkages, since LMP1 requires at least one of these domains within NEMO.

Lastly, the inherent requirement for upstream adapters may dictate the ability of Δ133–224 and 1–372* mutants to support LMP1-mediated but not Tax- or TNFα-mediated NF-κB activation. Unlike LMP1, Tax directly binds to NEMO. NEMO residues 69–151 are required for the association with Tax. Therefore, the inability for Δ133–224 to support Tax-mediated NF-κB activation likely results from the inability of the two proteins to interact (49). NEMO residues 133–224 are predicted to adopt an α-helical conformation. In the crystal structure of residues 150–272 in complex with Kaposi's Associated Herpes Virus (KSHV) v-FLIP, NEMO residues 192–252 form a parallel intramolecular coiled coil and mediate the interaction with v-FLIP (50). Amino acids 150–192 were not visible in the same structure and therefore, may be more flexible. The elimination of a flexible linker domain in NEMO may make the Δ133–224 mutant adopt a conformation that promotes NF-κB signaling after LMP1 expression through enhanced protein interaction with upstream adapters.

TNFα-mediated NF-κB activation also differs from LMP1 TES2-mediated activation in the requirement for RIP (20, 51–53). Our preliminary data indicate that neither Δ133–224 nor 1–372* binds to RIP, which may explain the failure of TNFα to stimulate in A45 or 2C cells. We also observe that TRAF6 interaction is intact with both Δ133–224 and 1–372*, consistent with the newly described TRAF6 interaction site at the NEMO amino terminus (54). Therefore, TRAF6 binding to NEMO may be sufficient for LMP1-mediated NF-κB activation.

The studies herein show that LMP1 TES2 mirrors other stimuli in that NEMO and the NEMO UBAN domain are required for IKK activation. These data also indicate that LMP1 differs from many stimuli in that the structural requirements for

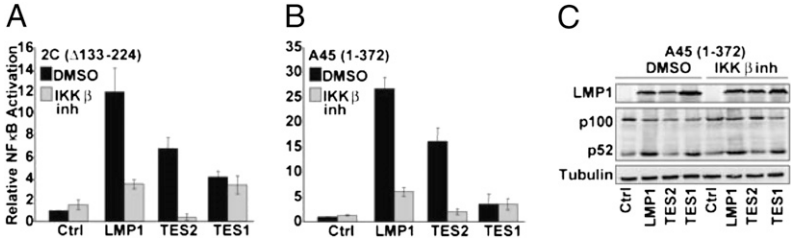

Fig. 5. LMP1 and LMP1 TES2 activate IKKβ in NEMO Δ133–224- and 1–372-expressing cells. (*A*) 2C (Δ133–224) or (*B*) A45 (1–372) Jurkat cells were treated with DMSO or an IKKβ inhibitor IV (10 uM) 6 h posttransfection with reporter plasmids and either PGK2 (Ctrl), Flag-LMP1 (LMP1), Flag LMP1 TES2 (TES2), or Flag LMP1 TES1 (TES2 mutant YYD_{386} to ID_{386}). (*C*) IKKβ inhibitor IV had no effect on IKKα-mediated p100 processing. Lysates from *B* were examined for LMP1, p100/p52 NF-κB2, and tubulin expression by Western blot analysis.

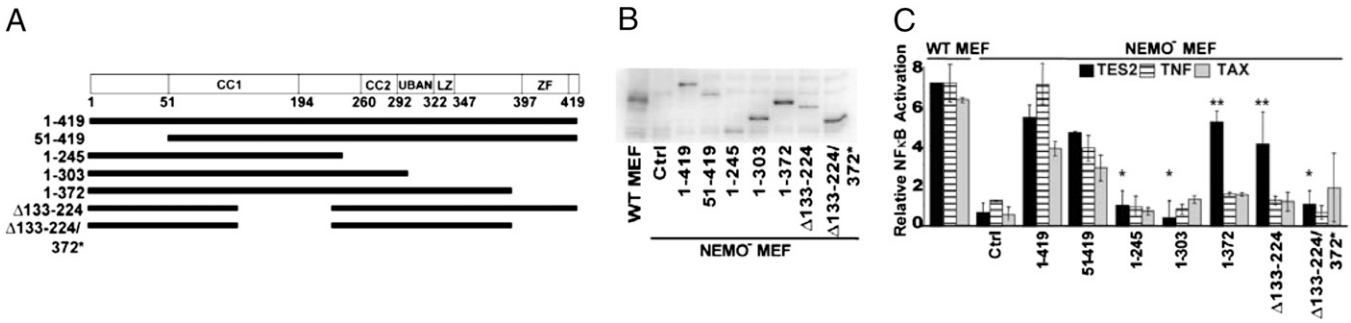

Fig. 6. LMP1 TES2-mediated NF-κB activation requires 303–372 encompassing the NEMO UBAN domain; Δ133–224 and 373–419 are individually dispensable for LMP1 TES2 signaling. Both are required for TNFα and TAX. (*A*) Schematic of FLAG-HA-NEMO constructs used to reconstitute NEMO⁻ MEFs indicating the positions of coiled-coil domain 1 (CC1), CC2, the ubiquitin binding in ABIN and NEMO domain (UBAN), leucine zipper (LZ), and zinc finger (ZF). (*B*) Anti-HA Western blot analysis of WT MEFs or NEMO- MEFs stably expressing PGK2 (Ctrl) or NEMO constructs from *A* as indicated. (*C*) LMP1 TES2-, TNFα-, and TAX-mediated NF-κB reporter assays in the indicated cell lines from *B*. Assay is as in Fig. 1. *Significant ($P < 0.01$). **Not significant ($P > 0.05$) in Student *t* test comparison with 1–419 reconstituted NEMO⁻ MEFs.

domains within NEMO are different from TNFα, TPA, Tax, and CD40. Thus, NEMO uses unique domains to coordinate NF-κB activation in response to distinct stimuli.

Materials and Methods

Cell Lines and Culture Conditions. The WT and NEMO⁻ MEF cell lines were provided by Michael Karin (University of California, San Diego). All MEF and HEK293 cell lines were maintained in Dulbecco's modified Eagle's serum supplemented with 10% Fetalplex animal serum complex, 2 mM L-glutamine, and 50 μM β-mercaptoethanol. The A45 and 2C NEMO mutant Jurkat cells were provided by Brian Seed (Massachusetts General Hospital, Boston, MA) and Shao-Cong Sun (University of Texas MD Anderson, Houston, TX) (21). Parental (clone 10.10.3) or NEMO-mutant Jurkat cells were cultured in RPMI supplemented with 10% Fetalplex animal serum complex (Gibco) and 2 mM L-glutamine.

Plasmids. PGK2 was generated by replacing the CMV promoter in pCDNA3 with the murine phosphoglycerate kinase (PGK) promoter as a Bgl2-Hind III fragment. Flag-LMP1, TES1 ($YYD_{384-386}ID$), and TES2 ($P_{204}A/Q_{206}A$) were cloned as BamH1-Sma1 fragments; 3× κB-luciferase (22), PGK-β-galactosidase (β-gal), pCMV-ΔN-IκBα pCMV4-IκBα-SSAA ($S_{32}A/S_{36}A$) (23), and pSG5 LMP1-CD40CT (8) have been described. The NEMO expression plasmid was purchased from Origene and modified with the Flag HA tags (*SI Materials and Methods*).

Transfections of Plasmids. Jurkat cells were transfected with the Nucleofector system (Amaxa). Jurkat cells were split 3 d before transfection; 5 μg plasmid DNA were introduced into 5×10^6 Jurkat cells by electroporation (Kit V, program S18; Amaxa). MEFs were split 2 d before transfection; 2×10^6 MEFs were electroporated with 5 μg plasmid DNA using 100 μL PBS and program A23.

Cloning of NEMO. Total RNA was extracted from WT, A45, and 2C Jurkat cells using TRIzol Reagent (Invitrogen); 1 μg total RNA from each sample was used as template for reverse transcription in a 50-μL reaction with oligo$(dT)_{20}$ and SuperScript III reverse transcriptase (SuperScript III First-Strand Synthesis System for RT-PCR; Invitrogen) at 55 °C for 50 min followed by 85 °C for 10 min. After treatment with 1 μL RNase H for 20 min at 37 °C, the RNA was used for RT-PCR. Parallel reactions to which no RT was added were used as controls. From the sequence for NEMO (Ensembl:ENSG00000073009), gene-specific primers were designed and used to amplify by PCR. The PCR reactions were performed using the PCR Enhancer System (GibcoBRL) [PCR buffer (–MgCl2), 10 μL RT reaction, 1.2 μM primer each, 0.2 mM dNTP each, 3 mM $MgCl_2$, 2.5 units Taq DNA Polymerase, 5 μL PCR Enhancer Solution]. PCR reactions of RNA treated without RT did not amplify products.

Knockdown of NEMO or TRAF6 by siRNA. Jurkat cells were transfected with a pool of four siRNAs specific for NEMO or TRAF6 (Thermo Scientific). The sequences of siRNAs used for knock were as follows: NEMO: AACAGGAGGUGAUCGAUAA, GAAGCGGCAUGUCGAGGUC, GAAUGCAGCUGGAAGAUCU, and GGAAGAGCCAACUGUGUGA; TRAF6: GGAGACAGGUUUCUUGUGA, GAUAUGAUGUAGAGUUUGA, GGCCAUAGGUUCUGCAAAG, and GCGCUUGCACCUUCAGUUA; control: AUGAACGUGAAUUGCUCAA, UAAGGCUAUGAAGAGAUAC, AUGUAUUGGCCUGUAUUAG, and UAGCGACUAAACACAUCAA.

Reporter Assays. Cells were harvested and washed with PBS and lysed with reporter lysis buffer (Promega). Cleared cell extracts were used to determine luciferase (Promega) and β-gal activities (Tropix), and protein expression was determined by Western blotting.

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