Lymphotoxin-β receptor mediates NEMO-independent NF-κB activation

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Abstract Lymphotoxin-β receptor (LTβR) is a member of the tumor necrosis factor receptor (TNFR) superfamily that activates nuclear factor-κB (NF-κB) through the IκB kinase (IKK) complex, the core of which is comprised of IKK1, IKK2 and NF-κB essential modulator (NEMO). We demonstrate here that the LTβR signaling to NF-κB activation does not necessarily require NEMO, which is essential for TNFR signaling. In the absence of NEMO, the p50 and RelB, but not RelA subunits of NF-κB are found in the nuclear DNA binding complexes induced by the LTβR signaling. Our results thus disclose NEMO-independent NF-κB activation by LTβR.
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Key words: IκB kinase; Lymphotoxin-β receptor; Nuclear factor-κB essential modulator; Nuclear factor-κB; RelB

1. Introduction

Lymphotoxin-β receptor (LTβR), a member of the tumor necrosis factor receptor (TNFR) superfamily, was identified as a molecule required for the development of lymphoid organs [1–5]. LTβR knockout mice showed a phenotype that included the lack of lymph nodes and Peyer's patches, and a disorganized splenic architecture [2]. LTβR binds specifically to two ligands: the membrane form of lymphotoxin LTα1β2 [4] and LIGHT, a recently identified member of the TNF superfamily [6]. These ligands activate nuclear factor-κB (NF-κB) following receptor ligation [7]. In addition, ectopic expression of LTβR, like other members of the TNFR superfamily, leads to the activation of NF-κB [8]. LTβR-mediated activation of NF-κB is supposed to be important for the development of lymphoid organs, because disruption or mutation of the NF-κB-inducing kinase (NIK) gene in mice resulted in a similar phenotype as was observed in LTβR-knockout mice [9]. However, the molecular mechanisms that underlie the NF-κB signaling pathway mediated by LTβR are still unclear.

IκB kinase (IKK) activation is a crucial step in the stimulation of NF-κB, which regulates many genes that participate in the immune, inflammatory, oncogenic or apoptotic processes [10,11]. The IKK complex is composed of two catalytic subunits, IKK1/α and IKK2/β, and a regulatory subunit, NF-κB essential modulator (NEMO)/IKKγ [12–22]. Activation of IKK leads to a phosphorylation of p105, p100 and the IκB family proteins, resulting in nuclear translocation of NF-κB hetero/homodimers [10]. It is known that IKK activation is indispensable for NF-κB activation in response to stimulation by various cytokines including TNF-α and interleukin-1β (IL-1β). Previous studies showed that the stimulation with agonistic antibody against LTβR failed to phosphorylate IκBα in mouse embryo fibroblasts (MEFs) derived from mice lacking IKK1 [23]. Thus, IKK activation is indispensable for LTβR-mediated NF-κB activation. We reported previously that stimulation by cytokines, such as TNF-α, lipopolysaccharide (LPS), or IL-1β, failed to activate IKK in NEMO-deficient cells [22]. However, it is not known if NEMO is required for the LTβR-induced IKK activation. To address this issue, we used NEMO-deficient rat fibroblasts previously used for complementation cloning of NEMO [22].

The cytoplasmic domain of LTβR, like other members of the TNFR superfamily, does not contain consensus amino acid sequences characteristic of domains with enzymatic activity. Thus, it is likely that NF-κB activation through LTβR is initiated by the proteins interacting with LTβR. The TNFR-associated factor (TRAF) family proteins were identified as signal transducers that bound to several members of the TNFR superfamily and other cytokine receptors, leading to the activation of NF-κB and mitogen-activated protein kinases (MAPKs) [24]. TRAF2, TRAF5 and TRAF6 were shown to activate NF-κB and to be involved in NF-κB activation mediated by these receptors [24]. Previous reports showed that the cytoplasmic domain of LTβR bound to TRAF2, 3, 4, and 5 [8,24–28]. However, the functional significance of these TRAFs in LTβR-mediated NF-κB signaling is not fully understood. We assessed how the TRAFs contribute to the NF-κB activation by LTβR, using MEFs derived from mice lacking both TRAF2 and TRAF5 [25].

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Abbreviations: EMSA, electrophoretic mobility shift assay; IκB, inhibitor of NF-κB; IKK, IκB kinase; IL-1β, interleukin-1β; LTβR, lymphotoxin-β receptor; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MAP3K, mitogen-activated protein kinase kinase kinase; MEF, mouse embryo fibroblast; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; TNFR, tumor necrosis factor receptor; TRAF, TNFR-associated factor

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2. Materials and methods

2.1. Materials

Anti-NEMO antibody was described previously [22]. Anti-actin antibody (C-2), anti-IKK1 antibody (H744), anti-IKK2 antibody (H-470), anti-p52 antibody (K-27) and anti-c-Rel antibody (N) were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-Flag antibody (M2) was purchased from Sigma (St. Louis, MO, USA). Anti-p50, anti-RelA and anti-RelB sera were kindly provided by Dr. Israël (Institut Pasteur, Paris, France). Recombinant wild type and S32A/S36A mutant GST-IκBα (1–72) proteins were prepared as described previously [22]. All the reagents were purchased from Sigma unless otherwise noted.

2.2. Plasmids

Igκ-ConAluc and EF1-lacZ vector were described previously [29]. A *Sac*I/*Bgl*II DNA fragment containing the cytomegalovirus early enhancer-promoter, U3-deleted murine leukemia virus long terminal repeat and packaging signal was excised from pRxhCD25iN [30], and subcloned into the same enzymatic site of pMX-IRES-EGFP [31], generating pMRX-IRES-EGFP. The puromycin resistance gene was amplified by PCR using pPUR (Clontech) as a template. The EGFP gene of pMRX-IRES-EGFP was replaced by the puromycin resistance gene, generating pMRX-IRES-puro. The cDNA of LTβR was amplified by PCR using pFlag/huLTβR [33] as a template. This cDNA was inserted into the pcDNA3-Flag or pMRX-IRES-puro vector. These vectors are referred to as pcDNA3 Flag-LTβR and pMRX-Flag-LTβR-puro, respectively. A cDNA fragment of TNFR1 amplified by PCR using pADB-TR55 [34] as a template was inserted into the pcDNA3-Flag vector, generating pcDNA3-Flag-TNFR1. All these PCR-amplified fragments were verified by sequencing.

2.3. Cell culture, transfection of plasmids and infection of retrovirus

5R is a NEMO-deficient subline of Rat-1 as described previously [22]. MEFs lacking IKK1 and IKK2 were kindly provided by Dr. Verma (Salk Institute, San Diego, CA, USA) [15]. MEFs lacking TRAF2 and TRAF5 were described previously [25]. PLAT-E cells were described previously [32]. Rat-1, 5R, MEFs and PLAT-E cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 U/ml of penicillin G and 100 μg of streptomycin. Rat-1 and 5R cells were transfected using the calcium phosphate coprecipitation method. MEFs and PLAT-E cells were transfected using fugene 6 transfection reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. For production of retroviruses, culture supernatants of PLAT-E cells transfected with pMRX-IRES-puro or pMRX-Flag-LTβR-IRES-puro were collected 72 h after transfection. Rat-1 and 5R cells were infected in the presence of 10 μg/ml of polybrene.

2.4. Reporter gene assay

Cells were transfected with 250 ng of Igκ-ConAluc and 250 ng of EF1-lacZ along with 100 ng of effector plasmids. Cells were lysed in lysis buffer (25 mM Tris–HCl, 8 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1% Triton X-100, 15% glycerol). Luciferase activities were determined as previously described and normalized on the basis of β-galactosidase activity [22].

2.5. Preparation of cytoplasmic and nuclear extracts

Cells were suspended in hypotonic buffer (10 mM HEPES [pH 7.8], 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and 0.1 mM ethylenediaminetetra-acetate (EDTA)) supplemented with protease and phosphatase inhibitors, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml aprotinin, 100 μM Na_3VO_4 and 20 mM β-glycerophosphate. After a 10 min incubation at 4°C, Nonidet P-40 was added to 1%. A soluble and an insoluble fraction were separated by a centrifugation. The supernatant was recovered as cytoplasmic extract. The nuclear pellet was washed with hypotonic buffer and resuspended in extraction buffer (50 mM HEPES [pH 7.8], 50 mM KCl, 350 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 2.5% glycerol). After a 30 min incubation at 4°C, with occasional agitation, DNA pellets were eliminated by centrifugation. The supernatant was recovered as nuclear extract.

2.6. Kinase assay

Cytoplasmic extracts (400 μg) were subjected to immunoprecipitation with anti-IKK1 or IKK2 antibody in TNT buffer (Tris–HCl 20 mM [pH 7.5], NaCl 200 mM, Triton X-100 1%) and collected on protein G-Sepharose beads. Kinase reactions were conducted for 30 min at 30°C in the presence of 5 μCi of [γ-^{32}P]ATP and wild type or S32A/S36A mutant GST-IκBα(1–72) protein as substrates. The reaction products were resolved on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels and revealed by autoradiography.

2.7. Western blot analysis

Cytoplasmic extracts (50 μg) were fractionated on 8–12% SDS–polyacrylamide gels, transferred onto Immobilon membranes (Millipore), and blots were revealed with an enhanced chemiluminescence detection system (ECL, Amersham).

2.8. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (5 μg) were incubated in 20 μl of binding buffer (10 mM HEPES [pH 7.8], 100 mM NaCl, 1 mM EDTA, 2.5% glycerol), 1 μg of poly[d(I-C)], and 0.5 ng ^{32}P-labeled κB probe derived from the H-2K^b promoter [35] or ^{32}P-labeled Oct-1 probe [36] and incubated for 30 min at room temperature. For supershift assays, nuclear extracts were incubated with specific antibodies for 30 min on ice before incubation with the labeled probe. Samples were run on a 5% polyacrylamide gel containing 2.5% glycerol in 0.5×TBE and revealed by autoradiography.

3. Results

3.1. LTβR can induce NF-κB-dependent transcription independently of NEMO

To investigate a role of NEMO in the LTβR-mediated NF-

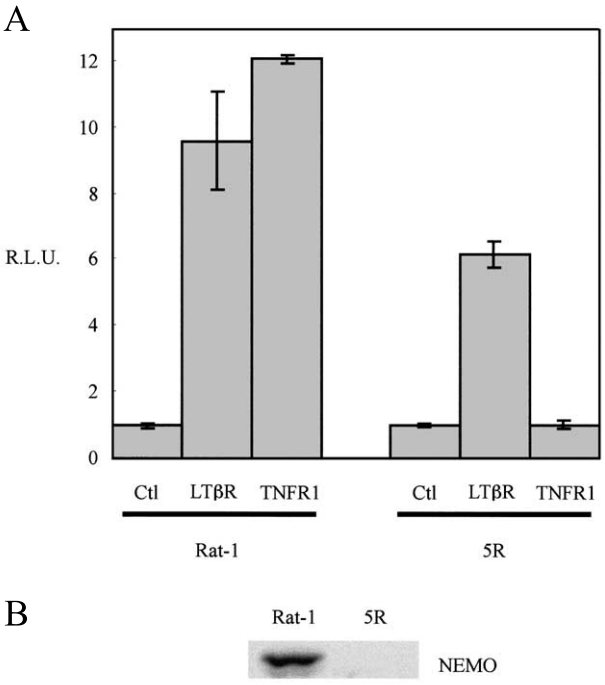

Fig. 1. LTβR, but not TNFR1, induces NF-κB-dependent transcription independently of NEMO. A: Rat-1 and 5R cells were transfected with 100 ng of pcDNA3-Flag (control; Ctl), pcDNA3-Flag-TNFR1 or pcDNA3-Flag-LTβR along with 0.25 μg of Igκ-ConAluc and EF1-lacZ. The cells were harvested and lysed in lysis buffer 36 h after transfection. The cellular extracts were subjected to reporter gene assay. The κB-dependent luciferase activity was normalized based on the β-galactosidase activity. The values shown are means ± S.D. from three separate transfections. The experiment was repeated twice with similar results being obtained. B: NEMO expression in Rat1 and 5R cells detected by anti-NEMO polyclonal antibodies.

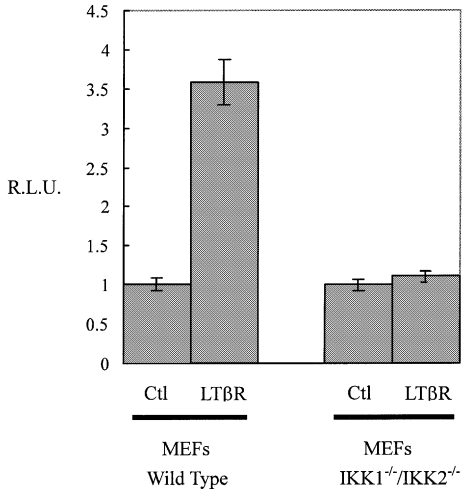

Fig. 2. IKK activation was required for LTβR-induced κB-dependent transcription. Wild type or IKK1/IKK2 double knockout MEFs were transfected with 100 ng of pcDNA3-Flag (control; Ctl) or pcDNA3-Flag-LTβR along with 0.25 μg of Igκ-ConAluc and EF1-lacZ. The cells were harvested and lysed in lysis buffer 22 h after transfection. Relative luciferase units were determined as described in Fig. 1. The values shown are means ± S.D. from three separate transfections. The experiment was repeated twice with similar results being obtained.

κB signaling pathways, we used 5R cells, which lack NEMO and were used in complementation cloning of NEMO (Fig. 1B). Reporter gene assays showed that ectopic expression of LTβR and TNFR1 induced κB-dependent transcription to similar extents in Rat-1 expressing wild type NEMO (Fig. 1A). Notably, LTβR induced κB-dependent transcription in 5R cells, albeit to a lesser extent (Fig. 1A). In contrast, TNFR1-induced κB-dependent transcription was severely impaired in 5R cells (Fig. 1A). Thus, LTβR, when ectopically expressed, does not require NEMO for NF-κB activation.

3.2. LTβR signaling induces IKK activation in the absence of NEMO

The results that LTβR did not necessarily require NEMO for NF-κB activation raised a possibility that ectopic expression of LTβR might activate NF-κB through pathways that did not involve IKK1 or IKK2 as was observed in UV-treated cells [37]. To assess a requirement of IKK activation for the LTβR-mediated NF-κB signaling, we tested MEFs deficient in both IKK1 and IKK2 for NF-κB activation by LTβR. Reporter gene assays revealed that LTβR failed to induce κB-dependent transcription in the absence of IKK1 and IKK2 (Fig. 2). This suggested that LTβR activated IKKs in the absence of NEMO, and prompted us to determine a kinase activity induced by LTβR in 5R cells. Immunoprecipitation of IKK1 followed by in vitro kinase reaction demonstrated significantly elevated IKK activity in Rat-1 as well as in 5R cells infected with retroviruses capable of expressing LTβR (Fig. 3). Lack of phosphorylation of a mutant substrate established the specificity of the experiment. Kinase assay coupled with immunoprecipitation of IKK2 revealed weak IKK activation by LTβR compared to that by TNF-α (data not shown) in Rat-1 and 5R cells expressing LTβR. Thus, LTβR can preferentially activate IKK1 in a NEMO-independent manner.

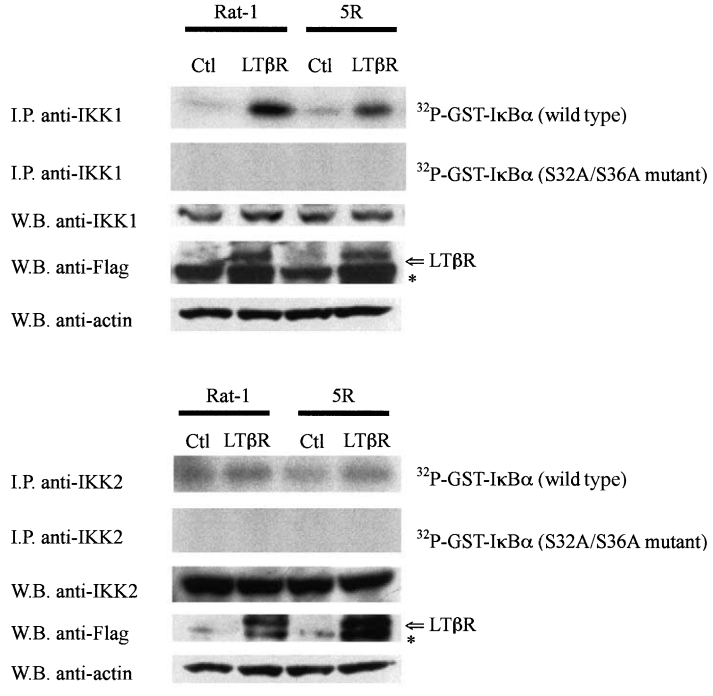

Fig. 3. LTβR increased IKK activity independently of NEMO. Rat-1 and 5R cells were infected with retroviruses produced by transfection of PLAT-E cells with either pMRX-IRES-puro (control; Ctl) or pMRX-Flag-LTβR-IRES-puro. The cells were harvested 48 h after infection. The cytoplasmic extracts were subjected to kinase assay and Western blot analysis. The endogenous IKK complex was immunoprecipitated by anti-IKK1 or IKK2 antibody and subjected to in vitro kinase reaction. Wild type or S32A/S36A mutant GST-IκBα protein was used as substrate. To control for equal amounts of IKK, Western blot analysis was performed, using anti-IKK1 or anti-IKK2 antibody respectively. Expression of Flag-LTβR was assessed by Western blot analysis with anti-Flag monoclonal antibody. The asterisks indicate a non-specific band. As a loading control, amounts of actin were shown by Western blot analysis with anti-actin antibody. These experiments were carried out twice. The results were essentially reproducible.

3.3. LTβR directs nuclear translocation of p50 and RelB, but not of RelA in the absence of NEMO

EMSA showed increased NF-κB DNA binding activity in Rat-1 and 5R cells expressing LTβR (Fig. 4A). To determine the subunit composition of the DNA binding complexes, we performed supershift assays using specific antibodies or antiserum against p50, p52, RelA, RelB or c-Rel, which are common components of NF-κB (Fig. 4B). Anti-p50 antiserum supershifted almost completely the faster migrating complexes in Rat-1 and 5R cells. Anti-RelB antiserum also supershifted the faster migrating complexes in Rat-1 and 5R cells expressing LTβR, while it had no appreciable effect on complexes induced in Rat-1 cells stimulated with TNF-α for 30 min (Fig. 4C), indicating the specificity of the antiserum and a distinct mechanism of NF-κB activation by LTβR. Anti-RelA antiserum weakly supershifted the slowly migrating complexes in Rat-1 cells, but not those at the corresponding position in 5R cells, although the RelA subunit was similarly expressed in Rat-1 and 5R cells (Fig. 4D). Anti-p52 antibody (K-27; Santa Cruz) or anti-c-Rel antibody (N; Santa Cruz) did not supershift the NF-κB complex either in Rat-1 or in 5R cells (Fig. 4B).

3.4. TRAFs are required for LTβR-induced κB-dependent transcription

We finally asked if the observed NEMO-independent IKK

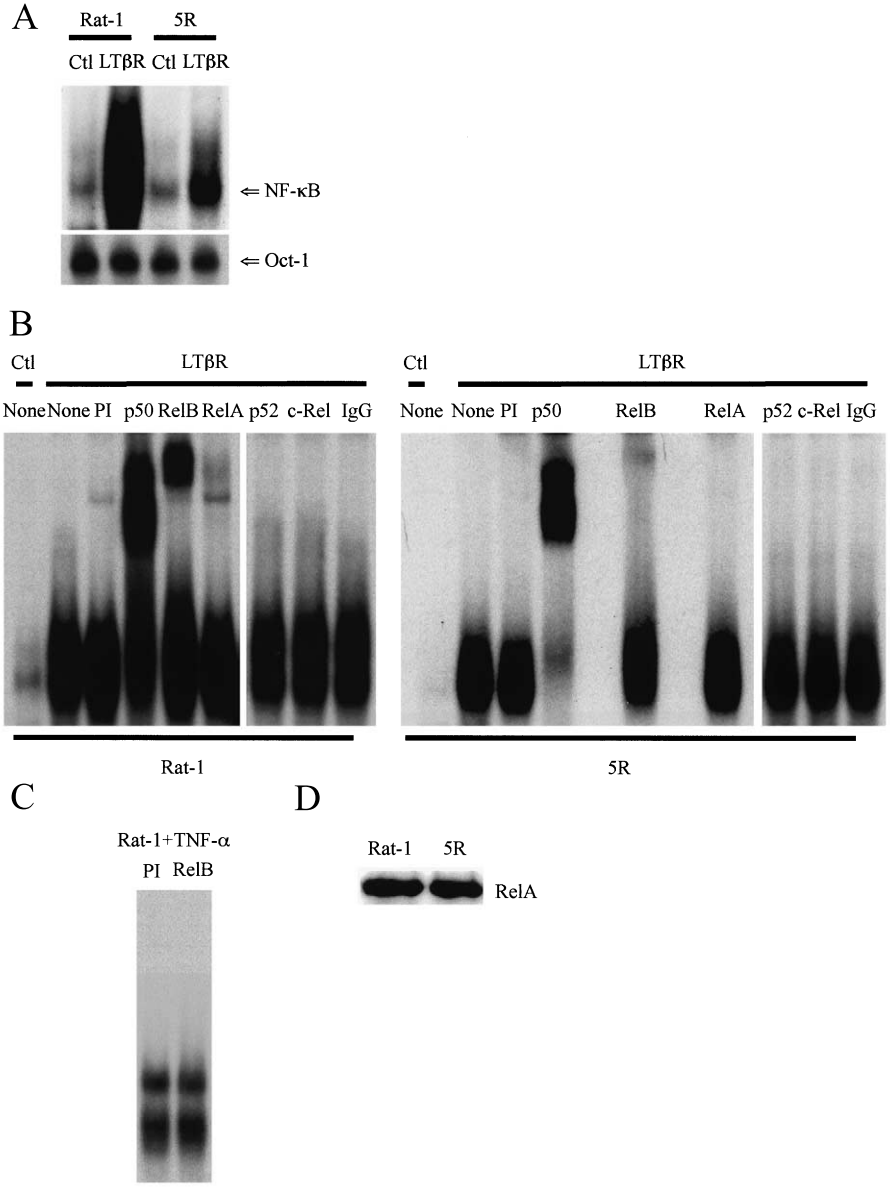

Fig. 4. LTβR induced nuclear localization of RelB and p50 independently of NEMO. Nuclear extracts were prepared from the cells used in Fig. 3. A: Nuclear extracts prepared from control or LTβR-expressing cells were incubated with ^{32}P-labeled κB or Oct-1 probe and subjected to EMSA. B: The extracts were incubated with ^{32}P-labeled κB probe in the absence or presence of pre-immune serum (PI), anti-p50 serum, anti-RelA serum, anti-RelB serum, anti-p52 antibody, anti-c-Rel antibody or control rabbit IgG, and then subjected to supershift assay. C: Nuclear extract of Rat-1 cells stimulated with TNF-α for 30 min was incubated with either PI or anti-RelB antiserum and ^{32}P-labeled κB probe, and then analyzed by EMSA. D: RelA expression in Rat-1 and 5R cells detected by anti-RelA serum. These experiments were carried out twice. The results were essentially reproducible.

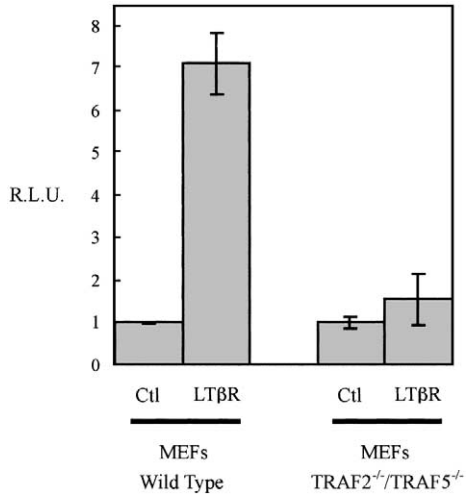

Fig. 5. LTβR-induced κB-dependent transcription was severely impaired in TRAF2 and TRAF5 double knockout MEFs. Wild type or TRAF2/TRAF5 double knockout MEFs were transfected with 100 ng of pcDNA3-Flag (control; Ctl) or pcDNA3-Flag-LTβR along with 0.25 μg of Igκ-ConAluc and EF1-lacZ. The cells were harvested and lysed in lysis buffer 22 h after transfection. Relative luciferase units were determined as described in Fig. 1. The values shown are means ± S.D. from three separate transfections. The experiment was repeated twice with similar results being obtained.

activation is linked to any particular upstream regulators. Recent studies showed that TRAF2, 3, 4 and 5, mediators of cytokine receptor-mediated signaling, could bind to LTβR. Since TRAF2 and TRAF5 have been shown to activate NF-κB, we examined the requirement of TRAF2 and TRAF5 in the LTβR-mediated NF-κB signaling. Reporter gene assays revealed that LTβR-induced κB-dependent transcription was readily detected in wild type MEFs, but was severely impaired in MEFs derived from mice deficient in both TRAF2 and TRAF5 (Fig. 5).

4. Discussion

Targeted disruption of IKK1 and IKK2 revealed that IKK activation was indispensable for NF-κB activation through a variety of cell surface receptors [13–15,19]. We had previously identified NEMO as a regulatory subunit of the IKK complex [22]. In this study, we showed that the LTβR signaling could activate IKK in a NEMO-independent manner, resulting in NF-κB activation. As far as we know, this is the first report showing that a cytokine receptor protein can activate NF-κB through IKK in the absence of NEMO. In contrast, TNFR1 was unable to activate NF-κB in 5R cells. Thus, the LTβR-induced NF-κB signaling is different from that induced by TNFR1 at least in the usage of NEMO.

The dependence on NEMO in IKK activation appears to be linked to the usage of the IKK catalytic subunits. There is growing evidence showing that IKK1 and IKK2 are differentially regulated by cytokine receptor-specific intracellular signal transducers and play distinct roles in the NF-κB signaling pathway [9,10,23,24]. Previous studies revealed that TNFR1 failed to activate NF-κB in IKK2-deficient cells. Signaling by other cytokines, such as LPS and IL-1β, also required IKK2 to activate NF-κB [38]. Moreover, the Tax protein of human T cell leukemia virus type I preferentially phosphorylates IKK2. We and other researchers demonstrated that NEMO played an indispensable role for the cytokine- or Tax-induced NF-κB activation [20–22]. On the other hand, LTβR signaling to NF-κB activation was reported to depend predominantly on IKK1 and failed to induce phosphorylation of IκBα in IKK1-deficient cells [23]. Indeed, LTβR-induced IKK activity revealed by immunoprecipitation of IKK2 was considerably weak (Fig. 3). A recent study by Hu et al. [39] demonstrated a role for IKK1 in keratinocyte differentiation that does not depend on its kinase activity or binding to NEMO. Our present results of NEMO-independent IKK activation by LTβR suggest that the NEMO dependence is linked to differential activities of IKK1 and IKK2.

MAP3Ks (mitogen-activated protein kinase kinase kinases) are believed to constitute important upstream regulators of IKK in the NF-κB signaling pathway [9,23,40,41]. NIK was identified as TRAF2-interacting MAP3K that phosphorylated IKK [40] and was found to mediate the LTβR-induced NF-κB activation, because mice deficient or mutant in NIK showed a phenotype similar to that of LTβR-deficient mice [9]. We also observed that a dominant negative form of NIK significantly impaired κB-dependent transcription induced by LTβR in Rat-1 and 5R cells (data not shown). On the other hand, a recent genetic study disclosed that MAP3K-3 is an essential mediator of the TNF-α-induced NF-κB activation [41]. Thus, it is plausible that differential usage of MAP3K may determine the dependence of NEMO and the IKK subunit(s) activation.

Mammalian cells are known to express five NF-κB family members: RelA, RelB, c-Rel, p100/p52 and p105/p50 [10]. In the present study, we showed that the LTβR-induced NF-κB complexes contained the RelB and p50 subunits in NEMO-deficient cells. RelB differs in its regulation from the other two NF-κB activators, RelA and c-Rel, which are controlled by IκB proteins [10,42]. Previous studies revealed that p100 is associated with RelB and inhibits its nuclear localization, but IκBα, IκBβ, IκBε or p105 are not [43]. Processing of p100 is regulated by IKK1 and NIK, both of which are indispensable for the LTβR-induced NF-κB activation [43–45]. In addition, ectopic expression of NIK caused NF-κB activation in 5R (data not shown), consistent with a previous report by Xiao et al. [46] that showed NEMO-dependent and -independent inducible p100 processing by Tax and NIK, respectively. Thus, it appears likely that the p100 processing induced by NIK couples with a NEMO-independent nuclear translocation of RelB in cells expressing LTβR. Notably, we found that LTβR expression induced nuclear translocation of the RelA subunit in Rat-1 cells, but not in 5R cells. These results indicated that NEMO plays an essential role for nuclear translocation of the RelA subunit following phosphorylation and degradation of the IκB proteins and that the LTβR signals to the IKK complex diverge into NEMO-dependent and -independent pathways.

Finally, we asked how TRAF2 and TRAF5 are involved in the LTβR-mediated NF-κB signaling pathway, since they are known to be required for the TNFR1 signaling to NF-κB [25]. TRAF2- or TRAF5-deficient mice showed secondary lymphoid tissue development [47], which was deficient in LTβR-knockout mice. It was supposed to be difficult to determine the individual role of each TRAF in the LTβR-induced NF-κB activation, because TRAF2 and TRAF5 could be redun-

dant in this signaling pathway as observed for TNF-α [25]. To assess the requirement of these TRAFs, we used MEFs deficient in both TRAF2 and TRAF5 in the present study and showed that the LTβR-induced NF-κB activation was greatly impaired in these cells. Thus, it seems likely that the NEMO-independent and -dependent pathways from LTβR to IKK activation diverge at a step downstream of TRAF2 and/or TRAF5. Nevertheless, a tiny reporter gene activation by LTβR expression in TRAF2 and TRAF5 double knockout MEFs and a previous report that a truncated form of LTβR lacking the putative TRAF binding domain still activated NF-κB leave open the questions whether certain molecule(s) other than TRAF2 and TRAF5 may mediate IKK activation by LTβR [8] and whether such activity has any link to the NEMO-dependent or -independent LTβR signaling.

Elucidation of the mechanism of the NEMO-independent IKK activation by LTβR will facilitate a better understanding of IKK activation, which should eventually enable us to know how different modes of IKK activation finally result in diverse gene regulation by NF-κB.

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