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Induction of the Alternative NF-κB Pathway by Lymphotoxin αβ (LTαβ) Relies on Internalization of LTβ Receptor

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Several tumor necrosis factor receptor (TNFR) family members activate both the classical and the alternative NF-κB pathways. However, how a single receptor engages these two distinct pathways is still poorly understood. Using lymphotoxin β receptor (LTβR) as a prototype, we showed that activation of the alternative, but not the classical, NF-κB pathway relied on internalization of the receptor. Further molecular analyses revealed a specific cytosolic region of LTβR essential for its internalization, TRAF3 recruitment, and p100 processing. Interestingly, we found that dynamin-dependent, but clathrin-independent, internalization of LTβR appeared to be required for the activation of the alternative, but not the classical, NF-κB pathway. In vivo, ligand-induced internalization of LTβR in mesenteric lymph node stromal cells correlated with induction of alternative NF-κB target genes. Thus, our data shed light on LTβR cellular trafficking as a process required for specific biological functions of NF-κB.

The tumor necrosis factor receptor (TNFR)/tumor necrosis factor ligand (TNFL) superfamily forms a complex network of cytokines and receptors that are important for biological functions ranging from cell homeostasis and inflammation to lymphoid organ development (20). This family is subdivided according to structural features within the cytosolic tail such as the presence of a death domain (DD) and/or tumor necrosis factor receptor-associated factor (TRAF) binding sites (6, 11). The DD is involved in the recruitment of other death domain-containing proteins like FADD and TRADD, which connect the receptor to downstream signaling pathways leading to cell death or cell survival. Likewise, the recruitment of adaptor proteins like TRADD (30, 38). TRAF proteins connect TNFR to at least two NF-κB signaling pathways. The latter control many of the proinflammatory and antiapoptotic roles associated with NF-κB through binding to a specific cis regulatory region called the κB site (37, 39).

An additional biological outcome accounts for TNFR members by the fact that a subclass (e.g., TNFRI and HVEM) activates solely the classical NF-κB pathway whereas other TNFRs (e.g., lymphotoxin β receptor [LTβR] and CD40) activate both the classical and the alternative (or noncanonical) NF-κB pathways (8, 51). The alternative pathway involves the activation of the NF-κB-inducing kinase (NIK), which activates IKKα, and both phosphorylate the inhibitor p100, leading to its subsequent polyubiquitination and partial proteosomal degradation into p52 (46, 56). In particular, it was demonstrated that neither LTβR nor BAFF-R required NEMO or IKKβ for inducing p100 processing (7–9). Ultimately, NIK and IKKα activate the dimer p52/RelB, which controls a set of genes involved in secondary lymphoid organ development, B cell survival, and osteoclastogenesis (45, 53). Indeed, NIK- and IKKα-deficient mice share a panel of developmental abnormalities reminiscent of mice deficient in LTβR, BAFF-R, or RANK (13, 15, 25, 46, 47, 49, 58). Deregulation of the alternative NF-κB pathway has also been associated with malignancy. For instance, transgenic mice expressing inducers of the alternative pathway such as BAFF or LTα1β2 display lymphoid malignancies and hepatocellular
carcinoma development, respectively (2, 18, 28). On the other hand, elevated expression of NIK and/or loss of expression of its negative regulators is a signature found in multiple myeloma and B cell lymphoma (1, 26, 42).

Thus, NIK appears to play a central role in many biological functions, but the molecular determinants that dictate its activation are still poorly characterized. The current model depicts TRAF3 as a bridge between TRAF2-associated c-IAP1/2 E3 ligase complex and the N-terminal domain of NIK promoting its constitutive K48-linked polyubiquitination and proteasomal degradation. Upon stimulation of CD40, TRAF3 is polyubiquitinated by c-IAP1/2 and degraded by the proteasome, allowing the stabilization and accumulation of NIK (30, 52, 60).

Therefore, TRAF3 recruitment has been proposed as a hallmark of the TNFR-induced alternative NF-κB pathway (17). However, HVEM, a TNFR that binds TRAF3, fails to activate the alternative pathway (5, 33). Thus, it is likely that the capacity to recruit TRAF3 is necessary but not sufficient for inducing the alternative NF-κB pathway.

Thus, the molecular mechanisms linking the outcome of TRAF-associated TNFR and the activation of p100 processing are far from being fully understood and need further biochemical and biological characterization. In this study, we have addressed how LTβR activates both the classical and the alternative NF-κB pathways. We found that activation of these two pathways is spatially and temporally regulated by LTβR trafficking.

**MATERIALS AND METHODS**

**Plasmids, cloning, and mutagenesis.** Expression vectors and sequences of primers used for cloning and mutagenesis are available upon request. PCR amplification of cDNAs was performed with Goldstar DNA polymerase (Eurogentec, and mutagenesis of LTβR was performed using the QuikChange site-directed mutagenesis XL kit (Stratagene) according to the manufacturer’s instructions.

**Abs and reagents.** The following commercially available antibodies (Abs) were used for several applications: p100/p52 (05-361) and anti-ubiquitin Lys48-specific antibody (05-1307) from Millipore; phospho-p100 (4810), phospho-Isoc (9246), anti-NIK (4994), and Myc tag (2276) from Upstate Cell Signaling; antihemagglutinin (anti-α-HA) (sc-805), LTβR-N15 (sc-8573), TRAF2 (sc-218), TRAF2 (sc-7187), TRAF3 (sc-1282), TRAF3 (sc-948), TRAF3 (sc-7220), and c-Jun (sc-7975) from Santa Cruz Biotechnology; actin (69100) from MP Biomedical; HA (MMS-101R) from Covance; Flag M2 (F3165) and Beads Red anti-Flag M2 (F2426) from Sigma; h-LTβR (AF629) from R&D Systems; glutathione S-transferase (GST) tag (71097-3) from Novagen; EEA1 (610456); AP50 (61260) from BD Bioscience; anti-dynamin-2 (PA1-661) from Thermo Scientific; and anti-NIK (ab6111) from Abcam.

Anti-Isoc monoclonal antibody (MAB) 10 was a gift from Ron Hay (University of St. Andrews, St. Andrews, Scotland). Anti-murine LTβR, mCAM-1, mVMCAM-1, mMAdCAM-1, C4D5, and mp100/p52 were previously described (3, 9). The following reagents were purchased from the indicated companies: cholroquine (C6628), bafilomycin A1 (B1793), N-ethylmaleimide (E1271), and n-octyl-β-D-glucopyranoside (2936826-83-8) from Sigma; diethio-bis(succinimidyl propionate) (DSP) (22585) from Thermo Scientific; recombinant LTα1β2 (678-LY) from R&D Systems; digitonin (300410) from Calbiochem; and Topro-3 iodide from Invitrogen.

**Mice and lymph node explants.** C57BL/6 (H-2b) and R1/b (H-2b) mice (backcrossed for more than 10 generations in a C57BL/6 background) (54) were bred and maintained under specific-pathogen-free conditions in the Biomedical Service Unit at the University of Birmingham according to Home Office and local ethics committee regulations. The day of vaginal plug detection was designated E0. freshly isolated fetal lymph nodes were isolated at the indicated time and either analyzed directly or explanted in organ cultures and incubated for 72h in the presence of an agonistic antibody to LTβR at 2 μg/ml (3).

**Cell lines, transfection, and retroviral infection.** HEK 293, HEK 293T, HeLa, and HeLa Tet/On Advanced (Clontech) cells and LTβR knockout (KO) MEFs were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotics, and 1% L-glutamine. For transient transfections, cells were seeded at 70 to 80% confluence and transfected using Lipofectamine 2000 (Invitrogen) or FuGENE HD (Roche Applied Sciences). For analyzing LTβR-mediated p100 processing into HEK 293T cells, 6-well plates were used and 100 ng of LTβR expression vector was transfected per well. Retrovirus was collected from the supernatant of Phoenix Amphi packaging cells transfected with pRetroX-Tight-HA-pα2-D76A/W421A or pBabe-puro-LTβR encoding wild type (wt) or ΔI 389-395, ΔI 345-358, or ΔI 359-368 mutant and used to infect HeLa Tet/On and HEK 293T cells or LTβR KO MEFs, respectively. Single clones were obtained after antibiotic selection, and expression of AP2α-ΔN (dominant negative) was achieved by adding 2 μg/ml of doxycycline for the indicated period.

**siRNA knockdown experiments.** Knockdown experiments for targeting CHC or Dn2 in HeLa cells were carried out with On Target Plus Smart pools M-004001 and L-004007, respectively (Dharmacon). The experiments were performed according to the protocol described by Motley et al. with minor modifications (36). HeLa cells were transfected with either small interfering RNA (siRNA) green fluorescent protein (GFP) as negative control or siRNA CHC. For the first round of transfection, cells were plated in 6-cm dishes and incubated for 4 h in serum/antibiotic-free medium in the presence of 12 μg Oligofectamine (Invitrogen) and 120 pmol of siRNA. Cells were trypsinized 24 h posttransfection and seeded into a 10-cm dish. After the second round of transfection on day 3, the cells were split into 6-well plates for further analyses on day 5. Knockdown experiments for targeting human AP2α-ΔN in HeLa Tet/On rat AP2α-ΔN were performed with two siRNAs (GCCGAGGAGUUAAAGAUU and AGUUU GAGCUU/AUGAAGUA) designed to efficiently target human AP2α-ΔN but not rat AP2α (Dharmacon). The cells were transfected twice on days 1 and 3, with siRNA AP2α-ΔN or with GFP siRNA as negative control (Dharmacon). Doxycycline was added at 2 μg/ml in the medium over the 5 days of the experiment.

**Immunoblotting, IP, and cell extract fractionation.** Whole-cell extracts were prepared by lysing cells in 0.5% SDS containing 5 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na3VO4), 20 mM β-glycerophosphate (βGP), and Complete protease inhibitor (Roche). Cells were separated by SDSPAGE and transferred onto nitrocellulose membranes (Millipore) followed by incubation with specific primary antibodies and secondary horseradish peroxydase (HRP)- conjugated antibodies (Dako). For coimmunoprecipitation (co-IP) assays, cells were analyzed after stimulation with LTβR antibody or 40 h posttransfection. Cells were lysed in TNT buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 0.5 to 1% Triton X-100, 5 mM NaF, 1 mM Na3VO4, 20 mM βGP, and Complete protease inhibitor (Roche)). Cell extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore) followed by incubation with specific primary antibodies and secondary horseradish peroxydase (HRP)- conjugated antibodies (Dako). For coimmunoprecipitation (co-IP) assays, cells were analyzed after stimulation with LTβR antibody or 40 h posttransfection. Cells were lysed in TNT buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 0.5 to 1% Triton X-100, 5 mM NaF, 1 mM Na3VO4, 20 mM βGP, and Complete protease inhibitor (Roche)). Cell extracts were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore) followed by incubation with specific primary antibodies and secondary horseradish peroxydase (HRP)- conjugated antibodies (Dako). For coimmunoprecipitation (co-IP) assays, cells were analyzed after stimulation with LTβR antibody or 40 h posttransfection. Cells were lysed in TNT buffer...
immunoprecipitation experiment (1st IP) by incubation with either control beads or Flag-M2 beads. The immunoprecipitates were then used for detecting Flag-NIK and K48-linked polyubiquitinated NIK. Quantification of signals was performed with the Image J software.

Oligomerization of LTβR and GST pulldown. For studying the ability of LTβR to form trimers, we performed transient transfections of HEK 293T cells with a combination of HA-, Flag-, and Myc-tagged LTβR expression vectors. A first immunoprecipitation was performed using Flag-M2 agaroose beads. The immunoprecipitated material was then selectively released overnight by competition with an excess of 3× Flag peptide (Sigma). The supernatants were subjected to a second immunoprecipitation with either an anti-HA or an anti-Myc antibody for 2 h at 4°C. The immunoprecipitated materials were analyzed by immunoblotting with an anti-Myc or an anti-HA antibody, respectively. To analyze aggregation of ectopic LTβR, HEK 293T cells were collected 40 h post-transfection, washed twice with phosphate-buffered saline (PBS), and then incubated for 30 min at room temperature in PBS containing 1 mM dithiobis[succinimidyl propionate] (DSP; Pierce/Thermo Fisher Scientific). After cross-linking, the cells were washed once in PBS and incubated for 15 min in 20 mM Tris-HCl (pH 7.4)–PBS to stop the reaction. The cells were split in SDS buffer, and protein extracts were analyzed by immunoblotting for LTβR expression. pEXN-4T wt LTβR or mutant expression vectors were transferred into Escherichia coli BL21. Bacterial cultures were grown to an A600 of 0.6 and were induced with 0.5 mM isopropyl-β-d-thiogalactopyranoside for 4 h at 37°C. After washing in PBS, the bacterial pellets were sonicated in NENT buffer (250 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.5, 1% Nonidet P-40). The lysates were cleared by centrifugation and incubated with glutathione-Sepharose 4B beads for 1 h at 4°C. After three washes in NENT buffer, the beads were collected in TGH buffer (250 mM NaCl, 1 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM MgCl2, 8.2% glycerol, 0.1 mM EDTA). Expression of GST fusion proteins was estimated by SDS-PAGE and Coomassie blue staining.

For GST fusion protein interactions with 35S-TRAF proteins, an aliquot of glutathione-Sepharose beads containing 1 μg of GST fusion protein was incubated with 7.5 μl of in vitro-translated TRAF for 1 h at 4°C in NENT buffer. Beads were washed twice in NENT buffer and then boiled in SDS loading buffer for 5 min. Bound proteins were analyzed by SDS-PAGE and then by autoradiography. For analyzing the binding of overexpressed mammalian TRAF proteins, cell lysates were prepared from transfected 293T cells in TGH buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], and protease inhibitors). One microgram of GST fusion protein was incubated with 150 μg of cell lysates for 3 h at 4°C. Beads were washed five times in TGH buffer and then boiled in SDS loading buffer for 5 min. Bound proteins were analyzed by SDS-PAGE and immunoblotting.

Flow cytometry. LTβR-expressing HEK 293 cells were analyzed 2 days post-transfection. Cells were fixed in 1% paraformaldehyde, permeabilized or not with 0.5% saponin, and incubated for 30 min with an anti-LTβR antibody. After washing in PBS-bovine serum albumin (BSA), cells were stained with a goat anti-HA antibody and a donkey anti-goat-antibody-phycocyanin (PE). To study the internalization of LTβR in MEFs or in HT29 cells, the cells were stimulated with 2 μg/ml of agonistic antibody to murine LTβR or 0.2 μg/ml of agonistic antibody to human LTβR and were incubated for the indicated period of time. After collection of the cell, the staining of cell surface and internalized LTβR was achieved with the same agonistic antibody followed by incubation with PE-labeled goat anti-rat secondary antibody or PE-labeled donkey anti-goat antibody. Samples were analyzed on a FACSCan II flow cytometer (BD Biosciences), and at least 10,000 events were acquired for each sample using FACS Diva software (BD Biosciences). Staining of mesenteric lymph node (mLN) stromal cells was performed as previously described (3).

Immunofluorescence microscopy. Transfected HEK 293T or HeLa cells were grown on glass coverslips. Two days post-transfection, cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.5% saponin–PBS for 20 min. 293T cells were then incubated for 1 h with anti-human LTβR antibody and subsequently incubated for 50 min with a donkey anti-goat-antibody–Alexa 546 (Invitrogen). Nuclei were stained with Topro-3 iodide (Invitrogen).

Transfected HeLa cells with HA-tagged wt LTβR, ΔS-LTβR, and ΔS ΔI 345–358 LTβR were stained with anti-HA sc-805 at a 1:50 dilution and an anti-rabbit antibody–Alexa 546, and nuclei were visualized with 4,6-diamidino-phenylindole (DAPI). For the colocalization of LTβR and endosomes, staining was performed on HT29 cells treated with the agonist antibody to LTβR. Following the indicated kinetic of stimulation, cells were fixed for 20 min with 2% p-formaldehyde and permeabilized for 15 min at room temperature with 50 μM digitonin. LTβR was detected with agonist primary antibody and a donkey anti-goat secondary antibody–Alexa 546. Early endosomes were detected with a mouse monoclonal anti-EEA1 antibody and a donkey anti-mouse-Alexa 488 secondary antibody.

Localization of endogenous TRAF3/NIK complex was achieved using the Duolink II Orange kit (Eurogentec, Belgium). Anti-rabbit plus and anti-goat minus antibodies were used as secondary antibodies for recognition of anti-TRAF3 H-20 (sc-948-G; Santa Cruz Biotechnology) and anti-NIK (ab6111; Abcam), and proteoglycan reaction was performed for 150 min. Nuclei were visualized with DAPI. Confocal microscopy was performed with either a Leica TCS SP2 microscope or an Olympus FV1000 microscope.

RESULTS

Molecular determinants of LTβR involved in induction of p100 processing. It has been previously shown that overexpression of LTβR is sufficient to activate NF-κB in a ligand-independent way (14). We constructed a panel of deletion mutants of the cytosolic tail of LTβR and evaluated their ability to induce the processing of p100 (Fig. 1A). All deletion mutants expressing at least the first 395 amino acids induced the processing of p100, whereas the shorter mutants did not (Fig. 1B). Thus, removal of the six residues upstream of amino acid 389 was sufficient to prevent LTβR-induced p100 processing. This region was characterized as a TRAF binding region involved in NF-κB activation (14). We narrowed down the critical region up to amino acid 392 (see Fig. S1A in the supplemental material). Surprisingly, alteration of acidic amino acids crucial for the recruitment of TRAF proteins into alanine (29) or internal deletion from amino acid 389 to 395 did not abrogate LTβR-induced p100 processing (Fig. 1C). Titration of wt LTβR and LTβR ΔI 389–395 expression level indicated that this TRAF binding site was dispensable for the induction of p100 processing (see Fig. S1B). These results suggested the requirement of another region upstream of amino acid 392 for LTβR-mediated p100 processing. Bioinformatics analysis of the cytosolic tail revealed another putative TRAF3 binding site, POQQS, at position 319 to 323 matching the consensus PXOX(S/T). However, the substitutions Q321A and S323A did not inhibit LTβR-induced p100 processing (see Fig. S1C). By generating additional internal deletions, we identified two mutants, named ΔI 345–358 and ΔI 359–368, missing critical regions for the processing of p100 (Fig. 1D). Additional shorter internal deletions along the region 345 to 368 displayed an alteration of LTβR-induced p100 processing to the same extent as ΔI 345–358 and ΔI 359–368 mutants (see Fig. S1D). In order to confirm our results under physiological conditions, we generated 293T clones stably expressing wt and mutant LTβR for which ligand inducibility was maintained (Fig. 1E). While agonistic anti-LTβR treatment allowed the induction of p100 processing in wt and ΔI 389–395 LTβR-expressing cells, cells expressing ΔI 345–358 LTβR failed to respond (Fig. 1F and data not shown). Similar results were obtained when we complemented LTβR KO MEFs with the same expression vectors (see Fig. S1E and F).

Conformation of LTβR dictates the avidity of its bipartite binding sites toward specific TRAF proteins. So far, a single TRAF binding site has been characterized and located at position 387 to 396 of human LTβR (14, 29). However, we demonstrated that this region was dispensable for the induction of the alternative NF-κB pathway (Fig. 1C). Therefore, we hypothesized that LTβR may contain another TRAF binding site required for the induction of p100 processing. We analyzed the
FIG. 1. Identification of the cytoplasmic domain involved in LTβR-mediated p100 processing. (A) Schematic representation of human full-length and deletion mutant LTβR. The black bar, the gray bar, and TM represent the extracellular domain (amino acids 1 to 121), the cytosolic tail (amino acids 242 to 425), and the transmembrane domain (amino acids 222 to 241), respectively. (B to D) HEK 293 cells were transiently transfected with different sets of LTβR deletion mutants of the cytosolic tail and with the triple D390E391E393/AAA mutant. (E) 293T cells were stably infected with retrovirus encoding the indicated Myc-tagged LTβR, and expression was analyzed with anti-Myc immunoblotting. (F) Stable clones were stimulated for 6 h with an agonistic (Ago) anti-LTβR antibody prior to analysis by Western blotting of the processing of p100 into p52.
recruitment of in vitro-translated $^{35}$S-TRAF2 and $^{35}$S-TRAF3 with recombinant GST-LTβR. We detected TRAF association with wt GST-LTβR but not with the mutant GST-LTβR-AAA, which contains point mutations disrupting TRAF recruitment within the region 387 to 396 (29) (Fig. 2A). Similarly, both GST-ΔI 345–358 and GST-ΔI 359–368 failed to bind in vitro-translated TRAF proteins or HA-TRAF3-containing 293 cell extracts (and TRAF2) (data not shown). Thus, the amino acid stretch 345 to 368, similarly to the region 387 to 396 of human LTβR, is required for the binding of TRAF proteins when
expressed as GST fusion proteins. Analysis of amino acid content of the region 345 to 368 did not reveal any conserved consensus TRAF binding site within either mouse or human LTβR (Fig. 2B). When we overexpressed wt and mutant LTβR into 293 cells, we observed that disruption of the region 389 to 395, 378 to 388, 359 to 368, or 345 to 358 completely abolished the recruitment of TRAF2 and TRAF5. However, removal of the regions 389 to 395 and 378 to 368 did affect the recruitment to TRAF3, but a significant residual binding was still observed despite this deletion (Fig. 2C and D). Hence, on one hand, when the cytosolic tail of LTβR is fused to GST, any mutation or deletion within one of the two TRAF binding sites is sufficient to abrogate the binding of TRAF2 or TRAF3. On the other hand, when full-length LTβR is expressed in 293 cells, the region 345 to 358 is sufficient for the recruitment of TRAF3 and the induction of p100 processing.

**Internalization of LTβR is required to induce the processing of p100.** Trimerization of TNFR is a prerequisite for triggering their downstream pathways. Thus, we addressed whether the LTβR mutants deficient for the induction of p100 processing were able to trimerize. We transfected 293 cells with three differently tagged wt or mutant LTβR ΔI 345–358 and ΔI 359–368 constructs (Flag, HA, and Myc). The resulting cell lysates were subjected to a double immunoprecipitation (IP) procedure (see Materials and Methods), and the immunoprecipitated materials were analyzed by Western blotting for expression of the third tagged LTβR. Under those conditions, wt LTβR was able to trimerize (Fig. 3A; see also Fig. S3A in the supplemental material). Interestingly, mutants LTβR ΔI 345–358 and ΔI 359–368 were still able to form trimeric complexes, while other TNFR-related proteins, such as Trail-R3 and TNFRII, did not coimmunoprecipitate (co-IP) in single-IP or double-IP procedures (Fig. 3B; see Fig. S3B). We also analyzed the ability of wt LTβR and mutants ΔI 345–358, ΔI 359–368, and Δ389 to form aggregates upon overexpression in 293 cells. Thirty-six hours posttransfection, cells were cross-linked with a membrane-permeable cross-linker (DSP) and LTβR aggregation was analyzed by Western blotting in the absence or presence of DTT (reversed cross-link). All mutants defective for p100 processing were as efficient as wt LTβR in forming high-molecular-weight aggregates (Fig. 3C). Overall, our data showed that deletions ΔI 345–358 and ΔI 359–368 did not affect the ability of LTβR to multimerize. Meanwhile, when we monitored by flow cytometry the outcome of cell surface expression of wt and mutants ΔI 345–358 and ΔI 359–368 in both nonpermeabilized (NP) and permeabilized (P) 293 transfected cells, we observed striking differences. As expected, a pool of wt LTβR was detected at the cell surface of nonpermeabilized 293 cells. However, the intensity of fluorescence was elevated in permeabilized cells, indicating that part of wt LTβR was present within an intracellular compartment (Fig. 3D). In contrast, both LTβR mutant ΔI 345–358 and mutant ΔI 359–368 were exclusively localized at the cell surface, since no further increase of fluorescence intensity was detected upon cell permeabilization. We confirmed by confocal microscopy that wt LTβR displayed a perinuclear staining whereas both mutant ΔI 345–358 and mutant ΔI 359–368 were exclusively seen in the plasma membrane (Fig. 3E). We also analyzed by Western blotting the cellular distribution into soluble and insoluble Triton X-100 fractions of active wt LTβR and LTβR-AAA and inactive ΔI 345–358 and ΔI 359–368 LTβR, for the processing of p100. Our results revealed that internalized wt LTβR and LTβR-AAA were mainly present in the insoluble fraction while the mutants ΔI 345–358 and ΔI 359–368 were mainly found in the soluble fraction (Fig. 3F).

We next explored the ability of LTβR to recruit TRAF2 and TRAF3 from the intracellular compartment. We first constructed signal sequence-deficient (ΔS) expression vectors for wt LTβR, ΔI 345–358, and ΔI 359–368 and checked their cellular localization in nonpermeabilized (NP) and permeabilized (P) cells. In all cases, we detected LTβR expression only in permeabilized cells, confirming the inability of LTβR to migrate to the cell surface (Fig. 4A). We then looked at the cellular location of these signal sequence-defective mutants by confocal microscopy. We observed a punctate staining of LTβR ΔS wt mainly localized in the perinuclear compartment. However, the staining was largely diffuse when the region 345 to 358 of LTβR was absent, indicating that this stretch of the receptor directs its specific intracellular location (Fig. 4B). We then showed that LTβR ΔS wt could recruit endogenous TRAF2 and TRAF3 as wt LTβR. However, the recruitment was impaired in the absence of the region 345 to 358 or 359 to 368 irrespective of the presence of the signal sequence (Fig. 4C). These results highlight a dual role of the region 345 to 368 of LTβR in mediating internalization from the plasma membrane (Fig. 3D and E) and TRAF recruitment from an internal cellular compartment. In addition, we observed that the ability of LTβR ΔS wt to induce the processing of p100 was severely impaired for the mutants ΔS/ΔI 345–358 and ΔS/ΔI 359–368 (Fig. 4D).

Overall, our results reveal that internalization of LTβR and binding of intracellular TRAF2 and TRAF3 are a prerequisite for the induction of p100 processing.

**Internalization of LTβR in vivo correlates with induction of the alternative NF-κB pathway.** We next addressed the physiological relevance of our findings in biological settings for which an LTβR-induced alternative pathway is known to play a role through production of p52/RelB. Therefore, we isolated mesenteric lymph nodes (mLNs) from embryonic day 14 (E14) from wt and RelB-deficient mice and explanted them ex vivo in fetal organ culture. At that stage, mLNs stromal cells had not yet fully matured and most CD45− cells were ICAM-1int VCAM-1int (3). However, treatment of wt explants with an agonistic antibody to LTβR for 3 days allowed ICAM-1int VCAM-1int cells to commit into ICAM-1high VCAM-1high mature stromal “organizer” cells (Fig. 5A). During the maturation process, these cells expressed MadCAM-1 at a high level. Interestingly, this transition was abrogated in mLNs from RelB−/− embryos. Thus, the absence of LTβR-mediated MadCAM-1 upregulation in RelB−/− stromal cells revealed that MadCAM-1 expression was a reliable readout for the activation of the alternative pathway ex vivo. Therefore, we extended our analyses in vivo by collecting mLNs from wt embryos at day E15 and day E17. At day E15, we detected ICAM-1int VCAM-1int cells but barely any ICAM-1int VCAM-1high cells (Fig. 5B). Nevertheless, at day E17, part of the pool of ICAM-1int VCAM-1int cells committed to the ICAM-1high VCAM-1high phenotype. Despite the fact that both populations expressed LTβR, only ICAM-1high VCAM-1high cells were positive for MadCAM-1. In particular, LTβR
FIG. 3. LTβR defective for p100 processing is sequestered into the plasma membrane. (A) HEK 293T cells were transfected with three differently tagged LTβRs (HA, Flag, and Myc tagged), and double immunoprecipitations were performed to analyze the trimerization of wt LTβR (see the supplemental material for details). (B) The same procedure as in panel A was applied for internal deletion mutants ΔI 345–358 and ΔI 359–368. (C) HEK 293T cells were transfected with wt LTβR, ΔI 345–358, ΔI 359–368, and ΔI389. The cross-linker DSP was used prior to immunoprecipitation and immunoblotting of LTβR under nonreduced (−DTT) and reduced (+DTT) conditions. (D) Flow cytometry analysis of HEK 293 cells mock transfected or transfected with expression vector for wt LTβR, LTβR ΔI 345–358, or LTβR ΔI 359–368 and stained for cell surface LTβR (nonpermeabilized [NP]) or cell surface and intracellular LTβR (permeabilized [P]). MFI (mean fluorescence intensity) represents the value of one measurement out of three independent experiments. (E) Localization of wt LTβR, LTβR ΔI 345–358, and LTβR ΔI 359–368 in HEK 293 cells. Arrows indicate the perinuclear compartment. (F) Cell fractionation of LTβR into Triton X-100-soluble and -insoluble fractions from HEK 293 cells transfected with the indicated LTβR constructs.
FIG. 4. Perinuclear location of LTβR is a prerequisite for the recruitment of endogenous TRAF proteins and induction of p100 processing. (A) Flow cytometry analysis of HEK 293 cells mock transfected or transfected with expression vector for LTβR ΔS wt, LTβR ΔS/ΔI 345–358, or LTβR ΔS/ΔI 359–368 and stained for cell surface LTβR (nonpermeabilized) or cell surface and intracellular LTβR (permeabilized). MFI (mean fluorescence intensity) represents the value of one measurement out of three independent experiments. (B) HeLa cells transiently transfected with the indicated HA-tagged construct were stained for LTβR (in red) and nuclei (DAPI). Arrows indicate the punctate perinuclear staining of LTβR. (C) HEK 293 cells were mock transfected or transfected with LTβR expression vectors encoding either wt, ΔI 345–358, ΔI 359–368, or their signal sequence (ΔS)-defective counterpart. Immunoprecipitated LTβR was analyzed by Western blotting for the recruitment of endogenous TRAF2 and TRAF3. The asterisks represent the cross-reactivity with Ig heavy chains. (D) Extracts from cells transfected with signal sequence (ΔS)-defective mutants were used to analyze the processing of p100 by Western blotting.
cell surface expression in the ICAM-1high VCAM-1high MAdCAM-1− cell population was reduced compared to that in ICAM-1int VCAM-1int MAdCAM-1− cells. However, this decrease was not due to a down-modulation of LTβR transcript since the mRNA levels of expression were similar in ICAM-1int VCAM-1int and ICAM-1high VCAM-1high cells (Fig. 5C). We conclude from these experiments that the activation of the alternative NF-κB pathway in vivo correlates with a downregulation of LTβR cell surface expression.

LTβR-induced stabilization of intracellular NIK and activation of the alternative NF-κB pathway rely on dynamin-2 GTPase activity. We further analyzed the mechanisms that control the internalization of endogenous LTβR after binding of its natural ligands. First, we performed flow cytometry analyses on human epithelial colon carcinoma HT29 cells and observed that the level of cell surface LTβR dropped as soon as 5 min after treatment with LTβR1 or LIGHT (Fig. 6A and data not shown). This early internalization of ligand-bound LTβR was conserved across species, since treatment of MEFs with an agonistic antibody resulted in a similar pattern of LTβR internalization (see Fig. S6A in the supplemental material). We confirmed the internalization of activated LTβR by performing confocal analyses of HT29 cells treated for different periods of time with an agonistic antibody to LTβR. LTβR was associated with a shift from a cell membrane staining (untreated cells) to a punctate pattern (LTβR stimulated), while the early endosome-associated protein EEA1 staining showed the typical punctate profile (Fig. 6B). We observed that, over time, activated LTβR accumulated in early endosomes and colocalized with EEA1. Several routes of internalization converge to early endosomes, and a role for AP2/clathrin complexes was highlighted for TNFR members such as TNFR1 or Fas (44). Interestingly, we found that LTβR is a bona fide partner of the adaptor complex AP2. Indeed, we observed that activation of LTβR led to a fast recruitment of the AP2μ2 subunit, which was almost concomitant with...
TRAF3 recruitment (Fig. 6C; see also Fig. S6B). This result prompted us to analyze the connection between the AP2 and LTβR-mediated NF-κB activation. Therefore, we analyzed the responsiveness of LTβR-activated HeLa cells transfected with either siRNA control or siRNA AP2β2. Despite a potent inhibition of endogenous AP2β2 expression combined with expression of a dominant negative rat AP2β2, neither IκBα degradation nor p100 processing was altered following

FIG. 6. Dynamin-dependent internalization of LTβR is required for the activation of the alternative but not the classical NF-κB pathway. (A) Flow cytometry analysis of cell surface LTβR expression in untreated and LTα1β2-treated HT29 cells. (B) Colocalization of LTβR and EEA1 to early endosomes. HT29 cells were stimulated with an agonistic antibody to LTβR for the indicated time period prior to immunostaining. (C) Recruitment of endogenous AP2μ2 subunit to immunoprecipitated LTβR. (D) Inducible (+ Dox [doycycline]) rat dominant negative (DN) AP2μ2-expressing HeLa cells transfected with control or siRNA AP2μ2 and stimulated with an agonistic anti-LTβR antibody. (E) HeLa cells transfected with siRNA clathrin heavy chain (CHC) and treated as indicated for the assessment of IκBα degradation and p100 processing. (F) The same cells were transfected with siRNA dynamin-2 and treated as in panel E for the analysis of classical and alternative NF-κB pathways. (G) HeLa cells were treated with the Smac mimetic CmpA prior to staining with the Duolink technology and the indicated antibodies. The endogenous NIK/TRAF3 complex appears in red, and nuclei appear in blue (DAPI).
LTßR stimulation (Fig. 6D). Similar results were obtained with siRNA CHC (Fig. 6E). Hence, LTßR-mediated p100 processing appeared to be AP2/clathrin independent.

Since dynamin participates in clathrin-dependent as well as clathrin-independent endocytosis, we extended our analysis by using siRNA dynamin-2. Under these conditions, LTßR-induced NIK stabilization, phosphorylation, and depletion of p100 were completely abrogated (Fig. 6F). Conversely, the activation of the classical pathway remained intact since the pattern of IkBa degradation was similar. Thus, although depletion of dynamin-2 does not prevent recruitment of signaling proteins to the cytosolic tail of LTßR for the induction of the classical NF-κB pathway, the receptor is not able to alleviate the constitutive degradation of NIK. Thus, we speculated that NIK must be kept in check within an intracellular compartment. To address this question, we used a method that allowed us to observe endogenous NIK/TRAF3 within the cell. Since NIK is constantly degraded by c-IAP1/2, we first pretreated or not HeLa cells with the Smac mimetic compound A (CmpA) for 2 h. This approach allowed us to stabilize NIK without disrupting its binding to TRAF3. Then, using the Duolink technology, we observed that endogenous NIK/TRAF3 complex was physically localized within punctate cytosolic bodies (Fig. 6G). Thus, it is likely that dynamin-2 participates in the transport of activated LTßR in close proximity to NIK/TRAF3 bodies to allow TRAF3 recruitment and activation of the alternative NF-κB pathway.

We next extended our analyses by using a noncompetitive inhibitor of the GTPase activity of dynamin, named Dynasore (32). We observed that preincubation of HeLa cells with Dynasore fully abrogated the stabilization of NIK and the processing of p100 in response to LTßR stimulation (Fig. 7A). These observations could be extended to other cell lines as well as to human primary fibroblasts (Fig. 7B and C).

Conversely, Dynasore inhibited neither the early phase of TRAF2 recruitment to activated LTßR nor the activation of the classical pathway since IkBa phosphorylation and degradation were similar in control and Dynasore-treated cells (Fig. 7D and E).

Altogether, these results confirmed our findings using the genetic approach with siRNA and revealed that the GTPase activity of dynamin-2 is crucial for the induction of the alternative NF-κB pathway.

**TRAF3 degradation is secondary to LTßR-mediated p100 processing.** The current model is that TRAF3 recruitment and polyubiquitination by c-IAP1/2 occur at the cytoplasmic membrane-anchored receptor, leading to subsequent proteasomal TRAF3 degradation and NIK-induced p100 processing (52, 60). However, we observed that LTßR internalization is absolutely required for the activation of the alternative NF-κB pathway. Thus, we hypothesized that TRAF3 might be targeted for K48 polyubiquitination within the intracellular compartment. We established a system in which TRAF2, TRAF3, NIK, and HA-tagged ubiquitin were transiently coexpressed into 293T cells in the absence or presence of LTßR ΔS wt. Following blockade of the proteasome, we were able to efficiently immunoprecipitate, from SDS-denatured and renatured protein extracts, equal amounts of TRAF3 and NIK, in the absence and presence of LTßR ΔS wt (Fig. 8A). However, when we probed the same membranes with an anti-ubiquitin K48 antibody, we did not detect any K48-linked polyubiquitinated TRAF3 adducts. Conversely, in the absence of LTßR ΔS wt, we observed that NIK was constitutively polyubiquitinated. Nevertheless, in the presence of LTßR ΔS wt, the level of K48-linked polyubiquitinated NIK was drastically reduced without a concomitant TRAF3 K48-linked polyubiquitination. These results suggested that proteasomal degradation of TRAF3 would not be the only mechanism regulating its steady state. Indeed, blockade of the proteasome only marginally prevented TRAF2 and TRAF3 degradation upon LTßR stimulation, despite a potent inhibition of IkBa degradation and p100 processing (Fig. 8B). Moreover, inactivation of c-IAP1 by Smac mimetic (CmpA) did not prevent further TRAF3 degradation upon costimulation of LTßR (Fig. 8C). These results indicated that other mechanisms might account for LTßR-mediated TRAF3 degradation. We next analyzed the role of lysosomes for the degradation of TRAF2 and TRAF3 by using two different inhibitors of vacuolar ATPase activity, such as bafilomycin A1 and chloroquine. HeLa cells were treated for different periods of time with an agonistic antibody to LTßR in the absence or presence of bafilomycin A1 or chloroquine, and the phosphorylation and degradation of IkBa, as well as the processing of p100, were analyzed by Western blotting. Under both conditions, phospho-IkBα appeared as soon as 15 min after stimulation followed by an almost complete degradation of IkBa within 30 min. The efficiencies of p100 processing were also comparable between untreated and bafilomycin A1- or chloroquine-treated cells (Fig. 8D and data not shown). These results indicate that LTßR-mediated lysosomal degradation of TRAF2 and TRAF3 is dispensable for the activation of both the classical and the alternative NF-κB pathway. Overall, our results strongly suggest that internalized LTßR displaces intracellular TRAF3 from NIK, allowing its stabilization as previously proposed (41).

Altogether, we propose a model depicting ligand-bound LTßR complex outcomes and in which dynamin-dependent...

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**FIG. 8. Intracellular LTßR activates the alternative NF-κB pathway independently of TRAF degradation.** (A) HEK 293 cells were transiently transfected (+) or not (−) with the indicated expression vectors. Cells were lysed in 1% SDS and diluted up to 0.1% prior to the first round of immunoprecipitation (1st IP) with a control antibody (Ctrl Ab) or an anti-TRAF3 antibody. The immunoprecipitated material was analyzed by immunoblotting for TRAF3 and K48-linked TRAF3. The supernatants from the 1st IP were incubated with control beads or anti-Flag-coated beads. The immunoprecipitated materials were analyzed by immunoblotting for NIK and K48-linked ubiquitinated NIK. The asterisks represent the cross-reactivity with Ig heavy chains. (B) HeLa cells were stimulated with the agonist (Aggo) to LTßR in the absence of DMSO (vehicle) or the proteasome inhibitor MG132, and cell extracts were analyzed by immunoblotting for the indicated proteins. (C) HeLa cells were treated with compound A (CmpA) or the Ago to LTßR alone or in combination as indicated, and the indicated proteins were analyzed by immunoblotting. (D) HeLa cells were stimulated as indicated with the Ago to LTßR in the absence (vehicle) or presence of bafilomycin A1. Total cell extracts were analyzed by Western blotting for the indicated proteins.
internalization uncouples the activation of the classical and the alternative NF-κB pathways (Fig. 9).

**DISCUSSION**

The biological functions fulfilled by members of the TNFR family rely on distinct signaling pathways for which recruitment of different TRAF proteins plays key roles. In this study, we identified an uncharacterized TRAF binding site spanning amino acid 345 to 368 of human LTβR. We showed that this region was as important as the triad D390/D391/E393 of LTβR for the recruitment of TRAF2 or TRAF3 in GST pulldown experiments (29). Of note, the primary sequence of amino acids 345 to 368 did not reveal any features of canonical TRAF2 or TRAF3 binding sites. Interestingly, other unconventional TRAF binding sites have also been characterized for TNFR2 (PLGVPDAGMKPS) and NIK (ISIIAQA), for which the recruitment of TRAF2 and TRAF3 appeared to be indirect and direct, respectively (16, 19, 30). Our data revealed that the affinity of the two TRAF binding sites of LTβR might fluctuate according to the oligomerization status of LTβR. Indeed, deletion of one of the two TRAF binding sites was sufficient to disrupt the recruitment of TRAF2 and TRAF3 to LTβR if expressed as a GST fusion protein. However, when LTβR was expressed as a native protein in eukaryotic cells, the region 345 to 368 retained some TRAF3 binding activity despite the deletion of the other TRAF binding site. It is reasonable to suggest that aggregation of LTβR increases the local concentration of LTβR, allowing it to increase its avidity toward TRAF3. The outcome of TRAF proteins following recruitment to TNFR varies from one receptor to another, involving degradation through either the proteasome or into lysosomes, as well as cellular relocalization to restricted cellular compartments (48). Recently, it was proposed that LTβR-mediated TRAF3 proteasomal degradation was required for stabilizing and accumulating NIK (41). However, under conditions for which overexpressed LTβR solely induced p100 processing, we did not observe an accumulation of K48-linked polyubiquitinated TRAF3, while the pool of K48-linked polyubiquitinated NIK was strongly reduced. Furthermore, it was shown that LTβR-mediated depletion of TRAF3 was required not only for the induction of the alternative pathway but also for the

FIG. 9. Model of LTβR trafficking and NF-κB activation. The binding of LTα1β2 to LTβR leads to its trimerization. This event allows a fast recruitment of TRAF proteins through the bipartite TRAF binding site (amino acids 389 to 395 in blue and 345 to 368 in red). This process then connects the receptor to the induction of IκBα degradation by the proteasome (classical NF-κB pathway). In the meantime, the complex AP2 in association with clathrin regulates an NF-κB-independent function of LTβR. While LTα1β2 accumulates at the cell surface of inducer cells, LTβR trimers form clusters on targeted cells. This process likely triggers the internalization of LTβR, which relies on the cytosolic region 345 to 368 and the presence of dynamin-2. Endocytic vesicles released from the plasma membrane expose the tail of LTβR toward the cytosol. This event allows LTβR to compete with intracellular NIK for the binding of its inhibitory complex TRAF3/TRAF2/c-IAP1/2. As a consequence, the constitutive proteasomal degradation of NIK (dashed line) is alleviated. Thus, NIK accumulates (solid line) and activates IκKα, and both events trigger the processing of p100 and the generation of p52/RelB. TRAF3/TRAF2/c-IAP1/2 complex is then degraded into lysosomes.
classical NF-κB pathway in MEFs, as well as in some colon epithelial cell lines (4, 59). Thus, proteasomal degradation of TRAF3 is associated not only with NIK stabilization. This statement can be also extended to signaling pathways downstream of CD40. Indeed, CD40-induced K48-linked polyubiquitination and proteasomal degradation of TRAF3 are strictly dependent on TRAF2 and c-IAP1/2 (21, 35, 52, 60). Based on these findings, a model had been proposed in which activated CD40 recruits TRAF2/TRAF3-c-IAP1/2 at the cell surface for promoting TRAF3 proteasomal degradation and NIK stabilization (52, 60). However, CD40-mediated K48-linked polyubiquitination and proteasomal degradation of TRAF3 are also required prior to cell membrane release of a MEKK1-containing complex that activates Jun N-terminal kinase (JNK) (34). Again, c-IAP1/2-mediated TRAF3 polyubiquitination is engaged in two distinct pathways involving MEKK1 and NIK. Therefore, assessing K48-linked TRAF3 polyubiquitination is not a readout strictly associated with an activation of NIK. Overall, TRAF3 appears to be a multitask protein that acts mainly as an inhibitor. It is likely that different pools of TRAF3-containing complexes exist, and according to the cell type and the duration of stimulation, TRAF3 is recruited and degraded at different locations to activate distinct pathways. We further observed that lymphotixin-induced TRAF2 and TRAF3 degradation also occurred in the lysosomal compartment. However, potent inhibition of TNFR-mediated lysosomal TRAF degradation did not alter the extent of p100 processing, suggesting that this type of degradation is likely secondary to NIK stabilization.

Internalization of TNFR has been mainly considered a mechanism participating in recycling and/or degradation. In this study, we identified a new function assigned to LTβR-mediated cell membrane receptor internalization, or LTβR internalization, or LTβR recombinant decoy receptors (18). Conversely, in LTβR-expressing melanoma cells, activation of NIK is driven in a ligand-independent way (12). In this particular case, the use of decoy LTβ would be useless and other strategies should be envisioned for preventing LTβR-mediated cell proliferation. The development of molecules that specifically block LTβR internalization, or other receptors, might be a promising research avenue for inflammatory disorders and cancer treatment.

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