Lymphotoxin β Receptor Triggering Induces Activation of the Nuclear Factor κB Transcription Factor in Some Cell Types*

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NFκB is a pleiotropic transcription factor capable of activating the expression of a great variety of genes critical for the immunoinflammatory response. Tumor necrosis factor α (TNFα) and lymphotoxin α (LTα, originally TNFβ) are potent nuclear factor κB (NFκB) activators in various cell types. The LTα molecule, in addition to being secreted as a soluble trimer, can also form membrane-anchored heterotrimers with the LTβ chain, another member of the TNF family. The LTα1β2 heterotrimer binds a specific receptor, called the LTβ receptor (LTβ-R), which is also a member of the TNF receptor family. Here, we show that engagement of LTβ-R with a soluble form of LTα1β2 or with a specific anti-LTβ-R agonistic monoclonal antibody CBE11 quickly induces activation of NFκB in HT-29 and WiDr human adenocarcinomas. LTβ-R triggering activates NFκB and induces proliferation in WI-38 human lung fibroblasts. No NFκB activation is observed in human umbilical vein endothelial cells, correlating with the inability of LTβ-R activation to induce expression of NFκB-dependent cell surface adhesion molecules. Thus, like several other members of the TNF receptor family, the LTβ-R can activate NFκB following receptor ligation in some but not all LTβ-R-positive cells.

For more than a decade, the TNF-LT1 system has been known to be composed of two related inflammatory cytokines, TNFα (cachectin or tumor necrosis factor α) and LT or LTα (lymphotoxin α, also called TNFβ) which are active as soluble trimers (1–5). Both ligands engage two receptors, TNF-R55 (TNF-R55) and TNF-R75 (TNF-R75) (5), and appeared historically to have similar biological activities. Recently, however, the discovery of membrane-bound forms of these two ligands revealed interesting differences (6). TNFα can be retained on the cell membrane as an uncleaved type II membrane protein, whereas LTα was found to be processed yet anchored to the membrane by association with a 33-kDa protein named LTβ (6). LTβ is a member of the TNF-LT family (7), and its gene maps to the TNF-LT locus (8). Protein biochemistry studies showed that the LTα chain which composes the functional LT trimer (renamed LTα3) can also form membrane-anchored heterotrimers with the LTβ chain, in predominately a LTα1β2 form with only minor amounts of the LTα2β1 form (9). Moreover, a specific receptor (LTβ-R) for the LTα and LTβ heterotrimers with homology to the TNF receptors has been identified and is a new member of the nerve growth factor and TNF receptor family (10). Interestingly, LTβ-R is expressed in a wide range of cell types, except lymphocytes, whereas, conversely, the expression of the ligand is restricted to activated lymphocytes (8–10). Relatively little is known about the function of LTβ-R. The aberrant development of lymph nodes observed in the LTα knockout mice (11, 12), which is not observed in the TNF-R55 or TNF-R75 knockout mice (13–15), and the ability of soluble decoy forms of the LTβ-R to block lymph node development2 indicates a role for surface LTα-LTβ complexes in the development of the peripheral lymphoid system. Additionally, LTα and LTβ signaling appears to be required for the expression of various cell surface adhesion proteins on cells in the marginal zones bordering the white pulp regions of the spleen (16).2–4 It is a reasonable assumption that the LTβ-R mediates these activities. Intriguingly, these observations suggest possible parallels between TNF and LT actions in terms of their abilities to control expression of surface proteins involved in cell-cell adhesion on endothelial cells and marginal zone cells in the spleen, respectively.

To understand the biological consequences of LTβ-R signaling, we have analyzed in depth two different areas based on the known effects of TNF signaling. First, we have shown previously that receptor activation by either soluble ligands or agonistic antireceptor monoclonal antibodies induces the death of some adenocarcinoma cell lines (17). Second, in striking contrast to the TNF-R system, LTβ-R activation was shown to have little effect on the display of adhesion molecules on endothelial cells.5 In light of these various observations, it would be useful to investigate which signal transduction pathways are affected by LTβ-R activation. Currently, little is known about LTβ-R activation other than the observation that the cytoplasmic domain of LTβ-R, like that of CD40, can bind to TNF receptor-associated factor 3, a member of a newly defined group of signal transduction molecules (18).

NFκB is a pleiotropic transcription factor that is especially involved in the regulation of the expression of numerous genes critical for the immunoinflammatory response, e.g., interleukin 6 and 8, vascular cell adhesion molecule 1, and E-selectin (reviewed in Ref. 19). Many of these genes are expressed in fibroblasts and endothelial cells and play pivotal roles in host defense. The system is also exploited by some viruses, including human immunodeficiency virus, to drive their expression.

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1 The abbreviations used are: TNF, tumor necrosis factor; LT, lymphotoxin; TNF-R, tumor necrosis factor receptor; LTβ-R, lymphotoxin β receptor; EMSA, electromobility shift assay; NFκB, nuclear factor κB; HUVEC, human umbilical vein endothelial cell; FACS, fluorescence-activated cell sorting.

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(19, 20). Signaling through five members of the TNF receptor family has been shown to activate NFκB, i.e. TNF-R55 (21–23), TNF-R75 (24, 25), CD30 (26, 27), Fas (28, 29), and CD40 (25, 30, 31). Interestingly, signaling by two members of this family, CD30 and the TNF receptors, has also been shown to enhance human immunodeficiency virus expression (26, 32–34). Recent results suggest that LTβ-R, as well, triggers human immunodeficiency virus replication in infected monocytic cells. In this report we show that LTβ-R signaling leads to NFκB activation in a cell type-restricted manner.

EXPERIMENTAL PROCEDURES

Cell Lines—The human colon adenocarcinomas WiDr and HT-29 and the human fibroblasts WI-38 were obtained from the American Type Culture Collection and grown in minimum Eagle’s medium–Earle’s salts medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS), 2 mM glutamine, 100 unit/ml penicillin, 100 mg/ml streptomycin, 0.5 mM sodium pyruvate, 0.5×100×nonessential amino acid solution (BioWhittaker, Walkersville, MD) at 37°C in 5% CO2. A consistently responsive subclone of the HT-29 line, called HT-29-14, was used in all of our experiments. WiDr and HT-29 cells are thought to be derived from the same patient (35). Human umbilical vein endothelial cells (HUVECs) were isolated by the method of Gimbrone (36) and serially passaged under the conditions modified and described by Thornton et al. (37). Normal human dermal fibroblasts and their optimized medium were purchased from Clonetech Corp. (San Diego, CA).

Reagents—Soluble recombinant LTα3 (LTα3 trimer) and LTα1β2 molecules were prepared as described previously (38). Briefly, S9 insect cells were infected with two recombinant baculoviruses that encode LTα and a soluble version of LTβ. Culture supernatants were passed over a series of TNF-R55 and LTβ-R affinity columns to purify LTα3 and LTα2β1. Human LTα3 was prepared as previously detailed (39). The various LT trimers were also prepared with a LTα50N mutation that eliminates TNF-R binding (38). The LTβ-R-human IgG1 fusion protein has been described previously (9). The mouse anti-human LTβ-R antibodies CBE11 and BDA8 have also been described previously (17). A control mouse IgG monoclonal antibody MOPC 21 was obtained from Organon Pharmaceuticals. Antibodies against p50, p65, RelB, c-Rel, and p52 NFκB subunits were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared as described by Digman et al. (40) with minor modifications. Briefly, cells were harvested and washed twice with phosphate-buffered saline. 1.5 to 2×10^6 cells were resuspended in a hypotonic lysis buffer composed of 10 mM Hepes, pH 7.9, 1.5 mM MgCl2, and 10 mM KC1 (buffer A) on ice. After a 20-min incubation, cells were homogenized by 20 strokes with a loose-fitting Dounce homogenizer. Nuclei were collected by centrifugation for 4 min at 6500 rpm at 4°C, and nuclear proteins were extracted with 3 pellet volume of high salt buffer (calibrated to contain 10 μg of protein as determined using the Bio-Rad protein assay for 45 min at ambient temperature in the presence of 2 μg of poly(dI-dC) (Pharmacia Biotech Inc.) and 30 μg of bovine serum albumin (41, 42).

In the case of supershift experiments, nuclear extracts were incubated 30 min with the labeled oligonucleotide at room temperature and then incubated an additional 30 min with an antibody specific for one member of the rel family (2 μg/reaction mix) at 4°C before loading the samples on the gel. Samples were electrophoresed on native 4% non-gradient or gradient polyacrylamide gels from Integrated Separation Systems (Natick, MA) as indicated in the figure legends.

Proliferation Assays—WI-38 cells were cultured in a 96-well plate in 100 μl of medium (1× cells/well) in the presence of varying amounts of human TNFα, LTα3, LTα1β2, a mouse anti-human LTβ-R antibody CBE11, or a control mouse IgG. After a 72-h incubation at 37°C, cells were pulsed 6 h with 1 μCi/well (in 50 μl) [methyl-3H]thymidine (DuPont NEN) and harvested using a Tomtec (Orange, CT) cell harvester. The radioactivity was measured in a Betaplate liquid scintillation counter (Pharmacia Biotech).

Flow Cytometry—HUVEC and WI-38 cells were detached with 5 mM EDTA in phosphate-buffered saline, and cells were washed twice with phosphate-buffered saline, 0.05% NaN3, and 1% bovine serum albumin (FACS buffer). Cells were incubated 30 min on ice with 10 μg/ml of mouse anti-human LTβ-R (BD8) or a control mouse IgG, washed once in FACS buffer, and incubated 30 min on ice with phosphodiethanolamine-conjugated donkey anti-mouse Ig (Jackson ImmunoResearch, West Grove, PA) at 5 μg/ml in FACS buffer. Cells were washed twice in FACS buffer and analyzed on a FACScan cytometer (Becton Dickinson). Cells were gated using forward versus side scatter to exclude dead cells and debris.

RESULTS AND DISCUSSION

NFκB activation was analyzed by EMSA using two related human adenocarcinoma cell lines, HT-29 (clone 14) and WiDr, which have been shown to be sensitive to LTα1β2 in a cytotoxicity assay in the presence of interferon γ (17). In a first set of experiments, WiDr cells in culture were stimulated for various periods of time (from 5 min to 12 h) with LTα1β2, CBE11 (a mouse anti-human LTβ-R, agonist antibody), or a mouse IgG as a negative control. LTβ-R signaling in WiDr or HT-29 cells resulted in a fast activation of NFκB, with a maximal signal occurring after 15 min of stimulation (Fig. 1, A and B, respectively). LTβ-R triggering is specifically mediating NFκB activation because, NFκB was activated with the anti-human LTβ-R agonistic monoclonal antibody CBE11 and not an isotype-matched control monoclonal antibody (Fig. 1A). The kinetics of activation of NFκB through LTβ-R were very similar to the kinetics observed for TNFα, LTα3, CD40, and Fas in other cell types (28, 43, 44), suggesting similar mechanisms of activation. NFκB activation was transient and disappeared after 12 h of stimulation (Fig. 1A). NFκB is a heterodimeric complex of p50 and p65 subunits, which are proteins of the Rel-NFκB family (19). Homodimeric or heterodimeric combinations with other members of this family such as RelB, c-Rel, and p52 are referred to as NFκB like (19, 45). The question of which NFκB dimer types were activated was addressed by supershift analysis using specific rabbit antisera to the p50, p65 (RelA), RelB, c-Rel, and p52 NFκB subunits or a control antibody. The NFκB bands resulting from TNFα, LTα3 or CBE11 stimulation, were supershifted by anti-p50 and anti-p65 antibodies (Fig. 1B). The supershift for p50 appeared weaker than for p65. LTα1β2 also activated a p50-p65 dimer in these cells (data not shown).

Similar results were seen using the same WiDr extracts, except that only the anti-p65 antibody supershifted the NFκB band obtained by the same three different stimuli (data not shown). These results suggest that LTα1β2 signaling through LTα1β2 activated predominantly the same type of NFκB dimer in a given cell type, i.e. p50-p65 NFκB dimer in HT-29 cells, and probably a p65-p65 dimer in WiDr, presumably using similar

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Moreover, LTα1β2 with a LTα D50N mutation (LTα1D50Nβ2), which would prevent the binding of a possible LTα3 contaminant to the TNF receptor p55, also activated NFκB with an intensity similar to that of LTα1β2 (Fig. 2C). Anti-human LTβ-R CBE11 antibody also specifically activated NFκB in WI-38 cells (Fig. 2C). Therefore, like the HT29-WiDr system, NFκB can also be activated in WI-38 cells specifically by LTβ-R cross-linking.

In addition to its well defined ability to induce cell death, TNFα has been shown to induce the proliferation of fibroblasts (46). In light of these dual effects on cell growth and death depending on cell type, we wished to determine whether LTβ-R signaling could also affect proliferation in a fibroblastoid cell line. Proliferation assays were performed using WI-38 fibroblasts stimulated for 72 h with TNFα, LTα3, LTα1β2, CBE11, and a mouse IgG as control. As shown in Fig. 3A, TNFα, LTα3, LTα1β2, and CBE11, but not the control antibody, stimulated WI-38 cell proliferation, demonstrating that the stimulation of LTβ-R induces proliferative signals in these cells. Both wild type LTα1β2 and LTα1β2 with a LTα D50N mutation equally stimulated the proliferation of WI-38 cells (Fig. 3B), again indicating specific LTβ-R signaling. The proliferative effects of LTβ-R signaling on nontransformed cells appear to cell type specific, since LTβ-R triggering has no effect on HUVEC proliferation, whereas TNFα inhibits their proliferation (47, 48). Specific stimulation of human LTβ-R triggers NFκB activation in WiDr and HT-29 cells. LTα1β2 and CBE11 failed to activate NFκB in HUVECs, which express reasonable levels of LTβ-R, whereas TNFα and LTα3 were fully potent. These observations are consistent with the inefficiency of LTα1β2 or CBE11 to induce the NFκB-dependent expression of E-selectin or vascular cell adhesion molecule 1 in HUVECs (50) and tend to exclude the LTβ-R system as a regulatory element in the endothelium-mediated inflammatory response. These observations are also supported by the results of systemic injection of LTα1β2 in mice, which showed no lethality in the dose range at which TNFα or LTα3 are lethal (51). The ability of LTβ-R to activate NFκB could depend, in part, on LTβ-R expression levels in the cell lines used in our experiments, which are higher in WiDr, HT-29, and WI-38 cells compared with HUVECs (Fig. 2B). However, previous work in the TNF system had shown that variations in receptor density alone cannot explain the responsiveness of various cells to TNFα (1), and we suspect that also in the LT system, a lower receptor density does not account for the nonresponsive behavior of HUVECs. These NFκB results and the proliferation studies indicate that both LTβ-R signaling and function are cell type specific.

These studies also reveal an interesting phenomenon. It appears that varying levels of receptor cross-linking are required for the activation of various signal transduction pathways. For example, soluble CBE11 was not very potent in killing WiDr or HT-29 cells, whereas immobilized CBE11 was fully active in the same cytotoxicity assays (1), presumably by creating a superior level of receptor cross-linking in the latter case. However, in the case of NFκB activation in these cells, soluble CBE11 was active. Likewise, soluble CBE11 was significantly less active than LTα1β2 in the activation of NFκB in WI-38 cells (Fig. 3A). These signaling differences between soluble and cell surface ligands and soluble or immobilized agonist antibodies are examples of the complexity of receptor triggering, which has been previously observed for the TNF receptor system (3, 49, 50). Receptor cross-linking has been shown to be critical for signaling in the TNF and Fas system (3, 51, 52), and a similar observation was made for LTβ-R, using Fab fragments of agonistic antibodies (17). It is possible that the signal transduction elements mediating, for example,

pathways. As was noted for Fas-induced NFκB activation (29), minor amounts of some other NFκB forms appear in the WiDr cells (Fig. 1A). The p65-p50 form of NFκB appears to be the common dimer activated by CD40 (25, 31), CD30 (26, 27), Fas (28), TNF-R55 (23), TNF-R75 (25), and LTβ-R, although CD40 (31) and CD30 (26) can activate additional NFκB isoforms in lymphoid cells.

Because TNFα and NFκB play fundamental roles in regulating the endothelium and fibroblasts, we examined the ability of the various LT forms to activate NFκB in HUVECs and the nontransformed human diploid fibroblast cell line WI-38. Both HUVECs and WI-38 cells express LTβ-R on their cell surface (Fig. 2B). As expected, LTα3 activated NFκB in HUVECs and WI-38 cells (Fig. 2A, and C). LTα1β2 and CBE11 also activated NFκB in WI-38 cells (Fig. 2C) but not in the HUVECs (Fig. 2A). Activation of NFκB by LTα1β2 in WI-38 cells was inhibited by addition of soluble human LTα1β2 and not by addition of soluble human TNF-R55 (Fig. 2C), indicating a LTβ-R specific event.
NFκB activation versus cell death require varying levels of oligomerization to trigger. Presumably immobilized antireceptor antibody and ligand would oligomerize the receptor to a greater extent than the theoretical dimers resulting from soluble monoclonal antibodies. Although this model is plausible, a completely conflicting pattern was observed with HepG2 cells, in which a monoclonal anti-TNF-R55 antibody was active, whereas TNFα was inactive (50). Clearly, further analysis of the events stemming from differing levels of receptor oligomerization and the varying lifetimes of the oligomerized states is required to understand the receptor activation.

No correlation appears to exist between NFκB activation and cell proliferation or death (28, 29, 53, 54). Our data are also consistent with this hypothesis, since in vitro LTb-R-mediated death in WiDr and HT-29 cells is interferon-γ dependent, whereas NFκB activation and the ability to induce WI-38 proliferation is interferon-γ independent. These results suggest that different intracellular mechanisms are responsible for these phenomena. On the other hand, some common signaling pathway must be used by these receptors, since stimulation with TNFα, LTα3, LTα1β2, or CBE11 leads to the activation, with similar kinetics, of the same type of NFκB dimer in HT-29 or WiDr cells. Presumably, multiple signal transduction path-

**Fig. 2.** EMSA analysis of NFκB activation in WI-38 cells and HUVECs and FACS analysis of cell surface LTβ-R expression. A, HUVECs in culture were stimulated for 30 min with 20 ng/ml TNFα, 100 ng/ml LTα3, 500 ng/ml LTα1β2, 1 μg/ml CBE11 anti-LTβ-R, or 1 μg/ml mouse IgG1 (MOPC 21) as a control. Under each group, samples indicated as unstimulated refer to cells without any treatment. The analysis was performed as indicated in Fig. 1A. Samples were run on a 4–20% gradient gel. Arrows, NFκB bands. B, cytofluorometric analysis of human LTβ-R expression on HUVECs and WI-38, WiDr, and HT-29 cells. Cells were stained with BDA8 anti-LTβ-R monoclonal antibody (black profiles) or an irrelevant mouse IgG as control (white profiles), followed by phosphatidylethanolamine-conjugated donkey anti-mouse Ig. Relative cell numbers (vertical) and fluorescence intensity (log) are presented. C, WI-38 cells in culture were stimulated for 15 min with 100 ng/ml LTα3 or 500 ng/ml LTα1β2 in the presence or absence of 10 μg/ml mouse LTαβ-human Fc or human TNF-R55-human Fc, 500 ng/ml LTα1D50Nβ2 (LTα1β2 containing a LTα D50N mutation), and 1 μg/ml CBE11 anti-LTβ-R. The analysis was performed as indicated in Fig. 1A. Samples were run on a 4% acrylamide gel.

**Fig. 3.** Effect of TNF and LTβ receptor activation on the proliferation of WI-38 cells. A, WI-38 cells in culture were stimulated for 72 h with various concentrations of TNFα ( ), LTα3 ( ), LTα1β2 ( ), and CBE11 ( ) agonist antibody against the human LTβ receptor and an irrelevant mouse IgG1 (MOPC 21) as control ( ). The cells were then incubated for 4 h with [methyl-3H]thymidine (1 μCi/well) and harvested. The incorporated radioactivity was measured and expressed as the mean and S.D. of triplicates. B, WI-38 cells in culture were stimulated for 72 h with various concentrations of LTα1β2 ( ) or LTα1β2 containing a LTα D50N mutation ( ). Then cells were incubated with [methyl-3H]thymidine (1 μCi/well) and analyzed as described in A.
ways are initiated following receptor cross-linking. The ability of the CBE11 monoclonal antibody to inhibit WiDr tumor growth in vivo is interdependent on the immune system (19). Cell type-specific transcription factor, and its properties are extensively studied in activated lymphocytes, whereas the receptor is expressed on nonlymphoid cells (55). Therefore, v in vitro activation of NF-κB through LTβ-R probably occurs exclusively following cell-cell contact between lymphocytes and nonlymphoid cells. NF-κB is a ubiquitous transcription factor regulating numerous genes (56). Cell type-specific activation of NF-κB might therefore represent a unique and restricted way of local communication between activated lymphocytes transiting into the lymphoid organs and inflamed tissues and their stromal environment.

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