The Lymphotoxin-β Receptor Is an Upstream Activator of NF-κB-mediated Transcription in Melanoma Cells*

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The pleiotropic transcription factor nuclear factor-κB (NF-κB) regulates the transcription of genes involved in the modulation of cell proliferation, apoptosis, and oncogenesis. Furthermore, a host of solid and hematopoietic tumor types exhibit constitutive activation of NF-κB (Basseres, D. S., and Baldwin, A. S. (2006) 25, 6817–6830). However, the mechanism of this constitutive activation of NF-κB has not been elucidated in the tumors. We have previously shown that NF-κB inducing kinase (NIK) and its association with Inhibitor of κB kinase αβ are elevated in melanoma cells compared with their normal counterpart, leading to constitutive activation of NF-κB. Moreover, expression of dominant negative NIK blocked this base-line NF-κB activity in melanoma cells. Of the three receptors that require NIK for activation of NF-κB, only the lymphotoxin-β receptor (LTβ-R) is expressed in melanoma. We show in this manuscript that for melanoma there is a strong relationship between expression of the LTβ-R and constitutive NF-κB transcriptional activity. Moreover, we show that activation of the LTβ-R can drive NF-κB activity to regulate gene expression that leads to enhanced cell growth. The inhibition by LTβ-R shRNA resulted in decreased NF-κB promoter activity, decreased growth, and decreased invasiveness as compared with control. These results indicate that the LTβ-R constitutively induces NF-κB activation, and this event may be associated with autonomous growth of melanoma cells.

The transcription factor NF-κB plays critical roles in diverse physiological processes and numerous human pathologies (1–3). Understanding the mechanisms that regulate NF-κB activity is critical for developing therapeutic strategies for many human diseases (2, 3). We and others have previously demonstrated that targeting NF-κB with a small molecule inhibitor of IκB kinase-β (IKKβ) inhibits the growth of melanoma cells in vitro and in vivo (4). However, broad targeting of NF-κB as a therapeutic measure for treatment in the clinic has its drawbacks due to effects on the immune system.

NF-κB-inducing kinase (NIK) is an upstream kinase in the NF-κB activation pathway and preferentially phosphorylates IKKα over IKKβ, leading to the activation of IKKα kinase activity (5, 6). It belongs to a family of serine/threonine kinases including NIK, MAPK kinase kinase (MEKK1–4), apoptosis signal-regulating kinase 1 (ASK1), Raf and others (7). Originally NIK was identified as a binding partner for the tumor necrosis factor (TNF) receptor-associated factor 2 inflammatory receptor and was demonstrated to be a key element of the NF-κB signaling pathway in response to TNF, interleukin-1, and CD95 (8). Initial studies of the function of NIK, in which the consequences of overexpression of this kinase or its mutants were assessed, suggested that NIK mediates activation of the canonical NF-κB pathway and does so in response to multiple inducers with many different physiological functions (9). However, later studies of mice of the aly strain, which express a non-functional NIK mutant, challenged the notion that NIK has a functional role in the activities of most of these inducers (10). Furthermore, a complete disruption of the NIK gene (nik−/−) leads to a phenotype that is reminiscent of lymphotoxin-β receptor (LTβ-R) knock-out mice (11). Indeed, cells from nik−/− mice showed a normal response to TNF-α or interleukin-1 treatment, whereas NF-κB activation in reaction to LTβ was impaired. Thus, it was suggested that NIK participates selectively in the activation of NF-κB by a restricted set of ligands that specifically affect the development and function of lymphocytes. The aly/aly mice show a cell-autonomous defect in B-cell function in addition to the traits associated with the LTβ-R−/− phenotype (12, 13). Recent studies have shown two other ligand/receptor pairs

The abbreviations used are: IKK, IκB kinase-β; NIK, NF-κB-inducing kinase; LTβ-R, lymphotoxin-β receptor; ELISA, enzyme-linked immunosorbent assay; MTT, thiazolyl blue tetrazolium bromide; TNF, tumor necrosis factor; FACS, fluorescent-activated cell sorting; VCAM, vascular cell adhesion molecule; HEM, normal human epidermal melanocyte; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcription; PBS, phosphate-buffered saline; shRNA, short hairpin RNA; SDF1, stromal cell-derived factor 1; LIGHT, lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells; MMP, matrix metalloproteinase.

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are also involved in NIK signaling, CD40/CD40L and Baff/ BaffR, which are expressed primarily on B cells (14).

The LTβ-R, a member of the tumor necrosis factor receptor superfamily, is expressed on the surface of many cell types, including cells of epithelial and myeloid lineages. To date, there are two known cytokines capable of transducing signal through LTβ-R. The first is lymphotoxin αβ2, a cell bound heterotrimeric complex of LTα and LTβ. The second cytokine, LIGHT, is a membrane-anchored homotrimeric complex capable of binding LTβ-R and herpes simplex virus entry mediator HVEM (15). It has been shown that signaling triggered by LTβ-R is required for the genesis of Peyer’s patches and lymph nodes based on the observation that Peyer’s patches and lymph nodes are deficient in LTβ-R gene-deleted mice (11, 16). It has been shown that LTβ-R can bind TNF receptor-associated factor (TRAF) 2, 3, and 5 but not TRAF6 (17–19).

We have demonstrated previously that NIK basal expression as well as IKK-associated NIK activity is higher in melanoma cells compared with normal melanocytes (20). In the present report we demonstrate that LTβ-R is an upstream modulator of the NF-κB pathway in melanoma cell lines and melanocytic lesions and, thus, may be an important receptor for progression and metastasis of human melanomas.

MATERIALS AND METHODS

Plasmids and Reagents—Rabbit lymphotoxin-β receptor antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). LTβ-R receptor agonistic antibody was obtained from Jeffrey Browning, Biogen (Cambridge, MA) (36), and the LTβ receptor-Fc chimera as well as the LIGHT ELISA kit was obtained from R&D Systems (629-LR) (Minneapolis, MN). The NF-κB luciferase reporter vector contains five tandem repeats of the NF-κB element 5′ to the transcription initiation site and is contained in pLUC-MCS reporter vector (Stratagene; La Jolla, CA). Thiazolyl blue tetrazolium bromide (MTT) reagent was purchased from Sigma-Aldrich.

Cell Culture and Transfection—The human melanoma cell lines Hs294T and SKMel28 cells were seeded at 3 × 10^5 cells/well in 24-well plates and cultured in 1 ml of DMEM/F-12, 0.2% FBS. LTβ-R agonist antibody (2 and 10 μg/ml) and LTβ-R-Fc (2 and 10 μg/ml) were supplemented in culture media of some wells to evaluate their effects on cell growth. Triplicate culture wells were set up for each treatment. At each time point, 100 μl of a 5 mg/ml MTT solution was added to each well and incubated at 37 °C for 4 h. The plates were placed in TH-4 swinging bucket holders and centrifuged at 1000 rpm for 5 min in a Beckman centrifuge. The supernatant media were removed. Then 700 μl of DMSO were added to each well and incubated for a further 15 min on a rotator. The plates were centrifuged again, and 650 μl of supernatant of each well was transferred to a cuvette and optical density at 570 nm was measured. The growth curve over a period of 72 h was plotted.

RT-PCR—Cells were washed three times with PBS followed by cell lysis with TRizol Reagent (Invitrogen). Total RNA was purified following the instructions of the manufacturer by phase separation and precipitation with chloroform and isopropyl alcohol, respectively. After washing once with 75% ethanol and brief drying, RNA was dissolved in RNase-free water. RT was performed using Invitrogen SuperScript II RT-PCR kit, and 5 μl of total RNA was reverse-transcribed into cDNA with oligo(dT) as primer. One microliter of the cDNA mix and specific primers (for human LTβ-R, sense 5′-CTACCTGAC- CATTGCCAGCTGTG-3′ and antisense 5′-CAGGGGAAG- TATGATGGCCTTCG-3′; for human LTα, primer 1 sense 5′-CCACCCCTACACCTCCTCCT-3′, primer 2 sense 5′-C TAAAACCTGCTGCTACCT-3′, and antisense 5′-CAGG AG-GCTCAGAAGACACAGTACT-3′; for human LTβ, sense 5′-GACTGGGTTCAGACACGTG-3′ and antisense 5′-TC AGAACGCCTGTTCCCT-3′) were used for further PCR amplification of human LTβ-R, LTα and LTβ with TaqDNA polymerase (Sigma) at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1:1000 dilution. The antibodies were visualized with either horseradish peroxidase-conjugated goat antimouse or anti-rabbit IgG (Roche Diagnostics) using enhanced chemiluminescence (Pierce).

Immunohistochemistry and Immunofluorescence—Paraffin-fixed tissue samples were obtained from melanoma and normal tissues of patients using protocols approved by the Vanderbilt University Institutional Review Board. All melanocytic lesion diagnoses on tissues used in these studies were made independently by dermatopathologists who were not involved in the study. Criteria for the diagnosis of dysplastic nevi were those adopted by the 1992 NIH Consensus Conference. This diagnosis requires both (a) architectural disorder and (b) melanocytic atypia, which is classified as mild, moderate, or severe. Immunohistochemistry and immunofluorescence was performed using standard protocol (21), then immunostained for LTβ-R with goat-anti LTβ-R (1:50, Santa Cruz, Temecula, CA). All primary antibody incubations were performed in a moisture chamber overnight at 4 °C. Secondary antibodies were donkey anti-rabbit Texas Red and anti-goat Alexa 488. The slides were fixed with Vectashield and sealed with clear nail polish. Stained sections were viewed and photographed using a fluorescence microscope. The excitation wavelength was 549 nm for Texas Red and 488 nm for Alexa 488.

MTT Assay—Hs294T and SKMel28 cells were seeded at 3 × 10^5 cells/well in 24-well plates and cultured in 1 ml of DMEM/F-12, 0.2% FBS. LTβ-R agonist antibody (2 and 10 μg/ml) and LTβ-R-Fc (2 and 10 μg/ml) were supplemented in culture media of some wells to evaluate their effects on cell growth. Triplicate culture wells were set up for each treatment. At each time point, 100 μl of a 5 mg/ml MTT solution was added to each well and incubated at 37 °C for 4 h. The plates were placed in TH-4 swinging bucket holders and centrifuged at 1000 rpm for 5 min in a Beckman centrifuge. The supernatant media were removed. Then 700 μl of DMSO were added to each well and incubated for a further 15 min on a rotator. The plates were centrifuged again, and 650 μl of supernatant of each well was transferred to a cuvette and optical density at 570 nm was measured. The growth curve over a period of 72 h was plotted.

Immunoblot Analysis—Whole cell extracts were obtained according to our standard protocol and probed with appropriate antibodies as described previously (20). The LTβ-R antibody was used at a 1:1000 dilution. The antibodies were visualized with either horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Roche Diagnostics) using enhanced chemiluminescence (Pierce).

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LTβ-R Gene Knockdown by shRNA—Human melanoma cell line Hs294T was cultured in DMEM/F-12 containing 10% FBS. Lentiviral shRNAmir vector pGIPZ with either targeting sequence for knocking-down human LTβ-R (clone ID: V2LHS_134097) or non-silencing control sequence was obtained from Vanderbilt Microarray Shared Resource and transfected into Hs294T cells with FuGENE 6 transfection reagent following the manufacturer's instruction. After 48 h cells were cultured in 0.5 mg/ml puromycin-containing media for selecting pGIPZ vector-expressing cells. By gradually increasing the concentration of puromycin to 3 mg/ml and after 3–4 weeks of culture, vector-expressing cells were collected and subjected to ELISA assay for LIGHT expression. For each cell line, three samples were collected from triplicate wells, and LIGHT concentrations were indicated as the mean ± S.E.

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Increased LTβ-R expression in melanoma cell lines. A, i, NHEM and melanoma cells were cultured in the absence of serum overnight, and the next morning whole cell extracts were prepared for immunoblotting. An increased expression of LTβ-R was observed in Hs294T, SKMel5, WM115, and SKMel28 melanoma cell lines as compared with normal NHEM cells. The same blots were re-probed with β-actin for normalization. ii, the bands of LTβ-R and β-actin were scanned, and the band density was analyzed by imaging software, FluorChem 8900, and normalized by the expression of β-actin in each cell line. The figure shows the mean ± S.E. of three experiments.

B, i, total RNA from melanoma cell lines were obtained and subjected to semi-quantitative RT-PCR for LTβ-R expression. ii, the bands of LTβ-R PCR product were scanned, and the band density was analyzed by imaging software, FluorChem 8900, and normalized by the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each cell line. The figure shows the mean ± S.E. of three experiments.
swabs, and the cells that had traversed to the reverse side of the inserts were rinsed with PBS, fixed in 4% formaldehyde for 30 min at room temperature, and stained with 1% crystal violet overnight at room temperature. Cells were counted under a light microscope (at 200× power), and invasive cell numbers were the averages of those from five areas on each slide. Each slide was evaluated independently by two researchers who were blinded to the pathology report.

### TABLE 1

**Immunostaining of normal and melanoma patients for LTβ-R**

Four levels of LTβ-R expression in tissue slides were documented based on the following criteria: score 0, no positive staining of any tumor cells on the slide; score 1, low positive staining on most of the tumor cells on the slide; score 2, moderate staining on most tumor cells; score 3, strong staining on most of the tumor cells on the slide. When there was heterogeneity on the staining intensity of different cell populations on the slide, the score in between the 0, 1, 2, or 3 reflects the average score across this population of cells. Each slide was evaluated independently by two researchers who were blinded to the pathology report.

| Patient no. | Histological diagnosis       | LTβ-R staining |
|-------------|-----------------------------|----------------|
| 1           | Normal nevus                | 1              |
| 2           | Normal nevus                | 1              |
| 3           | Normal nevus                | 2              |
| 4           | Normal nevus                | 1              |
| 5           | Normal nevus                | 1.75           |
| 6           | Normal nevus                | 1              |
| 7           | Normal nevus                | 2.5            |
| 8           | Normal nevus                | 2.75           |
| 9           | Compound nevus              | 1.5            |
| 10          | Compound nevus              | 1.5            |
| 11          | Compound nevus              | 2              |
| 12          | Intradermal nevus           | 1.75           |
| 13          | Intradermal nevus           | 1.75           |
| 14          | Dysplastic nevus            | 2              |
| 15          | Dysplastic nevus            | 2.5            |
| 16          | Severe dysplastic nevus     | 1.75           |
| 17          | Primary melanoma            | 1              |
| 18          | Primary melanoma            | 1.5            |
| 19          | Primary melanoma            | 1              |
| 20          | Primary melanoma            | 0.25           |
| 21          | Primary melanoma            | 1.25           |
| 22          | Metastatic melanoma         | 2.5            |
| 23          | Metastatic melanoma         | 3.0            |
| 24          | Metastatic melanoma         | 3.0            |
| 25          | Metastatic melanoma         | 3.0            |
| 26          | Metastatic melanoma         | 3.0            |

### RESULTS

**Lymphotoxin-β Receptor Expression Is Up-regulated in Melanoma Cells**—To examine the possibility that NF-κB activation in melanoma is linked to the expression of LTβ-R in melanoma cells, we looked for a correlation between the LTβ-R and NF-κB activation. Four different melanoma cell lines and normal control NHEMs were studied. Cells were deprived of serum overnight, then expression of LTβ-R was assayed by immunoblotting cell lysates using LTβ-R-specific antibody (Fig. 1A). Interestingly, the four melanoma cell lines, Hs294T, SKMel-5, WM115, and SKMel-28, exhibit high LTβ-R expression compared with NHEM cells. To further test whether the increase of LTβ-R expression is at the mRNA expression level, we used semi quantitative RT-PCR. Interestingly, in correlation with the protein levels based upon Western blot analysis, the RNA levels of LTβ-R were much higher in Hs294T and SKMel5 but moderate in WM115 and SKMel28 and not detected in NHEMs (Fig. 1B). These data suggest that melanoma cells have higher LTβ-R expression, which could potentially lead to activation of downstream pathways. Our prior studies have demonstrated that WM115, SKMel28, Hs294T, and SKMel5 cells exhibit moderate to high constitutive IKK activ-
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FIGURE 3. A, LTβ-R activates NF-κB activation in melanoma cells. The SKMel 28 cells were cotransfected with NF-κB luciferase reporter construct and the respiratory syncytial virus-β-galactosidase expression construct and either left untreated or treated with agonistic LTβ-R antibody (Ab) for different time points (12, 24, and 36 h). Cells were then harvested, and luciferase and β-galactosidase activity were measured. The relative luciferase activity (RLU) represents the luciferase activity of the sample that was normalized by β-galactosidase activity from three different experiments. The results are reported as the mean ± S.D. of induction (*, P = 0.05; **, P = 0.01). B, LTβ-R signaling stimulates cell proliferation. Cells were incubated in culture medium containing 0.2% FBS overnight and then incubated with or without 2 or 10 μg of agonistic LTβ-R antibody for 3 days before the MTT proliferation assay was performed as described under "Materials and Methods" (n = 0.002). C, the Hs294T cells were co-transfected with NF-κB luciferase reporter construct and the respiratory syncytial virus-β-galactosidase expression construct and either left untreated or treated with agonistic LTβ-RFc chimera for different time points (0, 12, and 24 h). Cells were then harvested, and luciferase and β-galactosidase activity were measured. The mean ± S.D. of luciferase activity is shown after normalization with β-galactosidase activity for each sample. D, effects of LTβ-RFc chimera on melanoma cell proliferation. As described in Fig. 3B, the cells were incubated in culture medium containing 0.2% FBS overnight and then incubated with 10 μg/ml concentrations of either LTβ-RFc chimera or control TNF receptor 1:Fc chimera for 3 days. MTT assay was performed to determine cell proliferation. Cells treated with 10 μg/ml isotropic IgG were used as a control. The mean ± S.D. of A570 nm of each treatment is shown.

NF-κB Activity Is Induced on Activation of LTβ-R—We have previously demonstrated that NIK is involved in the up-regulation of constitutive IKK kinase activity in melanoma cells including Hs294T, SKMel5, and WM115 cell lines. In contrast, SKMel28 cells have lower constitutive IKK activity (22). To examine whether activation of LTβ-R signaling can induce NF-κB activity, we treated SKMel28 melanoma cells with LTβ-R agonist antibody, and NF-κB promoter luciferase assays were performed. A significant increase in NF-κB luciferase activity was observed 24 h after treatment with agonist, thus demonstrating that LTβ-R can activate the NF-κB signaling pathway (likely through activation of NIK) in melanoma cells (Fig. 3A).

LTβ-R NIK-NF-κB Pathway Induces Cell Proliferation—LTβ-R has been linked to cellular transformation, proliferation, and apoptosis. To test whether this activated LTβ-R-NIK pathway is involved in functions of NF-κB in melanoma cells, we examined the effect of activation or inhibition of LTβ-R signaling pathway on cell proliferation. For these experiments we used Hs294T cells and treated them with the agonistic LTβ-R-specific monoclonal antibody (2 or 10 μg). Cell proliferation was assessed using the MTT assay (Fig. 3B). We observed an increase in cell proliferation with the agonist antibody as compared with untreated Hs294T cells, confirming that the LTβ-R signaling pathway may be associated with enhanced viability/growth of human melanoma cells.

Activation of the LTβ-R in Melanoma Appears to Be Ligand-independent—LIGHT as well as LTα1β2 are known ligands of LTβ-R. However, only LIGHT has been reported to be expressed by melanoma cells. To determine whether the melanoma cells studied here secrete LIGHT, we performed an
ELISA assay for LIGHT in a number of melanoma cell lines. Even though LIGHT was detected in the conditioned medium of HEK293 cells stably transfected with LIGHT expression vector, we could not detect LIGHT protein in the culture medium, in cell lysates, or on the cell surface of melanoma cells by FACS (data not shown). Thus, it appears that LIGHT might not be the endogenous ligand that constitutively activates the LTβ-R and NIK in melanoma cells in culture. Moreover, we were unable to detect LTβ expression by RT-PCR. We were also unable to detect LTα1/β2 by FACS analysis of the melanoma cells that exhibit the constitutive activation of NIK (data not shown).

We further performed experiments to determine whether melanoma cells produce a ligand for the LTβ-R by treating cells with a soluble receptor chimera that has the ability to bind ligands for the LTβ-R and prevent their activation of the receptor (23) (Fig. 3, C and D). Our experiments showed that the chimera had no effect on the NF-κB luciferase activity or cell growth of Hs294T cells, arguing against autocrine activation of the receptor through a secreted or membrane-bound ligand. Altogether, these data suggest that the level of expression of the receptor in Hs294T melanoma cells is sufficient to result in constitutive activation of the receptor downstream signaling pathways.

Inhibition of LTβ-R Expression Inhibits NF-κB Promoter Activity—To determine whether LTβ-R protein expression has a causal role in NF-κB activation, we stably inhibited the expression levels of LTβ-R expression in a Hs294T cells using small interfering RNA (lentiviral-shRNA vector) based inhibition of gene expression. Inhibition of LTβ-R expression was confirmed in a polyclonal population of Hs294T cells at the RNA and protein level by RT-PCR and Western blotting (Fig. 4A, i). Non-silencing shRNA vector was used as a control, and comparisons were made to a polyclonal population of Hs294T cells expressing.

![Image](https://example.com/image.png)

**FIGURE 4.** Inhibition of LTβ-R expression decreases NF-κB promoter activity in Hs294T cells. All the experiments were performed at least three times with reproducible results. A, i, inhibition of human (h) LTβ-R mRNA and protein expression levels in LTβ-R-knockdown Hs294T cells (Hs294T/V2L) as compared with control Hs294T cells (Hs294T/non-silencing) by RT-PCR and Western blot. Expression of β-actin in the cell samples was used as a control. ii, cell surface expression of LTβ-R on these two cell lines was compared by FACS analysis. Identical signal pattern from phycoerythrin-IgG control was detected from both Hs294T/V2L and Hs294T/non-silencing cells. Therefore, only one representative IgG control curve is shown in the graph (dotted line). iii, to
the non-silencing shRNA. The down-regulated expression of LTβ-R on the cell surface of the Hs294T/V2L cell line with LTβ-R knock down was also confirmed by FACS analysis (Fig. 4 A, ii). NF-κB luciferase activity was then determined in non-silencing vector stably transfected control cells or LTβ-R knockdown cells (Fig. 4 A, iii). As we would expect, blocking the LTβ-R pathway resulted in a reduction in NF-κB activity as compared with control cells, suggesting NF-κB activity is increased by LTβ-R pathway in melanoma cells.

This result was further confirmed by treatment with LTβ-R agonist antibody (2 μg/ml), which induced NF-κB luciferase activity for 0–36 h post-stimulation in Hs294T/non-silencing cells but not in Hs294T/V2L LTβ-R shRNA cells (Fig. 4, B and C). Treatment with 10 ng/ml TNF-α was used as positive control, and the induction was similar in Hs294T/non-silencing cells and Hs294T/V2L LTβ-R shRNA cells. To confirm these results, the same lentiviral-shRNA vectors were used to establish stable LTβ-R knockdown cell line (MeWo/V2L) and control cell line (MeWo/non-sil) (Figs. 4, D and E), then NF-κB luciferase activity was determined. Similar results as Hs294T cells further confirmed that NF-κB activation is inhibited in LTβ-R-deficient MeWo cells.

We have previously reported that melanoma cells expressed very high levels of CXC chemokines (22). Here, we show that loss of expression of the LTβ-R results in a decline in VCAM and CXCL12 mRNA expression based upon real time PCR (Fig. 4F). Because VCAM is transcriptionally regulated through the canonical NF-κB pathway and CXCL12/SDF1 is regulated through the non-canonical NF-κB pathway (32), these data suggest that in melanoma cells knocking down LTβ-R expression suppresses the constitutive activation of both pathways to NF-κB activation.

We further investigated whether LTβ-R expression is critical for constitutive proliferation of melanoma cells. Ten thousand Hs294T melanoma cells from LTβ-R knockdown cell lines, Hs294T/V2L or MeWo/V2L, as well as their control cell lines, Hs294T/non-sil or MeWo/non-sil, were seeded in 6-well tissue culture plates and incubated in DMEM/F-12, 1% FBS for 3 days. The number of cells in each well was determined by FACS analysis. As shown in Fig. 5A, the growth over the 3-day time frame of Hs294T/V2L or MeWo/V2L cells was significantly decreased (45 or 33%) as compared with their respective controls. These results indicate that loss of LTβ-R expression with its associated signaling has a negative impact on constitutive proliferation of melanoma cells.

To further demonstrate a causal link between receptor expression with malignancy, we tested whether inhibition of lymphotoxin-β expression has a causal role for the invasiveness of Hs294T melanoma cells. In Hs294T cells, knocking down the expression of the LTβ-R with an shRNA inhibited cell invasion by 67% as compared with the nonspecific shRNA (Fig. 5B). To explore the process by which these LTβ-R knock-down cells became less invasive, the secretion and activation of MMP-2 and MMP-9 in conditioned media was examined by gelatin zymography. Our results demonstrate that the concentration of latent MMP-9 in conditioned media from Hs294T/V2L and Hs294T/non-silencing cells was substantially decreased and almost undetectable as compared with Hs294T parental cells (Fig. 5C). Additionally, the concentration of latent MMP-2 was also decreased in Hs294T/V2L and Hs294T/non-silencing cells as compared with Hs294T parental cells and HT1080 control cells. Taken together, the above data suggest that the inhibition of lymphotoxin-β receptor expression contributes to the invasive effects of these cells.

**DISCUSSION**

NIK seems to be capable of activating both the alternative and the canonical NF-κB activation pathways; however, this participation is inducer-specific (5, 24). Thus, NIK might function in a way that is restricted by participating in one of the mechanisms of signaling by a receptor but not in others, thus contributing to the function of this receptor only in particular cell types and differentiation states. We have previously demonstrated a constitutive up-regulation of NIK expression and an association of NIK with IKK in melanoma cell lines, which is associated with high levels of NF-κB activity and enhanced CXCL1 expression in these cells (20).

NIK becomes activated upon phosphorylation of Thr-559 within its kinase domain. Subsequently, NIK phosphorylates IKKα/IKKβ, which in turn phosphorylates IkB, resulting in IkB ubiquitination and degradation followed by NF-κB (RelA/p50) activation. NIK also activates the non-canonical NF-κB pathway by phosphorylating IKKα, which subsequently phosphorylates p100. Phosphorylated p100 is ubiquitinated and degraded to p52, which binds RelB, and translocates into the nucleus to activate gene expression. The subcellular distribution of NIK to different compartments has been postulated to be an important means of regulating the function of this kinase. Moreover, nuclear NIK is capable of activating NF-κB, whereas this effect is diminished by nucleolar localization (26). Although NIK is known to shuttle between the cytosol and nucleus, in breast cancer cells endogenous NIK localized primarily to the nucleus, emphasizing that nuclear NIK might have important functional roles and might be causally contributing to the malignancy (26).
Because dominant negative NIK completely blocked constitutive NF-κB activity in melanoma cells (20), we chose to determine the molecule upstream of NIK responsible for its activation. We studied LTβ-R as a probable upstream modulator of NIK in melanoma cells since gene knock out studies have shown that NIK is indispensable for NF-κB activation in the LTβ-R signaling, although the TNF-α- and interleukin-1β-mediated NF-κB activation signaling remains intact in NIK-deficient cells. Furthermore, mice lacking NIK display a phenotype that was similar to LTβ-R-deficient mice (10). To test the involvement of LTβ-R as the upstream activator of NIK and, thus, NF-κB, we investigated the LTβ-R expression in different cell lines and tissues. Interestingly, we observed a correlation between constitutive IKK activity and LTβ-R expression in melanoma cell lines. LTβ-R expression is much higher in the three cell lines exhibiting higher constitutive IKK activity. In contrast, NHEM and melanoma cell lines such as SKMel-28 had low LTβ-R expression and lower constitutive IKK activity (22). We have previously reported that SkMel-28 cells exhibit a lower IKK kinase activity and about a 50% reduction in phosphorylation of NIK as compared with Hs294T cells (22). Although there is a 3-fold reduction in the CXCL1 luciferase activity in SK-Mel 28 cells as compared with Hs294T, this level of reduction in the NF-κB luciferase reporter activity between the two cell lines was not as apparent, suggesting that other effectors such as reduced nuclear shuttling of the activated NF-κB might result in the maintenance of NF-κB in SK-Mel 28 cells despite reduced IKK kinase activity and reduced phosphorylation. This possibility is supported by the finding of nearly equal levels of p65 in the nucleus of SK-Mel 28 and Hs294T cells (22). Taken together it appears that LTβ-R contributes to NF-κB activation in melanoma cells and tumors. Other studies have also demonstrated that LTβ-R can indirectly activate NF-κB by inducing phosphorylation of p65/RelA at Ser-536, which is mediated by NIK-IKKα (27).

To determine whether the LTβ-R is an upstream modulator in melanoma cells, we treated Hs294T melanoma cells with the agonistic LTβ-R antibody to induce this pathway. Interestingly, we saw an increased cell growth in response to this antibody activation of the LTβ-R signaling. Because the LTβ-R agonist antibody could activate NF-κB promoter activity in SKMel28 cells with lower basal IKK activity, we speculate that the increased cell proliferation induced by LTβ-R signaling is mediated by an increase in NIK-NF-κB activity in melanoma cells. Furthermore, we demonstrated here that activation of LTβ-R as a probable upstream modulator of NIK-dependent luciferase activity (p ≤ 0.05) (20), these data suggest that the LTβ-R acts through NIK to stimulate constitutive NF-κB activity in melanoma. Furthermore, we demonstrated here that activation of LTβ-R signaling can induce melanoma cell growth, suggesting that the LTβ-R-NIK-NF-κB pathway may be associated with enhanced viability/growth of human melanoma cells. Therefore, one would expect that dominant negative NIK or NIK knockdown will inhibit the growth of melanoma.
tumors. We have previously reported that suppression of NF-κB with small molecule inhibitors of IKK will inhibit proliferation of melanoma cells in vitro and in vivo (4). Moreover, ribozyme knockdown of IKKa or IKKβ inhibits the growth of melanoma tumors in vivo (28).

In this report we show that an upstream activator of NIK, the LTβ-R, is expressed in metastatic melanoma cell lines and lesions, and this receptor contributes to the constitutive NF-κB activity of melanoma cells. LIGHT as well as LTαβ2 are known ligands for the LTβ-R. A recent study by Mortarini et al. (29) demonstrated that LIGHT/TNFSF14 is constitutively expressed in human melanoma cells. We postulated that an autocrine LTβ-R signaling feedback loop could potentially cause melanoma cells to over-activate the NIK-NF-κB pathway, which would then result in the increased expression of the CXCL1 and CXCL8 chemokines. To evaluate the production of LIGHT by the melanoma cells studied here, we performed an ELISA for LIGHT on melanoma cell extracts and melanoma-conditioned medium. However, we did not detect LIGHT in melanoma cells or their conditioned medium, suggesting LIGHT might not be the endogenous ligand for the LTβ-R. Furthermore, we were unable to detect LTαβ2 expression by RT-PCR or FACS analysis. In addition, we treated cells with a soluble receptor chimera that has the ability to bind ligands for the LTβ-R and prevent their activation of the receptor (23). These experiments showed that the chimera had no effect on the NF-κB luciferase activity or cell growth of Hs294T cells. Combined together, our data argue against endogenous activation of the receptor through an autocrine loop and suggest that the NF-κB signaling targets NIK in melanoma cells (20, 21). How-

ever, the possibility of cross-talk among them remains to be determined. Recently it was shown that treatment of cancer cell lines with TNF-α decreases PTEN expression. In addition, overexpression of TNF-α downstream signaling targets NIK and p65 nuclear factor NF-κB lowers PTEN expression, suggesting that TNF-α-induced down-regulation of PTEN is mediated through a TNF-α/NIK/NF-κB pathway (34). Down-regulation of PTEN by NIK/NF-κB results in enhanced activation of the phosphatidylinositol 3-kinase protein-NIK pathway and augmentation of TNF-α-induced phosphatidylinositol 3-kinase/Akt stimulation. Of note, we and others have observed a decreased expression of PTEN in metastatic melanoma (25, 35). It is likely that activation of LTβ-R-NIK-NF-κB signaling can lead to down-regulation of PTEN and, thus, activation of phosphatidylinositol 3-kinase/Akt signaling in metastatic melanomas. A clear understanding of the molecular mechanisms involved in the constitutive NF-κB activation in tumor cells will allow the targeting of critical components and new opportunities for therapeutic intervention.

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