

# The Lymphotoxin- $\beta$ Receptor Is an Upstream Activator of NF- $\kappa$ B-mediated Transcription in Melanoma Cells\*

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Punita Dhawan<sup>†§1</sup>, Yingjun Su<sup>†1</sup>, Yee Mon Thu<sup>‡</sup>, Yingchun Yu<sup>‡</sup>, Paige Baugher<sup>‡</sup>, Darrel L. Ellis<sup>¶</sup>, Tammy Sobolik-Delmaire<sup>‡</sup>, Mark Kelley<sup>§</sup>, Timothy C. Cheung<sup>||</sup>, Carl F. Ware<sup>||</sup>, and Ann Richmond<sup>†\*\*\*2</sup>

From the \*\*Department of Veterans Affairs, Nashville, Tennessee 37212, †Department of Cancer Biology, ‡Surgical Oncology Research Laboratories, Department of Surgery, and ¶Division of Dermatology, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee 37232, and ||Division of Molecular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, California 92121

The pleiotropic transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B (p50/p65)) 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- $\kappa$ B (Basseres, D. S., and Baldwin, A. S. (2006) 25, 6817–6830). However, the mechanism for this constitutive activation of NF- $\kappa$ B has not been elucidated in the tumors. We have previously shown that NF- $\kappa$ B-inducing kinase (NIK) protein and its association with Inhibitor of  $\kappa$ B kinase  $\alpha\beta$  are elevated in melanoma cells compared with their normal counterpart, leading to constitutive activation of NF- $\kappa$ B. Moreover, expression of dominant negative NIK blocked this base-line NF- $\kappa$ B activity in melanoma cells. Of the three receptors that require NIK for activation of NF- $\kappa$ B, only the lymphotoxin- $\beta$  receptor (LT $\beta$ -R) is expressed in melanoma. We show in this manuscript that for melanoma there is a strong relationship between expression of the LT $\beta$ -R and constitutive NF- $\kappa$ B transcriptional activity. Moreover, we show that activation of the LT $\beta$ -R can drive NF- $\kappa$ B activity to regulate gene expression that leads to enhanced cell growth. The inhibition by LT $\beta$ -R shRNA resulted in decreased NF- $\kappa$ B promoter activity, decreased growth, and decreased invasiveness as compared with control. These results indicate that the LT $\beta$ -R constitutively induces NF- $\kappa$ B activation, and this event may be associated with autonomous growth of melanoma cells.

The transcription factor NF- $\kappa$ B plays critical roles in diverse physiological processes and numerous human pathologies (1–3). Understanding the mechanisms that regulate NF- $\kappa$ B activity is critical for developing therapeutic strategies for many human diseases (2, 3). We and others have previously demon-

strated that targeting NF- $\kappa$ B with a small molecule inhibitor of I $\kappa$ B kinase- $\beta$  (IKK $\beta$ )<sup>3</sup> inhibits the growth of melanoma cells *in vitro* and *in vivo* (4). However, broad targeting of NF- $\kappa$ B as a therapeutic measure for treatment in the clinic has its drawbacks due to effects on the immune system.

NF- $\kappa$ -inducing kinase (NIK) is an upstream kinase in the NF- $\kappa$ B activation pathway and preferentially phosphorylates IKK $\alpha$  over IKK $\beta$ , leading to the activation of IKK $\alpha$  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- $\kappa$ B 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- $\kappa$ B 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*<sup>-/-</sup>) leads to a phenotype that is reminiscent of lymphotoxin- $\beta$  receptor (LT $\beta$ -R) knock-out mice (11). Indeed, cells from *nik*<sup>-/-</sup> mice showed a normal response to TNF- $\alpha$  or interleukin-1 treatment, whereas NF- $\kappa$ B activation in reaction to LT $\beta$  was impaired. Thus, it was suggested that NIK participates selectively in the activation of NF- $\kappa$ B 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 *lt $\beta$ -R*<sup>-/-</sup> phenotype (12, 13). Recent studies have shown two other ligand/receptor pairs

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<sup>1</sup> These two authors contributed equally to this work.

<sup>2</sup> To whom correspondence should be addressed: Dept. of Cancer Biology, PRB 432, Vanderbilt University School of Medicine, 23rd Ave. S at Pierce, Nashville, TN 37232. Tel.: 615-343-7777; Fax: 615-936-2911; E-mail: ann.richmond@vanderbilt.edu.

<sup>3</sup> The abbreviations used are: IKK, I $\kappa$ B kinase- $\beta$ ; NIK, NF- $\kappa$ B-inducing kinase; LT $\beta$ -R, lymphotoxin- $\beta$  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; NHEM, 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 LT $\beta$ -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 LT $\beta$ -R. The first is lymphotoxin  $\alpha$ 1 $\beta$ 2, a cell bound heterotrimeric complex of LT $\alpha$  and LT $\beta$ . The second cytokine, LIGHT, is a membrane-anchored homotrimeric complex capable of binding LT $\beta$ -R and herpes simplex virus entry mediator HVEM (15). It has been shown that signaling triggered by LT $\beta$ -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 LT $\beta$ -R gene-deleted mice (11, 16). It has been shown that LT $\beta$ -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 LT $\beta$ -R is an upstream modulator of the NF- $\kappa$ B 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- $\beta$  receptor antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). LT $\beta$ -R receptor agonistic antibody was obtained from Jeffrey Browning, Biogen (Cambridge, MA) (36), and the LT $\beta$  receptor-Fc chimera as well as the LIGHT ELISA kit was obtained from R&D Systems (629-LR) (Minneapolis, MN). The NF- $\kappa$ B luciferase reporter vector contains five tandem repeats of the NF- $\kappa$ B 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, SKMel5, SKMel28, and WM115 were obtained from American Type Culture Collection (Manassas, VA). Normal human epidermal melanocytes (NHEMs) established from foreskin were obtained from the Skin Disease Research Center core facility at Vanderbilt University Medical Center. Retinal pigmented epithelial cells and melanoma cells were grown as described previously (21). NHEM cells were cultured in medium 154 supplemented with human melanocyte growth supplement (Cascade Biologics, Portland, OR). One day before transfection, the cells were seeded in 6-well cell culture plates to provide a final density of 40–60% confluence ( $\sim 3 \times 10^5$  cells/well). Cells were transfected using FuGENE 6 transfection reagent (Roche Diagnostics). Fold stimulation was calculated for each sample by dividing the normalized luciferase activity by the value obtained from the control transfection containing empty parental expression vectors (pCMV). The luciferase stimulation assays were performed on cells cultured in low serum (0.2% FBS) or serum-free medium.

**Immunoblot Analysis**—Whole cell extracts were obtained according to our standard protocol and probed with appropriate antibodies as described previously (20). The LT $\beta$ -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).

**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 LT $\beta$ -R with goat-anti LT $\beta$ -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  $3 \times 10^5$  cells/well in 24-well plates and cultured in 1 ml of DMEM/F-12, 0.2% FBS. LT $\beta$ -R-agonist antibody (2 and 10  $\mu$ g/ml) and LT $\beta$ -R-Fc (2 and 10  $\mu$ g/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  $\mu$ l 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  $\mu$ l of DMSO were added to each well and incubated for a further 15 min on a rotator. The plates were centrifuged again, and 650  $\mu$ l 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  $\mu$ l of total RNA was reverse-transcribed into cDNA with oligo(dt) as primer. One microliter of the cDNA mix and specific primers (for human LT $\beta$ -R, sense 5'-CTACCTGAC-CATCTGCCAGCTGTG-3' and antisense 5'-CAGGGAAG-TATGGATGGGCCTTCG-3'; for human LT $\alpha$ , primer 1 sense 5'-CCACCCTACACCTCCTCCTT-3', primer 2 sense 5'-CT-CAAACCTGCTGCTCACCT-3', and antisense 5'-CGAAG-GCTCCAAAGAAGACAGTACT-3'; for human LT $\beta$ , sense 5'-GACTGGGGTTTCAGAAGCTG-3' and antisense 5'-TC-AGAAACGCCTGTTCCCTTC-3') were used for further PCR amplification of human LT $\beta$ -R, LT $\alpha$  and LT $\beta$  with TaqDNA polymerase (Sigma) at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for

45 s for 30 cycles. The products were analyzed on a 2% agarose gel containing ethidium bromide.

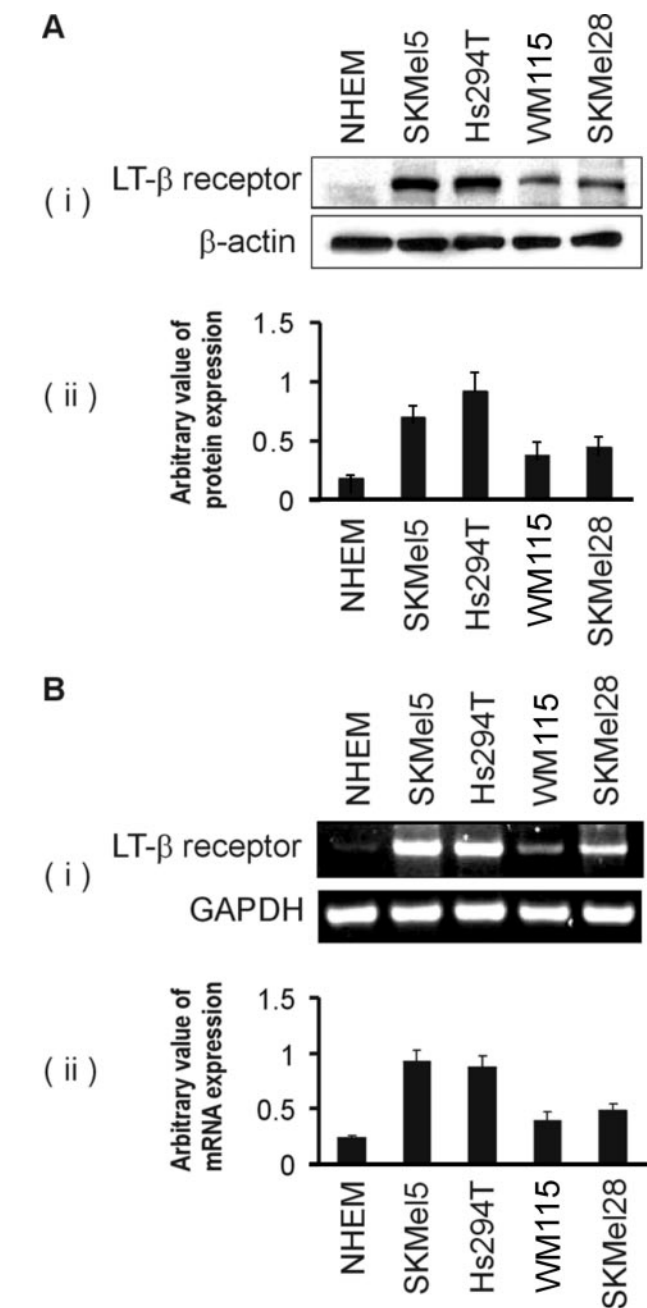
**ELISA for LIGHT**—Human LIGHT expressions in the conditioned media of melanocyte and melanoma cell cultures were assessed by an ELISA kit (R & D Systems). All the procedures were followed according to the kit instructions. Briefly, three melanocyte and three melanoma cell lines were cultured in 12-well plates in DMEM/F-12 media containing 10% FBS overnight. The media were then switched to serum-free media, and cells were cultured overnight. The culture media 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.

**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-encoded GFP expression was observed in all Hs294T cells. Expression of LTβ-R in the above two vector-transfected cells was characterized by RT-PCR, Western blot, and fluorescent-activated cell sorting (FACS) analysis.

**FACS on LTβ-R Expression**—One million melanoma cells were harvested by incubating cells with PBS-based dissociation buffer (Invitrogen) for 15 min. Cells were washed twice with cold PBS, 0.1% BSA and resuspended in 100 μl of PBS, 0.1% BSA containing either 1:50-diluted phycoerythrin (PE)-conjugated mouse anti-human LTβ-R monoclonal antibody (Abcam Inc., Cambridge, MA) or PE-conjugated isotypic IgG control (BD Biosciences Pharmingen). After incubation on ice for 1 h, cells were washed with PBS, 0.1% BSA twice, resuspended in 500 μl of PBS, 0.1% BSA containing 1:1000 diluted 7-aminoactinomycin D (Molecular Probes, Eugene, OR), and incubated for 15 min followed by FACS analysis.

**Real-time PCR**—Total RNA was isolated from the cells using an RNeasy mini kit (Qiagen). One microgram of the total RNA was reverse-transcribed using iScript cDNA synthesis RT-PCR kit (Bio-Rad) according to the user manual. The resulting cDNA was then amplified through quantitative real-time PCR by using iQ Real Time Sybr Green PCR supermix (Bio-Rad) and Bio-Rad IcylerIQ Multicolor Real-time PCR detection system. cDNA levels were normalized to actin amplified with the primers 5'-caccacaccttacaatgag-3' and 5'-atagcagcctggatagc-3'. The primers for VCAM and SDF1 were as published in PMID 127135952 and PMID 17108103, respectively (VCAM, 5'-CATGGAATTCGAACCCAAACA-3', and 5'-GGCTGACCAAGACGGTTGTATC-3'; SDF1, 5'-GATTGTAGCCCCGGCTGAAGA-3', and 5'-TTCGGGTCAATGCACACTTGT-3').

**Invasion Assay**—Transwells (8-μm pore size, 6.5 mm in diameter) from Costar (Cambridge, MA) were coated with 100 μl of 100 μg/ml collagen I and then left in an incubator for 2 h. Cells were trypsinized, washed with PBS, resus-



**FIGURE 1. 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 Western blot films were scanned, and LTβ-R band density was analyzed by imaging software, FluorChem 8900, Alpha Innotech Inc., and normalized by the expression of 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.

pended in 0.2% bovine serum albumin serum-free medium, and then seeded in transwells (100,000 cells per transwell). Cells were allowed to grow on transwells for 72 h. Cells remaining inside the inserts were removed with cotton

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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 $\times$  power), and invasive cell numbers were the averages of those from five areas on each

insert. Each invasion assay was done in triplicate and repeated three times.

**Gelatin Zymography Assay**—Cell cultures were seeded at a density of 150,000 cells in a 6-well dish in DMEM/F-12 media containing serum. The following day the cells were placed in serum-free media, and after 48 h the conditioned medium was collected and analyzed for proteinase activity. Protein concentration was measured by the Bradford assay (Bio-Rad). Activity of gelatinases (MMP-2 and MMP-9) was evaluated by gelatin zymography. Briefly, 15  $\mu$ l of conditioned media or reaction mixture was mixed with 15  $\mu$ l of Laemmli sample buffer without reducing agent. The conditioned media was loaded on 10% SDS-polyacrylamide gels containing 0.1 mg/ml gelatin and run at 100 V for 4 h in non-reducing conditions. After electrophoresis, gels were washed with 2.5% Triton-X-100 twice for 15 min at room temperature. The gels were incubated at 37  $^{\circ}$ C in substrate buffer (50 mM Tris-HCl, pH 7.5, 10 mM CaCl<sub>2</sub>) for 24 h. Gels were stained with 0.5% Coomassie Blue, 50% methanol, 10% acetic acid, and destained in 50% methanol and 10% acetic acid. Proteolytic activities of latent and activated gelatinase were visualized as clear bands against the blue background of stained gelatin.

**Statistical Analysis**—Student's *t* test for paired samples was used to determine statistical significance. Differences were considered statistically significant at  $p \leq 0.05$ .

**TABLE 1**

### Immunostaining of normal and melanoma patients for LT $\beta$ -R

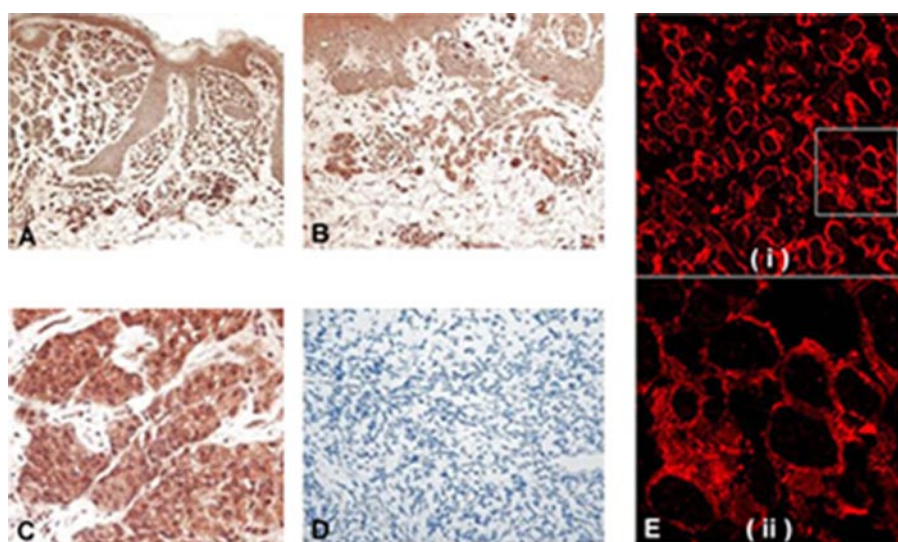
Four levels of LT $\beta$ -R expression in tissue slides were documented based on the following criteria: score 0, no positive staining of any tumor cells on the slide; score 1, low positive staining on most of the tumor cells on the slide; score 2, moderate staining on most tumor cells; score 3, strong 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	LT $\beta$ -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 nevi	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- $\beta$  Receptor Expression Is Up-regulated in Melanoma Cells**—To examine the possibility that NF- $\kappa$ B activation in melanoma is linked to the expression of LT $\beta$ -R in melanoma cells, we looked for a correlation between of the LT $\beta$ -R and NF- $\kappa$ B activation. Four different melanoma cell lines and normal control NHEMs were studied. Cells were deprived of serum overnight, then expression of LT $\beta$ -R was assessed by immunoblotting cell lysates using LT $\beta$ -R-specific antibody (Fig. 1A).

Interestingly, the four melanoma cell lines, Hs294T, SKMel-5, WM115, and SKMel-28, exhibit high LT $\beta$ -R expression compared with NHEM cells. To further test whether the increase of LT $\beta$ -R expression is at the mRNA expression level, we used semiquantitative RT-PCR. Interestingly, in correlation with the protein levels based upon Western blot analysis, the RNA levels of LT $\beta$ -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 LT $\beta$ -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-



**FIGURE 2. Paraffin-embedded sections from biopsy specimens of patients were immunostained with antibody for LT $\beta$ -R expression.** The red color indicates a positive immunolocalization of LT $\beta$ -R expression. The LT $\beta$ -R expression was not very strong in normal nevi (A) or nevi with mild dysplasia (B). Melanomas stain strongly for LT $\beta$ -R expression (C,  $\times 200$ ). The negative control for immunostaining using second antibody alone is shown in D ( $\times 200$ ). E shows confocal immunolocalization of the LT $\beta$ -R on tissue sections from metastatic melanoma; i,  $\times 200$ ; ii, inset showing magnification.

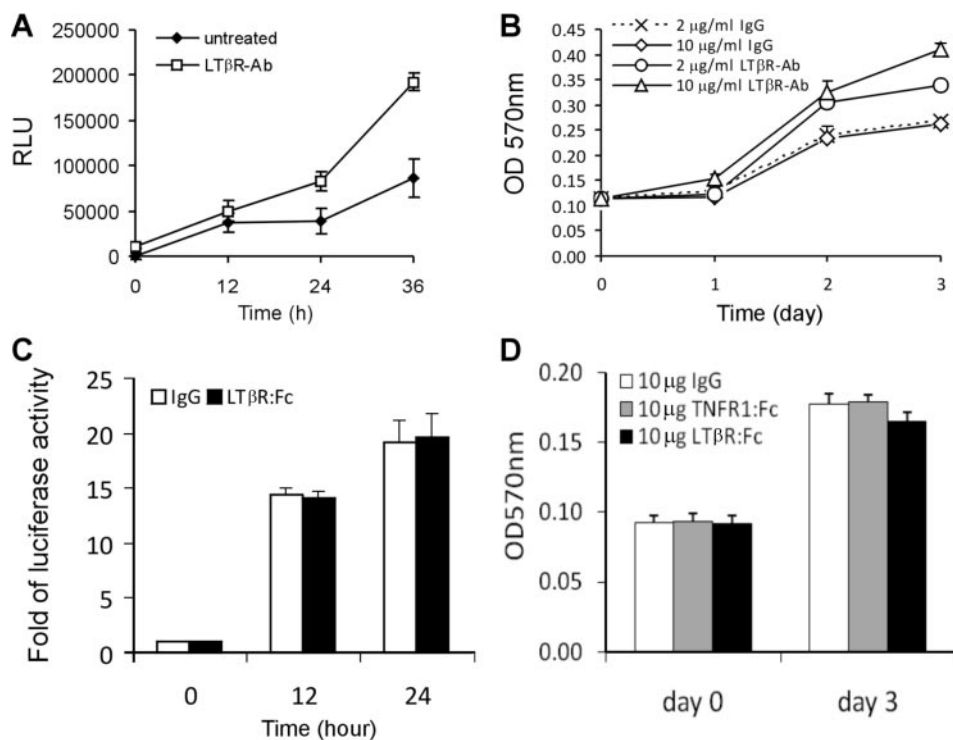


FIGURE 3. A, LT $\beta$ -R activates NF- $\kappa$ B activation in melanoma cells. The SKMel 28 cells were cotransfected with NF- $\kappa$ B luciferase reporter construct and the respiratory syncytial virus- $\beta$ -galactosidase expression construct and either left untreated or treated with agonistic LT $\beta$ -R antibody (Ab) for different time points (12, 24, and 36 h). Cells were then harvested, and luciferase and  $\beta$ -galactosidase activity were measured. The relative luciferase activity (RLU) represents the luciferase activity of the sample that was normalized by  $\beta$ -galactosidase activity from three different experiments. The results are reported as the mean  $\pm$  S.D. of induction (\*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ). B, LT $\beta$ -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  $\mu$ g of agonistic LT $\beta$ -R antibody for 3 days before the MTT proliferation assay was performed as described under "Materials and Methods" ( $p = 0.002$ ). C, the Hs294T cells were co-transfected with NF- $\kappa$ B luciferase reporter construct and the respiratory syncytial virus- $\beta$ -galactosidase expression construct and either left untreated or treated with agonistic LT $\beta$ -R-Fc chimera for different time points (0, 12, and 24 h). Cells were then harvested, and luciferase and  $\beta$ -galactosidase activity were measured. The mean  $\pm$  S.D. of luciferase activity is shown after normalization with  $\beta$ -galactosidase activity for each sample. D, effects of LT $\beta$ -R-Fc 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  $\mu$ g/ml concentrations of either LT $\beta$ -R-Fc chimera or control TNF receptor 1:Fc chimera for 3 days. MTT assay was performed to determine cell proliferation. Cells treated with 10  $\mu$ g/ml isotopic IgG were used as a control. The mean  $\pm$  S.D. of  $A_{570\text{ nm}}$  of each treatment is shown.

ity compared with NHEM cells (22). In addition, the basal DNA binding activity of NF- $\kappa$ B as analyzed by electrophoretic mobility shift assay analysis is constitutively high in most of the melanoma cell lines (Hs294T, SKMel-5, WM115, and WM852) (20) as compared with normal control cells. Thus, it appears that there is a correlation between the increased IKK activity, NF- $\kappa$ B binding activity, and LT $\beta$ -R expression in these four melanoma cell lines.

To further extend our *in vitro* studies to human patient samples, we tested the expression of LT $\beta$ -R in 26 human patient samples, including normal nevi, compound nevi, intradermal nevi, dysplastic nevi, a severely dysplastic nevus, primary melanoma, and metastatic melanoma lesions (Table 1). The paraffin-embedded tumor biopsies of pigmented lesions were immunostained using a LT $\beta$ -R-specific antibody. Four levels of LT $\beta$ -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. In agreement with our cell line data, the LT $\beta$ -R expression was much higher in metastatic melanoma tumor tissues (representative sample) (Fig. 2C) as compared with normal nevi or dysplastic nevi (Fig. 2, A and B). Fig. 2D represents a negative control, and confocal immunolocalization of the LT $\beta$ -R on tissue sections from metastatic melanoma is depicted in Fig. 2E.

**NF- $\kappa$ B Activity Is Induced on Activation of LT $\beta$ -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 LT $\beta$ -R signaling can induce NF- $\kappa$ B activity, we treated SKMel28 melanoma cells with LT $\beta$ -R agonist antibody, and NF- $\kappa$ B promoter luciferase assays were performed. A significant increase in NF- $\kappa$ B luciferase activity was observed 24 h after treatment with agonist, thus demonstrating that LT $\beta$ -R can activate the NF- $\kappa$ B signaling pathway (likely through activation of NIK) in melanoma cells (Fig. 3A).

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**LT $\beta$ -R NIK-NF- $\kappa$ B Pathway Induces Cell Proliferation**—NF- $\kappa$ B has been linked to cellular transformation, proliferation, and apoptosis. To test whether this activated LT $\beta$ -R-NIK pathway is involved in functions of NF- $\kappa$ B in melanoma cells, we examined the effect of activation or inhibition of LT $\beta$ -R signaling pathway on cell proliferation. For these experiments we used Hs294T cells and treated them with the agonistic LT $\beta$ -R-specific monoclonal antibody (2 or 10  $\mu$ g). 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 LT $\beta$ -R signaling pathway may be associated with enhanced viability/growth of human melanoma cells.

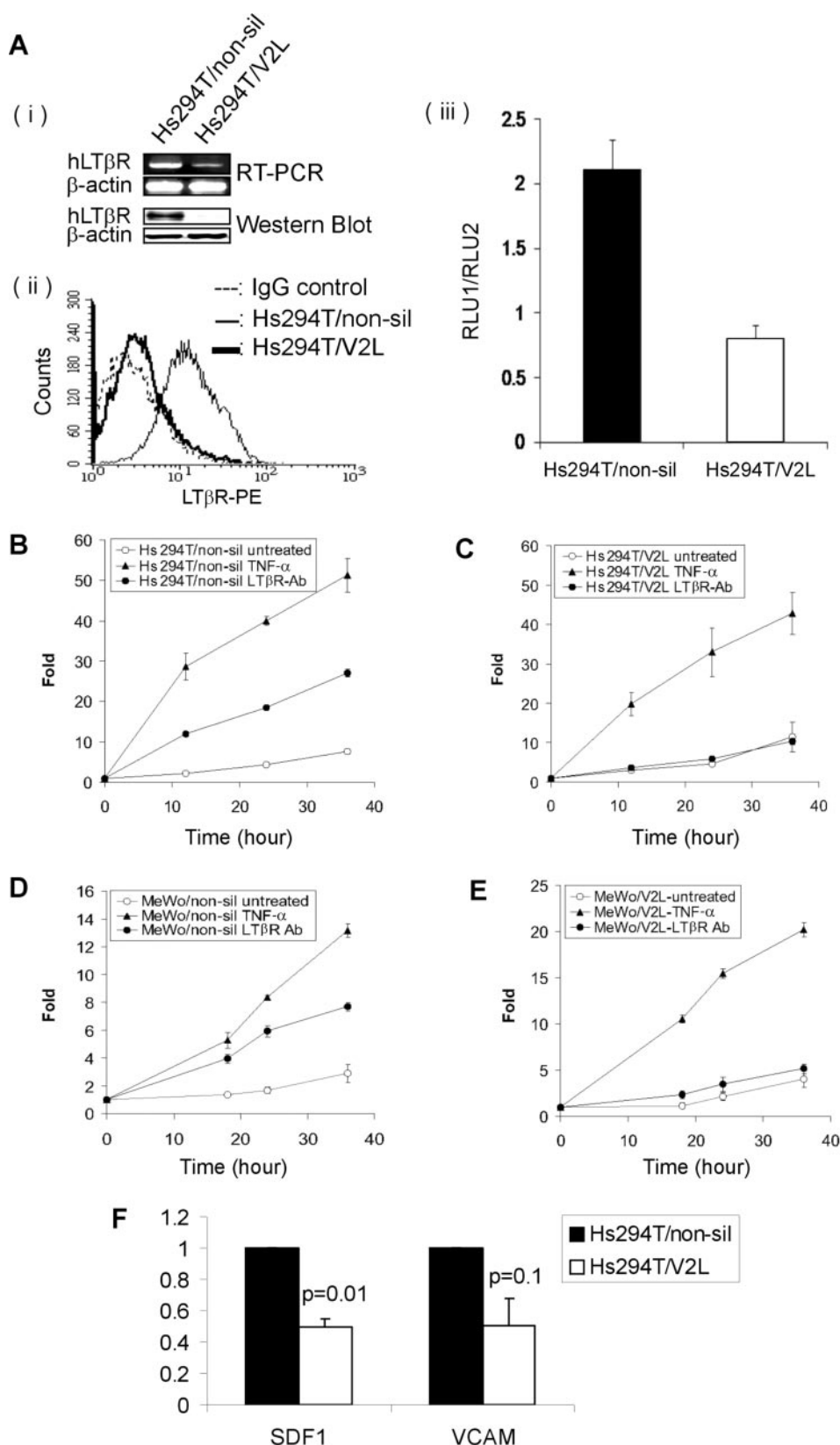
**Activation of the LT $\beta$ -R in Melanoma Appears to Be Ligand-independent**—LIGHT as well as LT $\alpha$ 1 $\beta$ 2 are known ligands of LT $\beta$ -R. However, only LIGHT has been reported to be expressed by melanoma cells. To determine whether the melanoma cells studied here secrete LIGHT, we performed an

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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 LT $\beta$ -R and NIK in melanoma cells in culture. Moreover, we were unable to detect LT $\beta$  expression by RT-PCR. We were also unable to detect LT $\alpha_1/\beta_2$  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 LT $\beta$ -R by treating cells with a soluble receptor chimera that has the ability to bind ligands for the LT $\beta$ -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- $\kappa$ B 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 LT $\beta$ -R Expression Inhibits NF- $\kappa$ B Promoter Activity**—To determine whether LT $\beta$ -R protein expression has a causal role in NF- $\kappa$ B activation, we stably inhibited the expression levels of LT $\beta$ -R expression in a Hs294T cells using small interfering RNA (lenticiral-shRNA vector) based inhibition of gene expression. Inhibition of LT $\beta$ -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).



**FIGURE 4. Inhibition of LT $\beta$ -R expression decreases NF- $\kappa$ B promoter activity in Hs294T cells.** All the experiments were performed at least three times with reproducible results. *A, i*, inhibition of human (*h*) LT $\beta$ -R mRNA and protein expression levels in LT $\beta$ -R-knockdown Hs294T cells (Hs294T/V2L) as compared with control Hs294T cells (Hs294T/non-silencing) by RT-PCR and Western blot. Expression of  $\beta$ -actin in the cell samples was used as a control. *ii*, cell surface expression of LT $\beta$ -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 LT $\beta$ -R on the cell surface of the Hs294T/V2L cell line with LT $\beta$ -R knock down was also confirmed by FACS analysis (Fig. 4 A, *ii*). NF- $\kappa$ B luciferase activity was then determined in non-silencing vector stably transfected control cells or LT $\beta$ -R knockdown cells (Fig. 4 A, *iii*). As we would expect, blocking the LT $\beta$ -R pathway resulted in a reduction in NF- $\kappa$ B activity as compared with control cells, suggesting NF- $\kappa$ B activity is increased by LT $\beta$ -R pathway in melanoma cells.

This result was further confirmed by treatment with LT $\beta$ -R agonist antibody (2  $\mu$ g/ml), which induced NF- $\kappa$ B luciferase activity for 0–36 h post-stimulation in Hs294T/non-silencing cells but not in Hs294T/V2L LT $\beta$ -R shRNA cells (Fig. 4, B and C). Treatment with 10 ng/ml TNF- $\alpha$  was used as positive control, and the induction was similar in Hs294T/non-silencing cells and Hs294T/V2L LT $\beta$ -R shRNA cells. To confirm these results, the same lentiviral-shRNA vectors were used to establish stable LT $\beta$ -R knockdown cell line (MeWo/V2L) and control cell line (MeWo/non-sil) (Figs. 4, D and E), then NF- $\kappa$ B luciferase activity was determined. Similar results as Hs294T cells further confirmed that NF- $\kappa$ B activation is inhibited in LT $\beta$ -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 LT $\beta$ -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- $\kappa$ B pathway and CXCL12/SDF1 is regulated through the non-canonical NF- $\kappa$ B pathway (32), these data suggest that in melanoma cells knocking down LT $\beta$ -R expression suppresses the constitutive activation of both pathways to NF- $\kappa$ B activation.

We further investigated whether LT $\beta$ -R expression is critical for constitutive proliferation of melanoma cells. Ten thousand Hs294T melanoma cells from LT $\beta$ -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 LT $\beta$ -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- $\beta$  expression has a causal role for the invasiveness of Hs294T melanoma cells. In Hs294T cells, knocking down the expression of the LT $\beta$ -R with an shRNA inhibited cell invasion by 67% as compared with the nonspecific shRNA (Fig. 5B). To explore the process by which these LT $\beta$ -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- $\beta$  receptor expression contributes to the invasive effects of these cells.

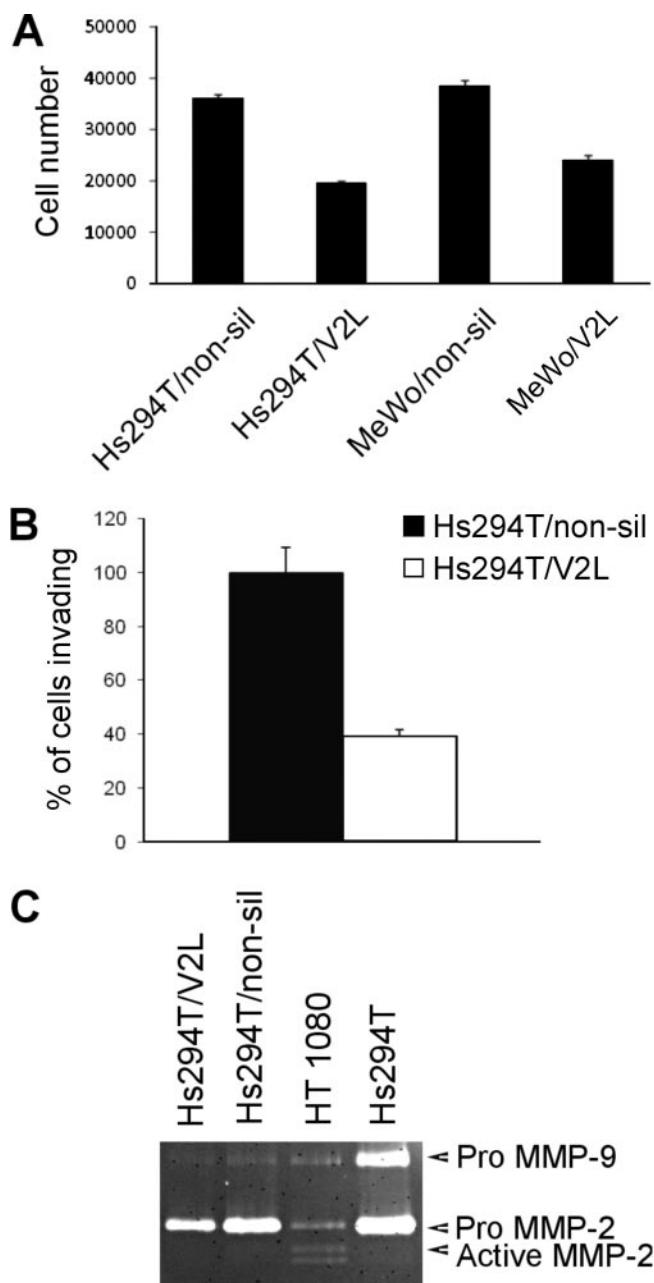
## DISCUSSION

NIK seems to be capable of activating both the alternative and the canonical NF- $\kappa$ B 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- $\kappa$ B activity and enhanced CXCL1 expression in these cells (20).

NIK becomes activated upon phosphorylation of Thr-559 within its kinase domain. Subsequently, NIK phosphorylates IKK $\alpha$ /IKK $\beta$ , which in turn phosphorylates I $\kappa$ B, resulting in I $\kappa$ B ubiquitination and degradation followed by NF- $\kappa$ B (RelA/p50) activation. NIK also activates the non-canonical NF- $\kappa$ B pathway by phosphorylating IKK $\alpha$ , 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- $\kappa$ B, 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).

compare constitutive NF- $\kappa$ B activity in both Hs294T/non-sil and Hs294T/V2L cell lines, a dual luciferase system (Promega) was used for firefly and *Renilla* luciferase assays. Specifically,  $1 \times 10^5$  cells of each cell line were co-transfected with 0.9 mg/ml NF- $\kappa$ B-luciferase and 0.6 mg/ml *Renilla*-TK-luciferase plasmid DNA using FuGENE 6 transfection reagent (Roche Diagnostics). The NF- $\kappa$ B luciferase activity (*RLU1*) was normalized by *Renilla*-TK-luciferase activity (*RLU2*) and mean  $\pm$  S.E. of activities were shown. The experiments were repeated 3 times, and about 62% decrease in NF- $\kappa$ B luciferase activity was found in Hs294T/V2L cells relative to Hs294T/non-silencing cells. B and C, LT $\beta$ -R agonist antibody (2  $\mu$ g/ml)-induced NF- $\kappa$ B luciferase activity for 0–36 h post-stimulation in Hs294T/non-silencing cells (B) and Hs294T/V2L LT $\beta$ -R shRNA cells (C). Treatment with 10 ng/ml TNF- $\alpha$  and 2  $\mu$ g/ml isotopic IgG were used as positive and negative controls, respectively. A representative graph is shown in which the mean  $\pm$  S.E. of activity values from triplicates is indicated. LT $\beta$ -R agonist antibody (Ab) (2  $\mu$ g/ml)-induced NF- $\kappa$ B luciferase activity for 0–36 h post-stimulation in MeWo/non-silencing cells (D) and LT $\beta$ -R knockdown cell line, MeWo/V2L (E). Treatments with TNF $\alpha$  (10 ng/ml) and isotopic IgG (2  $\mu$ g/ml) were included as positive or negative controls, respectively. The experiments were performed three times, and a representative graph is shown in which the mean  $\pm$  S.E. of activity values from triplicates is indicated. F, NF- $\kappa$ B targeted genes SDF1 and VCAM transcripts were diminished in Hs294T with LT $\beta$ -R knock down (V2L) compared with the non-silencing vector control (*sil*). Quantitative real-time PCR was performed using the cDNA synthesized from the total RNA of the cells. Actin levels were used to normalize the cDNA level in each sample. This experiment was repeated three times.

## LT $\beta$ -R Modulates Melanoma Growth and Metastasis through NF- $\kappa$ B



**FIGURE 5.** A, effects of LT $\beta$ -R on the constitutive proliferation of melanoma cells. Hs294T/non-silencing, Hs294T/V2L, MeWo/non-silencing, or MeWo/V2L cells were seeded in 6-well tissue culture plates at density of  $1 \times 10^5$  cells/well and incubated for 3 days in 4 ml/well of DMEM/F-12, 1% FBS. After trypsinization, cells from each well were washed twice with PBS, 1% BSA and resuspended in 1 ml of PBS, 1%BSA containing 1:1000 diluted 7-aminoactinomycin D and incubated for 10 min. Cell suspension samples were pipetted followed by immediate counting of cell number for 30 s. Triplicates were set up for each cell line, and the experiments were repeated three times. The mean  $\pm$  S.E. of cell number of triplicates are indicated. B, Hs294T/non-silencing, Hs294T/V2L cells were plated in collagen I-coated transwells (100,000 cells/transwell) and allowed to grow for 3 days. Invasive cell numbers were scored as described under "Materials and Methods," and the results are presented as -fold change to Hs294T/V2L cells. The data shown are the mean -fold change  $\pm$  S.D. C, gelatin zymography of conditioned media from melanoma cell lines. Cells were cultured in 6-well plates in DMEM/F-12 media containing 10% FBS. The following day the cells were placed in serum-free media for 48 h. The conditioned media was collected and run on a 10% SDS-PAGE gel (containing 0.1% gelatin). Conditioned media from HT1080 cells is shown as a control, and bands corresponding to MMP-2 and MMP-9 are indicated with arrows. For each cell line three samples were collected from triplicate wells with a representative group shown here.

Because dominant negative NIK completely blocked constitutive NF- $\kappa$ B activity in melanoma cells (20), we chose to determine the molecule upstream of NIK responsible for its activation. We studied LT $\beta$ -R as a probable upstream modulator of NIK in melanoma cells since gene knock out studies have shown that NIK is indispensable for NF- $\kappa$ B activation in the LT $\beta$ -R signaling, although the TNF- $\alpha$ - and interleukin-1 $\beta$ -mediated NF- $\kappa$ B activation signaling remains intact in NIK-deficient cells. Furthermore, mice lacking NIK display a phenotype that was similar to LT $\beta$ -R-deficient mice (10). To test the involvement of LT $\beta$ -R as the upstream activator of NIK and, thus, NF- $\kappa$ B, we investigated the LT $\beta$ -R expression in different cell lines and tissues. Interestingly, we observed a correlation between constitutive IKK activity and LT $\beta$ -R expression in melanoma cell lines. LT $\beta$ -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 LT $\beta$ -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 phospho-I $\kappa$ B $\alpha$  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- $\kappa$ B 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- $\kappa$ B might result in the maintenance of NF- $\kappa$ B in SK-Mel 28 cells despite reduced IKK kinase activity and reduced I $\kappa$ B $\alpha$  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 LT $\beta$ -R contributes to NF- $\kappa$ B activation in melanoma cells and tumors. Other studies have also demonstrated that LT $\beta$ -R can indirectly activate NF- $\kappa$ B by inducing phosphorylation of p65/RelA at Ser-536, which is mediated by NIK-IKK $\alpha$  (27).

To determine whether the LT $\beta$ -R is an upstream modulator in melanoma cells, we treated Hs294T melanoma cells with the agonistic LT $\beta$ -R antibody to induce this pathway. Interestingly, we saw an increased cell growth in response to this antibody activation of the LT $\beta$ -R signaling. Because the LT $\beta$ -R agonist antibody could activate NF- $\kappa$ B promoter activity in SKMel28 cells with lower basal IKK activity, we speculate that the increased cell proliferation induced by LT $\beta$ -R signaling is mediated by an increase in NIK-NF- $\kappa$ B activity. In addition, inhibition of the LT $\beta$ -R pathway by knocking down expression of LT $\beta$ -R results in reduction in the NF- $\kappa$ B promoter activity as monitored by NF- $\kappa$ B luciferase reporter assay. Together with our previous demonstration that overexpression of kinase-dead mutants of NIK (KK-AA) significantly suppressed the basal NF- $\kappa$ B-dependent luciferase activity ( $p \leq 0.05$ ) (20), these data suggest that the LT $\beta$ -R acts through NIK to stimulate constitutive NF- $\kappa$ B activity in melanoma. Furthermore, we demonstrated here that activation of LT $\beta$ -R signaling can induce melanoma cell growth, suggesting that the LT $\beta$ -R-NIK-NF- $\kappa$ B 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- $\kappa$ B with small molecule inhibitors of IKK will inhibit proliferation of melanoma cells *in vitro* and *in vivo* (4). Moreover, ribozyme knockdown of IKK $\alpha$  or IKK $\beta$  inhibits the growth of melanoma tumors *in vivo* (28).

In this report we show that an upstream activator of NIK, the LT $\beta$ -R, is expressed in metastatic melanoma cell lines and lesions, and this receptor contributes to the constitutive NF- $\kappa$ B activity of melanoma cells. LIGHT as well as LT $\alpha$ 1 $\beta$ 2 are known ligands for the LT $\beta$ -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 LT $\beta$ -R signaling feedback loop could potentially cause melanoma cells to over-activate the NIK-NF- $\kappa$ B 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 LT $\beta$ -R. Furthermore, we were unable to detect LT $\alpha$ 1 $\beta$ 2 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 LT $\beta$ -R and prevent their activation of the receptor (23). These experiments showed that the chimera had no effect on the NF- $\kappa$ B 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 elevated expression of LT $\beta$ -R in melanoma cells is sufficient for activation of downstream signaling. Our demonstration that knocking down the LT $\beta$ -R in melanoma cells markedly suppressed the constitutive NF- $\kappa$ B luciferase activity in these cells, and the growth of melanoma tumor cells supports this autoactivation model further. Support for this model comes from studies in multiple myeloma where overexpression of the LT $\beta$ -R is thought to be associated with activation of the NF- $\kappa$ B pathway (30).

The NIK-NF- $\kappa$ B pathway is important for the expression of the CXC chemokine, CXCL1, in melanoma tumors. LT $\beta$ -R signaling contributes to this constitutive up-regulation of NF- $\kappa$ B-mediated transcription of chemokines. Activation of LT $\beta$ -R induces CXCL-8 expression, and NF- $\kappa$ B and AP-1 are essential components of this LT $\beta$ -R-induced CXCL8 expression (31). We have previously reported that melanoma cells expressed very high levels of CXC chemokines (CXCL1 and CXCL8) as compared with NHEMs (22). These chemokines are regulated by the NF- $\kappa$ B pathway, and when the NF- $\kappa$ B pathway is inhibited, expression of the chemokine is inhibited (22). Here we show here that loss of expression of the LT $\beta$ -R results in a decline in VCAM and SDF-1 mRNA expression based upon real time PCR. Because the VCAM expression is dependent upon activation of the canonical NF- $\kappa$ B pathway and SDF-1 expression is dependent upon the non-canonical pathway, the suggestion is that both the canonical and non-canonical pathways to NF- $\kappa$ B activation are mediated by the LT $\beta$ -R in melanoma cells. Ligation of the LT $\beta$ -R has been previously shown to activate both of these pathways (32).

To demonstrate a causal link between receptor expression with a malignancy phenotype, we further tested the role of LT $\beta$ -R expression in cell invasion and MMP activity. Inhibition of LT $\beta$ -R expression in Hs294T results in a 3-fold decrease in cell invasion as well as a decrease in expression of both MMP-2 and MMP-9. These data suggest that LT $\beta$ -R expression is linked to MMP expression, which affects the invasive properties.

Recently, NIK was shown to play a crucial role in osteopontin-induced NF- $\kappa$ B activation, urokinase plasminogen activator secretion, and pro-matrix metalloproteinase-9 activation, ultimately controlling cell motility, invasiveness, and tumor growth (33).

We have shown in our previous studies that NIK as well as the phosphatidylinositol 3-kinase/Akt pathways are important for the activation of NF- $\kappa$ B in melanoma cells (20, 21). However, the possibility of cross-talk among them remains to be determined. Recently it was shown that treatment of cancer cell lines with TNF- $\alpha$  decreases PTEN expression. In addition, overexpression of TNF- $\alpha$  downstream signaling targets NIK and p65 nuclear factor NF- $\kappa$ B lowers PTEN expression, suggesting that TNF- $\alpha$ -induced down-regulation of PTEN is mediated through a TNF- $\alpha$ /NIK/NF- $\kappa$ B pathway (34). Down-regulation of PTEN by NIK/NF- $\kappa$ B results in enhanced activation of the phosphatidylinositol 3-kinase/Akt pathway and augmentation of TNF- $\alpha$ -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 LT $\beta$ -R-NIK-NF- $\kappa$ B 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- $\kappa$ B activation in tumor cells will allow the targeting of critical components and new opportunities for therapeutic intervention.

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