Expression level of Bcl-XL critically affects sensitivity of hepatocellular carcinoma cells to LIGHT-enhanced and interferon-γ-induced apoptosis

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Abstract. The molecular mechanisms of apoptosis caused by IFN-γ (interferon gamma)/LIGHT (lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpes virus entry mediator on T cells) have not been studied in detail. The present study was undertaken to gain insights into the signaling pathways involved in apoptosis induced by IFN-γ/LIGHT in hepatocellular carcinoma (HCC) cell lines. Cell proliferation assay, flow cytometry, Western blotting, gene transfer and RNA interference were used in this study. LIGHT enhanced IFN-γ-mediated apoptosis in Hep3B cells. IFN-γ/LIGHT-induced apoptosis was inhibited by blocking peptides to the lymphotoxin ß receptor (LT-ß R), and not by the herpes virus entry mediator (HVEM). Expression of LT-ß R remained unchanged after cytokine treatments. IFN-γ/LIGHT treatment resulted in the down-regulation of Bcl-XL and the activation of caspase-9 and caspase-3 as well as the decrease of phosphorylation of STAT3. HepG2 and SMMC-7721 cells, which showed high levels of endogenous Bcl-XL, displayed resistance to IFN-γ/LIGHT-induced apoptosis. Overexpression of Bcl-XL in Hep3B cells increased the resistance to IFN-γ/LIGHT induced apoptosis while the down-regulation of Bcl-XL in HepG2 and SMMC-7721 cells by RNA interference decreased the resistance. Our study provides important mechanistic insights into IFN-γ/LIGHT-induced apoptosis in HCC cells and may help to select better therapeutic strategies for certain cancers with distinct Bcl-XL expression.

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Introduction

LIGHT is a new cell surface-bound member of the tumor necrosis factor (TNF) superfamily which participates in multiple biological functions through ligand-receptor signaling. LIGHT induces differentiation of the human rhabdomyosarcoma cell line (RD cells) to smooth muscle cells and stimulates these RD cells to secrete interleukin-8 and RANTES (regulated on activation, normal T cell expressed and secreted) (1). Interestingly, by blocking the activation of both caspase-3 and caspase-8, LIGHT also acts as an anti-apoptotic agent against TNF-α-mediated liver injury (2). It has also been proposed that LIGHT is a potent initiator of T cell co-stimulation signals (3-5) affecting cytotoxic T lymphocyte (CTL)-mediated tumor rejection (5), allograft rejection (6) and graft versus host disease (7). In addition, an enhanced level of LIGHT may accelerate the progression of acquired immune deficiency syndrome (AIDS) in human immunodeficiency virus (HIV)-infected individuals (8) and promote lipid metabolism, inflammatory response, and thrombus formation in atherosclerotic plaques (9).

LIGHT also enhances interferon-γ (IFN-γ) induced apoptosis (10-12) but the mechanisms remain unclear. LIGHT forms a membrane-anchored homotrimeric complex which is capable of binding to both lymphotoxin ß receptor (LT-ß R) and HVEM (11,12). LT-ß R alone has been found sufficient for the induction of IFN-γ/LIGHT-induced apoptosis in HT-29 cells (13), but in MDA-MB-231 human breast carcinoma cells, both HVEM and LT-ß R are required to achieve the apoptotic effect of IFN-γ/LIGHT (12). TR6, a third receptor for LIGHT, suppresses the apoptotic effect of LIGHT and transduces signals from LIGHT to T cells to enhance T cell proliferation (14,15). Proteins associated with the LIGHT/LT-ß R complex have been identified by mass spectrometry, including TNF receptor-associated factors (TRAF) 2 and 3,

cellular inhibitor of apoptosis protein 1 (cIAP1) and second mitochondria-derived activator of caspase (Smac). Based on this model, the LIGHT/LT-ß R complex triggers the mitochondria-mediated apoptosis pathway via TRAF3 stimulated release of Smac from mitochondria (16). Overexpression of Bcl-2 also enhances IFN-γ/LIGHT-mediated apoptosis through Bcl-2 cleavage (17). The Bcl-2 cleavage fragment plays a pro-apoptotic role triggering the apoptosis cascade by a process in which the activation of caspase-3 is not required. In this study, we utilized hepatocellular carcinoma cells (HCC) to further investigate the anti-tumor effect of IFN-γ/LIGHT and the possible mechanisms. We demonstrated that the down-regulation of Bcl-XL resulting from the decrease of phosphorylated STAT3 is critical for IFN-γ/LIGHT-induced apoptosis, and that HCC cells with high levels of endogenous Bcl-XL are more resistant to IFN-γ/LIGHT-induced apoptosis. These findings are in line with previous reports on other tumor cell lines (18-20), suggesting a broad anti-tumor effect of IFN-γ/LIGHT-induced cell death in cells with low levels of endogenous Bcl-XL expression.

Materials and methods

Cell culture and reagents. HCC cell lines Hep3B (21), HepG2 (22) (The American Type Culture Collection, Rockville, MD, USA) and SMMC-7721 cells (23) (The Cell Bank of the Chinese Academy of Sciences, Shanghai, P.R. China) were used in the study. All cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% (v/v) fetal bovine serum (FBS) plus 1% glutamine (Gibco, Grand Island, NY, USA). A pSuper.neo + EGFP vector was purchased from OligoEngine (Seattle, WA, USA). A recombinant LIGHT soluble protein that only contained the extracellular region of human LIGHT and caspase inhibitors Z-DEVD-fmk, Z-IETD-fmk and Z-LEHD-fmk was purchased from R&D System (Minneapolis, MN, USA). A recombinant IFN-γ protein was obtained from Peprotech (Rocky Hill, NJ, USA). HVEM and LT-ß R blocking peptide, HVEM, LT-ß R, STAT3 and pTyrSTAT3 (phosphorylation of ^{705}Tyr) goat polyclonal antibodies were products of Santa Cruz Biotechnology (CA, USA). Polyclonal antibodies against caspase-3 (only recognizing procaspase-3), caspase-8 and caspase-9 (only recognizing procaspase-9), Bcl-XL, Bak and Bid were purchased from Neomark (Fremont, CA, USA). The bicinchoninic acid (BCA) protein assay kit was a product of Pierce Biotechnology (Rockford, IL, USA).

Construction and infection of adenovirus vectors. The human LIGHT overexpressing adenoviral vectors were constructed according to He *et al* (24). Briefly, cDNA for human LIGHT was inserted into a shuttle vector *pAdTrack-CMV* following the standard procedures. The resultant plasmid was linearized by enzyme digestion and subsequently co-transfected into *E. coli* BJ5183 with an adenoviral backbone plasmid, *pAdEasy-1*. Finally, recombinant adenoviruses were propagated on packaging 293 cells and purified by cesium chloride density gradient centrifugation and subsequent dialysis. The recombinant adenovirus carrying full LIGHT cDNA was defined as Ad-LIGHT and the mock recombinant adenovirus was named Ad. HCC cells were plated in 6- or 24-well plates, and adenoviral infection was performed the next day for 4 h with the virus diluted in DMEM to desired multiplicity of infection (MOI).

Construction of plasmid vectors and generation of stable transfectants. The double-stranded DNA template encoding small interfering RNAs (siRNAs) for STAT3 (25) and Bcl-XL (26) was synthesized. The sequences were STAT3: 5'GATC CCCGCAGCAGCTGAACAACATGTTCAAGAGACATG TTGTTCAGCTGCTGCTTTTTA-3' (forward), 5'AGC TTAAAAAGCAGCAGCTGAACAACATGTCTCTTGAAC ATGTTGTTCAGCTGCTGCGGG-3' (reverse); Bcl-XL: 5'GATCCCCCAGGGACAGCATATCAGAGTTCAAGAG ACTCTGATATGCTGTCCCTGTTTTTA-3' (forward), 5'AGCTTAAAAACAGGGACAGCATATCAGAGTCTCTT GAACTCTGATATGCTGTCCCTGGGG-3' (reverse). The pSuper.neo-EGFP vector, in which short hairpin RNAs (shRNA) are expressed under the control of the H1 promoter, was constructed according to Gao *et al* (25). The negative control vector that expresses a hairpin siRNA with limited homology to any known sequences in the human genome was provided with the vector kit. The cDNA encoding full-length human Bcl-XL was cloned into pIRES2-EGFP (Clontech, Mountain View, CA, USA). pIRES2-EGFP was used as a mock vector.

All vectors mentioned above were transfected into HCC cells using LipofectAMINE™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Stably transfected cells were selected with 800 ng/ml G418 (Sigma, St. Louis, MO, USA), followed by confirmation with Western blott analysis.

Cell viability assay. Cell viability was determined by MTT quantitative colorimetric assay. The cells were seeded in 96-well plates. After different treatments, 20 µl 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well and the plates were incubated at 37˚C for 4 h. The cells were then lysed by the addition of 150 µl dimethyl sulfoxide (DMSO) per well and mixed with a microplate shaker for approximately 5 min. The optical density of each sample was determined by measuring the absorbance at 492 nm versus 650 nm using an enzyme linked immunosorbent assay reader (BioRad, Hercules, CA, USA).

Membrane receptors and LIGHT analysis. Cells were harvested and incubated with Abs for HVEM, LT-ß R or LIGHT at 10 µg/ml in binding buffer at 4˚C for 30 min, followed by one wash and staining with phycoerythrin conjugated bovine anti-goat IgG at a final concentration of 20 µg/ml. The cell surface fluorescence intensity was analyzed by FACScan flow cytometry (BD Biosciences, San Jose, CA, USA).

Analysis of apoptosis. The extent of apoptosis was evaluated by the Annexin V/propidium iodide (PI) method. Briefly, $1x10^6$ cells from each treatment group were incubated with Annexin V-FITC solution (R&D System) at 4˚C for 10 min and washed three times with cold phosphate buffered saline (PBS) and then stained with PI at room temperature for 10 min.

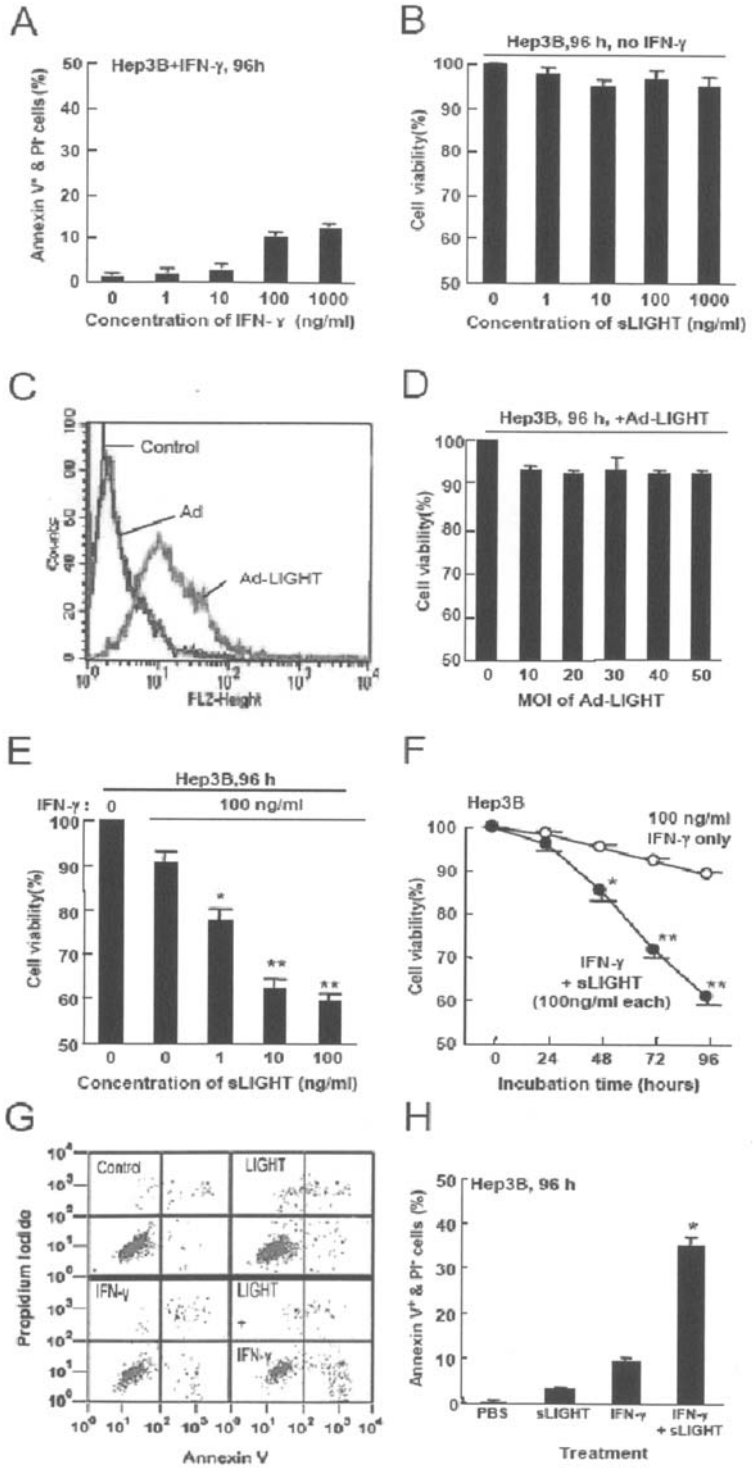

Figure 1. LIGHT enhances the apoptosis of Hep3B cells induced by IFN-γ. (A) Hep3B cells were treated with IFN-γ (0, 1, 10, 100 and 1000 ng/ml) and the percentage of cell apoptosis was analyzed by Annexin V/PI staining. The experiments were performed in triplicate and in at least two separate experiments. The bar indicates the standard error. (B) Hep3B cells were treated with sLIGHT (0, 1, 10, 100 and 1000 ng/ml) and cell viability was determined at 96 h by MTT assay. The mean number of viable cells in the 0 ng/ml group was set as 100% and the relative viability of other samples was calculated accordingly. The experiments were performed in triplicate and in at least two separated experiments. The bar indicates the standard error. (C) Hep3B cells were infected with adenovirus expressing full-length human LIGHT cDNA (Ad-LIGHT) or vector virus carrying no transgene (Ad). Cells positive for LIGHT expression on the plasma membrane were marked with goat anti-human LIGHT polyclonal Ab and identified by flow cytometry. Uninfected cells were used as the control. (D) Hep3B cells were infected with Ad-LIGHT at 10 to 50 multiplicity of infectivity (MOI) for 24 h and further cultured for 96 h. Cell viability was determined. The mean number of viable cells in the untreated group was set as 100% and the relative viability of other samples was calculated accordingly. The experiments were performed in triplicate and in at least two separate experiments. The bar indicates the standard error. (E) Hep3B cells were incubated with IFN-γ (100 ng/ml) and different concentrations of LIGHT (0, 1, 10 and 100 ng/ml) for 96 h. Cell viability was determined. (F) Hep3B cells were incubated with IFN-γ (100 ng/ml) alone (○) or IFN-γ (100 ng/ml) plus LIGHT (100 ng/ml) (●) for 24, 48, 72, or 96 h. Cell viability was determined at each time-point. $^{*}p<0.05$; $^{**}p<0.01$, versus group treated with IFN-γ (100 ng/ml) alone (E) and versus group treated with IFN-γ (100 ng/ml) alone at each time point (F). (G, H) Hep3B cells were incubated with either phosphate buffered saline (PBS), or LIGHT protein (100 ng/ml) alone, or IFN-γ (100 ng/ml) alone, or LIGHT (100 ng/ml) plus IFN-γ (100 ng/ml) for 96 h. Cell apoptosis was analyzed by Annexin V/PI staining. The percentage of apoptotic (Annexin-positive and PI-negative) cells in each group is plotted in G. The numbers shown in H are mean values of three independent experiments. $^{*}p<0.05$, versus group treated with IFN-γ (100 ng/ ml) alone.

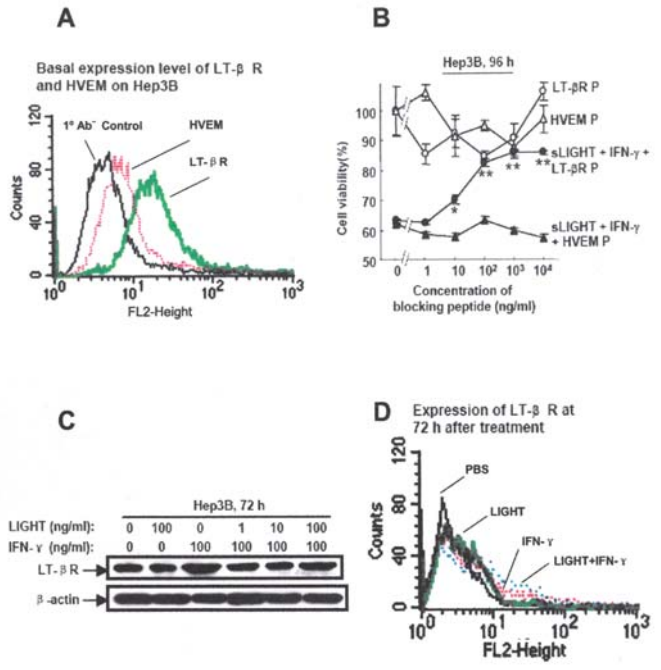

Figure 2. Expression of LT-ß R and HVEM on Hep3B cells and their participation in IFN-γ/LIGHT treatment. (A) Hep3B cells were stained with goat polyclonal antibody against HVEM or LT-ß R and the expression level of each receptor was determined by flow cytometry. Cells treated solely with secondary antibodies were used as an unstained negative control. (B) Hep3B cells were incubated either with various doses of HVEM blocking peptide alone (HVEM P), LT-ß R blocking peptide alone (LT-ß R P), or sLIGHT (100 ng/ml) plus IFN-γ (100 ng/ml) in the presence of various amounts of HVEM P or LT-ß R P for 96 h. The viability of treated cells was then determined. The mean number of viable cells in untreated Hep3B was set as 100% and the relative viability of other samples was calculated accordingly. The experiments were performed in triplicate, and in at least two separate experiments. The bar indicates the standard error. $^{*}p<0.05$; $^{**}p<0.01$, versus group treated with sLIGHT (100 ng/ml) plus IFN-γ (100 ng/ml) without the blocking peptide. (C) Hep3B cells were treated with various concentrations of LIGHT and/or IFN-γ (as indicated) for 72 h. Cell lysates were prepared and Western blotting was performed to determine the expression level of LT-ß R in all treatments. ß-actin was used as an internal control for total protein loading. (D) Hep3B cells were treated with LIGHT (100 ng/ml) and/or IFN-γ (100 ng/ml) for 72 h. Then cells were stained with goat polyclonal antibody against LT-ß R, and the receptor expression level was determined by FACS analysis. Cells treated with PBS were used as negative controls.

The fluorescence intensity of Annexin V-FITC and PI of individual cells was analyzed by FACScan.

Western blott analysis. Cells were treated with various compounds and lysed for 30 min at 4˚C in lysis buffer. Protein concentration was measured spectrophotometrically using the BCA protein assay. Equal amounts of proteins were subjected to SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes (Hybond, Amersham Pharmacia Biotech, Freiburg, Germany). After blocking with Tris buffered saline (TBS) containing 5% skim milk and 0.5% Tween-20, the membranes were reacted with appropriate antibodies (1:1000 for LT-ß R, 1:800 for HVEM, 1:400 for Bcl-XL, Bak, Bid, caspase-3, caspase-8, and caspase-9, 1:800 for STAT3 and 1:400 for pTrySTAT3) in TBS containing 3% bovine serum albumin and 0.2% Tween-20. After 3 washes, blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 60 min and developed with enhanced chemiluminescence reagents (Santa Cruz Biotechnology), followed by exposure to X-ray film (Kodak, Rochester, NY, USA).

Statistical analysis. Student's t-test was used to analyze the differences between groups as mentioned in the figure legends. A p-value of <0.05 was considered statistically significant.

Results

LIGHT enhances IFN-γ-mediated apoptosis of Hep3B cells. IFN-γ alone induced a moderate dose-dependent increase of apoptosis of Hep3B cells (Fig. 1A). Therefore, a single concentration of 100 ng/ml of IFN-γ was chosen for the rest of this study. As with IFN-γ, LIGHT alone in the form of the recombinant soluble protein (sLIGHT) or the full-length protein expressed by transfecting an adenovirus vector carrying the full-length cDNA insertion of human LIGHT protein (Ad-LIGHT) did not significantly induce apoptosis of Hep3B cells (Fig. 1B, C and D).

When Hep3B cells were treated with sLIGHT and IFN-γ together, significant decrease in cell viability was observed (Fig. 1E and F). After 96-h incubation, IFN-γ plus 1, 10 and 100 ng/ml sLIGHT significantly reduced Hep3B cell viability to 77.5%, 63.8% and 59.7%, respectively (Fig. 1E). This effect was also time dependent. After the treatment with IFN-γ alone, the cell viability decreased gradually over time to ~90% at 96 h, whereas after the treatment with IFN-γ and sLIGHT (100 ng/ml each) together, the viability changed slightly after 24 h, decreased to 85.5% at 48 h and reduced significantly to 71.3% and 60.6% at 72 h and 96 h respectively (Fig. 1F).

Analysis of cells with Annexin V/PI staining demonstrated that the observed cell death in these treatments was apoptotic rather than necrotic (Fig. 1G). The treatment with sLIGHT (at 100 ng/ml) or IFN-γ alone produced only 3.3% or 9.4% apoptotic cells respectively after 96-h incubation while the treatment with both cytokines produced markedly increased apoptotic cells (35.6%) (Fig. 1H).

LT-ß R, but not HVEM, participates in IFN-γ/LIGHT-induced apoptosis of Hep3B cells. LIGHT interacts with two receptors, HVEM and LT-ß R. Both receptors were identified as being expressed by Hep3B cells by FACS analysis (Fig. 2A).

In order to identify which receptor was involved in the cytotoxic effect of IFN-γ/LIGHT in Hep3B cells, soluble HVEM and LT-ß R blocking peptides were added to the cell culture to investigate whether IFN-γ/LIGHT-induced apoptosis could be reversed (Fig. 2B). The HVEM or LT-ß R blocking peptide alone showed no affect on cell death. However, the LT-ß R blocking peptide, but not HVEM, demonstrated a dose-dependent effect of blocking the IFN-γ/LIGHT-induced apoptosis of Hep3B cells. The effect of LT-ß R blocking peptide was significant at 10 ng/ml and the cell viability increased by >80% when the peptide concentration was >100 ng/ml. However, the LT-ß R blocking peptide failed to completely recover the cell viability of IFN-γ/LIGHT-treated Hep3B cells to the level of the untreated control group (86%), even at a concentration of up to $1x10^4$ ng/ml.

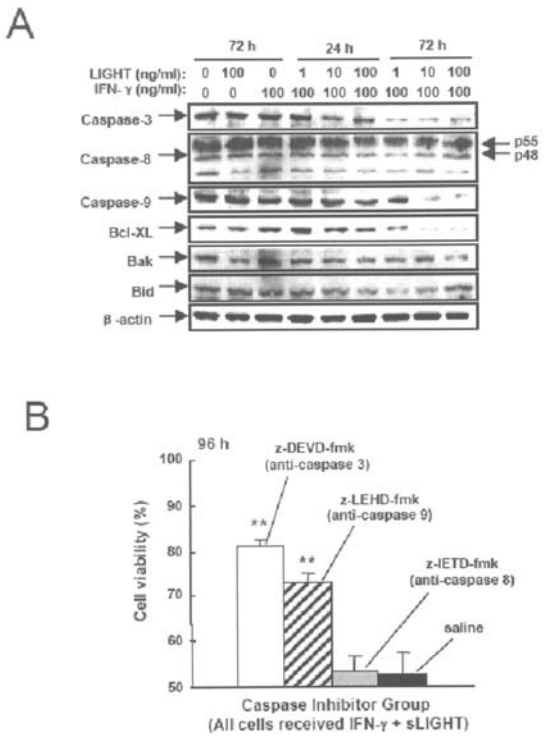

Figure 3. Expression levels of caspase-3, caspase-8, caspase-9, Bcl-XL, Bak and Bid in Hep3B cells treated with IFN-γ/LIGHT. (A) Hep3B cells were treated with 100 ng/ml IFN-γ and various concentrations of LIGHT for 24 h or 72 h as indicated. Cell lysates containing 30 μg of protein were subjected to 12% Tris-glycine gel electrophoresis followed by Western blotting with rabbit polyclonal antibodies against caspase-3, caspase-8, caspase-9, Bcl-XL, Bak, Bid, respectively. Cells treated with 100 ng/ml IFN-γ or 100 ng/ml LIGHT alone and untreated cells were used as the controls. ß-actin was used as an internal control for total protein loading. (B) Hep3B cells ($2x10^5$) treated with 100 ng/ml sLIGHT and 100 ng/ml of IFN-γ were also incubated with 100 μM of caspase-3 inhibitor Z-DEVD-fmk, caspase-9 inhibitor Z-IEHD-fmk, caspase-8 inhibitor Z-IEHD-fmk, or normal saline for 96 h. Cell viability was then measured. The viability of untreated (without any cytokine and inhibitor) cells was set at 100%, and the relative viability of other samples was calculated accordingly. The experiments were performed in triplicate, and in at least two separated experiments. The bar indicates the standard error. $^{**}p<0.01$, versus group treated with normal saline.

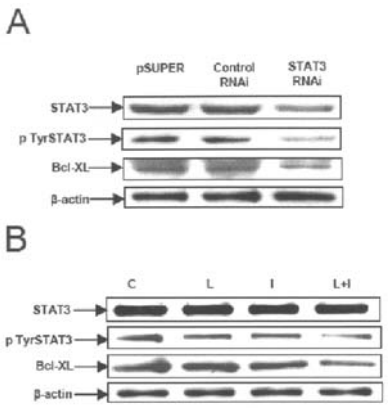

Figure 4. IFN-γ/LIGHT may down-regulate Bcl-XL through the decrease of pTyrSTAT3 in Hep3B cells. (A) Hep3B cells were transfected with pSUPER plasmid, the plasmid carrying STAT3 specific siRNA, or the plasmid carrying scrambled control siRNA, and stable clones were selected. Western blott analysis was used to examine the levels of Bcl-XL in different cells. Expression of ß-actin is shown to indicate equal total protein loading. (B) Hep3B cells were treated with 100 ng/ml IFN-γ and LIGHT for 72 h (L+I) as indicated. Cell lysates containing 30 μg protein were subjected to 10% Tris-glycine gel electrophoresis followed by Western blott assay with goat polyclonal antibodies against STAT3, pTyrSTAT3, and Bcl-XL, respectively. Cells treated with 100 ng/ml IFN-γ (I) or 100 ng/ml LIGHT (L) alone and untreated cells (C) were used as the controls. ß-actin was used as an internal control for total protein loading.

The expression levels of LT-ß R before and after the treatment with LIGHT and/or IFN-γ were examined by Western blotting and flow cytometry, and were found unchanged under all treatments (Fig. 2C and D).

Alterations of apoptosis cascade components. Little is known about the signal transduction pathway of IFN-γ/LIGHT-induced apoptosis. In order to explore this mechanism in HCC cells, alterations of a series of apoptosis cascade components were examined.

The protein levels of apoptosis initiator caspase-9 (involved in mitochondria pathway), apoptosis effector caspase-3, as well as anti-apoptotic protein Bcl-XL, were found diminished 24 h to 72 h after the combined treatment with LIGHT and IFN-γ, and the levels of the two caspases did not change after the treatment with either LIGHT or IFN-γ alone. On the other hand, the protein levels of apoptosis initiator caspase-8 (involved in the death receptor pathway), and Bcl-2 family members, Bak and Bid, remained largely constant after all treatments (Fig. 3A).

The function of caspases was further confirmed by the inhibitory experiment. IFN-γ/LIGHT-induced apoptosis was significantly reversed by caspase-3 inhibitor Z-DEVD-fmk and caspase-9 inhibitor Z-LEHD-fmk but not by caspase-8 inhibitor Z-IETD-fmk (Fig. 3B). This observation was consistent with the Western blott analysis which showed that caspase-3 and caspase-9, but not caspase-8, were involved in IFN-γ/LIGHT-induced apoptosis of Hep3B cells (Fig. 3A).

The down-regulation of Bcl-XL is attributed to the decrease of pTyrSTAT3 induced by IFN-γ/LIGHT. STAT3 has been shown to participate in oncogenesis through the up-regulation of genes encoding apoptosis inhibitors, such as Bcl-XL, in some cancer cell lines (27). To assess the relationship between the down-regulation of Bcl-XL and the decrease of pTyr STAT3, Hep3B cells were transfected with STAT3 specific siRNA. Results showed that the expression and phosphorylation of STAT3 were significantly decreased in the cells transfected with a plasmid carrying STAT3 specific siRNA compared with mock-transfected cells and in cells transfected with the plasmid carrying negative control siRNA. Correspondingly, Bcl-XL was significantly reduced in Hep3B cells transfected with STAT3 specific siRNA compared with the controls (Fig. 4A). These data indicate that the decrease of pTyrSTAT3 may lead to the down-regulation of Bcl-XL in Hep3B cells.

In order to confirm that the down-regulation of Bcl-XL induced by IFN-γ/LIGHT is the result of the decrease of STAT3 phosphorylation, alterations of STAT3 or the phosphorylation of STAT3 were examined after Hep3B cells were treated with LIGHT and/or IFN-γ. The level of STAT3 remained unchanged in all treatment groups. However, the pTyrSTAT3 and Bcl-XL diminished after the combined

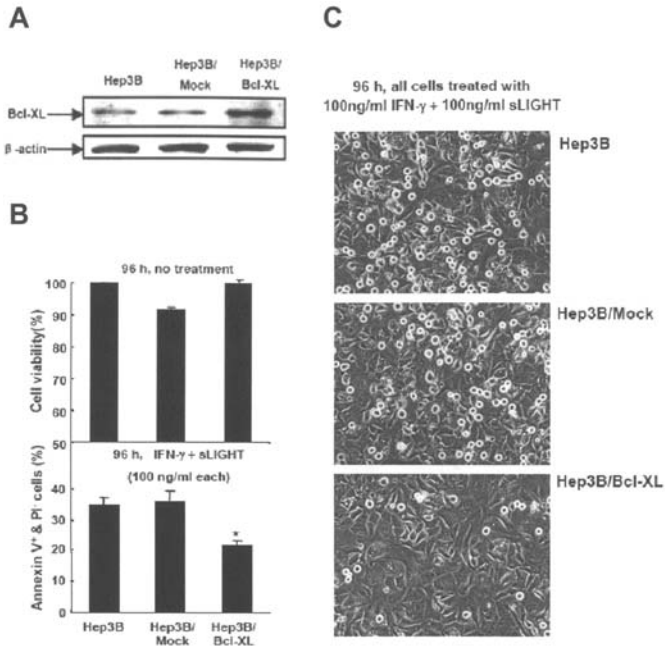

Figure 5. Overexpression of Bcl-XL reduces IFN-γ/LIGHT-induced apoptosis in Hep3B cells. (A) Cell lysates (containing 30 μg of total protein) from Hep3B, Hep3B/mock and Hep3B/Bcl-XL cells (without any treatment) were subjected to 12% Tris-glycine gel electrophoresis followed by Western blott assay with goat polyclonal antibodies against Bcl-XL or ß-actin. Expression of ß-actin is shown to indicate equal total protein loading. (B) Cell viability was determined after Hep3B, Hep3B/mock, or Hep3B/Bcl-XL cells were cultured for 96 h (without any treatment). The mean number of parental Hep3B cells was set at 100%. The experiments were performed in triplicate, and in at least two separate experiments. The bar indicates the standard error. (C) Hep3B, Hep3B/mock, or Hep3B/Bcl-XL cells were incubated with IFN-γ (100 ng/ml) and sLIGHT (100 ng/ml) for 96 h. Apoptosis was then analyzed by Annexin V/PI staining. The percentage of apoptotic cells was plotted. The numbers were mean values of three independent experiments. *p<0.05, versus Hep3B group. (D) Cell morphology under the conditions as described in C was photographed under an optic microscope (x40).

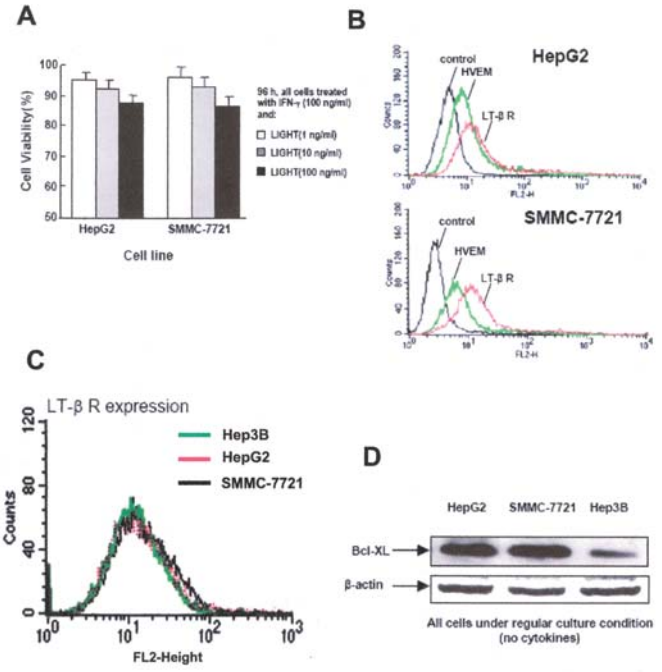

Figure 6. Resistance of HepG2 and SMMC-7721 cells to IFN-γ/LIGHT induces apoptosis and high endogenous expression of Bcl-XL. (A) HepG2 and SMMC-7721 cells were treated with IFN-γ (100 ng/ml) and sLIGHT (1, 10, and 100 ng/ml) for 96 h, and cell viability was assayed. Viability of untreated control cells was set at 100%, and viability relative to the control was presented. The experiments were performed in triplicate, and in at least two separate experiments. The bar indicates the standard error. (B) HepG2 and SMMC-7721 cells were stained with goat polyclonal antibodies against HVEM and LT-ß R, and the expression level of each receptor was then determined by FACS analysis. Cells treated solely with secondary antibodies were used as an unstained negative control. (C) Hep3B, HepG2 and SMMC-7721 cells were stained with a goat polyclonal antibody against LT-ß R and the receptor expression level was then determined by FACS analysis. (D) Western blott analysis was performed on cell lysates from HepG2, SMMC-7721 and Hep3B cells with a goat polyclonal antibody against Bcl-XL. Expression of ß-actin is shown to indicate equal total protein loading.

treatment with LIGHT and IFN-γ for 72 h, but not after the treatment with LIGHT or IFN-γ alone (Fig. 4B).

Overexpression of Bcl-XL increased resistance of Hep3B cells to IFN-γ/LIGHT-induced apoptosis. To further test the role of Bcl-XL in IFN-γ/LIGHT-induced apoptosis of human HCC cells, a Hep3B derived cell line stably expressing Bcl-XL (Hep3B/Bcl-XL) was established. Overexpression of Bcl-XL in Hep3B/Bcl-XL cells was confirmed by Western blott analysis (Fig. 5A). Untreated Hep3B/Bcl-XL cells grew at a similar rate to Hep3B cells or Hep3B mock transfected cells (Hep3B/Mock) (Fig. 5B). When treated with IFN-γ/LIGHT (100 ng/ml each) for 96 h, approximately 35% of parental Hep3B or Hep3B/mock cells underwent apoptosis, whereas only approximately 20% of Hep3B/Bcl-XL cells underwent apoptosis (Fig. 5C), suggesting that overexpression of Bcl-XL reduced the sensitivity of Hep3B cells to IFN-γ/LIGHT-induced apoptosis. Cell morphological changes also supported the finding that overexpression of Bcl-XL inhibited IFN-γ/LIGHT-induced apoptosis of Hep3B cells (Fig. 5D).

HepG2 and SMMC-7721 cells are more resistant to LIGHT/IFN-γ-induced apoptosis and have higher expression of Bcl-XL. To determine whether LIGHT/IFN-γ induces apoptosis of other HCC cell lines, HepG2 and SMMC-7721 cells were cultured with IFN-γ (100 ng/ml) and sLIGHT (1, 10 and 100 ng/ml) for 96 h. At the end of the culture, however, only approximately 12% of the cells were found dead after the dual cytokine treatment at the highest concentration (Fig. 6A), which was significantly less than the percentage of IFN-γ/LIGHT-induced cell death in Hep3B cells (up to ~40%) (Fig. 1E). Thus, HepG2 and SMMC-7721 appeared to have lower susceptibility to IFN-γ/LIGHT-induced apoptosis. To investigate whether this was due to overexpression of Bcl-XL, the levels of Bcl-XL protein in HepG2 and SMMC-7721 were evaluated by Western blotting and the results showed that these two cell lines contained much higher levels of Bcl-XL protein than Hep3B cells under the normal culture condition (Fig. 6D). In contrast, both HepG2 and SMMC-7721 cells expressed HVEM and LT-ß R (Fig. 6B), and no difference was observed in the level of LT-ß R expression among Hep3B, HepG2, and SMMC-7721 cells (Fig. 6C).

Down-regulation of Bcl-XL level via specific siRNA sensitizes HepG2 and SMMC-7721 cells to IFN-γ/LIGHT-

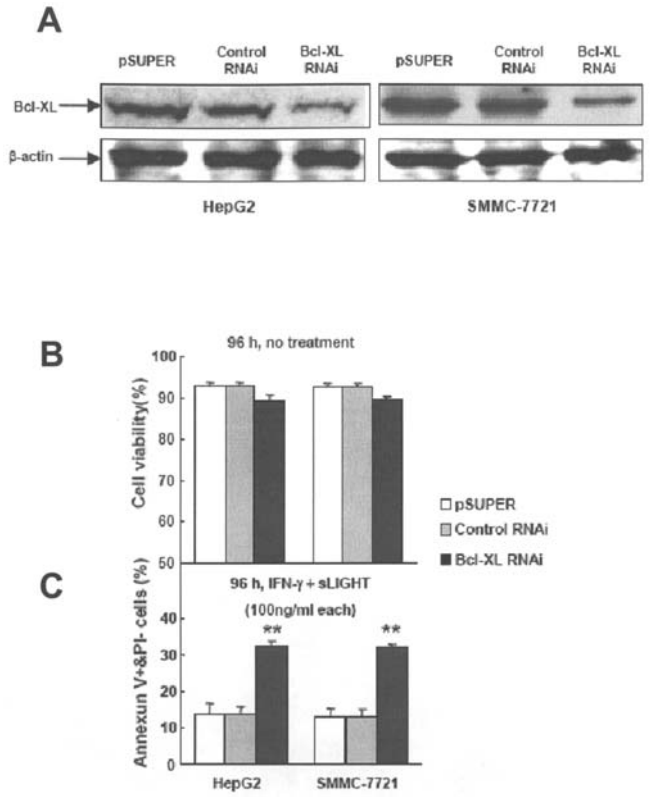

Figure 7. Down-regulation of Bcl-XL through specific siRNA increases the susceptibility of HepG2 and SMMC-7721 cells to IFN-γ/LIGHT-induced apoptosis. (A) HepG2 and SMMC-7721 cells were transfected with pSUPER plasmid, the plasmid carrying Bcl-XL specific siRNA, or the plasmid carrying scrambled control siRNA, and stable clones were selected. Western blott analysis was used to examine the level of Bcl-XL in different cells. Expression of ß-actin is shown to indicate equal total protein loading. (B) Cell viability was determined after cells described in A were cultured for 96 h without treatment. Untransfected parental cells were used as the controls for 100% viability. (C) Cells described in A were incubated with IFN-γ (100 ng/ml) and sLIGHT (100 ng/ml) for 96 h. Apoptosis was analyzed by Annexin V/PI staining. The percentage of apoptotic cells was plotted. The numbers are mean values of three independent experiments. $^{**}p<0.01$, versus HepG2 or SMMC-7721 group.

induced apoptosis. To further assess the relationship between overexpression of Bcl-XL and the resistance of HepG2 and SMMC-7721 cells to IFN-γ/LIGHT-induced apoptosis, the Bcl-XL gene expression in these two cell lines was stably reduced by specific siRNA and the effect of IFN-γ/LIGHT treatment was re-evaluated.

The Bcl-XL protein levels in HepG2 and SMMC-7721 cells, stably transfected with a plasmid expressing Bcl-XL specific siRNA were significantly lower than those in cells transfected with a backbone plasmid or a plasmid carrying scrambled control siRNA (Fig. 7A). A slight reduction in cell viability (~7%) without cytokine treatment was observed in Bcl-XL specific siRNA transfected cells compared with the mock or control siRNA transfected cells (Fig. 7B). With IFN-γ/LIGHT dual cytokine treatment however, a dramatic increase of cell apoptosis was observed in both HepG2 and SMMC-7721 cells stably expressing Bcl-XL specific siRNA, compared with the mock or control siRNA transfected cells (~32% in Bcl-XL siRNA groups vs ~13% in controls) (Fig. 7C).

Discussion

In the present study, we found that cytokine LIGHT enhanced IFN-γ-mediated apoptosis of cells in a human HCC cell line, Hep3B. However, in other HCC cell lines with high levels of endogenous Bcl-XL such as HepG2 and SMMC-7721, IFN-γ/LIGHT failed to show a significant cytotoxicity effect. Hep3B cells gained more resistance to IFN-γ/LIGHT-induced apoptosis by overexpressing Bcl-XL protein while HepG2 and SMMC-7721 cells became more susceptible to IFN-γ/LIGHT-induced apoptosis by knocking-down Bcl-XL protein expression. IFN-γ/LIGHT-induced apoptosis in Hep3B cells involved LIGHT receptor LT-ß R, but not HVEM. The activation of caspase-9 and caspase-3, but not caspase-8, and the decrease of phosphorylation of STAT3 resulted in the down-regulation of Bcl-XL expression which appeared to be the most critical factor in IFN-γ/LIGHT-induced apoptosis.

The fact that neither LIGHT nor IFN-γ alone induced significant apoptosis in Hep3B cells, and that the combined treatment with these two cytokines induced a marked apoptotic effect indicated that the enhancement of IFN-γ-mediated apoptosis in Hep3B cells by LIGHT is probably a synergistic effect. However the mechanisms by which LIGHT synergizes with IFN-γ and Bcl-XL is down-regulated remain unclear. A previous study reported that LIGHT or IFN-γ/LIGHT induced lower intercellular adhesion molecule-1 (ICAM-1) expression in a STAT1-deficient fibrosarcoma cell line U3A than in 2fTGH fibrosarcoma cells (28). However, when U3A1-1 cells were STAT1 reconstituted, the LIGHT-induced ICAM-1 expression pattern was restored to a similar level as in 2fTGH (28). IFN-γ is known to induce the activation of JAK1 and JAK2 and subsequent activation of STAT1. STAT1 then induces the expression of the pro-apoptotic IRF-1 protein, which mediates apoptosis (29). Thus, LIGHT may enhance IFN-γ-mediated apoptosis through enhancing the activation of the STAT1 signaling pathway. Interestingly, STAT3 is another transcription factor which is up-regulated during IFN-γ treatment, and the activated STAT1 and STAT3 inhibit each other (30). STAT3 has also been shown to be decreased after IFN-γ/LIGHT treatment. It is possible that IFN-γ/LIGHT activates STAT1 synergistically, which then inhibits STAT3 activation and down-regulates the expression of Bcl-XL in HCC cells. Further studies are ongoing in our laboratories to examine this hypothesis as well as the related signal transduction interactions.

Although accumulating evidence shows that STAT3 contributes to oncogenesis by upregulating Bcl-XL, the NF-κB signaling pathway is also an important pathway to elevate the expression of Bcl-XL (31) and to prolong cell survival (32). The down-regulation of Bcl-XL mediated by IFN-γ/LIGHT may result from a decreased activity of NF-κB. Previous studies have shown that the suppression of NF-κB activation by IFN-γ contributes to HT-29 cell death induced by IFN-γ/LIGHT (18). It has also been shown that STAT3 and NF-κB act with each other to regulate the expression of the target gene. For example, during the formation of the STAT3 complex, NF-κB p65 is essential for the synergistic induction of the serum amyloid A (SAA) by IL-1+IL-6 stimulation (33). Thus, it is possible that there is a crosstalk between the

STAT3 and NF-κB pathway in the down-regulation of Bcl-XL induced by IFN-γ/LIGHT.

LT-ß R appears to be the receptor involved in LIGHT enhancement of the IFN-γ-mediated apoptosis in Hep3B cells, since in this study, only the LT-ß R blocking peptide, and not the HVEM blocking peptide, reversed the apoptosis induced by IFN-γ/LIGHT although both HVEM and LT-ß R were expressed in Hep3B cells. Studies on LIGHT receptors have shown a large diversity of their biological functions. A previous study reported that a prostatic adenocarcinoma cell line PC-3 which expresses LT-ß R but not HVEM was resistant to LIGHT-mediated cytotoxicity (12). However, in HT29 cells, LT-ß R was found to be necessary and sufficient for apoptosis induced by LIGHT (13), which is consistent with our findings. In this study, a high dose of LT-ß R blocking peptide did not completely block the apoptosis induced by IFN-γ/LIGHT, probably because the LT-ß R blocking peptide only inhibited the activity of LIGHT, but not that of IFN-γ. However, it is also possible that receptors other than LT-ß R may interact with LIGHT and transduce an apoptotic signal.

Death receptor, mitochondria and endoplasmic are the three major apoptotic pathways which have been identified so far (34,35). IFN-γ/LIGHT-induced apoptosis of HCC cells involves the mitochondria pathway, as shown by the activation of caspase-9 and the down-regulation of Bcl-XL in our study. On the other hand, caspase-8, an initiator of the death receptor pathway, showed no alteration in the apoptosis of HCC cells induced by IFN-γ/LIGHT. In addition, LIGHT cannot bind to the death receptor, and the cytoplasmic domain of LT-ß R does not contain consensus sequences homologous to the death domain. A previous study demonstrated that IFN-γ alone induced a slight activation of caspase-8 in A549 cells (36), which is consistent with our observation in Hep3B cells. Based on these findings, we conclude that IFN-γ/LIGHT may not transduce the apoptotic signal through the death receptor in Hep3B cells. It was also observed in our study that caspase-3 and -9 inhibitors did not inhibit the apoptosis completely. Aden *et al* (22) and Chen *et al* (37) proposed that a caspase-independent apoptosis pathway might exist through which reactive oxygen species (ROS) and other inducers could bypass the alteration of caspases.

Bcl-XL has been shown to antagonize apoptosis induced by various stimuli and play a strong protective role against apoptosis, thus it facilitates tumor formation, progression and resistance to therapies (38). In our study, Bcl-XL was down-regulated during apoptosis induced by LIGHT and IFN-γ in Hep3B cells. Enforced overexpression of Bcl-XL in Hep3B cells increased the resistance to IFN-γ/LIGHT. Moreover, a higher endogenous Bcl-XL level contributed to the resistance of HepG2 and SMMC-7721 cells to the apoptosis induced by LIGHT and IFN-γ, and the artificial knocking down of Bcl-XL expression in these two cell lines by RNA interference recovered the susceptibility to the dual cytokine treatment. We hence conclude that Bcl-XL plays a central and counter-active role in the apoptosis of HCC cells induced by IFN-γ/LIGHT.

Of clinical relevance, Bcl-XL is overexpressed in one-third of human HCC tissues and may function as an important apoptosis antagonist during tumor growth *in vivo* (39). Therefore, analysis of Bcl-XL expression may identify patients who are less likely to benefit from IFN-γ/LIGHT treatment but may be candidates suitable for alternative strategies such as blocking the activity of Bcl-XL.

In conclusion, LIGHT enhances IFN-γ-induced apoptosis of HCC cells in which Bcl-XL plays a central and counteractive role. Bcl-XL overexpression inhibits IFN-γ/LIGHT-induced apoptosis in Hep3B cells which have low levels of endogenous Bcl-XL, while Bcl-XL specific siRNA restores the sensitivity to IFN-γ/LIGHT treatment of HepG2 and SMMC-7721 cells which have high levels of endogenous Bcl-XL. These data provide important mechanistic insights into IFN-γ/LIGHT-induced apoptosis in HCC cells, and may help to select better therapeutic strategies for certain cancers with a distinct Bcl-XL expression.

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