Molecular Immunology 62 (2014) 96–103

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Herpesvirus entry mediator (HVEM) attenuates signals mediated by the lymphotoxin β receptor (LTβR) in human cells stimulated by the shared ligand LIGHT

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ARTICLE INFO

Article history:
Received 25 March 2014
Received in revised form 6 June 2014
Accepted 8 June 2014
Available online 28 June 2014

Keywords:
LIGHT
Herpesvirus entry mediator (HVEM)
Lymphotoxin beta receptor (LTβR)
TNF receptor associated factor (TRAF)
Apoptosis
NF-kappa B (NF-κB)

ABSTRACT

Signals mediated by members of the tumor necrosis factor receptor superfamily modulate a network of diverse processes including initiation of inflammatory responses and altering cell fate between pathways favoring survival and death. Although such pathways have been well-described for the TNF-α receptor, less is known about signaling induced by the TNF superfamily member LIGHT and how it is differentially altered by expression of its two receptors LTβR and HVEM in the same cell. We used cell lines with different relative expression of HVEM and LTβR to show that LIGHT-induced signals mediated by these receptors were associated with altered TRAF2 stability and RelA nuclear translocation. Production of the inflammatory chemokine CXCL10 was primarily mediated by LTβR. Higher expression of HVEM was associated with cell survival, while unopposed LTβR signaling favored pathways leading to apoptosis. Importantly, restoring HVEM expression in cells with low endogenous expression recapitulated the phenotype of cells with higher endogenous expression. Together, our data provide evidence that relative expression of HVEM and LTβR modulates canonical NF-κB and pro-apoptotic signals stimulated by LIGHT.

1. Introduction

The TNF receptor superfamily (TNFRSF) includes more than 25 receptors that interact with nearly 20 ligands to influence cellular responses (Schrofelbauer and Hoffmann, 2011). The best studied TNFRSF member, TNF-R1, can form at least two distinct signaling complexes after interacting with the ligand TNF-α, with functional outcomes in a cell dependent on a web of complex downstream interactions that may lead to diverse influences on cell survival (Silke, 2011). Other TNFRSF members have also been found to alter the balance of inflammatory and survival responses in certain cells, often in response to stimulation by different ligands (Watts, 2005).

The TNFRSF members lymphotoxin β receptor (LTβR) and herpesvirus entry mediator (HVEM) each interact with the pro-inflammatory molecule LIGHT (lymphotoxin-related inducible ligand that competes for glycoprotein D binding to HVEM on T cells) (Harrop et al., 1998; Mauri et al., 1998). LTβR and HVEM may also interact with different isoforms of lymphotoxin α (LTα), LTα1β2 or LTα3, respectively (Mauri et al., 1998; Ware, 2005), while HVEM but not LTβR also binds B- and T-lymphocyte attenuator (BTLA) and CD160 (Cai et al., 2008; Sedy et al., 2005). LTβR and HVEM are expressed in similar cell types, including epithelial cells and certain immune cells (Browning and French, 2002). LIGHT, LTα, LTβ, BTLA, and CD160 are produced by a variety of immune cells including macrophages, T cells, B cells, and NK cells (Cai et al., 2008; Sedy et al., 2005; Morel et al., 2000; Paya et al., 1988; Ware et al., 1992; Gramaglia et al., 1999; Gonzalez et al., 2005).

Studies of functional outcomes in cells after LTβR or HVEM engagement have generally focused on the individual receptors. Use of LTα1β2 or agonist antibodies to activate LTβR signaling leads to NF-κB activation, inflammatory cytokine production, and growth arrest or cell death in some but not all LTβR-positive cells (Mackay et al., 1996; Degli-Esposti et al., 1997; Browning et al., 1996). Similarly, a mutant form of LIGHT capable of binding HVEM but not LTβR does not activate cell death pathways (Rooney et al., 2000),

Abbreviations: LIGHT, lymphotoxin-related inducible ligand that competes for glycoprotein D binding to HVEM on T cells; HVEM, herpesvirus entry mediator; LTβR, lymphotoxin beta receptor; TNFRSF, TNF receptor superfamily; BTLA, B- and T-lymphocyte attenuator.

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http://dx.doi.org/10.1016/j.molimm.2014.06.013
0161-5890/

while an analogous mutant capable of binding LTβR but not HVEM induces cell death (Chen et al., 2003). Using HVEM-specific agonists, signaling through this receptor promotes survival in epithelial cell lines (Cheung et al., 2009). These studies generally used specific agonists of either LTβR or HVEM, and did not focus on the combined effect of signaling through both molecules on the responding cell at the same time with the same agonist.

Upon ligand interaction, the intracellular domains of LTβR and HVEM bind TNF receptor associated factor (TRAF) family members (Kim et al., 2005), specifically TRAF2 (Cheung et al., 2009), which acts as a central hub for activation and inhibition of NF-κB, JNK, and caspase 8 (Ea et al., 2006; DiDonato et al., 1997). While TRAF2 signaling itself may not have a strong biological effect, TRAF2 activation or degradation can synergize with other signals, such as those stimulated by IFN-γ or TNF-α. For example, TRAF2-activated NF-κB binds the NF-κB promoter element of CXCL10, but does not itself drive CXCL10 production. The CXCL10 promoter contains two elements, an NF-κB binding element and interferon stimulated response element (ISRE) (Clarke et al., 2010). After TNF-α and IFN-γ treatment, STAT1 and TRAF2-activated NF-κB bind the promoter of CXCL10 and synergistically activate transcription of CXCL10 (Yeruva et al., 2008). Similarly, degradation of TRAF2 is insufficient to activate caspase 8 to promote apoptosis; other signals, such as those mediated by TNF-α, are required (Vince et al., 2008).

Given the complexities of TNFRSF signaling and the overlapping ligands and signal transduction pathways used by LTβR and HVEM, we studied the effect of co-expression of these receptors on LIGHT-induced signals in human cell lines. We show here that, consistent with prior studies, LIGHT induces chemokine production and pro-inflammatory signals in cells in which LTβR expression dominates that of HVEM, leading to chemokine production, TRAF2 degradation, caspase 8 activation, and polyADP ribose polymerase (PARP) cleavage. In cells with balanced LTβR and HVEM expression, TRAF2 stability is increased, RelA nuclear translocation is decreased, and there is less caspase cleavage, favoring cell survival. Thus, cells may vary expression of the different surface receptors detecting LIGHT to regulate overlapping signaling pathways that modulate cell fate during inflammatory responses.

2. Materials and methods

2.1. Cell lines, media and reagents

HeLa, HT-29, 293T, and U937 cells were maintained in 1 × DMEM with 10% FCS. Cells were treated with 10 ng/mL recombinant human IFN-γ, 10 ng/mL TNF-α, 100 ng/mL recombinant human LIGHT (Peprotech), or no stimulation. Plasmids used included the pBEC10 plasmid expressing HVEM (Montgomery et al., 1996), NF-κB and ISRE luciferase reporter plasmids (pNF-κB-Luc and pISRE-Luc, Agilent Technologies), and the full ORF of LIGHT cloned into pcDNA3 (Mauri et al., 1998). Mutations in the LIGHT ORF (G119E, R228E, and G119E/R228E) were introduced by quick-change mutagenesis.

2.2. Receptor quantitation

Cells and anti-mouse calibration beads (Bang Laboratories) were incubated with anti-trinitrophenyl (anti-TNP), anti-HVEM (Santa Cruz) or anti-LTβR (Biolegend) for 45 min in 1% BSA in PBS. Following incubation, the cells were washed and incubated with anti-mouse Alexafluor 647 (Invitrogen) for 45 min in 1% BSA in PBS. The cells were washed with PBS and fluorescence measured per cell. Receptor number per cell was calculated using a standard curve generated from calibration beads, according to the manufacturer instructions. This method provides quantitative assessment of each receptor from individual calibration curves, allowing direct comparison of surface levels for different proteins, and is not influenced by differences in fluorescence of antibodies used for detection of different proteins (as might occur with measurements of direct fluorescence intensity). Similar methods have been previously applied to determination of surface levels of viral entry receptors (Krummenacher et al., 2004).

2.3. Luciferase reporter assay

HT-29 or HeLa cells were plated into a 6 well plate at 1×10^6 cell per well. Cells were transfected with either pNF-κB-Luc or pISRE-Luc using Lipofectamine 2000 (Invitrogen) according to manufacturer instructions and incubated overnight. The following day the cells were split into 12 well plates and incubated overnight. After incubation, the cells were treated with either 10 ng/mL IFN-γ, 100 ng/mL LIGHT, both in combination, or left untreated. After 24 h, the cells were washed with PBS containing 0.1 g/L $MgCl_2$ and 0.1 g/L $CaCl_2$ (PBS-ABC) and lysed in 1 × passive lysis buffer (Promega). Firefly and Renilla luciferase activity was measured from lysates using the Promega Dual Luciferase Reporter Assay.

2.4. Cytokine measurements

HeLa, and HT-29 cells were plated in 12 well plates at a density of 3×10^5 cells per well overnight. The following day the cells were treated with either 10 ng/mL IFN-γ, 100 ng/mL LIGHT, both in combination, or left untreated. After 24 h, the supernatants were harvested and spun down at 14,000 × *g* for 5 min to pellet cell debris. CXCL10 (R&D systems or Peprotech) was detected from supernatants by ELISA according to manufacturer instructions.

2.5. Nuclear fractionation

1×10^7 HT-29 or HeLa cells were treated with or without 100 ng/mL LIGHT for 24 h. The cells were washed with PBS-ABC and lysed in 500 μL of cytoplasmic extraction buffer (10 mM KCl, 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) and placed on ice for 20 min. 25 μL of 10% NP-40 was added to each lysate, and samples were vortexed and then centrifuged at 14,000 × *g* for 10 min. The supernatants were removed and the pellet containing the nuclei resuspended in 100 μL of nuclear extraction buffer (0.4 M NaCl, 20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA) and incubated for 30 min on ice. The samples were spun at 14,000 × *g* for 10 min and the supernatants were collected. To concentrate the nuclear fraction, 400 μL dH_2O, 500 μL methanol, and 200 μL chloroform were added and samples were vortexed, centrifuged for 10 min at 14,000 × *g*, and the upper layer discarded above the phase separation. 400 μL of methanol was added, samples were again vortexed and centrifuged at 14,000 × *g* for 10 min, and supernatants were discarded and the pellet resuspended in 50 μL of lysis buffer.

2.6. Western blot analysis

Nuclear fractions were electrophoresed on a 10% polyacrylamide gel. Samples were transferred to nitrocellulose and blocked in 5% milk in 1 × TBST (50 mM Tris.HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Blots for RelA were incubated in 5% milk in 1 × TBST with 1:500 rabbit anti-RelA (Biolegend). Blots for JNK were incubated with 5% BSA in 1 × TBST with 1:250 rabbit total JNK (Cell Signaling) or 1:250