Modulation of TNFSF expression in lymphoid tissue inducer cells by dendritic cells activated with Toll-like receptor ligands

Sinsuk Han, Jihye Koo, Jingyu Bae, Soochan Kim, Song Baik & Mi-Yeon Kim*
Department of Bioinformatics and Life Science, The College of Natural Science, Soongsil University, Seoul 156-743, Korea

INTRODUCTION

Toll-like receptors (TLRs), which recognize structurally conserved components among pathogens, are mainly expressed by antigen-presenting cells such as dendritic cells (DCs), B cells, and macrophages. Recognition through TLRs triggers innate immune responses and influences antigen-specific adaptive immune responses. Although studies on the expression and functions of TLRs in antigen-presenting cells have been extensively reported, studies in lymphoid tissue inducer (LTI) cells have been limited. In this study, we observed that LTI cells expressed TLR2 and TLR4 mRNA as well as TLR2 protein and upregulated OX40L, CD30L, and TRANCE expression after stimulation with the TLR2 ligand zymosan or TLR4 ligand LPS. The expression of tumor necrosis factor superfamily (TNFSF) members was significantly upregulated when cells were cocultured with DCs, suggesting that upregulated TNFSF expression may contribute to antigen-specific adaptive immune responses. [BMB reports 2011; 44(2): 129-134]

RESULTS

Expression of TLRs on LTI cells, DCs, B cells, and macrophages

To determine whether or not LTI cells express TLRs, the mRNA expression of myeloid differentiation primary response gene 88 (MyD88), TLR2, TLR4, and TLR9 was examined (Fig. 1A). Since MyD88 is an adapter protein used by all TLRs except TLR3, its expression was measured and detected in all of the cells. LTI cells showed the highest expression of MyD88 compared to DCs, B cells, and macrophages, indicating that LTI
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TLR2, TLR4, and TLR9. In comparison, LTi cells expressed TLR2 and TLR4 but not TLR9.

Although LTi cells express TLR4 mRNA, their response to LPS is not definite since they do not express CD14, which promotes LPS binding to TLR4 (2). Therefore, the protein expression of LTi cells was examined by flow cytometry (Fig. 1B). The expression levels of TLR2 and TLR4 on LTi cells were 42.1% and 8.0%, respectively, whereas the expression levels on antigen-presenting cells were approximately two times higher than those on LTi cells. The expression levels of TLR2 and TLR4 on DCs were 74.0% and 17.8%, those on B cells were 95.4% and 30.7%, and those on macrophages were 94.9% and 37.4%, respectively.

Effects of zymosan and LPS on TNFSF expression on LTi cells
To determine the effects of ligands for TLR2 (zymosan) and TLR4 (LPS) on LTi cells, splenocytes were stimulated with zymosan or LPS, and TNFSF protein expression on LTi cells was analyzed. LTi cells cultured with zymosan upregulated the expression of OX40L (from 40.3% to 63.3%), CD30L (from 61.9% to 80.5%), and TRANCE (from 78.0% to 84.4%) (Fig. 2A). Surprisingly, LTi cells cultured with LPS upregulated TNFSF expression to a level comparable or slightly higher than those cultured with zymosan; OX40L+ cells were 68.9%, CD30L+ cells were 82.5%, and TRANCE+ cells were 84.3%. The same results were observed in an in vivo experiment (Fig. 2B). LTi cells isolated from zymosan- or LPS-injected mice showed higher expression of OX40L, CD30L, and TRANCE molecules than those isolated from PBS-injected control mice.

However, when purified LTi cells were stimulated with LPS or zymosan, the cells did not upregulate TNFSF expression (data not shown). In addition, the number of LTi cells in the presence or absence of TLR ligands did not change (Fig. 3), whereas those of DCs, B cells, and macrophages increased after activation with TLR ligands, except for that of DCs stimulated with zymosan.

Fig. 1. TLR expression on LTi cells, DCs, B cells, and macrophages. (A) mRNA expression of MyD88, TLR2, TLR4, and TLR9. Relative mRNA expression of genes was normalized to β-actin signals. The results show the average expression of three separate experiments. (B) Flow cytometric analysis of TLR2 and TLR4 expression on LTi cells, DCs, B cells, and macrophages. Numbers above bracketed lines in histograms indicate the percent of positive staining for each Ab. Filled histograms show isotype-matched control Abs. Data are representative of five experiments.

Fig. 2. Effects of zymosan and LPS on TNFSF expression on LTi cells. (A) Flow cytometry analysis of OX40L, CD30L, and TRANCE expression on LTi cells cultured with zymosan (50 μg/ml) or LPS (1 μg/ml) in vitro. Numbers above bracketed lines in histograms indicate the percent of positive staining for each Ab. Filled histograms show isotype-matched control Abs. Results are representative of seven separate experiments. (B) Flow cytometry analysis of surface expression of LTi cells in vivo. Mice were injected intraperitoneally with zymosan (150 μg), LPS (5 μg), or PBS as a control. Three hours after zymosan injection or 18 hours after LPS injection, the spleens were taken and analyzed for the expression of OX40L, CD30L, and TRANCE on LTi cells. Filled histograms show isotype-matched control Abs. Results are representative of three separate experiments.

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Fig. 3. Effects of TLR ligands on cell numbers of DCs, B cells, macrophages, and LTi cells. Splenocytes were cultured with zymosan (50 μg/ml) or LPS (1 μg/ml) in vitro for 18 hours, and the absolute cell number of each cell population was calculated. Y axis shows the percentage of absolute cell numbers compared to the control, which was cultured without TLR ligands (100%). The results are the average of three separate experiments. Error bar shows the standard deviation.

Fig. 4. TNFSF expression on LTi cells cocultured with DCs, B cells, macrophages, or macrophage-culture supernatant (S/N) in the presence of zymosan (50 μg) or LPS (1 μg) for 18 hours. OX40L (A), CD30L (B), and TRANCE (C) expression on LTi cells was analyzed by flow cytometry. Axes show the mean fluorescence intensity (MFI) of TNFSF expression. The results are average of four separate experiments. Error bar shows the standard deviation.

TNFSF expression on LTi cells cocultured with TLR-expressing cells
To investigate which cells influence TNFSF expression on LTi cells, LTi cells were cocultured with TLR-expressing DCs, B cells, macrophages, or macrophage-culture supernatant in the presence of zymosan or LPS (Fig. 4). When LTi cells were cocultured with DCs, TNFSF expression was markedly increased; OX40L expression on LTi cells cultured with DCs was increased 2.2 fold, as determined by mean fluorescence intensity (MFI) (from 293 to 631), in the presence of zymosan, and 2.3 fold (from 254 to 581) in the presence of LPS (Fig. 4A). CD30L expression was increased 4.0 fold (from 121 to 479) in the presence of zymosan and 2.8 fold (from 168 to 470) in the presence of LPS (Fig. 4B). TRANCE expression was increased 2.2 fold (from 258 to 579) in the presence of zymosan and 2.7 fold (from 209 to 554) in the presence of LPS (Fig. 4C). In comparison, when LTi cells were cocultured with B cells, alteration of expression was minimal; OX40L and TRANCE expression was increased 1.3-1.4 fold in the presence of LPS, whereas CD30L expression was increased 1.4 fold in the presence of zymosan. In addition, coculture with macrophages or macrophage-culture supernatant, which contains large amounts of inflammatory cytokines, did not produce significant changes.

The upregulation of TNFSF molecules on LTi cells cultured with DCs led us to examine the expression of TNFRSF molecules, OX40, CD30, and RANK, on DCs in the presence of zymosan or LPS. However, upon stimulation with zymosan or LPS, DCs did not modulate their expression of TNFRSF molecules (data not shown), although the expression of costimulatory molecules, CD40, CD80, and CD86, was significantly upregulated, as previously reported (19, 20). In addition, it was confirmed that DCs activated with zymosan or LPS upregulated IL-6 and IL-23 expression, as previously reported (21, 22).

DISCUSSION
TLRs are expressed on antigen-presenting cells such as DCs, B cells, and macrophages, and on activated/memory T cells (23, 24). After recognizing conserved microbial components through TLRs, cells trigger innate immune responses by producing proinflammatory cytokines and influencing Th1 and Th17 responses and antibody responses (1-5). Although the function of TLRs in antigen-presenting cells has been extensively studied, the expression and function of TLRs in splenic LTi cells are not well known. Recently, it was reported that human LTi-like cells express IL-5, IL-13, and IL-22 after activation with TLR2 ligands (18), and mouse LTi-like cells in the spleen and mucosa produce IL-17 and IL-22 upon stimulation with TLR5 ligand (17).

In this study, we investigated whether or not LTi cells isolated from mouse spleen express TLRs and respond to TLR
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ligands. We found that LTi cells expressed mRNA for MyD88, TLR2, and TLR4 but not TLR9. However, the cells did not express TLR4 on their surface, indicating that LTi cells may respond to TLR2 ligands such as zymosan, Pam3CSK, and peptidoglycan. To determine whether or not TLR2 and TLR4 on LTi cells respond to TLR ligands, splenocytes were cultured with the TLR2 ligand zymosan or TLR4 ligand LPS. LTi cells cultured with zymosan or LPS significantly upregulated TNFSF expression, suggesting that LTi cells responded to microbial components.

Since TLR4 was not detected on the LTi cell surface and the cell number did not increase after activation with zymosan or LPS, it is possible that other cells capable of responding to TLR ligands influenced protein expression on LTi cells. To examine this possibility, LTi cells were cocultured with TLR-expressing, antigen-presenting cells. The results show that the cells cocultured with DCs remarkably upregulated TNFSF expression, whereas those cocultured with other cells did not exhibit altered expression. These data indicate that DCs activated with TLR ligands strongly influenced TNFSF expression on LTi cells. Although DCs did not upregulate TNFRSF expression, it is possible that cytokines secreted by DCs influenced LTi cell surface expression. It is known that OX40L and TRANCE expression on LTi cells is upregulated by TL1A, a TNF-like ligand (25), whereas DCs activated with TLR ligands do not induce TL1A; instead DCs stimulated through FcγR induce TL1A (26). In addition, IL-7 upregulates CD30L on LTi cells, whereas DCs do not express IL-7 and require a 5-day culture (27). Although many studies have shown that DCs activated with TLRs express IL-23 (21, 22), we confirmed that IL-23 does not upregulate OX40L expression (28) but instead induces a large amount of IL-22 secretion to promote TH17 responses. This suggests that DCs activated with TLR ligands modulate TNFSF expression on LTi cells by another mechanism that remains to be determined.

Taken together, these data suggest that LTi cells respond to microbial components of TLR2 and TLR4 in a DC-dependent manner and that their upregulated TNFSF expression may influence adaptive immune responses.

MATERIALS AND METHODS

Preparation of cells
All experiments were performed in accordance with the regulations of the Soongsil University Institutional Animal Care and Use Committee. Cell suspensions for the isolation of LTi, DCs, and B cells were made from the spleens of 6-week-old C57BL/6 mice, as described previously (27). Briefly, spleens were cut into small fragments and then cultured in serum-free RPMI containing 5 mg/ml of collagenase/dipase (Roche, Mannheim, Germany) for 45 min at 37°C. Digested fragments were crushed between gauze. After depletion of red blood cells, cell suspensions were cultured with 1 μg/ml of LPS (Sigma-aldrich, St. Louis, MO) or 50 μg/ml of zymosan (Sigma-aldrich) for 18 hours.

Coculture with DCs, B cells, or macrophages
To coculture LTi cells with DCs or B cells, splenocytes were positively enriched using MACS anti-mouse CD11c or anti-mouse B220 microbeads (Miltenyi Biotec Ltd., Bergisch Glandbach, Germany), respectively. A total of 1 × 10^6 CD11c or B220-depleted cells as LTi cells were cocultured with 1 × 10^6 CD11c+ DCs or 1 × 10^6 B220+ B cells. To coculture LTi cells with macrophages, 6 × 10^6 spleenocytes were cocultured with 1 × 10^6 RAW264.7 cells. To culture cytokines secreted by macrophages, RAW264.7 cells were cultured with LPS or zymosan for 18 hours, after which the culture supernatant was added to the LTi cell culture at a ratio of 1:1.

Flow cytometry
mAbs for CD4 (clone GK1.5), CD8 (clone 53-6.7), and B220 (clone RA3-6B2) were purchased from BD Biosciences (San Jose, CA), and mAbs for CD3 (clone 145-2C11), CD11c (clone HL3), F4/80 (clone BM8), IL-17A, TLR2 (clone 6C2), and TLR4 (clone MT5510) were purchased from ebioscience (San Diego, CA). mAb for IL-22 (clone 140301) was obtained from R&D systems (Minneapolis, MN). Biotinylated mAbs against OX40 (clone OX-86), OX40L (clone RM134U), CD30 (clone mCD30.1), CD30L (clone RM153), RANK (clone R12-31), and TRANCE (clone IK22/5) were obtained from ebioscience. As the second-step staining reagents for biotinylated mAbs, streptavidin-phycocerythrin was purchased from BD Biosciences.

In order to detect IL-22 and IL-17A, cells were cultured for 4 hours in the presence of Golgi-stop™ (BD Biosciences) and then stained with surface antibodies for CD3, CD4, CD11c, and B220, followed by intracellular IL-22 or anti-IL-17A (eBioscience) using a Cytotox/Cytopem™ kit according to the manufacturer's instructions (BD Biosciences).

Quantitative PCR
For mRNA expression analysis, spleen cell suspensions were FACs sorted into CD3+ CD11c- B220+ CD4+ cells for LTi cells, CD11c+ cells for DCs, B220+ cells for B cells, and F4/80+ cells for macrophages using a MoFlo cell sorter (Cytomation, Fort Collins, CO). Sorted cells were then prepared for quantitative real-time PCR of cDNA (Takara, Shiga, Japan) as described previously (28). The relative amount of β-actin signals was calculated as 2^-ΔΔCt × 10^4. The specific primer sequences were synthesized by Bioneer (Daejeon, Korea).

β-actin (forward CGTGGAAAAGATGACCCAGATCA) (reverse TGTAGCAGCAGAAGGGCATAG)

MyD88 (forward AGAACAGACAGACTATCGGCT) (reverse CGCGCACACCTTTTCTCAAT)

TLR2 (forward GCAAACGCTGTTCTGCTCAG) (reverse AGTCGCTCCCTCTATTGTATT)

TLR4 (forward AGTCGCTCCCTCTATTGTATT) (reverse GA TGCCATTGTGTCCACCA)

TLR9 (forward ATGTTTCCTCCGTCGAAGGACT) (reverse G
AGGCTTCAGCTCACAGGG)

Injection of LPS or zymosan
Mice were injected intraperitoneally with 5 μg of LPS or 150 μg of zymosan in 200 μl of PBS. Three hours after zymosan injection or 18 hours after LPS injection, spleens were taken for analysis.

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