CXCL13 production by an established lymph node stromal cell line via lymphotoxin-beta receptor engagement involves the cooperation of multiple signaling pathways

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Abstract

Non-hematopoietic mesenchymal stromal cells in secondary lymphoid organs play pivotal roles in tissue organization and immune responses by exhibiting specialized features such as the production of lymphoid homeostatic chemokines. However, the maturational process of stromal cells mediated by lymphotoxin-beta receptor (LTβR) signaling, a key for stromal maturation, remains unclear. Taking advantage of a stromal cell line established from mouse lymph node, which can produce a homeostatic chemokine, CXC chemokine ligand (CXCL) 13, by the engagement of LTβR but not by tumor necrosis factor (TNF) receptor (TNFR), we analyzed the details of intracellular signaling events during the maturational process. The activation of both canonical and non-canonical nuclear factor-κB (NF-κB) pathways was essential for CXCL13 induction; however, an excessive amount of non-canonical RelB–p52 complex was still insufficient for CXCL13 gene expression. Under RelB–p52-over-expressed conditions, TNFα could induce a markedly high amount of CXCL13 production, indicating that the downstream of TNFR contains an additional key component of signaling. We also found that protein kinase C activity plays a critical role in this process in addition to the NF-κB pathways. Taken together, it is suggested that the maturation of lymphoid stromal cells mediated by LTβR is accomplished by the cooperation of multiple signaling cascades.

Introduction

Secondary lymphoid organs (SLOs) are important anatomic locations in which lymphocytes are accumulated for the induction of efficient adaptive immune responses (1). Several distinct types of non-hematopoietic mesenchymal cell lineages designated as stromal cells support the tissue architecture of the SLO (2–5). Recent findings have compelled researchers to consider that stromal cells play pivotal roles in immune responses by producing various factors, including cytokines, chemokines and adhesion molecules, which regulate the motility and homeostasis of immune cells (6–8). In addition, stromal cells seem indispensable for the organogenesis and tissue remodeling of SLOs (9). However, the molecular and cellular biological nature of these cells remain poorly understood.

Many studies using knockout mice have indicated that signals directing the activation of transcription factor nuclear factor-κBs (NF-κBs) mediated by lymphotoxins (LTs) and tumor necrosis factor (TNF) α are essential for the organogenesis and construction of tissue architecture of SLOs (9–11). The NF-κB family consists of five members, RelA, RelB, cRel, NF-κB1 (p50) and NF-κB2 (p52), which function as heterodimers composed of combinations of these members (11). TNF receptor (TNFR) transmits a signal that leads to the phosphorylation and degradation of inhibitor of NF-κB (IκB), which in turn triggers the translocation of RelA–p50 complex from the cytoplasm to nucleus (canonical pathway) and initiates a broad spectrum of inflammatory gene expression (11, 12). On the other hand, lymphotoxin-beta receptor (LTβR), a specific receptor for LTα1β2, triggers the phosphorylation and processing of p100 (NF-κB2 gene product) followed by the generation of p52 via NF-κB-inducing kinase (NIK) and IκB-kinase α and finally activates RelB–p52...
complex (non-canonical pathway) in addition to the canonical pathway (11, 13). The non-canonical pathway is known to regulate a restricted set of genes, including CXC chemokine ligand (CXCL) 13, which is a crucial chemokine for the organogenesis of SLOs (13–15). It is widely believed that lymphoid tissue inducer cells or lymphocytes, which produce LTα and/or TNFα, transmit a signal to stromal cells expressing LTβR and TNFαR followed by triggering the NF-κB-dependent expression of responsible genes required for SLO formation (9); however, this scenario is still hypothetical because it is assembled from evidence based on the phenotypical analysis of various knockout mice and huge numbers of biochemical examinations. Virtually no comprehensive verification of the cascades has been demonstrated in the strictly defined context of stromal cells as a sequential event from receptor engagement to gene expression and the production of functional protein. It is also unclear whether target genes of the non-canonical pathway such as CXCL13 could be induced simply by p100 processing and subsequent generation of the RelB–p52 complex or by the consequence of a cooperative effect with additional signaling components.

We previously established stromal cell lines from the mouse lymph node and showed that they preserved several features of stromal cells in SLOs (16, 17). One of these cell lines, BLS12, had a unique property to secrete CXCL13 efficiently upon LTβR engagement and closely resembled a recently identified stromal subset common to SLOs or organizer stromal cells in the anlagen (18); therefore, this cell line provides an opportunity to analyze the detailed activity inside lymphoid stromal cells. In this study, we took advantage of BLS12 cells to examine the LTβR-induced intracellular cascade toward the expression of CXCL13 and showed that protein kinase C (PKC) activity, as well as the activation of NF-κB pathways, play a critical role in this process. Our findings suggest that the maturation of lymphoid stromal cells mediated by LTβR is accomplished by the cooperation of multiple signaling pathways.

Materials and methods

Cell culture

BLS12 cells were maintained in 10% FCS DMEM medium supplemented with antibiotics as described previously (16). BLS12 cells stably transfected with IκBαSR or NIK have also been described (16, 18). Cells were stimulated with 10 ng ml–1 murine TNFα (Peprotech, Rocky Hill, NJ, USA) and/or 0.5–0.75 μg ml–1 goat anti-mouse LTβR antibody (R&D Systems, Minneapolis, MN, USA) for several hours or days. For pharmacological inhibition of PKC activity, cells were incubated with medium containing various concentrations of bisindolylmaleimide-I (BIM-I), Go6983, Go6976 and Ro-32-0432 (Calbiochem/Merck, Darmstadt, Germany) for 1–24 h or myristoylated pseudosubstrates (PS) for PKCα/β (Calbiochem) or PKCζ/λ (Biosource/Invitrogen, Carlsbad, CA, USA) for 24 h.

Retrovirus-mediated gene transfer

BLS12 cells were transfected with genes using a pMX-puro or -hyg retrovirus vector system (16, 19). pMX-hyg vector was constructed by replacing the puromycin resistance gene with the hygromycin resistance gene cassette. pMX-puro-p100AGRR was provided by D.V. Novack (20). Complementary DNA (cDNA) encoding human p52 was excised from p100AGRR by EcoRI and XhoI digestion and sub-cloned into pCMV-3Tag vector (Stratagene, La Jolla, CA, USA) in frame with a C-terminal 3xMyc tag. Subsequently, p52-Myc cDNA fragment was ligated into pMX-puro. Mouse RelB cDNA provided by J. Stavnezer (21) was sub-cloned into pCMV-3Tag vector in frame with an N-terminal 3xFlag tag and Flag-RelB fragment was subsequently ligated into pMX-hyg. BLS12 cells infected with retroviruses were selected by 5–10 μg ml–1 puromycin or 130 μg ml–1 hygromycin.

ELISA

CXCL13 produced by BLS12 cells in culture supernatants was detected using a sandwich ELISA system (Duoset; R&D Systems) according to the manufacturer’s recommendations.

Reverse transcription–PCR

Total RNA was extracted from BLS12 cells using TRIzol reagent (Invitrogen) and cDNA was synthesized using SuperScript III reverse transcriptase and oligo(dT)12-18 primer (Invitrogen). Four- or five-fold serial dilutions of cDNA were amplified by PCR with ExTag DNA polymerase (Takara Bio, Otsu, Japan) and the following specific primer pairs: GAPDH, 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTACCA-CCTTCCTG-3′; CXCL13, 5′-TTGAACCTCACCTCCAGGCA-3′ and 5′-CTTCAGGCACTCTTCCTTT-3′; CXCL10, 5′-AAACCGTACGCTGACT-3′ and 5′-TTACCTGTTCAGCCTTTCTC-3′; RelB, 5′-GCAGAAAGATGTTAAGGAGCCCTTTTA-GAC-3′; CXCL12, 5′-AAACACGTACGTGACT-3′ and 5′-TTACCTGTTCAGCCTTTCTC-3′; NIK, 5′-ATGAAAGGAGGT-CCACAAAC-3′ and 5′-GGAAGCGGGAAGAATCAG-3′; NF-κB2, 5′-GCCTAGCCAGAGATGGA-3′ and 5′-GCAGGACACCCAGATTATA-3′; NIK, 5′-ATGAAAGGAGGT-CCACAAAC-3′ and 5′-GGAAGCGGGAAGAATCAG-3′; NIK, 5′-TACCCAGATGTAGATCCAGG-3′ and 5′-AGATCTTGT-GGCCCTGTCAGA-3′.

Western blotting

Whole-cell lysates were prepared by lysing 1 × 106 cells in 200 μl radio immuno protein assay (RIPA) buffer; 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonylfluoride, 1% Nonidet P-40, 50 mM NaF, 1% sodium deoxycholate, 0.5 mM Na2VO4, 1 μg ml–1 aprotinin, 1 μg ml–1 leupeptin and 1 μg ml–1 pepstatin A. After insoluble materials were removed by centrifugation, lysates were boiled with the appropriate amount of SDS-PAGE loading buffer. Equal amounts of total cell lysates were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA). The membranes were soaked in a blocking solution (1% skim milk and 0.05% Tween 20–PBS) for 1 h and then incubated with primary antibodies for 1 h. After washing with Tween 20–PBS, membranes were incubated with appropriate HRP-conjugated secondary antibodies (The Jackson Laboratory, Bar Harbor, ME, USA) for 1 h. Specific bands were visualized by an ECL-Plus (GE Healthcare, Little Chalfont, UK). NF-κB p52 (sc-298), RelB (sc-226), RelA (sc-109) and tubulin (sc-5286) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).
CA, USA). Phospho-p44/42 MAP kinase (Thr202/Tyr204), p44/42 MAP kinase, iκBα and phospho-iκBα (Ser32) antibodies were from Cell Signaling Technology (Danvers, MA, USA), PKCα and PKCδ antibodies were from BD Biosciences (San Jose, CA, USA). Lamin-B1 antibody was from Zymed/Invitrogen.

**Immunoprecipitation**

Cells were lysed with RIPA buffer on a rotator for 1 h at 4°C. After insoluble materials were removed by centrifugation, the soluble supernatants were precleared with protein G Sepharose 4 Fast Flow (GE Healthcare). Samples were then immunoprecipitated with anti-Myc antibody (MBL, Nagoya, Japan) and 20 μl of protein G Sepharose beads. Protein G-bound protein complexes were washed intensively with RIPA buffer and boiled in loading buffer for SDS-PAGE. Immunoprecipitated proteins were detected by western blotting.

**Results**

LTβR-dependent CXCL13 production by BLS12 cells requires both canonical and non-canonical NF-κB pathways

Our previous study demonstrated that the treatment of BLS12 cells with an agonistic anti-LTβR antibody can trigger the production of CXCL13 in the culture supernatant (18) (Fig. 1A). The addition of TNFα markedly enhanced anti-LTβR antibody-dependent CXCL13 production, although TNFα alone had no effect. The production of CXCL13 protein well correlated with the mRNA expression in this system (Fig. 1B). In contrast, an inflammatory chemokine, CXCL10, a typical target gene of the canonical pathway, was induced by TNFα or anti-LTβR antibody, while CXCL12 was constitutively expressed in BLS12 cells.

We next examined the intracellular signaling components at several time points after BLS12 stimulation (Fig. 1C). As is well known, TNFα induced the rapid phosphorylation and degradation of iκBα (20 min after stimulation in our assay), and thereafter, the protein level recovered within an hour as a result of the up-regulation of iκBα gene expression (11), indicating transient activation of the canonical pathway after TNFR engagement. No remarkable enhancement of p100 processing coupled with the production of p52 was observed by TNFα. Instead, p100 and RelB were clearly augmented after 6 h of TNFα treatment. By contrast, LTβR engagement led to weak phosphorylation and slight reduction of iκBα at 20 min, while p100 processing and p52 generation, as well as a slight increase of RelB, were observed after 6 h. Strikingly, simultaneous stimulation with TNFα and anti-LTβR antibody resulted in the further accumulation of p100, RelB and p52. Collectively, since the canonical pathway is known to regulate the expression of RelB and NF-κB2 (p100/p52) genes, strong activation of the canonical pathway by TNFα could enhance RelB and p100 expressions without the production of p52. In contrast, LTβR signaling induced intense activation of the non-canonical pathway but weak activation of the canonical pathway. Simultaneous stimulation of TNFR and LTβR exhibits a synergistic effect on the activation of both pathways.

To clarify the role of the canonical pathway, a phosphorylation-deficient mutant of iκBα (iκBαSR), which is known to block the nuclear translocation of RelA–p50 but not RelB–p52 complex, was introduced into BLS12 cells by retrovirus vector. In transfected cells, stimulation-induced expressions of CXCL13 and CXCL10 were completely suppressed (Fig. 2A and B). Importantly, the basal expressions of both RelB and NF-κB2 under steady-state conditions were significantly reduced, whereas the expressions of LTβR and NIK were unchanged (Fig. 2B). Basal protein levels of RelB and p100 were also markedly diminished in this transfectant (Fig. 2C), indicating that the canonical pathway is essential for basal expression as well as stimulation-dependent up-regulation of RelB and NF-κB2. Therefore, as some reports have already suggested (12, 13, 15), regulating the production of the non-canonical pathway components is one of the important roles of the canonical pathway. We next introduced p100AGRR, a processing-resistant mutant of p100, into BLS12 cells to block the non-canonical pathway. In this transfectant, CXCL13 production was strongly inhibited, while CXCL10 was significantly
induced especially in TNFα + anti-LTβR antibody treatment, demonstrating that CXCL13 but not CXCL10 expression is affected by p100ΔGRR (Fig. 3). The p100ΔGRR transfectant stimulated by TNFα or anti-LTβR antibody showed slightly lower expression of CXCL10 compared with the control cells. This might reflect potentially inhibitory effect of p100 to the canonical pathway under a certain condition (20, 22). Together, these results indicate that the activation of both canonical and non-canonical pathways play indispensable roles in CXCL13 induction.

Excessive RelB and p52 are still insufficient for CXCL13 induction and an additional signaling pathway seems to be required

In general, RelA, RelB and p50 proteins are present abundantly in the cytoplasm, whereas the amount of p52 protein is maintained in relatively low levels in a steady state and only raised by adequate stimuli that trigger the processing of the precursor protein p100 (13, 15, 23), which would be a rate-limiting step for the activation of the non-canonical pathway. To address whether p100 processing followed by the generation of p52 itself is sufficient for LTβR-dependent CXCL13 production, BLS12 cells were infected with
a retrovirus vector encoding p52, which is expected to bypass the p100 processing step; however, such a transfectant did not produce CXCL13 under unstimulated conditions (Fig. 4), suggesting that the generation and accumulation of p52 alone is insufficient for CXCL13 induction. It is worth noting that anti-LTβR antibody or TNFα + anti-LTβR antibody led to a markedly high level of CXCL13 secretion by this transfectant compared with control cells, indicating that there is a clear enhancing effect on CXCL13 gene expression by p52 over-expression. More interestingly, treatment of TNFα alone also induced a large amount CXCL13 production in p52-transfected BLS12 cells, similar to TNFα + anti-LTβR antibody treatment. Under these conditions, the levels of endogenous p100, p52 and RelB were comparable to the control (Fig. 4C). Over-expression of RelB alone did not show such a phenomenon, while cells transfected with both RelB and p52 displayed a pattern of CXCL13 production resembling to p52-transfected cells. Therefore, the induction of CXCL13 requires more than the accumulation of non-canonical RelB–p52 complex, suggesting that additional signaling pathways play a pivotal role in this process. Importantly, as the over-expression of both RelB and p52 without any stimulation was unable to induce CXCL13, the effect of TNFα on CXCL13 production at least in RelB–p52-transfected cells is unlikely due to the up-regulation of RelB and p100 by activation of the canonical pathway.

LTβR-dependent CXCL13 expression involves PKC activity

To identify the signaling pathways participating in the LTβR-dependent induction of CXCL13, we treated BLS12 cells with various pharmacological inhibitors and found that PKC inhibitors, including BIM-1, Gö6983 (Fig. 5A and B), Gö6976 and Ro-32-0432 (data not shown), markedly inhibited the production of CXCL13. For further analysis, we selected Gö6983, which exhibited a clear dose-dependent inhibition of CXCL13 production and almost complete blockade was observed at 30 μM. Gö6983 showed no significant influence on the transcripts of RelB and NF-κB2, and the increase of RelB and p52 proteins due to stimulations was comparable to the control experiment (Fig. 5C and D), suggesting that RelA/p50 activity at least for the induction of RelB and NF-κB2 is unaffected by the inhibition of PKCs in BLS12 cells. However, it is worth noting that the treatment of anti-LTβR antibody or TNFα + anti-LTβR antibody in the presence of Gö6983 led to marked accumulation of p100 (described later).

We previously have demonstrated that the forced expression of NIK in BLS12 cells causes constitutive CXCL13 production (18). In NIK-over-expressing cells, PKC inhibitors suppressed CXCL13 production in a dose-dependent manner (Fig. 6A), suggesting that PKC functions downstream of NIK. Moreover, PKC inhibitors were able to suppress TNFα-dependent CXCL13 production by RelB–p52-over-expressing BLS12 cells (Fig. 6B), suggesting that PKC activity is also a signaling component downstream of TNFR. TNFα-dependent CXCL13 production under RelB–p52-over-expressing conditions was markedly inhibited by cell-permeable PS for PKCα/β or PKCζ/λ (Fig. 6C); therefore, PKC activity in the production of CXCL13 is composed of more than one subtype of PKC. We confirmed that the protein levels of PKCζ/β as well as PKCζ/λ were unchanged in IkBαSR-expressing BLS12 cells compared with the control, irrespective of the presence or absence of TNFα stimulus (data not shown). In addition, TNFα-induced up-regulation of PKC activity, which is detected as the PKC-dependent activation of ERK MAP kinase (24, 25), was also unaffected in IkBαSR transfectant (data not shown), indicating that PKC pathways mediated by at least the above subtypes is fundamentally independent of the NF-κB canonical pathway.

PKC participates in CXCL13 expression other than p100 processing and nuclear translocation of RelB–p52 complex

Gö6983 treatment caused no significant alteration in the amounts of total, cytoplasmic or nuclear RelB and p52 after anti-LTβR antibody stimulation compared with the control experiment, except for the accumulation of p100 (Fig. 5D–F). In particular, in the presence of Gö6983, nuclear p100 was markedly increased at 24–48 h post-stimulation. Since some reports have shown that p100 can bind RelA–p50 as well as RelB–p52 complexes and suppress their transcription-inducing activity (20, 22), it raises the possibility that the
accumulation of nuclear p100 might inhibit CXCL13 expression via suppressing RelB–p52 activity. However, the following evidence excludes this possibility, at least in our experimental setting. First, although the protein level of p100 is markedly increased in BLS12 cells stimulated with TNFα and anti-LTβR antibody, strong CXCL13 production is readily observed. Second, over-expression of RelB and p52 in BLS12 cells clearly augmented p100 expression under steady-state conditions and TNFα further enhanced the accumulation of p100 (Figs 4C and 7). Even in such a situation, however, CXCL13 production induced by TNFα was extremely high and G66983 was able to inhibit it without further accumulation of p100 (Fig. 7A–C). In addition, the formation of RelB–p52 complex and the amount of nuclear p100 were constant, irrespective of G66983 treatment (Fig. 7D and E). Therefore, it is unlikely that the expression of CXCL13 is affected by nuclear p100 accumulation in this cell context. Taken together, PKC activity is required for CXCL13 expression other than p100 processing or the increase of RelB.

Discussion

When receiving external signals through TNFR or LTβR, which activates NF-kB, stromal cells in SLOs probably express specific features for constructing tissue structure and supporting immune reactions. Analysis of the details of the ‘maturational process’ in the context of strictly defined stromal cells has been limited because of the absence of a suitable experimental system. In this study, we showed that stromal cell line BLS12, which can proliferate efficiently in vitro and preserves
PKC inhibitors suppress CXCL13 production in NIK- or RelB–p52-transfected BLS12 cells. (A) BLS12 cells stably transfected with NIK were treated with DMSO, BIM-I or G06983 for 48 h. CXCL13 production was examined by ELISA. (B) BLS12 cells stably transfected with vector or RelB–p52 were pre-treated with DMSO, BIM-I or G06983 for 1 h prior to TNFα stimulation for 24 h and CXCL13 production was examined. (C) Transfectant for RelB–p52 was stimulated with TNFα for 24 h in the presence of myristoylated PS for PKCa/β or ζ/δ, and CXCL13 production was examined.

Fig. 6. PKC inhibitors suppress CXCL13 production in NIK- or RelB–p52-transfected BLS12 cells. (A) BLS12 cells stably transfected with NIK were treated with DMSO, BIM-I or G06983 for 48 h. CXCL13 production was examined by ELISA. (B) BLS12 cells stably transfected with vector or RelB–p52 were pre-treated with DMSO, BIM-I or G06983 for 1 h prior to TNFα stimulation for 24 h and CXCL13 production was examined. (C) Transfectant for RelB–p52 was stimulated with TNFα for 24 h in the presence of myristoylated PS for PKCa/β or ζ/δ, and CXCL13 production was examined.

Signals for CXCL13 production in stromal cell
phosphorylation or the activation of other transcription factors or regulators. In addition, RelA–p50 complex might directly involve in CXCL13 gene transcription in cooperation with RelB–p52 complex. These are important issues to be addressed in the future. Besides PKC inhibitors, we also found pharmacological inhibitors that clearly inhibit LTβR-induced CXCL13 expression (H. Suto and T. Katakai, unpublished observation), suggesting that several different signaling components are involved in this process besides NF-κB pathways.

Fig. 7. G66983 inhibits TNFα-induced CXCL13 production by BLS12 cells over-expressing RelB and p52. (A and B) BLS12 cells stably transfected with RelB and p52 were stimulated with TNFα for 24 h in the presence of DMSO or G66983 (10 or 30 μM), and CXCL13 secretion (A) or transcripts (B) was examined. (C–E) G66983 does not influence the total amounts, complex formation and nuclear translocation of p52 and RelB. RelB–p52-transfected BLS12 cells were stimulated with TNFα in the presence of DMSO or G66983 (10 or 30 μM) for 24 h and examined for p100/p52, RelB and tubulin. (D) RelB–p52-over-expressing cells were stimulated with TNFα in the presence of DMSO or G66983 (30 μM) for 1 or 24 h. Cell lysates were immunoprecipitated with control mouse IgG or anti-Myc antibody and blotted with RelB antibody.
We thank H. Wajant for...

In summary, the maturation of SLO stromal cells mediated by LTβR and/or TNFR is accomplished by the cooperation of multiple signaling pathways, not only NF-κB canonical and non-canonical pathways but also additional signaling components (Fig. 8). PKC activity is a critical mediator in addition to NF-κBs.

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**Abbreviation**

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<tr>
<td>BIM-I</td>
<td>bisindolylmaleimide-I</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>IκB</td>
<td>inhibitor of NF-κB</td>
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<td>LT</td>
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<td>LTβR</td>
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**References**


