Thymic Stromal Lymphopoietin Is Produced by Dendritic Cells

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Thymic Stromal Lymphopoietin Is Produced by Dendritic Cells

Mohit Kashyap,* Yrina Rochman,* Rosanne Spolski,* Leigh Samsel,† and Warren J. Leonard*

Thymic stromal lymphopoietin (TSLP) is a type 1 cytokine that contributes to lymphopoiesis and the development of asthma and atopic dermatitis. TSLP acts on multiple lineages, including dendritic cells (DCs), T cells, NKT cells, eosinophils, and mast cells, mediating proliferation and survival and linking innate and adaptive immune responses. TSLP is produced by a range of cells, including epithelial cells, fibroblasts, stromal cells, and keratinocytes. DCs are important primary targets of TSLP, and we unexpectedly demonstrated that DCs also produce TSLP in response to TLR stimulation and that this is augmented by IL-4. Moreover, we demonstrated that when mice were challenged with house dust mite extract, lung CD11c+ DCs expressed TSLP mRNA at an even higher level than did epithelial cells. These data suggested that DCs not only respond to TSLP but also are a source of TSLP during pathogen and/or allergen encounter. The Journal of Immunology, 2011, 187: 000–000.

Thymic stromal lymphopoietin (TSLP) was originally identified as a growth-promoting factor produced by mouse thymic stromal cells that supported the development of immature B cells to the B220+/IgM+ stage (1). TSLP is a type I cytokine that acts via a receptor containing IL-7Rα and a TSLP-specific subunit, TSLP receptor (TSLPR) (2, 3); it signals via JAK1 and JAK2 to mediate the activation of STAT5A and STAT5B (4). TSLPR is most similar to the common cytokine receptor γ-chain, γc, which is a component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (5) and whose mutation results in X-linked SCID in humans (6).

TSLP has been implicated in the development of asthma (7), atopic dermatitis (8), and inflammatory arthritis (9). Moreover, it is required for normal worm expulsion during helminth infection (10) and for the inflammatory process that promotes cancer progression and metastasis (11–13). In addition to its actions on B cells, TSLP acts on other lineages, including dendritic cells (DCs) (14), T cells (4, 15, 16), mast cells (17), NKT cells (18), and eosinophils (19). TSLP can act via DCs to regulate the activation, differentiation, and homeostasis of T cells (8), but it also has direct effects on T cells, promoting their survival and proliferation in response to TCR activation (15, 16, 20). Interestingly, TSLPR knockout (KO) mice have a defective allergic inflammatory response in the lung, but this can be reversed by adoptive transfer of wild-type (WT) CD4+ T cells (7), underscoring a key role for the action of TSLP on these cells.

TSLP is expressed by a range of cell types, including epithelial cells, fibroblasts, keratinocytes, IgER-activated mast cells, protease-activated basophils, and human CD68+ macrophages, whereas it is not produced by other lympho-hematopoietic cells, including neutrophils, B cells, T cells, monocytes, myeloid DCs (mDCs), plasmacytoid DCs (pDCs), and endothelial cells (14, 21–23). Because DCs are involved in the initiation of immune responses, we decided to re-explore the possibility that DCs might also produce TSLP. Activation of DCs via TLRs is known to induce the expression of MHC class II, CD80, and CD86 and to induce the secretion of inflammatory cytokines and chemokines that are important for the adaptive immune response (24–27). We now show that TLR signals also induce the production of TSLP by bone marrow-derived DCs and that this is augmented by IL-4, with mDCs producing much higher levels of TSLP compared with pDCs. TSLP expression was also induced by murine splenic DCs, human monocytes, and monocyte-derived DCs (MDDCs). Moreover, when lung cells were sorted into epithelial cells or DCs, TSLP mRNA was expressed by the epithelial cells and by the DCs, and levels were enhanced when mice were challenged with house dust mite (HDM) extract, which induces allergic inflammation in the lung (28). Therefore, our study revealed that, in addition to their response to TSLP, DCs can produce TSLP in response to pathogens/allergens, with increased production in the presence of IL-4. The production of TSLP by DCs might bypass the need for epithelial cell-derived TSLP, potentially creating an autocrine loop wherein DCs both produce and respond to TSLP, as well as serving as a source of TSLP for other immune cells.

Materials and Methods

Cell culture, activation, and FACS analysis

Bone marrow cells from WT or Stat6 KO BALB/c mice (Jackson Laboratory) were cultured with GM-CSF (20 ng/ml; PeproTech) or Fms-related tyrosine kinase 3 ligand (Flt-3L) (100 ng/ml; PeproTech) for 8 d in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and streptomycin. On day 8, GM-CSF cell cultures were stimulated with allopurinol-conjugated CD11c Abs (BD Biosciences and R&D Systems) to assess purity (>90% pure). Also on day 8, FLT-3L cell cultures were...
Coulter). Cells were activated with polyinosinic-polycytidylic acid (poly-I:C) (10 μg/ml), single-stranded polyuridine (1 μg/ml), and flagellin (1 μg/ml) (Invivogen) or for varying periods of time with 10 μg/ml zymosan (Invivogen), 1 μg/ml LPS (Sigma), or 0.1 μg CpG (Alexis Biochemicals), with or without 10 ng/ml IL-4 (PeproTech), and TSLP mRNA and protein expression was evaluated. Splenic DCs were isolated using mouse pan-DC beads (Miltenyi Biotec). These cells were 80–85% pure based on staining with CD11c and B220 (BD Biosciences) and were activated with LPS or CpG for varying time points. Human monocytes were isolated using CD14 beads (Miltenyi Biotec) and cultured with human GM-CSF (50 ng/ml) and IL-4 (10 ng/ml) (PeproTech) for 7 d. The cells were washed and then stimulated with LPS and zymosan for 4 or 16 h to evaluate TSLP mRNA and protein expression, respectively. Monocyte and MDDC purity was assessed by staining with PE–Cy7–CD14 (BD Pharmingen) and FITC–TCRβ (BD Pharmingen) to identify T cells.

RNA isolation and real-time PCR

RNA was extracted using TRIzol reagent (Invitrogen) or RNeasy kit (Qiagen), and reverse-transcribed using the Omniscript cDNA synthesis kit (Qiagen), and cytokine cDNAs were identified by a fluorogenic 5′-nuclease PCR assay and an ABI Prism 7900HT sequence detection system (Perkin Elmer). One microgram of RNA was used for RT-PCR, and 0.2 μg this product was amplified using mouse or human TSLP, TNF-α, thymus and activation-regulated chemokine (TARC), MIP-1α, pulmonary surfactant-associated protein B (SFTPB), and IL-8 TaqMan FAM-MGB primers (Applied Biosystems), and mRNA levels were measured using standard curves relative to mouse or human RPL7 mRNA. The primers used for mouse Rpl7 were 5′-TACCAAGCGACTGGTCAGA-3′ and 5′-GGGGTGTGTTCT-3′, and the TagMan FAM-TAMRA probe was 5′-TGACATGTCGACAGGAGGAGATT-3′; the primers for human RPL7 were 5′-AGCGTTGATGTGCTGATCTC-3′ and 5′-CTCTTTGAAGCGTTTCCA-3′, and the TagMan FAM-TAMRA probe was 5′-ATACGGCATCCTGTCAAGGATTGAT-3′.

**ELISA**

A total of 1 or 2 × 106 mouse DCs was cultured with medium alone or medium containing LPS, CpG, or zymosan with or without IL-4 for 16 h. BAL fluid, lung supernatant, and in vitro culture supernatants were collected, and the concentration of TSLP, TARC, and MIP-1α was determined using an ELISA kit (R&D Systems and Antigenex).

**Western blot**

A total of 6–10 × 106 freshly isolated monocytes or 7-d-old MDDCs was stimulated with LPS or zymosan for 12 h plus 4 h with Golgi plug (Sigma) (total stimulation time = 16 h). The cells were then lysed and subjected to Western blot analysis with sheep anti-human TSLP primary Ab for TSLP.
Statistics

Results are expressed as mean ± SD. The p values < 0.05, as determined by the Student t test using Prism 4 software, were considered significant.

Results

Mouse bone marrow-derived and splenic DCs express TSLP, and this expression is augmented by IL-4

To determine whether DCs can produce TSLP, we first analyzed GM-CSF–induced mouse bone marrow-derived DCs. Treatment of these cells with the TLR ligands zymosan, LPS, and CpG, which signal via TLR2/6, TLR4, and TLR9, respectively, each significantly induced TSLP mRNA (Fig. 1A); as expected, these stimuli also significantly induced TNF-α mRNA (Fig. 1B), analogous to a previous report (27). Poly-I:C, flagellin, and single-stranded polyuridine, which signal via TLR3, TLR5, and TLR7, respectively, had weaker effects on both TSLP (Fig. 1C) and TNF-α (Fig. 1E) induction, with only flagellin significantly inducing TSLP mRNA, albeit more modestly than zymosan, LPS, and CpG (Fig. 1A). Zymosan (Fig. 1C), LPS (Fig. 1D), and CpG (Fig. 1E) each induced TSLP mRNA within 4 h, but these agents differ in their kinetics, with peak TSLP mRNA expression occurring earliest in response to zymosan. In addition to the production of TSLP mRNA by bone marrow-derived DCs, TSLP mRNA was produced by splenic DCs (Fig. 1F). Because IL-4 was reported to augment TSLP production by keratinocytes, fibroblasts, and epithelial cells when combined with TNF-α, IL-1β, or poly-I:C (29, 30), we evaluated the effect of IL-4 on TSLP expression. IL-4 augmented zymosan-, LPS-, and CpG-induced TSLP mRNA expression (Fig. 1G) and protein secretion (Fig. 1H) from bone marrow-derived DCs. Consistent with a key role for STAT6 in IL-4 signaling, IL-4 did not induce TSLP mRNA (Fig. 1G) or protein (Fig. 1H) in bone marrow-derived Stat6−/− DCs stimulated with LPS, CpG, or zymosan. This defect did not result from diminished viability of the cells in the absence of STAT6 (data not shown). Together, these results demonstrated that TSLP expression by DCs is induced by TLR stimulation and suggested that, during pathogen/allergen exposure, the recruitment of Th2 cells that produce IL-4 may amplify allergic inflammatory responses by augmenting production of TSLP by DCs.

TSLP production by mDCs but not by pDCs

To further explore the range of DCs that can produce TSLP, we cultured bone marrow cells with either GM-CSF for 8–12 d to produce mDCs (31) or with FLT-3L to generate pDCs (32). In response to LPS or CpG, TSLP mRNA expression was potently induced in mDCs, but little, if any, TSLP was induced in pDCs (Fig. 2A). In contrast, expression of MIP-1α, a chemokine known to be produced by both mDCs and pDCs (33), was increased in both populations of DCs (Fig. 2B). Correspondingly, LPS and CpG induced TSLP protein secretion by mDCs but not pDCs (Fig. 2C), whereas MIP-1α protein was secreted by both cell types (Fig. 2D). The detection of TSLP protein in the supernatant reflected secretion rather than release from dying cells, because LPS and

FIGURE 2. GM-CSF– but not FLT-3L–derived DCs produce TSLP. RT-PCR was used to measure TSLP (A) and MIP-1α (B) mRNA levels relative to RPL7 control mRNA in bone marrow-derived DCs that were activated with LPS or CpG for 4 h. mDCs were induced by culturing for 8 d in GM-CSF, and pDCs were induced by culturing with FLT-3L for 8 d and then isolating CD11c+/B220− cells by cell sorting with Abs to CD11c and B220. C and D, TSLP and MIP-1α protein levels, as measured by ELISA, after 16 h of LPS or CpG stimulation of DCs that had been differentiated. E, Percentage of live cells after 16 h stimulation, as measured by Annexin V and 7-aminoactinomycin D staining. Data are from three independent experiments for TSLP and two independent experiments for MIP-1α. *p < 0.05, **p < 0.01, compared with untreated samples.

FIGURE 3. Human peripheral blood monocytes and MDDCs produce TSLP and IL-8 mRNA in response to LPS and zymosan. Freshly isolated monocytes (CD14hiCD1c−) (A) and MDDCs (CD14loCD1c+) that had been grown for 7 d with GM-CSF plus IL-4 (B) were stimulated with LPS and zymosan, and TSLP (C) and IL-8 (D) mRNA levels were determined by RT-PCR. Data are representative of three independent experiments. E, Freshly isolated monocytes and 7-d GM-CSF plus IL-4–derived MDDCs were either left unstimulated or stimulated with LPS and zymosan, and Western blot analysis was performed. Data are from one of two independent experiments. **p < 0.01, compared with untreated control.
CpG did not diminish the viability of the different DC populations (Fig. 2E). Thus, mDCs, but not pDCs, are producers of TSLP.

**Human peripheral blood monocytes and MDDCs also express TSLP**

Monocytes from human blood develop into DCs when cultured with GM-CSF plus IL-4, and these MDDCs can function as potent APCs (34, 35). Therefore, we used freshly isolated human monocytes (Fig. 3A) and MDDCs (Fig. 3B) and found that TSLP mRNA was induced in both populations by either LPS or zymosan (Fig. 3C). As expected, as a control, IL-8 mRNA expression was also induced in both populations, but the basal LPS- and zymosan-induced levels in MDDCs were lower than in monocytes (Fig. 3D). Western blot analysis of monocytes and MDDCs revealed upregulation of TSLP protein upon LPS and zymosan stimulation (Fig. 3E). Hence, human monocytes and MDDCs also express TSLP.

**Lung epithelial cells and DCs produce TSLP in response to HDM challenge in vivo**

Above, we examined the ability of different DC populations to produce TSLP after being challenged in vitro. We next used intratracheally administered HDM extract as an in vivo stimulus (*Materials and Methods*). As expected, HDM induced an inflammatory response, with increased cells in the BAL fluid (Fig. 4A), with a higher percentage of neutrophils (Fig. 4B) and T cells (Fig. 4C), but a lower percentage of DCs (Fig. 4D), in the BAL fluid of HDM-challenged mice. Both TARC (Fig. 4E), a chemokine responsible for recruiting Th2 cells (36), and TSLP (Fig. 4F) were significantly higher in the lung after HDM challenge. To assess the source of production of TARC and TSLP, we isolated epithelial cells and DCs by cell sorting. Interestingly, basal levels of TSLP (Fig. 4G) and TARC (Fig. 4H) mRNAs were greater in CD324^+CD11c^+ DCs than in CD324^+CD11c^- epithelial cells, and HDM further induced mRNA levels of TSLP (Fig. 4G) and TARC (Fig. 4H) in both populations of cells. As expected, mRNA encoding pulmonary SFTPB was only expressed by the epithelial cells (Fig. 4I), confirming that the effects that we observed did not result from contamination of DCs by epithelial cells. These results established that HDM-mediated lung inflammation is associated with the production of TSLP by lung DCs, as well as by primary epithelial cells.

**Discussion**

In this study, we demonstrated that multiple activators of TLRs, including zymosan, LPS, and CpG, could each induce expression of TSLP in mouse bone marrow-derived DCs and splenic DCs. Interestingly, TLR-induced TSLP production by bone marrow-derived DCs was augmented by IL-4; as anticipated, this was via a STAT6-dependent mechanism. Increased TSLP expression was also observed in both freshly isolated human monocytes and MDDCs. The production of TSLP by DCs suggests possible autocrine effects on DCs; moreover, DCs may serve as a source of TSLP for other responsive lineages, such as CD4^+ T cells, to promote TSLP-dependent allergic inflammatory responses, such as asthma and atopic dermatitis. The production of TSLP by DCs was not anticipated, because DCs have been viewed only as critical targets for this cytokine. DCs produce proinflammatory cytokines, including TNF-α, IL-1β, and IL-6 upon TLR ligation (27). Therefore, we hypothesized that TSLP would also be produced by DCs resident in the gut, lung, and skin, organs that have proximity to the external environment upon TLR ligation by allergens/pathogens. In this regard, TLR pathways have been implicated in the pathogenesis of asthma (28, 30), but a link between these pathways and the production of TSLP by DCs has not been reported. Our data that lung CD324^+CD11c^- DCs induced TSLP mRNA upon HDM challenge suggested that production of TSLP by DCs could play a role in the development of asthma, with implications for other DC-dependent inflammatory diseases, as well.

In summary, TSLP was originally reported to be produced by epithelial cells and keratinocytes and to act on DCs, which, in turn, can activate T cells, thereby helping to bridge innate and adaptive immune response (8). We previously showed that functional receptors for TSLP are also expressed on T cells and other

**FIGURE 4.** Lung DCs produce TSLP upon HDM challenge. BALB/c mice were injected with HDM, as described in *Materials and Methods*. BAL fluid cellularity was determined (A), and the percentages of neutrophils (B), T cells (C), and DCs (D) were determined by flow cytometric staining with Ly-6G/CD11b (B), TCRβ (C), and CD11c/CD11b (D). Data are combined from five independent experiments, with 3–5 mice/group, yielding a total of 18 mice. TARC (E) and TSLP (F) protein levels were measured by ELISA in lung homogenate. Data are from one of three similar experiments, each of which had three to five mice/group. G–I, TSLP and TARC mRNA levels are induced by HDM in lung DCs. CD324^-CD11c^- (epithelial cell) and CD324^-CD11c^+ (DC) populations of total pooled cells from five mice were isolated by cell sorting from PBS- or HDM-treated mice, mRNA was isolated, and RT-PCR was performed for TSLP, TARC and pulmonary SFTPB. Data are expressed as the ratio of TARC, TSLP, or SFTPB mRNA to that of the RPL7 housekeeping gene and are from three independent experiments. *p < 0.05, **p < 0.01, HDM versus PBS.
lineages and that TSLPR+ T cells are critical for OVA-induced lung inflammation (7). Moreover, TSLP promotes T cell survival (4, 20). Previously, it was shown that DCs respond to TSLP, but in this study, we showed that DCs also produce this cytokine. Thus, the production of TSLP by DCs may allow the direct activation of the adaptive immune response, complementing and possibly circumventing the requirement for epitHELial cell/keratinocyte-derived TSLP in the activation of T cells and promoting T cell-dependent immune responses.

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Disclosures

W.J.L. and R.S. are inventors on patents and patent applications related to TSLP.

References


