

Thymic Stromal Lymphopoietin Induces Chemotactic and Prosurvival Effects in Eosinophils

Implications in Allergic Inflammation

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Thymic stromal lymphopoietin (TSLP) is highly expressed by bronchial epithelial cells and skin keratinocytes in allergic diseases. TSLP acts as a master switch for allergic inflammation through the activation of dendritic cells and mast cells for initiating inflammatory type 2 T-helper lymphocyte responses. To elucidate the immunological cascades of epithelium/keratinocyte–eosinophil–mediated allergic inflammation, we examined the modulating effects of TSLP on human eosinophils. Expression of TSLP receptor complex was detected by RT-PCR, flow cytometry, and Western blot. Adhesion molecules, cytokine, and chemokines were quantitated by flow cytometry or ELISA. Intracellular signal transduction molecules were measured by Western blot and flow cytometry. We observed that human eosinophils constitutively expressed functional heterodimeric TSLP receptor complex comprising TSLP-binding chain TSLPR and IL-7R α chain. TSLP could significantly delay eosinophil apoptosis, up-regulate cell surface expression of adhesion molecule CD18 and intercellular adhesion molecule-1, but down-regulate L-selectin, enhance eosinophil adhesion onto fibronectin, and induce the release of inflammatory cytokine IL-6 and chemokines CXCL8, CXCL1, and CCL2 (all $P < 0.05$). All these effects were concentration dependent and TSLP specific. TSLP regulated the above effects through the activation of extracellular signal-regulated protein kinase, p38 mitogen-activated protein kinase, and NF- κ B signaling pathway, but not signal transducer and activator of transcription 5 and 3, which were usually activated in other effector cells upon TSLP stimulation. Collectively, the above findings elucidate the proallergic mechanisms of TSLP via the activation of distinct intracellular signaling pathways in eosinophils.

Keywords: allergic inflammation; cytokines; eosinophils; signal transduction; thymic stromal lymphopoietin

Allergic diseases, such as asthma and atopic dermatitis (AD), are prevalent in developed countries, and have been increasing worldwide (1, 2). Eosinophils are the principal effector cells in late-phase immune reactions of allergic diseases, which are characterized by delayed apoptosis, accumulation and infiltration of eosinophils in bronchial epithelium, and AD lesion. Activated eosinophils can release cytotoxic granular proteins and lipid mediators, causing direct tissue damage. Eosinophils are also capable of producing and releasing a variety of

CLINICAL RELEVANCE

Thymic stromal lymphopoietin (TSLP) acts as a master switch for allergic inflammation through the activation of dendritic cells and mast cells for initiating inflammatory Th2 responses in allergic disease such as allergic asthma. This is the first report demonstrating that TSLP can stimulate human eosinophils through the extracellular signal-regulated protein kinase, p38 mitogen-activated protein kinase, and NF- κ B-dependent but signal transducer and activator of transcription (STAT)3- and STAT5-independent signaling transduction pathways for the prolonged survival, induction of adhesion molecules, and allergic inflammation-related cytokines and chemokines. Results therefore shed light on the regulatory mechanisms by which pro-allergic TSLP switches on the allergic inflammation via the activation of distinct intracellular signaling pathways in eosinophils.

proinflammatory cytokines and chemokines that can modulate cell adhesion system and attract different types of immune effector cells for aggravating inflammation (3, 4).

Thymic stromal lymphopoietin (TSLP) is a novel IL-7-like cytokine, mainly produced by keratinocytes, epithelial cells, smooth muscle cells, allergen-activated basophils, and IgE-primed mast cells (5–11). TSLP receptor (TSLPR) is a heterodimer comprising TSLP-specific binding chain and the IL-7R α chain, primarily expressed on CD11c⁺ dendritic cells (DCs), mast cells, preactivated CD4⁺, and CD8⁺ T cells (12–15). TSLP can promote different stages of B cell development, expand autologous CD4⁺ T cells, and induce the proliferation and differentiation of CD4⁺CD25⁺FOXP3⁺ regulatory T cells (16–20). Recent evidence has emphasized its crucial roles in allergic disorders. TSLP is expressed in acute and chronic lesions of patients with AD, but is not detected in normal skin (21). TSLP mRNA is highly expressed in epithelial and submucosa of patients with asthma and the bronchoalveolar lavage concentration of TSLP is much higher in patients with asthma and chronic obstructive pulmonary disease compared with control subjects (22, 23). Overexpression of TSLP in skin resulted in AD-like syndrome in the murine model, and TSLPR^{−/−} mice exhibited reducing airway inflammation in ovalbumin-induced asthma, whereas induced expression of TSLP in keratinocytes can aggravate ovalbumin-mediated allergic asthma (24–27). Moreover, anti-TSLP and anti-TSLPR antibodies could prevent T helper (Th) type 2 lymphocyte-mediated allergic skin inflammation and airway inflammation (28, 29). All the above findings indicate that TSLP play important roles in mediating allergic inflammation.

TSLP has been demonstrated to be involved in immune cell activation. It potentially activates immature human CD11c⁺ myeloid DCs to up-regulate costimulatory molecules, OX40L,

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CD80, and CD86, and Th2 chemokines, CCL17 and CCL22, for the induction of inflammatory Th2 responses (21). In the early-phase allergic reaction, TSLP can synergistically react with inflammatory cytokines, IL-1 β and TNF- α , to stimulate mast cells to release high levels of inflammatory Th2 cytokines and chemokines (30). The synergistic effects of TSLP with IL-1 β and TNF- α or novel Th2 cytokine IL-33 are also responsible for the activation of CD34⁺ progenitor hematopoietic cells, which can differentiate to other immune effector cells (31). Eosinophils produce various inflammatory mediators and immunoregulatory cytokines for the late-phase asthmatic response (3, 4). However, the effects of TSLP on eosinophils have not been reported.

Ablation of lymphocytes does not completely abrogate allergic diseases, implying that other immune effector cells may be involved in allergy (24, 26, 30, 32). Eosinophils have been shown to play an essential role in inflammation in mice, induced by intradermal administration of TSLP (33). In addition, large numbers of eosinophils have been found to infiltrate beneath skin epidermis in AD and bronchial epithelial cells in allergic asthma, whereas keratinocytes and epithelial cells can produce high-level expression of TSLP in allergic inflammation (3, 21–23, 34). This evidence, therefore, implies that TSLP can exhibit direct effects on eosinophils. To further elucidate the pathophysiological link between the activated epithelial cells/keratinocytes and eosinophil-mediated inflammation in allergic asthma and AD, we examined the effects of TSLP on eosinophil activation and the underlying intracellular regulatory mechanisms.

MATERIALS AND METHODS

Reagents

Recombinant human TSLP and IL-25, goat anti-human TSLPR antibody, sheep anti-human TSLP antibody, and control IgG were purchased from R&D Systems (Minneapolis, MN). Peptidoglycan was purchased from InvivoGen (San Diego, CA). Inhibitor κ Ba (IkBa) phosphorylation inhibitor BAY11-7082, mitogen-activated protein kinase (MAPK) kinase/extracellular signal-regulated kinase (ERK) inhibitor, U0126, c-Jun N-terminal kinase (JNK) inhibitor, SP600125, p38MAPK inhibitor, SB203580, phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, and Janus kinase (JAK) inhibitor, AG490, were purchased from Calbiochem (San Diego, CA). SB203580 was dissolved in water, whereas other inhibitors were dissolved in DMSO. In all studies, the concentration of DMSO was 0.1% (vol/vol). Rabbit anti-human signal transducer and activator of transcription (STAT) 3 and STAT5, anti-phospho-STAT3 (Ser 727), anti-phospho-STAT3 (Tyr 705), and anti-phospho-STAT5 (Tyr 694) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-pan ERK, anti-phospho-ERK1/2, anti-p38MAPK, anti-phospho-p38MAPK, anti-IkBa, and anti-phospho-IkBa were purchased from BD Pharmingen (San Diego, CA).

Isolation of Human Blood Eosinophils and Neutrophils from Buffy Coat

Peripheral blood eosinophils were purified as previously described (35). Fresh human buffy coat obtained from nonallergic, healthy volunteers of the Hong Kong Red Cross Blood Transfusion Service was diluted 1:2 with PBS at 4°C and centrifuged with an isotonic Percoll solution (density, 1.082 g/ml; GE Healthcare Bio-Sciences Corp., Piscataway, NJ) for 30 minutes at 1,000 $\times$ g. The eosinophil-rich granulocyte fraction was collected and incubated with anti-CD16 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 45 minutes. CD16-positive cells were depleted by passing through an LS+ column (Miltenyi Biotec) within a magnetic field. With this preparation, the drop-through fraction of eosinophils was at least 99% pure, as assessed by Hemacolor rapid blood smear stain (E Merck Diagnostica, Darmstadt, Germany). CD16⁺ neutrophils were collected by positive selection using an LS+ column. The isolated eosinophils

and neutrophils were cultured in RPMI 1,640 medium supplemented with 10% FBS and 20 mM Hepes (Invitrogen Life Technologies, Carlsbad, CA).

Endotoxin-Free Solutions

Cell culture medium was purchased from Invitrogen Life Technologies, free of detectable LPS (<0.1 endotoxin U/ml). All other solutions were prepared with pyrogen-free water and sterile polypropylene plasticware. No solution contained detectable LPS, as determined by the Limulus amoebocyte lysate assay (sensitivity limit 12 pg/ml; Biowhitaker Inc., Walkersville, MD).

Apoptosis Assay

Apoptosis of eosinophils was assessed by the TACS Annexin V-FITC assay (Trevigen Inc., Gaithersburg, MD) with flow cytometry (FACS-Calibur; BD Biosciences Corp., San Diego, CA). The population of viable cells was characterized by low mean fluorescence intensity (MFI) of both FITC-Annexin V and propidium iodide. For the DNA ladder assay, genomic DNA was harvested with the quick apoptotic DNA ladder detection kit (BioVision Inc., Mountain View, CA) and analyzed by electrophoresis with 2% agarose gel stained with ethidium bromide.

Assay for Chemokines, Cytokines, and Eosinophilic Cationic Protein

Concentrations of inflammatory cytokine, IL-6, and chemokines, CXCL8 and CCL2, in culture supernatant were measured with ELISA reagents from BD Pharmingen. Concentration of CXCL1 was measured using ELISA reagent from R&D Systems. The release of ECP was determined by fluorescence enzyme immunoassay (AutoCAP Analyzer; Pharmacia Diagnostics AB, Uppsala, Sweden).

Western Blot Analysis

Eosinophils were lysed with lysis buffer (20 mM Tris-HCl [pH 8.0], 120 mM NaCl, 1% Triton X-100, 10 mM EDTA, 1 mM EGTA, 0.05% 2-mercaptoethanol, 1 $\times$ protease inhibitors). Cell debris was removed by centrifugation at 14,000 $\times$ g for 15 minutes, and equal amount of proteins was heat denatured and subjected to SDS-PAGE (10%). After electrophoresis, the proteins were electrotransferred onto PVDF membrane (GE Healthcare). The membrane was blocked with 5% skimmed milk and probed with primary antibody at 4°C overnight. After washing, the membrane was incubated with secondary antibody coupled to horseradish peroxidase (GE Healthcare) for 1 hour at room temperature. Antibody-antigen complexes were then detected with an ECL chemiluminescent detection system (GE Healthcare).

RT-PCR

Total RNA was extracted with TRI-Reagent (Molecular Research Center Inc., Cincinnati, OH) and treated with DNase I to exclude genomic DNA contamination (Ambion Inc., Austin, TX). Extracted RNA was reverse transcribed into first-strand complementary DNA with the First-Strand cDNA Synthesis Kit (Applied Biosystems Inc., Foster City, CA), and then PCR was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems). The primers were as follows: TSLPR forward, 5'-TGAGATTTTCGTGGCATCAG-3', and reverse, 5'-GACCAGTCGCTTGGGTATGT-3', yielding a 238-bp product; human IL-7R α , forward, 5'-GAAGGTTGGAGAAAAGAGTC-3', and reverse, 5'-CAAAATGCTGATGGTTAGTAAG-3', yielding a 418-bp product; β -actin, forward, 5'-CTTCCTTCCTGGGCATG GAG-3', and reverse, 5'-TGGAGGGGCCGACTCGTCA-3', yielding a 300-bp product. After amplification a PTC 100 thermal cycler (MJ Research Inc., Waltham, MA), PCR products were electrophoresed on 2% agarose gel in Tris-acetate-EDTA buffer and stained with ethidium bromide. The electrophoretic bands were documented with the Gene Genius Gel Documentation System (Syngene Inc., Cambridge, UK).

Immunofluorescence Staining and Flow Cytometry

Immunofluorescence staining was performed as previously described (35). For assessing cell surface molecules, eosinophils were washed and resuspended with cold PBS supplemented with 0.5% BSA. After

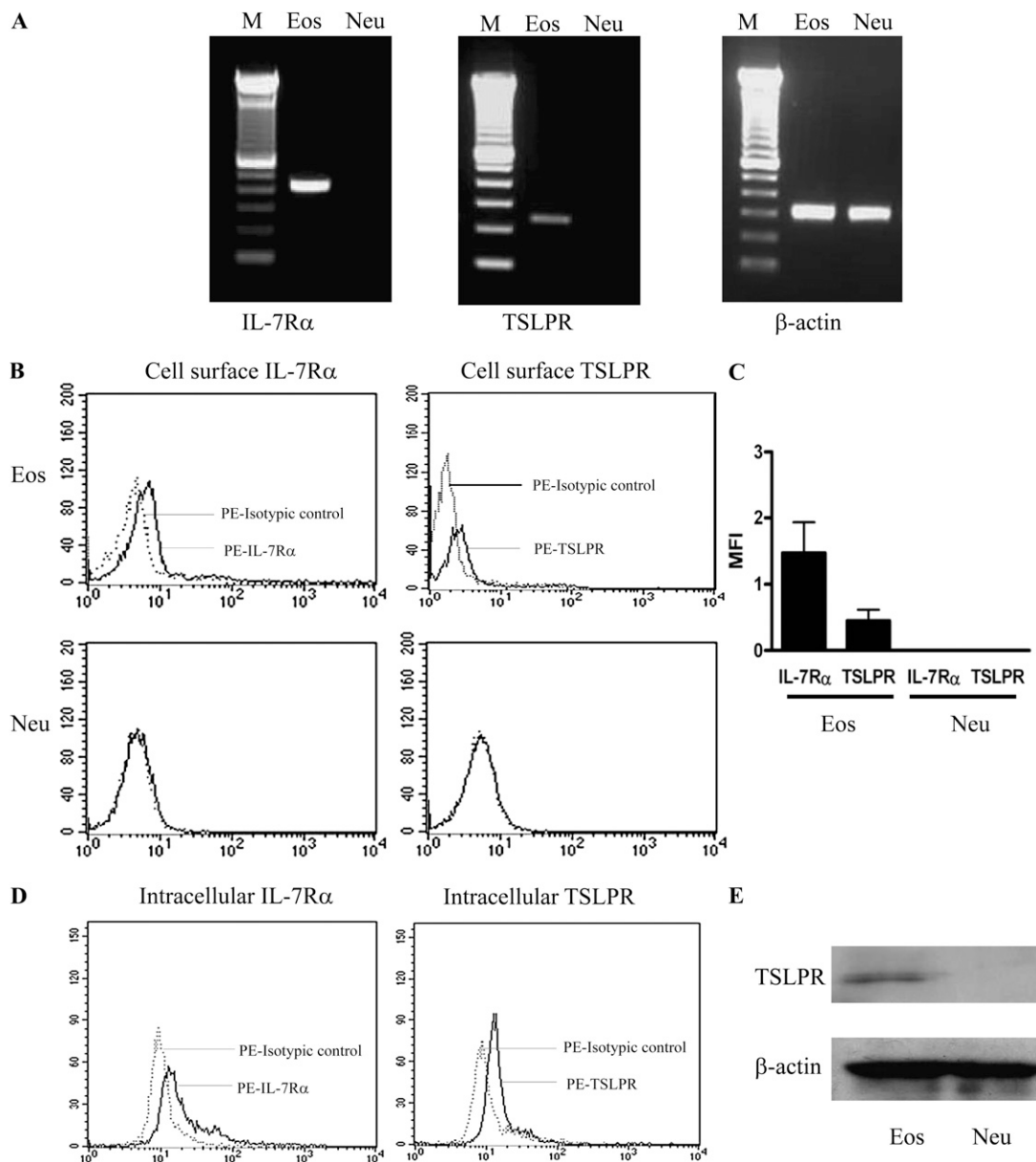


Figure 1. Expression of the heterodimeric receptor for thymic stromal lymphopoietin (TSLP) of eosinophils. (A) Total RNA was extracted from eosinophils and neutrophils (1×10^6 cells), followed by RT-PCR analysis for IL-7Rα, TSLP receptor (TSLPR), and β-actin (house keeping gene). (B) Representative histograms of cell surface expression of TSLPR and IL-7Rα on eosinophils and neutrophils were determined by flow cytometry. Triplicate experiments were performed with essentially identical results, and representative figure is shown. (C) Quantitative results of flow cytometric analysis of cell surface expression of IL-7Rα and TSLPR on neutrophils and eosinophils are presented with arithmetic mean ($\pm$ SD) (mean fluorescence intensity [MFI]) of three independent experiments). Results have been normalized by subtracting appropriate isotypic control. (D) Representative histograms of the intracellular expression of TSLPR and IL-7Rα in eosinophils. Triplicate experiments were performed with essentially identical results, and representative figure is shown. (E) Representative Western blot analysis of TSLPR protein expression of human eosinophils and neutrophils is shown from triplicate experiments with essentially identical result.

Total proteins were extracted from eosinophils and neutrophils (1×10^6 cells). β-Actin was used as a control to ensure an equal amount of loaded protein. *Dotted line*, isotypic controls, phycoerythrin (PE)-conjugated mouse IgG1, or PE-conjugated mouse IgG2a; *solid line*, PE-conjugated anti-human IL-7Rα or PE-conjugated anti-human TSLPR antibodies. M, 100-bp molecular size marker; Eos, eosinophils; Neu, neutrophils.

blocking with 2% human pooled serum for 20 minutes, cells were incubated with phycoerythrin (PE)-conjugated anti-human TSLPR and PE-conjugated mouse IgG2a (Biolegend Corp., San Diego, CA), FITC-conjugated anti-human L-selectin (R&D Systems), PE-conjugated anti-human IL-7Rα, PE-conjugated anti-human intercellular adhesion molecule (ICAM)-1, FITC-conjugated anti-human CD18, and FITC- or PE-conjugated mouse IgG1 (BD Pharmingen) antibodies for 45 minutes at 4°C in the dark. After washing, the cells were resuspended in 1% paraformaldehyde (as fixative) and subjected to analysis. To determine the intracellular expression of TSLPR, IL-7Rα, and phosphorylated signaling molecules, eosinophils were fixed with 4% paraformaldehyde for 10 minutes at 37°C. After centrifugation, cells were permeabilized in ice-cold methanol for 30 minutes and then stained with PE-conjugated anti-phospho-ERK1/2 (T202/Y204) and PE-conjugated anti-phospho-p38MAPK (T180/Y182) antibodies (BD Pharmingen) for 45 minutes at 4°C in the dark. Unconjugated mouse anti-human phospho-IκBα, followed by secondary FITC-conjugated

anti-mouse IgG antibodies, were used for the intracellular staining. Cells were then washed, resuspended, and subjected to analysis. Expression of adhesion molecules and phosphorylated signaling molecules of 10,000 viable cells was analyzed by FACSCalibur flow cytometer with CellQuestPro software (BD Biosciences).

Adhesion Assay

The adhesion assay was performed as previously described with slight modification (36). Eosinophils (1×10^6 cells/ml) with different treatments were cultured in 24-well plates coated with fibronectin (BD Bioscience) at 37°C, in a 5% CO₂, humidified atmosphere for 16 hours. After incubation, nonadherent cells were removed and the plates washed three times with 37°C PBS. Eosinophil adherence in each well was assessed by counting the number of eosinophils adhered to the fibronectin-coated well in four high-power fields (magnification, 100×) under an inverted microscope.

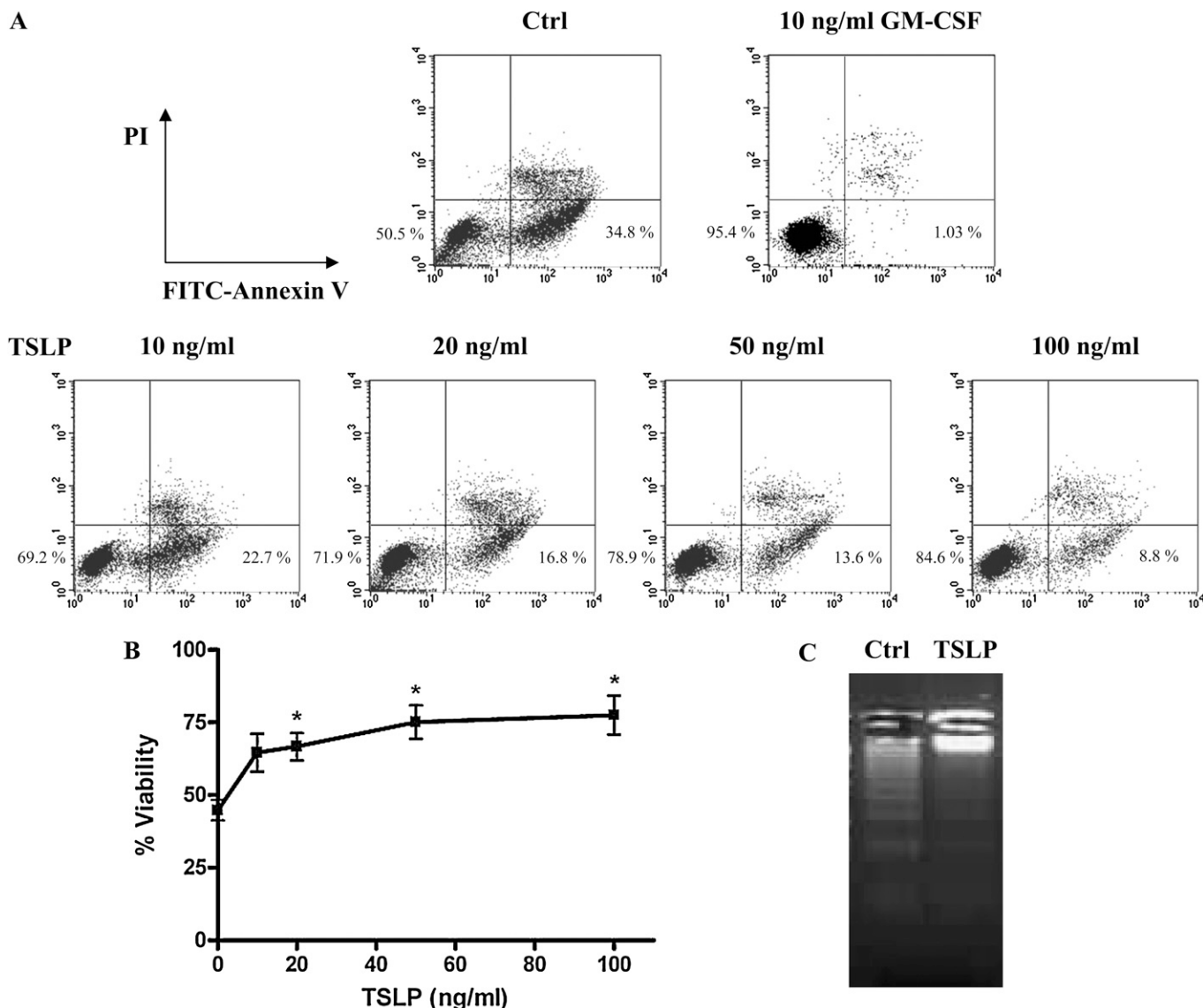


Figure 2. Effect of TSLP on the survival of eosinophils. Eosinophils (5×10^5 /well) were incubated with TSLP (0–100 ng/ml) or granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/ml) for 48 hours, and then analyzed by flow cytometry with annexin V–FITC and propidium iodide (PI) staining or DNA ladder assay. (A) Representative dot plots show early apoptotic cells in the lower right quadrant (annexin V–FITC–positive), late apoptotic (necrotic) cells in the upper right quadrant (annexin V–FITC–positive and PI–positive), and viable cells in the lower left quadrant (double negative). (B) Results of percent viability are expressed as the arithmetic mean ($\pm$ SD) from triplicate experiments. * $P < 0.05$ when compared with medium control (Ctrl). (C) DNA ladder assay of eosinophils with or without treatment with TSLP (50 ng/ml) for 24 hours.

Statistical Analysis

All data were expressed as mean ($\pm$ SD). Differences between groups were assessed by one-way ANOVA analysis. A P value less than 0.05 was considered significant. All analyses were performed with the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, version 16.0 (SPSS Inc., Chicago, IL).

RESULTS

Human Eosinophils Express TSLP Heterodimeric Receptors

Because neutrophils are involved in allergic reaction (37), both eosinophils and neutrophils were examined for TSLPR complex expression. IL-7R α mRNA was highly expressed in freshly isolated eosinophils, and TSLPR was also expressed in eosinophils, although the expression level was lower than that of IL-

7R α . On the other hand, there was no detection of mRNA expression of heterodimeric TSLPR complex in neutrophils (Figure 1A). Further investigation by flow cytometric analysis showed that both subunits were constitutively expressed on the surface of eosinophils, but not on the surface of neutrophils. Consistent with mRNA level, the expression of IL-7R α was higher compared with the TSLPR subunit (Figures 1B and 1C). We could not detect IL-7R α expression on neutrophils, which was consistent with a previous report indicating that neutrophils do not express IL-7R α (38). Because of the low surface expression of TSLPR, further investigation by intracellular staining by flow cytometry was performed. This showed that the intracellular expression level of TSLPR in eosinophils was much higher than that of surface expression, whereas intracellular expression of IL-7R α was similar to surface expression (Figure 1D). Western blot also confirmed that eosinophils, but

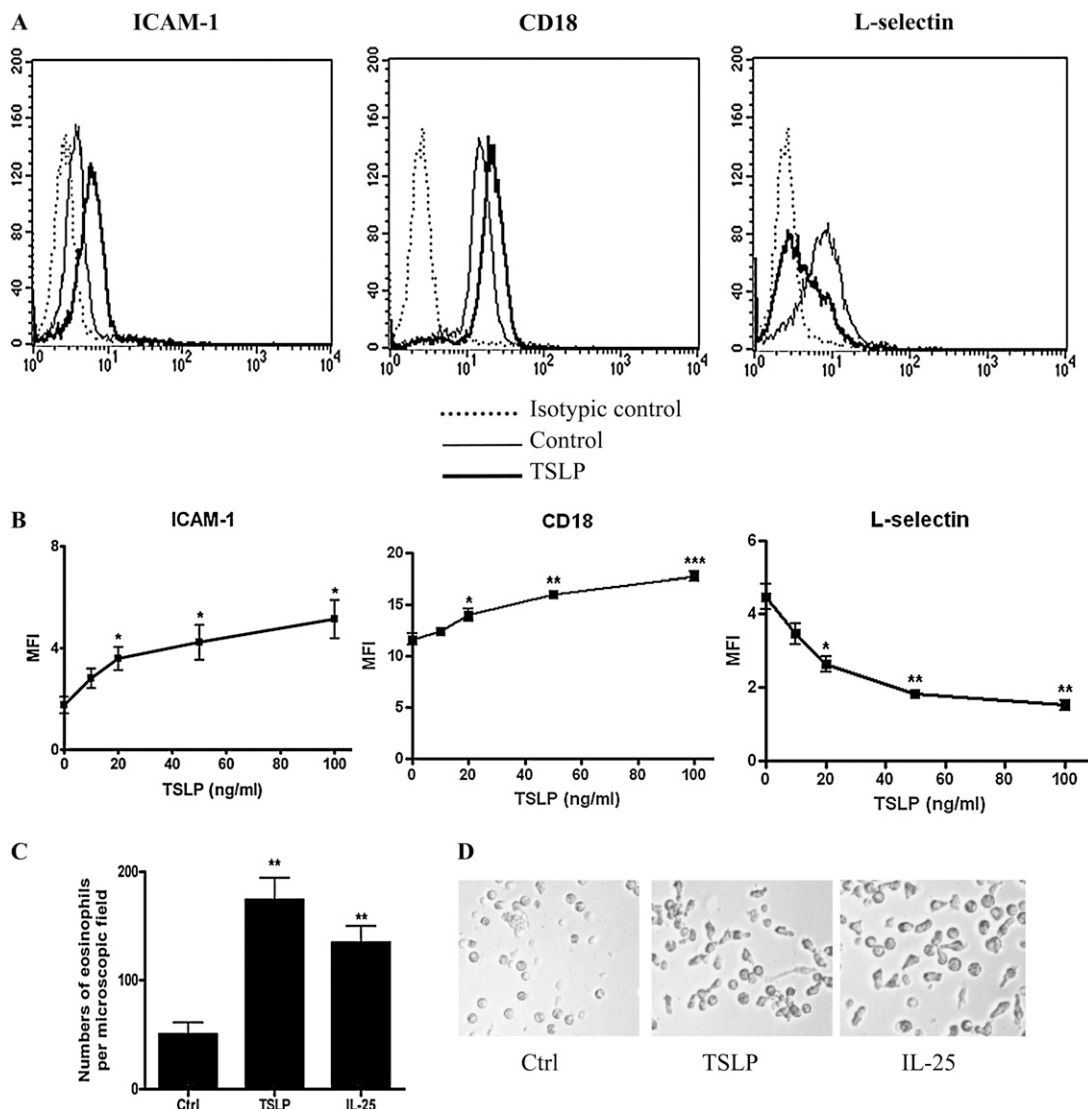


Figure 3. Effect of TSLP on the cell surface expression of adhesion molecules. (A) Eosinophils (5×10^5 /well) were cultured with TSLP (50 ng/ml) for 16 hours in a 24-well plate. Surface expression of adhesion molecules of 10,000 cells was analyzed by flow cytometry. Representative histograms of cell surface expression of intercellular adhesion molecule (ICAM)-1, CD18, and L-selectin on eosinophils are shown. Dotted line, isotypic control; gray line, medium control; black line, TSLP treatment. (B) Surface expression of adhesion molecules are shown as MFI. Results have been normalized by subtracting appropriate isotypic control, and are expressed as the arithmetic mean ($\pm$ SD) of three independent experiments. (C) Eosinophils were stimulated with TSLP (50 ng/ml) and IL-25 (50 ng/ml) for 16 hours in fibronectin-coated wells. Results are expressed as the arithmetic mean ($\pm$ SD) of the number of cells adhered onto fibronectin-coated wells in four random 100 $\times$ fields of quadruplicate experiments. (D) Eosinophils were incubated with medium, TSLP (50 ng/ml), or

IL-25 (50 ng/ml) for 16 hours. Photomicrographs show the morphology of eosinophils with 400 $\times$ magnification. Ctrl, control. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared with medium control.

not neutrophils, constitutively expressed TSLPR subunit (Figure 1E). Together, the expression of TSLPR complex on eosinophils indicates that TSLP could exert direct effects on eosinophils.

TSLP Can Enhance Eosinophil Survival

TSLP could sustain human CD11c⁺ DCs and human acute myeloid leukemia-derived cell line, MUTZ-3, viability and reduce their apoptosis (6, 21). However, TSLP showed no significant survival effect on freshly isolated human CD4⁺ T cells, although TSLP could increase proliferation of T cell receptor-activated CD4⁺ T cell (14). We therefore investigated whether TSLP could also enhance eosinophil survival. Results in Figures 2A and 2B show that approximately 50% of untreated eosinophils remained viable after a 48-hour incubation, whereas TSLP significantly reduced the apoptosis of eosinophils and increased the percentage of viable cells in a dose-dependent manner ($\sim 80\%$ of viable eosinophils at 50 ng/ml TSLP; all $P < 0.05$). However, the effect of TSLP on enhancing eosinophil survival was less potent than that of granulocyte-macrophage colony-stimulating factor (GM-CSF), as the viability mediated by 50 ng/ml TSLP was still less than that mediated by 10 ng/ml GM-CSF treatment (Figures 2A and 2B). To further demon-

strate that the antiapoptotic effect was specifically mediated by TSLP, anti-human TSLP neutralization antibody was used. The anti-human TSLP neutralization antibody inhibited the enhancing survival effect mediated by TSLP, whereas purified isotypic control antibody had no significant effect on eosinophil viability (see Figure E1 in the online supplement). The antiapoptotic effect of TSLP was further confirmed by DNA ladder fragmentation, the early characteristic of apoptosis. In agreement with the annexin V-propidium iodide assay result, spontaneous DNA fragmentation was obvious in untreated eosinophils, whereas eosinophils with TSLP (50 ng/ml) treatment significantly reduced apoptosis-induced DNA laddering fragmentation (Figure 2C). Therefore, TSLPR heterodimer expressed on eosinophils were functional for maintaining eosinophil survival.

TSLP Can Modulate the Cell Surface Expression of Adhesion Molecules on Human Eosinophils

The transendothelial migration of eosinophil to specific tissues involves a variety of adhesion molecules and their counter ligands (3, 4). Therefore, the modulatory effects of TSLP on the cell surface expression of adhesion molecules on eosinophils were investigated. Figures 3A and 3B show that TSLP

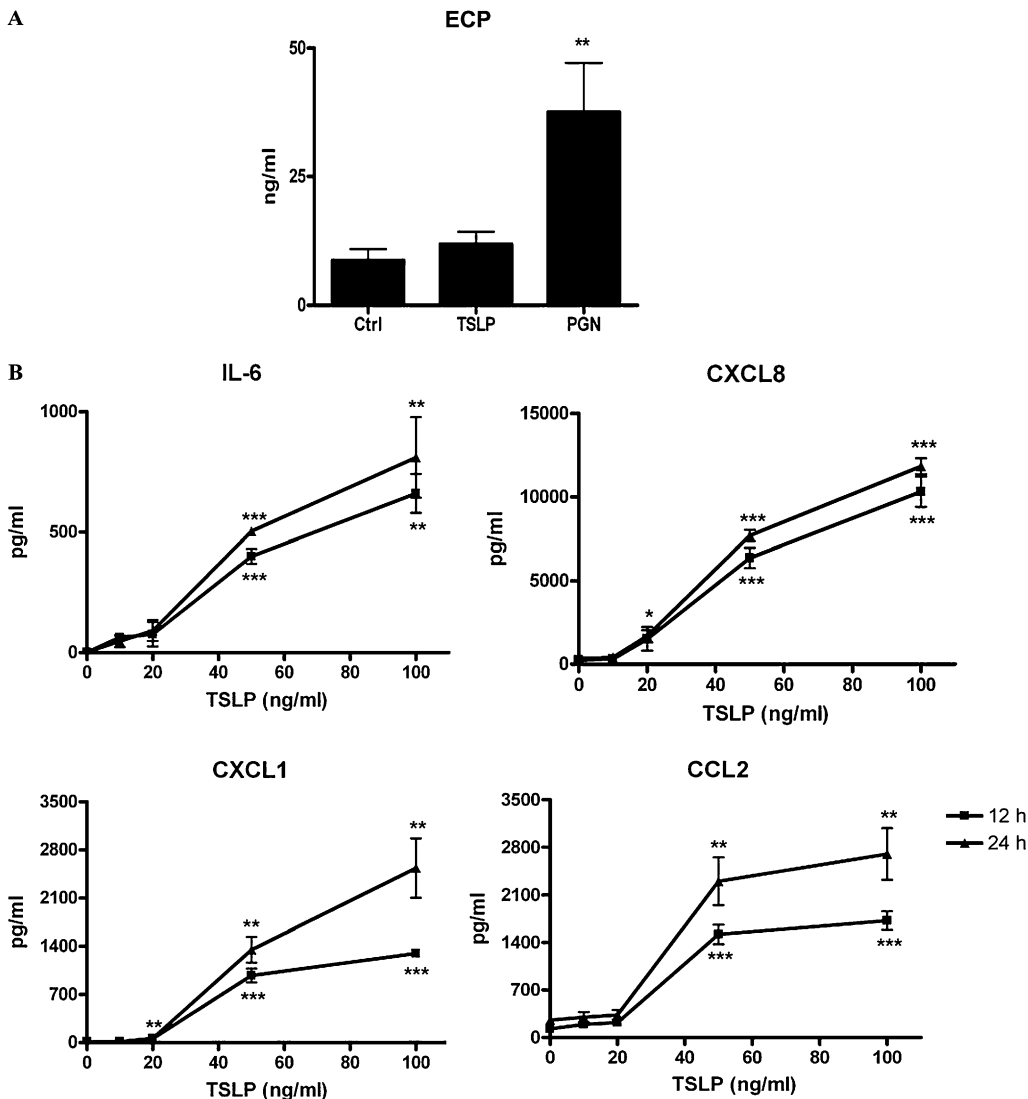


Figure 4. Effects of TSLP on eosinophilic cationic protein (ECP), cytokine, and chemokine release from eosinophils. (A) Eosinophils (1×10^6 cells) were cultured with TSLP (50 ng/ml) or peptidoglycan (PGN) (1 μ g/ml) for 4 hours, and the release of ECP was measured by fluorescence enzyme immunoassay. (B) Eosinophils (5×10^5 /well) were cultured with TSLP (0–100 ng/ml) for 12 or 24 hours. Expression of IL-6, CXCL8, CXCL1, and CCL2 were determined by ELISA. Results are expressed as the arithmetic mean ($\pm$ SD) from three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared with medium control.

could significantly up-regulate the surface expression of ICAM-1 and CD18, while down-regulating L-selectin expression in a dose-dependent manner. Changes of eosinophil adhesion molecules modulated by TSLP could be completely reversed by anti-human TSLP neutralization antibody, but not by control antibody (Figure E2). As shown in Figure 3C, TSLP could significantly increase the number of eosinophils adhered onto fibronectin-coated wells, with IL-25 treatment serving as the positive control (36). Morphological analysis showed that untreated eosinophils maintained the round shape, whereas TSLP-treated eosinophils were found to exhibit elongated shape, similar to that of IL-25-treated eosinophils (Figure 3D). Therefore, the altered expression profile of adhesion molecules indicates that TSLP may facilitate tissue eosinophilia.

TSLP Can Potently Induce the Release of Cytokines and Chemokines from Human Eosinophils

Eosinophils can undergo degranulation to release toxic cationic proteins, which cause direct tissue damage involving in the pathophysiology of allergic diseases (4). However, we could not detect any release of ECP from eosinophils upon TSLP stimulation (Figure 4A) in which peptidoglycan was used as the positive control (39). Actually, TSLP could not induce mast cell degranulation or the release of lipid mediators (30).

Aside from degranulation, eosinophils can synthesize and release a number of cytokines and chemokines (4). Figure 4B shows that TSLP directly activates eosinophils to significantly release inflammatory cytokine IL-6, and chemokines CXCL8, CXCL1, and CCL2 in a dose-dependent manner (0–100 ng/ml) at 12 and 24 hours. The most potent induction was found to be CXCL8, with a 10,000-fold increase, followed by CCL2, CXCL1, and IL-6. The releases at 12 hours of incubation were obviously lower than that at 24 hours. All this cytokine and chemokine production could be suppressed by anti-human TSLP neutralization antibody, but not by control antibody (Figure E3). This indicates that TSLP could specifically induce the release of cytokines and chemokines from human eosinophils. Eosinophils are capable of releasing IL-5, GM-CSF, IL-13, IL-1 β , IL-4, TNF- α , and CXCL5 upon stimulation (3, 4). However, we found that these cytokines in TSLP-treated eosinophils were either undetectable or the same as the medium control determined by ELISA or cytometric bead array (data not shown).

TSLP Could Not Activate STAT5 or STAT3 Signaling Pathway

Previous findings showed that STAT5 was activated in TSLP-treated pre-B cell line, NAG8/7, acute myeloid leukemia cell line, MUTZ-3, and preactivated human CD4 $^+$ and CD8 $^+$ T cells (6, 13–15). In addition, tyrosine phosphorylation of STAT5

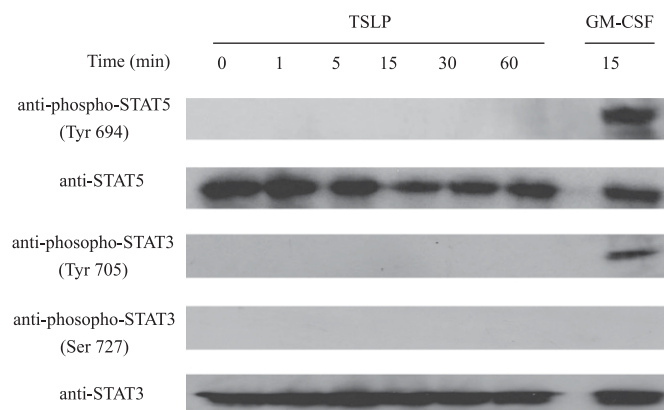


Figure 5. Effect of TSLP on signal transducer and activator of transcription (STAT) 3 and STAT5 activities in eosinophils. Eosinophils (1×10^6 cells) were cultured with or without TSLP (50 ng/ml) or GM-CSF (10 ng/ml) for different indicated incubation times. Total cellular proteins were extracted from eosinophils for the detection of phospho-STAT5 (Tyr 694), total STA5, phospho-STAT3 (Tyr 705), phospho-STAT3 (Ser 727), and total STAT3 with the indicated antibodies by Western blot analysis. Experiments were performed in three independent replicates with essentially identical results, and representative results are shown.

and STAT3 occurred upon TSLP stimulation in pro-B cell line, Ba/F3, transfected with TSLPR heterodimer (13). In our study, TSLP (50 ng/ml) could not obviously induce tyrosine phosphorylation of STAT5 and STAT3 in eosinophils, whereas the positive control GM-CSF (10 ng/ml) significantly induced tyrosine phosphorylation of STAT5 (Tyr 694) and STAT3 (Tyr 705) within 15 minutes (Figure 5). Another potential phosphorylation site, Ser 727, was reported in STAT3 activation (40, 41); however, phosphorylation of STAT3 Ser 727 could not be detected upon either TSLP or GM-CSF stimulation (Figure 5). GM-CSF-induced phosphorylation of STAT3 Ser 727 in eosinophils has actually not been reported. These results, therefore, suggest that other signal pathways may be involved in TSLP-mediated eosinophil activation.

TSLP Activates MAPK and NF- κ B Signaling Pathways

Although TSLP-mediated signaling pathways have rarely been reported, except in STAT5 and STAT3 activation, signal transduction via MAPK and NF- κ B plays important roles in cellular responses, including cell proliferation, differentiation, and survival (35, 36, 39, 42). Western blot analysis showed that phosphorylation of ERK and p38MAPK were detected upon TSLP stimulation, and NF- κ B was also activated, as demonstrated by I κ B α phosphorylation (Figure 6A). The result from intracellular staining with flow cytometry further confirmed that ERK, p38MAPK, and NF- κ B pathways were activated in eosinophils when stimulated by TSLP (Figure 6B). For activation kinetics, these three signaling molecules were all rapidly activated, peaked within 1 or 5 minutes, and returned to baseline within 30 or 60 minutes (Figures 6A and 6B). Although there was a slight difference in the activation kinetic results between Western blot and flow cytometry, the signaling responses for eosinophils induced by TSLP were rapid. However, we could not detect JNK, Akt, or JAKs activity in eosinophils upon TSLP stimulation (data not shown).

To further explore the relationship between NF- κ B, ERK, and p38MAPK activation upon TSLP stimulation, selective, specific inhibitors were used. BAY11-7082, SB203580, and U0126 inhibit the phosphorylation of I κ B α , p38MAPK, and

MAPK kinase 1/2 (upstream kinase of ERK), respectively. The optimal concentration was adopted with the highest inhibitory effects without any cytotoxic effects. BAY11-7082 profoundly inhibited TSLP-mediated I κ B α phosphorylation, but had no effect on the phosphorylation of ERK or p38MAPK. U0126 specifically inhibited phosphorylation of ERK, but had no effects on phosphorylation of p38MAPK or I κ B α . Similarly, SB203580 potently inhibited the phosphorylation of p38MAPK only, but not the phosphorylation of ERK or I κ B α (Figure 6C). These signaling pathways were, therefore, independent, without cross-talk in TSLP-mediated eosinophil activation.

TSLP-Mediated Activation of Eosinophils Involves MAPK and NF- κ B Pathways

As shown in Figure 7A, blocking the p38MAPK pathway with inhibitor SB203580, ERK pathway with U0126, and NF- κ B pathway with BAY11-7082 reduced TSLP-induced eosinophil survival to the basal level (all $P < 0.05$). For the changes of adhesion molecules, TSLP-induced CD18 and ICAM-1 expression could be significantly abrogated by all three inhibitors, BAY11-7082, U0126, and SB203580, whereas TSLP-induced L-selectin down-regulation could only be reversed by inhibitors U0126 and SB203580 (Figure 7B, all $P < 0.05$). For cytokine and chemokine release, TSLP-mediated induction of IL-6, CXCL1, and CCL2 could also be significantly suppressed by BAY11-7082, U0126, and SB203580, albeit to differing degrees, whereas blocking the NF- κ B signaling pathway with BAY11-7082 had no effect on CXCL8 production, and only U0126 and SB203580 showed significant inhibition on TSLP-induced CXCL8 (Figure 7C, all $P < 0.05$). In addition, JAK inhibitor, AG490, PI3K/Akt inhibitor, LY294002, or JNK inhibitor, SP600125, did not exert any significant effect on eosinophil survival or cytokine and chemokine production induced by TSLP (Figure 7). In summary, our results indicate that the three signaling pathways, ERK, p38MAPK, and NF- κ B, were involved in TSLP-mediated eosinophil activation.

DISCUSSION

To further characterize the immunological mechanisms of TSLP for initiating and sustaining allergic inflammation, we investigated the direct activating effects of TSLP on eosinophils, the principal effector cells in late-phase allergic response. We found that eosinophils constitutively expressed mRNA and protein of both TSLPR heterodimer, TSLPR, and IL-7R α , and the intracellular expression level of TSLPR was much higher than that of surface expression, whereas neutrophils showed negative expression of both subunits (38). TSLP could dose-dependently enhance eosinophil survival by reducing eosinophil apoptosis, which indicates that TSLPR heterodimer on eosinophil was functional. Upon stimulation, TSLP could not induce ECP release, but instead induced significant *in vitro* release of inflammatory cytokine, IL-6, CXC chemokines, CXCL8 and CXCL1, and CC chemokine, CCL2. Moreover, both transcriptional inhibitor, actinomycin D, and protein synthesis inhibitor, cycloheximide, significantly suppressed the release of cytokines and chemokines upon TSLP stimulation (Figure E4). The above results, therefore, demonstrate that TSLP actually induced newly synthesized IL-6, CXCL8, CXCL1, and CCL2, rather than the release of preformed substances in eosinophils. Inflammatory cytokine IL-6 can induce the tissue remodeling and fibrosis in eosinophil-associated diseases (43). IL-6 also induces the synthesis of acute-phase proteins, and mediates various inflammatory responses. Furthermore, IL-6 has been reported as a critical inducer for the polarization of a novel subset of T

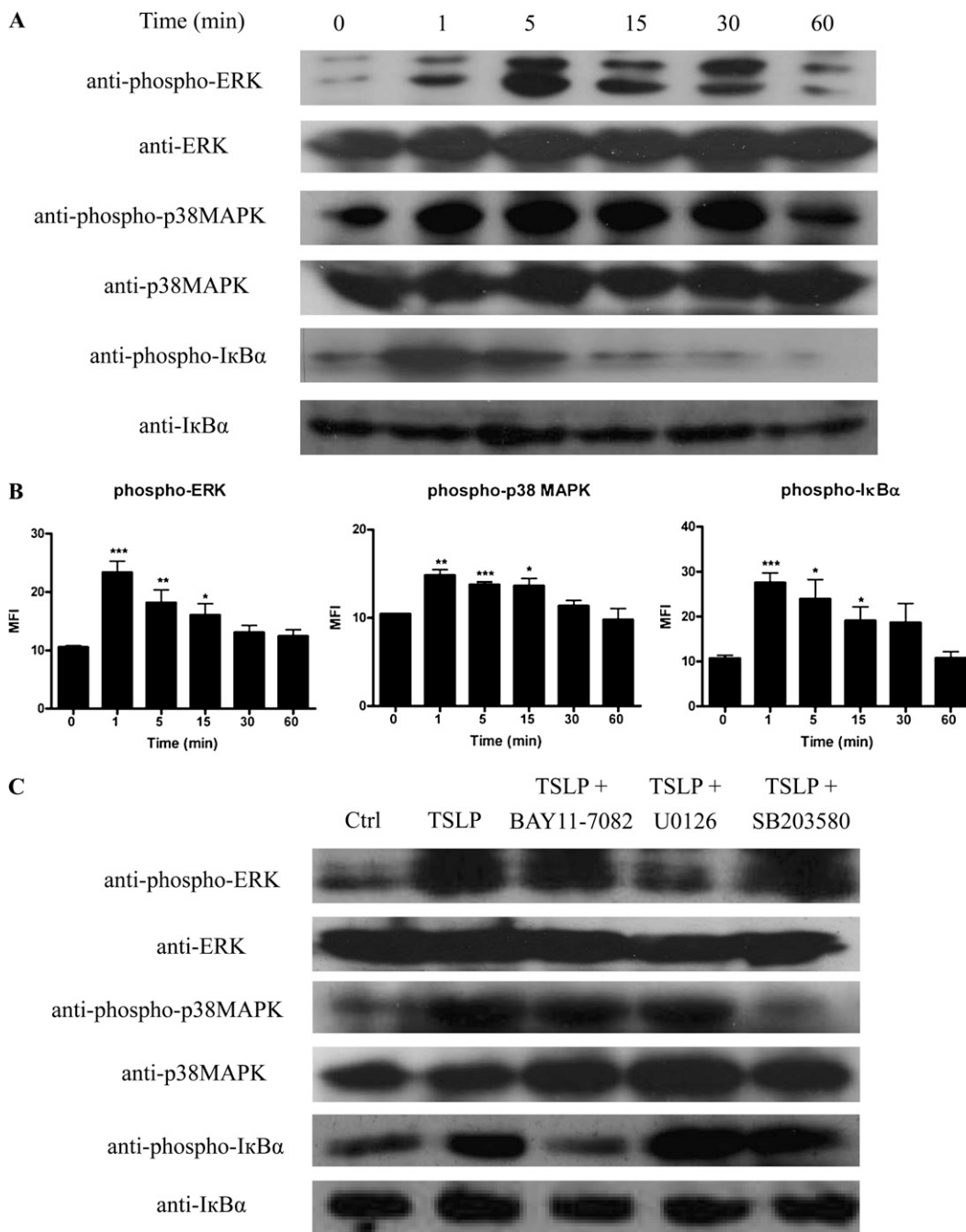


Figure 6. Effects of TSLP on intracellular extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and NF- κ B activities. Eosinophils were incubated with or without TSLP (50 ng/ml) for different incubation times. (A) Total cellular proteins were extracted from eosinophils for the detection of phospho-ERK, phospho-p38MAPK, and phospho-inhibitor- κ B α (I κ B α) with the indicated antibodies by Western blot analysis. (B) The amounts of intracellular phosphorylated signaling molecules in 10,000 permeabilized eosinophils were measured by flow cytometry. Results of phospho-ERK, phospho-p38MAPK, and phospho-I κ B α are shown in MFI, and are expressed as the arithmetic mean ($\pm$ SD) of three independent experiments. * P < 0.05, ** P < 0.01, and *** P < 0.001 when compared with medium control. (C) Eosinophils were pretreated with -inhibitor BAY11-7082 (1 μ M), U0126 (2.5 μ M), and SB203580 (2.5 μ M) for 1 hour, followed by stimulation with TSLP (50 ng/ml) for a further 5 minutes. Phospho-ERK, phospho-p38MAPK, and phospho-I κ B α were detected by Western blot. Experiments were performed in three independent replicates with essentially identical results, and representative results are shown.

helper lymphocytes, Th17, which has been suggested to play pathogenic role in AD and allergic asthma (44). For chemokines, both CXCL8 and CXCL1 are potent chemoattractants for neutrophils and basophils (45, 46), whereas the expression and secretion of CCL2 is well correlated to the infiltration of monocytes, basophils, eosinophils, and memory T lymphocytes and natural killer cells (47). TSLP-induced release of CCL2, CXCL8, and CXCL1 from eosinophils can therefore mediate the recruitment, infiltration, and activation of the above immune effector cells in the microenvironment of inflammatory sites, thereby amplifying inflammatory responses in allergic diseases.

In this study, TSLP significantly up-regulated the cell surface expression of ICAM-1 and CD18, but suppressed L-selectin in a dose-dependent manner. ICAM-1 has been well demonstrated to potentiate inflammatory process in childhood asthma (48). The

interaction of ICAM-1 and integrins is essential for the recruitment and transendothelial migration of eosinophils (3, 4). The up-regulation of both ICAM-1 and CD18 expression is, therefore, important for the recruitment and transmigration of eosinophils into inflammatory sites. Our finding of the down-regulation of L-selectin in TSLP-treated eosinophils concurs with previous studies showing that activated eosinophils could down-regulate L-selectin (36). Unlike most of the other adhesion molecules, the function of the selectin family is uniquely restricted to the interaction of leukocytes with the vascular endothelium. L-selectin mediates the initial attachment of eosinophils onto endothelial cells before their firm adhesion and diapedesis at sites of inflammation (3, 4). As eosinophils pass through the diverse beds of vascular endothelium, a differential use of various adhesion molecules is observed (3, 4, 36). Similar to Th2 cytokine, IL-25, TSLP can significantly enhance the

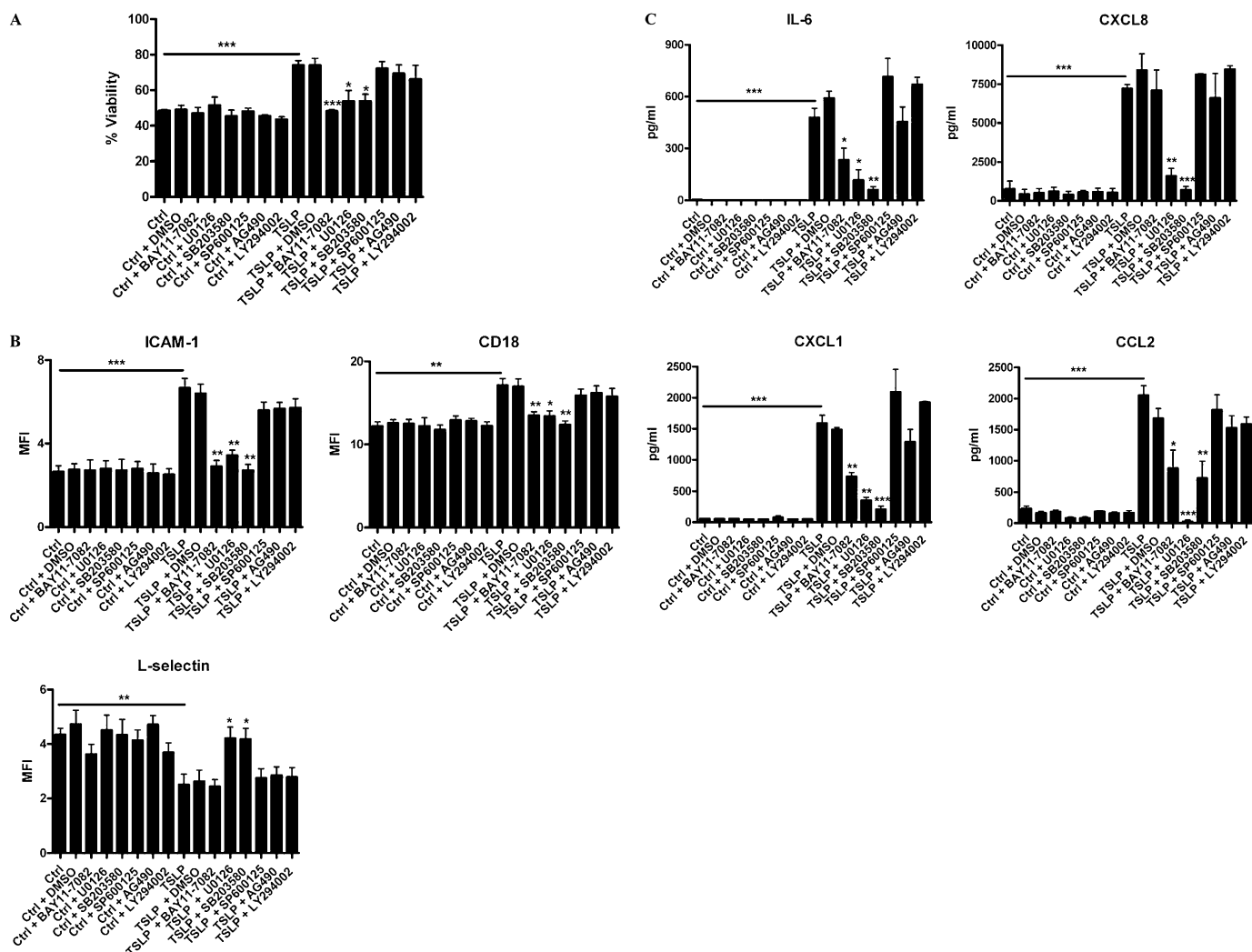


Figure 7. Effects of signaling inhibitors on viability, adhesion molecule expression, and cytokine and chemokine release of eosinophils. Eosinophils were pretreated with AG490 (2 μ M), BAY11-7082 (1 μ M), LY294002 (2 μ M), U0126 (2.5 μ M), SB203580 (2.5 μ M), and SP600125 (2 μ M) for 1 hour, followed by incubation with or without TSLP (50 ng/ml) in the presence of inhibitors for a further (A) 48 hours for viability assay, (B) 16 hours for adhesion molecule expression, and (C) 24 hours for cytokine and chemokine release. Results are expressed as the arithmetic mean ($\pm$ SD) from three independent experiments. DMSO (0.1%) was used as the vehicle control. * P < 0.05, ** P < 0.01, and *** P < 0.001 when compared between groups denoted by horizontal lines or groups of inhibitor treatment and TSLP alone. Ctrl, control.

adhesion of eosinophils onto fibronectin, one important component of extracellular matrix. Together with the induced changes in cellular morphology and cytoskeletal arrangement of eosinophils (Figure 3D), TSLP may facilitate local site eosinophilia (36). Large numbers of eosinophils accumulate beneath skin epidermis in AD and bronchial epithelial cells in asthma (3, 34). Although we did not provide evidence that TSLP could directly chemoattract eosinophils, TSLP indeed modulates eosinophil adhesion molecules, and thus facilitates eosinophil transendothelial migration and tissue eosinophilia. Another important mechanism for TSLP-mediated eosinophilia at skin and bronchial airway is probably due to the local production of eosinophil chemokine, eotaxin-2, by TSLP-activated DCs (11, 21).

According to previous studies, TSLP could activate STAT5 through JAK-STAT-independent pathway (11). In contrast to the above studies, we found that TSLP could not activate STAT3 or STAT5 in eosinophils, as demonstrated by the negative tyrosine and serine phosphorylation. The reason may be that eosinophils are terminally differentiated cells, and lack the potential for proliferation and cell division (3). Indeed,

STAT5 and STAT3 activation by TSLP stimulation is involved in the proliferation of human acute myeloid leukemia-derived cell line, MUTZ-3, and T cell receptor-activated CD4⁺ T cells (6, 13). Although STAT5 was phosphorylated by TSLP in bone marrow-derived pre-B cell line, TSLP-mediated STAT5 activation is insufficient to induce cellular proliferation (49). In view of the above findings, we may infer that other signaling cascades must be involved to compensate for STAT5 in mediating cell proliferation or other cell functions. MAPK and NF- κ B pathways were involved in regulating adhesion molecule expression and cytokine and chemokine release of activated eosinophils upon exposure to various stimuli, such as Th17 cytokines, IL-25, and Toll-like receptor ligands (35, 36, 39). In this study, ERK, p38MAPK, and NF- κ B were also activated in TSLP-treated eosinophils. However, these signaling pathways were activated independently, without cross-talk. Inhibitor experiments further elucidated the intracellular signaling mechanisms regulating the induction of cytokine/chemokine release and maintenance of survival. The consistent results from signaling molecule activation and inhibitor suppression

experiments further confirmed that ERK, p38MAPK, and NF- κ B were activated and involved in TSLP-mediated eosinophil function. Because TSLP can directly activate ERK, p38MAPK, and NF- κ B intracellular signaling pathways in eosinophils, the effect is markedly different from that of other cell types reported before (11, 20). In addition, PI3K/Akt pathway was reported to be activated in mouse CD8⁺ T cells by murine TSLP (15). However, we could not detect any Akt activation upon human TSLP stimulation. This may also raise the issue that the mechanisms modulated by TSLP were cell specific—at least specific for the terminally differentiated eosinophils.

Endobronchial secretion of asthma-related Th2 cytokine, IL-13, at the local inflammatory site has been reported to be at least 100-fold higher than in the circulation (50). Moreover, recombinant TSLP expressed by *Escherichia coli* used in this study has a lower activity than native TSLP, due to the differences in glycosylation and post-translational modification. Therefore, the recombinant TSLP concentration (50 ng/ml) used in this study may activate eosinophils at sites of inflammation, but probably not in the circulation. Actually, the optimal concentration of TSLP (50 ng/ml) used in our experiments is similar to that in previous publications (14, 15, 28).

To our knowledge, this is the first report to demonstrate that TSLP can modulate human eosinophils through ERK-, p38MAPK-, and NF- κ B-dependent, but STAT3- and STAT5-independent signaling transduction pathways. Together with previous studies of TSLP, we further elucidated TSLP-mediated immunophysiological mechanisms in allergic inflammation and the underlying signaling transduction (11). Upon challenge by allergens, viruses, or bacteria, TSLP is induced from epidermal keratinocytes, bronchial epithelial cells, smooth muscle cells, stromal cells, or mast cells. Local immature DCs are immediately activated by TSLP to up-regulate cell surface costimulatory molecules, MHC-II, CD80, CD86, and OX40L, which drive inflammatory Th2 cell differentiation (20, 21, 30). Meanwhile, activated DCs release chemokines IL-8 and eotaxin-2, which attract neutrophils and eosinophils, as well as CCL17 and CCL22, which attract inflammatory Th2 cells (21). In addition, TSLP can directly stimulate locally retained and newly attracted eosinophils by delaying the apoptosis and inducing the release of IL-6, CXCL8, CXCL1, and CCL2, leading to the subsequent infiltration of inflammatory cells, activation of Th17 cells, and aggravation of inflammation. Together with local inflammatory cytokines, TSLP can stimulate mast cells to produce IL-5, IL-13, IL-6, and GM-CSF for triggering IgE production, augmenting Th2 immune response and eosinophilia (30). Regarding intracellular signal transduction, we have elucidated distinct signaling mechanisms in which ERK, p38MAPK, and NF- κ B are selectively activated by TSLP in eosinophils. In view of recent advances in the application of MAPK and NF- κ B inhibitors as potential anti-inflammatory agents in asthma (42), our present study should provide new clues for the development of novel treatment for TSLP-mediated allergic diseases. Follow-up studies should be conducted to further investigate whether ERK, p38MAPK, and NF- κ B are also involved in TSLP-activated DCs, mast cells, and CD4⁺ T cells in allergic inflammation.

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