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EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Thymic stromal lymphopoietin stimulates the formation of eosinophil extracellular traps

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Keywords

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Abstract

Background: Thymic stromal lymphopoietin (TSLP) that is released by epithelial cells upon certain environmental triggers activates cells of the innate and adaptive immune system resulting in a preferential T helper 2 immune response. By releasing eosinophil extracellular traps (EETs), eosinophils achieve an efficient extracellular bacterial killing. Eosinophil extracellular traps release, however, has been observed in both infectious and noninfectious eosinophilic diseases. Here, we aim to investigate whether eosinophils generate functional EETs as a direct response to TSLP, and further to study the extra- and intracellular mechanisms involved in this process as well as TSLP receptor (TSLPR) expression by eosinophils *in vitro* and *in vivo*.

Methods: Thymic stromal lymphopoietin receptor expression on blood and tissue eosinophils was assessed by immunoblotting, flow cytometry, and immunofluorescence staining. Purified eosinophils were stimulated with recombinant human TSLP. The release of extracellular DNA in association with eosinophilic cationic protein (ECP) was detected by fluorescence staining techniques and confocal microscopy. In addition, cell survival, cell adhesion, production of reactive oxygen species (ROS), and the inhibition of bacterial growth by TSLP-stimulated eosinophils were measured.

Results: Thymic stromal lymphopoietin receptor was observed on peripheral blood eosinophils as well as on tissue infiltrating eosinophils in skin diseases. TSLP did not affect eosinophil survival, but induced the formation of EETs consisting of mitochondrial DNA in association with ECP in a concentration- and time-dependent manner. Eosinophil extracellular trap release could be inhibited by blocking either cell adhesion or ROS production. While eosinophils prevented the growth of both *Staphylococcus aureus* and *Staphylococcus epidermidis*, the latter were unable to elicit EET formation and eosinophils required additional TSLP stimulation to achieve this antibacterial activity.

Conclusions: thymic stromal lymphopoietin directly stimulates eosinophils to produce EETs. Our observations link epithelial TSLP expression triggered by environmental factors with pathogen defense mechanisms involving eosinophils.

Abbreviations

C5a, complement factor 5a; DAPI, 4,6-diamino-2-phenylindole; DNase, deoxyribonuclease; DPI, diphenylene iodonium chloride; EET, eosinophil extracellular trap; ECP, eosinophil cationic protein; IL, interleukin; IL-7Ra, IL-7 receptor alpha; LPS, lipopolysaccharide; PI, propidium iodide; ROS, reactive oxygen species; TLR, Toll-like receptor; TSLP, thymic stromal lymphopoietin; TSLPR, thymic stromal lymphopoietin receptor.

Eosinophils play an important role in host defense, immunoregulation, and tissue remodeling (1–3). Recently, the release of mitochondrial DNA together with toxic granule proteins forming eosinophil extracellular traps (EETs) has been reported (4). Eosinophil extracellular traps are able to kill bacteria efficiently and have been observed in both infectious and autoimmune intestinal diseases (4). EETs have also been observed in noninfectious diseases of the skin (5) and airways

(6). To generate EETs, eosinophils require priming by IL-5 or IFN- γ , and subsequent stimulation of Toll-like receptors (TLR), complement, or chemokine receptors (4).

Thymic stromal lymphopoietin (TSLP) is produced by epithelial cells at barrier surfaces such as the intestine, airways, and skin (7). Elevated TSLP expression has been found in atopic diseases, such as atopic dermatitis (AD) (8), bronchial asthma (9), and allergic rhinitis (10). Strong epidermal TSLP expression was also observed in eosinophilic skin diseases such as allergic contact dermatitis, urticaria, and eosinophilic dermatitis, and in autoimmune bullous diseases (11). Thymic stromal lymphopoietin was shown to be released by keratinocytes via protease-activated receptor (PAR)-2 activation (12) and mechanical injury (13), as well as by TLR-2/TLR-6 stimulation by *Staphylococcus aureus* (14), or TLR-3 stimulation mediated by extracellular double-stranded RNA (15). Thymic stromal lymphopoietin is known to target dendritic cells (16, 17), T cells (18, 19), natural killer T cells (20), mast cells (21), and basophils (22, 23) driving preferentially T helper 2 type inflammation. Recently, TSLP was also reported to upregulate surface adhesion molecules and chemokine release by eosinophils expressing the TSLPR/IL-7R α receptor complex (24).

On the basis of the observations that TSLP initiates inflammation following epithelial barrier injury and that eosinophils participate in pathogen defense by generating EETs, we aimed to investigate whether TSLP might not itself be responsible for activating eosinophils to release EETs. Further, we characterized the conditions under which TSLP-stimulated eosinophils produce EETs as well as the intra- and extracellular mechanisms involved.

Material and methods

Cell isolation

Blood eosinophils were purified from healthy donors and patients with atopic diseases or hypereosinophilic syndromes by negative selection from peripheral blood using the EasySep human eosinophil enrichment kit (StemCell, Grenoble, France). Unwanted cells were targeted for removal with tetrameric antibody complexes recognizing CD2, CD3, CD14, CD16, CD19, CD20, CD36, CD56, CD123, glycophorin A, and dextran-coated magnetic particles. To isolate basophils using the EasySep human basophil enrichment kit (StemCell), tetrameric antibody complexes recognizing CD2, CD3, CD14, CD15, CD16, CD19, CD24, CD34, CD36, CD45RA, CD56, glycophorin A, and dextran-coated magnetic particles were used to remove the unwanted cells from peripheral blood mononuclear cell (PBMC) fraction. The purity of isolated eosinophils and basophils was 98% as assessed by Diff-Quik (Medion GmbH, Dudingen, Switzerland) staining and light microscopy.

Peripheral blood neutrophils were purified from healthy individuals by Ficoll-Hypaque centrifugation. Briefly, PBMCs were separated by centrifugation on Ficoll-Hypaque (Seromed-Fakola AG, Basel, Switzerland). The lower layer consisting mainly of granulocytes and erythrocytes was treated with erythrocyte lysis solution (155 mM NH₄Cl, 10 mM

KHCO₃, and 0.1 mM EDTA, pH 7.3) (25). The resulting cell populations contained >95% neutrophils assessed by staining of cells with Diff-Quik (Medion GmbH) staining and light microscopy. Monocytes were collected from the PBMC fraction. PBMCs were washed twice with PBS and incubated with RPMI, 10% FCS, and 1% penicillin/streptomycin (Invitrogen, Paisley, UK) for 30 min at 37°C in plastic flasks. Adherent monocytes were collected, washed twice with PBS, and used for subsequent experiments.

Written informed consent was obtained from all blood donors, and the study was approved by the ethics committee of the Canton of Bern.

Eosinophil activation

Eosinophils were seeded on 12-mm glass cover slips and stimulated with 0.1–100 ng/ml recombinant human TSLP (R&D Systems, Minneapolis, MN, USA) for 5–60 min. For control experiments, we treated the slides with 10 U/ml deoxyribonuclease (DNase; Roche Diagnostics, Rotkreuz, Switzerland) and additionally pretreated the eosinophils with polymyxin B (Calbiochem, Lucerne, Switzerland) or polyclonal anti-TSLP antibody (Ab) (R&D Systems). Combined 10 ng/ml IL-5 (R&D Systems) and 100 nM C5a (MBL International, Woburn, MA, USA) stimulation served as a positive control (4).

Confocal laser scanning microscopy

Isolated blood cells were either fixed with 4% paraformaldehyde or observed in live-cell microscopy experiments. For DNA detection, slides were treated with either 1 μ g/ml propidium iodide (PI) or 0.5 μ M SYTO13 or mounted with mounting medium containing 4,6-diamino-2-phenylindole (DAPI; all Invitrogen) (4, 26). Eosinophilic cationic protein (ECP) colocalizing with extracellular DNA was determined by immunofluorescence using an anti-ECP mAb (Pharmacia Diagnostics AB, Uppsala, Sweden) (4). To determine TSLPR expression, isolated granulocytes were stained with monoclonal antibodies to TSLPR (R&D Systems), CD16 (Santa Cruz Biotechnology, Heidelberg, Germany), or CD193/CCR3 (Biolegend, San Diego, CA, USA). Mitochondrial DNA was visualized with MitoSox Red (Invitrogen). Immunofluorescent staining was also performed on 6- μ m formaldehyde-fixed and paraffin-embedded skin sections using the following primary antibodies: anti-TSLPR (Novus Biologicals, Littleton, CO, USA), anti-ECP (Pharmacia), anti-TSLP (R&D Systems), and appropriate secondary antibodies labeled with Alexa Fluor 488 and Alexa Fluor 555 or Cy3, respectively (all Invitrogen). After washing with phosphate-buffered saline, the specimens were mounted in a drop of fluorescent mounting medium (DAKO, Glostrup, Denmark). Slides were analyzed by confocal laser scanning microscopy (LSM 510; Carl Zeiss Micro Imaging, Jena, Germany).

Immunoblotting

Electrophoresis was conducted in Run Blue 4–20% SDS gels (Expedeon, Cambridgeshire, UK), and the separated proteins

were transferred onto polyvinylidene difluoride membranes (Millipore, Tullagreen, Ireland). The membranes were incubated with blocking solution (phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk) overnight at 4°C. The membranes were then incubated with 1 µg/ml of antihuman TSLPR (Novus Biologicals) or IL-7Rα (Millipore, Temecula, CA, USA) antibodies, respectively, in blocking solution for 1 h at room temperature. As a loading control, stripped filters were incubated with anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mAb diluted 1 : 5000 (Millipore). Filters were washed in PBS-Tween (0.1%) for 30 min and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies diluted 1 : 5000 (GE Healthcare, Buckinghamshire, UK) at room temperature for 1 h. After additional washing, the blots were developed by an enhanced chemiluminescence technique (ECL kit; GE Healthcare) according to the manufacturer's instruction.

Flow cytometry

Isolated cells were treated with blocking solution containing 10% FCS, 10% polyvalent human IgG (CSL Behring, Bern, Switzerland), and 1% human IgG (Jackson Immuno Research, West Grove, PA, USA) in PBS for 20 min. Five micrograms per millilitre biotinylated antihuman TSLPR or isotype control antibody (both R&D Systems) was added, and the cells were incubated for 30 min at 4°C followed by staining with streptavidin-PE (Biolegend). For IL-7Rα expression analysis, a PE-labeled Ab (BD Biosciences, San Jose, CA, USA) was used. Receptor expression was analyzed by cytometric analysis (FACSCalibur; BD Bioscience) and quantitated using FlowJo software (Ashland, OR, USA).

Adhesion assay

Eosinophils (1×10^6 /ml) were incubated in serum-free X-VIVO (Lonza, Verviers, Belgium) with and without monoclonal anti-CD18 (Ancell, Bayport, MN, USA) and anti-CD11b Abs (Santa Cruz) on ice for 30 min and stimulated with or without TSLP for 30 min at 37°C, and EET formation was determined. To measure adherence, cells were washed twice with PBS and stained with Syto13 (Invitrogen). Optical density was measured using SpectraMaxM2 plate reader (Bucher Biotech, Basel, Switzerland) after 10-min incubation at 37°C. As negative controls, mouse IgG1 F(ab)₂ and mouse IgG1 antibody (both Jackson ImmunoResearch) were used.

Determination of cell death

Eosinophil death was assessed by the uptake of calcein AM and ethidium homodimer-1 (both from Invitrogen) after 20-min stimulation with 20 ng/ml TSLP by live-cell imaging using confocal laser scanning microscopy. Phorbol 12-myristate 13-acetate (PMA, 100 nM; Calbiochem) was used as a positive control. For flow cytometric analysis, eosinophils were stimulated with 10, 20, and 50 ng/ml TSLP (R&D

Systems) or 25 ng/ml IL-5 (R&D Systems). Cell death was assessed by the uptake of 1 µM ethidium bromide (Invitrogen) at 24, 48, and 72 h (26).

ROS measurement

Isolated eosinophils were placed in black 96-well microplates (Greiner Bio-One GmbH, Frickenhausen, Germany), incubated with 1 µM dihydro-rhodamine-123 (DHR; Sigma, Buchs, Switzerland) for 30 min, and stimulated with either 20 or 50 ng/ml TSLP, IL-5 plus C5a, or 100 nM PMA as a positive control. To block ROS generation by NADPH oxidase, 5 µM of the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI; Calbiochem) was added to eosinophils for 10 min prior to stimulation. Reactive oxygen species (ROS) generation was measured at different time points by SpectraMaxM2 plate reader (Bucher Biotech) (27).

Bacterial strains and culture conditions

Staphylococcus aureus ATCC 25923 and *Staphylococcus epidermidis* ATCC 14990 were cultured in brain-heart infusion (BHI) medium (Sigma) at 37°C with shaking. Fresh overnight bacterial cultures were diluted 1 : 100 in BHI medium and then grown to mid-logarithmic growth phase (OD₆₀₀ = 0.7) for subsequent experiments.

Bacterial killing assay

Bacterial killing assay was performed as previously described (28). Briefly, human eosinophils (1×10^6 /ml) were resuspended in RPMI and stimulated with TSLP or IL-5 + C5a in the presence or absence of 10 U/ml DNase for 30 min at 37°C, 5% CO₂; thereafter, they were infected with bacteria resuspended in Hanks' balanced salt solution (HBSS; Invitrogen) and incubated for 60 min at 37°C with an up-and-down rolling motion. 400 µl ice-cold HBSS was added and centrifuged at 100 g, 10 min, 4°C. Supernatants were collected, diluted, plated in BHI agar (Sigma), and grown overnight before counting the colonies. For visualization, bacteria (10×10^6 /ml) were incubated in 82 µg/ml fluorescein-5-isothiocyanate (FITC isomer I; Invitrogen) for 30 min on ice. Eosinophils (2×10^5 /100 µl) were seeded on cover slips and either left untreated or were stimulated with TSLP for 30 min at 37°C. FITC-labeled bacteria (1×10^6 /100 µl) were added to the eosinophils and incubated for 1 h at 37°C. Cells were stained with MitoSox Red, fixed with 4% paraformaldehyde, and mounted with mounting medium containing DAPI. Slides were sealed and analyzed by confocal laser scanning microscopy.

Statistical analysis

Data are presented as mean levels ± SEMs. For multiple comparisons, one-way analysis of variance was applied followed by Tukey's test. All statistical analyses were carried out using Graph Pad Prism 5 software (La Jolla, CA, USA). *P* values <0.05 were considered statistically significant.

Results

TSLP stimulated eosinophil extracellular trap formation

To investigate whether TSLP triggers the formation of EETs, purified blood eosinophils were stimulated with TSLP at different concentrations. The results showed that eosinophils upon TSLP stimulation released extracellular DNA (Fig. 1A) in a time (Fig. 1C)- and concentration (Fig. 1D)-dependent manner. The greatest proportion of DNA-releasing eosinophils was observed upon stimulation with 20 ng/ml TSLP after 30 min; hence, these conditions were used for further experiments. Without TSLP stimulation, no DNA release was detected (Fig. 1A). To confirm that the effect of TSLP was specific, we neutralized TSLP by a blocking antibody. Indeed, when eosinophils were incubated with TSLP together with blocking anti-TSLP antibody, no DNA release was observed (Fig. 1A,D). To exclude any potential effect of lipopolysaccharide, TSLP was preincubated with polymyxin B. This treatment had no effect on TSLP-induced DNA release (Fig. 1A,D). However, a brief treatment with DNase completely abolished the extracellular DNA structures (Fig. 1B). As IL-5 and C5a have previously been shown to

be potent triggers of DNA release, we investigated whether either or both factors could further enhance the effect of TSLP. Preincubation of eosinophils with IL-5, C5a, or both failed to increase TSLP-induced DNA release (Fig. 1E). Moreover, the same proportion of eosinophils releasing DNA was observed upon priming with IL-5 and subsequent stimulation with C5a, or following stimulation with TSLP exclusively (Fig. 1E). Eosinophils isolated from healthy donors or patients with atopic diseases or hypereosinophilic syndrome exhibited no differences in DNA release upon TSLP stimulation (data not shown), suggesting that priming or prior *in vivo* stimulation has no effect on TSLP-induced DNA release *in vitro*.

As the extracellular DNA structures were reported to contain toxic granular proteins in addition to DNA, TSLP-stimulated eosinophils were also stained by PI in combination with an anti-ECP mAb. In the extracellular structures, we identified both DNA and ECP, thus indicating that they indeed represent EETs (Fig. 2A). Unstimulated eosinophils demonstrated no EET formation and thus retained their DNA and ECP within the cell (Fig. 2B). Interestingly, TSLP-stimulated eosinophils after having released EETs still retained their

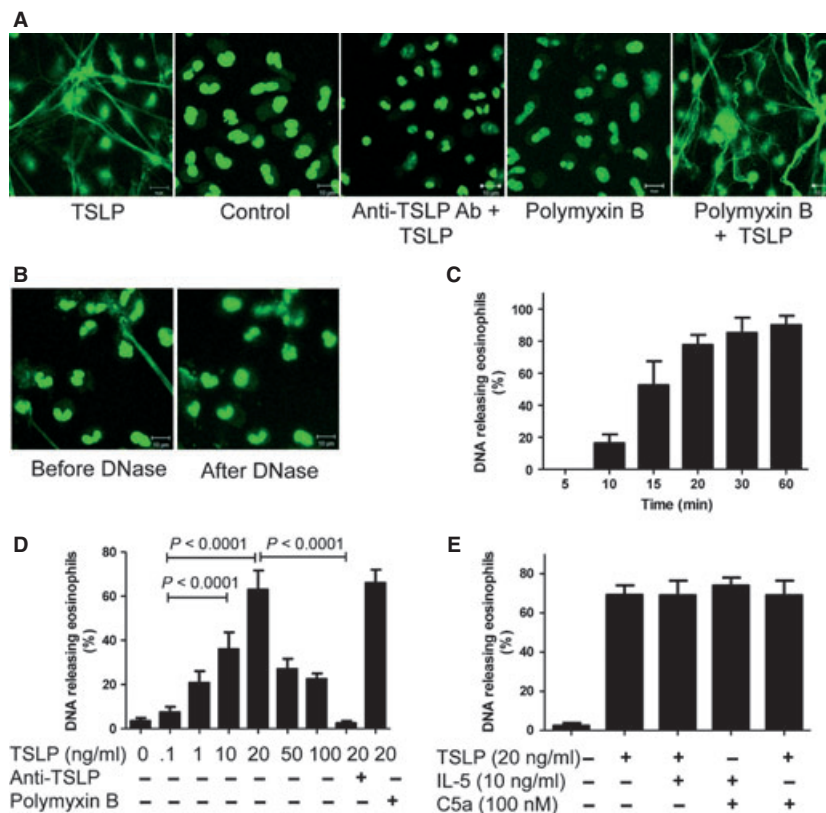


Figure 1 Release of DNA traps from eosinophils upon thymic stromal lymphopoietin (TSLP). (A) Thymic stromal lymphopoietin (20 ng/ml for 30 min) stimulated eosinophils to release DNA traps (DNA stained with Syto13, green). This effect was blocked by preincubation with anti-TSLP Ab and was not attributed to lipopolysaccharide because polymyxin B treatment was without effect. (B) Deoxyribonuclease abolished DNA traps. (A and B: Magnification

×1000) (C) Increasing numbers of eosinophils show DNA release over time with a maximum after 60 min ($n = 4$). (D) Concentration-dependent increase in eosinophil DNA release with a maximum at 20 ng/ml TSLP stimulation ($n = 5$). (E) Thymic stromal lymphopoietin-induced DNA release was not further enhanced by additional stimulation with IL-5 or C5a and was comparable with that obtained after IL-5 plus C5a stimulation ($n = 6$).

nuclei but exhibited markedly reduced cytoplasmic DNA and ECP (Fig. 2A). When neutrophils were stimulated with TSLP, no extracellular DNA release was observed (Fig. 2C).

Eosinophils expressed TSLP receptor *in vitro* and *in vivo*

To verify a functional response by eosinophils to TSLP stimulation, we investigated the TSLPR expression on blood and

tissue eosinophils. As demonstrated by confocal microscopy, in the population of blood granulocytes, TSLPR expression was present exclusively on eosinophils but not neutrophils as identified by morphology (Fig. 3A). Eosinophils identified by staining with an monoclonal anti-CCR3 Ab also demonstrated the expression of TSLPR, whereas CD16-positive neutrophils did not express TSLPR (Fig. 3B,C). Moreover, consistent with previous reports (24), TSLPR and the

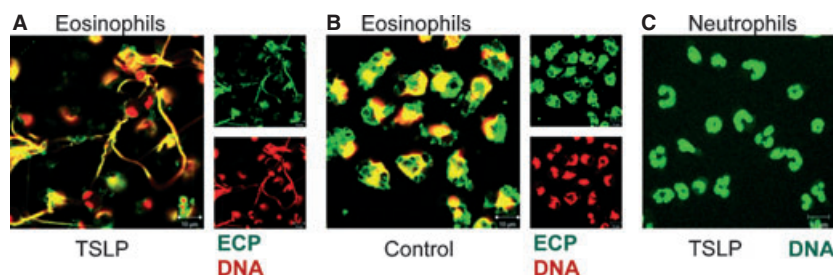


Figure 2 Eosinophil extracellular traps contain DNA and granule proteins. (A) After thymic stromal lymphopoietin (TSLP) (20 ng/ml for 30 min) stimulation, eosinophils release DNA (stained with PI, red) together with eosinophilic cationic protein (ECP) (green). (B)

Unstimulated eosinophils retain both DNA and ECP in the cell (yellow overlay). (C) Neutrophils do not release DNA upon TSLP stimulation (DNA stained with Syto13, green). Representative images of three independent experiments are shown.

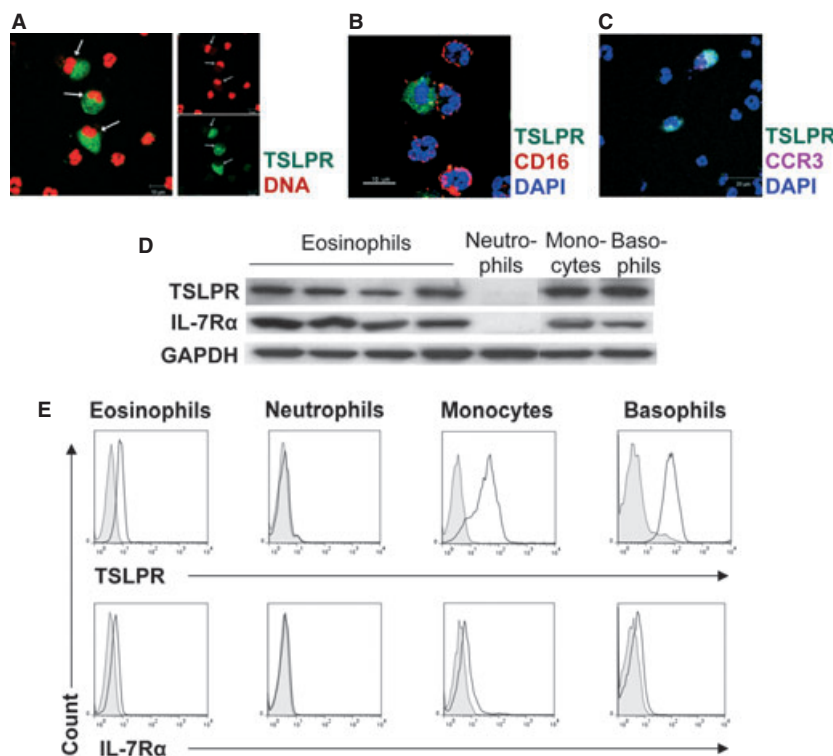


Figure 3 Thymic stromal lymphopoietin receptor (TSLPR) expression on eosinophils. (A) Among isolated granulocytes, TSLPR (green) was expressed on eosinophils as assessed by morphology (arrows). (B) Eosinophils with double-lobed nuclei bear TSLPR (green), whereas neutrophils stained with an anti-CD16 antibody (red) lacked TSLPR. (C) Eosinophils identified by CCR3 expression

exhibited TSLPR (double-stained cells). Magnification $\times 1000$. (D) Immunoblot revealed TSLPR and IL-7R α expression on eosinophils, monocytes, and basophils, but not neutrophils. (E) Thymic stromal lymphopoietin receptor expression on eosinophils, monocytes, and basophils as assessed by flow cytometry. Representative data of three independent experiments are shown.

IL-7R α chain that together form a heterodimeric receptor complex (29) were found on eosinophils as well as monocytes and basophils. However, as assessed by immunoblotting (Fig. 3D) and flow cytometry (Fig. 3E), the receptor complex was absent on neutrophils isolated from peripheral blood. To investigate whether tissue eosinophils express TSLPR and thus might be potential targets for TSLP, we performed immunofluorescence staining on specimens of eosinophilic skin diseases in which both TSLP expression and EETs had previously been detected (5, 11). Considerable numbers of skin infiltrating eosinophils (over 90%) expressed TSLPR (Fig. 4A). The highest numbers of TSLPR-bearing eosinophils were noted in Wells' syndrome (WS) and bullous pemphigoid (BP) with 463 and 622 cells per mm², respectively, compared with 164 TSLPR+ eosinophils per mm² in AD (Fig. 4B). Staining with isotype control antibodies revealed no significant staining (data not shown). Furthermore, we observed an increased expression of TSLP in these specimens compared to normal skin (Fig. 4C).

DNA release depends on adhesion and ROS production

Our experiments suggested that TSLP was able to induce EETs in isolated blood eosinophils in the absence of any additional agonist. We next investigated whether TSLP alone is sufficient for DNA release or whether eosinophils require additional stimulation via adhesion molecules. After blocking

adhesion molecules on eosinophils with antibodies inhibiting the integrins CD18 and CD11b, DNA release upon TSLP stimulation was completely abolished (Fig. 5A,B). The effect of anti-CD18 and anti-CD11b antibodies on inhibiting eosinophil adhesion was confirmed by measuring the optical density (Fig. 5C). As there is evidence that ROS are involved in extracellular DNA release (4), we investigated whether DNA release following TSLP treatment also shows ROS dependence. Inhibition of ROS production with DPI prior to TSLP stimulation completely blocked DNA release (Fig. 5A, B). The quantitation of ROS production demonstrated that ROS levels measured upon TSLP stimulation are comparable to those induced upon combined IL-5 and C5a stimulation of eosinophils (Fig. 5D).

Stimulation with TSLP at concentrations 10–50 ng/ml for 30 min did not affect eosinophil viability as determined by microscopy and live/dead staining (Fig. 6A) or ethidium incorporation measured by flow cytometry (Fig. 6B), indicating that the observed DNA release is not associated with cell death. Furthermore, we did not observe any effect of TSLP on eosinophil survival as assessed by flow cytometry (Fig. 6B). The extracellular DNA released by eosinophils has been reported to derive from mitochondria (4). We employed MitoSox Red dye which enters viable cells and specifically visualizes mitochondria by emitting fluorescent signals depending on superoxide production; we also obtained evidence that the DNA released upon TSLP stimulation

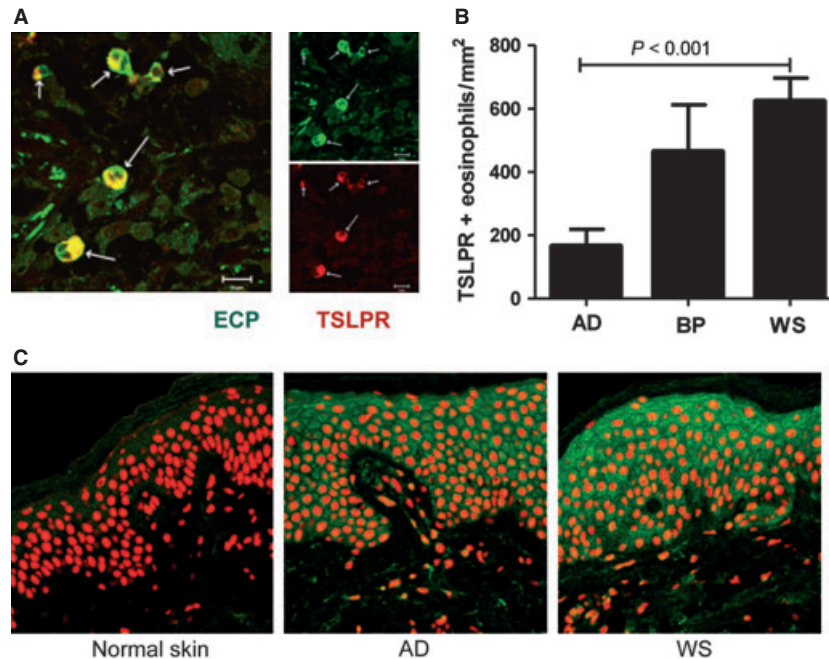


Figure 4 Thymic stromal lymphopoietin receptor (TSLPR) expression in eosinophilic skin diseases. (A) Immunofluorescence staining with antibodies to eosinophilic cationic protein (green) and TSLPR (red) showed TSLPR-bearing tissue eosinophils in atopic dermatitis. (B) Quantitation of TSLPR-expressing eosinophils in atopic dermatitis (AD, $n = 11$), bullous pemphigoid

(BP, $n = 4$), and Wells' syndrome (WS, $n = 3$). Numbers of positive cells per mm² were calculated based on the counts in 10 fields per specimen at a magnification $\times 1000$. (C) Thymic stromal lymphopoietin is strongly expressed by epidermal keratinocytes in AD and WS as compared with normal skin. Magnification $\times 400$.

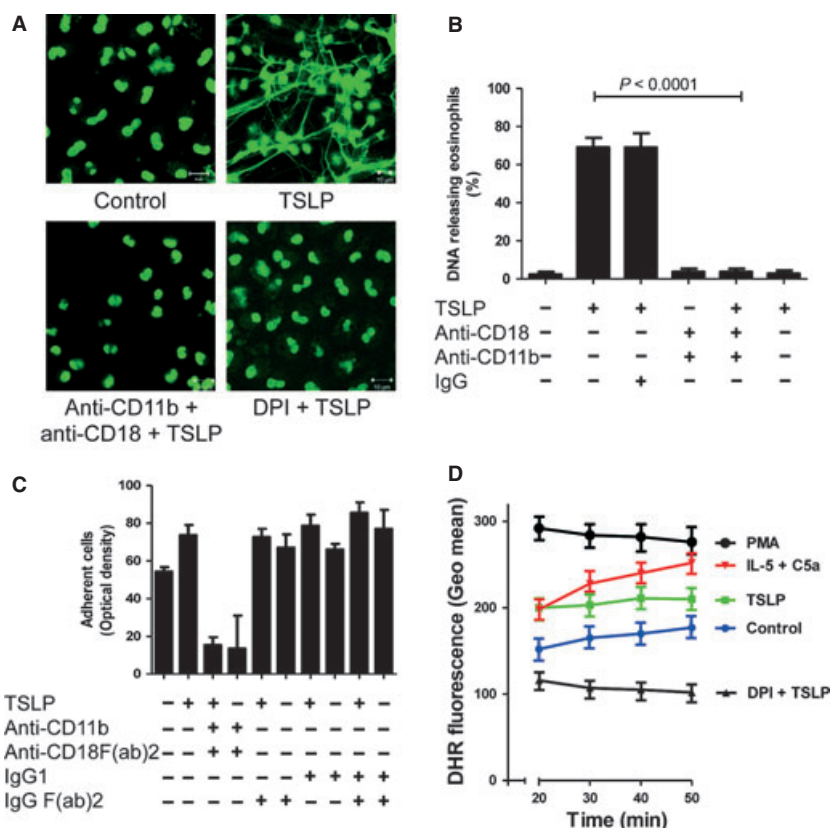


Figure 5 Eosinophil extracellular trap (EET) release is dependent on adhesion and production of reactive oxygen species (ROS). (A) Blocking adhesion with antibodies to CD11b and CD18 or blocking ROS generation with diphenylene iodonium chloride (DPI) completely inhibited EET release upon thymic stromal lymphopoietin (TSLP) stimulation. (B) Quantitation of eosinophils releasing EETs after TSLP stimulation (20 ng/ml for 30 min) in the presence or absence of antibodies to CD11b, CD18, control

represents mitochondrial DNA (Fig. 6C). After eosinophils had released DNA, mitochondria largely emptied of DNA remained (Fig. 6C).

TSLP-stimulated eosinophils prevent the growth of *Staphylococcus epidermidis*

Next, we investigated whether TSLP-mediated EETs exhibit antibacterial activities (4). When eosinophils were cocultured with *S. aureus*, they immediately generated EETs in the absence of any additional stimuli, most likely via TLR2 stimulation (30). The EETs under these conditions were functional as they entrapped *S. aureus* and significantly inhibited their growth (Fig. 7A,C). In contrast, eosinophils exposed to *S. epidermidis* showed no EET release and had little effect on the bacteria in the surrounding medium (Fig. 7A,C). However, following concurrent TSLP stimulation, eosinophils generated EETs and also significantly inhibited *S. epidermidis* growth (Fig. 7A). To investigate whether the antibacterial effect was dependent on EET

Ab, or DPI ($n = 6$). (C) Eosinophil adherence was blocked by antibodies to CD11b and CD18, but not by TSLP or control antibodies, as assessed by measuring optical density ($n = 3$). (D) Reactive oxygen species production in eosinophils is increased in TSLP (20 ng/ml) stimulated as compared with unstimulated eosinophils and is comparable with that upon priming with IL-5 plus C5a stimulation. Preincubation with DPI blocked ROS production ($n = 7$).

formation, we added DNase to the system completely abolishing the inhibitory effect on bacterial growth, hence suggesting that EET generation was indeed required for the antibacterial activity (Fig. 7B).

Discussion

Here, we report for the first time that TSLP directly stimulates eosinophils to release EETs. This mechanism provides a link between the injury to an epithelial barrier and an increased TSLP expression with subsequent pathogen defense response by eosinophils. Thus, our findings seem likely to be relevant in eosinophilic inflammation.

TSLP was identified as potent trigger of eosinophils, which released DNA to the same extent as observed following IL-5 and C5a stimulation. DNA release was a rapid event occurring within minutes after TSLP stimulation in a time-dependent manner. Eosinophil extracellular traps were induced by TSLP at concentrations shown to be released by epithelial cells (21). DNA release upon TSLP was specific

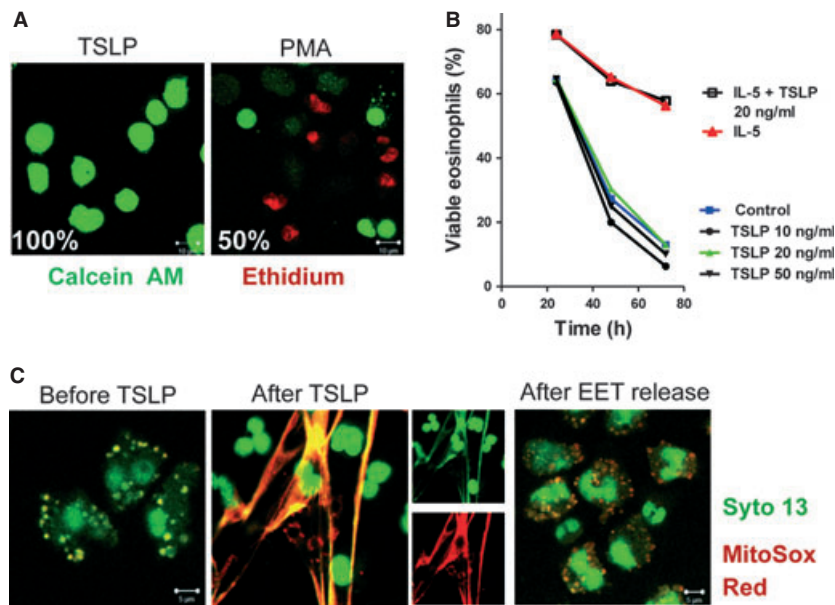


Figure 6 Thymic stromal lymphopoietin (TSLP) induced the release of mitochondrial DNA. (A) Live-cell imaging showed no effect of short-term TSLP stimulation (20 ng/ml for 30 min) on eosinophil viability in contrast to PMA (100 nM). (B) Thymic stromal lymphopoietin at concentrations 10–50 ng/ml did not affect eosinophil survival in contrast to prolonged survival upon IL-5 stimulation over

3 days as assessed by ethidium bromide uptake and flow cytometry ($n = 10$). (C) Eosinophils contained DNA (Syto13) in the nuclei (green) and mitochondria (stained with MitoSox, overlay yellow). Thymic stromal lymphopoietin induced the release of mitochondrial DNA (green and red). After DNA release, mitochondria (red) lacked DNA, but nuclei did not. Magnification $\times 1000$.

and dependent on TSLPR expression. Considering that TSLP is released upon disruption of the epithelial barrier (12–14, 31), it seems logical that TSLP itself is able to initiate a direct and rapid defense reaction. We observed TSLP-mediated activation of eosinophils followed by subsequent release of EETs, which had previously been shown to play a role in host defense (4). Especially in the gut, where eosinophils are resident, such a mechanism might be expected to play a role (32). Eosinophil extracellular traps had been reported to be composed of DNA and toxic granule proteins, suggesting a targeted attack of the eosinophils against pathogens in parallel with limited collateral tissue damage (4). Here, we could show that TSLP-induced EETs contain both DNA and ECP. Thus, eosinophils can release toxic granule proteins that stick to the DNA nets in response to TSLP stimulation.

Consistent with a previous report (24), we could demonstrate the expression of both components of the heterodimeric TSLP receptor complex, TSLPR and IL-7R α , on eosinophils. Although eosinophils expressed lower levels of TSLPR compared with monocytes and basophils, the expression was sufficient to respond functionally to EET release upon TSLP stimulation. Like eosinophils, neutrophils have the capacity to release extracellular DNA traps, the so-called NETs, upon priming with GM-CSF and subsequent stimulation via TLR4 or C5a (26). However, because neutrophils lack both TSLPR and IL-7R α , they did not release DNA upon TSLP stimulation. In addition to our *in vitro* observations, we also demonstrate TSLPR expression on eosinophils *in vivo* using biopsies derived from patients

with different eosinophilic skin diseases. Interestingly, in eosinophilic skin diseases, for example, BP and WS, in which we demonstrated strong epidermal TSLP expression as well as TSLPR-bearing eosinophils, we detected multiple EETs in the tissue (5), suggesting a functional interaction between TSLP and eosinophils under these inflammatory conditions.

TSLP has been reported to play a role under physiologic conditions by maintaining gut immune homeostasis as well as in parasitic and allergic inflammation (7, 33). In response to microbial stimulation, intestinal epithelial cells produced TSLP and thus induced tolerogenic dendritic cells, which subsequently primed naive T cells to produce TGF- β (34). In helminthic infection, TSLP-induced T helper 2 cytokine expression was shown to be required for worm expulsion (35). Overexpression of TSLP in the skin leads to an AD-like disease with characteristic histological features including T-cell and eosinophil infiltration (36). It should be noted that exactly at the same sites where enhanced TSLP expression was detected, eosinophils were not only present, they were also found to generate EETs, that is, in the gut, skin, and lung (4–7). Therefore, both this colocalization and the functional response of eosinophils to TSLP *in vitro* strongly suggest that TSLP exerts a direct effect on eosinophils, resulting in EET formation and perhaps other functions.

Interestingly, whereas eosinophils generated EETs following stimulation with *S. aureus*, no extracellular DNA traps were found upon exposure to *S. epidermidis*, perhaps owing

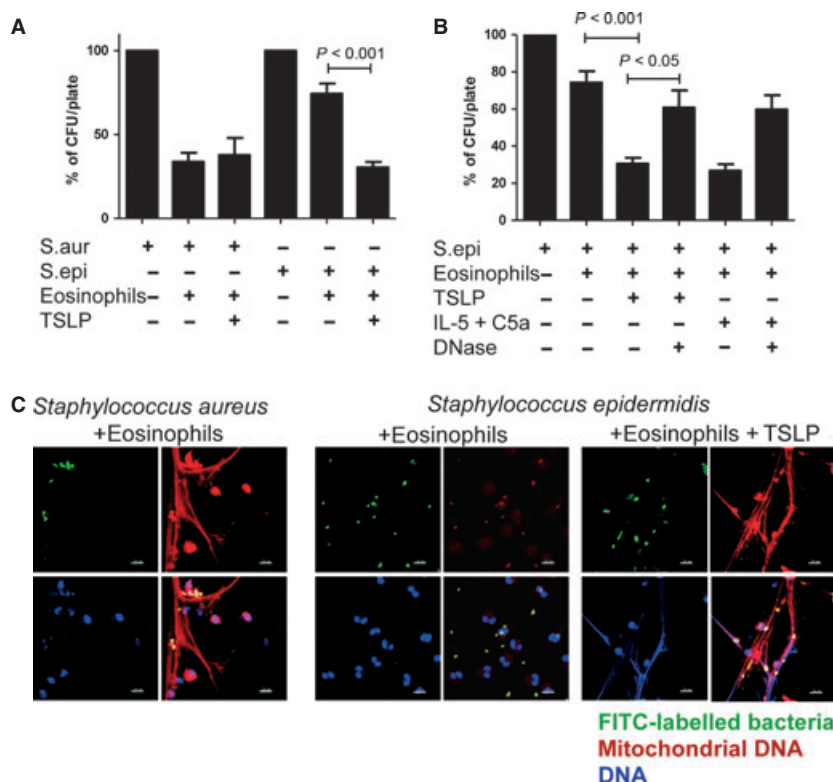


Figure 7 Thymic stromal lymphopoietin (TSLP) stimulated eosinophils to form eosinophil extracellular traps (EETs) and inhibit growth of *Staphylococcus epidermidis*. (A) TSLP significantly increased the capacity of eosinophils to block the growth of commensal *S. epidermidis*, but had no additional inhibitory effect on *Staphylococcus aureus* ($n = 3$). (B) Growth inhibition of

S. epidermidis by eosinophils upon TSLP stimulation was as effective as IL-5 plus C5a stimulation and was abolished by deoxyribonuclease ($n = 3$). (C) Upon stimulation with *S. aureus*, eosinophils released DNA traps (red, blue) capturing the bacteria (green). However, eosinophils required TSLP stimulation to produce EETs (red, blue) trapping *S. epidermidis* (green) ($n = 2$). Magnification $\times 1000$.

to activation of different signaling pathways (37). The failure of EET formation in the presence of *S. epidermidis* was associated with the absence of any eosinophil antibacterial activity. However, following stimulation with TSLP, eosinophils were able to inhibit the growth of *S. epidermidis*. *Staphylococcus epidermidis* represents a commensal bacterium, which may invade the host if the barrier function of the skin is interrupted (38). As under these conditions keratinocytes express and release TSLP (13), the antibacterial effect of eosinophils may prevent further invasion by *S. epidermidis* and, perhaps, other bacteria. On the other hand, the anti-*S. epidermidis* effect of eosinophils may lead to a new balance of the skin flora, allowing the colonization with pathogenic microbes. For instance, the reduction in *S. epidermidis*, which is an important source of antimicrobial peptides, might result in a propagation of *S. aureus*, which plays a key pathogenic role in AD (39, 40). Moreover, the observation that *S. aureus* directly stimulates EET generation underlines the role of eosinophils as innate immune cells participating in the defense against pathogenic microbes (4).

In our experiments, it became evident that the effect of TSLP on DNA release is dependent on the adhesion of eosinophils. CD11b/CD18-dependent adhesion has been

shown to play an important role in eosinophil activation, intracellular signaling, degranulation, and superoxide production (41, 42). In neutrophils, an integrin-dependent ROS production by mitochondria with subsequent activation of a signal transduction cascade has been described (43). Recently, it has been reported that eosinophils upregulate adhesion molecules upon TSLP stimulation (24). Thus, it seems likely that TSLP has dual functions on eosinophils in the tissue by promoting first adhesion, which then fulfills a prerequisite for EET release. The observation that adhesion is essential points to the possibility that TSLP-induced EET formation is restricted to tissue eosinophils and does not occur in the circulation under normal conditions. EETs were found to be released by viable eosinophils, and TSLP did not induce cell death. Noteworthy is, however, that TSLP at the concentrations used to stimulate EET release did not prolong eosinophil survival. This is in contrast to the previous observations stating enhanced eosinophil survival upon 48-h TSLP stimulation (24). Moreover, the generation of EETs was dependent on ROS production, indicating an active cellular response. Consistent with the previous reports on NETs and EETs (4, 26), we could show that the released DNA is of mitochondrial origin.

Taken together, our results indicate that eosinophils respond to TSLP stimulation by releasing mitochondrial DNA together with toxic granule proteins resulting in EET formation. This innate immune mechanism is likely to play a role in host defense at epithelial surfaces, where TSLP is released following barrier disruption and where eosinophil infiltration can often be observed. These new insights into eosinophil activation may add to a better understanding of the role of eosinophils under physiologic and pathologic conditions. Furthermore, TSLP represents an interesting potential target to modulate eosinophil activation.

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Conflict of interest

The authors declare that they have no conflict of interest.

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