Allergology International xxx (2016) 1–9

Contents lists available at ScienceDirect

Allergology International

journal homepage: http://www.elsevier.com/locate/alit

Original article

The effect of calprotectin on TSLP and IL-25 production from airway epithelial cells

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ARTICLE INFO

Article history:
Received 22 February 2016
Received in revised form
7 June 2016
Accepted 24 June 2016
Available online xxx

Keywords:
Calprotectin
Eosinophilic chronic rhinosinusitis
IL-25
Th2-type inflammation
TSLP

Abbreviations:
AR, allergic rhinitis; DAMPs, damage-associated molecular patterns; ECRS, eosinophilic chronic rhinosinusitis; IL, interleukin; IT, inferior turbinate; NECRS, non-eosinophilic chronic rhinosinusitis; NHBE, normal human bronchial epithelial; NPs, nasal polyps; PNE, primary nasal epithelial; RAGE, receptor for advanced glycation end products; TLR, Toll-like receptor; TSLP, thymic stromal lymphopoietin; UT, uncinate process tissues

ABSTRACT

Background: Calprotectin is a heterodimer complex of the S100A8 and S100A9 proteins, and has various functions as an innate mediator at the sites of inflammation.
The aim of this study was to elucidate the roles of calprotectin in the eosinophilic chronic rhinosinusitis (ECRS).
Methods: Allergen-induced production of calprotectin was evaluated in cultured normal human bronchial epithelial (NHBE) cells by ELISA and RT-PCR. We then examined the roles of calprotectin on *Alternaria alternata* (*Alternaria*)-induced production of thymic stromal lymphopoietin (TSLP) and IL-25 in NHBE cells. The extracellular concentration and allergen-induced secretion of calprotectin in cultured primary nasal epithelial (PNE) cells were examined and compared between patients with ECRS and non-eosinophilic chronic rhinosinusitis (NECRS).
Results: *Alternaria*, house dust mites, protease from *Staphylococcus aureus*, papain, trypsin, poly-inosinic:polycytidylic acid and lipopolysaccharide stimulated calprotectin production in the cultured NHBE cells. The combination of calprotectin and ATP stimulated the production of TSLP and IL-25 in NHBE cells, and calprotectin stimulated *Alternaria*-induced production of TSLP and IL-25, which was suppressed by blocking P2 purinergic receptors and by treatment with siRNA for S100A8, S100A9 or calprotectin receptors (Toll-like receptor 4 or receptor for advanced glycation end products). Allergen-induced calprotectin production was significantly stimulated in PNE cells from patients with ECRS.
Conclusions: These results indicate that calprotectin enhances the allergen-induced Th2-type inflammatory responses in airway epithelial cells via the secretion of TSLP and IL-25, and that calprotectin secreted by the epithelial cells may be involved in the pathogenesis of ECRS.
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Introduction

Calprotectin is a heterodimer complex of the S100A8 and S100A9 proteins, which are members of the S100 family of proteins. S100 family proteins play various roles in inflammation, barrier function, cancer and innate immunity. Intracellularly, calprotectin promotes phagocyte migration by promoting the polymerization and stabilization of tubulin microfilaments.[1] Extracellular calprotectin is released by neutrophils, monocytes and epithelial cells, and exhibits proinflammatory functions, such as antimicrobial activity against bacteria and fungi, and chemoattractant activity for neutrophils and monocytes.[2] Calprotectin stimulates the production of proinflammatory cytokines, such as IL-8 and tumor necrosis factor (TNF)-α, in keratinocytes and airway epithelial cells.[3,4] Calprotectin also has anti-inflammatory and tissue-protective functions as it can inhibit matrix metalloproteinase activity and the production of reactive oxygen species (ROS).[5–7] Thus, calprotectin may have diverse functions as an innate mediator at the sites of inflammation.

Toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE) are specific receptors for calprotectin.[8,9] Calprotectin is found in inflammatory tissues and exudates, including nasal polyps (NPs) and nasal lavage fluids,[10] and is

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Peer review under responsibility of Japanese Society of Allergology.

http://dx.doi.org/10.1016/j.alit.2016.06.011
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thought to be involved in the pathogenesis of various inflammatory diseases, such as rheumatoid arthritis, autoimmune diseases, inflammatory bowel diseases and anti-neutrophil cytoplasmic autoantibody-associated vasculitis.[11–15] However, the role of calprotectin in upper airway Th2-type inflammation is not well understood.

Newly discovered epithelial-derived cytokines, i.e., thymic stromal lymphopoietin (TSLP) and IL-25, induce Th2 cytokine-dependent inflammation, and play key roles in innate and adaptive immune responses in airway mucosa.[16] Allergens, such as *Alternaria alternata* (*Alternaria*) and house dust mites (HDM), stimulate the production of TSLP and IL-25 in airway epithelial cells via protease-activated receptor (PAR)-2,[17,18] and TSLP and IL-25 contribute to the initiation and development of allergic inflammation in allergic rhinitis, asthma and atopic dermatitis.[19–22] Increased expression levels of TSLP and IL-25 have been reported in the NPs of patients with chronic rhinosinusitis (CRS), and were shown to exacerbate airway inflammation, and contribute to CRS disease progression.[23,24]

We hypothesized that calprotectin also plays an important role in the pathogenesis of eosinophilic chronic rhinosinusitis (ECRS). In the present study, we evaluated the effects of allergens on the release of calprotectin from airway epithelial cells, and the role of calprotectin in the secretion of TSLP and IL-25 induced by an airborne allergen, *Alternaria*. Finally, we examined the intracellular concentration and allergen-induced secretion of calprotectin in cultured primary nasal epithelial (PNE) cells of CRS patients.

Methods

Human subjects

Sixty-six patients with nasal or paranasal sinus diseases were enrolled in this study (age, 20–77 years; mean age, 49.1 years) (Table 1). Total IgE, allergen-specific IgE and blood eosinophil counts were performed for all the patients. Computed tomography (CT) assessment by Lund–Mackay score, the number of the nasal polyp and endoscopy were performed for the CRS patients. The CRS patients were classified into ECRS and non-eosinophilic chronic rhinosinusitis (NECRS) groups based on JESREC study.[25] ECRS was defined histologically as an averaged eosinophil count of more than 70 per microscopic field (400× magnification) in three fields of the subepithelial area of NPs; these were counted by two of the authors independently under light microscopy.[25] The diagnosis of asthma was based on the criteria of the Global Initiative for Asthma published in 2006.[26] Allergic rhinitis was diagnosed based on the Practical Guidelines for the Management of Allergic Rhinitis in Japan published in 2009.[27]

Inferior turbinates (IT) were obtained from patients with allergic rhinitis (AR) or patients with deflected nasal septum during conchotomy. NPs of patients with NECRS or ECRS, and uncinate process tissues (UT) of patients with NECRS or control subjects were obtained during endoscopic sinus surgery. None of the patients had been treated with systemic corticosteroids or topical corticosteroids for at least 4 weeks prior to the surgery. Informed consent was obtained from all subjects before sampling. This clinical protocol was approved by the Shiga University of Medical Science Institutional Review Board for Clinical Investigation.

Reagents

Crude allergens of *Alternaria* and HDM were purchased from Greer Laboratories (Lenoir, NC, USA). Protease from *Staphylococcus aureus* (*S. aureus*) was obtained from Abnova (Taipei, Taiwan). Polyinosinic:polycytidylic acid (poly(I:C)) was obtained from InvivoGen (San Diego, CA, USA). Trypsin from bovine pancreas, lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, adenosine 5′-triphosphate disodium salt hydrate (ATP), adenosine 5′-triphosphate periodate oxidized sodium salt (oATP), trans-epoxysuccinyl-L-leucylamide (4-guanidino) butane (E-64), 4-amidinophenylmethanesulfonyl fluoride (APMSF) and nonidet P-40 were obtained from Sigma–Aldrich (St. Louis, MO, USA). Papain from *Carica papaya* and suramin were obtained from Calbiochem (Charlottesville, VA, USA). Recombinant calprotectin was obtained from Hycult Biotech (Uden, Netherlands). Small interfering RNA (siRNA) for TLR4, S100A8, and S100A9, and Allstars Negative Control siRNA were obtained from Qiagen (Hilden, Netherland), and siRNA for RAGE was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture, treatment, and transfection

Normal human bronchial epithelial (NHBE) cells were obtained from Lonza (Walkersville, MD, USA) and were then transfected with the catalytic component of telomerase, the human catalytic subunit of the telomerase reverse transcriptase gene, which was kindly provided by Professor H. Kita (Mayo Clinic, Rochester, MN, USA). PNE cells were obtained by scratching the UT or NPs by curettage with a Rhinoprobe (Arlington Scientific, Inc., Springville, UT, USA). The NHBE cells or PNE cells were suspended in supplemented basal epithelial growth medium (BEGM) (Lonza, Basel, Switzerland) and cultured in a 24-well tissue culture plate (Falcon; Corning, Inc., NY,

Table 1
Patient characteristics.

Subject characteristics		IT		UT	UT or NP	NP
		Control	AR	Control	NECRS	ECRS
Total		8 (6M/2F)	17 (9M/8F)	10 (8M/2F)	15 (11M/4F)	16 (14M/2F)
Average age (range)		50.4 (38–66)	33.4 (20–64)	54.7 (46–76)	58.3 (20–77)	53.3 (26–75)
Asthma (n)		0	5	1	2	8
Atopy (n)		0	17	5	5	14
Blood eosinophils (%)		2.13 ± 1.33	5.17 ± 3.27**	4.10 ± 2.27	5.01 ± 5.01	8.42 ± 5.14*
Total IgE (U/ml)		76.2 ± 66.9	254.2 ± 239.2	209.4 ± 209.4	250.3 ± 258.4	343.6 ± 190.2*
Specific IgE (U/ml)	HDM	0.13 ± 0.01	17.5 ± 2.22**	6.21 ± 3.04	1.29 ± 4.01	7.11 ± 2.27
	Alternaria	0.1	0.15 ± 0.01	0.14 ± 0.11	0.13 ± 0.10	0.70 ± 0.29
CT score					8.47 ± 7.17	15.8 ± 6.62*
Nasal polyps (%)	Mono				20% (3/15)	0% (0/16)
	Multi				40% (6/15)	100% (16/16)

IT, inferior turbinate; UT, uncinate process; NP, nasal polyp; AR, allergic rhinitis; ECRS, eosinophilic chronic rhinosinusitis; NECRS, non-eosinophilic chronic rhinosinusitis; F, female; M, male.
P values were evaluated from the Mann–Whitney U test comparing 2 groups (IT) and the Kruskal–Wallis test comparing all 3 groups (UT/NP). $^{*}P < 0.05$ and $^{**}P < 0.01$.

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USA). After incubating confluent cells for 24 h in BEGM without supplements, *Alternaria* (200 μg/ml), HDM (100 μg/ml), protease from *S. aureus* (1 μg/ml), trypsin (10 nM), papain (10 nM), poly(I:C) (10 μg/ml), LPS (10 μg/ml), ATP (2 mM), or calprotectin (2, 10, 20 μg/ml) was added, and the cells were incubated for an additional 24 h. In some experiments, PNECs were seeded (3×10^4 cells/well) in a 24-well tissue culture plate and grown until they were 80% confluent by the BEGM® without steroid. Cultured PNECs were also stimulated for 24 h with HDM (100 μg/ml), *Alternaria* (200 μg/mL), protease from *S. aureus* (1 μg/mL).

Previous reports had noted that high concentrations of fungal extracts or proteases would produce morphologic changes and desquamate epithelial cells.[28] At the relatively low concentrations listed above, we did not observe changes in morphology in the NHBE cells for up to 24 h. In some experiments, oATP (100 μM), suramin (300 μM), APMSF (100 μM) or E-64 (50 μM) was added to the NHBE cells 30 min before incubation.

To transfect NHBE cells, HiPerFect (Qiagen) was used according to the manufacturer's instructions. The cells were transfected with siRNA against TLR4 (5 nM), RAGE (50 nM), S100A9 (5 nM), or S100A8 (5 nM), or the Allstars Negative Control siRNA (5 nM). Knockdown of the target gene mRNA was confirmed by real-time RT-PCR.

Calprotectin and cytokine production and release in NHBE cells and PNE cells

Cell lysates were prepared by treating unstimulated cultured PNE cells with nonidet P-40. Cell lysates and cell-free supernatants were collected and stored at −20 °C until use. ELISA kits were used to determine the concentrations of TSLP and IL-25 (USCN Life Science Inc., Wuhan, China), calprotectin (BioLegend, San Diego, CA, USA) and IL-8 (R&D Systems, Minneapolis, MN, USA). The sensitivity limits of the TSLP, IL-25, calprotectin and IL-8 kits were 5.7 pg/ml, 5.4 pg/ml, 0.62 ng/ml and 31.25 pg/ml, respectively.

RNA isolation and real-time RT-PCR

Total RNA was purified using a PureLink RNA Mini Kit (Thermo Fisher Scientific, MA, USA). cDNAs were synthesized from 1 μg of each purified RNA sample using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). Amplification and detection of specific products were performed using the LightCycler 480 Real-Time PCR system (Roche). The reaction was incubated at 45 °C for 60 min and was stopped by heating to 85 °C for 5 min. The real-time RT-PCR mixture contained 1 μl of cDNA, 10.0 μl of LightCycler 480 Probe Master (Roche), 0.2 μl of probes, and 0.1 μl of two primer sets: S100A8 (forward primer: GCCAAGCCTAA CCGCTATAA; reverse primer: ATGATGCCCACGGACTTG), S100A9 (forward primer: GTGCGAAAAGATCTGCAAAA; reverse primer: TCAGCTGCTTGTCTGCATTT), TLR4 (forward primer: CTCTCCTGCGTG AGACCAG; reverse primer: TCCATGCATTGATAAGTAATATTAGGA), RAGE (forward primer: GGATGAAGGATGTGAGTGACC; reverse primer: CCCACAGAGCCTGCAGTT) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer: AGCCACATCGCTC AGACAC; reverse primer: GCCCAATACGACCAAATCC). The reaction mixtures were topped up to a final volume of 20 μl with sterile water. The real-time RT-PCR protocol was as follows: denaturation by a hot start at 95 °C for 10 min, followed by 40 cycles of a two-step program (denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min). Transcription was normalized to that of GAPDH in each sample, and expressed as relative expression compared with the parallel NHBE cells cultured in the absence of stimuli.

Detection of calprotectin by immunohistochemistry and confocal microscopy

NHBE cells were cultured on Lab-Tek 2 chamber slides (Fisher, Rochester, NY, USA). The slides were fixed and permeabilized using Cytofix/Cytoperm reagents for 20 min at 4 °C, and then washed with BD Perm/Wash™ buffer (BD Pharmingen, San Diego, CA, USA) for 30 min at room temperature. Fixed cells were blocked with 5% normal rabbit serum (Sigma) for 1 h and stained overnight with rabbit anti-human calprotectin or normal rabbit IgG (control IgG) at 4 °C. For immunofluorescence, the cells were incubated with fluorescein-conjugated goat anti-rabbit IgG for 2 h at room temperature, washed in BD Perm/Wash™ buffer for 30 min, and mounted in Vectashield® Mounting Medium with DNA-binding dye, the nucleus-specific dye 4′-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Fluorescent images were visualized using an LSM510 confocal microscope (Carl Zeiss, Jena, Germany), and digital images (512 × 512 pixels, 800× magnification) were captured using the KS400 Image Analysis System (Carl Zeiss). The threshold for each negative control image was calibrated to a baseline value without positive pixels. All images were processed using the Zeiss LSM Image Browser (Carl Zeiss).

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM) from the indicated number of replicates. Two-sided differences between two samples were analyzed with the Mann–Whitney U test. Data of three-group comparisons were evaluated by Kruskal–Wallis one-way analysis. Values of $P < 0.05$ were considered significant.

Results

Calprotectin production and mRNA expression in NHBE cells are induced by various allergens, prototypic proteases and TLR agonists

To investigate the mechanisms of calprotectin production in airway epithelial cells, we examined the effects of environmental allergens, prototype proteases and TLR ligands on calprotectin production in NHBE cells. *Alternaria*, HDM, protease from *S. aureus*, papain, trypsin, poly(I:C) and LPS all caused a significant increase in the concentration of calprotectin in the cell-free supernatants (Fig. 1a). The concentrations of calprotectin continued to increase after incubation with *Alternaria* or poly(I:C) for up to 24 h (Fig. 1b).

We next performed an immunofluorescence study of calprotectin in NHBE cells. Calprotectin was weakly detected in the cytoplasm of resting NHBE cells. After incubation with *Alternaria* for 24 h, calprotectin was strongly expressed in the cytoplasm of NHBE cells (Fig. 1c). *Alternaria* did not affect the cell morphology of NHBE cells. Calprotectin is a heterodimer complex of the S100A8 and S100A9 proteins. The mRNA expressions of S100A8 and S100A9 increased significantly in NHBE cells after 8 h of incubation with *Alternaria* (Fig. 1d, e). We then used a gene knockdown approach, and NHBE cells were transfected with siRNAs specific for S100A8 or S100A9, or a control siRNA. Transfection with the specific siRNAs, but not the control siRNA, significantly suppressed the expression of the target mRNA (Fig. 2a). These knockdown NHBE cells were then incubated with *Alternaria* for 24 h, and *Alternaria*-induced calprotectin production was significantly inhibited in the S100A8- or S100A9-knockdown cells (Fig. 2b). We then examined whether the protease activity of allergens, such as *Alternaria*, HDM and protease from *S. aureus*, are involved in allergens-induced calprotectin production. When allergens were pretreated with APMSF, a

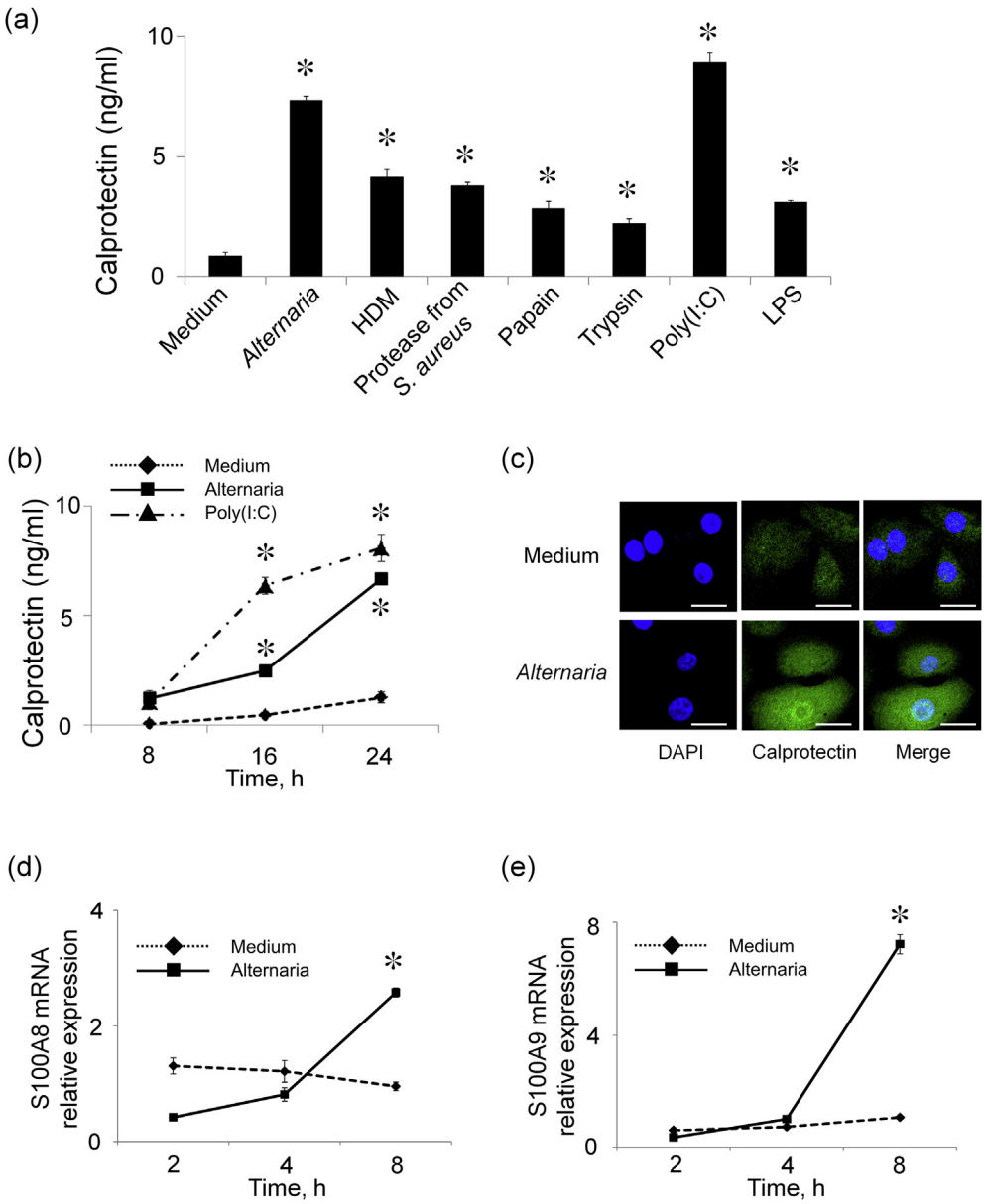

Fig. 1. Calprotectin secretion and mRNA expression in NHBE cells. **(a)** Calprotectin levels in cell-free supernatants from cells incubated for 24 h with *Alternaria* (200 μg/ml), HDM (100 μg/ml), protease from *S. aureus* (1 μg/ml), papain (10 nM), trypsin (10 nM), poly(I:C) (10 μg/ml) or LPS (10 μg/ml). $^*P < 0.05$; n = 4. **(b)** Calprotectin levels after 8, 16 or 24 h of incubation with medium alone (control), poly(I:C) (10 μg/ml) or *Alternaria* (200 μg/ml). $^*P < 0.05$; n = 4. **(c)** Immunofluorescent staining of calprotectin in NHBE cells incubated with medium alone (upper panels) or *Alternaria* (lower panels) (200 μg/ml) for 24 h. Left panels show nuclear staining with DAPI. Center panels show FITC images with anti-calprotectin antibody. Right panels show overlays of the left and middle panels. Scale bars indicate 50 μm. **(d)** S100A8 mRNA expression in NHBE cells after 2, 4 or 8 h of incubation with medium alone or *Alternaria* (200 μg/ml). $^*P < 0.05$; n = 4. **(e)** S100A9 mRNA expression in NHBE cells after 2, 4 or 8 h of incubation with medium alone or *Alternaria* (200 μg/ml). $^*P < 0.05$; n = 4.

serine protease inhibitor, or E-64, a cysteine protease inhibitor, calprotectin concentrations significantly decreased in the cell-free supernatants, suggesting that the protease activity of allergens are important in calprotectin production (Fig. 2c).

Calprotectin and ATP stimulate TSLP and IL-25 production in NHBE cells

We examined the effects of calprotectin on TSLP and IL-25 production in NHBE cells. When NHBE cells were treated with calprotectin alone (2, 10 or 20 μg/ml), TSLP and IL-25 production was not stimulated. However, when NHBE cells were treated with the combination of calprotectin (2, 10 or 20 μg/ml) and ATP (2 mM), TSLP and IL-25 production was significantly stimulated in a dose-dependent manner (Fig. 3a, b). In contrast, IL-8 production was stimulated by calprotectin alone (10 and 20 μg/ml) (Fig. 3c). Calprotectin (2 μg/ml) also significantly stimulated *Alternaria*-induced production of TSLP and IL-25 in NHBE cells (Fig. 3d, e).

Mechanism of Alternaria-induced TSLP and IL-25 production in NHBE cells

Alternaria stimulated the production of TSLP and IL-25 in NHBE cells. To examine the role of the ATP pathway in *Alternaria*-induced TSLP and IL-25 production, NHBE cells were incubated with *Alternaria* and antagonists for P2 purinergic receptors, oATP or suramin.

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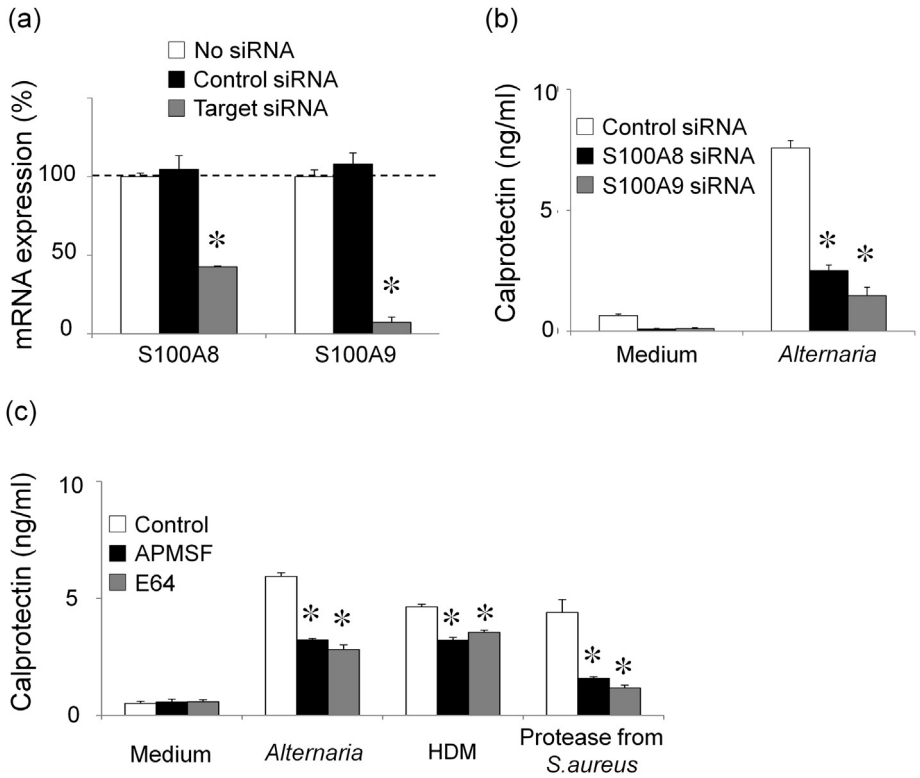

Fig. 2. Allergens-induced calprotectin production. **(a)** The mRNA expression levels of S100A8 and S100A9 in NHBE cells transfected with siRNA against S100A8 (5 nM) or S100A9 (5 nM), or control siRNA (5 nM) for 48 h. Data are expressed as a ratio to the mock-transfected cells without siRNA. $^*P < 0.05$ compared with control cells without siRNA; n = 4. **(b)** Effects of siRNA against S100A8 and S100A9 on *Alternaria*-induced calprotectin production. $^*P < 0.05$; n = 4. **(c)** Effects of a serine protease inhibitor, APMSF (100 μM), and a cysteine protease inhibitor, E-64 (50 μM), on allergens-induced calprotectin production. $^*P < 0.05$; n = 4.

P2 receptor antagonists were added to the NHBE cells 30 min before incubation with *Alternaria*. Both oATP and suramin significantly inhibited *Alternaria*-induced TSLP and IL-25 production (Fig. 4a, b), indicating that the ATP pathway is involved in *Alternaria*-induced TSLP and IL-25 production.

TLR4 and RAGE are specific receptors for calprotectin. We knocked down TLR4 or RAGE by transfecting NHBE cells with siRNA for TLR4 or RAGE, respectively, and control cells were transfected with control siRNA. In the knockdown cells, the expression of the target mRNA was significantly suppressed when compared with the expression in the control cells (Fig. 5a). *Alternaria*-induced TSLP and IL-25 production in cell-free supernatants were significantly inhibited in TLR4- or RAGE-knockdown NHBE cells (Fig. 5b, c). Moreover, *Alternaria*-induced TSLP and IL-25 production was significantly inhibited in the S100A8- or S100A9-knockdown NHBE cells (Fig. 5d, e). These results indicate that calprotectin is involved in *Alternaria*-induced production of TSLP and IL-25 in NHBE cells.

Calprotectin concentrations in cell lysates and in cell-free supernatants of cultured PNE cells

The clinical characteristics of the patients are shown in Table 1. We examined the concentrations of calprotectin in cell lysates and in cell-free supernatants of cultured PNE cells from the IT of AR patients or control subjects and from the NPs of NECRS patients or ECRS patients and from the UT of NECRS patients or control subjects. The intracellular calprotectin concentration in unstimulated PNE cells from ECRS patients was significantly lower than those from NECRS patients or control subjects (Fig. 6a). There were no difference between AR patients and control subjects (Fig. 6b). However, allergen (*Alternaria*, HDM or protease from *S. aureus*)-induced calprotectin production was significantly stimulated in PNE cells from ECRS patients when compared with those from NECRS patients even though the levels of unstimulated calprotectin release were identical among the groups (Fig. 6c). The allergen-induced calprotectin production from IT was no difference between AR patients and control subjects (data not shown). These results suggest that an increased production of calprotectin may be involved in the pathogenesis of ECRS.

Discussion

Our study provides the first evidence that calprotectin plays an important role in Th2-type airway inflammation. We showed that: 1) airborne allergens, such as *Alternaria* and HDM, stimulate the production and release of calprotectin in cultured NHBE cells; 2) the protease activity of allergens is crucial in this process; 3) the combination of calprotectin and ATP induces the production of TSLP and IL-25 in NHBE cells; 4) calprotectin stimulates the *Alternaria*-induced production of TSLP and IL-25, which is suppressed by blocking P2 purinergic receptors and by siRNA for S100A8, S100A9, TLR4 or RAGE; and 5) allergen-induced calprotectin production is increased in the PNE cells from ECRS patients. These results indicate that calprotectin enhances the allergen-induced Th2-type inflammatory responses in airway epithelial cells via the secretion of TSLP and IL-25, and that calprotectin produced by the epithelial cells may be involved in the pathogenesis of ECRS.

S100 protein, ATP, uric acid and high-mobility group box 1 (HMGB1) protein are all examples of damage-associated molecular patterns (DAMPs). DAMPs, also known as alarmins, are intracellular molecules that are involved in cellular functions under normal homeostasis, but are released outside of the cell after cell death,

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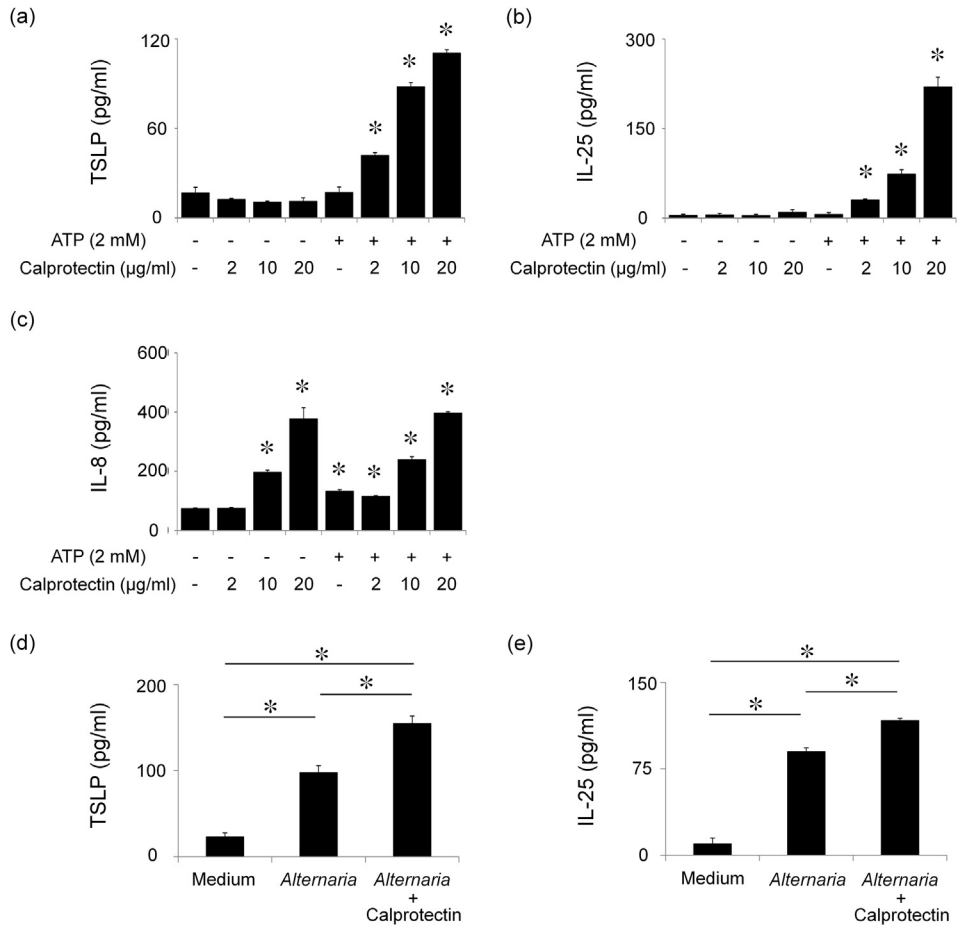

Fig. 3. Effects of calprotectin and ATP on the levels of TSLP, IL-25 and IL-8 in cell-free supernatants from NHBE cells. **(a–c)** Effects of calprotectin and ATP (24 h of incubation) on the production of TSLP, IL-25 and IL-8. $^*P < 0.05$; n = 4. **(d, e)** Effects of calprotectin (2 μg/ml) (24 h of incubation) on *Alternaria*-induced production of TSLP and IL-25. $^*P < 0.05$; n = 4.

signaling tissue damage.[29] Recently, several studies have reported the relationship between DAMPs and allergic inflammation; for example, significantly high concentrations of HMGB1 were found in the sputa and nasal secretions of patients with asthma, nasal allergy or CRS.[30] RAGE is a specific receptor of HMGB1, and HDM-induced allergic responses, including eosinophil infiltration and Th2 cytokine production, were significantly suppressed in RAGE−/− mice.[31] Increased levels of ATP were observed in the bronchoalveolar lavage fluids of patients with asthma, and neutralization of the ATP inhibited Th2 sensitization to inhaled antigens and ongoing Th2-type airway inflammation.[32] Moreover, we also confirmed that calprotectin was induced in airway epithelial cells after exposure to airborne allergens, indicating that the combination of calprotectin and ATP as secreted DAMP molecules can directly induce TSLP and IL-25 production via TLR4 and RAGE in airway epithelial cells. Taken together, these results suggest that DAMPs play a role in the development and exacerbation of airway allergic inflammation.

Recently, it was revealed that several mediators and receptors, including PAR-2, TLR4 and RAGE, are involved in the initiation and development of innate immune responses against environmental allergens. The protease activity of allergens and PAR-2 signaling

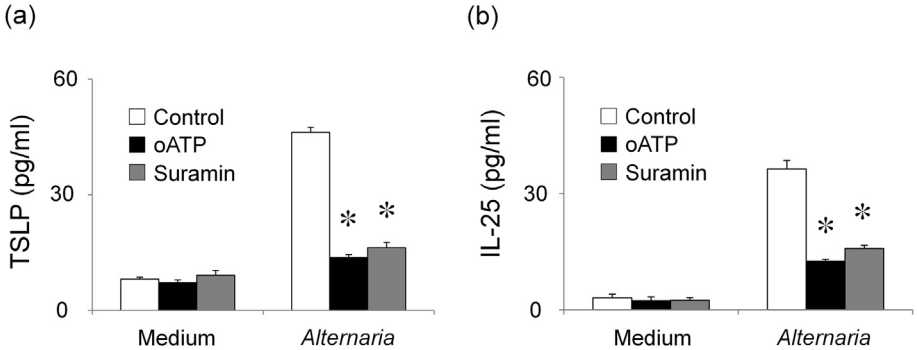

Fig. 4. Effects of P2 purinergic receptor antagonists, oATP (100 μM) and suramin (300 μM), on *Alternaria*-induced production of TSLP **(a)** and IL-25 **(b)** in NHBE cells. $^*P < 0.05$; n = 4.

Please cite this article in press as: Kato T, et al., The effect of calprotectin on TSLP and IL-25 production from airway epithelial cells, Allergology International (2016), http://dx.doi.org/10.1016/j.alit.2016.06.011

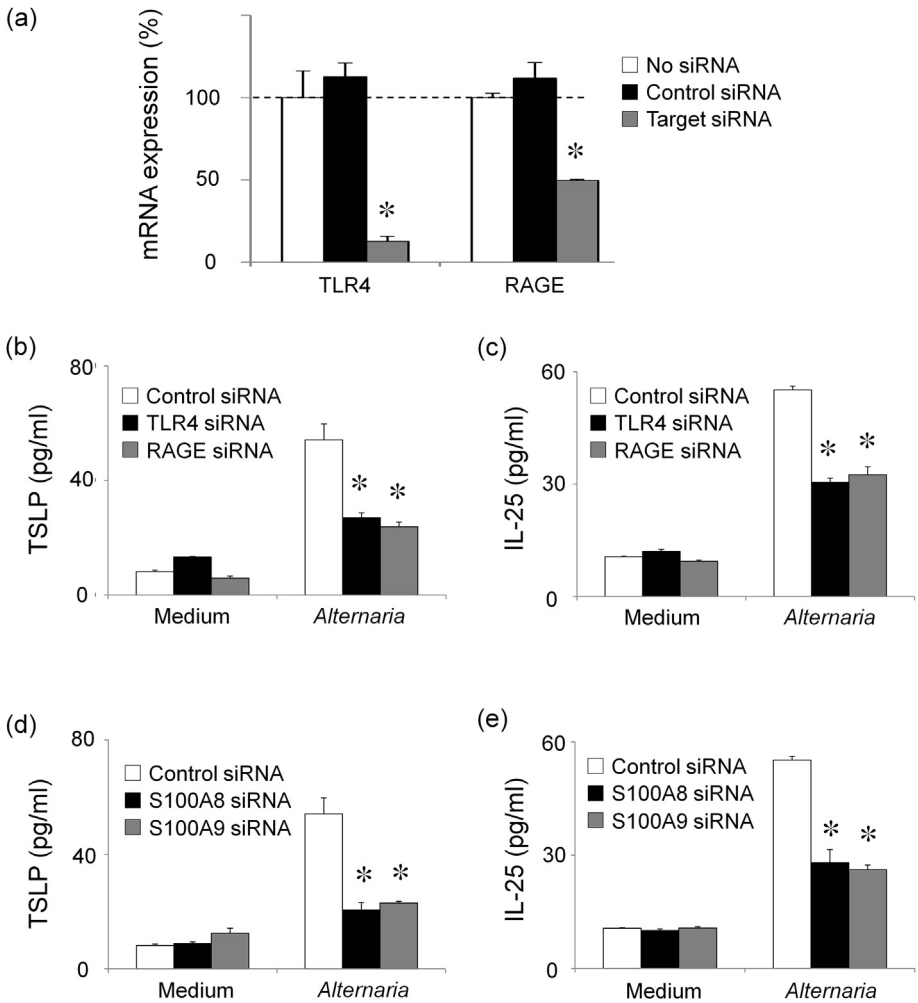

Fig. 5. *Alternaria* induced TSLP and IL-25 via calprotectin production in NHBE cells. **(a)** The mRNA expression levels of TLR4 and RAGE in NHBE cells transfected with siRNA against TLR4 (5 nM), RAGE (50 nM) or control siRNA (5 nM) for 48 h. Data are expressed as a ratio to the mock-transfected cells without siRNA. $^*P < 0.05$ compared with control cells without siRNA; n = 4. **(b, c)** Effects of siRNA against TLR4 and RAGE on *Alternaria*-induced production of TSLP and IL-25. $^*P < 0.05$; n = 4. **(d, e)** Effects of siRNA against S100A8 (5 nM) and S100A9 (5 nM) on *Alternaria*-induced production of TSLP and IL-25. $^*P < 0.05$; n = 4.

stimulate TSLP and IL-25 secretion in epithelial cells.[17,18] In addition, we revealed that other innate immune-stimulatory molecules, such as TLR4 and RAGE, were also involved in the *Alternaria*-induced TSLP and IL-25 production. TLRs, such as TLR2 and TLR4, play important roles in both innate and adaptive immunity to fungi.[33] Interestingly, a non-enzymatic mite allergen, Der p 2, likely stimulates an innate immune response through TLR4 by molecular mimicry of a lipid-recognition protein, MD-2.[34] Inhalation of a TLR4 antagonist targeting exposed epithelial cells suppressed the salient features of asthma, including bronchial hyperreactivity.[35] In NHBE cells, HMGB1 bound to TLR4 and RAGE, and also induced the expression and secretion of TSLP via RAGE by activating p38 MAPK and ERK1/2.[36] Another investigator also reported that protease allergens, such as papain, induce the expression of IL-25 and TSLP via the MAPK pathway in primary lung epithelial cells of mice.[17] As such, several receptors, including PARs, TLRs and RAGE, may be involved in both the recognition and initiation of immune responses to environmental allergens. Interestingly, analyses of PAR-2 and TLR4 signal transduction suggest that these receptors may physically interact and cooperate to produce inflammatory responses.[37] Taken together, these findings suggest that innate immune receptors, such as PARs, TLRs and RAGE, cooperate and regulate allergen-induced responses in airway epithelial cells.

A high concentration of calprotectin over 0.1 μM (>3.6 μg/ml) has been shown to promote the expression of pro-inflammatory mediators, including IL-6, IL-8 and TNFα, in human keratinocytes.[3] High extracellular concentrations of calprotectin are found in the serum and at inflammatory sites of patients with autoimmune disease, such as arthritis, systemic lupus erythematosus and Crohn's disease.[12–14] In this study, high concentrations of calprotectin (>10 μg/ml) alone induced IL-8 production, not IL-25 and TSLP production. However, the calprotectin by adding ATP induced TSLP and IL-25 production. These results indicated that the combination of ATP and calprotectin is involved in Th2-type airway inflammatory responses. Further studies will be needed to thoroughly understand the physiological relevance of ATP and calprotectin.

A previous report also showed that the calprotectin level was lower in the epithelium of polyp tissues and the nasal lavage fluid of chronic rhinosinusitis with nasal polyp patients when compared to healthy controls.[10] However, the calprotectin concentration was significantly increased in polyp tissues.[10,38] They discussed the diminished calprotectin in nasal epithelial cells induces a lack of innate host defense as an antimicrobial or antifungal effect in patients of CRS. Similarly, in the present study, the calprotectin level was significantly decreased in the unstimulated epithelial cells of

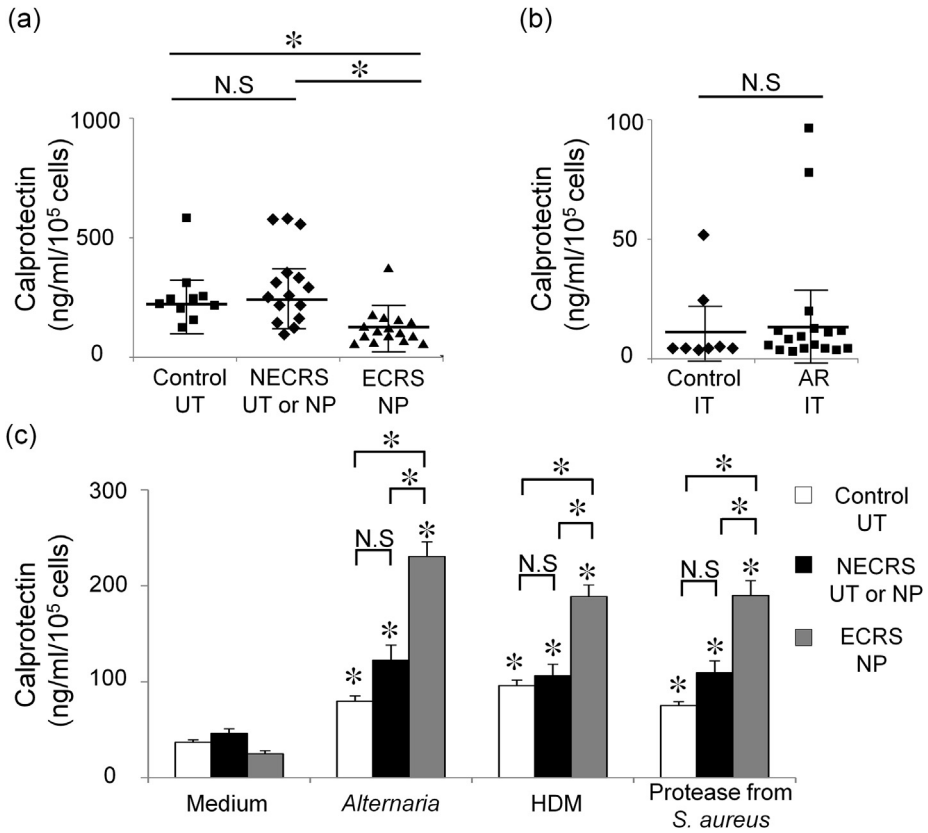

Fig. 6. Calprotectin concentrations in cell lysates and in cell-free supernatants of cultured PNE cells. **(a)** Calprotectin concentrations in cell lysates of unstimulated PNE cells from UT or NPs. $^*P < 0.05$ (control: n = 10, NECRS: n = 15, ECRS n = 16). **(b)** Calprotectin concentrations in cell lysates of unstimulated PNE cells from IT. $^*P < 0.05$ (control: n = 8, AR: n = 17). **(c)** Calprotectin concentrations in cell-free supernatants of cultured PNE cells stimulated by 24 h of incubation with *Alternaria* (200 μg/ml), HDM (100 μg/ml) or protease from *S. aureus* (1 μg/ml). $^*P < 0.05$ (control: n = 4, NECRS: n = 8, ECRS n = 8).

the nasal polyps from ECRS patients. However, allergen-induced calprotectin production was significantly increased in the PNE cells from ECRS patients when compared to those from NECRS patients or the control subjects. Previous studies suggested that soluble gp130 (sgp130), a natural inhibitor of IL-6 transsignaling, is increase in polyp tissues from CRS with NPs (CRSwNP) patients.[39] Sgp130 Levels in polyp tissues from CRSwNP patients were positively correlated with aging. In addition, calprotectin levels in nasal lavage fluids were negatively correlated with aging.[10,40,41] Because IL-6 trans-signaling is one of calprotectin production pathway,[40] the increased sgp130 may decrease calprotectin expression in the epithelial cells via inhibition IL-6 trans-signaling. In contrast, increased production of calprotectin against the protease activity of allergens in the NPs of ECRS may be derived to the dysfunction of mucosal barrier or the decreased antimicrobial responses, but not to IL-6 trans-signaling. Further studies are needed to investigate the factors regulating calprotectin.

In the present study, calprotectin was found to be in the allergen-induced production of TSLP and IL-25 in airway epithelial cells. Although the pathogenesis of ECRS remains unclear, increased protein levels and mRNA expression of TSLP and IL-25 have been reported in the epithelial cells of NPs from ECRS patients. Increased epithelial tissue expression of TSLP and IL-25 has also been confirmed by immunofluorescent staining.[42] These results indicate that epithelial cell-derived TSLP and IL-25 may be involved in the development of ECRS, and that calprotectin enhances and modulates these inflammatory responses in airway epithelial cells.

In conclusion, these findings provide new mechanistic insight into the development of allergen-induced immune responses and the pathogenesis of ECRS, suggesting that calprotectin may be one of the key players in airway Th2-type inflammation.

Acknowledgements

We thank Professor Hirohito Kita (Mayo Clinic, Rochester, MN, USA) for kindly providing the NHBE cells with telomerase activity.

Conflict of interest

JH is an employee of Shiseido Co., Ltd. The rest of the authors have no conflict of interest.

Authors' contributions

TK contributed to study design, research, data collection and wrote the manuscript. HK contributed to study design, research, wrote the manuscript. KM contributed to data collection. JH contributed to study design. TS contributed to wrote the overall organization.

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Please cite this article in press as: Kato T, et al., The effect of calprotectin on TSLP and IL-25 production from airway epithelial cells, Allergology International (2016), http://dx.doi.org/10.1016/j.alit.2016.06.011