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Alternaria extract activates autophagy that induces IL-18 release from airway epithelial cells

Hiroki Murai^{a,b}, Shintaro Okazaki^a, Hisako Hayashi^a, Akiko Kawakita^a, Koa Hosoki^b, Motoko Yasutomi^a, Sanjiv Sur^b, and Yusei Ohshima^{a,*}

^aDepartment of Pediatrics, University of Fukui, 23-3 Matsuoka Shimo-Aizuki, Eiheiji-cho, Yoshida-gun, Fukui, 911-1193, Japan

^bDepartment of Internal Medicine, University of Texas Medical Branch at Galveston, 301 University Blvd, Galveston, TX, 77555, United States of America

Abstract

Alternaria alternata is a major outdoor allergen that causes allergic airway diseases. *Alternaria* extract (ALT-E) has been shown to induce airway epithelial cells to release IL-18 and thereby initiate Th2-type responses. We investigated the underlying mechanisms involved in IL-18 release from ALT-E-stimulated airway epithelial cells. Normal human bronchial epithelial cells and A549 human lung adenocarcinoma cells were stimulated with ALT-E in the presence of different inhibitors of autophagy or caspases. IL-18 levels in culture supernatants were measured by ELISA. The numbers of autophagosomes, an LC3-I to LC3-II conversion, and p62 degradation were determined by immunofluorescence staining and immunoblotting. 3-methyladenine and bafilomycin, which inhibit the formation of preautophagosomal structures and autolysosomes, respectively, suppressed ALT-E-induced IL-18 release by cells, whereas caspase 1 and 8 inhibitors did not. ALT-E-stimulation increased autophagosome formation, LC-3 conversion, and p62 degradation in airway epithelial cells. LPS-stimulation induced the LC3 conversion in A549 cells, but did not induce IL-18 release or p62 degradation. Unlike LPS, ALT-E induced airway epithelial cells to release IL-18 via an autophagy dependent, caspase 1 and 8 independent pathway. Although autophagy has been shown to negatively regulate canonical inflammasome activity in TLR-stimulated macrophages, our data indicates that this process is an unconventional mechanism of IL-18 secretion by airway epithelial cells.

Keywords

Autophagy; airway epithelial cells; allergy; asthma; interleukin-18; *Alternaria*

*Corresponding author: Yusei Ohshima, Department of Pediatrics, University of Fukui, 23-3 Matsuoka Shimo-Aizuki, Eiheiji-cho, Yoshida-gun, Fukui, 910-1193, Japan. Fax: +81-776-61-8129, yohshima@u-fukui.ac.jp (Y. Ohshima).

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1. Introduction

A number of epidemiological studies have shown that the fungus *Alternaria alternata* is the source of a common allergen that causes allergic airway diseases, including bronchial asthma, and that sensitivity to this *Alternaria alternata* allergen is associated with asthma severity, bronchial hyperresponsiveness, and fatal asthma exacerbations [1–3]. We recently demonstrated that an *Alternaria* extract (ALT-E) could induce airway epithelial cells to release interleukin (IL)-18 and thereby directly initiate Th2 differentiation by naive CD4⁺ T-cells [4]. Although originally discovered as a factor that induced IFN- γ production by Th1 cells, IL-18 also has the potential to induce IL-4 and IL-13 production by T cells, natural killer (NK) cells, NKT cells, mast cells, and basophils [5–6]. Increased IL-18 expression and serum levels have been reported in humans with allergic airway diseases [7–8]. A functional polymorphism of the IL-18 gene was also shown to be associated with the severity of bronchial asthma [9]. Taken together, these results suggest that IL-18 might play an important role in the pathophysiology of *Alternaria*-associated asthma.

IL-18 is a pro-inflammatory cytokine that belongs to the IL-1 family and is produced in a biologically inactive precursor form, pro-IL-18 [10]. Due to a lack of a signal peptide, as with IL-1 β , mature IL-18 is secreted after pro-IL-18 is cleaved by the cysteine protease caspase 1. Caspase 1 activity is controlled by inflammasomes, which are multiprotein signaling complexes that detect microbial-derived molecular signatures or endogenous danger signals [11]. Several lines of evidence suggest that there is a non-canonical IL-1 β and IL-18 activation pathway that involves caspase-8 and receptor interacting protein (RIP) kinases [12–13]. Furthermore, in some inflammatory conditions, IL-1 β and IL-18 processing might occur extracellularly due to the actions of a number of proteases, including neutrophil proteinase-3, mast cell chymase, matrix metalloproteinases, cathepsin G, and granzyme A [14–16].

Macroautophagy (often simply referred to as autophagy) is a cellular process that involves the recycling of cellular proteins and the removal of intracellular microorganisms through the lysosome machinery [17–18]. Autophagy is a multistage process that comprises several steps: (1) formation of autophagosomes; (2) fusion of these autophagosomes with lysosomes to form autolysosomes; (3) degradation of autolysosome contents; and (4) utilization of these degradation products [19]. Autophagy is known to control inflammation by removing endogenous inflammasome agonists and by autophagic degradation of inflammasome components, which results in reduced inflammasome and caspase 1 activation [18–20]. Additionally, autophagy-based unconventional secretion, or so-called autosecretion, has been shown to enable leaderless cytosolic proteins, such as IL-1 β and IL-18, to be delivered extracellularly without entering the endoplasmic reticulum (ER)-to-Golgi secretory pathway [21–22].

In this study, we investigated whether autophagy and inflammasome pathways were involved in IL-18 release from ALT-E-stimulated airway epithelial cells. We show that ALTE-stimulation can induce airway epithelial cells to release IL-18 via an autophagy dependent and caspase1 and 8 independent pathway.

2. Materials and Methods

2.1. Reagents

Caspase 1, 8 inhibitors and 3-methyladenine were purchased from Calbiochem (Gibbstown, NJ). C75, Bafilomycin A1, Pam3CSK4, MALP2, Poly (I:C), and LPS were from Sigma-Aldrich (St. Louis, MO). Flagellin, FSL-1, imiquimod, single stranded RNA40, and ODN 2006 were from InvivoGen (San Diego, CA).

2.2. Cell culture

Normal human bronchial epithelial (NHBE) cells were obtained from Cambrex Bio Science (Walkersville, MD) and A549 cells, a human lung adenocarcinoma line, were from the American Type Culture Collection (Manassas, VA). A549 cells were cultured at 37°C with 5% CO₂ in RPMI 1640 supplemented with 10% FBS, penicillin G sodium (100 units/ml), and streptomycin sulfate (100 µg/ml) (Invitrogen, Carlsbad, CA). NHBE cells were maintained in BEGM Bullet Kit medium (Lonza, Switzerland). Cells at 2–4 passages were used for experiments.

NHBE cells or A549 cells ($\approx 2 \times 10^4$ cells/well) were subcultured in 96-well culture plates at 37°C overnight. Prior to stimulation, cells were pre-treated for 1 hour with different inhibitors, including indicated concentrations of caspase-1 inhibitor VI, caspase 8 inhibitor, 3-methyladenine, C75, or Bafilomycin A1. Cells were then stimulated for 15 minutes with either 30 µg/ml of Alternaria extract (ALT-E), 1 µg/ml of Pam3CSK4, 200 ng/ml of MALP2, 50 µg/ml of Poly (I:C), 10 µg/ml of LPS, 1 µg/ml of Flagellin, 1 µg/ml of FSL-1, 5 µg/ml of imiquimod, 100 µg/ml of single stranded RNA40, or 5 µg/ml of ODN 2006. Immediately after stimulation, cell culture supernatants were collected for IL-18 measurements. Morphological changes of ALT-E stimulated A549 cells were examined under an inverted phase contrast microscope.

2.3. Cytokine measurements

To quantify cytokine levels in cell culture supernatants, multiplex suspension protein array analysis was performed using a Bio-Plex Pro Human Cytokine 21-Plex Panel and a Bio-Plex Pro Human Cytokine 27-Plex Panel (Bio Rad Laboratories, Inc., Hercules, CA), according to the manufacturer's protocol. IL-18 concentrations were determined by ELISA. Antibody pairs and standard recombinant human IL-18 for ELISA were purchased from Medical and Biological Laboratory, Co. (Nagoya, Japan). The antibody pairs did not recognize pro-IL-18.

2.4. Live cell imaging for autophagosome formation

A549 cells were stained using a Cyto-ID autophagy detection kit (Enzo, NY), according to the manufacturer's protocol. Autophagosome formation in cells that were stimulated with 10 µg/ml of ALT-E was continuously monitored using a Leica TCS SP II confocal laser scanning microscope (Wetzlar, Germany).

2.5. Western blotting

Total cell proteins were extracted using Complete Lysis-M reagent (Roche Diagnostics Japan, Tokyo, Japan) and were electrophoresed by SDS-PAGE (30 µg protein/lane). Proteins

were transferred to PVDF membranes (Millipore, Billerica, MA) and immunoblotted with specific primary antibodies: anti-human LC3 or p62 antibodies (Cell Signaling Technologies, Danvers, MA); and an anti-human α -actin antibody (Sigma Aldrich). Blots were then incubated with appropriate HRP-conjugated secondary antibodies and bands were disclosed using an ECL-Plus detection system (GE Healthcare Japan, Tokyo, Japan).

2.6. Enzyme activity assay

Caspase 1/ICE fluorometric assay kits were from R&D Systems (Minneapolis, MN). A total of 1×10^6 A549 cells were stimulated with 30 μ g/ml of ALT-E for indicated periods. Then, 100 μ g of cell lysate proteins were used for the enzyme activation assay, according to the manufacturer's protocol, with fluorescent emissions measured with an Infinite 200 (Tecan, Mannedorf, Switzerland).

2.7. Statistical analysis

Results are given as means $\pm$ SEM's. Statistical comparisons between different group results were made using either unpaired *t* tests (two groups) or one way ANOVA (more than two groups). A *p*-value of < 0.05 was considered significant.

3. Results

3.1. ALT-E-induced IL-18 release is independent of caspase 1 and 8 activation

We first investigated which cytokines were released from ALT-E stimulated NHBE cells by multiplex cytokine array analysis. Among the cytokines investigated, IL-18 levels in culture supernatants were dramatically increased after stimulation with ALT-E (Fig 1A). We next examined whether conventional inflammasome pathways were involved in IL-18 release from airway epithelial cells in response to ALT-E stimulation using different inhibitors. As shown in Figure 1B and 1C, 5–20 μ M caspase-1 inhibitor VI or caspase 8 inhibitor failed to inhibit IL-18 release from ALT-E-stimulated A549 cells, suggesting that IL-18 release was mediated by another pathway. A near-infrared fluorescent probe selective for caspase 1 revealed that caspase 1 was not activated in A549 cells, even after 60 minutes of stimulation with ALT-E (Figure 1D) Furthermore, C75 [23], a fatty acid synthase inhibitor that is known to induce caspase 3-dependent apoptosis, also failed to affect IL-18 release from ALT-E-stimulated A549 cells (Fig. 1E). Together these results suggest that neither the canonical NLRP3 inflammasome-caspase 1 pathway nor the non-canonical caspase 8 inflammasome are involved in ALT-E-mediated IL-18 release by these cells [11].

3.2. ALT-E-induced IL-18 release is mediated by autophagy activation

Building on these unexpected observations, we next examined the role autophagy in ALTE-induced IL-18 release. In contrast to the results described above, the PI3-kinase inhibitor 3-methyladenine (3-MA) and bafilomycin A1, which inhibit preautophagosomal structure formation and autophagosome-lysosome fusion, respectively [19], significantly suppressed the release of IL-18 from ALT-E-stimulated A549 cells and NHBE cells (Fig 2A, B, and C). Incubation of A549 cells with ALT-E rapidly induced swelling and rounding of cells in 30 min, resulting in cell death. The morphological changes were inhibited in the presence of 3-

MA but not caspase 1 inhibitor VI, suggesting that ALT-E-induced cell death might be mediated by activation of autophagy (Figure 2D).

To verify the involvement of autophagy activation, we analyzed autophagosome formation and the levels of autophagy indicator proteins, including LC3-I, LC3-II, and p62 [19]. As shown in Figure 3, ALT-E rapidly augmented autophagosome formation in A549 cells. Moreover, the ratio of LC-3II/LC-3I expression, which reflects the LC3-I to LC3-II conversion, was increased and p62 degradation associated with autolysosome formation was induced at 15 minutes after stimulation with ALT-E.

3.3. LPS does not induce IL-18 release and fails to progress autophagy flux

ALT-E might include various pathogen-associated molecular patterns, such as Toll like receptor (TLR) ligands, which could have induced autophagy and stimulated IL-18 release by cells. However, none of the examined TLR agonists, such as lipopolysaccharide (TLR4), Pam3CSK4 (TLR1 and 2), MALP2 (TLR2 and 6), Poly (I:C) (TLR3), Flagellin (TLR5), FSL-1 (TLR2/6), imiquimod (TLR8), single stranded RNA40 (TLR7, 8), ODN 2006 (TLR9) induced IL-18 release from A549 cells (Fig 4A).

LPS is known to activate autophagy in mouse peritoneal macrophages [24]. To explore the relevance of autophagy to IL-18 release, we examined whether LPS could activate autophagy in A549 cells. Notably, LPS-stimulation enhanced the ratio of LC-3II/LC-3I expression but not p62 degradation (Fig. 4B and 4C), which might suggest that LPS could induce autophagosome formation but failed to progress autophagy flux to autolysosome formation.

4. Discussion

In this study, we found that ALT-E-stimulation activated an autophagy-based unconventional secretion pathway in airway epithelial cells and thereby induced the extracellular release of IL-18 independent of caspase 1 and 8 activation.

A number of reports have shown negative effects of autophagy on IL-1 β and IL-18 activation by inflammasomes and that caspase 1 had a dominant effect on degenerative autophagy [18,20,25]. In contrast to degenerative autophagy, a contribution of secretory autophagy to the extracellular delivery of IL-1 β and IL-18 has been detected in murine bone marrow-derived macrophages as a transient autophagy-dependent secretory process [22]. Although this latter mechanism of IL-1 β and IL-18 secretion was inflammasome dependent, a role for caspase 1 in secretion was not determined in that study.

Based on our results, secretory autophagy rather than degenerative autophagy may have operated in IL-18 release from ALT-E-stimulated airway epithelial cells independent of caspase 1 activation. LPS did not induce p62 degradation or IL-18 release, whereas ALT-E induced p62 degradation and IL-18 release. Given that p62 degradation occurs in autophagosomes [19], the fusion step of autophagosomes with lysosomes might be essential for processing and secretion of a mature form of IL-18 by secretory autophagy in airway epithelial cells.

We previously demonstrated that ALT-E also caused airway epithelial cells' death with features of necrosis rather than those of apoptosis [4]. A novel form of pro-inflammatory programmed cell death, pyroptosis, is known to result in pro-inflammatory cytokine processing [26]. However, this death pathway depends on caspase 1. Autophagy has been implicated in association with caspase-independent cell death in apoptosis-compromised cells, which leads to necrosis and necroptosis. Because autophagy inhibitors but not a caspase 1 inhibitor prevented against the progression of ALT-E-induced airway epithelial cell death, autophagy might also be involved in cell death.

It was previously reported that autophagy increased in epithelial cells of the asthmatic airway and that genetic polymorphisms of the ATG5 gene were associated with asthma and reduced FEV1 measurements [27, 28]. Furthermore, autophagy likely plays a major role in the interplay between respiratory viral infections and asthma [29]. Our results suggest that autophagy is also involved in the pathophysiology of *Alternaria*-associated asthma through IL-18 production and epithelial cell damage. Thus, autophagy could be a new therapeutic target in those with asthma.

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Highlights

- Alternaria activates autophagy that induces IL-18 release from epithelial cells.
- This IL-18 release is independent of the canonical activation of the inflammasome.
- Autophagy might be involved in the pathophysiology of Alternaria-associated asthma.

Figure 1.

ALT-E selectively induces IL-18 release from cells and is not suppressed by caspase inhibitors. (A) NHBE cells were incubated with PBS or ALT-E. Cytokine concentrations in culture supernatants collected after stimulation for 15 min were analyzed by multiplex suspension protein arrays (n = 4). Heat map shows relative cytokine concentrations, which are indicated on a scale ranging from red to blue with dark red indicating the highest and dark blue indicating the lowest concentrations. (B, C, E) A549 cells were pre-treated with the indicated concentrations of inhibitors for caspase 1 (B), and caspase 8 (C) or C75 (E), and then incubated with (closed bars) or without (open bars) ALTE. IL-18 concentrations in culture supernatants collected after stimulation for 15 min were determined by ELISA. Results are means ± SEM's (n = 4) nd; not detected, * $p < 0.05$, ** $p < 0.01$. (D) Caspase 1 activity in cell lysates of A549 cells stimulated with ALT-E for indicated periods were measured by a caspase 1 fluorometric assay. Caspase-1 activities were expressed as fold changes relative to non-stimulated cells.

Figure 2.

ALT-E-induced IL-18 release and morphological changes are suppressed by autophagy inhibitors. A549 cells (A, B) and NHBE cells (C) were incubated with (closed bars) or without (open bars) ALT-E. Cells were pre-treated with the indicated concentrations of inhibitors for 3-MA (A, C) or bafilomycin (B). IL-18 concentrations in culture supernatants collected after stimulation for 15 min were determined by ELISA. Results are means $\pm$ SEM's (n = 4). nd; not detected, * $p < 0.05$, ** $p < 0.01$. A549 cells were cultured with ALT-E for 15 min in the presence or absence of 10 μ M caspase 1 inhibitor or 10 mM 3-MA (D). Changes in cell morphology were observed by a phase contrast microscope.

Figure 3.

ALT-E-stimulation activates an autophagy flux. A549 cells were stimulated with ALT-E for the indicated times. Autophagosome formation in these cells was detected with a Cyto-ID autophagy detection kit (A). Equal amounts of whole cell lysates were separated on SDS-PAGE and Western blots were prepared to evaluate the LC3-I to LC3-II conversion (B) and p62 degradation (C). Blots were stripped and re-blotted to assess actin expression. Results shown are representative of 4 experiments. Results are means $\pm$ SEM's (n = 4). * p < 0.05, ** p < 0.01.

Figure 4.

Toll like receptor agonists do not induce IL-18 release or p62 degradation. A549 cells were stimulated with the indicated TLR agonists for 15 min (A). IL-18 concentrations in culture supernatants were determined by ELISA. A549 cells were stimulated with 1 mg/ml of LPS for the indicated times (B, C). Equal amounts of whole cell lysates were separated on SDS-PAGE and Western blots were prepared to evaluate the LC3-I to LC3-II conversion (B) and p62 degradation (C). Blots were stripped and re-blotted to assess actin expression. Results shown are representative of 4 experiments. Results are means $\pm$ SEM's (n = 4). * $p < 0.05$, ** $p < 0.01$