Chapter 4

Free-Living Amoeba *Acanthamoeba* Triggers Allergic Inflammation of Airways

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1. Introduction

An estimated 300 million people worldwide have asthma, and 250,000 deaths are attributed annually to the disease. From 2001 to 2009, the number of people diagnosed with asthma grew by 4.3 million. Allergic airway inflammation is closely related to airway hyperresponsiveness (AHR), the production of mucus, and airway remodeling. This inflammation is mediated by the T helper type 2 (Th2)-cell response, the upregulation of interleukins (IL)-4, 5, and 13, which are produced by activated CD4+ T cells, and by elevated immunoglobulin E (IgE) production. Asthma has long been associated with atopy, a predilection for producing antigen-specific IgE antibodies against environmental allergens capable of mediating hypersensitivity reactions, particularly immediate skin reactions [1, 2]. Many environmental proteases are believed to be allergens that elicit allergic airway inflammation. Allergens from house dust mites [3], cockroaches [4], fungi [3], and pollens have been reported to contain cysteine, serine, and aspartic proteases [5].

Recently, asthma patients with high serum IgE levels, but who do not react to known allergens in skin prick tests, have been identified, suggesting the presence of unknown environmental allergens [6]. We hypothesize that free living amoeba (FLA) are undiscovered aeroallergens. One of the FLA, *Acanthamoeba*, is an opportunistic protozoan broadly detected throughout the environment. The amoeba can cause severe human diseases, including amoebic keratitis (it can lead to blindness) in health person and fatal encephalitis in AIDS patients [7]. *Acanthamoeba* species have been isolated from swimming pools, public sewage, water supplies, air-conditioning units, sediments, air, compost, soils, contact lenses and their storage cases [8]. In addition, *Acanthamoeba* have been isolated from human bodies especially nasal cavities, pharyngeal swabs, lung tissues, and skin [8-10]. Perhaps it is not unsurprisingly that we have been found anti-*Acanthamoeba* antibodies from many of healthy individuals tested, this

indicated that exposure to the amoeba is common [11]. *Acanthamoeba* exist as trophozoites or cysts. Trophozoites are the metabolically active form, consuming nutrients via phagocytosis, while unfavorable environmental conditions lead to the formation of cysts. In addition, a lot of proteases, including cysteine and serine proteases, have been detected from *Acanthamoeba* excreted/secreted (ES) proteins. These proteases are significant determinants of protozoan pathogenicity and host cell invasion. It has been proposed that proteases play a central role in various processes, such as host cell invasion and way out, cyto-adherence, morphological differentiation, digestion of host proteins, stimulation immune response, and escape from host immune responses [12-16]. However, in spite of their ubiquitous existence in the environment and expression of a lot of proteases capable of eliciting allergic airway inflammation, no report exploring this connection has been published to date.

2. *Acanthamoeba* trophozoites elicited a strong allergic airway inflammation response

Airway allergens are experimentally confirmed by the ability to elicit allergic airway inflammation when it was inhaled. Ovalbumin (OVA) is one of commonly used the experimental allergens, but is unable to elicit allergic airway inflammation if directly administered by inhalation without any allergens. By contrast, pollens and fungal-derived allergens can easily elicit allergic responses when inhaled through the airway tract [17-19]. Therefore, if repeated administration of *Acanthamoeba* into the airway tract, allergic response can be occurred in the airway. Park et al. reported that repeated inoculation of *Acanthamoeba* trophozoites to a mouse model elicited allergic airway inflammation [20]. In order to test the ability of *Acanthamoeba* to trigger allergic inflammation, they administrated trophozoites form of *Acanthamoeba* (5×10^4) into the nose of mice and evaluated immunological and pathological responses (Fig. 1A). In the mice inoculated with *Acanthamoeba* trophozoites, a dose-dependent increase in AHR to methacholine was observed (Fig. 1B). These mice also presented inflammatory cell infiltrations, and the numbers of neutrophils, eosinophils, (Fig. 1C) and lymphocytes were increased in the broncho-alveolar lavage fluid (BALF) (Fig. 1C). They also suggested that enormous inflammatory cell infiltration, hyperplasia of goblet cell and epithelial cell was found in the lung of *Acanthamoeba* nasally administrated mice [20]. Also, It were increased that the levels of Th2 cytokines (IL-4, IL-5, and IL-13) in the BALF and in the supernatant of culture medium of T cells in lung draining lymph node (LLN), in the *Acanthamoeba* administrated mice, compared with those of the controls. However, IFN-γ levels and IL-17 cytokine levels were unchanged in LLN and BALF by *Acanthamoeba* infection. The immunoglobulin (Ig) E level and *Acanthamoeba*-specific IgE level were significantly increases in total serum of *Acanthamoeba* infected mice (Fig. 1D).

It is possible for a person to come into contact with as many as 100 trophozoites at a time from *Acanthamoeba* contained tap water [21]. In order to evaluate whether such a dose of *Acanthamoeba* could elicit airway allergic inflammation, they introduced one hundred trophozoites of *A. lugdunensis* intranasally (Fig. 1E). Histology revealed some infiltrated of inflammatory cells, and mild hyperplasia of epithelial cells in the lung after administration and mucin expression

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in administrated mice was also higher than in control. A few *Acanthamoeba* were detected in alveoli, and quite a few eosinophils were observed around *Acanthamoeba* (Fig. 1F). Although, IL-5 levels in the LLNs and BALF were elevated following *Acanthamoeba* nasally treatment (Fig. 1G), but IL-13 and IL-4 levels were unchanged. Total levels of IgE in the serum and anti-*Acanthamoeba* IgE were unchanged by low dose *Acanthamoeba* treatment.

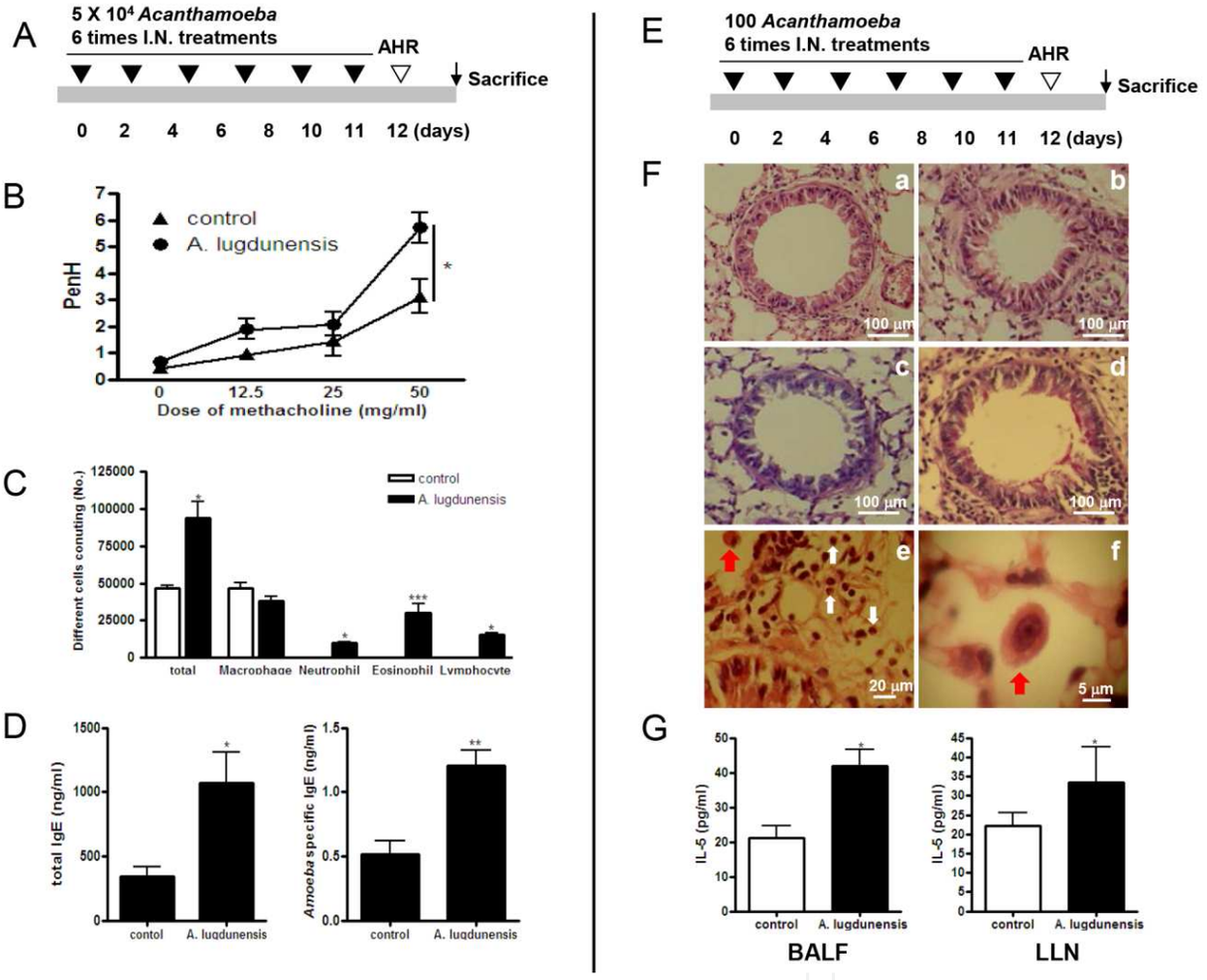

Figure 1. ***Acanthamoeba*** **elicit airway allergic inflammation in mice [20]**. Allergic airway inflammation was induced by inoculating mice with high (A–D) or low (E–G) doses of *Acanthamoeba*. (A) Intranasal inoculation schedule for the high-dose (4×10^5 *Acanthamoeba* trophozoites) model. (B) Airway resistance values in response to methacholine (0 - 50 mg/mL). (C) Differential cell count in 800 μL bronchoalveolar lavage (BAL) after Diff-Quik staining. (D) Total and *Acanthamoeba*-specific IgE levels were measured in serum by ELISA. (E) Intranasal inoculation schedule for the low-dose (100 *Acanthamoeba* trophozoites) model. (F) Tissue inflammation observed in stained lung sections (a and c, PBS-treated; b and d, *Acanthamoeba*-infected; a and b, H&E stained; c and d, PAS-stained; red arrows, *Acanthamoeba* trophozoites; white arrows, eosinophils). (G) Cytokine concentrations in BAL fluids (BALF) and in the culture medium of CD3-stimulated lymphocytes isolated from lung draining lymph nodes. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n=7, three independent experiments).

3. *Acanthamoeba* produced strong ES proteases that could induce severe allergic inflammation in airway through Protease Activated Receptor 2 (PAR2)

Proteases of *Acanthamoeba* are required for the their life cycle maintenance [7, 14]. It is well known that *Acanthamoeba* excretory and secretory (ES) protein contained abundant serine protease. Serine protease is necessary for the encystation and excystation of *Acanthamoeba* [22]. Serrano-Luna et al. identified 17 proteins with proteolytic activity in *Acanthamoeba* [14]. They demonstrated that proteolytic activity of ES proteins attributed primarily to the serine proteases and secondly to cysteine proteases, using protease inhibitors [14]. Subtilisin, one of the serine proteases, have been detected from ES proteins of *Acanthamoeba*, and also it was known as an inducer of asthma, [22, 23]. In addition, subtilisin has been detected from various organisms, and it can stimulate specific antibodies production in mice, and elicit various allergic response [24, 25].

Park et al., introduced protease-containing ES protein samples of *Acanthamoeba* into the nasal of mice six times, and observed the functional and immunological changes to the respiratory system. Lungs of ES protein-administered mice showed abundant infiltration of immune cell around the airway tracts, elevated mucin expression, and hyperplasia of goblet cells [20]. Levels of Th2 cytokines (IL-5, IL-4 and IL-13) were higher in the LLNs and BALF from the ES protein administrated group than those of control groups. Protease activity from the ES protein preparation was able to digest gelatin (Fig. 2A). This activity was abolished by introduction of PMSF (serine protease inhibitor), but not affected by cysteine protease inhibitor E-64, but some protein bands that had weaker protease activity were inhibited. The metallo protease inhibitor, matrix metallopeptidase (MMP)-9, did not inhibit the activity of most proteases (Fig. 2A). *Acanthamoeba* ES proteins treatment increased the critical factors (*TARC*, *TSLP*, *MDC*, *IL-25*, *eotaxin* gene expression) for Th2 response initiation and development in lung epithelial cells, and also led to increased levels of PARs in MLE12 cells (Fig. 2B & 2C) [20].

Recently PARs is known as belong to seven-transmembrane domain G protein coupled receptors [26]. They are activated through proteolytic cleavage of their N-terminal "tethered ligand" domains [27]. PAR1, 2, 3 and PAR4 have been cloned. They can be activated by thrombin (PAR1, PAR3, and PAR4), also can be activated by neutrophil protease 3, mast cell tryptase, trypsin, and several serine proteases (PAR2) [28]. Park et al. treated ES proteins with serine protease inhibitor (PMSF) and evaluated airway inflammation. The results showed that Pre-treatment with PMSF lead to a significant decrease in most values of the inflammation index, relative to administration of untreated ES proteins. The airway hyperresistant response (AHR) to methacholine following ES protein administration was likewise decreased by PMSF pre-treatment [20]. In addition, the infiltration of immune cells was lower in the PMSF-treated group, compared with ES protein-treatment alone; most notably, the number of eosinophils significantly decreased. In evaluation of the airway allergic inflammation induced by *Acanthamoeba* on PAR2 deficient (KO) mice, infiltration of immune cell around the airway tracts, elevated mucin expression, and hyperplasia of

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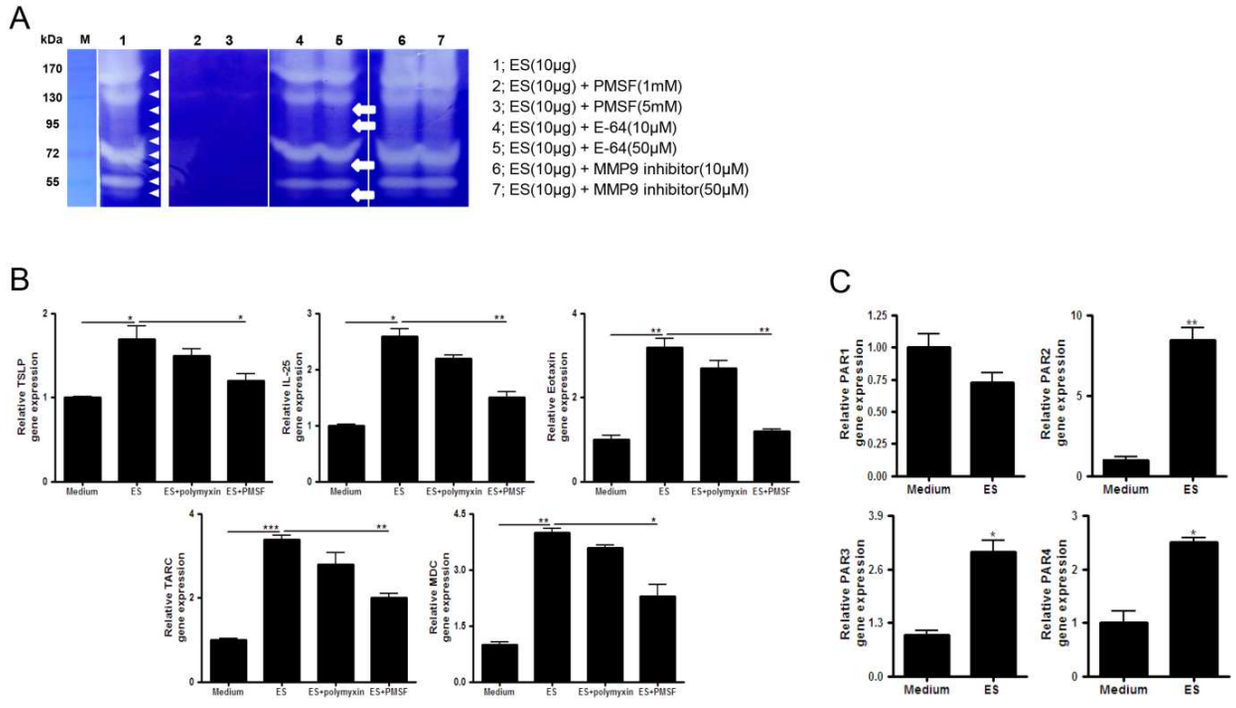

Figure 2. Excreted/secreted (ES) proteins elicit T helper type 2 related chemokine and cytokine production [20]. (A) ES proteins were treated with various protease inhibitors. Samples were incubated for 2 h and assayed by zymography on 0.1% gelatin SDS-PAGE gels (lane 1, 10 μg ES proteins; lane 2, with 1 mM PMSF; lane 3, with 5 mM PMSF; lane 4, with 10 μM E-64; lane 5, with 50 μM E-64; lane 6, with 10 μM MMP-9 inhibitor; lane 7, with 50 μM MMP-9 inhibitor; arrowhead, protease activity from ES proteins; arrow, protease activity inhibited by E64). (B) Th2-related chemokine gene expression (*TSLP*, *TARC*, *MDC*, eotaxin, and *IL-25*) was measured in MLE12 cells after incubation with 1 μg/mL of ES proteins (ES) for 2 h, or pre-treatment with 0.1 mM PMSF and 10 μg/mL polymyxin B (polymyxin) for 2 h. (C) The fold-change in PAR mRNA levels in MLE12 cells treated with ES proteins relative to those treated with medium, detected by real-time RT-PCR.

lung goblet cells were observed in PAR2 KO mice like as WT mice. However, Th2 cytokine level in the LLNs and BALF were lower in PAR2 KO mice treated with ES proteins than those of WT mice [20].

4. ES proteins activate dendritic cells (DCs) and the differentiation of Th2 cells

PARK et al., suggested that *Acanthamoeba* ES proteins strongly stimulated DCs and enhanced the expression of CD80, CD86, CD40, and MHC II. (Fig. 3). Once ES protein stimulated DCs were co-incubated with $CD4^+CD25^-CD62L^+$T cells (naïve T cells), the number of $CD4^+$ Th2 cells (IL-4-secreting $CD4^+$ T cell) increased after co-incubation the DCs and naïve T cells (Fig. 4A). In addition, Th2 cytokines (IL-5, IL-4, and IL-13) production by $CD4^+$ T cells increased in culture supernatants of co-incubated with ES protein stimulated DCs (Fig. 4B). In addition, naïve T cells co-incubated with *Acanthamoeba* ES protein stimulated DCs had high levels of, transcription factor, GATA-3 gene expression (Fig. 4C).

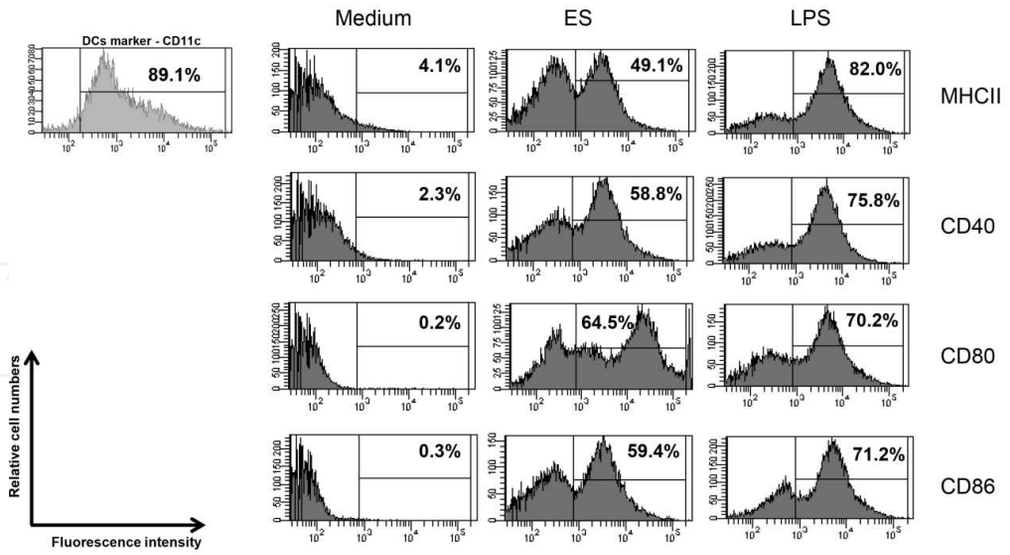

Figure 3. Acanthamoeba ES proteins activate BMDCs [20]. Expression of cell surface markers (CD40, CD80 CD86, and MHCII,) on mouse BMDCs pulsed with ES proteins or LPS for 48 h, compared with untreated cells.

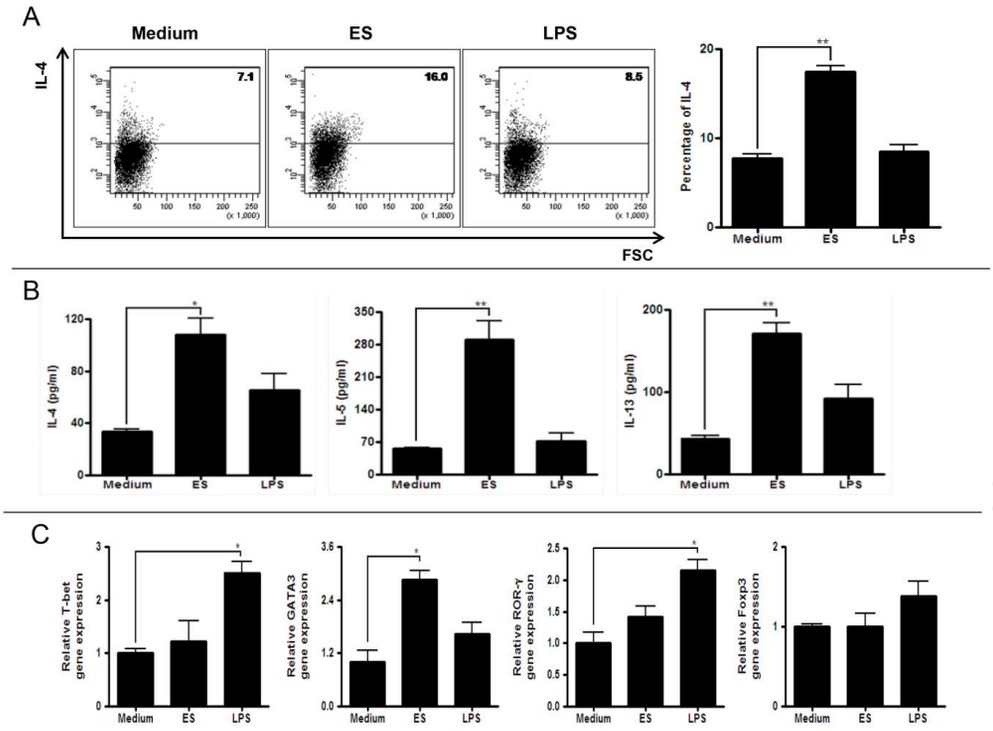

Figure 4. Differentiation of T helper type 2 (Th2) cells from naïve T cells after co-cultivation with ES protein-activated BMDCs [20]. (A) Naïve T cells were cultured with BMDCs stimulated by ES proteins or LPS, or non-stimulated BMDCs for 3 d in the presence of anti-CD3 antibodies. After gating with $CD4^+$ T cells, IL-4-producing T cells were counted. (Medium, naïve T cells with non-stimulated BMDCs; ES, naïve T cells with ES protein-stimulated BMDCs; LPS, naïve T cells with LPS-stimulated BMDCs). (B) Cytokine levels in the supernatants from co-cultures of naïve T cells and BMDC, measured by ELISA. (C) Gene levels were evaluated by real time-RT PCR from naïve T cell/BMDC co-cultures.

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Enhancement of IL-5, IL-4, IL-13, and CXCL1 (eotaxin) are critical for the induction of allergic asthma by Th2 cells [29, 30]. Furthermore, CCR3, CCR4, and CCR8 was expressed on Th2 cells. Imai et al., suggested that TARC, MDC, and high-affinity CCR4 ligands can induce Th2 cells migration to the selective sites [31]. Therefore, production of serine protease activity contained *Acanthamoeba* ES proteins might stimulate DCs, and promoting the differentiation of $CD4^{+}CD25^{-}CD62L^{+}$T (naive T) cells to Th2 cells.

5. *Acanthamoeba* antigens are detected in house dust, and significantly high level of anti *Acanthamoeba* IgE in asthma patients

Park et al., demonstrated that after samples of house dust were reacted with total serum from *Acanthamoeba* infected or uninfected mice, and the total dust reacted IgG1 levels in serum of infected mice were higher than those of control mice [20]. These results indicate that *Acanthamoeba* can be contaminated from domestic environments, like as house dust mite DerP1 allergen. Therefore, it is no wonder that almost all healthy persons have anti-*Acanthamoeba* IgG [10, 11]. They also screened *Acanthamoeba*-specific IgE levels in patients with asthma in order to know whether *Acanthamoeba* can be related with asthma in humans. The asthma patients have significantly higher IgE levels ($p = 0.028$) than those of healthy persons [20]. According to all of results proposed that *Acanthamoeba* could be a novel human airway allergen.

6. Conclusion

Acanthamoeba trophozoites and ES proteins stimulated allergic airway inflammation, and extended Th2 responses via PAR2 signaling and DC activation in a mouse asthma model. Furthermore, patients with asthma had higher anti-*Acanthamoeb*a IgE titers than those of healthy persons. In order to aid the diagnosis, we needed further studies to identify the specific ES allergens from Acanthamoeba.

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