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CD4$^+$ T cells from food allergy model are resistant to TCR-dependent apoptotic induction

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ABSTRACT

Background: CD4$^+$ T cell polarization plays a critical role in the pathogenesis of allergy. How to modulate the skewed CD4$^+$ T cell polarization is less clear. The specific immunotherapy (SIT) is the only specific remedy for the treatment of allergic diseases; the therapeutic effect is to be improved.
Objectives: This study aims to investigate the role of interleukin (IL)-18 in enhancing the therapeutic effect of SIT.
Methods: A peanut allergy mouse model was developed and treated with SIT or/and IL-18. CD4$^+$ T cell apoptosis was assessed by flow cytometry. The expression of Fas ligand (FasL) was observed by quantitative real time RT-PCR and Western blotting. Interferon-γ in the culture medium was determined by enzyme-linked immunosorbent assay. The fasL gene promoter methylation in CD4$^+$ T cells was assessed by methylation specific PCR.
Results: The results showed that lower levels of IL-18 were detected in allergic mice; administration of IL-18 significantly enhanced the therapeutic effect of SIT on suppressing the allergic inflammation in the mouse intestine. In the cell culture studies, IL-18 increased the TCR-dependent CD4$^+$ T cell apoptosis, the expression of FasL in CD4$^+$ T cells, the production of Interferon-γ and the demethylation of the FasL promoter in CD4$^+$ T cells.
Conclusions: Administration of IL-18 enhances the effect of SIT on suppressing allergic inflammation in the mouse intestine via enhancing the TCR-dependent CD4$^+$ T cell apoptosis.

1. Introduction

The pathogenesis of allergy is unclear. Most therapeutic remedies for allergic diseases are limited to control the clinical symptoms currently, only the antigen specific immunotherapy (SIT) is a specific remedy for the treatment of allergic diseases. Yet, the efficiency of SIT is to be improved.

CD4$^+$ T cells play an important role in the pathogenesis of allergic diseases. CD4$^+$ T cells include T helper (Th)1, Th2, Th17 and regulatory T cells, in which Th2 cells are the major cell types contributing to the pathological changes of allergic diseases [1]. In some other cases, Th1 cells also contribute to the pathogenesis of allergy, such as allergic asthma [2]. The skewed Th2 cell polarization is one of the major pathological features of allergy. Upon the activation of the TCR (T cell receptor) by specific antigens, the antigen specific Th2 cells proliferate and produce proinflammatory cytokines, such as IL-4, IL-5 and IL-13, to induce allergic inflammation in the local tissues. In general, after activation, T cells may undergo the programmed cell death, the apoptosis, to be eliminated. Such a condition is designated the activation-induced cell death (AICD) [3]. Whether the AICD mechanism is dysfunction in antigen specific Th2 cells under an allergic environment is less clear. To adjust the skewed Th2 polarization is refractory currently.

IL-18 is encoded by the *IL18* gene [4]. It is produced by keratinocytes, macrophages, monocytes, dendritic cells, osteoblastic stromal cells, epithelial cells, T cells, microglia and ependymal cells [4]. IL-18 is a pleiotropic cytokine involving in both Th1 and Th2 responses. One of the major functions of IL-18 is to induce interferon (IFN)-γ [4]. IL-18 is involved in the pathogenesis of a number

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of inflammatory diseases, such as inflammatory bowel disease [5] and neural inflammation [6]. IFN-γ is a signature cytokine of Th1 response, it is proposed that the administration of IFN-γ can counterbalance Th2 responses to suppress the allergic inflammation [7]; the underlying mechanism is not fully understood yet.

The functions of IFN-γ on inducing apoptosis is recognized [8], but the mechanisms are poorly understood, likely involving in multiple pathways [8]. Fas and Fas ligand (FasL) is a couple of molecules to induce apoptosis, which is the major mechanism of AICD [9]. It is reported that IFN-γ can modulate the expression of FasL [10]. Thus, we hypothesize that IL-18 enhances the therapeutic effect of SIT on allergic inflammation via modulating the AICD in antigen specific $CD4^+$ T cells. In this study, we observed that IL-18 significantly enhanced the effect of SIT on suppressing allergic inflammation of the intestine in a peanut allergy mouse model. The cell culture studies showed that IL-18 increased the expression of FasL in $CD4^+$ T cells via the promoter demethylation, which enhanced the AICD by inducing the TCR-dependent $CD4^+$ T cell apoptosis.

2. Materials and methods

2.1. Reagents

Antibodies of FasL, CD28 were purchased from Santa Cruz Biotech China branch (Shanghai, China). The Annexin V kit was purchased from Sigma Aldrich China branch (Shanghai, China). Reagents of qRT-PCR and Western blotting were purchased from Invitrogen China branch (Shanghai, China). Magnetic bead-conjugated antibodies were purchased from Miltenyi Biotech China branch (Shanghai, China). DNA purification kit was purchased from Promega China branch (Beijing, China). The EpiXploreTM Methyl Detection kit was purchased from Clontech China branch (Beijing, China). The Ara h2 protein was obtained from AbBioTec (Guangzhou, China). ELISA kits of IFN-γ, IL-4, IL-18, recombinant IL-18 and neutralizing anti-IFN-γ mAb was purchased from R&D Systems China branch (Shanghai, China). Anti-CD3 coated 96-well plates were purchased from BD Bioscience China branch (Shanghai, China).

2.2. Mice

Male BALB/c mice (6–8 week old) were purchased from the Guangzhou Experimental Animal Center (Guangzhou, China) and maintained in a pathogen free environment. The experimental procedures were approved by the Animal Ethic Committee at Shenzhen University.

2.3. Peanut allergy mouse model development

Following published procedures [11,12] with modification (the peanut extracts were replaced by Ara h2), BALB/c mice were gavage-fed with a recombinant peanut allergen, Ara h2 (0.1 mg/mouse) mixed in cholera toxin (20 μg/ml) in 0.3 ml saline, once a week for 4 consecutive weeks. On the fifth week, the mice were orally challenged with Ara h2 (1 mg/mouse in 0.3 ml saline) and sacrificed next day. Samples of the blood, small intestinal segments were collected from each mouse immediately after death; the samples were processed to assess the allergic inflammation following our established procedures [12,13].

2.4. SIT and IL-18 administration

Following the published procedures [14] with modification (the egg protein using in the previous studies was replaced by Ara h2), the sensitized mice were treated with oral SIT. Briefly, Ara h2 was gavage-fed with doses of 1 mg (days 1 and 2), 2 mg (days 3 and 4), 3 mg (days 5–7), 4 mg (days 8 and 9), and 5 mg (days 10–14). Control mice were treated with saline. A group of mice was treated with both SIT and recombinant IL-18 (rIL-18; 1 μg/mouse; i.p. in 0.1 ml saline) together with each treatment of SIT.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Levels of IFN-γ, IL-18 and IL-4 were determined by ELISA with commercial reagent kits following the manufacturer's instruction. The Ara h2-specific IgE levels were determined by an in-house ELISA following our established procedures. Briefly, the 96-well plates were coated with Ara h2 (20 μg/ml; 0.2 ml/well) at 4 °C overnight and washed with phosphate buffered saline (PBS). After blocking with 1% bovine serum albumin (BSA) for 30 min, the serum (diluted 10 folds; in duplicate) or BSA (using as a negative control) was added to each well (0.1 ml/well) and incubated for 1 h at room temperature, washed with PBS, and followed by incubation with the anti-mouse IgG (10 ng/ml; conjugated with horseradish peroxidase). After washing with PBS, tetramethylbenzidine (TMB; 0.1 ml/well) was added to each well and incubated for 15 min; the reaction was stopped by adding 2 M H_2SO_4 (0.05 ml/well). The plates were read by an ELx808 absorbance microplate reader (BioTek, Shanghai, China) at 450 nm. The results were presented as the optical density (OD). The OD value of the negative control wells was subtracted from each readout of sample wells.

2.6. Counting mast cells and eosinophils in the intestinal mucosa

A segment of the jejunum was excised from each mouse and processed for counting mast cells and eosinophils with the procedures we reported previously [12,13].

2.7. Isolation of immune cells

The small intestine and spleen was collected from each mouse. Lamina propria mononuclear cells (LPMC) and spleen cells were isolated with our established procedures [12,13]. The $CD4^+$ T cells and dendritic cells (DC) were isolated with a commercial $CD4^+$ T cell or DC isolation kit following the manufacturer's instruction. The purity of the isolated $CD4^+$ T cells and DC was greater than 98% as checked by flow cytometry.

2.8. Antigen specific $CD4^+$ T cell proliferation assay

The isolated $CD4^+$ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and cultured with DC (T cell:DC = 5:1) in the presence of Ara h2 (the specific allergen) or BSA (using as a control) for 72 h. The cells were analyzed by the CFSE-dilution assay. The results were presented as the frequency of proliferating cells in a bar graph (the flow cytometry histograms were not presented, but available upon request).

2.9. Observation of mouse diarrhea and recording the core temperature

After oral challenge with the specific antigen, the mice were observed for 2 h for diarrhea. The core temperature was recorded at 30 min

2.10. Activation of TCR of $CD4^+$ T cells

$CD4^+$ T cells were isolated from the spleen of naïve mice as described above. The cells were cultured in the presence of phytohaemagglutinin (5 ng/ml) overnight for a preliminary activation, and