



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.

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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, 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 EpiXplore™ 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 μ g/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 μ g/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 μ g/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 H₂SO₄ (0.05 ml/well). The plates were read by an EL808 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 phytohemagglutinin (5 ng/ml) overnight for a preliminary activation, and

then cultured in anti-CD3 Ab coated 96-well plates in the presence of anti-CD28 Ab (2 µg/ml) for 3 days. To test the effect of IL-18 on CD4⁺ T cell apoptosis, cells were treated with the above Ab in the presence of rIL-18 (100 ng/ml) in the culture.

2.11. Assessment of apoptotic cells

The activated CD4⁺ T cells were stained with an Annexin V reagent kit following the manufacturer's instruction; then, the cells were stained with propidium iodide (PI; 5 µg/ml, 15 min). The cells were analyzed by flow cytometry. The data were analyzed with software Flowjo (Tree Star Inc., Ashland, OR).

2.12. Quantitative real time RT-PCR (qRT-PCR)

The total RNA was extracted from the cells with Trizol reagent. The cDNA was synthesized with a reverse transcription kit. qPCR was performed in a MiniOpticon™ Real-Time PCR Detection System (Bio-Rad, Shanghai, China) with the SYBR® Green Real-Time PCR Master Mixes. The primers using in this study include: FasL (NCBI: NM_010177.4), forward: tcagatgcaagtgtgtgggt; reverse: gggtatctcatgggctgtg. The results were calculated with the $2^{-\Delta\Delta Ct}$ method.

2.13. Western blotting

The total proteins were extracted from the cells. The proteins were fractioned by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 30 min, incubated with the primary antibodies (50–100 ng/ml) for 1 h at room temperature and followed by incubation with the secondary antibodies (conjugated with horseradish peroxidase) for 1 h. The membrane was washed with Tris-buffered saline with triton X-100 after each incubation. The membrane was developed with enhanced chemiluminescence (ECL). The results were photographed using the UVP BioSpectrum Imaging System (Upland, CA, USA).

2.14. FasL promoter methylation analysis

Genomic DNA was isolated with the Wizard genomic DNA purification kit. Methylation analyses were performed by bisulfite conversion of genomic DNA using the EpiXplorer™ Methyl Detection kit. The primer sequences used to amplify the FasL promoter region (NCBI: DQ846745.1) were: (i) methylation primer: forward (ATGTGAGATATTTTTTTAGTCGA) and reverse (AATCTTCGAAATCCAATTAACGT); (ii) unmethylation primer: forward (GATGTGGA GATATTTTTTTAGTTGA) and reverse (AATCTTCAAATCCAATTAACATC).

2.15. Statistics

The data are presented as mean ± SD. The difference between two groups was determined by ANOVA. $P < 0.05$ was set as a significant criterion.

3. Results

3.1. Levels of serum IL-18 are suppressed in mice with Ara h2 allergy

We developed an intestinal allergy mouse model with Ara h2 as the specific allergen. The serum was collected and analyzed by ELISA to assess the levels of IL-18. The results showed that the levels of IL-18 were 69.6 ± 14.5 pg/ml in allergic mice ($n = 6$) vs

controls (175.3 ± 23.5 pg/ml; $n = 6$, $p < 0.01$). The mice were treated with saline). The results implicate that intestinal allergy may alter the levels of IL-18.

Both CD4⁺ T cells and CD8⁺ T cells produce IL-18. To clarify if intestinal allergy alters the production of IL-18 in CD4⁺ T cells or/and CD8⁺ T cells, we analyzed LPMC by flow cytometry. The results showed that the frequency of CD8⁺ T cells of allergic mice was slightly more than control mice, but did not reach the significant levels (Fig. 1B, E and H); however, the frequency of CD4⁺ T cells was much more in allergic group than control group (Fig. 1B, E and H, $p < 0.01$). The gated cell populations of Fig. 1B and E were further analyzed for IL-18 expression. The results showed that the frequency of IL-18⁺ CD8⁺ T cells was not significantly different between control mice (Fig. 1A and G) and allergic mice (Fig. 1D and G) while in CD4⁺ T cells, the frequency of IL-18⁺ cells was significantly less ($p < 0.01$) in allergic group (Fig. 1F and J) as compared with control mice (Fig. 1C and J). The results indicate that allergic reactions down regulate IL-18 expression in CD4⁺ T cells in the intestinal mucosa which are not apparently altered ($p > 0.05$) in CD8⁺ T cells.

3.2. IL-18 enhances the therapeutic efficiency of SIT

We treated peanut allergy mice with oral SIT or/and administration (i.p.) of IL-18 in a two-week protocol. The results showed that, upon Ara h2 challenge, allergic inflammation in the intestine and allergic symptoms were induced in allergic mice including high serum Ara h2-specific IgE (Fig. 2A), serum levels of IL-4 (Fig. 2B), marked infiltration of mast cells and eosinophils in the intestinal mucosa (Fig. 2C), Ara h2-specific CD4⁺ T cell proliferation (Fig. 2D), drop of the core temperature (Fig. 2E) and diarrhea (Fig. 2F). Treating with SIT moderately improved ($p < 0.01$) the above allergic symptoms and inflammation, which was further improved in the mice treated with both SIT and IL-18 (Fig. 2). The results indicate that administration with IL-18 significantly enhances the therapeutic effect of SIT on mouse peanut allergy.

3.3. IL-18 increases TCR-dependent CD4⁺ T cell apoptosis

To gain insight into the mechanism by which IL-18 enhances the effect of SIT on peanut allergy, we employed the well-established model of polyclonal TCR activation of CD4⁺ T cells by stimulating CD4⁺ T cells with anti-CD3/CD28 Ab (antibody) in the culture with or without the presence of rIL-18. As shown by flow cytometry data, exposure to anti-CD3/CD28 Ab significantly induced the naïve CD4⁺ T cell apoptosis, which was not further enhanced by the addition of rIL-18. In the CD4⁺ T cells isolated from Ara h2-sensitized mouse intestine, the frequency of apoptotic CD4⁺ T cells was markedly less ($p < 0.01$) than that in naïve CD4⁺ T cells after the exposure to anti-CD3/CD28 Ab; however, which were markedly increased after the addition of rIL-18; exposure to rIL-18 alone did not alter the CD4⁺ T cell apoptosis (Fig. 3). The results imply that the TCR-dependent CD4⁺ T cell apoptosis is compromised by allergy, which can be reconciled by the addition of IL-18 to the culture.

3.4. IL-18 modulates FasL expression in CD4⁺ T cells

Activation of the Fas/FasL pathway is the major mechanism of AICD [15]. Based on the results of Fig. 3, we inferred that IL-18 might modulate the expression of FasL in CD4⁺ T cells to enhance the TCR-dependent CD4⁺ T cell apoptosis. To test the hypothesis, we assessed the expression of FasL in CD4⁺ T cells after treating with the same procedures of Fig. 3. The results showed that activation of TCR significantly increased the expression of FasL in naïve CD4⁺ T cells, but it was much less in CD4⁺ T cells from sensitized

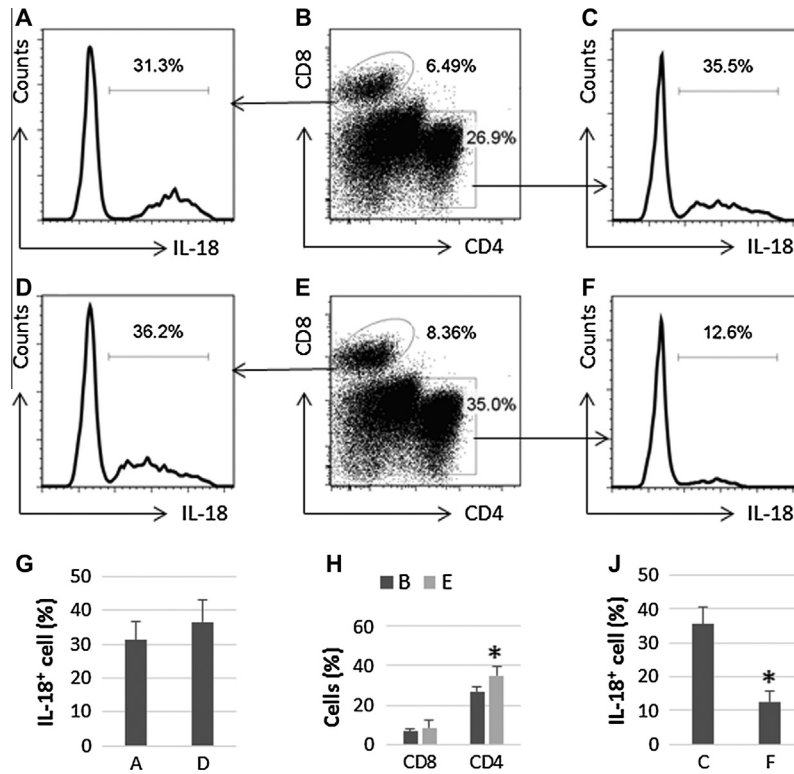


Fig. 1. Allergic reactions alter IL-18 expression in intestinal CD4⁺ T cells. LPMC were isolated from control mice and allergic mice respectively, and analyzed by flow cytometry. B and E, the dot plots indicate the frequency of CD4⁺ and CD8⁺ T cells; the summarized data are presented in panel H. A and D, the gated cells of B and E were further analyzed; the histograms of A and D indicate the frequency of IL-18⁺ CD8⁺ T cells (pointed by arrows); the summarized data are presented in panel G. C and F, the histograms indicate the frequency of IL-18⁺ CD4⁺ T cells in A and D (pointed by arrows), which is summarized in panel J. Data of G, H and J are presented as mean $\pm$ SD; * p < 0.01, compared with group C. The data represent 3 separate experiments.

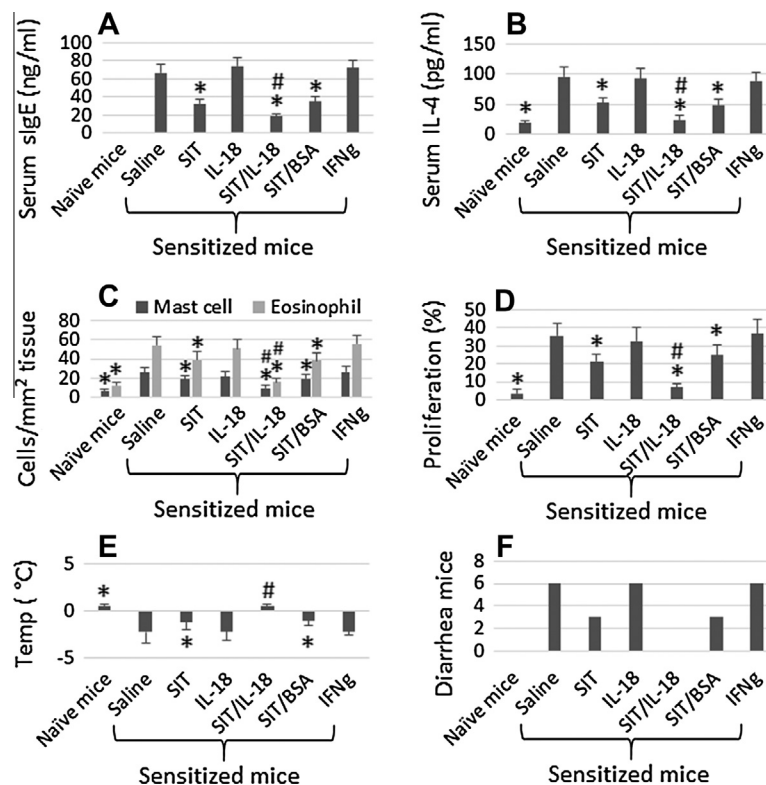


Fig. 2. Administration IL-18 enhances the therapeutic effect of SIT. Peanut Ag-sensitized mice were treated with SIT or SIT/IL-18 (20 μ g/kg; i.p.) as denoted on the X axis. BSA: An irrelevant control protein. IFN γ : Mice were treated with IFN- γ (500 ng/mouse, i.p. with the same time points of SIT). * p < 0.01, compared with saline group. # p < 0.01, compared with SIT group. Each group consisted of 6 mice. Samples from individual mice were processed separately. Each experiment was repeated 6 times.

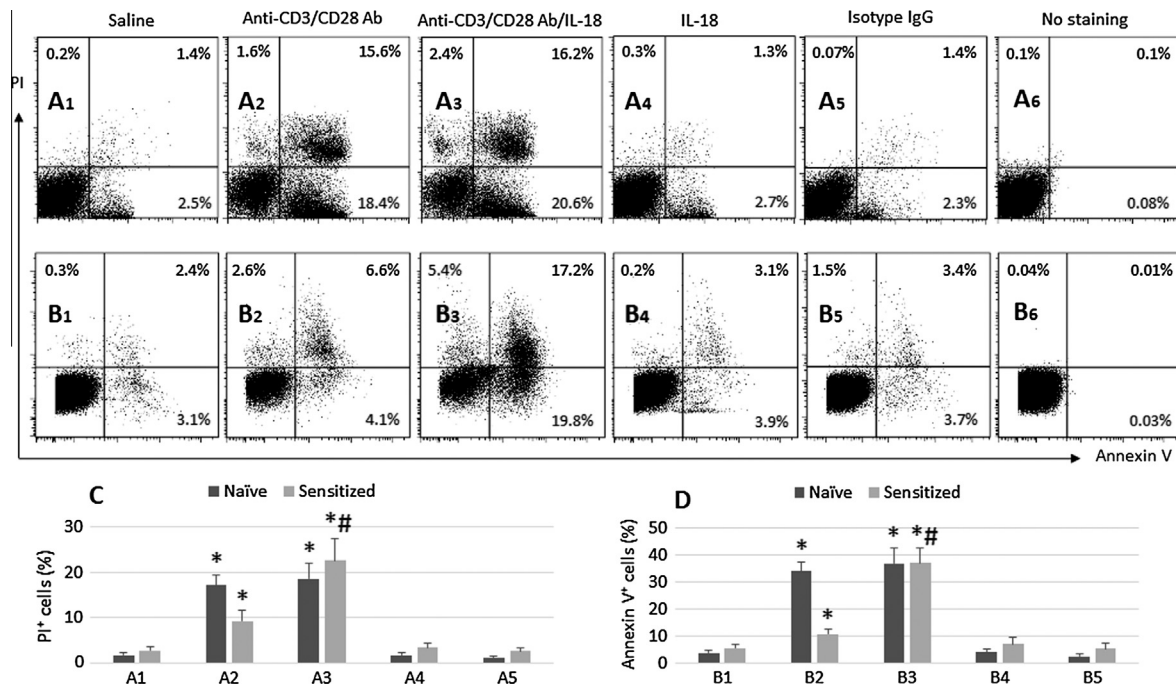


Fig. 3. Administration of IL-18 enhances TCR-dependent CD4⁺ T cell apoptosis. CD4⁺ T cells were isolated from the intestine of naïve mice (A) and sensitized mice (B), and treated as denoted above the sub-panels. The group labels on the X axis of C and D are the same as those in dot plots. **p* < 0.01, compared with group A1. #*p* < 0.01, compared with group B1. Each group consists of 6 mice. The data (mean ± SD) represent 6 experiments (see Fig. 2 for the reagent doses).

mice; however, concomitant exposure to both anti-CD3/CD28 Ab and IL-18 induced comparable amount of FasL in CD4⁺ T cells from sensitized mice to naïve CD4⁺ T cells. Exposure to IL-18 alone did not induce detectable changes of FasL expression. The presence of a neutralizing anti-IFN- γ Ab in the culture abolished the increase in the FasL expression induced by anti-CD3/CD28 Ab and IL-18 (Fig. 3A–C). To corroborate the results, we assessed the expression

of FasL on the surface of the CD4⁺ T cells by flow cytometry. The results were comparable to the results of qRT-PCR and Western blotting (Fig. 4D). The results indicate that CD4⁺ T cells from sensitized mice express less amount of FasL in response to TCR activation; IL-18 can enhance the TCR-dependent FasL expression in CD4⁺ T cells from sensitized mice, it is probably mediated by IFN- γ (we further investigated it below).

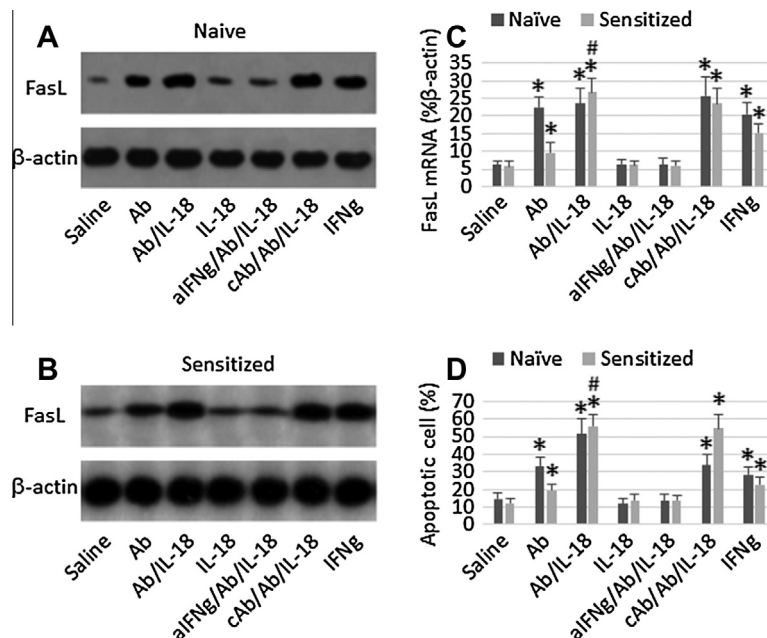


Fig. 4. Administration of IL-18 promotes FasL expression in CD4⁺ T cells. CD4⁺ T cells were isolated from the small intestine of naïve mice (Naïve) and sensitized mice (Sensitized), and treated in the culture as denoted below the immune blots. The expression of FasL of CD4⁺ T cells was analyzed by Western blotting (A and B), qRT-PCR (C) and flow cytometry (D). Ab: Anti-CD3/CD28 Ab. IL-18: 100 ng/ml. aIFN γ : Anti-IFN- γ (200 ng/ml). IFN γ : IFN- γ (20 ng/ml). cAb: Isotype IgG. **p* < 0.01, compared with saline group. #*p* < 0.01, compared with Ab group. Each group consists of 6 mice. The data represent 6 separate experiments.

3.5. IL-18 enhances TCR-dependent FasL expression in CD4⁺ T cells via IFN- γ

We next took further insight into the mechanism by which IL-18 enhanced the TCR-dependent FasL expression in CD4⁺ T cells. Firstly, we measured the levels of IFN- γ in the culture medium of CD4⁺ T cells. As shown by ELISA, IFN- γ was significantly increased in the culture medium after the TCR-activation by anti-CD3/CD28 Ab in naïve group, which was much less in sensitized group. The addition of IL-18 to the culture significantly increased the IFN- γ levels in sensitized group, but did not further increase in naïve group (Fig. 5).

Epigenetic modification of target genes plays an important role in the regulation of cell phenotyping and functioning. The IL-18-induced FasL expression in CD4⁺ T cells via modulating the FasL promoter methylation has not been investigated. To this end, we observed the FasL promoter methylation in the CD4⁺ T cells after exposure to IL-18. In naïve CD4⁺ T cells, the amounts of the methylated FasL promoter DNA were much more than the unmethylated FasL promoter. After stimulating with anti-CD3/CD28 Ab, the amounts of the unmethylated FasL promoter DNA were markedly increased; it was not further increased after exposure to IL-18 in the culture. In CD4⁺ T cells from allergic mice, the saline-treated group showed more methylated FasL promoter than unmethylated gene. After stimulating by anti-CD3/CD28 Ab, the amounts of the unmethylated FasL promoter DNA were not increased much. The addition of IL-18 together with anti-CD3/CD28 Ab to the culture markedly increased the amounts of the unmethylated FasL promoter DNA, which did not occur when the CD4⁺ T cells were stimulated with IL-18 alone. The presence of a neutralizing anti-IFN- γ Ab abolished the increase in unmethylated amounts of the FasL promoter DNA in both naïve group and sensitized group treated with anti-CD3/CD28 Ab and IL-18. To corroborate the effect of IFN- γ on the FasL promoter DNA unmethylation, a group of cells was stimulated by rIFN- γ in the culture; it significantly increased the amounts of unmethylated FasL promoter and reduced the amounts of methylated FasL in CD4⁺ T cells (Fig. 6). The results indicate that IL-18 can increase the amounts of unmethylated FasL promoter DNA in CD4⁺ T cells; IFN- γ plays an important role in the process.

4. Discussion

In line with the published data that both CD4⁺ T cells and CD8⁺ T cells produce IL-18 [16,17], the present study observed that the expression of IL-18 in both CD4⁺ T cells and CD8⁺ T cells of intestinal mucosa; intestinal allergy suppressed the expression of IL-18 mainly in CD4⁺ T cells, but not in CD8⁺ T cells. Administration of IL-18 significantly enhanced the effect of SIT on suppression of allergic inflammation as well as the antigen specific CD4⁺ T cell

polarization in the intestine of a peanut allergy mouse model. In the cell culture studies, the results showed that IL-18 enhanced the TCR-dependent apoptosis in CD4⁺ T cells from allergic mice, which was mediated by IFN- γ , the latter increased the expression of FasL in CD4⁺ T cells via a mechanism of the FasL promoter demethylation. Intestinal allergy compromises the AICD mechanism via suppression of IL-18 in antigen specific CD4⁺ T cells.

AICD is an important mechanism to maintain the homeostasis of the T cell immunity [18]. Fas/FasL interaction is the major pathway of AICD. Our data are in line with previous reports [19] by showing a significant increase in apoptotic CD4⁺ T cells from naïve mice as well as the increase in FasL expression after the polyclonal TCR activation. However, in the CD4⁺ T cells from allergic mice, the activation of the TCR induced much fewer apoptotic CD4⁺ T cells and less FasL expression than naïve group. The phenomenon implicates that CD4⁺ T cells from allergic subjects are resistant to the TCR-dependent apoptotic induction; it may be one of the mechanisms of the sustaining skewed Th2 cell polarization in allergic diseases; the notion is supported by previous studies that also indicate that activated T cells are resistant to TCR-mediated apoptosis in asthmatics [20].

IL-18 is a pleiotropic cytokine. Because of its IFN- γ -inducer property, IL-18 is categorized to the proinflammatory cytokines [21]. Published data show that IL-18 can influence either the Th1 or Th2 response, depending on the cytokine milieu [22]. The present data show that upon exposure to IL-18 in the culture, CD4⁺ T cells from allergic mice produced high levels of IFN- γ . It is noteworthy that the increase in IFN- γ in the culture is TCR-dependent; the CD4⁺ T cells did not produce IFN- γ after exposure to IL-18 alone. The results implicate that using IL-18 alone may not attenuate the allergic inflammation induced by Ara h2. The inference is supported by the results of the peanut allergy mouse model study, in which using IL-18 alone had no detectable effect on suppressing the allergic inflammation in the intestine. Others indicate that using IL-18 even exacerbate the ongoing allergic asthma [23]. Thus, it is necessary to use both SIT and IL-18 concurrently to treat subjects with allergic disorders to avoid the potential side effect and to enhance the therapeutic effect of SIT on allergic inflammation.

The results show that the CD4⁺ T cell apoptosis are in parallel to the IFN- γ production of CD4⁺ T cells in the culture in response to the TCR activation, which implicates that IFN- γ is involved in the TCR-dependent CD4⁺ T cell apoptosis. Indeed, further results indicate that upon the TCR activation, the expression of FasL is increased in naïve CD4⁺ T cells as well as the CD4⁺ T cells of allergic mice after stimulating by IL-18; the increase in FasL was abolished by the presence of a neutralizing anti-IFN- γ antibody. FasL plays a critical role in the AICD via the apoptotic pathway [9]. However, such an important mechanism of CD4⁺ T cells of subjects with allergic disorders does not function properly as observed in the present study. Our data indicate that this mechanism can be restored by the addition of IL-18 to the CD4⁺ T cells together with TCR-activation. Thus, in line with previous suggestions that IL-18 and IFN- γ may counterbalance the skewed Th2 response [24], the present results implicate a potential of IL-18 to facilitate the therapeutic effect of SIT on allergic diseases. However, it should be cautious to make sure the subjects do not have inflammatory bowel disease when using IL-18 to treat allergic disorders in the intestine since IL-18 may potentiate inflammation in the context of Crohn's disease by facilitating neutrophil transepithelial migration [25].

The present data further indicate that exogenous IL-18 indirectly suppresses Th2 response mediated by IFN- γ . Not only inducing Th0 cells to differentiate into Th1 cells, IFN- γ also suppresses Th2 cell recruitment and suppresses Th2 cytokines [26]. One of the underlying mechanisms of such suppression is

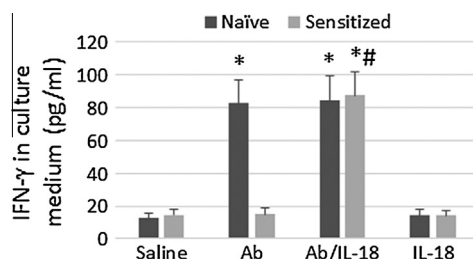


Fig. 5. Administration of IL-18-induced IFN- γ in CD4⁺ T cells. The treatment of CD4⁺ T cells is denoted on the X axis. IL-18: 100 ng/ml. The culture media were analyzed by ELISA. * $p < 0.01$, compared with saline group. # $p < 0.01$, compared with Ab group. Each group consists of 6 mice. The data represent 6 separate experiments.

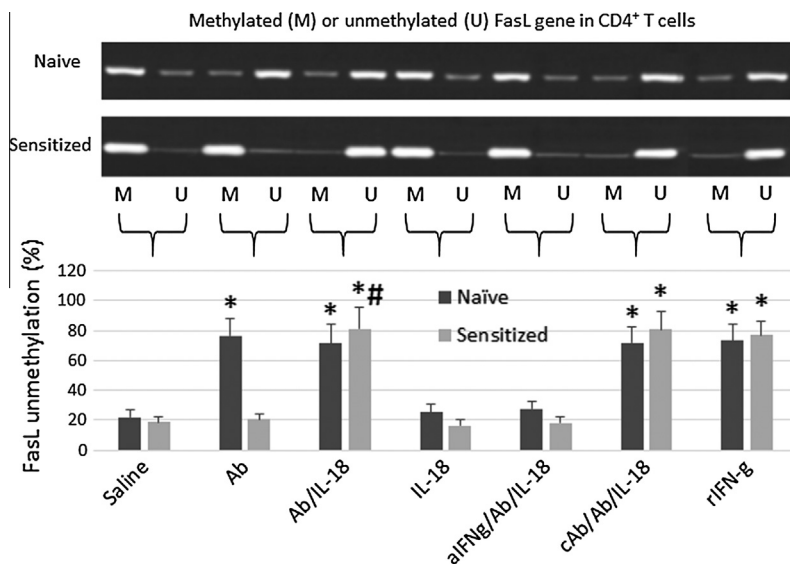


Fig. 6. Administration of IL-18 modulates FasL gene methylation. The treatment of CD4⁺ T cells is denoted on X axis. The nuclear extracts were analyzed by methylation specific PCR. Ab: Anti-CD3/CD28 Ab. IL-18: 100 ng/ml. aIFNγ: Anti-IFN-γ (200 ng/ml). IFNγ: IFN-γ (20 ng/ml). cAb: Isotype IgG. The bars indicate the ratio of unmethylated FasL DNA ($=U \div (U + M) \times 100$). * $p < 0.01$, compared with saline group. # $p < 0.01$, compared with Ab group. Each group consists of 6 mice. The data represent 6 separate experiments.

via promoting FasL expression in Th2 cells, which further induces Th2 cell apoptosis [27]. The underlying mechanism may be via increasing STAT1 phosphorylation, which up regulates FasL expression [28]. Our data provide further explanation that IL-18/IFN-γ induces the FasL promoter demethylation.

In summary, the present data show that administration of IL-18 can enhance the therapeutic effect of SIT on allergic inflammation in the intestine via a mechanism that IL-18 induces IFN-γ production in CD4⁺ T cells; the IFN-γ in turn induces FasL expression in CD4⁺ T cells, which induces the TCR activation-dependent CD4⁺ T cell apoptosis.

Author contribution

WH, CY, LX, MZZ, HH and QJ performed experiments, analyzed experimental data and reviewed the manuscript. ZL and PCY designed the project and supervised the experiments. PCY wrote the manuscript.

Declare of the conflict of interest

None to declare.

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