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Interleukin-18 correlates with interleukin-4 but not interferon-γ production in lymphocyte cultures from atopic dermatitis patients after staphylococcal enterotoxin B stimulation

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Abstract

Background: *Staphylococcus aureus (S. aureus)* triggers exacerbation of atopic dermatitis (AD) and causes chronic inflammation through the action of various proteins such as staphylococcal enterotoxin B (SEB). SEB has a role in activating interleukin (IL)-18, an important regulator of interferon (IFN)-γ and IL-4, in regards to a therapeutic strategy.

Objective: To determine the correlation of IL-18 level with the IL-4 and IFN-γ level in lymphocyte cultures from AD patients following SEB stimulation.

Method: Twenty patients with AD based on the Hanifin and Rajka criteria and 20 healthy subjects as a control group were selected. A 5 ml blood sample from each subject was taken for lymphocyte culture. The culture was stimulated with SEB for two days and the outcomes were assessed by enzyme-linked immunosorbent assays (ELISA) to evaluate the levels of IL-18, IL-4, and IFN-γ.

Results: In the AD group, the levels of IL-18, IL-4, and IFN-γ in lymphocyte cultures with SEB were significantly increased compared with non-SEB exposed cells (each $p<0.001$); similar results were found in the control group. The level of IL-18 was significantly elevated in lymphocyte cultures with SEB stimulation in AD vs. control ($p<0.05$) and without SEB in AD vs. control ($p<0.05$). Furthermore, IL-18 levels were significantly correlated with IL-4 levels and score atopic dermatitis (SCORAD) values in AD patients with SEB ($r=0.41$, $p<0.05$; and $r=0.70$, $p<0.05$, respectively); on the in contrary, there was no correlation between IL-18 and IFN-γ levels ($p=0.469$).

Conclusions: Our results suggest that IL-18 is correlated with increased of IL-4 levels in SEB-stimulated AD lymphocyte cultures.

Keywords: atopic dermatitis, interleukin-18, interleukin-4, interferon-γ, lymphocyte culture, staphylococcal enterotoxin B

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Introduction

Atopic dermatitis (AD), also known as atopic eczema, is a chronic, relapsing, highly inflammatory skin disease characterized by pruritic eczematous skin lesions that usually presents in people with respiratory allergy. The prevalence of AD in children is 10% to 20%, and in adults is 1% to 3%.[1] The causes and mechanisms of AD are not completely understood.[2]

Antigen-specific T cells play an important role in AD pathogenesis at a cellular level,[3] and have been found in lesional skin of AD to produce different cytokines.[4,5] T helper (Th) 1 cells secrete interleukin (IL)-2 and interferon (IFN)-γ,[6] while Th2 cells secrete IL-4, IL-5, and IL-13.[6,7] IL-4 is the major factor regulating immunoglobulin (Ig) E production by B cells, and is required for optimal Th2 differentiation.[8] Furthermore, IL-4

inhibits the production of IFN-γ.[1]

IL-18 is a pleiotropic cytokine which can influence either the Th1 or Th2 response, depending on the cytokine milieu and the genetic background.[9] It is produced by macrophages,[10] and levels are increased in AD patients.[11] This cytokine is produced by a wide range of cells, such as dendritic cells, monocytes and keratinocytes,[12] and works synergistically with IL-12. A high level of IL-18 and the presence of IL-12, IL-2, and other microbial agents significantly elevates IFN-γ production.[13]

Staphylococcus aureus (S. aureus) is found in 90% people with active AD, and can worsen this skin disease. Thus, *S. aureus* appears to be particularly important in the pathogenesis of AD.[14] *S. aureus* is able to secrete exotoxins with superantigenic properties, such as staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), and toxic shock syndrome toxin-1 (TSST-1).[15] SEB has the capability to bind directly to T cells without previously interacting with macrophages.[14] A previous study showed that SEB promotes IL-18 and IFN- γ production in whole blood.[13]

Related to the roles of IL-18 in AD as a proinflammatory cytokine, IL-18 has a significant impact on controlling the Th1/Th2 balance. Synergistically with IL-12, IL-18 promotes IFN-γ production by Th1; conversely, IL-18 has also been shown to increase the production of IL-4 and IL-13 and to stimulate the synthesis of IgE.[16]

Until now, there have been few studies concerning the role of IL-18 and which cytokines are affected by IL-18. Hence, the aim of this study was to determine the correlation between IL-18 and both IL-4 and IFN-γ production in lymphocyte cultures derived from AD patients after SEB exposure.

Methods

Patients

This study was performed in accordance with the Declaration of Helsinki ethical guidelines and was approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Padjadjaran, Hasan Sadikin General Hospital, Bandung, West Java, Indonesia. An oral description of the study was provided and subsequently written informed consent was obtained from each participant.

This study was an in vitro experiment performed on the lymphocyte cultures of AD patients and healthy subjects as the control group. Cultured lymphocytes were stimulated with SEB, then the IL-18, IL-4, and IFN-γ levels were measured. A schematic diagram of the study is provided in **Figure 1**. In the AD group, subject needed to fulfill the Hanifin and Rajka criteria prior to enrollment. Twenty subjects in each group aged more than two years old were recruited from the Dermatology Clinic at Hasan Sadikin General Hospital. The subjects were obtained using a consecutive sampling technique and were excluded from the study if they used systemic and/or topical corticosteroids, antibiotics or other immunosuppressive agents such as cyclosporine, tacrolimus, azathioprine, etc. within the last two weeks before the study started. All included subjects displayed atopic manifestations, such as asthma, allergic rhinitis or hay fever.

Lymphocytes Isolation

About 5 ml of blood was collected in a tube with heparin as an anticoagulant. Lymphocytes were separated from the other blood components using the Ficoll-Hypaque density gradient method. The blood was diluted in 5 ml of Hank's solution (Sigma-Aldrich, Missouri, USA) in a 1:1 proportion and mixed well. About 5 ml of Ficoll-Hypaque (Sigma-Aldrich, Missouri, USA) was carefully added to the bottom of the tube, such that the blood remained on top and was not mixed. The tubes were centrifuged for 15 min at 1800 rpm. The upper solutions were discarded, leaving approximately 1.5 cm above the white ring of lymphocytes. Then, the lymphocytes were harvested and transferred to another tube. Next, a wash was performed by adding Hank's solution to a final volume of 15 ml. The tubes were centrifuged for 10 min at 1400 rpm and the supernatants were discarded. This washing procedure was repeated twice. The pellet was resuspended in 1 ml of RPMI medium (Sigma-Aldrich, Missouri, USA) containing 15% human serum. The lymphocytes were cultured with medium and 15% human serum. SEB (R&D Systems, Minneapolis, USA) (50 µl) was added to the lymphocyte cultures and incubated at 37°C in 5% CO_2 incubator for two days.

Enzyme-linked immunosorbent assay (ELISA) for IL-18, IL-4, and IFN-γ

The IL-18, IL-4, and IFN-γ levels were measured from lymphocyte culture supernatants using human IL-18, IL-4, and IFN-γ ELISA kits, respectively, according to the manufacturer's instructions (Biolegend, California, USA). The levels of the various cytokines in pg/ml were used for data analysis.

Statistical analysis

Results are expressed as mean±SD and statistically significant differences are defined as a p value less than 0.05. The Wilcoxon test was used to compare differences between SEB exposed and untreated cells. The Mann-Whitney test was used to compare differences between the AD and control groups. The Pearson correlation test was applied for the correlation analysis of IL-18 with IL-4 and score atopic dermatitis (SCORAD) values.

Results

Demographic details

The data from 20 subjects in each group were reviewed and analyzed descriptively. The patients with AD included 7 males and 13 females, ranging in age from 7-55 years old. The control group of non-AD patients with no complaint of any dermatologic disorders was comprised of 9 males and 11 females, ranging in age from 7-48 years old.

Score atopic dermatitis (SCORAD) index in AD patients

Score atopic dermatitis (SCORAD) was used to assess the severity of AD in this study. **Table 1** shows that AD patients generally presented with moderate or severe AD. SCORAD values less than 15, between 15 and 40 and more than 40 are considered to indicate mild, moderate, and severe AD.

IL-18, IL-4, and IFN-γ levels

As shown in **Table 2**, the AD group showed significant elevations in IL-18 levels compared to control subjects without SEB exposure ($p<0.05$). The SEB-exposed lymphocyte cultures showed a similar result between the AD and control groups ($p<0.05$) based on the Mann-Whitney test. We also assessed IL-4 and IFN-γ levels, but found no significant differences between the AD and control groups after SEB exposure. Similar results were found between the AD and control groups without SEB stimulation.

The mean IL-18, IL-4, and IFN-γ levels in lymphocyte cultures with SEB stimulation were significantly higher than those without SEB stimulation in the AD group (each $p<0.05$). However, there was no significant difference in IL-18 levels without and with SEB stimulation in the control group. Interestingly, in the control group, significant increases in IL-4 and IFN-γ levels were found in with SEB stimulation compared to without SEB stimulation (both $p<0.05$) (**Table 3**).

Table 1. Severity scoring of AD in AD patients

The severity of AD	SCORAD index	AD patients (n=20)
Mild	<15	3 (15%)
Moderate	15-40	9 (45%)
Severe	>40	8 (40%)

AD = atopic dermatitis, SCORAD = score atopic dermatitis

Elevation in IL-18 correlates with IL-4 level

As shown in Table 4, there was a positive correlation between IL-18 and IL-4 levels in the AD group after SEB stimulation ($r=0.411$). Furthermore, after SEB stimulation, the

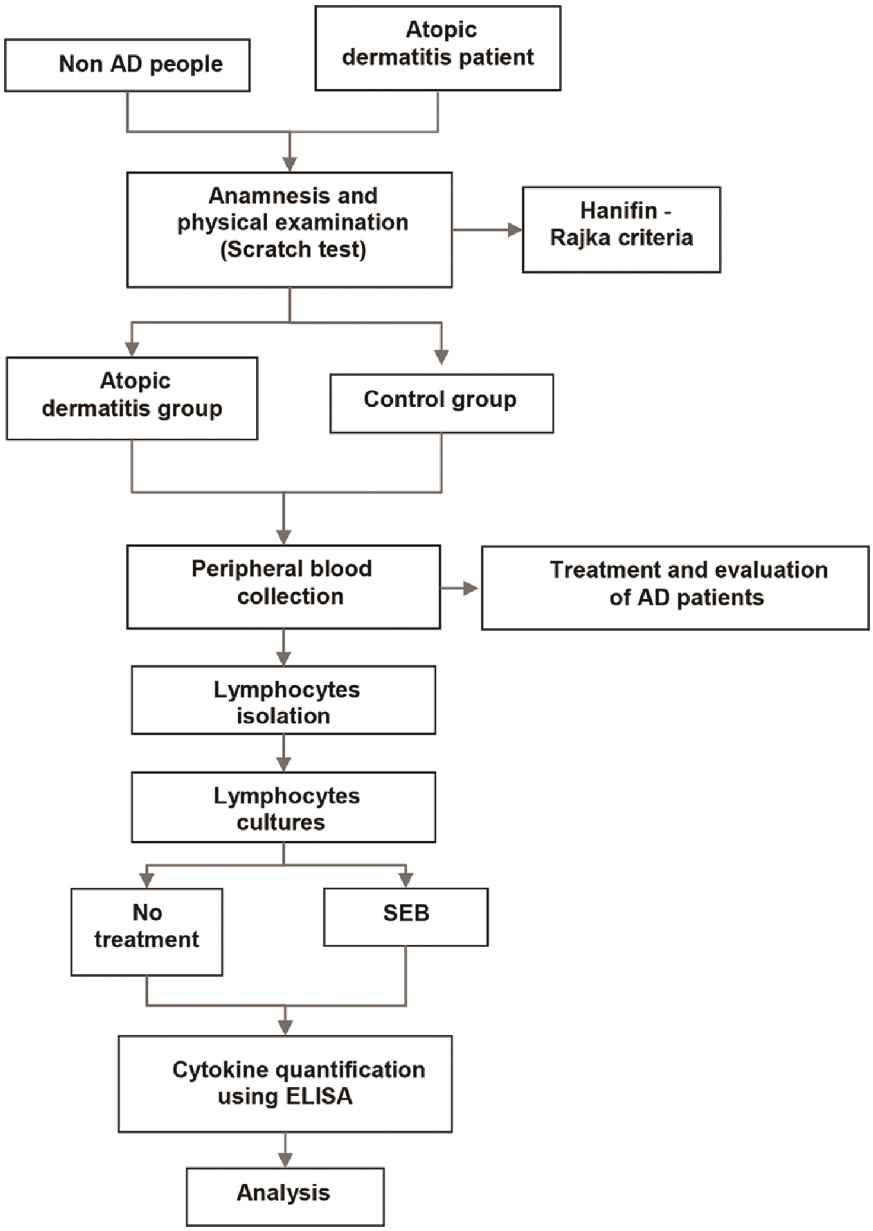

Figure 1 Schematic of research in atopic dermatitis patients.

Table 2. Comparison of IL-18, IL-4, and IFN-γ levels in lymphocyte cultures of AD and control group

	AD	Control	p value
IL-18			
Without exposure	54.63±42.18	33.36±29.75	0.036 *
With SEB	99.38±112.74	54.19±60.58	0.041 *
IL-4			
Without exposure	0.32±0.53	0.32±0.13	0.416
With SEB	2.43±3.20	3.02±2.31	0.066
IFN-γ			
Without exposure	12.75±8.17	19.76±25.95	0.588
With SEB	1293.17±1037.28	1419.20±919.72	0.808

Data are expressed as mean±standard deviation
* p value by Mann-Whitney analysis, statistical significant was accepted at p<0.05
AD = atopic dermatitis, SEB = staphylococcal enterotoxin B

Table 3. Comparison of IL-18, IL-4, and IFN-γ levels in lymphocyte cultures of non-exposed and SEB-exposed group

	Without exposure	With SEB	p value
IL-18			
AD	54.63±42.18	99.38±112.74	<0.001 *
Control	33.36±29.75	54.19±60.58	0.145
IL-4			
AD	0.32±0.53	2.43±3.20	<0.001 *
With SEB	0.32±0.13	3.02±2.31	<0.001 *
IFN-γ			
AD	12.75±8.17	1293.17±1037.28	<0.001 *
With SEB	19.76±25.95	1419.20±919.72	<0.001 *

Data are expressed as mean±standard deviation
* p value by Wilcoxon analysis, statistical significant was accepted at p<0.05
AD = atopic dermatitis, SEB = staphylococcal enterotoxin B

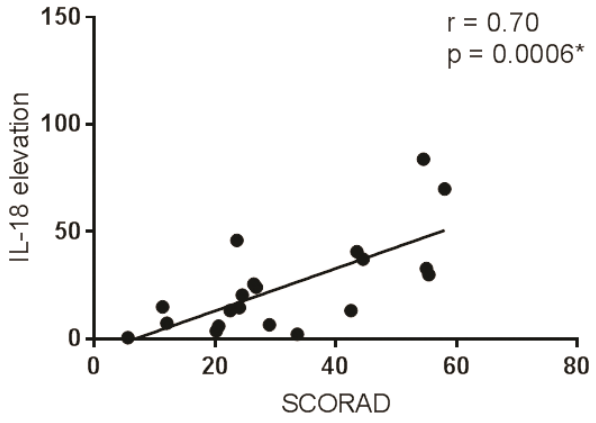

Figure 2. Correlation of IL-18 elevation and SCORAD after SEB stimulation in AD group showed a statistically significant correlation (r=0.70, p=0.0006). Pearson correlation analysis was used and * p value is significant if <0.05.

Table 4. Correlation of IL-18 elevation between IL-4 and IFN-γ after SEB stimulation in AD and control group

Correlation of elevation (%) †	Control		AD	
	Correlation Coefficient (r)	p value	Correlation Coefficient (r)	p value
IL-18 and IL-4	0.024	0.460	0.411	0.036 *
IL-18 and IFN-γ	-0.073	0.380	-0.018	0.469

* p value by Pearson correlation analysis, statistical significant was accepted at p <0.05
† % of elevation = 100x(with SEB value – without SEB value)/without SEB value.
AD = atopic dermatitis, SEB

AD group showed a significant correlation between IL-18 and IL-4 levels (p<0.05), but not with IFN-γ. In the control group, the correlation between IL-18 and IL-4 after SEB stimulation in lymphocyte cultures showed no significant differences; a similar result was also found for the correlation between IL-18 and IFN-γ. Thus, the IL-18 level increased along with the level of IL-4, but only in AD patients.

Elevation in IL-18 correlates with SCORAD

The correlation between IL-18 with AD severity was further evaluated using Pearson correlation coefficient (r) values. A statistically significant correlation between IL-18 levels in SEB-stimulated lymphocyte cultures and SCORAD was found for AD patients (r=0.70; p<0.05) (**Figure 2**). These data indicate that the level of IL-18 is correlated with AD severity.

Discussion

The level of IL-18 is thought to play a role in the development of AD. It expressed by macrophages, dendritic cells, monocytes and keratinocytes.[10,12] IL-18 receptor α (IL-18Rα) is expressed on the surface of natural killer (NK) cells, $CD8^+$ T lymphocytes, activated $CD4^+$ T cells and $CD19^+$ peripheral blood B lymphocytes.[17] Previous immunofluorescent microscopy studies determined that the major cell type responding to IL-18 is $IL\text{-}18R^+$ $CD4^+$ T cells. Thus, IL-18 is chemotactic for T lymphocytes.[18]

This study showed the IL-18 level in AD lymphocyte cultures was higher significantly than in lymphocyte cultures from control subjects (**Table 2**). This result is in accordance with a study by El-Mezzein *et al.*[19] that reported higher IL-18 levels in peripheral blood mononuclear cell (PBMC) culture supernatants from AD patients than in those from control subjects. This was considered to be an effect of increased T lymphocyte proliferation, which results from IL-18 binding to its receptor on the surface of T lymphocytes.

In the AD group, when we stimulated lymphocyte cultures with SEB, we found that the IL-18 level increased significantly compared with unstimulated cultures (**Table 3**). A previous study reported that there is a relationship between the severity of skin lesions and sensitization to SEB in adult patients with AD.[20] Inoue *et al.* also stated that IL-18 levels in the horny layer are significantly higher in the skin lesions of AD patients

associated with *S. aureus* colonization than in healthy subjects.[21] Terada *et al.*[22] observed that *S. aureus* antigen could increase the level of IL-18, contributing to the development of lesions; when IL-18 was blocked or absent, this inhibited *S. aureus* antigen-induced AD.

The disease severity of AD patients was quantified using SCORAD values.[16] Our study shows that AD patients generally present with moderate or severe AD (**Table 1**). A study by Patel *et al.* in 2001 found that the average SCORAD value for children with AD is 40.5; most of these patients had *S. aureus* colonized on the skin.[23] Novak *et al.* also showed that a single nucleotide polymorphism of IL-18 is associated with AD, with an average SCORAD index of 47.9.[16] However, Yang *et al.* found that the SCORAD index does not reflect the subjective disease severity experienced by patients.[24]

As shown in **Figure 3**, IL-18 levels were correlated with SCORAD values. This indicates that IL-18 might be a marker for AD exacerbation, and that the inhibition of IL-18 might prevent the occurrence of AD. It may be possible to treat AD patients through the inhibition of IL-18 production or blockade of the IL-18 receptor on T cells. IL-18 has been shown to be a regulator of Th1 and Th2 cytokine production in atopy, which aggravates the symptoms of AD. IL-18 stimulates Th2 cells to produce IL-4, which induces the production of immunoglobulin E (IgE) by B cells; IL-18 also stimulates Th1 cells to produce IFN-γ.[9]

A recent study showed that IL-4 levels are significantly different between SEB-stimulated and unstimulated cells. This result was also shown in a study by Kuscnaroff *et al.*[25] in mice, in which the IL-4 level was elevated after SEB injection. Another study also showed that IL-4 expression is increased after SEB stimulation.[26] A significant difference in IFN-γ was also found between SEB-stimulated and unstimulated cells in this study, for both AD and control subjects (**Table 3**). Patou *et al.*[26] and Campbell *et al.*[27] also found that SEB exposure can increase the level of IFN-γ.

The role of IL-18 in AD patients exposed to SEB is still questionable. IL-18 induces the production of both IL-4 and IFN-γ.[16] However, the acute phase of AD is initially caused by Th2 cytokines, especially IL-4, IL-5, and IL-13, while in the chronic phase, Th1 cytokines are dominant, especially IL-12, IFN-γ, and IL-18.[28] These statements led us to question which cytokines are most affected by IL-18 in the lymphocytes of AD patients stimulated with SEB, i.e. IL-4 or IFN-γ.

The present study shows a significant correlation between the increases in both IL-18 and IL-4 levels in the AD group, whereas the correlation was non-significant in the control group. Different results were shown in the correlation between the increase in IL-18 and IFN-γ levels. In both the AD and control groups, a negative correlation coefficient and non-significant results were found between IL-18 and IFN-γ levels (**Table 4**). This result suggests that IL-18 plays a role in increasing the levels of IL-4 in AD, but not those of IFN-γ.

Another study has shown that SEB exposure induces IL-18 production, followed by an increase in IL-4, which could raise IgE levels, resulting in acute exacerbation.[29] This can be explained by an earlier study showing that there was no increase in IL-12 expression after SEB stimulation in PBMC culture.[16] Thus, Th1 responses could not be stimulated by IL-18 without IL-12. However, IL-18 could induce IL-4 secretion by Th2 cells in AD patients stimulated by SEB, similar to the results of this study.

Conclusions

IL-18 is believed to contribute to the pathogenesis of AD. Our results suggest that IL-18 is correlated with IL-4 production after SEB stimulation in lymphocyte cultures derived from AD patients. Thus, based on this study, it may be possible to inhibit the production of IL-18 or block the IL-18 receptor in T cells as a new therapeutic target for AD. However, further research is needed to delineate the pathogenesis of AD in the context of other cytokines.

Conflict of interest

There are no conflicts of interest.

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