

Contribution of IL-18 to eosinophilic airway inflammation induced by immunization and challenge with *Staphylococcus aureus* proteins

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Abstract

We previously reported that intranasal challenge with ovalbumin (OVA) plus IL-18 induces airway hyperresponsiveness (AHR) and eosinophilic airway inflammation in mice with OVA-specific T_H1 cells. These two conditions can be prevented by neutralizing anti-IFN- γ and anti-IL-13 antibodies, respectively. The mice develop AHR and eosinophilic airway inflammation after challenge with OVA plus LPS instead of IL-18 and endogenous IL-18 is known to be involved. In contrast, IL-18 does not facilitate these changes in mice possessing OVA-specific T_H2 cells. Here, we investigated whether IL-18 is involved in the development of asthma in mice immunized and challenged with bacterial proteins. Upon intranasal exposure to protein A (SpA) derived from *Staphylococcus aureus*, mice immunized with SpA exhibited AHR and peribronchial eosinophilic inflammation if IFN- γ or IL-13 were present, respectively. The CD4⁺ T cells from draining lymph nodes (DLNs) of the SpA-immunized and -challenged mice produced a robust IFN- γ and IL-13 in response to immobilized anti-CD3 antibodies. Treatment with neutralizing anti-IL-18 antibodies prevented asthmatic inflammation concomitant with their impaired potential to express IFN- γ and IL-13. Furthermore, naive mice that received the CD4⁺ T cells from DLNs of SpA-immunized mice developed airway inflammation depending upon the presence of IL-18. Immunodeficient mice that received human PBMCs, which had been stimulated with SpA *in vitro*, developed dense peribronchial accumulation of human CD4⁺ T cells upon SpA challenge. Neutralizing anti-human IL-18 antibodies protected against this airway inflammation. These results suggest the importance of IL-18 for the development of asthmatic inflammation associated with airway exposure to bacterial proteins.

Keywords: airway hyperresponsiveness, asthma, eosinophilic inflammation, IL-18, *Staphylococcus aureus*

Introduction

Bronchial asthma is complex syndrome characterized by airway hyperresponsiveness (AHR) and reversible airflow obstruction with airway inflammation and mucus formation (1–8). Bronchial asthma is believed to be mediated by T_H2 cells and their cytokines. IL-13 produced by the T_H2 cells can principally

account for almost all the above pathogenic responses (2, 9). However, other subsets of CD4⁺ T cells, such as T_H1, T_H17, regulatory T (Treg) and CD1d-restricted NKT cells, are now recognized to play a role in the modulation of airway allergic inflammation (10). T_H2 cell-directed therapy has limited

efficacy (11), suggesting that bronchial asthma develops by diverse immunological mechanisms. Respiratory infections caused by bacteria frequently activate the T_H1 -cell response through activation of Toll-like receptors (12, 13) and are associated with the initiation and/or exacerbation of bronchial asthma in humans (14, 15). These clinical studies strongly suggest that some types of bronchial asthma may be explained by the activation of T_H1 -cell responses. However, intranasal challenge with ovalbumin (OVA) alone cannot evoke asthma in mice carrying OVA-specific T_H1 cells (16, 17), indicating that the T_H1 -cell response alone is not sufficient enough to induce these pathological alterations. We have demonstrated that intranasal challenge with exogenous IL-18 or bacterial LPS induces IL-18 production in mice. In conjunction with OVA, this can induce robust asthma in mice immunized with OVA and the T_H1 adjuvant, CFA (16–18). OVA initiates OVA-specific T_H1 cells to produce IFN- γ but not IL-13, whereas OVA with IL-18 is capable of activating these T_H1 cells to produce larger amounts of IFN- γ , as well as IL-13, IL-9 and various chemokines that recruit eosinophils and other leukocytes (16). Persistent stimulation with IL-18 and the antigen alters the T_H1 cells, resulting in them producing both IFN- γ and IL-13 (19). With respect to their potential to produce both pro-inflammatory and pro-atopic cytokines/chemokines, we designated T_H1 cells that were re-stimulated with antigen and IL-18 as super T_H1 cells (16, 18, 19). In asthmatic mice possessing super T_H1 cells, IL-13 is responsible for the eosinophilic airway inflammation and remodeling. AHR is caused by IFN- γ , but not IL-13 (16, 17), which is in contrast to T_H2 cell-initiated asthmatic alterations where IL-13 plays a common and critical role (20, 21). Thus, IL-18 is likely to be involved in the development of bacterial infection-associated asthma. However, it is entirely unknown whether bacteria or their products by themselves can trigger super T_H1 cell type bronchial asthma.

The site of a pathogenic infection often determines the phenotype of infection-associated atopic diseases presumably by recruiting and activating pathogen-specific effector T cells and by inducing IL-18 release from the site. Infection with bacteria such as *Staphylococcus aureus* sometimes exacerbates atopic dermatitis in humans (22). We recently observed that consecutive and topical application of *S. aureus* protein A (SpA) (23) induces atopic dermatitis-like skin alterations in naive NC/Nga mice that have a genetically impaired skin barrier (19). The $CD4^+$ T cells prepared from the DLNs of mice with SpA-induced dermatitis express a cytokine profile characteristic of super T_H1 cells. Administration of neutralizing anti-IL-18 antibodies protects against dermatitis as well as super T_H1 -cell development (19). Based on these observations, we assumed that the mice carrying SpA-specific T_H1 cells were highly vulnerable to asthma upon intranasal challenge with SpA. To test this hypothesis, we generated a novel asthmatic inflammation mouse model to determine the requirement of IL-18 in the development of SpA-induced asthma. Severely immunodeficient mice that had been inoculated with SpA-stimulated human PBMCs exhibited airway inflammation following intranasal challenge with SpA. Treatment with neutralizing anti-human IL-18 antibodies prevented this airway inflammation. Thus, IL-18 could be a potential target for the treatment of asthmatic inflammation associated with bacterial infection.

Methods

Animals and reagents

Female BALB/c mice and BALB/c *nu/nu* mice were purchased from CLEA Japan (Osaka, Japan). C57BL/6 background *Rag2^{-/-}C γ ^{-/-}* mice were from Taconic Farms (Hudson, NY, USA). All animals were bred and/or maintained in specific pathogen-free conditions at the animal facilities of Hyogo College of Medicine and were used at 6–10 weeks of age. Animal experiments were performed in accordance with the guidelines of the National Institutes of Health, as specified by the animal care policy of Hyogo College of Medicine. SpA from *S. aureus* Cowan I was purchased from CalbioChem (La Jolla, CA, USA). Recombinant murine IL-18 was purchased from MBL (Nagoya, Japan). Anti-mouse CD3 ϵ mAb (2C11), anti-mouse CD4 mAb (GK1), anti-human CD4 mAb (RPA-T4) and anti-human CD45 mAb (HI30) were from BD Biosciences Pharmingen (San Diego, CA, USA). Neutralizing anti-IFN- γ mAb was partly purified from the ascites fluid collected from BALB/c *nu/nu* mice inoculated intraperitoneally with hybridoma 6A2 purchased from the American Type Culture Collection (Manassas, VA, USA) (17, 19). Soluble IL-13R α 2-Fc was purchased from R&D Systems (San Diego, CA, USA) (17, 19). Rabbit polyclonal anti-mouse IL-18 antibodies were prepared in our laboratory (19). We generated a neutralizing anti-human IL-18 mAb as described previously (24).

Induction of asthma

The experimental protocol for asthma induction was the same as described in our previous report except we used SpA instead of OVA (17) (Supplementary Figure 1 is available at *International Immunology* Online). Briefly, BALB/c mice were immunized with SpA (500 μ g) in CFA, followed by a boost with SpA in incomplete Freund's adjuvant (IFA) at day 14. For the adoptive cell transfer study, $CD4^+$ T cells isolated from DLNs of the immunized and boosted mice were labeled with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) and 6-CFSE. The CFSE-labeled cells (1×10^7) were administered intravenously into naive BALB/c mice (17). Two weeks following the SpA boost or after $CD4^+$ T cell transfer, mice were exposed intranasally to 50 μ l of SpA (250 μ g) in PBS for three consecutive days. In some experiments, neutralizing anti-mouse IL-18 antibodies (500 μ g) were injected intraperitoneally into the mice at 1 day before and 1 day after intranasal exposure to SpA (17, 19). Anti-IFN- γ mAb (100 μ g) or IL-13R2 α -Fc (20 μ g) was intranasally administered as outlined previously (17). Mice were sacrificed at 24 h after the final intranasal exposure of SpA.

Invasive measurement of AHR

Invasive measurement of AHR was assessed as an increase in pulmonary resistance (RLung) in response to aerosolized β -methacholine as described previously (17). RLung was measured by Pulmos-II (MIPS, Osaka, Japan) hardware and software (MIPS).

Preparation of $CD4^+$ lymph node cells and lung homogenate

$CD4^+$ T cells from the DLN were purified by magnetic-activated cell sorting (17). Lungs were homogenized with

1 ml of lysis buffer according to the method described previously (19, 25).

Cytoplasmic staining for IFN- γ and IL-13

Cells were isolated from mediastinal lymph nodes of SpA-immunized mice after consecutive 3-day challenge with SpA and were incubated with immobilized anti-CD3 mAb and 100 U ml⁻¹ of IL-2 in the presence or absence of rIL-18 (100 ng ml⁻¹) for 48 h. Cytoplasmic staining of the cells for IFN- γ and IL-13 were performed using antigen-presenting cells (APC)-anti-CD4 mAb (RM4-5), FITC-anti-IFN- γ mAb (XMG1.2) and PE-anti-IL-13 mAb (eBio13A).

Preparation of human PBMCs

PBMCs from healthy volunteers (26) were cultured with 100 μ g ml⁻¹ of SpA for 4 days. Experimental protocols for the use of human PBMCs were approved by the College Review Board of Hyogo College of Medicine.

Establishment of mice implanted with human PBMCs

SpA-stimulated human PBMCs (1×10^7) were transplanted intravenously into *Rag2*^{-/-}*C γ* ^{-/-} mice (27). One-week post-transplantation, we isolated lymphocytes from the peripheral blood and spleen of the recipient mice and analyzed proportions of human CD45⁺ cells in each preparation by flow cytometry. We used mice that contained >5% human CD45⁺ cells in their peripheral blood because they also contained >10% dual CD45⁺/CD4⁺ cells in their spleen (described below). The mice that received human SpA-stimulated PBMCs were then exposed intranasally to SpA for three consecutive days. In order to block the action of human IL-18, anti-human IL-18 mAb (300 μ g) was intranasally administered 1 h before SpA exposure. Twenty-four hours following the final administration of SpA, lungs were sampled for histological and confocal microscopic studies.

Preparation of bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was collected (17) and total cell number was determined in each sample. Cytospin preparations of BALF were stained with Dif-Quik (Baxter Healthcare Corp., Miami, FL, USA). Eosinophils and neutrophils were distinguished from each other by their difference in staining.

Histology

Lung specimens were fixed in 10% buffered formalin and sections were stained with hematoxylin and eosin (17). Fields of view on a microscope were selected at random and printed in large scale to distinguish eosinophils from other cell types. Eosinophils and the total number of nucleated cells in each field of view were counted. The mean $\pm$ SD of 10 fields of view per sample were calculated.

Confocal laser microscopic analysis

Frozen sections were fixed and incubated with FITC- or PE-conjugated mAb, followed by evaluation using a laser confocal microscope (model IX81; Olympus, Tokyo, Japan) (19).

Detection of cytokines and chemokines

Concentrations of IL-4, IL-13, tumor necrosis factor- α and IFN- γ in culture supernatants were determined with appropriate ELISA kits (Genzyme, Cambridge, MA, USA). Mouse IL-18 was measured by an ELISA kit from MBL. The concentrations of various mouse chemokines were measured with a Bio-Plex Cytokine assay kit (Bio-Rad, Hercules, CA, USA).

Statistics

Three to five mice were used for each experimental group. Data are expressed as the mean $\pm$ SD of triplicate samples. Significance between experimental and control groups was determined via an unpaired Student's *t*-test. A *P* value <0.05 was considered significant. Two to three experiments were performed per assay, and the representative data were shown.

Results

SpA-induced asthmatic inflammation

We examined whether intranasal challenge with SpA induces asthma-like airway inflammation in SpA-immunized mice. We immunized BALB/c mice subcutaneously with SpA in the T_H1 adjuvant, CFA, followed by a booster with SpA in IFA 2 weeks later. Twenty-eight days after the initial immunization, we administered SpA through a nasal tract for three consecutive days and examined the severity of asthmatic inflammation by measuring AHR, analyzing BALF preparations and lung histology (Supplemental Figure 1 is available at *International Immunology* Online). Invasive measurement of AHR revealed that SpA-immunized mice exhibited substantial AHR upon intranasal SpA challenge (Fig. 1A). None of the mice exhibited AHR after treatment with PBS (Fig. 1A) and naive mice were free from AHR even after SpA challenge (Fig. 1A). Thus, SpA immunization and SpA challenge are both required for the development of AHR. Following intranasal challenge with SpA, SpA-immunized mice demonstrated an increase in the number of eosinophils and neutrophils in BALF (Fig. 1B). These increases were not observed after treatment with PBS (Fig. 1B). The severity of AHR, consistent with our previous observations (16, 17), coincided with the cell numbers of eosinophils in BALF. This was also the case for the density of eosinophilic inflammation around the airway. Histological analysis revealed that only SpA-immunized mice developed severe inflammation around the airway following challenge with SpA, but not with PBS (Fig. 1C, E and F). Furthermore, intranasal challenge with SpA, but not PBS or OVA, induced eosinophilia (Supplementary Figure 2 is available at *International Immunology* Online), suggesting that SpA works in an antigen-specific manner. Naive mice exhibited only modest lung inflammation, if any, after intranasal SpA challenge (Fig. 1D). Eosinophils accumulated around the airway of the SpA-immunized and -challenged mice but not in mice treated with the other combinations of immunogens (Fig. 1J). Taken together, these results indicate that SpA-immunized and -challenged mice fulfill the clinical signs of asthmatic inflammation and are a suitable mouse model for bacterial infection-associated asthma-like inflammatory illnesses.

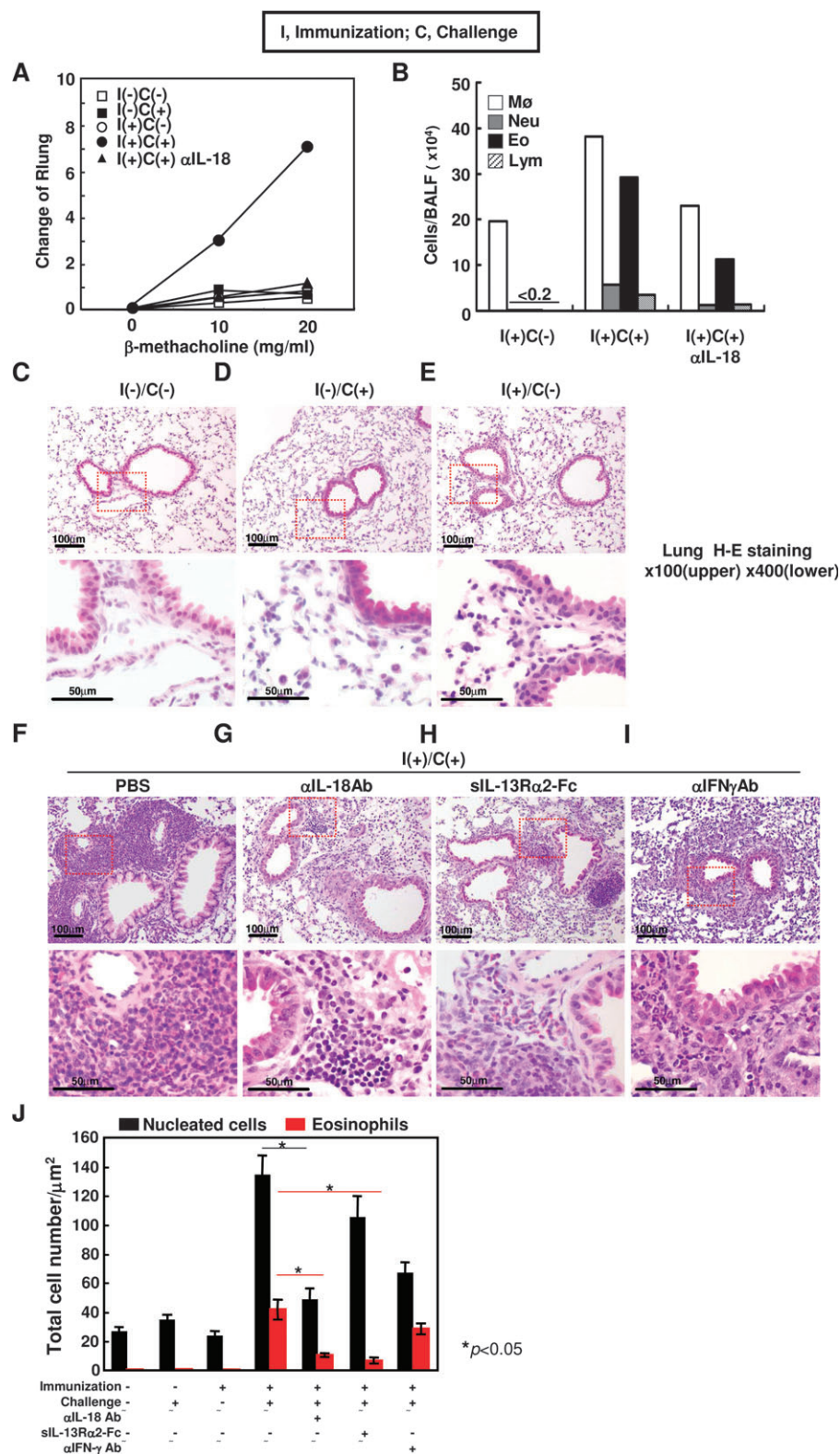


Fig. 1. Requirement of endogenous IL-18 for SpA-induced bronchial asthma. SpA-immunized mice 'I(+)' or naive mice 'I(-)' were intranasally challenged with SpA (250 μ g/50 μ l) 'C(+)' or PBS (50 μ l) 'C(-)'. Neutralizing anti-IL-18 antibodies (500 μ g) (A, B, G and J), soluble IL-13R α -Fc (sIL-13R α -Fc) (20 μ g) (H) or neutralizing anti-IFN- γ (α IFN γ antibody) (100 μ g) was administered twice into SpA-immunized mice intravenously, (I) 1 day before or after 3-day intranasal challenge with SpA (α IL-18 antibody). Twenty-four hours after the last SpA challenge, invasive measurement of AHR (A), cellular analysis of BALF (B) and histological analysis of lung specimens (C-I) were performed. (C-I) Upper panels are at low magnifications, and the lower panels are high magnification images of the areas indicated by a red-dotted square in the corresponding

Requirement of IL-18 for SpA-induced asthma

As previously reported, SpA-induced atopic dermatitis develops in an IL-18-dependent manner (19). We investigated whether IL-18 plays a pivotal role in the development of SpA-induced asthmatic inflammation. To test this, we administered neutralizing anti-IL-18 antibodies into the SpA-immunized mice 1 day before and 1 day after the initial intranasal SpA challenge (Supplementary Figure 1 is available at *International Immunology* Online). Administration of neutralizing anti-IL-18 antibodies profoundly reduced AHR (Fig. 1A) and significantly hampered respiratory inflammation and eosinophilia (Fig. 1B, G and J). Consistently (2, 9, 16), blocking the action of IL-13, but not IFN- γ , protected against eosinophilia in the airway (Fig. 1H–J). Conversely and consistently, blockade of IFN- γ , but not of IL-13, prevented AHR (16) (Supplementary Figure 3 is available at *International Immunology* Online). It would appear that endogenous IL-18 seems to be important in the development of SpA-induced asthmatic airway inflammation due to IL-13 production.

Super T_H1 -cell differentiation

As IL-13 was profoundly involved in inflammation of the airways (Fig. 1H and J), we examined whether this experimental immunization/challenge protocol induces the development of CD4⁺ DLN cells into super T_H1 cells or into IL-13-secreting T_H2 cells. We stimulated CD4⁺ DLN cells with immobilized anti-CD3 mAb and measured the concentrations of T_H2 and super T_H1 cytokines. CD4⁺ DLN cells from SpA-immunized and -challenged mice produced larger amounts of IFN- γ and IL-13, but little IL-4, compared with SpA-immunized mice without SpA challenge (Fig. 2A), indicating their development into super T_H1 cells, but not T_H2 cells, during intranasal SpA challenge. Furthermore, we examined whether both IL-13 and IFN- γ are produced by a single CD4⁺ cell isolated from SpA-challenged and SpA-immunized mice. We isolated cells from mediastinal lymph nodes of SpA-challenged and SpA-immunized mice and incubated the cells with plate-bound anti-CD3 in the presence or absence of exogenous IL-18. We found a very small proportion of IL-13⁺IFN- γ ⁺ CD4⁺ T cells after TCR stimulation alone (Fig. 2B). However, upon TCR and IL-18 stimulation, the proportion of IL-13⁺IFN- γ ⁺ CD4⁺ T cells was significantly elevated (Fig. 2B), suggesting that super T_H1 -cell differentiation occurs in SpA-immunized mice after intranasal challenge with SpA. At the same, this stimulation induced an increase in two other populations: IL-13-producing cells and IFN- γ -producing cells (Fig. 2B). Thus, three populations, consisting of IL-13-producing cells, IFN- γ -producing cells and IL-13 plus IFN- γ -producing cells, contribute to induction of SpA-induced bronchial asthma.

Next, we investigated the roles of endogenous IL-18 in super T_H1 -cell development. CD4⁺ DLN cells prepared from the mice additionally treated with anti-IL-18 antibodies produced much less IFN- γ and IL-13 than those from SpA-

induced asthmatic mice (Fig. 2A), suggesting the possibility that IL-18 release during SpA challenge participates in super T_H1 -cell differentiation. To test this possibility, we examined whether IL-18 is produced in the asthmatic lung. SpA immunization alone failed to induce significant increase in IL-18 concentration within the lung tissue (Fig. 2C). SpA immunization and challenge seemed to increase IL-18 levels significantly in the lung (Fig. 2C). These results suggest that airway constituents such as respiratory epithelial cells and/or alveolar macrophages might release IL-18 in response to SpA.

Induction of chemokines attracting eosinophils and neutrophils in the lungs

As IL-18 is capable of inducing chemokine production from epithelial cells, T_H1 cells and super T_H1 cells (16, 18), we examined whether IL-18 could induce expression of chemokines in the lung, particularly chemokines recruiting eosinophils and neutrophils, during intranasal SpA challenge. Lung homogenates from mice immunized with SpA only contained almost basal amounts of chemokines attracting eosinophils, including CCL5 (RANTES) and CCL11 (Eotaxin), and neutrophils, such as CXCL1 (KC) and CCL2 (MCP-1), when compared with naive mice (Fig. 3). However, it was only after SpA challenge that pulmonary levels of CCL5, CCL11, CCL2 and CXCL1 were significantly elevated (Fig. 3). This was also the case for the chemokines attracting diverse types of leukocytes, such as, CCL3 (MIP-1 α) and CCL4 (MIP-1 β), as well as the pro-inflammatory cytokines, IL-1 β and IL-6 (Fig. 3). In contrast, T_H2 cytokines, IL-4 and IL-5 were not induced after SpA challenge (Fig. 3). As expected, treatment with neutralizing anti-IL-18 antibodies during intranasal exposure to SpA significantly reduced chemokine expression levels (Fig. 3). Thus, the expression of these chemokines could be induced by IL-18.

Importance of SpA-activated super T_H1 cells in the development of airway inflammation

We examined whether super T_H1 cells are effector cells of SpA-induced asthmatic inflammation. To test this, we transferred CD4⁺ DLN cells from SpA-immunized mice into naive mice, followed by intranasal administration of SpA for three consecutive days. Upon daily exposure to PBS, mice receiving the CD4⁺ DLN cells demonstrated an intact response to methacholine treatment and evaded airway inflammation (Figs 1A and C and 4A and C). Upon exposure to SpA, these mice exhibited obvious AHR (Fig. 4A) and airway inflammation (Fig. 4C), prompting us to investigate whether donor CD4⁺ DLN cells migrated into the airway as a response to SpA challenge in order to exert their effector functions. We labeled the donor cells with CFSE, injected them into naive recipient mice and analyzed their localization in the recipient lung after SpA challenge. Many CFSE-labeled cells had migrated into the lung (Fig. 4B). Most of the

upper panels. Upper scale bars indicate 100 μ m, while the lower ones represent 50 μ m. Lung sections were stained with hematoxylin and eosin (H&E). The mean $\pm$ SD of total nucleated cells per square micrometer (black bars) and eosinophils per square micrometer (red bars) in 10 fields of view selected at random are shown (J). M ϕ , macrophages; Neu, neutrophils; Eo, eosinophils; Lym, lymphocytes. Data are representative of three independent experiments with five mice per group.

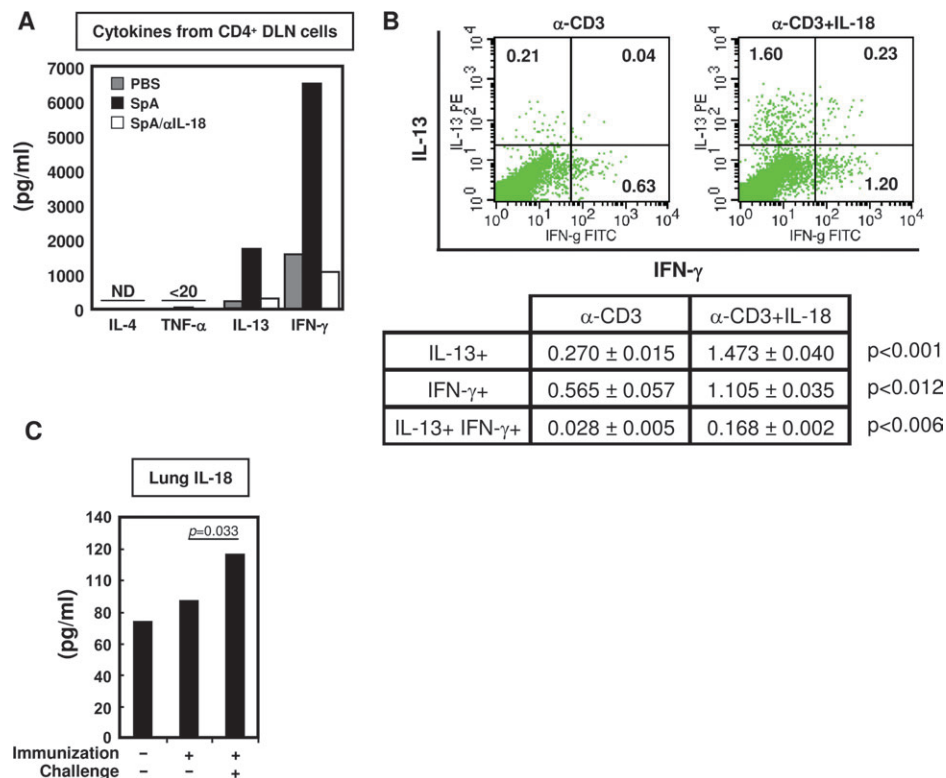


Fig. 2. IL-18-dependent differentiation toward super T_H1 cells. (A) DLN cells were prepared from variously treated mice as shown in the legend to Fig. 1A, and $CD4^+$ T cells (1×10^5) were incubated on immobilized anti-CD3 ϵ mAb. Various cytokine concentrations in each supernatant were measured by ELISA. (B) Mediastinal lymph node cells were prepared from SpA-challenged SpA-immunized mice and were incubated with immobilized anti-CD3 ϵ mAb alone (α -CD3) or anti-CD3 ϵ mAb plus IL-18 for 48 h, followed by cytoplasmic staining for IFN- γ and IL-13. (C) Lung homogenates were prepared from naive mice, SpA-immunized mice and SpA-immunized and -challenged mice, for the measurement of IL-18 by ELISA. Data are representative of three independent experiments with five mice in each group.

pulmonary $CD4^+$ T cells co-expressed CFSE (Fig. 4B), indicating that the donor $CD4^+$ T cells but few of the recipient cells, accumulated in the lung. Upon exposure to PBS, few CFSE-labeled cells or $CD4^+$ T cells were observed in the recipient lung (data not shown). These results demonstrate that SpA-specific $CD4^+$ DLN cells migrate and are fully activated after being exposed to SpA, eventually leading to the development of asthmatic inflammation. Blocking the action of IL-18 protected the mice from AHR and airway inflammation (Fig. 4A and C), suggesting that SpA-induced IL-18 in the airway in combination with administered SpA might differentiate the donor cells toward super T_H1 cells, thereby becoming highly pathogenic effector cells.

Involvement of human IL-18 in SpA-induced airway inflammation in transiently humanized mice

Finally, we investigated whether IL-18 is a therapeutic target for the treatment of airway inflammation in humans, associated with bacterial infection. First, we tried to generate mice transiently carrying human immune competent cells. We incubated PBMCs from healthy donors with SpA *in vitro*, transferred them into immunodeficient mice without T cells, B cells and NK cells and investigated whether human PBMCs settled in the recipient mice by calculating proportions of human $CD45^+$ hematopoietic cells (27) in peripheral immune tissues.

Human $CD45^+$ cells were robustly observed in the spleen and peripheral blood of the recipient mice at day 7 after PBMC transfer (Fig. 5). About one-third of human $CD45^+$ cells co-expressed the human CD4 marker (Fig. 5).

Like the $CD4^+$ DLN cell-transplanted mice (Fig. 4), the SpA-stimulated humanized mice developed airway inflammation upon intranasal challenge with SpA, concomitant with dense accumulation of human $CD4^+$ T cells around the airway (Fig. 6C and D). This result indicated that SpA-stimulated human $CD4^+$ T cells migrated into the airway and presumably evoked pulmonary inflammation in response to exogenous SpA and endogenous IL-18. Upon PBS exposure, however, the host mice showed weak airway inflammation with modest but apparent accumulation of human $CD4^+$ cells (Fig. 6A and B). We investigated the role of human IL-18 and confirmed that anti-human IL-18 mAb (24) potentially neutralized human IL-18 (Supplementary Figure 4 is available at *International Immunology* Online). This mAb prevented SpA-induced airway inflammation in these mice by attenuating airway accumulation of human $CD4^+$ T cells (Fig. 6E and F). Therefore, human cell-derived IL-18 might fully activate human SpA-specific T_H1 cells to become pathogenic effector cells. Indeed, human PBMCs could release super T_H1 cell-inducing cytokines, such as IL-12 and IL-18, and super T_H1 cytokines, such as IL-13 and IFN- γ , in response to SpA *in vitro* (Supplementary Figure 5 is

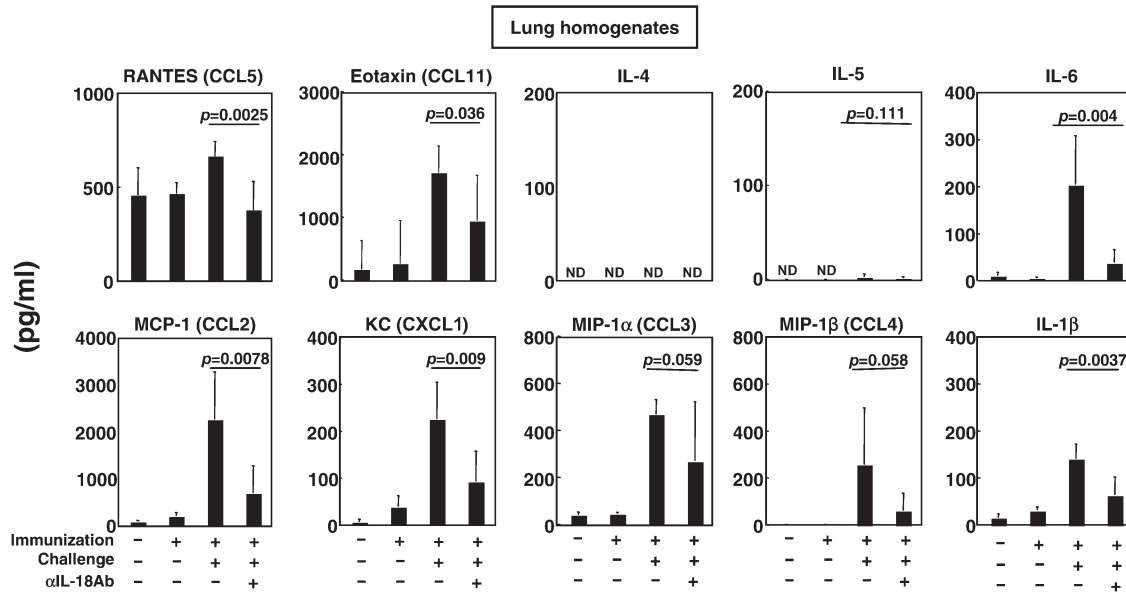


Fig. 3. IL-18 involvement in the induction of chemokines for neutrophils and eosinophils. Lung homogenates were prepared from the mice of three experimental groups (five mice per group) as shown in the legend to Fig. 1A and naive mice (five mice per group). The concentration of CCL2 (MCP-2), CCL3 (MIP1 α), CCL4 (MIP1 β), CCL5 (RANTES), CCL11 (Eotaxin), CXCL1 (KC), IL-1 β , IL-4, IL-5 and IL-6 were measured by BioPlex®. Data are representative of three independent experiments.

available at *International Immunology* Online). Taken together, these results suggest that blocking the action of IL-18 is a potent therapeutic regimen for human airway inflammation initiated and/or exacerbated by bacterial infection.

Discussion

This study showed that endogenous IL-18 is critical for the development of SpA-induced asthmatic inflammation in mice. Upon intranasal exposure to SpA, mice immunized with SpA developed bronchial asthma-like airway inflammation (Fig. 1), concomitant with super T_H1-cell development and elevation of lung pro-inflammatory cytokine/chemokine production (Figs 2A–B and 3). Notably, lung IL-18 levels were significantly elevated in the SpA-induced asthmatic mice (Fig. 2C), and all these responses were prevented by inhibiting the action of IL-18 (Figs 1–3). Thus, IL-18 released from the lung exposed to SpA, together with SpA-presenting APC, might enter into mesenteric lymph node, in which SpA-specific T_H1 cells might develop toward super T_H1 cells. Furthermore, SpA-activated CD4⁺ T cells, when transferred into naive mice, prepared the host mice to be highly responsive to intranasal administration of SpA, inducing bronchial asthma-like symptoms dependent upon endogenous IL-18 (Fig. 4). Finally, the humanized mice developed airway inflammation in a manner dependent on human IL-18 after intranasal SpA challenge (Fig. 6). IL-18 is important for *S. aureus*-associated asthmatic inflammation in mice and perhaps in humans.

We generated an airway inflammation model of temporally humanized mice by intranasal challenge with the bacterial protein, SpA. *Rag2*^{-/-}*C γ* ^{-/-} mice injected with human PBMC transiently possessed human hematopoietic cells in their peripheral lymphoid organs (Fig. 5). Due of their lack of T, B

and NK cells, the recipient mice could not recognize human donor cells as antigens. In contrast, human donor cells consisting of those types of lymphocytes had the potential to be activated by recognizing xenogeneic recipient cells in host mice. In fact, mice having received human PBMCs spontaneously exhibited non-specific inflammatory changes in their lungs and livers as compared with control *Rag2*^{-/-}*C γ* ^{-/-} mice (Figs 1C and 6A; Supplementary Figure 6 is available at *International Immunology* Online). Despite apparent infiltration with human hematopoietic cells in the steady state (Fig. 6B), the basal lung inflammatory change was minimal (Fig. 6A), and the host mice survived without ill effects until sacrificed. This may be partly due to the inability of host cells to respond to IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 signals. Therefore, it could be said that these transiently humanized mice have limited inflammatory responses without SpA challenge. However, intranasal SpA challenge induced severe airway inflammation in the mice that received SpA-stimulated PBMCs (Fig. 6C and D). Human CD4⁺ T cells were densely recruited into airway after SpA challenge (Fig. 6C). Administration of neutralizing anti-human IL-18 significantly inhibited the development of airway inflammation by diminishing the accumulation of the donor cells (Fig. 6E and F). SpA-specific human CD4⁺ T cells activated by both SpA and IL-18 likely induced the development of airway inflammation by releasing human cytokines and chemokines that recruit human PBMCs. Additionally, some of human chemokines [e.g. CCL5 (RANTES)] might act on murine cells to migrate as well. Further study is required to identify the human factors involved in this airway inflammation. Nonetheless, our results strongly suggest that IL-18 is a potent clinical target for the treatment of bronchial asthma associated with *S. aureus* colonization or infection.

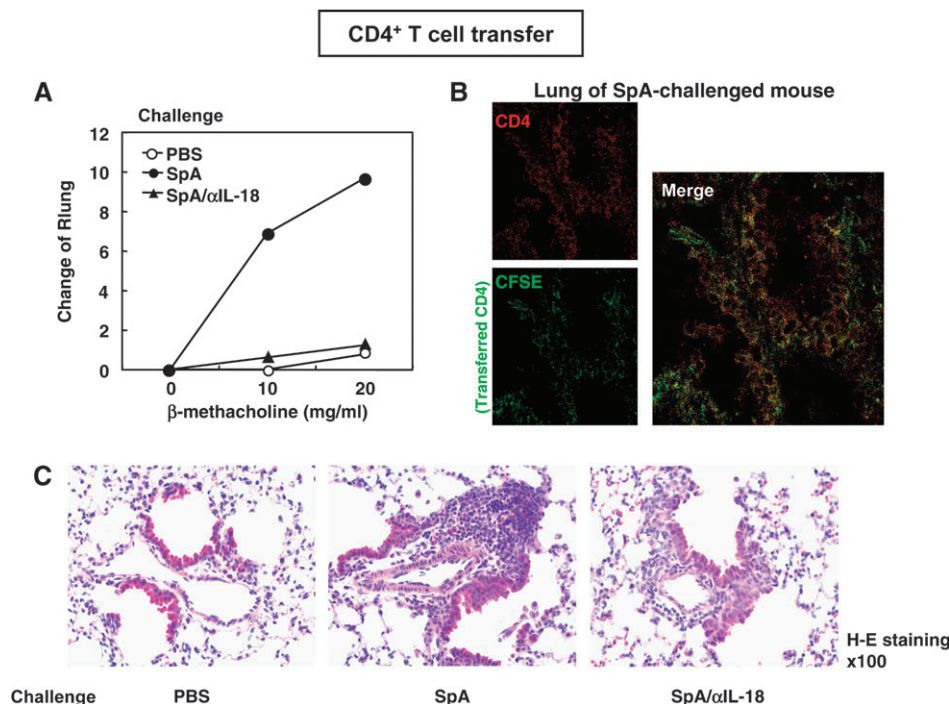


Fig. 4. SpA-sensitized CD4⁺ T cells can transfer disease susceptibility. CD4⁺ DLN cells isolated from mice immunized and boosted with SpA were labeled with CFSE and transferred into naive mice (1×10^7 per mouse). Recipient mice received intranasal challenge with SpA (250 μ g/50 μ l) (SpA), PBS or SpA and neutralizing anti-IL-18 antibodies (500 μ g) (SpA/αIL-18). Twenty-four hours after the last SpA challenge, invasive measurement of AHR (A), confocal microscopic analysis for pulmonary CFSE (green) and CD4 (red) expression (B) and histological analysis (H&E) (C) were performed. Data are representative of three independent experiments with five mice.

It is unclear how human T cells recognize SpA in the transiently humanized mice. After *in vitro* stimulation with SpA, human PBMCs produced IFN- γ and IL-13 (Supplementary Figure 3 is available at *International Immunology* Online), and the CD4⁺ T-cell population expanded (data not shown), suggesting that human APCs possess the potential to present SpA to the CD4⁺ T cells in order to activate them. It was assumed that APCs included in the human donor cell preparation might serve as APCs for the SpA-specific human effector CD4⁺ T cells in humanized mice as well. Alternatively, SpA-specific human effector cells might recognize SpA presented by xenogeneic mouse APCs by the mechanisms currently poorly understood.

We did not measure AHR of transiently humanized mice for a number of reasons. First, the recipients are *Rag2*^{-/-}*C γ* ^{-/-} mice, which lack responsiveness to IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Second, in general, mice are poor responders to human cytokines. Third, C57BL/6 mice, a background of *Rag2*^{-/-}*C γ* ^{-/-} mice, are resistant to T_H2 type and super T_H1 cell type asthma (16). Fourth, is that transiently humanized mice are not homogeneous in terms of the degree of repopulation with human CD4⁺ T cells.

Bronchial asthma is now recognized to have diverse immunopathogenesis. Recently, we demonstrated that intranasal challenge with OVA, plus the IL-18-inducible bacterial LPS, initiates robust bronchial asthma in mice immunized with OVA/CFA. Neutralization of IL-18 during OVA plus LPS challenges inhibits AHR in OVA-specific T_H1 cell-bearing mice, suggesting that OVA plus LPS activates T_H1 cells via endogenous IL-18. In contrast, the role of endogenous IL-18

in allergic T_H2/IgE-dependent asthma is not clearly defined. One report demonstrated only partial contribution of IL-18 to OVA inhalation-induced chronic allergic airway inflammation with remodeling (20). Another report demonstrated that IL-18 does not affect AHR and airway inflammation in allergic bronchial asthma (21), contrasting strikingly to the clear pathological role of endogenous IL-18 in non-allergic super T_H1 cell-dependent asthma (17).

Various types of cells, including macrophages and epithelial cells, can produce IL-18 (28). In this study, we observed elevation of lung IL-18 in the SpA-immunized mice only after intranasal SpA challenge (Fig. 2B), suggesting that IL-18 might be derived from airway constituents, such as respiratory epithelial cells and/or alveolar macrophages. Despite our intensive efforts, we could not observe obvious release of IL-18 from either type of cells after *in vitro* stimulation with SpA.

Recent reports have clearly shown that respiratory epithelial cells play a pivotal role in the development of T_H2 type murine asthma induced by airway exposure to the house dust mite, a common allergen of human asthma (29–31). It was believed that an antigen-specific T_H2-cell response develops only under the limited condition of immunization with the protein in combination with T_H2-cell adjuvant. Beyond this dogma, it was shown that multiple intra-tracheal challenges with house dust mite alone could trigger a T_H2-cell response without prior immunization with antigen/T_H2 adjuvant complex. Mice lacking TLR4 expression on their respiratory epithelial cells, but not hematopoietic cells, were able to evade T_H2 type asthma. Because house dust mites possess intrinsic TLR4 agonists, TLR4 on respiratory

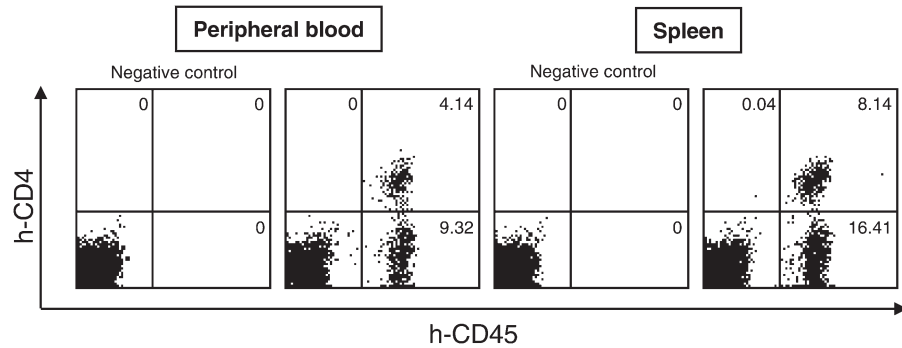


Fig. 5. Mice injected with human PBMCs. Seven days after SpA-stimulated PBMC (1×10^7) were injected into mice, the *Rag2*^{-/-}*Cγ*^{-/-} mice were analyzed for repopulation with human lymphocytes in their peripheral blood and spleen. Cells were incubated with PE-conjugated anti-human CD4 and FITC-labeled anti-human CD45 or a corresponding isotype-matched mAb (negative control). Data are representative of four independent experiments with five mice.

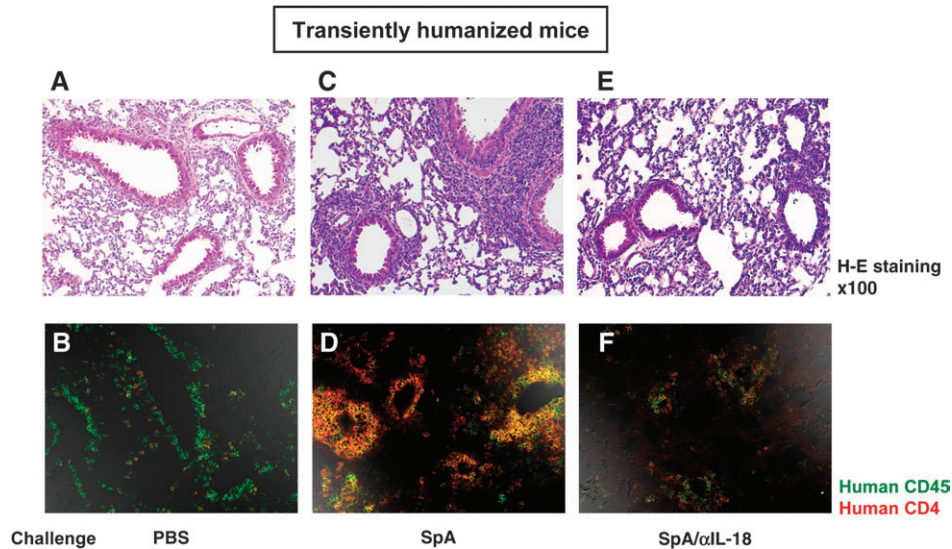


Fig. 6. Blocking the action of human IL-18 prevents SpA-induced airway inflammation in transiently humanized mice. Mice having received SpA-stimulated PBMC (1×10^7) were intranasally treated with PBS (A and B) or challenged with SpA (250 μ g/50 μ l) (SpA; C and D) or with SpA and neutralizing anti-human IL-18 antibody (300 μ g) (SpA/αIL-18; E and F). Twenty-four hours after the last SpA challenge, lung specimens were sampled for histological analysis [hematoxylin and eosin (H&E)] (A, C and E) and for localization analysis of human CD4⁺CD45⁺ T cells (B, D and F). Data are representative of two independent experiments with five mice.

epithelial cells might recognize the agonist and produce T_H2 -activating cytokines, such as IL-25, TSLP and IL-33 (29, 32, 33), eventually resulting in the development of T_H2 type asthma. As previously reported, daily topical application of SpA without T_H1 adjuvant can induce atopic dermatitis, in which super T_H1 -cell development plays a critical role (19). Intriguingly, murine epidermal cells can release super T_H1 cell-activating IL-18 in response to SpA (34). Thus, SpA, like house dust mites in the airway, seems to exert dual actions as a T-cell antigen and adjuvant in the skin. These observations together with our present results suggest that respiratory mucosa and skin, particularly respiratory epithelial cells and epidermal cells, respectively, are the sites required for activation of T_H2 and/or super T_H1 cells. A similar mechanism might also be responsible for the airway asthmatic inflammation induced by SpA.

Accumulated evidence suggests the involvement of IL-18 in atopic diseases in humans. Patients with atopic dermatitis

and bronchial asthma have higher levels of serum IL-18 than healthy volunteers (35, 36). In particular, serum IL-18 levels are shown to coincide with the disease severity of atopic dermatitis. Furthermore, gain-of-function polymorphisms of *IL-18* are observed in patients with atopic dermatitis and bronchial asthma (37–39). This implies that IL-18 is preferentially produced after airway and/or dermal colonization with microbes in those patients, eventually leading to the development of exposure site-specific super T_H1 cell-dependent allergic diseases.

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