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Topical suplatast tosilate (IPD) ameliorates Th2 cytokine-mediated dermatitis in caspase-1 transgenic mice by downregulating interleukin-4 and interleukin-5

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Conflicts of interest
None declared.

Summary

Background Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by elevated serum levels of IgE. AD is associated with Th2 cytokines including interleukin (IL)-4, IL-5, IL-13 and IL-10. Systemic administration of suplatast tosilate (IPD) is currently used to treat Th2 cytokine-mediated AD.

Objectives To evaluate the effect of topical IPD on skin lesions of AD using a genetically engineered AD mouse model (K14/caspase-1 transgenic mouse: KCASP1Tg).

Methods IPD ointment (3%) and white petrolatum (WP) were applied to KCASP1Tg mice every other day from 6 to 14 weeks after birth. Histopathological analysis of skin lesions and measurement of mRNA expression of cytokines in skin lesions and spleen cells were carried out. We also compared changes in serum parameters between IPD-treated and WP-treated KCASP1Tg mice.

Results WP-treated mice developed dermatitis at 8 weeks after birth. However, skin lesions in IPD-treated mice were limited. Histopathologically, skin lesions in WP-treated KCASP1Tg mice showed marked inflammatory changes with increased mast cell infiltration. However, mice treated with IPD showed minimum skin lesions with scarce mast cell infiltration. WP-treated KCASP1Tg mice had significant elevation in the serum levels of histamine, IgE and IL-18 as compared with IPD-treated KCASP1Tg mice. mRNA expression of IL-4 and IL-5 in the skin lesions from WP-treated KCASP1Tg mice was significantly higher than in those from IPD-treated mice. In the spleen, the expression of IL-4, IL-5 and interferon-γ was significantly increased in WP-treated KCASP1Tg mice as compared with their IPD-treated counterparts.

Conclusions This study shows that topical therapy with IPD inhibits the expression of IL-4 and IL-5 and ameliorates skin manifestations in an AD mouse model, suggesting the potential usefulness of topical IPD for the treatment of AD.

Atopic dermatitis (AD) or atopic eczema is a chronic inflammatory skin disease with frequent recurrence. The prevalence of AD in schoolchildren ranges from 7% to 20% in Western countries, Australia and Japan.[1–3] Patients with AD commonly have xerosis (dry skin) and a personal or family history of atopy, asthma, allergic rhinitis and allergic conjunctivitis. AD is a type of eczema characterized by an IgE-mediated inflammatory reaction. Eosinophilia and high serum levels of IgE are characteristic laboratory abnormalities. Specific IgE for environmental antigens can be identified in more than 80% of patients with AD.[1] These manifestations have been attributed to impaired T-cell function. T cell-mediated inflammatory responses have recently been categorized into Th1 and Th2 types according to T-cell cytokine expression profile.[4] Th2-type reactions are mediated by interleukin (IL)-4, IL-5, IL-13 and IL-10. AD is considered to be a Th2-mediated disease with high serum IgE involvement.[5] Peripheral blood mononuclear cells from patients with AD have increased capacity to produce IL-4, IL-5 and IL-13 but limited capacity to produce interferon (IFN)-γ.[6–8] The effects of Th2 cytokines are

counteracted by Th1 cytokines, which include IFN-γ and IL-12. Under normal immune conditions, there is a balance between Th1 and Th2 cytokines. However, recent investigations suggested that, although Th2 cytokines predominate during the acute phase of AD, Th1-type cytokines are dominant in the late stages of the disease.[9,10] Therefore, switching from a Th2 to a Th1 condition may constitute an alternative therapeutic approach in AD.

We have previously established a K14/caspase-1 transgenic mouse (KCASP1Tg) that overexpresses caspase-1 in the skin, and this mouse has been used as a model of AD.[11–13] KCASP1Tg is a keratin 14 promoter-driven caspase-1-transgenic mouse that constitutively secretes endogenous IL-1β and IL-18 in the epidermis. KCASP1Tg mice develop recalcitrant itching dermatitis from 8 weeks of age. The dermatitis starts in the face and then spreads to the extremities and trunk; it is characterized by acute erosive lesions at the beginning of the disease, followed by chronic lichenoid lesions in the chronic stage. Histopathological examination shows infiltrating mononuclear cells around dermal vessels and dermal fibrosis. Toluidine blue staining shows a dense infiltration of mast cells, and the plasma levels of histamine are markedly elevated. These findings confirm the occurrence of AD, and fulfil all major criteria of Hanifin and Rajka for the diagnosis of AD:[14] presence of pruritus, typical morphology and distribution, chronic or chronically relapsing dermatitis, and personal or family history of atopic diseases. They also satisfy 11 of the 22 minor criteria.[11,13] This mouse model also fulfils the diagnostic criteria for AD defined by the U.K. Working Party,[15] except for history of asthma or hay fever.

Suplatast tosilate (IPD) is an immunomodulator that suppresses eosinophil infiltration, IgE production[16,17] and allergic inflammation by suppressing IL-4 and IL-5 production.[18,19] The efficacy of systemic administration of IPD has been previously reported in AD and nasal allergies.[20–22] The efficacy of IPD is dose dependent, its immunomodulatory action being stronger at high concentration. The occurrence of side-effects depends on the drug delivery system. Therefore, topical application of IPD may be more effective and safer than systemic administration. To investigate the topical effect of IPD, an ointment containing 3% IPD was prepared and evaluated in an AD mouse model, KCASP1Tg.

Materials and methods

Atopic dermatitis mouse model

KCASP1Tg mice were used as a model of AD.[11–13] Wild-type C57/BL6 mice were used as a control. Age-matched male animals were used in all experiments.

Topical treatment

IPD was suspended in white petrolatum (WP) at a concentration of 3% and used as an ointment. WP alone was used as control. KCASP1Tg and wild-type mice aged 6 weeks were treated with 1 g of topical IPD or WP every other day. There were three mice per group.

Clinical manifestations

Skin manifestations were evaluated as the percentage of whole-body involved skin surface area. The surface areas of the involved skin and the whole body surface were evaluated by marking on lucent plastic wraps, and were examined every 2 weeks from 6 to 14 weeks.

Histopathological study

Skin samples were taken after 8 weeks of treatment. Samples were embedded with paraffin and sections were prepared for routine haematoxylin and eosin and toluidine blue staining. The remaining samples were used for mRNA extraction.

Serum levels of IgE, histamine and interleukin-18

Serum was separated by centrifugation from mice following treatment for 8 weeks. Plasma histamine levels were measured by radioimmunoassay (SRL, Osaka, Japan). Serum levels of IgE were measured by enzyme-linked immunosorbent assay (ELISA) according to methods described previously.[23] IL-18 concentration was determined by an ELISA kit from MBL (Nagoya, Japan).

mRNA expression of cytokines

mRNA of skin lesions was prepared as previously reported.[11] Skin samples were snap frozen in liquid nitrogen. RNA was extracted using commercial kits (Nippon Gene, Toyama, Japan) and used for polymerase chain reaction amplification. The primer pairs specific for IL-4, IL-5 and IFN-γ were as follows: IL-4: 5′-GCT AGT TGT CAT CCT GCT CTTC-3′ and 5′-GCT CTT TAG CTT TCC AGG AAG TC-3′; IL-5: 5′-GAC AAGCAATGAGACACGATGAGG-3′ and 5′-GAACTCTGCAGGT AATCCAGG-3′;[24] and IFN-γ: 5′-AAC GCT ACA CAC ACT GCA TCTTGG-3′ and 5′-GAC TTC AAA GAG TCT GAGG-3′.[24]

Statistical analysis

Statistical analysis was carried out using the Mann–Whitney U-test. $P < 0{\cdot}05$ was considered as statistically significant. Results are shown as mean ± SD.

Results

Cutaneous manifestations

KCASP1Tg mice treated with WP developed erosive dermatitis that started on the face at 8 weeks of age. The skin lesions then spread to the ears, neck, extremities and trunk. Epithelialization of the erosive lesions was followed by the occurrence of hairless lichenoid dermatitis (Fig. 1a). Mice intensively scratched

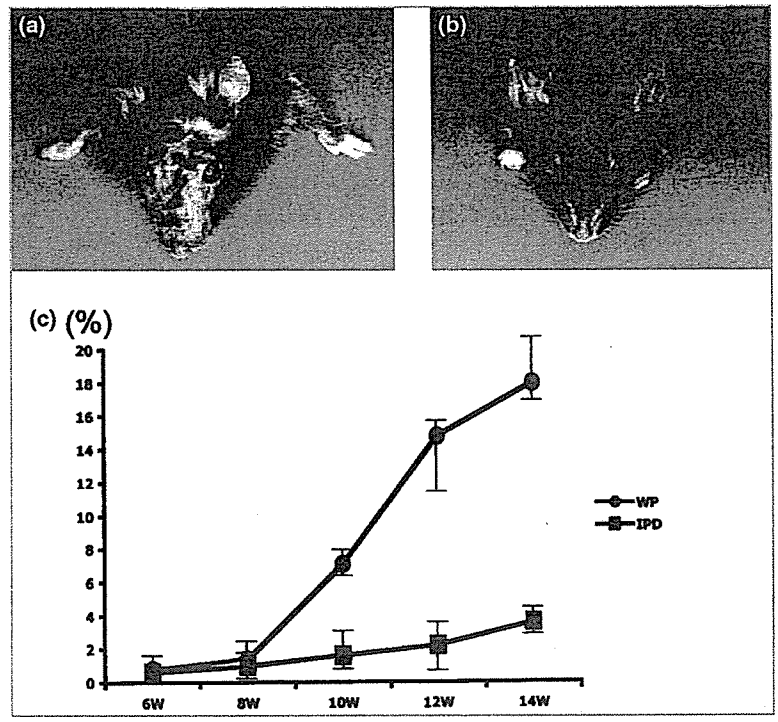

Fig 1. Dermatitis was severe in KCASP1Tg mice treated for 8 weeks with white petrolatum (WP). Remarkable dermatitis was detected in the face and neck (a). In contrast, the grade of dermatitis was slight and limited to the periocular region in KCASP1Tg mice treated for 8 weeks with topical IPD (b). The mean ± SD percentage of involved skin area was lower in IPD-treated KCASP1Tg mice than in WP-treated KCASP1Tg mice (c).

the skin lesions. In contrast, IPD-treated KCASP1Tg mice developed only modest periocular dermatitis (Fig. 1b). The percentage skin area involved was lower in IPD-treated KCASP1Tg mice than in WP-treated KCASP1Tg mice (Fig. 1c) and dermatitis was limited to the periocular region and ears after 8 weeks of treatment in IPD-treated KCASP1Tg mice. Wild-type control C57/BL6 mice treated with WP or IPD did not show any inflammatory dermatitis, and the percentage skin area involved was 0% during their time course (data not shown).

Histopathological findings

WP-treated KCASP1Tg mice showed marked inflammatory reactions in skin lesions (Fig. 2a). The epidermis was acanthotic with hyperkeratosis. There was a significant mononuclear cell infiltration with marked fibrosis in the dermis. However, there were no inflammatory reactions or epidermal acanthosis in skin lesions from IPD-treated mice (Fig. 2b).

Skin mast cell infiltration

Dermal mast cell infiltration was evaluated by counting the number of toluidine blue-positive cells in five different fields (×40). The mean ± SD number of infiltrating mast cells was significantly increased in skin lesions from WP-treated KCASP1Tg mice (43·3 ± 8·4 per field) as compared with wild-type mice (10·5 ± 1·7 per field) ($P < 0·01$). The number of mast cells was significantly reduced in skin lesions from IPD-treated mice (16·4 ± 2·2 per field) as compared with WP-treated KCASP1Tg mice ($P < 0·01$) (Fig. 3).

Serum levels of histamine, IgE and interleukin-18

A high serum level of histamine (540 nmol L^{-1}) was detected in KCASP1Tg mice treated with WP for 8 weeks (Fig. 4a). However, the serum level of histamine was significantly decreased in IPD-treated mice (42 nmol L^{-1}). The serum level of IgE (28·17 μg mL^{-1}) was significantly elevated (Fig. 4b) in WP-treated KCASP1Tg mice but it was below the detection threshold in IPD-treated mice. The serum level of IL-18 was also significantly increased in WP-treated KCASP1Tg mice (5671 pg mL^{-1}) (Fig. 4c), but it was lower in IPD-treated mice (847 pg mL^{-1}).

Cytokine mRNA expression

mRNA expression of IL-4 and IL-5 was increased in skin lesions from WP-treated KCASP1Tg mice (Fig. 5), but these cytokines were undetectable in lesions from IPD-treated KCASP1Tg mice. Similarly, mRNA expression of IL-4, IL-5 and IFN-γ was markedly elevated in spleen cells from WP-treated mice, but expression was decreased in spleen cells from IPD-treated mice; mRNA levels of IL-5 and IFN-γ were detectable but they were significantly reduced as compared with WP-treated mice.

Discussion

In this study to investigate the topical effect of IPD, an ointment containing 3% IPD was prepared, and the effect was evaluated in an AD mouse model, KCASP1Tg. The KCASP1Tg

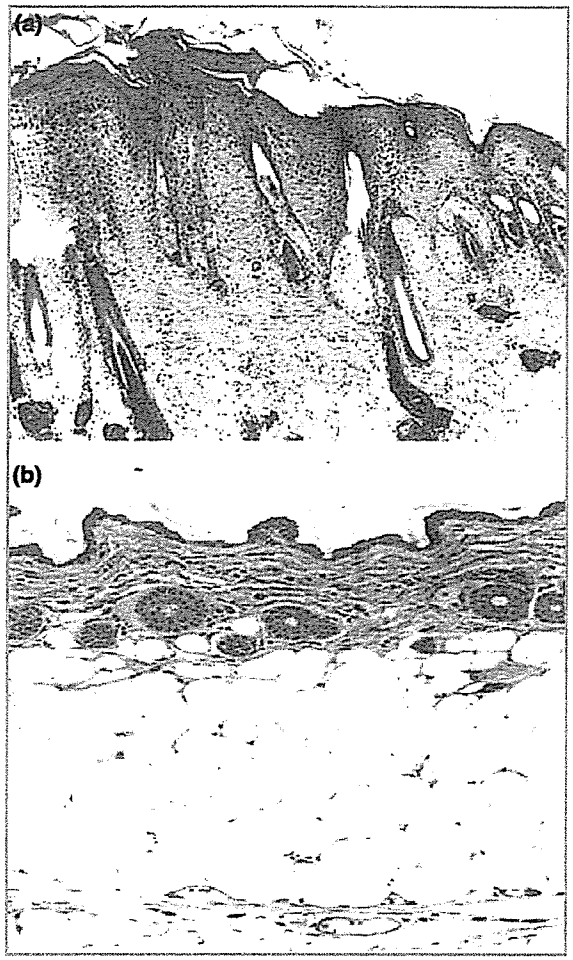

Fig 2. Skin lesions in white petrolatum-treated KCASP1Tg mice showed marked dermatitis with intense dermal and epidermal cell infiltration (a). IPD-treated KCASP1Tg mice showed only modest dermatitis (b).

mice used in the present study are a newly established mouse model of AD. KCASP1Tg mice secrete active forms of IL-18 and IL-1β in the epidermis, and these cytokines cause persistent itching dermatitis.[11] Increased serum levels of IgE can be detected in our mouse AD model. This abnormal circulating level of IgE depends on CD4+ T-cell activation and IL-4 expression.[11,12] Topical treatment with IPD induced a significant suppression of cutaneous manifestations in KCASP1Tg mice as compared with mice receiving placebo. Topical IPD also completely inhibited histopathological changes of dermatitis in KCASP1Tg mice. The findings that the number of infiltrating mast cells in the dermis and the serum levels of histamine and IgE were decreased after treatment suggest that topical IPD also has a systemic effect.

There may arise a possible concern regarding systemic absorption of topical IPD. However, we have performed preliminary experiments for systemic absorption of topical IPD on rats with tape-stripped ulcers in comparison with oral ingestion. Although cutaneous absorption of IPD was detectable, the plasma concentration of IPD was far lower than that measured following oral ingestion (data not shown). Therefore, the amelioration of Th2 cytokine-mediated dermatitis in this AD mouse model suggests an effect of topical IPD. IPD is not a histamine receptor antagonist, and thus clinical improvement is not a result of direct suppression of the effects of histamine. Overall, these findings suggest that the beneficial effects of IPD depend on its inhibitory activity on cytokine expression.

In the present study, increased mRNA expression of IL-4 and IL-5 but not IFN-γ was detected in skin lesions from WP-treated mice. However, skin samples from IPD-treated mice did not show expression of IL-4, IL-5 or IFN-γ. These observations suggest that topical IPD suppresses allergic inflammation by down-regulating Th2 cytokines. Although IPD inhibited Th2 cytokine expression, it did not affect Th1 cytokine expression as demonstrated by the unaffected level of IFN-γ after treatment. To evaluate the systemic effects of topical IPD, the expression of cytokines by spleen cells was assessed. The spleen cells from

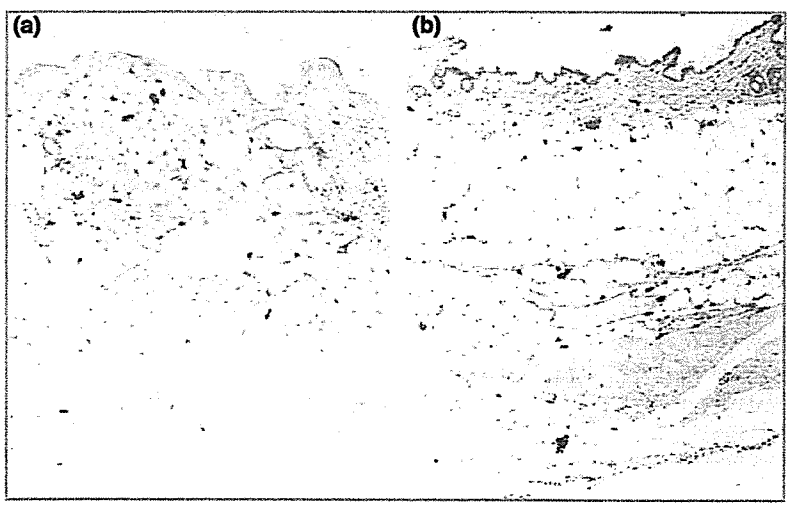

Fig 3. Skin lesions in white petrolatum-treated KCASP1Tg mice showed dermal mast cell infiltration (mean ± SD 43·3 ± 8·4 per field) (a). Skin lesions of IPD-treated KCASP1Tg mice showed a limited number of mast cells in the dermis (mean ± SD 16·4 ± 2·2 per field) (b).

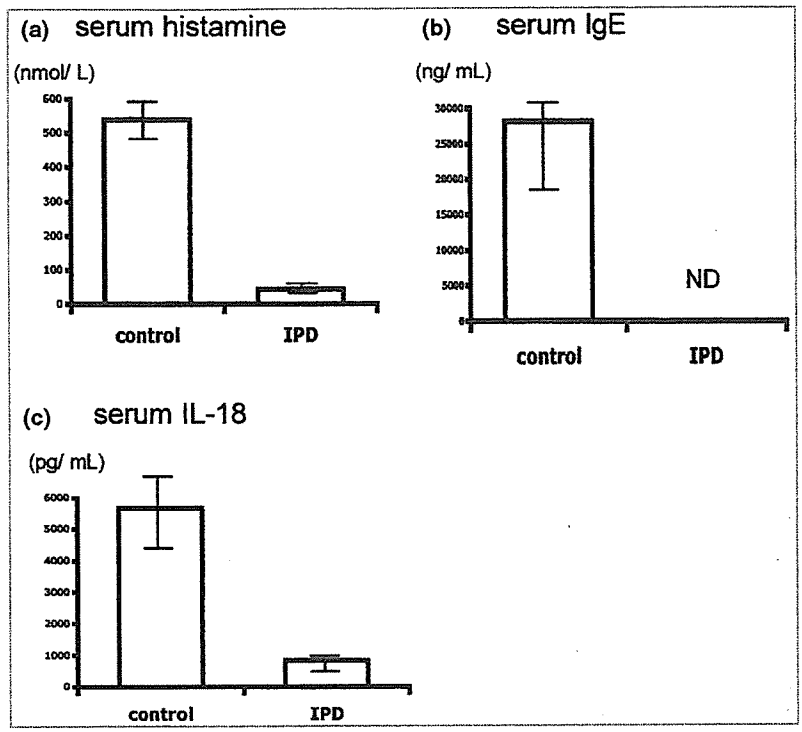

Fig 4. The serum level of histamine in white petrolatum (WP)-treated KCASP1Tg mice was high (540 nmol L^{-1}) after 8 weeks of treatment, but it was low (42 nmol L^{-1}) in IPD-treated mice (a). The serum level of IgE in WP-treated KCASP1Tg mice was high (28·17 μg mL^{-1}) after 8 weeks of treatment, but it was not detectable (ND) in IPD-treated mice (b). Serum interleukin (IL)-18 level in WP-treated KCASP1Tg mice was elevated (5671 pg mL^{-1}) after 8 weeks of treatment, but it was low (847 pg mL^{-1}) in IPD-treated mice (c). Results are shown as mean ± SD.

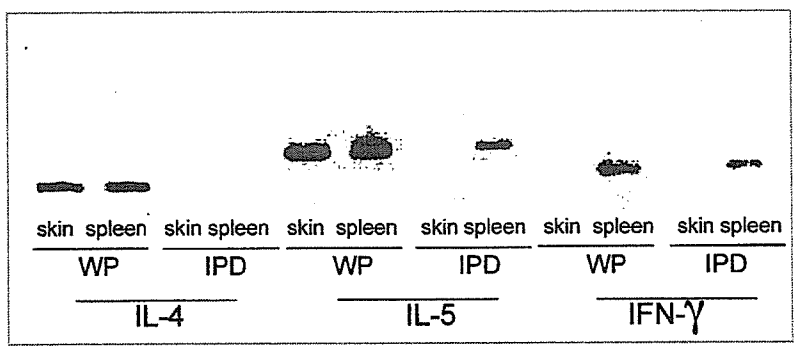

Fig 5. Cytokine mRNA expression in the skin and spleen cells from white petrolatum (WP)- and IPD-treated mice. High expression of interleukin (IL)-4 and IL-5 mRNA was observed in skin lesions from KCASP1Tg mice treated for 8 weeks with WP. However, IL-4 and IL-5 were undetectable in the skin from IPD-treated KCASP1Tg mice. High levels of IL-4, IL-5 and interferon (IFN)-γ mRNA were detected in spleen cells from WP-treated mice. However, IL-4 mRNA was undetectable in spleen cells from IPD-treated mice. Levels of IL-5 and IFN-γ mRNA were detected but they were lower than those detected in WP-treated mice.

mice treated wth WP expressed significant mRNA levels of IL-4, IL-5 and IFN-γ. Unlike in cutaneous lesions, both Th1 and Th2 T cells are activated in the systemic circulation of KCASP1Tg mice. However, topical IPD was able to give effective suppression of both Th1 and Th2 cytokines in our AD model. Particularly noticeable was the complete systemic inhibition of IL-4 expression by topical IPD. Does topical IPD regulate systemic Th2 shifting or is it able to affect the systemic immune system directly? Results of our previous skin-graft experiments in KCASP1Tg mice suggested that skin lesions are the cause of the systemic Th2 shift.[25] Therefore, it is likely that correction of skin lesions ameliorates the systemic immune response.

In addition, topical IPD may also regulate IFN-γ expression in the skin. A Th1 shift has been proposed as the mechanism by which IPD benefits patients with AD. However, Th1 cytokines, in particular IFN-γ, may also have detrimental effects. IFN-γ plays a role in chronic stages of AD, and its overexpression may induce lethal effects in KCASP1Tg mice. Topical IPD corrected the Th2/Th1 imbalance in the epidermis and systemic circulation in our AD model.

Systemic administration of IPD is indicated in cases of AD.[18,19,26] However, topical therapy in AD may have several advantages over systemic administration. For example, topical application of drugs may be better tolerated and may allow

better penetration of the drugs into skin lesions of AD. We found no side-effects in our study; therefore, topical IPD may be a novel therapeutic modality for AD.

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Contribution of IL-18-induced innate T cell activation to airway inflammation with mucus hypersecretion and airway hyperresponsiveness

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Keywords: bronchial asthma, IL-4, IL-13, innate type 2, mucin

Abstract

Human bronchial asthma is characterized by airway hyperresponsiveness (AHR), eosinophilic airway inflammation, mucus hypersecretion and high serum level of IgE. IL-18 was originally regarded to induce T_h1-related cytokines from T_h1 cells in the presence of IL-12. However, our previous reports clearly demonstrated that IL-18 with IL-2 promotes T_h2 cytokines production from T cells and NK cells. Furthermore, IL-18 with IL-3 stimulates basophils and mast cells to produce T_h2 cytokines. Thus, we examined the capacity of IL-2 and IL-18 to induce AHR, airway eosinophilic inflammation and goblet cell metaplasia. Intranasal administration of IL-2 and IL-18 induces AHR, mucus hypersecretion and eosinophilic inflammation in the lungs of naive mice. $CD4^+$ T cells are prerequisite for this IL-2 plus IL-18-induced bronchial asthma, because $CD4^+$ T cells-depleted or Rag-2-deficient (Rag-$2^{-/-}$) mice did not develop bronchial asthma after IL-2 plus IL-18 treatment. Both STAT6$^{-/-}$ mice and IL-13-neutralized wild-type mice failed to develop AHR, goblet cell metaplasia and airway eosinophilic inflammation, while IL-$4^{-/-}$ mice almost normally developed, suggesting that IL-13 is a major causative factor and IL-4 mainly enhances the degree of AHR and eosinophilic inflammation. Both IL-4 and IL-13 equally induce eotaxin in mouse embryonic fibroblasts. However, only IL-13 blockade inhibited asthma symptoms, suggesting that IL-13 but not IL-4 is produced abundantly and plays a critical role in the pathogenesis of bronchial asthma in this model. As airway epithelial cells store robust IL-18, IL-18 might be critically involved in pathogen-induced bronchial asthma, in which pathogens stimulate epithelial cells to produce IL-18 without IL-12 induction.

Introduction

Bronchial asthma is characterized by airway hyperresponsiveness (AHR), eosinophilic airway inflammation, airway remodeling, airway mucus hypersecretion and high serum levels of IgE (1–4). AHR and mucus oversecretion are often linked to asthma symptoms and morbidity. Although the mechanisms underlying these features are complex, it is widely accepted that T_h2 cells, which produce a limited repertoire of cytokines including IL-4, IL-5, IL-9 and IL-13, are responsible for inducing these characteristic features of bronchial asthma (5–7). Indeed, $CD4^+$ T cells producing IL-4, IL-5, IL-9 and IL-13 were identified in the bronchoalveolar lavage (BAL) and airway biopsy (8,9) of asthma patients. Experimental animal studies also revealed that T_h2 cells induce airway eosinophilia, goblet cell metaplasia with mucus oversecretion and AHR (10). Among the T_h2-related cytokines, IL-13 is suggested to play a critical role in induction of AHR and mucus oversecretion (11–14). Indeed, blockade of IL-13 markedly inhibits allergen-induced AHR, eosinophilic inflammation and goblet cell metaplasia in animal models (11,12). Furthermore, direct intranasal administration of IL-13 into naive mice induces mucus hypersecretion, airway eosinophilic infiltration and AHR (11,12).

In contrast, T_h1 cells had generally been regarded to protect against bronchial asthma by damping the activity of T_h2 cells via IFN-γ (15,16). However, our recent studies revealed that naive mice transferred with antigen-specific T_h1 cells exhibit bronchial asthma after challenge with both antigen and IL-18 but not with antigen alone (17). We found that T_h1 cells

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become very pathological, when they are stimulated with Ag and IL-18, possibly by production of IL-13 and IFN-γ. Our study substantiated further the previous study that administration of a combination of IL-13 and IFN-γ induces severe airway inflammation independently of antigenic challenge (18).

IL-18 was originally identified as a factor that enhances IFN-γ production from T_h1 cells in the presence of anti-CD3 and IL-12 (19–21). However, our recent studies and those of others demonstrated that IL-18 directly promotes T_h2 cytokines production from T cells, NK cells, basophils and mast cells, resulting in IgE production (22–27). Usually, secretion of cytokines by T cells is the result of antigen-stimulation. Therefore, cytokines can play a critical role in regulation of an acquired immune response. However, T cells stimulated with IL-12 plus IL-18 exhibit T_h1 response (28). Furthermore, T cells also show T_h2 response when stimulated with IL-2 and IL-18 in the absence of Ag stimulation (25,26). Since IL-18 induces T_h1 or T_h2 cytokines production by T cells without TCR engagement, we could designate these TCR-independent T cell activation pathways as innate type 1 or type 2 T cell activation, respectively (28–30). As airway epithelial cells store robust IL-18 (31), we could assume the possibility that some types of infectious agents might stimulate bronchial epithelial cells to produce IL-18. Thus, it is very important to determine the biological relevance of IL-18-induced innate T cell activation pathway in the pathogenesis of bronchial asthma.

Here, we have demonstrated that intranasal administration of IL-2 and IL-18 into naive mice induces them to express various mRNAs for cytokines (IL-4, -5, -9, -13, -17) and chemokines (MIP-2, GCP-2 and eotaxin) in their lungs and to develop dense infiltration of eosinophils and neutrophils in the peribronchial and perivenular region, goblet cell metaplasia with mucus hypersecretion and AHR, which are prominent features of bronchial asthma. These data suggest the biological relevance of IL-18 in antigen-independent bronchial asthma following viral or bacterial airway infection.

Methods

Animals and reagents

Specific pathogen-free (SPF) female BALB/c mice and BALB/c-background STAT6-deficient ($STAT6^{-/-}$) mice were purchased from Jackson Laboratory. BALB/c $IL\text{-}4^{-/-}$ and $Rag\text{-}2^{-/-}$ mice were obtained from Taconic (Germantown, NY, USA). All mice were bred under SPF condition at the animal facilities of Hyogo College of Medicine (Nishinomiya, Japan) and were used at 8–10 weeks of age. Animal experiments were conducted according to the Guideline for Animal Experiments at Hyogo College of Medicine. Recombinant human IL-2 was kindly provided by Ajinomoto Co. Inc. (Kawasaki, Japan). Recombinant mouse IL-5, IL-13 and IL-18 were purchased from Genetics Institute Inc. (Cambridge, MA, USA) and MBL (Nagoya, Japan), respectively. Recombinant mouse IL-4 was obtained and purified from a recombinant baculovirus (AcMNPV.IL-4) prepared in our laboratory.

In vivo treatment of mice

Animals were daily exposed intranasally to various combinations of IL-18 (0–1 μg day^{-1}) and IL-2 (500 U day^{-1}) in 50 μl of PBS for 1–4 days. Control mice were exposed to PBS alone. Mice were analyzed at 24 h after the final exposure to PBS alone or cytokines. To deplete $CD4^+$ T cells, BALB/c mice were intraperitoneally injected four times (−14, −10, −7, −4 days before cytokine treatment) with mAb to CD4 (clone, GK1.5; 0.5 mg day^{-1}). For the blockade of IL-13 *in vivo*, 20 μg of $sIL\text{-}13R\alpha_2$-Fc or 20 μg of control human IgG (Genetics Institute Inc.) were daily administered intranasally as the mixed form with IL-2 and IL-18 for 4 days.

Measurement of AHR

We measured AHR to β-methacholine (Mch) inhalation in mice by using Pulmos-I (MIPS, Osaka, Japan) hardware and software as described in our previous report (17). We placed a mouse in a chamber and exposed it to aerosols of saline (baseline) first and then to increased concentrations of Mch (5, 10 and 20 mg ml^{-1}). After each 2 min exposure, we measured enhanced pause, a dimensionless index that reflects changes in amplitude of pressure waveform and expiratory time, for 3 min.

BAL

BAL was performed with three aliquots of 1.0 ml of PBS per mouse. Total cell counts were performed. Cytospin preparations of BAL fluid (BALF) were stained with Diff-Quik (Baxter Healthcare Corp., Miami, FL, USA), and differentials were performed based on morphology and staining characteristics.

Histology

Lungs were prepared for histology by perfusion of the animal via the right ventricle with 10 ml of PBS, then fixed in 10% buffered formalin, cut into 3-μm sections and stained with hematoxylin and eosin or predigested periodic acid Schiff.

Preparation of embryonic fibroblasts

Mouse embryonic fibroblasts were prepared from E14 embryos generated by BALB/c mice. Briefly, the heads were removed, and the carcasses were finely minced with scissors and digested by incubation in PBS containing 0.1% trypsin (GIBCO-BRL, Grand Island, NY, USA) and 0.02% EDTA (NACALAI TESQUE, INC., Kyoto, Japan) for 20 min at 37°C. Trypsin was inactivated by washing the cells twice using DMEM supplemented with 10% FBS, 2-ME (50 μM), L-glutamine (2 mM), penicillin (100 U $ml)^{-1}$ and streptomycin (100 μg ml^{-1}) and the cells were plated onto tissue culture dishes. After 3–4 days, when the growth of fibroblasts was established, embryo fragments were removed. Embryonic fibroblasts were maintained as a monolayer culture, and cells from the second or third passage were used.

In vitro culture

Splenic $CD4^+$ T cells from BALB/c mice were purified by MicroBeads (anti-mouse CD4, clone RM4-5; Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched $CD4^+$ T cells (10^5/0.2 ml $well^{-1}$) were cultured with medium alone or various combinations of IL-2 (200 pM) and IL-18 (50 ng ml^{-1}) for 4 days in RPMI 1640 supplemented with 10% FBS, 2-ME (50 μM), L-glutamine (2 mM), penicillin (100 U ml^{-1}) and

streptomycin (100 μg ml^{-1}). Supernatants were harvested and tested for IL-4 and IL-13 contents by ELISA. Mouse embryonic fibroblasts (5×10^4/0.2 ml $well^{-1}$) were cultured with complete DMEM alone, various combinations of IL-4 (103 U ml^{-1}) and IL-13 (10 ng ml^{-1}) or IL-5 (10 ng ml^{-1}) for 2 days. Supernatants were harvested and tested for eotaxin contents by ELISA.

Homogenized lungs

BALB/c mice were daily exposed intranasally to IL-18 (1 μg day^{-1}) and IL-2 (500 U day^{-1}) for 1–4 days. Lungs were removed at 8 h after the final exposure to IL-2 and IL-18 and homogenized with 1 ml of 0.1% TritonX100 containing 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg ml^{-1} aprotinin and 2 μg ml^{-1} leupeptin by Bead Smash 12 (WAKENYAKU, Kyoto, Japan) for 1 min, and centrifuged at 9100 × *g* for 5 min at 4°C. The obtained supernatants were tested for IL-4 and IL-13 contents by ELISA.

Reverse Transcription–PCR analysis

Animals were daily exposed intranasally to IL-18 (1 μg day^{-1}) and IL-2 (500 U day^{-1}) in 50 μl of PBS for 1–4 days. Lungs were removed at 4 h after the final exposure to IL-2 and IL-18 and total RNA was extracted using Trizol reagent, treated with DNase I and reverse transcribed using Superscript II RT and oligo $(dT)_{12-18}$ primer (all four reagents from Invitrogen, Carlsbad, CA, USA). For amplification of IL-4 and IL-13 cDNA, after initial denaturation step at 94°C for 2 min, 34 cycles were performed at 94°C for 1 min followed by 55°C for 1 min and 68°C for 1 min, and then further extension at 68°C for 7 min. For amplification of IL-9 cDNA, PCR assays were performed for 34 cycles (94°C for 1 min followed by 58°C for 1 min and 68°C for 1 min). For amplification of IL-5, MIP-2, eotaxin-2, RANTES and GCP-2 cDNA, PCR assays were performed for 34 cycles (94°C for 1 min followed by 60°C for 1 min and 68°C for 1 min). For amplification of eotaxin and β-actin cDNA, PCR assays were performed for 30 cycles (94°C for 1 min followed by 62°C for 1 min and 68°C for 1 min). For amplification of IL-17 cDNA, PCR assays were performed for 34 cycles (94°C for 15 s followed by 58°C for 30 s and 68°C for 1 min). At the end of cycles, samples were stored at 4°C until analyzed. After amplification, PCR products were separated by electrophoresis in 1.7% agarose gels and visualized by UV light illumination. Primer sequences were as follows: IL-4, 5′-GAA TGT ACC AGG AGC CAT ATC, 3′-CTC AGT ACT ACG AGT AAT CCA; IL-5, 5′-ATG GAG ATT CCC ATG AGC AC, 3′-GTC TCT CCT CGC CAC ACT TC; IL-9, 5′-TAC ATC CTT GCC TCT GTT TT, 3′-CAG AAA TGA CAG TGT GTT GC; IL-13, 5′-ACA GCT CCC TGG TTC TCT CA, 3′-GCT ACT TCG ATT TTG GTA TCG G; IL-17, 5′-TCT CAT CCA GCA AGA GAT CC, 3′-AGT TTG GGA CCC CTT TAC AC; eotaxin, 5′-CTC CAC AGC GCT TCT ATT CC, 3′-CTT CTT CTT GGG GTC AGC AC; GCP-2, 5′-CCA CCC ACG GAT TTT CTT TA, 3′-GGT CCC CAT TTC ATG AGA GA; eotaxin-2, 5′-CTG TGA CCA TCC CCT CAT CT, 3′-TAT GTG CCT CTG AAC CCA CA; RANTES, 5′-GTG CCC ACG TCA AGG AGT AT, 3′-ATT TCT TGG GTT TGC TGT GC; MIP-2, 5′-GAA GTC ATA GCC ACT CTC AAG GGG C, 3′-CAA CTC ACC CTC TCC CCA GAA AC; β-actin, 5′-GTA CGA CCA GAG GCA TAC AGG, 3′-GAT GAC GAT ATC GCT GCG CTG.

Results

IL-2 plus IL-18-induced mucus hypersecretion, airway inflammation and AHR

We first examined the effect of IL-2 and IL-18 treatment on airway inflammation and AHR. We daily treated naive BALB/c mice with intranasal administration of IL-2 and/or IL-18 for 4 days. Histopathological analysis of the lung revealed that administration of IL-18 (1 μg day^{-1}) and IL-2 (500 U day^{-1}) strongly induced peribronchial and perivenular infiltration with eosinophils and mononuclear cells, and goblet cell metaplasia with mucus hypersecretion in the airways (Fig. 1A and B). However, treatment with each component alone did not induce or only weakly induced these changes (Fig. 1A). Titration study indicated that IL-18, in the presence of IL-2 (500 U day^{-1}), dose-dependently induced airway inflammation and mucus production (Fig. 1A). Kinetic study revealed that 1 day or 2 days treatment with IL-18 (1 μg day^{-1}) and IL-2 (500 U day^{-1}) was not sufficient to induce airway inflammation and goblet cell metaplasia. However, 3 or 4 days treatment induced overt airway inflammation and goblet cell metaplasia (data not shown). Furthermore, mice received IL-2 and IL-18 >3 days through their nasal tracts exhibited AHR after nasal exposure to various concentrations of Mch, whereas mice received PBS, IL-2 or IL-18 alone did not exhibit (Fig. 1C), suggesting that intranasal administration of IL-2 and IL-18 induces naive mice to develop mucus hypersecretion, airway inflammation and AHR.

To understand the mechanisms underlying IL-2 and IL-18-induced AHR, we examined the numbers of inflammatory cells infiltrating lung tissues of mice after their exposure to PBS, IL-2 and/or IL-18 for 4 days (Fig. 1D). Analysis of BALF revealed marked increases in the numbers of eosinophils and neutrophils after administration of both IL-2 and IL-18 (Fig. 1D), suggesting that IL-2 and IL-18 induce infiltration of eosinophils and neutrophils in the lungs. These results taken together strongly indicated that intranasal administration of IL-2 and IL-18 induced mucus hypersecretion, airway infiltration with eosinophils and neutrophils and AHR, which are prominent pathological and physiological features of bronchial asthma.

$CD4^+$ T cell-dependent bronchial asthma in IL-2 plus IL-18-treated mice

To determine whether this IL-2 plus IL-18-induced bronchial asthma is dependent on the function of $CD4^+$ T cells, we administrated IL-2 and IL-18 into BALB/c wild-type mice depleted of $CD4^+$ T cells by the pretreatment with anti-CD4 antibody or Rag-$2^{-/-}$ mice, lacking both T cells and B cells. Anti-CD4 treatment significantly impaired IL-2 plus IL-18-induced goblet cell metaplasia with mucus production (Fig. 2A), AHR upon Mch challenge (Fig. 2B) and the number of eosinophils and neutrophils in BALF (Fig. 2C). IL-2 plus IL-18-administered Rag-$2^{-/-}$ mice showed no mucus production, AHR and airway inflammation (Fig. 2). These results clearly indicated that IL-2 plus IL-18-induced bronchial asthma by activation of $CD4^+$ T cells.

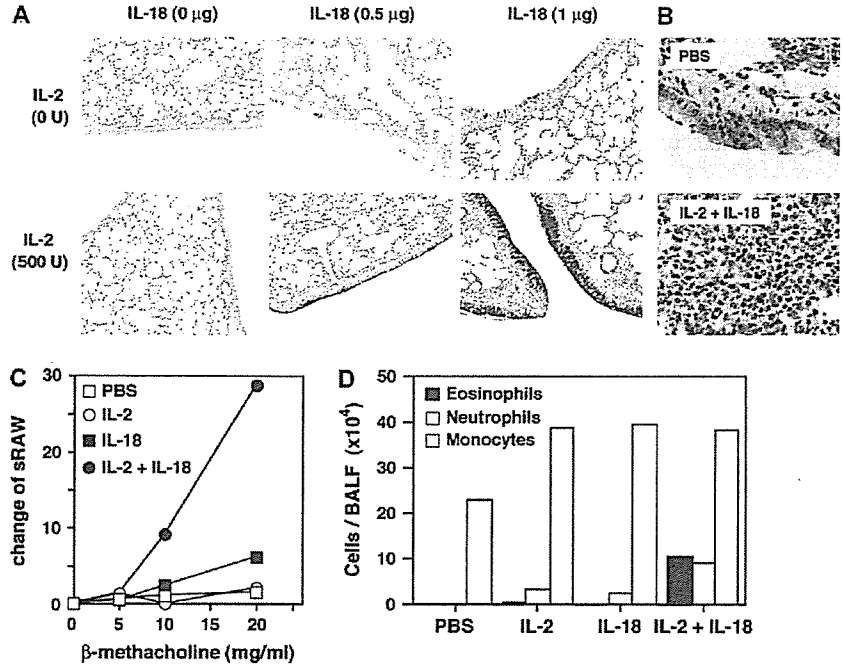

Fig. 1. IL-2 plus IL-18-induced goblet cell hyperplasia, AHR and airway inflammation in naive mice. (A and B) BALB/c mice (6–8 mice per group) were daily exposed intranasally to PBS alone, or IL-18 (0.5–1 μg day $^{-1}$) and/or IL-2 (500 U day $^{-1}$) in 50 μl of PBS for 4 days. Lungs were prepared for histology by perfusing the animal via the right ventricle with 10 ml of PBS, then fixed in 10% buffered formalin, cut into 3-μm sections and stained with predigested periodic acid Schiff (A) or hematoxylin and eosin (B). Original magnification: ×100. (C) AHR in response to increased concentrations of inhaled β-methacholine was measured in a whole-body plethysmograph. (D) Inflammatory cell composition of BALF from mice daily exposed intranasally to PBS alone, or IL-18 (1 μg day $^{-1}$) and/or IL-2 (500 U day $^{-1}$) for 4 consecutive days. Cell differential percentages were determined by light microscopic evaluation of cytospin preparation. Data are expressed as absolute numbers of cells. Representative results of five animals were shown.

IL-13 induces mucus hypersecretion, airway inflammation and AHR

To determine which cytokines mediate IL-2 plus IL-18-induced goblet cell metaplasia with mucus hypersecretion, airway infiltration with eosinophils and neutophils and AHR, we intranasally administered IL-2 and IL-18 into STAT6$^{-/-}$ mice, IL-4$^{-/-}$ mice or BALB/c wild-type mice given a soluble IL-13Rα$_2$-human Fc fusion protein (sIL-13Rα$_2$-Fc), which selectively binds to and neutralizes IL-13 (17,26). As shown in Fig. 3, STAT6$^{-/-}$ mice completely evaded IL-2 and IL-18-induced bronchial asthma, suggesting the critical involvement of IL-4 and/or IL-13 in bronchial asthma. However, IL-4$^{-/-}$ mice normally developed goblet cell metaplasia (Fig. 3A), whereas wild-type mice treated with sIL-13Rα$_2$-Fc failed to do so (Fig. 3A), suggesting that IL-13 is indispensable for inducing mucus overproduction. IL-4$^{-/-}$ mice diminished but still exhibited AHR and airway eosinophilic inflammation (Fig. 3B and C), whereas wild-type mice treated with sIL-13Rα$_2$-Fc did not exhibit AHR and airway eosinophilic infiltration (Fig. 3B and C), suggesting the importance of IL-13 in the pathogenesis of bronchial asthma. In Fig. 3(D), we demonstrated that splenic CD4$^+$ T cells become highly IL-13-producing cells when they are stimulated with IL-2 and IL-18 *in vitro*. Compared with IL-13 production (29.8 ± 6.8 ng ml^{-1}), IL-2 plus IL-18-stimulated T cells produced IL-4 poorly (94.5 ± 7.5 pg ml^{-1}). Furthermore, compared with the degree of IL-4 induction, IL-2 and IL-18 more strongly induced IL-13 in the lungs (Fig. 3E). These results suggest the possibility that IL-2 plus IL-18 induces goblet cell metaplasia, AHR and eosinophilic infiltration principally by strong IL-13 induction and only partly by IL-4 due to poor IL-4 induction.

Expression of mRNAs for cytokines and chemokines in lung of IL-2 plus IL-18-treated mice

To evaluate further which cytokines and/or chemokines regulate IL-2 plus IL-18-induced airway eosinophilic and neutrophilic inflammation, lungs were tested for the expression of mRNA for cytokines and chemokines at various time points after intranasal administration of IL-2 and IL-18. mRNAs for cytokines (IL-4, IL-5, IL-9, IL-13 and IL-17) and chemokines (MIP-2, GCP-2, eotaxin and eotaxin-2) were measured by RT-PCR. Normally these cytokines/chemokines mRNAs are below the detection level in the lungs of naive mice. However, mRNAs for IL-4, IL-5, IL-9 and IL-13 became detectable at day 1 and gradually increased in the lungs of both wild-type and STAT6$^{-/-}$ mice after intranasal administration of IL-2 and IL-18 (Fig. 4). IL-5 and IL-9 mRNAs were apparently expressed in STAT6$^{-/-}$ mice. Nevertheless, these mice did not develop AHR and eosinophilic airway inflammation (Fig. 3B and C), suggesting that these two cytokines are not involved in IL-2 plus IL-18-induced asthma. mRNAs for IL-17 and CXC chemokines (MIP-2 and GCP-2) also became detectable at

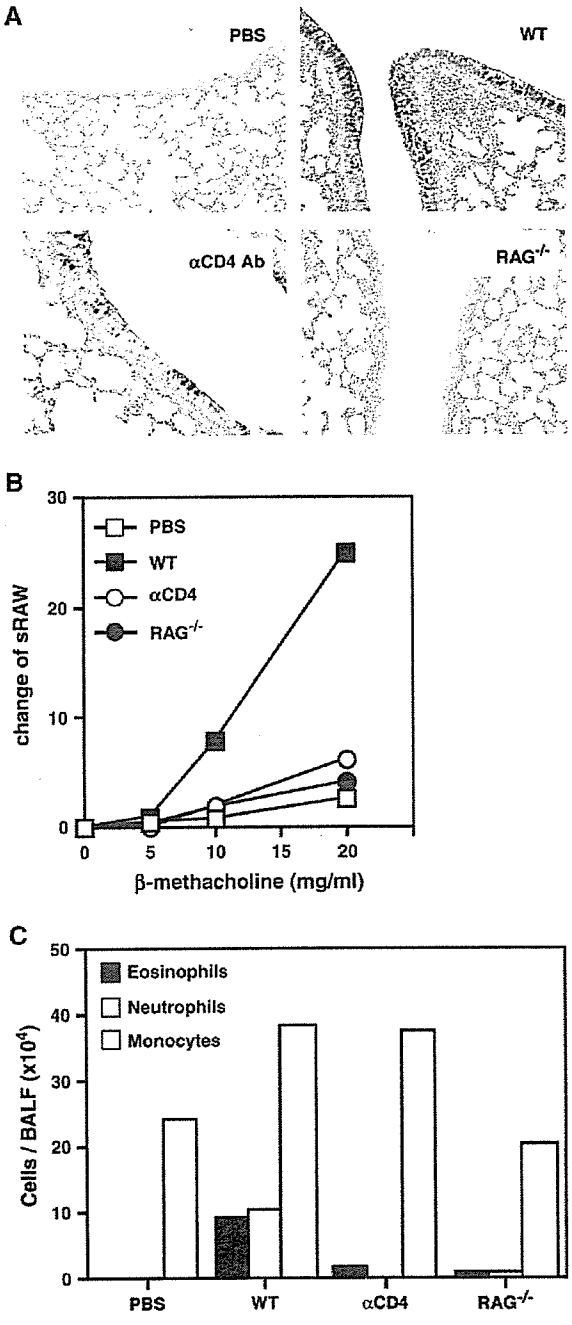

Fig. 2. IL-2 plus IL-18-induced CD4+ T cell-dependent goblet cell hyperplasia, AHR and airway inflammation. BALB/c wild-type (WT) and BALB/c-background Rag-2 $^{-/-}$ (RAG $^{-/-}$) mice were daily exposed intranasally to IL-18 (1 μg day $^{-1}$) and IL-2 (500 U day $^{-1}$) for 4 days. To deplete CD4+ T cells, BALB/c mice received anti-CD4 (GK1.5; 0.5 mg day $^{-1}$) antibody at 4, 7, 10 and 14 days before intranasal administration of IL-2 and IL-18. Twenty-four hours after the final exposure to IL-2 plus IL-18, lungs were stained with predigested periodic acid Schiff (A), AHR in response to increasing concentrations of inhaled β-methacholine (B), and inflammatory cell composition of BALF (C) were examined as described in Fig. 1. Representative results of six animals were shown.

day 1 and sustained their expressions until day 4 in both wild-type and STAT6$^{-/-}$ mice treated with intranasal administration of IL-2 and IL-18 (Fig. 4). MIP-2 and GCP-2 are potent chemoattractants for neutrophils (32,33). IL-17 is produced principally by CD4+ T cells and bears the potential to increase neutrophil recruitment into the airways by releasing several CXC chemokines, including GCP-2 and MIP-2 in bronchial epithelial cells (34,35). Thus, IL-2 and IL-18-induced airway neutrophilic infiltration even in STAT6$^{-/-}$ mice could be explained by the function of IL-17 and/or CXC chemokines (MIP-2 and GCP-2). Most striking difference in the pattern of expression of mRNAs between wild-type and STAT6$^{-/-}$ mice is the expression of eotaxin, which is the most potent chemoattractant for eosinophils (Fig. 4). Eotaxin mRNA expression became detectable at day 1 and are persistently high up to day 4. STAT6$^{-/-}$ mice failed to increase this message, suggesting that IL-2 and IL-18 induce this message by STAT6 activation (Fig. 4). In addition to eotaxin, we have measured mRNAs for eotaxin-2 and RANTES, which are also known to recruit eosinophils. Like eotaxin, eotaxin-2 mRNA expression was promptly and STAT6-dependently induced and its expression was persistently high up to day 4. However, mRNA for RANTES was constitutively and highly expressed in the lungs of naive mice and was not up-regulated by IL-2 plus IL-18 treatment (Fig. 4). These results explain why STAT6$^{-/-}$ mice treated with IL-2 plus IL-18 lacked airway eosinophilic infiltration, even though they express IL-5 mRNA expression in their lungs.

We finally compared the activity of IL-4, IL-5 or IL-13 to induce eotaxin expression in cultured fibroblasts. As reported (36,37), IL-4 and IL-13 showed comparable effect on eotaxin induction from cultured fibroblasts but failed to exhibit additive inducing effect (IL-4, 605.9 ± 37.9 pg ml^{-1}; IL-13, 467.7 ± 33.4 pg ml^{-1}; IL-4 + IL-13, 599.5 ± 36.0 pg ml^{-1}). In contrast, IL-5 could not induce this chemokine expression. These results substantiated further the observation that IL-2 and IL-18 induce eosinophilic infiltration in the lungs by dominant IL-13 induction with poor IL-4 in lung-infiltrating CD4+ T cells.

Discussion

Patients with extrinsic bronchial asthma develop AHR when challenged with corresponding allergen. Thus, allergen acts as a causative factor in T_h2 cell-mediated bronchial asthma. However, bronchial asthma patients often exhibit AHR following infection with bacteria or viruses (38). It is well-known evidence that infection with *Mycoplasma pneumoniae* or influenza A often induces asthma-like AHR even in the non-allergic patients (39–41). Therefore, it is very important to reveal the mechanism of how these infections exacerbates bronchial asthma independently of Ag. Since IL-18 is deeply associated with induction or exacerbation of bronchial asthma and is also markedly induced following several infections, in this report we tested the pathological effect of IL-18 on AHR and airway inflammation.

IL-18 is a unique cytokine that regulates both T_h1 and T_h2 responses (23). IL-18 and IL-12 act synergistically to promote IFN-γ production (19,28,42). However, IL-18 with IL-2 shows the capacity to induce production of T_h2 cytokines (IL-4, IL-5, IL-9 and IL-13) from CD4+ T cells (25,26). Thus, IL-18 induces T_h1 or T_h2 cytokines production from T cells dependently of its

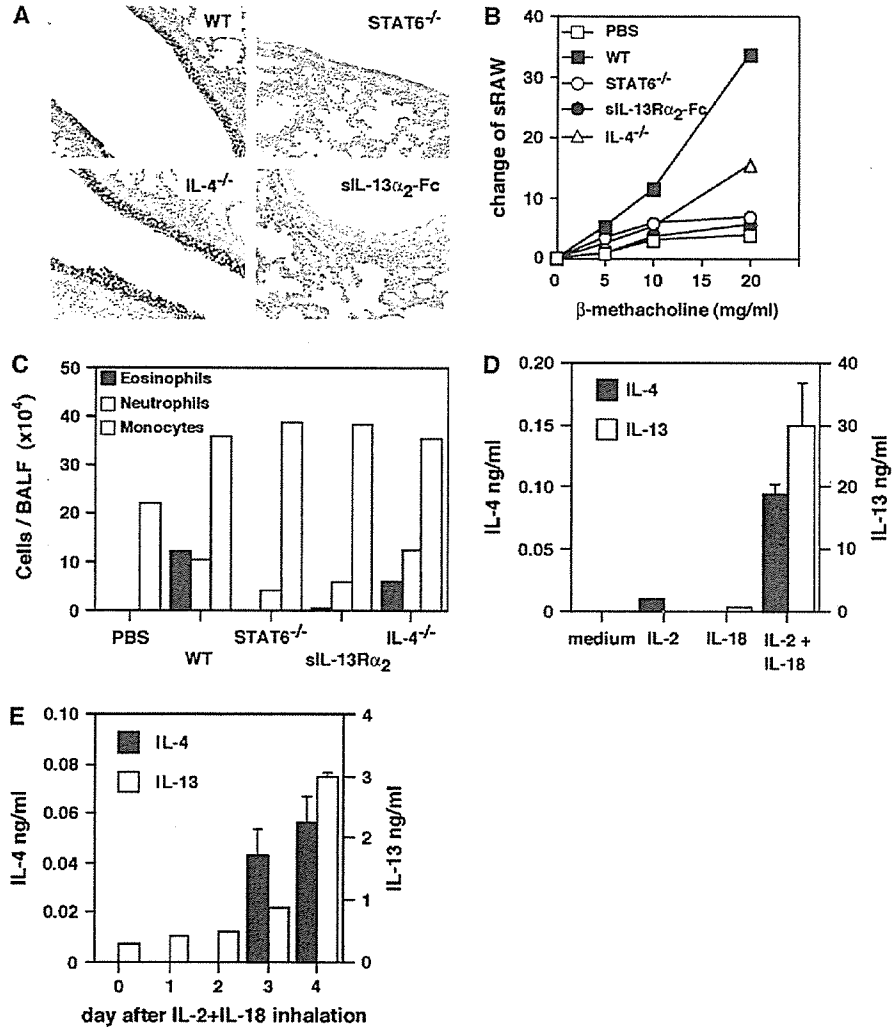

Fig. 3. IL-2 plus IL-18-induced STAT6-dependent goblet cell hyperplasia, AHR and airway inflammation. BALB/c wild-type (WT), BALB/c-background STAT6 $^{-/-}$ and IL-4 $^{-/-}$ mice were daily exposed intranasally to IL-18 (1 μg day $^{-1}$) and IL-2 (500 U day $^{-1}$) for 4 days. For the blockade of IL-13 *in vivo*, 20 μg of sIL-13Rα$_2$-Fc or 20 μg of control human IgG were daily administered intranasally as the mixed form with IL-2 and IL-18 for 4 days. Twenty-four hours after the final exposure to IL-2 plus IL-18, lungs were stained with predigested periodic acid Schiff (A), AHR in response to increasing concentrations of inhaled β-methacholine (B), and inflammatory cell composition of BALF (C) were examined as described in Fig. 1. Representative results of six animals were shown. (D) Splenic CD4$^+$ T cells (10^6/0.2 ml well $^{-1}$) from BALB/c mice were cultured with medium alone or various combinations of IL-2 (200 pM) and IL-18 (50 ng ml $^{-1}$). After 4 days of culture, supernatants were harvested and tested for IL-4 and IL-13 contents by ELISA. Results are geometric means + SD. (E) Wild-type mice were daily exposed intranasally to IL-18 (1 μg day $^{-1}$) and IL-2 (500 U day $^{-1}$) for 1–4 days. Lungs were removed at 8 h after the final exposure to IL-2 and IL-18, and homogenized and centrifuged as described in Methods. The obtained supernatants were tested for IL-4 and IL-13 contents by ELISA. Results are geometric means + SD.

cytokine milieu. In general, T_h1 or T_h2 cells require TCR engagement to produce T_h1- or T_h2-related cytokines, respectively. As noted above, IL-18 plus IL-12- or IL-18 plus IL-2-stimulated T cells produce T_h1- or T_h2-related cytokines, respectively, without TCR engagement. Therefore, we previously proposed that there are two types of T cell activation pathway, acquired type (TCR-dependent) activation and innate type (TCR-independent) activation pathways (29). It is well accepted that acquired T cell activation pathway plays a critical role in an acquired immune response, particularly bronchial asthma. In contrast, it is still unclear whether the innate T cell activation pathway is indeed involved in the pathogenesis of bronchial asthma. Therefore, we sought to determine the pathological effect of IL-18-mediated innate T cell activation pathway for induction of bronchial asthma.

In this study, we have shown that daily intranasal administrations of IL-2 and IL-18 into naive mice for four consecutive days induce them to develop goblet cell metaplasia with mucus hypersecretion, airway eosinophilic as well as neutrophilic infiltration and AHR, which are prominent features of bronchial asthma (Figs. 1 and 2). Then, we tried to elucidate the underlying mechanism of how this treatment induces

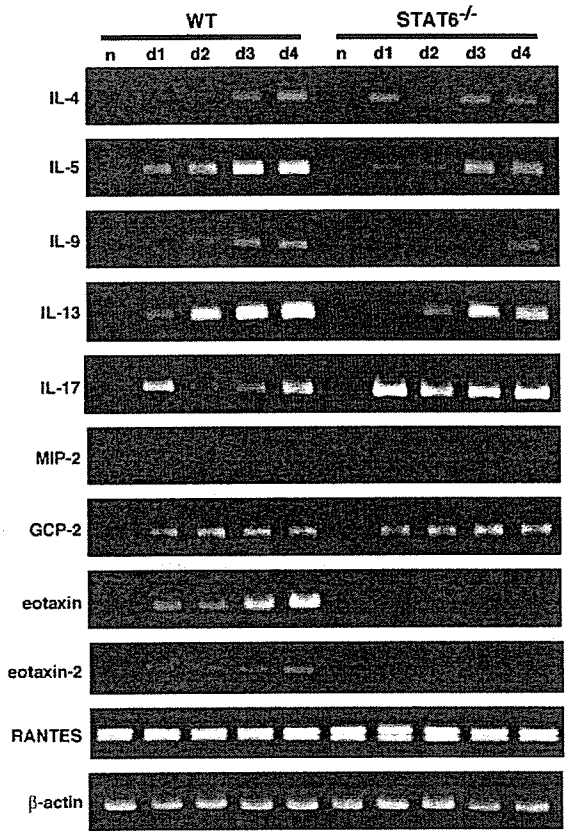

Fig. 4. Expression of mRNAs for cytokine and chemokine in lung from IL-2 plus IL-18-inhalated mice. Wild-type and STAT6 $^{/}$ mice were daily exposed intranasally to IL-18 (1 μg day ^{1}) and IL-2 (500 U day ^{1}) for 1–4 days. Lungs were removed at 4 h after the final exposure to IL-2 and IL-18 and total RNA was extracted. Expression of mRNAs for cytokine (IL-4, IL-5, IL-9, IL-13 and IL-17) and chemokine (MIP-2, GCP-2, eotaxin, eotaxin-2 and RANTES) were measured by RT-PCR.

bronchial asthma. We found that IL-2 and IL-18 act on $CD4^+$ T cells to produce IL-13, which in turn induces goblet cell metaplasia with mucus hypersecretion and eosinophilic infiltration in the lungs. Mucus hypersecretion is completely inhibited by IL-13-neutralization. Furthermore, IL-$4^{-/-}$ mice normally developed goblet cell metaplasia and eosinophilic infiltration in response to intranasal administration of IL-2 and IL-18. Thus, IL-2 and IL-18 induce goblet cell metaplasia and eosinophilic inflammation principally by IL-13 induction. Indeed and importantly, *in vitro* $CD4^+$ T cell activation with IL-2 and IL-18 induces IL-13 production strongly and IL-4 production weakly (Fig. 3D). In contrast to the poor IL-4 induction by IL-2 and IL-18, this combination strongly induced IL-13 in the lungs (Fig. 3E). Thus, IL-2 and IL-18 mainly induced bronchial asthma via IL-13. However, IL-$4^{-/-}$ mice partially reduced AHR (Fig. 3B), suggesting that IL-2 plus IL-18-stimulated T cells partly induce AHR via IL-4.

In animal models of allergic asthma, blockage of IL-13 markedly inhibits allergen-induced AHR (11,12). IL-$13^{-/-}$ mice sensitized and challenged with antigen fail to develop AHR, although they develop severe airway inflammation (43). Furthermore, IL-13 delivery to the airway of naive mice causes AHR without antigen (11,12). All of these reports demonstrate the essential role of IL-13 in the development of AHR. However, other studies suggest that IL-4 by itself can induce AHR. Indeed, IL-4 delivery to the airway of naive Rag-$2^{-/-}$ mice causes AHR (12). Like IL-13, IL-4 affects epithelial cells and smooth muscle cells to induce AHR (44,45). Furthermore, in lung epithelial cells, both IL-4 and IL-13 inhibit the activity of inducible nitric oxide synthesis and decrease production of nitric oxide, a bronchodilator (46). Our data indicated the strong contribution of IL-13 to bronchial asthma. However, contribution of IL-4 to IL-2 plus IL-18-induced asthma seems to be very weak, perhaps due to the low ability of IL-2 plus IL-18-stimulated T cells to produce IL-4.

We also found that intranasal administration of IL-2 and IL-18 induces various mRNAs for cytokines (IL-4, IL-5, IL-9, IL-13 and IL-17) and chemokines (MIP-2 and GCP-2) in lungs of wild-type and STAT6$^{-/-}$ mice (Fig. 4). Wild-type mice developed eosinophilic infiltration, which was inhibited by neutralization of IL-13. STAT6$^{-/-}$ mice did not develop eosinophilic infiltration, even though they normally expressed mRNAs for IL-5 and RANTES, suggesting that STAT6 activation is responsible for inducing eosinophilic infiltration. Wild-type mice expressed eotaxin and eotaxin-2, while STAT6$^{-/-}$ mice did not (Fig. 4), suggesting that induction of eotaxin and eotaxin-2 is dependent upon STAT6 activation. IL-13 or IL-4 equally induced eotaxin in cultured fibroblasts. In contrast, IL-5 failed to induce this chemokine. Beside this, IL-4 or IL-13 can induce the endothelial expression of vascular cell adhesion molecule 1 (VCAM-1) (47), which binds to integrin α4β1 (VLA-4) expressed on eosinophils (48). Thus, IL-13 induces eosinophilic infiltration by induction of eotaxin and VCAM-1 in a STAT6-dependent manner. IL-4 also has the same potential. Nevertheless, neutralization of IL-13 alone almost completely inhibited AHR, eosinophilic infiltration and mucus secretion (Fig. 3). Based on these evidences, we concluded that IL-2 and IL-18 induce eosinophilic infiltration by strong promotion of IL-13 induction from T cells.

IL-2 and IL-18 also induced neutrophilic infiltration. In contrast to airway eosinophilia, IL-2 plus IL-18-induced airway neutrophilia is not dependent on STAT6 (Fig. 3). It has been reported that IL-17 bears the potential to increase neutrophil recruitment into the airways by releasing several different CXC chemokines including GCP-2, Gro-α and IL-8 in human bronchial epithelial cells or MIP-2 in mouse bronchial epithelial cells, respectively (34,35). All of these CXC chemokines are potent neutrophil chemotactants (33). In this study, IL-2 plus IL-18-administered STAT6$^{-/-}$ mice showed airway neutrophilia (Fig. 3) and increased expression of mRNAs for IL-17, MIP-2 and GCP-2 (Fig. 4). Taken together, IL-2 plus IL-18 induced airway neutrophilia by induction of IL-17 and/or CXC chemokines (MIP-2 and GCP-2).

Recently, we have demonstrated that T_h1 cells have the capacity to produce both T_h1 cytokines and T_h2 cytokines when stimulated with Ag and IL-18 and become very pathological cells when they show such phenotype in the lung (17). Mice transferred with Ag-specific T_h1 cells exhibit neither AHR nor respiratory tract eosinophilic inflammation when challenged with intranasal administration of Ag. However, they suffer from severe bronchial asthma upon challenge

with Ag plus IL-18, with comparable levels of AHR and eosinophilic inflammation to those in mice transferred with T_h2 cells and challenged with Ag later (17). The former type of asthma is categorized as acquired type T_h1 asthma to distinguish this from T_h2-induced bronchial asthma. Consistent with previous reports, neutralization of IL-13 inhibits AHR and eosinophilic infiltration in T_h2-induced bronchial asthma. In contrast, blockade of IL-13 does not reduce IL-18-induced T_h1 asthma, although this treatment markedly reduces eosinophilic infiltration in the airway (17), suggesting that molecules other than IL-13 contribute to the development of Ag plus IL-18-induced AHR in T_h1 mice.

In the present study, we have revealed that neutralization of IL-13 almost completely inhibited IL-2 plus IL-18-induced bronchial asthma (Fig. 3). However, it is important to know the effect of endogenous IFN-γ. Thus, we administered IL-2 plus IL-18 into wild-type BALB/c and BALB/c-background IFN-$\gamma^{-/-}$ mice. Both wild-type and IFN-$\gamma^{-/-}$ BALB/c mice showed similar degree of mucus production in the lungs. Furthermore, they showed comparable level of AHR upon Mch challenge. However, IL-2 plus IL-18-adminstered IFN-$\gamma^{-/-}$ mice exhibited ~1.5-fold increase in the number of eosinophils in their BALF (data not shown). These results taken together indicate that compared with strong IL-12 plus IL-18 effect on IFN-γ production from T cells, IL-2 plus IL-18 poorly induced T cell IFN-γ production, resulting in little effect on AHR, mucus production and eosinophilic infiltration. Thus, IL-2 plus IL-18 mainly induces AHR via IL-13 but not IFN-γ. Importantly, intranasal administration of IL-2 plus IL-18 into mice induces bronchial asthma independently of antigenic challenge. Therefore, IL-18 is critically involved in the pathogenesis of both acquired type and innate type bronchial asthma, suggesting IL-18 as a therapeutic target for the treatment of both types of bronchial asthma.

Acknowledgements

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Abbreviations

AHR	airway hyperresponsiveness
Mch	methacholine
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
IL-13Rα$_2$-Fc	IL-13Rα$_2$-human Fc fusion protein

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Plasma interleukin (IL)-18 (interferon-γ-inducing factor) and other inflammatory cytokines in patients with gouty arthritis and monosodium urate monohydrate crystal-induced secretion of IL-18

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Abstract

To determine whether levels of interleukin (IL)-18, together with those of IL-1β, tumor necrosis factor-α, IL-6, and IL-8, are elevated in the plasma of patients with gouty arthritis, the plasma concentrations of those cytokines were measured in 31 males with gouty arthritis. Further, $CD14^+$ cells were obtained from human blood and thioglycolate medium-induced peritoneal cells obtained from caspase 1-deficient mice, and then separately cultured in the presence of monosodium urate monohydrate (MSU) crystals. In addition, in an animal in vivo experiment, MSU crystals were injected into subcutaneous air pouches of IL-18-deficient mice. The plasma concentrations of IL-18, IL-6, and IL-8 were elevated in the presence of gouty arthritis in the gout patients. In the in vitro study, the presence of MSU crystals stimulated $CD14^+$ cells (monocytes) to secrete IL-18 and increased the activity of caspase 1 in $CD14^+$ cells, whereas there was no significant effect on IL-18 messenger RNA in $CD14^+$ cells and only a slight induction of IL-18 secretion from thioglycolate medium-induced caspase 1-deficient peritoneal cells. In the in vivo experiment, MSU crystals injected into the air pouch promoted neutrophil accumulation along with an increase in concentrations of keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-1α in air-pouch fluids in both IL-18-deficient and wild-type mice. However, there was no increase in the concentration of IL-18 in air-pouch fluids in either mouse strain. Our results suggest that plasma IL-18, IL-6, IL-8, and C-reactive protein (CRP) levels reflect local inflammation associated with gouty arthritis, though IL-18 does not play an important role in neutrophil accumulation. Further, they suggest that MSU crystals accelerate the processing of IL-18 from an inactive to active form via the activation of caspase 1.

Keywords: Interleukin-18 (interferon-γ-inducing factor); Cytokine; Urate; Gout; Caspase; Gouty arthritis

1. Introduction

Gout is a disease that is manifested by an increase in serum urate concentration, recurrent attacks of acute arthritis, deposits of monosodium urate monohydrate (MSU) in and around the joints of the extremities, renal disease involving the interstitial tissues and blood vessels, and uric acid nephrolithiasis. During attacks of acute arthritis, MSU crystals are found in leukocytes in synovial fluids, indicating that they are involved with induction of those attacks. In previous studies [1,2], MSU crystals were demonstrated to stimulate

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synovial cells, monocytes-macrophages, and neutrophils to produce a variety of different cytokines including tumor necrosis factor (TNF-)-α, interleukin (IL)-8, IL-1β, IL-6, and monocyte chemotactic factor, which induce acute inflammation. Among them, TNF-α, IL-8, IL-1β and IL-6 are produced by activated macrophages. Okamura et al. reported that the cytokine IL-18 induces the production of interferon-γ from activated Th 1 cells, especially in the presence of IL-12 [3]. Thereafter, IL-18 was demonstrated to be one of the major products of monocytes-macrophages and similarly induced in a number of infectious diseases. In addition, it was recently shown that IL-18 promotes neutrophil accumulation [4].

Since MSU crystals may stimulate macrophages, we hypothesized that they also stimulate synovial membranes and monocyte-macrophages to secrete IL-18 in the joints and that IL-18 play a role in gouty arthritis along with other cytokines. However, there is no known study that has demonstrated that MSU crystals stimulate monocytes-macrophages to produce IL-18. Therefore, we measured plasma IL-18 levels together with those of IL-1β, TNF-α, IL-6, and IL-8 to determine if they are elevated in patients with MSU crystal-induced acute arthritis. Further, in an in vitro study to investigate the mechanism of urate crystal-induced production of IL-18, we determined whether MSU crystals induce human monocytes to produce IL-18 and stimulate Brewer's thioglycolate medium-induced macrophage to produce IL-18 in caspase 1-deficient mice. We also examined whether IL-18 plays a role in MSU crystal-induced neutrophil accumulation in IL-18-deficient mice, since IL-18 is known to promote neutrophil accumulation [4].

2. Materials and methods

2.1. Subjects

A total of 31 male outpatients (aged 32–55 years old, mean 45 ± 12 years) with primary gout, diagnosed according to the criteria outlined by the American Rheumatism Association [5], were included in the present study. We did not determine whether MSU crystals were present in the inflamed joints in any of the subjects at the beginning of the study. All of those who are free of gouty arthritis had normal values in routine laboratory tests, which included AST, ALT, serum creatinine, and fasting blood glucose levels. Twelve of the subjects had been treated with allopurinol and 15 with benzbromarone, while 4 had received no drugs for asymptomatic hyperuricemia. In addition, 13 subjects had been treated with a diet regimen for mild hypertriglyceridemia. Blood samples were taken from 8 subjects during periods with and without gouty arthritis, as well as from 8 during a period of gouty arthritis and from 15 during a period without gouty arthritis. Blood was drawn using a heparinized syringe within 48 h of onset from subjects with active gouty arthritis, (on the worst day of inflammation in 15 of 16 cases), while in those without, blood was taken during a period when there was no clinical evidence of gouty arthritis. Of those suffering from acute arthritis, none had received colchicine or nonsteroidal anti-inflammatory drugs. For the paired samples, the lag time between the two samples was 26 ± 6 days (mean ± SD). Inflamed joints during the period of acute arthritis were the knee in 2 cases, ankle in 2 cases, and MP of the first toe in 12 cases. None of the patients had tophus. The blood samples were separated by centrifugation to obtain plasma, which was stored at −70 °C until use.

2.2. Reagents

Uric acid was purchased from Wako Pure Chemicals Ltd (Osaka, Japan) and MSU crystals were produced by a method previously described [6], with slight modifications. Briefly, uric acid was treated for 2 h at 200 °C, and then uric acid solution (0.03 M, pH 7.5) was prepared by dissolution of equimolar quantities of the uric acid and sodium hydroxide, with sodium chloride (0.1 M final concentration) added to accelerate the formation of triclinic MSU crystallization. Endotoxin was not detected at a concentration of 10 mg of MSU crystals/ml in PBS (<0.8 pg/ml endotoxin by limulus amoebocyte assay measured by Special Laboratory Research (Tokyo, Japan)). They were 10.1 ± 5.3 μm (mean ± SD). The same lot of MSU crystals was used throughout the study. Z-Val-Ala-Asp (Ome)-CH_2F was obtained from Peptide Institute Inc. (Osaka, Japan).

2.3. Preparation of $CD14^+$ cells

Blood samples were obtained from five normal subjects using heparinized syringes and laid over a Ficoll-Paque gradient (Pharmacia, Piscataway, NJ) to isolate peripheral blood mononuclear cells (PBMC) by centrifugation. Following centrifugation, $CD14^+$ cells (monocytes) were purified from the isolated PBMC by positive selection with magnetic beads conjugated to anti-CD14 (Miltenyi Biotech, Auburn, CA, USA).

The recovery rate of $CD14^+$ cells was 95%. Therefore, the $CD14^-$ PBMC cells ($CD14^-$ cells) contained a small number of $CD14^+$ cells (0.85% of $CD14^-$ PBMC cells determined by fluorescence activated cell sorting).

2.4. Cell culture conditions

$CD14^+$ and $CD14^-$ (0.5×10^6) cells were plated in flat-bottomed 48-well plates (Iwaki Glass, Tokyo, Japan) in 0.25 ml of RPMI 1640 containing penicillin–streptomycin and 20% fetal cow serum, in the absence or presence of the MSU crystals (0.25, 0.5 or 0.75 mg/ml). In addition, $CD14^+$ cells (0.5×10^6) were plated in flat-bottomed 48-well plates (Iwaki Glass) in 0.25 ml of RPMI 1640 containing penicillin-streptomycin and 20% fetal cow serum in the presence of the MSU crystals (0.75 mg/ml) with the addition of DMSO (1 μl/ml final concentration) containing the caspase 1 inhibitor Z-Val-Ala-Asp (Ome)-CH_2F (0, 0.4, 0.8, or 2.0 μmol/l final concentration). These cells were cultured for 24 h in 5% CO_2 in an incubator, then the culture medium was collected and centrifuged at 1500 × g for 10 min, after which a supernatant was obtained. In addition, to measure the activity of caspase 1 in $CD14^+$ cells, cells (0.5×10^6) were plated in

a flat-bottomed 48-well plate (Iwaki Glass, Tokyo, Japan) in 0.25 ml of RPMI 1640 containing penicillin-streptomycin and 20% fetal cow serum in the absence or presence of the MSU crystals (0.75 mg/ml), and cultured for 3 h.

2.5. Cytokines and caspase 1 activity

Human and mouse IL-18 levels were measured using a sandwich-type ELISA with commercially available Human IL-18 and Mouse IL-18 ELISA kits (BMI Medical and Biological Laboratories Co. Ltd., Nagoya, Japan), respectively. The lower detection limits of human and mouse IL-18 were 12.5 and 5 pg/ml, respectively. The assay kit for human IL-18 uses a human IL-18 calibrator (recombinant protein) and two monoclonal antibodies against two different epitopes of human IL-18, while the kit for mouse IL-18 uses a mouse IL-18 calibrator (recombinant protein) and two monoclonal antibodies against two different epitopes of mouse IL-18. Human IL-1β, TNF-α, IL-6 and IL-8 levels were determined using commercially available ELISA kits (BioSource International Inc., CA, USA). The activity of caspase 1 in $CD14^-$ cells was determined using the commercially available Carboxyfluorescein FLICA Apoptosis Detection Kit Caspase 1 FLICA (Serotec Ltd, Oxford, UK). Mouse IL-1β, IL-6, keratinocyte-derived chemokine (KC), and macrophage inflammatory protein (MIP)-1α were measured using commercially available ELISA kits (R&D Systems Minneapolis, MN, USA).

2.6. Cytokine expression in cells

RNA was obtained from incubated cells by a chloroform–phenol extraction method, after which cDNA was reverse transcribed from the total RNA obtained. The sense primer for IL-18 was 5′-GAA GAT TCA AAT TGC ATC TTAC-3′ and the anti-sense primer was 5′-GCT TGA ATC TAA ATT ATC AGT C-3′, while the sense primer for β-actin was 5′-TGA CGG GGT CAC CCACACTGTGCCCATCTA-3′ and the anti-sense primer was 5′-CTA GAAGCA TTT GCGGTG GAC GAT GAA GGG-3′. These primers were used under the following conditions. cDNA was denatured at 94 °C for 5 min, followed by 40 cycles of denaturation for IL-18 and 25 cycles of denaturation for β-actin at 94 °C for 30 s, then annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, with a final extension step of 7 min. PCR was carried out in a volume of 25 μl containing 50 mM KCl, 10 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each of dATP, dCTP, dGTP, dTTP, 0.1 μg of cDNA, 10 pmol of each primer, and 1.5 U of Taq polymerase in a DNA thermal cycler (Perkin Elmer Cetus). IL-18 mRNA was quantified using a human IL-18 competitive PCR assay after equalizing the amount of total RNA from monocytes incubated with and without MSU crystals by the method of Kotake et al. [7], using a human β-actin competitive PCR assay. DNA competitors to β-actin and IL-18 cDNA were constructed using a Takara competitive DNA construction kit (Takara Biomedics, Tokyo, Japan), and competitive PCR assays were performed with various concentrations of the competitor, as described in the Protocols section of the kit.

2.7. Mice

Specific pathogen-free female C57BL/6 mice (6–8 weeks old) were purchased from SLC (Shizuoka, Japan). Female caspase 1-deficient mice with a C57BL/6 background [8] and female IL-18-deficient mice with a C57BL/6 background [9] were used in the experiments.

2.8. Treatment of mice

Peritoneal exudate cells were elicited in wild-type and caspase 1-deficient mice by peritoneal injection of 3 ml of Brewer's thioglycolate medium (Difco, Detroit, MI, USA). Cells were harvested at 72 h after injection by washing the peritoneal cavity with a lavage composed of 5 ml of ice-cold sterile PBS. The peritoneal exudate cells (1×10^6) (TM-induced peritoneal cells) were plated in flat-bottomed 48-well plates (Iwaki Glass) in 0.5 ml of RPMI 1640 containing penicillin–streptomycin and 20% fetal cow serum, in the presence or absence of MSU crystals (0.1, 0.2 or 0.4 mg/ml). These cells were cultured for 24 h in 5% CO_2 in an incubator, then the culture medium was collected and centrifuged at $1500 \times g$ for 10 min, after which the supernatant was obtained.

Subcutaneous air pouches were created in wild-type and IL-18-deficient mice according to a method previously described [10]. Three milliliters of air was injected into subcutaneous tissue in the back, followed by a second injection of 5 ml of air after 3 days. Seven days after the first injection, MSU crystals (3 mg in a volume of sterile PBS) were injected into the air pouches and pouch fluid samples were harvested by injecting 3 ml of PBS 8 h after administering the MSU crystals. The infiltrated cells were then counted using a hemocytometer and stained with Wright–Giemsa solution to perform a differential leukocyte count. In addition, the pouch fluid samples were centrifuged at $1500 \times g$ for 10 min to obtain supernatant specimens.

2.9. Statistical analyses

Values are expressed as the median with range in the study of samples obtained from gouty patients and significant differences between the variables were analyzed using a Mann–Whitney *U*-test. In other studies, values are expressed as the mean ± SD and significant differences between the variables were analyzed using ANOVA following Boneferroni correction. Significance was defined as $P < 0.05$.

3. Results

3.1. Plasma CRP, IL-18, IL-1β, TNF-α, IL-6, and IL-8 levels in patients with gout

Plasma C-reactive protein (CRP) was higher in the presence of gouty arthritis than in its absence ($P < 0.05$) (Table 1). In the 8 patients from whom blood samples were obtained in both the presence and absence of gouty arthritis, plasma CRP was also higher in the presence of gouty arthritis

Table 1
Plasma concentrations of CRP and cytokines in patients with gout

	Presence of gouty arthritis ($n = 16$)	Absence of gouty arthritis ($n = 23$)
CRP level (mg/l)	19 (4–75)*	<3 (<3)
IL-18 (pg/ml)	291 (204–522)**	174 (66–356)
IL-6 (pg/ml)	1.57 (0.3–21.50)*	0.33 (0.05–2.14)
IL-8 (pg/ml)	1.87 (0.92–13.53)*	1.32 (0.20–5.70)
IL-1β (pg/ml)	0.41 (0.1–2.35)	0.48 (0.1–1.23)
TNF-α (pg/ml)	<0.9 (<0.9 5.53)	<0.9 (<0.9–1.38)

Values are expressed as the median with range. Significant differences between the variables were analyzed using a Mann-Whitney U-test. *$P < 0.05$ and **$P < 0.01$, as compared with the respective value in the absence of gouty arthritis.

(26 mg/l, range 4–69 mg/l vs. below 3 mg/l, range <3 mg/l) ($P < 0.05$). However, in the presence of gouty arthritis ($n = 16$), the level of plasma CRP was not significantly correlated with the level of plasma IL-18 (data not shown).

Plasma IL-18 was higher in the presence of gouty arthritis ($n = 16$) than in its absence ($n = 23$) ($P < 0.01$) (Table 1). Further, plasma IL-6 and IL-8 levels were also higher in the presence of gouty arthritis (both $P < 0.05$) (Table 1). In contrast, the level of plasma IL-1β was not significantly different between the presence and absence of gouty arthritis in all subjects. TNF-α levels were beyond the limit of detection (>0.9 pg/ml) in 4 of 16 patients in the presence of acute arthritis (1.08–5.53 pg/ml) and below the limit of detection (<0.9 pg/ml) in the other 12 (Table 1), and also beyond the limits of detection in 7 of 23 patients without the presence of acute arthritis (1.00–1.38 pg/ml) and below the limits of detection in the other 16 (Table 1). Thus, the difference in TNF-α level was not significant between the presence and absence of gouty arthritis (Table 1).

In the 8 subjects from whom paired blood samples could be obtained, plasma IL-18 was significantly higher in the presence of gouty arthritis (297 pg/ml, range 204–437 pg/ml vs. 179 pg/ml, range 139–356 pg/ml) ($P < 0.05$). In addition, plasma IL-6 was significantly higher in the presence than the absence of gouty arthritis (2.15 pg/ml, range 0.92–3.45 pg/ml vs. 1.22 pg/ml, range 0.52–4.84 pg/ml) ($P < 0.05$). On the other hand, plasma IL8, IL-1β and TNF-α were not different between the presence and absence of gouty arthritis, respectively, in the 8 subjects from whom paired blood samples were obtained (data not shown).

3.2. IL-18 production by MSU crystal-stimulated CD14$^+$ cells over time

In the presence of 0.75 mg/ml of MSU crystals, the concentration of IL-18 in the supernatant of the culture medium containing CD14$^+$ cells increased and reached a plateau after 12 h (Fig. 1).

3.3. IL-18 production by MSU crystal-stimulated CD14$^+$ cells and CD14$^-$ cells

In the presence of 0.25, 0.5, and 0.75 mg/ml of MSU crystals, the concentration of IL-18 was increased in the supernatant of the culture medium containing CD14$^+$ cells after 24 h (Table 2), while in their absence, it was below the limit of detection (<12.5 pg/ml) (Table 2). On the other hand, in the presence of 0.75 mg/ml of MSU crystals, IL-18 was below the limit of detection in the supernatant of culture medium containing CD14$^-$ cells after 24 h.

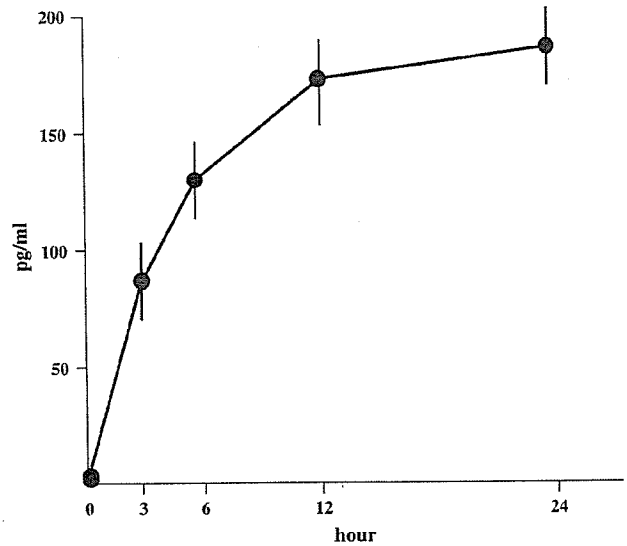

Fig. 1. IL-18 production by MSU crystal-stimulated CD14$^+$ cells ($n = 5$).

3.4. IL-18 and IL-1β production by MSU crystal-stimulated CD14$^+$ cells in the presence and absence of caspase, inhibitor Z-Val-Ala-Asp (Ome)-CH$_2$F

The MSU crystal (0.75 mg/ml)-induced production of IL-18 by CD14$^+$ cells was inhibited by 47%, 64%, and 84% in the presence of 0.4, 0.8 and 2.0 μmol/l, respectively, of the caspase 1 inhibitor Z-Val-Ala-Asp (Ome)-CH$_2$F, while that of IL-1β was also inhibited by 67%, 93%, and 99%, respectively, indicating that the MSU crystal-induced production of IL-18 and IL-1β was ascribable to the activation of caspase 1 by MSU crystals (Table 3). In contrast, the MSU crystal-induced production of TNF-α was not inhibited in the presence of the caspase 1 inhibitor (Table 3).

Table 2
IL-18 production by MSU crystal-stimulated CD14$^+$ cells and CD14$^-$ cells ($n = 5$)

MSU crystals (mg/ml)	Concentration of IL-18 (pg/ml)	
	CD14$^+$ cells	CD14$^-$ cells
0	ND	ND
0.25	75.2 ± 14.5**	ND
0.5	127.3 ± 19.2**	ND
0.75	224.5 ± 37.8**	ND

Values are expressed as the mean ± SD. ND denotes not detected (below 12.5 pg/ml). Significant differences between the variables were analyzed using ANOVA. **$P < 0.01$, as compared with the reference value without the addition of MSU crystals in medium containing CD14$^+$ cells.

Table 3
Inhibition of MSU crystal-induced IL-18 production by the caspase 1 inhibitor Z-Val-Ala-Asp (Ome)-CH_2F ($n = 5$)

Caspase 1 inhibitor (μmol/l)	MSU crystal-stimulated $CD14^+$ cells		
	IL-18 concentration (pg/ml)	IL1β concentration (pg/ml)	TNF-α concentration (pg/ml)
0	197.1 ± 22.1	272.9 ± 35.1	117.7 ± 19.8
0.4	105.0 ± 14.1*	90.8 ± 9.3*	117.9 ± 25.2
0.8	71.0 ± 11.9*	18.6 ± 3.5*	108.6 ± 21.6
2.0	31.1 ± 9.5*	3.2 ± 1.1*	112.1 ± 16.9

Values are expressed as the mean ± SD. Significant differences between the variables were analyzed using ANOVA. *$P < 0.05$, as compared with the reference value without the addition of caspase 1 inhibitor.

3.5. Caspase 1 activity

The activity of caspase 1 in $CD14^+$ cells in the presence of MSU crystals (0.75 mg/ml) was increased by 3.74 ± 0.73-fold ($P < 0.01$) ($n = 5$) and 3.07 ± 0.64-fold ($P < 0.01$) ($n = 5$), at 1 and 3 h respectively, after the beginning of incubation, as compared with that in the absence of MSU crystals.

3.6. IL-18 messenger RNA (mRNA) in $CD14^+$ cells

We performed a competitive PCR assay of IL-18 after equalizing the amount of total RNA from monocytes incubated with and without MSU crystals using human β-actin (data not shown) at 3, 6, and 12 h after beginning the incubation of $CD14^+$ cells. The expressions of the IL-18 PCR product and competitor were nearly equal at a competitor concentration of 10^4 copies/tube at all of the time points, with a representative result shown in Fig. 2. These findings showed that MSU crystals (0.75 mg/well) did not increase the expression of IL-18 mRNA in $CD14^+$ cells (0.2×10^6), suggesting a role of post-transcriptional regulation of IL-18 by MSU crystals.

3.7. IL-18 produced by TM-induced peritoneal cells (peritoneal macrophages) in caspase 1- deficient mice

In a preliminary study, an increase in the concentration of IL-18 in peritoneal macrophage-containing culture medium was observed after the addition of MSU crystals. However, the concentration of IL-18 in the culture media did not increase when the concentration of MSU crystals in the culture media was greater than 0.4 mg/ml. Therefore, we performed this experiment with the concentrations of MSU crystals in the culture media within a range of 0–0.4 mg/ml. The concentrations of IL-18 in the supernatants of culture media containing TM-induced peritoneal cells (1×10^6 cells/well) increased in the presence but not in the absence of MSU crystals in wild-type mice (Table 4). On the other hand, the concentrations of IL-18 in the supernatants of culture media containing TM-induced peritoneal cells (1×10^6 cells/well) slightly increased in the presence of MSU crystals and were not detected in the absence of MSU crystals in caspase 1-deficient mice (Table 4).

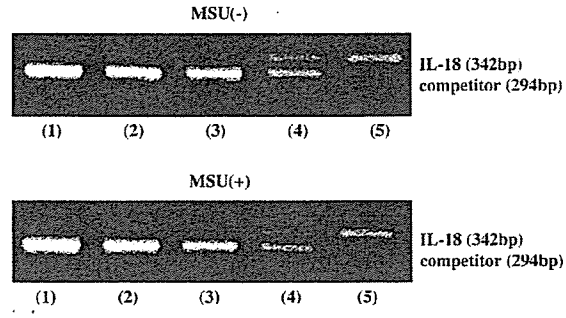

Fig. 2. Competitive PCR of IL-18 mRNA. Total RNA was extracted from MSU crystal-stimulated $CD14^+$ cells and control $CD14^-$ cells after 12 h of culture. IL-18 mRNA was quantified using a human IL-18 competitive PCR assay after equalizing the amount of total RNA of $CD14^+$ cells incubated with and without MSU crystals, using the method of Kotake et al. [6], with a human β-actin competitive PCR. For a competitive PCR assay of IL-18, 0.5 μg of total RNA of $CD14^+$ cells incubated with MSU crystals and the adjusted amount of total RNA incubated without MSU were applied. The final PCR products were subjected to 3% agarose gel electrophoresis and stained with ethidium bromide. The amounts of the competitor template added per tube were 10^7, 10^6, 10^5, 10^4, and 10^3 copies/tube (lanes 1–5).

These results suggest that caspase 1 plays an important role in the MSU crystal-induced secretion of IL-18.

3.8. Infiltrating cells, neutrophils, and cytokines in air pouches

The numbers of infiltrating cells and neutrophils in air pouches of wild-type and IL-18-deficient mice at 8 h after injections of PBS alone and PBS containing MSU crystals (3 mg) are shown in Table 5. The numbers were not significantly different between wild-type and IL-18-deficient mice.

In addition, the concentrations of IL-18 at 8 h after the injection of PBS alone and PBS containing MSU crystals (3 mg) were below the limit of detection (< 5 pg/ml) in all pouch fluid supernatant samples (Table 5). The concentrations of IL-1β, KC, MIP-1α, and IL-6 in air pouch fluid supernatants from both wild-type and IL-18 deficient-mice after the injections of PBS alone and PBS containing MSU crystals (3 mg) are shown in Table 5. The concentrations of IL-1β, KC, MIP-1α, and IL-6 following the injection of PBS

Table 4
IL-18 produced by MSU crystal-stimulated TM-induced peritoneal cells (peritoneal macrophage) in caspase 1- deficient mice

MSU crystals (mg/ml)	IL-18 (pg/ml)	
	Wild-type mice ($n = 4$)	Caspase 1-deficient mice ($n = 4$)
0	ND	ND
0.1	10.5 ± 3.0**	ND
0.2	19.3 ± 3.4**	8.0 ± 1.4
0.4	37.8 + 8.1**	8.7 ± 0.5

Values are expressed as the mean ± SD. ND, not detected (<5 pg/ml). Significant differences between the variables were analyzed using ANOVA. **$P < 0.01$, as compared with the respective value in caspase 1-deficient mice.