

Contribution of IL-18 to atopic-dermatitis-like skin inflammation induced by *Staphylococcus aureus* product in mice

Makoto Terada^{*†}, Hiroko Tsutsui^{†‡§}, Yasutomo Imai^{†¶}, Koubun Yasuda^{*‡}, Hitoshi Mizutani^{†¶}, Kiyofumi Yamanishi^{†¶}, Masato Kubo^{**}, Kiyoshi Matsui^{*}, Hajime Sano^{*}, and Kenji Nakanishi^{†‡††}

^{*}Division of Rheumatology and Clinical Immunology, Department of Internal Medicine, and Departments of [†]Immunology and Medical Zoology and [‡]Dermatology, Hyogo College of Medicine, Nishinomiya 663-8501, Japan; [§]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Kawaguchi 332-0012, Japan; [¶]Department of Dermatology, Mie University School of Medicine, Tsu 514-8507, Japan; and ^{**}Laboratory for Signal Network, RIKEN Research Center for Allergy and Immunology, RIKEN Yokohama Institute, Yokohama, Kanagawa 230-0045, Japan

Communicated by Tadamitsu Kishimoto, Osaka University, Osaka, Japan, April 10, 2006 (received for review November 30, 2005)

Atopic dermatitis (AD) is a common inflammatory skin disease of unknown etiology. Cutaneous infection with microbes such as *Staphylococcus aureus* and/or skin cleansing with detergent exacerbates clinical AD. Here, we generated an AD animal model by destroying skin barrier function with detergent and subsequent topical application of protein A from *S. aureus* (SpA). NC/Nga mice, which genetically have reduced skin barrier function, and BALB/c mice having intact skin barrier function, were susceptible to this combination and developed severe and moderate AD, respectively, associated with dermal accumulation of eosinophils and mast cells. Both types of mice showed an increase in serum levels of IL-18, but not IgE. The epidermis of the NC/Nga mice rapidly expressed T helper type 1 (Th1)-associated chemokines, including ligands for CXCR3 and CCR5, after application of both SpA and detergent, but not after application of detergent alone. Although treatment with detergent induced moderate Th1 cell response, additional SpA treatment was a prerequisite for induction of the differentiation of naive T cells toward unique Th1 cells, termed "super Th1 cells," capable of producing both Th1 (IFN- γ) and T helper type 2 cytokine (IL-13), as well as IL-3, and expressing CXCR3 and CCR5. Induction of super Th1 cells required IL-18 stimulation. Blockade of IL-18 prevented AD development, whereas blockade of IL-3 partially prevented AD development, suggesting a contribution of IL-18-dependent IL-3 production to AD with cutaneous mastocytosis. *il18*^{-/-} BALB/c mice similarly evaded SDS/SpA-induced AD. Thus, IL-18 might be important for the development of infection-associated AD by induction of IL-3 from super Th1 cells.

IL-3 | protein A | IFN- γ | super Th1 cells | intrinsic atopic dermatitis

Atopic dermatitis (AD) is a common inflammatory skin disease. Major features of AD are pruritus, chronic relapsing course, and genetic predisposition (1–3). AD is divided into two types: (i) AD associated with IgE-mediated responses, affecting 70–80% of patients and termed "extrinsic AD," and (ii) AD without IgE-mediated responses, affecting 20–30% of patients and termed "intrinsic AD." Patients with extrinsic AD show preferential deviation toward T helper type 2 (Th2) responses, together with accumulation of Th2 chemokines such as CCL17 (thymus- and activation-regulated chemokine) and CCL22 (monocyte-derived chemokine) in the cutaneous lesions (1, 2). However, recent clinical studies have revealed that the immunological aspects of the skin lesions are quite different among the clinical stages of AD (1–3). Nonetheless, the immunopathological bases for intrinsic AD are still unknown.

Recently, we showed that transgenic mice that oversecrete IL-18 from their epidermal cells spontaneously develop AD-like dermatitis under specific pathogen-free (SPF) conditions, and that the deletion of *il18*, but not *stat6*, which encodes a signaling molecule necessary for Th2 and IgE responses, protects against the development of AD (4, 5). This finding suggests that excessive cutaneous

IL-18 release is a causative factor for intrinsic-type AD. Recent clinical studies have revealed that IL-18 production levels closely parallel disease severity (6–8). Therefore, it is important to clarify whether, and how, endogenous IL-18 contributes to the development of AD when skin is exposed to natural infectious agents.

Although it is well documented that cutaneous infection with *Staphylococcus aureus* exacerbates clinical AD (1, 3), the underlying mechanism is not fully understood. Recently, we demonstrated that protein A (SpA), a surface molecule and virulent factor of *S. aureus* (9), stimulates mouse epidermal cells to secrete IL-18 (10). However, cutaneous application of SpA alone did not induce major skin alterations in C57BL/6 mice, despite the fact that it produced elevated serum levels of IL-18 and IgE (5, 10). This outcome led us to assume that additional factors are required for the development of AD. Because skin cleansing with detergent aggravates clinical AD (1, 11), skin barrier destruction seems to be a second important factor in AD development. Inasmuch as NC/Nga mice, which have a genetically impaired skin barrier due to reduced ceramide production (12), frequently develop AD-like dermatitis after exposure to mites (13–15), we assumed that genetic skin barrier dysfunction was a third prerequisite for the development of AD. Here, we generated an intrinsic AD mouse model by daily application of SpA, after destruction of the skin barrier with a subclinical dose of SDS, a detergent (16). Neutralizing anti-IL-18 Abs could completely protect against SDS/SpA-induced AD. Furthermore, *il18*^{-/-} BALB/c mice evaded development of AD under SDS/SpA challenge. Our present results clearly demonstrate the importance of both detergent and SpA for the development of AD and provide insight into the immunopathological bases for AD. These findings also point to a possible therapeutic regimen for intrinsic AD that would target IL-18.

Results

SpA-Induced AD-Like Dermatitis. Daily application of SpA after treatment with a subclinical dose of SDS dose-dependently induced AD-like dermatitis in NC/Nga mice under SPF conditions (Figs. 1A and 2A). However, application of SpA without prior SDS treatment did not induce any skin alterations (Figs. 1A and 2A), indicating a requirement for both SpA and SDS. Because 4% SDS and 200 μ g of SpA induced the highest levels of skin alteration in all NC/Nga

Conflict of interest statement: No conflicts declared.

Abbreviations: AD, atopic dermatitis; Th2, T helper type 2; SPF, specific pathogen-free; SpA, *Staphylococcus aureus* protein A; Th1, T helper type 1; LN, lymph node.

[§]Present addresses: Department of Microbiology, Hyogo College of Medicine, Nishinomiya 663-8501, Japan; and Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Kawaguchi 332-0012, Japan.

^{††}To whom correspondence should be addressed at: Department of Immunology and Medical Zoology, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya, 663-8501 Japan. E-mail: nakaken@hyo-med.ac.jp.

© 2006 by The National Academy of Sciences of the USA

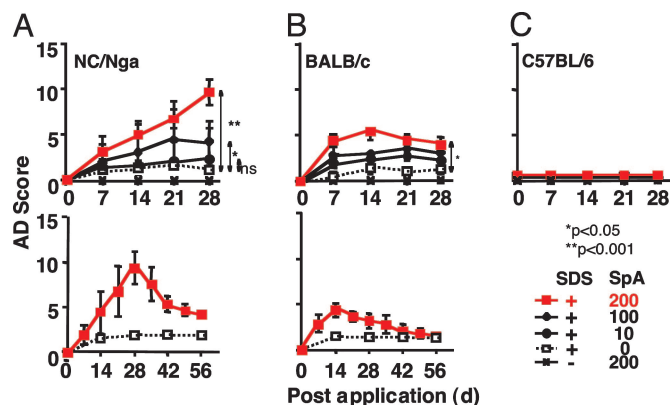


Fig. 1. Comparison of susceptibility to SDS/SpA-induced AD. NC/Nga (A), BALB/c (B), and C57BL/6 (C) mice were treated daily with (+) or without (–) 4% SDS and subsequently with various doses (μ g) of SpA for the indicated days after challenge. AD scores were determined as described in ref. 13. ns, not significant. A and B Lower show some of the same data on a longer time scale.

mice, we used this combination in our study. The AD score (13) peaked at 4 weeks and then declined gradually, but still to a level higher than basal level (Fig. 1A). Thus, NC/Nga mice are highly susceptible to the combined application of SDS and SpA. BALB/c mice, although they evaded spontaneous skin alterations under conventional conditions, exhibited dermatitis phenotypically similar to that in NC/Nga mice, but with much lower AD scores (Fig. 1B). In BALB/c mice, the AD score peaked at 2 weeks after application and then declined to the lowest levels equivalent to those in mice treated with SDS alone (Fig. 1B). Thus, BALB/c mice are moderately susceptible to combined application of SDS and SpA, whereas C57BL/6 mice are resistant (Fig. 1C). Because NC/Nga mice developed severe and persistent AD-like clinical AD, we used this strain of mouse in our analysis. Sequential treatment with SDS and BSA or ovalbumin instead of SpA did not induce skin alterations in NC/Nga mice (data not shown), suggesting that SpA does not simply serve as a foreign antigen (Ag). Thus, we found that SDS/SpA-induced AD depended on genetic predisposition, including skin barrier dysfunction (12).

Inflammatory Skin Alterations with Mast Cell Accumulation. Histological analyses revealed epidermal hyperplasia (acanthosis) and inflammatory changes in the dermis of the skin sites of SDS/SpA-treated mice (Fig. 2*B*). The dermis was densely infiltrated with various subtypes of leukocytes, including eosinophils and mast cells (Fig. 2*B*). Focal accumulations of eosinophils were observed in the s.c. layer (Fig. 2*B Inset*). In contrast, mice treated with SDS alone showed only minor skin alteration (Fig. 2*B*).

The skin sites showed increased T cell numbers and MHC class II levels (Fig. 2*B*), suggesting cutaneous accumulation of IFN- γ (17). However, mice treated with SDS alone had nearly intact skin (Fig. 2*B*).

Plasma histamine levels closely paralleled the degree of mastocytosis observed (Fig. 2 *C* and *D*), indicating the presence of activated mast cells. However, serum levels of IgE were not elevated (Fig. 2*E*). Collectively, these results indicated that SDS/SpA-induced dermatitis is a good intrinsic animal model for AD and suggested that IgE is not principally involved in the development of this dermatitis.

Differentiation Toward Super T Helper Type 1 (Th1) Cells Producing Th1 Cytokine, IL-3, and IL-13, but Not IL-4. To address the mechanism underlying the up-regulated MHC class II expression, we investigated whether CD4⁺ T cells differentiated toward Th1 cells. CD4⁺ T cells from the regional lymph nodes (LNs) of treated mice at days

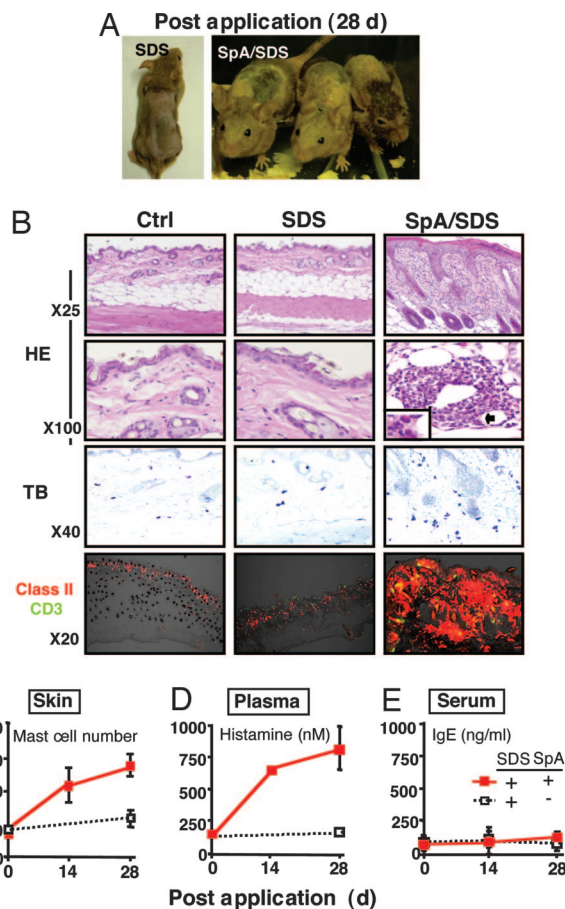


Fig. 2. SDS/SpA-induced AD. NC/Nga mice were treated daily with 4% SDS and subsequently with 200 μ g SpA. (A) Representative photographs at day 28. (B) Cutaneous sections prepared from mice treated with SpA and/or SDS for 28 days or from untreated mice (Ctrl) were stained with hematoxylin and eosin (HE) or toluidine blue (TB). The arrow denotes eosinophils, magnified in *Inset*. Frozen skin sections were incubated with phycoerythrin-conjugated MHC class II and FITC-conjugated anti-CD3. (C) Mast cells were counted in the skin sections. (D and E) Plasma and serum were sampled, and histamine (D) and IgE (E) were measured by ELISA.

1 and 2 produced larger amounts of IFN- γ than did those from untreated mice (Fig. 3A). The cells produced basal levels of IL-4 and IL-13, indicating rapid differentiation toward Th1 cells. Intriguingly, CD4⁺ T cells from mice treated for 7 days and longer gained the capacity to produce much larger amounts of IL-13 and IL-3 as well (Fig. 3A), indicating that the CD4⁺ T cells further differentiate into the unique cells that can produce IFN- γ , IL-3, and IL-13, but not IL-4. We propose to designate this subpopulation as “super Th1 cells” with regard to their unique potential to produce both Th1- and Th2-related proinflammatory cytokines, each of which is involved in various inflammatory tissue diseases (17–20). In contrast, CD4⁺ LN cells from SDS-treated mice showed a simple and moderate shift to Th1 cells (Fig. 3A), as was also the case for CD4⁺ LN cells from ovalbumin/SDS-treated mice that had no apparent dermatitis (data not shown). These results clearly indicate that the regional LN cells develop toward super Th1 cells after SDS/SpA application and suggest that the Th1-driving condition attainable with SDS treatment was insufficient for the development of AD.

Next, we investigated whether IL-12 and IL-18, prototype cytokines for Th1 cell development (21), are accumulated in treated mice. Regional LNs expressed *il12* after SDS/SpA challenge (Fig. 3B). Intriguingly, SDS by itself can induce *il12* expression (Fig. 3B),

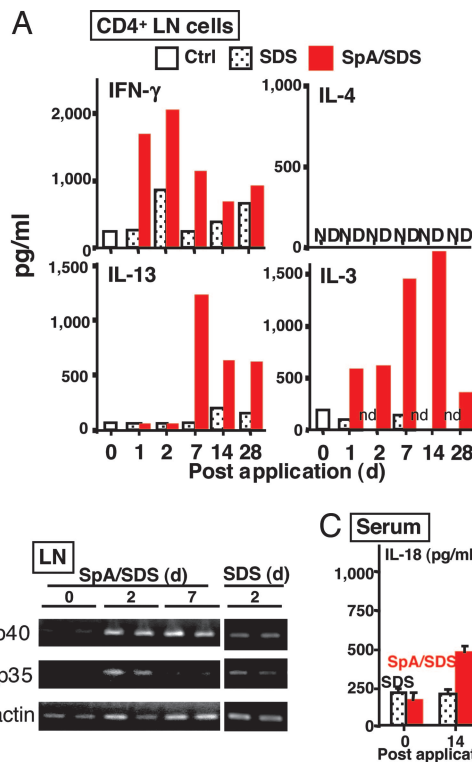


Fig. 3. Differentiation of regional LN CD4⁺ cells into super Th1 cells. NC/Nga mice were treated daily with SDS or SDS/SpA. CD4⁺ cells were isolated from the cervical and axillary LNs, pooled, and incubated with immobilized anti-CD3 for 48 hr. Concentrations of IFN- γ , IL-3, IL-4, and IL-13 were determined by ELISA (A). The cervical and axillary LNs (B) and sera (C) were sampled, and expressions of *il12 p40* and *il12 p35* (B) and IL-18 levels (C) were determined by RT-PCR and ELISA, respectively. Photographs are representative of three independent experiments with similar results. ND, not detected; nd, not done.

indicating the importance of SDS for IL-12 production. Consistent with our previous report (10), in NC/Nga (Fig. 3C), BALB/c, and C57BL/6 mice (data not shown), IL-18 accumulated in the circulation after application of SDS/SpA but not SDS alone.

Requirement of IL-18 for Super Th1 Cell Development. Our observations led us to assume that IL-18, together with IL-12, commits naive CD4⁺ T cells to the formation of super Th1 cells. To evaluate this assumption, we incubated naive NC/Nga CD4⁺ LN cells under Th1 condition supplemented with IL-18 (Th1 + IL-18), as described in *Methods*, and examined the cells' production of IFN- γ , IL-3, and IL-13 upon stimulation with anti-CD3. The CD4⁺ T cells primed under Th1 + IL-18 produced all of the cytokines except IL-4 (Fig. 4A), demonstrating and mimicking the potential for IL-18 to supershift toward super Th1 cells *in vivo*. Notably, super Th1 cells produced larger amounts of IL-3 and IL-13, but still no IL-4, after challenge with immobilized anti-CD3 + IL-18 (Fig. 4A). Consistent with our previous report (22), 8.5% of CD4⁺ T cells primed under Th1 + IL-18, but none of those primed under simple Th1 condition, expressed both Th1 cytokines (e.g., IFN- γ) and Th2 cytokines (e.g., IL-13) by cytoplasmic cytokine staining (data not shown). In a separate experiment, we observed that IFN- γ ⁺ cells purified from super Th1 cells by capturing secreted IFN- γ (22) produce IL-3 and IL-13 in response to immobilized anti-CD3 + IL-18 (data not shown). Thus, anti-CD3 stimulation synergizes with IL-18 to produce IL-3 and IL-13 selectively from super Th1 cells. BALB/c CD4⁺ LN cells differentiated into super Th1 cells when primed under Th1 + IL-18, confirming this as a super Th1 cell-inducing

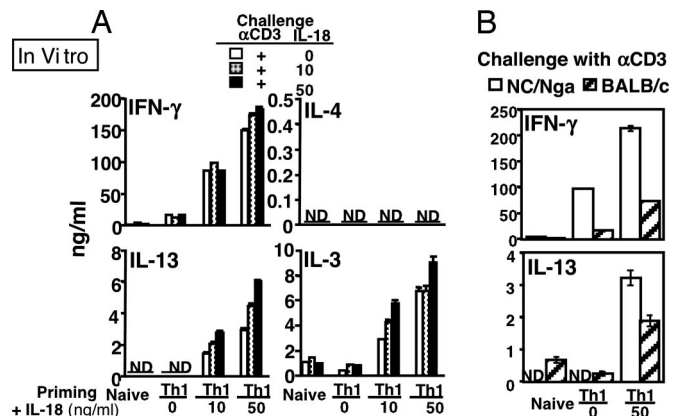


Fig. 4. Requirement of IL-12 and IL-18 for super Th1 cell development. CD4⁺ LN cells from naive NC/Nga (A and B) or BALB/c (B) mice were stimulated with various doses of IL-18 under Th1 cell conditions. The cells were freshly challenged with immobilized anti-CD3 with (A) or without (B) various doses of IL-18 for 48 hr, and cytokine concentrations were measured. ND, not detected.

condition (Fig. 4B). All of these results indicate that IL-18 is an inducer and activator of super Th1 cells.

Rapid Induction of IFN- γ , IL-13, IL-3, and Th1 Chemokines in the Skin Sites of Treated Mice. It is important to note that the treated skin sites quickly began to express the super Th1-associated cytokines and chemokines required for the recruitment of super Th1 cells (23, 24). We measured levels of these cytokines in the skin site homogenates. Concentrations of IFN- γ , IL-13, and IL-3, but not IL-4, were increased (Fig. 5A), indicating that the super Th1 cells are recruited and produce these cytokines *in vivo*.

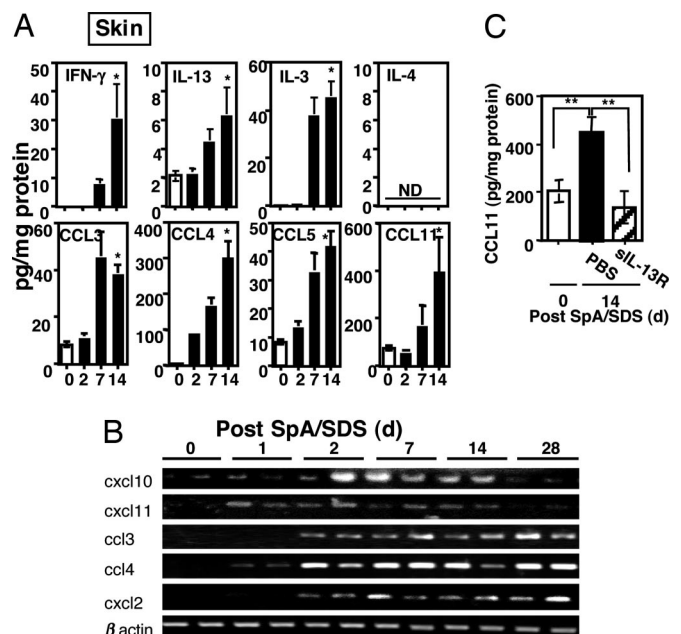


Fig. 5. Rapid production of chemokines in skin sites. (A) Cytokines and chemokine levels in skin homogenates were measured. Asterisks indicate significant increase ($P < 0.05$) of the individual molecules as compared with those at day 0. (B) Total RNA was extracted from the skin specimens and RT-PCR was performed. Photographs are representative of three independent experiments with similar results. (C) Mice were treated with or without SDS/SpA (open bar) and additionally treated with soluble IL-13R α 2-Fc (sIL-13R, hatched bar) or vehicle (PBS, filled bar). CCL11 concentrations in the skin homogenates at day 14 were measured. **, $P < 0.01$.

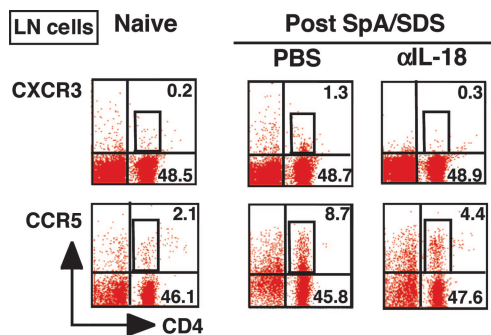


Fig. 6. IL-18-dependent CXCR3 and CCR5 expression on CD4⁺ LN cells. LN cells were prepared from naive mice or from PBS- or anti-IL-18-treated SD/SpA-challenged mice at day 2 or day 7 and examined for their expression of CD4/CXCR3 and CD4/CCR5 by FACS. Numbers in the upper right corner of each graph indicate the percentage of cells expressing both CD4 and corresponding chemokine receptor gated in a square. Numbers in the lower right corner indicate the percentage of CD4⁺ cells negative for these receptors.

SDS/SpA application simultaneously induced rapid production of CCL3 [macrophage inflammatory protein (MIP)-1 α], CCL4 (MIP-1 β), CCL5 [regulated on activation normal T cell expressed and secreted (RANTES)], and CCL11 (eotaxin) (Fig. 5 *A* and *B*), which can recruit various subtypes of leukocytes, including mast cells and eosinophils. This change was also the case for *ccl10* (*ip10*), *ccl11* (*i-tac*), and *ccl2*, which can recruit macrophages, T cells, and neutrophils (Fig. 5*B*). However, treatment with SDS alone did not induce these chemokine messages (data not shown). These results indicated the importance of SpA for induction of the expression of chemokines.

Because IL-13 is a potent stimulus for CCL11 production (25),

we investigated possible involvement of IL-13 in the elevated CCL11 expression. Treatment with IL-13 antagonist inhibited the elevation of cutaneous CCL11 (Fig. 5C), indicating that IL-13 is required for the production of CCL11.

IL-18 Induction of Chemokine Receptor Expressions on Super Th1 Cells.

Next, we investigated the mechanism underlying the recruitment of super Th1 cells into the skin sites. Because CXCR3 and CCR5 recognize CXCL10/CXCL11 and CCL3/CCL4/CCL5, respectively, we analyzed CXCR3 and CCR5 expression on the regional LN CD4⁺ cells. Both CXCR3 and CCR5 expressions were induced on the LN CD4⁺ cells after SDS/SpA application (Fig. 6).

We then examined the possible involvement of IL-18 in these chemokine receptor inductions. IL-18 blockade prevented induction of CXCR3 and CCR5 (Fig. 6), indicating the requirement of IL-18 for CXCR3 and CCR5 inductions on super Th1 cells.

Contribution of Endogenous IL-18 to the Development of AD. We

tested whether endogenous IL-18 is required for the development of SDS/SpA-induced AD. Mice treated with neutralizing anti-IL-18 had profoundly reduced AD scores, with little epidermal hyperplasia; little infiltration with T cells, eosinophils, and neutrophils; and basal levels of mast cells and MHC class II (Fig. 7A and B). To formally establish the essential role of endogenous IL-18, we applied SDS/SpA to *il18*^{-/-} BALB/c mice. Wild-type BALB/c mice exhibited AD-associated histological changes resembling those in NC/Nga mice, whereas *il18*^{-/-} BALB/c mice were nearly free from these changes (Fig. 7C), again pointing to IL-18 as a causative factor. These results indicated that endogenous IL-18 is essential for the development of SDS/SpA-induced AD.

Involvement of IL-3 in the Induction of Cutaneous Mastocytosis.

Because differentiation toward Th1 cells precedes differentiation toward super Th1 cells (Fig. 3A) and fundamentally requires IFN- γ

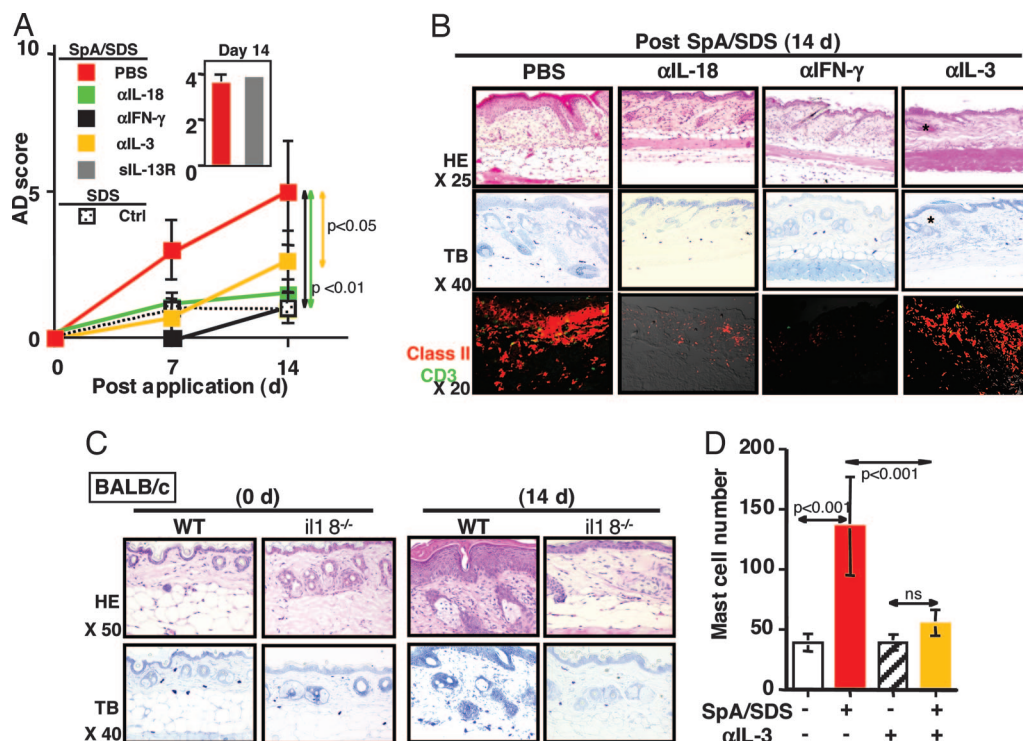


Fig. 7. IL-18 as a potent therapeutic target for SDS/SpA-induced AD. (A, B, D) NC/Nga mice were administered PBS, anti-IL-18 Abs (α IL-18), anti-IFN- γ Abs (α IFN- γ), or anti-IL-3 Abs (α IL-3) i.p. during SDS/SpA challenge. For control study, NC/Nga mice were treated with SDS alone. (A *Inset*) NC/Nga mice were administered PBS (red bar) or soluble IL-13R α 2-Fc (sIL-13R, gray bar) i.p. (A) AD sores were determined. (B) Histological and immunohistological analyses were performed. Asterisks indicate the sites with epidermal hyperplasia of anti-IL-3-treated, SDS/SpA-challenged mice. (C) Wild-type or *il18*^{-/-} BALB/c mice were examined on day 0 (*Left*) or were treated daily with SDS/SpA for 14 days (*Right*). (D) Mast cell numbers were counted. ns, not significant.

(17), we investigated the role of IFN- γ . Treatment with neutralizing anti-IFN- γ protected against the development of AD concomitant with inhibition of elevated MHC class II expression (Fig. 7*A* and *B*), indicating the requirement of IFN- γ for AD development. To investigate how super Th1 cells are involved, we examined the roles of cytokines produced by the super Th1 cells but poorly by Th1 cells. Although treatment with IL-13 antagonist profoundly inhibited the elevation of CCL11 (Fig. 5*C*), this treatment did not reduce the AD scores until day 14 (Fig. 7*A Inset*), indicating a minor role for IL-13. Because IL-3 induces mast cell proliferation/activation (20) synergistically with IL-18 (26), we investigated the possible involvement of IL-3. Blockade of IL-13 profoundly inhibited mastocytosis (Fig. 7*D*) and substantially protected against AD development (Fig. 7*A* and *B*). On the basis of all our observations, we conclude that super Th1 cells contribute to the development of AD at least partly by producing IL-3 in response to IL-18.

Discussion

We generated an intrinsic AD mouse model by the application of detergent and subsequently SpA. SDS treatment induced the expression of IL-12, and possibly other cytokines such as IL-23. Topical application of SpA induces the release of IL-18 from keratinocytes. We have provided evidence that IL-18 contributes to intrinsic AD development by inducing and activating super Th1 cells that have the potential to produce IFN- γ , IL-13, and IL-3.

Th1 responses negatively regulate Th2 responses, and vice versa. Theoretically, AD might be classified into two types: Th1 and Th2. However, individual AD patients often have mixed-type immunity (3). As in clinical AD, NC/Nga mice can develop Th1, Th2, or mixed-type AD. The mice develop Th2-type AD when kept under conventional conditions (13, 14); however, they show mixed-type AD after application of SDS/SpA under SPF conditions. In the strict sense, our SDS/SpA-induced AD model can be classified as a super Th1 type (Figs. 3*A* and 4). Therefore, it is intriguing to note that this super Th1 cell subset alone may explain the complicated immunopathology of some types of clinical AD.

Coproduction of IL-13 and IFN- γ is a hallmark for certain types of clinical AD. Indeed, lymphocytes from intrinsic AD patients produce greater amounts of IFN- γ and IL-13 than those from healthy individuals (27). There are two possible explanations for this outcome. One is that these patients have both Th1 and Th2 cells. Alternatively, the patients may have super Th1 cells. It is quite important to distinguish the latter from the former. Our present results suggest that susceptibility to intrinsic AD is at least partly determined by the intrinsic nature of T lymphocytes to preferentially develop into super Th1 cells. In fact, highly susceptible (NC/Nga) and moderately susceptible (BALB/c) mice showed robust and moderate differentiation toward super Th1 cells, respectively (Fig. 4*B*), whereas resistant C57BL/6 mice failed to develop super Th1 cells (data not shown). Notably, in both highly and moderately susceptible strains, IL-18 is commonly essential for the development of AD (Fig. 7). For patients who have predominantly super Th1 cells, a treatment targeting IL-18 might be very beneficial.

The results of our present study provide evidence that a Th1-skewing milieu can induce allergic inflammatory diseases by differentiating naive CD4⁺ T cells into super Th1 cells, in collaboration with IL-18. Although they are a major effector cell population involved in IgE-mediated allergic inflammatory diseases, mast cells can be activated by IL-3 and IL-18 in the absence of crosslinkage of their Fc ϵ RI (20, 28–30). Super Th1 cells can be involved in dermal mastocytosis through production of IL-3 in response to Ag and IL-18 (Figs. 3*A*, 4, 5*A*, and 7). Thus, it might be IL-3 that links the Th1-driving circumstances to mast cell involvement in allergic inflammatory diseases.

Super Th1 cells characteristically produce IL-13, a central effector cytokine for the development of inflammatory tissue diseases such as bronchial asthma (18, 31–33). However, IL-13 is not

intensely involved in the development of SDS/SpA-induced AD, at least in its acute phase (Fig. 7*A*). IL-13 is a potent stimulator and activator of transforming growth factor β 1, a fibrogenic cytokine (31). Therefore, it is plausible that IL-13 produced by super Th1 cells is involved in the late-occurring dermal fibrosis of SDS/SpA-induced AD.

SDS/SpA-treated mice showed major clinical features of AD, indicating the suitability of SDS/SpA-induced dermatitis as an AD model. Furthermore, skin lesions of SDS/SpA-treated mice closely resemble those of clinical AD in terms of dense infiltration of eosinophils and mast cells (3, 34) and elevated histamine levels (35). SDS/SpA-treated mice showed little increase in serum IgE (Fig. 1*E*), suggesting that our model characterizes an intrinsic AD. Although it is important to know that dermal mastocytosis and elevated histamine levels, both of which are major features of this model, are more prominent in clinical intrinsic AD when compared with extrinsic AD, no appropriate clinical reports properly address this issue. Further clinical studies are needed to determine whether this model represents an intrinsic AD in the strict sense.

Notably, SpA cannot be replaced by conventional protein Ags (data not shown), suggesting that SpA might perform several functions that ovalbumin or BSA cannot. First, SpA can stimulate keratinocytes to release IL-18 (10). Second, SpA seems to directly cause rapid expression of chemokines in the skin sites (Fig. 5), as it does in the respiratory tract (9). Thus, SpA can function as a professional AD-inducing protein in certain situations, which presents the possibility that molecules that can induce IL-18 and chemokine release from epidermal cells might function as an intrinsic AD-inducing factor. Further study is needed to validate such conclusions.

We also observed that IL-18 blockade, or absence, inhibited SDS/SpA-induced AD. This finding suggests the importance of IL-18 as a therapeutic target molecule for certain atopic diseases.

Methods

Mice. NC/Nga, BALB/c, and C57BL/6 mice were purchased from Oriental Yeast (Osaka). *il18*^{−/−} BALB/c mice have been described elsewhere (21). Five to seven female mice, 6–10 weeks of age, were used in each *in vivo* study as experimental and control groups. All mice were kept under SPF conditions and received humane care, as outlined in the *Guide for the Care and Use of Laboratory Animals* (36).

Reagents. SpA from *S. aureus* Cowan I was purchased from Calbiochem. Anti-CD28, anti-CD3, anti-CD4, and anti-MHC class II were from Pharmingen. Hybridomas producing neutralizing anti-IFN- γ mAb (R6A2) or anti-IL-4 mAb (11B11) were from American Type Culture Collection. Neutralizing polyclonal anti-IL-18 Abs were described elsewhere (22). Neutralizing polyclonal anti-IL-3 Abs, soluble IL-13R α 2-Fc chimera, rIL-12, and rIL-2 were from R & D Systems. Recombinant mouse IL-18 was from MBL (Nagoya, Japan).

Induction of AD. Mice were treated with 4% SDS in sterile distilled water on a 5-cm² area of their shaven backs, and 30 min later with SpA in sterile distilled water, once a day. In some experiments, mice received anti-IL-18 (500 μ g), anti-IFN- γ (500 μ g), soluble IL-13R α 2-Fc (100 μ g), or anti-IL-3 (500 μ g) on day 0 and every 5 days after challenge with SDS/SpA. We used *in vivo* a neutralizing dose of anti-IL-3 Abs or IL-13R α 2-Fc in accordance with the supplier's instructions or on the basis of our previous study (22). Serum, plasma, skin specimens, and axillary and cervical LNs were sampled. In some experiments, skin homogenates were prepared as described in ref. 10. A clinical AD score was determined as described in ref. 13.

Histological Study. Sections from skin specimens were stained as detailed in ref. 4. In some experiments, mast cell numbers in 10

Table 1. Primer sequences for cytokines and chemokines

Cytokine/ chemokine	Sense primer	Antisense primer	No. of PCR cycles
IL-12p35	AGACGCTTTGATGATGACC	TCACTCTGAAGGGTCTGCT	35
IL-12p40	CGTGCTCATGGCTGGTGCAAG	GAACACATGCCCACTTGCTG	35
CXCL10	ACCATGAACCCAAGTGCTGCCGTC	GCTTCACTCCAGTTAAGGAGCCCT	35
CXCL11	TCTGCTGTCTTGGAAACATGC	CGTGTGCCTCGTGATATTTG	35
CXCL2	GAAGTCATAGCCACTCTCAAGGGGC	CAACTCACCTCTCCCAGAAAC	35
CCL3	CCTCTGTCACTGCTCAACA	CTGCCTCAAGACTCTCAGG	35
CCL4	CCCACTTCTGCTGTTTCTC	GGGAGACACGCTCTATAA	35
β -Actin	GATGACGATATCGTGCCTG	GTACGACCAGAGGCATACAGG	28

low-power fields selected randomly were counted (Figs. 2C and 7D).

Confocal Laser Microscopic Analysis. Frozen sections from freshly isolated skin specimens were fixed and incubated with FITC-and/or phycoerythrin-conjugated mAbs ($\times 50$), followed by evaluation using a laser scanning confocal microscope (model IX81; Olympus, Tokyo).

In Vitro Super Th1 Cell Differentiation. Magnetic cell sorting system-enriched CD4⁺ LN cells from untreated mice were incubated with 10 ng/ml rIL-12, 10 units/ml IL-2, and 10 μ g/ml anti-IL-4 in the presence of rIL-18 in complete RPMI medium 1640 in an anti-CD3- (5 μ g/ml) and anti-CD28 (1 μ g/ml)-coated dish. Two-round cell culture was undertaken, and the cells collected were freshly incubated with immobilized anti-CD3 plus IL-18 for 48 hr.

Expression of CXCR3/CCR5 on CD4⁺ LN Cells. Cervical and axillary LN cells prepared from NC/Nga mice at day 2 or 7 after application were incubated with allophycocyanin-conjugated anti-CD4 and phycoerythrin-conjugated anti-CXCR3 (Pharmingen) or with a battery of goat anti-CCR5 (Zymed) and FITC-conjugated anti-goat IgG, respectively (10).

ELISA for Cytokines, Chemokines, IgE, and Histamine. Concentrations of IL-4, IL-13, IL-3, and IFN- γ were determined with ELISA kits (Genzyme) or Mouse 23 Plex Panel (Bio-Rad). Histamine and IL-18 levels were determined with ELISA kits from Immunotech (Marseille, France) and MBL, respectively. Serum IgE was measured as described in ref. 4.

RT-PCR. Total RNA was extracted, and RT-PCR was performed (10). Primers for cytokines and chemokines, and the number of PCR cycles, are shown in Table 1.

Statistical Analysis. Data are shown as the mean $\pm$ SD of triplicate samples. Significance of differences between the experimental and control groups was examined by the unpaired Student's *t* test. The data are representative of three independent experiments with similar results.

We thank Drs. N. Hayashi and J. Sawaki at Hyogo College of Medicine for enthusiastic discussion and Ms. S. Yumikura-Futatsugi and Ms. N. Nakano for excellent technical assistance. This work was supported in part by grants, including a Hitec Research Center Grant, from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

- Leung, D. Y. M. & Bieber, T. (2003) *Lancet* **361**, 151–160.
- Kupper, T. S. & Fuhlbrigge, R. C. (2004) *Nat. Rev. Immunol.* **4**, 211–222.
- Leung, D. Y. M., Boguniewicz, M., Howell, M. D., Nomura, I. & Hamid, Q. A. (2004) *J. Clin. Invest.* **113**, 651–657.
- Konishi, H., Tsutsui, H., Murakami, T., Yumikura-Futatsugi, S., Yamanaka, K., Tanaka, M., Iwakura, Y., Suzuki, N., Takeda, K., Akira, S., et al. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 11340–11345.
- Tsutsui, H., Yoshimoto, T., Hayashi, N., Mizutani, H. & Nakanishi, K. (2004) *Immunol. Rev.* **202**, 115–138.
- Tanaka, T., Tsutsui, H., Yoshimoto, T., Kotani, M., Matsumoto, M., Fujita, A., Wang, W., Higa, S., Kishimoto, T., Nakanishi, K. & Suemura, M. (2001) *Int. Arch. Allergy Immunol.* **125**, 236–240.
- Ohnishi, H., Kato, Z., Watanabe, M., Fukutomi, O., Inoue, R., Teramoto, T. & Kondo, N. (2003) *Allergol. Int.* **52**, 123–130.
- Novak, N., Kruse, S., Potreck, J., Maintz, L., Jennecke, C., Weidinger, S., Fimmer, R. & Bieber, T. (2005) *J. Allergy Clin. Immunol.* **115**, 828–833.
- Gómez, M., Lee, A., Reddy, B., Muir, A., Soong, G., Pitt, A., Cheung, A. & Prince, A. (2004) *Nat. Med.* **10**, 842–848.
- Nakano, H., Tsutsui, H., Terada, M., Yasuda, K., Matsui, K., Yumikura-Futatsugi, S., Yamanaka, K., Mizutani, H., Yamamura, T. & Nakanishi, K. (2003) *Int. Immunol.* **15**, 611–621.
- Kiriya, T., Sugiura, H. & Uehara, M. (2003) *J. Dermatol.* **30**, 708–712.
- Aioi, A., Tonogaito, H., Suto, H., Hamada, K., Ra, C., Owaga, H., Maibach, H. & Matsuda, H. (2001) *Br. J. Dermatol.* **144**, 12–18.
- Matsuda, H., Watanabe, N., Geba, G. P., Sperl, J., Tsudzuki, M., Hiroi, J., Matsumoto, M., Ushio, H., Saito, S., Askenase, P. W. & Ra, C. (1997) *Int. Immunol.* **9**, 461–466.
- Vestergaard, C., Yoneyama, H., Murai, M., Nakamura, K., Tamaki, K., Terashima, Y., Imai, T., Yoshie, O., Irimura, T., Mizutani, H. & Matsushima, K. (1999) *J. Clin. Invest.* **104**, 1097–1105.
- Yagi, R., Nagai, H., Igo, Y., Akimoto, T., Arai, T. & Kubo, M. (2002) *J. Immunol.* **168**, 2020–2027.
- Tsai, J.-C., Shen, L.-C., Sheu, H.-M. & Lu, C.-C. (2003) *Arch. Dermatol. Res.* **295**, 169–174.
- Boehm, U., Klamp, T., Groot, M. & Howard, J. C. (1997) *Annu. Rev. Immunol.* **15**, 749–795.
- Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, Y., Karp, C. L. & Donaldson, D. D. (1998) *Science* **282**, 2258–2261.
- Kuperman, D. A., Huang, X., Koth, L. L., Chang, G. H., Dogganov, G. M., Zhu, Z., Elias, J. A., Sheppard, D. & Erle, D. J. (2002) *Nat. Med.* **8**, 885–889.
- Shelburne, C. P. & Ryan, J. J. (2001) *Immunol. Rev.* **179**, 80–93.
- Takeda, K., Tsutsui, H., Yoshimoto, T., Adachi, O., Yoshida, N., Kishimoto, T., Okamura, H., Nakanishi, K. & Akira, S. (1998) *Immunity* **8**, 383–390.
- Sugimoto, T., Ishikawa, Y., Yoshimoto, T., Hayashi, N., Fujimoto, J. & Nakanishi, K. (2004) *J. Exp. Med.* **199**, 535–545.
- Rossi, D. & Zlotnik, A. (2000) *Annu. Rev. Immunol.* **18**, 212–242.
- Rot, A. & von Andrian, U. H. (2004) *Annu. Rev. Immunol.* **22**, 891–928.
- Zhu, Z., Homer, R. J., Wang, Z., Chen, Q., Geba, G. P., Wang, J., Zhang, Y. & Elias, J. A. (1999) *J. Clin. Invest.* **103**, 779–788.
- Yoshimoto, T., Tsutsui, H., Tominaga, K., Hoshino, K., Okamura, H., Akira, S., Paul, W. E. & Nakanishi, K. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 13962–13966.
- Simon, D., Borelli, S., Braathen, L. & Simon, H. (2002) *Allergy* **57**, 431–435.
- Hamaguchi, Y., Kanakura, Y., Fujita, J., Takeda, S., Nakano, T., Tarui, S., Honjo, T. & Kitamura, Y. (1987) *J. Exp. Med.* **165**, 268–273.
- Matsuura, N. & Zetter, B. R. (1989) *J. Exp. Med.* **170**, 1421–1426.
- Galli, S. J., Kalensnikoff, J., Grimaldeston, M. A., Piliponsky, A. M., Williams, C. M. M. & Tsai, M. (2005) *Annu. Rev. Immunol.* **23**, 749–786.
- Wynn, T. A. (2003) *Annu. Rev. Immunol.* **21**, 425–456.
- Ford, J. G., Rennick, D., Donaldson, D. D., Venkayya, R., McArthur, C., Hansell, E., Kurup, V. P., Warnock, M. & Grünig, G. (2001) *J. Immunol.* **167**, 1769–1777.
- Wills-Karp, M. (2004) *Immunol. Rev.* **202**, 175–190.
- Mihm, M. C., Jr., Soter, N. A., Dvorak, H. F. & Austen, K. F. (1976) *J. Invest. Dermatol.* **67**, 305–312.
- Steinhoff, M., Neisius, U., Ikoma, A., Fartasch, M., Heyer, G., Skov, P. S., Luger, T. A. & Schmelz, M. (2003) *J. Neurosci.* **23**, 6176–6180.
- Institute of Laboratory Animal Resources, National Research Council (1996) *Guide for the Care and Use of Laboratory Animals* (Natl. Acad. Press, Washington, DC).