Table 1 Characterization of mast cells and basophils

	Mast cells	Basophils
Development	Bone marrow	Bone marrow
Distribution	Peripheral tissues	Blood
Survival time	Long (weeks)	Short (2–5 days)
Surface marker	FcεRIα$^+$, CD117 (c-kit)$^+$	FcεRIα$^+$, DX5$^+$, CD34$^+$

conditional depletion systems. Recently, new types of genetically modified mouse strains have been developed, and previously proposed functions of mast cells and basophils have been revisited extensively. In this review, we summarize these recent studies of mast cells and basophils and focus on their role in cutaneous immune response.

Mast cell-specific depletion model

The selective depletion of mast cells or basophils *in vivo* is a useful approach through which the role of these cell types during immune responses is addressed. Classical models used to study the mast cell functions are based on Kit-mutant mouse strains. In addition to their mast cell defect, *Kit$^{W/Wv}$* mice have multiple hematopoietic abnormalities that include compromised fitness of the hematopoietic stem and progenitor cells (10), severe macrocytic anemia (11), impaired T development in the thymus (11), and a shift in intraepithelial T cells in the gut in favor of T-cell receptor (TCR) $\alpha\beta^+$ cells and against TCR $\gamma\delta^+$ cells (12). Importantly, this *Kit* mutant is neutropenic, which may be a major factor affecting immune responses in this strain (13).

New mouse models have recently been developed to avoid these problems. Several groups have reported the generation of mice expressing Cre recombinase under the control of mast cell protease genes (14–16) (Table 2). In 2011, four laboratories used their mice or additionally generated lines to obtain Kit-independent mast cell-deficient mouse strains (6, 17–19). These strains differ in the selected gene loci as well as in the methods to drive ectopic gene expression in mast cells (targeted knock-in or transgenic overexpression) and in depletion mechanisms. Dudeck et al. generated a mast cell depletion model, which is based on *mast cell protease (Mcpt) 5-Cre* transgenic mice crossed with *i diphtheria toxin receptor (iDTR)* line (17). In the *Mcpt5-Cre$^+$iDTR$^+$* mice, a single injection of DT results in a complete loss of peritoneal mast cells but not skin mast cells. For the depletion of mast cells in ear skin, *Mcpt5-Cre$^+$iDTR$^+$* mice need additional local injection of DT (17). The constitutive mast cell depletion model was generated with *Mcpt5-Cre* mice crossed with the *R-DTA* line (20). Both models exhibited no depletion in other cell types. Lilla et al. (19) crossed mice carrying the *Cpa3-Cre* transgene with mice in which the first exon of myeloid cell leukemia sequence 1 (Mcl-1) is flanked by LoxP sites and generated constitutive mast cell deficiency. These mice exhibit reductions in mast cell number in all tissues but also marked reduction in basophils, splenic neutrophilia, and macrocytic anemia. Feyerabend et al. (18) also generated a mast cell deficiency model using *Cpa3-Cre* transgenic mice, which exhibit reductions in mast cells and basophils. Considering the phenotype of these mast cell depletion models, Cpa3 might play some roles in the development of both mast cell and basophils. We also reported Mas-TRECK Tg mice using DTR transgene under the control of 5′ enhancer, promoter, and intronic enhancer of IL-4 (6). After DT treatment of the mice, both mast cells and basophils are depleted, but basophils are restored earlier than mast cells.

Basophil-specific depletion model

Due to the lack of any natural mouse mutants with basophil deficiencies, antibodies have often been used to study the contribution of basophils in different experimental settings. These antibodies recognize either FcεRI (clone MAR-1) or the orphan-activating receptor CD200 receptor 3 (CD200R3) (clone Ba103), which are both mainly expressed by basophils and mast cells. Although both antibody clones can efficiently deplete basophils, they can also activate mast cells (21, 22). Furthermore, the depletion of basophils by Ba103 is FcR dependent and might therefore activate myeloid cells and natural killer (NK) cells (23). MAR-1 also depletes a subset of FcεRI-expressing dendritic cells (DCs) (24). Several new mouse strains with a constitutive or inducible depletion of basophils have recently been generated (Table 2). *Mcpt8* is a basophil-specific gene in the conserved chymase locus (25). Three groups generated the basophil depletion models by taking advantage of this gene regulation (21, 26, 27). In these mice, basophils, but not mast cells or other cell types, were depleted in blood and spleen. Mukai et al. (28) reported a different basophil depletion model, in which the N-terminal sequences for P1-Runx were replaced with neo^r gene, resulting in the absence of both P1-Runx1 transcriptions and protein. These mice showed a severe reduction in basophils, but not eosinophils, neutrophils, or mast cells. Sawaguchi et al. (29) reported Bas-TRECK Tg mice using DTR transgene under the control of 5′ enhancer, promoter, and intronic enhancer of IL-4. Whereas new mast cell depletion models exhibited marginal effects on basophil depletion, new basophil depletion models seem to have no effect on other immune cell depletion.

Mast cell and/or basophil involvement in several skin diseases

Various models of severe inflammatory autoimmune diseases reveal that neutrophil infiltration into the sites of local inflammation and tissue destruction critically depends on mast cells. Involvements of mast cells have been suggested in several animal disease models, such as psoriasis, rheumatoid arthritis (RA) (30–32), and bacterial infection (33, 34). These observations seem to be highly relevant in terms of our understanding of human diseases, as large numbers of activated mast cells infiltrate the tissues in the corresponding human diseases, such as allergic contact dermatitis, psoriasis, and RA (35–37). In addition to these diseases, mast cells are involved in multiple inflammatory and malignant diseases (Table 3).

Table 2 *Summary of mast cell- and basophil depletion models*

Mouse strain	Description	Model of depletion	Deleted population	Remarks	Refs
Mast cells					
Mcpt5-Cre	BAC transgene (129 kb, Cre inserted after the Mcpt5 start codon)	Cross with Cre-iDTR mice or R-DTA mice.	CTMCs (>90%)	MMCs and basophils are not deleted	(16, 17)
Cpa3-Cre	Promoter transgene	Mcl-1$^{fl/fl}$ mice causes impaired survival	CTMCs and MMCs (>90%), basophils (60–80%)	Mice develop splenic neutrophilia and macrocytic anemia	(19)
Cpa3^{Cre}	Knock-in of Cre before the first exon of Cpa3	Constitutive depletion	CTMCs and MMCs (100%), basophils (60% in the spleen)	NA	(18)
Mas-TRECK	DTR transgene (under the control of 5′ enhancer, promoter, and intronic enhancer of IL4)	DT injection	CTMCs, MMCs, and basophils (90–100%)	Basophils are restored two weeks after DT treatment	(6, 29)
Basophils					
Basoph8	Knock-in of IRES-YFP-Cre cassette before the Mcpt8 start codon	Cross with R-DTA mice	Basophils (>90%)	NA	(26)
Mcpt8-Cre	BAC transgene (228 kb, Cre inserted after the Mcpt8 start codon)	Constitutive depletion	Basophils (>90%)	NA	(21)
Mcpt8^{DTR}	Knock-in of IRES-DTR-EGFP cassette in 3′ UTR of Mcpt8	DT injection	Basophils (>90%)	NA	(27)
P1-Runx1	Knockout	P1-Runx1 seems to be important for the basophil lineage	Basophils (>90%)	NA	(28)
Bas-TRECK	DTR transgene (under the control of 5′ enhancer, promoter, and intronic enhancer of IL4)	DT injection	Basophils (>90%)	NA	(29)

BAC, bacterial artificial chromosome; BMMCs, bone marrow-derived mast cells; CTMCs, connective tissue mast cells; DTR, diphtheria toxin receptor; EGFP, enhanced green fluorescent protein; iDTR, inducible DTR; IL4, interleukin-4; IRES, internal ribosome entry site; P1-RUNX1, distal promoter-derived runt-related transcription factor 1; Mcpt, mast cell protease; MMCs, mucosal mast cells; NA, not applicable; NR, not reported; R-DTA, ROSA-diphtheria toxin-α; UTR, untranslated region; YFP, yellow fluorescent protein.

On the other hand, it is still largely unclear in which skin diseases basophils are involved. Recent studies have shown infiltration of basophils in several skin diseases, such as atopic dermatitis (AD), prurigo, and urticaria (38). It is notable that the skin lesions of bullous pemphigoid, classical eosinophilic pustular folliculitis (Ofuji's disease), and Henoch–Schönlein purpura also frequently show tissue basophilia (38–41) (Table 4).

The role of mast cells during AD pathogenesis

Atopic dermatitis is characterized by skin inflammation, impaired skin barrier function, and IgE-mediated sensitization to food and environmental allergens. The etiology of this disease is not yet understood completely, but it is multifactorial; the disease, moreover, is characterized by complex interactions between genetic and environmental factors. Recently, two major hypotheses have come to the fore as possible explanation for the pathogenesis of this heterogeneous disease: (I) One assumes that the primary defect is an immune dysregulation that causes Th2-predominant inflammation and IgE-mediated sensitization(42). In the other hypothesis (II), an intrinsic defect in skin barrier function such as a filaggrin mutation is underscored as a primary cause of the disease(43, 44). In the latter scenario, even the uninvolved phase of the disease presents cutaneous hypersensitivity of nonlesional skin, which results from a defective skin barrier that allows the penetration of allergens and microbial pathogens(44).

As most studies have shown increased numbers of mast cells in skin lesions in human AD and mouse AD models, it is generally assumed that the mast cells contribute to skin inflammation. However, few studies have directly addressed whether, to what extent, or by what mechanism, mast cells play a role in the development of AD pathogenesis. IL-31, a

Table 3 Dermatological diseases with evidence for mast cell involvement

Urticaria (92)
Localized mastocytoma (93)
Systemic mastocytosis (93)
Contact dermatitis (6)
Psoriasis and psoriasis arthritis (94, 95)
Atopic dermatitis (96)
Bullous autoimmune diseases: bullous pemphigoid (97)
Autoimmune vasculitis (98)
Systemic lupus erythematodes (99)
Systemic sclerosis and morphea (100)
Chronic graft-*vs*-host disease (101)
Morbus Morbihan and rosacea (102)
Skin infections: bacteria, fungi, parasites (Leishmania major) (103, 104)
Skin tumors: basal cell carcinoma, spinocellular carcinoma, angiosarcoma (105)

Table 4 Dermatological diseases with evidence for basophil infiltration

Atopic dermatitis (38)
Prurigo (38)
Urticaria (38)
Pemphigus vulgaris (38)
Drug eruption (38)
Henoch–Schönlein purpura (38)
Insect bite: tick bite (41)
Scabies (38)
Dermatomyositis (38)
Eosinophilic pustular folliculitis (40)
Leprosy: LL type (39)
Bullous pemphigoid (106)
Eosinophilic folliculitis (106)
Wells' syndrome (106)

4-helix-bundle cytokine, is a new candidate of itch mediator, preferentially produced by T cells skewed toward Th2 (45). Leukocytes in patients with AD expressed significantly higher IL-31 levels compared with those of control subjects, and serum IL-31 levels correlate with disease activity in AD (46–48). It was reported that the number of IL-31-positive mast cells was increased in the lesional skin with AD patients, and human mast cell lines upregulated IL-31 in the presence of antimicrobial peptides that were highly detected in the AD skin lesion (49, 50). These results provide evidence that mast cells may be involved in the pathogenesis of AD.

In mice, epicutaneous application of ovalbumin (OVA) protein is widely used for the induction of AD-like dermatitis. Although skin inflammation induced by epicutaneous application of OVA is comparable in wild-type and $Kit^{W/W-v}$ mice (51), skin inflammation induced by cutaneous sensitization with cedar pollen antigens was abolished in $Kit^{W/W-v}$ and $Kit^{Sl/Sl-d}$ mice (52). This cedar pollen dermatitis model was found to be independent of signal transducer and activator of transcription 6 (Stat6) and IgE but dependent on CRTH2 (chemo-attractant receptor homologous molecule expressed on Th2 cells), a PGD_2 receptor. However, mast-cell-reconstitution experiments have not been performed in either study. Interestingly, a recent study showed that FcεRI and FcγR are involved in a cutaneous OVA patch model(53). However, the involved cell type was not investigated in this study. Analysis using new cell-specific depletion models may answer this question.

The role of basophils during allergic skin response

The development of allergic reactions of the skin seems to be associated with basophil recruitment and activation (38). While the effector functions of basophils in allergic airway inflammation have been well studied (54), the roles of basophils in the allergic skin reactions remain largely unclear. Recent findings using basophil-specific depletion models have revealed the essential roles of basophils in allergic skin response.

The development of Th2 responses is one of the most pivotal steps during allergic skin reaction. Basophils have been proposed to play a crucial role for Th2 cell differentiation in mice (55–57). It has been reported that $CD49b^+$ $Fc\varepsilon RI^+$ c-Kit^- basophils migrate into draining LNs from the skin site of papain injection and thus act as antigen-presenting cells (APCs) by taking up and processing antigens (56). In addition, basophils express MHC class II and costimulatory molecules and secrete IL-4 and thymic stromal lymphopoietin (TSLP), which are critical for Th2 development. Therefore, basophils alone are considered to induce Th2 polarization from naïve T cells without requiring DCs under certain conditions. In contrast, another group has found that IL-4-producing basophils were recruited to the mediastinal LNs upon primary exposure to house dust mites. In this case, they contributed to the strength of the Th2 response in the lungs, but, in this model, basophils could not present antigens or express the chaperones involved in antigen presentation (24). Therefore, the authors claimed that DCs were necessary and sufficient for inducing Th2 immunity to house dust mites in the lungs without the requirement of basophils. It has consistently been reported that Th2 responses are severely impaired either after *Schistosoma mansoni* egg injection or during active *Schistosoma mansoni* infection by the depletion of $CD11c^+$ cells but not by the depletion of basophils using anti-FcεRIα antibody (58). Therefore, the role of basophils in the development of the Th2 response has been controversial.

We have demonstrated that basophils are responsible for cutaneous Th2 skewing to haptens and peptide antigens but not protein antigens using Bas-TRECK Tg mice, a new basophil-deficient model (7). Interestingly, basophils expressed MHC class II, CD40, CD80, CD86, and IL-4 in the hapten-induced cutaneous Th2 model. *In vitro* experiment, we showed that basophils could not take up or process OVA protein sufficiently using the DQ-OVA system. We assume that the discrepancy stems not from the different routes of antigen administrations but from the different types of

antigens used such as proteins, peptides, and haptens. Hapten antigens may bind to MHC class II on the surface of basophils directly, and peptides can be acquired and presented by basophils, while protein antigens are not presented efficiently by basophils because the protein is hardly digested by basophils. In fact, previous reports have demonstrated that basophils promote Th2 induction using OVA peptide but not OVA protein *in vitro* (56, 57). The protease allergen papain reaches the LNs after cutaneous immunization and induces MHC class II expression on basophils in accord with the preparation of OVA peptide antigens from OVA protein *in vivo* (56). Another group has reported that basophils pulsed with anti-2, 4-dinitrophenyl (DNP-IgE) exhibit enhanced Th2 skewing upon exposure to DNP-conjugated OVA by taking up DNP-OVA–IgE anti-DNP immune complexes (57). Although house dust mites also contain cysteine protease activity, they are not sufficient for Th2 induction as they do not upregulate MHC class II on basophils in this model even though cysteine protease may work to prepare peptide antigens from protein antigens *in vivo* (24).

Additionally, our group reported that basophils increase the frequency of IL-4-positive $CD4^+$ T cells by the aid of DCs (7). As basophils cannot take up or process protein antigens efficiently, DCs may prepare peptides to be presented by basophils or may promote basophils to produce IL-4 to skew Th2. In line with this, our study has demonstrated that Langerhans cells, an epidermal DC subset, mediate epicutaneous sensitization with OVA protein antigens to induce Th2-type immune responses (59). In addition, Th2 reaction was reduced upon sensitization with protein antigens or schistosome infections in a CD11c-depletion model (24, 58); therefore, DCs seem to be necessary for Th2 induction both *in vivo* and *in vitro* upon protein antigen exposure. Given that basophils were found in the vicinity of T-cells in the T-cell zone of the draining LNs, it is possible that basophils, T-cells, and DCs promote Th2 induction in a coordinated way. It would be intriguing to further evaluate whether DCs present peptides to basophils directly or by trogocytosis for the transfer of plasma membrane fragments from APCs to lymphocytes.

Although murine basophils have been reported to function as APCs, subsequent studies investigating the role of basophils as APCs in human subjects have been less clear. It has recently been demonstrated that human basophils also express MHC class II (60, 61) and that MHC class II expressing basophils were incapable of inducing antigen-specific T-cell activation or proliferation upon house dust mite allergen as antigens (61). Therefore, future studies are needed to determine the clinical significance of human basophils as APCs.

The role of mast cells during contact hypersensitivity

Contact hypersensitivity (CHS) has been widely used as a model to study cutaneous immune responses, as it is a prototype of delayed-type hypersensitivity mediated by antigen-specific T cells (62, 63). Contact hypersensitivity is classified into a sensitization phase and an elicitation phase. An essential step in the sensitization phase for CHS is the migration of hapten-bearing cutaneous DCs, such as epidermal Langerhans cells (LCs) and dermal DCs, into the skin-draining lymph nodes (LNs). After completing their maturation, matured DCs present antigens to naive T cells in the LNs, thus establishing the sensitization phase. In the subsequent challenge phase, re-exposure to the cognate hapten results in the recruitment of antigen-specific T cells and other non-antigen-specific leukocytes.

The functions of cutaneous DCs are modulated by keratinocyte-derived pro-inflammatory cytokines (64). The role of the different skin DC subsets in CHS (inducers, regulators, or functional redundancy) is a matter of active debate (65). In addition, dermal DCs, including Langerin $(CD207)^+$, may also play an important role in CHS (66).

Mast cells are a candidate DC modulator as they express and release a wide variety of intermediaries, such as histamine, TNF-α, and lipid mediators. It has been reported that activated human cord blood-derived mast cells induce DC maturation *in vitro* (67), that IgE-stimulated mast cell-derived histamine induces murine LC migration *in vivo* (68), and that MC-derived TNF-α promotes cutaneous murine DC migration *in vivo* in an IgE-independent manner (69), and coculture of mast cells and DCs results in upregulation of DC maturation markers, such as CD40, CD80, and CD86 (70). Moreover, mast cells were a requisite for the migration of plasmacytoid and $CD8^+$ subsets of DCs into the draining LNs (71). On the other hand, prostaglandin (PG) D_2 is abundantly produced by mast cells in response to allergens (72) and inhibits LC migration (73). Therefore, MCs might have bi-directional effects on DC activity in a context-dependent manner, and the question of the mechanisms by which DCs are modulated by mast cells is an important issue to pursue.

While basophils operate irrespective of the stage of CHS development (7), the role of mast cells in CHS remains controversial. In some studies, mast cell-deficient mice have exhibited a reduced inflammation in TNCB-induced CHS (74, 75). Other studies reported undiminished CHS induced with TNCB or DNFB (76, 77). Furthermore, a recent publication reported that mast cells have regulatory roles through their production of IL-10, as mast cell-deficient mice exhibited enhanced urushiol and DNFB-induced CHS (78). In these studies, however, mice carrying mutations in the stem cell factor or its receptor c-Kit were used as mast cell-deficient mice (C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ or WBB6F1-$Kit^{W/W\text{-}v}$). Although these mice lack mast cells, they also have various other immunological alterations, making it difficult to form a conclusion regarding the role of mast cells in CHS based solely on studies using these mice.

In new mast cell depletion models (7, 17), it has been reported that mice depleted of mast cells exhibited reduced CHS induced with FITC, oxazolone, or DNFB (7, 17). In addition, mast cell-specific depletion of IL-10 did not result in exacerbated CHS. Using Mas-TRECK Tg mice, we demonstrated that skin DC migration and/or maturation and T-cell priming in the sensitization phase were impaired (6). Mast cells stimulated DCs via ICAM-1 or lymphocyte function-associated antigen 1 interaction and by membrane-bound tumor

necrosis factor α on mast cells (6) (Fig. 1). Interestingly, activated DCs in turn increased Ca^{2+} influx in mast cells (6), suggesting that mast cells and DCs interact to activate each other. In the elicitation phase, mast cell deficiency resulted in an impaired CHS response, probably as a result of reduced vascular permeability caused by a loss of histamine release from mast cells (17).

To date, it remains unknown why there is such a discrepancy between the reports using stem cell factor-deficient or c-Kit-deficient models and those using conditional mast cell ablation models. One of the differences between these two models is the existence of melanocytes and hematopoietic stem cells. Recently, melanocytes were shown to express TLRs to modulate immune responses and to produce IL-1α and IL-1β (79, 80). In addition, because of the congenital absence of mast cells in $Kit^{W/Wv}$ and $Kit^{W-sh/W-sh}$ mice, a compensatory mechanism may exist such as the repopulation of the skin with basophils (81). Therefore, $Kit^{W/Wv}$ and $Kit^{W-sh/W-sh}$ mice may not necessarily be appropriate to evaluate the exclusive roles of mast cells. Consistent with the results using Mas-TRCK Tg mice, the CHS response was reduced in DT-treated *Mcpt5-Cre*$^{+}$*iDTR*$^{+}$ mice (17), which showed the attenuation of CHS response using DNFB and FITC as hapten. Thus, newly generated mast cell depletion mouse models provided evidence that mast cells promote the development of CHS irrespective of the type of haptens.

Basophil-dependent delayed-type hypersensitivity

A cutaneous delayed-type hypersensitivity reaction containing large basophil infiltrates was extensively studied in the 1970s (82). It was termed Jones-Mote hypersensitivity (JMH) in humans or cutaneous basophil hypersensitivity (CBH) in guinea pigs (83). CBH is distinct from the classical delayed-type hypersensitivity in several aspects (83). In general, CBH is elicited by the immunization of proteins in incomplete Freund's adjuvant (without mycobacterial components), whereas immunization using complete Freund's adjuvant (with mycobacterial components) is usually needed to elicit the classical hypersensitivity. CBH is characterized by erythema and a slight thickening; it peaks at 18–24 h after the antigen challenge and fades by 48 h. Classical delayed-type hypersensitivity, on the other hand, is characterized by erythema and induration; it reaches its maximal intensity within 24–30 h and remains indurated as long as 48–72 h (84).

In 2005, it was reported that basophils contributed to a novel type of chronic IgE-mediated allergic inflammation in mice (85). Although basophils are not essential for the immediate- and late-phase responses that occur after multivalent antigens are administered via a single subcutaneous injection into the ear, they are required for the IgE-mediated chronic allergic inflammation that follows. IgE-mediated chronic allergic inflammation (IgE-CAI) was found to be independent of mast cells and T cells but was dependent on an FcεRIα^{+}, CD49b^{+} cell population identified as basophils (85). Interestingly, although basophils accounted for only 1–2% of the cellular infiltrate at the site of the skin lesion, their depletion led to a dramatic reduction in inflammation associated with a decrease in the number of eosinophils and neutrophils and a marked reduction in ear thickness (85). Recent studies have shown that inflammatory monocytes recruited to IgE-CAI lesions acquire an anti-inflammatory M2 phenotype via basophil-derived IL-4 (86). Collectively, these results illustrate the potent inflammatory effects of small numbers of basophils

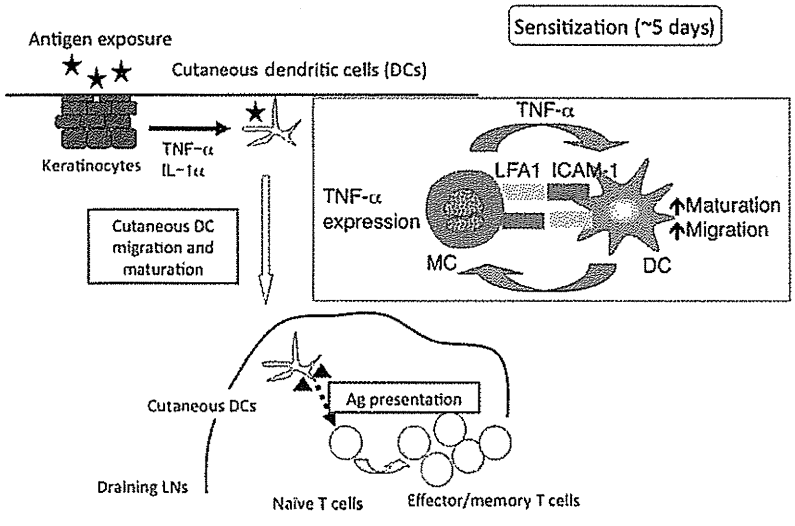

Figure 1 Schema of contact dermatitis. Upon exposure to antigens, keratinocytes produce pro-inflammatory cytokines, such as TNF-α and IL-1α. These cytokines promote DC maturation and migration into draining LNs. In addition, mast cells interact with DCs in the dermis and stimulate DCs via ICAM-1-LFA-1 interaction and membrane-bound TNF-α expressed by mast cells. DCs, in turn, induce calcium flux into MC by unknown mechanism. In the draining LNs, matured DCs present antigens to naïve T cells for the induction of memory/effector T cells and their expansion.

and suggest a novel, nonredundant role for basophils in the initiation and maintenance of chronic IgE-mediated inflammatory responses in mice.

Human basophils have some different roles when compared with mouse basophils. For instance, mouse basophils produce platelet-activating factor upon activation, which significantly contributes to the development of anaphylaxis in response to penicillin–IgG antibody complexes; however, human basophils do not respond to IgG immune complexes (87, 88). On the other hand, some findings from mouse basophils might shed light on the pathogenesis of human cutaneous diseases. Approximately 40% of patients with chronic idiopathic urticaria (CIU) have antibodies to FcεRIα. Some patients with CIU exhibited urticaria in response to IgG anti-IgE and/or FcεRIα antibodies that might activate mast cells or basophils (89). In addition, incubation of donor basophils with sera from patients with CIU and positive autologous serum skin test demonstrated a significant upregulation of CD203c (90). Furthermore, basophils in urticarial lesions of CIU are known to increase in number (38, 91). Consistently, basopenia in CIU appears to be due to the migration of basophils from the peripheral blood to urticarial lesions (38, 91). Therefore, the phenomenon seen in a mouse IgE-CAI model might explain the pathogenesis of human CIU.

Conclusion

Through studies with newly developed mast cell-deficient or basophil-deficient mice, our understanding of the mechanisms of cutaneous immune reaction has advanced significantly beyond our understandings a decade ago. At the same time, however, some key questions remain unanswered, such as what role basophils play in pathogenic processes, where they are detected in the lesional skin, and how DCs present peptides to basophils during Th2 skewing. In addition, there still remains a compelling need to determine whether these findings in mouse models are relevant to humans, especially about basophils, most of the current knowledge *in vivo* is based on the murine model. Further studies are needed to investigate the counterpart in human skin diseases in the future. However, the newly developed mast cell-deficient or basophil-deficient models can provide us with valuable information on the mechanisms of cutaneous diseases. Future studies focusing on this topic will enable us to develop novel therapeutic approaches to controlling cutaneous inflammatory diseases.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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ORIGINAL ARTICLE

See related commentary on pg 2071

IL-17A as an Inducer for Th2 Immune Responses in Murine Atopic Dermatitis Models

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Atopic dermatitis (AD) is generally regarded as a type 2 helper T (Th2)-mediated inflammatory skin disease. Although the number of IL-17A-producing cells is increased in the peripheral blood and in acute skin lesion of AD patients, the role of IL-17A in the pathogenesis of AD remains unclear. To clarify this issue, we used murine AD models in an IL-17A-deficient condition. In a repeated hapten application–induced AD model, skin inflammation, IL-4 production in the draining lymph nodes (LNs), and hapten-specific IgG1 and IgE induction were suppressed in IL-17A-deficient mice. Vγ4$^+$ γδ T cells in the skin-draining LNs and Vγ5$^-$ dermal γδ T cells in the skin were the major sources of IL-17A. Consistently, in flaky-tail (*Flg*$^{ft/ft}$ *ma/ma*) mice, spontaneous development of AD-like dermatitis and IgE induction were attenuated by IL-17A deficiency. Moreover, Th2 differentiation from naive T cells was promoted *in vitro* by the addition of IL-17A. Taken together, our results suggest that IL-17A mediates Th2-type immune responses and that IL-17A signal may be a therapeutic target of AD.

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INTRODUCTION

Skin is an essential immune organ that can elicit a variety of immune responses against foreign antigen exposure (Egawa and Kabashima, 2011). Atopic dermatitis (AD) is a pruritic chronic retractable inflammatory skin disease that is induced by a complex interaction between susceptibility genes encoding skin barrier components and stimulation by environmental antigens (Palmer *et al.*, 2006; Kabashima, 2012, 2013). AD is classically characterized by a type 2 helper T (Th2)–dominant condition, which seems to be mediated by epidermal Langerhans cells stimulated with thymic stromal lymphopoietin (TSLP) on keratinocytes (Soumelis *et al.*, 2002). TSLP also induces Th2 chemokines, including thymus- and activation-regulated chemokine/CCL17, and is involved in allergic responses, IgE production, and eosinophilia (Liu *et al.*, 2007; Nakajima *et al.*, 2012).

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Abbreviations: AD, atopic dermatitis; CHS, contact hypersensitivity; LN, lymph node; Th, T helper; TSLP, thymic stromal lymphopoietin

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IL-17A is known to be involved in host defense against various pathogens. The inappropriate production of IL-17A is considered to be involved in the development of Th17-mediated autoimmune and inflammatory conditions, such as multiple sclerosis, rheumatoid arthritis, and psoriasis (Iwakura *et al.*, 2011). Consistently, the IL-17A-Th17 pathway is a good therapeutic target of psoriasis (Miossec and Kolls, 2012). IL-17A is also involved in the pathogenesis of contact hypersensitivity (CHS) responses, as the CHS response was attenuated in IL-17A-deficient (IL-17A$^{-/-}$) mice via the impaired expansion of hapten-specific CD4$^+$ T cells in the sensitization phase (Nakae *et al.*, 2002; Honda *et al.*, 2013). Th17 axis in AD is known to be attenuated compared with psoriasis, reflecting the model for AD as Th2/Th22 and psoriasis as a Th1/Th17-polarized disease, respectively. This is reflected in part by low levels of downstream molecules in the Th17 pathway, including key antimicrobial peptides, which is related to the increased infection rate seen in AD (Ong *et al.*, 2002).

In contrast, others and we have reported that the number of IL-17A-producing cells is increased in the peripheral blood mononuclear cells of patients with severe AD and at the acute eczematous lesions of AD in humans (Toda *et al.*, 2003; Koga *et al.*, 2008). Consistently, IL-17A was highly detected in the spontaneously developed AD-like skin lesions in flaky-tail (*Flg*^{ft}) mice, which carry mutations in *filaggrin* and *matted* genes (Fallon *et al.*, 2009; Oyoshi *et al.*, 2009; Moniaga and Kabashima, 2011).

In addition to CD4$^+$ Th17 cells, subsets of CD8$^+$ T cells, γδ T cells, natural killer T cells, monocytes, natural killer cells, and lymphoid tissue inducer–like cells have been shown to produce IL-17A (Cua and Tato, 2010). Among them, γδ T cells

produce IL-17A in psoriasis skin lesions in murine models and human samples (Cai *et al.*, 2011; Laggner *et al.*, 2011). In murine skin, there exist at least two populations of γδ T cells: $V\gamma5^+$ γδ TCR^{high+} γδ T cells in the epidermis and $V\gamma5^-$ γδ TCR^{mid+} γδ T cells in the dermis. It has recently been revealed that $CCR6^+$ IL-23 receptor $(IL23R)^+$ γδ T cells are present in the murine dermis (Gray *et al.*, 2011), which secrete IL-17A and IL-22 by responding to IL-23 and IL-1β (Sutton *et al.*, 2009; Cua and Tato, 2010), and that IgE induction is impaired in γδ T cell–deficient mice (Strid *et al.*, 2011). Therefore, it is important to determine which cells produce IL-17A and to evaluate the role of IL-17A in the pathogenesis of AD.

RESULTS

IL-17A deficiency attenuated the development of hapten-induced AD-like skin lesions

Single hapten elicitation (hereafter, called acute CHS) is known to induce delayed-type hypersensitivity, as a murine model of human contact dermatitis (Honda *et al.*, 2013). On the other hand, repeated hapten exposure (hereafter, called chronic CHS) shifts immune responses from a typical Th1-dominated delayed-type hypersensitivity response to a chronic Th2-dominated late-phase and immediate-type hypersensitivity response, which are clinically relevant to the acute phase of AD in humans (Kitagaki *et al.*, 1997; Man *et al.*, 2008; Jin *et al.*, 2009; Hatano *et al.*, 2010). Consistently, recent studies have proposed that cutaneous exposure to hapten contributes to the induction of AD—this is called the hapten-atopy hypothesis (McFadden *et al.*, 2011). We applied this murine AD-like model to wild-type (WT) and $IL\text{-}17A^{-/-}$ mice on BALB/c background.

In acute CHS, ear swelling 24 and 48 hours after application (i.e., a delayed-type hypersensitivity reaction) was attenuated in $IL\text{-}17A^{-/-}$ mice, as reported previously (Nakae *et al.*, 2002); (Figure 1a, upper panel), whereas ear swelling without sensitization was comparable between WT and $IL\text{-}17A^{-/-}$ mice. In chronic CHS, WT mice exhibited prominent ear-swelling responses 1 and 6 hours after the last hapten application, which were significantly attenuated in $IL\text{-}17A^{-/-}$ mice (Figure 1a, lower panel). Histological examination of skin from WT mice revealed epidermal hyperplasia with inflammatory cell infiltration, which is similar to acute AD skin lesions (Figure 1b, Supplementary Table S1 online). These findings were less apparent in $IL\text{-}17A^{-/-}$ mice (Figure 1b; Supplementary Table S1 online). The total histological score of $IL\text{-}17A^{-/-}$ mice was significantly lower than that of WT mice (Figure 1c).

To evaluate the extent of induction of Th2 in $IL\text{-}17A^{-/-}$ mice, we analyzed cytokine mRNA expression levels of the skin and draining lymph nodes (LNs) by quantitative PCR analysis 6 hours after the last elicitation of chronic CHS and in the steady state (indicated as control). Consistent with the observation that IL-17A-producing cells are increased in human AD (Toda *et al.*, 2003; Koga *et al.*, 2008), the IL-17A expression levels of the skin and draining LNs were elevated after repeated hapten exposures (Figure 2a). The IL-4 mRNA expression levels of the skin and draining LNs in $IL\text{-}17A^{-/-}$ mice were significantly decreased when compared with those of WT mice (Figure 2a). On the other hand, the IFN-γ, IL-10, and IL-22 mRNA expression levels of the skin and draining LNs did not show any significant differences between WT and $IL\text{-}17A^{-/-}$ mice (Figure 2a).

It is known that IgE and IgG1 are Th2-dependent Igs and that IgG2a is a Th1-dependent Ig, respectively (Snapper *et al.*, 1988a, b). Serum 4-ethoxymethlene-2-phenyloxazolin-5-one-specific IgE, IgG1, and IgG2a levels in $IL\text{-}17A^{-/-}$ mice after chronic CHS were significantly lower than those in WT mice (Figure 2b). These data suggest that IL-17A is involved in the development of both acute CHS (Th1) and chronic CHS (Th2) models.

Th2 chemokine expression levels and IL-4-producing cells were attenuated by IL-17A deficiency

TSLP and CCL17/thymus- and activation-regulated chemokine have a pivotal role in Th2-mediated allergic response. We therefore analyzed mRNA expression levels of these genes 6 hours after the last elicitation of chronic CHS. TSLP and CCL17/thymus- and activation-regulated chemokine mRNA levels in the ear skin were increased in WT mice. After chronic CHS, the mRNA levels of TSLP and CCL17/thymus- and activation-regulated chemokine in $IL\text{-}17A^{-/-}$ mice were significantly lower than those in WT mice by 70 and 30%, respectively (Figure 3a and b). The above findings suggest that Th2 induction was impaired in $IL\text{-}17A^{-/-}$ mice. As such, we focused on the IL-4 induction via IL-17A signaling.

Consistent with the mRNA analysis (Figure 2a), the number of $IL\text{-}4^+$ cells was increased in chronic CHS by intracellular cytokine staining, and the number of $IL\text{-}4^+$ cells in $IL\text{-}17A^{-/-}$ mice was significantly lower than that in WT mice (Figure 3c). In addition, the number of $CD4^+$ $IL\text{-}4^+$ Th2 cells in $IL\text{-}17A^{-/-}$ mice was significantly lower than that in WT mice (Figure 3d). It is known that naive T cells express IL-17R (Ishigame *et al.*, 2009); therefore, there is a possibility that IL-17A may be involved in IL-4-producing Th2-cell differentiation. To dissect the direct action of IL-17A in Th2 differentiation, we incubated naive $CD4^+$ T cells under a Th2-skewing condition in the presence or absence of IL-17A. Under the Th2-skewing condition, the number of IL-4-producing cells in $CD4^+$ T cells was increased by IL-17A under different anti-CD3/CD28 stimulation conditions (Figure 3e). The above findings suggest that IL-17A may contribute to Th2 differentiation in the draining LN.

γδ T cells are the main producer of IL-17A

We examined the enhanced IL-17A mRNA expression after repeated hapten application as above (Figure 2a). Next, we confirmed this phenomenon using an intracellular cytokine staining assay. The numbers of IL-17A-producing cells in the skin-draining LNs (Figure 4a) and in the lesional skin (Figure 4b) were increased after repeated hapten application. The main producer of IL-17A in the skin-draining LNs was $V\gamma4^+$ γδ TCR^+ T cells (Figure 4a). In the skin, there exist at least two γδ T-cell subsets: $V\gamma5^+$ epidermal γδ T cells, and $V\gamma5^-$ dermal γδ T cells (Mabuchi *et al.*, 2011). After repeated hapten application, the number of $CD45^+$ $IL\text{-}17A^+$ cells was increased in the lesional skin, and the main producer of

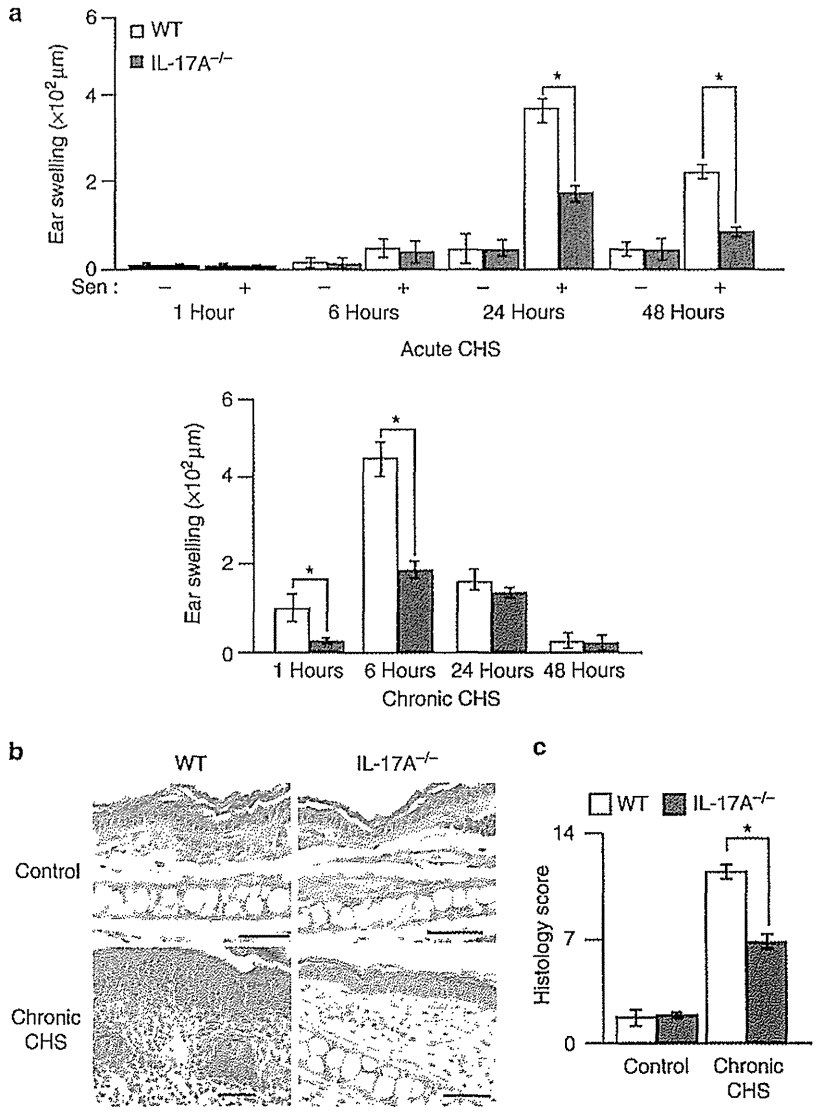

Figure 1. IL-17A deficiency attenuated the development of hapten-induced atopic dermatitis (AD)-like skin lesions. (a) Time course of antigen-specific ear-swelling responses of wild-type (WT) mice and IL-17A$^{-/-}$ mice after the last elicitation with 4-ethoxymethlene-2-phenyloxazolin-5-one (OX) in acute (upper) and chronic contact hypersensitivity (CHS; lower). In acute CHS, ear swelling of mice with (+) or without (−) sensitization (sens) was shown. (b) Histology (hematoxylin and eosin staining) of the ear skin of WT or IL-17A$^{-/-}$ mice 6 hours after the last application with OX in chronic CHS or untreated mice (control). Bar = 100 μm. (c) Total histology score. *P<0.05 (n=5 mice per group).

IL-17A was Vγ5$^-$ dermal γδ T cells (Figure 4b). Next, we analyzed single-cell suspensions from the epidermis. We detected major histocompatibility complex class II$^-$ IL-17A-producing cells in the epidermis, after repeated application, and the majority of them were Vγ5$^-$ γδ T cells (Figure 5a and b). This result suggests that IL-17A-producing dermal γδ T cells migrated into the epidermis after repeated hapten application.

IL-17A deficiency led to impaired Th2 induction in *Flg*^{ft} mice

Finally, we sought to evaluate the role of IL-17A in another murine AD-like model, *Flg*^{ft} mice, which mutated in *filaggrin* and *matted* genes. Tmem79 (ma/ma) mutation is responsible for the spontaneous dermatitis phenotype in *Flg*^{ft} mice (Sasaki *et al.*, 2013; Saunders *et al.*, 2013), and deficiency of filaggrin gene alone does not induce spontaneous dermatitis (Kawasaki *et al.*, 2012). The *Flg*^{ft} mice exhibited Th17-dominant milieu in the skin and skin-draining LNs (Fallon *et al.*, 2009; Oyoshi *et al.*, 2009; Moniaga and Kabashima, 2011). We crossed *Flg*^{ft} mice with IL-17A$^{-/-}$ mice on B6 background to evaluate the role of IL-17A in the development of spontaneous AD-like skin lesions in *Flg*^{ft} mice. Clinical appearance was mild in IL-17A$^{-/-}$ *Flg*^{ft} mice when compared with *Flg*^{ft} mice at the age of 20 weeks (Figure 6a, Supplementary Table S3 online). Consistently, the histological score of IL-17A$^{-/-}$ *Flg*^{ft} mice was lower than that of IL-17A$^{+/+}$ *Flg*^{ft} mice (Figure 6b; Supplementary Figure S2 online; Supplementary Table S2 online). We measured serum Th2-type Ig (IgE and IgG1) and Th1-type Ig (IgG2c) levels, and found that IL-17A deficiency

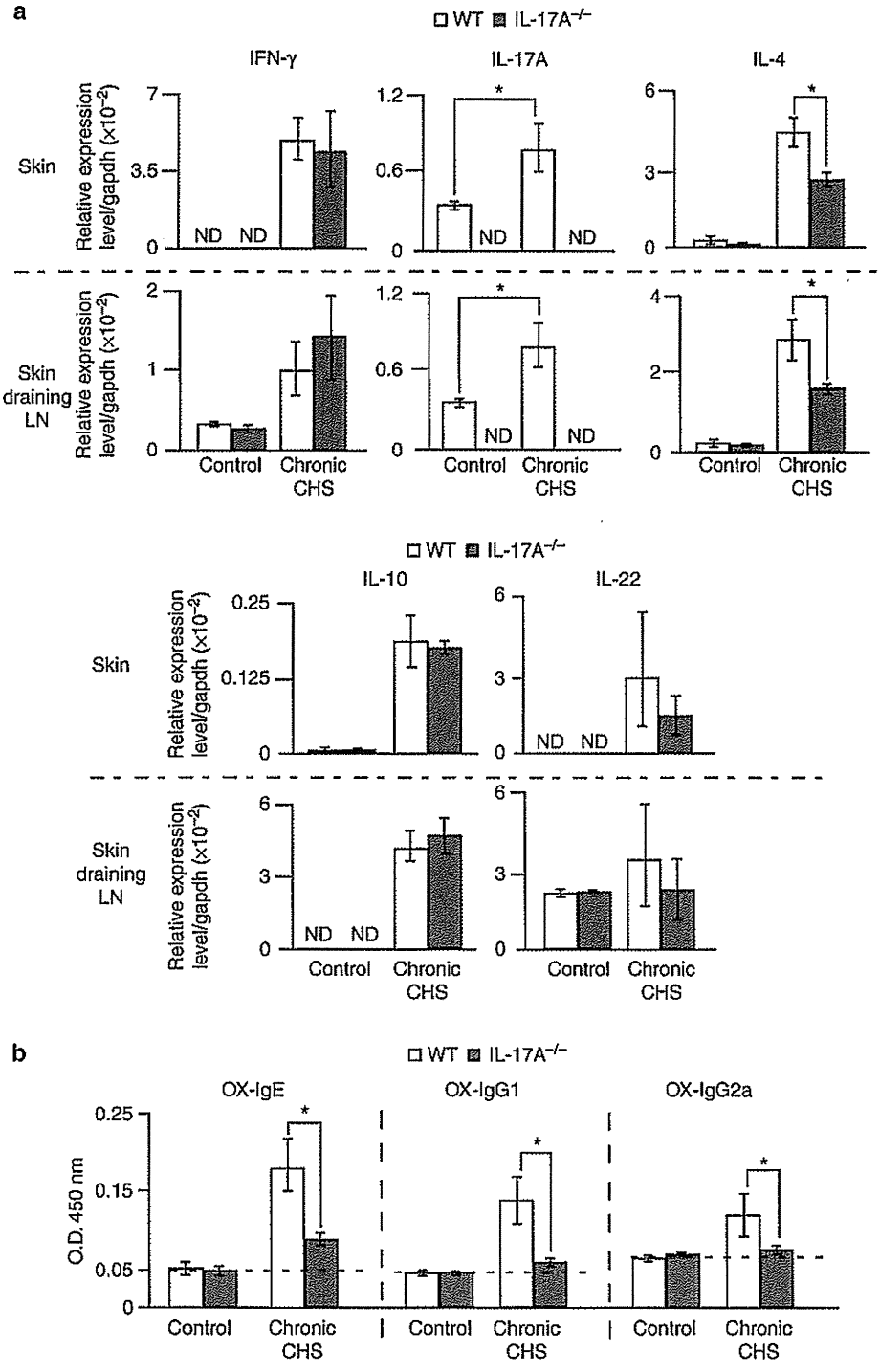

Figure 2. Cytokine expression levels and antigen-specific Ig production. (a) mRNA expression levels in the skin and skin-draining lymph nodes (LNs) 6 hours after the last elicitation of chronic contact hypersensitivity (CHS) or untreated mice (control). (b) Serum 4-ethoxymethlene-2-phenyloxazolin-5-one (OX)-specific IgE, IgG1, and IgG2a levels, as determined by ELISA. Optical density value for IgE, IgG1, and IgG2a levels were measured at a wavelength of 450 nm. *$P<0.05$ ($n=5$ mice per group). WT, wild type.

led to impaired IgE production in *Flg*^{ft} mice (Figure 6c), whereas the levels of IgG1 and IgG2c were comparable between these two groups (Figure 6c).

To further characterize the effect of IL-17A deficiency in *Flg*^{ft} mice, we analyzed the compositions of LN and spleen cells by means of flow cytometry. Although IL-17A deficiency

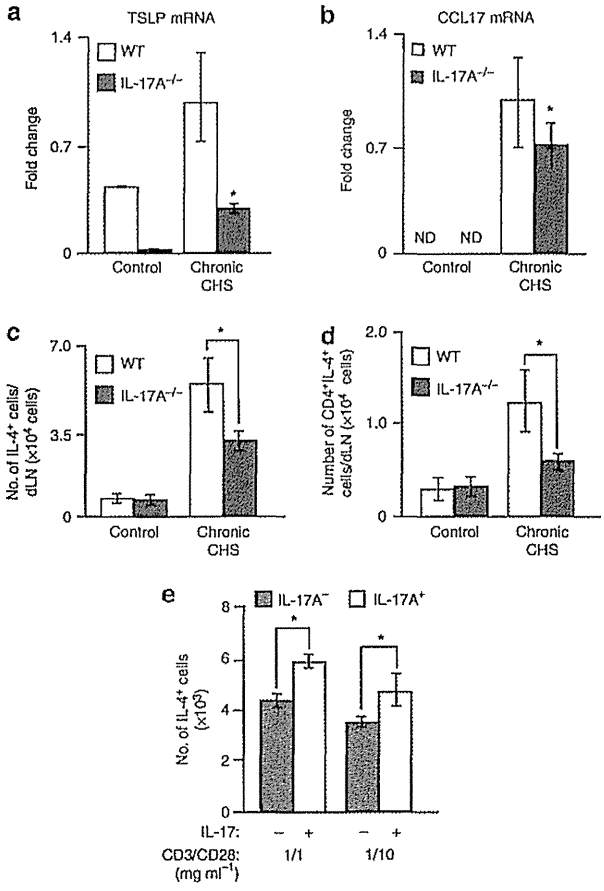

Figure 3. IL-17A deficiency attenuated type 2 helper T (Th2) chemokine expression and IL-4 induction. (a, b) Thymic stromal lymphopoietin (TSLP) and CCL17/thymus- and activation-regulated chemokines (TARC) mRNA expression levels of the ears 6 hours after the last elicitation of chronic contact hypersensitivity (CHS) or untreated mice (control) were normalized to *GAPDH* mRNA in the same sample. Results are presented as fold change relative to wild-type (WT) in chronic CHS, taken as one. (c, d) The number of total IL-4-producing cells (c) and IL-4-producing $CD4^+$ T cells (d) in the draining lymph nodes (LNs). (e) *In vitro* type 2 helper T (Th2) differentiation assay. The number of $IL\text{-}4^+$ cells per well is shown. $^*P<0.05$.

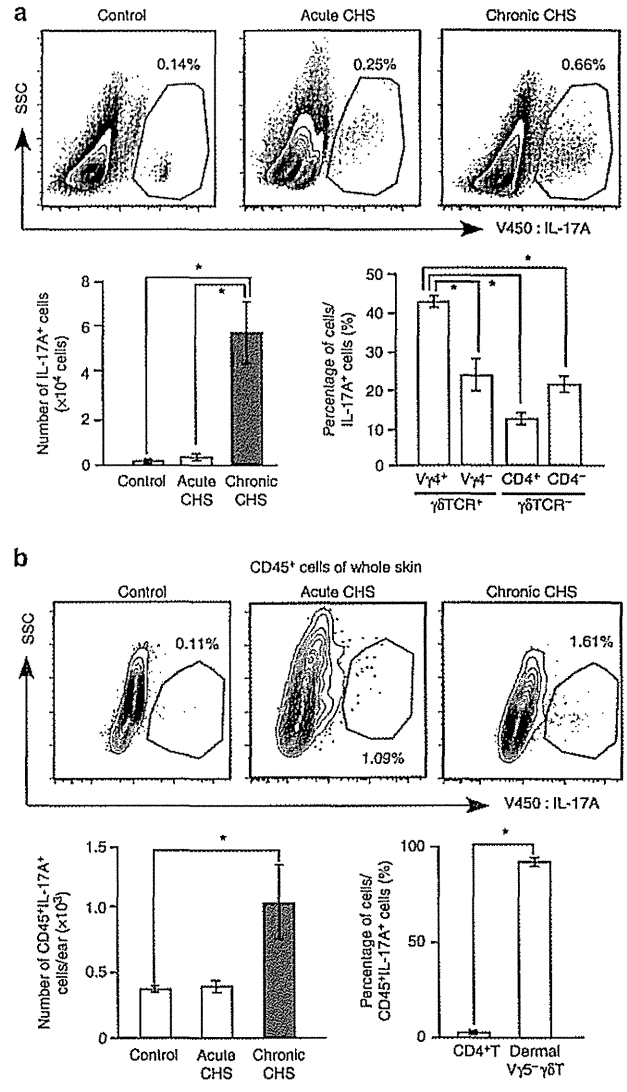

Figure 4. γδ T cells are the main producers of IL-17A. (a, b) Single-cell suspensions were collected 6 hours after the last elicitation of chronic contact hypersensitivity (CHS) for intracellular IL-17A staining. The number of IL-17A-producing cells in skin-draining lymph nodes (LNs; a) and $CD45^+$ $IL\text{-}17A^+$ cells in the lesional skin (epidermis and dermis) (b). The percentages of $V\gamma4^+$ or $V\gamma4^-$ γδ TCR^+ cells and $CD4^+$ or $CD4^-$ γδ TCR^+ cells (a) and $CD4^+$ and dermal $V\gamma5^-$ γδ T cells (b) among IL-17A-producing cells (right lower panel). $^*P<0.05$ ($n=5$ per group).

did not attenuate spleen cells in B6 and *Flg*^{ft} mice, the total cell numbers of LN cells and cell counts and frequencies of $CD4^+$ and $CD8^+$ T-cell subsets were significantly decreased in $IL\text{-}17A^{-/-}$ *Flg*^{ft} mice (Supplementary Figure S3a and b online).

We then prepared single-cell suspensions from LNs and stimulated them with phorbol 12-myristate 13-acetate/ionomycin for 4 hours to analyze the number of IL-4-producing $CD4^+$ T cells. The number of IL-4-producing $CD4^+$ T cells in $IL\text{-}17A^{-/-}$ *Flg*^{ft} mice was significantly decreased compared with $IL\text{-}17A^{+/+}$ *Flg*^{ft} mice (Figure 6d). These results suggest that IL-17A facilitates Th2 induction in *Flg*^{ft} mice. In our study, we observed that the IgE induction was attenuated in murine AD-like models. It has been reported that IL-17A promotes IgE production in B cells in humans *in vitro* (Doreau *et al.*, 2009; Milovanovic *et al.*, 2010). Consistently, IL-17A marginally promoted IgE production in B cells in a concentration-dependent manner under indicated anti-CD40 antibody and recombinant murine IL-4 concentrations (Figure 6e).

DISCUSSION

The roles of IL-17A in the induction of Th2 and the development of AD remain to be elucidated. In this study, we sought to clarify this issue using two AD-like murine models: a repeated hapten application–induced chronic CHS model and *Flg*^{ft} mice (Kitagaki *et al.*, 1997; Spergel *et al.*, 1998; Moniaga *et al.*, 2010). In the chronic CHS model, IL-17A-producing cells were increased in the lesional skin and draining LNs. The skin inflammation and IL-4 production

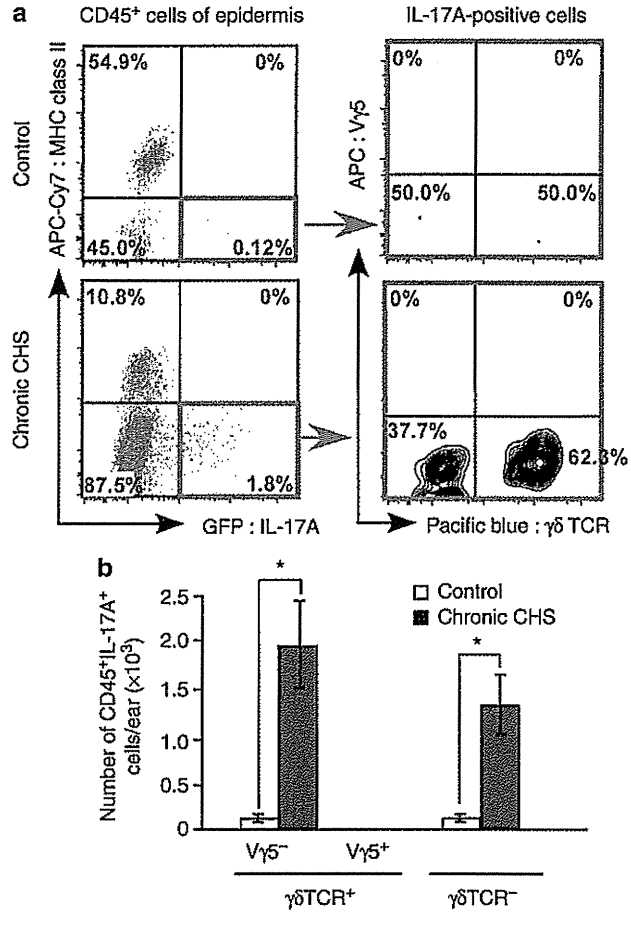

Figure 5. Dermal Vγ5 γδ T cells were detected in the epidermis after chronic contact hypersensitivity (CHS). (a) Single epidermal cell suspensions from the lesional skin of IL-17A enhanced green fluorescent protein (eGFP) reporter mice were collected 6 hours after the last elicitation of chronic CHS. FACS plots of IL-17A-positive cells (surrounded with red line, left panel) and characterization of IL-17A-positive cells (right panel). (b) $^*P<0.05$ ($n=5$ per group).

were suppressed by IL-17A deficiency. Consistently, in *Flg*^{ft} mice, AD-like dermatitis was attenuated by depletion of IL-17A. *In vitro* Th2 differentiation from naive T cells was enhanced by the addition of IL-17A. IL-17A deficiency decreased TSLP and CCL17 expressions in chronic CHS, which may contribute to the induction of Th2 indirectly. These findings suggest that IL-17A mediates the development of AD. The findings may be consistent with a recent report that IL-17A-producing Th2 cells represent the key pathogenic Th2 cells that promote the exacerbation of allergic asthma in humans (Wang *et al.*, 2010).

On the other hand, IL-4 has been considered to inhibit IL-17A production potentially (Eyerich *et al.*, 2009). Consistently, IL-17A-positive cells infiltrated into the lesions of AD more attenuated in chronic lesions than in acute lesions (Koga *et al.*, 2008). In addition, Th17 axis in AD is rather attenuated compared with psoriasis in humans. Therefore, although IL-17A seems to promote Th2, IL-4 as a Th2 cytokine may suppress IL-17A production as a feedback mechanism.

Intriguingly, the immune responses of IL-17A$^{-/-}$ mice are impaired in Th1-based acute CHS and Th2-based AD models (as shown in this study). Therefore, we speculate that IL-17A works as the enhancer of both Th1 and Th2 differentiation depending on the cytokine milieu, although it does not decide the direction of differentiation by itself. We therefore suppose that IL-17A may not promote Th2-dominated milieu under pure Th17 conditions in established Th17 diseases.

The effect of IL-17A on IgE induction is another issue to be addressed. We demonstrated that IL-17A deficiency decreased Th2-type IgE production in *Flg*^{ft} mice, whereas IL-17A deficiency decreased both Th1- and Th2-type Ig production in the chronic CHS model. This difference may stem from the differences of these models. *Flg*^{ft} mice exhibited Th2/Th17 conditions without induction of Th1, whereas the chronic CHS model exhibited Th2 conditions by shifting from Th1 conditions upon repeated hapten application. The mechanisms of IgE production and class switching from IgG1 to IgE remain unclear. The patients of classical-type hyper-IgE syndrome due to dominant-negative mutations in the *STAT3* (signal transducer and activator of transcription-3) gene exhibit high concentrations of IgE, as contrasted with normal serum levels of all other Igs (IgG, IgA, IgM, and IgD) and IgG subclasses (Minegishi, 2009). Moreover, a recent study demonstrated that *STAT3* expression on Th2 cells regulates class switch from IgG1 to IgE *in vitro* and *in vivo* (Mari *et al.*, 2013). Therefore, the dependency of *STAT3* for the induction of IgE may vary between the chronic hapten application–induced AD model and *Flg*^{ft} mice, which may explain the different phenotypes between these two groups.

Given that this study was conducted in mice, there are limitations when translating the findings to humans. These points may include the following: (1) we used the mouse model rather than human AD skin or blood samples; (2) we applied the hapten-induced murine AD-like model, which may induce nonspecific immune activation of several inflammatory axes as reported (He *et al.*, 2008; Jin *et al.*, 2009), and it might be difficult to draw conclusions in humans from this model; (3) in humans, the functional role for γδ T cells remains unclear in terms of IL-17 production; it has not been demonstrated in humans unlike mice. We agree that there still remain limitations in this study, but our findings suggest that IL-17A mediates Th2-type immune responses in the perspective of the development of AD, and that IL-17A signal may be a therapeutic target of AD.

MATERIALS AND METHODS

Mice

C57BL/6NCrSlc (B6) and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). IL-17A$^{-/-}$ mice on B6 and BALB/c genetic backgrounds were generated (Nakae *et al.*, 2002). Flaky-tail mice (STOCK a/a ma ft/ma ft/J; *Flg*^{ft} mice; Fallon *et al.*, 2009; Moniaga *et al.*, 2010) were backcrossed more than six generations onto B6 mice background. IL-17A enhanced green fluorescent protein reporter mice were kindly provided by Dr Masato Kubo. Seven- to 12-week-old female mice that were bred in specific pathogen-free facilities at Kyoto University were used for all experiments, which were approved

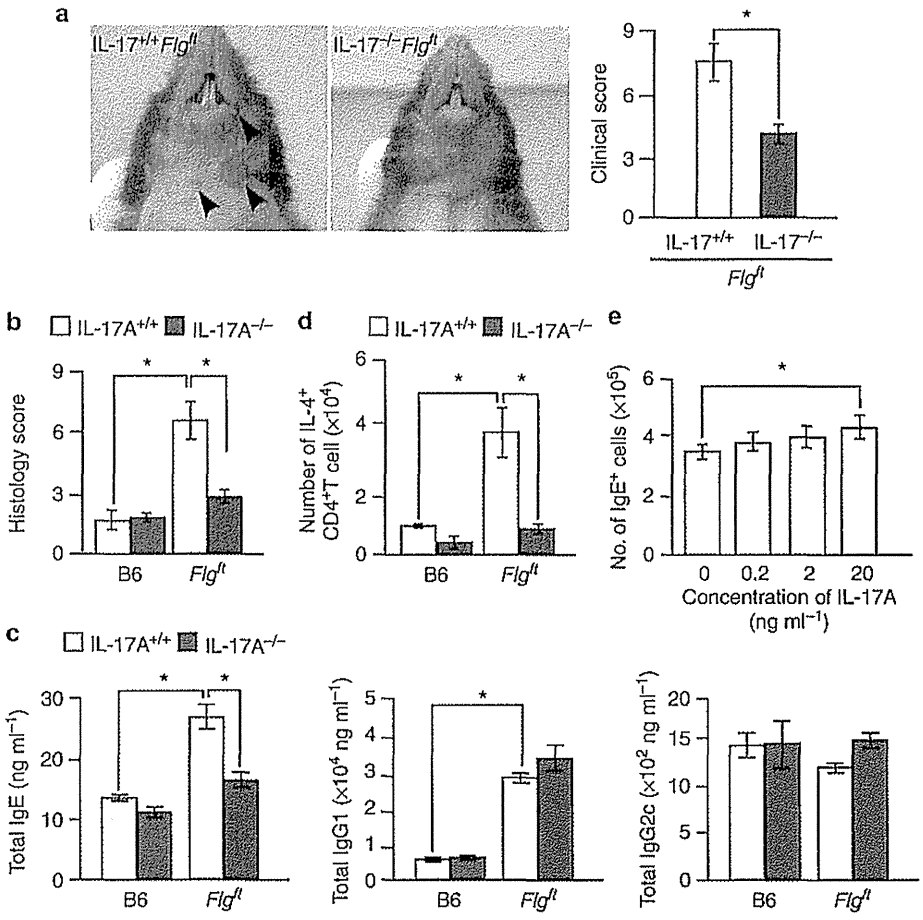

Figure 6. IL-17A deficiency attenuated cutaneous inflammation and type 2 helper T (Th2) induction in *Flg*^{ft} mice. (a, b) Clinical findings and total clinical scores (a) and histology scores (b) of IL-17$^{+/+}$ and IL-17$^{-/-}$ *Flg*^{ft} mice. Arrows indicate erosion of the skin. (c) Serum IgE, IgG1, and IgG2c levels. (d) The number of IL-4-producing CD4^{+} T cells. (e) Class switch recombination assay. The number of IgE-producing splenic CD19^{+} B220^{+} cells in various concentrations of IL-17A. *$P<0.05$ ($n=5$ per group).

by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Acute and chronic CHS

The acute CHS reaction to hapten is an animal model of human allergic contact dermatitis (Honda *et al.*, 2013). For sensitization, the shaved abdominal skin was applied with 25 μl of 3% 4-ethoxymethlene-2-phenyloxazolin-5-one (Wako, Osaka, Japan) solution in ethanol (day 0). For acute CHS induction, 5 days after the initial abdominal application when sensitization was established, 20 μl of 0.6% 4-ethoxymethlene-2-phenyloxazolin-5-one solution was applied to the ear (Supplementary Figure S1a online). For chronic CHS induction, 4-ethoxymethlene-2-phenyloxazolin-5-one solution was applied to the ear every other day until day 13 (Supplementary Figure S1b online). Ear thicknesses were measured using a micrometer before (0 h) and 1, 6, 24, and 48 h after elicitation. The differences between the indicated time points and 0 h were referred to as ear swelling. Immediate-type, late-phase, and delayed-type hypersensitivity reactions were manifested by ear-swelling responses at 1, 6, and 24 h after the last hapten application, respectively. Histology was obtained 6 h after the last hapten application (Supplementary Figure S1b online).

Quantitative reverse transcription–PCR analysis

We collected ears and cervical LNs 6 hours after the last elicitation to analyze cytokine mRNA expression levels. Total RNA was isolated with RNeasy kits and DNase I (Qiagen, Hilden, Germany). Complementary DNA was reverse transcribed using the Prime Script RT reagent kit (Takara Bio, Otsu, Japan). Quantitative PCR analysis was performed as reported previously (Nakajima *et al.*, 2010). All primers were obtained from Greiner Japan (Tokyo, Japan). The primer sequences were as follows: IFN-γ, 5′-GAA CTG GCA AAA GGA TGG TGA-3′ (forward), 5′-TGT GGG TTG TTG ACC TCA AAC-3′ (reverse); IL-4, 5′-GGT CTC AAC CCC CAG CTA GT-3′ (forward), 5′-GCC GAT GAT CTC TCT CAA GTG AT-3′ (reverse); IL-17A, 5′-CTC CAG AAG GCC CTC AGA CTA C-3′ (forward), 5′-GGG TCT TCA TTG CGG TGG-3′ (reverse); IL-10, 5′-GCT CTT ACT GAC TGG CAT GAG-3′ (forward), 5′-CGC AGC TCT AGG AGC ATG TG-3′ (reverse); CCL17, 5′-CAG GGA TGC CAT CGT GTT TCT-3′ (forward), and 5′-GGT CAC AGG CCG TTT TAT GTT-3′ (reverse). For each sample, triplicate test reactions and a control reaction lacking reverse transcriptase were analyzed for expression of the genes, and the results were normalized to those of "housekeeping" glyceraldehyde-3-phosphate dehydrogenase mRNA.

Th2 differentiation assay

Naive $CD4^+$ T cells were sorted with the autoMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany); the purity was >95%. For an *in vitro* Th2 differentiation assay, BALB/c naive $CD4^+$ $CD62L^+$ T cells were stimulated for 2 days with plate-bound anti-CD3 (1 $\mu g\,ml^{-1}$) and plate-bound anti-CD28 (1 or 10 $\mu g\,ml^{-1}$) in the presence of 10 $ng\,ml^{-1}$ IL-4, 10 $\mu g\,ml^{-1}$ anti-IFN-γ Ab, and 20 $ng\,ml^{-1}$ IL-2. Forty-eight hours after the primary stimulation, cells were washed and cultured for 72 hours under the Th2-skewing conditions without anti-CD3 or anti-CD28 Ab stimulation.

Class switch recombination assay *in vitro*

Splenocyte single-cell suspensions were isolated by $CD19^+$ selection via magnetic columns (Miltenyi Biotec). $CD19^+$ cells (5×10^5) were cultured in flat-bottomed 24-well plates and stimulated with 1 $\mu g\,ml^{-1}$ of anti-CD40 antibody (BD Bioscience, San Diego, CA) plus 50 $ng\,ml^{-1}$ of recombinant mouse IL-4 (R&D Systems, Minneapolis, MN) for 6 days to induce class switching to IgE. IgE-producing cells were analyzed by flow cytometry.

Statistical analysis

Unless otherwise indicated, data are presented as the means ± SD and are representative of three independent experiments. *P*-values were calculated according to the two-tailed *t*-test and the Mann–Whitney *U*-test for independent samples and the Wilcoxon test for related samples. *P*-values < 0.05 are considered to be significant and are marked by an asterisk in the figures.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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may also be anti-inflammatory by reducing nuclear factor kappa B gene expression.[7] Similarly, in addition to their inflammatory role, Langerhans cells are thought to have immunoregulatory functions.[8] Indeed, we see that langerin$^+$ cells are largely reduced in lesional than in nonlesional AD skin at baseline, and significantly increase on clinical reversal after 12 weeks of CsA treatment (Fig 1, *E*, and Table E3).

In addition to the RDGP, other possible mechanisms for disease recurrence in the same areas need to be considered, including (1) regional differences (increased humidity/friction, transepidermal water loss, pH, and lipids) that allow increased antigen penetration, (2) epigenetic modifications, and (3) microbiome differences.[9]

In summary, we have demonstrated that although the CsA RDGP is much smaller than the NB-UVB RDGP, important structural defects and residual inflammation remain and the overall size of the RDGP does not predict relapse kinetics. Given that NB-UVB and CsA have different courses of disease maintenance on discontinuing therapy, some elements in the RDGP of each treatment might explain relevant treatment- and disease-specific mechanisms.

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Proteome analysis of stratum corneum from atopic dermatitis patients by hybrid quadrupole-orbitrap mass spectrometer

To the Editor:

The significance of stratum corneum (SC) barrier has been strengthened especially since 2006, when *filaggrin* (*FLG*) mutations and very low expression of profilaggrin/filaggrin monomer were found in patients with atopic dermatitis (AD) as well as ichthyosis vulgaris (IV).[1] Many other SC constituents and proteases are also known to be involved in the barrier, as new susceptibility loci for AD were found by genomewide association study[2] and proteome analysis.[3] Although the SC barrier condition is crucial for the assessment of AD, comprehensive evaluation of its abnormalities in individuals remains to be addressed. It is also notable that AD can be divided into serum IgE-high extrinsic AD (EAD) with impaired barrier and serum IgE-normal intrinsic AD (IAD) with relatively preserved barrier.[4] Here, we sought to identify and quantify wide-ranging proteins by proteome analysis of SC samples obtained by using a noninvasive tape stripping technique.

This study was approved by the Ethical Committee of Hamamatsu University School of Medicine. *EAD* was defined as IgE levels of less than 400 kU/L or 200 < IgE ≤ 400 plus class 2 or more of IgE specific to *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, and *IAD* was defined as serum IgE levels of 200 kU/L or less or 200 < IgE ≤ 400 plus class 0 or 1 of the specific IgE.[5] Enrolled in this study were 8 patients with EAD (mean age, 28.1 ± 9.1 years; 5 men, 3 women; mean serum IgE level, 4260.6 ± 5278.9 kU/L), 4 patients with IAD (mean age, 47.8 ± 6.1 years; 1 man, 3 women; mean serum IgE level, 153.8 ± 64.2 kU/L), 3 patients with IV (mean age, 59.3 ± 22.1 years; 3 men), and 3 normal healthy subjects (mean age, 28.3 ± 3.2 years; 3 men). After obtaining informed consent, we collected SC by using a stripping technique with a cellophane tape (Nichiban Co, Tokyo, Japan) from the flexor surface of the forearm of the subjects. The SC-harvested tape was dipped in 10 mL of toluene. After removal of the insoluble tape backing, the sample was centrifuged and the precipitate was washed with toluene 6 times to remove residual adhesives. SC proteins were extracted from the dried sample by solubilization in 50 mM Tris-HCl (pH 6.8) containing 1% SDS and sonication. Acetone-purified extracts were reconstructed with 7 mol/L urea and 50 mM NH_4HCO_3. Ten microgram protein samples were denatured and digested by In-solution tryptic digestion and guanidination kit and purified by C18 spin columns. The samples were solved by 0.1% formic acid solution and analyzed by using Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, Mass). By the mass spectrometer and the

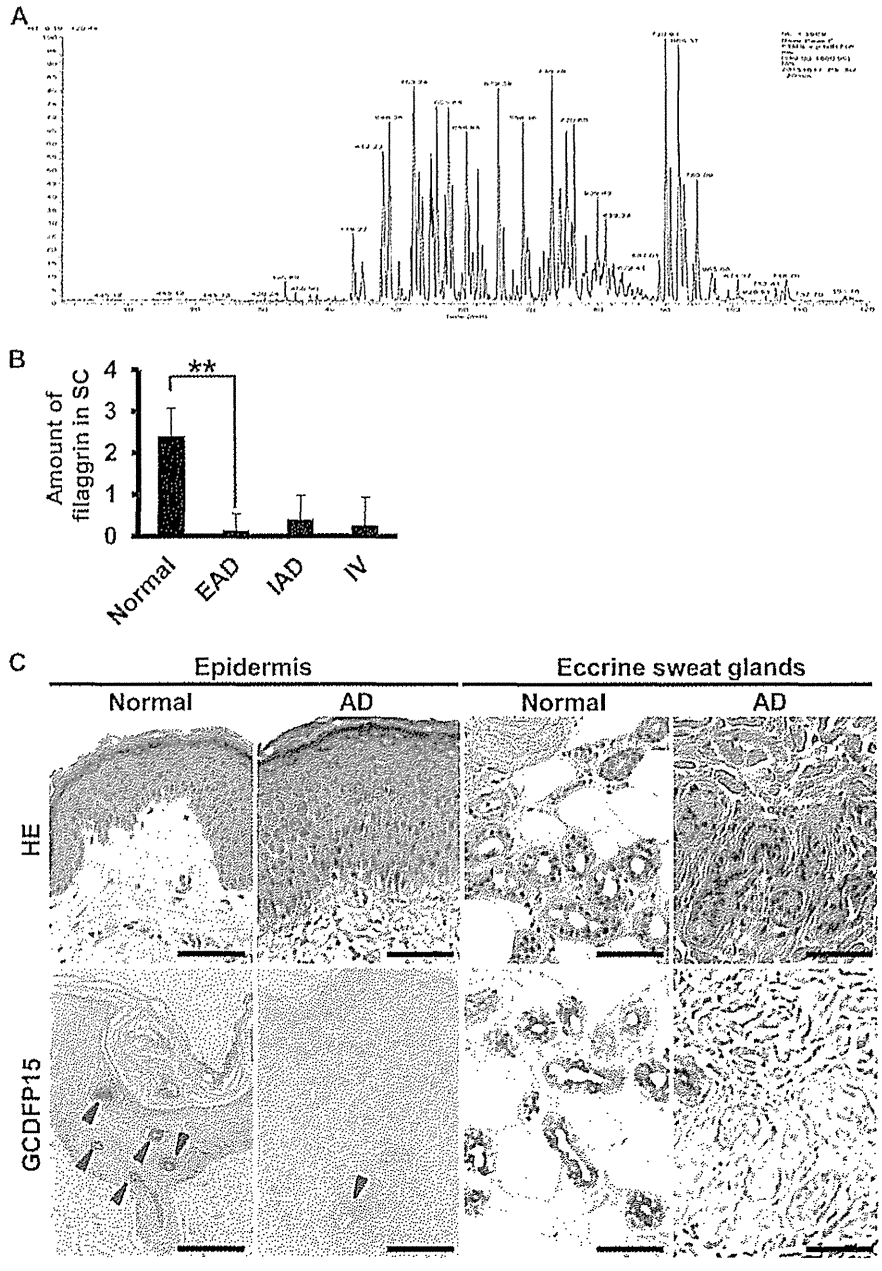

FIG 1. Characterization of patients with AD or IV by liquid chromatography (LC)/MS/MS analysis and immunohistochemistry. A, Representative LC/MS/MS analysis of SC. MS spectra were examined in the individual subjects. Note that nonspecific signals, such as polymer and contaminants, were not detected. Substances were detected by using the Mascot search engine (Matrix Science, London, UK; version 2.4) against SwissProt database of human. The amounts of individual proteins were semi-quantified by using the Proteome Discoverer v.1.4 software (Thermo Fisher Scientific). B, Comparison of FLG amounts between normal healthy subjects and patients with EAD, IAD, and IV. FLG quantity was decreased in patients with EAD, IAD, and IV than in normal subjects (**$P < .01$). C, Representative hematoxylin and eosin staining histopathology of the epidermis and dermal eccrine sweat glands in a normal subject and a patient with EAD. Scale bar is 100 μm *(top)*. Representative mmunohistochemical staining for GCDFP15 *(bottom)*. *Red arrowheads*, acrosyringium. Scale bar is 100 μm.

subsequent database analysis (Fig 1, *A*), we identified 421 proteins (see Table E1 in this article's Online Repository at www.jacionline.org). We divided the measurement of each substance by the amount of glyceraldehyde 3-phosphate dehydrogenase. The data were expressed as mean ± SD in each group. The log-transform values were compared between the subject groups by using ANOVA. Furthermore, multiple comparisons of each of the patient groups and healthy controls were conducted by using the Tukey test. See this article's Methods section in the Online Repository at www.jacionline.org.