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Prostanoid Receptors as Possible Targets for Anti-Allergic Drugs: Recent Advances in Prostanoids on Allergy and Immunology

Tetsuya Honda[1], Yoshiki Tokura[2], Yoshiki Miyachi[1] and Kenji Kabashima[*,1,3]

[1]*Department of Dermatology and* [3]*Center for Innovation in Immunoregulative Technology and Therapeutics, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan*

[2]*Department of Dermatology, University of Environmental and Occupational Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan*

Abstract: Prostanoids, consisting of prostaglandins and thromboxane, are cyclooxygenase metabolites of arachidonic acid released in various pathophysiological conditions which exert a range of actions mediated through their respective receptors expressed on target cells. Although it has been difficult to analyze the physiological role of prostanoids, recent developments in both the disruption of the respective gene and receptor selective compounds have enabled us to investigate the physiological roles for each receptor. It has been demonstrated that each prostanoid receptor has multiple functions, and that their expression is regulated in a context-dependent manner that sometimes results in opposite, excitatory and inhibitory, outcomes. The balance of prostanoid production and receptor expression has been revealed to be important for homeostasis of the human body. Here, we review new findings on the roles of prostanoids in allergic and immune diseases, focusing on contact dermatitis, atopic dermatitis, asthma, rheumatoid arthritis, and encephalomyelitis, and also discuss the clinical potentials of receptor-selective drugs.

Keywords: Prostanoid, atopic dermatitis, contact dermatitis, NSAID, prostaglandin, asthma, rheumatoid arthritis, encephalomyelitis, allergy.

INTRODUCTION

Allergic and immune diseases, including asthma, atopic dermatitis (AD), rhinitis, and autoimmune diseases are related to each other as steps in the 'atopic march,' and are found to be increasing in number [1]. They have been treated by suppressing inflammation mainly through steroid-based therapy that, unfortunately, has multiple side effects, such as obesity, hyperglycemia, and osteoporosis, among others. Recently, several advanced therapies for these allergic and immune diseases [2, 3] have been developed, but most of them are very expensive. Therefore, the development of more effective and inexpensive treatments with fewer side effects is in high demand.

When tissues are exposed to diverse pathophysiological stimuli, arachidonic acid (AA) is released from membrane phospholipids, and converted to lipid mediators, such as prostanoids, leukotrienes (LTs) and hydroxy-eicosatetraenoic acids (HETEs). Prostanoids are formed by the cyclooxygenase (COX) pathway, whereas LTs and HETEs are formed by the 5-, 12- and 15-lipoxygenase (LO) pathways. COX has two isoforms, COX-1 and COX-2. While COX-1 is constitutively expressed in cells, COX-2 requires specific stimulation, by substances such as acetone and the phorbol ester TPA [4]. This reaction results in the formation of an unstable endoperoxide intermediate prostaglandin (PG) H_2, which, in turn, is metabolized to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane (TX) A_2 by specific synthases.

Prostanoids are released from cells immediately after formation. Because they are chemically and metabolically unstable, they usually function only locally through membrane receptors on target cells [5]. Nine types and subtypes of membrane prostanoid receptors are conserved in mammals from mouse to human: two subtypes of the PGD receptor (DP; and the chemoattractant receptor homologous-molecule expressed on Th2 cells, CRTH2), four subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP) (Fig. 1). All are G protein–coupled rhodopsin-type receptors with seven transmembrane domains (Fig. 1). Main signal transduction mechanisms of these prostanoid receptors are through regulation of intracellular cyclic adenosine monophosphate (cAMP) concentration and intracellular free calcium concentration. DP, EP2, EP4 and IP are Gs coupled receptors and elevate intracellular cAMP concentration, while EP3 and CRTH2 are Gi coupled receptors and decrease intracellular cAMP. EP1, FP and TP are Gq and other G protein coupled receptors and increase intracellular calcium concentration [5]. However, most of them may couple to more than one G protein with each signaling pathway. Recently, individual prostanoid receptor gene-deficient mice have been used as models to dissect out the respective roles of each receptor in combination with the use of compounds that selectively bind to prostanoid receptors as agonists or antagonists [6]. These genetic and pharmacological approaches have revealed new roles for prostanoids and their receptors in allergic and immune diseases. In this review, we describe the current investigative status of prostanoids in allergic and immune diseases, especially focusing on skin disease, and discuss the clinical potentials of receptor-selective drugs.

*Address correspondence to this author at the Department of Dermatology and Center for Innovation in Immunoregulative Technology and Therapeutics, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawara, Sakyo, Kyoto 606-8507, Japan; Tel: + 81-75-751-3310; Fax: + 81-75-761-3002; E-mail: kaba@kuhp.kyoto-u.ac.jp
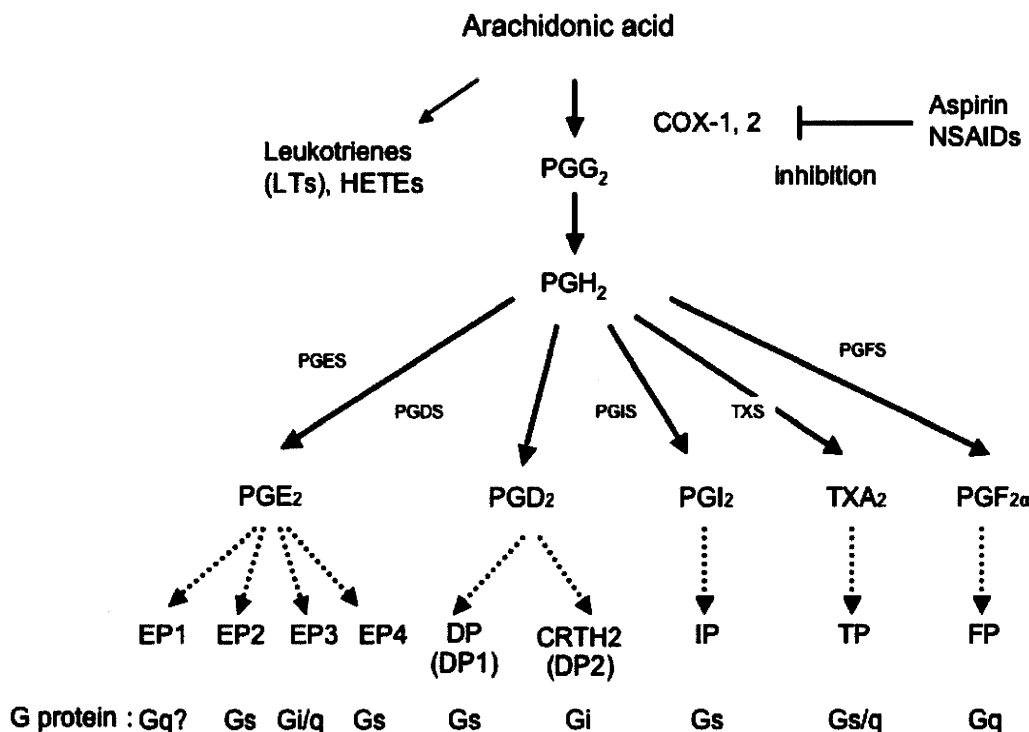
Fig. (1). Biosynthetic pathways of prostanoids. The formation of PGD_2, PGE_2, $PGF_{2\alpha}$, PGG_2, PGH_2, and PGI_2, and TXA_2, from arachidonic acid is shown. The first two steps of the pathway (*i.e.,* conversion of arachidonic acid to PGG_2 and then to PGH_2) are catalyzed by cyclooxygenase (COX), and the subsequent conversion of PGH_2 to each PG is catalyzed by the respective synthase as shown. All are G protein-coupled rhodopsin-type receptors.

PROSTANOID FORMATION IN THE SKIN

Human bodies are exposed to external stimuli continuously. As a representative organ, the skin plays an important role in self-defense during exposure to foreign antigens, and consists of many immune cells, such as keratinocytes (KCs), T cells, B cells, mast cells, eosinophils, fibroblasts, and two types of cutaneous dendritic cells (DCs), epidermal Langerhans cells (LCs) and dermal DCs (dDCs). In the normal human skin, immunohistochemical examinations have revealed that COX-1 is observed throughout the epidermis, whereas COX-2 exists in more differentiated, suprabasilar KCs and outer root sheath cells of hair follicles [7, 8].

Among prostanoids, PGE_2 is the main COX product in human epidermal homogenates [9]. PGD_2 has been detected in human skin [9], and PGD synthase is present predominantly in LCs, dDCs, dermal macrophages and mast cells, but not in KCs [10, 11]. Of this group of cells, mast cells have been found to be one of the major cellular sources of PGD_2. Only very low TX synthase activity has been found in the skin; however, high levels of TXB_2, as a metabolite of TXA_2, were detected in the cultured supernatant of LCs and DCs [12]. PGI_2 was detected in the skin of the murine atopic dermatitis model [13]. $PGF_{2\alpha}$, was observed in skin exudates of nickel allergy patients [14]. The above findings on the synthesis of prostanoids are summarized in Table 1.

Table 1. Expression of Prostanoid Synthases and Prostanoid Receptors in the Skin

	PGDS	PGES	PGFS	PGIS	TXS	DP	CRTH2	EP1	EP2	EP3	EP4	FP	IP	TP
Keratinocytes	m	m, h						h	h	h	h			
Langerhans cells	h	m, h			m	m, h		m	m	m	m			
Dendritic cells	h	m, h		m	m, h				m, h		m, h	m		
T cells							m, h(Th2)	m	m	m	m, h		m	m
B cells								m, h	m, h	m, h	m, h		m	
Macrophages	h	m, h		m	m, h		m	m	m	m	m, h			
Eosinophils	h	h	h		h	m	m		m, h		h			h
Mast cells, basophils	m, h				h	m, h	m, h	m	m	m	m			
Neutrophils		h			h	h	m		m, h		m			
Blood vessels		h	h	h				m, h	m, h	m, h	m, h		m, h	m, h

PG; Prostaglandin, s; synthase, m; mouse, h; human
Modified from the reference by Tilley *et al.*

PROSTANOID RECEPTOR EXPRESSION ON KERATINOCYTES AND IMMUNE CELLS

Adult human KCs express mRNA for all subtypes of PGE_2 receptors [15, 16] and the expression of all PGE_2 receptors have been detected in mouse KCs by immunohistochemistry [17]. Mouse LCs and DCs have been shown to express DP [18], EP1, 2, 3, 4 [19], and IP [20]. T cells are known to express EP1, 2, 3, 4 [21], IP [22] and TP [12]. PGE_2 suppresses T cell proliferation, T cell differentiation in the thymus, and IL-1 production by acting at EP2 and EP4 [23] *in vitro*. B cells express EP1, 2, 3, and 4 [21] and PGE_2 facilitates IgE class switching through EP2 and EP4 *in vitro* [24]. Mast cells express EP1, 2, 3, 4, DP, and IP [21, 25], and PGE_2 acts at EP3 to suppress degranulation [26]. Human eosinophils express EP2, EP4, DP, CRTH2 and TP [25, 27], and PGE_2 seems to prolong eosinophil survival [28, 29]. On the other hand, PGE_2 suppresses TNF-α production and enhances IL-6 production from neutrophils stimulated by lipopolysaccharide (LPS) through EP2 and EP4 [25, 30]. As summarized in Table **1**, prostanoids and their receptors are produced and expressed by a wide variety of cells in the skin. This varied expression pattern of prostanoids maintains the homeostasis of our body, which will be discussed as below.

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS AND PROSTANOIDS

The roles of prostanoids on allergy and immune diseases have been suggested by clinically monitoring the effects of non-steroidal anti-inflammatory drugs (NSAIDs), a set of COX inhibitors. It is well known that cutaneous immune responses are associated with an increase in prostanoid formation; however, the roles of prostanoids have been less well defined. This is presumably because the effects of NSAIDs are far less marked compared to those of steroids [31]. There are some skin diseases that are effectively treated with NSAIDs [3, 31, 32]; for example, ibuprofen piconol has an anti-inflammatory effect on acne vulgaris, by inhibiting leukocyte migration induced by *Propionibacterium acnes* [33]. However, NSAIDs are generally not useful for inflammatory skin diseases, such as contact dermatitis and AD [3, 31] largely because NSAIDs occasionally induce contact dermatitis themselves. Since NSAID is a COX inhibitor, which blocks formation of all prostanoids, observations obtained from NSAIDs neither indicate which type of prostanoid nor which class of prostanoid receptor is involved in a given process. Recent genetic and pharmacological approaches have revealed some unexpected findings regarding each prostanoid receptor. It is high time to reconsider the significance of each prostanoid receptor in allergic and immune diseases.

PROSTANOIDS IN CONTACT HYPERSENSITIVITY - SENSITIZATION PHASE

In order to evaluate the physiological roles of prostanoids in immune responses of the skin, the use of a contact hypersensitivity (CHS) model (in other words, allergic contact dermatitis) is an effective tool [34, 35]. Migration of cutaneous DCs to the lymph nodes is a crucial step in the initia-

tion of CHS. This activation of cutaneous DCs is initiated by KCs that secrete pro-inflammatory cytokines upon antigen application. Thus, by virtue of their specific cytokine secretion pattern, KCs determine the microenvironment for cutaneous DC maturation and migration (Fig. **2**).

PGE_2 produced by KCs upon antigen exposure acts at EP4 on cutaneous DCs to facilitate initiation of cutaneous immune responses by promoting the migration and maturation of cutaneous DCs, and the blockade of PGE_2-EP4 signaling attenuates the CHS response [19] (Fig. **2**). Interestingly, prostanoid activity producing the opposite effects has also been documented: PGD_2 induced by percutaneous infection with the helminth parasite *Schistosoma mansoni* specifically impedes the migration of LCs through the DP receptor [18], and administration of the DP agonist, BW245C, inhibits migration of LCs and attenuates OVA-induced dermatitis [36]. Stimulation of DP signaling also inhibits the migration of lung DC, which leads to the suppression of airway inflammation [37]. The PGI_2 receptor IP inhibits the proinflammatory cytokine production and T cell stimulatory function of DCs [38]. These activities of lipid mediators are not only limited to prostanoids: LC migration from the skin to the lymph nodes utilizes the LTC_4 transporter multidrug resistance-associated protein 1 [39]. The significance of these prostanoid receptors in pathophysiological conditions remained to be elucidated, but modulation of the signaling of these receptors may lead to a discovery of a possible candidate for the immune reaction.

Once cutaneous DCs migrate to draining lymph nodes, they present antigens to naïve T cells to prime them. Subsequently, the engagement of the antigen complex by T cell receptors triggers clonal expansion and differentiation of T cells. CD4$^+$ helper T (Th) cells are differentiated into at least three subsets: Th1, Th2 and Th17. Similarly, CD8$^+$ cytotoxic T (Tc) cells undergo differentiation into two subsets: Tc1 cells and Tc2 cells. Contact hypersensitivity is mainly mediated by Th1 cells and to some extent by Th17 cells [40]. Although the suppressive activity of PGE_2 on Th1 differentiation *in vitro* has been known since the 1980s, the *in vivo* role of PGE_2 on Th differentiation has only recently been addressed. In the sensitization phase of CHS, PGE_2 produced by DCs stimulate EP1 receptors on naïve CD4$^+$ and CD8$^+$ T cells and promote Th1 and Tc1 differentiation [41]. Accordingly, EP1-deficient mice showed reduced Th1 and Tc1 differentiation and CHS responses [41]. In addition to EP receptor signaling, IP signaling promotes Th1 and Tc1 differentiation through cAMP dependent mechanism [42]. Interestingly, it has been reported that IP deficient mice showed enhanced Th2 response such as elevated IgE concentration in serum in mouse OVA-induced asthma model [22], suggesting that lack of PGI_2-IP signaling might result in Th2 biased immune response through inhibition of Th1 differentiation in IP deficient mice. Prostanoids also regulate DC-T cell interaction in the priming of naïve T cells. Cutaneous DCs produce abundant TXA_2, which acts on naïve T cells to impair the DC-T cell interaction [12]. Predictably, TP-deficient mice or wild-type mice treated with a TP antagonist, S-145, during the sensitization period showed enhanced CHS responses, indicating that TP signaling negatively regulates the priming of T cells [12] (Fig. **2**).
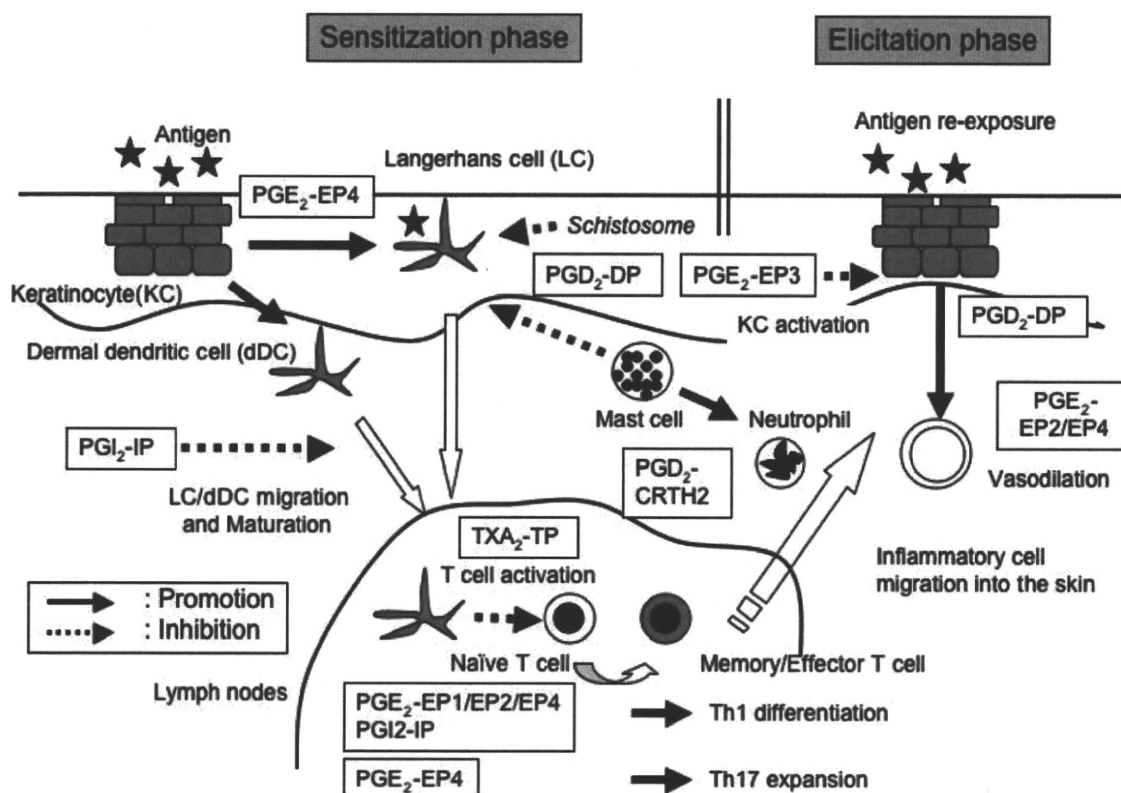
Fig. (2). Hypothesis on the role of prostanoids in the contact hypersensitivity response. During the sensitization period, antigen induces pro-inflammatory cytokine secretion by KCs, which enhances cutaneous DCs (LCs and dDCs) activation and migration to regional lymph nodes. In the lymph nodes, cutaneous DCs activate naïve T cells which differentiate into mature memory T cells. During antigen exposure to the skin, KCs produce PGE_2, and mast cells produce PGD_2. Moreover, *schistosomes* produce PGD_2 during helminthic infection. The PGE_2-EP4 pathway promotes, but PGD_2-DP and PGI_2-IP pathways inhibit cutaneous DC migration and maturation. TXA_2 produced by activated cutaneous DCs seems to act on naïve T cells to disrupt DC-T cell interaction. The PGE_2-EP1/EP2/EP4 pathways promote Th1 cell differentiation. The PGE_2-EP4 pathway also promotes Th17 cell expansion.

During the elicitation phase of the CHS response, secondary antigen exposure to the skin stimulates KCs to secrete pro-inflammatory cytokines, chemokines and other mediators, which activate the endothelial activation of blood vessels. This activation attracts memory T cell infiltration into the skin. Subsequently, antigen-loaded antigen-presenting cells activate memory T cells to induce mediator release. PGE_2 dilates blood vessels possibly through EP2 and EP4. PGE_2-EP3 signaling inhibits KCs activation and plays an anti-inflammatory role in CHS.

Regulatory T cells (Tregs) are another important T cell subset, which suppress the activation of effector T cells and play suppressive function in various disease, including CHS [43-45] It has been reported that PGE_2 promote Foxp3 mRNA expression throught EP2 and EP4 dependent mechanism *in vitro* [46]. However, the physiological role of these signaling in the skin immune response has yet to be elucidated.

6. PROSTANOIDS IN CONTACT HYPERSENSITIVITY – ELICITATION PHASE

After establishment of the sensitization phase, antigen re-challenge onto the skin stimulates KCs to produce memory T cell-attracting chemokines, such as CCL27, and neutrophil-attracting chemokines, such as CXCL1 and CXCL2, and to evoke inflammation, in a stage called the elicitation phase. It has been demonstrated that these chemokines are induced by PGE_2 [35, 47], and several prostanoid receptors are also involved in this phase. For example, PGD_2 promotes

neutrophil infiltration through CRTH2 and contributes to the progression of inflammation [48]. Accordingly, administration of CRTH2 antagonist attenuates the CHS response [49]. On the other hand, stimulation of the EP3 receptors on KCs inhibited the chemokine expression in KCs, and suppressed the CHS response [50]. Predictably, EP3-deficient mice showed increased CHS responses, suggesting that both endogenous and exogenous EP3 signaling plays an anti-inflammatory role at the elicitation site under certain conditions [50].

On the other hand, TPA is known to induce skin inflammation. After TPA application, acute edema is induced and followed by inflammatory cell infiltration, with this entire episode being induced by TNF−α and PGE_2 [51]. In fact, intradermal injection of PGE_2 into human skin causes erythema with vascular permeability changes [52-54]. Similar skin inflammation is induced by ultraviolet B, which is mediated through EP2 and EP4 receptors [55]. Such direct effects of prostanoids on blood vessels might affect the elicitation

phase of CHS, but the physiological role in this context remains unknown.

7. PROSTANOIDS IN AD

AD is a common pruritic and chronic inflammatory skin disease that is regarded as one of the T helper type 2 (Th2) diseases. In the dermis, a cellular infiltrate is present consisting of lymphocytes, monocytes and mast cells. Histamine is one of the mediators suspected to play an important role in Th2 disease processes, but its role in AD is uncertain because antihistamines improve the disease only partially, not dramatically [56, 57]. Therefore, in the search for potential mediators involved in the inflammatory processes of AD, mediators other than histamine have to be considered. In this respect, it would be of relevance to examine the potential role of prostanoids in the molecular pathology of AD. In biopsy specimens from patients with AD, PGE$_2$ has been determined in biologically active amounts in both lesional and perilesional skin [58]. In contrast, normal levels of eicosanoids were found in the uninvolved skin of these patients [58]. Using an ovalbumin-induced mouse AD model, COX-2 inhibition induced both enhanced eosinophil infiltration and elevated IL-4 expression in the skin lesion with elevated serum IgE and IgG1, suggesting that COX-2-derived prostanoids play protective roles in the development of AD. As TP-deficient mice exhibited an enhanced immune response with an increased serum IgE level on a repeated hapten application-induced murine AD model [12], TP signaling may be responsible for the worsened phenotype of COX-2-deficient mice.

PGE$_2$ has the capacities to induce wheal and flare reactions when injected into human skin [59] and to modulate the inflammatory responses elicited by other mediators [54, 60]. In contrast, one of the characteristics of AD is the elevation of IgE in the sera of patients, which is related to pruritus [61]. PGE$_2$ drives Ig class switching to IgE by acting at EP2 and EP4 on B cells under LPS and IL-4 stimulation *in vitro* [24]. The physiological role of PGE$_2$ on class switching in AD patients should be pursued in future studies.

PGD$_2$ is the major prostanoid produced by activated mast cells. PGD$_2$ has two types of receptors, DP and CRTH2. CRTH2 induces chemotaxis in Th2 cells, eosinophils and basophils with enhanced degranulation [62, 63]. In response to PGD$_2$, CRTH2 also induces Th2 cell and neutrophil migration into inflammatory skin sites [48]. Virtually all CRTH2$^+$ CD4$^+$ lymphocytes have a pure Th2 phenotype and occupy not all but a large proportion of circulating Th2 cells in both normal and AD subjects. In AD patients, a preferential increase of CRTH2$^+$ cells was noted within the disease-related cutaneous lymphocyte-associated antigen-positive CD4$^+$ T cell compartment [64]. There remains a need to clarify the respective roles of DP and CRTH2 in pathophysiological conditions.

Pruritus is also an important hallmark of AD as PGE$_2$ is known to evoke pruritus in AD patients [65]. And PGD$_2$, but not the CRTH2 agonist, 13, 14-dihydro-15-keto-PGD$_2$, reduced scratching behavior in NC/Nga AD model mice, suggesting that DP suppresses pruritic activity [66].

The above findings indicate that each prostanoid receptor plays its own role in a context-dependent manner (Fig. **3**).
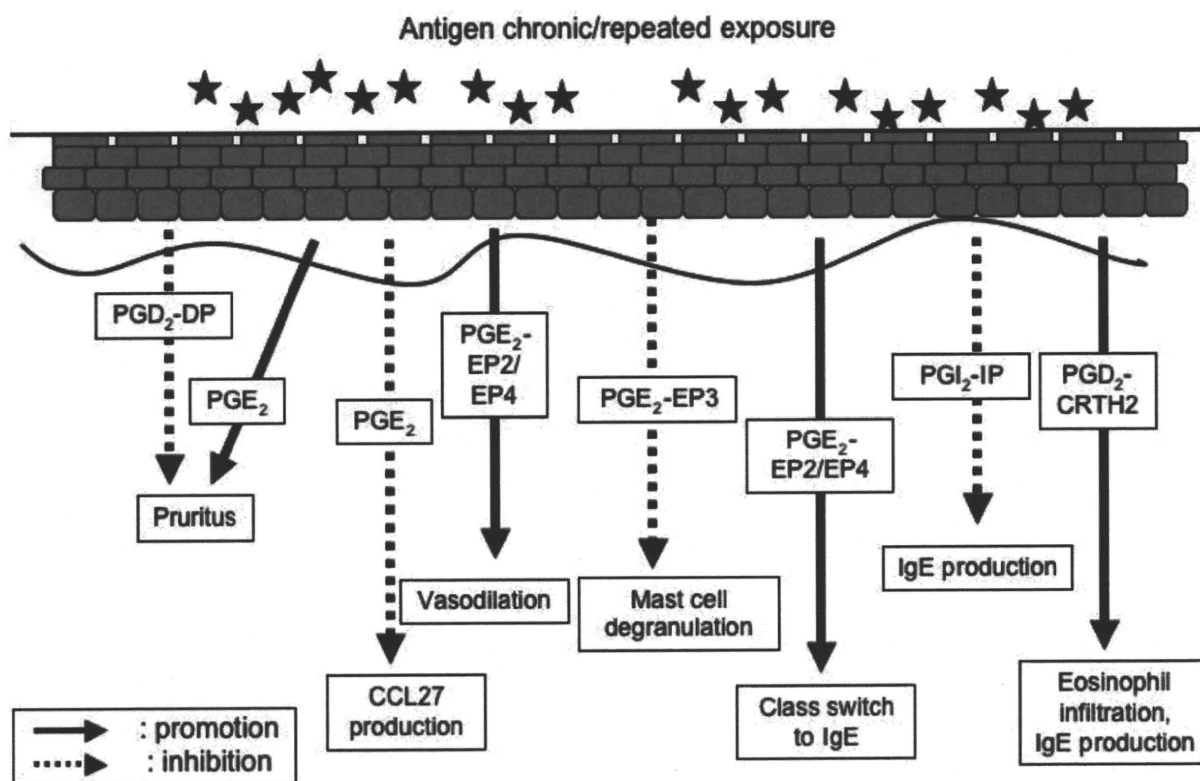

Fig. (3). Prostanoids in atopic dermatitis. A considerable amount of recent data has suggested that prostanoid receptors play some critical roles in the pathogenesis of AD in a context-dependent manner. Possible roles are summarized.

AD might be controlled by antagonists for DP, CRTH2, EP2, EP4, and/or IP, and by agonists for EP4 and/or TP.

8. PROSTANOIDS IN ASTHMA

PGD$_2$ is a major prostanoid produced by activated mast cells [67] and is released in large amounts during asthmatic attacks in certain patients [68]. It has also been reported that the increase of prostanoid in BALF were detected in a mouse asthma model [69, 70]. Although the role of PGD$_2$ in allergic asthma long remained unclear, an analysis using DP-deficient mice revealed that PGD$_2$-DP signaling stimulates chemokine expression on airway epithelial cells and facilitates Th2 cell and eosinophil accumulation in the lungs, and plays a central role in asthma [67]. Single nucleotide polymorphism analysis of the human DP gene (PTGDR) also suggests the importance of DP in the pathogenesis of asthma [71]. From these observations, it suggests that prostanoids facilitate asthmatic attacks through DP signaling both in mice and humans.

In addition to DP actions, an involvement of PGD$_2$-CRTH2 signaling has been reported in asthma. Administration of a CRTH2 antagonist reduced eosinophil accumulation in a mouse asthma model, and administration of a CRTH2 agonist augmented infiltrations of inflammatory cells into the lungs [72, 73], suggesting that PGD$_2$-CRTH2 signaling also mediate airway inflammation. However, CRTH2-deficient mice showed increased inflammatory cell infiltrations and IL-5 production from activated T lympho-

cytes [74], suggesting that CRTH2 signaling may regulate cytokine production in the development of asthma. Further analyses are needed to clarify whether an inhibition of CRTH2 signaling would have an overall beneficial effect.

On the other hand, the existence of prostanoid receptors that negatively regulate allergic reactions has been reported [26]. Among PGE receptor-deficient mice, EP3-deficient mice showed exaggerated airway inflammation, and administration of an EP3 agonist suppressed the inflammation by inhibiting mast cell activation and chemokine production from airway epithelial cells [26]. EP3 signaling is also reported to play an anti-inflammatory role in experimental allergic conjunctivitis [75]. These results indicate that PGE$_2$-EP3 signaling negatively regulates allergic inflammation. It has been well known that ingestion of aspirin (NSAIDs), which blocks prostanoid synthesis, sometimes induces severe bronchoconstriction in a proportion of subjects with asthma. Previously, such aspirin-induced asthmatic attacks (AIA) were explained by the diversion of arachidonic acid metabolism from the COX pathway to the LO pathway. However, the balance of PGD$_2$-DP signaling as promoter of airway inflammation and PGE$_2$-EP3 signaling as suppressor of airway inflammation may explain the mechanism of the AIA.

9. PROSTANOIDS IN RHEUMATOID ARTHRITIS

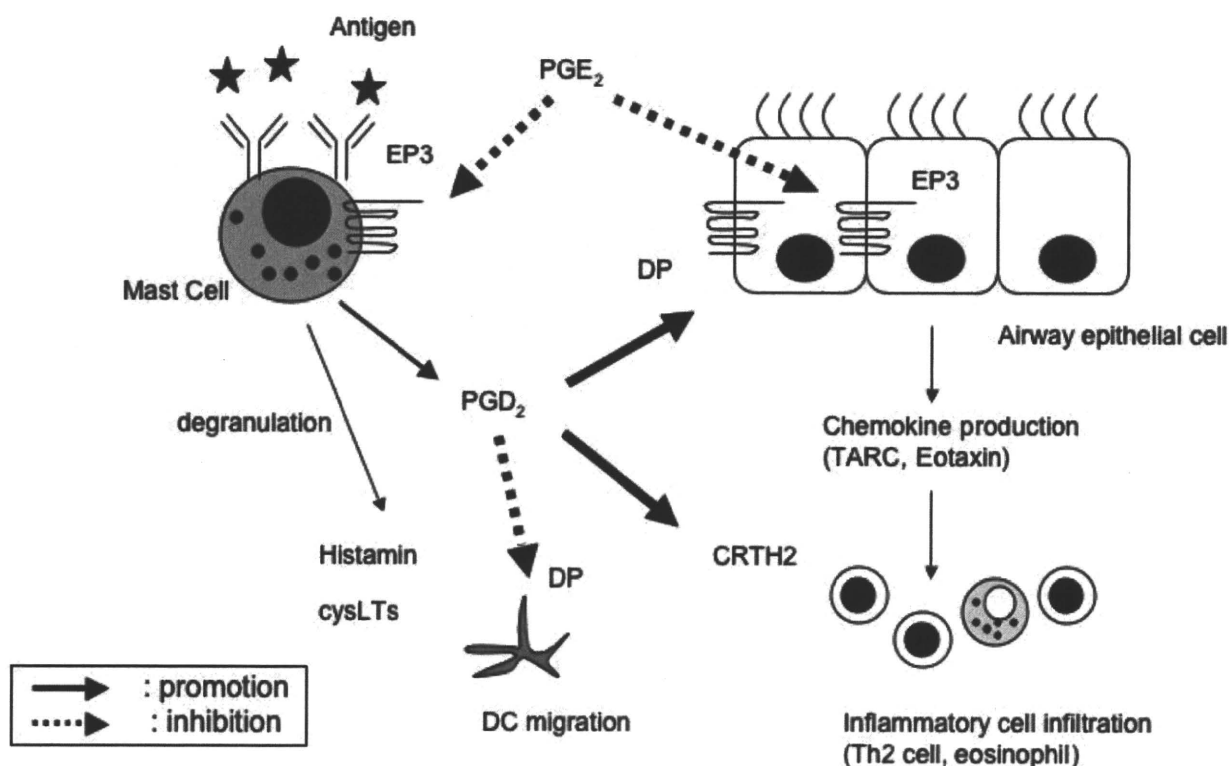Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints characterized by inflammatory cell

Fig. (4). Prostanoids in asthma. PGD$_2$ produced by mast cells act on airway epithelial cells through DP and facilitate inflammation by promoting chemokine production. PGD$_2$-DP signaling plays a suppressive role in the sensitization phase by inhibiting DC migration and activation. PGD$_2$ also acts on Th2 cells and eosinophils through CRTH2, and promotes accumulation of inflammatory cells. In contrast, PGE$_2$-EP3 signaling plays an anti-inflammatory role by inhibiting mast cell degranulation and chemokine production from airway epithelial cells.

infiltration, synovial hyperplasia and destruction of cartilage and bone. NSAIDs have been long and widely used for treatment of RA. Among PGs, PGE$_2$ has been suggested as a main PG type active in RA reactions. In fact, it has been reported that mice deficient in microsomal PGE synthase-1 showed reduced arthritic responses in mouse collagen-induced arthritis (CIA) [76]. It was later revealed that EP2 and EP4 mediate inflammation in CIA [77]. Furthermore, PGI$_2$-IP signaling plays a critical role in the development of CIA by enhancing the expression of arthritis related genes, such as IL-6 vascular endothelial growth factor-A, and the receptor activator of NF-kappa B ligand, in synovial fibroblasts [78]. Regulation of both PGI$_2$-IP signaling and PGE$_2$-EP2/EP4 signaling can be one of the potential targets in controlling joint inflammation.

10. PROSTANOIDS IN ENCEPHALOMYELITIS

Recently, EP2 and EP4 receptors have been reported to regulate Th1 and Th17 differentiation [79]. Both EP2 and EP4 signaling on naïve T cells promote Th1 differentiation through the phosphatidyl inositol-3 kinase pathway. *EP4 signaling also promote Th17 differentiation through the cAMP pathway, and blockade of EP4 signaling inhibited Th17 differentiation in CHS and mouse experimental encephalomyelitis (EAE) [79].* As Th17 cells are involved in many diseases, including not only CHS, but also psoriasis, rheumatoid arthritis, and encephalomyelitis among others, an antagonist of the EP4 receptor may become a useful drug target for regulating Th17-mediated diseases.

11. CONCLUSIONS

In this review, we have summarized current findings on the actions of prostanoids and their receptors in allergic and immune diseases of the skin. It is worthwhile to mention the role of prostanoid receptors on cutaneous DC function, where EP4 as promotor of DC migration and DP as inhibitor of DC migration mediate in opposite directions. NSAID treatment may therefore mask the complex effects of prosta-noids in the disease pathway. Clarification of each prostanoid pathway should widen our understanding not only of the actions of prostanoids but also of the delicate regulation of cutaneous immune reactions. At present, the studies on the role of allergic and immune diseases at the prostanoid receptor level were mostly conducted using experimental animals. The next question to address is to what degree this pathway contributes to initiation and progression of human diseases, and how effective the therapy directed to this signaling is. There are several ongoing efforts to develop better prostanoid receptor agonists and antagonists, and clinical trials involving these types of drugs may be able to clarify these issues. Selective manipulation of the actions mediated by each receptor may provide a novel therapeutic strategy for cutaneous allergic or inflammatory disorders.

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Letter to the Editor

Histamine modulates the responsiveness of keratinocytes to IL-17 and TNF-α through the H1-receptor

Interleukin (IL)-17-producing CD4+ helper T cells, Th17 cells, are involved in protection against bacterial pathogens and in the pathogenesis of various cutaneous inflammatory diseases, such as psoriasis and contact hypersensitivity [1]. In addition, a higher percentage of Th17 cells has been detected in the lesional skin and in the peripheral blood in the acute exacerbation phase of atopic dermatitis (AD), compared to normal controls [2,3]. It has been thought that IL-17 produced by Th17 cells infiltrating into the dermis acts on keratinocytes to produce inflammatory mediators, such as IL-8 and granulocyte macrophage colony-stimulating factor (GM-CSF) to chemoattract neutrophils and T cells and to activate Langerhans cells and endothelial cells, respectively [2,4], which initiates and enhances cutaneous inflammations.

Initiation of cutaneous inflammation correlates with rapid upregulation of pro-inflammatory mediators such as IL-1α, IL-1β, and tumor necrosis factor (TNF)-α. Recently, TNF-α has been paid special attention, since neutralizing anti-TNF-α therapy is effective in the treatment of a wide variety of diseases, including psoriasis. Other mediators, such as histamine, are thought to play important roles in AD and even in psoriasis, since mast cells are activated early in the developing psoriatic lesion and later increase in number in the upper dermis with concomitant expression of cytokines, histamine, and TNF-α [5]. However, the impact of mediators such as TNF-α and histamine on IL-17-induced inflammatory mediator production remains unclear. In this study, we evaluated the responsiveness of keratinocytes to IL-17 in the presence of inflammatory mediators such as TNF-α and histamine. In addition, we examined the effect of a histamine H1-receptor antagonist to identify the responsible role of the H1-receptor in this process.

A quantity of 4×10^4 normal human epidermal keratinocytes (NHEK; Kurabo, Osaka, Japan) was cultured in serum-free medium (Humedia-KG2, Kurabo) in 24-well plates for 4 days (70–80% confluence). Over the last 24 h, growth supplements were depleted. NHEK were then cultured with or without IL-17 (20 ng/ml; R&D Systems, Minneapolis, MN), histamine (10^{-4} M; Wako Pure Chemical, Osaka, Japan), and TNF-α (50 ng/ml; Miltenyi Biotec, CA, USA) alone or in combination for additional 3 days. The culture supernatants were collected and examined for IL-8 and GM-CSF using the cytometric bead array (CBA) system (BD Biosciences, San Diego, CA) according to the manufacturer's protocol. In addition, the mRNA of NHEK was extracted using the RNeasy mini kit (Qiagen, Tokyo, Japan) and mRNA levels of the H1-receptor were measured using real-time quantitative PCR (qPCR).

As shown in previous studies, IL-17 directly enhanced the production of IL-8 and GM-CSF on NHEK (Fig. 1A and B). In combination with TNF-α, IL-17 markedly increased IL-8 and GM-
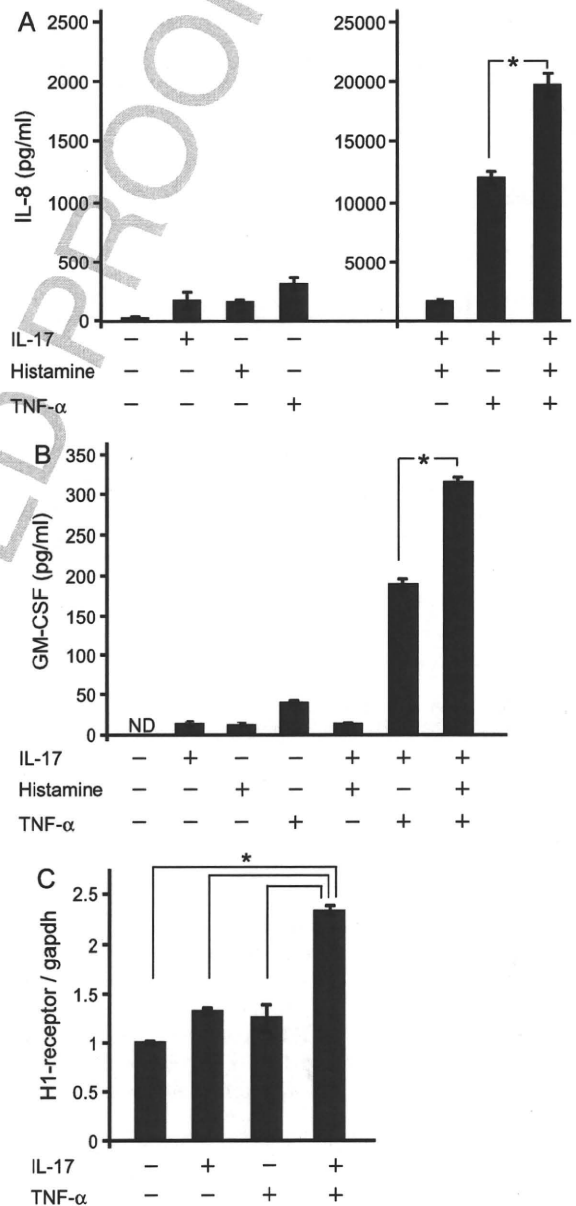

Fig. 1. The combination of TNF-α and histamine enhances the responsiveness of keratinocytes to IL-17.

NHEK were cultured in combination with IL-17, TNF-α, and/or histamine as indicated. The concentrations of IL-8 (A) and GM-CSF (B) in the culture supernatants were examined by CBA. (C) After cultivation, mRNA of NHEK was extracted for evaluating the expression level of the H1-receptor by qPCR. *, $P < 0.05$ between the indicated groups. Columns show the mean ± SD, and data are representative of three independent experiments with similar results. Statistical analysis was performed using ANOVA with the Tukey–Kramer multiple-comparison test.
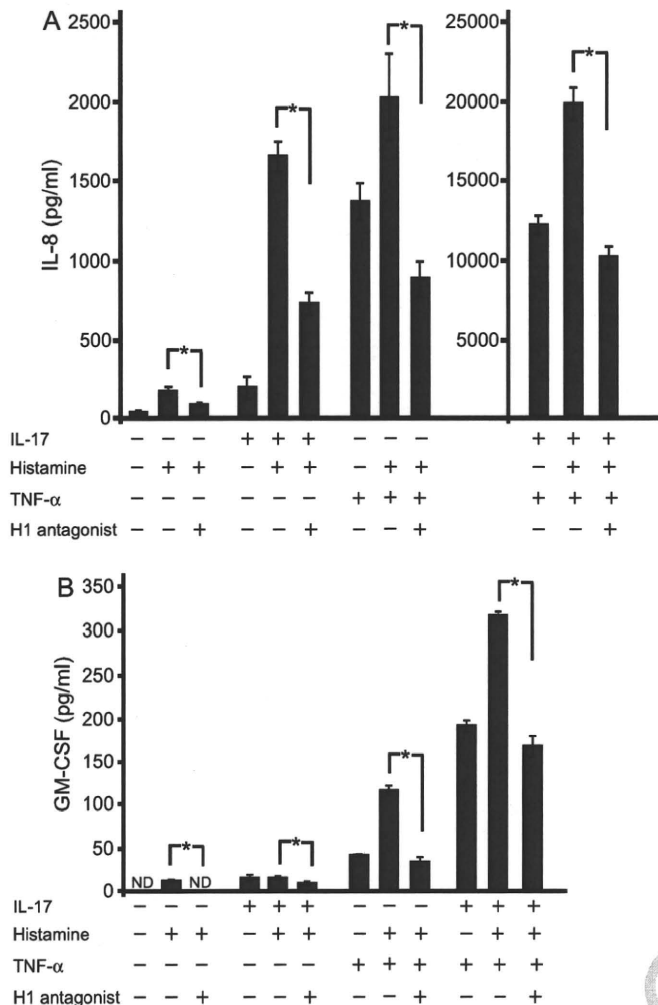
Fig. 2. Effect of an H1-receptor antagonist on the production of inflammatory mediators by IL-17-treated keratinocytes.
NHEK were cultured in combination with IL-17, TNF-α, histamine, and an H1-receptor antagonist as indicated. The concentrations of IL-8 (A) and GM-CSF (B) in the culture supernatants were examined by CBA. *, $P < 0.05$ between the indicated groups. Columns show the mean ± SD, and data are representative of three independent experiments with similar results. Statistical analysis was performed using ANOVA with the Tukey–Kramer multiple-comparison test.

CSF production. Although histamine marginally increased IL-8 and GM-CSF induced by IL-17 alone, when histamine was added to the culture medium with IL-17 and TNF-α in combination, histamine markedly augmented the production of IL-8 and GM-CSF. In addition, we found that the H1-receptor mRNA level was elevated by the presence of IL-17 and TNF-α in combination (Fig. 1C). This suggests that IL-17 and TNF-α intensified the effect of histamine through the H1-receptor.

Histamine elicits its wide variety of functions through the H1–H4 receptors [6]. To identify the role of the H1-receptor, a histamine H1-receptor antagonist carebastine (10^{-6} M, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), which is a carboxylated metabolite of ebastine and possesses anti-H1 receptor activity 10–100 times stronger than ebastine [7], was added into the relevant wells at the same time when other reagents were applied. Histamine-induced additional IL-8 and GM-CSF production concomitantly stimulated by IL-17 and/or TNF-α, was almost completely abrogated by the H1-receptor antagonist (Fig. 2A and B).

Previous studies have demonstrated that IL-17-induced inflammatory mediator production is augmented by the presence

of inflammatory cytokines such as TNF-α and interferon (IFN)-α *in vitro* [2,4,8] and in patients with inflammatory diseases, such as asthma, chronic obstructive pulmonary disease, and psoriasis [9,10]. In our study, we showed that the combination of IL-17, TNF-α, and histamine strongly induced the production of IL-8 and GM-CSF. In the skin lesion of AD and psoriasis, there is an infiltration of neutrophils, mast cells, and T cells. The cytokines they release locally act together in a synergistic and additive fashion on target cells [8]. Interestingly, the effect of histamine was somewhat enhanced when IL-17 and TNF-α were added in combination compared to when IL-17 alone was added, which suggests that the response of keratinocytes to histamine was enhanced due to the elevation of the H1-receptor expression level by IL-17 and TNF-α in combination. Taken together, histamine seems to enhance the effect of IL-17 on keratinocytes to produce inflammatory mediators in the presence of other pro-inflammatory cytokines, including TNF-α through the histamine H1-receptor. Therefore, anti-histamines might have beneficial effects on IL-17 and TNF-α-mediated diseases, such as psoriasis.

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Catharina Sagita Moniaga
Gyohei Egawa
Hiromi Doi
Yoshiki Miyachi
Kenji Kabashima*
Department of Dermatology, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

*Corresponding author at: Department of Dermatology, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawara, Sakyo-ku, Kyoto 606-8507, Japan. Tel.: +81 75 751 3310; fax: +81 75 761 3002
E-mail address: kaba@kuhp.kyoto-u.ac.jp (K. Kabashima)

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Molecular Pathogenesis of Genetic and Inherited Diseases

Flaky Tail Mouse Denotes Human Atopic Dermatitis in the Steady State and by Topical Application with *Dermatophagoides pteronyssinus* Extract

Catharina Sagita Moniaga,* Gyohei Egawa,*† Hiroshi Kawasaki,‡ Mariko Hara-Chikuma,* Tetsuya Honda,* Hideaki Tanizaki,* Saeko Nakajima,* Atsushi Otsuka,* Hiroyuki Matsuoka,§ Akiharu Kubo,‡ Jun-ichi Sakabe,¶ Yoshiki Tokura,¶ Yoshiki Miyachi,* Masayuki Amagai,‡ and Kenji Kabashima*†

From the Department of Dermatology,* and the Center for Innovation in Immunoregulative Technology and Therapeutics,† Graduate School of Medicine, Kyoto University, Kyoto; the Department of Dermatology,‡ Faculty of Medicine, Keio University, Tokyo; the Division of Medical Zoology,§ Jichi Medical University, Tochigi-ken; and the Department of Dermatology,¶ University of Occupational and Environmental Health, Fukuoka, Japan

The barrier abnormality, a loss-of-function mutation in the gene encoding filaggrin (*FLG*), which is linked to the incidence of atopic dermatitis (AD), is a recently discovered but important factor in the pathogenesis of AD. Flaky tail (*Flg^ft^*) mice, essentially deficient in filaggrin, have been used to investigate the role of filaggrin on AD. However, the relevancy of *Flg^ft^* mice to human AD needs to be determined further. In this study, we observed the clinical manifestations of *Flg^ft^* mice in the steady state and their cutaneous immune responses against external stimuli, favoring human AD. Under specific pathogen-free conditions, the majority of *Flg^ft^* mice developed clinical and histological eczematous skin lesions similar to human AD with outside-to-inside skin barrier dysfunction evaluated by newly devised methods. In addition, cutaneous hapten-induced contact hypersensitivity as a model of acquired immune response and a mite extract-induced dermatitis model physiologically relevant to a human AD were enhanced in *Flg^ft^* mice. These results suggest that the *Flg^ft^* mouse genotype has potential as an animal model of AD corresponding with filaggrin mutation in human AD. (*Am J Pathol 2010, 176:2385–2393; DOI: 10.2353/ajpath.2010.090957*)

Atopic dermatitis (AD), which affects at least 15% of children in developed countries, is characterized by eczematous skin lesions, dry skin, and pruritus.[1–3] Although the precise pathogenic mechanism of AD is as yet unknown, several accumulated lines of evidence suggest that a defective skin barrier to environmental stimuli may contribute to its pathogenesis. It has long been thought that the barrier abnormality in AD is not merely an epiphenomenon but rather is the "driver" of disease activity.[4] The evidence for a primary structural abnormality of the stratum corneum in AD is derived from a recently discovered link between the incidence of AD and loss-of-function mutations in the gene encoding filaggrin (*FLG*). Individuals carrying the *FLG* null allele variants tend to develop AD.[5–7]

Filaggrin protein is localized in the granular layers of the epidermis. Profilaggrin, a 400-kDa polyprotein, is the main component of keratohyalin granules.[8–10] In the differentiation of keratinocytes, profilaggrin is dephosphorylated and cleaved into 10 to 12 essentially identical 27-kDa filaggrin molecules, which aggregate in the keratin cytoskeleton system to form a dense protein-lipid matrix.[10] This structure is thought to prevent epidermal water loss and impede the entry of external stimuli, such as allergens, toxic chemicals, and infectious organisms. Therefore, filaggrin is a key protein in the terminal differentiation of the epidermis and in skin barrier function.[11]

Because AD is a common disease for which satisfactory therapies have not yet been established, understanding the mechanism of AD through animal models is an essential issue.[1,12] Flaky tail (*Flg^ft^*) mice, first introduced in 1958, are spontaneously mutated mice with

abnormally small ears, tail constriction, and a flaky appearance of the tail skin, which is most evident between 5 and 14 days of age.[13] Mice of the *Flg^ft* genotype express an abnormal profilaggrin polypeptide that does not form normal keratohyalin F granules and is not proteolytically processed to filaggrin. Therefore, filaggrin is absent from the cornified layers in the epidermis of the *Flg^ft* mouse.[14–16]

Recently, it has been revealed that the gene responsible for the characteristic phenotype of *Flg^ft* mice is a nonsense mutation of 1-bp deletion analogous to a common human *FLG* mutation.[15] These mice developed eczematous skin lesions after age 28 weeks under specific pathogen-free (SPF) conditions[17] and enhanced penetration of tracer perfusion determined by ultrastructural visualization,[16] and were predisposed to develop an allergen-specific immune response after epicutaneous sensitization with the foreign allergen ovalbumin (OVA).[15,17] On the other hand, general immunity through intraperitoneal sensitization with OVA was comparable between *Flg^ft* mice and control mice.[15,17]

Despite these recent advances, there still remain several issues with *Flg^ft* mice to be addressed. For example, serial close observation of clinical manifestations in reference to human AD will be informative. It is of value to evaluate the responses to external stimuli relevant to human AD, such as mite extracts, instead of OVA that has been used previously. A comparative study on the skin-mediated contact hypersensitivity (CHS) response and non-skin-mediated delayed-type hypersensitivity response is important to evaluate the impact of barrier dysfunction on immune responses *in vivo*. In addition, although it has now been determined that the barrier dysfunction is a key element in the establishment of AD, there is no established method to evaluate the outside-to-inside barrier function quantitatively.

In this study, we found that *Flg^ft* mice showed spontaneous dermatitis with skin lesions mimicking human AD in a steady state under SPF conditions: serial occurrence of manifestations as scaling, erythema, pruritus, and erosion followed by edema in this order. We also successfully evaluated outside-to-inside barrier dysfunction in *Flg^ft* mice quantitatively using a newly developed method. In addition, we determined that the Th1/Tc1-mediated immune response was enhanced by immunization through skin but not through non-skin immunization. Last, we induced severe AD-like skin lesions in *Flg^ft* mice by application of mites as a physiologically relevant antigen for human AD, which will be an applicable animal model of AD.

Materials and Methods

Mice

C57BL/6NCrSlc (B6) mice were purchased from SLC (Shizuoka, Japan). Flaky tail (STOCK *a/a ma ft/ma ft/J*; *Flg^ft* mice) mice have double-homozygous filaggrin (*Flg*) and matted (*ma*) mutations.[13,14] We used B6 mice as a control of *Flg^ft* mice because *Flg^ft* mice were described to

be outcrossed onto B6 mice at The Jackson Laboratory (Bar Harbor, ME)[13,14] (of note, although the strain was crossed with B6, it is not a B6 congenic strain but rather a hybrid stock that is probably semi-inbred). Female mice were used in all experiments unless otherwise stated; they were maintained on a 12-hour light/dark cycle at a temperature of 24°C and at a humidity of 50 + 10% under SPF conditions at Kyoto University Graduate School of Medicine. Routine colony surveillance and diagnostic workup verified that mice were free of Ectromelia virus, lymphocytic choriomeningitis virus, mouse hepatitis virus, Sendai virus, *Mycoplasma pulmonis*, cilia-associated respiratory bacillus, *Citrobacter rodentium* [*Escherichia coli* O115a,c:K(B)], *Clostridium piliforme* (Tyzzer's organism), *Corynebacterium kutscheri*, *Helicobacter hepaticus*, *Pasteurella pneumotropica*, *Salmonella* spp., parasites, intestinal protozoans, *Enterobius*, and ectoparasites. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Clinical Observation and Histology

The clinical severity of skin lesions was scored according to the macroscopic diagnostic criteria that were used for the NC/Nga mouse.[18] In brief, the total clinical score for skin lesions was designated as the sum of individual scores, graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe), for the symptoms of pruritus, erythema, edema, erosion, and scaling. Pruritus was observed clinically for more than 2 minutes.

For the histological portion of the study, the dorsal skin of mice was stained with H&E. Toluidine blue staining was used to detect mast cells, and the number of mast cells was calculated as the average from five different fields of each sample ×40 magnification).

Flow Cytometric Analysis and Quantitative RT-PCR

Cells from the skin-draining axillary and inguinal lymph nodes (LNs) and from the spleen were analyzed with flow cytometry. Fluorescent-labeled anti-CD4 and anti-CD8 antibodies were obtained from eBioscience (San Diego, CA) and used to stain cells. The total number of cells per organ and the number of cells in each subset were calculated through flow cytometry using the FACSCanto II system (Becton Dickinson, San Diego, CA). Quantitative RT-PCR was performed as described previously, using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a control.[19]

Total and Mite-Specific Serum IgE

Total serum IgE levels were measured with a mouse IgE ELISA Kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's protocols. For the measurement of mite-specific IgE levels, the same type of mouse IgE ELISA Kit was used with slightly modifications. Specifi-

cally, plates were coated and incubated with 10 μg/ml *Dermatophagoides pteronyssinus* (Dp) (Biostir, Kobe, Japan) diluted with coating buffer for 60 minutes. After a blocking period of 30 minutes, 100 μl of 5× diluted serum was added into each well and incubated for 2 hours. Anti-mouse IgE-horseradish peroxidase conjugate (1:15,000; 100 μL) was used to conjugate the antigen-antibody complex for 60 minutes at room temperature; from this point on the ELISA Kit was used according to the manufacturer's protocol. Absorbance was measured at 450 nm. The difference between the sample absorbance and the mean of negative control absorbance was taken as the result.

Skin Barrier Function

The dorsal regions of the skin were shaved in all mice before measurement. To evaluate inside-to-outside barrier function, transepidermal water loss (TEWL) was measured with a Tewameter Vapo Scan (Asahi Biomed, Tokyo, Japan) at 24°C and 46% relative humidity.

Outside-to-inside barrier function was assessed by means of fluorescein isothiocyanate isomer I (FITC) (Sigma-Aldrich, St. Louis, MO). The shaved dorsal skin of mice was treated with 100 μl of 1% FITC diluted in acetone and dibutyl phthalate (1:4); 3 hours later, this area was tape-stripped (Scotch tape, 3M, St. Paul, MN) nine times to remove the stratum corneum containing the remnant of FITC. The painted area (1.2 cm × 1.2 cm) was removed, and FITC concentration was measured. Each skin sample was soaked in PBS at 60°C for 10 seconds, after which the dermis and epidermis were separated. The epidermis was soaked in 500 μl of PBS, homogenized, and spun down at 2200 × g. The supernatant was collected, and fluorescence was measured at an excitation wavelength of 535 nm and an emission wavelength of 460 nm using an Arvo SX 1420 counter (Wallac, PerkinElmer, Waltham, MA). The fluorescence value was compared with a standard curve using FITC serial dilutions.

For the evaluation of fluorescence intensities of FITC penetrated into the epidermis, a 1 × 1 cm skin sample was taken after tape stripping, and a 10-μm Tissue-Tek (Sakura Finetek, Tokyo, Japan)-embedded section was analyzed using a BZ-9000 Biorevo digital microscope (Keyence, Osaka, Japan) at the same time exposure.

An *in situ* dye permeability assay with toluidine blue was performed using embryos at 18 days (littermates). Unfixed, untreated embryos were dehydrated by a 1-minute incubation in an ascending series of methanol (25, 50, 74, and 100%) and rehydrated with the descending same methanol series, washed in PBS, and stained with 0.01% toluidine blue.

Scratching Behavior

Scratching behavior was measured in detail using the Sclaba Real system (Noveltec, Kobe, Japan). Mice were put into the machine 20 minutes before measurement to allow them to adapt to the new environment. Ointment was then applied, and the number and duration of scratching sessions were counted according to the manufacturer's protocol for 15 minutes.[20]

Dermatitis Models

For the assessment of irritant contact dermatitis, 20 μl of 0.2 mg/ml phorbol myristate acetate (PMA) (Sigma-Aldrich) was applied to both sides of the ears. Ear thickness change was measured at 1, 3, 12, and 24 hours as well as 5 days after application.

To induce a CHS response, 25 μl of 0.5% 1-fluoro-2.4-dinitrobenzene (DNFB) (Nacalai Tesque, Kyoto, Japan) was painted on the shaved abdomens of mice for sensitization. Five days later, the ears were challenged with 20 μl of 0.2% DNFB, and ear thickness change was measured at 24 and 48 hours after application. Nonsensitized mice were used as a control. A delayed-type hypersensitivity response model was established using OVA (Sigma-Aldrich). Mice were sensitized with 200 μl of 0.5 mg/ml of OVA in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) intraperitoneally and challenged 5 days later with an injection of 20 μl of 1 mg/ml of OVA in incomplete Freund's adjuvant (Difco Laboratories) into the hind footpads. Footpad thickness was measured before and 24 hours after challenge. Nonsensitized mice were used as a control. Footpad swelling was calculated by (footpad thickness change of sensitized mice) − (footpad thickness change of nonsensitized mice). To induce murine AD-like skin lesions, 40 mg of 0.5% Dp in white petrolatum was topically applied to the ears and upper back twice a week for 8 weeks. Petrolatum without Dp was used as a control. One gram of Dp body product (Biostir) contained 1.78 mg of total protein with 2.47 μg of Dp protein (Der p1). Ear thickness and clinical scores were measured every week. Mite-specific IgE levels, TEWL, and histological appearance of eczematous skin were observed 12 hours after the final application.

Statistical Analysis

Data were analyzed using an unpaired two-tailed t-test. $P < 0.05$ was considered to be significant.

Results

Spontaneous Dermatitis of Flg[ft] Mice in the Steady State under SPF Conditions

As described previously,[14,15] the expression of the filaggrin monomer was barely detectable by Western blotting in the dorsal skin of *Flg[ft]* mice compared with that of B6 mice (data not shown). Here, we investigated the clinical manifestations seen in the skin of *Flg[ft]* mice raised in a steady state under SPF conditions and found that *Flg[ft]* mice developed spontaneous dermatitis (Figure 1A). The clinical severities of skin lesions, including pruritic activity, erythema, edema, erosion, and scaling, were scored. The total clinical scores of *Flg[ft]* mice increased with age
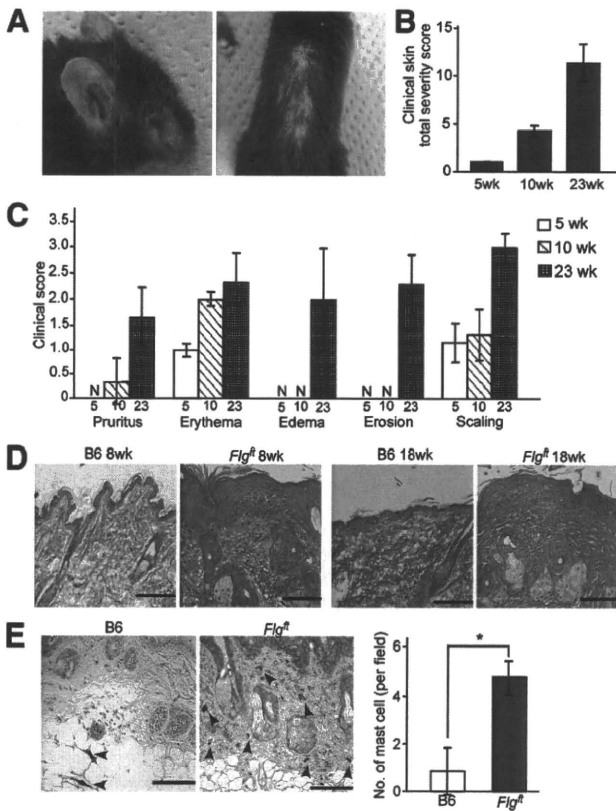
Figure 1. Spontaneous dermatitis in Flg^{ft} mice in SPF. **A:** Clinical photographs of 20-week-old Flg^{ft} mice. Total clinical severity scores (**B**) for each particular item (**C**) in 5-, 10- and 23-week-old Flg^{ft} mice. N, none. **D:** H&E-stained sections in 8- and 18-week-old mice. Scale bar = 100 μm. **E:** Toluidine blue staining of the skin from 8-week-old B6 and Flg^{ft} mice and the numbers of mast cells (**arrowheads**) per field are shown. *$P < 0.05$.

Figure 2. Skin barrier dysfunction in Flg^{ft} mice. **A:** TEWL through dorsal skin of 5-, 8-, and 16-week-old B6 and Flg^{ft} mice. **B:** Amount of FITC in the skin of B6 and Flg^{ft} mice after topical application. **C:** Fluorescence intensities of FITC of the skin after topical application. **Dashed white lines** indicate the border between the epidermis and the dermis, and the top of the epidermis. *$P < 0.05$.

(Figure 1B). The first manifestations to appear when mice were young were erythema and fine scaling; pruritic activity, erosion, and edema followed later (Figure 1C). In contrast, no cutaneous manifestation was observed in either B6 mice, studied as a control, or heterozygous mice intercrossed with Flg^{ft} and B6 mice kept under SPF conditions throughout the experimental period (data not shown). In addition, there was no apparent difference in terms of clinical manifestations between the genders of Flg^{ft} mice throughout the period (data not shown).

Histological examination of skin from Flg^{ft} mice revealed epidermal acanthosis, increased lymphocyte infiltration, and dense fibrous bundles in the dermis in both younger (8-week-old) and older (18-week-old) Flg^{ft} mice; none of these were observed in B6 mice (Figure 1D). In addition, toluidine blue staining to detect mast cells showed an increased number of mast cells, especially degranulated mast cells in the upper dermis, in Flg^{ft} mice (Figure 1E). No mouse or human mite bodies were detected in the sections. These data support the diagnosis of spontaneous clinical dermatitis in Flg^{ft} mice in the steady state under SPF conditions.

Defect of Skin Barrier Function in Flg^ft Mice

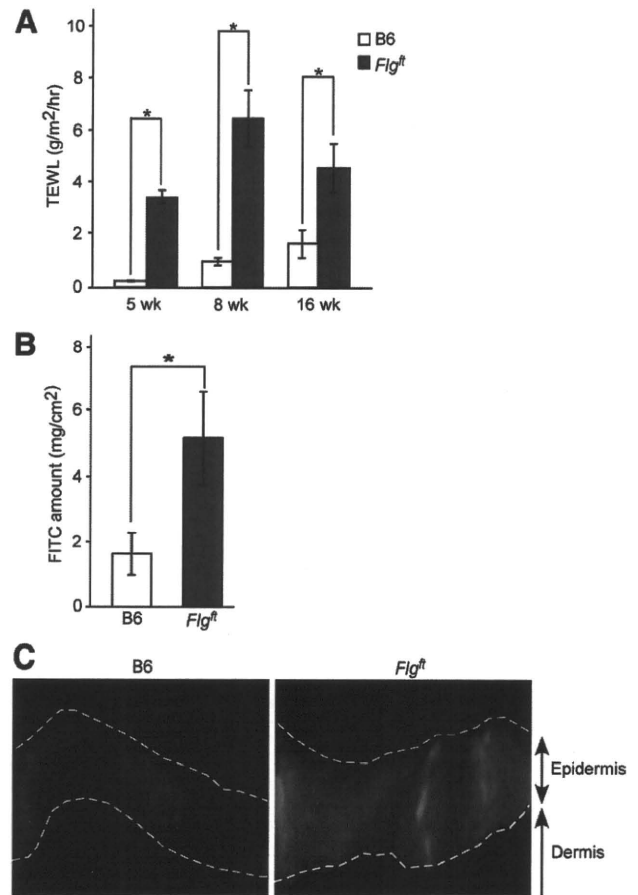Because barrier dysfunction is a common characteristic of AD,[4–7,21] we measured TEWL, an established indicator of barrier function.[21] TEWL was significantly higher in Flg^{ft} mice than in B6 mice from an early age (4 weeks) to an older age (16 weeks) (Figure 2A). Because TEWL is only a measure of water transportation through the skin from the inside to the outside of the body, another experimental method was necessary to evaluate outside-to-inside barrier function from the perspective of invasion of external stimuli. To address this issue, we measured FITC penetration through the skin from the outside. FITC solution was applied to the shaved dorsal skin of 8-week-old female mice; 3 hours later, the epidermis was separated and homogenized so that the FITC content could be measured with a fluorometer. The epidermis of Flg^{ft} mice contained a higher amount of FITC than that of B6 mice (Figure 2B). Neither group had FITC in the dermis after this procedure, however (data not shown). In addition, observation of fluorescence intensities in the epidermis of both mice showed stronger fluorescence in Flg^{ft} mice (Figure 2C). To further analyze the skin permeability, we examined the mouse embryos by toluidine blue solution and showed that the Flg^{ft} embryo was entirely dye-permeable compared with the control littermate (Supplemental Figure S1, see *http://ajp.amjpathol.org*). These data strongly indicate a defect in the skin barrier of Flg^{ft}
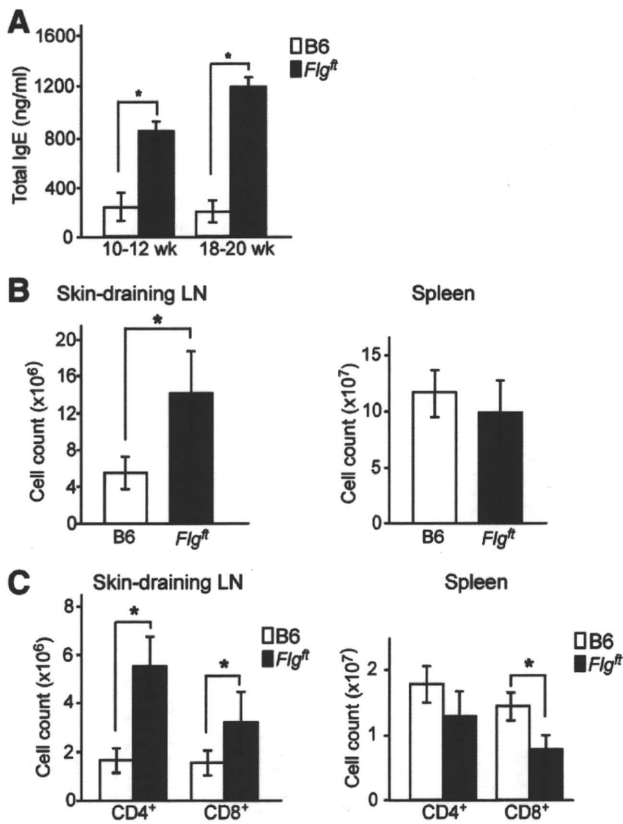
Figure 3. The immune status of *Flg^ft* mice in a steady state. **A:** Total serum IgE levels of B6 and *Flg^ft* mice as measured by enzyme-linked immunosorbent assay. **B** and **C:** Numbers of total cells (**B**), CD4^+ cells, and CD8^+ cells in the skin-draining LN and spleen (**C**). *$P < 0.05$.

Figure 4. Enhanced cutaneous immune responses in *Flg^ft* mice. **A** and **B:** Ear thickness change in B6 and *Flg^ft* mice after topical application of PMA as a model of irritant contact dermatitis (**A**), after DNFB challenge on the ears with or without sensitization (**B, left panel**) and the relative ear swelling (**B, right panel**) as a model of CHS. **C:** Delayed-type hypersensitivity response. B6 and *Flg^ft* mice were intraperitoneally sensitized with OVA, and challenged through subcutaneous injection to the footpad. Twenty-four hours later, footpad swelling change was measured. *$P < 0.05$.

mice, both from inside to outside and from outside to inside.

Immune Status in the Steady State

To further elucidate the immune status of *Flg^ft* mice in the steady state under SPF conditions, we measured the levels of total serum IgE, because increased severity of AD is known to be correlated with elevated serum IgE levels.[22] IgE levels were significantly higher in *Flg^ft* mice than in age-matched B6 mice in the steady state under SPF conditions (Figure 3A). To investigate this matter in greater detail, single cell suspensions from the skin-draining inguinal and axillary LNs and from the spleen were analyzed. The total mononuclear cell number of the LNs was significantly higher in *Flg^ft* mice than in B6 mice, but that of the spleen was comparable (Figure 3B). In addition, *Flg^ft* mice exhibited significantly higher numbers of CD4^+ and CD8^+ cells in the skin-draining LNs, but not in the spleen (Figure 3C). Thus, an enhanced immune reaction seems to be induced in *Flg^ft* mice by the condition of their skin.

To further analyze the immune condition of the skin, we measured the Th1 (interferon-γ [IFN-γ]), Th2 (interleukin [IL]-4 and IL-13), and Th17 (IL-17) cytokine mRNA levels of dorsal skin of 9-week-old mice in the steady state. The mRNA expression levels of IFN-γ, IL-4, and IL-13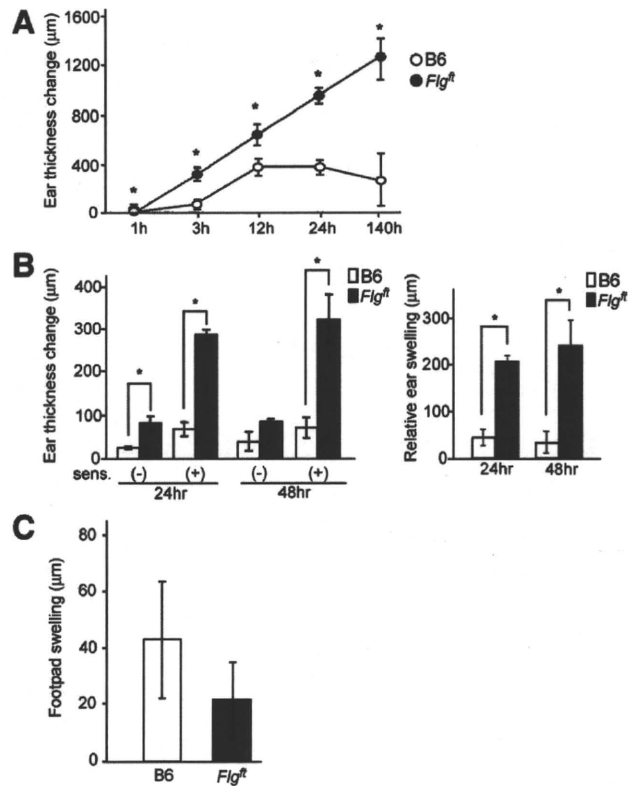 were similar between *Flg^ft* and B6 mice, but there was an enhancement in the IL-17 mRNA expression (data not shown) as reported previously.[17]

Enhanced Dermatitis in Flg^ft Mice under External Stimuli

To characterize the likelihood of various cutaneous immune responses, mice were exposed to various external stimuli. First, we studied the irritant contact dermatitis response to PMA as an irritant agent. When we applied PMA to the ears of B6 and *Flg^ft* mice, *Flg^ft* mice exhibited an enhanced ear swelling response compared with age-matched B6 mice throughout the experimental period (Figure 4A). Next, we measured the CHS response to DNFB. DNFB was applied to the abdominal skin for sensitization; 5 days later, the ears were challenged with the same hapten. The ear thickness change was more prominent in *Flg^ft* mice than in B6 mice (Figure 4B, left panel). On the other hand, the ear thickness change of mice without sensitization was higher for *Flg^ft* mice than B6 mice, suggesting that irritation contact dermatitis was enhanced in *Flg^ft* mice as expected. To avoid the involvement of this irritation in CHS, we next analyzed the relative ear swelling by subtracting the ear thickness change without sensitization from the ear thickness change with sensitization. The relative ear swelling was more exten-
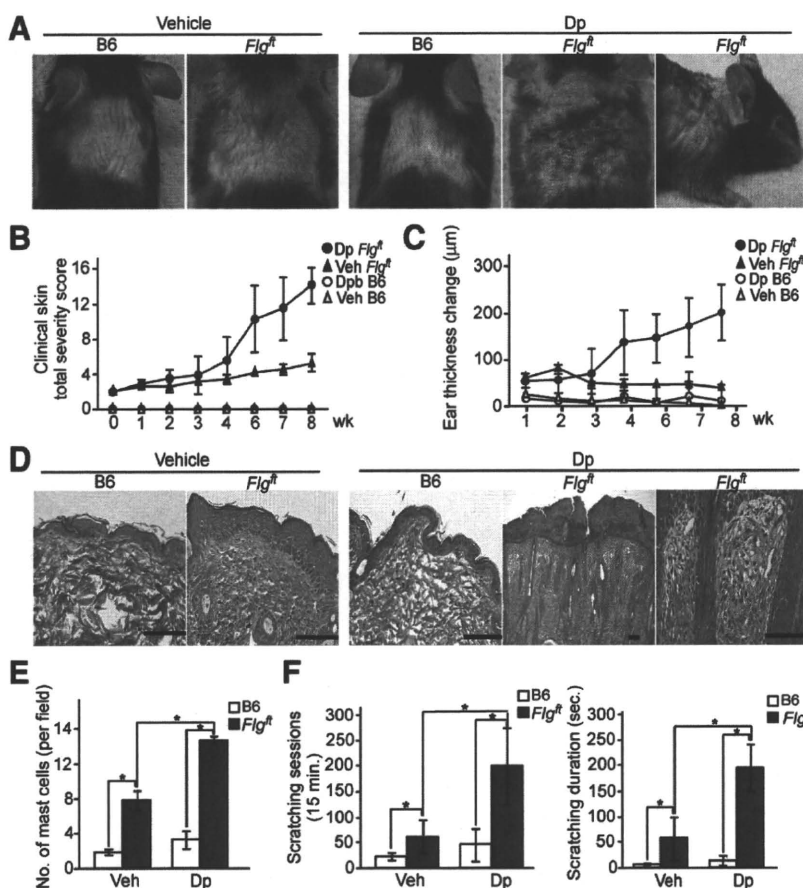
Figure 5. Mite (Dp)-induced dermatitis model. B6 and *Flg^ft* mice were topically treated with ointment with (Dp) or without (vehicle/Veh) Dp. **A–C:** Clinical photographs after the last application of Dp (**A**), clinical skin severity scores (**B**), and changes in ear thickness (**C**) at each indicated time point after application. **D** and **E:** Histological appearance of the skin (**D**) and the numbers of mast cells (**E**) after the last application. Scale bars = 100 μm. **F:** Scratching behavior: the number of scratching sessions (**left**) and the total duration of scratching (**right**) over 15 minutes after the last application. **G:** TEWL after the last application. **H:** Serum mite-specific IgE levels. *$P < 0.05$.

sive in *Flg^ft* mice than in B6 mice (Figure 4B, right panel). We then measured the relative amount of mRNA for IFN-γ, as a representative Th1 cytokine, to GAPDH as an endogenous control. The relative amount of IFN-γ was higher in the ears of *Flg^ft* mice than in those of B6 mice 12 hours after the challenge (0.27 ± 0.13 versus 0.019 ± 0.013, $n = 3$). To further assess the immune responses of *Flg^ft* mice, we elicited a delayed-type hypersensitivity response through nonepicutaneous sensitization and challenge. Mice were immunized intraperitoneally with OVA and challenged with a subcutaneous injection of OVA into the footpad. In contrast to the CHS response induced via the skin, the resulting footpad swelling in *Flg^ft* mice was lower rather than higher than that in B6 mice (Figure 4C). We also examined the production of mRNA levels of the spleen 3 days after intraperitoneal OVA injection, and it showed a similar level of IFN-γ between *Flg^ft* mice and B6 mice (relative mRNA amount to GAPDH: 0.011 ± 0.005 versus 0.016 ± 0.006, $n = 5$). Thus, Th1/Tc1 immune responses were enhanced in *Flg^ft* mice only when the stimuli operated via the skin, suggesting that the enhanced immune responses seen in *Flg^ft* mice depend on skin barrier dysfunction.

It has been reported that *Flg^ft* mice show an enhanced immune response to OVA.[15,17] Their reaction to clinically relevant allergens such as mites has not been evaluated, however. It has also been reported that BALB/c or NC/Nga mice develop an allergic cutaneous immune response to mite antigens when they are applied to the skin after vigorous barrier disruption by means of tape-strip-

ping or SDS treatment.[23,24] Accordingly, we sought to determine whether skin lesions could be induced in *Flg^ft* mice through the application of Dp ointment without any skin barrier disruption procedures to evaluate the physiological significance of filaggrin.

The application of Dp ointment to shaved backs and ears induced no cutaneous manifestation in B6 mice throughout the experimental period (Figure 5, A and B), but the same treatment induced dermatitis in *Flg^ft* mice, especially on the ears, face, and dorsal skin. Petrolatum alone, used instead of Dp ointment as a control, induced no skin manifestation (Figure 5, A–C). The clinical severity of Dp-induced dermatitis was scored; after 16 applications of Dp ointment over 8 weeks, *Flg^ft* mice had developed a very severe skin condition in contrast with the control groups. Consistently, ear swelling in response to Dp ointment was most prominent in *Flg^ft* mice (Figure 5C). Histological examination of H&E-stained sections of involved *Flg^ft* skin after 16 applications showed acanthosis, elongation of rete ridges, and dense lymphocyte and neutrophil infiltration in the dermis (Figure 5D), accompanied by an increased number of mast cells in the dermis (Figure 5E). We also measured the scratching behavior of *Flg^ft* mice treated with Dp using the Sclaba Real system. The number of scratching sessions and the total duration of scratching were significantly higher in *Flg^ft* mice than in B6 mice, even among those mice that had not been treated with Dp ointment (Figure 5F); treatment of *Flg^ft* mice with Dp ointment raised the number of scratching sessions and the total duration of scratching even higher.

We further evaluated barrier function by measuring TEWL in Dp-treated and untreated mice of each genotype; TEWL was higher in untreated *Flg^ft^* mice than in B6 mice, and Dp treatment of *Flg^ft^* mice raised TEWL even higher (Figure 5G). Finally, we examined mite-specific serum IgE levels after the last application and found that *Flg^ft^* mice had higher levels of Dp-specific IgE than B6 mice had (Figure 5H). Thus, the treatment of *Flg^ft^* mice with Dp ointment, even without prior barrier disruption, remarkably enhanced both the clinical manifestations and the laboratory findings that correspond to indicators of human AD.

Discussion

Here, we demonstrated that *Flg^ft^* mice exhibit spontaneous dermatitis with lymphadenopathy, elevated IgE levels, and skin barrier disruption in a steady state under SPF conditions. These outcomes are compatible with the features of human AD, which include chronic eczema, pruritus, and dry skin with elevated TEWL and serum IgE levels.[1-4,25,26] In addition, *Flg^ft^* mice exhibit enhanced susceptibility to irritant contact dermatitis, CHS, and mite-induced dermatitis compared with B6 mice; these characteristics are also reminiscent of human AD. These results suggest that the barrier defect in this strain of mice leads to spontaneous dermatitis and enhances cutaneous immune responses and inflammation.

Since the first introduction of *Flg^ft^* mice in 1972,[13] there have been only a few reports of these mice. The first report demonstrated that *Flg^ft^* mice without the *ma* mutation showed flaky skin as early as postnatal day 2 but became normal in appearance by 3 to 4 weeks of age without spontaneous dermatitis except for their slightly smaller ears.[13] Later, the lack of filaggrin in the epidermis was proposed in the commercially available strain of *Flg^ft^* mice used in this study, which has both *Flg* and *ma* mutations, as a model of ichthyosis vulgaris, and therefore the cutaneous inflammatory conditions from the perspective of AD was not discussed.[14] There have been three recent studies using *Flg^ft^* mice as a model of filaggrin deficiency: Fallon et al[15] used *Flg^ft^* mice from which the *ma* mutation had been eliminated with four additional backcrosses to B6 mice, and others used the commercially available *Flg^ft^* mice.[16,17] The first report showed only a histological abnormality without clinical manifestations,[15] the second report demonstrated spontaneous eczematous skin lesions after 28 weeks of age,[17] and the third report did not indicate any spontaneous dermatitis in *Flg^ft^* mice.[16] In our experiment, we observed a spontaneous dermatitis as early as 5 weeks of age with mild erythema and fine scales. These symptoms gradually exacerbated, accompanied by scratching, erosion, and edema, respectively, and became prominent at the age of 23 weeks. The discrepancies among these results seem to be related to the presence or absence of the *ma* mutation and/or variation in the genetic backgrounds of the different strains used and to environmental factors. It has been reported that Japan has higher morbidity for AD than other countries,[27,28] possibly attributable to environmental factors such as pollen.

It has been reported that TEWL, an indicator of inside-to-outside barrier function, is high in both AD patients with the *FLG* mutation[29] and *Flg^ft^* mice.[15] In consideration of the immunological defense by the skin, however, it is more important to assess outside-to-inside barrier function rather than inside-to-outside barrier function. In fact, outside-to-inside barrier dysfunction has recently been proposed as the most important aspect in the pathogenesis of AD.[9,26] Scharschmidt et al[16] reported increased bidirectional paracellular permeability of water-soluble xenobiotes by ultrastructural visualization in *Flg^ft^* mice, suggesting a defect of the outside-to-inside barrier. However, the quantitative measurement of this parameter has not been addressed. Here, we propose a new method for evaluating outside-to-inside barrier function quantitatively by measuring the penetrance of FITC through the skin. This method has a parallel correlation with the qualitative measurement of FITC penetrated in epidermis and an established method for skin permeability assay, the *in situ* dye staining method. Therefore, by using this new method, we were able to detect outside-to-inside barrier dysfunction in *Flg^ft^* mice quantitatively.

The skin abnormality associated with AD is well known to be a predisposing factor to sensitive skin[30,31] and allergic contact dermatitis,[32,33] but patients with AD produce a tuberculin response similar to that of healthy control subjects.[34,35] In humans, sensitive skin is defined as reduced tolerance to cutaneous stimulation, with symptoms ranging from visible signs of irritation to subjective neurosensory discomfort.[30,31] The question of whether human AD patients are more prone to allergic contact dermatitis than nonatopic individuals is still controversial.[33] To address this question, we evaluated skin responsiveness to PMA as an irritant and found that irritant contact dermatitis was enhanced in *Flg^ft^* mice. In addition, *Flg^ft^* mice showed an increased skin-sensitized CHS reaction, a form of classic Th1- and Tc1-mediated delayed-type hypersensitivity to haptens, emphasized by increased IFN-γ production. In contrast, when mice were sensitized intraperitoneally, no difference was observed between *Flg^ft^* and B6 mice *in vivo* or *in vitro*. This finding is consistent with the observation that humans with and without AD respond comparably to tuberculin tests[34,35] and suggests that skin barrier function regulates cutaneous immune conditions, which hints at a possible mechanism involved in human AD.

Clinical studies have provided evidence that a house dust mite allergen plays a causative or exacerbating role in human AD[36] and that a strong correlation exists between patients with *FLG* null alleles and house dust mite-specific IgE.[37] AD-like skin lesions can be induced by repeated topical application of a mite allergen in NC/Nga mice but not in BALB/c mice.[23] In the present study, we induced skin lesions that were clinically and histologically similar to AD, along with increased TEWL, increased scratch behavior, and increased levels of mite-specific IgE, in *Flg^ft^* mice through the application of Dp. Dp is a common aeroallergen that is frequently involved in induction of human AD. It has protease activities, spe-