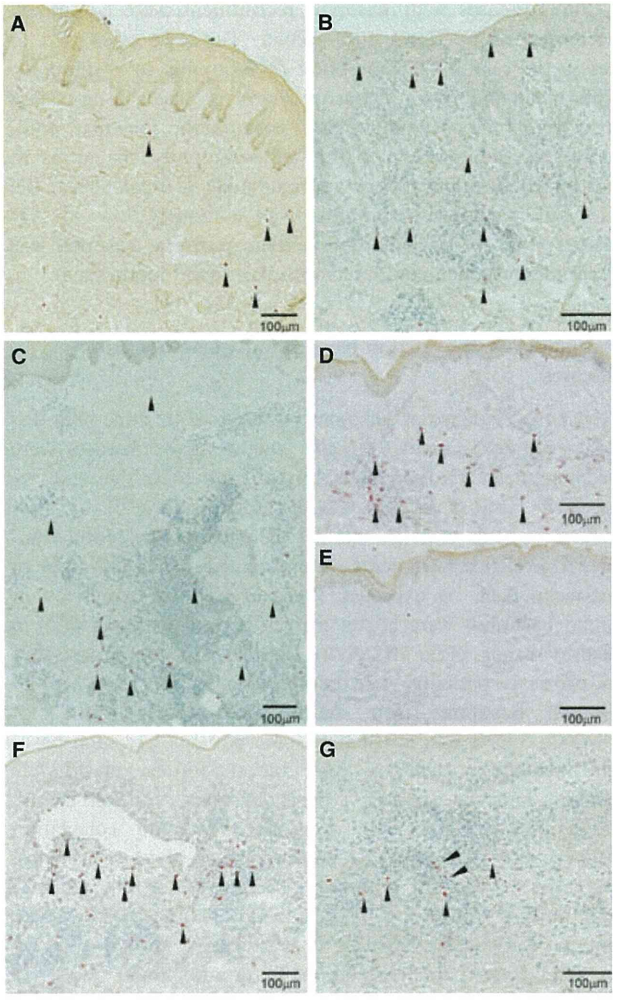

Figure 1 Representative features of BB1 immunostaining for human skin diseases. (A) Atopic dermatitis (lesional skin). (B) Skin site showing positive reaction to house dust mite allergen at 24 h in atopic dermatitis. A number of basophils were observed within both the epidermis and dermis. (C) Prurigo nodularis. (D) Urticarial lesions that persisted for more than 24 h. (E) Isotype control of (D). (F) Bullous pemphigoid. (G) Henoch–Schönlein purpura. Basophils are indicated by arrowheads.

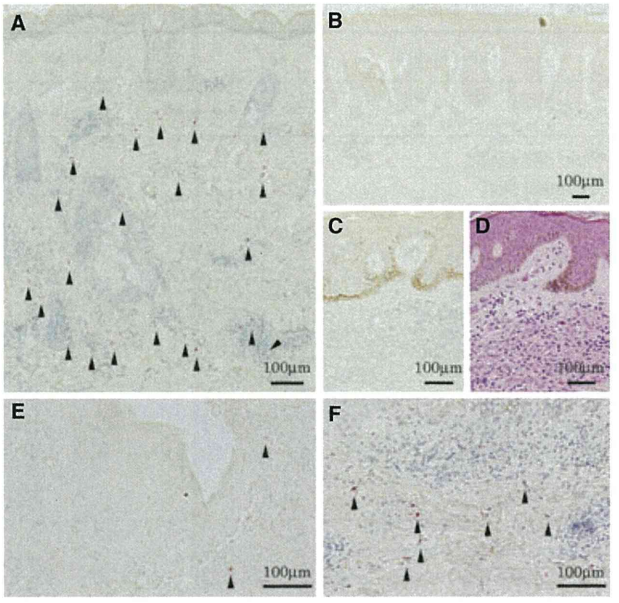

Figure 2 Basophil recruitment in eosinophilic pustular folliculitis and scabies. (A) A Number of basophils were observed around hair follicules in the entire dermis of eosinophilic pustular folliculitis (*arrow heads*). In contrast, basophilis were entirely absent in psoriasis vulgaris (B) and mastocytosis ((C) Immunohistochemical staining, (D) H&E staining). (E) Skin lesions around tick mouthparts of *Ixodes persulcatus*. (F) Scabies.

acanthosis (IgE-mediated chronic allergic skin inflammation; IgE-CAI) (21). These features appear to share phenotypical and histological similarities with those of prurigo commonly appearing as urticarial papules. Considering the evidence that basophils contribute as essential 'initiator cells' to the development of mouse IgE-CAI (22), one could assume that basophils also participate in the pathological mechanisms of relevant human skin diseases. Indeed, basophils were frequently observed in prurigo lesions. Basophils, rather than mast cells, may thus be a therapeutic target for prurigo reactions.

While insect bites by *Cimex lectularius* and scabies lesions showed basophil accumulation (Table 1 and Fig. 2F), few tissue basophils were seen around the tick mouthparts of *Ixodes persulcatus*, despite marked cellular infiltration by neutrophils, lymphocytes and eosinophils (Fig. 2E). This appeared to be due to the fact that the tick bite lesion tested in this study was obtained from a patient who did not have any past history of tick bites. A recent observation revealed that basophils contribute to the acquired resistance to ticks, but not to first infestation in mice (16). Our results also suggest that basophils are an important player in the acquired and protective immunity against *Cimex lectularius* and scabies.

In this study, basophils were generally detected in skin diseases where eosinophils were present, as evidenced by the marked number of basophils in bullous pemphigoid and eosinophilic pustular folliculitis. Both basophils and eosinophils express the common chemokine receptor CCR3. Ligands for CCR3, such as eotaxin-1 (CCL11) and-3 (CCL26), are produced by dermal fibroblasts in response to Th2-type cytokines, i.e., IL-4 and IL-13 (27, 28). Inflammation where the cytokine balance is biased toward Th2 immunity should enable concomitant basophil and eosinophil recruitment into skin lesions. Recent findings revealed that basophils, eosinophils, as well as Th2 cells express CRTH2, a receptor of prostaglandin D2 (PGD2). We have reported evidence suggesting the involvement of CRTH2 receptor in AD, urticaria, prurigo nodularis and eosinophilic pustular folliculitis (29, 30). CRTH2-signaling induces activation and chemotaxis in these cells (31). Thus, PGD2–CRTH2 inter-

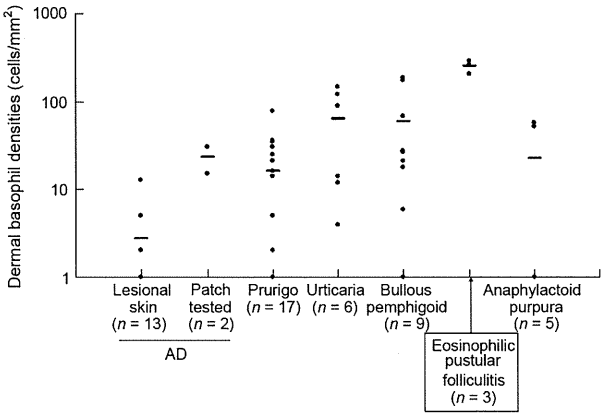

Figure 3 Dermal basophil densities in skin diseases. The density of BB1 (+) basophils was analyzed by the IMAGE PRO®PLUS software as described in the *materials and methods*. Horizontal bars indicate mean levels. AD atopic dermatitis.

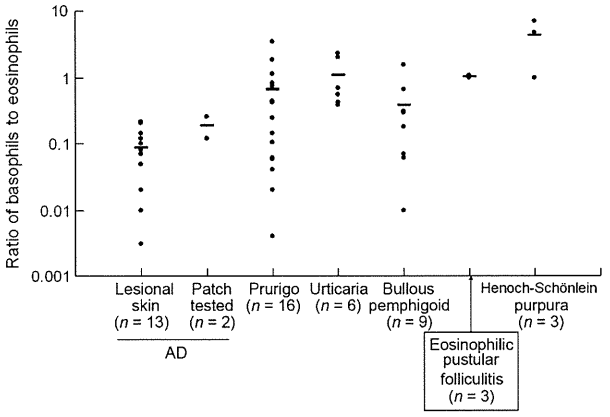

Figure 4 Ratios of the number of infiltrative basophils *vs.* eosinophils. Serial tissue sections were processed to BB1 immunostaining or H&E staining, and the number of basophils and eosinophils were counted. Results are expressed as the ratio of basophils/eosinophils.

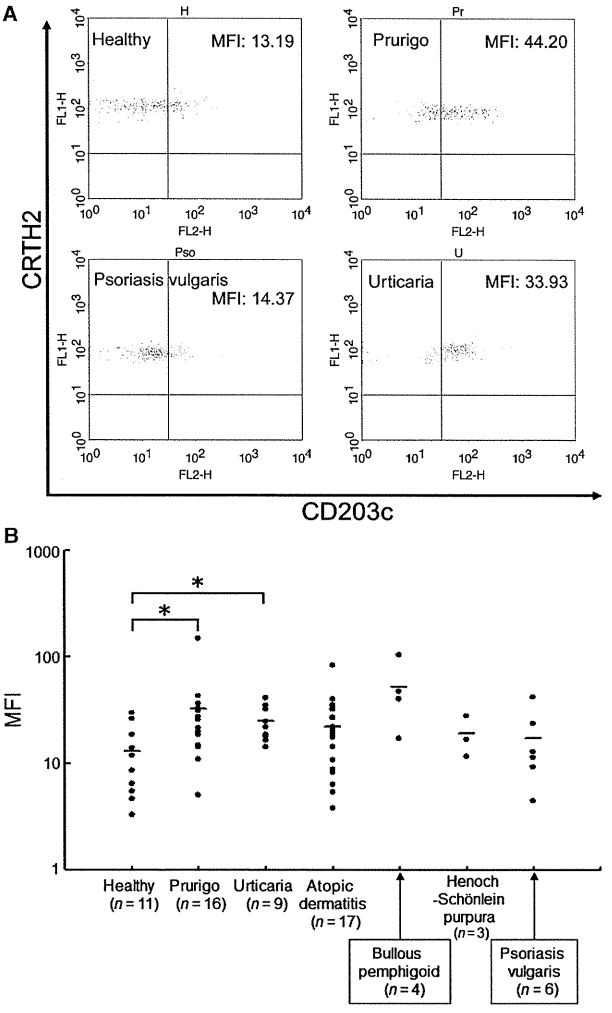

Figure 5 CD203c expression by blood basophils. (A) Representative figures of flow cytometric analysis for basophil CD203c. (B) Levels of mean fluorescence intensity (MFI) of CD203c on basophils from skin diseases. MFI is shown as data after subtraction of MFI of control samples. Horizontal bars indicate mean levels. $*P < 0.05$ compared with healthy donors.

action could be another pathway that promotes co-accumulation of basophils and eosinophils in Th2-predominant inflammation.

The ratios of tissue basophils/eosinophils varied among skin diseases (Fig. 4), despite sharing the aforementioned common chemoattractant receptors, i.e. CCR3 and CRTH2. Thus, different mechanisms appear to regulate recruitment of these two types of cells in each disease. In human atopic subjects, intra-dermal injection of CCL3/MIP-1α induced eosinophil, but not basophil, infiltration (32). A recent report demonstrated that CCR7, a chemokine receptor not observed in eosinophils, promotes IL-3-induced basophil rolling and adhesion to endothelial cells (33). Thus, the ratio of basophils to eosinophils could be influenced by the local production of CCL3/MIP-1α and/or IL-3.

BB1 Ab detects a basophil granule-associated protein (basogranulin) (23). Therefore, we might not be able to detect some of the degranulated basophils, resulting in the underestimation of infiltrative basophil numbers. This pitfalls may be also true for degranulated eosinophils. Variations of basophil numbers and of ratios of basophils to eosinophils may be due to differences in activation and degranulation status of these cells in each lesion of skin diseases.

In this study, we assessed the activation status of blood basophils. To this end, cell surface expression of CD203c was evaluated by flow cytometry (25). As expected, CD203c expression in patients with prurigo and urticaria was higher than that of healthy donors. There was also a trend toward increased CD203c in bullous pemphigoid, although it was not statistically significant. This was probably due to a small

sample size (n = 4). A prior report also demonstrated increased expression of basophil CD203c in chronic urticaria (34), while another study only detected elevated expression of basophil CD69, but not CD203c (35). Conversely, patients with psoriasis vulgaris where tissue basophils were absent did not show blood basophil activation. In AD patients, levels of basophil activation were comparable to those in healthy donors. This appeared to mirror the observation that basophil recruitment in skin lesions in AD was not necessarily prominent, unlike in patch-tested sites for allergens. However, it was noted that some AD patients showed high levels of basophil CD203c. Basophil activation may be promoted during immune reactions biased toward Th2. The wide variations in CD203c levels in AD may be due to the fact that AD individuals are not necessarily in the immunological state biased toward Th2 (26).

Henoch–Schönlein purpura is a type of vasculitis in the superficial plexus in the dermis (leukocytoclastic vasculitis). Vascular damage is principally mediated by neutrophils. Surprisingly, however, a number of basophils were detected in skin lesions, and appeared more prominent than eosinophil recruitment (Fig. 4). The contribution of basophils in the pathological mechanisms of Henoch–Schönlein purpura is uncertain, but it is unlikely that basophils directly participate in the vascular damage, as they were localized around vessels and intercollagenous spaces, but not adjacent to or inside vascular walls. In addition, we were unable to detect blood basophil activation in Henoch–Schönlein purpura (Fig. 5B), although this was assessed with only a small number of samples.

Collectively, basophils are recruited into skin lesions in a variety of inflammatory skin diseases even though blood basophilia is not seen. Further study is needed in order to verify the contribution of basophils to the pathological etiology of each disease.

Conflict of interest statement

We have no conflict of interest in relation to the publication of this paper.

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REVIEW ARTICLE

Newly appreciated roles for basophils in allergy and protective immunity

H. Karasuyama[1,2], K. Obata[1], T. Wada[1]*, Y. Tsujimura[1]* & K. Mukai[1†]

[1]Department of Immune Regulation and [2]JST, CREST, Tokyo Medical and Dental University Graduate School, Tokyo, Japan

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Correspondence
Hajime Karasuyama, Department of Immune Regulation, Tokyo Medical and Dental University Graduate School,
Tokyo 113-8519, Japan.
Tel.: +81-3-5803-5162
Fax: +81-3-3814-7172
E-mail: karasuyama.mbch@tmd.ac.jp

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*Present address: Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Ibaraki, Japan.

†Present address: Department of Pathology, Stanford University, Stanford, CA, USA.

Abstract

Basophils are evolutionarily conserved in many animal species, in spite of the fact that they account for <1% of peripheral blood leukocyte. This suggests that basophils have an indispensable and nonredundant role *in vivo*, even though they show some phenotypic similarity with tissue-resident mast cells. However, their functional significance remained uncertain long after Paul Ehrlich discovered them as blood-circulating cells with basophilic granules more than 130 years ago. The study of basophils has been far behind that of mast cells, owing to the rarity of basophils and the paucity of tools for their detection and functional analysis. Recent development of novel analytical tools, including basophil-depleting antibodies and genetically engineered mice deficient only in basophils, has greatly advanced basophil research and illuminated previously unrecognized roles of basophils. We now appreciate that basophils and mast cells play distinct roles in immune responses. Basophils have crucial roles in the development of acute and chronic allergic responses, the protective immunity against ecto- and endoparasites, and the regulation of acquired immunity, including the augmentation of humoral memory responses and the initiation of Th2 responses. Thus, basophils are no longer the neglected minority and are key players in the immune system.

Basophils are basophilic granulocytes and were first described at the end of the nineteenth century by Paul Ehrlich as blood-circulating cells with basophilic granules in their cytoplasm that stain in purple with basic dyes. They are very rare compared to neutrophils and represent <1% of peripheral blood leukocytes. In addition to basophilic granules in the cytoplasm, basophils share some important features with mast cells (1). Both basophils and mast cells express high-affinity IgE receptor FcεRI on their surface and release allergy-inducing chemical mediators such as histamine in response to various stimuli, including IgE/FcεRI-mediated one. Therefore, basophils have often erroneously been considered as minor relatives of mast cells (2). From the clinical point of view, basophils are much more accessible than tissue-resident mast cells. Accordingly, basophils are a practical and convenient surrogate for mast cells in *in vitro* allergy test, in that basophils isolated from allergic patients are incubated with allergen candidates to determine which allergen indeed stimulates basophils (3, 4). Basophils were some times considered even as blood-circulating precursors of tissue-resident mast cells. Although we now appreciate that basophils and mast cells are distinct cell lineages, the developmental origin of basophils still remains poorly defined, compared to that of other hematopoietic cells. A recent study in mice demonstrated a close lineage relationship between basophils and mast cells, by identifying common progenitors that can differentiate into basophils and mast cells but not other lineages (5). Curiously, the same study also identified basophil-committed monopotent progenitors, probably derived from granulocyte/monocyte progenitors (5), while another study identified mast cell-committed progenitors that are directly derived from multipotent progenitors (6). Even more compli-

cated, human basophils seem to have a closer relationship with eosinophils rather than with mast cells (7), based on the result of *in vitro* colony assay, the presence of granulocytes with a basophil–eosinophil hybrid phenotype in some leukemic patients, and two case reports of patients who lacked both basophils and eosinophils.

Basophils and mast cells differ in several important aspects, in spite of the above-mentioned similarity. Developmentally, basophils originate and mature in the bone marrow, circulate as terminally differentiated cells in the peripheral blood, and do not usually infiltrate peripheral tissues unless inflammation takes place. In contrast, mast cells mature in peripheral tissues, after their bone marrow-derived precursors enter the circulation and migrate into peripheral tissues. Mature mast cells reside in peripheral tissues and do not usually circulate in the peripheral blood. Thus, the anatomical localization differs between basophils and mast cells. They also differ in the life span: basophils are short-lived (approximately 2.5 days in mice) (8) while mast cells survive for weeks and months. Basophils do not proliferate after their maturation, unlike mast cells. These apparent differences between basophils and mast cells, together with the evolutionary conservation of basophils in many animal species (9), suggest that basophils and mast cells may have distinct roles *in vivo*. However, the functional significance of basophils remained an enigma long after their discovery by Paul Ehrlich. At last, recent studies have uncovered previously unappreciated roles of basophils and placed the once-overlooked basophils on the main stage of immunology (10–19). This review focuses on emerging roles of basophils in physiological and pathological settings, based mainly on animal studies performed in the past 5 years.

Recent technological advances in basophil research

The study of basophils has long been hampered not only by their rarity but also by the lack of tools for their detection and functional analyses. During the past 5 years, a series of groundbreaking tools have been developed and brought new era of basophil research.

Novel tools for definitive identification of basophils

One can readily stain and identify human basophils in blood smear with basic dyes as Paul Ehrlich described. However, this is not true for histochemical identification of tissue-infiltrating basophils in formalin-fixed and paraffin-embedded tissue sections, in sharp contrast to relatively easy identification of tissue-resident mast cells. This difficulty in basophil detection has rendered the basophil research far behind the mast cell research. Although successful identification of tissue-infiltrating basophils was reported with use of electron microscopy and epon-embedded tissue sections (20), more practical and convenient methods for basophil identification have long been sought. A panel of monoclonal antibodies (mAbs) specific to human basophils has been established for the immunohistochemical detection of basophil infiltration into peripheral tissues, including BB1 (basogranulin-specific), J175-7D4 (pro-major basic protein-specific), and 2D7 (21–23). We have recently established TUG 8, a mAb specific to mouse mast cell protease 8 (mMCP-8), which is suitable for immunohistochemical identification of mouse basophils (24). mMCP-8 is a granzyme B-like serine protease that is selectively expressed by basophils and stored in their secretory granules (24).

Recent studies have identified useful markers on the surface of human and mouse basophils, including CD49b (DX5), CD123 (IL-3 receptor α chain), CD200R3, CD203c, 2B4, CCR2, CCR3, and the intermediate level of CD45R expression (13, 25–30). These surface markers, in combination with other surface phenotypes such as FcϵRI$^+$ and c-Kit$^-$ as well as their characteristic side/forward scatter profile distinct from that of other granulocytes, allow basophils to be readily identified and isolated by flow cytometry, in spite of their rarity. This has greatly advanced the characterization and functional analysis of basophils.

Generation of basophil-deficient animal models

The discovery of natural mutant mice deficient in mast cells, such as WBB6F1-*Kit*$^{W/W\text{-}v}$ and C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, has greatly advanced our understanding of mast cell functions *in vivo* (31). In contrast, model animals deficient only in basophils have long been unavailable until recently. The development of basophil-depleting mAbs has partly overcome this obstacle (32–34). We found that *in vivo* administration of Ba103, a mAb specific to CD200R3 (29), leads to transient ablation of basophils (32), while others demonstrated that MAR-1, a mAb specific to FcϵRIα, also displays basophil-depleting activity (33, 34). Although these mAbs react with both basophils and mast cells *ex vivo*, they preferentially deplete circulating basophils and show minimal effect on the number of tissue-resident mast cells, when administered *in vivo* (Fig. 1A). Taking advantage of these basophil-depleting mAbs, previously unappreciated roles for basophils have been illuminated, as discussed below. We recently found that anti-asialo GM1 antibody efficiently depletes basophils *in vivo* in addition to natural killer cells (35).

A potential problem with the Ba103- or MAR-1-mediated basophil depletion is the unwanted side-effects on mast cells. Indeed, intravenous injection of Ba103 or MAR-1 stimulates mast cells *in vivo* and induces systemic anaphylaxis (32, 36), and repeated intraperitoneal administration of MAR-1 leads to reduced number of mast cells in the peritoneal cavity (33). Accordingly, when mast cells substantially contribute to the immune response of interest, the antibody-mediated basophil depletion cannot provide clear evidence for the role of basophils in the response. The phenotype of antibody-treated mice may be ascribed to basophil depletion, to deleterious effects on mast cells, or to both. To solve this potential problem, we recently created *Mcpt8*^{DTR} mice in that only basophils express the human diphtheria toxin receptor, and therefore administration of diphtheria toxin induce selective and transient ablation of basophils, without any deleterious effect on other cell lineages including mast cells (37) (Fig. 1 B). Others generated *Mcpt8Cre* transgenic mice that are

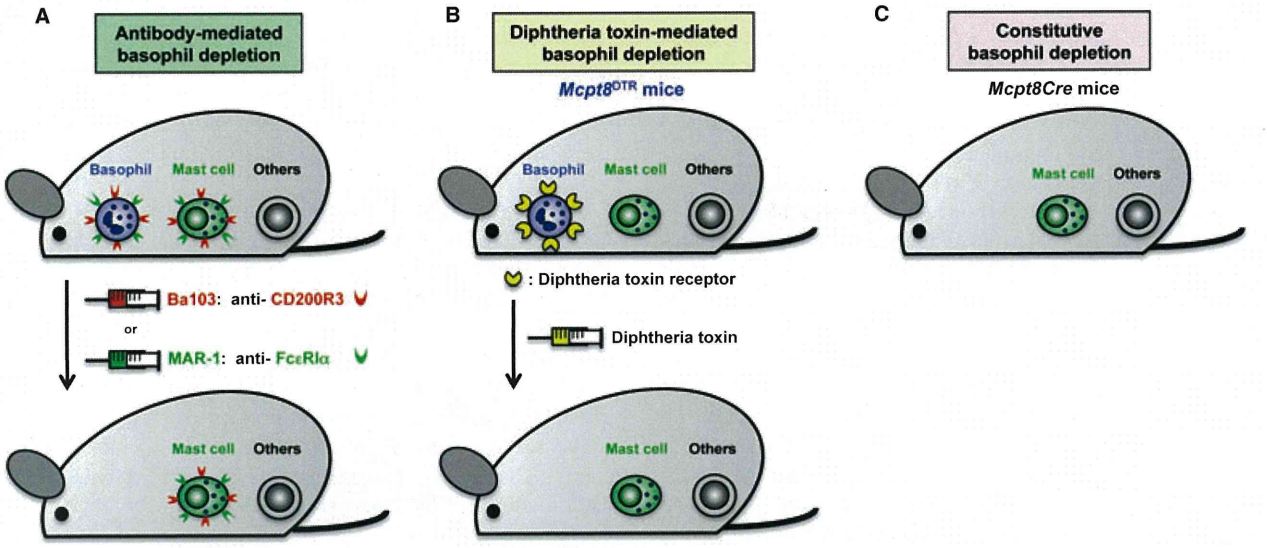

Figure 1 Generation of basophil-deficient mouse models. (A) Antibody-mediated basophil depletion. Treatment of mice with Ba103 (anti-CD200R3 mAb) or MAR-1 (anti-FcεRIα mAb) preferentially ablates basophils, even though both mAbs react with mast cells in addition to basophils. (B) Diphtheria toxin-mediated basophil depletion. *Mcpt8*^{DTR} mice express human diphtheria toxin receptor only on basophils, and therefore diphtheria toxin administration induces selective and transient depletion of basophils. (C) Constitutive basophil depletion. *Mcpt8Cre* transgenic mice constitutively lack most basophils.

constitutively deficient for basophils (36) (Fig. 1C). The establishment of these engineered mice with inducible or constitutive ablation of basophils has truly enabled the functional characterization of basophils, including clear distinction between basophil and mast cell in their functional roles *in vivo*.

Role of basophils in allergy

While basophils circulate in the peripheral blood under homeostatic conditions, they are often recruited to the affected tissues in allergic disorders, including asthma, atopic dermatitis and rhinitis (13, 38). However, no appropriate animal model of allergy was available until recently to address the issue whether basophils indeed play a crucial role or just redundant with mast cells in allergic responses. In the 1970s, extensive studies were performed on a delayed-onset cutaneous hypersensitivity reaction that is associated with a massive dermal infiltration of basophils (comprising 50–90% of the infiltrates), and was therefore designated cutaneous basophil hypersensitivity (CBH) in guinea pigs or Jones–Mote hypersensitivity in humans (39). In contrast to the classical delayed-type hypersensitivity reaction, CBH skin reaction is characterized by erythema and thickening without apparent induration, peaks 18–24 h after the antigen challenge, and fades by 48 h. Unexpectedly, the treatment of guinea pigs with anti-basophil antiserum before the antigen challenge did not diminish the CBH reaction even though basophils were efficiently depleted from the peripheral blood and the skin lesion (40). This disappointedly argued against the importance of basophils as initiators or effectors in the CBH reaction, and the enthusiasm for CBH and basophils faded out. As discussed below, recent studies have revisited the functional significance of basophils in allergic reactions and illuminated their crucial and nonredundant roles in mouse models of allergic disorders.

Basophils in delayed-onset allergy

It is well known that IgE and mast cells crucially contribute to immediate-type allergic reactions. We recently demonstrated that IgE is also involved in a delayed-onset allergic inflammation in the skin, where basophils but not mast cells play an essential role in the development of inflammation, providing the first clear evidence that basophils and mast cells play distinct roles *in vivo* (32, 41). In antigen-specific IgE transgenic mice or in normal mice that have been passively sensitized with antigen-specific IgE, a single subcutaneous administration of antigens in the ear skin elicits three consecutive waves of ear swelling (41, 42) (Fig. 2A). The first two waves, the early-phase ear swelling within 1 h after the antigen challenge and the late-phase ear swelling 6–10 h later, occur in a mast cell-dependent manner. The third wave is delayed-onset (starting on day 2 with a peak on day 4 post-challenge) and more intense ear swelling with massive infiltration of eosinophils in the skin lesions (Fig. 2B) as well as hyperplastic epidermis with hyperkeratosis. Therefore, we designated this delayed-onset cutaneous response IgE-mediated chronic allergic inflammation (IgE-CAI) (41).

Notably, IgE-CAI can be elicited even in mast cell- or T cell-deficient mice. Adoptive transfer of cells isolated from wild-type mice into FcεRI-deficient mice, that cannot mount IgE-CAI, identified FcεRI^{+} basophils to be responsible for the development of IgE-CAI (41). The essential role of

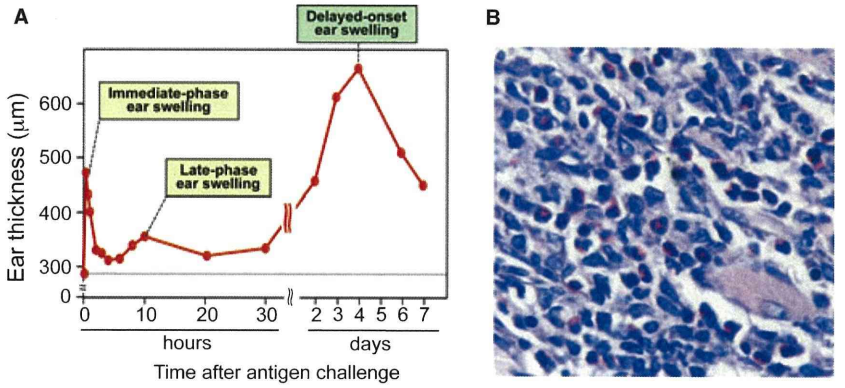

Figure 2 IgE-mediated chronic allergic inflammation (IgE-CAI). (A) Three consecutive waves of ear swellings are elicited when IgE transgenic mice or IgE-sensitized mice are challenged with subcutaneous injection of the corresponding antigen in the ear skin. The first (immediate-phase) and second (late-phase) ear swellings are immediate-type allergic reactions and occur in a mast cell-dependent manner. In contrast, the third one (IgE-CAI) is delayed-onset and dependent on basophils. (B) In a Giemsa-stained specimen of the IgE-CAI skin lesion, massive infiltration of cells, including eosinophils, is observed.

basophils in IgE-CAI was further highlighted by the observation that IgE-CAI is not elicited in the absence of basophils, such as in Ba103-treated mice (32), *Mcpt8Cre* mice (36), and diphtheria toxin-treated $Mcpt8^{DTR}$ mice (M. Egawa, K. Mukai, T. Wada, and H. Karasuyama, unpublished observation). Intriguingly, basophils represent only approximately 2% of the infiltrates in the IgE-CAI skin lesions (not like in the CBH reaction observed in guinea pigs), while eosinophils and neutrophils are abundant (41). The treatment of mice with Ba103 during the progress of dermatitis showed a therapeutic effect on the ongoing inflammation and lead to the reduction in ear swelling and in the number of eosinophils and neutrophils infiltrating the skin lesions, concomitantly with elimination of basophils from the site of inflammation (32). This strongly suggests that basophils may function as initiators as well as, or rather than, effectors of the allergic inflammation and contribute to the recruitment of other proinflammatory cells such as eosinophils and neutrophils (Fig. 3). Although it remains to be determined whether basophils play the similar role in human allergic responses, it would be reasonable to consider the possible contribution of basophils to the pathogenesis of chronic allergic inflammation in humans even when the frequency of basophils is low in affected tissues.

Basophils in immediate-type allergy

Anaphylaxis is an acute-onset, systemic, and potentially fatal allergic reaction, typically associated with IgE, mast

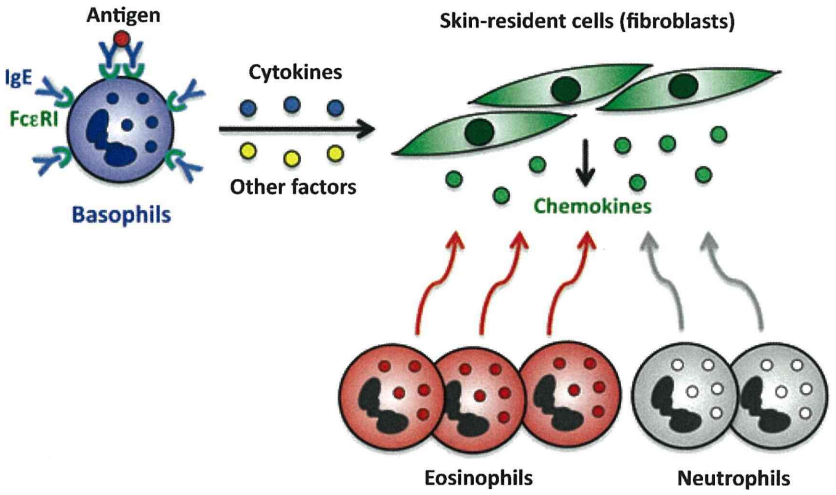

Figure 3 A crucial role for basophils in development of IgE-mediated chronic allergic inflammation (a proposed model). When IgE-armed basophils in the circulation migrate into the skin tissue and encounter the corresponding antigens, they are activated to secrete soluble factors, including cytokines, which in turn stimulate skin-resident cells such as fibroblasts. The skin-resident cells stimulated with basophil-derived factors produce large amounts of cytokines to attract proinflammatory cells such as eosinophils and neutrophils, leading to chronic allergic inflammation.

cells, and histamine (43). Because basophils can release histamine and leukotriene C4 in response to FcεRI-mediated stimulation (3), basophils have been considered likely contributors to IgE-mediated systemic anaphylaxis. However, given the fact that the absolute number of basophils in the body is far less than that of mast cells, it remains uncertain to what extent basophils contribute to systemic anaphylaxis. In this regard, we recently demonstrated that basophils, unlike mast cells, have no significant contribution to IgE-mediated systemic anaphylaxis in a mouse model (44). Instead, basophils are involved in IgG-mediated systemic anaphylaxis under our experimental conditions (44). Previous studies reported that the classical pathway of anaphylaxis, in which IgE, mast cells, and histamine are involved, cannot explain every case of anaphylaxis (45). Mice deficient in mast cells, IgE, or FcεRI still develop systemic anaphylaxis, while FcRγ-deficient mice fail to do so (46–49). This suggests that the alternative pathway of anaphylaxis exists, in which nonmast cells and IgG (instead of IgE) are involved (45). Indeed, passive sensitization with antigen-specific IgG confers on mast cell-deficient mice the ability to develop systemic anaphylaxis in response to the antigen challenge (44, 48, 49). We found that basophils efficiently captured antigen/IgG1-immune complexes on their surface immediately after the intravenous injection of antigens in mice that had been passively sensitized with penicillin V-specific IgG1 mAb (44). Ba103-mediated basophil depletion suppressed IgG1-mediated systemic anaphylaxis, demonstrating basophils contribute to IgG1-mediated systemic anaphylaxis (44). While the treatment of mice with anti-histamine before the antigen challenge showed little or no inhibitory effect on IgG1-mediated anaphylaxis, the treatment with a platelet-activating factor (PAF) antagonist almost completely abolished it, indicating that PAF rather than histamine is the major chemical mediator of the anaphylaxis (44). PAF is far more potent than histamine in induction of anaphylaxis. Thus, immune complex-activated basophils can induce severe systemic anaphylaxis through PAF release, in spite of the fact that the absolute number of basophils is far less than that of mast cells.

Macrophages reportedly play a critical role in another model of IgG- and PAF-mediated systemic anaphylaxis, in that mice are actively immunized with goat anti-mouse IgD antiserum and then challenged with goat IgG (46). Recent study demonstrated that neutrophils play a predominant role in a distinct model of IgG-mediated, active systemic anaphylaxis, in which IgG2, FcγRIV, and PAF are involved (50). In this model, neither macrophages nor basophils play the major role. Basophil-deficient *Mcpt8Cre* mice were shown to develop active anaphylaxis as well as passive IgG1-mediated anaphylaxis (36). It should be noted that the experimental condition employed varies from study to study, including the mouse strains and immunization protocols as well as types and doses of antigen and antibody. Thus, the mechanism underlying the IgG-mediated systemic anaphylaxis appears multifaceted, and not as simple as IgE-mediated one. Basophils, neutrophils and macrophages among others have potential to induce IgG-mediated systemic anaphylaxis through PAF release, and their relative contribution may depend on experimental settings.

It was thought that much higher amounts of antigen and antibody are needed to elicit IgG-mediated systemic anaphylaxis than IgE-mediated one, suggesting that IgG-mediated anaphylaxis may not occur in real life. We recently demonstrated that IgG-mediated anaphylaxis can be induced even under conditions of limited amounts of antibody and antigen in a mouse model using a cocktail of IgG1 mAbs (51). Moreover, a relatively small dose of antigen induces severe anaphylaxis through the combination of the IgE- and IgG-mediated pathways (51). Thus, IgG-mediated systemic anaphylaxis, particularly in conjunction with IgE-mediated one, may not rare in real life, in contrast to previous thought. In this regard, it is notable that treatment with the combination of anti-histamine and PAF antagonist efficiently suppressed systemic anaphylaxis in a mouse model of peanut allergy (52). Several case reports indicated human anaphylaxis that occurred in the apparent absence of detectable antigen-specific IgE or in the absence of a sign of mast cell activation (increase in serum tryptase levels) (53, 54). Serum PAF levels are well correlated with the severity of anaphylaxis in humans (55). These observations suggest that the alternative pathway of anaphylaxis may be operative in humans as well.

Role of basophils in protective immunity

The evolutionary conservation of basophils in many animal species (9) strongly suggests that a primary role of basophils is not an initiator or effector of allergic reactions and should be host-beneficial. Basophilia, increased number of basophils, is frequently observed in association with parasitic infections, suggesting that basophils contribute to protective immunity against parasites (56). Recent studies have clearly demonstrated that basophils indeed play crucial roles in protection from infections with ecto- and endo-parasites (19).

Ticks are blood-feeding ectoparasites and can transmit a variety of microorganisms that can cause serious infectious diseases in humans and animals (57). Lyme disease is a common tick-born disease, and its incidence has increased in the United States these days. Many animal species develop resistance to tick feeding once they experienced tick bites (58), leading to reduced number and body weight of engorged ticks in subsequent infestations. This acquired tick resistance greatly contributes to reducing pathogen transmission from infected ticks to host animals. Elucidation of cellular and molecular mechanisms underlying the acquired tick resistance would help develop efficient vaccination against tick-feeding and tick-born diseases. However, previous studies reported conflicting results regarding cells responsible for manifestation of tick resistance: basophils play an important role in guinea pigs (59) while mast cells instead of basophils do so in mice (60). This may suggest that basophils and mast cells have redundant roles in tick resistance, and their relative contribution varies in different species of host animals and ticks.

We have recently revisited this issue by using novel tools and illuminated the pivotal contribution of basophils to acquired tick resistance in mice (37). In accordance with the

earlier report (60), Giemsa staining of skin tissue sections failed to detect basophils infiltrating tick-feeding sites in mice, unlike in guinea pigs. However, immunohistochemical analysis with TUG8, a newly developed mAb specific to mMCP-8 (24), clearly demonstrated that basophils are recruited to and clustered around the tick mouthparts during the second, but not the first infestation with ticks (37), suggesting the possible involvement of basophils in acquired tick resistance. The selective basophil depletion before the second tick infestation, by means of diphtheria toxin treatment of *Mcpt8*^{DTR} mice, completely abolished the acquired tick resistance in the second infestation (37). We also confirmed the previous observation that mast cell-deficient mice fail to acquire tick resistance in the second infestation, indicating the essential role of mast cells. Thus, both basophils and mast cells play pivotal roles in the manifestation of acquired tick resistance. In the first infestation, mice raise antibodies, particularly of IgE class, against tick saliva antigens. In the second infestation, IgE-armed basophils are recruited to the tick-feeding sites and activated upon encounter with tick antigens, leading to the release of a variety of mediators, including proteases, which interfere with tick feeding. Mast cells contribute to the tick resistance in an antibody-independent manner (37). It remains to be determined how mast cells cooperate with basophils to manifest tick resistance.

Basophils also contribute to protection against helminthic endoparasites. Helminths are the most common infectious agents of humans in developing countries. In the mouse model of hookworm infection, basophil depletion results in impaired worm expulsion in secondary but not primary infection (36, 61), indicating a crucial role of basophils in protective immunity during repeated infections, as observed in tick infestation. In *Trichuris muris* infection, basophils contribute to worm expulsion in primary infection (62). In chronic infection with filarial nematodes, basophils amplify Th2-type immune responses, including IgE production, but do not contribute to worm expulsion in primary infection (63). Thus, the extent of basophil's contribution to the protection from helminthic infections varies, depending on the kinds of helminthes and types of infection.

Basophils are also involved in protective immunity to bacterial infections. In mice, basophils enhance humoral memory responses by activating B and T cells in the secondary immune response through their IL-4 and IL-6 production (33). Basophils also support the survival of plasma cells (64). Of note, basophil depletion before a second vaccination with pneumococcal antigens resulted in reduced production of antigen-specific IgGs and rendered mice more susceptible to *Streptococcus pneumoniae* infection (33). Human basophils also stimulate B cells to secrete IgM and to undergo class switching to IgG and IgA (65). Moreover, activated human basophils produce antimicrobial peptides such as β-defensin and cathelicidin, which inhibit bacterial replication (65). Notably, the basophil-mediated enhancement of B-cell activation and plasma cell survival is also attributed to the accelerated production of autoantibodies in a mouse model of systemic lupus erythematosus (66).

Role of basophils in regulation of Th2 cell differentiation

In addition to the role of basophils in the above-mentioned B-cell activation, recent studies have illuminated the role of basophils in initiating Th2 cell differentiation (34, 62, 67, 68) (Fig. 4). IL-4 has a crucial role in driving the differentiation of naïve T cells to Th2 cells, which produce Th2-type cytokines such as IL-4 and IL-13, and are involved in protective immunity against parasites and allergic responses (69). Studies in the early 1990s discovered that basophils from both humans and mice can rapidly secrete large quantities of IL-4 as well as IL-13 (3, 70, 71). The establishment of IL-4/GFP reporter mice revealed that basophils indeed produce IL-4 *in vivo* during parasitic infections (25, 26). Of note, basophils are the major source of IL-4 at the early phase of immune responses (72, 73). These findings suggest that basophils may be involved in providing initial IL-4 necessary for Th2 cell differentiation and mediating Th2 immune responses. However, it remained uncertain whether basophils and naïve CD4$^+$ T cells interact with each other in the lymph node, in which Th2 differentiation takes place. A recent study demonstrated that basophils indeed migrate into the draining lymph node after subcutaneous injection of papain, a protease that induces a strong Th2 response (34). Papain directly stimulates basophils to secrete IL-4 in an IgE-independent manner (34). Importantly, the antibody-mediated basophil depletion abol-

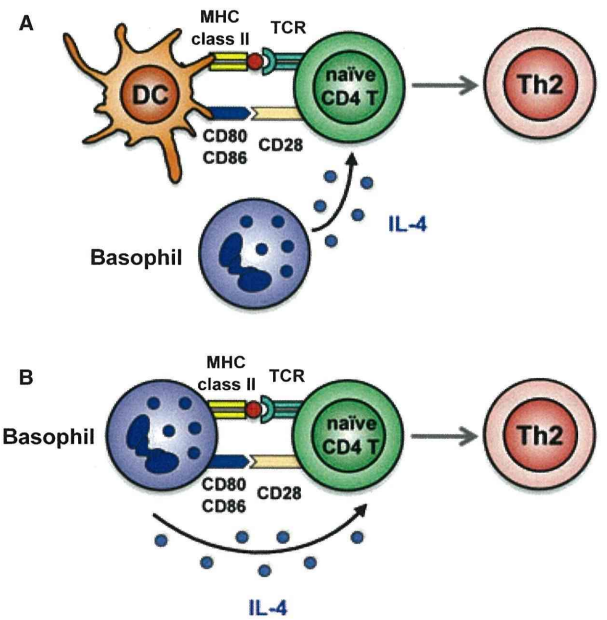

Figure 4 Roles for basophils in initiating Th2 cell differentiation. (A) Basophils function as IL-4 providers. Basophils are transiently recruited to the draining lymph node and provide IL-4 to naïve CD4$^+$ T cells to promote their differentiation to Th2 cells, while dendritic cells (DCs) function as antigen-presenting cell (APCs). (B) Basophils express MHC class II and co-stimulatory molecules and can function as both APCs and IL-4 providers to promote Th2 cell differentiation even in the absence of DCs.

ished Th2 cell differentiation in the lymph nodes (34), demonstrating that basophils critically contribute to papain-induced Th2 cell differentiation. Papain administration also induces migration of dermal dendritic cells (DCs) to the draining node. Therefore, it was proposed that DCs process and present antigens to naïve $CD4^+$ T cells, while basophils provide IL-4 necessary for Th2 differentiation (34) (Fig. 4A).

The role of basophils in mediating Th2 cell differentiation was further expanded by three independent studies showing that basophils can function as antigen-presenting cells (APCs) (62, 67, 68). Basophils express MHC class II (albeit to a lesser extent than do DCs and B cells) and costimulatory molecules such as CD80 and CD86. Therefore, basophils alone can induce Th2 cell differentiation through their functions as both APCs and IL-4 providers (Fig. 4B). Indeed, under experimental conditions employed, DC depletion had little or no impact on Th2 cell differentiation whereas basophil depletion abolished or diminished Th2 cell differentiation. The dual function of basophils in Th2 cell differentiation appears to be analogous to that of DCs in Th1 cell differentiation, in that DCs function as both APCs and IL-12 providers. This new paradigm that basophils are the genuine APCs for induction of Th2 responses got much attention and has brought basophils under the spotlight, but also met with severe criticism. The crucial role of DCs was demonstrated in papain-induced Th2 cell differentiation (36, 74) as well as in the induction of Th2 responses in asthma models (75, 76). Furthermore, Th2 responses to parasitic infections were not impaired by basophil depletion (36, 37, 73, 77). Thus, the relative contribution of basophils and DCs to Th2 cell differentiation appears to vary, depending on the model of Th2 responses employed (17, 69). It is notable that basophils can also act as APCs for $CD8^+$ T-cell differentiation (78), although the *in vivo* relevance of this finding remains to be clarified.

Concluding remarks

Basophils were often considered to be minor and redundant 'circulating mast cells' because of their phenotypic similarity with mast cells, and hence their functional significance remained questioned for a long time. As discussed in this review article, taking advantage of newly developed analytical tools, recent studies have illuminated previously unappreciated roles for basophils, that are distinct from those played by mast cells. Thus, a neglected minority gains new respect. Basophils play important and nonredundant roles in the regulation of acquired immunity, and the protective immunity against parasitic infections. Basophils are also critically involved in the development and deterioration of immune disorders such as allergy and autoimmunity in mouse models. Antibody-mediated basophil depletion showed therapeutic effects on these diseases (32, 44, 66). Treatment of mice with cyclosporine A or steroids almost completely suppressed basophil-mediated IgE-CAI even in the absence of T cells, suggesting that these immunosuppressants act on basophils (42). Considering the fact that the absolute number of basophils is much less than that of other effector cells, basophils and their products could be promising therapeutic targets for such immune disorders, even though further studies are essential to establish that the findings in mouse models are relevant to human diseases.

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Conflict of interest

The authors have no conflicting financial interests.

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Increased expression of mRNAs for IL-4, IL-17, IL-22 and IL-31 in skin lesions of subacute and chronic forms of prurigo

Prurigo lesions are divided into acute, subacute, and chronic forms [1]. Subacute prurigo tends to affect middle-aged women with symmetrical distribution. Chronic prurigo consists of small, irritable papules, preferentially on the abdomen and other sites. These two forms persist for a long period and may evolve into nodular lesions. Prurigo is severely itchy and characterized histologically by infiltrates of lymphocytes and eosinophils and acanthosis of the epidermis. Th2 cells expressing interleukin (IL)-4, IL-5 and IL-10 infiltrate into the lesional skin of subacute and chronic prurigos [2]. However, all features of prurigo cannot be ascribed to Th2 cytokines, and other cytokines may participate in the pathogenesis. IL-31 is pathogenic for pruritic skin diseases, such as atopic dermatitis and prurigo nodularis [3]. On the other hand, IL-22 is a critical cytokine for proliferation of epidermal keratinocytes [4]. We therefore investigated the expression of these cytokines in the skin lesions of prurigo.

The ethical committee of our university approved this study. Papulonodular lesions of subacute and chronic prurigos (*ca.* 3 mm in diameter) were biopsied and subjected to real-time polymerase chain (PCR) analysis. Seven patients (M:F = 4:3; mean age, 68.7 ± 10.9) were enrolled. As controls, skin specimens (center of lesional skin) of 7 patients with plaque psoriasis (M: F = 4:3; 65.5 years ± 15.9) and 5 normal individuals were used. Total RNA was extracted with an RNA extraction kit (Promega, Madison, WI). RNA was reverse transcribed and amplified by random hexamer with primers and probes from TaqMan (Applied Biosystems, Foster City, CA). The result for each gene was normalized to the quantity of β-actin (*ACTB*) detected in the sample. In parallel, we measured the percentage of IL-17$^+$CD4$^+$ T (Th17) cells in peripheral blood mononuclear cells (PBMC) from the patients, as described previously [5]. Intracellular IL-17 of PBMC was stained according to the protocol of Cytostain (Immunotech, Marseille, France), with some modifications.

The level of *IL4* mRNA expression was higher in prurigo skin than in normal and psoriatic skin, and there was no significant difference between normal and psoriatic skin *(figure 1A)*. *IL31* mRNA was expressed at a significantly higher level in prurigo than psoriasis and normal skin *(figure 1B)*. mRNAs for IL-17 *(figure 1C)* and IL-22 *(figure 1D)* were highly expressed in both prurigo and psoriasis lesions, compared to normal skin. A significant correlation was found between the expression levels of *IL4* and *IL31* ($P = 0.0429$), and a correlation tendency was observed between *IL17* and *IL22* ($P = 0.1096$) in patients with prurigo. The percentages of peripheral blood Th17 cells (IL-17$^+$CD3$^+$CD4$^+$CD8$^-$ cells) were also examined. *Figure 1E* represents a typical flowcytometric analysis of Th17 cells. The frequencies of Th17 cells were significantly higher in prurigo as well as in psoriasis than in normal control *(figure 1F)*.

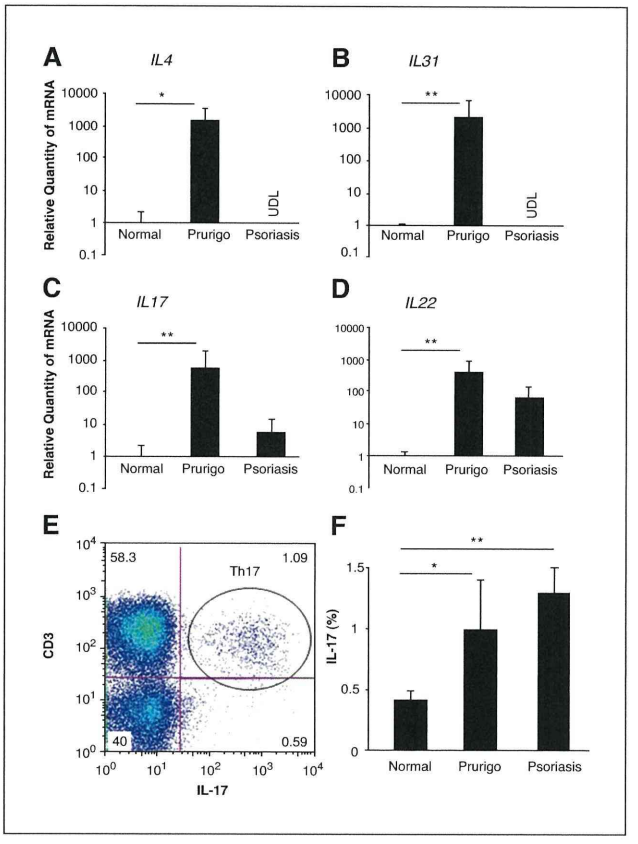

Figure 1. Expression of cytokines in the skin and percentages of Th17 cells in the blood. **A, B, C, D)** Biopsy specimens were taken from lesional skin of patients with prurigo and psoriasis, and from normal skin of healthy subjects. They were examined for the expression of the indicated four cytokines by real-time RT-PCR. The data represent the means ± SD. UDL: under detection level. In IL-4 of psoriasis, 6 cases were UDL and one case was 5.15. In IL-31 of psoriasis, 5 cases were UDL and 2 cases were 2.77 and 3.07. $*P = 0.024$, $**P < 0.01$. **E, F)** PBMC from the patients and normal volunteers were subjected to intracellular cytokine staining and flow cytometry. A representative flow cytometric data is shown **(E)**. The mean ± SD of Th17 cell percentages in each group **(F)**. $*P = 0.050$, $**P = 0.0019$.

In addition to the previous observation that Th2 cytokines are highly expressed in prurigo lesions [2], the present study showed that *IL31*, *IL17*, and *IL22* are also increased in prurigo. IL-31 is produced by a certain subset of Th2 cells and is involved in pruritic disorders [3], and *IL31* and *IL4* were correlated with each other in our prurigo study, as seen in atopic dermatitis [6]. Prurigo shared the high expression

levels of IL-17 and IL-22 with psoriasis. Epidermal keratinocytes proliferate in response to IL-22 [4], leading to acanthosis. Accordingly, we found that *IL17* and *IL22* were elevated in prurigo lesions. Prurigo may be characterized by the increment of IL-17/IL-22 and IL-31. Since these two categories of cytokines seem to be derived from different T cell populations, Th17/Th22 and Th2, it is considered that the characteristics of prurigo, i.e. pruritus and epidermal hyperplasia, are mediated by the unique combination of pathogenic T cells. ■

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Department of Dermatology,
University of Occupational and
Environmental Health Iseigaoka,
Yahatanishi-ku, Kitakyushu,
807-8555, Japan
<tokura@med.uoeh-u.ac.jp>

Kio PARK
Tomoko MORI
Motonobu NAKAMURA
Yoshiki TOKURA

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Nodular secondary syphilis

A 64-year-old man presented with an eighteen-day history of a non-pruritic eythematous eruption localized to his upper limbs, neck and back. He denied recent genital or buccal ulceration or heterosexual extramarital sexual intercourse as well as systemic complaints. His medical history was significant for dislipidemia and his only medication was simvastatin. Physical examination revealed multiple, non tender, firm, rubbery, erythematous nodules distributed on the upper limbs, back and a tumor in the neck *(figures 1A, B)*. No mucosal, palm and sole lesions, nor adenomegalies or alopecia were observed. Two biopsies were performed and sent for histological evaluation and culture. Histopathological examination revealed a dense nodular infiltrate mainly composed of small lymphocytes throughout the entire dermis *(figure 1C)*. Serum immunoelectrophoresis showed an Ig G K band that could be monoclonal. Meanwhile his wife had a diagnosis of HIV seropositivity while preparing for major surgery. Human immunodeficiency serology was positive. VDRL was reactive at a titer of 1: 512 as well as MHA-TP. This was confounding, since we could not rule out the presence of a concomitant lymphoproliferative process and syphilis. Cell-marker studies showed the infiltrate to be composed of an increased proportion of T cells, which were mainly CD 8 positive. B-cell compound showed equal expression of K and λ light chains. No clonal T-cell receptor gene rearrangement was observed. PCR testing for *Treponema pallidum* in the skin biopsy was positive. Histological specimen revision showed histiocytes with very few plasma cells. The periodic acid-Schiff and tissue culture showed no microorganisms. Blood tests revealed moderate hepatic cytolysis, hyperproteinemia and hypergammaglobulinemia. We diagnosed secondary syphilis mimicking a T-cell pseudolymphoma. The patient was treated with intramuscular benzathine penicillin 2.4 M units weekly for three weeks, with clearing of the lesions.

The classic presentation of secondary syphilis is that of a generalized papulosquamous eruption characteristically bilateral, symmetric, more pro-eminent on the upper limbs and in the early stages involving the palms and soles, accompanied by a flu-like prodrome with lymphadenopathy. Nodular presentation of secondary syphilis is extremely uncommon, and despite being first described more than 20 years ago only a few cases have been published in the literature [1]. The lesions can be localized on the face, mucous membranes, palms and soles. The presence of nodules can correspond to a late secondary stage of disease which is progressing to the third stage and may represent a specific hypersensivity reaction to treponemal infections [2]. Characteristic histological findings of secondary syphilis include a superficial and deep perivascular plasma cell infiltrate, endarteritis, inflammatory cell infiltrate obscuring the dermo-epidermal junction and epidermal hyperplasia [3]. However, the histological features of secondary syphilis have a wide range of patterns and often are nonspecific. As our case shows, it can be confusing clinically and histologically, mimicking a lymphoproliferative cutaneous process, either benign or malign (pseudolymphoma and lymphoma).

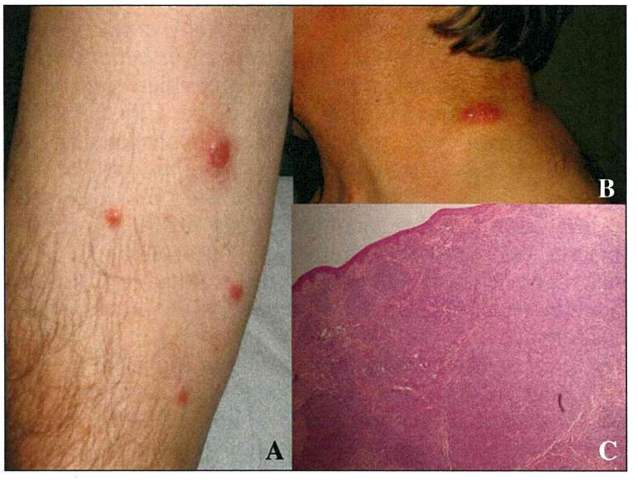

Figure 1. Clinical **(A, B)** and histological **(C)** features.

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Immunopathology and Infectious Diseases

FcεRI, but Not FcγR, Signals Induce Prostaglandin D2 and E2 Production from Basophils

Tsukasa Ugajin,* Takahiro Satoh,* Toru Kanamori,* Kosuke Aritake,† Yoshihiro Urade,† and Hiroo Yokozeki*

From the Department of Dermatology, Graduate School, Tokyo Medical and Dental University, Tokyo; and the Department of Molecular Behavioral Biology,† Osaka Bioscience Institute, Osaka, Japan*

Prostaglandin (PG) D2 and PGE2 are arachidonic acid metabolites that are generated though an isomerization reaction catalyzed by PG synthases. PGs have been implicated in immunologic reactions in addition to a wide range of physiological functions. It has long been thought that basophils, in contrast to mast cells, do not synthesize PGs, although they do release leukotrienes and platelet-activating factor. Here, we show that basophils function as a source of PGD2 and PGE2. *In vitro*-cultured basophils from mouse bone marrow produced both PGD2 and PGE2 in response to IgE + antigen (Ag), but not to IgG + Ag. Release of PGs was almost completely abrogated in cultured basophils from FcRγ-chain$^{-/-}$ mice, indicating the involvement of FcεRI. Basophils freshly isolated from bone marrow cells (primary basophils) were also capable of secreting PGD2 and PGE2. Although the amount of PGD2 released from primary basophils was lower than that from mast cells, the capability of primary basophils to generate PGE2 was more potent than that of mast cells. Transcripts and proteins for both hematopoietic-type PGD synthase and PGE synthase were detected in basophils. In addition, human basophils, like mouse basophils, also produced PGD2 through IgE-mediated stimulation. Thus, basophils could be an important source of PGD2/PGE2 and may contribute to allergic inflammation and immune responses. *(Am J Pathol 2011, 179:775–782; DOI: 10.1016/j.ajpath.2011.04.023)*

Prostaglandins, such as PGD2 and PGE2, are cyclooxygenase (COX) metabolites of arachidonic acids. They have a wide range of biological activities, including relaxation and contraction of smooth muscles, and modulation of neuronal activity.[1] PGD2 is principally produced by activated mast cells and, to a lesser extent, by T helper cell 2 (Th2) cells and dendritic cells[2–4] and exerts its effect through D prostanoid[5] and chemoattractant receptor-homologous molecule receptors expressed onTh2 lymphocytes (CRTH2).[6] A number of recent studies have shown that PGD2 is involved in inflammatory reactions. Mast cell-derived PGD2 suppresses IL-12 production by dendritic cells that induce Th2 responses *in vivo*.[7] PGD2 both activates and induces chemotaxis of Th2 cells, eosinophils, and basophils.[6] A large amount of PGD2 is detected in broncho-alveolar lavage fluid during allergen-induced airway inflammation.[8] Transgenic mice overexpressing human PGD synthase showed exacerbation of ovalbumin (OVA)-induced lung inflammation associated with pronounced eosinophilia and increased Th2 cytokine production.[9] In our previous studies, mice deficient in the *CRTH2* gene were characterized by alleviated IgE-mediated cutaneous responses, contact hypersensitivity reactions,[10] and cedar pollen dermatitis.[11] However, PGE2 is produced by a variety of cells, including fibroblasts and macrophages, and exerts its effects via prostaglandin E2 (EP)1, EP2, EP3, and EP4 receptors.[12] PGE2 acts on T cells to enhance production of Th2-type cytokines and to inhibit production of Th1 cytokines *in vitro*,[13] whereas another study showed that PGE2 facilitates Th1 differentiation via EP2 and EP4 signaling.[14] Furthermore, PGE2 enhances IL-23 production by dendritic cells via EP4[15] and facilitates Th17 expansion in the presence of IL-23 through EP2 and EP4 signaling.[14] Thus, PGD2 and PGE2 could be important mediators in immune and allergic responses.

Basophils represent <1% of peripheral blood leukocytes. Like mast cells, they express the high-affinity IgE receptor, FcεRI, on their cell surface and release chemical mediators after FcεRI cross-linking. Under physiological conditions, basophils principally circulate in periph-

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Address reprint requests to Takahiro Satoh, M.D., Ph.D., Department of Dermatology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, 113-8519, Japan. E-mail: tasa-1688.derm@tmd.ac.jp.

eral blood, whereas mast cells reside in peripheral tissues. During allergic inflammation, such as bronchial asthma and atopic dermatitis, basophils infiltrate into peripheral tissues.[16,17] Several lines of evidence have shown that basophils play a major role in allergic reactions and/or immune responses.[18–21] Basophils initiate IgE-mediated, chronic, cutaneous allergic inflammation (IgE-CAI).[18] They also elicit IgG- but not IgE-mediated anaphylaxis.[19] Basophils promote antigen-specific Th2 development[20] and augment humoral memory responses.[21] Basophils can also mediate protective immunity against helminthes and ticks.[22,23]

Basophils, in contrast to mast cells, have long been thought not to synthesize arachidonic acid metabolites other than leukotriene C4 and platelet activating factor (PAF).[24–28] However, a recent flow cytometric study showed that hematopoietic-type prostaglandin D synthase (H-PGDS), which isomerizes PGH2 into PGD2,[29] is localized intracellularly in human basophils,[3] although a detailed examination of PGD2 generation from basophils was not performed. In the present study, we aimed to clarify whether basophils are capable of producing PGs. Our study showed that both mouse and human basophils secrete PGD2 and/or PGE2 after the cross-linking of FcεRI receptors, implying a possible contribution of basophils to inflammatory and/or immune reactions by functioning as a productive source of PGD2 and PGE2.

Materials and Methods

Animals

C57BL/6 and BALB/c mice (6 to 10 weeks old) were purchased from Sankyo Labo Service (Tokyo, Japan). FcRγ-chain$^{-/-}$C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All experiments in this study were performed according to the Guideline for Animal Use and Experimentation as set out by Tokyo Medical and Dental University.

Cell Preparation and Culture

Bone marrow-derived basophils (BMBAs) were prepared by culturing bone marrow cells in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 10 ng/mL recombinant IL-3 (R&D Systems, Minneapolis, MN) for 10 days, followed by isolation of CD49b$^+$ cells with the use of a magnetically activated cell sorter system with biotinylated anti-CD49b and streptavidin microbeads (Miltenyi Biotec, Auburn, CA).

Bone marrow-derived mast cells (BMMCs) were obtained by culturing bone marrow cells in the presence of 10 ng/mL rIL-3 for 30 days (10, 20, and 30 days for Western blotting), followed by magnetic sorting of c-kit$^+$ cells with biotinylated anti-c-kit and streptavidin microbeads.

Primary basophils were prepared by enrichment of CD49b$^+$ cells from freshly isolated bone marrow cells with the use of the magnetically activated cell sorter system described above. CD49b$^+$ cells include ~20% basophils, as determined by flow cytometric analysis of CD49b and CD123.

Antibodies

Horseradish peroxidase–conjugated goat anti-rat IgG and rabbit anti-Actin IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Isotype-matched control antibodies (Abs; rat IgG2aκ and mouse IgG1κ), purified rat anti-mouse CD16/CD32 (2.4G2), and biotinylated monoclonal Abs (mAbs) specific for CD49b (Dx5) and c-kit (2B8) were from BD Pharmingen (San Diego, CA). Fluorescein isothiocyanate–conjugated anti-CD49b (Dx5) and phosphatidylethanolamin–anti-mouse CD123 (IL-3Rα) were purchased from eBioscience (San Diego, CA). Horseradish peroxidase–conjugated goat anti-rabbit IgG was from Dako (Glostrup, Denmark), and goat anti-human IgE (epsilon) IgG was from Invitrogen Life Technology (Carlsbad, CA). Anti-mMCP-8 (mouse mast cell protease 8) mAb (TUG8) was established by the first author (T.U.). Anti-H-PGDS mAb (7H4) and anti-mPGES (membrane-bound PGE synthase) mAb (6C6)[30] were established in Osaka Bioscience Institute (Osaka, Japan).

Florescent-Ab Conjugation

Anti-mMCP-8 mAb[31] was conjugated with Alexa 488, and both anti-H-PGDS mAb and anti-mPGES mAb[30] were conjugated with Alexa 647 by using the Alexa Fluor Monoclonal Antibody Labeling kit (Invitrogen).

Stimulation of Basophils and Mast Cells

For IgE-mediated stimulation, cells were sensitized with 0.5 μg/mL anti-TNP (trinitrophenyl)-IgE (IGEL-b4) in the presence of 10 ng/mL IL-3, then washed, and stimulated with 20 ng/mL TNP-OVA (Bioresearch Technologies, Novato, CA) or OVA (Sigma-Aldrich, St Louis, MO) for 30 minutes. For IgG-mediated stimulation, cells were stimulated with an immune complex composed of 20 μg/mL anti-TNP IgG (TIB-191, a generous gift from Dr. Karasuyama, Tokyo Medical and Dental University) and 40 μg/mL TNP-OVA or OVA for 30 minutes.

Isolation and Stimulation of Human Blood Basophils

Candidate blood donors were initially screened from healthy volunteers on the basis of the response of their basophils in a basophil activation test (Allergenicity kit; Beckman Coulter, Inc., Fullerton, CA), which assessed CD203c expression in response to IgE stimulation. Blood basophils were separated by Ficoll gradient centrifugation from venous whole blood anticoagulated with EDTA, followed by negative selection with the use of a Human Basophil Isolation Kit (Miltenyi Biotec). Basophil purity was >98%. Basophils were primed with 10 ng/mL human IL-3 (Sigma-Aldrich) for 6 hours, then washed, and stimulated with anti-human IgE (1 μg/mL) for 30 minutes.

Measurement of PGD2 and PGE2

The cells were harvested after stimulation. Culture supernatant fluids were collected, and PGs were purified as described previously. In brief, acidified culture supernatant fluids were added to solid phase extraction cartridges (C-18; Cayman Chemical, Ann Arbor, MI), followed by elution with ethyl acetate containing 1% methanol.[4] The concentrations of PGD2 and PGE2 were measured by using the ProstaglandinD2-Mox enzyme immunosorbent assay (EIA) kit (Cayman Chemical) and the Prostaglandin E2 EIA kit (Cayman Chemical), respectively.

High-Performance Liquid Chromatography with Tandem Mass Spectrometry

Negative ionspray tandem mass spectrometric measurements of PGs were conducted with an API 3200 (Applied Biosystems, Foster City, CA) equipped with NANOSPACE SI-2 system (SHISEIDO, Tokyo, Japan). Inertsil ODS-3 HPLC columns (GL Science, Torrance, CA) were used for high-performance liquid chromatography (HPLC) separations with a mobile phase consisting of 10% to 100% acetonitrile gradients 0.01 acetic acid at a flow rate of 200 μL/minute.

Immunoblotting

Cells were lysed in lysis buffer containing 0.5% Triton X-100 (MP Biochemical, Solon, OH) and a protease inhibitor cocktail (Sigma-Aldrich). Total cell lysates were subjected to SDS-PAGE (10% polyacrylamide), followed by immunoblotting with the indicated antibodies and horseradish peroxidase–conjugated secondary Abs. Proteins were visualized with the ECL Plus Western Blotting System (GE Healthcare, Buckinghamshire, UK).

Cytospin and Flow Cytometric Analyses

Cytospin preparations were fixed in methanol and treated with blocking solution containing 10% normal goat serum, 0.01% Triton X-100, and 0.1% NaN3 to prevent nonspecific Ab binding. The preparations were incubated with a combination of Alexa 488-conjugated anti-mMCP-8 mAb and Alexa 647-conjugated anti-H-PGDS mAb or Alexa 647-conjugated anti-mPGES1 mAb and were examined under a confocal laser scanning microscope (LSM510; Carl Zeiss, Oberkochen, Germany). For flow cytometric analyses, single-cell suspensions were treated with 2.4G2 and normal rat serum and were subsequently stained with the indicated Abs. Cells were analyzed with a FACSCaliber (BD Biosciences, Mountain View, CA).

Semiquantitative RT-PCR and Real-Time PCR Analysis

Total RNA was prepared from cells, and first-strand cDNA was synthesized with reverse transcription with oligo-dT primers. For semiquantitative RT-PCR, PCR was performed with a fivefold serially diluted cDNA template at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds for 35 cycles, followed by further extension at 72°C for 5 minutes. For real-time PCR, the expression of each gene was measured relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the use of SYBR Green dye (Applied Biosystems) with real-time PCR systems. The following primers (forward and reverse, respectively) were used for both PCR reactions: for *Hpgds*, 5′-ATCCACCAGAGCCTCGCAATAG-3′ and 5′-TCATCCAGCGTGTCCACCA-3′; for *Ptgds*, 5′-GACACAGTGCAGCCCAACTTTC-3′ and 5′-GGGCTACCACTGTCTTGCACATA-3′; for *Ptges1*, 5′-GGATGCGCTGAAACGTGGA-3′ and 5′-CAGGAATGAGTACACGAAGCC-3′; for *Ptges2*, 5′-CTCATCAGCAAGCGCCTCAA-3′ and 5′-GGTCTTTACCCACGGCTGTCA-3′; for *Ptges3,* 5′-ATCACATGGGTGGTGATGAGGA-3′ and 5′-AGGCGATGACAACAGCCCTTAC-3′; and for *GAPDH*, 5′-TTCACCACCATGGAGAAGGCCG-3′ and 5′-GGCATGGACTGTGGTCATGA-3′.

Results

BMBAs Release Both PGD2 and PGE2 in Response to IgE + Ag, but Not IgG + Ag

To begin to assess whether basophils release PGD2, BMBAs were prepared from mice and tested for their ability to generate PGD2. BMBAs primed with anti-TNP-IgE secreted PGD2 in response to TNP-OVA, indicating that BMBAs produce PGD2 after IgE-mediated stimulation (Figure 1A). Moreover, to our surprise, BMBAs also generated PGE2 in response to Ag-specific IgE + Ag, albeit to a lesser extent than PGD2 (Figure 1B). Basophils have been reported to release PAF, an arachidonic acid metabolite, on stimulation of IgG receptors, thereby eliciting systemic anaphylaxis.[19] Thus, we next explored whether BMBAs produce PGD2 and/or PGE2 via IgG-mediated stimulation. However, neither PGD2 nor PGE2 was released after stimulation of BMBAs with an immune complex of anti-TNP-IgG and TNP-OVA (Figure 1, C and D).

FcεRI Is Involved in IgE-Mediated PGD2/E2 Secretion

To confirm the involvement of the high-affinity IgE receptor, FcεRI, in PG secretion, we analyzed PGD2 and PGE2 generation in BMBAs prepared from FcRγ-chain$^{-/-}$ mice lacking FcεRI expression. Basophils from FcRγ-chain$^{-/-}$ mice were determined by their expression of CD49b and CD123 instead of CD49b and FcεRI, as previously reported.[32] We first confirmed that BMBAs differentiated normally even in the absence of the FcRγ-chain (Figure 1E). The PGD2 and PGE2 secretion observed from BMBAs of wild-type C57BL/6 mice was completely abrogated in BMBAs of FcRγ-chain$^{-/-}$ mice (Figure 1, F and G). These data indicated that PGD2 and PGE2 release from BMBAs depend on FcεRI-mediated, but not CD23-mediated, signals.

Primary Basophils also Produce both PGD2 and PGE2 in Response to FcεRI-Mediated Stimulation

We next attempted to verify whether primary basophils are also capable of producing PGD2 and/or PGE2. Primary basophils, that is, CD49b$^+$ cells freshly isolated from bone marrow, were stimulated with anti-TNP IgE and TNP-OVA. We first measured PGD2 and PGE2 in their supernatant fluid with the use of EIA as described earlier. Primary basophils produced more or less the same amount of PGD2 as that from BMBAs (Figure 2). However, PGE2 secretion from primary basophils was higher than that from BMBAs. The ability of primary basophils to produce PGD2 and PGE2 was further confirmed by analysis of PGs with the use of HPLC with tandem mass spectrometry. Both PGD2 and PGE2 were detected in basophil supernatant fluids by HPLC-tandem mass spectrometry analysis (Figure 3). This result clearly shows that basophils produce both PGD2 and PGE2. Primary basophils appear to be more potent for PGE2 production than BMBAs.

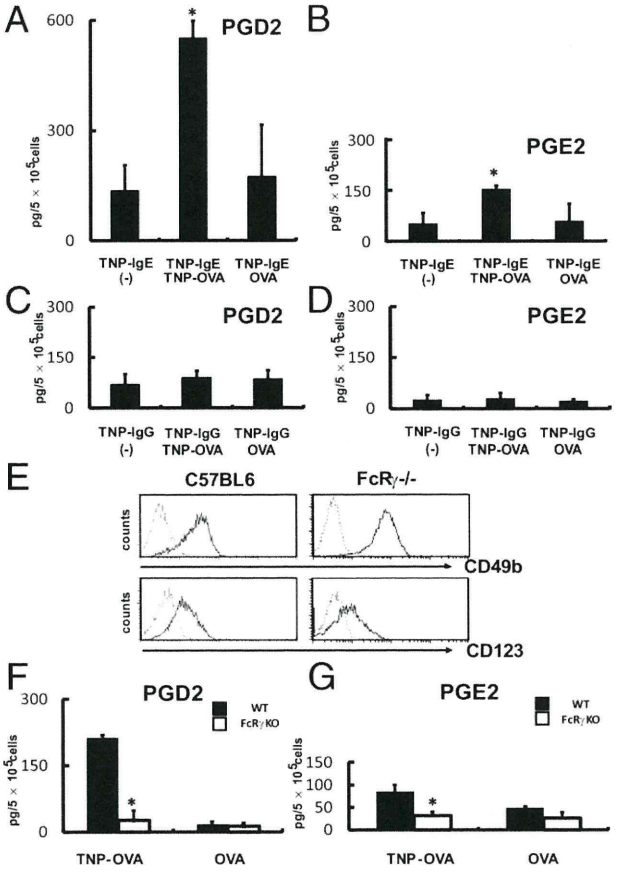

Figure 1. BMBAs secrete both PGD2 and PGE2 after cross-linking of FcεRI receptors. **A** and **B:** BMBAs prepared from bone marrow cells of BALB/c mice were sensitized with 0.5 μg/mL anti-TNP-IgE overnight, followed by stimulation with 20 ng/mL TNP-OVA or OVA for 30 minutes. The concentration of PGD2 and PGE2 in the supernatant fluids was determined with EIA. BMBAs produced both PGD2 and PGE2 in response to IgE-mediated stimulation. **C** and **D:** BMBAs were stimulated with 20 μg/mL anti-TNP-IgG and 40 μg/mL TNP-OVA for 30 minutes. **E:** BMBAs from C57BL/6 FcRγ-chain$^{-/-}$ mice were identified as CD49b$^+$ CD123$^+$ cells by flow cytometric analysis. Differentiation of basophils was not affected by the absence of the FcRγ chain. **F** and **G:** Neither PGD2 nor PGE2 was produced by BMBAs prepared from C57BL/6 FcRγ-chain$^{-/-}$ mice after *in vitro* stimulation with IgE+ Ag. *$P < 0.05$ (Student's *t*-test). Error bars indicate standard deviation.

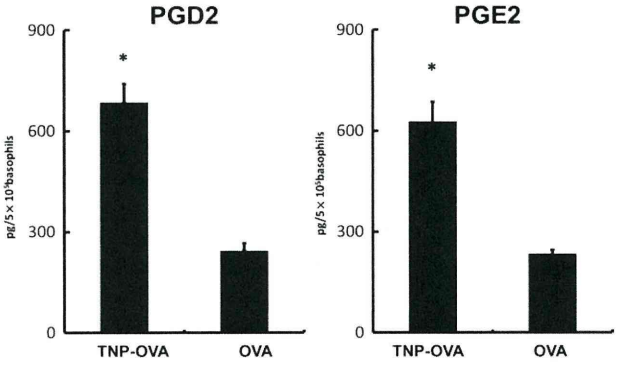

Figure 2. PGD2 and PGE2 production from primary basophils. Primary basophils (CD49b$^+$ cells freshly isolated from bone marrow cells) were sensitized with 0.5 μg/mL anti-TNP-IgE, and then stimulated with 20 ng/mL TNP-OVA or OVA for 30 minutes. The concentration of PGD2 and PGE2 in the supernatant fluids was determined with EIA. Primary basophils secreted comparable levels of PGE2 and PGD2. *$P < 0.05$, compared with OVA stimulation. (Student's *t*-test). Error bars indicate standard deviation.

PGD2 and PGE2 Production by BMMCs

Although mast cells are widely known to produce PGD2,[2,33] PGE2 production by mast cells has not been well studied. We next assessed the generation of both PGE2 and PGD2 by BMMCs. BMMCs prepared from BALB/c mice released ~1.5 times as much PGD2 as primary basophils in response to TNP-OVA (Figure 4) but, in contrast, did not secrete as much PGE2 as primary basophils.

Basophils Express H-PGDS and PGES

We next analyzed the expression of PGD2 and PGE2 synthesizing enzymes in murine basophils with the use of RT-

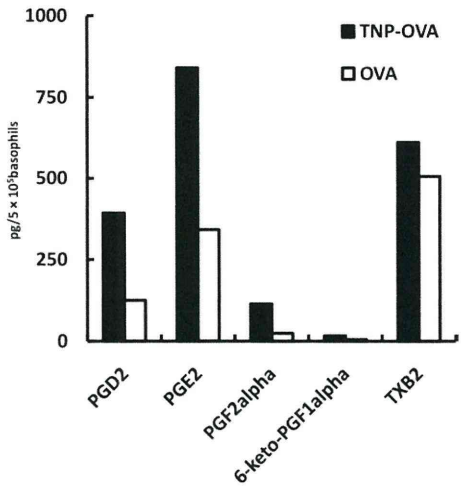

Figure 3. HPLC-tandem mass spectrometry analysis of PGs produced by primary basophils. Primary basophils sensitized with anti-TNP-IgE overnight were stimulated with TNP-OVA or OVA. The level of PGs in the supernatant fluids was determined with HPLC-tandem mass spectrometry. Primary basophils secreted both PGD2 and PGE2 but not PGF1/2 or TXB2. TXB2, thromboxan B2.

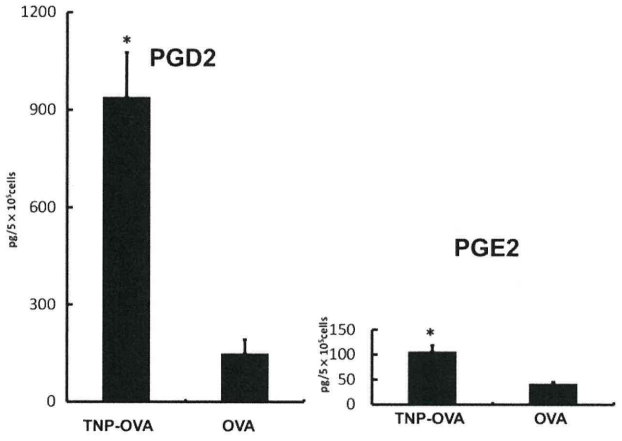

Figure 4. PGD2 and PGE2 production by BMMCs. BMMCs prepared from BALB/c mice sensitized with 0.5 μg/mL anti-TNP-IgE released a significant amount of PGD2 in response to 20 ng/mL TNP-OVA but a low level of PGE2. $^*P < 0.05$, compared with OVA stimulation (Student's *t*-test). Error bars indicate standard deviation.

PCR and Western blotting. PGD2 is synthesized by isomerization of PGH2 through PGDS.[29] PGDS is classified into two types, H-PGDS and lipocalin-type PGD synthase (L-PGDS).[29,34] PGE2 is synthesized by isomerization of PGH2 through PGES. PGES is classified into three types, glutathione-dependent membrane-bound PGES (mPGES1), glutathione-independent membrane-bound PGES (mPGES2), and cytosolic PGES (cPGES).[35–37] *Hpgds* transcripts that encode H-PGDS were readily detected in both BMBAs and BMMCs by RT-PCR. In contrast, *Ptgds* transcripts that encode L-PGDS were only detected in BMMCs (Figure 5, A and B). We analyzed H-PGDS protein expression in BMBAs and BMMCs at various stages of their development by immunoblotting. H-PGDS expression was relatively low in BMBAs ($CD49b^+$, $c\text{-}kit^-$) and in 10-day–cultured BMMCs ($CD49^-$, $c\text{-}kit^+$), but was increased in 20-day–cultured and 30-day–cultured BMMCs (Figure 6A), indicating that H-PGDS activity in BMMCs increased along with their differentiation. *Ptges1*, *Ptges2*, and *Ptges3* transcripts, which encode mPGES1, mPGES2, and cPGES, respectively, were all detected in BMBAs as well as in BMMCs (Figure 5, A and C).

Furthermore, bone marrow cells expressing mMCP-8, a specific marker for basophils,[31] were positive for both H-PGDS and mPGES1 by immunohistochemical analysis (Figure 6B). These results show that basophils express H-PGDS as well as PGES, thereby producing both PGD2 and PGE2.

Human Blood Basophils also Produce PGD2 but Not PGE2

A prior flow cytometric study reported the presence of H-PGDS in human blood basophils.[3] We therefore examined whether human basophils produce PGD2 and/or PGE2 after IgE-mediated stimulation. We primed human blood basophils isolated from several healthy donors with IL-3 and then stimulated them with anti-human IgE Ab for 30 minutes. Human basophils secreted PGD2 on stimulation (Figure 7). Unlike murine basophils, however, PGE2 generation from human basophils was barely detectable (data not shown).

Discussion

It has long been believed that basophils do not synthesize arachidonic acid metabolites except for leukotriene C4 and PAF.[27,28] However, in the present study, we demonstrated for the first time that mouse basophils produce both PGD2 and PGE2 after aggregation of FcεRI receptors. In addition, we found that human basophils are also capable of producing PGD2 after IgE-mediated stimulation.

The expression of the two types of PGDS, H- and L-PGDS, varies according to cell type.[29,34] Mast cells, antigen-presenting cells, and a small population of Th2 cells express H-PGDS.[2–4,33] L-PGDS is expressed in meningeal cells, epithelial cells of the choroid plexus, and oligodendrocytes in the brain.[38] In our study, the gene encoding H-PGDS, but not that encoding L-PGDS, was expressed in BMBAs. H-PGDS proteins were detected in BMBAs as well as in primary basophils. Thus, H-PGDS appears to be a major enzyme involved in PGD2 generation in basophils. BMMCs released a greater amount of PGD2 than BMBAs (Figures 1A and 2). The higher production of PGD2 by BMMCs relative to BMBAs

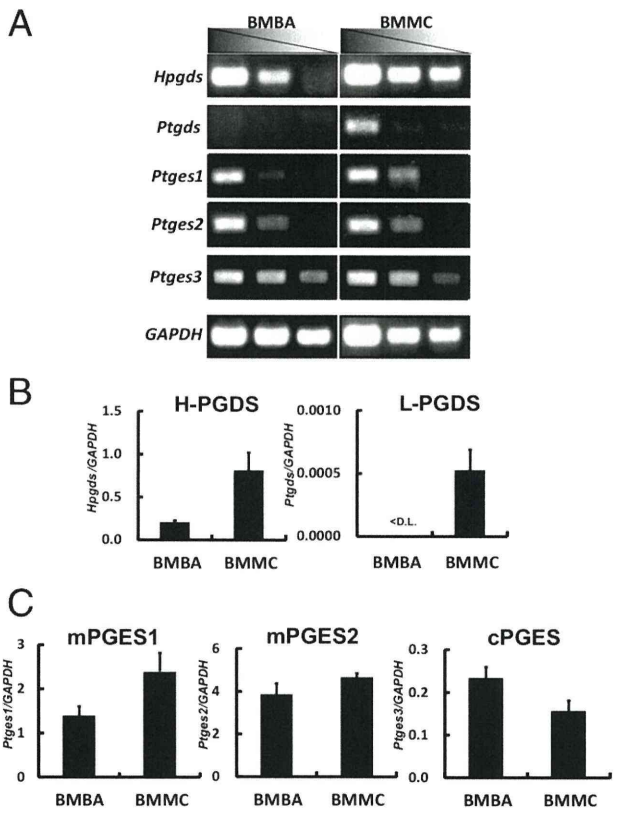

Figure 5. Expression of PG synthase (PGS) transcripts in BMBAs and BMMCs. The expression of the indicated genes in BMBAs and BMMCs was determined by quantitative RT-PCR analysis of total cellular RNA. **A:** Gel electrophoresis of the PCR products. The PCR templates were fivefold serially diluted. **B** and **C:** The expression of each gene was measured relative to GAPDH with the use of SYBR Green dye with real-time PCR systems. Error bars indicate standard deviation.