response to systemic indomethacin.[3,4] Indomethacin can even be used as a diagnostic tool for EPF,[5] although the underlying therapeutic mechanisms have not been fully elucidated.

Prostaglandin D_2 (PGD_2) is one of the cyclo-oxygenase metabolites of arachidonic acid. It is synthesized by the isomerization of PGH_2 through the enzymatic activity of PGD synthase. Two types of PGD synthase have been identified: haematopoietic-type PGD synthase (H-PGDS); and lipocalin-type PGD (L-PGDS).[6,7] H-PGDS is expressed by haematopoietic cells, such as mast cells,[8,9] T helper type 2 (Th2) cells,[10] dendritic cells,[11] eosinophils[12] and basophils.[13] These cells produce PGD_2 in response to a variety of stimuli. L-PGDS is principally present in meningeal cells, epithelial cells of the choroid plexus, and oligodendrocytes in the brain.[14] Prostaglandin D_2 shows a wide range of biological activities, including vasodilatation, bronchoconstriction, inhibition of platelet aggregation and regulation of the sleep–wake cycle.[14–18] A number of recent lines of evidence have indicated that PGD_2 is also involved in allergic inflammation. Mice that over-produce PGD_2 exhibit enhanced allergic lung inflammation with eosinophilia and Th2-type cytokine production.[19] Prostaglandin D_2 promotes skin inflammation of IgE-mediated chronic skin responses and the elicitation phase of contact hypersensitivity.[20,21]

Biological activities of PGD_2 are mediated by the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) and the D prostanoid receptor. These receptors are members of the G protein-coupled, seven transmembrane receptor family. In eosinophils, CRTH2 signals induce calcium mobilization and cell migration.[22] Eosinophil degranulation is also promoted by CRTH2 stimulation.[23] Expression of CRTH2 in eosinophils is increased in patients with atopic dermatitis, chronic urticaria or prurigo.[24] Antagonists of CRTH2 ameliorate skin inflammation in IgE-mediated chronic skin responses, contact hypersensitivity and cedar pollen dermatitis in accordance with reduced numbers of dermal eosinophils.[20,21,25]

Recent studies have identified indomethacin as a potent agonist of CRTH2,[26] causing PGD_2-like and eotaxin (CCL11) -like responses in eosinophils.[27] These findings are somewhat inconsistent, offering clinical evidence that indomethacin is a useful therapeutic tool for EPF[3,4] and other eosinophilic skin diseases, such as angiolymphoid hyperplasia with eosinophilia[28] and recurrent cutaneous eosinophilic vasculitis.[29] In this respect, our previous evidence might provide one explanation for this discrepancy.[30] Treatment of eosinophils and lymphocytes with indomethacin resulted in reduced cell surface expression of CRTH2 on these cells. However, functional modulations in eosinophils by indomethacin have yet to be fully determined. The present study sought to elucidate the pharmacological effects of indomethacin on eosinophil migration in response to PGD_2 and eotaxin (CCL11), to obtain insights into mechanisms for the amelioration of eosinophilic skin inflammation by indomethacin.

Materials and methods

Isolation of eosinophils

Peripheral blood anti-coagulated with EDTA was obtained from healthy volunteers with informed consent. After sedimentation of red blood cells using 6% Dextran-T500 (Sigma-Aldrich, St Louis, MO) in physiological saline, eosinophils (density > 1·085) were semi-purified by Percoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient centrifugation.[31] Eosinophils were further purified by negative selection with CD16 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of eosinophils was > 99%. This study was approved by the Ethics Committee of Tokyo Medical and Dental University (No. 887).

Chemotaxis assay

Twenty-five-microlitre aliquots of eosinophils at 4×10^7 to 6×10^7/ml (in PBS containing 10 mM HEPES, 0·1% BSA, 1 mM Ca^{2+}, 1 mM Mg^{2+} and 10 mM glucose) were placed on the top filter membrane of a 96-well micro-chemotaxis chamber with 5-μm pores (ChemoTx® Disposable Chemotaxis System; NeuroProbe, Gaithersburg, MD).[27,32] These plates were incubated at 37° in a humidified CO_2 incubator for 60 min. Cells in the upper and lower chambers were collected and stored at − 80° until use.

Migration of eosinophils was assessed by photometric assay using eosinophil peroxidase activity, as described previously with modifications.[33–36] In brief, eosinophil samples were thawed and mixed with an equal volume of 1% hexadecyltrimethylammonium bromide (Wako Pure Chemical Industries, Osaka, Japan) in 50-mM potassium phosphate buffer (pH 6·4) to entirely lyse eosinophils. Samples were then reacted with twice the volume of 4·5-mM *o*-phenylenediammonium dichloride in 50 mM HEPES containing 4·5 mM KBr and 3·3 mM H_2O_2 for 15 min. The reaction was stopped with 1M H_2SO_4. Optical density was read at 490 nm with a MicroReader (Model 680; Bio-Rad Laboratories, Hercules, CA). A standard curve was drawn by plotting the eosinophil peroxidase activities of serially diluted eosinophil samples for assessment of the region of linear response. Results were expressed as percentages of eosinophil migration [(cells in lower chambers)/(cells in lower chambers + cells in upper chambers) × 100].

Flow cytometric analyses

Single-cell suspensions in PBS/5% fetal calf serum were stained with FITC-conjugated CCR3 (R&D Systems,

Minneapolis, MN) and/or phycoerythrin-conjugated anti-CD294 (CRTH2; Miltenyi Biotec) antibodies. FITC-conjugated or phycoerythrin-conjugated mouse IgG1 antibodies (Dako, Glostrup, Denmark) were used as negative controls. Cells were analysed using a FACSCalibur flow cytometer (Becton Dickinson and Co., Franklin Lakes, NJ).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were incubated with mouse anti-human L-PGDS monoclonal antibody (1B7) or rabbit anti-human L-PGDS polyclonal antibody (kindly provided by Urade and Aritake; Osaka Bioscience Institute, Osaka, Japan) overnight following heat treatment with Dako Real Target Retrieval Solution (Dako) and inactivation of internal peroxidase activity with H_2O_2. These sections were then reacted with horseradish peroxidase-labelled EnVision polymer (Dako). Reactive products were visualized by diaminobenzidine.

Statistical analyses

An analysis of variance test followed by Scheffe's *F*-test was performed to assess the statistical significance of differences between means.

Results

Indomethacin and PGD_2 induce both chemokinetic and chemotactic responses in eosinophils via CRTH2 receptors

To begin to understand the direct effects of indomethacin on eosinophils, migration of eosinophils to indomethacin was assessed. Indomethacin (Wako Pure Chemical Industries) in the upper chambers induced weak eosinophil migration to medium (lower chambers), which became more marked when indomethacin was added to both upper and lower chambers (Fig. 1a). This indicated that indomethacin induced chemokinetic responses in eosinophils. In addition, restriction of indomethacin to only the lower chambers resulted in more significant migration

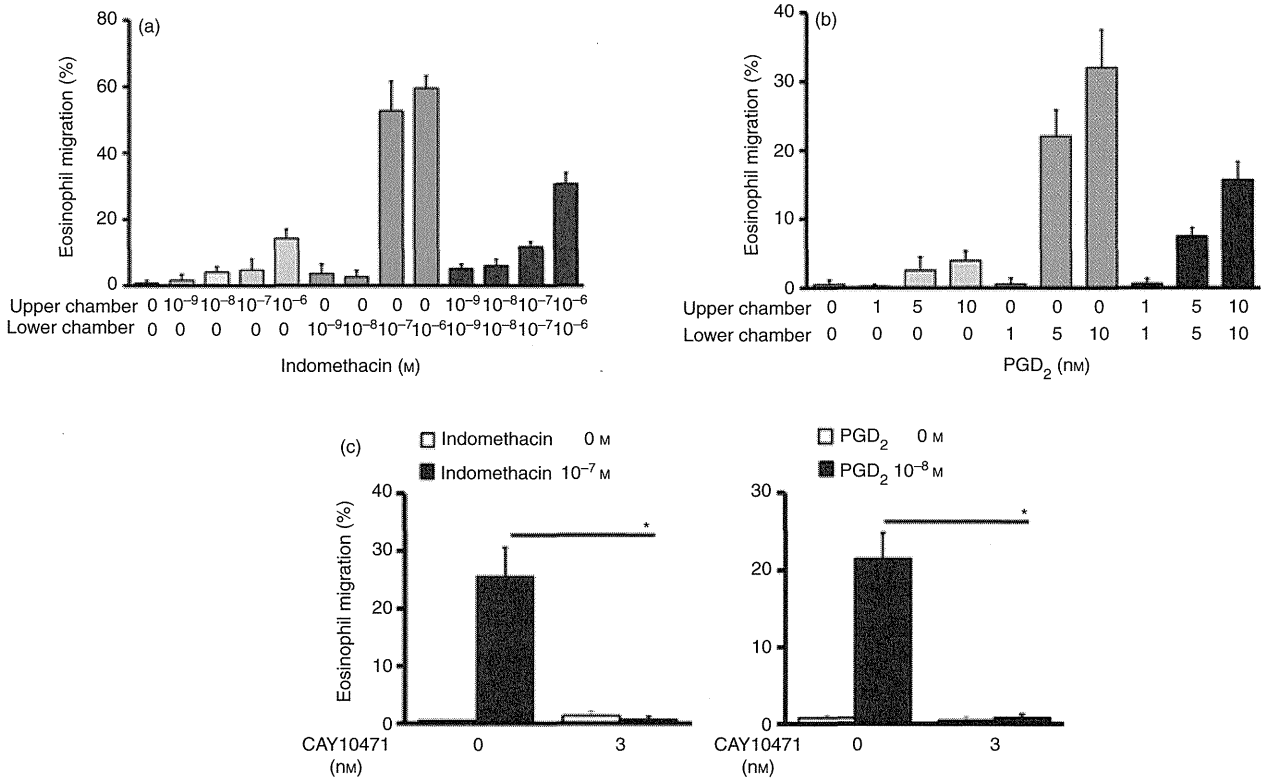

Figure 1. Indomethacin and prostaglandin D_2 (PGD_2) induced chemotaxis in eosinophils through chemoattractant receptor-homologous molecule expressed on T helper type 2 cells (CRTH2) receptors. Cells were mixed with either indomethacin, PGD_2, or medium, and then applied to the upper chamber. Eosinophil migration to the lower chamber containing either indomethacin, PGD_2, or medium was assessed by photometric assay using eosinophil peroxidase activity (a, b). Eosinophil migration in response to indomethacin and PGD_2 (lower chambers) was assessed in the presence of CAY10471 (3 nM), a CRTH2 antagonist, in the upper chamber (c). Representative results of two independent experiments are shown.

of eosinophils, in a dose-dependent manner. Indomethacin therefore has both chemokinetic and chemotactic effects on eosinophils.

Prostaglandin D_2 is known as a chemotactic factor for eosinophils.[22] However, another study denied PGD_2-induced chemotactic responses, but not chemokinetic responses.[23] This study therefore attempted to verify actual effects of PGD_2 on eosinophil migration. Eosinophils exhibited marked migration to PGD_2 (Cayman Chemical, Ann Arbor, MI) in the lower chambers, whereas PGD_2 in the upper chambers and in upper/lower chambers also induced weak and moderate cell migration, respectively (Fig. 1b). Checkerboard analysis clearly revealed the chemotactic action of PGD_2 on eosinophils (Table 1). These effects of indomethacin and PGD_2 were dependent on CRTH2, as cell migration was almost entirely abolished by pre-treatment with CAY10471 (Cayman Chemical), a specific CRTH2 antagonist (Fig. 1c).

Table 1. Checkerboard analysis for migration to prostaglandin D_2 (PGD_2)

	PGD_2 in upper chamber (nM)			
	0	1	5	10
PGD_2 in lower chamber (nM)				
0	0·4 ± 0·71	0·2 ± 0·3	2·5 ± 2·0	3·9 ± 1·4
1	0·5 ± 0·9	0·6 ± 0·8	3·3 ± 2·0	2·3 ± 0·6
5	22·1 ± 3·8	8·7 ± 2·2	7·4 ± 1·3	6·5 ± 1·0
10	32·0 ± 5·5	19·2 ± 3·1	19·9 ± 5·2	15·7 ± 2·6

[1]Percentage of migrated eosinophils.

Indomethacin pre-treatment inhibits eosinophil migration to PGD_2 and eotaxin (CCL11)

We next sought to determine the effects of indomethacin on eosinophil migration to PGD_2 and eotaxin (CCL11). Pre-treatment of eosinophils with indomethacin (37°, 90 min) inhibited PGD_2-induced chemotaxis in a dose-dependent manner, whereas low-dose indomethacin (10^{-10} M) appeared to exert weak stimulatory effects on eosinophil migration (Fig. 2a). Chemotactic response to eotaxin (CCL11) was more mildly inhibited by indomethacin than the response to PGD_2 (Fig. 2b). Again, a low dose of indomethacin weakly stimulated cell migration to eotaxin. On the other hand, the chemotactic response of eosinophils was not suppressed by pre-treatment with diclofenac (Wako Pure Chemical Industries), a cyclooxygenases inhibitor (Fig. 2c,d).

Previous studies have suggested that eotaxin and/or eotaxin-3 (CCL26), both of which are ligands for CCR3 on eosinophils, may contribute to eosinophil accumulation in EPF lesions.[37,38] Eotaxin synthesis, in principle, is stimulated by interleukin-4 and interleukin-13, which are

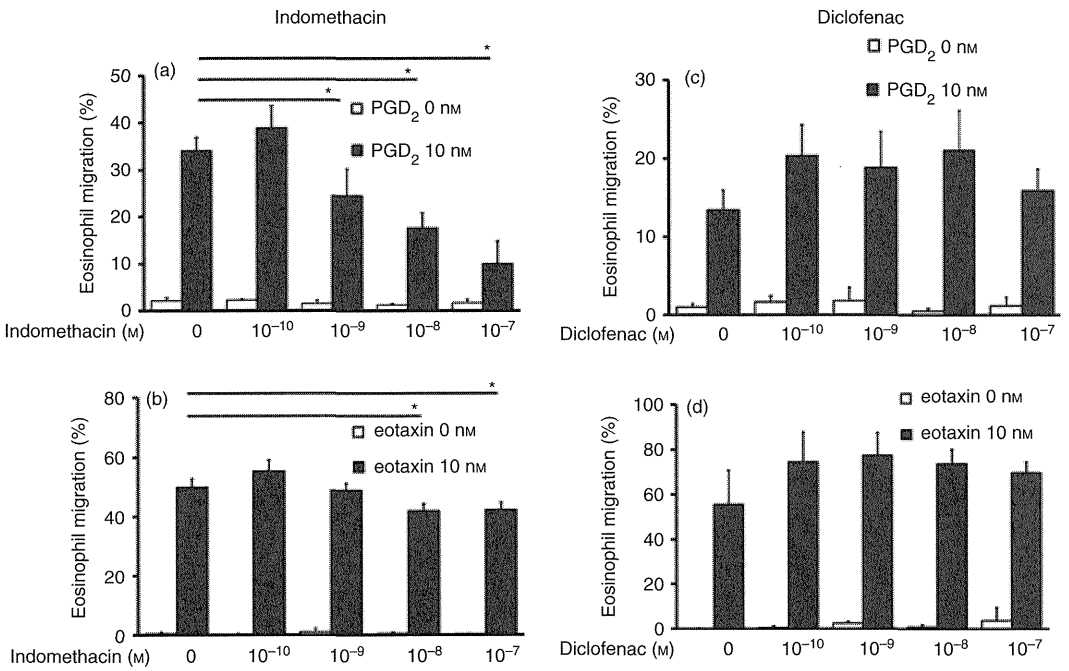

Figure 2. Eosinophil chemotaxis is inhibited by pre-treatment with indomethacin, but not diclofenac. Eosinophils were pre-treated with indomethacin or diclofenac for 90 min at 37°, followed by centrifugation and application to the upper chamber. Eosinophil migration to prostaglandin D_2 (PGD_2) (a, c) and eotaxin (CCL11) (b, d) in the lower chambers was assessed by eosinophil peroxidase photometric assay. *$P < 0.05$. Representative results of at least three independent experiments are shown.

Th2-type cytokines.[39,40] Th2-type immune responses are promoted by PGD_2[19,41] and a number of H-PGDS$^+$ cells have been demonstrated in EPF.[30,38] Prostaglandin D_2 and eotaxin may therefore co-exist in the lesional skin. We next assessed the effects of indomethacin on chemotaxis to PGD_2 in combination with eotaxin. Addition of eotaxin to PGD_2 in lower chambers showed enhancing effects on eosinophil migration (Fig. 3). Pre-treatment of eosinophils with indomethacin suppressed eosinophil chemotactic responses to PGD_2/eotaxin in a dose-dependent manner.

Indomethacin cancels priming effects of Δ^{12}-PGJ_2

As a plasma metabolite of PGD_2, Δ^{12}-PGJ_2 has been shown to exert priming effects on eotaxin-induced chemotaxis.[32] We confirmed this, as evidenced by increased eosinophil migration to eotaxin when Δ^{12}-PGJ_2 (5×10^{-8} M) was added to the upper chambers (Fig. 4a). This priming effect was almost completely inhibited by pre-treatment with a CRTH2 antagonist (CAY10471) (Fig. 4a), indicating that Δ^{12}-PGJ_2 exerted its actions via the CRTH2 receptor. Similarly, indomethacin, a CRTH2 agonist, completely cancelled the priming effects of Δ^{12}-PGJ_2 on eosinophil chemotaxis to eotaxin in a dose-dependent manner (Fig. 4b).

Down-modulation of CRTH2 and CCR3 by indomethacin

Treatment of eosinophils with indomethacin clearly down-modulated cell surface expression of CRTH2 (Fig. 5), consistent with our previous report.[30] Similarly, indomethacin suppressed CCR3 expression on eosinophils. However, these modulatory effects on CRTH2 and CCR3 were barely detectable when eosinophils were treated with low doses of indomethacin, i.e. at 10^{-8} M for CRTH2 (data not shown) and 10^{-7} M for CCR3 (Fig. 5, left lower panel). Suppressive effects of indomethacin on chemotaxis to PGD_2 and eotaxin therefore did not seem to be simply mediated by down-modulation of cell surface expressions of these receptors, but could instead be a result of altered functions.

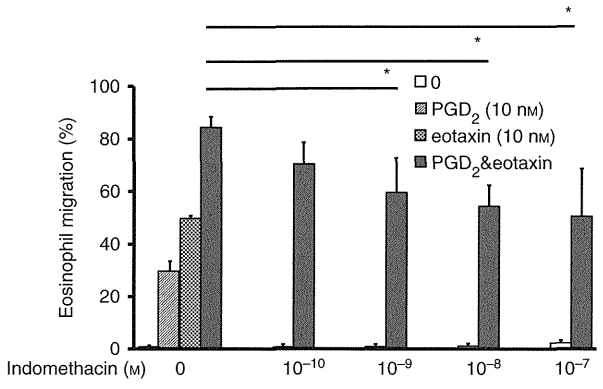

Figure 3. Effects of indomethacin on chemotaxis to prostaglandin D_2 (PGD_2) in combination with eotaxin. Eosinophils were pre-treated with indomethacin for 90 min at 37°, followed by centrifugation and application to the upper chamber. In lower chambers, both PGD_2 and eotaxin (CCL11) were added, and eosinophil migration was assessed. *$P < 0{\cdot}05$. Representative results of at least three independent experiments are shown.

Continuous exposure of eosinophils to indomethacin inhibits eosinophil migration in response to PGD_2, but enhances eotaxin-induced migration

Next, we were interested in the migration of eosinophils under continuous exposure to indomethacin. Indomethacin was added to both upper and lower chambers. As

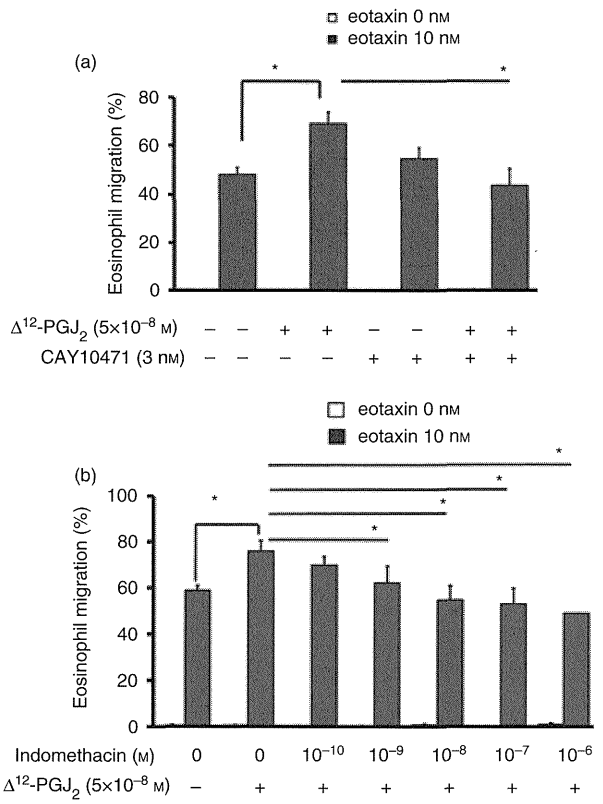

Figure 4. Chemoattractant receptor-homologous molecule expressed on T helper type 2 cells (CRTH2) -mediated eosinophil priming by Δ^{12}-prostaglandin J_2 (PGJ_2) was cancelled by indomethacin. (a) Eosinophils were pre-treated with CAY10471 for 10 min at 37°. After centrifugation, they were mixed with Δ^{12}-PGJ_2, then applied to the upper chamber. Chemotaxis to eotaxin (lower chamber) was assessed in the presence of Δ^{12}-PGJ_2. Pre-treatment with CAY10471 cancelled the priming effects of Δ^{12}-PGJ_2 on eosinophil migration to eotaxin. (b) Eosinophils were pre-treated with indomethacin for 90 min at 37°. After centrifugation, they were mixed with Δ^{12}-PGJ_2, then applied to the upper chamber, and subjected to chemotactic assay for eotaxin. *$P < 0{\cdot}05$. Representative results of at least three independent experiments are shown.

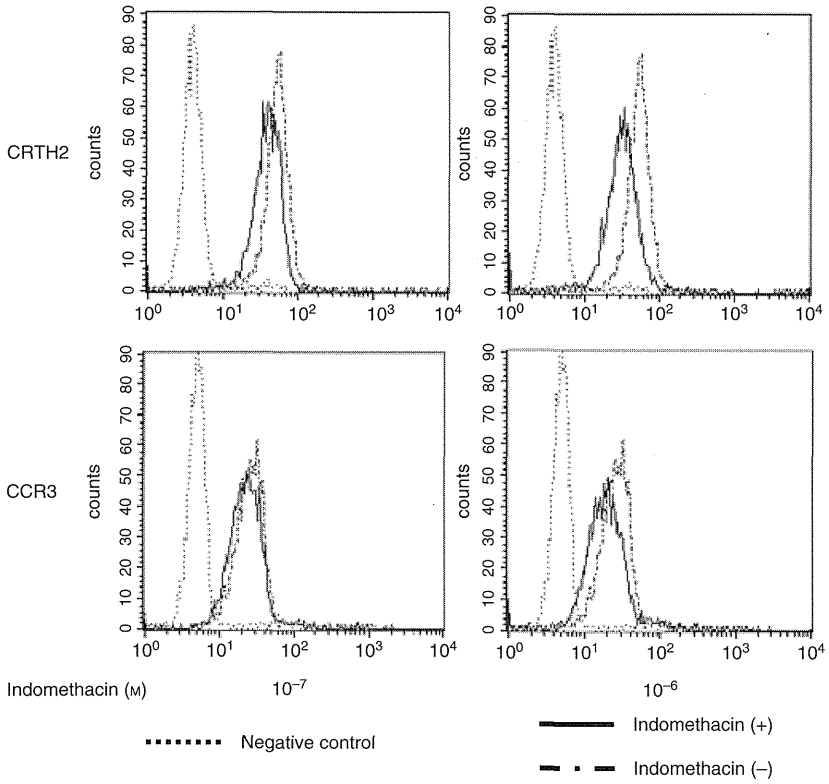

Figure 5. Down-modulation of chemoattractant receptor-homologous molecule expressed on T helper type 2 cells (CRTH2) and CCR3 by indomethacin. Eosinophils were incubated with indomethacin (10^{-7} or 10^{-6} M) for 90 min. Cell surface expression of CRTH2 and CCR3 was assessed by flow cytometry. Representative results of three independent experiments are shown.

expected, spontaneous migration to the lower chambers (chemokinesis) increased in parallel with doses of indomethacin (Fig. 6a), whereas migration in response to PGD_2 was markedly suppressed by continuous exposure of eosinophils to indomethacin. These findings were in striking contrast to the results of eotaxin-induced cell migration. Migration in response to eotaxin did not decrease, but rather increased at higher doses of indomethacin (Fig. 6b). Eosinophil migration to PGD_2/eotaxin decreased in the presence of indomethacin in a dose-dependent manner (Fig. 6c).

L-PGDS expression in lesional skin of EPF

Previous reports have demonstrated a number of inflammatory cells expressing H-PGDS in lesional skin of EPF.[30,38] However, the involvement of L-PGDS in EPF lesions has not been clarified. An immunohistochemical study using mouse monoclonal antibody (1B7) detected L-PGDS in hair follicle epithelium with eosinophilic pustules (Fig. 7a). Rarely, EPF affects palms and soles that lack the hair follicle apparatus. In such situations, the acrosyringium may be the site predominantly affected in these lesions.[2] Interestingly, epidermal keratinocytes surrounding eosinophilic pustules were positive for L-PGDS, together with positive staining in acrosyringeal ducts in the stratum corneum (Fig. 7c). More importantly, eccrine sweat glands exhibited a strongly positive reaction for L-PGDS (Fig. 7d). Similar data were obtained when rabbit polyclonal antibody to L-PGDS was used (data not shown).

Discussion

Indomethacin is an inhibitor of cyclo-oxygenases,[42] exerting therapeutic effects on certain eosinophilic diseases, particularly EPF.[3–5] The present study revealed inhibitory effects of indomethacin on eosinophil migratory functions *in vitro*, whereas diclofenac, another cyclo-oxygenase inhibitor, was unable to suppress eosinophil migration.

Prostaglandin D_2 induced both chemokinesis and chemotaxis in eosinophils via CRTH2 stimulation, and this was also the case for indomethacin. Nevertheless, indomethacin clearly inhibited eosinophil chemotaxis to PGD_2 within a therapeutic range (10^{-7}–10^{-6} M). The priming effects of Δ^{12}-PGJ_2, a plasma metabolite of PGD_2, on eotaxin-induced migration were also cancelled by indomethacin. These actions are probably attributable to homologous and functional desensitization of the CRTH2 receptor by the agonistic actions of indomethacin. Down-modulation of cell surface expression of CRTH2 on eosinophils by indomethacin could also contribute to low responsiveness to PGD_2. These findings could explain the apparently inconsistent evidence that indomethacin has an anti-inflammatory effect on eosinophilic diseases such as EPF despite being a CRTH2 agonist, rather than an antagonist. Th2 cells and basophils are known to express CRTH2.[22] Hence, not only eosinophils, but also Th2 cells

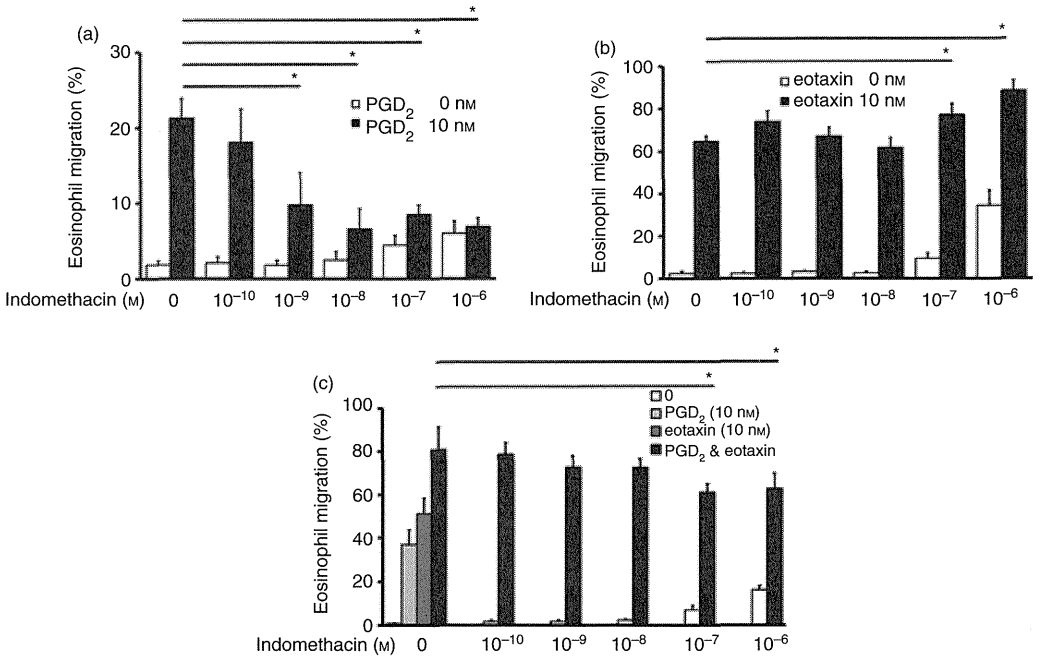

Figure 6. Effects of continuous exposure to indomethacin on eosinophil migration in response to prostaglandin D_2 (PGD_2) and eotaxin. Indomethacin was added to both upper and lower chambers. Migration to PGD_2 (a), eotaxin (b) and PGD_2/eotaxin (c) was assessed. Chemokinesis (eosinophil migration to medium, open column) markedly increased under exposure to indomethacin. On the other hand, eosinophil migration to PGD_2 significantly decreased, and eotaxin-induced migration was enhanced at high doses of indomethacin along with increased chemokinesis. (c) Eosinophil migration to PGD_2/eotaxin was dose-dependently suppressed under continuous exposure to indomethacin. $^*P < 0{\cdot}05$. Representative results of at least three independent experiments are shown.

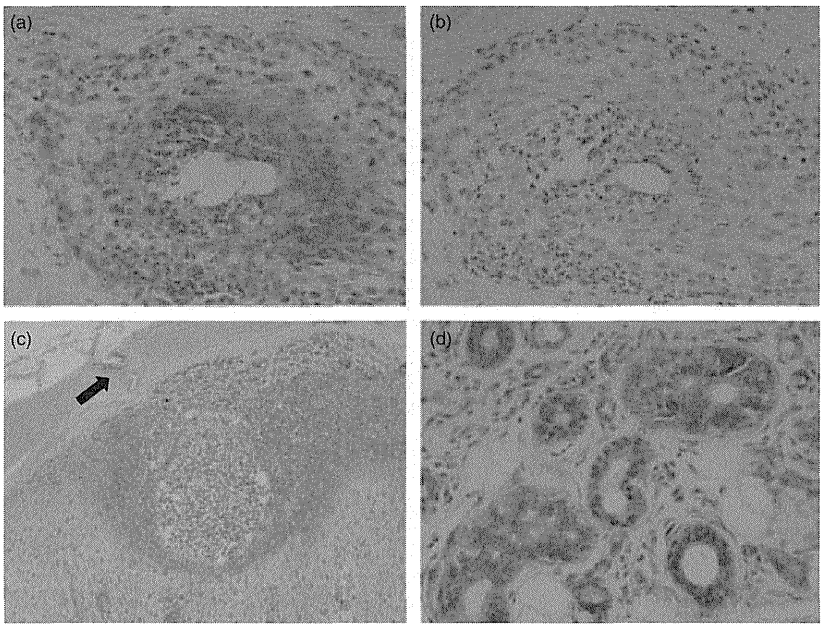

Figure 7. Immunohistochemical localization of lipocalin-type prostaglandin D synthase (L-PGDS) in skin lesions of eosinophilic pustular folliculitis (EPF). (a) Hair follicle epithelium with eosinophil infiltration was positive for L-PGDS. (b) Isotype control of (a). (c) Palmar lesions of EPF. Epidermal keratinocytes around pustules and eccrine ostia in the stratum corneum showed positive results for L-PGDS. (d) L-PGDS in eccrine ducts and glands. Basal (clear) cells in secretory portion and ductal cells were positive for L-PGDS.

and basophils could be targets of indomethacin. Our previous report found that a significant number of basophils infiltrate the skin lesions of EPF to a similar degree to eosinophils.[43]

Indomethacin also weakly suppressed eotaxin-induced eosinophil migration. This is possibly a result of cross-desensitization between CRTH2 and a receptor for eotaxin, CCR3.[44] Mutual and functional modulation between seven-transmembrane G protein-coupled chemoattractant receptors has also been demonstrated in C5a, *N*-formyl-methionyl-leucyl-phenylalanine and interleukin-8 receptors on neutrophils.[45]

Interestingly, low-dose indomethacin (10^{-10} M) had a weak priming effect on PGD_2-induced and eotaxin-induced eosinophil migration. Although the data shown here were not statistically significant (Fig. 2a,b), we observed a reproducible trend toward promotion of chemotactic responses by low-dose indomethacin in repeated experiments, some of which produced statistically significant results. Such findings suggest that CRTH2 stimulation by agonists results in a bell-shaped priming response for chemotaxis, and administration of doses of indomethacin below the therapeutic range may carry a potential risk of eosinophil activation that may lead to exacerbation of PGD_2-mediated or eotaxin-mediated inflammation.

To further elucidate the pharmacological actions of indomethacin, this study assessed eosinophil migration in response to PGD_2 and eotaxin in the presence of indomethacin in both upper and lower chambers. Continuous exposure of eosinophils to indomethacin exerted marked inhibitory effects on PGD_2-induced chemotaxis, but caused more apparent chemokinesis than that seen with indomethacin pre-treatment. Conversely, and intriguingly, eotaxin-induced cell migration was dose-dependently enhanced along with promotion of chemokinesis. These findings imply that indomethacin may not necessarily inhibit eotaxin-induced eosinophilic inflammation, due to the promotion of chemokinesis when eosinophils are persistently exposed to indomethacin. Whether this recapitulates the circumstances at local inflammatory sites *in vivo* of individuals administered with indomethacin is uncertain, but cross-desensitization of CCR3 receptor may not be the sole explanation for the therapeutic mechanisms of indomethacin. In general, indomethacin is not necessarily effective for Th2-predominant inflammation, including atopic dermatitis and bronchial asthma, where eotaxin is probably produced. Indomethacin can therefore be assumed to exert therapeutic effects in diseases where PGD_2–CRTH2 interactions offer greater contributions to the pathological mechanisms than Th2-type cytokines and chemokines such as eotaxin.

In one patient with EPF, we measured serum levels of PGD_2. During disease onset, the patient showed $4{\cdot}9 \times 10^{-10}$ M of PGD_2 in the blood, considerably higher than the PGD_2 levels of three healthy volunteers ($1{\cdot}8 \pm 0{\cdot}84 \times 10^{-10}$ M). Elevated PGD_2 levels normalized ($1{\cdot}1 \times 10^{-10}$ M) after successful treatment with indomethacin. Although a further study with larger sample size is required, these data suggest that PGD_2 is actually produced in the EPF. Indeed, in EPF lesions, our group and others detected H-PGDS-expressing cells in inflammatory infiltrates as cellular sources of PGD_2.[30,38] The present study also found that hair follicle epithelium stained positive by immunohistochemistry for L-PGDS, another enzyme synthesizing PGD_2, which may account for hair follicle accumulation of eosinophils. Interestingly, dermal eccrine glands/ducts, eccrine ostia in stratum corneum and epidermal keratinocytes around eosinophilic pustules were also positive for L-PGDS. Lipocalin-type PGDS is a multifunctional protein showing a lipocalin-type structure.[46] This protein functions as a PGD_2-producing enzyme, but also binds various lipophilic substances and can be secreted into various body fluids, such as cerebrospinal fluid and urine. In this respect, we lack direct evidence for local synthesis of L-PGDS by keratinocytes and eccrine apparatuses. However, the presence of L-PGDS may contribute to local production of PGD_2 and accumulation of CRTH2-expressing cells, such as eosinophils, Th2 cells and basophils in hair follicles and acrosyringium. These findings may be consistent with our recent finding that acrosyringium appears to be the principal site affected in EPF with palmoplantar lesions, where hair follicles are lacking.[2]

A recent study revealed that PGD_2 stimulates sebocytes to produce eotaxin-3 (CCL26) via peroxisome proliferator-activated receptor γ, but not CRTH2, leading to the accumulation of eosinophils in sebaceous hair follicles.[38] That study illustrated upstream PGD_2 signals and downstream CCR3 signals in the cascade of mechanisms underlying eosinophil accumulation. Hence, several inflammatory pathways (i.e. $CRTH_2$-dependent and independent pathways) appear to be involved in the pathological mechanisms underlying EPF. Therefore, it is assumed that indomethacin exerts its effects through desensitization of CRTH2 signals in eosinophils as well as through inhibition of PGD_2 synthesis in local tissue. The latter case may be limited to some types of eosinophilic inflammation where PGD_2 production is a major contributor in the pathogenesis of the disease.

Acknowledgements

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Disclosures

The authors declare that they have no conflicts of interest.

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α(1,3) Fucosyltransferases IV and VII Are Essential for the Initial Recruitment of Basophils in Chronic Allergic Inflammation

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Basophils act as initiator cells for the development of IgE-mediated chronic allergic inflammation (IgE-CAI). However, detailed mechanisms of initial recruitment of basophils into the skin have yet to be clarified. Selectins mediate leukocyte capture and rolling on the vascular endothelium for extravasation. Counter-receptor activity of selectins is regulated by α(1, 3) fucosyltransferases (FTs) IV and VII. To clarify the contribution of selectin ligands regulated by FTs for initial basophil recruitment, IgE-CAI was induced in mice deficient in *FT-IV* and/or *FT-VII* genes. Although FT-IV(−/−) and FT-VII(−/−) mice exhibited comparable skin responses to wild-type mice, the FT-IV(−/−)/FT-VII(−/−) mice showed significantly impaired inflammation. Although the transfer of basophils to FcRγ(−/−) mice induced IgE-CAI, this induction was completely absent when basophils from FT-IV(−/−)/FT-VII(−/−) mice were transferred. L-selectin, but not P- and E-selectin, blocking Abs inhibited skin inflammation *in vivo*. P-selectin glycoprotein-1 (PSGL-1) antibody also ameliorated skin inflammation, and basophils were bound to L-selectin in a PSGL-1-dependent manner, which was regulated by FT-IV/VII. Functional PSGL-1 generated by basophil FT-IV/VII and its subsequent binding to L-selectin could be one of the essential steps required for initial basophil recruitment and the development of IgE-CAI in mice.

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INTRODUCTION

Leukocyte recruitment from the vasculature to the inflammatory sites is a multistep process. The first step of extravasation is leukocyte capture and rolling along the endothelial surfaces, a process that is mediated by selectins. P- and E-selectins on the endothelial cells contribute to the primary capture of leukocytes via binding to their ligands. Conversely, L-selectin is constitutively expressed on most types of circulating leukocytes. L-selectin binds to its ligands on activated endothelial cells (Spertini *et al.*, 1992; Luscinskas *et al.*, 1994; Tu *et al.*, 1999), and also mediates binding to leukocytes already adhering to endothelial cells (secondary capture) (Guyer *et al.*, 1996; Walcheck *et al.*, 1996).

The glycans that contribute to selectin counter-receptor activity arise through glycosylation reactions in which the terminal steps are catalyzed by α(1,3) fucosyltransferases (FTs) (Lowe, 2002). Mice deficient in the *FT-VII* gene (FT-VII(−/−) mice) are characterized by absent P-, E-, and L-selectin ligand activities (Maly *et al.*, 1996). Although the contribution of FT-IV is somewhat subtle when FT-VII is expressed (Weninger *et al.*, 2000), the inflammation-dependent leukocyte recruitment is retained in the FT-VII(−/−) mice. However, it is extinguished in the FT-IV(−/−)/FT-VII(−/−) mice, indicating that FT-IV contributes to E-, P-, and L-selectin ligand generation (Homeister *et al.*, 2001).

Basophils represent <1% of the peripheral blood leukocytes. Under physiological conditions, basophils do not reside in the peripheral tissues. However, basophils can infiltrate into the skin during inflammatory conditions (Ito *et al.*, 2011). Despite the similarities of basophils and mast cells, recent studies have revealed unique functions for basophils, such as producing IL-4 and IL-13 (Redrup *et al.*, 1998; Sokol *et al.*, 2008; Watanabe *et al.*, 2008), and functioning as antigen-presenting cells that induce Th2 cells (Sokol *et al.*, 2009). Basophils also mediate protective immunity against helminthes and ticks (Voehringer, 2009; Wada *et al.*, 2010), in addition to being indispensable for IgG-mediated anaphylactic reactions in mice (Tsujimura *et al.*, 2008).

IgE-mediated chronic allergic inflammation (IgE-CAI) is a long-lasting inflammation that follows immediate-type reactions and late-phase responses. It is histopathologically characterized by numerous eosinophils and mast cells (Mukai *et al.*, 2005; Obata *et al.*, 2007). Although tissue basophils constitute only a minor population of total cellular infiltrate, they have a critical role in the development of IgE-CAI. After a depletion of basophils but not the mast cells, it has been

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Abbreviations: CHS, contact hypersensitivity; FT, α(1, 3) fucosyltransferase; IgE-CAI, IgE-mediated chronic allergic inflammation; mRNA, messenger RNA; PSGL-1, P-selectin glycoprotein-1; TNP, trinitrophenyl; WT, wild type

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shown that there is an almost complete abrogation of IgE-CAI (Obata *et al.*, 2007). Thus, basophils are now considered to initiate inflammation of IgE-CAI. Nevertheless, the current understanding of early events involving basophil recruitment to the skin remains limited. This study was designed to determine the requirements of selectin ligand activity for initial basophil recruitment to the skin controlled by FT-IV and -VII during IgE-CAI.

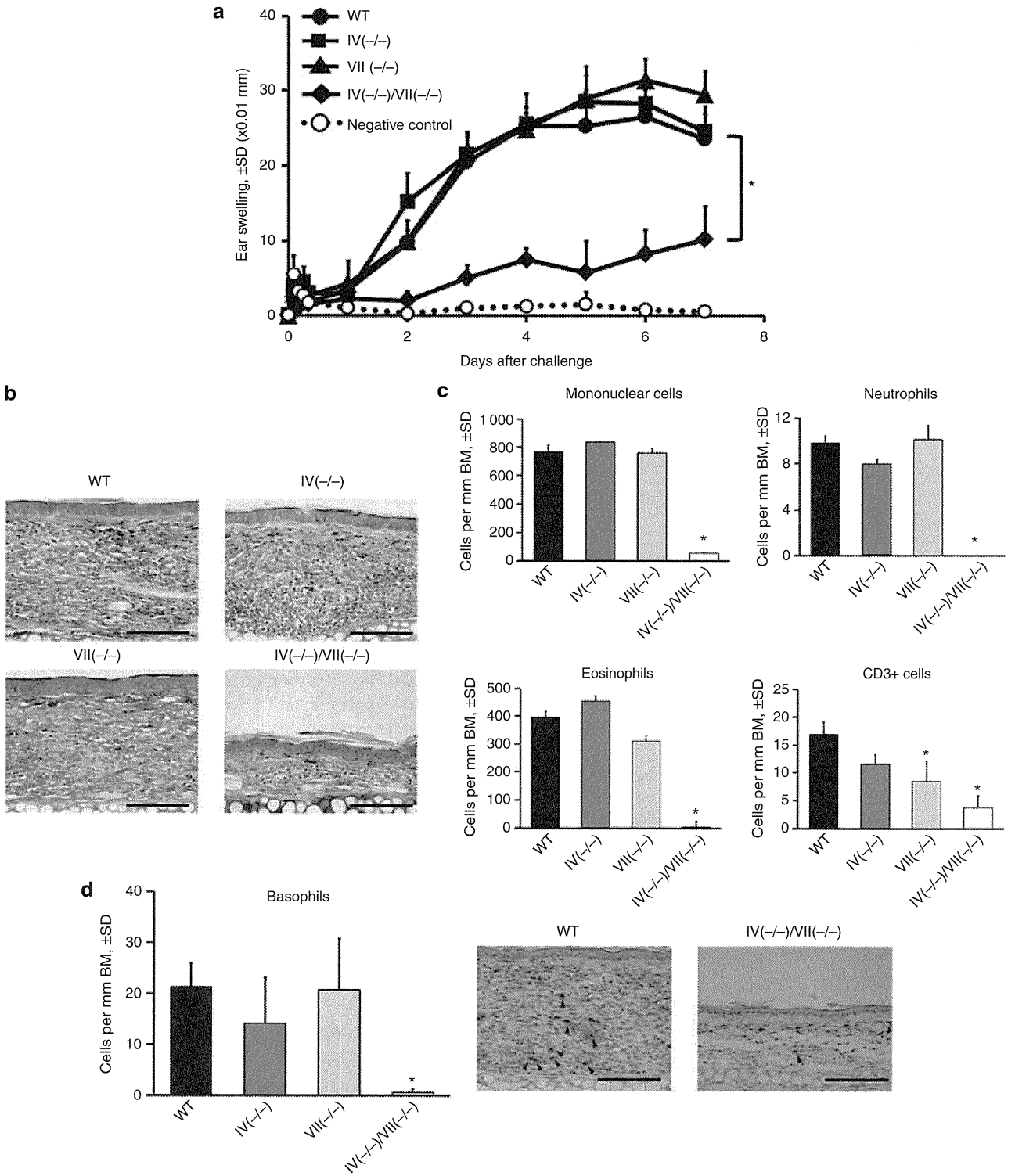

Figure 1. IgE-mediated chronic allergic inflammation (IgE-CAI) in α(1, 3) fucosyltransferase-IV (FT-IV)- and/or FT-VII-deficient mice. (**a**) IgE-CAI was induced in mice lacking FT-IV and/or FT-VII. Negative control mice were challenged with trinitrophenyl-OVA (TNP-OVA) without TNP-IgE injection. (**b**) Histopathological features of the skin (Giemsa's staining). (**c**) Cell populations in inflammatory skin. (**d**) Basophil numbers in inflammatory skin. Basophils were detected by mouse mast cell protease-8 mAb (arrows in the right panel). *$P<0.05$ compared with wild-type (WT) mice. BM, basement membrane. Bar = 100 μm.

RESULTS

Dependency of IgE-CAI on the collaborative functions of FT-IV and FT-VII

To determine selectins and FTs contribution to skin inflammation, IgE-CAI was induced in FT-IV(−/−), FT-VII(−/−), and FT-IV(−/−)/FT-VII(−/−) mice. FT-IV(−/−) mice exhibited levels of skin responses comparable to those seen in the wild-type (WT) mice. In addition, FT-VII deficiency also did not affect IgE-CAI. Nevertheless, FT-IV(−/−)/FT-VII(−/−) mice showed remarkably reduced skin responses (Figure 1a). Histological examination demonstrated that the number of dermal mononuclear cells, neutrophils, and eosinophils were similar among the WT, FT-IV(−/−), and FT-VII(−/−) mice, although they were significantly reduced in the FT-IV(−/−)/FT-VII(−/−) mice (Figure 1b and c). A similar trend was noted for the number of basophils as detected by a basophil-specific antibody (Ugajin *et al.*, 2009) (Figure 1d). Conversely, the number of CD3 (+) T cells apparently decreased in FT-VII(−/−) mice, with this decrease even more prominent in FT-IV(−/−)/FT-VII(−/−) mice (Figure 1c). These findings demonstrate that IgE-CAI is dependent on both FT-IV and FT-VII.

Indispensability of FT-VII in delayed-type skin responses

To obtain further insight into the function of FTs in skin inflammation, we induced contact hypersensitivity (CHS) responses in FT-deficient mice. Although the CHS of the FT-IV(−/−) animals was comparable to WT mice, there was a significantly reduced skin response observed in FT-VII(−/−) mice, unlike that seen for IgE-CAI. Consistent with a previous report (Smithson *et al.*, 2001), CHS was almost completely absent in FT-IV(−/−)/FT-VII(−/−) mice (Figure 2a). Similarly, as compared with the WT mice, delayed-type hypersensitivity reactions to sheep red blood cells (SRBCs) in FT-VII(−/−) and FT-IV(−/−)/FT-VII(−/−), but not FT-IV(−/−), mice were remarkably alleviated (Figure 2b).

Induction of IgE-CAI with basophils lacking both FT-IV and VII

On the basis of the fact that IgE-CAI is entirely dependent on basophils (Mukai *et al.*, 2005; Obata *et al.*, 2007), we attempted to determine the contribution of selectin ligands generated by basophil FTs to the development of skin responses. Basophil transfer from WT mice to irradiated FcRγ(−/−) mice lacking FcεRI successfully induced IgE-CAI (Figure 3a), which was consistent with a prior report (Mukai *et al.*, 2005). Basophil-enriched cell suspension consisted of ~20% primary basophils and ~80% other cells, including CD49b (+) natural killer (NK) cells. Nevertheless, NK cells, T cells, NKT cells, B cells, and

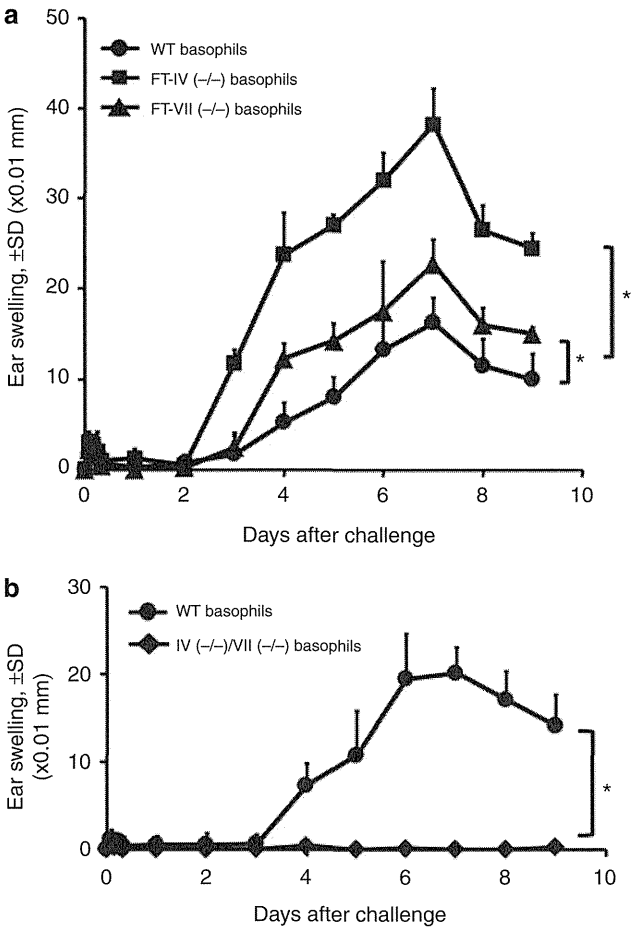

Figure 3. α(1, 3) Fucosyltransferase-IV/VII (FT-IV/VII) in basophils are indispensable for IgE-mediated chronic allergic inflammation (IgE-CAI). IgE-CAI was induced in FcRγ(−/−) mice that received primary basophils from wild-type (WT), FT-IV(−/−), FT-VII(−/−), and FT-IV(−/−)/FT-VII(−/−) mice. (**a**) Although basophils from FT-IV(−/−) and FT-VII(−/−) mice induced exacerbated IgE-CAI as compared with WT basophils, (**b**) FT-IV(−/−)/FT-VII(−/−) mice–derived basophils were incapable of inducing IgE-CAI. *$P<0.05$.

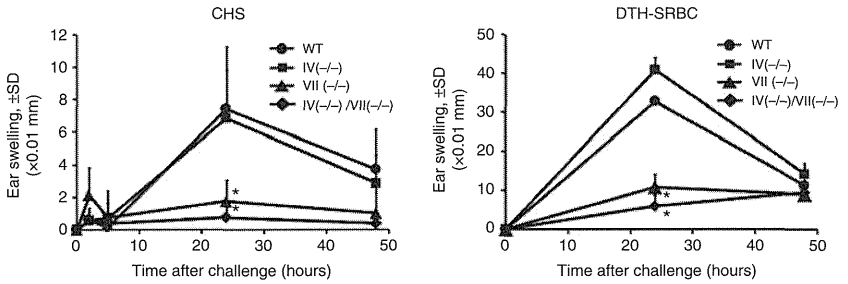

Figure 2. Delayed-type hypersensitivity (DTH) reactions in α(1, 3) fucosyltransferase-IV (FT-IV)- and/or FT-VII-deficient mice. Contact hypersensitivity (CHS) and DTH to sheep red blood cells (DTH-SRBCs) were induced in mice lacking FT-IV and/or -VII. *$P<0.05$ compared with wild-type (WT) mice.

dendritic cells are dispensable for IgE-CAI (Mukai *et al.*, 2005), and thus the development of IgE-CAI in FcRγ(−/−) mice in this experiment could be exclusively mediated by primary basophils. This was also confirmed by the results that IgE-CAI in mice receiving basophil-enriched cell suspension was remarkably alleviated when recipient mice were treated with basophil-depletion antibody (Ba103, kindly provided by Dr Karasuyama (Obata *et al.*, 2007)) (Supplementary Figure S1 online). Basophils from FT-VII(−/−) mice were also capable of inducing IgE-CAI, and interestingly there were higher induction levels as compared with those seen for the WT basophils. This exacerbation was even more marked when basophils were transferred from FT-IV(−/−) mice. Conversely, skin responses in FcRγ(−/−) mice that underwent transfers of primary basophils from FT-IV(−/−)/FT-VII(−/−) mice were completely absent (Figure 3b). Thus, IgE-CAI is entirely dependent on basophil selectin ligands that are collaboratively generated by FT-IV and FT-VII.

Expression of functional selectin ligands on basophils is not sufficient for the full development of IgE-CAI

As inflammatory cells, such as T cells, neutrophils, and eosinophils, have FT-IV and/or FT-VII and are recruited to the skin in a selectin-dependent manner (Homeister *et al.*, 2001; Smithson *et al.*, 2001; Satoh *et al.*, 2005), we examined the development of IgE-CAI by performing experiments designed to assess the contribution of selectin ligands generated by FT-IV/VII in cells other than basophils. WT basophils together with CD49b (−) bone marrow cells (effector cells) from either WT or FT-IV(−/−)/FT-VII(−/−) mice were transferred to irradiated FT-IV(−/−)/FT-VII(−/−) mice. Transfers with the CD49b (−) effector cells from WT mice resulted in a successful induction of IgE-CAI in FT-IV(−/−)/FT-VII(−/−) mice (Figure 4a). Although the CD49b (−) effector cells from FT-IV(−/−)/FT-VII(−/−) mice also induced IgE-CAI responses, induction levels were lower than those of the mice receiving WT mice–derived

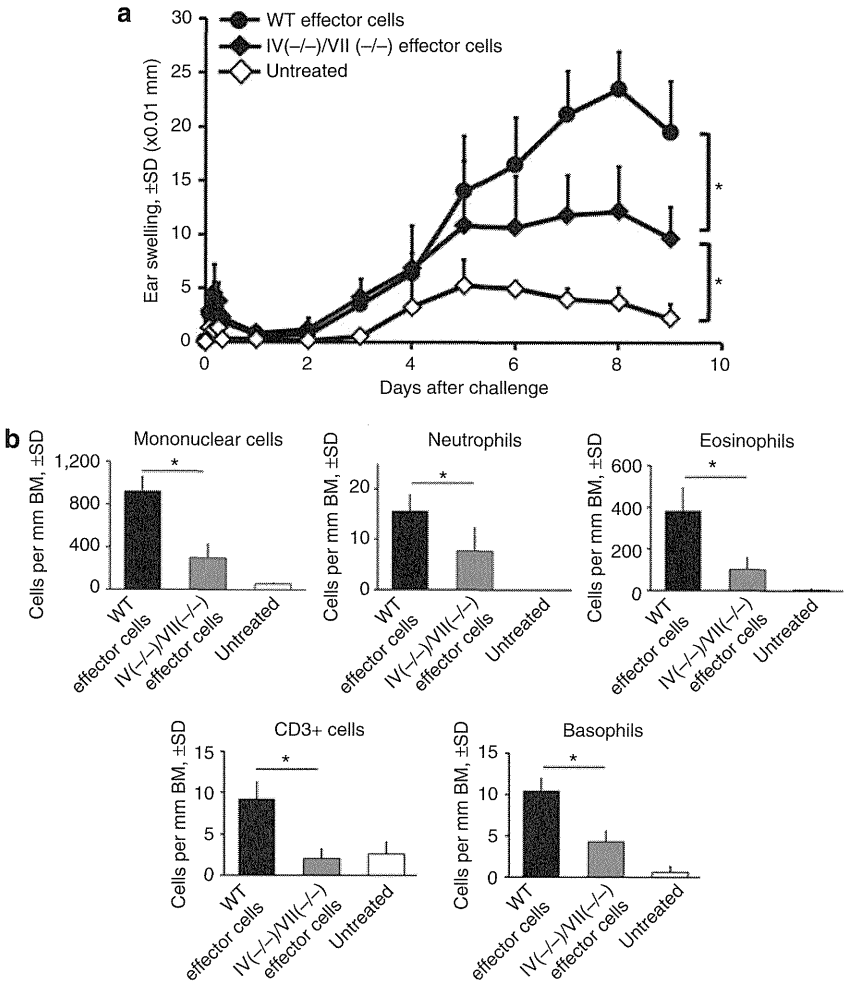

Figure 4. Selectin-dependent cooperative recruitment of basophils and effector cells. (**a**) Irradiated α(1, 3) fucosyltransferase-IV (FT-IV)(−/−)/FT-VII(−/−) mice received wild-type (WT) basophils in combination with CD49b(−) bone marrow cells (effector cells) from either WT or FT-IV(−/−)/FT-VII(−/−) mice. They were then immunized with trinitrophenyl-IgE (TNP-IgE) and challenged with TNP-OVA. The untreated group comprised FT-IV(−/−)/FT-VII(−/−) mice without cell transfer. (**b**) Cell populations in inflammatory skin. *$P<0.05$ compared with WT effector cells. BM, basement membrane.

CD49b (−) effector cells. When cell populations from inflammatory skin were analyzed, it was shown that, even in the presence of WT basophils, there was an impairment of the recruitment of mononuclear cells, neutrophils, CD3 (+) T cells, and eosinophils in mice transferred with FT-IV(−/−)/FT-VII(−/−) mice–derived CD49b(−) effector cells (Figure 4b). More importantly, when WT basophils were cotransferred with CD49b (−) effector cells from FT-IV(−/−)/FT-VII(−/−) mice, complete recruitment into the skin was not achieved. These data suggest that selectin-dependent recruitment of the effector cells appears to be necessary for sufficient responses of IgE-CAI and effective basophil infiltration to occur, even though functional selectin ligand generation in basophils by FT-IV/VII is essential for skin inflammation.

Binding of E- and P-selectins to basophils *in vitro*

Primary basophils expressed transcripts of FT-IV and FT-VII messenger RNA (mRNA; Figure 5a). This was in contrast to bone marrow–derived mast cells, which only expressed extremely low levels of FT mRNA. Although bone marrow–derived basophils had FT transcripts, the levels were much lower than those seen for the primary basophils. Flow cytometry results showed that E- and P-selectin chimeras could bind to primary basophils from WT but not to FT-IV(−/−)/FT-VII(−/−) mice *in vitro* (Figure 5b).

Blockade of E- and/or P-selectins and the amelioration of IgE-CAI

Given the evidence that basophil expression of both E- and P-selectin ligands was dependent upon FT-IV/VII expression, we next attempted to determine the contribution of E- and P-selectins to the actual basophil recruitment. To determine this, we initially examined the effects of blocking Abs against selectins on the development of IgE-CAI. Unexpectedly, we found that blocking of either the E- (clone 10E9.6, BD Bioscience Pharmingen (San Jose, CA), 100 μg per mouse, intravenous) or P- (clone RB40.34, BD Bioscience Pharmingen, 100 μg per mouse, intravenous) selectins did not result in amelioration of IgE-CAI (Supplementary Figure S2 online). Similarly, dual blocking of P- and E-selectins by coadministration of these two Abs also failed to suppress IgE-CAI. These results were in a striking contrast to prior reports demonstrating that the same antibody clones against P- and E-selectins clearly alleviated eotaxin-induced eosinophil accumulation (Satoh *et al.*, 2005) and cutaneous arthus reaction (Yanaba *et al.*, 2003).

Role of P-selectin glycoprotein-1 and L-selectin interaction in basophil recruitment and development of IgE-CAI

Leukocytes express L-selectin, which then interacts with inducible endothelial ligands and contributes to leukocyte rolling (Spertini *et al.*, 1991, 1992; Luscinskas *et al.*, 1994; Tu *et al.*, 1999). Counter-receptor activity of the L-selectin ligand on endothelial cells has been shown to be dependent on the modification by FT-IV and/or VII (Maly *et al.*, 1996; Tu *et al.*, 1999). However, the interaction of basophil L-selectin with ligands modified by endothelial FTs did not seem to be part of the essential pathway for the development of IgE-CAI, as

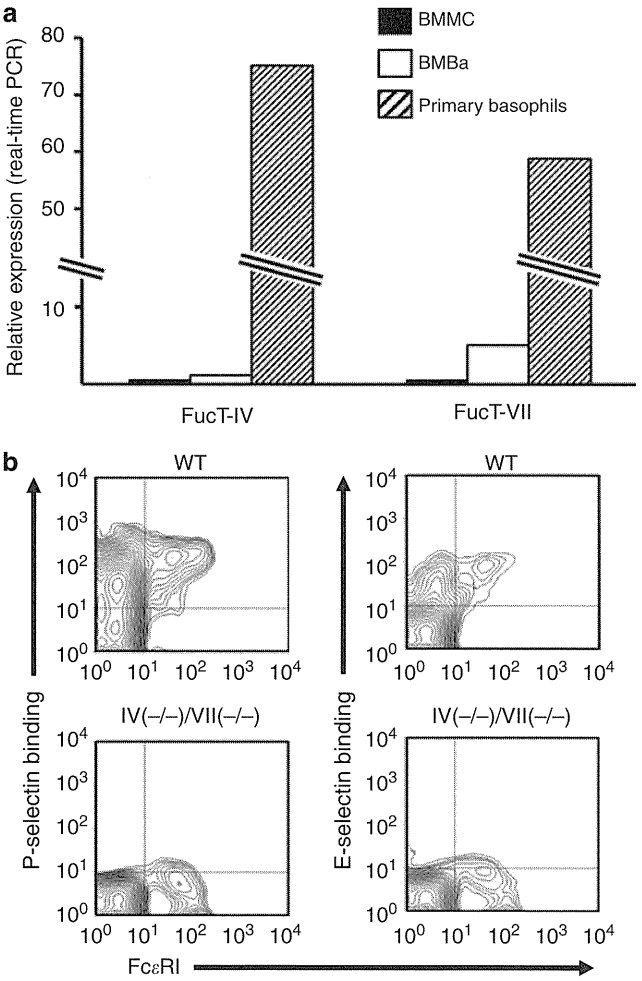

Figure 5. Expression of α(1, 3) fucosyltransferase (FT) messenger RNA (mRNA) and FT-dependent selectin binding in basophils. (**a**) Primary basophils were subjected to further purification by positive selection with CD123 (purity >99%). Transcripts for FT-IV and FT-VII mRNA were quantified by real-time PCR. (**b**) Binding of soluble P- and E-selectins to primary basophils assessed by flow cytometry. BMBa, bone marrow–derived basophil; BMMCs, bone marrow–derived mast cells.

basophils from WT mice were able to successfully induce skin inflammation in FT-IV(−/−)/FT-VII(−/−) mice lacking counter-receptor activity for L-selectin on endothelial cells (Figure 4a). Prior evidence has also shown that leukocyte PGSL-1, which is a major ligand for P-selectin, can function as a counter-receptor for L-selectin in an FT-dependent manner, thereby contributing to secondary tethering(Guyer *et al.*, 1996; Walcheck *et al.*, 1996). These findings led us to hypothesize that modification of P-selectin glycoprotein-1 (PSGL-1) by FTs in basophils combined with the subsequent binding to L-selectin was an essential pathway for the development of IgE-CAI. To test this hypothesis, we initially confirmed that primary basophils from both WT and FT-IV(−/−)/FT-VII(−/−) mice expressed PSGL-1 and L-selectin on their cell surface (Figure 6a). PSGL-1 Ab (4RA10, BD Bioscience Pharmingen) almost completely inhibited the *in vitro* binding

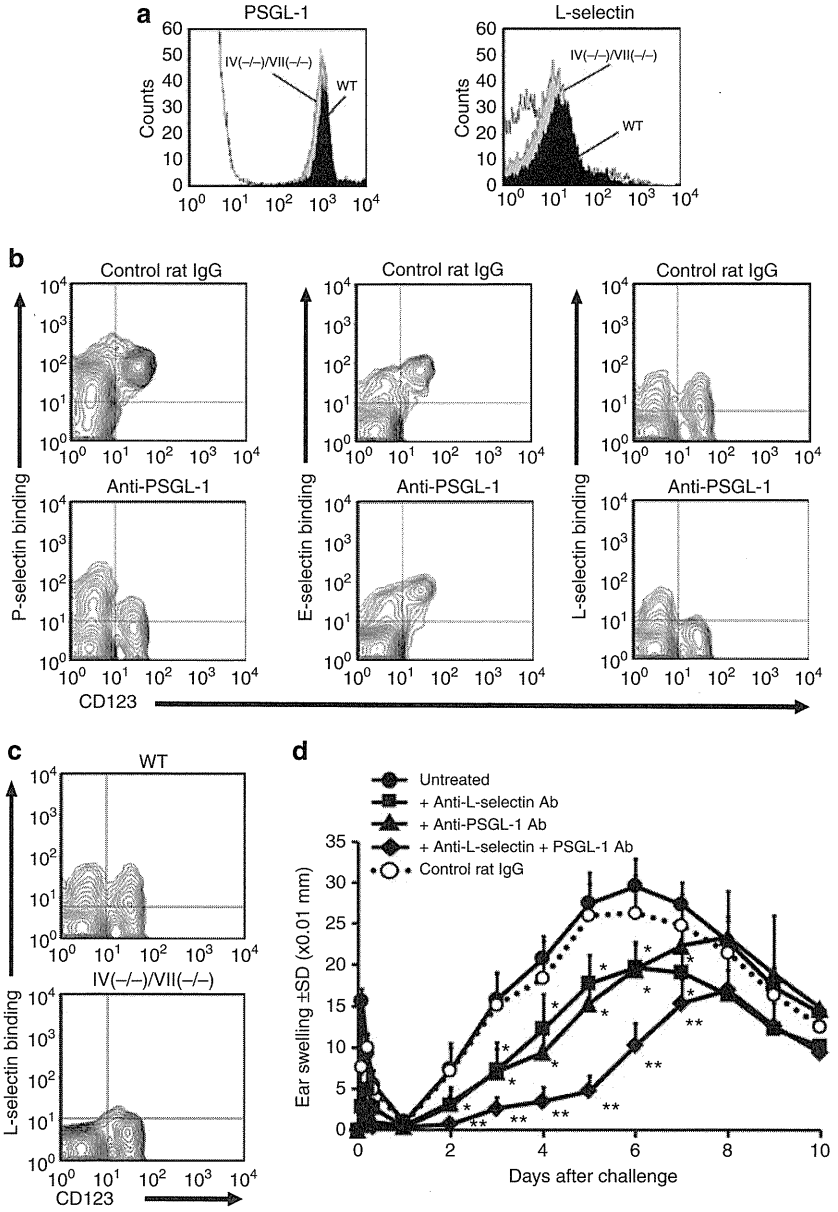

Figure 6. Basophil P-selectin glycoprotein-1 (PSGL-1)–L-selectin interaction involvement in IgE-mediated chronic allergic inflammation (IgE-CAI). (**a**) PSGL-1 and L-selectin expressions on basophils. (**b**) PSGL-1 Ab effect on selectin binding to primary basophils. (**c**) L-selectin binding to basophils from wild-type (WT) and α(1, 3) fucosyltransferase-IV (FT-IV)(−/−)/FT-VII(−/−) mice. (**d**) Effects of L-selectin and/or PSGL-1 Abs on IgE-CAI in WT mice. $*P<0.05$ compared with control IgG. $**P<0.05$ compared with L-selectin or PSGL-1 Ab alone.

of both the L- and P-selectins to the WT basophils (Figure 6b). Counter-receptor activity of PSGL-1 for L-selectin appears to be dependent on FT-VI/VII, as L-selectin failed to bind the primary basophils from FT-IV(−/−)/FT-VII(−/−) mice (Figure 6c). We then assessed whether blocking L-selectin could ameliorate IgE-CAI. As expected, the administration of the L-selectin blocking Ab (MEL-14, eBioscience, San Diego, CA) partly but significantly inhibited IgE-CAI. Similarly, PSGL-1 blocking Ab (4RA10) also inhibited IgE-CAI. When there was concomitant administration of these two Abs, further suppression of skin responses was also observed (Figure 6d).

DISCUSSION

Extravasation and recruitment of basophils to the skin are an essential step for the development of IgE-CAI (Mukai *et al.*, 2005; Obata *et al.*, 2007). This study examined the FT-IV/VII-dependent basophil recruitment and induction of IgE-CAI.

Although a single deficiency of the *FT-IV* or *FT-VII* genes did not affect IgE-CAI, greatly impaired skin responses were seen in the FT-IV(−/−)/FT-VII(−/−) mice. To elucidate the contribution of FTs in basophils during IgE-CAI, we transferred basophils into FcRγ(−/−) mice lacking FcεRI. Unlike those from WT mice, basophils from the FT-IV(−/−)/FT-VII(−/−)

mice failed to induce IgE-CAI in FcRγ(−/−) mice. These data confirm the critical contribution of basophils for the development of IgE-CAI (Mukai *et al.*, 2005) and suggest that impaired skin responses in FT-IV(−/−)/FT-VII(−/−) mice is largely due to the inability to recruit basophils into the skin.

Leukocytes other than basophils may also require selectins for their recruitment to the skin during IgE-CAI. Our results indicated that the transfer of WT basophils with basophil-depleted bone marrow cells (effector cells) from FT-IV(−/−)/FT-VII(−/−) mice were not able to fully develop IgE-CAI as compared with the WT basophils that were cotransferred with WT effector cells (Figure 4a). In addition, WT basophils themselves were not effectively recruited into the skin when in the presence of effector cells from FT-IV(−/−)/FT-VI(−/−) mice. Thus, it appears that some effector cells require FT-IV/VII-dependent modification of selectin ligands in order to be recruited to the skin. In addition, these cells appeared to increase the effectiveness of basophil recruitment to the skin. Once basophils are recruited into the skin, they can promote the accumulation of other effector cells. These cells, in turn, may then assist in further basophil recruitment into the skin.

Basophils from FT-IV(−/−)/FT-VII(−/−) mice did not show avidity to soluble E- and P-selectins, which indicates that these are dependent on the FT function (Figure 5b). However, IgE-CAI was unexpectedly not suppressed after the use of blocking Abs against P- and E-selectins, despite the inability of basophils from FT-IV(−/−)/VII(−/−) mice to induce IgE-CAI (Figure 3b). Conversely, blockade of L-selectin resulted in a moderate suppression of IgE-CAI. It is possible that PSGL-1 on basophils could be a counter-receptor of the basophil L-selectin. On the basis of our results that showed that WT basophils could successfully induce IgE-CAI in FT-IV(−/−)/FT-VII(−/−) mice, it appears that endothelial L-selectin ligands might not be essential for basophil recruitment. We demonstrated that L-selectin bound PSGL-1 *in vitro*, and this binding was dependent on the basophil FT-IV/VII. In addition, when we blocked PSGL-1, this alleviated IgE-CAI *in vivo*. These were similar to the level of suppression that was seen when using anti-L-selectin Ab. Thus, FT-mediated modification of basophil PSGL-1 and the binding to L-selectin appear to be one of the important steps required for the development of IgE-CAI.

Intriguingly, we also noted that coadministration of anti-PSGL-1 and L-selectin Abs was able to more efficiently inhibit IgE-CAI than the injection of a single Ab. Although we have not been able to completely assure that optimal doses of each antibody were used, this suggests that an adhesion pathway other than PSGL-1-L-selectin interaction might contribute to the development of IgE-CAI. Several lines of evidence have suggested that an L-selectin-dependent leukocyte–leukocyte interaction facilitates the subsequent direct interaction of leukocytes with endothelial selectins, which leads to the amplification of initial leukocyte recruitment (Alon *et al.*, 1996; Walcheck *et al.*, 1996; Sperandio *et al.*, 2003). In this respect, endothelial L-selectin ligands and P-selectin might assist in the capture and rolling of basophils and effector cells on the endothelial cells following the PSGL-1-L-selectin interaction, although the blocking of P-selectin alone is not sufficient for the inhibition of basophil recruitment and the development of IgE-CAI. The roles of E-, P-, and L-selectins in leukocyte capture and/or rolling on endothelial cells have been shown to be partially redundant, and these three selectins can also function synergistically (Ley *et al.*, 1993, 1995; Ley and Tedder, 1995; Lowe, 2002).

IgE-CAI offers a unique mouse model of skin inflammation, in that it is dependent on IgE and FcεRI of basophils, but independent of FcεRI of mast cells and other cells that usually have central roles in some human allergic inflammations (von Bubnoff *et al.*, 2003). In addition, the characteristics of mouse basophils differ from those of human basophils in many respects (Lee and McGarry, 2007). Another difference between humans and mice is seen in the regulatory functions of FTs. Human FT-VII, but not FT-IV, modifies PSGL-1 of leukocytes, leading to the expression of cutaneous lymphocyte–associated antigen, which acts as a functional selectin ligand and skin-homing receptor (Kieffer *et al.*, 2001). On the other hand, murine leukocytes express barely detectable levels of cutaneous lymphocyte–associated antigen epitope despite the expression of FT-VII, but still efficiently bind to E- and P-selectins. Murine FT-VII appears to fucosylate only a few quite specific glycans that interact preferentially with selectins (Kobzdej *et al.*, 2002). Thus, it would be difficult to consider the present findings for IgE-CAI in mice as directly applicable to human allergic skin diseases.

Collectively, basophil recruitment and development of IgE-CAI are entirely dependent on collaborative control by FT-IV and VII in the basophils. L-selectin binding to basophil PSGL-1 modified by the FTs could be a central event that ultimately leads to the subsequent inflammatory steps of IgE-CAI.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Sankyo Labo Service (Tokyo, Japan). FcRγ chain(−/−) C57BL/6 mice (Takai *et al.*, 1994) were kindly provided by Dr Takai of Tohoku University, Japan. FT-IV(−/−) mice, FT-VII(−/−) mice, and FT-IV(−/−)/FT-VII(−/−) mice (Maly *et al.*, 1996; Homeister *et al.*, 2001) were originally established at the University of Michigan (Dr Lowe), with colonies maintained at Case Western Reserve University (Dr Myers), which provided animals to our department. The use of animals was in full compliance with the Committee for Animal Experiments of Tokyo Medical and Dental University.

Antibodies

Isotype-matched control Ab (rat IgG2aκ), rat anti-CD16/CD32 (2.4G2), biotinylated anti-CD49b (Dx5), and PE-labeled anti-PSGL-1 (P-selectin glycoprotein-1) (2PH1) Abs were from BD Bioscience Pharmingen. PE/Cy5-labeled anti-L-selectin Ab (MEL-14) was from BioLegend (San Diego, CA). FITC-conjugated anti-CD49b (Dx5), FITC- and PE-conjugated anti-FcεRI Ab (MAR-1), FITC- and PE-labeled anti-mouse CD123 (IL-3Rα), and PE-labeled anti-c-kit (ACK2), biotinylated anti-c-kit (2B8) Abs were purchased from eBioscience. Anti-CD3e (M-20) was from Santa Cruz Biotechnology

(Santa Cruz, CA). Anti-mouse mast cell protease-8 mAb (TUG8) (Ugajin *et al.*, 2009) was provided by Dr Karasuyama of Tokyo Medical and Dental University.

Cutaneous inflammatory reactions

Trinitrophenyl (TNP)-specific IgE was purified from ascites of BALB/c-*nu/nu* mice by intraperitoneal injection of the IGEL b4 B cell hybridoma (ATCC, Rockville, MD; TIB141) (Rudolph *et al.*, 1981). IgE-CAI was induced by passive immunization of mice with TNP-specific IgE (150 μg per mouse, intravenous) (Mukai *et al.*, 2005). Mice were challenged 24 hours later with TNP-OVA (10 μg per ear, Biosearch Technologies, Novato, CA) on each ear lobe.

CHS reactions were induced by application of 50 μl of 0.5% DNFB (Nacalai Tesque, Kyoto, Japan) in acetone:olive oil (4:1) onto the ventral skin on day 0. On day 5, each ear lobe was challenged with 20 μl of 0.2% DNFB in acetone:olive oil (4:1). Ear thickness was measured using a dial thickness gauge (Ozaki, Tokyo, Japan) before and after the challenges.

DTH to SRBCs was induced by subcutaneous immunization with 100 μl of 20% SRBCs on the back on days −1 and 0. On day 5, 20 μl of 20% SRBC was injected into the footpad. Footpad thickness was measured before and after the challenges. Each group consisted of at least four mice.

Cell preparation

Primary basophils were prepared by enrichment of CD49b (+) cells from freshly isolated bone marrow cells using the MACS system with biotinylated anti-CD49b and streptavidin microbeads. As determined by flow cytometric analysis for CD49b and CD123 expression, the CD49b (+) cells included ~20% basophils.

Bone marrow–derived basophils were prepared by culturing bone marrow cells in RPMI 1640 supplemented with 10% fetal calf serum and 10 ng ml^{-1} rIL-3 (R&D Systems, Minneapolis, MN) for 10 days, followed by isolation of the CD49b (+) cells using an MACS system.

Bone marrow–derived mast cells were obtained by culturing bone marrow cells in the presence of 10 ng ml^{-1} rIL-3 for 4 weeks, followed by isolation of the c-kit+ cells.

Basophil transfer

Basophil transfer for inducing IgE-CAI in FcRγ chain(−/−) mice was performed using a previously described method (Mukai *et al.*, 2005). Briefly, CD49b (+) basophil–enriched bone marrow cells (primary basophils; 6×10^6 cells per recipient) were transferred into irradiated FcRγ(−/−) mice (6 Gy) together with CD49b(−) bone marrow cells (effector cells) from naive FcRγ chain(−/−) mice. Four days later, mice were passively immunized with TNP-IgE followed by challenge with TNP-OVA.

In vitro selectin binding assay of basophils

CD49b (+) basophil–enriched bone marrow cells were suspended in phosphate-buffered saline containing 5% fetal calf serum, 0.1% NaN_3, 1 mmol l^{-1} Ca^{2+}, and 1 mmol l^{-1} Mg^{2+}, followed by incubation with 10 μg ml^{-1} of murine P-, E-, or L-selectin-human IgG Fc chimera or control human IgG1 Fc (R&D Systems) for 40 minutes at 4 °C. After washing the cells, they were incubated with PE-F $(ab')_2$ goat anti-human IgG Fc Ab (Rockland, Gilbertsville, PA) for 30 minutes at 4 °C. They were then counterstained with FITC- FcεRI Ab or FITC- CD123 Abs. Selectin binding was examined using flow cytometric analysis with FACS Calibur (BD Biosciences, Mountain View, CA).

Real-time PCR

Quantitative real-time reverse-transcriptase PCR was performed with reverse-transcribed RNA by real-time monitoring of the increase in fluorescence of the SYBR Green dye (Brilliant SYBR Green QPCR Master Mix, Stratagene, La Jolla, CA) using the Mx3000P Real-Time PCR system (Stratagene). The primers for PCR were 5′-TGTGTCCGTC GTGGATCTGA-3′ and 5′-TTGCTGTTGAAGTCGCAGGAG-3′ for mouse GAPDH; 5′-CGCTGTGGGACCAATCTTGA-3′ and 5′-CCAGT GTTTGGCACCAGCA-3′ for mouse FT-IV; and 5′-AGATGCCCTGG TGGGCTTTAG-3′ and 5′-TCAGCCATGGGTCAAGGTAAGTC-3′ for mouse FT-VII.

Statistical analyses

A Student's *t*-test was used to assess statistical significance of the differences between the mean values. Analysis of the data for the time-course changes of the skin responses was performed by using the repeated measures analysis of variance test, followed by either a Student's *t*-test or Scheffe's F test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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

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