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Prostaglandin D₂ Plays an Essential Role in Chronic Allergic Inflammation of the Skin via CRTH2 Receptor

Takahiro Satoh, Rie Moroi, Kosuke Aritake, Yoshihiro Urade, Yasumasa Kanai, Koji Sumi, Hiroo Yokozeki, Hiroyuki Hirai, Kinya Nagata, Toshifumi Hara, Masanori Utsuyama, Katsuiku Hirokawa, Kazuo Sugamura, Kiyoshi Nishioka, and Masatake Nakamura

PGD₂ plays roles in allergic inflammation via specific receptors, the PGD receptor designated DP and CRTH2 (chemoattractant receptor homologous molecule expressed on Th2 cells). We generated mutant mice carrying a targeted disruption of the CRTH2 gene to investigate the functional roles of CRTH2 in cutaneous inflammatory responses. CRTH2-deficient mice were fertile and grew normally. Ear-swelling responses induced by hapten-specific IgE were less pronounced in mutant mice, giving 35–55% of the responses of normal mice. Similar results were seen in mice treated with a hemopoietic PGD synthase inhibitor, HQL-79, or a CRTH2 antagonist, ramatroban. The reduction in cutaneous responses was associated with decreased infiltration of lymphocytes, eosinophils, and basophils and decreased production of macrophage-derived chemokine and RANTES at inflammatory sites. In models of chronic contact hypersensitivity induced by repeated hapten application, CRTH2 deficiency resulted in a reduction by approximately half of skin responses and low levels (63% of control) of serum IgE production, although in vivo migration of Langerhans cells and dendritic cells to regional lymph nodes was not impaired in CRTH2-deficient mice. In contrast, delayed-type hypersensitivity to SRBC and irritation dermatitis in mutant mice were the same as in wild-type mice. These findings indicate that the PGD₂-CRTH2 system plays a significant role in chronic allergic skin inflammation. CRTH2 may represent a novel therapeutic target for treatment of human allergic disorders, including atopic dermatitis. The Journal of Immunology, 2006, 177: 2621–2629.

It is thought that PGD₂ exerts its physiological effects through the classical PGD receptor (DP), which is expressed in a variety of cell lineages (7). Signals from DP inhibit chemotaxis of eosinophils and dendritic cells (DC) in vitro (8, 9). Mice with targeted disruption of the DP gene exhibit reduced eosinophil infiltration into the lung and fail to develop airway hyperreactivity in response to allergen exposure (10). It is possible that DP-mediated signals are involved in allergic inflammation and, thus, another receptor for PGD₂ may be postulated. Indeed, we recently identified CRTH2 (chemoattractant receptor homologous molecule expressed on Th2 cells), which acts as a PGD₂ receptor with properties different from those of DP (11).

CRTH2 and DP are members of the G protein-coupled, seven-transmembrane receptor family. CRTH2 is coupled with Gₛ, whereas Gₛ is associated with DP (11). CRTH2 is expressed in eosinophils, basophils, and a subpopulation of Th2 cells and monocytes in humans (12). Upon treatment with PGD₂, these cells exhibit chemotaxis and/or Ca²⁺ mobilization (11, 13). An increase in the percentage of CRTH2⁺ cells among cutaneous lymphocyte-associated Ag-positive and CD4⁺ cells in blood was observed in patients with atopic dermatitis (14). The PGD₂-CRTH2 system is thus apparently involved in atopic dermatitis. However, the roles of CRTH2 in allergic inflammation, particularly in vivo, remain to be determined.

Atopic dermatitis is a common and distinctive chronic allergic disease that exhibits eosinophilia and increased IgE levels in blood. IgE has been shown to mediate immediate-type hypersensitivity via activation of mast cells, which release a series of chemical mediators including histamine and prostaglandins. Recent studies with murine models of skin inflammation have demonstrated that exogenous introduction of IgE or its gene induces immediate-type responses (ITRs) within a couple of hours, late-phase responses (LPRs) at 24 h, and very-late-phase responses (vLPRs)
(third-phase response) several days after challenge (15–18). Histopathological examination of vLPRs indicates epidermal hyperplasia and marked increases in lymphocyte and eosinophil cell numbers. These results clearly demonstrate the pathogenic involvement of IgE in chronic allergic inflammation as well as in ITR (15, 16). In addition, repeated challenge with haptons, the so-called chronic contact hypersensitivity (CHS) mouse model, induces an ITR followed by a late reaction with IgE elevation. These responses are accompanied by a cytokine shift from a Th1 profile to a Th2 profile (19, 20). The collective phenotypic appearance of these responses in mice resembles that of patients with atopic dermatitis.

To examine the link between CRTH2 and chronic allergic inflammation, we generated mice with targeted disruption of the CRTH2 gene and used mouse models of skin inflammation. Our results demonstrate that CRTH2-mediated signals play essential roles in IgE-mediated cutaneous responses and chronic CHS and suggest the possibility that CRTH2 may be a potent therapeutic target for the treatment of chronic allergic skin inflammations such as atopic dermatitis.

Materials and Methods

Generation of CRTH2-deficient mice

Several genomic clones containing the CRTH2 gene of the 129/sV mouse strain provided the 5'-NdeI-EcoRV 6.3-kb and 3'-DraI-BglII 3.0-kb fragments, which were used as the left and right vector arms, respectively. The targeting vector was constructed by inserting the left arm, the neoymycin cassette from ploxPneo-1, the right arm, and the diphtheria toxin cassette from pMC1DTPa (Kurabo) into the Smal site, the EcoRI-XhoI sites, and the KpnI sites of pBluescript II, respectively, and it was then introduced into mouse embryonic stem (ES) cells (provided by Dr. S. Azuma, Kitasato University, Sagamihara, Japan) from the 129/sV mouse strain by electroporation. ES cells with homologous recombination were microinjected into C57BL/6J blastocysts. Male chimeras were crossed with BALB/cJ females to generate germline heterozygous offspring. After backcrossing with wild-type BALB/cJ for 10 generations, homozygous CRTH2 mutants and wild-type animals were obtained by intercrossing heterozygotes. Genotypes were determined by Southern blot hybridization or PCR. The use of animals was in full compliance with the Committee for Animal Experiments of Tokyo Medical and Dental University, Tokyo, Japan.

DNA preparation and Southern blot hybridization

Genomic DNA from mouse tails and ES cells was prepared as described previously (21) with some modifications, digested with restriction enzymes, electrophoresed in a 0.7% agarose gel, transferred to a nylon membrane, and hybridized with a 5'- or 3'-probe labeled with [α-32P]dCTP (3000 Ci/mmol; Amersham Biosciences) at 55°C overnight. The membrane was washed three times in 2× SSC (3 M NaCl, 300 mM trisodium citrate) with 0.1% SDS at 65°C and subjected to autoradiography with a bioimaging analyzer (BAS 2000; FujiFilm).

RNA preparation and reverse transcription

Specimens were stored in RNAlater (Ambion) at 4°C until use. Total cellular RNA from lung and skin was isolated using RNAeasy mini kit (Qiagen). Reverse transcription was conducted in a reaction buffer containing hexanucleotide mixture (A, G, C, U, 62.5 U/ml; Boehringer Mannheim), dNTPs (0.125 mM each), human placenta RNase inhibitor (80 U; Takara Bio), reverse transcriptase (400 U; Moloney murine leukemia virus; Takara Bio), and 800 ng of total RNA.

Polymerase chain reaction

PCR was conducted in a reaction buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2) containing dNTPs (2.5 mM each), 1 U/μl Perfect Match (Stratagene), and 5 U/μl Taq DNA polymerase (Takara Bio). The reaction was initiated by denaturation at 94°C for 3 min followed by 28 cycles for GAPDH and 33 cycles for CRTH2 genes (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C). Genomic DNA (∼100 ng) or reverse-transcribed DNA (equivalent to 100 ng of total RNA) was used as a template. The primers used were: 5'-AGACACGTACTCGGATGGAA-3' and 5'-TCCTCGAGCTACTTTTGC-3' for the targeted mutant CRTH2 gene;

FIGURE 1. Targeted disruption of the CRTH2 gene. A, Schematic representations of the targeting vector, wild-type allele, and mutant allele. Ba, Bgl II, Nd, and Dr indicate restriction sites for BamHI, BglII, NdeI, and DraI, respectively. Boxes on lines indicate regions that are transcribed. The 5' and 3'-probes used for Southern blot analysis are shown. Lines with DNA sizes below the mutant allele indicate restriction fragments detected with either the 5' or 3'-probe. Plus (+) and minus (−) signs indicate the wild-type and mutant alleles, respectively. B, Representative genomic Southern blot analysis for the wild-type (+/+ ) and mutant (−/−) homozygotes and the heterozygote (+/−). Genomic DNAs extracted from tails were digested with BamHI and hybridized with the 3'-probe after gel separation and blotting. The 5.4- and 3.6-kb bands indicate the wild-type and mutant alleles, respectively. C, PCR genotyping of offspring from heterozygote intercrossing. The primer pairs used to detect the wild-type allele and the neomycin gene in the mutant allele generate 360- and 200-bp fragments, respectively. D, RT-PCR analysis of CRTH2 expression in lung. RNA was extracted from the lungs of wild-type (+) and mutant homozygotes (−) and heterozygotes (±) and subjected to RT-PCR with primers for CRTH2 and β-actin, generating 141- and 366-bp fragments, respectively. Lane c, control PCR without template.
IgE-mediated cutaneous reactions

Cutaneous inflammatory reactions

IgE-mediated cutaneous reactions were induced by s.c. injection into the dorsal site of the mouse ear lobe with 1.25 μg of anti-DNP-specific IgE (MP Biomedicals). Mice were then challenged 24 h later with 20 μl of 0.2% 2,4-dinitrofluorobenzene (DNFB) (Nakarai Tesque) in acetone:olive oil (4:1). Ear thickness was determined using a dial thickness gauge (Peacock) before and after challenge. Ear swelling was expressed as the mean increase in thickness above the basal control value.

FITC (Sigma) in acetone: dibutyl phthalate (1:1) was used to induce allergic contact sensitivity by application of 100 μl of 1% solution onto ventral skin on day 0 and day 1. On day 6, ear lobes were challenged with 20 μl of 1% FITC. Ear thickness was determined immediately before and after challenge.

Chronic allergic contact hypersensitivity was induced by application of 50 μl of 5% 2,4,6-trinitrochlorobenzene (TNCB) (Nakarai Tesque) in ethanol:acetone (3:1) onto the dorsal skin on day 0. On day 5, each ear lobe was challenged with 20 μl of 1% TNCB in acetone:olive oil (1:4). Application of 1% TNCB onto each ear lobe was repeated every 2 days from day 5 to day 25. Ear thickness was determined before and after each challenge.

Delayed-type hypersensitivity (DTH) was induced by s.c. immunization with 100 μl of 20% SRBC in the back on day 0. On day 5, 20 μl of 20% SRBC was injected into the footpad. Footpad thickness was determined immediately before and 24 h after the challenge. Control groups were sensitized with PBS without SRBC.

Measurement of PGD₂ in the skin

The amount of PGD₂ in the skin was measured as described previously (24). Briefly, ear lobes were homogenized in 2 ml of ethanol containing 0.25 N HCl using a Polytron homogenizer. [³H]PGD₂ (PerkinElmer) was added to each homogenate as a tracer to assess recovery during purification. An ethanol extract containing PGD₂ was added to Sep Pak C18 cartridges (Waters Associates), followed by elution with ethyl acetate and fractionation by HPLC. PGD₂ was measured using an assay kit (Cayman Chemical).

Preparation and analysis of epidermal sheets

Mice were sacrificed, and each ear lobe was split into dorsal and ventral halves using forceps. The dorsal halves were cut into small pieces, and these were incubated for 2 h at 37°C in PBS containing 10 mM EDTA. The epidermis was peeled away from the dermis using forceps and then fixed in acetone for 5 min. Epidermal Langerhans cells (LC) were stained with goat polyclonal anti-Langerin Ab (Santa Cruz Biotechnology) using a Histofine SAB-PO kit (Nichirei).

FIGURE 2. IgE-mediated cutaneous responses in CRTH2-deficient mice. A, Ear swelling induced by administration of Ag-specific IgE. Wild-type and CRTH2-deficient mice received DNP-specific IgE and were challenged with DNFB. Ear thickness was measured at the indicated times. * p < 0.05. At least four mice for each group were used in the assay. B, Histopathological features of the skin during vLPR. Tissue sections of swollen ear lobes during vLPR from mutant and wild-type mice were prepared and subjected to Giemsa staining. (original magnification: ×200). C, Cell populations in infiltrates from inflammatory skin. Stained sections were observed by light microscopy to count the number of lymphocytes, neutrophils, eosinophils, and mast cells. B.M., basement membrane. * p < 0.05. D, Basophils in inflammatory skin infiltrate. Single-cell suspensions from ear skin were stained with fluorescence-conjugated Abs for FceRI, c-kit, and Gr-1 and analyzed by flow cytometry. Cells with FceRI and without c-kit and Gr-1 were counted as basophils. Representative results from three independent experiments are shown. Error bars indicate SD.
Ear tissues were stained with anti-H-PGDS Ab and anti-rabbit IgG Ab and examined by microscopy. Arrowheads indicate cells positive for H-PGDS.

CD8 FITC-conjugated anti-CD4 Ab (clone RM4-5; BD Biosciences), anti-rabbit IgG (DakoCytomation). The sections were then incubated with tetramethylrhodamine isothiocyanate-conjugated anti-rabbit Ab, and were subjected to confocal laser-scanning microscopic analysis. Arrowheads indicate cells positive for respective molecules.

In some experiments, frozen tissue sections were incubated with anti-mouse H-PGDS Ab followed by incubation with biotinylated anti-rabbit IgG and Vectastain ABC reagent (Vector Laboratories). In some experiments, frozen tissue sections were incubated with anti-mouse H-PGDS Ab followed by incubation with tetramethylrhodamine isothiocyanate-conjugated anti-rabbit IgG (DakoCytomation). The sections were then incubated with FITC-conjugated anti-CD4 Ab (clone RM4-5; BD Biosciences), anti-CD8α Ab (clone 53-6.7; BD Biosciences), or anti-FcεRIα Ab (clone MAR-1; eBioscience). For double staining of LC, epidermal sheets were first stained with FITC-conjugated anti-I-A<sup>+</sup> Ab (clone AMS-32.1; BD Biosciences) followed by H-PGDS staining. Immunostained samples were examined under a confocal laser-scanning microscope (FluoView; Olympus).

**Immunohistochemistry for hemopoietic PGDS (H-PGDS)**

Tissue sections were deparaffinized, rehydrated, and incubated in absolute methanol with 0.3% H<sub>2</sub>O<sub>2</sub> to inactivate endogenous peroxidase activity. After treatment with trypsin, sections were incubated in PBS containing 10% normal goat serum, 0.01% Triton X-100, and 0.1% NaN<sub>3</sub> and treated with rabbit polyclonal anti-mouse H-PGDS Ab (raised in Osaka Bioscience Institute), followed by incubation with biotinylated anti-rabbit IgG and Vectastain ABC reagent (Vector Laboratories). In some experiments, frozen tissue sections were incubated with anti-mouse H-PGDS Ab followed by incubation with tetramethylrhodamine isothiocyanate-conjugated anti-rabbit IgG (DakoCytomation). The sections were then incubated with FITC-conjugated anti-CD4 Ab (clone RM4-5; BD Biosciences), anti-CD8α Ab (clone 53-6.7; BD Biosciences), or anti-FcεRIα Ab (clone MAR-1; eBioscience). For double staining of LC, epidermal sheets were first stained with FITC-conjugated anti-I-A<sup>+</sup> Ab (clone AMS-32.1; BD Biosciences) followed by H-PGDS staining. Immunostained samples were examined under a confocal laser-scanning microscope (FluoView; Olympus).

**Flow cytometric analysis of murine basophils in the skin**

Cells were obtained from ears by treatment with collagenase type 3 (115 U/ml; Worthington Biochemical) and stained with FITC-conjugated anti-mouse FcεRIα Ab (clone MAR-1), R-PE-conjugated anti-mouse CD11c Ab (clone 53-6.7; BD Biosciences), or anti-FcεRIα Ab (clone MAR-1; eBioscience). For double staining of LC, epidermal sheets were first stained with FITC-conjugated anti-I-A<sup>+</sup> Ab (clone AMS-32.1; BD Biosciences) followed by H-PGDS staining. Immunostained samples were examined under a confocal laser-scanning microscope (FluoView; Olympus).

**Measurement of cytokines and chemokines in the skin**

Punched ear lobes (8-mm diameter) from challenged mice were homogenized in PBS containing 0.1% Tween 20 (500 µl/tissue) and then centrifuged at 15,000 × g for 10 min. Concentrations of cytokines and chemokines in the supernatants were determined by sandwich ELISA. ELISA kits for murine IL-4, IL-5, and IFN-γ were purchased from Pierce, and the ELISA kits for IL-13, thymus- and activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC), RANTES, and IFN-γ-inducible protein 10 were from R&D Systems. The eotaxin enzyme immunoassay kit was obtained from Techno Laboratories.

**Measurement of IgE levels in serum**

Peripheral blood was obtained from the retroorbital plexus. Serum IgE levels were determined using a commercial sandwich ELISA kit (Yamasa) according to the manufacturer’s instructions.

**Statistical analyses**

Student’s t test was used to assess the statistical significance of differences between mean values. Statistical analyses of the results in Fig. 5 were performed by the Bonferroni multiple comparison test.

**Results**

**Generation and general properties of CRTH2-deficient mice**

Mice lacking the CRTH2 gene were generated by targeted disruption of the CRTH2 gene (Fig. 1A). Germline-transmitting chimeras were mated with wild-type BALB/c females, and CRTH2 heterozygotes were then backcrossed with BALB/c 10 times. The
resultant heterozygotes were intercrossed to generate CRTH2 homozygous mutant, heterozygous mutant, and wild-type animals. Genotypes were determined by Southern blot hybridization and PCR (Fig. 1, B and C). Offspring from heterozygote intercrosses were born at approximately the expected Mendelian ratios (57 wild-type, 145 heterozygote, and 51 homozygote mice) (1:2.5:0.9). RT-PCR analysis for CRTH2 mRNA showed little or no expression of CRTH2 transcripts in homozygotes in lungs in which CRTH2 mRNA was detected in wild-type animals (Fig. 1D). CRTH2-deficient homozygotes showed no significant abnormalities at birth or during postnatal growth. Intercrosses between mutant homozygotes yielded normal numbers of offspring, indicating that the CRTH2-deficient homozygotes were fertile.

**IgE-mediated cutaneous responses in CRTH2-deficient mice**

Mouse responses of IgE-mediated cutaneous inflammation have been shown to consist of at least three phases, ITR, LPR and vLPR, together with marked infiltration by eosinophils and lymphocytes (15–18). To examine the involvement of CRTH2 in IgE-mediated cutaneous responses in vivo, we assessed the ear-swelling responses of CRTH2-deficient mice. Mice were passively immunized with DNP-specific IgE and challenged with DNFB. The ear-swelling response after challenge was clear in wild-type mice, but all three phases of the response were significantly reduced in mutant mice (Fig. 2A). Among the three phases, the mostly marked reduction was seen for vLPR compared with wild-type mice (65%). Histological analyses of the challenged ears of CRTH2-deficient mice on day 5 showed a profound reduction in dermal cellular infiltrate when compared with wild-type mice (Fig. 2B). Similar reductions in ear swelling were seen in the CRTH2-deficient C57BL/6 strain (data not shown). These observations indicate that CRTH2 functions in the cutaneous responses induced by Ag-specific IgE.

The infiltrate included lymphocytes, eosinophils, neutrophils, and mast cells. These cell types were significantly decreased in the infiltrates of mutant mice in terms of cell number (Fig. 2, B and C). In humans, the CRTH2-mediated signal induces chemotaxis in basophils (11). In mice, basophils play a critical role in 2,4,6-trinitrophenyl-IgE-mediated cutaneous vLPR (25). We thus investigated the effects of CRTH2 on basophil recruitment to the skin. Flow cytometric analysis of dermal cells in vLPR demonstrated that basophil infiltration was also significantly reduced in mutant mice (Fig. 2D).

We then examined the production of PGD2 in the challenged ear lobes of CRTH2-deficient and wild-type mice. Challenge with DNFB resulted in a significant increase in PGD2 levels in wild-type mice on day 5 (vLPR), whereas mutant mice did not show any appreciable elevation of PGD2 (Fig. 3A). A significant difference in PGD2 levels at 3 and 24 h after challenge was not observed between mutant and wild-type mice (data not shown). Cells expressing H-PGDS were present during IgE-induced vLPR (Fig. 3B). H-PGDS expression in the dermis was seen in cells positive for FceRI (Fig. 3C), CD4, or CD8 (data not shown) by confocal laser-scanning microscopy. Moreover, H-PGDS was constitutively expressed in MHC class II-positive epidermal LC (Fig. 3C). Ag challenge may stimulate FceRI+ cells, T cells, and LC to produce PGD2, which, together with other chemical mediators, contributes to recruitment of CRTH2+ cells into inflammatory sites. Indeed, levels of CRTH2 mRNA in the skin were low but detectable before challenge and increased with time after challenge (Fig. 3D). This notion is consistent with the results observed in wild-type mice that were administered either HQL-79 (Cayman Chemical), an inhibitor of PGD synthase, or ramatroban (Bayer Yakuhin), an antagonist of CRTH2. Wild-type BALB/c mice were perorally administered with HQL-79 for 8 consecutive days (days 1 to 6) and immunized with DNP-specific IgE. vLPR was markedly inhibited by administration of HQL-79 (Fig. 4A). Similar results were seen with mice orally given ramatroban at a dose of 0.3 mg/kg (Fig. 4B). Collectively, these results demonstrate that PGD2 is involved in the pathogenesis of IgE-mediated cutaneous inflammation via CRTH2.

**Cytokine and chemokine production in CRTH2-deficient mice**

To gain insight into CRTH2-mediated inflammatory responses, we determined the production of cytokines and chemokines in skin lesions. The homogenates of swollen ears were subjected to measurement of IL-4, MDC, eotaxin, IFN-γ, RANTES, and TARC. IL-4 and IFN-γ production in CRTH2-deficient mice was comparable to that in wild-type mice. Levels of MDC and RANTES 6 days after challenge were significantly lower in CRTH2-deficient mice than in wild-type mice (Fig. 5). CRTH2-deficient mice produced lower levels of eotaxin than wild-type mice on day 6; however, this was not statistically significant. Three hours after challenge, mutant mice exhibited higher TARC production; however,

**FIGURE 4.** Involvement of PGD2 and CRTH2 in IgE-mediated cutaneous responses. A, Effects of HQL-79 on ear swelling induced by IgE. Wild-type mice were sensitized with DNP-IgE on day −1 and challenged by DNFB painting on day 0. HQL-79 was administered from day −1 to day 6 at the doses indicated in the figure, and ear thickness was determined at the indicated times. *, p < 0.05, as compared with positive control. B, Effect of ramatroban on IgE-mediated cutaneous responses. Wild-type mice were sensitized with DNP-specific IgE and challenged with DNFB. Ramatroban was administered at doses of 0.3, 3, and 30 mg/kg body weight for 8 days. Ear thickness was measured at the indicated times after challenge. Mice administered 3 and 30 mg/kg exhibited similar results. *, p < 0.05. Each group consisted of at least four mice.
afterward the differences were not significant. IL-5, IL-13, and IFN-γ-inducible protein 10 were undetectable in cutaneous preparations from both strains (data not shown). CRTH2-deficient mice exhibited defects in cytokine and chemokine production, and these defects were not biased toward Th2-type cytokines and chemokines.

**Acute CHS in CRTH2-deficient mice**

Because acute CHS to FITC is Th2 dependent (26) and is sensitive to the CRTH2 antagonist ramatroban (27), we examined the involvement of CRTH2 in CHS to that of FITC. CHS was induced in CRTH2-deficient mice by sensitization with FITC. Both mutant and wild-type mice showed FITC-induced CHS; however, ear swelling in mutant mice was slightly but significantly alleviated (Fig. 6A).

We then attempted to determine whether CRTH2 is involved in the migration of epidermal LC and DC in the afferent phase of FITC-induced CHS. In untreated skin, the number of LC in the epidermis of CRTH2-deficient mice was comparable to that in wild-type mice (Fig. 6B). Upon application of FITC, the number of FITC^+ LC and DC with CD11c in regional lymph nodes increased, but no significant differences were seen between mutant and wild-type mice as assessed by flow cytometry; the percentage of LC and DC among total cells in lymph nodes was 0.5 ± 0.088% in wild-type mice and 0.67 ± 0.131% in CRTH2-deficient mice, thus demonstrating that the reductions in acute CHS in CRTH2-deficient mice are not due to impaired LC and DC migration into regional lymph nodes during sensitization. This finding indicates that CRTH2 is not involved in LC migration, which agrees with the previous observation that PGD2 affects LC migration via DP (9).

**Chronic CHS in CRTH2-deficient mice**

Repeated application of hapten has been shown to induce chronic CHS, exhibiting an ITR instead of a delayed-type response and a

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**FIGURE 5.** Levels of cytokines and chemokines during cutaneous responses. Mutant and wild-type mice were challenged with DNFB following DNP-IgE injection. At the indicated times after challenge, ear skin specimens were collected by punching, and supernatants were prepared from skin homogenates. Amounts of cytokines and chemokines in the supernatants were determined by ELISA. One group at each time point consisted of 3–5 animals. Representative results from two independent experiments are shown. Data were analyzed by the Bonferroni multiple comparison test. *, p < 0.05. Error bars indicate SD.

**FIGURE 6.** Acute CHS induced by FITC administration. A, Ear swelling in FITC-induced acute CHS. Mice were sensitized with FITC on the ventral skin on days 0 and 1 and challenged with FITC on day 6. Ear thickness was measured 24 h after challenge. Four mice were used in each group, and representative results among three separate experiments are shown. *, p < 0.05. B, Histological analysis of epidermal LC. Untreated epidermis from mutant and wild-type mice was stained with anti-Langerin Ab and observed by light microscopy. The number of epidermal LC in CRTH2-deficient and wild-type mice was 1451.2 ± 203.79 and 1468.8 ± 204.18 cells/mm², respectively (original magnification, ×400).
shift in local cytokine production from a Th1 to a Th2 profile (19, 20, 28). To investigate the effects of CRTH2 on chronic CHS, mutant and wild-type mice were challenged with TNCB every 2 days from day 5 to day 25 following initial sensitization on day 0. Both strains exhibited similar shifts in the kinetics of cutaneous reactions. As seen with the acute-phase responses to FITC shown in Fig. 6A, ear-swelling responses 24 h after challenge on day 5 (acute CHS) were slightly weaker in CRTH2-deficient mice than in wild-type mice (Fig. 7A). Mutant mice exhibited reduced cutaneous reactions in chronic CHS in response to challenge on days 13 and 25 (Fig. 7A). Basal ear thickness increased gradually with challenge. This phenomenon is thought to result from accumulated inflammation induced by repeated challenge. The basal levels of the ear thickness in mutant mice on day 27 were significantly reduced when compared with those in wild-type mice (Fig. 7B). The levels of total serum IgE on day 27 in TNCB-induced CHS were significantly higher in wild-type mice than in CRTH2-deficient mice (Fig. 7C). These results imply that CRTH2 participates in the development of hapten-induced chronic CHS.

Effects of CRTH2 on DTH reaction and irritation dermatitis

The pathomechanisms of IgE-mediated cutaneous responses and chronic CHS in which tissue eosinophilia is prominent may be different from those of DTH reaction or irritation dermatitis. Based on the observation that the DTH reaction on the footpad in response to SRBC is independent of Th2-type response (29), we speculated that CRTH2 is not involved in the DTH reaction. Mutant mice administered SRBC exhibited significant footpad responses that were similar to those of wild-type mice (Fig. 8A).

Irritation dermatitis is dependent on mast cell-derived mediators (30); thus, we further examined whether the PGD2-CRTH2 system is involved in this dermatitis. Irritation dermatitis was induced by the application of croton oil, and ear swelling was compared between the two mouse strains. Interestingly, the lack of CRTH2 had no effect on the ear-swelling response to croton oil application in the two strains (Fig. 8B), which is presumably consistent with the previous observation that croton oil dermatitis is not dependent on Th2-mediated signals (29). These observations suggest that DTH and irritation dermatitis are mediated by cells that do not require CRTH2 function.

Discussion

PGD2 has been implicated in allergic reactions. This notion is based on the observations that overexpression of PGDS induces Th2-dominated lung inflammation with pronounced eosinophilia (6) and that DP gene disruption results in relief of allergic asthma (10). In addition, the activation of CRTH2 has been shown to aggravate allergic lung inflammation (22). However, the role of CRTH2 in allergic reactions remains uncertain.

In the present study, using CRTH2-deficient mice we clearly demonstrate for the first time that the PGD2-CRTH2 system plays a role in the IgE-mediated inflammatory response in the skin. CRTH2-deficient mice exhibited reduced skin responses. This reduced effect was most marked during vLPR. The same reduction was seen in mice administered the PGDS inhibitor HQL-79 or the CRTH2 antagonist ramatroban. These results unequivocally show that CRTH2 mediates IgE-dependent cutaneous inflammation. It is possible that DP may also be cooperatively involved in this event.

Histological analysis revealed reductions in the number of lymphocytes, eosinophils, neutrophils, and mast cells in infiltrates. In addition, basophil infiltration in the dermis was also reduced during vLPR in mutant mice. These observations indicate that various cells are affected by CRTH2 gene disruption. PGD2 may act as an attractant for a panel of cells positive for CRTH2. Alternatively,
CRTH2 may attract the first basophils during the early stages of skin inflammation, which then induces a further cascade of cell recruitment. A recent study illustrates that basophils are critical in the induction of 2,4,6-trinitrophenyl-IgE-mediated vLPR (25). Our results showing that mutant mice exhibited less ear swelling in response to DNP-specific IgE may be attributed, at least in part, to impaired basophil infiltration. This impairment is presumably associated with a lack of CRTH2-mediated signals.

PGD2 produced by allergic stimulation attracts CRTH2-positive cells, which include cells positive for H-PGDS, into inflammation sites. These cells were identified as FceRI+ mast cells or basophils and CD4+ and CD8+ cells seen during vLPR in the dermis (Fig. 3B) and are presumably the sources of the high levels of PGD2 during vLPR (Fig. 3A), which may further induce infiltration. This notion is consistent with the recent finding that 13,14-dihydro-15-keto-PGD2, which is a CRTH2 agonist, exacerbates skin inflammation induced by OVA (22).

We observed constitutive expression of H-PGDS in epidermal resident LC, as shown in the rat skin in which LC express PGDS (31). Constitutive expression of H-PGDS may contribute to basal levels of PGD2, which acts to recruit CRTH2-positive inflammatory cells into the skin for surveillance even under normal conditions. A recent report demonstrates that, under allergic conditions, IgE-mediated activation of mast cells induces LC migration toward lymph nodes in vivo (32). LC in lymph nodes may be potent sources of PGD2 for CRTH2+ cell homing, after which the cells migrate to inflammatory sites during IgE-mediated cutaneous responses.

Our results with CRTH2-deficient mice imply that CRTH2 may participate in the constitution of local cytokine and chemokine profiles in allergic inflammation. Mutant mice differed from wild-type mice in the production of MDC and RANTES induced by hapten challenge. The impaired recruitment of eosinophils may be due not only to the direct effects of the absence of PGD2-CRTH2 signaling but also to diminished production of RANTES. Although IL-5 levels in our ear specimens were below the detection limit, IL-5 may not be the cause of impaired cell recruitment, as IL-5 is reported to be unnecessary for eosinophil recruitment into the skin (33). It also appears likely that the reduced lymphocyte accumulation is partly associated with decreased MDC and RANTES production. In humans, CRTH2 is preferentially expressed in Th2 cells rather than in Th1 cells, thus resulting in elevated production of IL-4, IL-5, and IL-13 in human T cells by CRTH2 stimulation (34). Thus, one may assume that the disruption of the CRTH2 gene causes a shift from Th2 to Th1 cytokine and chemokine profiles in mice. Expression of CRTH2 in mice, however, is not biased toward Th2 cells, unlike the case in humans (35). Indeed, production of IL-4 and IFN-γ was not affected by the absence of CRTH2. In other words, changes in local cytokine and chemokine levels in CRTH2-deficient mice cannot simply be explained by impaired infiltration of Th2 cells. Our results suggest that the cytokines and chemokines regulated by CRTH2 are different in mice and humans.

Experimental chronic CHS is established by repeated application of hapten. Each application induces ear swelling, which occurs at earlier time points than those by other applications. In the present study, no differences were observed in the shift of ear swelling kinetics; however, the ear swelling in CRTH2-deficient mice was lower than that in wild-type mice. The differences appeared predominantly in the basal thickness of ears, which increased gradually after each application. Basal thickness in mutant mice was three-quarters that of wild-type mice on day 27. Mutant mice appeared to produce lower levels of IgE in sera than wild-type mice. At present, a functional link between CRTH2 and IgE level is unclear. Chronic CHS in mice is thought to be representative of human atopic dermatitis. This model is histologically characterized by epidermal hyperplasia and marked increases in dermal eosinophils (19, 20). These findings suggest that CRTH2 is a potential target for therapeutic improvement of chronic eczematous reactions.

The absence of CRTH2 suppressed acute CHS induced by either TNCB or FITC application. Similar suppression was also seen in FITC-mediated acute CHS in mice treated with ramatroban (27). The suppressive effects caused by ramatroban appeared to be greater than those observed with dysfunction of CRTH2. This observation might be attributable to the action of ramatroban on a thromboxane A2 receptor other than CRTH2. However, this may not be case, as a thromboxane A2 receptor antagonist had no effect on the responses. Alternatively, targeted disruption of the CRTH2 gene may have resulted in activation of alternative pathways, such as the DP receptor pathway, that may be partly able to compensate for the lack of the CRTH2-derived signals. This notion is supported by the recent study that, in an asthmatic model, DP signals appear to regulate production of Th2 cytokines (10).

After completion of this study, another study using an asthma model with CRTH2-deficient C57BL/6 mice reported enhanced eosinophil recruitment into the lung and production of IL-5 and IL-13 by splenocytes (36). Our preliminary asthma experiments did not show significant difference in infiltrates in the lung between our mutant and wild-type C57BL/6 mice (H. Hirai, unpublished results). In addition, we observed that splenocytes of mutant C57BL/6 mice produced the same amounts of IL-4, IL-5, and IFN-γ as those in wild-type mice upon CD3/CD28 stimulation. Similar results were seen in BALB/c background mice. At present, we do not know the reason for this inconsistency between the two groups, each of which established CRTH2-deficient mice independently. To draw final conclusions, further study including experiments with antagonists and agonists for CRTH2 will be required.

In summary, the present study shows that PGD2-CRTH2 interaction plays important roles in the development of chronic allergic inflammation in the skin, such as IgE-mediated vLPR and chronic CHS, and that CRTH2 is not involved in irritation dermatitis. CRTH2 may thus have potential as a therapeutic target for treatment of atopic dermatitis and other allergic skin diseases.

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