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Dual Functions of Prostaglandin D2 in Murine Contact Hypersensitivity via DP and CRTH2

Yoshihiro Yamamoto,* Shinobu Otani,* Hiroyuki Hirai,† Kinya Nagata,† Kosuke Aritake,‡ Yoshihiro Urade,‡ Shuh Narumiya,§ Hiroo Yokozeki,* Masataka Nakamura,¶ and Takahiro Satoh*

From the Department of Dermatology, Graduate School, Tokyo Medical and Dental University, Tokyo; the Department of Advanced Medicine and Development,† Bio Medical Laboratories, Inc., Saitama; the Department of Molecular Behavioral Biology,‡ Osaka Bioscience Institute, Osaka; the Department of Pharmacology,§ Faculty of Medicine, Kyoto University, Kyoto; and the Human Gene Sciences Center,¶ Tokyo Medical and Dental University, Tokyo, Japan*

Prostaglandin D2 (PGD2) exerts its effects through two distinct receptors: the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) and the D prostanoid (DP) receptor. Our previous study demonstrated that CRTH2 mediates contact hypersensitivity (CHS) in mice. However, the function of DP receptor remains to be fully established. In this study, we examine the pathophysiological roles of PGD2 using DP-deficient ($DP^{-/-}$) and CRTH2/DP-deficient ($CRTH2^{-/-}/DP^{-/-}$) mice to elucidate receptor-mediated PGD2 action in CHS. We observed profound exacerbation of CHS in $DP^{-/-}$ mice. $CRTH2^{-/-}/DP^{-/-}$ mice showed similar exacerbation, but to a lesser extent. These symptoms were accompanied by increased production of interferon-γ and IL-17. The increase in IL-17 producing $\gamma\delta$ T cells was marked and presumably contributed to the enhanced CHS. DP deficiency promoted the *in vivo* migration of dendritic cells to regional lymph nodes. A DP agonist added to DCs *in vitro* was able to inhibit production of IL-12 and IL-1β. Interestingly, production of IL-10 in dendritic cells was elevated via the DP pathway, but it was lowered by the CRTH2 pathway. Collectively, PGD2 signals through CRTH2 to mediate CHS inflammation, and conversely, DP signals to exert inhibitory effects on CHS. Thus, we report opposing functions for PGD2 that depend on receptor usage in allergic reactions. ***(Am J Pathol 2011, 179:302–314; DOI: 10.1016/j.ajpath.2011.03.047)***

Prostaglandin (PGD) D2 is an arachidonic acid metabolite that exerts a range of biological activities including vasodilatation, bronchoconstriction, and inhibition of platelet aggregation.[1–4] Prostaglandin D2 (PGD2) is synthesized by the isomerization of prostaglandin H2 (PGH2) through the enzymatic activity of PGD synthase. Two types of PGD synthase have been identified: lipocalin-type PGD synthase and hematopoietic-type PGD synthase (H-PGDS).[5,6] Although lipocalin-type PGD synthase is present in meningeal cells, epithelial cells of the choroid plexus, and oligodendrocytes in the brain, as well as being involved in the sleep-wake cycle,[7] H-PGDS is principally expressed in hematopoietic cells, such as mast cells.[8,9] On stimulation with antigens, mast cells rapidly secrete PGD2.[10,11] In addition, recent studies indicate that a small population of Th2-type cells and dendritic cells (DCs) harbor H-PGDS, producing PGD2 in response to a variety of stimuli.[12–14]

Mounting evidence suggests that PGD2 is involved in allergic inflammation. For example, PGD2 production is observed in bronchoalveolar lavage fluid from asthmatic patients.[15] Mice that overproduce PGD2 exhibit an enhanced allergic lung response, eosinophilia, and increased Th2-type cytokine production.[16] We have demonstrated that PGD2 plays an essential role in IgE-mediated skin responses in mice.[12] In contrast, another study that used H-PGDS-deficient and/or -transgenic mice revealed protective roles of PGD2 against delayed-type hypersensitivity responses of the skin.[17] A possible antipruritic potential of PGD2 in the scratching behavior of mice has also been proposed.[18,19] Thus, whether PGD2 facilitates or down-regulates allergic processes has yet to be determined.

PGD2 exerts its effects through the D prostanoid (DP) receptor and the chemoattractant receptor-homologous

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

molecule expressed on Th2 cells (CRTH2) receptor. DP and CRTH2 are members of the G protein-coupled, seven transmembrane receptor family. DP is coupled with the Gαs protein, whereas Gαi protein is associated with CRTH2.[20] DP-mediated signals inhibit DC migration.[21–23] In eosinophils, mobilization from bone marrow and chemotaxis are promoted by DP.[24] A DP agonist has also been shown to sustain the survival of eosinophils.[25] On the other hand, CRTH2 is expressed in eosinophils, basophils, and a subpopulation of Th2 cells, in particular, central memory Th2 cells and monocytes.[26–28] CRTH2 signals induce calcium mobilization and chemotaxis in eosinophils and basophils.[20] Eosinophil degranulation is promoted by CRTH2 stimulation.[25] In addition, CRTH2 signals enhance IL-4, IL-5, and IL-13 production from Th2 cells.[29] CRTH2 expression is increased in eosinophils of atopic dermatitis, chronic urticaria, and in prurigo patients.[30] CRTH2-expressing T cells are elevated in the blood of atopic dermatitis.[31]

Despite the different lines of evidence previously mentioned, the *in vivo* roles of DP in inflammation are somewhat controversial. Mice with targeted disruption of the *DP* gene exhibit reduced eosinophil infiltration into the lung and fail to develop airway hyperreactivity.[32] Inhibition of DP alleviates asthmatic responses in guinea pigs.[33] Conversely, a DP agonist has been shown to down-regulate murine models of atopic dermatitis[22] and allergic asthma.[34] Similarly, the roles of CRTH2 *in vivo* are controversial. Although studies using mice lacking the *CRTH2* gene have demonstrated alleviated responses in airway hyperreactivity,[35] nasal pollinosis,[36] IgE-mediated skin inflammation,[12] and cedar pollen dermatitis,[37] other groups have shown enhanced eosinophil recruitment into the lung in an asthmatic model of CRTH2-deficient mice.[38] The reasons for such discrepancies in the different animal models of allergic diseases cannot be fully explained, suggesting that the function of PGD2 in inflammation may be more complex than previously thought. Thus, to develop new therapeutic strategies, it is crucial to delineate the physiological roles of PGD2 in each step of the inflammatory pathway with respect to the involvement of individual receptors.

Contact hypersensitivity (CHS), a well-characterized model of allergic skin inflammation, is an immune response to a reactive hapten covalently coupled to a cell surface protein. DCs in the skin capture and process antigens. They migrate to regional lymph node (LN) cells and present antigens to T cells. Antigen-specific T cells are recruited to the skin in response to subsequent exposure to haptens. They mediate allergic responses through the release of a variety of cytokine and chemokine production from resident cells. This leads to the accumulation of effector cells, such as neutrophils and eosinophils. It has been revealed that Th1- and Th17-type immunity contributes to the development of CHS.[39–49] In particular, $CD8^{(+)}$ cells producing interferon (IFN)-γ and/or IL-17 play essential roles in the elicitation phase of CHS,[42–44] although Th2-type immune responses also seem to be involved.[50–52]

In the present study, we examined the respective roles of PGD2 receptors in CHS using genetically modified mice. Our results demonstrate that PGD2 exerts both allergic and anti-allergic roles in inflammation, which are mediated through the reciprocal functions of CRTH2 and DP, respectively. This suggests protective roles for PGD2 under normal physiological and pathogenic conditions.

Materials and Methods

Mice

BALB/c mice were purchased from Sankyo Labo Service (Tokyo, Japan). $CRTH2^{-/-}$ mice, $DP^{-/-}$ mice, and $H\text{-}PGDS^{-/-}$ mice have been previously described.[12,17,32] Receptor null ($CRTH2^{-/-}/DP^{-/-}$) mice were generated in Tokyo Medical and Dental University by crossing each of the receptor-deficient mice. Mice lacking PGD2 receptors and those that lack H-PGDS genes are on a BALB/c background. Mice were maintained under specific pathogen-free conditions in our animal facility. The use of animals was in full compliance with the Committee for Animal Experiments of Tokyo Medical and Dental University.

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (CD4-FITC, GK1.5), CD8 (CD8-FITC, 53–6.7), and γδ T cell receptor (TCR) (γδ TCR-FITC, UC7-13D5) antibodies (Abs), phycoerythrin (PE)-conjugated anti-mouse IL-13 (IL-13-PE, eBio 13A), and CD25 (CD25-PE, PC61.5) Abs, biotin-conjugated anti-mouse γδ TCR (γδ TCR-Biotin, GL-3) Ab, PE-Cy5-conjugated anti-mouse Foxp (Foxp3-PE-Cy5, FJK-16s) Ab, and anti-mouse CD28 (clone 37.51) Ab were purchased from eBioscience (San Diego, CA). FITC-conjugated anti-mouse $I\text{-}A^d$ ($I\text{-}A^d$ –FITC, AMS-32.1) and CD86 (CD86-FITC, GL1) Abs, and PE conjugated anti-mouse CD11c (CD11c-PE, HL3), IFN-γ (IFN-γ-PE, XMG 1.2), and CD16/CD32 (2.4G2) Abs were from BD Biosciences Pharmingen (Franklin Lakes, NJ). PE-conjugated IL-17 (IL-17-PE, TC11-18H10.1), anti-IL-17 (TC11-18H10.1), and IFN-γ (XMG 1.2) Abs were from BioLegend (San Diego, CA) , and anti-IL-13 (eBio 1316H) Ab was from R&D Systems (Minneapolis, MN).

Acute Cutaneous Inflammatory Reactions

Acute CHS was induced by applying 100 μL of 5% 2,4,6-trinitrochlorobenzene (TNCB) (Nacalai Tesque, Kyoto, Japan) in ethanol:acetone (3:1), or 100 μL of 0.5% 2,4-dinitrofluorobenzene (Nacalai Tesque) in acetone:olive oil (4:1). On day 5, each ear lobe was challenged with 20 μL of 1% TNCB in acetone:olive oil (1:4) or 0.2% 2,4-dinitrofluorobenzene in acetone:olive oil (4:1). Ear thickness was determined before and after each challenge. Control ears were challenged with the respective vehicles. Irritation dermatitis was induced by painting 20 μL of 1% croton oil (Nacalai Tesque) in acetone on mouse ear lobes. Ear thickness was measured with a dial thickness gauge (Peacock, Tokyo, Japan) immediately before and after painting and expressed as the mean increment in thickness above basal line control value.

Flow Cytometry

Single cell suspensions were prepared in PBS containing 5% fetal calf serum and 0.1% NaN_3. After blocking IgG receptors with anti-mouse CD16/CD32 monoclonal Ab, cells were stained with the first Abs against cell surface markers. Intracellular staining was performed by IntraStain kit (Dako Cytomation, Glostrup, Denmark).

TNP-Modified SCs

Spleen cells (SCs) obtained from naive mice were adjusted to 5×10^7 cells/mL and mixed with equal volumes of 10 mmol/L TNBS (2,4,6-trinitrobenzene sulfonic sodium salt) (Nacalai Tesque) in PBS. Cells were then incubated at 37°C for 30 minutes, washed three times and incubated with mytomycin C (Kyowa Hakko Kirin, Tokyo, Japan) at 50 μg/mL, at 37°C for 40 minutes, followed by three further washes with PBS.

Preparation and Stimulation of Immune LNs

Axillary and abdominal LNs were collected on day 5 from mice sensitized with TNCB (day 0). Single cell suspensions were prepared by teasing the LN. Immune LN cells (400 μL of 5×10^6 cells/mL) were incubated with 400 μL of 5×10^6 trinitrophenyl (TNP)-SC/mL in a 24-well plate for 24 hours and supernatants were collected and stored at −80°C until use. For the detection of intracellular cytokines, immune LN cells incubated with trinitrophenyl-spleen cells (TNP-SC) for 48 hours were stimulated with 50 ng/mL (phorbol 12-myristate 13-acetate, Sigma Aldrich, St. Louis, MO), 500 ng/mL ionomycin (Calbiochem, San Diego, CA) , and 5 μg/mL Brefeldin A (Sigma Aldrich) for a further 12 hours. They were then analyzed with a flow cytometer.

Preparation of Th Cells and $\gamma\delta$ T Cells

Naive CD4 T cells were isolated using $CD4^{(+)}$/CD62L (+) T cell isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were stimulated in CD3-coated plates (BD Biosciences Pharmingen) with anti-mouse CD28 Ab (1 μg/mL), and cultured for 4 days in the presence of IL-12 (5 ng/mL), IL-2 (10 ng/mL), and anti-mouse IL-4 Ab (10 μg/mL) for Th 1 cells, IL-4 (5 ng/mL), IL-2 (10 ng/mL), and anti-mouse IFN-γ Ab (10 μg/mL) for Th2 cells, and IL-6 (20 ng/mL), transforming growth factor-β (10 ng/mL), anti-mouse IL-4 Ab (10 μg/mL), and anti-mouse IFN-γ Ab (10 μg/mL) for Th17 cells. $\gamma\delta$ T cells were prepared using the TCR $\gamma\delta$ (+) T cell isolation kit (Miltenyi Biotec). The $\gamma\delta$ T cells were more than 95% pure.

Adoptive Cell Transfer

Immune LN cells were transferred into each ear lobe (1×10^6 cells/30 μL/ear) into naive mice that immediately received challenge.

In Vivo *Migration of Langerhans Cells and DCs*

Inguinal and axillary LN cells were collected from mice sacrificed at 24 hours after application of 1% FITC (Sigma Aldrich) in acetone:dibutyl phthalate (1:1) onto the abdominal skin. Cells were incubated with CD11c-PE and analyzed with a flow cytometer.

Stimulation of BMDC

Bone marrow cells (2×10^6 cells) collected by flushing femur and tibial bones were cultured in 10 mL RPMI-1640 containing 10% fetal calf serum and granulocyte macrophage-colony stimulating factor (20 ng/mL, PeproTech Ec, London, UK) in a 100-mm Petri dish. Three days later, 10 mL of medium containing GM-CSF (20 ng/mL) was added to the dish and cultured for another 4 days. Bone marrow-derived dendritic cells (BMDC) were prepared by positive selection with CD11c-microbeads (Miltenyi Biotech) and treated with 13,14-dihydro-15-keto-PGD2 (DK-PGD2, a CRTH2 agonist; Cayman Chemical Co., Ann Arbor, MI) or BW245C (a DP agonist, Cayman Chemical Co.).

Measurement of Cytokines and Chemokines

Punched ear lobes (8 mm in diameter) from challenged mice were homogenized in PBS containing 0.1% Tween 20 (500 μL/tissue), and then centrifuged at $2500 \times g$ for 10 minutes. Levels of cytokines and chemokines in the supernatants of homogenates were determined by sandwich enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay (ELISA) kits for murine IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IFN-γ, IP-10 (CXCL10), regulated upon activation, normal T cell expressed and secreted (RANTES) (CCL5), eotaxin (CCL11), thymus and activation-regulated chemokine (TARC) (CCL17), and macrophage-derived chemokine (MDC) (CCL22) were purchased from R&D Systems, and for IL-23 from eBioscience.

RNA Preparation and Reverse Transcription

Total cellular RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan). Reverse transcription mix (20 μL) consisted of 8 μL of 5× buffer (250 mmol/L Tris-HCl, pH 8.3, 375 mmol/L KCl, 50 mmol/L DTT, and 15 mmol/L $MgCl_2$), 4 μL of hexanucleotide mixture (62.5 A_{260} U/mL, Roche Diagnostics, Mannheim, Germany), 2 μL of dNTPs (2.5 mmol/L each), 4 μL of 20 U/μL of human placenta ribonuclease inhibitor (Takara Bio Inc., Shiga, Japan), and 2 μL of 200 U/μL reverse transcriptase (Moloney murine leukemia virus; Takara Bio Inc.). A total of 20 μL of reverse transcription mix and 20 μL of 40 ng/μL total RNA were combined in a tube and vortex-mixed. Reactions were performed at 37°C for 60 minutes. Reverse transcriptase was inactivated at 70°C for 10 minutes, and the samples were stored at −80°C.

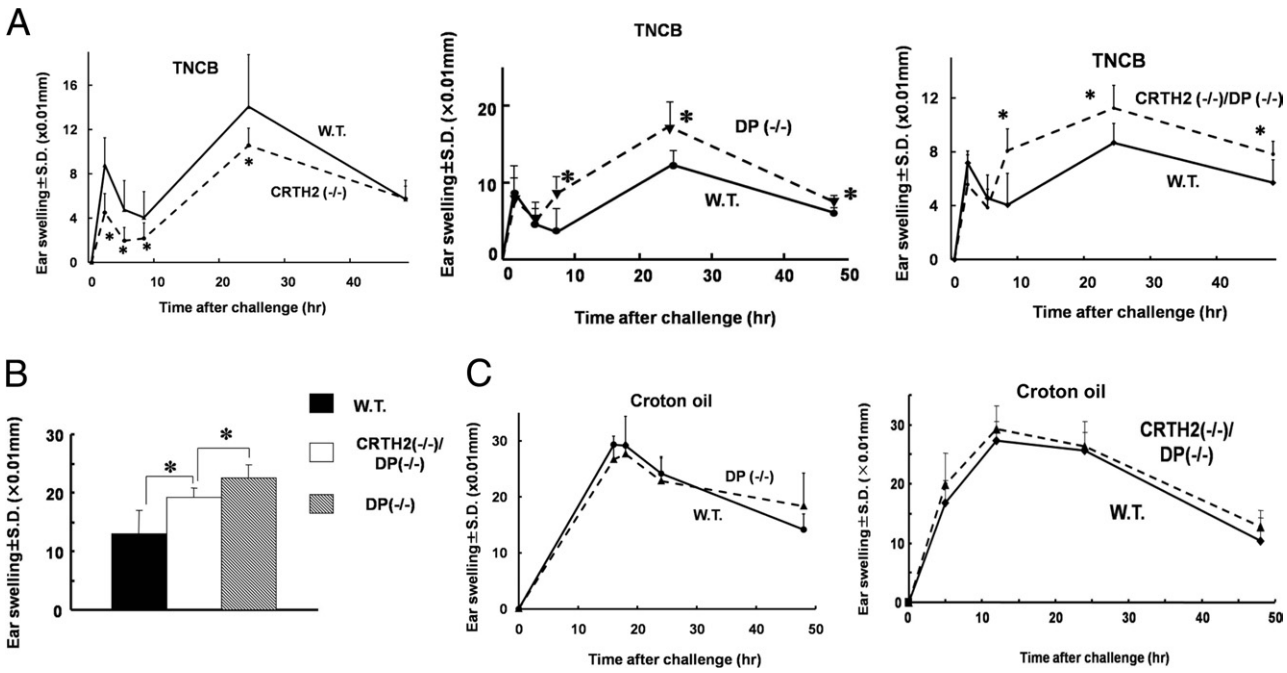

Figure 1. Contact hypersensitivity (CHS) responses in prostaglandin D2 (PGD2) receptor-deficient mice. **A:** Ear thickness in CHS responses to TNCB. WT, CRTH2$^{-/-}$, D prostanoid (DP)$^{-/-}$ and CRTH2$^{-/-}$/DP$^{-/-}$ mice were challenged with 2,4,6-trinitrochlorobenzene (TNCB). Ear thickness was measured at the times indicated. Numbers of mice in each group were four to six. Experiments were independently repeated at least three times. Means ± SD. **B:** Simultaneous comparison of ear thickness in CHS responses. Wild-type (W.T.), DP$^{-/-}$ receptor and chemoattractant receptor-homologous molecule expressed on Th2 cells CRTH2$^{-/-}$/DP$^{-/-}$ mice were challenged with TNCB. Ear thickness was measured 24 hours after challenge. **C:** Ear thickness in irritation dermatitis to croton oil. DP$^{-/-}$ mice and CRTH2$^{-/-}$/DP$^{-/-}$ mice were painted with croton oil and ear thickness was measured at the times indicated. $^{*}P < 0.05$.

PCR

PCR was performed in the reaction buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, and 1.5 mmol/L $MgCl_2$) containing dNTPs (2.5 mmol/L each), 0.5 U Perfect Match (Stratagene, La Jolla, CA), 2.5 UTaq DNA polymerase (Takara Bio Inc.), and 100 ng reverse transcribed RNA in a total volume of 50 μL. The reaction was initiated by denaturation at 94°C for 3 minutes and followed by 40 cycles of PCR reaction conditions of 1 minute at 94°C, 1 minute at 68°C and 1 minute at 72°C for the *CRTH2* gene and 1 minute at 94°C, 1 minute at 56°C and 1 minute at 72°C for the *DP* gene. Reverse-transcribed DNA (equivalent to 100 ng total RNA) was used as a template. The primers used were, 5′-TGGTCTCAACCAATCAGCAC-3′ and 5′-CTGTGGTTTGGAAGCTGGACC-3′ for the *CRTH2* gene (201 bp), 5′-ACCAGAGCCTAAAGGAACTG-3′ and 5′-AAGGCACGTCACCTTGCGC-3′ for the *DP* gene (173 bp), and 5′-TGTGTCCGTCGTGGATCTG-3′ and 5′-TTGCTGTTGAAGTCGCAGG-3′ for the *GAPDH* gene (150 bp).

Quantitative RT-PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye (Brilliant SYBR Green QPCR Master Mix, Stratagene) with the Mx3000P Real-Time PCR system (Stratagene). The PCR primers were: 5′-ATGGTACCGGGCACTACCTTTG-3′ and 5′-TGACAGTTCGCGCAGGATG-3′ for the *GATA3* gene (133 bp), 5′-AGGCTGCCTGCAGTGCTTCTA-3′ and 5′-GGACACTCGTATCAACAGATGCGTA-3′ for the *T-bet* gene (177 bp), 5′-CATCATCTCTGCAAGACTCATCGAC-3′ and 5′-TTTCCACATGTTGGCTGCAC-3′ for the *RORγt* gene (91 bp), and 5′-ACGCGCAAACATGAGTCCAG-3′ and 5′-CTCAGCAGCAGCAACAGCATC-3′ for the *IL-17* gene (63 bp).

Statistical Analyses

The Student's *t*-test was used to assess the statistical significance of differences between mean values. In data for time course changes of ear swelling responses, repeated measures analysis of variance test followed by Student's *t*-test or Scheffé's F test was performed.

Results

Acute Skin Inflammation in PGD2 Receptor-Deficient Mice

We have previously shown that CRTH2$^{-/-}$ mice exhibit less severe pathophysiological features of skin inflammation than wild-type (WT) mice.[12] We have again confirmed this in the present study, as shown in Figure 1A. To further understand the function of PGD2 through its receptors in skin inflammation, homozygous mice lacking the *DP* (DP$^{-/-}$) and *CRTH2/DP* (CRTH2$^{-/-}$/DP$^{-/-}$) genes were examined for symptoms of CHS after treatment with TNCB. Unexpectedly, because PGD2 has been considered to be an inflammatory mediator, skin responses in DP$^{-/-}$ mice were significantly exacerbated in comparison to those in WT mice (Figure 1A). A similar observation was associated with CHS after treatment with

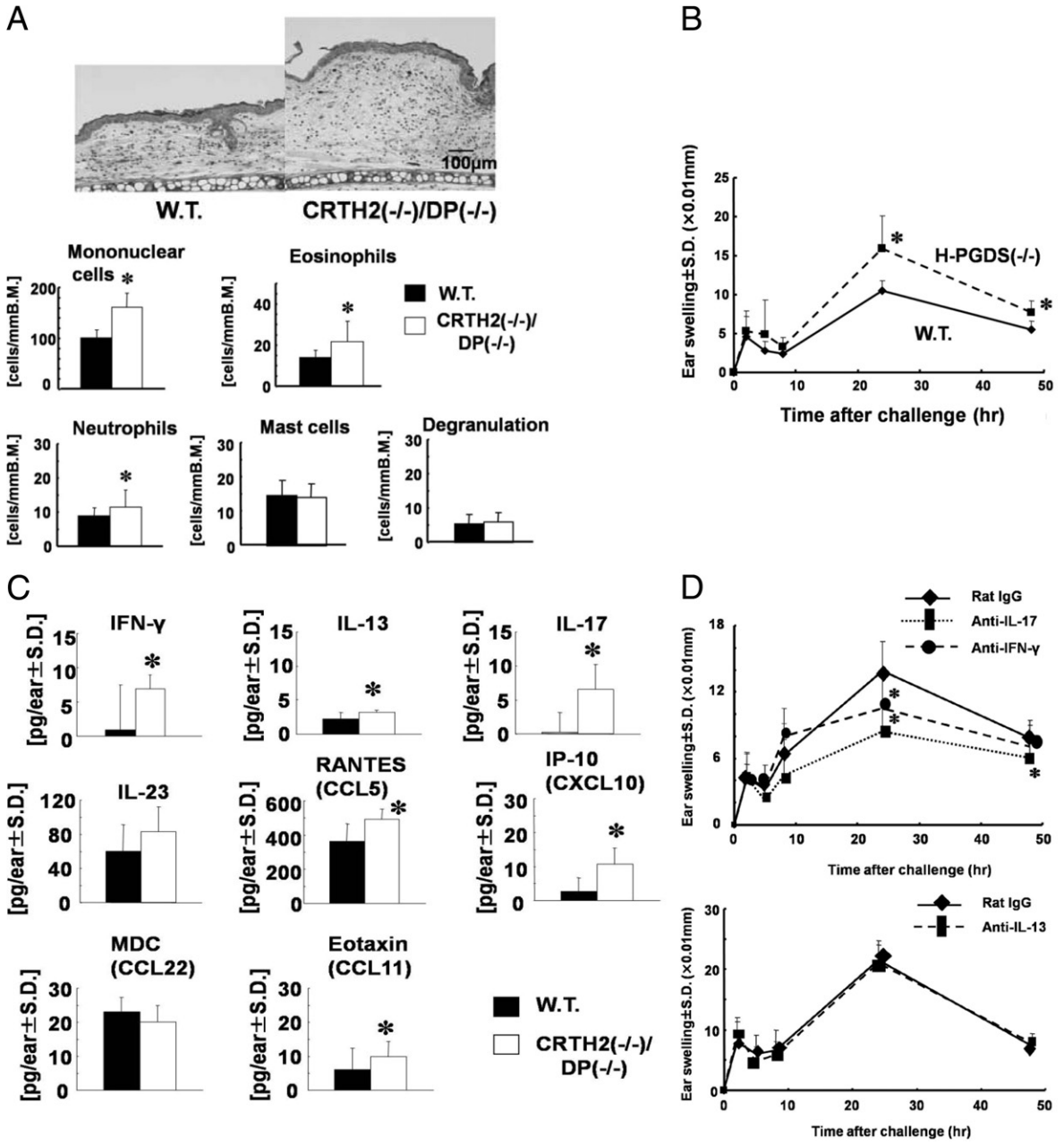

Figure 2. Modulation of parameters in contact hypersensitivity (CHS) in mutant mice. **A:** Histological features and cell populations in inflammatory skin. Wild-type (W.T.) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2$^{-/-}$)/D prostanoid (DP$^{-/-}$) mice were challenged with 2,4,6-trinitrochlorobenzene (TNCB) and inflammatory ears at 24 hours after challenge were subjected to histological observation and cell counting. Cells were counted in five distinct areas and determined as numbers per 1 mm of basement membrane (B.M.). Means ± SD. **B:** CHS responses in mice lacking the *H-PGDS* gene. W.T. and hematopoietic-type PGD synthase (H-PGDS$^{-/-}$) mice were challenged with TNCB. Ear thickness was measured at the times indicated. **C:** Cytokine and chemokine production in CHS. W.T. and CRTH2$^{-/-}$/DP$^{-/-}$ mice were challenged with TNCB. Ear lobes were punched 24 hours after challenge and levels of cytokines and chemokines were determined by enzyme-linked immunosorbent assay. Means ± SD. **D:** Ear thickness in CHS treated with anti-cytokine antibodies. CRTH2$^{-/-}$/DP$^{-/-}$ mice were sensitized with TNCB. Blocking Abs against cytokines interferon [(IFN)-γ, IL-13, and IL-17 at doses of 200 μg/mouse] were administered i.p. 1 day before challenge. Ear thickness was measured at the times indicated. *$P < 0.05$. Numbers of mice in each group were four to six. Representative results from at least two independent experiments are shown.

2,4-dinitrofluorobenzene (data not shown). Pathological phenotypes between DP$^{-/-}$ mice and WT mice were also seen in CRTH2$^{-/-}$/DP$^{-/-}$ mice (Figure 1A). DP$^{-/-}$ mice generally showed more severe skin inflammation than CRTH2$^{-/-}$/DP$^{-/-}$ mice (Figure 1B). On the other hand, irritation dermatitis induced by croton oil was not different in the mutant mice compared to WT (Figure 1C). Histological analysis of CHS in response to TNCB revealed that edema and cellular infiltrate comprising mononuclear cells, eosinophils, and neutrophils in the dermis were markedly increased in CRTH2$^{-/-}$/DP$^{-/-}$ mice and DP$^{-/-}$ mice than in WT mice (Figure 2A) and (see Supplemental Figure S1A at *http://ajp.amjpathol.org*). This suggests that PGD2 down-regulates allergic CHS responses via DP and that the DP pathway is dominant over the CRTH2 pathway, but irritation dermatitis is unaffected

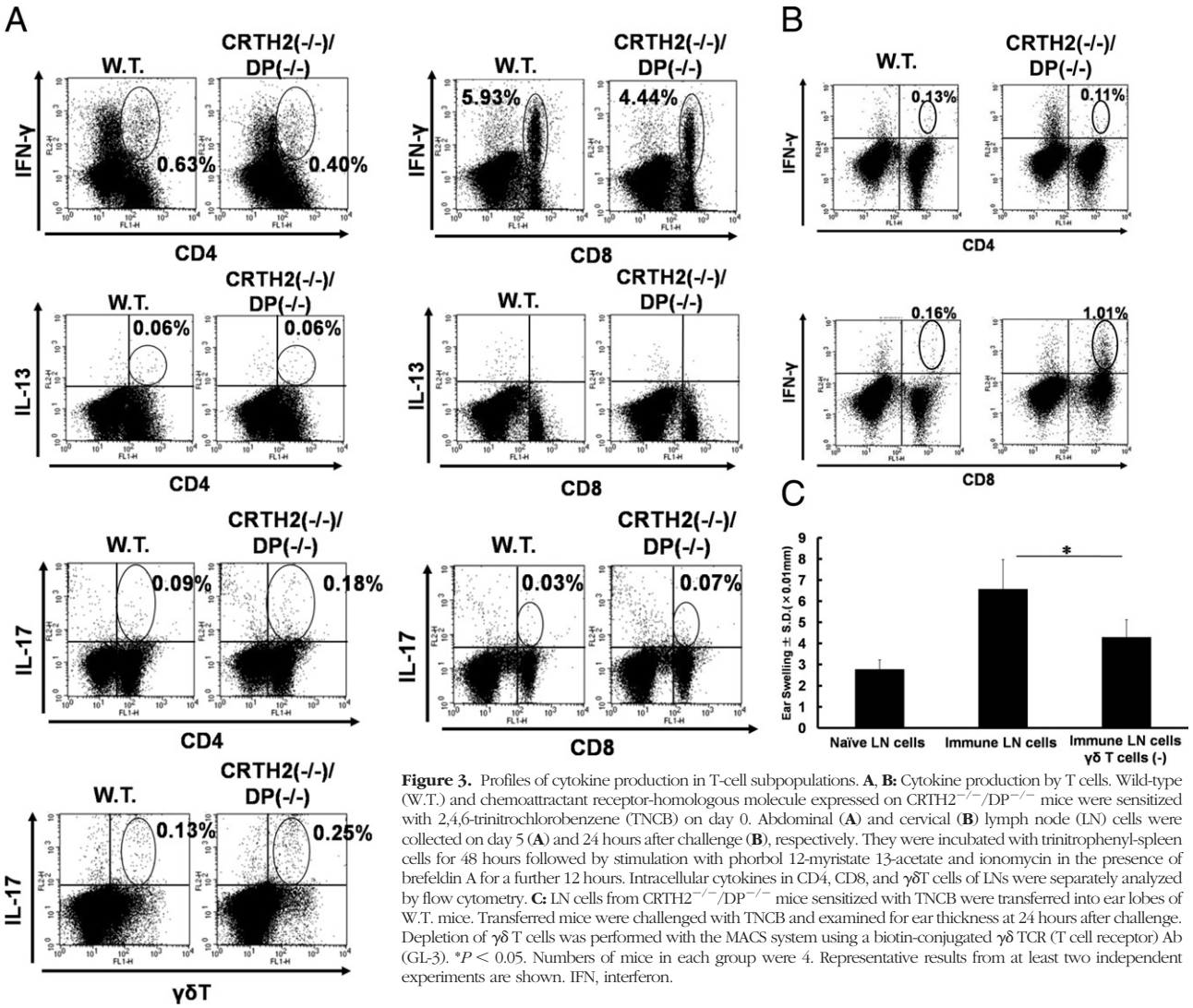

Figure 3. Profiles of cytokine production in T-cell subpopulations. **A**, **B:** Cytokine production by T cells. Wild-type (W.T.) and chemoattractant receptor-homologous molecule expressed on CRTH2$^{-/-}$/DP$^{-/-}$ mice were sensitized with 2,4,6-trinitrochlorobenzene (TNCB) on day 0. Abdominal (**A**) and cervical (**B**) lymph node (LN) cells were collected on day 5 (**A**) and 24 hours after challenge (**B**), respectively. They were incubated with trinitrophenyl-spleen cells for 48 hours followed by stimulation with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A for a further 12 hours. Intracellular cytokines in CD4, CD8, and γδT cells of LNs were separately analyzed by flow cytometry. **C:** LN cells from CRTH2$^{-/-}$/DP$^{-/-}$ mice sensitized with TNCB were transferred into ear lobes of W.T. mice. Transferred mice were challenged with TNCB and examined for ear thickness at 24 hours after challenge. Depletion of γδ T cells was performed with the MACS system using a biotin-conjugated γδ TCR (T cell receptor) Ab (GL-3). *$P < 0.05$. Numbers of mice in each group were 4. Representative results from at least two independent experiments are shown. IFN, interferon.

by PGD2. This notion can also be supported by the observation that H-PGDS$^{-/-}$ mice suffered from enhanced CHS (Figure 2B) and from comparable irritation dermatitis responses (data not shown).

Contribution of IL-17 and IFN-γ to CHS

To gain insight into the pathological mechanisms, the production of cytokines and chemokines during CHS responses was compared between CRTH2$^{-/-}$/DP$^{-/-}$ mice and WT mice. Levels of IL-17 and IFN-γ in inflammatory regions were significantly higher in the mutant mice than in WT mice (Figure 2C). Production of IL-13 increased slightly in mutant mice. IL-4 was below the detection limit. Similar cytokine production profiles were seen with DP$^{-/-}$ mice in response to TNCB (see Supplemental Figure S1B at *http://ajp.amjpathol.org*). The contribution of at least IL-17 and IFN-γ to CHS was also evident, because treatment with Abs that neutralizes IL-17 or IFN-γ reduced the inflammation phenotypes of CRTH2$^{-/-}$/DP$^{-/-}$ and DP$^{-/-}$ mice (Figure 2D) and (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). Neutralization of IL-13 did not affect inflammation. Ear swelling responses were also accompanied by increased chemokine production, namely RANTES (CCL5), IP-10 (CXCL10), and eotaxin (CCL11), but not macrophage-derived chemokine (CCL22).

To identify the cell sources of increased IL-17 and IFN-γ production, regional immune LNs were examined for their cytokine profiles. LN cells were prepared from mice 5 days after sensitization. CRTH2$^{-/-}$/DP$^{-/-}$ mice exhibited an increase in the prevalence of IL-17 cells, but not IFN-γ (+) or IL-13 (+) cells, when compared to WT mice (Figure 3A). In addition to CD4- and CD8-positive T cells, γδ T cells produced IL-17; notably, approximately half of the IL-17 cells were γδ T cells. As the total cell numbers of LNs from sensitized CRTH2$^{-/-}$/DP$^{-/-}$ mice were much higher than LNs from sensitized WT mice ($7.8 \pm 2.0 \times 10^6$ cells/LN versus $4.9 \pm 1.5 \times 10^6$ cells/LN; $P < 0.05$), it is likely that the increased numbers of IL-17 cells in CRTH2$^{-/-}$/DP$^{-/-}$ mice are significant. DP$^{-/-}$ mice again showed increased CD4$^+$, CD8$^+$, and γδ (+) cells expressing IL-17 (see Supplemental Figure S3 at *http://ajp.amjpathol.org*).

LN cells were further examined for their production of IL-17 by stimulation *in vitro* with TNP-SC. CRTH2$^{-/-}$/DP$^{-/-}$ LN cells produced higher levels of IL-17 in the culture supernatants than those from WT mice (33.4 ± 10.4 pg/mL versus 11.9 ± 6.1 pg/mL; $P < 0.05$). In contrast, no appreciable difference in the numbers of IFN-γ (+) cells or IL-13 (+) cells was seen between CRTH2$^{-/-}$/DP$^{-/-}$ mice and WT mice. Profiles of cervical LN cells from mice 24 hours after ear challenge also showed similar results, except that IFN-γ-producing CD8$^{(+)}$ cells, but not CD4$^{(+)}$ cells, were significantly increased in CRTH2$^{-/-}$/DP$^{-/-}$ mice (Figure 3B).

Implication of γδ T Cells in CHS

The increase in IL-17 γδ T cells in CRTH2$^{-/-}$/DP$^{-/-}$ mice and DP$^{-/-}$ mice prompted us to examine the roles of these cells in the elicitation of CHS. LN cells from CRTH2$^{-/-}$/DP$^{-/-}$ mice immunized with TNCB were transferred into the ear lobes of WT mice, followed by TNCB challenge and CHS examination. Transfer of immune LN cells induced a marked CHS response compared to naive cell transfer. After depletion of γδ T cells from the immune LN cells, ear lobes that received the transferred cells showed significant reductions in CHS (Figure 3C). These results, together with the observation of suppressed skin responses by anti-IL-17 Ab, suggest that IL-17 derived from γδ T cells, at least in part, contributes to the exacerbation of CHS in CRTH2$^{-/-}$/DP$^{-/-}$ mice.

CRTH2-Mediated Proliferation of γδ T Cells Producing IL-17

The increase in γδ T cells in the mutant mice also promoted us to examine the effects of distinct PGD2 signals on IL-17 production. We first established that γδ T cells expressed CRTH2 and DP at the mRNA level (Figure 4A). For this we used γδ T cells from regional LNs of sensitized mice because IL-17 γδ T cells from immune LN cells grew more efficiently than those from the spleen when in the presence of IL-23 (25.1% versus 11.3%). IL-17 was secreted from γδ T cells in response to IL-23 without T cell receptor engagement (Figure 4B), as previously reported.[53,54] Unexpectedly, a CRTH2 agonist DK-PGD2, but not a DP agonist BW245C, enhanced IL-23-dependent IL-17 production in a dose-dependent manner (Figure 4B). A DK-PGD2-dependent increase in IL-17 production was not seen in γδ T cells from CRTH2$^{-/-}$ mice, thereby excluding the possibility of nonspecific effects of DK-PGD2 (Figure 4C). The enhancement of IL-17 protein generation was accompanied by an increased IL-17 mRNA synthesis (Figure 4D). IL-23 has been shown to increase expression of RORγt mRNA in IL-17-producing γδ T cells.[55] We found that DK-PGD2 treatment strongly enhanced RORγt mRNA expression (Figure 4D).

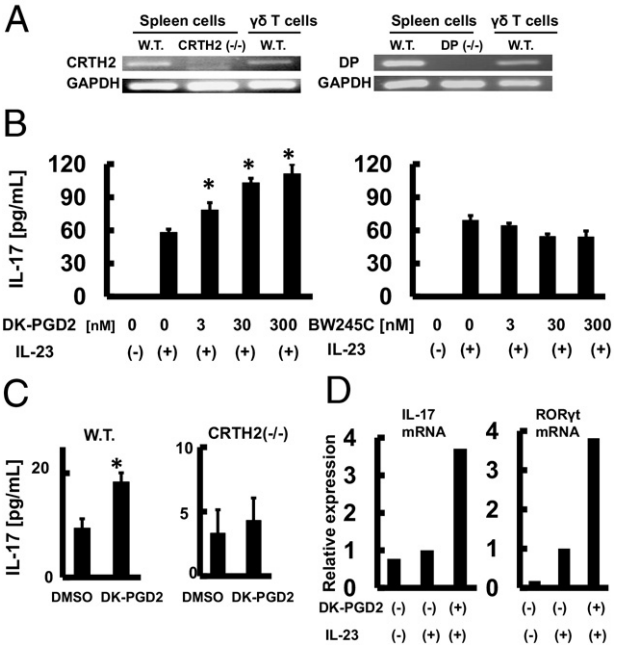

Figure 4. IL-17 production by γδ T cells. **A:** Expression of chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) and D prostanoid (DP) in γδ T cells. γδ T cells were isolated from wild-type (W.T.) mice and examined for their expression of both CRTH2 and DP mRNA by RT-PCR. The purity of γδ T cells was always >95% as assessed by γδ TCR-FITC antibody staining (UC7-13D5). Spleen cells from W.T. mice DP$^{-/-}$ and CRTH2$^{-/-}$ mice were used as positive and negative controls, respectively. **B** and **C:** γδ T cells were isolated from W.T. (**B**, **C**) and CRTH2$^{-/-}$ mice (**C**), and stimulated with DK-PGD2 (**B**, **C**) or BW245C (**B**) in the presence of IL-23 (50 ng/mL). IL-17 levels were determined by enzyme-linked immunosorbent assay. Error bars indicate SD. **D:** Expression of IL-17 and RORγt mRNA in γδ T cells. γδ T cells were isolated from W.T. mice and stimulated with DK-PGD2 and IL-23. Levels of mRNA for IL-17 and RORγt were determined by real-time RT-PCR. *$P < 0.05$. Representative results from at least three independent experiments are shown.

Involvement of PGD2 Signals in Regulatory T Cell and Th Cell Development

The contribution of regulatory T cells to the exacerbation of skin inflammation in CRTH2$^{-/-}$/DP$^{-/-}$ mice and DP$^{-/-}$ mice was assessed. As a previous report indicated that DP signals promote the development of regulatory T cells,[34] we found this a relevant point worth investigating. However, we could not detect any increase in CD4$^{(+)}$/Foxp3$^{(+)}$ cells, either in the spleen or in regional LNs of the mutant mice (Figure 5A). Similar data were obtained when CD25 (+)/Foxp3$^{(+)}$ cells in CD4 T cells were analyzed (data not shown).

The effects of PGD2 receptor agonists on the development of T cell subsets were also studied. Naive CD4 T cells were differentiated in vitro into Th1, Th2, and Th17 cells in the presence of the PGD2 receptor agonists. However, PGD2 receptor stimulation had no effect on T-bet, GATA-3, or RORγt mRNA expression (Figure 5B).

Enhanced Migration of LCs/DCs in Mutant Mice

PGD2 has been demonstrated to inhibit migration of DCs, which are important for priming T cells in regional LN.[21–23] Therefore, we asked the question of how *in vivo*

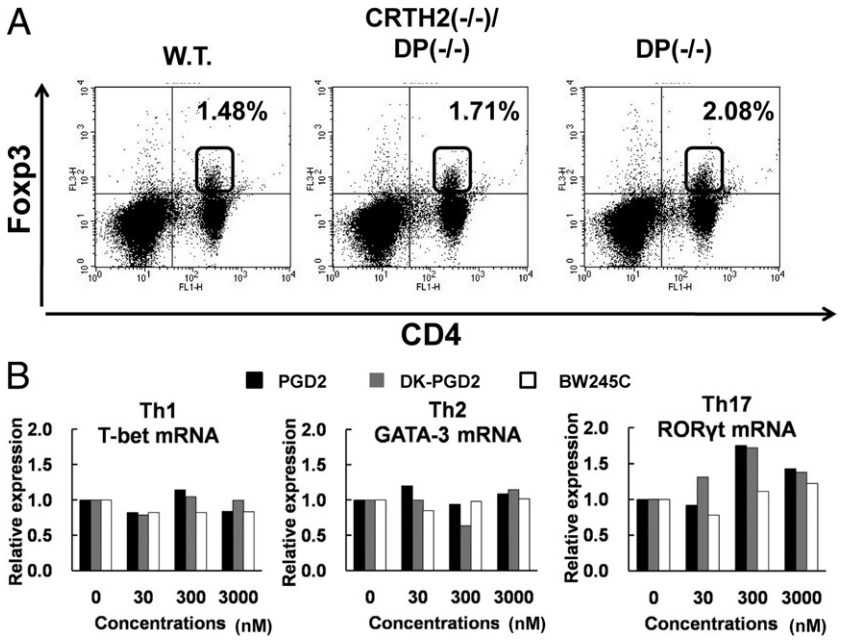

Figure 5. Effects of prostaglandin D2 (PGD2) signals on T-cell development. **A:** Foxp3 expression in $CD4^{(+)}$ T cells of PGD2 receptor-deficient mice. Immune lymph node (LN) cells were prepared from wild-type (W.T.), D prostanoid $(DP)^{-/-}$ and chemoattractant receptor-homologous molecule expressed on Th2 cells $(CRTH2)^{-/-}/DP^{-/-}$ mice sensitized with 2,4,6-trinitrochlorobenzene (TNCB) and then analyzed by flow cytometry after staining with anti-Foxp3 and CD4 antibodies. **B:** Effects of PGD2 on the expression of T-cell transcription factors. Th1, Th2, or Th17 cells were induced as described in *Materials and Methods* and incubated for 4 hours with the respective PGD2 agonists. mRNA was extracted and examined for T-bet, GATA-3, and RORγt by real-time RT-PCR. Representative results from at least three independent experiments are shown.

migration of Langerhans cells (LCs)/DCs affects CHS exacerbation in $CRTH2^{-/-}/DP^{-/-}$ and $DP^{-/-}$ mice when IL-17 and IFN-γ are increased. Regional LN cells of mice painted with FITC on the ventral skin were examined for LC/DC numbers by flow cytometric analysis. Although the numbers of epidermal LCs in untreated $CRTH2^{-/-}/DP^{-/-}$ mice were comparable to those in WT mice (data not shown), $CRTH2^{-/-}$ $DP^{-/-}$ mice showed higher numbers of $FITC^{(+)}/CD11c^{(+)}$ cells in regional LN than those in FITC-treated WT mice (Figure 6A). As LC/DC migration was not affected by CRTH2 deficiency,[12] we postulated that enhanced migration of LCs/DCs could be largely due to DP deficiency. As expected, single DP deficiency resulted in enhanced migration of LCs/DCs *in vivo*. In studies on the effects of PGD2 receptor deficiency on DC maturation, we found that CD86 and MHC class II ex-

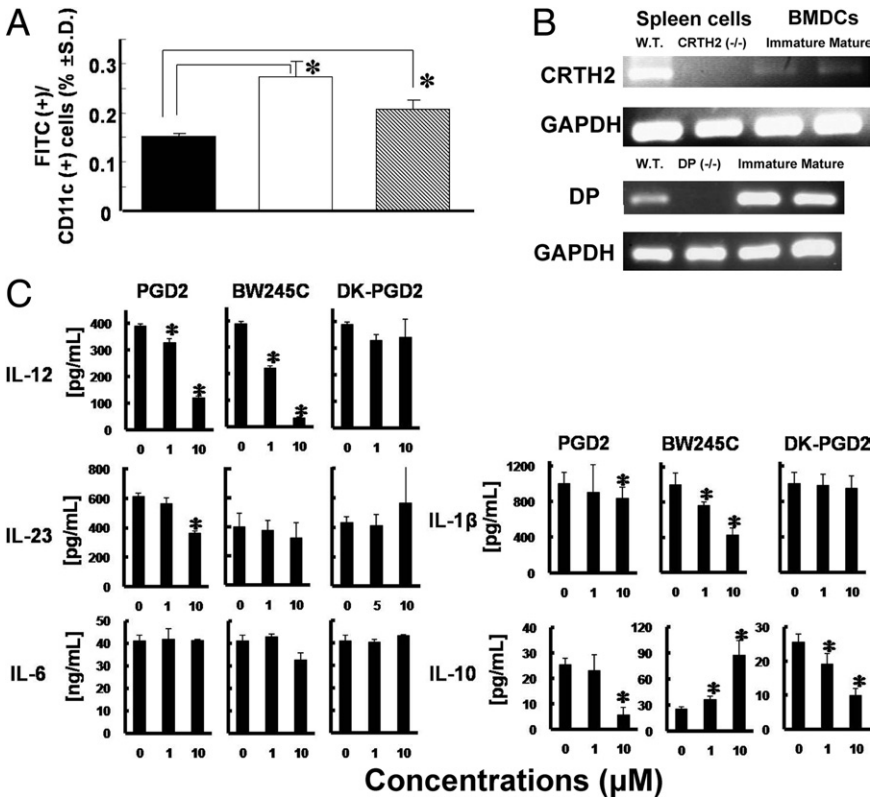

Figure 6. Modulation of dendritic cell (DC) function by prostaglandin D2 (PGD2) receptor signals. **A:** Migration of DC in mutant mice. Wild-type (WT; black bar), $DP^{-/-}$ (grey bar), and chemoattractant receptor-homologous molecule expressed on $CRTH2^{-/-}/DP^{-/-}$ (white bar) mice were sensitized with fluorescein isothiocyanate (FITC), and 24 hours later, draining lymph node (LN) cells were isolated. $FITC^{(+)}/CD11c^{(+)}$ DCs in LN cells were assessed by flow cytometry. **B:** Expression in CRTH2 and DP in bone marrow-derived dendritic cells (BMDC). The BMDC were subjected to mRNA extraction, followed by real-time PCR with primers specific to CRTH2, DP, and GADPH. Spleen cells from W.T. and $CRTH2^{-/-}/DP^{-/-}$ mice were used as positive and negative controls, respectively. Maturation of DC induced by lipopolysaccharide (LPS) (1 μg/mL, 20 hours) was assessed by the increased expression of major histocompatibility complex class II and CD86 (data not shown). **C:** Effects of PGD2 on cytokine production. BMDC (5×10^5 cells/mL) were incubated with PGD2, BW245C, or DK-PGD2 for 4 hours, followed by stimulation with LPS (1 μg/mL) for an additional 16 hours. Enzyme-linked immunosorbent assay was used to measure cytokines in the supernatants. Error bars indicate standard deviation. $^*P < 0.05$. Representative results from at least three independent experiments are shown.

pression on CD11c$^{(+)}$ DCs was similar among WT mice, CRTH2$^{-/-}$ DP$^{-/-}$ mice and DP$^{-/-}$ mice (see Supplemental Figure S4 at http://ajp.amjpathol.org). These results indicate that PGD2 negatively regulates LC/DC migration via DP during sensitization without modulation of DC maturation.

Next we attempted to clarify the roles of PGD2 receptors in cytokine production from DCs. After confirmation of CRTH2 and DP expression at the mRNA level (Figure 6B), BMDCs were treated with an agonist specific to either CRTH2 (DK-PGD2) or DP (BW245C). BW245C inhibited production of IL-12 (a Th1-polarizing cytokine) from BMDC, as seen with PGD2 (Figure 6C). This is consistent with previous reports.[56–57] The CRTH2 agonist had little or no effect on IL-12 production. Production of IL-23, a cytokine promoting the expansion of Th17 cells and IL-17-$\gamma\delta$ T cells,[53,54,58] was minimally inhibited by PGD2, but not by BW245C. In contrast, BW245C, but not DK-PGD2, markedly suppressed IL-1β, which is another cytokine promoting the development of Th17 cells[59] and expansion of IL-17-$\gamma\delta$ T cells.[54] None of the agonists affected IL-6 production. Interestingly, signals from the PGD2 receptors exerted reciprocal effects on IL-10 production. BW245C markedly enhanced IL-10 generation from BMDC, whereas DK-PGD2 exerted suppressive effects on IL-10 production.

Modulation of T Cell-Activating Capacity of DCs by PGD2 Receptor Signals

BMDCs were treated with either BW245C (DP agonist, 10 μmol/L) or DK-PGD2 (CRTH2 agonist, 10 μmol/L) for 4 hours followed by haptenization with TNBS (1 mmol/L). Immune LN cells were stimulated with these haptenated BMDCs (10:1) for 24 hours. BW245C-treated BMDCs stimulated immune LN cells to produce less IFN-γ than vehicle-treated DCs (902 $\pm$ 134.2 pg/mL versus1234 $\pm$ 98.6 pg/mL; $P < 0.05$). Treatment of BMDCs with DK-PGD2 did not affect IFN-γ production from LN cells. IL-17 generation from LN cells was not influenced by BMDC treated with BW245C or DK-PGD2 (data not shown).

Reciprocal Effects of PGD2 on the Afferent and Efferent Phases of CHS

Based on the modulatory effects of PGD2 on migration and cytokine production in DCs, it was assumed that PGD2 principally down-regulates the sensitization step of CHS. To test this notion, WT mice were treated with HQL-79 (an H-PGDS inhibitor, Cayman Chemical Co.) during the afferent phase and then examined for CHS. We found that the inhibitor exacerbated ear swelling responses (Figure 7A). In the efferent phase, HQL-79 significantly inhibited the CHS response. These observations were confirmed by experiments with agonists for each receptor, which were directly injected into the ear lobes. PGD2 and DK-PGD2 were found to enhance ear-swelling responses (Figure 7B). In contrast, BW245C suppressed the elicitation phase of CHS. Stimulatory roles of CRTH2 were further confirmed by the observation that ramatroban (Cayman Chemical Co.), a CRTH2 antagonist, exerted inhibitory effects by its administration p.o. during the efferent phase (Figure 7C). These results clearly indicate that PGD2 signaling through CRTH2 has stimulatory functions in the elicitation phase of CHS, whereas DP signaling potentially has suppressive effects in the efferent phase.

Discussion

In the present study, we have clarified the physiological roles of PGD2 in CHS by assessing the function of its receptors. Although PGD2 has been recognized as an important inflammatory mediator, lack of PGD2 production, or both CRTH2 and DP expression, showed little (if any) alleviation of skin responses, and rather induced significant exacerbation. Therefore, PGD2 as a whole appears to provide negative regulatory signals to the development of CHS. On the other hand, irritation dermatitis is unlikely to be regulated by PGD2, indicating immune-regulatory roles of PGD2 in allergic skin inflammation.

Enhanced inflammation in DP$^{-/-}$ mice and CRTH2$^{-/-}$/DP$^{-/-}$ mice might be principally mediated by increased production of IFN-γ and IL-17. The major source of increased IFN-γ could be CD8$^{(+)}$ T cells, as demonstrated by flow cytometric analysis of cervical LN cells, although the increase in IFN-γ-producing cells in regional LNs of sensitization areas was not seen. Prior evidence indicates the critical contribution of CD8$^{(+)}$/IFN-γ cells (Tc1) to inflammation at the elicitation phase of CHS.[39–40] IL-17 appears to be generated by CD4$^{(+)}$/IL-17 cells (Th17) and CD8$^{(+)}$/IL-17 cells (Tc17 cells) (Figure 3A), both of which are shown to contribute to the sensitization and elicitation phases of CHS, respectively.[42,45] In addition, it was somewhat surprising that approximately half of the IL-17-producing cells were $\gamma\delta$ T cells. In this study, we demonstrated that $\gamma\delta$ T cells participate in the elicitation of CHS responses in the mutant mice (Figure 3C). Prior studies have also revealed the pathogenic roles of IL-17 $\gamma\delta$ T cells in inflammatory reactions; $\gamma\delta$ T cells that produce IL-17 have been shown to play critical roles in the development of experimental autoimmune encephalomyelitis,[54] and to be implicated in neutrophil infiltration in response to *Escherichia coli*.[53]

We found that a single deficiency in the *DP* gene always showed greater exacerbation of skin inflammation than the double deficiency in CRTH2$^{-/-}$/DP$^{-/-}$ mice (Figure 1C). This might be due to the contribution of the CRTH2 deficiency, which could have alleviated skin inflammation.

In this study, we have further clarified the immunological events regulated by each receptor signal in terms of function and development of DC and T cells. Under these experimental conditions, we found that DP signals were not involved in DC maturation, as we did not observe any appreciable changes in CD86 or MHC class II expression, in contrast to the results of Hammad et al.[34] Instead, our results indicate that PGD2 through its interaction with DP inhibits the migration of DCs into regional LNs. In

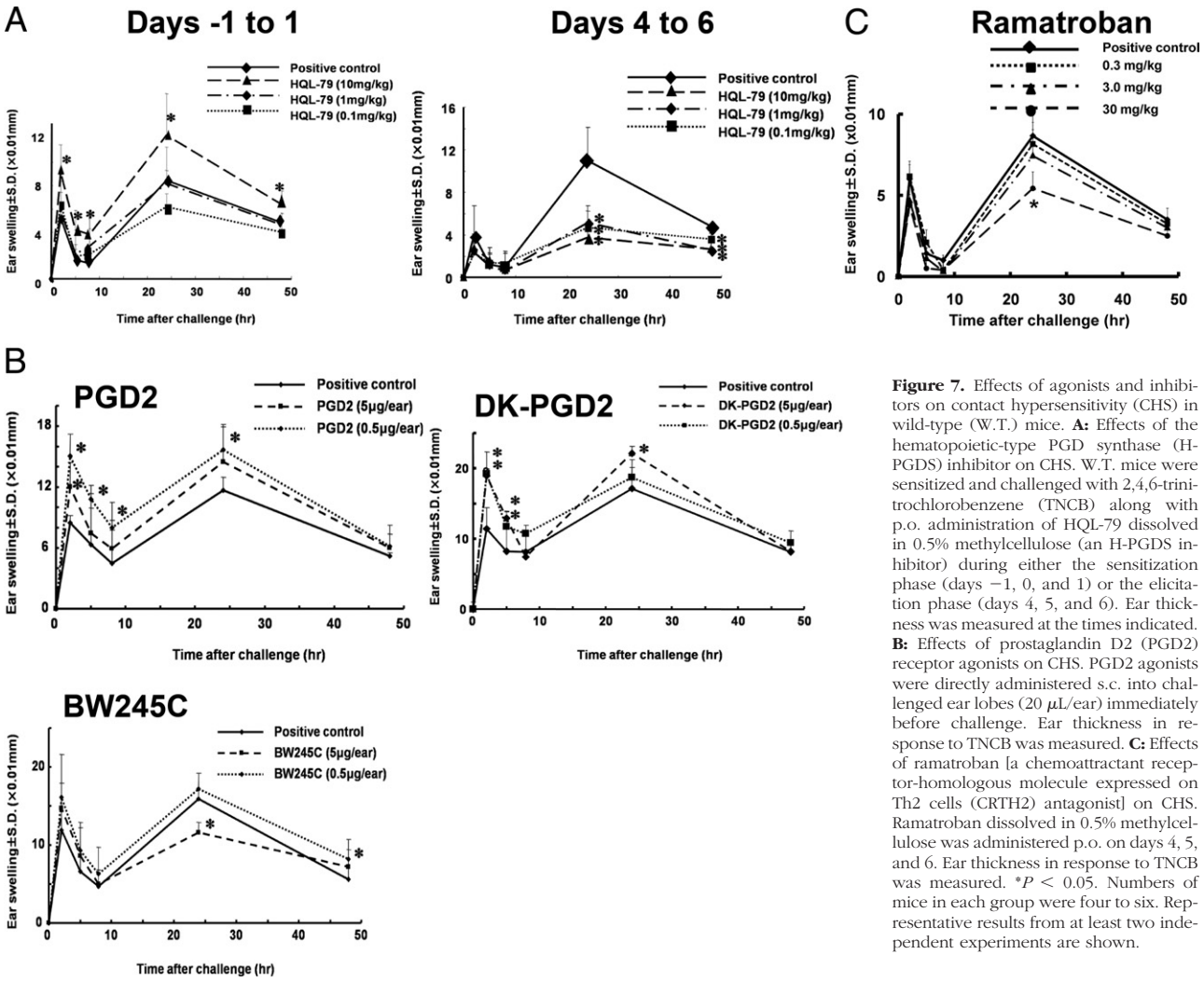

Figure 7. Effects of agonists and inhibitors on contact hypersensitivity (CHS) in wild-type (W.T.) mice. **A:** Effects of the hematopoietic-type PGD synthase (H-PGDS) inhibitor on CHS. W.T. mice were sensitized and challenged with 2,4,6-trinitrochlorobenzene (TNCB) along with p.o. administration of HQL-79 dissolved in 0.5% methylcellulose (an H-PGDS inhibitor) during either the sensitization phase (days −1, 0, and 1) or the elicitation phase (days 4, 5, and 6). Ear thickness was measured at the times indicated. **B:** Effects of prostaglandin D2 (PGD2) receptor agonists on CHS. PGD2 agonists were directly administered s.c. into challenged ear lobes (20 μL/ear) immediately before challenge. Ear thickness in response to TNCB was measured. **C:** Effects of ramatroban [a chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) antagonist] on CHS. Ramatroban dissolved in 0.5% methylcellulose was administered p.o. on days 4, 5, and 6. Ear thickness in response to TNCB was measured. $^*P < 0.05$. Numbers of mice in each group were four to six. Representative results from at least two independent experiments are shown.

addition, the DP signals were able to suppress IL-12 generation in DCs. Treatment of DCs with DP agonist led to suppression of stimulating IFN-γ production from immune LN cells. DP signals also inhibited IL-1β production in DCs. IL-1β has been shown to support development and expansion of Th17 and $\gamma\delta$ T cells that produce IL-17.[54,59] Although *in vitro* treatment of DCs with DP agonist did not affect IL-17 generation by LN cells, data not shown here demonstrated that CD11c$^{(+)}$ DCs prepared from immune LN cells of DP$^{-/-}$ mice were more capable of stimulating LN cells (WT mice) to produce IL-17 production when compared with CD11c$^{(+)}$ DCs from WT mice (1127 ± 84.2 versus 878 ± 126.3 pg/mL). Thus, the exacerbated phenotypes seen in DP$^{-/-}$ mice and CRTH2$^{-/-}$/DP$^{-/-}$ mice can be attributed to enhanced Th1 and Th17 immunity, induced by DP deficiency in LCs/DCs. More interestingly and probably importantly, because of the inhibitory function of IL-10 in DC and in CHS,[60–62] DP signals are critically linked to the enhancement of IL-10 production in BMDC. In contrast, CRTH2 signals suppress IL-10 production. Notably, the mode of action of PGD2 on the regulation of IL-10 production seems distinct from that of IL-12 and IL-1β production. This is because PGD2 regulates IL-10 production through both CRTH2 and DP, but only the PGD2-mediated DP pathway affects the production of IL-12 and IL-1β.

An earlier report demonstrated that DP signals in DC promote their capability to induce regulatory T cells.[34] Mice treated with a DP agonist had higher numbers of regulatory T cells than control mice during an asthmatic response. However, in this study there were no significant changes in the prevalence of regulatory T cells in CRTH2$^{-/-}$/DP$^{-/-}$ mice or DP$^{-/-}$ mice (Figure 5A). In contrast to extrinsic stimulation of DP, endogenous levels of PGD2 provided to DC *in vivo* during sensitization may be insufficient for the induction of regulatory T cells. Thus, the absence of the *DP* gene may not result in modulation of regulatory T cell development.

We did not observe any direct effects of PGD2 agonists on CD4 T cell development (ie, Th1, Th2, or Th17 cells). Unexpectedly, however, DK-PGD2 (a CRTH2 agonist) enhanced IL-17 production from $\gamma\delta$ T cells. Data not shown here demonstrated that local levels of IL-17 in CRTH2$^{-/-}$ mice were lower than those in WT mice. The CRTH2-mediated expansion of IL-17-producing $\gamma\delta$ T

cells may account for milder skin inflammation in CRTH2$^{-/-}$ mice than in WT mice. Presumably the same is true for the difference between CRTH2$^{-/-}$/DP$^{-/-}$ mice and DP$^{-/-}$ mice. Nevertheless, it seems inconsistent that CRTH2$^{-/-}$/DP$^{-/-}$ mice showed exacerbated skin reactions in association with increased IL-17 γδ T cells than in WT mice. The balance between CRTH2 and DP is likely to determine the final response. The absence of DP on inflammation might overcome the effects of CRTH2 deficiency.

In addition to the reciprocal functions of CRTH2 and DP, we demonstrated phase-dependent roles of PGD2 during the development of CHS. The inhibition of PGD2 by HQL-79 (an inhibitor of H-PGDS) rendered opposite effects depending on the phases of inflammation. For example, during the sensitization phase the shortage of PGD2 resulted in the exacerbation of skin responses, whereas blocking PGD2 during the elicitation phase alleviated such responses. The exacerbation is at least partly due to a lack of DP signals, which affects LC/DC migration and IL-10 production, further confirming the findings of a prior report.[21] Stimulatory function of PGD2 in the efferent phase of CHS was further confirmed by local administration of PGD2 during challenge reaction, which exacerbated skin inflammation. The facilitation of inflammation by PGD2 might be mediated by CRTH2 signals as ramatroban, a CRTH2 antagonist, which exhibited suppressive effects when administered during elicitation that was consistent with prior reports.[63,64] Nevertheless, BW245C down-regulated the skin reactions, indicating that DP is capable of providing braking signals, even in the elicitation phase, whereas PGD2 positively contributes to the inflammation as its total outcome. These findings could be important in regard to a clinical point of view. Co-administration of a CRTH2 antagonist and a DP agonist should efficiently improve skin inflammation. In contrast, a DP agonist and/or H-PGDS inhibitor may increase the risk of sensitization to environmental allergens.

Although we have not been able to show actual sources of PGD2 in CHS, DC in sensitization areas could be a strong candidate.[14] Epidermal DC, in particular Langerhans cells that produce PGD2, may act on neighboring DCs and/or dermal DCs, which could interfere with their migration to LNs via DP. This could be one of the natural regulatory mechanisms to protect individuals from excessive sensitization of environmental allergens. On the other hand, in the inflammatory skin, mast cells and T cells, and particularly Th2 cells, may function as PGD2-producing cells.[13] It is important to note that PGD2 rapidly down-regulates cell surface CRTH2 expression on T cells and eosinophils.[65,66] As a result, the predominance of DP signals in these cells and the termination of inflammation by braking signals from DP may follow.

PGD2 is probably secreted to protect the host from environmental allergens via initiating and terminating inflammation.

Acknowledgments

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