Prostaglandin D_2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on T_H2 cells

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Background: Activation of the group 2 innate lymphoid cell (ILC2) population leads to production of the classical type 2 cytokines, thus promoting type 2 immunity. Chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2), a receptor for prostaglandin D_2 (PGD_2), is expressed by human ILC2s. However, the function of CRTH2 in these cells is unclear.
Objectives: We sought to determine the role of PGD_2 and CRTH2 in human ILC2s and compare it with that of the established ILC2 activators IL-25 and IL-33.
Methods: The effects of PGD_2, IL-25, and IL-33 on the cell migration, cytokine production, gene regulation, and receptor expression of ILC2s were measured with chemotaxis, ELISA, Luminex, flow cytometry, quantitative RT-PCR, and QuantiGene assays. The effects of PGD_2 under physiologic conditions were evaluated by using the supernatant from activated mast cells.
Results: PGD_2 binding to CRTH2 induced ILC2 migration and production of type 2 cytokines and many other cytokines. ILC2 activation through CRTH2 also upregulated the expression of IL-33 and IL-25 receptor subunits (ST2 and IL-17RA). The effects of PGD_2 on ILC2s could be mimicked by the supernatant from activated human mast cells and inhibited by a CRTH2 antagonist.
Conclusions: PGD_2 is an important and potent activator of ILC2s through CRTH2 mediating strong proallergic inflammatory responses. Through IgE-mediated mast cell degranulation, these innate cells can also contribute to adaptive type 2 immunity; thus CRTH2 bridges the innate and adaptive pathways in human ILC2s. (J Allergy Clin Immunol 2014;133:1184-94.)

Key words: *Group 2 innate lymphoid cell, PGD_2, chemoattractant receptor-homologous molecule expressed on T_H2 cells, IL-25, IL-33, innate type 2 immunity, adaptive type 2 immunity*

Innate lymphoid cells (ILCs) are emerging as a novel family of hematopoietic effectors that are heterogeneous in their location, cytokine production, and effector functions.[1-3] They lack specific antigen receptors and lineage markers and serve critical roles in innate immune responses to microorganisms, lymphoid tissue formation, and tissue remodeling.[2] ILCs can be categorized into 3 subsets (group 1 ILCs [ILC1s], group 2 ILCs [ILC2s], and group 3 ILCs [ILC3s]) based on phenotypic and functional characteristics.[4]

ILC2s are ILCs that produce type 2 cytokines (IL-4, IL-5, IL-9, and IL-13) and are dependent on GATA3 and retinoic acid receptor–related orphan receptor α for their development and function.[5-11] This group of cells is found in the blood, spleen, intestine, liver, skin, fat-associated lymphoid clusters, and lymph nodes of mice and have also previously been termed natural helper cells, nuocytes, or innate helper 2 cells by different groups,[5,12-14] but the overall term ILC2 is now accepted.[4] They express IL-17RB (IL-25R) and ST2 (IL-33R) receptors and respond to IL-25 (IL-17 family member) and IL-33 (IL-1 family member). Such cells are thought to contribute to protection against parasites and also promote allergic inflammation.[15] Lung-resident ILC2s in mice have been shown to restore epithelial integrity and lung function by producing amphiregulin, a wound-healing regulator.[16] Airway infection with H3N1 induced airway hyperreactivity by stimulating alveolar macrophages to produce IL-33 and therefore activating ILC2s.[17] Similarly,

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Abbreviations used
CRTH2: Chemoattractant receptor-homologous molecule expressed on T_H2 cells
CSF-1: Macrophage colony-stimulating factor
cysLT: Cysteinyl leukotriene
$CysLT_1$: Cysteinyl leukotriene receptor 1
EC_{50}: Median effective concentration
ILC: Innate lymphoid cell
ILC2: Group 2 innate lymphoid cell
PGD_2: Prostaglandin D_2

intranasal administration of IL-25 and IL-33 in mouse asthma models induces ILC2 infiltration into the lungs and airway hyperreactivity.[18,19] The human counterpart of mouse ILC2s was recently discovered in human peripheral blood, lung tissue, and fetal gut and skin and has been found in increased numbers in inflamed nasal polyps and skin.[16,20-22] ILC2s observed within lesional atopic dermatitis skin is compatible with a role in pathogenesis because increased production of IL-13 is well established in atopic skin, leading to downregulation of antimicrobial peptides and filaggrin.[21,23,24] This human ILC population was found also to express chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2).[20] A recent report showed that prostaglandin D_2 (PGD_2) induced ILC2s to produce IL-13 through activation of CRTH2 in a synergistic manner with IL-25/IL-33.[22] However, understanding of the role of CRTH2 in these cells is still limited.

CRTH2 is a G protein–coupled receptor for PGD_2, a major mediator released from activated mast cells.[25] Before the discovery of ILC2s, CRTH2 was known to be abundant on eosinophils, basophils, and T_H2 cells. Emerging evidence suggests that the activation of CRTH2 leads to proinflammatory responses in leukocytes, including chemotaxis of eosinophils, basophils, and T_H2 cells[25-27]; T_H2 cytokine production,[28,29] which is enhanced by cysteinyl leukotrienes (cysLTs)[30]; and proinflammatory protein expression.[28,31] Our previous studies also demonstrated that the signaling of CRTH2 suppresses T_H2 cell apoptosis.[32] Allergic responses mediated by IgE, mast cells, T_H2 cells, and eosinophils are dramatically reduced in mice in which CRTH2 is genetically ablated or by small-molecule CRTH2 antagonists.[33-35] Antagonism of CRTH2 is currently being tested as a useful approach to control allergic diseases.[36]

In this study we investigated the role of CRTH2 in human ILC2s isolated *ex vivo*. We found that CRTH2 plays a critical role in proinflammatory responses of ILC2s, including cell migration and diverse cytokine production. Activation of CRTH2 also upregulated the IL-33 and IL-25 receptors (ST2 and IL-17RA), and the combination of PGD_2, IL-33, and IL-25 enhanced some ILC2 responses. These novel observations define CRTH2 as a key trigger for ILC2 activation and thus places it at the center of a tissue inflammation network.

METHODS

ILC2 cell preparation and culture

Skin immune cells were isolated from the human skin biopsy specimens of healthy donors. The tissue was cut and then digested in collagenase P at 37°C overnight. After washing with 10 mmol/L EDTA solution, cell suspensions were obtained by passing through tissue strainers. Mononuclear cells were isolated from the cell suspensions with Ficoll-Paque PLUS gradient. PBMCs were isolated from leukocyte cones (National Blood Service, Bristol, United Kingdom) by using Lymphoprep gradient.

ILC2s were prepared from mononuclear cells and cultured by using a modified method described previously.[20] Briefly, $CD3^+$ T cells were predepleted with CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany); otherwise, the mononuclear cells were labeled with an antibody mixture (see Table E1 in this article's Online Repository at www.jacionline.org). Lineage-negative (CD3, CD4, CD8, CD14, CD19, CD56, CD11b, CD11c, FcεRI, and CD123), $CD45^{high}$, $CD127^+$, and $CRTH2^+$ cells were sorted on a MoFlo XDP cell sorter (Beckman Coulter, Fullerton, Calif) and cultured with 100 IU/mL IL-2, 10% heat-inactivated human serum, 1× L-glutamine, 1× penicillin/streptomycin, and gamma-irradiated PBMCs (from 3 healthy volunteers) in RPMI 1640 (Sigma, St Louis, Mo). Half of the medium was replaced with fresh medium every 2 to 3 days. The irradiated cells were degraded within 1 to 2 weeks of culture, and the purity of the ILC2s was confirmed by using fluorescence-activated cell sorting before use. The cells were changed to fresh medium without IL-2 before treatment.

Use of human tissue samples was conducted under the ethical approval of the Oxford Clinical Research Committee.

Human mast cell culture and activation

Human mast cells were cultured from $CD34^+$ progenitor cells and treated with human IgE (Chemicon International, Temecula, Calif) and goat anti-human IgE (1 μg/mL, Sigma) in the presence or absence of diclofenac (10 μmol/L), as described previously.[37] Supernatants of the cells were collected and measured for PGD_2 and IL-13 with ELISA or stored at −80°C until used as mast cell supernatants for the treatment of ILC2s.

Chemotaxis assays

For measurement of cell migration, ILC2s were resuspended with RPMI 1640 media; 25 μL of cell suspension and 29-μL test samples prepared in RPMI 1640 or mast cell supernatants were applied to the upper and lower chambers, respectively, in a 5-μm pore sized 96-well ChemoTx plate (Neuro Probe, Gaithersburg, Md). After incubation (37°C for 60 minutes), the migrated cells in the lower chambers were collected and mixed with a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, Wis) and quantified by using a FLUOstar OPTIMA luminescence plate reader (BMG LabTech, Cary, NC).

Luminex assays

After ILC2 treatments for 4 hours, the concentrations of selected human cytokines in the supernatants were measured by using a Procarta Human Cytokine Immunoassay kit (Affymetrix, Santa Clara, Calif) with magnetic beads, according to the manufacturer's instruction. Results were obtained with a Bio-Plex 200 System (Bio-Rad Laboratories, Hercules, Calif).

QuantiGene Plex assays

After various treatments for 2.5 hours, the mRNA levels of selected genes in ILC2s were measured by using a QuantiGene 2.0 Plex Assay kit (Affymetrix) with magnetic beads, as per the manufacturer's instruction. Results were quantified with a Bio-Plex 200 System (Bio-Rad Laboratories).

Quantitative RT-PCR

Quantitative RT-PCR was conducted, as described previously.[30] Primers and probes (Roche, Mannheim, Germany) used are listed in Table E2 in this article's Online Repository at www.jacionline.org.

ELISA

Concentrations of cytokines in the supernatants of ILC2s or mast cells were assayed with ELISA kits (R&D Systems, Minneapolis, Minn). PGD_2 levels in the supernatants of mast cells were assayed with a PGD_2-MOX enzyme

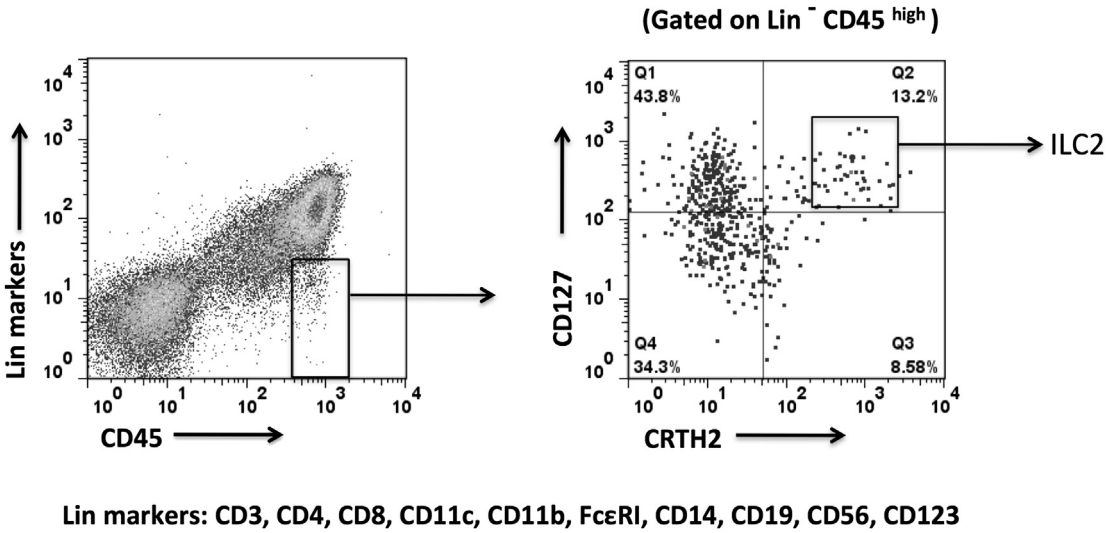

FIG 1. ILC2 isolation. ILC2s isolated from human skin were lineage marker–negative (CD3, CD4, CD8, CD14, CD19, CD56, CD11c, CD11 b, FcεRI, T-cell receptor γδ, T-cell receptor αβ, and CD123), $CD45^{high}$, IL-7Rα^{+}, and $CRTH2^{+}$. Isotype controls are shown in Fig E1.

immunoassay kit (Cayman Chemicals, Ann Arbor, Mich). Results were measured in a FLUOstar OPTIMA luminescence plate reader (BMG LabTech).

Flow cytometric analysis

ILC2s were fluorescently labeled with antibodies (see Table E1) and acquired by using Summit software on a CyAn flow Cytometer (Beckman Coulter).

Statistics

Data were analyzed by using 1-way ANOVA, followed by the Newman-Keuls test. *P* values of less than .05 were considered statistically significant.

RESULTS

CRTH2 mediates chemotaxis of human ILC2s

To understand the role of CRTH2 in human ILC2s, we compared the effect of PGD_2 with the effects of IL-33 and IL-25 on ILC2 migration. Lineage-negative, $CD45^{high}$, $CD127^{+}$, and $CRTH2^{+}$ ILC2s were isolated from human skin biopsy specimens and peripheral blood of healthy adult donors (Fig 1 and see Fig E1 in this article's Online Repository at www.jacionline.org) and tested with dose titrations of PGD_2, IL-33, and IL-25 in chemotaxis assays (Fig 2, *A*). Both PGD_2 and IL-33 caused ILC2 migration in a dose-dependent manner, peaking at approximately 100 nmol/L for PGD_2 and 30 ng/mL for IL-33. The chemoattractant effect of IL-25 on ILC2s was very weak. The maximum response achieved with PGD_2 was 4.75-fold higher than that achieved with IL-33. The ILC2s cultured from skin and blood showed similar responses to PGD_2, IL-25, and IL-33.

To confirm the receptor mediating ILC2 migration induced by PGD_2 was CRTH2, we used the selective CRTH2 antagonist TM30089. ILC2 migration triggered by PGD_2 (30 nmol/L) was completely inhibited by TM30089 (1 μmol/L; Fig 2, *B*).

The effects of combinations of these stimulators were examined to further elucidate the contribution and interaction of PGD_2, IL-33, and IL-25 on ILC2 migration (Fig 2, *C*). Concentrations of stimulators less than the peak in their dose curves (5 nmol/L for PGD_2 and 10 ng/mL for IL-33 and IL-25) were used for these combination tests to avoid saturation of the response. No additive effect was detected when the stimulators were combined. The cell migration in response to the combinations of PGD_2 and IL-33 or PGD_2, IL-33, and IL-25 at these doses appeared to be mainly mediated by CRTH2 because the responses were largely inhibited by TM30089.

Activation of human ILC2s through CRTH2 induces type 2 cytokine production

One of the most striking features of ILC2s is their ability to produce type 2 cytokines.[5] Cells were stimulated with increasing concentrations of PGD_2 for 2.5 hours for mRNA analysis or for 4 hours for protein analysis to investigate the role of CRTH2 in type 2 cytokine production in human ILC2s (Fig 3, *A*). The treatment increased cytokine expression at the levels of both mRNA and secreted protein in a dose-dependent manner (Fig 3, *A*). The median effective concentration (EC_{50}) of PGD_2 for IL-4, IL-5, and IL-13 production at the mRNA level was 88, 178, and 111 nmol/L, respectively, and that at the protein level was 195, 118, and 82.6 nmol/L, respectively. Type 2 cytokine production induced by 100 nmol/L PGD_2 was completely blocked with 1 μmol/L TM30089 (Fig 3, *B*).

It has been reported that IL-33 and IL-25 promote type 2 cytokine production from ILC2s.[5,38,39] ILC2s were treated with PGD_2, IL-33, or IL-25 alone (at concentrations close to their relative EC_{50}) or in combination for 4 hours to define the effect of the combination of PGD_2, IL-33, and IL-25 on type 2 cytokine production (Fig 3, *B*). Both IL-33 and IL-25 evoked type 2 cytokine production from ILC2s. In contrast, IL-33 had no effect on $Lin^{-}CD127^{+}CRTH2^{-}$ cells (see Fig E2 in this article's Online Repository at www.jacionline.org). However, the efficacy of both IL-33 and IL-25 at this time point was weaker than that of PGD_2 in ILC2s from both skin and blood. Interestingly, the combination of IL-33 and IL-25 at these doses did not enhance stimulation compared with either IL-33 or IL-25 alone; however, the combination of these cytokines, particularly IL-25 with PGD_2, enhanced cytokine production with an apparent synergistic effect.

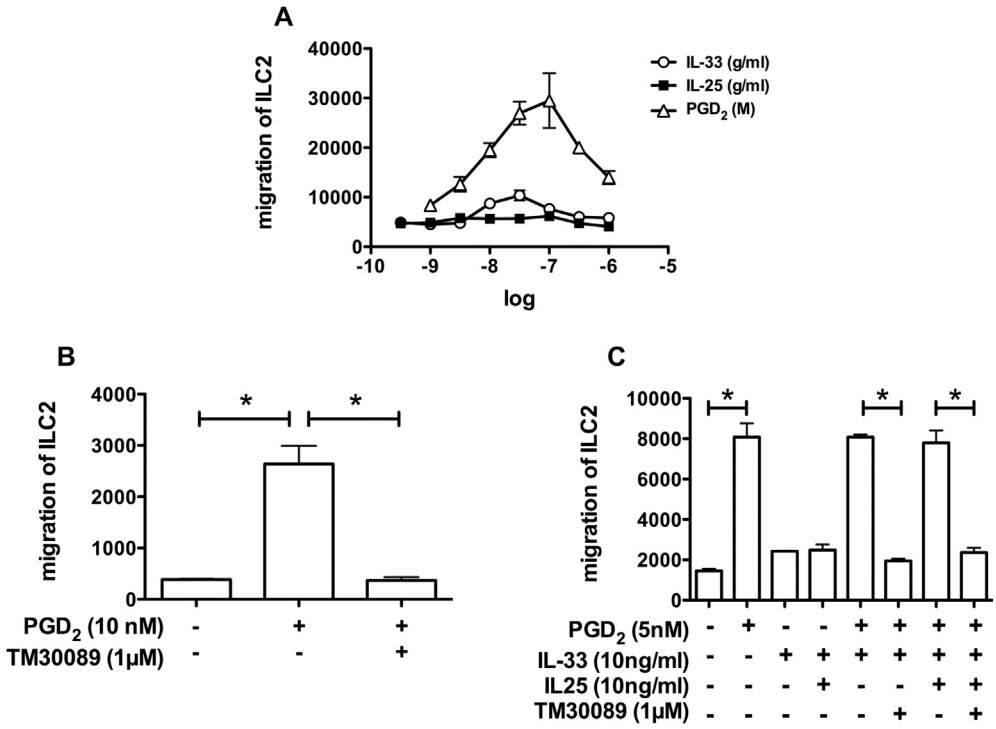

FIG 2. Migration of ILC2s (**A** and **B,** skin; **C,** blood) to PGD_2 is mediated by CRTH2. Fig 2, *A*, Migration after stimulation with IL-25, IL-33, or PGD_2. Fig 2, *B*, Migration after exposure to PGD_2 in the absence or presence of TM30089. Fig 2, *C*, Migration in response to PGD_2, IL-33, or IL-25 alone or in combination with or without TM30089. $*P < .05$ (n = 3).

The contribution of PGD_2 in these combination treatments was effectively blocked by TM30089.

Activation of human ILC2s through CRTH2 regulates other cytokine production

The effect of CRTH2 on other cytokine production was investigated to further understand the proinflammatory role of CRTH2 in ILC2s (Fig 4). Cells were incubated with increasing concentrations of PGD_2 for 4 hours, and protein levels of IL-3, IL-8, IL-9, IL-17A, IL-17F, IL-21, GM-CSF, macrophage colony-stimulating factor (CSF-1), and IFN-γ were measured. PGD_2 induced the production of IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1 in a dose-dependent manner (Fig 4, *A*). The EC_{50} of PGD_2 for IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1 was 79.8, 65.7, 47.4, 43, 132.5, and 29.2 nmol/L, respectively. No IL-17A, IL-17F, or IFN-γ was detected (data not shown). As for type 2 cytokines, the production of IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1 induced by PGD_2, both at mRNA and protein levels, was enhanced by combination with IL-33 and IL-25. This enhancement was particularly significant for IL-8, IL-9, and GM-CSF production (Fig 4, *B*). In contrast, the mRNA level of IFN-γ was downregulated by PGD_2 at nanomolar concentrations (Fig 4, *C*). The regulatory effects of PGD_2 on these cytokines, whether activating or inhibitory, were reversed by TM30089 (1 μmol/L; Fig 4, *B* and *C*).

PGD_2 upregulates IL-33 receptors but downregulates CRTH2 expression in human ILC2s

To explore the potential interaction between IL-33/IL-25–mediated and PGD_2-mediated immune responses, we examined the effect of these activators on the expression of their receptors in ILC2s (Fig 5). After 2.5 hours of stimulation with PGD_2, mRNA levels for ST2 was increased significantly, mRNA levels for the IL-17RA subunit of the IL-25 receptor were also upregulated slightly, and mRNA levels for CRTH2 were reduced markedly (Fig 5, *A*, and see Fig E3 in this article's Online Repository at www.jacionline.org). The effect on the expression of the IL-17RB subunit of the IL-25 receptor was minor. Treatment with IL-33 or IL-25 alone had no significant effect on the expression of these receptors at this time point; however, the combination of PGD_2, IL-33, and IL-25 enhanced the upregulation of ST2 mRNA (Fig 5, *A*). The CRTH2-dependent regulation of these receptors was inhibited by TM30089.

To verify the regulation of these receptors at the protein level, the expression of ST2 and CRTH2 on the cell surface of ILC2s was analyzed by using fluorescence-activated cell sorting after treatment with PGD_2 (150 nmol/L) in the presence or absence of TM30089 (1 μmol/L; Fig 5, *B* and *C*). ST2-positive cells increased from 14.3% to 25.2% after 4 hours of treatment with PGD_2, and this was inhibited by TM30089 (Fig 5, *B*). Decreased expression of CRTH2 was detected after 6 hours of treatment with PGD_2, and the blockade of CRTH2 activity by using TM30089 inhibited this downregulation (Fig 5, *C*).

Human mast cell–derived PGD_2 triggers ILC2s through CRTH2

Mast cells are the major source of PGD_2 during allergic responses.[40,41] The effect of endogenously synthesized PGD_2 from activated human mast cells on ILC2s was examined to confirm the activation of CRTH2 in ILC2s under physiologic

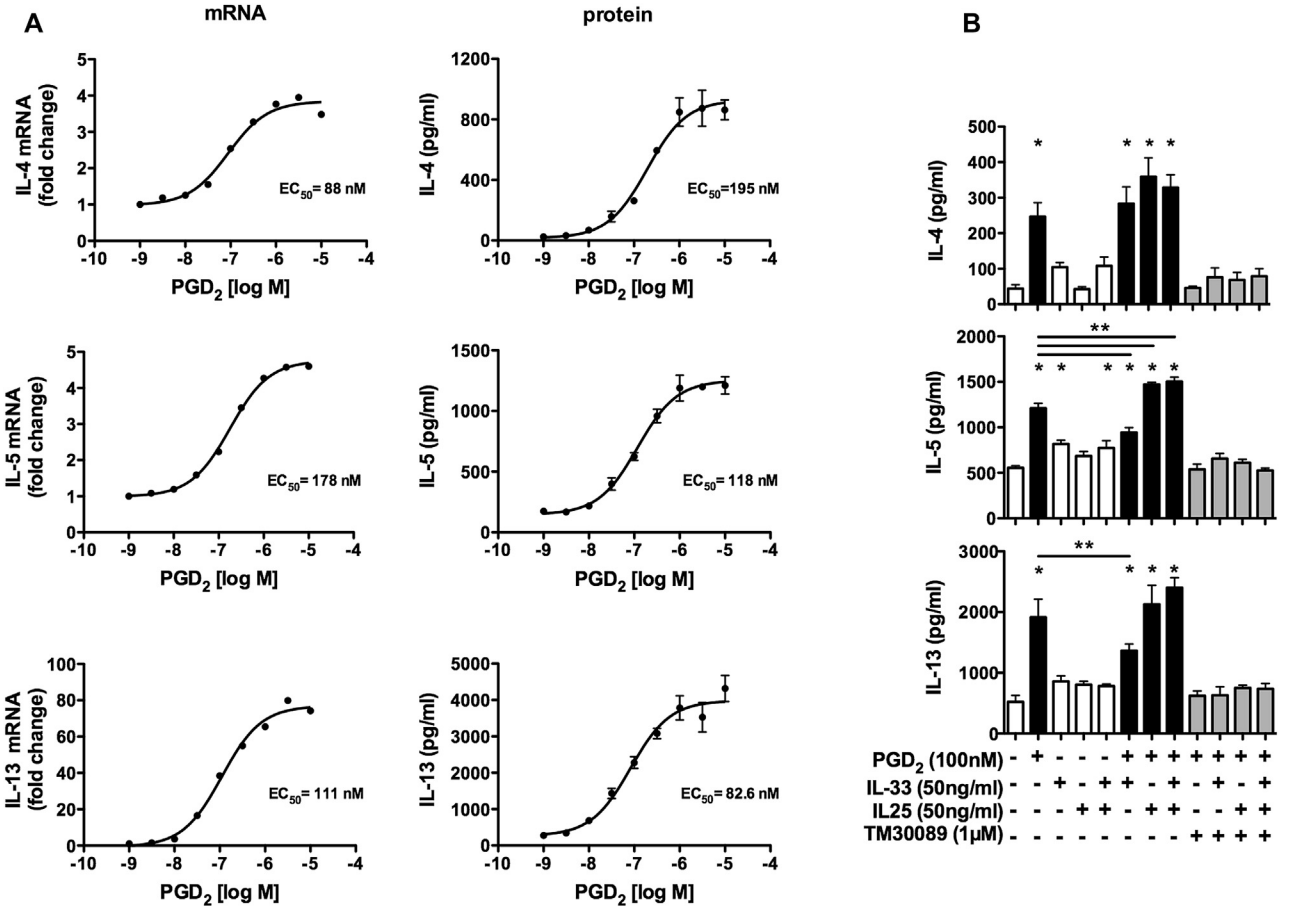

FIG 3. CRTH2 mediates type 2 cytokine production in ILC2s (skin) in response to PGD_2. **A,** mRNA levels of cytokines in cells (mRNA) and cytokine concentrations in supernatants (protein) after incubation with various concentration of PGD_2. **B,** Concentrations of cytokines released after treatments are as indicated. $*P < .05$ between control and other treatments and $**P < .05$ between PGD_2 and indicated treatments (n = 3).

conditions. Only low levels of PGD_2 (<0.1 ng/2 × 10^6 cell/mL) were detectable in supernatants from resting mast cells. After activation with IgE followed by anti-IgE antibody cross-linking, mast cell cultures produced high PGD_2 levels (>11 ng/2 × 10^6 cell/mL; Fig 6, *A*). Cotreatment of IgE/anti-IgE–activated mast cells with diclofenac (10 μmol/L), an inhibitor of COX-2, during the period of anti-IgE stimulation abolished PGD_2 production (<0.2 ng/2 × 10^6 cell/mL; Fig 6, *A*). Only very low levels of IL-13 (<200 pg/2 × 10^6 cell/mL) could be detected in any of these mast cell supernatants.

The supernatants of these mast cell treatments were used to test the effects of endogenous PGD_2 in human ILC2s. Notably, the capacities of the supernatants to activate ILC2s were dependent on the PGD_2 levels in the supernatants (Fig 6 and see Fig E4 in this article's Online Repository at www.jacionline.org). The supernatant containing high levels of PGD_2 (supernatant 2) but not the supernatant derived from the resting mast cells (supernatant 1) induced strong cell migration (Fig 6, *B*) and type 2 cytokine production (Fig 6, *C*). Treatment of ILC2s with supernatant 2 also caused the production of other proinflammatory cytokines (IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1; see Fig E4). Blockade of PGD_2 synthesis with diclofenac (supernatant 3) removed most of the capacity to stimulate ILC2s, particularly for type 2 cytokines (Fig 6, *B* and *C*), although the effect of diclofenac on production of IL-3, IL-9, and CSF-1 was not significant (see Fig E4). These ILC2 cell responses to supernatant 2 were blocked by TM30089 (Fig 6, *B* and *C*, and see Fig E4). BWA868C, an antagonist for D prostanoid receptor (another PGD_2 receptor), and montelukast, an antagonist for cysteinyl leukotriene receptor 1 ($CysLT_1$), were used to further confirm the receptor involved (see Fig E5 in this article's Online Repository at www.jacionline.org). Montelukast, but not BWA868C, inhibited production of IL-3, IL-13, and GM-CSF significantly in ILC2s in response to supernatant 2, and combination of TM30089 and montelukast blocked the response completely.

Similar to the results from experiments with exogenous PGD_2, the supernatant from activated mast cells upregulated the mRNA of ST2 mRNA significantly and IL-17RA weakly and downregulated CRTH2 mRNA in ILC2s (Fig 6, *D*). These effects were also inhibited by TM30089.

DISCUSSION

Activation of group 2 ILCs leads to the production of classical type 2 cytokines, thus promoting type 2 immunity. Increased numbers of ILC2s have been observed in inflamed tissues, such as allergic lung tissue in mice[18,19] and nasal polyps[20] and skin[21] in

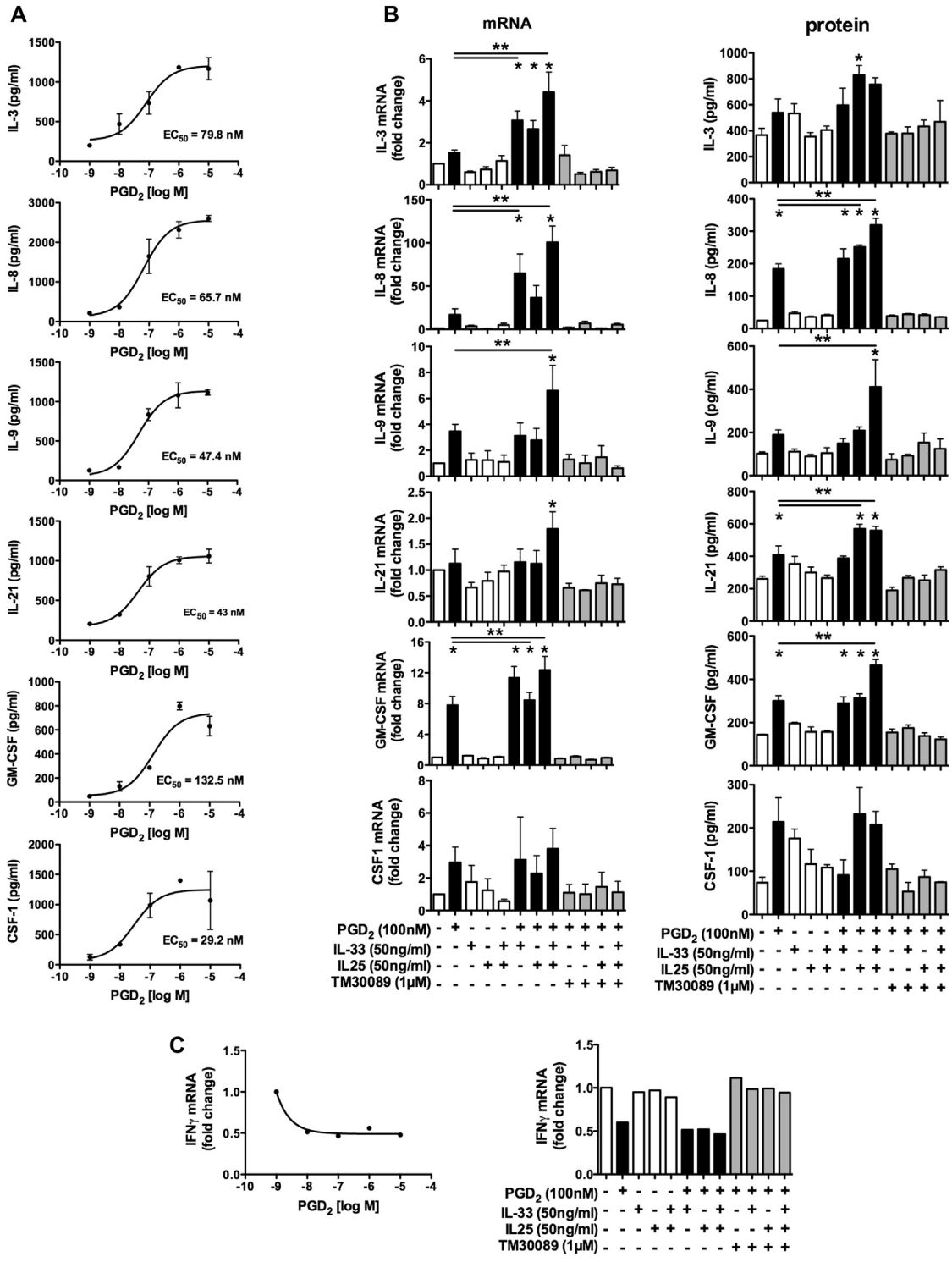

FIG 4. Activation of CRTH2 evokes proinflammatory cytokine production in ILC2s (skin). **A,** Cytokine concentrations after stimulation with PGD_2. **B,** mRNA levels of cytokines (mRNA) and concentrations of cytokines (protein) after treatments, as indicated. **C,** mRNA level of IFN-γ after treatments. $*P < .05$ between control and other treatments and $**P < .05$ between PGD_2 and indicated treatments (n = 3).

human subjects. It has been recently shown that CRTH2 is expressed in human ILC2s and that the activation of this receptor leads to IL-13 release from the cells.[20,22] Here we have shown that PGD_2 elicits many strong proinflammatory responses in *ex vivo* ILC2s isolated from human skin and blood. In contrast to Kim et al,[21] who did not identify $CD161^+CRTH2^+$ ILC2s in healthy human skin, we managed to isolate these cells from the normal human skin, although they were in low proportion. PGD_2 induced migration of these cells and promoted production of type 2 cytokines (IL-4, IL-5, and IL-13) and many other proinflammatory

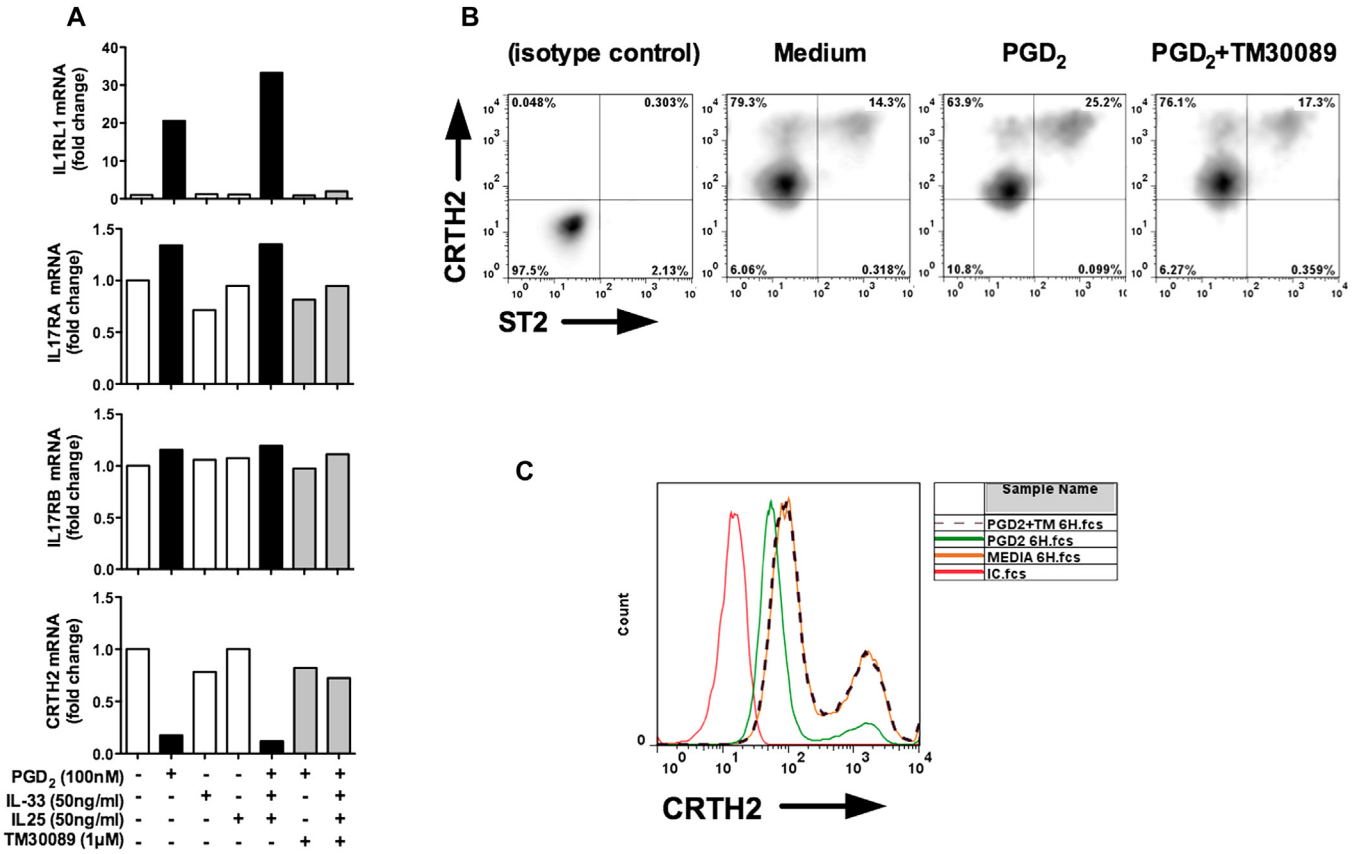

FIG 5. Activation of CRTH2 modulates receptor expression in ILC2s (**A,** blood; **B** and **C,** skin). Fig 5, *A*, mRNA levels of receptor genes after treatments (n = 3). The expression of ST2 (Fig 5, *B*) and CRTH2 (Fig 5, *C*) in ILC2s after incubation with medium or PGD_2 with or without TM30089 for 4 (Fig 5, *B*) or 6 (Fig 5, *C*) hours, as determined by using fluorescence-activated cell sorting, is shown.

cytokines (IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1). The stimulatory effect of PGD_2 was mediated by CRTH2 because it was inhibited completely by a specific CRTH2 antagonist TM30089.[32] These proinflammatory roles of CRTH2 in ILC2s could be confirmed under pathophysiologic conditions by using endogenously synthesized PGD_2 from human mast cells activated through IgE binding. Therefore our study reveals a potent mechanism for ILC2 activation in type 2 immunity.

A number of studies have recently identified the epithelium-derived cytokines IL-25 and IL-33 as critical activators of ILC2-mediated innate immunity against parasite infection and responses to allergen challenge.[15,42,43] Lack of these cytokines delays the onset of type 2 responses mediated by ILC2s in mouse models.[5,44,45] In our studies of human ILC2s, administration of IL-33 initiated cell migration and type 2 cytokine production. IL-25 also induced cytokine production, although the effect on chemotaxis was marginal. However, the efficacy of IL-25 and IL-33 was weaker than that of PGD_2 during the tested time points, suggesting that PGD_2 could be another important activator of ILC2s. As reported by Barnig et al,[22] combination treatment with PGD_2, IL-33, and IL-25 enhanced cytokine production by ILC2s, although no synergistic effect on chemotaxis was seen. Interestingly, activation of CRTH2 strongly upregulated expression of the IL-33 receptor ST2 and moderately upregulated the IL-25 receptor subunit IL-17A. Therefore IL-25, IL-33, and PGD_2 could act in concert in ILC2-mediated immune responses.

ILC2s are enriched at sites of inflammation after parasitic infection or allergic challenge,[14,18-20] but the mechanism involved in their recruitment remains obscure. IL-33 caused ILC2 migration in a dose-dependent manner, although the efficacy of IL-33 was weaker than that of PGD_2. The migration of ILC2s toward PGD_2 was completely inhibited by a CRTH2 antagonist, implying that CRTH2 is an important chemoattractant receptor in human ILC2s. Neither IL-25 nor IL-33 potentiated the migration of ILC2s in response to PGD_2, suggesting that if the 3 activators coexisted in inflamed tissue, PGD_2 could serve as a dominant contributor to the recruitment cascade of ILC2s.

It is well established that activation of ILC2s is characterized by the production of high levels of type 2 cytokines that in turn affect antibody class-switching, recruitment of inflammatory effector cells (eg, eosinophils, basophils, and mast cells), and goblet cell hyperplasia leading to mucus production, all of which contribute to the immune responses to parasite infection, allergen challenge, and tissue damage.[1,6,7] In this study we demonstrated that ILC2s are capable of producing many other proinflammatory cytokines after activation, including IL-3, IL-8, IL-21, GM-CSF, and CSF-1. These cytokines could also play important roles in orchestrating ILC2-mediated immune responses. IL-3 can be critical for the growth and differentiation of $CD34^+$ progenitor cells into basophils and mast cells and monocytes into dendritic cells.[46,47] IL-8 is a potent chemokine for neutrophils,[48,49] a cell type that is associated with severe asthma.[50,51] IL-21 can induce

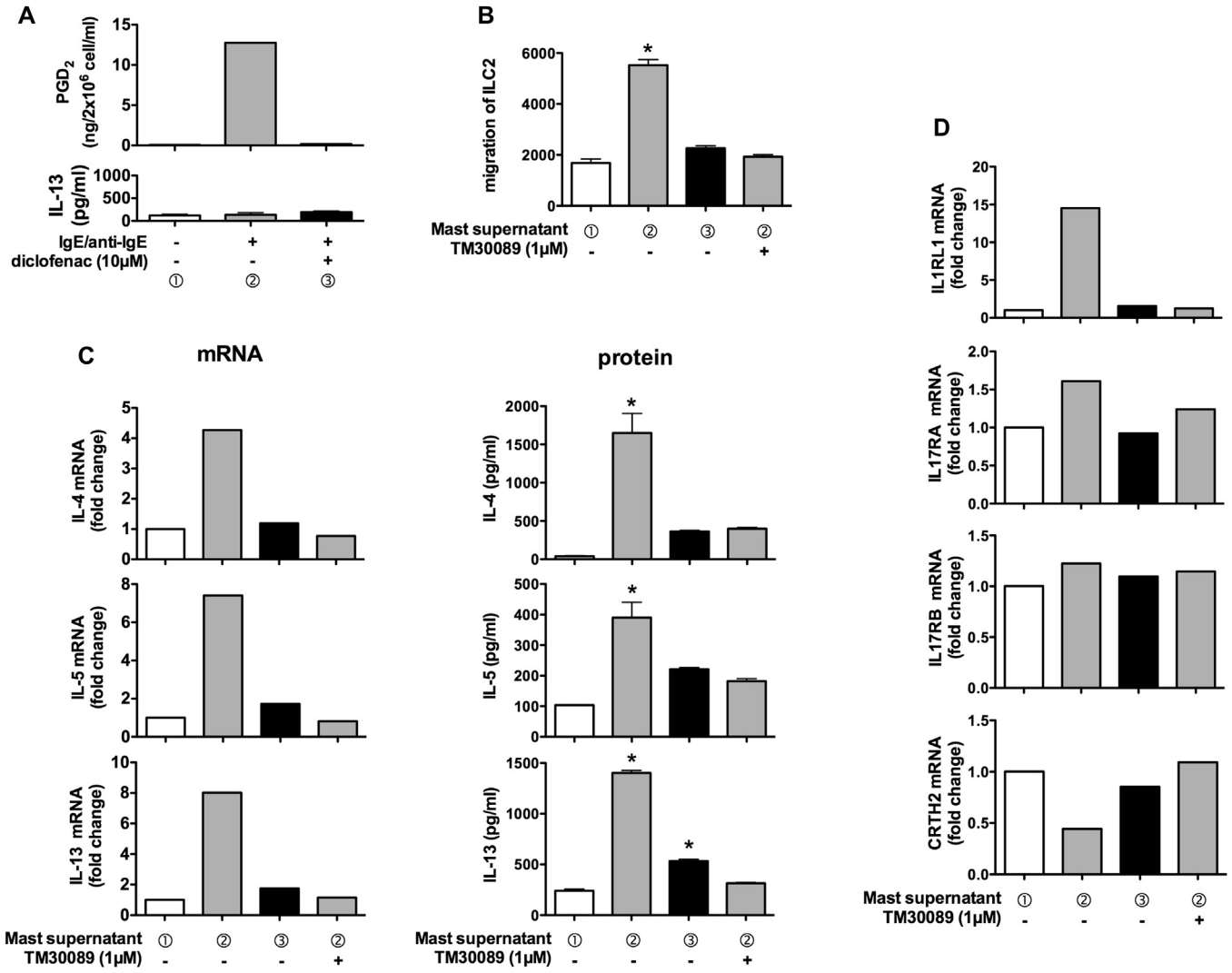

FIG 6. Effect of mast cell supernatants on activation of ILC2s (skin) is mediated by CRTH2. **A,** Levels of PGD_2 and IL-13 in supernatants of mast cells treated with medium *(white bars)* or IgE/anti-IgE antibody with *(black bars)* or without *(gray bars)* diclofenac. Supernatants were assigned as supernatants 1 to 3. **B,** ILC2 migration after exposure to supernatants with or without TM30089. **C** and **D,** mRNA and protein levels of cytokines (Fig 6, *C*) and mRNA levels of receptors (Fig 6, *D*) in ILCs after incubation with supernatants with or without TM30089 for 3 hours. $*P < .05$ (n = 2).

inflammation in mice through regulation of recruitment of neutrophil and monocyte populations[52] and is also involved in the pathogenesis of allergic disorders and autoimmune diseases (including inflammatory bowel diseases, rheumatoid arthritis, psoriasis, and systemic lupus erythematosus) by controlling the growth, survival, differentiation, and function of T and B cells.[53-57] GM-CSF and CSF-1 also contribute to allergic and autoimmune diseases.[58,59] GM-CSF is critical for eosinophil and neutrophil survival and their activities.[60,61] Overexpression of GM-CSF in mice enhances and anti–GM-CSF antibodies inhibit allergic sensitization and airway inflammation.[62-64] IL-3 and GM-CSF are coordinately induced with IL-4, IL-5, IL-9, and IL-13, and their genes also cluster on the same chromosome locus, 5q31-33, a major susceptibility locus for asthma and atopy.[65] In contrast, the activation of CRTH2 downregulated gene transcription levels of IFN-γ in ILC2s, suggesting that CRTH2 signaling could potentially favor viral infection. In fact, an unexpected efficacy in reduction of viral infection by one CRTH2 drug has been observed in clinical trials.[66] Therefore through activation of CRTH2, ILC2s might be involved in other as yet unrecognized immune responses.

PGD_2 is the major arachidonic acid metabolite released from mast cells during allergic responses.[40,41,67] High concentrations of PGD_2 are detected in the airways of asthmatic patients challenged with allergen,[68] and increased activation of the PGD_2 pathway has been found in patients with severe asthma.[69] To determine whether CRTH2-mediated activation of ILC2s was functioned under physiologic conditions, we examined the effect on ILC2s of endogenously synthesized PGD_2 from human mast cells. The ILC2 cell responses to mast cell supernatants were similar to those seen to exogenously synthesized PGD_2. The only difference was that some responses to the mast cell supernatants could not be completely blocked by the CRTH2 antagonist or by inhibition of PGD_2 synthesis. This could be caused by the

presence of other active mediators released from activated mast cells in the supernatant, which drive production of specific cytokines. Our data with montelukast suggested that cysLTs are also important ILC2 stimulators. Mast cells are found mainly in epithelial barriers, such as skin and mucosal tissues, and increase in number after exposure to allergens.[70] In mouse skin ILC2s migrated specifically toward and interacted with skin-resident mast cells,[14] and ILCs were also found in proximity to tissue mast cells in human lungs.[22] Therefore ILC2s can also contribute to mast cell–mediated type 2 immunity.[11] Although multiple stored or *de novo*–synthesized inflammatory mediators are released from activated mast cells,[37] it is striking that ILC2 migration and type 2 cytokine production in response to mast cell supernatant can be inhibited mostly by CRTH2 antagonism (Fig 6), making it likely that PGD_2/CRTH2 serves as a dominant link between activated mast cells and activation of ILC2s. Mast cells orchestrate adaptive type 2 immunity to helminths or allergen through IgE/FcεRI-dependent activation.[71] However, mast cells can also be nonspecifically activated in IgE/FcεRI-independent ways by substances such as peptides, basic compounds, anaphylatoxins, dextrans, and cytokines.[71-73] Many studies have revealed the critical role of PGD_2/CRTH2 in adaptive type 2 immunity, particularly in mast cell–mediated activation of T_H2 cells and eosinophils.[25,26,28,29,31] Here we further extend their role to the activation of ILC2s. Beyond this, PGD_2 production can also be induced by innate responses, such as macrophages activated by double-stranded RNA through Toll-like receptor 3.[74] Therefore ILC2 activation induced by PGD_2 could be mediated by either innate or adaptive immune pathways.

Our previous study revealed that the type 2 cytokine production in human T_H2 cells mediated by CRTH2 was markedly enhanced by another group of mast cell mediators, cysLTs.[30] A recent report has described that ILC2s in lungs of mice express $CysLT_1$, which regulates type 2 cytokine production.[75] We have also confirmed the expression of $CysLT_1$ in human ILC2s (data not shown). The combination of TM30089 and montelukast enhanced their inhibitory effect on cytokine production in ILC2s in response to mast cell supernatant. This suggests that CRTH2 and leukotriene receptors could also act synergistically in mast cell–mediated human ILC2 activation. Furthermore, by producing cytokines (IL-3, IL-4, and IL-13), activation of ILC2s could in turn enhance mast cell activation. Given the association with tissue mast cells and allergic skin disease, it might be that the inhibition of PGD_2-mediated recruitment and activation of ILC2s through CRTH2 might provide a therapeutic opportunity for atopic dermatitis.

In conclusion, the current study highlights the important proinflammatory role of CRTH2 and its ligand, PGD_2, in human ILC2s, and potential roles of ILC2s in IgE/mast cell/CRTH2–mediated adaptive immune cascades. In addition to IL-25 and IL-33, PGD_2 is clearly another important and potent driving force in ILC2 activation. It can directly stimulate ILC2s through CRTH2 and can also potentiate IL-25/IL-33–mediated innate responses. Through IgE-mediated mast cell degranulation, ILC2s can contribute to both innate and adaptive type 2 immunity, and through upregulation of IL-33/IL25 receptors and synergistic interaction with these receptors, CRTH2 plays a pivotal role in bridging innate and adaptive pathways in ILC2s.

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Key messages

- **PGD_2 activates human ILC2s through CRTH2 and induces strong proinflammatory responses, which can serve as a potential therapeutic opportunity for IgE/mast cell/ILC2–mediated allergic inflammation.**
- **Through sensing IgE-mediated mast cell degranulation, ILC2s can contribute to both innate and adaptive type 2 immunity.**
- **Through upregulation of IL-33/IL-25 receptors and synergistic interaction to these receptors, CRTH2 plays a pivotal role in bridging innate and adaptive pathways in human ILC2s.**

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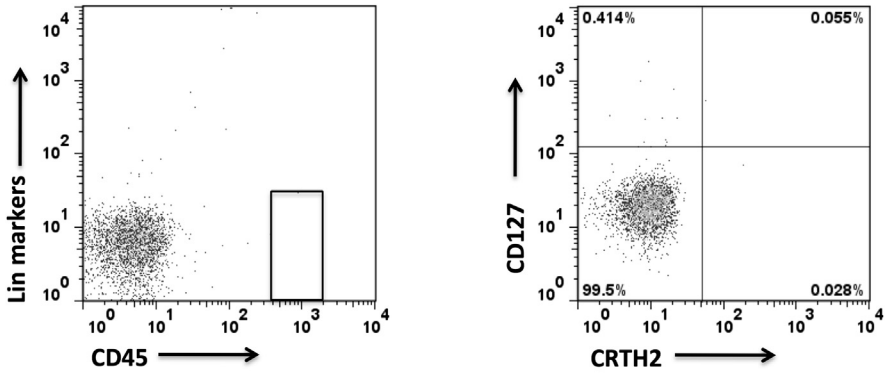

FIG E1. Isotype controls for ILC2 isolation (Fig 1).

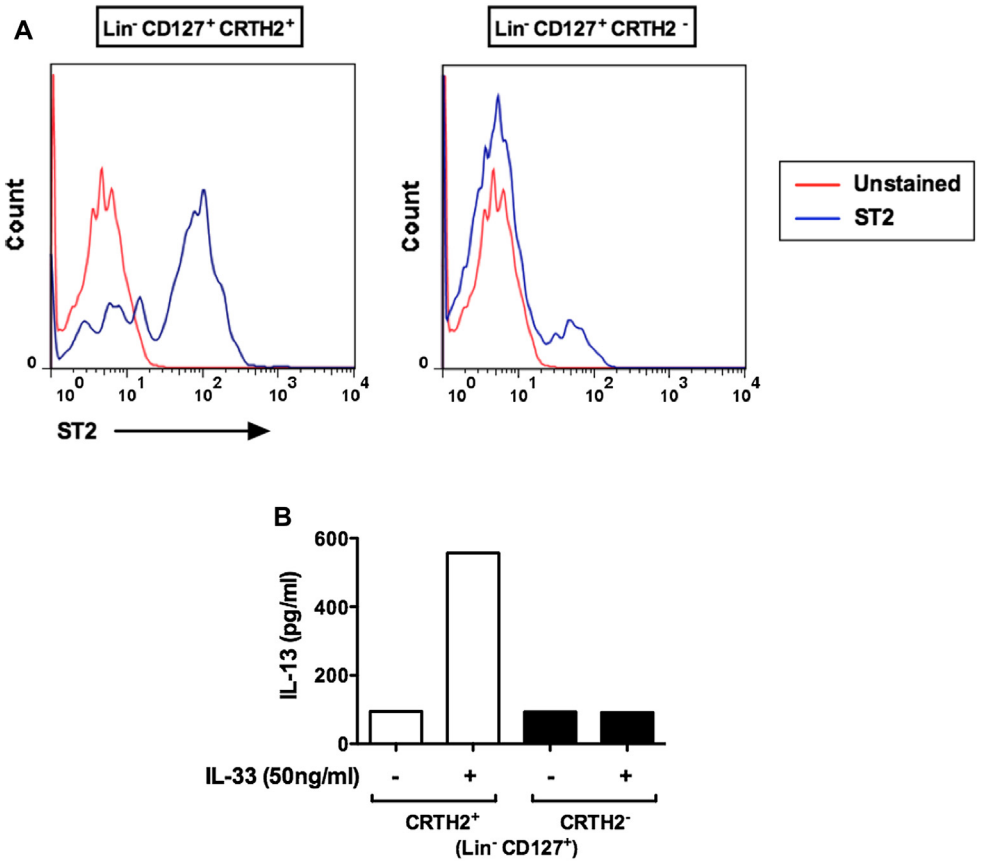

FIG E2. Comparison of Lin$^-$CD127$^+$CRTH2$^-$ and Lin$^-$CD127$^+$CRTH2$^+$ cells from human skin. **A,** Expression of ST2 *(blue line)* on CRTH2$^-$ cells was much lower than expression on CRTH2$^+$ cells. The *red line* shows unstained cells. **B,** Lin$^-$CD127$^+$CRTH2$^+$ *(white columns)* but not Lin$^-$CD127$^+$CRTH2$^-$ cells *(black columns)* responded to IL-33 stimulation by IL-13 production (n = 2).

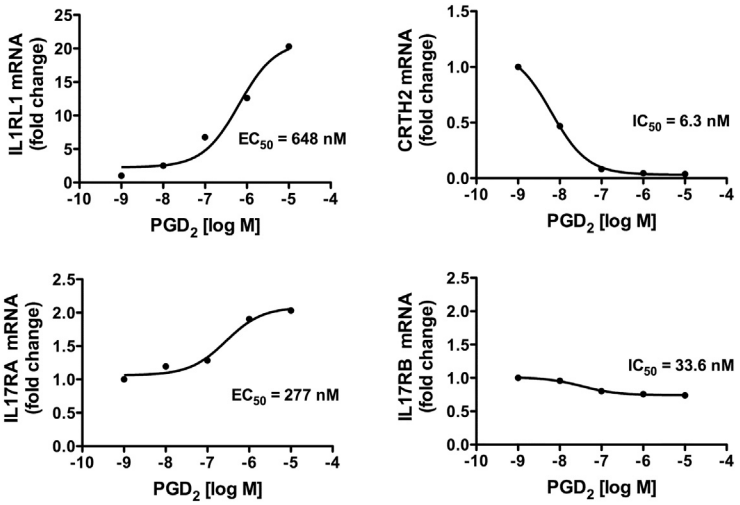

FIG E3. Expression of ST2, IL-17RA, and CRTH2 in ILC2s (skin) is regulated by PGD_2 in a dose-dependent manner. The mRNA level of ST2, CRTH2, IL-17RA, and IL-17RB in the cell pellets of ILC2s after stimulation with various concentrations of PGD_2 is shown. The mRNA levels in the cells treated with 1 nmol/L PGD_2 were treated as 1-fold (n = 2).

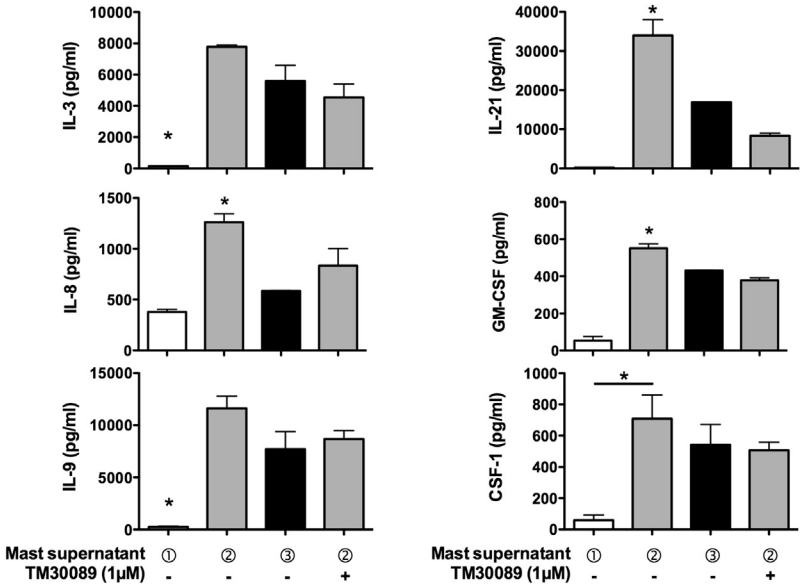

FIG E4. CRTH2 mediates proinflammatory cytokine production in ILC2s (skin) in response to supernatants from activated mast cells. Concentrations of IL-3, IL-8, IL-9, IL-21, GM-CSF, and CSF-1 in supernatants after ILC2 incubation with 1:1.5 diluted supernatants of mast cells treated with medium *(white bars)* or IgE/anti-IgE antibody with *(black bars)* or without *(gray bars)* diclofenac in the presence or absence of TM30089 for 3 hours. $*P < .05$ (n = 2).

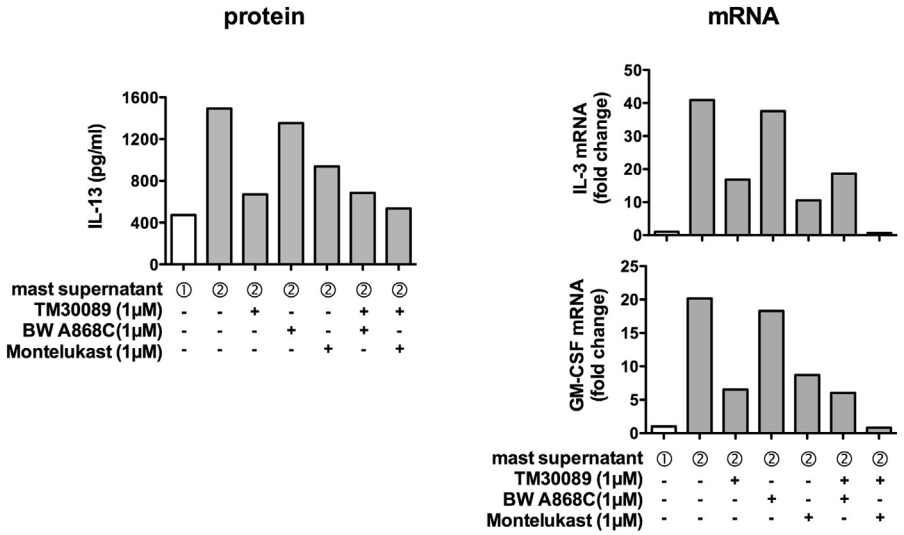

FIG E5. Cytokine production by ILC2s (skin) in response to supernatants from activated mast cells is inhibited by CysLT$_1$ antagonist partially but not by D prostanoid receptor antagonist. The effects of TM30089, BWA868C, montelukast, and their combination on the production of IL-13 (protein), IL-3, and GM-CSF (mRNA) in ILC2s treated with the supernatant from IgE/anti-IgE-activated mast cells *(gray bars)* were examined with ELISA or quantitative RT-PCR (n = 1).

TABLE E1. Antibody list used for ILC2 purification

Antigen	Clone	Supplier
CD3	SK7	BD Biosciences, San Jose, Calif
CD19	SJ25C1	BD Biosciences
CD123	FAB301C	R&D Systems, Minneapolis, Minn
CD11b	DCIS1/18	Abcam, Cambridge, United Kingdom
CD11c	BU15	Abcam
CD8	RPA-T8	BioLegend, San Diego, Calif
FcεRI	AER-37 (CRA-1)	BioLegend
CD14	MφP9	BD Biosciences
CD4	MEM-241	Abcam
CD45	H130	BioLegend
CD56	B159	BioLegend
CRTH2	BM16	Miltenyi Biotec, Bergisch Gladbach, Germany
IL-7Rα	A019D5	BioLegend
ST2	Ab72778	Abcam

TABLE E2. Primers and probes used for quantitative RT-PCR

Gene	Primer	Probe no.
IL4	5′-CACCGAGTTGACCGTAACAG-3′ 5′-GCCCTGCAGAAGGTTTCC-3′	16
IL5	5′-GGTTTGTTGCAGCCAAAGAT-3′ 5′-TCTTGGCCCTCATTCTCACT-3′	25
IL13	5′-AGCCCTCAGGGAGCTCAT-3′ 5′-CTCCATACCATGCTGCCATT-3′	17
IL17A	5′-TGGGAAGACCTCATTGGTGT-3′ 5′-GGATTTCGTGGGATTGTGAT-3′	8
IL17F	5′-GGCATCATCAATGAAAACCA-3′ 5′-TGGGGTCCCAAGTGACAG-3′	10
IFNG	5′-GGCATTTTGAAGAATTGGAAAG-3′ 5′-TTTGGATGCTCTGGTCATCTT-3′	21
CRTH2	5′-CCTGTGCTCCCTCTGTGC-3′ 5′-TCTGGAGACGGCTCATCTG-3′	43
IL1RL1	5′-TTGTCCTACCATTGACCTCTACAA-3′ 5′-GATCCTTGAAGAGCCTGACAA-3′	56
IL17RA	5′-CATCCTGCTCATCGTCTGC-3′ 5′-GCCATCGGTGTATTTGGTGT-3′	85
IL17RB	5′-GCCCTTCCATGTCTGTGAAT-3′ 5′-CCGGCCTTGACACACTTT-3′	64
GAPDH	5′-AGCCACATCGCTCAGACAC-3′ 5′-GCCCAATACGACCAAATCC-3′	60

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.