



IFN- γ and TNF- α potentiate prostaglandin D2-induced human eosinophil chemotaxis through up-regulation of CRTH2 surface receptor

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ARTICLE INFO

Article history:

Received 26 February 2010

Received in revised form 21 July 2011

Accepted 21 July 2011

Available online 9 August 2011

Keywords:

IFN

TNF

CRTH2

Eosinophils

Allergic rhinitis

ABSTRACT

Prostaglandin D2 (PGD2) receptor CRTH2, is a pro-inflammatory molecule involved in eosinophil recruitment to the allergic airway. We investigated the expression of CRTH2 in eosinophil from allergic rhinitis patients (AR) and tested the modulatory role of several TH1 and TH2 cytokines closely related to the allergic immunological response, on the expression of CRTH2 receptor, utilizing human eosinophil cell line (Eo1-1). The expression of CRTH2 was tested by immunohistochemistry and flow cytometry (FACS). Chemotaxis was performed in micro-chemotaxis chambers. It is shown that the expression of CRTH2 by eosinophils was significantly higher in the nasal tissue and peripheral blood of AR patients, when compared to control subjects. PGD2 exhibited a typical bell shape dose response in attracting eosinophil from AR patients with optimal activity at 10^{-7} M. Eo1-1 cell surface expression of CRTH2 was significantly up-regulated by 10 ng/ml IFN- γ and TNF- α . The percentage of Eo1-1 cells expressing the receptor increased by IFN- γ and TNF- α from $12.74\% \pm 2.66$ to $55\% \pm 8$ and $33.8\% \pm 9.4$, respectively. PGD2-induced Eo1-1 chemotaxis was not blocked by SB203580, H-89 Dihydrochloride, Bisindolylmaleimide, or Genistein. PGD2-induced Eo1-1 chemotaxis was potentiated by IFN- γ and TNF- α without changing the signal transduction pathway. Correlation of our results to peripheral blood eosinophils from allergic rhinitis patients confirmed that 3 hour pretreatment of eosinophils by 10 ng/ml IFN- γ and TNF- α , increased the mean fluorescence intensity (MFI) of CRTH2 from 8.23 to 9.68 and 9.38, respectively, and potentiated PGD2-induced eosinophil chemotaxis. Our results demonstrate a novel synergism between PGD2, IFN- γ and TNF- α , in eosinophil chemotaxis.

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1. Introduction

Mast cell-derived PGD2 is an important mediator involved in eosinophilic airway inflammation [1,2]. One of the two recognized receptors for PGD2 is CRTH2 that is a seven-transmembrane G-protein-coupled receptor (GPCR). It is composed of 395 amino acids residues with lower homology to other protanoid receptors [3]. Its expression by eosinophil in nasal mucosa speculates a possible role for CRTH2 in eosinophilic nasal inflammation [4,5]. Furthermore, this novel chemoattractant receptor-homologous molecule (CRTH2) expressed by T_H2 cells and eosinophil gained a lot of attention as promoter for PGD2 induced eosinophilia in allergic airway [6–9].

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Recently, in an allergic rhinitis murine model it has been demonstrated that the PGD2–CRTH2 interaction is elevated following pollen sensitization. This resulted into specific IgE and IgG1 production, nasal eosinophilia and IL-4 and IL-5 production by submandibular lymph node cells. Additionally, CRTH2 mRNA in nasal mucosa was significantly elevated in Cry j 1-sensitized mice [10].

Immunological responses have the tendency to either induce T_H1 cytokine profile or T_H2 cytokine profile. T_H2 predominantly secretes IL-4 and IL-5 while T_H1 predominantly secretes IL-2, TNF- α and IFN- γ . Both subtypes share the secretion of IL-3, IL-10 among others. On the other hand the primary function of IL-1, IL-7 and IL-15 is to promote maturation and survival of T cells. However, these latter cytokines have been shown to possess pro-inflammatory actions in eosinophilic allergic airway diseases such as AR and asthma [11–13]. In type 1 hypersensitivity immunological response, it is the T_H2 profile that dominates the scene, resulting into a pool of cytokines at the site of inflammation, many of which work in synergism recruiting eosinophils. On the other hand, the role of T_H1 cytokines in eosinophil recruitment

is a modulatory one [14–19]. Nonetheless, little is known about the effect of different T_H1 and T_H2 cytokines in CRTH2 modulation in eosinophils.

Accordingly, the current study was designed to investigate the effects of several cytokines closely related to the immunological response on Eo1-1 expression of CRTH2. This cell line is ideal to study proteins and the ability of n-butyrate to induce their differentiation to mature eosinophils also makes them ideal for the study of allergic inflammation in diseases such as allergic rhinitis and asthma. Functional correlation of the results was further studied in freshly isolated peripheral blood eosinophil from allergic rhinitis patients.

2. Materials and methods

2.1. Patients and control subjects

After obtaining their consent, a total of 26 patients and 10 healthy control subjects participated in the current study. All patients were allergic to aeroallergens as confirmed by skin tests and radioallergo-sorbant test (RAST). These patients presented with poorly controlled AR symptoms to our rhinology clinic. As for the immunohistochemical studies, samples from the middle turbinate were obtained from AR

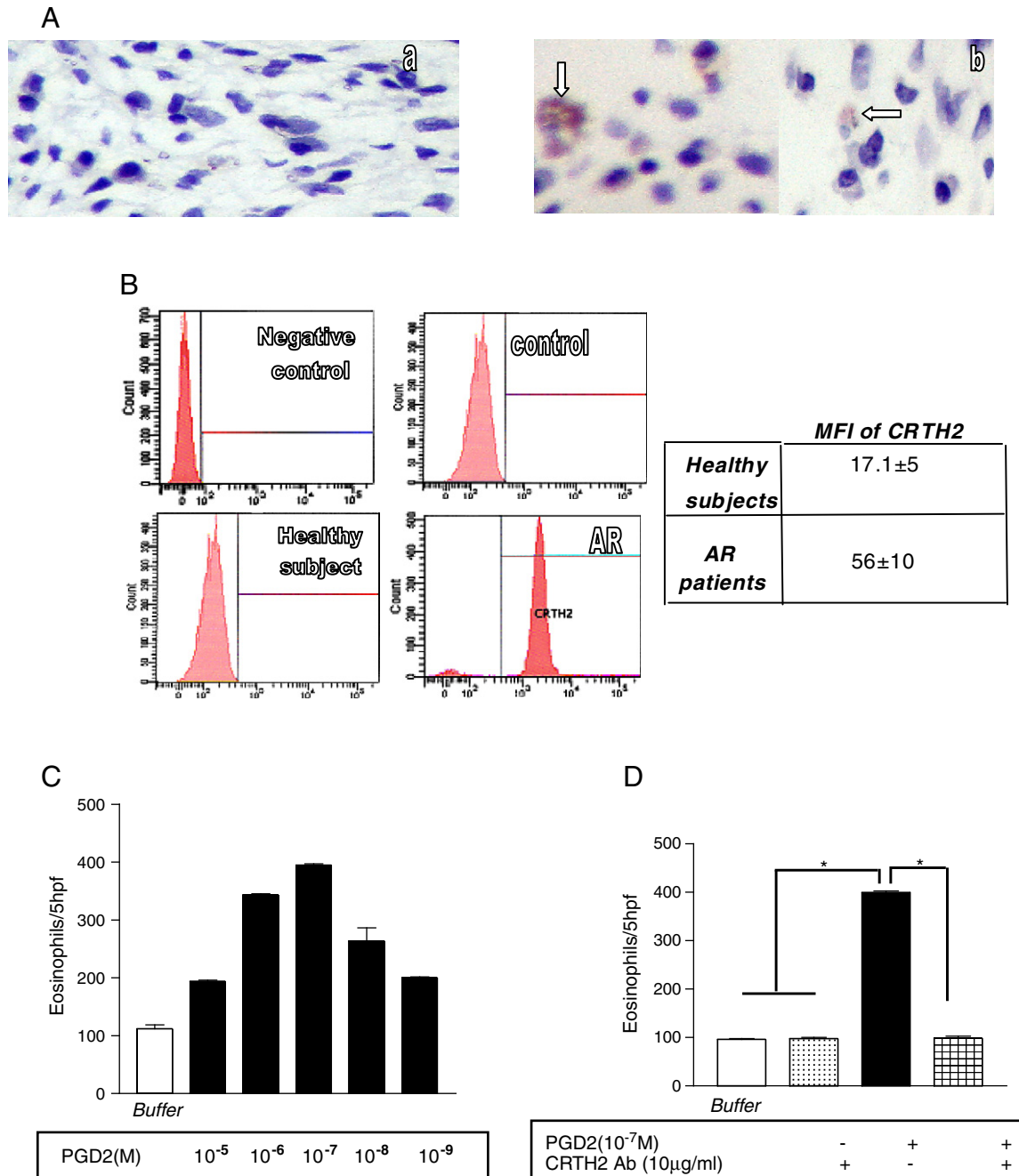


Fig. 1. (A) Immunohistochemistry analysis of CRTH2 expression by infiltrating eosinophils from middle turbinate mucosa of (a) control subject representative of 5 ($n=5$), and (b) AR patient representative of 5 ($n=5$). (B) FACS analysis of CRTH2 receptor surface expression from peripheral blood eosinophils. Histograms are of 2 subject's representative of 12 showing similar results. (C) PGD2 eosinophilotactic dose response from AR patients. (D) Effect of anti-CRTH2 blocking Ab in PGD2-induced eosinophil chemotaxis. Results are the mean $\pm$ SEM of 6 independent experiments performed in triplicate.

Table 1

(A) Effect of different kinase inhibitors on PGD₂-induced eosinophil chemotaxis. (B) Priming of EoL-1 chemotaxis by 10 ng/ml IFN- γ against PGD₂ in the presence and absence of different kinases inhibitors. (C) Priming of EoL-1 chemotaxis by 10 ng/ml TNF- α against PGD₂ in the presence and absence of different kinases inhibitors. Results are the mean $\pm$ SEM of 5 independent experiments.

Stimulant	Eosinophils/5 hpf
(A)	
Buffer	98 $\pm$ 2.6
PGD ₂ 10 ⁻⁷ M	341 $\pm$ 13.5
PGD ₂ + SB203580	358.4 $\pm$ 7
PGD ₂ + H-89 Dihydrochloride	369.4 $\pm$ 2.1
PGD ₂ + Bisino-lylmaleimide	335.4 $\pm$ 12.3
PGD ₂ + Genistein	405.8 $\pm$ 8.7*
(B)	
Buffer	99 $\pm$ 3
PGD ₂ 10 ⁻⁹ M	198 $\pm$ 5
PGD ₂ + IFN- γ	310 $\pm$ 7.2
PGD ₂ + IFN- γ + SB203580	305 $\pm$ 4.2
PGD ₂ + IFN- γ + H-89 Dihydrochloride	314 $\pm$ 6
PGD ₂ + IFN- γ + Bisino-lylmaleimide	299 $\pm$ 7.3
PGD ₂ + IFN- γ + Genistein	350 $\pm$ 10*
(C)	
Buffer	102 $\pm$ 4
PGD ₂ 10 ⁻⁹ M	200 $\pm$ 5
PGD ₂ + TNF- α	300 $\pm$ 7
PGD ₂ + TNF- α + SB203580	305 $\pm$ 12
PGD ₂ + TNF- α + H-89 Dihydrochloride	299 $\pm$ 8
PGD ₂ + TNF- α + Bisino-lylmaleimide	307 $\pm$ 12
PGD ₂ + TNF- α + Genistein	360 $\pm$ 10*

patients undergoing functional endoscopic surgery for complicated chronic rhinosinusitis.

2.2. Cell preparation

Eosinophils were isolated by Percoll (66%) solution separation. Eosinophils were further purified (98%) by negative selection immunomagnetic cell separation (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), using anti-CD16 magnetic beads.

2.3. EoL-1 cell line

Human eosinophilic leukemia (EoL-1) cell line (Riken BioResource Center, Japan) was used in the current biochemical study. Cell differentiation into mature eosinophils was induced by histone deacetylase inhibitors, n-butyrate precisely as described earlier [20].

2.4. Cell culture

EoL-1 cells (6×10^5 cells) were incubated at 37 °C–5% CO₂ with 1 ml RPMI-1640 supplemented with 100 U penicillin/ml and 100 μ g streptomycin/ml (Lonza, Verviers, Belgium), and 10% of inactivated fetal calf serum (Lonza) in 24 well plate (BD Biosciences). Cells were stimulated with 1 ng/ml IL-2 or 10 ng/ml IL-1 β , IL-3, IL-5, IL-7, IL-10, IL-15, TNF- α or IFN- γ (R&D Systems). After 24 h culture, cells were washed twice with PBS containing 0.2% serum albumin bovine (Sigma) and marked for cytometry.

2.5. FACS analysis

CRTH2 expression on EoL-1 and eosinophil was analyzed by FACS (FACS CANTO II BD Systems). Briefly, stimulated or unstimulated cells were incubated with anti-CRTH2 antibody (BD Biosciences) for 30 min in the dark for surface expression or cells were first permeabilized by 0.1% saponin (Sigma Aldrich) for 15 min before being incubated with the anti-CRTH2 antibody, for the total expression. The cells were then washed twice and conserved in 1% paraformaldehyde and then analyzed for their fluorescence intensity.

2.6. Chemotaxis experiments

Chemotaxis assays were performed in triplicate in a 48-well microchemotaxis Boyden chamber incubated in 5% CO₂ at 37 °C for 90 min. Aliquots of 29 μ l of the chemotactic agent PGD₂ (Cayman Chemical) at different molar concentrations were placed in the lower wells and 50 μ l of eosinophils (10⁶ cells/ml) or EoL-1 cells were placed in the upper wells. The two chambers were separated by a 5.0 μ m pore polycarbonate membrane (Nuclepore, Whatman, Middlesex, UK). Migrated cells adherent to the lower surface were counted in 5 selected high power fields/well under a light microscope (5 hpf; $\times 400$). As for the priming experiments, cells were first preincubated with 10 ng/ml IFN- γ or TNF- α , before the induction of their chemotaxis. As for the blocking experiments, the cells were pretreated with anti-human CRTH2 receptor antibody (R&D Systems), H-89 Dihydrochloride (VWR-CALBIOCHEM) a PKA inhibitor, Bisindolylmaleimide (VWR-CALBIOCHEM) a PKC inhibitor, SB203580 (VWR-CALBIOCHEM) a P38 MAP-Kinase inhibitor or Genistein (Sigma) a tyrosine kinase inhibitor for 1 h and during chemotaxis incubation period.

2.7. Immunohistochemistry

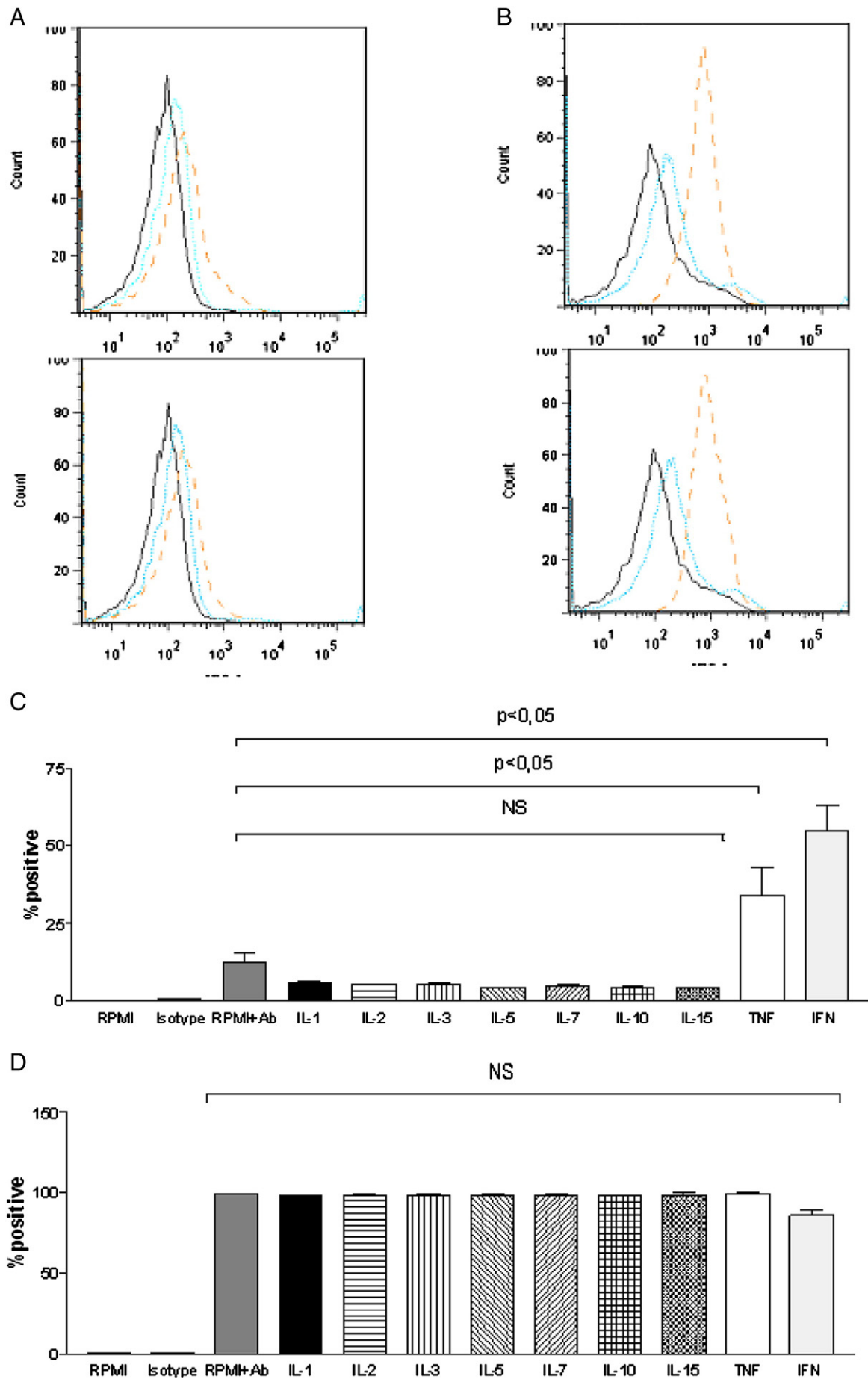
Sections of nasal specimens underwent immunoperoxidase staining using antibodies directed against CRTH2 (1:25) (HPA014259; Sigma). The sections were deparaffinized in xylene and rehydrated in methanol. Endogenous peroxidases were blocked by 5% H₂O₂ treatment. For better antigen retrieval of CRTH2, the samples were boiled in a microwave oven for 23 min in citrate buffer. Samples were then washed with PBS. Samples were then incubated with the primary antibody at room temperature for 1 h. After washings, the revelation was performed with the use of appropriate secondary antibody, the EnVision anti-rabbit kit (CRTH2; Dako A/S) according to the supplier's recommendations. Immunoreactivity was visualized by a treatment with diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA), and the slides were counterstained with Mayer's hematoxylin. For staining intensity, (–) represented samples in which the staining was undetectable, whereas (+) and (++) denoted samples with weak and strong staining, respectively.

2.8. Measurement of total CRTH2 protein expression

2.8.1. Preparation of cell extracts

For preparation of whole cell extracts, cells were pelleted by centrifugation and washed twice with phosphate-buffered saline. The cell pellets were re-suspended in cold RIPA lysis buffer (Tris HCl pH 7.6 25 mM; NaCl 150 mM; NP40 1%; sodium deoxycholate 1%; sodium dodecyl sulfate 0.1%; Pierce) supplemented with protease inhibitors (Complete, Roche) and subsequently swirled for 10 min on ice. The

Fig. 2. Expression of CRTH2 receptor by EoL-1 cells. (A) The levels of CRTH2 expressed on the cell surface of EoL-1, and (B) EoL-1 cells differentiated to mature eosinophils. Solid line = negative control (antibody isotype), dotted line = spontaneous expression while the dashed line = the expression after 24 h stimulation with 10 ng/ml IFN- γ (upper panel), or 10 ng/ml TNF- α (lower panel). (C) Percentage of EoL-1 cells expressing surface CRTH2 receptor spontaneously and in response to different cytokines or (D) total expression. Results are the mean $\pm$ SEM of 8 independent experiments. Statistical significance is by Mann–Whitney test.



extracts were then centrifugated at 14,000 g for 15 min at 4 °C. The supernatants were analyzed for protein content by the Bio-Rad protein assay based on Bradford method (Bio-Rad).

2.8.2. Western blotting

Western blot analysis was performed on proteins extracted after 24 h of treatment with TNF- α or IFN- γ at concentration of 10 ng/ml. Samples were separated on a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Roche). The anti-CRTH2 rabbit polyclonal antibody (ABCAM ab59382) was used at a 1:200 dilution. The secondary anti-rabbit antibody coupled with horseradish peroxidase (Amersham) at a 1:3000 dilution was detected by chemiluminescence with the ECL system (Pierce).

3. Statistical analysis

Statistical analysis was performed by Mann–Whitney and paired *t*-test. *P* value <0.05 was considered significant.

4. Results

4.1. Involvement of CRTH2 expression in allergic rhinitis

Fig. 1A demonstrates immunohistochemical studies of nasal tissue obtained from the middle turbinate as a part of the surgical procedure, from patients undergoing endoscopic sinus surgery, for allergic chronic rhinosinusitis (ACRS), and from control subjects operated for concha bullosa (pneumatized middle turbinate) with no allergy. Infiltrating eosinophils were strongly positively stained (++) for CRTH2 in ACRS samples compared to control subjects in whom cellular staining for CRTH2 was undetectable (–). Further, Fig. 1B shows that peripheral blood eosinophils from AR patients expressed significant amounts of CRTH2 molecules on their surface when compared to control healthy subjects. MFI was 17.1 ± 5 for the spontaneous expression from the control subjects, and 56 ± 10 for AR patients. These results indicate the involvement of CRTH2 in the pathophysiology of eosinophilic inflammation in AR.

4.1.1. PGD2 induced eosinophil chemotaxis

To characterize the functional role of CRTH2 in inducing eosinophil chemotaxis against its ligand PGD2, we next performed chemotaxis assays in modified Boyden chambers. PGD2 exhibited typical bell shape chemotactic curve with optimal activity at 10^{-7} M against eosinophils of AR patients (Fig. 1C). This effect was mediated by CRTH2 receptor, since CRTH2 receptor antagonist blocked PGD2-induced eosinophils chemotaxis (Fig. 1D). Comparative study using Eo1-1 cells and the optimal dose of PGD2 was performed to explore the possible signal transduction mechanism involved in eosinophil chemotaxis against PGD2. As shown in Table 1A, none of the agents used in our assay; SB203580, H-89 Dihydrochloride, Bisindolylmaleimide, or Genistein, did block PGD2-induced Eo1-1 chemotaxis. However, we observed that genistein rather increased Eo1-1 migration. These results indicate that PKC, PKA, P38-MAPkinase and tyrosine kinase are not involved in PGD2 signaling via CRTH2 in Eo1-1.

4.2. Expression of CRTH2 receptor by Eo1-1 and its modulation by different cytokines

Preliminary studies demonstrated the ability of Eo1-1 to spontaneously express CRTH2 in a similar fashion as Eo1-1 cells differentiated into mature eosinophils and thus was ideal for the current study. Fig. 2A and B shows the histograms of CRTH2 expression by Eo1-1 and Eo1-1 differentiated to mature eosinophils. As can be seen there was up-regulation of the surface expression by both IFN- γ (upper panel) and TNF- α (lower panel). None of the other tested

cytokines did up-regulate the surface expression (histograms not shown). Moreover, all tested cytokines including IFN- γ and TNF- α did modulate the total expression of CRTH2 (data not shown). Fig. 2C shows that the mean percentage of Eo1-1 cells spontaneously expressing surface CRTH2 was $12.74\% \pm 2.66$. However, after 24 h culture of Eo1-1 in the presence of IL-1 β , IL-2, IL-3, IL-5, IL-7, IL-10, IL-15, TNF- α or IFN- γ , the percentage of cells expressing the surface receptor was significantly up-regulated by IFN- γ and TNF- α to $55\% \pm 8$ and $33.8\% \pm 9.4$, respectively. On the other hand all other tested cytokines induced a rather non significant reduction in the percentage of cells expressing CRTH2 in their surfaces. Fig. 1D shows no effect of tested cytokines in modulating the percentage of cells total expression of CRTH2.

4.2.1. Total CRTH2 protein expression by Eo1-1 cells after 24 h treatment with IFN- γ and TNF- α

To confirm our flow cytometry results that indicated that IFN- γ and TNF- α induced the dynamic surface expression without affecting the rate of synthesis of the total CRTH2 protein, western blotting from Eo1-1 whole extracts was performed. As seen in Fig. 3, after 24 h treatment neither IFN- γ nor TNF- α increased the total CRTH2 protein contents.

4.3. Potentiation of PGD2-induced eosinophil chemotaxis by IFN- γ and TNF- α

To correlate our biochemical results obtained in Fig. 2 to a more clinical application, we performed similar experiments utilizing freshly isolated eosinophils from AR patients. Our primary studies confirmed the ability of IFN- γ and TNF- α to increase the MFI of CRTH2. The time course experiments demonstrated an effect starting at 1 h with optimal activity at 3 h and lasting up to 24 h (data not shown). Therefore we next investigated the priming effect of IFN- γ and TNF- α on PGD2-induced eosinophil after 3 h treatment period. As demonstrated in Fig. 4A, IFN- γ and TNF- α induced exaggerated migratory response of eosinophils against a sub-optimal dose of PGD2 (10^{-9} M). The MFI of CRTH2 surface expression on eosinophils of allergic rhinitis patients at 3 h incubation was 8.2 for control cells, 9.6 for cells treated with 10 ng/ml IFN- γ and 9.3 for cells treated with 10 ng/ml TNF- α (Fig. 4B).

4.4. Effect of IFN- γ and TNF- α on the signal transduction pathway involved in PGD2 induced Eo1-1 chemotaxis

Finally, to investigate whether IFN- γ and TNF- α also induced a change in the signal transduction of PGD2-induced Eo1-1 chemotaxis, we repeated the chemotaxis assays using the same kinases inhibitors. Table 1B and C shows that PGD2-induced Eo1-1 chemotaxis was still not sensitive to SB203580, H-89 Dihydrochloride, Bisindolylmaleimide, or Genistein. These results indicate that IFN- γ and

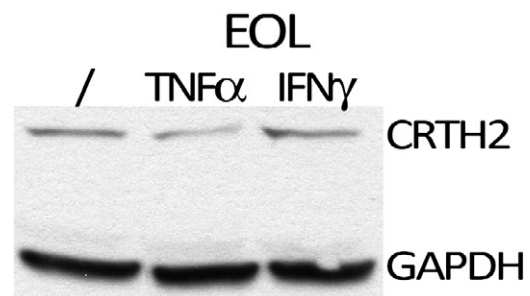


Fig. 3. Expression of CRTH2 in Eo1-1 cells after TNF α or IFN γ treatment. Western blot was performed on whole cell extracts from Eo1-1 cells. Treatment was for 24 h. (/) = no treatment (buffer); GAPDH was used as control.

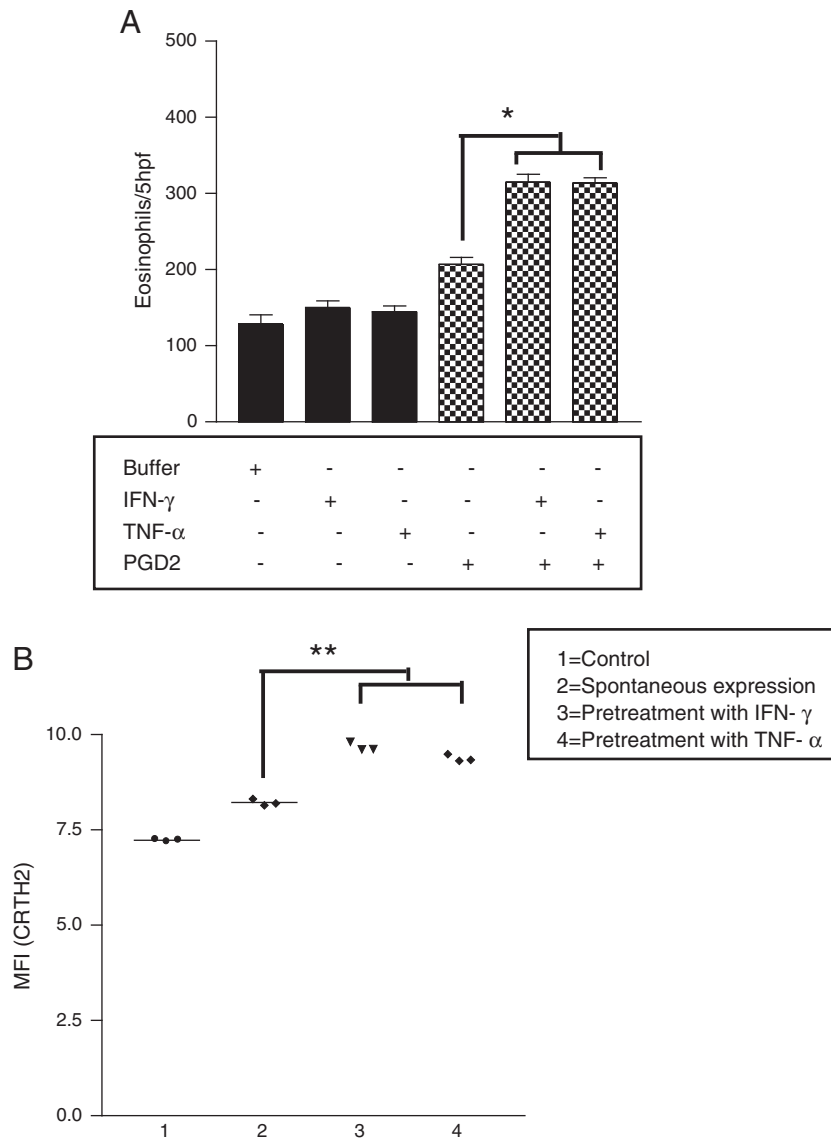


Fig. 4. Priming effect of 10 ng/ml IFN- γ and TNF- α on 10^{-9} M PGD2-induced eosinophils chemotaxis and CRTH2 expression from AR patients. (A) Chemotaxis. Results are the mean $\pm$ SEM of 3 independent experiments performed in triplicate. (B) Fluocytometry. Results are the MFI of CRTH2 expression on eosinophils ($n=3$).

TNF- α did not cause a shift in the signal transduction pathway towards PKC, PKA, P38 MAP-kinase or tyrosine kinase activity.

5. Discussion

Eosinophilia is a hallmark in allergic rhinitis and asthma, and is blamed for the chronicity of the disease. Therefore, the current interest in airway eosinophilic inflammation is to discover the molecular events resulting in eosinophil recruitment and hence to develop an effective mode of therapy. Herein, we show that CRTH2, the prostaglandin receptor that has been reported to be up-regulated in nasal tissues of subjects with allergic rhinitis, has the ability to be up-regulated in vitro by IFN- γ and TNF- α .

Western blotting experiments confirmed that the amount of CRTH2 total protein was not modulated neither by IFN- γ nor by TNF- α . This indicates that IFN- γ and TNF- α have the ability to induce the dynamic surface expression without altering the rate of synthesis of CRTH2 molecules.

Interestingly, all other tested cytokines induced non-significant reduction of the surface expression without altering the total expression. This indicates that Eol-1 stimulation with interleukins (IL-1 β , -2, -3, -5,

-7, -10 and -15) closely related to the immune response, is possibly associated with the dynamic internalization of CRTH2 receptors but has no effect on the rate of the molecules synthesis. Taken collectively, the dynamics of CRTH2 expression or internalization seems to be regulated by interleukins and cytokines of both T_{H1} and T_{H2} immunological responses.

IFN- γ and TNF- α were able to prime eosinophils from AR patients to exaggerated chemotactic response to suboptimal dose of PGD2. This gives a better correlation to their possible roles in the pathophysiology of AR. In this context, IFN- γ and TNF- α did not cause a change in the signal transduction in eosinophils against PGD2. This may indicate that their priming effect on eosinophil chemotaxis was primarily through the up-regulated surface expression of CRTH2.

For years the literature regarding the pathophysiology of allergic airway eosinophilic inflammation concentrated mainly on the effects of the T_{H2} induced cytokines. However, recently some articles also demonstrated a novel modulatory role for T_{H1} mediated cytokines in eosinophilic allergic inflammation. Herein, we show that T_{H1} cytokines play an important immunomodulatory role in PGD2-induced eosinophilic inflammation through up-regulation of CRTH2

expression. If so, we propose that CRTH2 antagonists and/or anti-TNF- α and anti-IFN- γ may be useful therapeutic targets for eosinophilic inflammation of the allergic airways. More studies are required to further characterize the biochemical events involved in TNF- α and IFN- γ induced expression of CRTH2 in eosinophils and to explore the exact signal pathway(s) involved in PGD₂-induced eosinophil chemotaxis.

Acknowledgments

This work is supported in part by the Centre Hospitalier Universitaire de Liege grant number 4717 and in part by the Pulmonology Laboratory, GIGA Research.

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