

Induction of cyclooxygenase-2 expression by allergens in lymphocytes from allergic patients

Pedro Chacón¹, Antonio Vega¹, Javier Monteseirín^{1,3}, Rajaa El Bekay², Gonzalo Alba², José Luis Pérez-Formoso¹, Alberto Msartínez⁵, Juan. A. Asturias⁵, Ramón Pérez-Cano⁴ and José Conde¹

¹ Servicio de Inmunología y Alergia, Hospital Universitario Virgen Macarena, Universidad de Sevilla, Sevilla, Spain

² Departamento de Bioquímica Médica y Biología Molecular, Universidad de Sevilla, Sevilla, Spain

³ Clínica Sagrado Corazón, Sevilla, Spain

⁴ Departamento de Medicina, Universidad de Sevilla, Sevilla, Spain

⁵ Departamento R&D, Bial-Arístegui, Bilbao, Spain

Cyclooxygenase (COX) is a key enzyme in prostaglandin (PG) synthesis. Up-regulation of COX-2 expression is responsible for increased PG release during inflammatory conditions and is thought to be also involved in allergic states. In this study, we demonstrate that in human T, B and natural killer lymphocytes from allergic patients, COX-2 expression became induced upon cell challenge with specific allergens and that this process is presumably IgE dependent and occurs after CD23 receptor ligation. This induction took place at both mRNA and protein levels and was accompanied by PGD₂ release. IgE-dependent lymphocyte treatment elicited, in parallel, an activation of the MAPK p38 and extracellular signal-regulated kinase 1/2, an enhancement of calcineurin (CaN) activity, and an increase of the DNA-binding activity of the nuclear factor of activated T cells and of NF-κB, with a concomitant decrease in the levels of the cytosolic inhibitor of κB, IκB. In addition, specific chemical inhibitors of MAPK, such as PD098059 and SB203580, as well as MG-132, an inhibitor of proteasomal activity, abolished allergen-induced COX-2 up-regulation, suggesting that this process is mediated by MAPK and NF-κB. However, induction of COX-2 expression was not hampered by the CaN inhibitor cyclosporin A. We also examined the effect of a selective COX-2 inhibitor, NS-398, on cytokine production by human lymphocytes. Treatment with NS-398 severely diminished the IgE-dependently induced production of IL-8 and TNF-α. These results underscore the relevant role of lymphocyte COX-2 in allergy and suggest that COX-2 inhibitors may contribute to the improvement of allergic inflammation through the reduction of inflammatory mediator production by human lymphocytes.

Received 4/8/04

Revised 28/1/05

Accepted 3/6/05

[DOI 10.1002/eji.200425572]

Key words:

allergy
· inflammation
· cyclooxygenases
· lymphocytes

Introduction

Among the mediators involved in the development and pathophysiology of allergic diseases, an important role is played by arachidonic acid metabolites, such as prostaglandins (PG), whose levels are elevated in fluids from patients suffering asthma [1]. Biologically active PG are the products resulting from the enzymatic activity of PGH₂ synthase [cyclooxygenase (COX)]. COX exists in two different enzyme isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in most mammalian tissues and is involved in homeostatic

The first three authors contributed equally to this study.

Correspondence: Dr. Francisco Javier Monteseirín Mateo, Asunción 17, 3.º Izqda, 41011 Sevilla, Spain

Fax: +34-954-907-041

e-mail: fmonteseirinm@meditex.es

Abbreviations: CaN: calcineurin · COX: cyclooxygenase ·

CsA: cyclosporin A · EMSA: electrophoretic mobility shift

assay · ERK: extracellular signal-regulated kinase ·

GAPDH: glyceraldehyde-3-phosphate dehydrogenase ·

IκB: inhibitor of κB · MEK: MAPK/ERK kinase · NF-AT: nuclear

factor of activated T cells · NSAID: non-steroidal anti-inflammatory drugs

regulation; in contrast, COX-2 is undetectable but can be induced by pro-inflammatory stimuli, including cytokines, growth factors, tumour promoters, hormones and bacterial endotoxin [2]. COX-2 is associated with inflammation, and its expression has been found increased in the airway epithelium, submucosal inflammatory infiltrate and induced sputum of allergic asthmatic patients [3–5].

The molecular mechanism by which COX-2 gene induction is mediated remains unclear. Previous studies have pointed out a connection between this induction and the activation of MAPK-dependent pathways [6, 7]. MAPK activities have also been associated with allergic inflammation [8, 9]. The transcriptional activation of COX-2 is mediated by the binding of transcription factors to regulatory elements in the COX-2 promoter, such as nuclear factor of activated T cells (NF-AT) [10, 11] or NF- κ B [12, 13]. Available data also reveals an important role of NF-AT and NF- κ B in allergic asthma [14, 15].

Conventional non-steroidal anti-inflammatory drugs (NSAID) are therapeutic agents that inhibit the enzymatic COX-1 and -2 activities [16]. New research has developed selective and specific COX-2 inhibitors without gastrointestinal or haematological side effects. Recent reports have shown that these drugs reduce allergen-elicited inflammation symptoms in rat animal models [17].

Lymphocytes play an important role in the induction and maintenance of the allergic inflammatory cascade [18], with production, after allergen exposure, of allergen-specific antibodies of the IgE class [19] or release of cytokines [20]. Recent studies have indicated that in human T lymphocytes, COX-2 expression becomes induced after TCR activation, with concomitant production of PG [21, 22], whereas COX-1 is constitutively expressed. However, no data have so far been reported on the presence of COX in the other lymphocytes subsets, *i.e.* B and NK cells.

In the present work, we set out to analyse whether the challenge with environmental allergens of human lymphocytes from allergic patients sensitised to these allergens was able to regulate COX-2 gene expression in these cells. Here, we show for the first time that an IgE-dependent mechanism induces COX-2 expression in cultured lymphocytes (B, T, and NK subtypes), in a process that is accompanied by PGD₂ release. Additionally, we have investigated the main IgE-binding receptor involved. We also present experimental evidence on the role of MAPK [p38 and extracellular signal-regulated kinase (ERK)1/2], calcineurin (CaN), NF-AT and NF- κ B signalling pathways in allergen-elicited COX-2 induction. Finally, we examine the effect of a selective COX-2 inhibitor, NS-398, on the IgE-mediated activation (measured as IL-8 and TNF- α production) of human lymphocytes. These findings define a new role for

lymphocyte COX-2 as a generating source of PG during the allergic state. Moreover, our results suggest that COX-2-selective NSAID could have important applications in anti-allergic therapy, given their capacity to regulate cytokine production by allergen-activated human lymphocytes.

Results

Induction of COX-2 expression by specific allergens in human PBL

Lymphocytes from allergic patients specifically sensitised to D₁ were cultured in the presence of this allergen extract, and the expression of COX-2 protein was analysed by Western blotting after treatment. Fig. 1 shows that whereas COX-2 was essentially undetectable in untreated PBL, after allergen stimulation, COX-2 protein was expressed in both a dose- (Fig. 1A) and a time-dependent (Fig. 1B) manner. Fig. 1C illustrates the specificity of the response to allergens. PBL from a patient specifically allergic to T₉, G₃, and E₂ showed a clear response when they were cultured in the presence of these allergens. However, when cells were cultured with M₆ or D₁ allergens (to which the patient was not sensitised), no COX-2 expression was detected. The response of PBL from healthy subjects to E₂, T₉, G₃, M₆, and D₁ allergens is shown in Fig. 1D. In this case, all tested allergens were ineffective in influencing COX-2

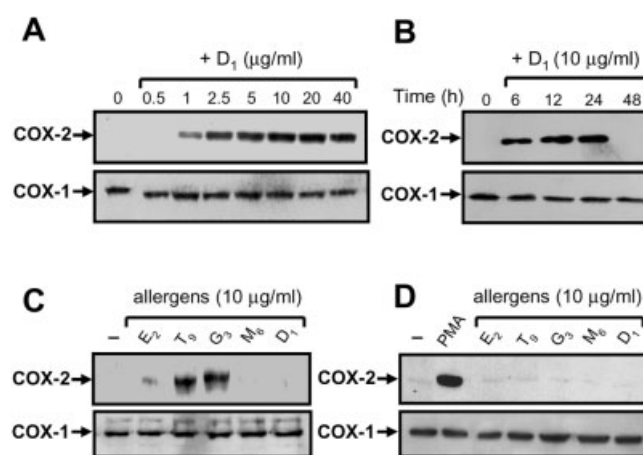


Fig. 1. Induction of COX-2 protein expression by specific allergens in human PBL. D₁ allergen extract dose-dependent (A) or time-dependent (B) COX-2 protein expression in PBL from a patient sensitised to this allergen. (C) Specific activation of lymphocytes from a patient allergic to E₂, G₃, and T₉ and non-sensitised to M₆ and D₁. (D) Response to allergens by lymphocytes from a healthy donor, incubated with the indicated allergens or with PMA (100 nM). Similar results were obtained in at least three other subjects studied for each experiment.

expression, in contrast to what was observed when cells were treated with PMA, a potent inducer of COX-2 in lymphocytes [10, 21]. In all cases, COX-1 expression was not affected by these treatments.

Role of IgE in the COX-2 induction by allergens

To investigate the participation of IgE in the allergen-induced COX-2 expression, we examined the binding of specific IgE on the surface of PBL. As is shown (Table 1), only allergic subjects ($n = 5-10$) had a positive correlation between the measurements of serum- and lymphocyte surface-eluted specific IgE. None of healthy subjects ($n = 1-4$) had detectable levels of specific IgE to allergens in any case. Moreover, none of the patients and healthy donors had specific IgG to allergens in the supernatant eluted from the lymphocyte surface (data not shown).

Fig. 2A shows that anti-human IgE antibody treatment mimicked the effect of allergens on COX-2 expression. Unspecific goat IgG antibody was inefficient (Fig. 2A, last lane). Also, no COX-2 expression was detected when IgE molecules were stripped from the lymphocyte surface (Fig. 2B).

Since there are three defined types of IgE receptors (FcεRI, FcεRII/CD23 and galectin-3) [23, 24], experiments were performed in order to identify the main IgE receptor/s involved in allergen-induced COX-2 expression. Fig. 2C illustrates that COX-2 induction was mainly observed when the antibody 9P.25 (directed against CD23) was used. Conversely the addition of the antibody CRA1 (directed against the FcεRI α chain) had no effect, and the effect of the A3A12 (directed against galectin-3) challenge was hardly detectable. As isotype control, we used mouse IgG1.

Table 1. Presence of specific IgE bound to lymphocyte surface and specific IgE in the serum, from the same donors.

No.	cell surface specific IgE					serum specific IgE				
	D ₁	E ₂	G ₃	T ₉	M ₆	D ₁	E ₂	G ₃	T ₉	M ₆
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	+	+	+	+	-	+	+	+	+	-
6	+	-	+	+	+	+	-	+	+	+
7	+	-	-	-	+	+	-	-	+	+
8	-	-	+	+	-	-	-	+	+	-
9	-	-	+	-	-	-	-	+	-	-
10	+	-	-	-	-	+	-	-	-	-

+ = positive; - = negative

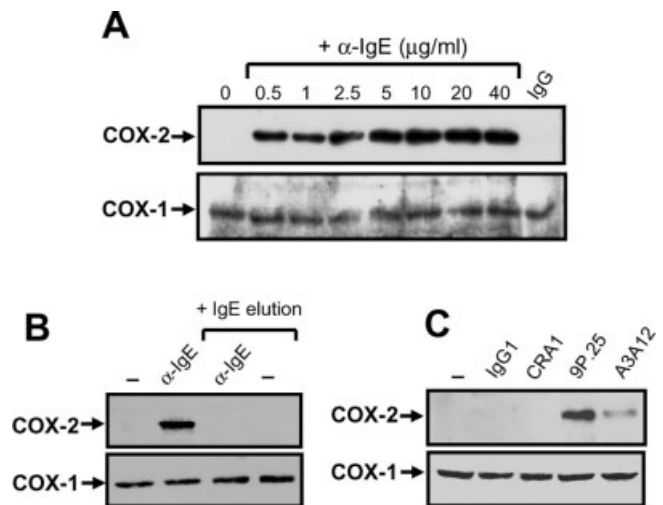


Fig. 2. Role of IgE in COX-2 induction by allergens. (A) Effect of anti-IgE antibody (α -IgE) or goat IgG (10 μ g/mL) on COX-2 expression by PBL from an allergic donor, after 18 h of treatment. (B) Cells from an allergic patient were left untreated (lanes 1, 2) or were treated (lane 3) to elute the surface Ig and then challenged with anti-IgE antibody (10 μ g/mL; lanes 2, 3). (C) Cells from an allergic subject were incubated with CRA1, 9P.25 or A3A12 antibodies (5 μ g/mL) or mouse IgG1 (5 μ g/mL) for 18 h. In all cases, COX-2 and COX-1 protein levels were analysed.

IgE-dependent induction of COX-2 takes place in T, B, and NK lymphocytes

After two-colour FACS analysis, we identified specific lymphocyte subsets in which COX-2 was up-regulated by an IgE-dependent mechanism. Fig. 3A shows that the allergen/anti-IgE antibody treatment resulted in an enhanced expression of intracellular COX-2 protein, which took place in all subsets analysed, with the greatest responsiveness to stimulation being attributable to B and NK cells. Neither goat IgG antibody nor a set of different allergens to which the patients had not been sensitised were able to induce COX-2 (data not shown). Fig. 3B illustrates, by Western blotting, the response of the three purified cell populations to the anti-CD23 or anti-galectin-3 antibodies. As is shown, the greatest induction was detected in B and NK cells after treatment with the 9P.25 antibody. As above, anti-galectin-3 antibody treatment had hardly any effect.

An IgE-dependent mechanism-promoted release of PGD₂

Cells from donors sensitised to T₉ were cultured in the presence of this allergen, and an enzymatic product of COX, PGD₂, was analysed in the culture supernatants. As illustrated in Fig. 4, the exposure to allergen elicited an increased, time- (Fig. 4A) and dose-dependent (Fig. 4B) PGD₂ release. As shown in Fig. 4C, T, B and NK

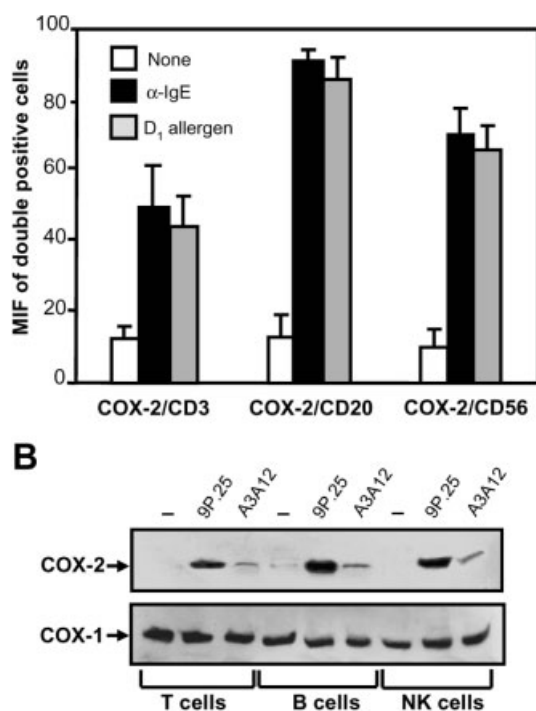


Fig. 3. IgE-dependent COX-2 protein expression takes place in T, B and NK lymphocytes. (A) Flow cytometry analysis of COX-2 expression in PBL isolated from D₁-sensitised patients. Cells were left untreated or were treated with anti-IgE antibody (α -IgE) or D₁ allergens for 18 h. Then, two-colour FACS analysis of CD3/CD20/CD56 and COX-2 expression was performed. The results represent the mean intensity of fluorescence (MIF) of double-positive stained cells. The values shown are the means $\pm$ SEM of three separate experiments. (B) Western blotting analysis of COX-1 and COX-2 expression in highly purified T, B and NK lymphocytes, incubated for 18 h in the absence or presence of 5 μ g/mL 9P.25 or A3A12 antibodies.

lymphocytes experienced an increased production of PGD₂ after challenge with anti-CD23 antibody. The levels of PGD₂ detected for each population increased by 2.5-fold, 3.5-fold and 3.0-fold, respectively, compared to basal levels seen in unstimulated cells. The addition of the anti-galectin-3 antibody to the culture medium produced a hardly detectable effect.

Role of MAPK in the control of allergen-dependent COX-2 expression

Because MAPK proteins have been implicated in the regulation of COX-2 [6, 7, 12], we evaluated the potential role of them in the allergen-dependent COX-2 expression. Fig. 5A, B shows the activation of the MAPK ERK1/2 and p38 after D₁ treatment in PBL from patients sensitised to this allergen extract. On the other hand, no c-Jun N-terminal kinase (JNK) activation was found upon allergen treatment (data not shown).

Specific chemical inhibitors of these MAPK pathways were tested. Fig. 5C and D show, respectively, that both

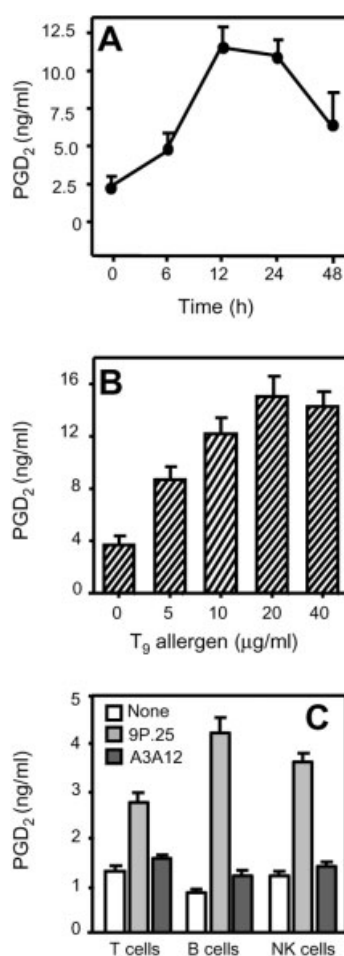


Fig. 4. Analysis of IgE-mediated PGD₂ production by human lymphocytes. PBL from T₉-sensitised patients were incubated with this allergen for the times (A) and at the doses (B) indicated. (C) Highly purified T, B and NK lymphocytes from an allergic patient were left untreated or were treated with 5 μ g/mL 9P.25 or A3A12 antibodies for 12 h. Levels of PGD₂ in the culture medium supernatants were analysed. The values shown are the means $\pm$ SEM from three independent assays for each experiment in which each measurement was performed in triplicate.

the MAPK/ERK kinase (MEK) inhibitor PD098059 [7], at 25 μ M, and the p38 inhibitor SB203580 [6], at 0.1 μ M, blocked both T₉- and G₃-induced COX-2 expression in PBL from donors sensitised to the first or the second allergen extract. Similar to the allergens, PD98059 and SB203580 also inhibited the COX-2 expression induced by anti-CD23 antibody (Fig. 5E).

Allergen up-regulates COX-2 mRNA, role of MAPK

The effect on COX-2 induction was analysed at the mRNA level by RT-PCR analysis. Fig. 6A illustrates the kinetics of D₁-induced COX-2 mRNA expression in PBL from a patient sensitised to this allergen. The specificity of COX-2 mRNA induction was analysed for its response

to the G₃, D₁ and T₉ allergens in PBL from a patient who had a positive sensitivity to G₃ and D₁ and a negative sensitivity to T₉. Fig. 6B shows that the pattern of induction of mRNA expression, or its lack thereof, paralleled the previous sensitisation of the donor. COX-2 mRNA expression also increased in PBL in response to anti-IgE or anti-CD23 antibodies and to PMA (Fig. 6C). We next examined the effect of PD098059 and SB203580. As shown in Fig. 6D, the MEK inhibitor at 25 μ M was not able to totally abolish the presence of COX-2 mRNA, whereas the same dose strongly inhibited COX-2 protein accumulation (Fig. 5C). In contrast, the p38 inhibitor SB203580 at 0.1 μ M blocked the allergen-dependent COX-2 induction at both the mRNA (Fig. 6E) and the protein (Fig. 5D) level.

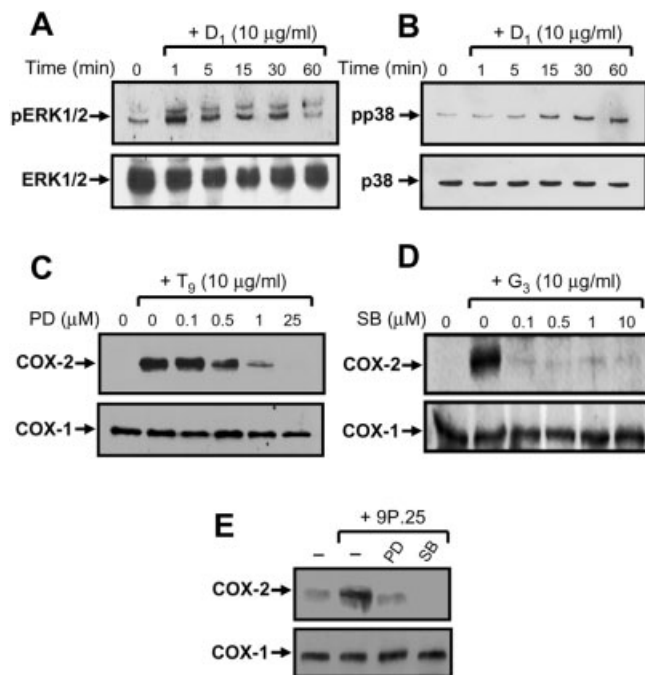


Fig. 5. Involvement of MAPK in allergen-dependent COX-2 protein expression. (A, B) PBL from a patient sensitised to the D₁ allergen were incubated with this allergen for the times indicated, and phosphorylated ERK1/2 (pERK1/2) (A) or p38 (pp38) (B) was subsequently detected. Total (phosphorylated + unphosphorylated forms) ERK1/2 and p38 are also shown (C). Cells from a T₉-sensitised patient were pre-incubated for 45 min with PD098059 (PD) at the doses indicated, and then treated with T₉ allergen for 18 h. Expression levels of COX-2 and COX-1 proteins were analysed. (D) Cells from a G₃-sensitised patient were pre-incubated for 45 min with SB203580 (SB) and then challenged with G₃ allergen for 18 h. Expression levels of COX-2 and COX-1 proteins are shown. (E) PBL from an allergic patient were pre-incubated for 45 min with PD098059 or SB203580 and then challenged with 9P.25 antibody for 18 h. Results illustrated are representative of a total of three separate experiments.

Involvement of transcription factors NF- κ B and NF-AT in the induction of COX-2 expression by specific allergens

We next studied the involvement of the transcription factors NF- κ B and NF-AT, both of which have binding sites in the COX-2 promoter [10, 25], in allergen-elicited COX-2 expression. Fig. 7A shows that stimulation with D₁ of PBL from a patient sensitised to this allergen extract resulted in an enhanced NF- κ B DNA-binding activity. The efficiency of this factor's binding was evidenced by competition with unlabelled probe. The specificity of this response was further underscored by the degradation of the inhibitor of κ B (I κ B)- α , occurring in the cytosol after treatment of cells with allergens (Fig. 7B). Since the degradation of I κ B is accounted for by the proteasome, we assayed the effect of the proteasome inhibitor MG-132 [26] on allergen-dependent COX-2 expression. As illustrated in Fig. 7C, D, pretreatment of PBL with this inhibitor strongly attenuated D₁- or anti-CD23 antibody-induced COX-2

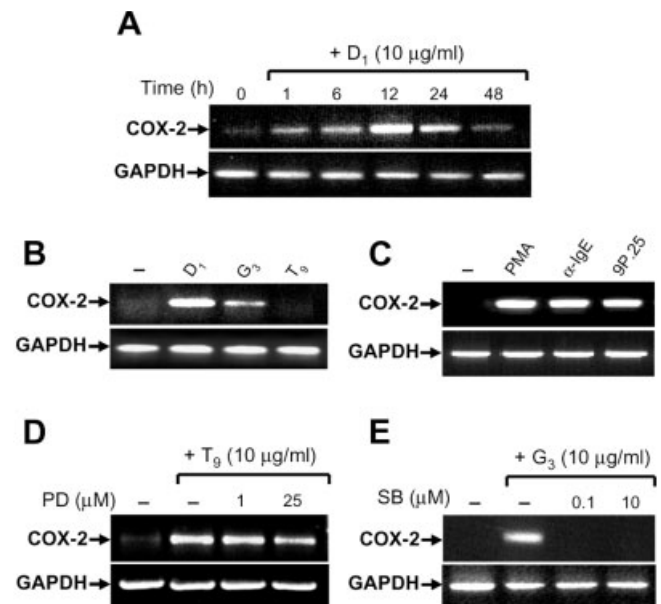


Fig. 6. IgE-dependent induction of COX-2 mRNA expression. Effect of MAPK inhibitors. (A) PBL from a patient sensitised to the D₁ allergen were either left untreated or were treated with D₁ for the times indicated. (B) PBL from a patient sensitised to D₁ and G₃ allergens were incubated with 10 μ g/mL G₃, D₁, or T₉ allergen. (C) Lymphocytes were incubated with 10 μ g/mL anti-IgE antibody (α -IgE), 100 nM PMA or 5 μ g/mL 9P.25 antibody. (D) PBL from a patient sensitised to T₉ allergen were pre-incubated for 45 min in the presence or absence of PD098059 (PD) at the indicated doses, and then challenged for 8 h with T₉ allergen. (E) Lymphocytes from a patient sensitised to G₃ were pre-incubated with the indicated doses of SB203580 (SB) for 45 min and then challenged with G₃ allergen for 8 h. In all cases, COX-2 mRNA or GAPDH mRNA were assayed by RT-PCR. Similar results were obtained in two additional experiments.

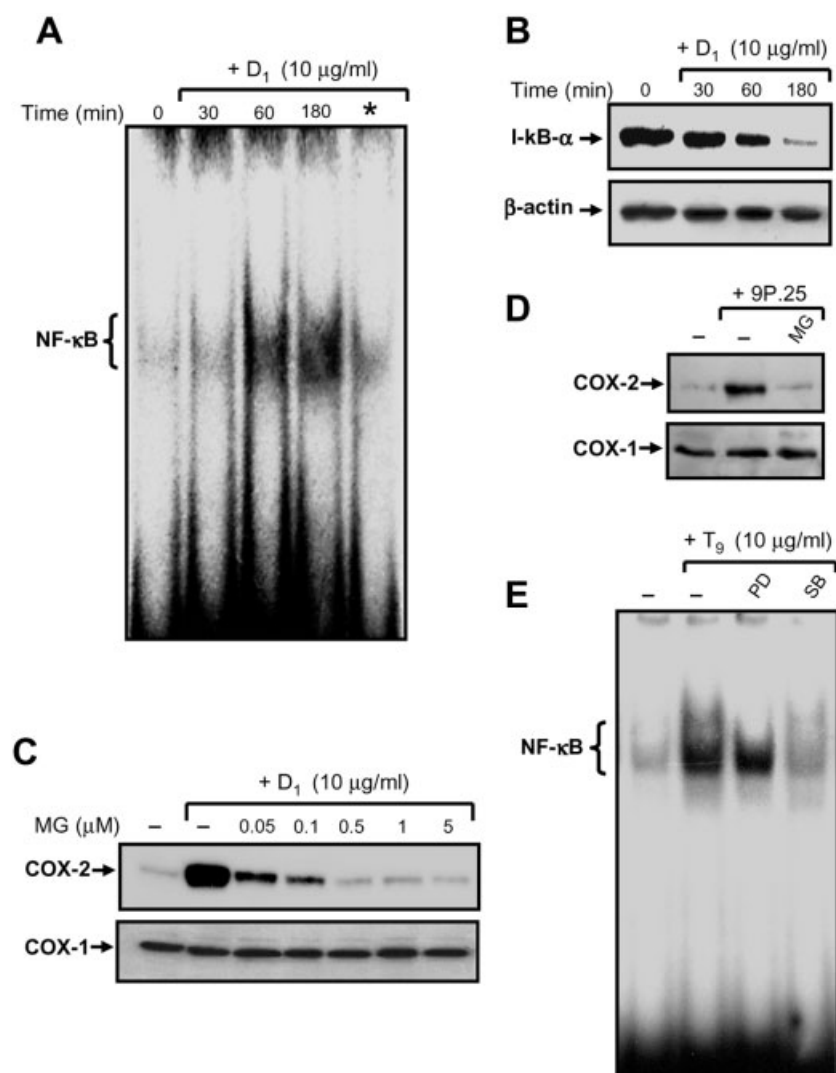


Fig. 7. Role of NF- κ B in allergen-dependent COX-2 induction. (A) Cells from a D₁-allergic patient were treated with 10 μ g/mL D₁ allergen for the indicated times, and NF- κ B DNA-binding activity was determined by EMSA in nuclear extracts. The retarded band is shown (bracket). The asterisk indicates that the binding reaction was performed for 180 min in the presence of a 100-fold molar excess of unlabelled probe. (B) I κ B- α levels analysed by Western blotting in cytosolic extracts from the same PBL as in (A). (C, D) COX-2 protein expression in PBL from a D₁-allergic patient pretreated with MG-132 for 2 h at the indicated doses, and then treated with D₁ (C) or 9P.25 antibody (D) for 18 h. (E) NF- κ B DNA-binding activity in PBL from a T₉ allergen-sensitized patient pretreated or not with 10 μ M SB203580 (SB) or 25 μ M PD098059 (PD) for 45 min before being challenged with the T₉ allergen. Two additional experiments were performed with results similar to those shown.

expression. Because the IgE-dependent COX-2 mRNA induction was prevented by the p38 inhibitor SB203580, we next determined whether MAPK (p38 and/or ERK1/2) activation was involved in the regulation of NF- κ B activation by allergens. As shown (Fig. 7E), when PBL from a T₉-sensitized donor were cultured in the presence of this allergen, after pretreatment with SB203580 or PD098059, NF- κ B DNA-binding activity was significantly reduced in the presence of SB203580, but not of PD098059.

Regarding NF-AT, Fig. 8A illustrates that the G₃ allergen increased the formation of NF-AT/DNA complexes in lymphocytes from a patient sensitized to this

allergen. The specificity of this binding was evidenced by competition with unlabelled probe.

Absence of CaN involvement in allergen/anti-IgE antibody-dependent COX-2 expression

Since a close relationship has been shown between NF-AT and CaN [27], CaN phosphatase activity was analysed in PBL from atopic donors treated with anti-IgE antibody. Fig. 8B illustrates that the activity was increased after treatment. Cyclosporin A (CsA), an immunosuppressor that inhibits CaN activity, had no effect on anti-IgE or anti CD23 antibody-dependent

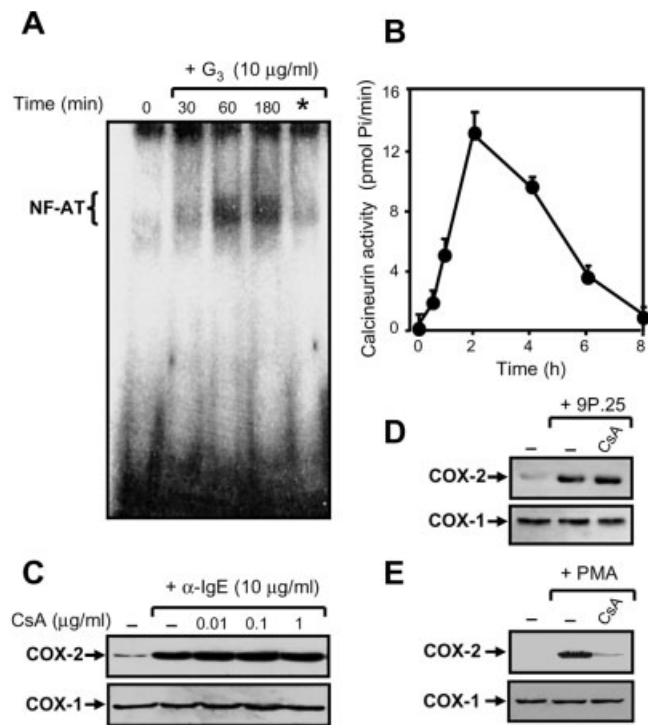


Fig. 8. Role of NF-AT and CaN in IgE-dependent COX-2 expression. (A) PBL from a G_3 -allergic patient were left untreated or were treated with G_3 allergen for the indicated times. NF-AT DNA-binding activity was determined in nuclear extracts by EMSA, and the retarded band is indicated (bracket). The asterisk indicates that the binding reaction was performed for 180 min in the presence of a 100-fold molar excess of unlabelled probe. (B) Time-dependent CaN activity assays by anti-IgE antibody-challenged (10 μ g/mL) PBL from allergic patients. Plotted values are the means $\pm$ SEM from three separate experiments in which each measurement was performed in duplicate. (C–E) PBL were pre-incubated for 2 h with CsA at the doses indicated (ng/mL) and then incubated for 18 h with the indicated amounts of anti-IgE (α -IgE) (C) or 9P.25 antibody (D) or 100 nM PMA (E). COX-2 protein expression was analysed by Western blotting. Two additional experiments were separately performed with similar results.

COX-2 induction (Fig. 8C and D, respectively). However, as previously reported [10, 21], this drug was able to abolish PMA-promoted COX-2 induction (Fig. 8E).

Effect of COX-2 inhibition on lymphocyte IL-8 and TNF- α production

When T, B and NK subsets of lymphocytes from allergic patients are challenged by an IgE-dependent mechanism, an increased release of the inflammatory cytokines IL-8 and TNF- α in the culture supernatants is detected (P.C. and J.M., unpublished observations). After treatment with the anti-galectin-3 antibody, maximal TNF- α production mainly occurred in NK cells ($\sim$ 4.5-fold compared to basal levels) (Fig. 9A), whereas maximal IL-8 production was observed in B cells ($\sim$ 4.0-fold

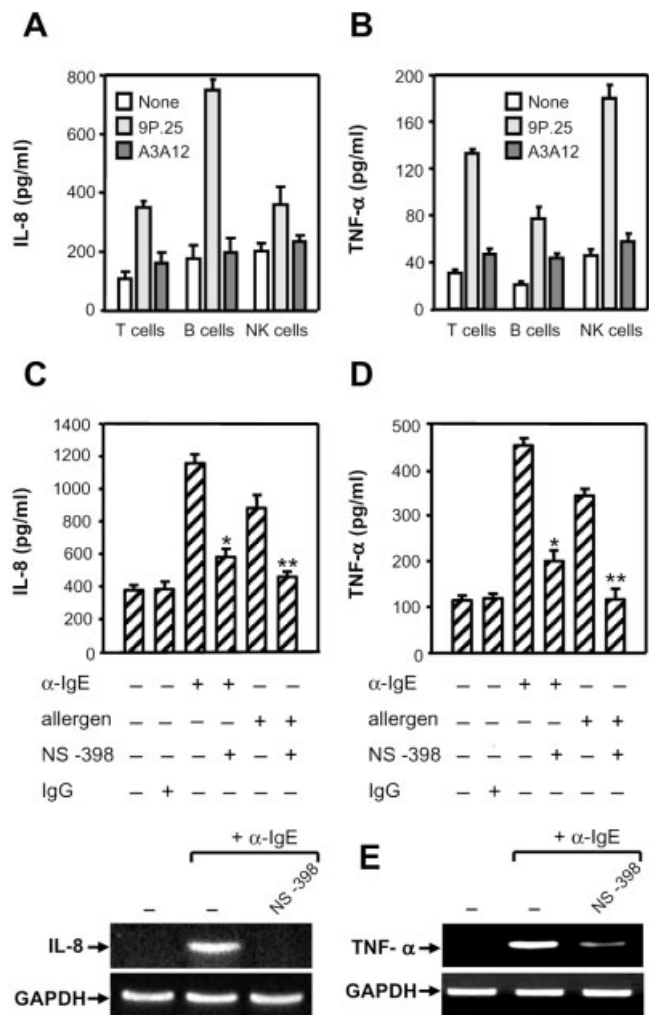


Fig. 9. IgE-dependent cytokine production by human lymphocytes. Effect of COX-2 inhibitor NS-398. (A, B) Highly purified T, B and NK lymphocytes, obtained from allergic patients, were incubated for 24 h in the absence or presence of 5 μ g/mL 9P.25 antibodies. (C, D) PBL from T_9 -allergic patients were left untreated or were stimulated with 10 μ g/mL T_9 α λεργεν, αντι-IγE αντιβोधν (α -IgE) or goat IgG for 24 h. Where indicated, the cells were pre-incubated for 45 min with 50 μ M NS-398. Culture supernatants were processed by ELISA for IL-8 (A, C) or TNF- α (B, D). Values are expressed as the mean $\pm$ SEM from three independent assays in which each measurement was performed in triplicate (* p < 0.001 compared with anti-IgE-treated cells; ** p < 0.001 compared with allergen-treated cells). (E) PBL from allergic patients were left untreated or were treated with anti-IgE antibody (α -IgE) (10 μ g/mL) for 8 h. Where indicated, the NS-398 inhibitor (50 μ M) was added 45 min before treatment. IL-8 mRNA, TNF- α mRNA, or GAPDH mRNA was assayed by RT-PCR. The figure is representative of two independent experiments.

compared to basal levels) (Fig. 9B). On the other hand, treatment with the anti-galectin-3 antibody hardly affected the release of both cytokines.

We next assayed the physiological role of COX-2 induction in PBL, evaluating the effects of the selective NSAID and the specific COX-2 inhibitor, NS-398 [28], on

the IgE-dependent production of these two lymphokines. As shown in Fig. 9C, D, pretreatment with NS-398 (specific COX-2 inhibitor) strongly diminished the release of both IL-8 and TNF- α proteins by cells from patients sensitised to T₉ cultured in the presence of this allergen extract or of anti-IgE antibody. IgG had no effect. As is shown in Fig. 9E, this compound, in a similar fashion to protein production, reduced anti-IgE-induced expression of IL-8 and TNF- α mRNA accumulation.

Discussion

In this work, we demonstrate for the first time that an allergen-mediated mechanism was able to elicit, in PBL from asthmatic patients sensitised to these allergens, a fine and specific induction of COX-2 protein expression, associated with an increase of COX-2 mRNA. The involvement of IgE molecules in the allergen-mediated effects may be envisaged by three main aspects: (i) We demonstrated the presence of allergen-specific IgE molecules bound to the surface of lymphocytes from allergic patients and the absence of allergen-specific IgG. (ii) Anti-IgE antibody challenge triggered a response equivalent to that elicited by the allergens, and this effect was not observable when IgE was stripped from the cell surface. (iii) Ligation of the low-affinity IgE receptor Fc ϵ RII/CD23 with monoclonal antibodies evoked an up-regulation of COX-2. Taken together, our results suggest that allergens activate lymphocytes to induce COX-2 through the ligation of specific IgE molecules, which in turn would be bound to their CD23 receptors. This mechanism would explain why the allergens were ineffective on lymphocytes from healthy donors or from allergic patients sensitised to different allergens than those tested. In these cases, allergens could not bind to preexistent specific IgE receptor complexes located at the cell surface, and COX-2 induction would not take place. Because only a COX-2 induction has been described after Fc ϵ RI cross-linking in mouse cultured mast cells [29], our suggested model provides the first evidence of a CD23-mediated COX-2 up-regulation.

Although the capacity of human T lymphocytes to induce COX-2 synthesis has been reported [10, 11, 21, 22], no studies have analysed this protein in B and NK lymphocytes. For this reason, our results also demonstrate, for first time, that COX-2 can be up-regulated in these two lymphocyte subsets upon their promoted activation by allergens via CD23 cross-linking.

The relationship between allergy and COX-2 expression has previously been studied in different human cell types. Some authors have found an increased expression of COX-2 in the airway epithelium, in induced sputum [3, 5] or in submucosal inflammatory infiltrates of

asthmatic patients [4]. Immunochemical analysis pointed at macrophages [4] and eosinophils [5] as the main cells expressing COX-2. Also, in cultured smooth muscle cells, COX-2 expression is markedly induced by pro-inflammatory cytokines whose levels are increased in asthmatic airways [30]. However, COX-2 expression in lymphocytes from allergic patients has not been analysed until now. Our finding of an allergen-dependent COX-2 induction in human PBL raises further questions on the contribution of these cells to an inflammatory process such as allergy, suggesting an important role in allergic conditions for lymphocyte COX-2 and the eicosanoids it generates. In this regard, an increase in serum levels of PGD₂, a potent bronchoconstrictor and vasodilator, has been associated with allergic diseases [31]. Also, mice deficient in PGD₂ receptors showed reduced accumulation of lymphocytes and eosinophils in the lungs after allergen challenge [31], because PGD₂ selectively induces chemotaxis in T lymphocytes, eosinophils, and basophils [22]. Although mast cells are known to be the major producers of PGD₂ [29], recent studies have demonstrated that T lymphocytes also release comparable levels of PGD₂ [22]. In agreement with this, we have observed that culture in the presence of allergen or anti-CD23 antibody induced PGD₂ release by human T lymphocytes. However, our results also extend the production of PGD₂ to B and NK lymphocyte populations.

In addition to PGD₂ release, we have demonstrated that an IgE-mediated activation induced the expression and release of TNF- α and IL-8 by human lymphocytes. These findings are in agreement with previous studies reporting an IgE-dependent mechanism, elicited by allergens, of TNF- α [32] or IL-8 production [33] by mononuclear cells and neutrophils, respectively. Evidence for the implication of these mediators, whose levels have been found elevated in bronchoalveolar lavage fluid and airways of asthmatic patients, in the pathology of allergy has been reported [34]. Both cytokines are potent cellular chemoattractant molecules at the inflammation sites. Thus, IL-8 induces chemotaxis of neutrophils and lymphocytes [35] or eosinophils [36]. TNF- α augments cellular influx by up-regulating the expression of adhesion molecules in inflammatory leukocytes, increasing their adhesion to surfaces, as *e.g.* in airways [37]. Additionally, our data show that the production of IL-8 and TNF- α by lymphocytes after IgE-mediated activation was prevented by the COX-2 inhibitor NS-398, which is in line with previous observations [21, 38]. In this context, our results could explain the ameliorating effect of NS-398 observed in an animal model of allergic inflammation [17], probably through the reduction of chemoattractant inflammatory molecules such as TNF- α or IL-8.

Another goal of our study was to unveil the potential signalling pathways triggered by the challenge of human PBL with allergens. Numerous reports have indicated a role of MAPK, particularly ERK1/2 and p38, in the regulation of COX-2 expression in monocytes [6], neuroblastoma cells [12] and epithelial cells [7], by LPS, IL-1 β and TNF- α , respectively. On the other hand, an association between MAPK activity and allergic inflammation has been found previously in the lungs of asthmatic mice [9] or in the bronchoalveolar lavage fluids from asthmatic patients [8]. In agreement with these results, our work shows that the exposure of PBL from patients allergic to specific allergens promoted p38 and ERK1/2 activation. Moreover, present data demonstrate that the p38-specific inhibitor SB203580 provoked a strong down-regulation of allergen-induced COX-2 mRNA and protein expression in human lymphocytes. In contrast, the MEK inhibitor PD098059 abolished COX-2 protein expression, but only slightly affected mRNA levels. The decrease in allergen-induced COX-2 mRNA levels promoted by SB203580 suggests that the p38 MAPK pathway may modulate the transcription and/or stabilisation of the COX-2 gene, as previously reported [6]. The relevance of the NF- κ B binding sites in the COX-2 promoter is well established in macrophages [39], tumour cells [12] and epithelial cells [13], stimulated by LPS, IL-1 β and TNF- α , respectively. Moreover, NF- κ B plays a pivotal role in allergy [15], and an activation of NF- κ B has been found in mononuclear cells of patients with atopic asthma [32]. In this light, the present work shows evidence of the participation of NF- κ B in IgE-dependent COX-2 induction in human lymphocytes. We have detected, by electrophoretic mobility shift assay (EMSA), that NF- κ B DNA-binding activity was enhanced by allergen treatment, with an associated degradation of cytoplasmic I κ B. Our study also shows that the proteasome inhibitor MG-132 abrogates allergen- or anti-CD23-induced COX-2 expression. The MAPK p38 and ERK1/2 have been shown to mediate NF- κ B transactivation in TNF- α -mediated COX-2 induction in epithelial cells [7] through the activation of I κ B kinase (IKK). In our case, only p38 appeared necessary for NF- κ B activation since the MEK inhibitor PD098059 did not abolish the stimulation of COX-2 expression by allergens. A relationship between COX-2 expression and NF-AT activation has been suggested in T lymphocytes [10, 11] and endothelial cells [40]. In these cells, the inhibitor of CaN [27], CsA, potently abrogates the NF-AT-dependent COX-2 induction caused by Ca²⁺-mobilising agents. Furthermore, an important role of the CsA-sensitive transcription factor NF-AT in the allergic response has been established [14]. In contrast with these observations, our data reveal that although anti-IgE antibody/allergen treatment increased CaN activity

and NF-AT DNA-binding activity, CsA was not able to abolish the stimulus-promoted COX-2 induction in human lymphocytes. However, a possible participation of NF-AT in this process cannot be ruled out. A similar inability of CsA to abolish the COX-2 induction mediated by Cot kinase-dependent NF-AT activation has been described [11]. The authors have suggested that the activity of this factor is regulated downstream of CaN activation or through the operation of parallel pathway(s). In our model, similar modulatory mechanisms could be involved in NF-AT activation.

In summary, we present evidence of a new mechanism of COX-2 induction in human PBL (T, B and NK cells), elicited by allergens and mediated by IgE molecules (through CD23 ligation), and provide information on the intracellular signal transduction pathways involved in this process. Our data allow us to envision lymphocytes as a source of inflammatory mediators (PG and cytokines) in allergic reactions, and suggest that selective COX-2 inhibitors, by acting as modulators of lymphocyte functions, may constitute potentially attractive drugs for their use in anti-allergic therapy.

Materials and methods

Chemicals and reagents

The allergens, commercially available, freeze-dried and standardised extracts including D₁ (*Dermatophagoides pteronyssinus*), G₃ (*Dactylis glomerata*), T₉ (*Olea europaea*), E₂ (*Dog epithelium*), and M₆ (*Alternaria alternata*), were purchased from Bial-Arístegui (Bilbao, Spain). Purified goat anti-human IgE antibody was from Caltag Laboratories (Burlingame, CA). Goat or mouse IgG antibodies, PD098059, SB203580, MG-132, CsA and anti- β -actin antibody were from Sigma Chemical Co. (Madrid, Spain). NS-398 was from Calbiochem-Novabiochem (San Diego, CA). [γ -³²P]ATP, PCR primers, the double-stranded probes [5'-GGAGGAAAAC-TGTTTCATACAGAAGGCGT-3', encompassing the distal NF-AT binding site (underlined) of the human IL-2 promoter, and 5'-AGTGAGGGGACTTTCCAGGC-3', containing a consensus NF- κ B site (underlined)] were from Amersham Pharmacia-Biotech (Barcelona, Spain). FITC-conjugated and unconjugated antibodies to human COX-2 were obtained from Cayman Chemical (Ann Arbor, MI). The antibodies against phosphorylated p38 (Thr¹⁸⁰/Tyr¹⁸²), phosphorylated ERK1/2 (Thr²⁰²/Tyr²⁰⁴) and total p38 were from New England Biolabs (Beverly, MA). The antibodies against COX-1 (sc-7950), the total forms of ERK1/2 (sc-93) and against I κ B- α (sc-371) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PE-conjugated or unconjugated antibodies against cell surface antigens (CD14, CD3, CD20, CD56 and CD203c), their IgG₁ isotypes, coated magnetic anti-IgG and anti-CD23 antibodies (clone 9P.25) were from Coulter-IZASA (Barcelona, Spain). Anti-galectin-3 antibody (clone A3A12) and anti-Fc ϵ R1 α chain antibody (clone AER-37/CRA1) were purchased from Abcam

(Cambridge, UK) and eBioscience (San Diego, CA), respectively. The reagents used had endotoxin levels of ≤ 0.01 ng/mL, as tested by the *Limulus* lysate assay (Coatest, Mölndal, Sweden).

Patients and controls

The groups studied included adult atopic patients with bronchial asthma and healthy non-atopic volunteer controls, with a sex distribution ratio (female/male) of 0.9. All subjects were lifelong non-smokers. Asthma was diagnosed on the basis of criteria previously described [41]. The patients gave positive results for the skin prick test (Bial-Aristegui) and for specific IgE (HYTEC 288, Hycor Biomedical-IZASA, Barcelona, Spain) tests to at least one common allergen. The subjects did not receive any treatment or specific hyposensitisation. None of them had experienced episodes of asthma for at least 3 months, and none had experienced respiratory tract infections in the 4 wk before blood sampling. The healthy group had no history of allergy or bronchial symptoms and was negative for the skin prick and specific IgE tests toward a battery of inhalant allergens (house-dust mites, pollens, moulds, and animal danders). This study was previously approved by the Hospital Virgen Macarena Ethics Committee, and each subject gave prior informed consent.

Lymphocyte isolation, cell culture, and treatment

Human PBL were isolated as described [42]. Contaminating monocytes were depleted by a magnetic cell separation system (MACS) with anti-CD14 antibody incubation followed by the addition of anti-IgG antibody-coated magnetic beads. For the purification of lymphocyte subsets, after depletion of monocytes, cells were separated by MACS with a mixture of anti-CD3, anti-CD20 or anti-CD56 mAb (depending on population), followed by the addition of anti-IgG antibody-coated magnetic beads. The cell preparations were $>98\%$ CD3⁺, $>98\%$ CD20⁺ and $>98\%$ CD56⁺, respectively. Contamination with basophils was excluded by staining with anti-CD203c antibody.

Cells were cultured in RPMI 1640 medium supplemented with 10% v/v foetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, and maintained at 37°C in an atmosphere of 5% CO₂ and 95% O₂. For stimulation treatments, the cells were incubated with stimulus at 37°C. The inhibitors PD098059, SB203580 and NS-398 were added 45 min, and CsA and MG-132 2 h, prior to stimulation. None of these reagents affected the viability of the cells at the concentrations used, as confirmed by the Trypan blue dye exclusion test. Lymphocyte viability was in all cases greater than 96%. In all cases, vehicle controls for the inhibitors were assayed.

Cell lysis and Western blotting

Cell lysis for assaying COX expression and MAPK activation was performed as described [10, 43]. Western blotting was carried out as indicated [43, 44].

Dissociation of lymphocyte-bound immunoglobulins and IgE receptor/s cross-linking

Immunoglobulins were dissociated from the lymphocyte surfaces as described [33], and the obtained supernatants were used for specific IgE and IgG determinations (HYTEC 288).

When IgE receptor cross-linking experiments were performed, immunoglobulins were first eluted, and then the cells were incubated with anti-IgE receptor antibodies.

COX-2 expression analysis by flow cytometry

For intracellular detection of COX-2 by flow cytometry, PBL (2×10^6 cells) were incubated with antibodies against human lymphocyte markers, i.e. CD3, CD20 and CD56, or the IgG₁-PE isotype control antibody. PBL were then fixated and permeabilised using the Perm-cell kit (Caltag Laboratories), following the manufacturer's indications. FITC-conjugated anti-human COX-2 or IgG₁ isotype control antibodies were added, and after incubation for 30 min, the samples were washed, resuspended in PBS and analysed by flow cytometry (Coulter-IZASA, Barcelona, Spain).

RNA extraction and RT-PCR analysis

RNA extraction and reverse transcription were performed as described [42]. cDNA was amplified by PCR using specific primers for COX-2 [21], IL-8 [33], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [21], and TNF- α (5'-AAGCCTGTAGCCCATGTTGT-3' and 5'-CCAGATAGATGGGCTCATAC-3'). The reactions were performed by 25–30 cycles of 94°C for 1 min, 58–60°C for 1 min, and 72°C for 1 min. Sizes of the expected fragments were: COX-2 (300 bp), IL-8 (265 bp), TNF- α (330 bp) and GAPDH (605 bp).

CaN phosphatase assay

CaN phosphatase activity was measured as described [43].

EMSA

Nuclear extracts were prepared and EMSA were performed as described [44].

PG and IL release

PG or IL levels in culture supernatants from lymphocytes (10^6 – 10^7 cells/300–1500 µL) were determined in replicate wells using the Prostaglandin D₂-Mox Enzyme Immunoassay kit (Cayman) and the IL-8 or TNF- α sandwich (Coulter-IZASA), according to the manufacturer's instructions.

Statistical analysis

Statistical data are expressed as the mean $\pm$ SEM. The Student's unpaired *t*-test or one-way ANOVA was used to make comparisons between groups.

Acknowledgements: This work was financed by grants from the Junta de Andalucía, Fundación SEIAC, and Bial-Aristegui.

References

- Wenzel, S. E., Arachidonic acid metabolites: mediators of inflammation in asthma. *Pharmacotherapy* 1997. **17**: 3S–12S.
- Smith, W. L., Dewitt, D. L. and Garavito, R. M., Cyclooxygenases: Structural, cellular and molecular biology. *Annu. Rev. Biochem.* 2000. **69**: 145–181.
- Pang, L., Pitt, D., Petkova, D. and Knox, A. J., The COX-1/ COX-2 balance in asthma. *Clin. Exp. Allergy* 1998. **28**: 1050–1058.
- Taha, R., Olivenstein, R., Utsumi, T., Ernst, P., Barnes, P. J., Rodger, I. W. and Giaid, A., Prostaglandin H synthase 2 expression in airway cells from patients with asthma and chronic obstructive pulmonary disease. *Am. J. Crit. Care Med.* 2000. **161**: 636–640.
- Profita, M., Sala, A., Bonnano, A., Riccobono, L., Siena, L., Melis, M. R., Di Giorgi, R. et al., Increased prostaglandin E2 concentrations and cyclooxygenase-2 expression in asthmatic subjects with sputum eosinophilia. *J. Allergy. Clin. Immunol.* 2003. **112**: 709–716.
- Dean, J. J. E., Brook, M., Clack, A. R. and Saklatvala, J., p38 Mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. *J. Biol. Chem.* 1999. **274**: 264–269.
- Chen, C.-C., Sun, Y.-T., Chen, J.-J. and Chang, Y.-J., Tumour necrosis factor- α -induced cyclooxygenase-2 expression via sequential activation of ceramide-dependent mitogen-activated protein kinases, and I κ B kinase 1/2 in human alveolar epithelial cells. *Mol. Pharmacol.* 2001. **59**: 493–500.
- Fahy, O., Hammed, H., Senechel, S., Pestel, J., Tonnel, A. B., Wallert, B. and Tsiocopoulos, A., Synergistic effect of diesel organic extracts and allergen Derp1 on the release of chemokines by peripheral blood mononuclear cells from allergic subjects: involvement of the MAP kinase pathway. *Am. J. Respir. Cell. Mol. Biol.* 2000. **23**: 247–254.
- Duan, W., Chan, J. H., Wong, C. H., Leung, B. P. and Wong, W. S., Anti-inflammatory effects of mitogen-activated protein kinase inhibitor U0126 in an asthma mouse model. *J. Immunol.* 2004. **172**: 7053–7059.
- Iñiguez, M. A., Martínez-Martínez, S., Punzón, C., Redondo, J. M. and Fresno, M., An essential role of the nuclear factor of activated T cells in the regulation of the expression of cyclooxygenase-2 gene in human T lymphocytes. *J. Biol. Chem.* 2000. **275**: 23627–23635.
- De Gregorio, R., Iñiguez, M. A., Fresno, M. and Alemany, S., Cot kinase induces cyclooxygenase-2 expression in T cells through activation nuclear factor of activated T cells. *J. Biol. Chem.* 2001. **276**: 27003–27009.
- Fiebich, B. L., Mueksh, B., Boehringer, M. and Hüll, M., Interleukin-1 β induces cyclooxygenase-2 and prostaglandin E₂ synthesis in human neuroblastoma cells: Involvement of p38 mitogen-activated protein kinase and nuclear factor κ B. *J. Neurochem.* 2000. **75**: 3018–3025.
- Chen, C.-C., Sun, Y.-T., Chen, J.-J. and Chiu, K. T., TNF- α -induced cyclooxygenase-2 in human lung epithelial cells: Involvement of the phospholipase C- γ 2, protein kinase C- α , tyrosine kinase, NF- κ B-inducing kinase, and I- κ B kinase pathway. *J. Immunol.* 2000. **165**: 2719–2728.
- Viola, J. P. and Rao, A., Role of the cyclosporin-sensitive transcription factor NFAT1 in the allergic response. *Mem. Inst. Oswaldo Cruz* 1997. **92**: 147–155.
- Barnes, P. J. and Adcock, I. M., NF- κ B: A pivotal role in asthma and new target for therapy. *Trends Biochem. Sci.* 1997. **18**: 46–50.
- Vane, J. R., Introduction: mechanisms of action of NSAIDs. *Br. J. Rheumatol.* 1996. **35**: 1–3.
- Niki, H., Tominaga, Y., Watanabe-kobayashi, M., Mue, S. and Ohuchi, K., Possible participation of cyclooxygenase-2 in the recurrence of allergic inflammation in rats. *Eur. J. Pharmacol.* 1997. **320**: 193–200.
- Romagnani, S., The role of lymphocytes in allergic diseases. *J. Allergy Clin. Immunol.* 2000. **105**: 399–408.
- Yanagihara, Y., Mechanism and regulation of human IgE synthesis. *Allergy Clin. Immunol. Int.* 1998. **10**: 193–197.
- Del Prete, G. F., De Carli, M., D'Elia, M., Maestrelli, P., Ricci, M., Fabri, L. and Romagnani, S., Allergen exposure induces the activation of allergen-specific Th₂ cells in the airway mucosa of patients with allergic respiratory disorders. *Eur. J. Immunol.* 1993. **23**: 1445–1449.
- Iñiguez, M. A., Punzón, C. and Fresno, M., Induction of cyclooxygenase-2 on activated T lymphocytes: Regulation of T cell activation by cyclooxygenase-2 inhibitors. *J. Immunol.* 1999. **163**: 111–119.
- Tanaka, K., Ogawa, K., Sagamura, K., Nakamura, M., Takano, S. and Nagata, K., Differential production of prostaglandin D₂ by human helper T cells subsets. *J. Immunol.* 2000. **164**: 2277–2280.
- Novak, N., Kraft, S. and Bieber, T., Ig-E receptors. *Curr. Opin. Immunol.* 2001. **13**: 721–726.
- Liu, F. T., S-type mammalian lectins in allergic inflammation. *Immunol. Today* 1993. **14**: 486–490.
- Appleby, S. B., Ristimäki, A., Nelson, K., Narko, K. and Hla, T., Structure of the human cyclooxygenase-2 gene. *Biochem. J.* 1994. **302**: 723–727.
- Palombella, V. J., Rando, O. J., Goldberg, A. L. and Maniatis, T., The ubiquitin-proteasome pathway is required for processing the NF- κ B1 precursor protein and the activation of NF- κ B. *Cell* 1994. **78**: 113–125.
- Clipstone, N. A. and Crabtree, G. R., Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* 1992. **357**: 695–697.
- Futaki, N., Takahashi, S., Yokamura, M., Arai, I., Higuchi, S. and Otomo, S., NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity *in vitro*. *Prostaglandins* 1994. **47**: 55–59.
- Murakami, M., Bingham, C. O., Matsumoto, R., Austen, K. F. and Arm, J. P., IgE-dependent activation of cytokine-primed mouse cultured mast cells induces a delayed phase of prostaglandin D₂ generation via prostaglandin endoperoxidase synthase-2. *J. Immunol.* 1995. **155**: 4445–4453.
- Belvisi, M. G., Saunders, M. A., Haddad, B., Hirst, S. J., Yacoub, M. H., Barnes, P. J. and Mitchell, J. A., Induction of cyclooxygenase-2 by cytokines in human cultured airway smooth muscle cells: novel inflammatory role of this cell type. *Br. J. Pharmacol.* 1997. **120**: 910–916.
- Matsuoka, T., Hirata, M., Tanaka, H., Takashashi, Y., Murata, T., Kabashima, K., Sugimoto, Y. et al., Prostaglandin D₂ as mediator of allergic asthma. *Science* 2000. **287**: 2013–2017.
- Mitsuta, K., Matsuse, H., Fukushima, C., Kawano, T., Tomari, S., Obase, Y., Goto, S. et al., Production of TNF- α by peripheral blood mononuclear cells through activation of nuclear factor κ B by specific allergen stimulation in patients with atopic asthma. *Allergy Asthma Proc.* 2003. **24**: 19–26.
- Monteseirín, J., Chacón, P., Vega, A., El Bekay, R., Álvarez, M., Alba, G., Conde, M. et al., Human neutrophils synthesize IL-8 in an IgE-mediated activation. *J. Leukoc. Biol.* 2004. **76**: 692–700.
- Broide, D. H., Lotz, M. and Cuomo, A. J., Cytokines in symptomatic asthma. *J. Allergy Clin. Immunol.* 1992. **89**: 958–967.
- Larsen, C. G., Anderson, A. O., Appella, E., Oppenheim, J. J. and Matsushima, K., The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science* 1989. **243**: 1464–1466.
- Shute, J., Interleukin-8 is a potent eosinophil chemoattractant. *Clin. Exp. Allergy* 1994. **24**: 203–206.
- Thornhill, M. H., Wellicome, S. M., Mahiouz, D. L., Lanchbury, J. S., Kyan-Aung, U. and Haskard, D. O., Tumor necrosis factor combines with IL-4 or IFN- γ to enhance endothelial cell adhesiveness for T cells. The contribution of vascular adhesion molecule-1 and independent binding mechanisms. *J. Immunol.* 1991. **146**: 592–598.
- Kimura, T., Iwase, M., Kondo, G., Watanabe, H., Ohashi, M., Ito, D. and Nagumo, M., Suppressive effect of selective cyclooxygenase-2 inhibitor on cytokine release in human neutrophils. *Inter. Immunopharmacol.* 2003. **3**: 1519–1528.
- D'Acquisto, F., Luvone, T., Rombolá, L., Sautebin, L., Di Rosa, M. and Carnuccio, R., Involvement of NF- κ B in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages. *FEBS Lett* 1997. **418**: 175–178.
- Robida, A. M., Xu, K., Ellington, M. L. and Murphy, T. J., Cyclosporin A selectively inhibits mitogen-induced cyclooxygenase-2 gene transcription in vascular smooth muscle cells. *Mol. Pharmacol.* 2000. **58**: 701–708.

- 41 American Thoracic Society, Definitions and classifications of chronic bronchitis, asthma and emphysema. *Am. Rev. Respir. Dis.* 1962. **85**:762–768.
- 42 Álvarez-Maqueda, M., El Bekay, R., Alba, G., Monteseirín, J., Chacón, P., Vega, A., Martín-Nieto, J. et al., 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J2 induces heme oxygenase-1 gene expression in a reactive oxygen species-dependent manner in human lymphocytes. *J. Biol. Chem.* **279**: 21929–21937.
- 43 Carballo, M., Márquez, G., Conde, M., Martín-Nieto, J., Monteseirín, J., Conde, J., Pintado, E. and Sobrino, F., Characterization of calcineurin in human neutrophils. Inhibitory effect of hydrogen peroxide on its enzyme activity and on NF- κ B DNA binding. *J. Biol. Chem.* 1999. **274**: 93–100.
- 44 El Bekay, R., Álvarez, M., Monteseirín, J., Alba, G., Chacón, P., Vega, A., Martín-Nieto, J. et al., Oxidative stress is a critical mediator of the angiotensin II signal in human neutrophils: involvement of mitogen-activated protein kinase, calcineurin, and the transcription factor NF- κ B. *Blood* 2003. **102**: 662–671.