

Rhinitis, Sinusitis, and upper airway disease

Modulation by aspirin of nuclear phospho-signal transducer and activator of transcription 6 expression: Possible role in therapeutic benefit associated with aspirin desensitization

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Background: Aspirin-exacerbated respiratory disease is characterized by asthma, nasal polyps, and intolerance to aspirin with overexpression of leukotriene (LT) C₄ synthase and cysteinyl leukotriene receptors. Through an unknown mechanism, aspirin desensitization is an effective treatment.

Objective: We hypothesized that aspirin desensitization blocks IL-4-induced expression of these LT activities through inhibition of signal transducer and activator of transcription 6 (STAT6)-mediated transcription.

Methods: Nuclear extracts were derived from THP-1 and normal human monocytes resting and cocultured with aspirin before IL-4 stimulation. Quantitative PCRs were conducted. Electrophoretic mobility shift assays were performed by using oligomers for STAT6 sites within the LT receptor and synthase promoters. Western blots of nuclear extracts were probed by using anti-phospho-STAT6 antibodies.

Results: Upregulation of LT receptor mRNA by IL-4 was negated by aspirin and ketorolac but not by sodium salicylate. The STAT6 site in the LT receptor and synthase promoters was confirmed by using mobility shift assays by competing with unlabeled STAT6 consensus probes and supershifts with anti-STAT6 antibodies. Aspirin and ketorolac decreased the IL-4-inducible expression of nuclear STAT6 observed in mobility shift assays and Western hybridization.

Conclusion: The LT receptor and synthase promoters have STAT6-binding sites that are engaged by IL-4-induced nuclear extracts and inhibited by aspirin. Assuming that normal monocytes behave like monocytes from patients with aspirin-exacerbated respiratory disease, inhibition of IL-4-STAT6 might explain a mechanism in aspirin desensitization daily treatment, resulting in downregulation of production and responsiveness to cysteinyl leukotrienes. This biologic activity of aspirin likely reflects COX inhibition because it was shared with

ketorolac but not sodium salicylate. (*J Allergy Clin Immunol* 2009;■■■:■■■-■■■)

Key words: Aspirin-exacerbated respiratory disease, cysteinyl leukotrienes, signal transduction, IL-4

Aspirin-exacerbated respiratory disease (AERD) is a syndrome characterized by asthma that is often severe and persistent and can be associated with aggressive airway remodeling,¹ extensive hyperplastic eosinophilic sinusitis with nasal polyp formation,² and intolerance to aspirin and other nonselective inhibitors of COX.³⁻⁵ AERD is associated with profound overproduction and overresponsiveness to cysteinyl leukotrienes (CysLTs). Overproduction of CysLTs reflects increased expression of the enzyme leukotriene (LT) C₄ synthase (LTC₄S), which is especially upregulated in the lungs⁶ and nasal polyps⁷ of subjects with AERD. Because of their overexpression of LTC₄S, subjects with AERD demonstrate constitutive overproduction of CysLT, as can be demonstrated in bronchoalveolar lavage fluid and exhaled breath condensate,⁸ as well as through quantification of urinary LTE₄.⁹ Upregulation of LTC₄S permits the further profound surge in CysLT production after the mast cell and eosinophil activation that occurs when these patients ingest aspirin and other nonselective nonsteroidal anti-inflammatory drugs (NSAIDs).^{10,11}

CysLTs function through their ability to interact with at least 2 receptors. CysLT type 1 receptors (CysLT₁R) are prominently expressed on airway smooth muscle, eosinophils, and other immune cells,¹² and these receptors primarily mediate the CysLT-induced bronchospasm associated with aspirin reactions.^{13,14} Subjects with AERD also demonstrate profound increased sensitivity to CysLTs,¹⁵ reflecting their marked upregulation in CysLT₁R.¹⁶

Both the pathway responsible for CysLT synthesis and the expression of CysLT receptors are tightly regulated by cytokines, including, prominently, IL-4. Induction of LTC₄S in mast cells is a biologic activity of IL-4 (but not IL-13),¹⁷ and similarly, IL-4 induced CysLT₁R on numerous target cells relevant to AERD.^{18,19}

Aspirin desensitization is an effective treatment for AERD.^{3,20,21} The molecular mechanism of the beneficial effects of aspirin has not been determined but could be related to the ability of this compound to inhibit the biologic activities of IL-4. Consistent with this concept are the observations that successful aspirin desensitization is associated with downregulation of both CysLT₁R expression^{15,16} and LT synthesis.²² Aspirin directly inhibits T-cell IL-4 expression.²³ Alternatively, engagement of the IL-4 receptor by IL-4 induces the activation of signal transducer and activator of transcription 6 (STAT6) through the Janus kinases.²⁴⁻²⁶ STAT6 activation is critical for many of the

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Abbreviations used

AERD: Aspirin-exacerbated respiratory disease
 CysLT: Cysteinyl leukotriene
 CysLT1R: Cysteinyl leukotriene type 1 receptor
 EMSA: Electrophoretic mobility shift assay
 LT: Leukotriene
 LTC₄S: Leukotriene C₄ synthase
 mPGES-1: Microsomal prostaglandin E synthase 1
 NF-κB: Nuclear factor κB
 NSAID: Nonsteroidal anti-inflammatory drug
 pSTAT6: Phospho-signal transducer and activator of transcription 6
 STAT6: Signal transducer and activator of transcription 6

biologic activities of IL-4, including induction of the IgE isotype switch. A STAT6 site in the CysLT1R promoter has been identified and shown to be critical for its IL-4-mediated transcription regulation.²⁷ Similarly, a putative STAT6 site has been identified in the LTC₄S gene (Bing Lam, unpublished data). Studies demonstrate that aspirin inhibits the activation of STAT6.²⁸ This might not be specific to STAT6 but might reflect broader influences of aspirin (and other NSAIDs) on cell transduction pathways, including nuclear factor of activated T cells- and nuclear factor κB (NF-κB)-dependent transcription.²⁹⁻³¹ These observations suggested that aspirin might produce its clinical utility in subjects with AERD through direct inhibition of the IL-4-activated STAT6 pathway. Anaphylactoid reactions reflect inhibition of COX and, as such, also occur with other COX inhibitors.³² Although desensitization can be induced to other COX inhibitors, it is not established whether the therapeutic benefit that follows desensitization reflects COX inhibition or some of these other off-target anti-inflammatory effects of NSAIDs and salicylates. We therefore investigated the inhibition by aspirin and other NSAIDs of the STAT6-mediated regulation of the CysLT1R and LTC₄S genes.

METHODS**Cell culture**

THP-1 mononuclear phagocytic cells were purchased from American Type Tissue Culture (Manassas, Va) and grown in complete RPMI 1640 medium (Invitrogen, Carlsbad, Calif) supplemented with HEPES (10 mmol/L), penicillin (100 U/mL)/streptomycin (100 μg/mL, Invitrogen), L-glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), glucose (4.5 g/L), sodium bicarbonate (1.5 g/L), β-mercaptoethanol (0.05 mmol/L), and 10% FBS (HyClone, Logan, Utah). Cells were maintained at 37 °C in 5% CO₂.

Aspirin/NSAID/salicylate modulation of gene expression

THP-1 cells were evaluated for putative IL-4-inducible genes, including CysLT1R, LTC₄S, FcεRIα, IL-4 receptor, and CCL11 (eotaxin-1). Cells (10⁶) were subsequently preincubated with or without aspirin (0.1-10 mmol/L), lysine aspirin (10 mmol/L), ketorolac (10 mmol/L; Bedford Laboratories, Bedford, Ohio), or sodium salicylate (10 mmol/L; Sigma, St Louis, Mo) for 15 minutes and then exposed to IL-4 (10 ng/mL; BD Biosciences, San Jose, Calif) for 16 hours. Aspirin was solubilized as a 1 mol/L stock solution in ethanol and diluted into culture media, and all other NSAIDs were solubilized directly as 1 mol/L stock solutions in culture media. These concentrations were chosen from preliminary studies involving dose-response curves that demonstrated optimal activity or from published studies.²⁸ Expression of CysLT1R and FcεRIα was determined by means of quantitative RT-PCR. Total RNA was extracted from cells by using a SV Total RNA Isolation kit (Promega, Madison,

Wis). Conversion of the mRNA to cDNA was performed with a Taqman Reverse Transcription kit (Roche, Branchburg, NJ), as previously described.³³ Briefly, 200 ng of RNA was added to each reaction along with oligo dT primers, 5.5 mmol/L MgCl₂, 2 mmol/L deoxyribonucleoside triphosphates, RNasin, and reverse transcriptase. Reactions went through 1 cycle of 10 minutes at 25 °C, 30 minutes at 48 °C, and 5 minutes at 95 °C in a Bio-Rad iCycler thermocycler (Bio-Rad, Hercules, Calif). The cDNA was amplified by means of PCR with SYBR Green detection (Bio-Rad) by using primer pairs as previously described³⁴ for CysLT1R and LTC₄S, as well as the housekeeping genes β-actin and EF-1α. Primer pairs for each reaction were synthesized by Integrated DNA Technologies (Coralville, Iowa). Quantification of changes in receptor expression induced by cytokines was performed by using the comparative cycle threshold (CT) method. Briefly, the amount of target, normalized to an endogenous reference and relative to a calibrator was calculated by $2^{-\Delta\Delta CT}$ as follows:

$$\Delta\Delta CT = (\text{Threshold cycle unstimulated gene of interest} - \text{Threshold cycle unstimulated housekeeping gene}) - (\text{Threshold cycle stimulated gene of interest} - \text{Threshold cycle stimulated housekeeping gene}).$$

The comparative CT method was validated by showing that the efficiencies of target and reference amplification were equal across a range of mRNA concentrations.

Monocytes

Monocytes were enriched from healthy subjects who were not aspirin sensitive under a protocol approved by the Human Investigations Committee at the University of Virginia and after informed consent was provided by the subject. PBMCs were isolated through Ficoll-Hypaque density gradient centrifugation from heparinized venous blood. CD14⁺ monocytes were enriched (>90%) by means of negative selection with magnetic bead affinity chromatography (Miltenyi Biotec, Auburn, Calif). Monocytes were resuspended in complete medium with 10% autologous serum and stimulated as described for THP-1 cells.

Nuclear extracts

Relevant nuclear extracts were obtained from THP-1 mononuclear cells or healthy donor monocytes. Cells were either left in a resting state or exposed to aspirin, lysine aspirin, ketorolac, or sodium salicylate (10 mmol/L each) for 15 minutes followed by IL-4 (10 ng/mL for 30 minutes). Nuclear extracts were prepared by using a commercial kit according to the manufacturer's directions (Nuclear extraction kit, Sigma).

Electrophoretic mobility shift assay

DNA oligomers containing the putative STAT6 sites within the CysLT1R and the LTC₄S promoters were synthesized (Integrated DNA Technologies) by using the following sequences:

CysLT1R sequence:

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5' - TCGA AATAATTTCTGAGAATTCTAT - 3'
      |||
3' - TTATTAAGGACTCTTAAGATAAGCT - 5'
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LTC₄ Synthase sequence:

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5' - TCGA CTGAGGGACTTCC TGAGAAGT - 3'
      |||
3' - GACTCCCTGAAGGACTCTTC AAGCT - 5'
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³²P-deoxycytidine triphosphate internal radiolabeling of double-stranded DNA probes was performed as previously described.^{35,36} Labeled probe was electrophoresed on a 6% nondenaturing polyacrylamide gel to separate labeled probe from free nucleotide and unlabeled double-stranded oligonucleotides that were incompletely extended. The probe was eluted from the gel and isolated over a Centriscap separation column (Princeton Separations, Adelphia, NJ), and each probe was counted to determine the radioactivity. From the

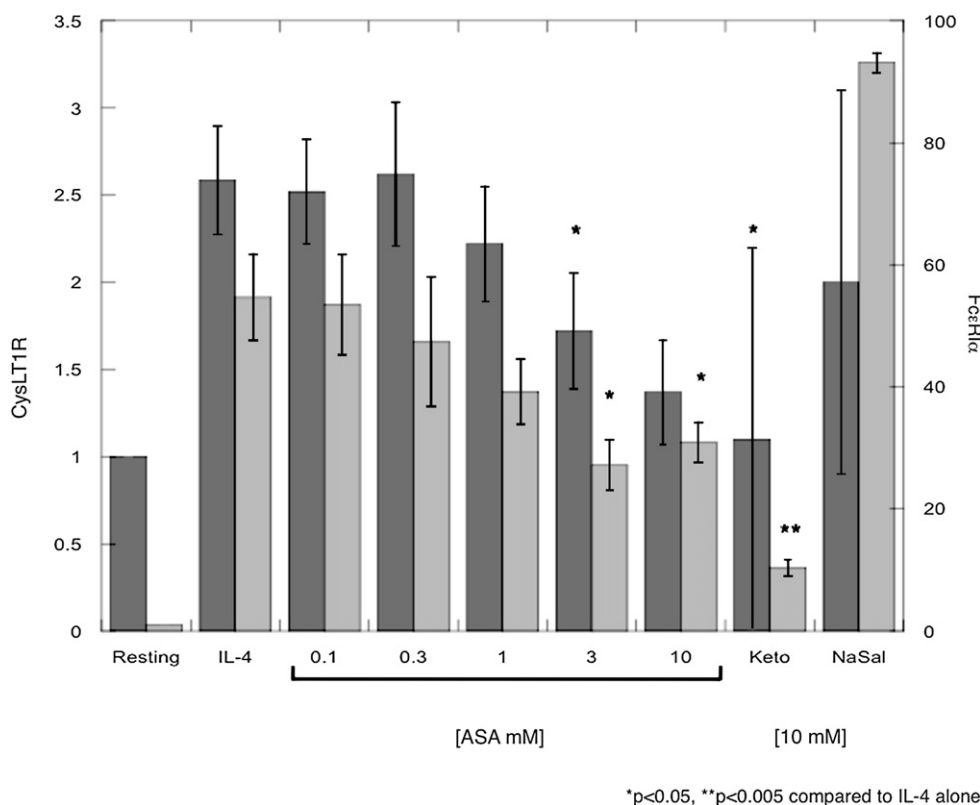


FIG 1. Modulation by aspirin and other NSAIDs of CysLT1R and FcεRIα IL-4-induced transcript expression. Gene expression was induced by IL-4 (10 ng/mL), and its inhibition by aspirin (ASA; 0.1-10 mmol/L), ketorolac (10 mmol/L), and sodium salicylate (10 mmol/L) was evaluated by means of quantitative PCR. Data are expressed as the fold increase compared with unstimulated THP-1 cells. Dark gray bars, CysLT1R; light gray bars, FcεRIα. * $P < .05$ compared with IL-4 alone.

counts, the femtomole of each probe was calculated. Electrophoretic mobility shift assays (EMSAs) were electrophoresed on a 6% nondenaturing polyacrylamide gel. Specific binding of STAT6 to the CysLT1R and LTC₄S promoters was evaluated by means of competition with 30- to 1000-fold molar excess of unlabeled STAT6 consensus double-stranded oligonucleotides derived from the ϵ heavy chain promoter (5'-GTATTTCCAGAAAAGGAAC-3') and mutant (base changes are underlined) consensus probes (5'-GTATTTCCGGTTAAAAGGAAC-3'; Santa Cruz Biotechnology, Santa Cruz, Calif). Supershift assays were performed with anti-STAT6, anti-phospho-STAT6 (pSTAT6), and, as controls, anti-Stat4 and anti-NF- κ B antibodies (Santa Cruz Biotechnology).

Western hybridization of nuclear extracts

Nuclear extracts were obtained as previously described. Protein concentration was determined for each sample, and equal amounts of protein were added to 2 $\times$ Laemmli Sample Buffer (Bio-Rad) and heated for 3 minutes at 95 °C. Samples were run on a 10% SDS polyacrylamide gel (ISC BioExpress, Kaysville, Utah) and transferred to a nitrocellulose membrane for 18 hours at 40 V. The membrane was blocked with TBST buffer (50 mmol/L Tris-HCl [pH 7.6], 150 mmol/L NaCl, and 0.05% Tween-20) with 5% nonfat dry milk before addition of anti-pSTAT6 antibodies (Santa Cruz Biotechnology). The membrane was washed before addition of the goat anti-rabbit horseradish peroxidase secondary antibody (Bio-Rad). Blots were developed by using the Supersignal West Pico Chemiluminescent kit (Pierce, Rockford, Ill).

Statistical analysis

Data for quantitative PCR are expressed as means $\pm$ SEMs. Statistical significance between resting and IL-4-stimulated cells (with and without

aspirin) was determined by using the Mann-Whitney test for nonparametric data with JMP 6.02 software (SAS Institute, Inc, Cary, NC) on a Macintosh computer. A P value of less than .05 was considered to indicate statistical significance.

RESULTS

Aspirin modulation of gene expression

Our initial studies were performed by using the human mononuclear phagocytic cell line THP-1 as a representative cell line known to express pSTAT6 in response to IL-4. We screened our THP-1 cells for increased expression of CysLT1R, LTC₄S, FcεRIα, IL-4 receptor, CD23, and CCL11 (eotaxin-1) transcripts in response to IL-4 by means of quantitative PCR. In preliminary experiments we optimized time course and dose response for IL-4 induction of gene expression. Subsequent experiments were performed with 10 ng/mL IL-4 for 16 hours. IL-4-inducible expression of CysLT1R and FcεRIα mRNA was measured (Fig 1), and the upregulation of CysLT1R and FcεRIα by IL-4 was significantly reduced in a dose-dependent fashion by aspirin. Data represent means $\pm$ SEMs of at least 3 replicate experiments. Although LTC₄S expression in mast cells¹⁷ has been shown to be induced by IL-4, upregulation by IL-4 was not observed in the THP-1 cell line.

Modulation of gene expression by NSAIDs

Quantitative PCR results for FcεRIα and CysLT1R transcripts were also evaluated in the additional presence of lysine aspirin, sodium salicylate, and ketorolac to determine whether modulation of gene expression was specific to aspirin (Fig 1). Inhibition of

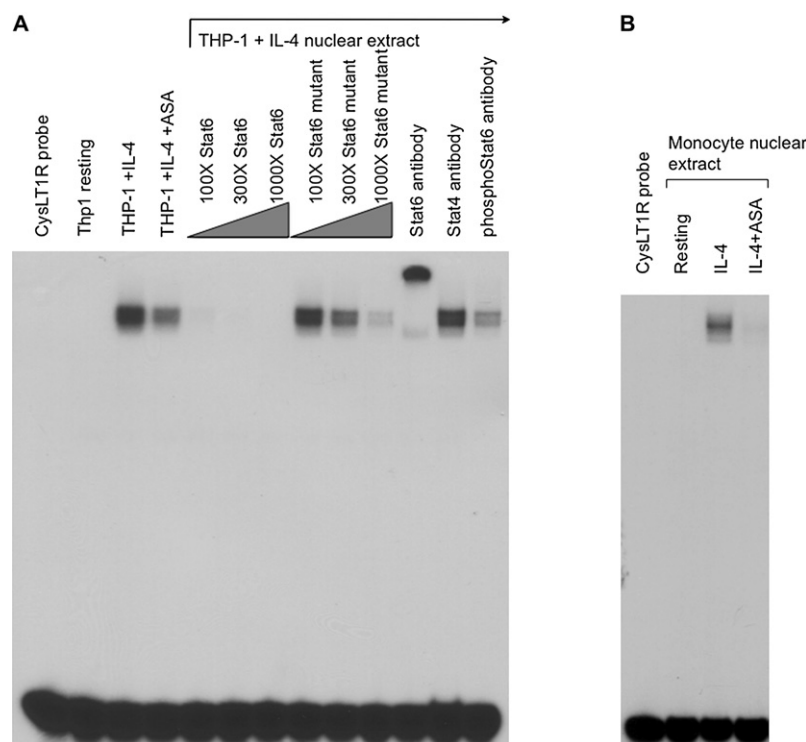


FIG 2. EMSA for the STAT6 site in the CysLTR1 promoter. **A**, Nuclear extracts were obtained from THP-1 mononuclear cells under unstimulated, IL-4-stimulated (10 ng/mL), and IL-4-stimulated in the presence of aspirin (ASA; 10 mmol/L) conditions. STAT6 binding was evaluated by means of competition with 30- to 1000-fold molar excess of unlabeled STAT6 consensus but not a mutant probe, as well as by supershifting with anti-STAT6, anti-pSTAT6, and anti-Stat4 antibodies. **B**, Relevance to normal tissue was evaluated by using nuclear extracts derived from enriched peripheral blood-derived mononuclear phagocytes.

transcript expression was limited to aspirin, lysine aspirin, and ketorolac. Data represent means $\pm$ SEMs of 5 replicate experiments.

EMSAs

EMSAs were initially performed on the STAT6 site, which is reported to be present within the CysLTR1R promoter and critical for transcription activity.²⁷ We evaluated the presence of activated STAT6 within nuclear extracts derived from THP-1 mononuclear phagocytic cells. Nuclear extracts from IL-4-stimulated (10 ng/mL for 30 minutes) but not from resting THP-1 cells produced a mobility shift (Fig 2, A). That this was produced by the presence of STAT6 within these nuclear extracts was confirmed by the ability of a molar excess of unlabeled DNA derived from the STAT6 sequence within the ϵ heavy chain promoter, but not a mutant form of this consensus sequence, to compete for binding of this nuclear extract-derived factor. The IL-4-inducible presence of STAT6 was confirmed by means of supershifting the protein-DNA complex with an anti-STAT6 antibody and by means of inhibition of STAT6 binding with an anti-pSTAT6 antibody that binds to the DNA-binding domain of STAT6. No change in binding was observed with the control anti-Stat4 antibody. The addition of aspirin (10 mmol/L) 15 minutes before stimulation with IL-4 precluded expression of activated STAT6 within the nuclear extracts (Fig 2, A). Induction of STAT6 by IL-4 and prevention of its expression within the nucleus by aspirin was confirmed in monocytes derived from a healthy donor to confirm the physiologic relevance of these observations (Fig 2, B). Although there is no reason to believe that monocytes from subjects with

AERD and those from non-aspirin-sensitive asthmatic subjects would behave differently, such experiments have not been performed by us. In separate experiments (a representative result of 3 similar experiments is displayed in Fig 3) inhibition of nuclear pSTAT6 expression was shown for aspirin and ketorolac but less so with lysine aspirin and sodium salicylate. Similarly, we demonstrated induction by IL-4 of STAT6 within nuclear extracts capable of binding to the putative STAT6 site defined within the LTC₄S gene promoter and prevention through preincubation with aspirin (Fig 4). Identity as STAT6 was again confirmed both by means of competition with a STAT6 consensus sequence and by the supershifting observed with anti-STAT6 antibody.

Western hybridization

Western blots of the nuclear extracts used for the EMSA studies probed with anti-pSTAT6 confirmed that IL-4 treatment of THP-1 cells induced expression of pSTAT6 protein and directed its migration into the nucleus (Fig 5). Similar to what was observed with the EMSAs, coculture of THP-1 cells with aspirin, lysine aspirin, or sodium salicylate for 15 minutes before IL-4 stimulation markedly reduced the appearance of pSTAT6 protein within the nuclear extracts. With ketorolac, pSTAT6 was no longer detectable in the nuclear extract by means of immune staining.

IL-4 modulation of prostaglandin pathway

It is possible that COX products from monocytes could act in an autocrine or paracrine manner and that this might explain the lack

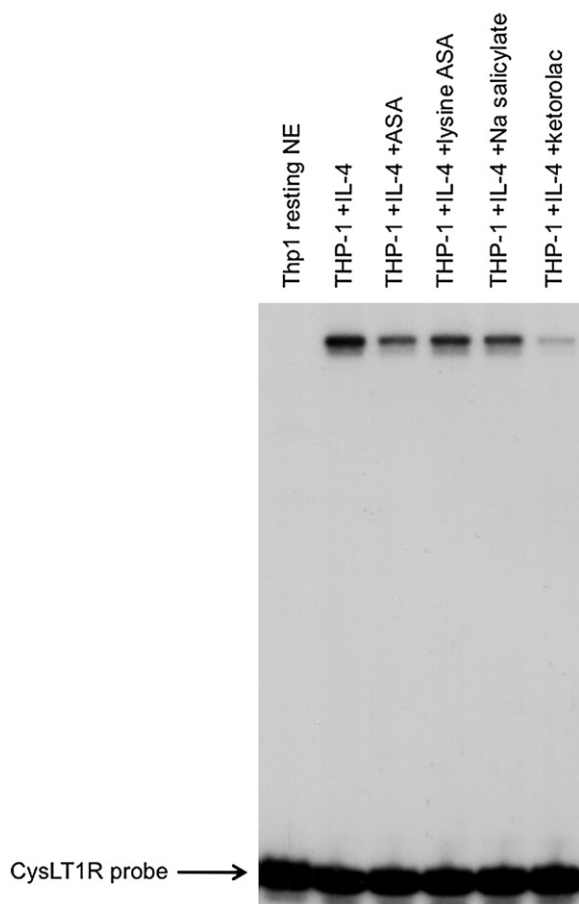


FIG 3. Modulation of STAT6 EMSA results by aspirin (ASA) and other NSAIDs. EMSAs were performed as previously described. Nuclear extracts (NE) were obtained from THP-1 mononuclear cells in the resting state, stimulated with IL-4 (10 ng/mL), and stimulated with IL-4 in the additional presence of aspirin (10 mmol/L), lysine aspirin (10 mmol/L), sodium salicylate (10 mmol/L), or ketorolac (10 mmol/L).

of effect observed with sodium salicylate. The effect of IL-4 on basal expression of COX-1, COX-2, and microsomal prostaglandin E synthase 1 (mPGES-1) mRNA in monocytes was examined (see Methods in this article's Online Repository at www.jacionline.org). IL-4, but not IL-13, was able to decrease expression of COX-2 and mPGES-1 (see Table E1 in this article's Online Repository at www.jacionline.org). When monocytes were stimulated with LPS or IL-1 β , mPGES-1 mRNA levels increased significantly, and this increase was again inhibited by IL-4 (see Fig E1 in this article's Online Repository at www.jacionline.org). The net result was that addition of IL-4 to monocytes prevented basal or stimulated release of prostaglandin E₂ (see Fig E2 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Aspirin desensitization is an effective treatment for AERD and has been associated with diminished need for nasal endoscopic surgery, improved sense of smell, fewer bouts of acute sinusitis, reduced need for oral corticosteroids, and less severe asthma.^{3,20,21} However, the mechanism underlying this efficacy is not understood. Severity of AERD is associated with the marked overproduction of CysLTs, reflecting in large part

overexpression of LTC₄S,^{6,7} and, similarly, the marked overresponsiveness to these proinflammatory mediators, reflecting in large part overexpression of CysLT1R.^{15,16} This is a central mechanism of AERD, as shown by the clinical efficacy of LT pathway inhibitors in these patients.³⁷ Successful aspirin desensitization is associated with downregulation of CysLT sensitivity and both CysLT1R expression^{15,16} and LT synthesis.²²

The mechanism by which aspirin might block these LT effects is not immediately obvious. Aspirin (and other NSAIDs) is known to have off-target effects, including modulation of nuclear trafficking of numerous transcription factors, including nuclear factor of activated T cells, NF- κ B, and STAT6.²⁹⁻³¹ Many of these off-target effects of aspirin only occur at significantly higher concentrations than those required for COX-1 or COX-2 inhibition.³⁰ The concentration of aspirin known to be effective after aspirin desensitization (1300 mg/d) is somewhat higher than that required to inhibit COX-1 or COX-2, which is consistent with the concept that the aspirin could be acting through one of these COX-independent pathways. The expression of both CysLT1R and LTC₄S is mediated by IL-4¹⁷⁻¹⁹ in a STAT6-dependent fashion (Bing Lam, unpublished data).²⁷ We therefore investigated the ability of aspirin to block the STAT6-dependent IL-4-inducible expression of CysLT1R and LTC₄S.

These studies confirmed the IL-4-dependent induction of CysLT1R on THP-1 cells (Fig 1). However, in contrast to mast cells, we did not see induction of LTC₄S. Although mononuclear phagocytic cells can synthesize and secrete CysLTs, in comparison with mast cells, basophils, and eosinophils, they are considered only minor sources³⁸; however, monocytes from subjects with AERD might show enhanced induction of LTC₄S. In subjects with AERD, mast cells and eosinophils are considered the major source of the surge of CysLTs observed,¹¹ and monocytes are not known to be specifically targeted by aspirin. As cancerous cells, THP-1 might not be reflective of physiologic mononuclear phagocytic cells. Thus our failure to demonstrate IL-4-inducible LTC₄S in THP-1 cells might reflect either specific inhibitors or the absence of relaxed chromatin in these cells, which are capable of responding to the induced pSTAT6. The more general significance of inhibition of IL-4-inducible activities by aspirin can be deduced, however, from our observation regarding reduced Fc ϵ RI α expression.

Our EMSA studies confirm the presence of these STAT6 sites within both the CysLT1R and LTC₄S promoters (Figs 2 and 4). The presence of a mobility shift specifically mediated by STAT6 was confirmed by the ability of a molar excess of unlabeled DNA probes comprising the STAT6 site within the ϵ heavy chain promoter to block binding but not a mutated version of this STAT6 probe. That this shift was produced by pSTAT6 was further confirmed by using anti-STAT6 and anti-pSTAT6 antibodies.

The mechanism by which aspirin blocks STAT6 expression is not known but has been suggested to involve nuclear trafficking and recycling of transcription factors.³⁰ The current studies do not distinguish whether aspirin acts to block either induction of pSTAT6 or trafficking of newly activated pSTAT6 into the nucleus. Our Western hybridization data do confirm the absence of functional pSTAT6 protein within the nuclei of aspirin-treated cells (Fig 4). These results were extended to other NSAIDs, including ketorolac, but not sodium salicylate. Ketorolac has been successfully used in the diagnosis of aspirin intolerance,³² and these observations suggest a plausible basis for it to have clinical efficacy in subjects with AERD.

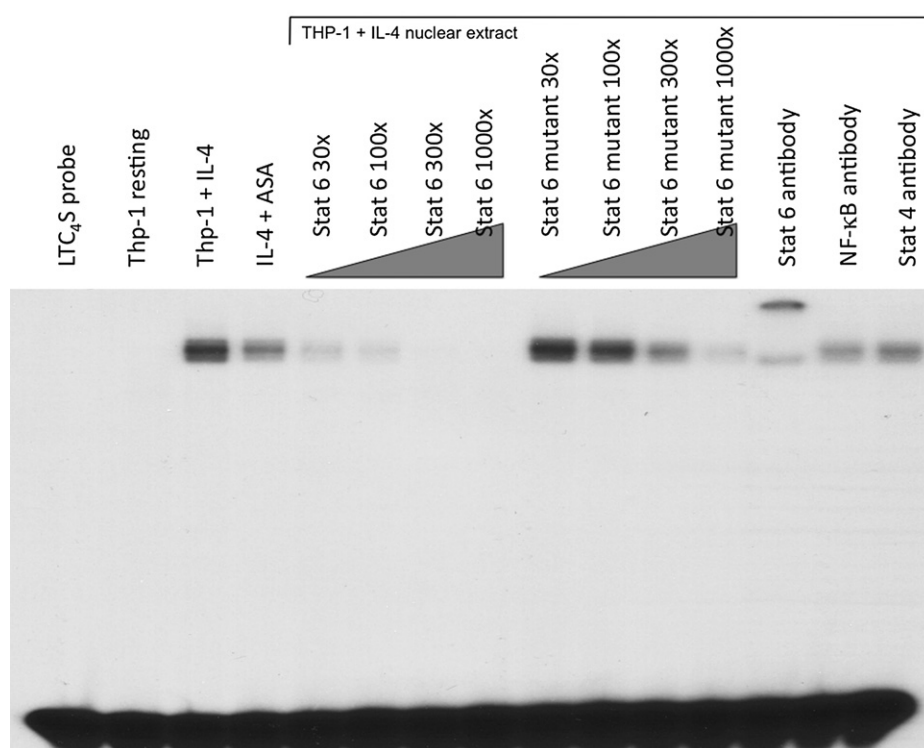


FIG 4. EMSAs for the STAT6 site in LTC₄S promoter. EMSAs were performed as described for CysLT1R. Confirmation of STAT6 engagement of consensus promoter sequence was confirmed by means of competition with 30- to 1000-fold molar excess of unlabeled STAT6 consensus but not a mutant probe, as well as by supershifting with anti-STAT6 but neither of the control (NF- κ B or Stat4) antibodies. ASA, Aspirin.

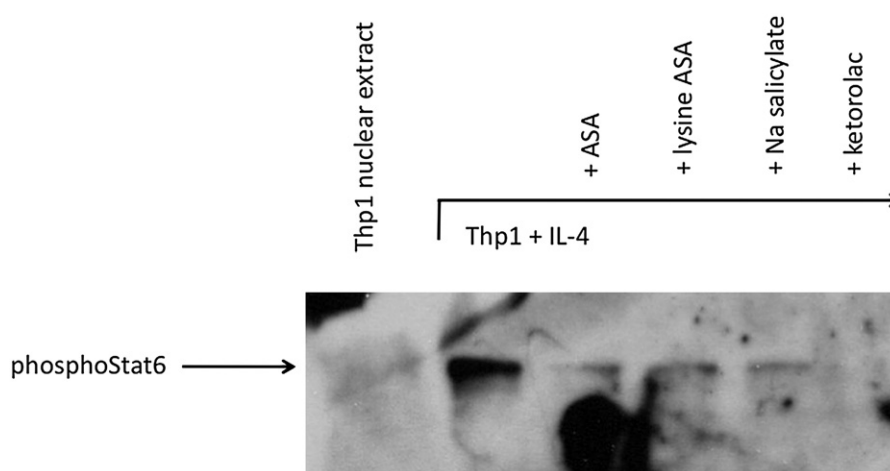


FIG 5. Western hybridization of nuclear extracts. Nuclear extract proteins were separated on an SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The presence of pSTAT6 was determined by means of probing with an anti-pSTAT6 antibody and chemiluminescent detection.

Countering the argument that aspirin might function in subjects with AERD as an IL-4–STAT6 antagonist is the lack of an obvious recognition of its utility in aspirin-tolerant asthma or even allergic rhinitis, disorders that also involve IL-4 and STAT6 expression. It could be disputed that if aspirin is an effective anti-IL-4 agent, it should be effective in all asthmatic subjects. A subset of asthmatic subjects who have aspirin-responsive asthma is recognized,³⁹ and aspirin might have efficacy in patients with chronic sinusitis

without aspirin intolerance (Donald Stevenson, unpublished data). Intriguingly, aspirin is reportedly protective against the development of asthma, as shown in a randomized clinical trial of apparently healthy adult women, in which the administration of 100 mg of aspirin on alternate days reduced the relative risk of a newly reported diagnosis of asthma.⁴⁰ AERD is a distinct disorder from aspirin-tolerant asthma, and these subjects are unique in their production of and sensitivity to LTs. Efficacy of aspirin

use in subjects with AERD might therefore reflect the heightened importance of these LT-dependent and plausibly aspirin-responsive mechanisms in subjects with AERD in comparison with subjects with aspirin-tolerant asthma.

In conclusion, the CysLT₁ receptor and LTC₄S promoters have STAT6-binding sites that are engaged by IL-4–induced nuclear extracts and inhibited by aspirin. Aspirin desensitization might provide effective therapy for AERD, at least in part, through mitigation of STAT6 expression, thereby downregulating LT pathways. In the future, it will be interesting to study monocytes from subjects with AERD and compare the responses with those seen in healthy subjects and see whether there are inherent differences in the responses to aspirin and other NSAIDs or whether this is a general phenomenon of all monocytes.

Clinical implications: Aspirin desensitization is an effective treatment for AERD. Our studies demonstrated that aspirin inhibits the IL-4–activated STAT6 pathway and suggest that this might be the basis for its clinical utility in subjects with AERD.

REFERENCES

- Mascia K, Haselkorn T, Deniz YM, Miller DP, Bleecker ER, Borish L. Aspirin sensitivity and severity of asthma: evidence for irreversible airway obstruction in patients with severe or difficult-to-treat asthma. *J Allergy Clin Immunol* 2005;116:970-5.
- Mascia K, Borish L, Patrie J, Hunt J, Phillips CD, Steinke JW. Chronic hyperplastic eosinophilic sinusitis as a predictor of aspirin-exacerbated respiratory disease. *Ann Allergy Asthma Immunol* 2005;94:652-7.
- Szczeklik A, Stevenson DD. Aspirin-induced asthma: advances in pathogenesis and management. *J Allergy Clin Immunol* 1999;104:5-13.
- Szczeklik A, Nizankowska E. Clinical features and diagnosis of aspirin induced asthma. *Thorax* 2000;55(suppl 2):S42-4.
- Berges-Gimeno MP, Simon RA, Stevenson DD. The natural history and clinical characteristics of aspirin-exacerbated respiratory disease. *Ann Allergy Asthma Immunol* 2002;89:474-8.
- Cowburn AS, Sladek K, Soja J, Adamek L, Nizankowska E, Szczeklik A, et al. Overexpression of leukotriene C₄ synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest* 1998;101:834-46.
- Perez-Novo CA, Watelet JB, Claeys C, Van Cauwenberge P, Bachert C. Prostaglandin, leukotriene, and lipoxin balance in chronic rhinosinusitis with and without nasal polyposis. *J Allergy Clin Immunol* 2005;115:1189-96.
- Antczak A, Montuschi P, Kharitonov S, Gorski P, Barnes PJ. Increased exhaled cysteinyl-leukotrienes and 8-isoprostane in aspirin-induced asthma. *Am J Respir Crit Care Med* 2002;166:301-6.
- Daffern PJ, Muilenburg D, Hugli TE, Stevenson DD. Association of urinary leukotriene E₄ excretion during aspirin challenges with severity of respiratory responses. *J Allergy Clin Immunol* 1999;104:559-64.
- Sladek K, Szczeklik A. Cysteinyl leukotrienes overproduction and mast cell activation in aspirin-provoked bronchospasm in asthma. *Eur Respir J* 1993;6:391-9.
- Fischer AR, Rosenberg MA, Lilly CM, Callery JC, Rubin P, Cohn J, et al. Direct evidence for a role of the mast cell in the nasal response to aspirin in aspirin-sensitive asthma. *J Allergy Clin Immunol* 1994;94:1046-56.
- Lynch KR, O'Neill GP, Liu Q, Im DS, Sawyer N, Metters KM, et al. Characterization of the human cysteinyl leukotriene CysLT₁ receptor. *Nature* 1999;399:789-93.
- Dahlen SE, Malmstrom K, Nizankowska E, Dahlen B, Kuna P, Kowalski M, et al. Improvement of aspirin-intolerant asthma by montelukast, a leukotriene antagonist: a randomized, double-blind, placebo-controlled trial. *Am J Respir Crit Care Med* 2002;165:9-14.
- White A, Ludington E, Mehra P, Stevenson DD, Simon RA. Effect of leukotriene modifier drugs on the safety of oral aspirin challenges. *Ann Allergy Asthma Immunol* 2006;97:688-93.
- Arm JP, O'Hickey SP, Spur BW, Lee TH. Airway responsiveness to histamine and leukotriene E₄ in subjects with aspirin-induced asthma. *Am Rev Respir Dis* 1989;140:148-53.
- Sousa AR, Parikh A, Scadding G, Corrigan CJ, Lee TH. Leukotriene-receptor expression on nasal mucosal inflammatory cells in aspirin-sensitive rhinosinusitis. *N Engl J Med* 2002;347:1493-9.
- Hsieh FH, Lam BK, Penrose JF, Austen KF, Boyce JA. T helper cell type 2 cytokines coordinately regulate immunoglobulin E-dependent cysteinyl leukotriene production by human cord blood-derived mast cells: profound induction of leukotriene C₄ synthase expression by interleukin 4. *J Exp Med* 2001;193:123-33.
- Thivierge M, Stankova J, Rola-Pleszczynski M. IL-13 and IL-4 up-regulate cysteinyl leukotriene 1 receptor expression in human monocytes and macrophages. *J Immunol* 2001;167:2855-60.
- Early SB, Barekzi E, Negri J, Hise K, Borish L, Steinke JW. Concordant modulation of CysLT receptor expression by IL-4 and IFN-gamma on peripheral immune cells. *Am J Respir Cell Mol Biol* 2007;36:715-20.
- Sweet JM, Stevenson DD, Simon RA, Mathison DA. Long-term effects of aspirin desensitization—treatment for aspirin-sensitive rhinosinusitis-asthma. *J Allergy Clin Immunol* 1990;85:59-65.
- Stevenson DD, Simon RA. Selection of patients for aspirin desensitization treatment. *J Allergy Clin Immunol* 2006;118:801-4.
- Juergens UR, Christiansen SC, Stevenson DD, Zuraw BL. Inhibition of monocyte leukotriene B₄ production after aspirin desensitization. *J Allergy Clin Immunol* 1995;96:148-56.
- Cianferoni A, Schroeder JT, Kim J, Schmidt JW, Lichtenstein LM, Georas SN, et al. Selective inhibition of interleukin-4 gene expression in human T cells by aspirin. *Blood* 2001;97:1742-9.
- Oakes SA, Candotti F, Johnston JA, Chen YQ, Ryan JJ, Taylor N, et al. Signaling via IL-2 and IL-4 in JAK3-deficient severe combined immunodeficiency lymphocytes: JAK3-dependent and independent pathways. *Immunity* 1996;5:605-15.
- Miloux B, Laurent P, Bonnin O, Lupker J, Caput D, Vita N, et al. Cloning of the human IL-13R alpha1 chain and reconstitution with the IL4R alpha of a functional IL-4/IL-13 receptor complex. *FEBS Lett* 1997;401:163-6.
- Chen XH, Patel BK, Wang LM, Frankel M, Ellmore N, Flavell RA, et al. Jak1 expression is required for mediating interleukin-4-induced tyrosine phosphorylation of insulin receptor substrate and STAT6 signaling molecules. *J Biol Chem* 1997;272:6556-60.
- Woszczek G, Pawliczak R, Qi HY, Nagineni S, Alsaaty S, Logun C, et al. Functional characterization of human cysteinyl leukotriene 1 receptor gene structure. *J Immunol* 2005;175:5152-9.
- Perez GM, Melo M, Keegan AD, Zamorano J. Aspirin and salicylates inhibit the IL-4- and IL-13-induced activation of STAT6. *J Immunol* 2002;168:1428-34.
- Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* 1994;265:956-9.
- Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J* 2001;15:2057-72.
- Aceves M, Duenas A, Gomez C, San Vicente E, Crespo MS, Garcia-Rodriguez C. A new pharmacological effect of salicylates: inhibition of NFAT-dependent transcription. *J Immunol* 2004;173:5721-9.
- White A, Bigby T, Stevenson D. Intranasal ketorolac challenge for the diagnosis of aspirin-exacerbated respiratory disease. *Ann Allergy Asthma Immunol* 2006;97:190-5.
- Steinke JW, Bradley D, Arango P, Crouse CD, Frierson H, Kountakis SE, et al. Cysteinyl leukotriene expression in chronic hyperplastic sinusitis-nasal polyposis: importance to eosinophilia and asthma. *J Allergy Clin Immunol* 2003;111:342-9.
- Negri J, Early SB, Steinke JW, Borish L. Corticosteroids as inhibitors of cysteinyl leukotriene metabolic and signaling pathways. *J Allergy Clin Immunol* 2008;121:1232-7.
- Hagman J, Travis A, Grosschedl R. A novel lineage-specific nuclear factor regulates mb-1 gene transcription at the early stages of B cell differentiation. *EMBO J* 1991;10:3409-17.
- Fitzsimmons D, Hodsdon W, Wheat W, Maira SM, Wasylyk B, Hagman J. Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. *Genes Dev* 1996;10:2198-211.
- Dahlen B, Nizankowska E, Szczeklik A, Zetterstrom O, Bochenek G, Kumlin M, et al. Benefits from adding the 5-lipoxygenase inhibitor zileuton to conventional therapy in aspirin-intolerant asthmatics. *Am J Respir Crit Care Med* 1998;157:1187-94.
- Boyce JA. Eicosanoids in asthma, allergic inflammation, and host defense. *Curr Mol Med* 2008;8:335-49.
- Szczeklik A, Nizankowska E. Asthma improved by aspirin-like drugs. *Br J Dis Chest* 1983;77:153-8.
- Kurth T, Barr RG, Gaziano JM, Buring JE. Randomised aspirin assignment and risk of adult-onset asthma in the Women's Health Study. *Thorax* 2008;63:514-8.

METHODS

Cytokine/chemokine determination

Prostaglandin E₂ (Cayman Chemical, Ann Arbor, Mich) levels were measured in supernatants from cell cultures by using ELISA kits, according to the manufacturer's instructions. The sensitivity of this assay was less than 7.8 pg/mL. Concentrations were calculated on the basis of standard curves by using the KC4 Kineticalc for Windows software (Bio-Tek, Winooski, Vt).

Reverse transcription of mRNA

Total RNA was extracted from cells as described. Primers for COX-1, COX-2, and mPGES-1 are as follows: COX-1 forward, 5'-GTAGACCTCGGCCA

CATTATG-3'; COX-1 reverse, 5'-GGGGTAGTGCATCAACACAG-3'; COX-1 probe, 5'-FAM-CTGGAGCGTCAGTATCAACTGCGGCTC-BHQ1-3'; COX-2 forward, 5'-GTGGACTTAAATCATATTACGGTGAA-3'; COX-2 reverse, 5'-ATCATCTCTGCCTGAGTATCTTTG-3'; COX-2 probe, 5'-CY5-AAACTCTGGCTAGACAGCGTAAACTGCG-BHQ2-3'; mPGES-1 forward, 5'-CTTCCTCGTGGGCCGTGT-3'; and mPGES-1 reverse, 5'-TGGGCCAGGGTGTAGGTC-3'

Detection of products for mPGES-1 and β -actin was performed by using syber-green supermix (Bio-Rad), whereas detection of COX-1 and COX-2 was performed by using fluorescently labeled probes. Primer pairs and probes for each reaction were synthesized by Integrated DNA Technologies.

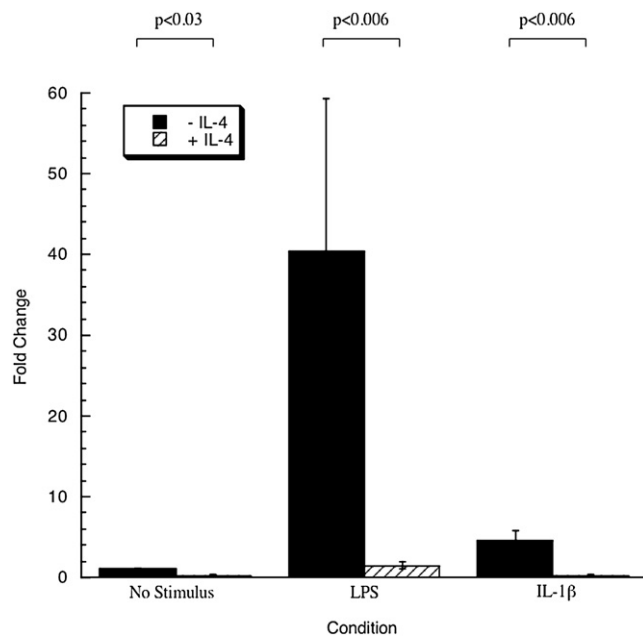


FIG E1. IL-4 inhibition of stimulated mPGES-1 mRNA expression. IL-4 was added to monocytes (10 ng/mL) alone or with either LPS (1 μ g/mL) or IL-1 β (10 ng/mL). Cells were incubated for 16 hours before RNA was collected. After reverse transcription of 200 ng of RNA, PCR was performed with primers specific for mPGES-1 and the housekeeping gene β -actin as a positive control. Products were quantified by using real-time PCR with syber-green detection and reported as a fold change in comparison with unstimulated monocytes.

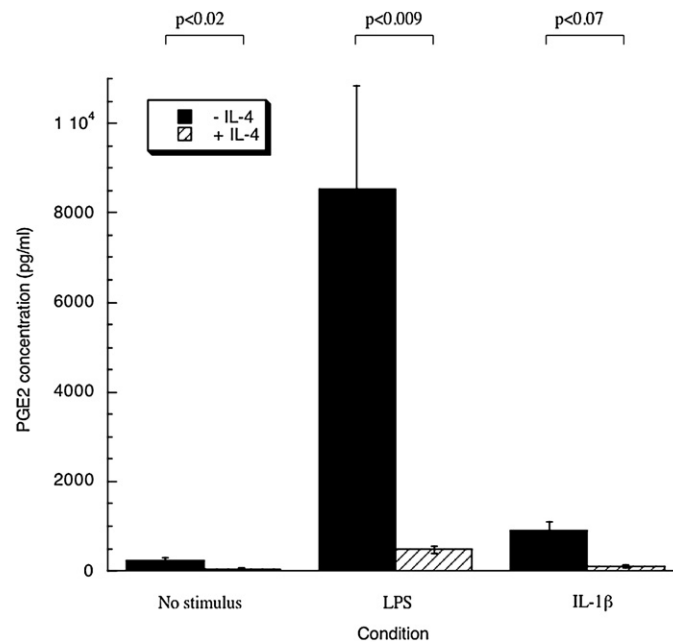


FIG E2. IL-4 inhibits monocyte prostaglandin E₂ (PGE₂) secretion. IL-4 was added to the cells (10 ng/mL) alone or with either LPS (1 μg/mL) or IL-1β (10 ng/mL). Cells were incubated for 24 hours before supernatants were collected. Prostaglandin E₂ levels were measured by means of ELISA and reported as picograms per milliliter.

TABLE E1. Quantitative PCR expression of COX-1/COX-2/mPGES-1: modulation by IL-4 and IL-13

Cell type	IL-4			IL-13		
	COX-1	COX-2	mPGES-1	COX-1	COX-2	mPGES-1
Monocytes	1.1 ± 0.7	0.3 ± 0.2*	0.2 ± 0.1*	1.4 ± 0.6	1.5 ± 0.9	1.5 ± 0.8

Data are presented as a fold change in comparison with unstimulated cells, the value of which was set at 1.

* $P < .01$.