

Nonsteroidal anti-inflammatory–induced inhibition of signal transducer and activator of transcription 6 (STAT-6) phosphorylation in aspirin-exacerbated respiratory disease

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Background: Aspirin desensitization provides long-term clinical benefits. The exact mechanisms of aspirin desensitization are not clearly understood.

Objective: We sought to evaluate the effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on T-cell activation of the IL-4 pathway in aspirin-sensitive patients with asthma and control subjects.

Methods: A total of 11 aspirin-sensitive patients with asthma, 10 aspirin-tolerant patients with asthma, and 10 controls without asthma were studied. PBMCs were stimulated with an anti-CD3 antibody and IL-4 or IL-12, with and without the presence of NSAIDs. The expression of phosphorylated signal transducers and activators of transcription 6 (pSTAT6), phosphorylated signal transducers and activators of transcription 4, and IL-4 was detected in CD4 T cells by flow cytometry.

Results: Stimulation with a combination of anti-CD3 and IL-4 induced pSTAT6 in CD4 T cells from all subjects. The induction of pSTAT6 was significantly higher in aspirin-sensitive patients with asthma than in controls subjects. The increase in pSTAT6 was inhibited in a dose-dependent manner by aspirin and indomethacin and minimally by sodium salicylate. This inhibition was strongest in aspirin-sensitive patients. Two-group comparisons showed significant differences in pSTAT6 inhibition by all concentrations of indomethacin and aspirin: between aspirin-sensitive and aspirin-tolerant groups and between aspirin-sensitive and control groups. No differences were found between aspirin-tolerant and control groups at all 3 concentrations. The inhibition of pSTAT6 was associated with reduced IL-4 expression.

Conclusions: NSAIDs inhibited signal transducers and activators of transcription 6 signaling in CD4 T cells. This inhibition was significantly higher in aspirin-sensitive patients than in aspirin-

tolerant subjects and was associated with reduced expression of IL-4. These findings have implications for clinical benefits of aspirin desensitization in aspirin-sensitive patients with asthma. (*J Allergy Clin Immunol* 2016;■■■:■■■-■■■.)

Key words: Aspirin-exacerbated respiratory disease, IL-4 signaling inhibition, pSTAT6 inhibition, mechanism of aspirin desensitization

It has been almost 100 years since Widal et al¹ first described aspirin challenges and desensitization for patients with the syndrome of asthma, nasal polyposis, and aspirin intolerance. Later, following works by Samter and Beers,² the condition was termed Samter's triad because of the association of these 3 overlapping conditions. Subsequent studies have shown that aspirin desensitization therapy provides significant clinical improvement in nasal symptoms and a reduction in corticosteroid use.³ Symptom improvement has been reported to occur as early as 4 weeks after the initiation of aspirin therapy, and long-term benefits include significant reductions in the number of sinus infections and operations, hospitalizations for asthma, and use of systemic steroids.^{4,5} Despite these data supporting the clinical benefit, the exact mechanisms of aspirin desensitization are still not clearly understood.

Aspirin-exacerbated respiratory disease (AERD) is characterized by a high level of tissue, as well as blood eosinophils, suggesting a type 2 immune response (including a T_H2 response) disorder. Recently, Steinke et al⁶ reported an increased expression of IFN- γ mRNA transcripts in the nasal polyp tissue derived from subjects with AERD, as compared with aspirin-tolerant patients and control subjects. Eosinophils were the source of IFN- γ . The AERD tissue expressed high levels of not only IFN- γ but also IL-4. Interestingly, the level of IL-5 was not elevated. The noted findings primarily reflected the cytokine milieu derived from tissue eosinophils.⁶ The simultaneous presence of IL-4 and IFN- γ in nasal polyps from subjects with AERD is similar to that observed in atopic dermatitis, another T_H2 disease with a mixed T_H1/T_H2 cytokine milieu. Thus, AERD may represent a mixed T_H1/T_H2 immune response disease.

AERD is associated with increased cysteinyl-leukotriene production. Urinary leukotriene E₄ (LTE₄) levels correlate with the severity of aspirin response.⁷ During desensitization, urine LTE₄ levels initially increase following aspirin ingestion but subsequently decrease to basal levels with continued treatment. Cysteinyl-leukotriene receptor 1 numbers also decrease following desensitization.⁸⁻¹¹ Recently, there has been progress in further elucidating immunologic differences in patients with AERD and differential responses following aspirin desensitization. We have shown that aspirin desensitization was associated with

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Abbreviations used

AERD: Aspirin-exacerbated respiratory disease
 LTE₄: Leukotriene E₄
 MFI: Mean fluorescence intensity
 NSAID: Nonsteroidal anti-inflammatory drug
 pSTAT6: Phosphorylated signal transducers and activators of transcription 6
 STAT4: Signal transducers and activators of transcription 4
 STAT6: Signal transducers and activators of transcription 6
 Jak1: Janus kinase 1

suppression of IL-4 and matrix metalloproteinase-9.¹² We conducted the following study to examine the mechanisms underlying the mechanism of reduced IL-4 production following aspirin desensitization. We evaluated the *in vitro* effects of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) on peripheral blood T-cell activation in aspirin-sensitive and aspirin-tolerant patients with asthma, as well as in healthy controls.

METHODS**Subjects**

Subjects were recruited from our outpatient clinic at National Jewish Health in Denver, Colo. Subjects were selected on the basis of their history of AERD and included a diagnosis of asthma, chronic hyperplastic sinus disease with nasal polyposis, and a convincing history of aspirin sensitivity. Patients with AERD had already agreed to undergo aspirin challenge and desensitization in consultation with their primary physician before recruitment. Asthma was based on physician diagnosis. All subjects had a previous positive methacholine and historical reversibility of at least 12% and 200 cc, respectively, and were not on oral steroids. A total of 11 aspirin-sensitive patients with asthma underwent aspirin challenge and desensitization as described previously, although in this experiment we studied baseline data as described below.¹² We also studied 10 aspirin-tolerant patients with asthma as well as 10 controls without asthma. Controls without asthma included 6 subjects with allergic rhinitis without asthma and 4 healthy subjects.

Blood collection

Protocol for serum blood draw was approved through the Institutional Review Board of National Jewish Health (HS no. 2591). Approximately 50 mL of blood was collected in EDTA anticoagulated (lavender top) tubes from each subject on completion of appropriate consent. Blood was collected before aspirin challenge and was kept at room temperature until processing within 1 to 3 hours of collection.

Aspirin inhibition and IL-4 stimulation

PBMCs were isolated by using Ficoll-Hypaque (Histopaque-1077; Sigma-Aldrich, St Louis, Mo) gradient centrifugation. Cell culture plates were coated with 2 μ g/mL of antihuman CD3 antibody (clone OKT3; eBioscience, San Diego, Calif). PBMCs were cultured in the coated plates in RPMI 1640 with 10% FBS. NSAID stock solutions of 30 mM, 100 mM, and 300 mM were made by dissolving aspirin (acetylsalicylic acid), indomethacin, and sodium salicylate (Sigma-Aldrich) in ethanol as a vehicle. Following overnight culture, equal volumes of the appropriate NSAID stock solution, or ethanol, were added to PBMC cell cultures for final concentrations of 0.3 mM, 1 mM, and 3 mM. This concentration (1%) of ethanol did not affect cell viability, signal transducers and activators of transcription 6 (STAT6) phosphorylation, or IL-4 production in our experiments. After an additional 24 hours, PBMCs were stimulated with 20 ng/mL of human recombinant IL-4 for 20 minutes. In select experiments, IL-12, instead of IL-4, was used. For measurement of intracellular

IL-4, the culture with IL-4 was extended for 3 days. One sample of PBMCs from these cultures was then washed extensively, fixed with 4% paraformaldehyde solution for 15 minutes, and processed for staining and flow cytometry as described below. In another set of experiments, PBMCs were cultured as above for 2 days after the addition of IL-4. RNA was isolated and used for measurement of mRNA for IL4 by real-time PCR as described previously.¹³ The primers for IL-4 were as follows: forward, ACTTGAACAGCCTCACA GAG; reverse, TTGGAGGCAGCAAAGATGTC; beta actin forward, AGAGC TACGAGCTGCCTGAC, reverse, AGCACTGTGTTGGCGTACAG.

Intracellular staining and analysis by flow cytometry

Fixed PBMCs were permeabilized by adding 90% methanol on ice for 30 minutes. Cells were then stained with fluorescein isothiocyanate-labeled anti-phospho-STAT6 (anti-phosphorylated signal transducers and activators of transcription 6 [pSTAT6]) antibody (clone 18), phycoerythrin-labeled anti-pSTAT4 (clone 38) (both from BD Biosciences, San Jose, Calif), phycoerythrin/Cy7-labeled anti-IL-4, and antigen-presenting cell-labeled anti-CD4 antibody (clone RPA-T4) (both from BioLegend, San Diego, Calif). Samples were then analyzed by flow cytometry (CyAn ADP analyzer; Beckman Coulter, Brea, Calif). Data analysis was completed using the FlowJo software (v 7.6.5; Tree Star, Inc, Ashland, Ore) as described previously.^{13,14}

Statistical analyses

Inhibition as measured by STAT6 phosphorylation was modeled using linear mixed models. Percent inhibition was log transformed before analysis. Predictors of interest were group (aspirin-sensitive, aspirin-tolerant, and healthy controls), concentration (0.3, 1, and 3 mM), and the interaction between group and concentration. The interaction term was kept in all models to allow for model flexibility. All models contained a random intercept for each subject. Analyses were completed using Statistical Analysis System version 9.4. Additional statistical analyses for differences between groups were performed using the software GraphPad Prism 6 (San Diego, Calif). Nonparametric tests were used for all analyses.

RESULTS

We isolated PBMCs, prestimulated overnight with an anti-CD3 antibody and then incubated with 2 strong COX inhibitors (aspirin and indomethacin) and a weak COX inhibitor (sodium salicylate) for 24 hours. After this period, the cells were activated with IL-4 for 20 minutes and then assayed for phosphorylation of various signaling molecules by flow cytometry. Fig 1 shows representative flow cytograms for pSTAT6 and pSTAT4 in CD4 T cells and non-CD4 PBMCs. There was very little pSTAT6 in nonstimulated cells under basal conditions (Fig 1, A). Stimulation with a combination of anti-CD3 and IL-4 induced pSTAT6, which was reflected by an increase in the percentage of pSTAT6+ CD4 T cells, as well as by the increase in mean fluorescence intensity (MFI) (Fig 1, C). This increase occurred in both CD4 T cells and non-CD4 PBMCs. For this article, we analyzed pSTAT6 data for the CD4 T-cell population only. The increase in pSTAT6 (both percentage of positive cells and their MFI) was inhibited in a dose-dependent manner by aspirin (Fig 1, D-F). Isotype antibody controls are shown in Fig 1, G. The effect of aspirin was not specific for STAT6 because it also inhibited anti-CD3/IL12-induced phosphorylation of signal transducers and activators of transcription 4 (STAT4) (Fig 1, H and I). Using this approach, we studied inhibition, by 2 potent COX-1 inhibitors (aspirin and indomethacin) and 1 weak inhibitor (sodium salicylate), of signaling through the IL-4 pathway in 11 aspirin-sensitive, 10 aspirin-tolerant, and 10 control subjects.

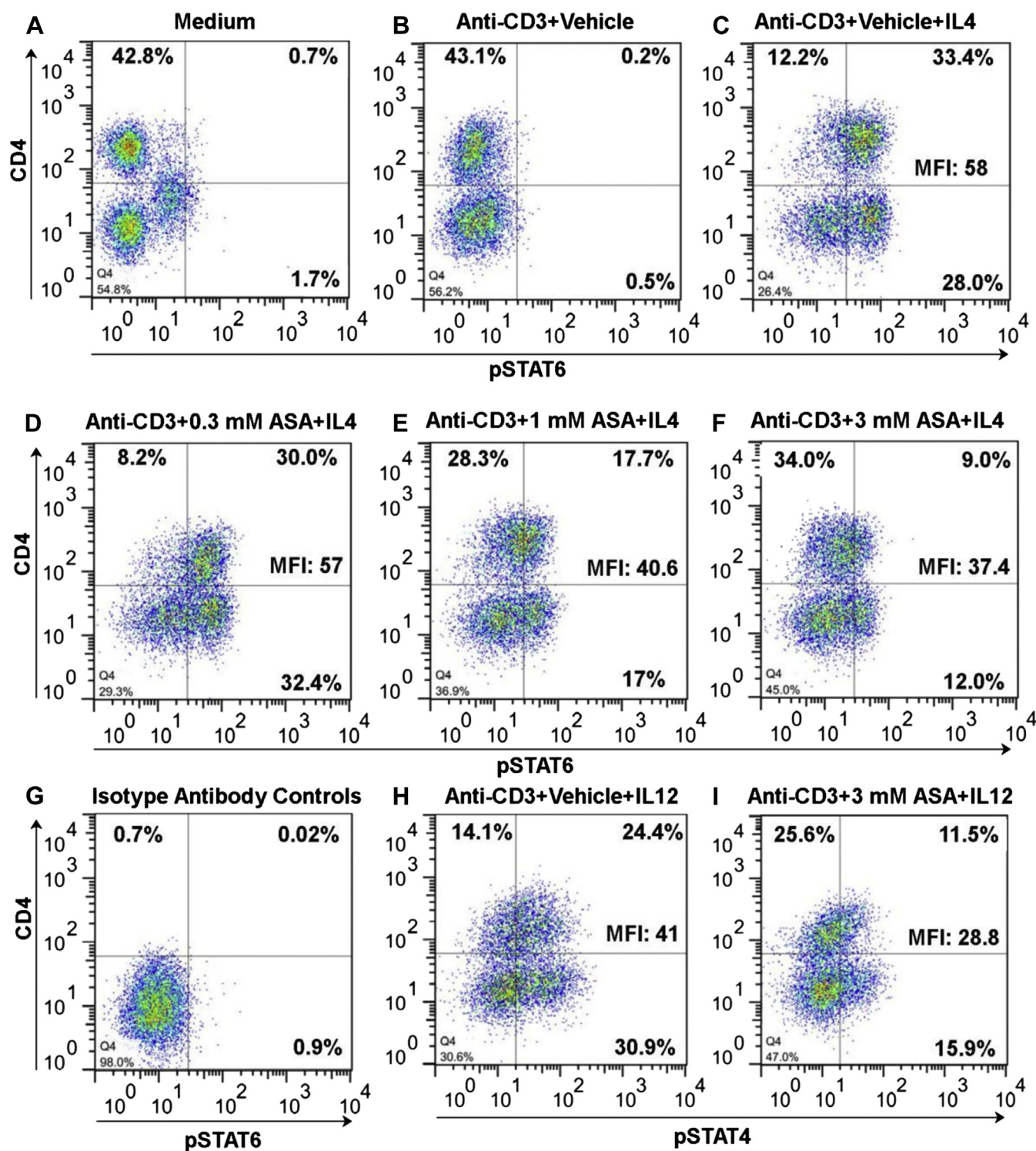


FIG 1. Representative flow cytograms from experiments performed with blood from 21 patients with asthma and 10 control subjects without asthma. Effect of aspirin on pSTAT6 in blood CD4 T cells. PBMCs stimulated with an anti-CD3 antibody and IL-4 or IL-12 in the presence of aspirin (ASA) or vehicle (ethanol) for 48 hours. The expression of pSTAT6: with medium only (**A**); anti-CD3 + medium (**B**); anti-CD3 + medium + IL-4 (**C**); anti-CD3+ medium + 3 doses of ASA (0.3 mM, 1 mM, and 3 mM) (**D-F**); isotype antibody control (**G**); pSTAT4 expression: anti-CD3 + medium + IL-12 (**H**); anti-CD3 + medium + IL-12 + 3 mM ASA (**I**).

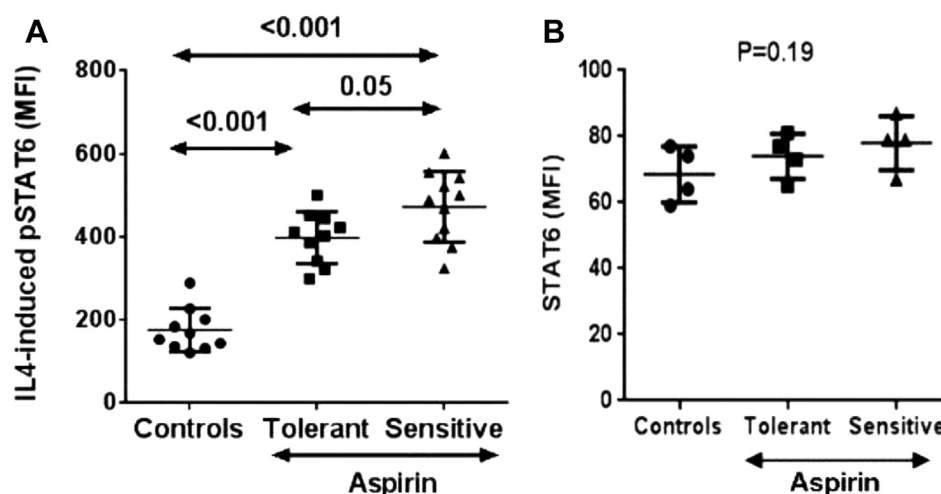


FIG 2. Comparison of IL-4-induced pSTAT6 (**A**) and baseline STAT6 (**B**) in CD4 T cells from aspirin-sensitive and aspirin-tolerant patients with asthma and control subjects. PBMCs were cultured as described in Fig 1. The MFI of pSTAT6 and STAT6 from IL-4-stimulated CD4 T cells was compared among the 3 study groups. *P* values for 2-group comparison by Mann-Whitney *U* test are shown in the graph. The *P* value for multiple-group comparison by Kruskal-Wallis test was less than .001. Each symbol represents a single subject.

IL-4 induced pSTAT6 in all study subjects. However, this induction was significantly higher in aspirin-sensitive patients with asthma than in aspirin-tolerant patients with asthma and controls subjects (Fig 2, A). IL-4-induced pSTAT6 expression was also higher in aspirin-tolerant patients than in control subjects. The MFI of baseline STAT6 expression was largely similar among the 3 study groups (Fig 2, B). Indomethacin and aspirin inhibited pSTAT6 phosphorylation in all study subjects in a dose-dependent manner (Fig 3, A-E, Groups with concentration interactions: $P = .002$ for indomethacin, $P = .06$ for aspirin, and $P = .9$ for sodium salicylate). The inhibition was strongest in aspirin-sensitive patients and modest ($\leq 15\%$) in aspirin-tolerant patients with asthma and healthy controls. From the linear mixed model, significant differences were found in pSTAT6 MFI at all concentrations of indomethacin between aspirin-sensitive and aspirin-tolerant groups ($P = .0004$ for 0.3 mM, .0001 for 1 mM, and $<.0001$ for 3 mM) and between aspirin-sensitive and control subjects ($P = .009$ for 0.3 mM, .0003 for 1 mM, and $<.0001$ for 3 mM). No significant differences were found between aspirin-tolerant and control groups at all 3 concentrations. Aspirin exhibited a similar pattern as indomethacin with significant differences in pSTAT6 MFI between aspirin-sensitive and aspirin-tolerant groups at 0.3 ($P = .002$), 1 ($P = .0007$), and 3 mM ($P = .0002$). When comparing aspirin-sensitive patients to controls, significant differences were found at 0.3, 1, and 3 mM concentrations (with *P* values of .003, .0005, and $<.0001$, respectively). There were no significant differences between aspirin-tolerant patients and controls at all 3 concentrations.

We examined pSTAT6 in CD4 T-cell gated populations by flow cytometry. However, we cultured a mixed population of blood mononuclear cells in our experiments. Thus, the effect of the COX-1 inhibitors on T-cell STAT6 could be indirect and mediated by other cells. To address this issue, we isolated CD4 T cells by negative selection using magnetic beads and then cultured them with anti-CD3 and IL-4 with aspirin or vehicle. In CD4 T cells from 3 aspirin-sensitive patients, stimulation with anti-CD3 antibody and IL-4 in the presence of the vehicle induced pSTAT6

in $33.0\% \pm 11\%$ cells. This frequency of pSTAT6+ cells went down to $22.2\% \pm 11\%$ in the presence of 3 mM of aspirin. This level of inhibition (33%) was slightly lower than that observed (44%) in the mononuclear cell population. STAT6 is phosphorylated downstream of Janus kinase 1 (Jak1) and Jakus kinase 3. Aspirin inhibited Jak1 phosphorylation in both aspirin-sensitive and aspirin-tolerant patients, although the degree of inhibition was much higher in aspirin-sensitive patients (Fig 3, F). STAT6 phosphorylation leads to induction of IL-4 production in T cells. In a next step, we examined whether aspirin inhibited IL-4 production in CD4 T cells on activation. For IL-4 induction, PBMCs from 4 aspirin-sensitive patients were stimulated with anti-CD3 and IL-4 for 3 days in the presence or absence of aspirin. This stimulation induced variable levels of IL-4 in the study subjects (Fig 4, A and B). Aspirin at 3 mM concentration inhibited IL-4 + CD4 T-cell number and MFI of IL-4 staining in all 3 subjects. To confirm whether this inhibition occurred at the transcription level, we examined mRNA expression by real-time PCR in 3 aspirin-sensitive subjects. Aspirin inhibited the expression of IL4 mRNA in all 3 subjects (Fig 4, C).

DISCUSSION

The previously reported findings demonstrating a decrease in IL-4 after aspirin desensitization led to the current interrogation of the T-cell IL-4 signaling pathway, specifically looking at the inhibition of phosphorylation of STAT6 by aspirin, indomethacin, and sodium salicylate. The first 2 drugs are potent COX-1 inhibitors, whereas sodium salicylate is a weak inhibitor. The severity of the respiratory reaction to NSAIDs in AERD directly correlates with their potency of COX-1 inhibition.¹⁵ Our results show that both aspirin and indomethacin inhibited STAT6 phosphorylation in a dose-dependent manner. Sodium salicylate was a weak inhibitor. Although the inhibition of the kinases by aspirin and indomethacin was dose-dependent, the degree of inhibition was rather modest in aspirin-tolerant patients and healthy controls. As a matter of fact, the inhibition in these study groups did not exceed 15% of the baseline. However, both

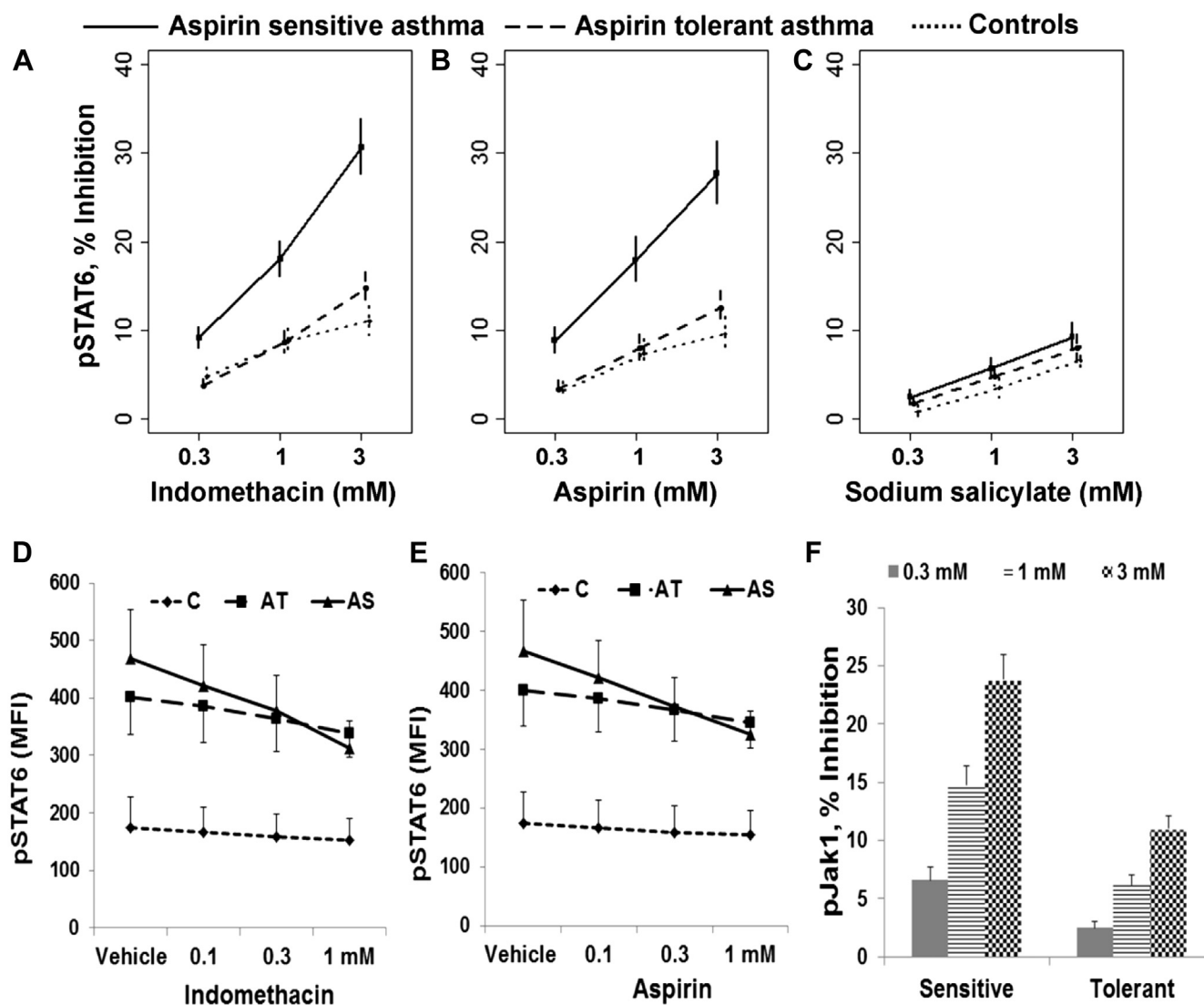


FIG 3. A-E, Comparison of the effect of indomethacin, aspirin, and sodium salicylate on CD4 T-cell pSTAT6 expression in aspirin-sensitive (N = 11) and aspirin-tolerant (N = 10) patients with asthma and control subjects (N = 10). F, Effect of aspirin on phosphorylated Jak1 in aspirin-sensitive (N=11) and aspirin-tolerant (N=10) patients with asthma. $P = .01$ for 0.1 mM aspirin and $P < .01$ for other doses of aspirin (Mann-Whitney U test).

numerically, and perhaps more importantly, biologically significant inhibition was observed only in aspirin-sensitive patients.

Aspirin has previously been shown to inhibit STAT6 phosphorylation and reduced binding to promoter site of the IL-4-inducible genes, cysteinyl-leukotriene receptor 1 and Fc epsilon RIa.^{16,17} However, there are important differences between our study and the 2 previous studies. The first used a B-cell lymphoma (M12) cell line, and the authors used relatively high concentrations (5-20 mM) of aspirin and sodium salicylate to inhibit STAT6.¹⁶ In the second study, a monocytic cell line THP1 and blood monocytes were used for the experiments.¹⁷ We used lower concentrations of the COX-1 inhibitors and examined their effect on blood T cells. More importantly, we focused on aspirin sensitivity of T cells from aspirin-sensitive and aspirin-tolerant patients with asthma, and from healthy controls, whereas the other 2 studies did not. This is important because STAT6 regulates TH2 cell function in allergic inflammation. Sodium salicylate was found to be equally inhibitory for STAT6 in the first study. We

observed rather modest but dose-dependent inhibition of pSTAT6 with sodium salicylate in our study. The discrepancy in the strength of inhibition could be due to differences in the dose and the cell type used. STAT6 signaling leads to the production of IL-4 in T cells. Accordingly, aspirin has been shown to inhibit IL-4 production in blood CD4 T cells from healthy donors.¹⁸ We confirmed this finding in T cells from aspirin-sensitive patients with asthma. In support, aspirin treatment of mice *in vivo* has been shown to reduce IL-4 and IL-10 production by tumor tissue.¹⁹ Furthermore, single nucleotide polymorphism of Janus kinases and STATs have been shown to be associated with the beneficial effect of aspirin in colorectal and breast cancer in humans.^{20,21}

The inhibition of STAT6 by both aspirin and indomethacin is likely due to the inhibition of Jak1, which functions upstream as reported previously.¹⁶ Jak1 also phosphorylates STAT4. Accordingly, we observed inhibition of STAT4 phosphorylation. The inhibition of STAT4 phosphorylation is likely to have

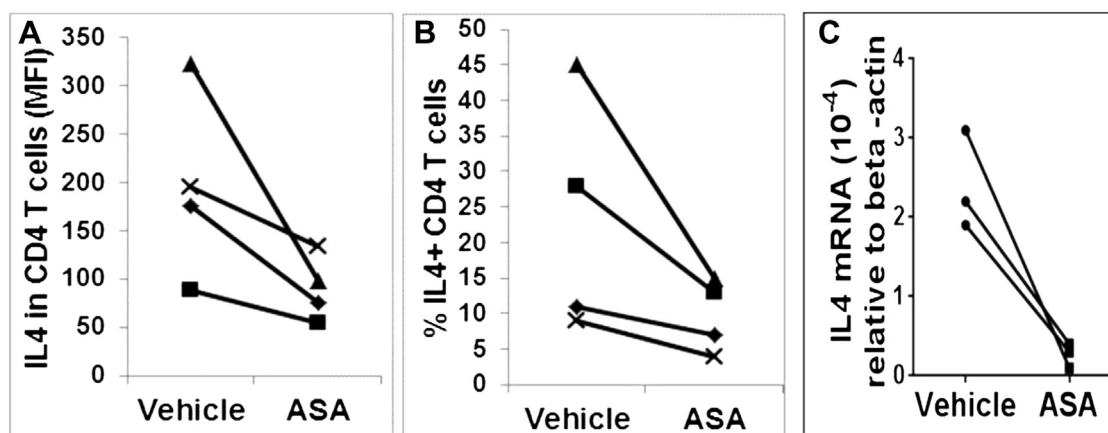


FIG 4. Effect of aspirin on IL-4 production by CD4 T cells. **A**, PBMCs from 3 patients with aspirin-sensitive asthma were cultured with anti-CD3 and IL-4 for 3 days in the presence of aspirin (3 mM) or vehicle (ethanol). Washed cells were fixed, permeabilized, and stained for CD4 and intracellular IL-4 by flow cytometry. **A**, MFI of IL4+ cells. **B**, Percentage of IL4+ cells. **C**, PBMCs were cultured as above for 2 days. RNA was isolated and used to measure IL4 mRNA by real-time PCR. Results are presented in reference to β -actin mRNA in the samples.

implications for type 1 inflammation, such as rheumatoid arthritis. The strong inhibition of pSTAT6 by indomethacin and aspirin and not by sodium salicylate suggests that a decreased COX-1 activity and prostaglandin synthesis and/or increased leukotriene synthesis might have contributed to this inhibition. Aspirin-sensitive patients with asthma have increased levels of cysteinyl-leukotrienes in blood, urine, bronchoalveolar lavage, exhaled breath condensate, and nasal washings.^{7,22-26} Potential mechanisms by which cysteinyl-leukotrienes could affect Janus kinase signal transducer and activator of transcription activation include inhibition of IL-4 receptor expression and/or activation of inhibitors of Jak kinases (eg, tyrosine phosphatases and suppressor cytokine signaling).²⁷

This finding has implications for aspirin desensitization in patients with AERD. Daily administration of aspirin has been shown to improve respiratory symptoms in patients with AERD following aspirin desensitization.^{3-5,28,29} Its mechanism remains largely unknown. Our results show that aspirin-sensitive patients have a heightened IL-4-induced pSTAT6 response in T cells. They are also susceptible to an exaggerated aspirin-mediated inhibition of pSTAT6.

What is currently not known is that whether this greater degree of inhibition of IL-4 is unique to T cells or all IL-4-producing cells such as mast cells and eosinophils. Lysine-aspirin, a water-soluble derivative of aspirin, has recently been shown to flux calcium and promote degranulation of eosinophils. This is a very intriguing finding, which may explain the mechanism of aspirin-induced respiratory symptoms. Interestingly, ketorolac, a strong inhibitor of COX and a trigger for AERD, did not manifest the same property. This raises concerns about the relevance of eosinophil activation by lysine-aspirin.³⁰ In our experiment, STAT6 inhibition by aspirin, indomethacin, and sodium salicylate paralleled their potency of COX inhibition.

Although STAT6 inhibition by aspirin has previously been reported in macrophage and B-cell lines, this is the first study demonstrating STAT6 inhibition in CD4 T cells, a primary source of IL-4, thus providing a mechanistic explanation for the observed reduction in sputum IL-4 following aspirin desensitization described in our previous article. This study also shows a

heightened STAT6 inhibition by aspirin in patients with AERD, which may explain the beneficial effect of aspirin desensitization in these patients. The diagnosis of aspirin-sensitive asthma relies on a documented history of aspirin-induced respiratory and other systemic symptoms. In the absence of such a history, an aspirin challenge is required to diagnose AERD because there are no reliable *in vitro* tests. An incremental aspirin challenge is typically a cumbersome 2-day procedure that requires a specialized medical center. As mentioned previously, the LTE₄ level in a 24-hour urine sample is frequently elevated in AERD. Our study identified 2 abnormal blood test results—heightened STAT6 phosphorylation in CD4 T cells and increased STAT6 inhibition by aspirin in patients with AERD. It would be interesting to determine in future studies whether a combination of these 2 blood tests and the urinary LTE₄ test would provide a more reliable and sensitive laboratory test for the diagnosis of AERD.

Key messages

- Aspirin desensitization has previously been shown to significantly reduce airway IL-4 in subjects with AERD. This article reports *in vitro* findings of pSTAT-6 inhibition in AERD with aspirin and indomethacin.
- Baseline induction of pSTAT-6 with *in vitro* stimulation is much higher in subjects with AERD relative to aspirin-tolerant and control subjects.
- Weak COX-1 inhibitors such as sodium salicylate do not inhibit similarly to aspirin or indomethacin.

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