Selective inhibition of interleukin-4 gene expression in human T cells by aspirin

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Introduction

Aspirin (acetylsalicylic acid [ASA]) is the oldest and most widely used nonsteroidal anti-inflammatory drug (NSAID). Although several other classes of NSAIDs have become available since the introduction of ASA in 1899, this agent and structurally related salicylates still provide the mainstay of therapy for inflammatory musculoskeletal disorders. In addition, these compounds have been shown to be effective in the management and prevention of an increasingly diverse array of noninflammatory conditions, including coronary and cerebral ischemia and gastrointestinal cancer.1,2

Both the therapeutic properties of NSAIDs and their side effects have been ascribed to their ability to inhibit generation of prostaglandin (PG) and thromboxane by interfering with the intracellular enzyme cyclo-oxygenase (COX).3 It is widely accepted that the anti-inflammatory actions of NSAIDs are mediated by inhibition of the inducible COX isoform COX-2, whereas their detrimental effects on gastric mucosa viability and platelet function are due mostly to inhibition of COX-1.4 The relative effectiveness of several NSAIDs against the 2 isozymes varies considerably.5 In particular, although ASA is a relatively effective, irreversible inhibitor of COX-1, its effects on COX-2 activity are negligible.5 This probably explains why higher doses of ASA are required in the treatment of chronic inflammatory diseases than are sufficient to inhibit PG generation in different experimental models in vitro.6 However, the in vivo anti-inflammatory and antinecrosis activity of nonacetylated salicylates, which are poor overall inhibitors of both COX-1 and COX-2, is almost superimposable to that of ASA or even more potent NSAIDs, such as diclofenac.7 Indeed, given the short serum half-life of ASA (15 minutes), the serum concentrations of salicylic acid (SA), its major nonacetylated metabolite, are better predictors of therapeutic effectiveness than the concentrations of ASA itself.6

In the light of these observations, it has been speculated that inhibition of PG production cannot fully account for the therapeutic potential of ASA and related salicylates.6 Indeed, several studies showed that these compounds have a spectrum of biochemical and pharmacologic effects that are not related to COX inhibition and are not shared with other NSAIDs.8-15 A major finding was the discovery that ASA and SA can interfere with the activation of critical transcription factors, such as nuclear factor (NF)-κB (NF-κB) and activator protein 1 (AP-1).8,10 On the other hand, salicylates were reported to activate mitogen-activated protein kinases and enhance interferon (IFN) signaling.11,13,16,17 These overall effects of salicylates are compounded by their ability to induce the release of potent anti-inflammatory mediators, such as adenosine and 15-epi-lipoxin A₄.9,14 Taken together, these observations suggest that the multiple therapeutic effects of ASA derive from its ability to regulate a network of biochemical and cellular events more complex than was initially thought.

It has long been speculated that the effectiveness of ASA in chronic inflammation, as well as in several apparently unrelated clinical conditions, might be at least partly accounted for by its effects on immune responses.18 However, data on this issue are sparse and contradictory. In particular, few studies have assessed the effects of ASA or other NSAIDs on T-cell differentiation and function. Decreased production of immunomodulating PGs, such as prostaglandin E₂ (PGE₂), in accessory cells may account for the
ability of ASA and other NSAIDs to bolster T helper (Th) 1–driven cellular immune responses.19,20 ASA and ibuprofen can enhance mitogen-induced T-cell proliferation and the expression of interleukin-2 (IL-2), IFN-γ.21,22 Macrophage-derived IL-1β, tumor necrosis factor-α, and IFN-α.23,24 Conversely, by inhibiting NF-κB and perhaps other mechanisms, high concentrations of salicylates interfere with Th1-cell differentiation and effector responses.25–27 This is in keeping with the idea that NF-κB activation is a preferential requirement for expression of cytokine genes in Th1 cells and macrophages.28–30

In this study, we examined the effect of ASA on the expression of effector cytokines in purified human CD4 + T cells. The study is the first to find that therapeutic concentrations of ASA can significantly and selectively inhibit the expression of the Th2-associated cytokine IL-4. This effect of ASA is not associated with reduced cell viability or detectable apoptotic changes and is apparently not due to inhibition or acetylation of COX isozymes in T cells, since it is not shared with other NSAIDs and can be reproduced in experiments using identical concentrations of the nonacetylated salicylate SA. Our findings in purified CD4 + T cells provide the first evidence that these cells may be a direct target of ASA and related compounds. We found that inhibition of IL-4 expression by ASA and SA occurs at the transcriptional level and is apparently not due to inhibition or acetylation of COX isozymes in the transcriptional level and is associated cytokine IL-4. This effect of ASA is not associated with significantly and selectively inhibit the expression of the Th2-associated cytokines IL-4 and IL-13.

Materials and methods

Cell isolation and culture

Peripheral blood T cells (PBT) were enriched by elutriation of residual cells from unidentified healthy donors undergoing hemapheresis (Oncology Center, Johns Hopkins Medical Institutions, Baltimore, MD).31 These preparations, which were analyzed for expression of leukocyte markers with an Epics Profile II cytometer (Beckman Coulter, Fullerton, CA), comprised an average of about 45% CD3 + CD4 + cells, 20% CD3 + CD8 +, 5% CD19 +, and 3% CD14 -. CD3 + CD4 + cells were purified to at least 97% by using the StemSep CD4 + T-cell enrichment cocktail (StemCell Technologies, Vancouver, BC) and magnetically activated cell-sorter LS + columns (Miltenyi, Auburn, CA). PBT and the Jurkat human T-cell line (donated by Dr Jack L. Strominger, Harvard University, Boston, MA) were cultured in complete medium consisting of 90% RPMI 1640 (Biofluids, Rockville, MD), 20% fetal bovine serum (Hyclone, Logan, UT), 10% L-glutamine (Corning, Corning, NY), and 40 μg/mL gentamicin.

Cell stimulation and cytokine detection

PBT (2 × 10^6/0.5 mL) were stimulated with A23187 and phorbol myristate acetate (PMA; Calbiochem, La Jolla, CA) or murine monoclonal antibodies against human CD3 (clone HIT3a) and CD28 (CD28.2; Pharmingen, San Diego, CA). Enriched PBT (2 × 10^6/mL) were primed by 6-day incubation in complete medium containing 5 μg/mL phytohemagglutinin (PHA; Calbiochem) with or without 50 ng/mL IL-2 (R&D, Minneapolis, MN). At the end of the incubation, cells were harvested, washed 3 times, and seeded in 24-well plates (10^5/mL) for restimulation. ASA, SA, indomethacin (IM), flurbiprofen (FBF), sulfasalazine (SSA), or dimethyl sulfoxide (DMSO) carrier (Sigma, St Louis, MO) were added 15 minutes before stimulation. Cytokine titers were measured in supernatants collected 18 to 20 hours after stimulation by using Cytoscreen human IL-4 detection limit, 7.8 pg/mL, Cytoscreen human IFN-γ (15.6 pg/mL), human IL-2 (1.1 U/mL) enzyme-amplified sensitivity immunosorbent assay (ELISA) kits (Immunotech, Marseille, France). Cell viability was assessed by using trypsin blue or propidium iodide exclusion. In selected experiments, cells treated with ASA, SA, or mouse antihuman CD95 (BioSource) were analyzed for expression of early apoptotic markers by staining with annexin V–fluorescein isothiocyanate (FITC; ApoAlert; ClonTech, Palo Alto, CA).

RNA isolation and analysis

The expression of cytokine transcripts was analyzed by reverse transcription–polymerase chain reaction (RT-PCR) of total RNA extracted 6 hours after stimulation with the Trizol reagent (Life Technologies).32 The primers used were 5′-TCCCAACTGCTTCCCCCTCCTG-3′ (forward IL-4), 5′-TGCTTGCTGCTTGGAAGACTG–3′ (reverse IL-4), 5′-AAGGCTCCCTGCTGGCATGGG-3′ (forward IL-13), 5′-GGGGACCTCTCTTTGTTGTGTT–3′ (reverse IL-13), 5′-GCATCCAAAGAGTGGTAGGACACATC–3′ (forward IFN-γ), 5′-CGACCTCTGAAACAGCATCCTGACT–3′ (reverse IFN-γ), 5′-CATGCCCCAAAGGCCCCAGAG–3′ (forward IL-2), and 5′-GCTGCTCTCATCAAGTCAATCAACACATG–3′ (reverse IL-2) (all from Genosys, The Woodlands, TX).

Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Stratagene (La Jolla, CA). IL-4 transcripts were quantified by real-time RT-PCR.33 Total RNA (10 ng/sample) was subjected to RT-PCR using the GeneAmp Gold kit (Applied Biosystems, Foster City, CA). Quantitative determination of the amplified products was done with an ABI Prism 7700 System (Applied Biosystems). The forward and reverse primers were 5′-CGAATGGAACAGATGTTCCA-3′ and 5′-CAGGGCCCAGAGGT-TCC-3′, respectively (Applied Biosystems). The detection probe, labeled with the reporter dye 6-carboxy-fluorescein (FAM, 6-FAM), and the quencher dye 6-carboxytetramethyl-rhodamine (lumiphenylalanine (HPlu), 582 nm) at the 3′ end, was 5′-TCCATTCTGAAACGCGCTGACA-3′ (Applied Biosystems). GAPDH was monitored by using reagents from Applied Biosystems. Cycle threshold (CT) values were calculated for IL-4 and GAPDH. The relative IL-4 transcript levels in treated (T) and control (C) samples were expressed as 2−ΔΔCT, in which ΔΔCT = ΔCT(T) − ΔCT(C), and ΔCT = CT(T) − CTGAPDH, for each experimental condition.

Transient transfections and analysis of reporter gene expression

The IL-4.265, IL-4.225, IL-4.145, IL-4.95, and IL-4.65 chloromphenicol acetyltransferase (CAT) plasmids were constructed by insertion in the HindIII and XhoI sites of pCAT-Basic (Promega, Madison, WI) of PCR-generated fragments spanning base pairs (bp) −265, −225, −145, −95, and −65, respectively, to +55 of the human IL-4 gene.33 The IL-2.152X CAT plasmid (IL-2.312), including bp −312 to +55 of the human IL-2 gene, was donated by Dr Gerald R. Crabtree (Stanford University, Stanford, CA).34 Plasmids (1 μg) were transfected into 10^6 Jurkat cells by 48-hour incubation in 3 mL complete medium containing 5 μg/mL SuperFect (Qiagen, Valencia, CA).35 Cells were treated as indicated 20 hours before harvest. Expression of CAT was measured by using a commercial ELISA (Roche, Indianapolis, IN) and normalized by considering the total protein in each sample (Bio-Rad, Hercules, CA).

Electrophoretic mobility shift assays

Jurkat cells (2.5 × 10^6/condition) were lysed in 10 mM HEPES (pH 7.9), 30 mM potassium chloride (KCI), 1 mM dithiothreitol (DTT), 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/mL leupeptin, 1 μg/mL aprotinin, and 0.075% Nonidet P40 (Sigma). Nuclei were extracted in 20 mM HEPES (pH 7.9), 420 mM KCl, 1 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 μg/mL leupeptin, 1 μg/mL aprotinin, and 20% glycerol. Protein concentrations were measured by using the Bradford reagent (Bio-Rad). The following oligonucleotides spanning IL-4 promoter elements (Figure 5B) were synthesized (Genosys): 5′-TTACCTGGTCTGGATGGCAATTTC–3′ (IL-4.145), 5′-TTTTCCTGCTGAAAGAAAGAGGT- GTCTGACA-3′ (IL-4.145), and 5′-GAGAGGGCTCTGGATGGCAATTTC–3′ (IL-4.145). These oligonucleotides, as well as a 22 mer spanning a consensus NF-κB element from the mouse immunoglobulin κ light-chain gene (Promega), were labeled by random hexamer priming using γ-phosphorus
32-deoxyctidine triphosphate (Amersham Pharmacia, Piscataway, NJ). Then, 5 to 20 fmol of the probe (10,000-30,000 cpm) was incubated (30 minutes, 25°C) with 5 μg nuclear extracts in 12 mM HEPES (pH 7.9), 50 mM KCI, 0.5 mM magnesium chloride, 0.24 mM EDTA, 4 mM DTT, 12% glycerol, 0.1 mg/mL bovine serum albumin, and 30 μg/mL poly(dI-dC) (Amersham Pharmacia). Where indicated, the binding reactions were incubated (30 minutes, 4°C) with rabbit antiserum for NF of activated T cells (NFAT) 1 (Upstate, Lake Placid, NY), CCAAT-binding factor (CBF) A (Accurate, Westbury, NY), Ets-1 and Ets-2, and NF-κB p65 and p50 (Santa Cruz Biotechnology, Santa Cruz, CA). DNA-protein complexes were resolved by 4% native polyacrylamide gel electrophoresis in 45 mM Tris (pH 8.2), 45 mM boric acid, and 1 mM EDTA and then visualized by using autoradiography.

**Results**

**Aspirin selectively inhibits IL-4 secretion from CD4+ T cells**

We studied the effects of ASA on the secretion of effector cytokines from freshly isolated CD4+ PBT. Suppression of PG production in monocytes may contribute to the reported enhancing effects of NSAIDs, including ASA, on T-cell proliferation and IFN-γ and IL-2 expression. Although both COX-1 and COX-2 have been reported to be expressed in human T cells, the importance of this finding is not clear. To minimize the contribution of monocytes or other accessory cells to the possible effects of ASA on T-cell activation, we purified CD3+CD4+ cells to at least 97% by means of negative selection from enriched PBT preparations.

CD4+ PBT were treated with increasing concentrations of ASA (10-3-3 × 10-3 M) 15 minutes before stimulation with the Ca2+ ionophore A23187 (0.5 μg/mL) and the protein kinase C (PKC) agonist PMA (25 ng/mL). These concentrations of ASA were chosen on the basis of the plasma levels known to be therapeutic in patients with chronic inflammatory conditions. Figure 1A shows the effect of 10-3 M ASA on IL-4, IL-13, IFN-γ, and IL-2 secretion in these experiments. ASA did not affect the production of IL-13, IFN-γ, and IL-2, but it significantly inhibited IL-4 secretion by 47% ± 2.4% in 3 experiments; P < .05. As shown in Figure 1B, 10-3 M ASA was the lowest concentration that significantly inhibited IL-4 expression in these cultures, whereas none of the concentrations tested affected IL-2 secretion.

Similar results were obtained with enriched preparations containing about 45% CD4+ PBT and substantial percentages of accessory cells. Figure 1C shows the effect of 10-3 M ASA on IL-4 expression in these preparations, stimulated with either A23187 (0.25 μg/mL) and PMA (25 mg/mL) or with anti-CD3 (3 μg/mL) and anti-CD28 monoclonal antibodies (5 μg/mL). A short incubation (15 minutes) with this concentration of ASA consistently and significantly inhibited IL-4 secretion, irrespective of the stimulants used. Although ASA did not affect IL-2 secretion in cells stimulated with A23187 and PMA, it enhanced expression of this cytokine induced by anti-CD3 and anti-CD28 or by A23187 alone (data not shown). Figure 1D shows that 3 × 10-3 M ASA inhibited to a comparable degree IL-4 expression in mitogen-primed enriched PBT stimulated with A23187 and PMA. Although expression of IL-4 was at a lower level in these cultures than in freshly isolated PBT, presumably because of the antagonizing effects of Th1-associated cytokines, ASA was at least as effective at inhibiting IL-4 production in cells cultured under Th2-biased conditions, such as inclusion of IL-4 (10 ng/mL) in the priming medium (Figure 1D). However, the baseline cytokine profiles—in particular, the frequencies of IL-4-expressing cells compared with IFN-γ-expressing cells—were not significantly affected in PBT primed in the presence of 10-3 M ASA (data not shown).

**COX inhibition and acetylation play no role in IL-4 inhibition by aspirin**

To assess whether inhibition of PG generation might account for IL-4 inhibition by ASA, we compared the effects of different COX inhibitors on expression of this cytokine in purified CD4+ PBT. Figure 2 shows that among several NSAIDs, only SA, the weakest inhibitor of COX-1 and COX-2 in this pharmacologic group, inhibited IL-4 expression to an extent comparable to that of ASA. In striking contrast to this finding, the potent COX inhibitors FBP and IM, used at concentrations in or above their respective COX-inhibitory and therapeutic ranges, did not affect IL-4 expression (Figure 2). Neither of these compounds significantly affected IL-2 secretion.

In these experiments, ASA or other NSAIDs did not affect cell viability, as assessed by trypan blue or propidium iodide exclusion. Even longer incubations (up to 72 hours) of enriched or purified CD4+ PBT with 10-3 M ASA or SA did not affect the proliferative responses of these cells to nonspecific mitogens (data not shown). To verify whether the inhibitory effects of ASA and SA on IL-4 expression were associated with induction of apoptotic changes in

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**Figure 1. Selective inhibition of IL-4 secretion in CD4+ PBT treated with ASA.**

(A) IL-4, IL-13, IFN-γ, and IL-2 secretion in PBT (purified to ≥ 97% CD3+CD4+) treated with a therapeutic concentration of ASA (10-3 M) or the corresponding amount of DMSO carrier (0.1%) 15 minutes before 20-hour stimulation with A23187 (0.5 μg/mL) and PMA (25 mg/mL). Shown is the mean ± SEM percentage of IL-4 (615.7 ± 70.5 pg/mL), IL-13 (253.2 ± 110.5 pg/mL), IFN-γ (1.1 ± 0.1 ng/mL), and IL-2 (158.2 ± 67.5 U/mL) secretion from DMSO-treated controls in 3 independent experiments done in duplicate. The asterisk indicates P < .05 (Wilcoxon test) relative to controls. (B) Purified PBT (≥ 97% CD3+CD4+) were treated with increasing concentrations of ASA (10-4-3 × 10-3 M) or corresponding amounts of DMSO before stimulation with A23187 (0.5 μg/mL) and PMA (25 mg/mL). The mean ± SEM percentage of control IL-4 (595.1 ± 28.9 pg/mL) and IL-2 (364.4 ± 39.7 U/mL) secretion in 4 independent experiments done in duplicate is shown. The asterisk indicates P < .05 (Wilcoxon test) relative to DMSO-treated controls. (C) IL-4 concentrations in the supernatants of enriched PBT preparations (33%-54% CD3+CD4+) treated with 10-3 M ASA or 0.1% DMSO before stimulation with anti-CD3 (μCD3): 3 μg/mL and anti-CD28 (μCD28): 5 μg/mL monoclonal antibodies or with A23187 (0.5 μg/mL) and PMA (25 mg/mL). The mean ± SEM result from 3 independent experiments done in duplicate is shown. The asterisk indicates P < .05 (Wilcoxon test) relative to DMSO-treated controls. (D) IL-4 concentrations in the supernatants of enriched PBT primed with PHA (5 μg/mL; control) in the presence or absence of IL-4 (50 ng/mL), then treated with ASA (10-3 M) or DMSO (0.1%), 15 minutes before restimulation with A23187 (0.5 μg/mL) and PMA (25 mg/mL). The mean ± SEM result from 3 independent experiments done in duplicate is shown. The asterisk indicates P < .05 (Wilcoxon test) relative to DMSO-treated controls.
IL-4 gene inhibition by aspirin is not associated with reduced NF-κB activation

Significant inhibition of IL-4 expression was observed consistently in cells treated with 10⁻³ M ASA. A concentration 2.5 to 5 times higher was previously reported to be necessary for comparable inhibition of NF-κB DNA-binding and transcriptional activities. To determine whether lower concentrations of salicylates might affect NF-κB activation under our experimental conditions, we conducted electrophoretic mobility shift assays (EMSAs) using nuclear extracts from Jurkat cells treated with ASA or the related compound SSA, a known potent and selective inhibitor of NF-κB. Figure 4A shows an experiment in which stimulation with A23187 (0.5 μg/mL) and PMA (10 ng/mL) resulted in formation of 2 complexes on an oligonucleotide spanning a consensus NF-κB element from the κ-light-chain gene (lane 2). Consistent with previous findings, neither complex formed when extracts from cells stimulated with A23187 alone were used (lane 5). Antibodies raised against the p65 (lane 3) and p50 (lane 4) NF-κB subunits interfered with formation of both complexes. Treatment with concentrations of ASA that significantly inhibited IL-4 expression (Figure 4A) showed the effect of 2 × 10⁻³ M did not affect formation of either complex (lane 6).

In contrast, a similar dependency of IL-4 promoter inhibition by aspirin is not associated with reduced NF-κB activation

Aspirin inhibits IL-4 gene expression at the transcriptional level

Decreased IL-4 secretion was paralleled by reduced accumulation of IL-4 message in CD4⁺ PBT treated with ASA. Figure 3A shows the inhibitory effect of 10⁻³ M ASA on IL-4 RNA expression in cells stimulated with 0.5 μg/mL A23187 and 10 ng/mL PMA. Also shown is that this concentration of ASA did not affect IL-13, IFN-γ, or IL-2 expression in these experiments. The degree of IL-4 RNA inhibition by ASA was analyzed by using real-time quantitative RT-PCR. In 3 such experiments, IL-4 RNA levels were decreased by 56.4% ± 7.3% and 71.2% ± 6% in CD4⁺ PBT treated with 10⁻³ M and 3 × 10⁻³ M ASA, respectively (Figure 3B). A comparable decrease was observed in cells treated with the same concentrations of SA (data not shown), whereas 10⁻⁵ M IM had no effect (Figure 3B). Similar results were obtained in the Jurkat human T-cell line (data not shown).

To investigate the molecular mechanisms mediating the inhibitory effect of salicylates on IL-4 gene expression, we analyzed the effects of these compounds on IL-4 promoter activity in transiently transfected Jurkat cells. As shown in Figure 3C, ASA (10⁻⁵–10⁻³ M) caused concentration-dependent decrease of CAT expression driven by a 265-bp IL-4 promoter fragment in Jurkat cells stimulated with A23187 (0.5 μg/mL), whereas it did not affect constitutive IL-4 promoter activity. The degree and concentration dependency of IL-4 promoter inhibition by ASA closely matched our findings on IL-4 secretion and transcript expression in PBT (Figures 1B and 3B). In contrast, ASA did not inhibit, but slightly enhanced, IL-2 promoter activity in Jurkat cells stimulated with A23187 and PMA (Figure 3C). Enhancement of IL-2 promoter activity was especially obvious at lower concentrations of ASA and after suboptimal stimulation (data not shown).

Figure 2. Comparison of the effects of different NSAIDs on IL-2 and IL-4 secretion from CD4⁺ PBT. Purified PBT (≥ 97% CD3⁻CD4⁺) were treated with optimal concentrations of ASA (10⁻³ M), SA (10⁻³ M), IM (10⁻¹ M), and FBP (10⁻³ M) or corresponding amounts of DMSO carrier (up to 0.1%) before stimulation with PMA (10 ng/mL) and A23187 (0.5 μg/mL). The mean ± SEM percentage of control IL-4 (●, 572.4 ± 26.2 pg/mL) and IL-2 (□, 194.8 ± 43.4 U/mL) secretion in 4 independent experiments done in duplicate is shown. The asterisk indicates P < .05 (Wilcoxon test) relative to DMSO-treated controls.

Figure 3. Inhibition of IL-4 gene transcription in CD4⁺ T cells by salicylates.

(A) Purified PBT (≥ 97% CD3⁻CD4⁺) were treated with ASA (10⁻³ M) or the corresponding amount of DMSO carrier (0.1%) before stimulation with A23187 (0.5 μg/mL) and PMA (10 ng/mL) for 6 hours. Shown are the results of RT-PCR of total RNA extracted in a typical experiment using primers specific for the indicated cytokine gene transcripts. Shown as an RNA integrity and loading control is the expression of transcripts of the GAPDH gene. Similar results were obtained in 3 identical experiments using ASA or SA. (B) Real-time RT-PCR analysis of IL-4 RNA expression in purified PBT incubated in the presence of the indicated concentrations of ASA or with 10⁻³ M IM before stimulation with A23187 (0.5 μg/mL) and PMA (10 ng/mL) for 6 hours. Standard curves for IL-4 and GAPDH were generated by serial dilutions of PBT total RNA in separate experiments (data not shown). The relative IL-4 RNA levels were calculated. Shown is the mean ± SEM percentage of control IL-4 expression (corresponding to 2⁻ΔΔCt × 100) in 3 independent experiments done in duplicate. The asterisk indicates P < .05 relative to DMSO-treated controls. (C) Jurkat T cells transiently transfected with the IL-2.265 or IL-2.512 CAT reporter plasmids were treated (15 minutes) with the indicated concentrations of ASA (10⁻⁵ M; ■, 10⁻³ M; □, 10⁻⁵ M) or corresponding amounts of DMSO (□) before 20-hour stimulation with A23187 (0.5 μg/mL) with (IL-2) or without (IL-4) PMA (10 ng/mL). Data are expressed as fold induction of intracellular CAT relative to unstimulated controls and are the mean ± SEM results from 4 independent experiments done in duplicate. The asterisk indicates P < .05 (Wilcoxon test) relative to DMSO-treated controls.
concentration of SSA (2 × 10^{-3} M) was, as reported previously, sufficient to repress NF-κB binding in Jurkat cells completely (lane 7).

The effect of SSA on NF-κB activation was not paralleled by an increased inhibitory effect, relative to ASA, on IL-4 promoter activity (Figure 4B). ASA and SA were the strongest inhibitors of IL-4 promoter-driven transcription among a panel of structurally related compounds. In these experiments, SSA and 2,5-dihydroxybenzoic acid, a major hydroxylated ASA metabolite in vivo, inhibited promoter activity by about 40%, whereas other salicylates were completely ineffective (data not shown). Figure 4B shows the effects of identical concentrations of ASA and SSA (2 × 10^{-3} M) on A23187-induced CAT expression in Jurkat cells transfected with the IL-4.265 construct. SSA was significantly less effective than ASA in inhibiting transcriptional activity of this construct. IL-4 promoter activity was not affected in cells treated with 5-aminosalicylic acid (5-ASA), the salicylate moiety of SSA previously found to be a relatively ineffective inhibitor of NF-κB activation. Similar results were obtained in an analysis of IL-4 secretion in PBT preparations (data not shown).

Identification of a salicylate-targeted region in the human IL-4 promoter

To map the promoter element mediating IL-4 gene inhibition by ASA, we generated a panel of IL-4 promoter deletions for use in reporter studies. ASA and SA (10^{-3} M) were equally effective at inhibiting the activation of IL-4 promoter constructs truncated at bp −265 through −145 (data not shown). Figure 5A shows that CAT expression was significantly inhibited in Jurkat cells transfected with a construct carrying an IL-4 promoter fragment spanning bp −145 to +55 (IL-4.145) and treated with ASA or SA (10^{-3} M) but not FBP (10^{-5} M). The same concentrations of ASA and FBP but not SA up-regulated the transcriptional activity of an IL-4 promoter construct (IL-4.95) lacking bp −145 to −96 (Figure 5A). This indicates that an element mediating inhibition of IL-4 transcription by ASA and SA is located between bp −145 and −96 of the human IL-4 promoter and that a COX-related mechanism might account for increased transcriptional activity of the IL-4.95 construct in cells treated with ASA or FBP.

The sequence of the salicylate-targeted region of the human IL-4 promoter is shown in Figure 5B. Integrity of this region appears to be critical for maximal IL-4 promoter activity in murine and human T cells. An inverted CCAAT box at bp −114 is the only element in this region of the IL-4 promoter that has been characterized, and it binds the ubiquitous and constitutive factor CBF, also known as NF-Y. However, sequence analysis revealed the presence of additional putative binding sites for several plausible candidates for IL-4 gene regulation in differentiated T cells (Figure 5B). These include an 80% conserved Ets-binding site at bp −128, an E box (CANNTG) at bp −103, and an E2 box at bp −119 that is known to be recognized by multi–zinc-finger factors such as ZEB. Although factors binding to such elements have been found to be involved in T-cell pathophysiology and the development of immune and inflammatory responses, none have yet been characterized as a regulator of IL-4 expression or a salicylate target.

To understand the relative contribution of each of these elements and their cognate factors to IL-4 transcriptional regulation by salicylates, we generated 3 oligonucleotides spanning overlapping sequences in a region including bp −155 to −90 of the IL-4 promoter (Figure 5B). In EMSAs using nuclear extracts from Jurkat cells, treatment with ASA (10^{-3} M) interfered with the
binding of an A23187-induced complex (Figure 5C, arrow) to an oligonucleotide spanning bp −135 to −110 (IL-4.135; lane 4) but did not substantially affect any of the complexes forming on bp −115 to −90 of the IL-4 promoter (IL-4.115; lane 8). ASA did not affect the pattern of complex formation in EMSAs using an IL-4.155 probe or an oligonucleotide centered on the CCAAT box (IL-4.CCAAT; data not shown). Similar results were obtained with extracts from A23187- and PMA-stimulated, ASA- or SA-treated PBT (data not shown). To further characterize the ASA-responsive complex forming on IL-4.135, we tested several antibodies, including IgG specific for the factors Ets-1 and Ets-2, NFAT-1, and CBF, in EMSAs using extracts from activated Jurkat cells or PBT. None of these affected complex formation in these experiments (data not shown).

**Discussion**

This study is the first to find that therapeutic concentrations of ASA significantly inhibit IL-4 gene expression in activated CD4⁺ T cells. Inhibition of IL-4 secretion in cells treated with ASA was not associated with reduced viability, impaired basic biochemical and molecular functions, or expression of early apoptotic markers. Indeed, depending on the stimulants used, IL-2 production was not inhibited or was moderately enhanced after treatment with ASA. Similarly, ASA did not affect expression of the effector cytokines IL-13 and IFN-γ in CD4⁺ PBT. The results of our experiments in transiently transfected Jurkat T cells indicate that the effect of ASA on IL-4 expression is exerted at the transcriptional level. Our findings cannot be accounted for by the reported inhibitory effects of ASA on PG generation or NF-κB activation, and they suggest involvement of an alternative mechanism of action of salicylates.

To our knowledge, our experiments using purified PBT and Jurkat cells provide the first evidence that CD4⁺ T cells may be a direct target of ASA and related compounds. The hypothesis that inhibition of immunomodulatory PGs mediates the effect of ASA on IL-4 production in these cells was not validated by our experiments using structurally unrelated COX inhibitors (Figures 2 and 5A). Moreover, T cells have been reported to produce negligible amounts of known COX products, with almost 100-fold lower PG₂ E₃ levels than required to modulate cytokine production. Previous studies using peripheral blood mononuclear cell or unfractionated PBT preparations showed that submillimolar concentrations of ASA can enhance several aspects of mitogen-induced T-cell activation, including proliferative responses and the expression of IFN-γ and IL-2. Although these effects of ASA have been related to its ability to suppress production of monocytic-derived PGs, prolonged (96-hour) incubation with several nonsalicylic NSAIDs was found to enhance mitogen-induced T-cell proliferation and IL-2 production in the absence of monocyes. However, this finding was not confirmed in subsequent studies of long-term (≥ 72-hour) in vitro effects of ASA and SA on monocyte-depleted PBT preparations. In our study, which focused on the short-term effects of ASA on isolated CD4⁺ T cells, IL-2 expression and promoter activity were enhanced only under specific stimulation conditions, and lower concentrations of ASA (10⁻⁵⁻¹₀⁻⁴ M) appeared to be more effective, presumably because of the concentration-dependent involvement of opposite mechanisms of action.

None of the concentrations of ASA used in our study significantly affected production of IL-13 and IFN-γ in CD4⁺ PBT. Taken together, these findings indicate that IL-4 is a preferential target of salicylates in these cells, thus pointing to the existence of unique molecular pathways that regulate expression of this cytokine gene in human T cells. Several reports indicated that NSAIDs, including ASA and SA, can induce an increase in intracellular Ca²⁺ and the transient activation of PKC in T cells. Indeed, these 2 events are thought to be necessary and sufficient for antigen- and mitogen-dependent T-cell activation and cytokine gene expression. Indeed, one study found that FBP can activate the Ca²⁺- or PKC-dependent factors NFAT, AP-1, and NF-κB in unfractinated PBT. However, the relation between these findings and the results of our study is unclear. Although these phenomena have been cited to explain the comitogenic potential of ASA and other NSAIDs, they are not necessarily associated with T-cell activation and IL-2 production, presumably because of their intrinsically transient nature. Furthermore, the idea that NSAIDs, particularly ASA and SA, function as adjuvants in host immune responses was challenged in studies showing the COX-independent inhibitory effect of ASA on the activation of NF-κB and other critical transcriptional activators in T cells and other types of cells.

ASA inhibits NF-κB nuclear translocation at much higher concentrations (≥ 2.5 x 10⁻⁵ M) than its reported concentration that inhibits by 50% COX-1 (≥ 2 x 10⁻⁶ M) and COX-2 (≥ 3 x 10⁻⁴ M). Indeed, higher doses of ASA than are required to inhibit PG generation are used to treat chronic inflammatory diseases. However, salicylate-related toxicity (eg, tinnitus) can occur with SA plasma levels as low as 1.2 x 10⁻³ M. Significant inhibition of IL-4 expression or promoter activity in our study was obtained with ASA or SA concentrations of 10⁻³ M or lower (Figure 3B), well within the reported therapeutic range for these compounds (0.8-1.7 x 10⁻³ M). In this study, we confirmed that these concentrations of ASA are not sufficient to affect NF-κB DNA-binding and transcriptional activity (Figure 4A). On the other hand, the structurally related compound SSA, although it caused, as reported previously, almost complete inhibition of NF-κB DNA binding (Figure 4A), was a significantly less effective inhibitor of IL-4 promoter activity than ASA or SA (Figure 4B).

ASA effectively inhibited IL-4 promoter activation in cells stimulated with a Ca²⁺ ionophore (Figure 3B). Although the engagement of Ca²⁺-delivered signals is necessary and sufficient for maximal activation of the IL-4 gene in several T-cell lines and clones, including Jurkat cells, our study confirmed that PKC coactivation by PMA is a stringent requirement for NF-κB induction in these cells (Figure 4A). Although NF-κB is a necessary activator of the IL-2 gene, it contributes significantly to IL-4 down-regulation by PMA. In our study, however, ASA and SA inhibited IL-4 expression without affecting IL-2 expression, at a concentration (3 x 10⁻¹³ M) reported to cause at least 50% inhibition of NF-κB–induced transcription. Taken together, our findings indicate that a factor other than NF-κB is involved in the inhibitory effect of salicylates on IL-4 transcription.

The mechanisms regulating IL-4 gene expression in human and murine CD4⁺ T cells have been the focus of intensive investigation during the past few years. Members of the NFAT family of transcription factors are thought to have a critical role in the activation of antigen-dependent IL-4 gene expression in T cells. Although the known NFAT molecular species appear to be expressed at similar levels in Th1 and Th2 cells, NFAT-directed IL-4 transcription is preferentially induced in Th2 cells, presumably because of the involvement of lineage-restricted coactivators. NFAT, however, is not a reasonable candidate for a molecular target of salicylates in our experimental system. Ca²⁺- and calcineurin-dependent activation of NFAT also appears to...
be a requirement for expression of IFN-γ, IL-2, and perhaps IL-13.\textsuperscript{50,52,56,57} Moreover, increased NFAT nuclear mobilization and DNA binding after treatment with FBP was observed in isolated PBT.\textsuperscript{49} We previously found that ASA can up-regulate NFAT DNA binding after treatment with FBP was observed in isolated experiments using PBT or Jurkat cells.\textsuperscript{58} These effects, shared with other COX inhibitors, such as IM and FBP, likely account for increased transcriptional activity of a minimal IL-4 promoter construct (IL-4.95; Figure 5A) but are clearly dissociated from COX-independent inhibition of IL-4 expression, in analogy with results of previous studies of COX-2 gene regulation.\textsuperscript{59,60}

Taken together, our observations are consistent with the idea that a previously unrecognized transcriptional target accounts for the inhibitory effect of salicylates on IL-4 expression. In vitro and in vivo evidence suggests that the proximal 88 bp of the IL-4 promoter, including the NFAT-binding P0 and P1 elements (Figure 5B), are sufficient to mediate proper lineage-restricted and mitogen- or antigen-induced IL-4 expression in distinct Th subsets.\textsuperscript{55,61} However, our finding that IL-4 gene inhibition by salicylates involves DNA-protein interactions in a region upstream of bp −95 is in agreement with the idea that multiple promoter elements contribute to IL-4 gene activation in differentiated T cells.\textsuperscript{33,35,43,44}

We found that ASA affects formation of an inducible complex on a discrete region of the human IL-4 promoter between bp −135 and −110. On the basis of our findings and sequence homology, we infer that an Ets family member other than Ets-1 and Ets-2, a zinc-finger protein such as ZEB, or both, may contribute critically to formation of this complex.\textsuperscript{55-57} Although this observation provides a mechanistic basis for the inhibitory effects of salicylates on IL-4 transcription in T cells, additional studies are necessary to determine the precise location and sequence of a discrete ASA-responsive element on this region of the IL-4 promoter as well as the identity and function of its cognate factor or factors.

IL-4, the prototypic cytokine expressed in Th2 cells, plays a pivotal role in the regulation of hematopoiesis and immune and inflammatory responses and is involved in the pathogenesis of a wide spectrum of disease conditions.\textsuperscript{62-65} Although suppression of Th1 responses by means of NF-κB inhibition occurs at ASA doses well above the therapeutic range,\textsuperscript{25-27} lower concentrations of ASA can enhance (by means of inhibition of monocyte PG generation) the in vitro and ex vivo expression of at least some Th1 cytokines and inherently counteract Th2 responses.\textsuperscript{21,22,24} Therefore, NSAIDs have been evaluated as possible adjuvants of Th1-driven antiviral responses,\textsuperscript{17,66} and in the management of Th2-associated and IL-4–associated conditions such as atopic asthma or rhinitis.\textsuperscript{57,68} It was shown that topical treatment with lysis-ASA or SA, but not IM, can prevent the early asthmatic response to inhaled allergen,\textsuperscript{69} a finding consistent with the critical role of IL-4 in the development of such reactions.\textsuperscript{70} IL-4 and Th2 responses are also involved in vernal conjunctivitis,\textsuperscript{71} juvenile rheumatoid arthritis,\textsuperscript{72} Kawasaki disease,\textsuperscript{73} and other conditions in which the effectiveness of salicylates is well documented.\textsuperscript{74-76} Our study, which showed direct inhibition of IL-4 production in T cells independent of COX and NF-κB activity, provides a new rationale for and creates new perspectives on the therapeutic applications of ASA and related compounds.

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