Maturation of Human Dendritic Cells as Sulfasalazine Target

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Aim. Sulfasalazine, a nonsteroidal anti-inflammatory drug, is effective in treating some autoimmune diseases, but its mechanism of action is unclear. To determine whether dendritic cells could be a possible target of the drug, we studied the effects of sulfasalazine and its metabolites, aminosalicylate and sulfapyridine, on in vitro maturation (terminal differentiation) of human myeloid dendritic cells.

Methods. We prepared immature dendritic cells by incubating CD14-positive cells in the presence of granulocyte-macrophage colony-stimulating factor and interleukin (IL)-4. The cells were matured by addition of tumor necrosis factor (TNF)-α, IL-1β, and prostaglandin E2 in the presence of sulfasalazine or its metabolites—aminosalicylate and sulfapyridine, or their combinations. We quantified the effect of drugs on the dendritic cell characteristics, such as stimulation of autologous and allogeneic pan-T cell proliferation, surface marker phenotype, IL-12 p40 subunit secretion, and activation of nuclear transcription factor (NF)-κB.

Results. Dendritic cells treated with sulfasalazine (1.25 µmol/L or 2.5 µmol/L) could not stimulate T cells (p<0.028, two-sided paired t-test). In distinction to drug-free maturing dendritic cells, 2.5 µmol/L sulfasalazine upregulated the levels of CD14 and CD68 and downregulated the levels of CD40, CD80, and CD83 (for all CD markers, p<0.03 for difference between measurements in the absence and the presence of sulfasalazine). From concentration-dependent changes in CD83 expression, we found an apparent ID50 = 1.5 µmol/L sulfasalazine. The apparent ID50 value for aminosalicylate-inhibited maturation was 4 µmol/L. Sulfapyridine had no effect. At 1.25 µmol/L, sulfasalazine largely inhibited NF-κB activation in dendritic cells.

Conclusion. Maturing human dendritic cells are hundred-fold more sensitive to sulfasalazine than T cells and NK cells and the most sensitive human cells described so far. Thus, dendritic cell maturation is an important target of sulfasalazine. Because of the role of dendritic cells in (auto)immunity, inhibition of their maturation might provide a target for further optimization of sulfasalazine therapy.

Key words: antigens, CD; autoimmunity; dendritic cells; mesalamine; NF-kappa B; sulfapyridine; sulfasalazine

Dendritic cells play the key role in priming and boosting immunity by virtue of their unique ability to recruit and educate naïve T cells (1). Inappropriate presentation of proper (“self”) antigens by dendritic cells can break tolerance and may partake in pathogenesis of autoimmunity (2). Thus, dendritic cells could provide an important link in the vicious circle of autoimmunity comprised of inflammatory tissue damage, internalization and processing of damaged tissue, dendritic-cell mediated antigen presentation, and recruitment of effector cells that damage the tissue. Dendritic cells internalize damaged tissue most efficiently when they are immature; they present processed antigens when they are mature (terminally differentiated), and can express high levels of antigen-presenting molecules, costimulatory molecules, cytokines, and chemokines (1). Therefore, inhibition of dendritic cell maturation could play an important role in the mechanism of immunosuppression.

Recently, we have postulated that dendritic cells are important targets of anti-inflammatory action of salicylates, and we found that salicylates inhibit the maturation of dendritic cells; in vivo, this effect could disrupt the cycle of autoimmunity (3). Maturation of dendritic cells is inhibited by both acetylated and non-acetylated salicylates to a similar extent, indicating that the inhibition of cyclooxygenase(s) does not play a major role, but that inhibition of nuclear transcription factor κB (NF-κB) and possibly other transcription factors could be involved (3). The notion that the process of maturation is the main target of salicylates is corroborated by the finding that, after maturation, dendritic cells are largely refractory to these drugs (3).

A potent salicylate derivative is sulfasalazine, a drug effective in the treatment of inflammatory bowel disease (4), rheumatoid arthritis (5), and other autoimmune diseases (6,10). Its effect on dendritic cells is not
known. To determine whether sulfasalazine affects dendritic cells, we measured the effects of the drug on the ability of immature dendritic cells to acquire mature phenotype, secrete IL-12, and stimulate T cells. We found that sulfasalazine inhibited these features with the apparent ID<sub>50</sub> values of 1.5 μM/mL, revealing that sulfasalazine is thousand-fold more potent than aspirin and salicylate (3). To determine the role of its metabolites, aminosalicylate and sulfapyridine, we quantified the effects of each constituent on dendritic cell phenotype. Sulfapyridine was inactive, aminosalicylate was active, and intact sulfasalazine was the most active.

**Materials and Methods**

**Isolation of CD14-positive Cells and Culture of Dendritic Cells**

Immature dendritic cells were prepared from buffy coats from the blood of normal healthy volunteers, as described elsewhere (3). CD14-positive cells were isolated by immunomagnetic separation (Miltenyi Biotec, Auburn, CA, USA) and cultured at 1x10<sup>6</sup> cells/mL in X-VIVO-15 medium (Bio-Whittaker, Walkersville, MD, USA) supplemented with human AB serum (10%; Sigma, St. Louis, MO, USA), penicillin (100 U/mL; Gibco BRL, Gaithersburg, MD, USA), streptomycin (100 μg/mL; Gibco BRL), granulocyte-macrophage colony-stimulating factor (GM-CSF, 800 IU/mL; R&D; Minneapolis, MN, USA), and IL-4 (1000 IU/mL; R&D). On days three and five, one milliliter of the medium (modified by an increase of GM-CSF to 1600 IU/mL) was added to each well. On day seven, non-adherent immature dendritic cells were collected by pipetting, counted, centrifuged, resuspended in the medium containing GM-CSF (800 IU/mL) and IL-4 (1000 IU/mL), and plated at 5x10<sup>5</sup> per mL in 24-well plates. The “cocktail” of inflammatory mediators (TNF-α, 1100 IU/mL; R&D); IL-1β, 1870 IU/mL; R&D); and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), 1.0 μg/mL (Sigma), with or without salicylates, was added to cells at the time of plating. Cells were incubated for three days and then analyzed. Alternatively, the cells were incubated for three days with 10 μg/mL of a CD40-specific monoclonal antibody (clone EA5; Biosource, Camarillo, CA, USA) substituted for TNF-α, IL-1β, and PGE<sub>2</sub>.

**Drugs and Incubation Conditions**

Sulfasalazine (2-hydroxy-5-[4-[(2-pyridinylamino)sulfonyl]-phenyl]azo]benzoic acid), p-aminosalicylic acid (4-amino-2-hydroxybenzoic acid), and sulfapyridine (4-amino-N2-pyridinylbenzenesulfonamide) were all produced by Sigma. Each drug was dissolved in NaOH (0.1 mol/L), diluted to 50 mmol/L in X-VIVO 15 medium, and pH was adjusted to 7.4. Samples of these stock solutions were kept at −20°C, thawed immediately before use, and diluted to final concentrations in the cell culture medium. Cells were incubated in a humidified atmosphere containing 5% carbon dioxide (CO<sub>2</sub>) at 37°C.

**Autologous and Allogeneic T Cell Proliferation**

Dendritic cells were washed twice in X-VIVO-15 medium containing 1.0% human AB serum, penicillin, streptomycin, and 2-mercaptoethanol (50 μmol/L; Gibco BRL) and irradiated with 3,000 rad from a cesium-137 source. CD3-positive autologous or allogeneic (pooled from nine individuals) target leukocytes were irradiated with 3,000 rad from a cesium-137 source. CD3-positive autologous or allogeneic (pooled from nine individuals) target leukocytes were irradiated with 3,000 rad from a cesium-137 source. CD83 (HB15A; Immunotech, Westbrook, ME, USA) and CD86 (BU63; Ancell, Bayport, MN, USA) were conjugated to fluorescein. Antibodies specific for HLA Class I (G46-2.6; Pharmingen), CD80 (L107.4; Becton Dickinson), and CD83 (HB15A; Immunotech, Westbrook, ME, USA) were conjugated to phycoerythrin.

**Antibodies and Flow Cytometry**

The levels of membrane markers were quantified by flow cytometry with the aid of fluorescein-labeled antibodies. Cells were harvested by scraping followed by rinsing the dishes with phosphate-buffered saline (PBS), collected by centrifugation and resuspended in PBS. Antibody was added and incubated on ice for 20 min in the dark. Cells were washed with 2.0 mL PBS, re-suspended in 200 μL of PBS, and analyzed immediately or fixed by the addition of paraformaldehyde to the final concentration of 1.0%. Bound antibodies were evaluated on 10,000 cells per assaay on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed by CellQuest analysis software (Becton Dickinson). Data were analyzed for geometric mean fluorescence intensity or percentage of positive cells in comparison to unstained cells or cells stained with isotype controls.

**Immunoglobulin G isotype control (MOPC21/3421; Biosource) was conjugated to fluorescein or phycoerythrin. Antibodies specific for HLA-DR (B-F1; Biosource), CD14 (B-AB; Biosource), and C86 (B6; Ancell, Bayport, MN, USA) were conjugated to fluorescein. Antibodies specific for HLA Class I (G46-2.6; Pharmingen), CD80 (L107.4; Becton Dickinson), and CD83 (HB15A; Immunotech, Westbrook, ME, USA) were conjugated to phycoerythrin.

**Measurement of Secreted Interleukin-12**

Conditioned medium (100 μL) was collected from each well 48 hours after initiation of differentiation, centrifuged to remove debris, and stored at −70°C. After thawing, 50 μL were withdrawn for IL-12 measurement by sandwich-ELISA (Endogen, Westbury, MA, USA) specific for the p40 unit, according to manufacter's instructions.

**Electrophoretic Mobility Shift Assay**

We determined the level of activated NF-kB by the standard electrophoretic mobility shift assay (12). Twelve hours after stimulation with the “cocktail” of inflammatory mediators with or without salicylates, cells were collected by scraping, washed with PBS, and lysed for 30 min on ice in a buffered high-salt detergent (20 mmol/L HEPES, pH 7.9, 350 mmol/L NaCl, 20% w/v glycerc, 1% w/v NP-40 detergent, 1 mmol/L MgCl<sub>2</sub>, 0.5 mmol/L EDTA, 0.1 mmol/L EGTA, 0.5 mmol/L TDT, 0.1% PMSF, 1% aprotinin). The lysate was centrifuged at 13,000×G for 5 min in the cold. Protein concentrations were measured by the Micro BCA Protein Assay (Pierce, Rockford, IL, USA). Equal amounts of the lysate, containing 20 μg of protein, were used to assess NF-kB activation by binding to a 32P-labeled NF-kB–specific oligonucleotide, according to the manufacturer's protocol (Promega, Madison, WI, USA). As a control, either unlabeled competitor or unlabeled noncompetitor oligonucleotide was included in the reaction mixture. Samples were incubated at room temperature for 25 min, electrophoresed on polyacrylamide gels (6%), and visualized by autoradiography.

**Statistics**

All experiments were repeated with samples from two to five individual donors with similar results. All assays were performed in at least triplicate samples. Probability that the mean values of two experimental groups were identical was tested by the two-tailed t-test for paired samples. The level of significance was set at p = 0.05.

**Results**

**Sulfasalazine-Treated Dendritic Cells Do Not Stimulate T Cells**

To determine the effects of sulfasalazine on the function of dendritic cells matured with TNF-α, IL-1β and PGE<sub>2</sub>, we incubated such cells with allogeneic (Fig. 1A) and autologous T cells (Fig. 1B), and mea-
Figure 1. A. Allogeneic T cell proliferation (measured as incorporated radioactivity, mean ± standard deviation) stimulated by immature dendritic cells (dotted line), mature dendritic cells (broken line), and dendritic cells differentiated by immature dendritic cells (dotted line), mature dendritic cells differentiated for three days in the presence of sulfasalazine (1.25 µmol/L, closed circle). B. Autologous T cell proliferation stimulated by dendritic cells differentiated for three days in the presence of graded concentrations of sulfasalazine and pulsed with tetanus toxoid.

Figure 2. Expression (mean ± standard deviation) of CD14 (closed circle), CD40 (triangle), CD68 (closed rhomb), CD80 (square) and CD83 (open circle) as a function of sulfasalazine concentration. Open symbols, left ordinate; closed symbols, right ordinate. The corresponding values for the differences between no drug and sulfasalazine concentration: CD14, 0.018; CD40, 0.007; CD68, 0.015; CD80, 0.0136; and CD83, 0.013. Inhibiting Dendritic Cell Maturation

Because sulfasalazine is degraded into pharmacologically active aminosalicylate and sulfapyridine (cf. ref. 14), we determined the role of these molecules in modulating dendritic cell maturation. Consequently, in experiments similar to those in Figure 2, we incubated immature dendritic cells with maturing cytokines in the presence of graded concentrations of sulfasalazine, aminosalicylate or sulfapyridine, or equimolar amounts of aminosalicylate and sulfapyridine. We measured the levels of CD14, a monocyte/macrophage marker (Fig. 3A), and of CD83, a marker of mature dendritic cells (Fig. 3B). Aminosalicylate exerted effects qualitatively similar to those of sulfasalazine, whereas sulfapyridine had no effect. There was no difference between the cells incubated with aminosalicylate alone and those incubated with additional equimolar sulfapyridine. The shape of the concentration dependence curves for CD83 allowed us to estimate the ID₅₀ value of 1.5 µmol/L sulfasalazine; aminosalicylate inhibited maturation with an ID₅₀ value around 4 µmol/L (Fig. 3B). Thus, we concluded that aminosalicylate was the main pharmacophore in sulfasalazine, but sulfasalazine was more potent (p = 0.034 for the difference in the levels of CD14 at 2.5 µmol/L sulfasalazine and 2.5 µmol/L aminosalicylate; for the respective difference in CD83, p = 0.011). Apparently, aminosalicylate was more effective when chemically linked to sulfapyridine, forming thus intact sulfasalazine.

We measured the levels of IL-12p40 secreted by the cells (Figs. 3A and 3B) as an additional marker of dendritic cell maturity (1). Levels of IL-12p40 declined with increasing concentrations of sulfasalazine and aminosalicylate in a manner similar to the change in CD83.

Aminosalicylate is Similar But Not Identical to Sulfasalazine in Inhibiting Dendritic Cell Maturation

To characterize the effects of sulfasalazine on dendritic cell maturation, we determined the levels of CD14, CD40, CD68, CD80, and CD83 as a function of drug concentration. The levels of these molecules changed as a function of sulfasalazine concentration (Fig. 2). P values for the differences between no drug and 2.5 µmol/L sulfasalazine were: CD14, 0.018; CD40, 0.007; CD68, 0.015; CD80, 0.0136; and CD83, 0.030 (for easier comparison of Figs. 2 and 3, all p values are reported for differences with or at 2.5 µmol/L drugs). Also, we determined the expression of HLA-A/B/C, HLA-DR, and CD86 in the absence and presence of 1.25 µmol/L sulfasalazine. The drug suppressed the expression of these molecules to the extent similar to suppression of CD40, CD80, and CD83 (data not shown). Incubation with sulfasalazine did not affect cell viability as determined by trypan blue exclusion and binding of annexin-V (data not shown; ref. 13). These data indicate that sulfasalazine inhibited the differentiation of dendritic cells.

Phenotype of Sulfasalazine-treated Dendritic Cells Resembles Immature Dendritic Cells

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in the levels of CD83 (Fig. 3C; p = 8 × 10^{-4} for the difference at 2.5 µmol/L). We determined the apparent ID_{50} ≈ 1 µmol/L for sulfasalazine and ID_{50} = 4 µmol/L for aminosalicylate (Fig. 3C). Sulfapyridine had no effect.

Sulfasalazine Inhibits NF-κB with Efficiency Similar to Inhibition of Maturation Markers

NF-κB mediates the key transcription events during dendritic cell maturation (15). Aspirin and salicylate inhibit activation of this transcription factor with ID_{50} values similar to those observed for inhibition of dendritic cell maturation. To determine the effects of sulfasalazine on NF-κB in dendritic cells, we measured the effects of the drug on the levels of activated NF-κB (Fig. 4). We found that sulfasalazine at 1.25 µmol/L largely inhibited NF-κB activation. Thus, sulfasalazine-induced NF-κB inhibition was similar to the inhibition of membrane markers and IL-12p40.

Discussion

Sulfasalazine alone or in combination with other drugs effectively treats inflammatory and autoimmune diseases (4,6-10). Upon oral administration, the drug is cleaved into 5-aminosalicylate and sulfapyridine. Sulfasalazine and sulfapyridine can reach concentrations up to 100 µmol/L in the circulation and synovial fluid, whereas aminosalicylate is absorbed poorly and reaches much lower concentration (cf. ref. 14). Sulfasalazine, sulfapyridine, and aminosalicylate affect different cellular and molecular targets differently. For example, sulfasalazine, unlike aminosalicylate or sulfapyridine, inhibits NF-κB, induces apoptosis in T cells, and arrests phagocytosis and DNA synthesis in human white blood cells (17).
On the other hand, sulfasalazine and salicypridine, but not aminosalicylate, inhibit chemotaxis of endothelial cells (18). In contrast to salicypridine, 5-aminosalicylate and derivatives are clinically effective in inflammatory bowel disease (cf. refs. 4, 19, 20). Although in some systems NF-κB is not required for sulfasalazine action (21), many effects of sulfasalazine and aminosalicylate are mediated by inhibition of NF-κB (22).

Dendritic cell maturation requires NF-κB activation (15). Previously, we have shown that aspirin and salicylate inhibit maturation of dendritic cells at concentrations achievable in patients on high-dose aspirin therapy (3). This effect is independent of cyclooxygenase inhibition, but is correlated with inhibition of NF-κB (3). Here we show that sulfasalazine inhibits dendritic cell maturation a thousand-fold more potently than aspirin and salicylate (ID₅₀/sulfasalazine < 2 μmol/L, ID₅₀/aspirin = 2.5 mmol/L; ref. 3) and that NF-κB is inhibited accordingly. Apparently, the activity of sulfasalazine depends on 5-aminosalicylate, as salicypridine had no effect. Aminosalicylate was less potent than sulfasalazine, indicating that the salicypridine moiety contributes to the overall activity of sulfasalazine, possibly by facilitating passage across the cell membrane.

In this study, we quantified the effect of drugs on several dendritic cell characteristics; stimulation of autologous and allogeneic pan-T cell proliferation, surface marker phenotype, and secretion of IL-12 p40 as a soluble marker of dendritic cell maturity. Finally, we followed the effects of the drugs on activation of NF-κB. According to all these criteria, the effects of sulfasalazine on dendritic cells are remarkable. Sulfasalazine inhibited much of the expression of CD83, a marker of mature dendritic cells. The accompanying partial inhibition of antigen-presenting HLA-A/B/C and HLA-DR molecules as well as of CD40, CD80, and CD86, normally highly expressed in mature dendritic cells, might impair the ability of dendritic cells to communicate with immune effector cells (1,23). These changes occurred at the drug concentrations below 1 μmol/L, qualifying dendritic cells as the most sensitive human cells described so far (compare the data in Table 1, ref. 14). Others found that human T cells and NK cells were more than hundred-fold less sensitive to sulfasalazine than dendritic cells in this study, despite similar culture conditions and duration of incubation with the drugs (16 hours to three days; cf. ref. 14). A three-day incubation with sulfasalazine inhibited proliferation of stimulated human peripheral blood mononuclear cells with an estimated ID₅₀ > 25 μmol/L. (17). The relative insensitivity of other cells to inhibition by sulfasalazine supports the notion that dendritic cell maturation might be an important, if not the most important, target in anti-inflammatory and antirheumatic action of sulfasalazine. Our finding of the similarity in the effects of sulfasalazine and aminosalicylate, a drug mostly ineffective in other human cells (14), further corroborates the unique sensitivity of dendritic cells.

We induced the maturation of dendritic cells by a mixture of TNF-α, IL-1β, and PGE₂. At the concentrations we used, these substances provide strong and sustained inflammatory signals that result in high yields and rather homogeneous populations of CD83-positive cells matured in minimum time (11,24). However, even under these optimized conditions, sulfasalazine and aminosalicylate effectively inhibited dendritic cell maturation. This observation points to the possibility that sulfasalazine could prevent dendritic cell maturation in an inflammatory milieu in vivo.

The other reason that the observed effects are remarkable resides with the very role of dendritic cells in immune response. Dendritic cells are the sole antigen-presenting cells that can prime naïve T cells to recognize hitherto unrecognized antigens (1). Consequently, presentation of antigens characteristic of tissues can break their tolerance. This feature of dendritic cells has been used to initiate therapeutic immunity against cancer (cf. ref. 23). Also, it can provide the critical step in pathogenesis of autoimmunity (2,26-30). Thus, timely inhibition of dendritic maturation might prevent development of (auto)immunity. The exquisite sensitivity of dendritic cells to sulfasalazine indicates the need for further investigation into the optimal timing of drug administration. It is possible that the best opportunity to affect the natural history of autoimmune disease is to administer the drug as early as possible. The putative “dendritic cell stage” of disease might be more sensitive to the drug than the later phases characterized by the less sensitive (auto)antigen-specific T cell clones.

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References

8 Le Roux JF, Robin H, Doan S, Prisant O, Belayachi N, Hoang-Xuan T. Traitement des pemphigoides oculaires


21 Cronstein BN, Montesinos MC, Weissmann G. Salicylates and sulfasalazine, but not glucocorticoids, inhibit leukocyte accumulation by an adenosine-dependent mechanism that is independent of inhibition of prostaglandin synthesis and p105 of NF-κB. Proc Natl Acad Sci USA 1999;96:6377-81.


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