

Sulfamethoxazole and Its Metabolite Nitroso Sulfamethoxazole Stimulate Dendritic Cell Costimulatory Signaling¹

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Different signals in addition to the antigenic signal are required to initiate an immunological reaction. In the context of sulfamethoxazole allergy, the Ag is thought to be derived from its toxic nitroso metabolite, but little is known about the costimulatory signals, including those associated with dendritic cell maturation. In this study, we demonstrate increased CD40 expression, but not CD80, CD83, or CD86, with dendritic cell surfaces exposed to sulfamethoxazole (250–500 μ M) and the protein-reactive metabolite nitroso sulfamethoxazole (1–10 μ M). Increased CD40 expression was not associated with apoptosis or necrosis, or glutathione depletion. Covalently modified intracellular proteins were detected when sulfamethoxazole was incubated with dendritic cells. Importantly, the enzyme inhibitor 1-aminobenzotriazole prevented the increase in CD40 expression with sulfamethoxazole, but not with nitroso sulfamethoxazole or LPS. The enzymes CYP2C9, CYP2C8, and myeloperoxidase catalyzed the conversion of sulfamethoxazole to sulfamethoxazole hydroxylamine. Myeloperoxidase was expressed at high levels in dendritic cells. Nitroso sulfamethoxazole immunogenicity was inhibited in mice with a blocking anti-CD40L Ab. In addition, when a primary nitroso sulfamethoxazole-specific T cell response using drug-naïve human cells was generated, the magnitude of the response was enhanced when cultures were exposed to a stimulatory anti-CD40 Ab. Finally, increased CD40 expression was 5-fold higher on nitroso sulfamethoxazole-treated dendritic cells from an HIV-positive allergic patient compared with volunteers. These data provide evidence of a link between localized metabolism, dendritic cell activation, and drug immunogenicity. *The Journal of Immunology*, 2007, 178: 5533–5542.

Severe T cell-mediated drug hypersensitivity reactions are a major problem both in drug development and in the clinic. The antibiotic sulfamethoxazole (SMX)³ is associated with hypersensitivity in 1–3% of the exposed population (1), which rises to ~50% of HIV patients (2). Because of this, and because of the availability and known protein reactivity of its metabolites (3), SMX is commonly used to investigate the chemical and cellular basis of drug hypersensitivity (4–9).

SMX is readily oxidized by several enzymes, including CYP2C9 (10) and myeloperoxidase (MPO) (11), to an unstable hydroxylamine intermediate (SMX-NHOH) that auto-oxidizes to a protein-reactive nitroso species (SMX-NO; Fig. 1) (3, 12). SMX-NO can bind covalently to cysteine residues on cellular protein (4, 13–16) and this binding, above a threshold, causes direct toxicity (5, 16) but also provides an antigenic signal to SMX-NO-specific T cells (9, 17). Furthermore, Ag-specific T

cells have been isolated and cloned from hypersensitive patients and have been characterized in terms of their functionality and phenotype (6–8, 18, 19).

Although it has been understood for some time that T cells require an Ag-specific signal via the MHC:TCR interaction for activation, known as signal 1, only recently has the existence of a second obligatory signal, or signal 2, been recognized (Fig. 1). Signal 2 is a series of exogenous and endogenous signals that mature dendritic cells, leading to enhanced costimulation (20, 21). Stimuli that lead to maturation of dendritic cells include conserved microbial products which interact with pattern-recognition receptors, such as TLRs (22). This may be of relevance to the increased risk of hypersensitivity reactions associated with viral infections (23, 24), such as EBV (25), human herpes virus-6 (23), and HIV (26). Clearly viral infections are important in some, but not all, drug hypersensitivity reactions because the majority of such reactions occur in the absence of viral infections. However, the nature of the accessory signal in such instances is unclear.

It is known that dendritic cell maturation signals can also be provided by necrotic cell death through the release of intracellular molecules into the extracellular matrix (27, 28), apoptotic cell death (29), or the induction of oxidative stress (30, 31). Indeed, studies with contact sensitizers have demonstrated that small protein-reactive compounds can induce dendritic cell maturation (30, 32, 33) at concentrations associated with significant cell death (34). It is interesting to note, therefore, that lymphocytes from hypersensitive patients are more susceptible to cell death in response to reactive drug metabolites than control cells (35–38). Furthermore, patients with HIV infection have a decreased capacity to detoxify SMX-NO (39, 40) and lymphocytes from these patients are more susceptible to SMX metabolite-induced cell death (41).

The role of SMX in generating signal 1, either directly or via drug metabolism and haptentation, has been well-studied (5–9, 13,

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³ Abbreviations used in this paper: SMX, sulfamethoxazole; SMX-NHOH, sulfamethoxazole hydroxylamine; SMX-NO, nitroso sulfamethoxazole; MPO, myeloperoxidase; ABT, 1-aminobenzotriazole; rh, recombinant human; GSH, reduced glutathione; SI, stimulation index; COX-2, cyclooxygenase-2.

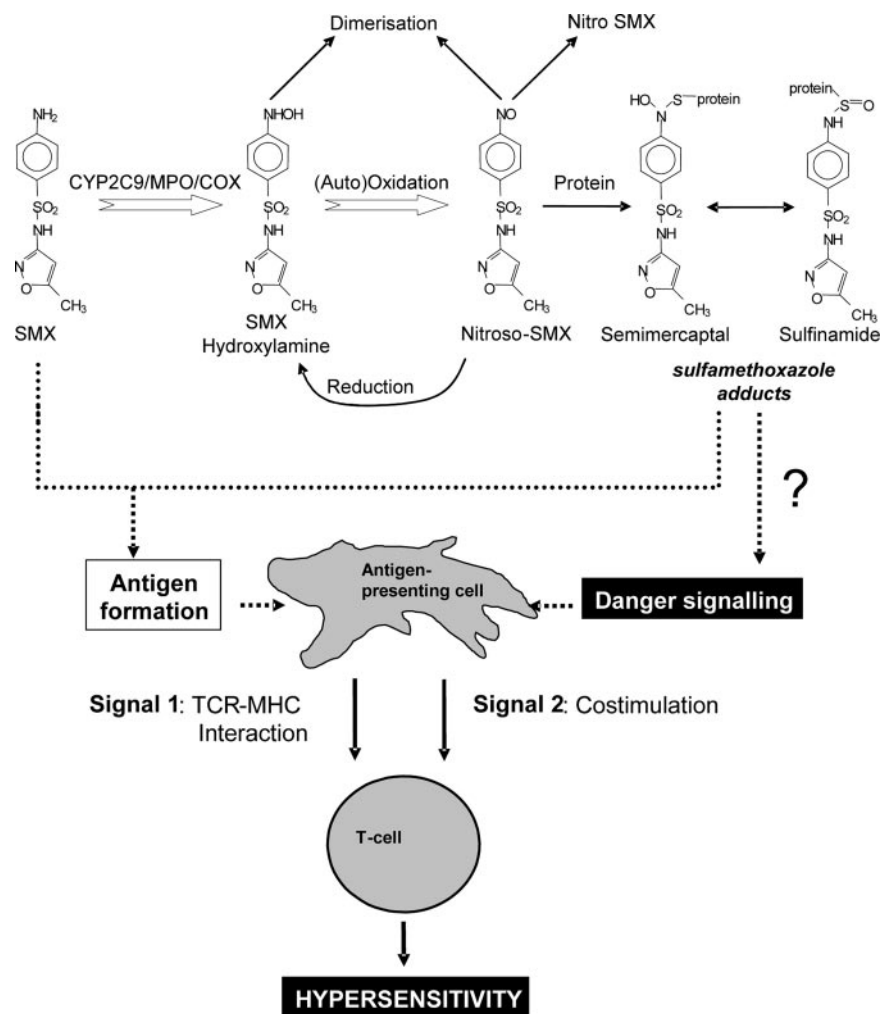


FIGURE 1. Schematic representation depicting the chemical and immunological fate of oxidative SMX metabolites. This figure was adapted from Sanderson et al. (43) with permission from the *American Association of Pharmaceutical Scientists Journal (AAPJ)*.

14, 19), but nothing is known about the role of the drug with regard to signal 2, despite some speculation (21, 42–45).

In this study, we examined the effect of SMX and SMX-NO on the costimulatory potential of dendritic cells and explored the possibility that localized dendritic cell metabolism and subsequent covalent binding of SMX metabolites to protein might be associated with immunologically relevant changes in functionality.

Materials and Methods

Materials

Hydroxylamine and nitroso metabolites of SMX were synthesized as described previously (3), and determined by HPLC, mass spectrometry, and nuclear magnetic resonance to be >99% pure. Recombinant human (rh) IL-2, rhIL-4, and rhGM-CSF were obtained from PeproTech. FITC-labeled anti-CD40, CD80, CD83, and CD86 Abs, and the leucoperm fixation and permeabilization kit were all obtained from Serotec. Anti-mouse CD154 (MR1) was purchased from BD Biosciences. Anti-human CD40 Abs 82111 and 82102 were supplied by R&D Systems. [^3H -CH $_3$]Thymidine was obtained from Moravak. Lymphoprep was obtained from Nycomed. The Annexin V^{FITC} kit was obtained from Bender MedSystems. All rCYP-expressing supersomes were obtained from BD Biosciences. DNase I and diethyl pyrocarbonate-treated water were obtained from Ambion. RiboGreen RNA quantification kit was obtained from Molecular Probes. RNeasy 96 total RNA extraction kit and RLT lysis buffer were obtained from Qiagen. Oligo(dT)_{12–18} primer, 2'-deoxynucleoside 5'-triphosphate mix and the Superscript II reverse transcriptase kit were obtained from Invitrogen Life Technologies. Oligonucleotide fluorogenic probes and TaqMan Universal PCR Mastermix were obtained from Applied Biosystems. All other chemicals, components, and oligonucleotide primers were obtained from Sigma-Aldrich.

Generation of dendritic cells from healthy volunteers

PBMC were isolated from heparinized venous blood of healthy human volunteers by centrifugation on a density gradient of Lymphoprep. PBMC were cultured in 24-well plates for 4 h followed by repeated washing to remove nonadherent cells. Adherent cells were then cultured in 1 ml of RPMI 1640 medium, supplemented with 10% FBS, HEPES buffer (25 mM), L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), IL-4 (800 U/ml), and GM-CSF (800 U/ml). Half of the medium was replaced with fresh complete medium on days 1, 3, and 5, and immature dendritic cells were ready for use on day 6. Purity of dendritic cell populations was determined by FACS analysis with an anti-human CD11c Ab and was consistently >95%.

Generation of dendritic cells from sulfamethoxazole-exposed patients

Dendritic cells were generated from the blood of one HIV-infected patient who was acutely hospitalized with a SMX hypersensitivity reaction (rash and fever) and from four SMX-exposed nonhypersensitive HIV-infected volunteers. Approval for the study was obtained from the North Manchester Local Research Ethics Committee and informed consent was obtained from each participant.

Drug treatment and analysis of cell surface markers

Phenotype analysis of drug-exposed dendritic cells was performed by flow cytometry. Immature dendritic cells were incubated with SMX, SMX-NO (both 0.5–4000 μM ; made up in DMSO, final concentration <0.5%) or LPS (*Escherichia coli* 0111; 1 $\mu\text{g}/\text{ml}$) for 24 h, before being washed, incubated with FITC-labeled mouse anti-human CD40, CD80, CD83, or CD86 Abs for 30 min at 4°C. Cells were washed repeatedly and fluorescence was measured using an EPICS-XL flow cytometer (Coulter Electronics). For

some experiments, 1-aminobenzotriazole (ABT) (1 mM) was added to the cells 30 min before the addition of drugs, or polymyxin B (20 μ g/ml) was preincubated with the drug for 30 min before addition to the cells.

Toxicity studies

Immature dendritic cells were incubated with SMX or SMX-NO (0.01–1000 μ M) for 24 h. Cell recovery was determined by counting with trypan blue and was consistently >95%. The populations were then analyzed for apoptotic or necrotic cells using annexin V propidium iodide dual staining methodology. Briefly, cells were stained with rhAnnexin V^{FITC} (1 μ g/ml) for 10 min before being washed and incubated with propidium iodide (35 μ M). The cells were then analyzed for apoptotic and necrotic cell death by flow cytometry.

Glutathione content of treated dendritic cells

Immature dendritic cells were incubated with SMX or SMX-NO (1–1000 μ M) for 24 h. The cells were then washed and lysed with 10 mM HCl and an aliquot was taken for protein content determination by the method of Bradford et al. (46). Total glutathione and oxidized glutathione levels were determined by a microtiter plate assay according to the method of Vandeputte et al. (47). Reduced glutathione (GSH) levels were determined by subtracting oxidized glutathione from total glutathione and were expressed as nanomoles of GSH per milligram of protein.

Measurement of the irreversible binding of sulfamethoxazole metabolites to dendritic cells

Immature dendritic cells were incubated with SMX (0.1–50 μ M) or SMX-NO (50–4000 μ M) for 24 h. The cells were then washed repeatedly to remove unbound SMX and stained using a rabbit anti-SMX IgG Ab (3 μ l) for 30 min at 4°C. Following subsequent washing steps, cells were incubated with FITC-conjugated anti-rabbit IgG for a further 30 min at 4°C. Certain populations were fixed and permeabilized using a Leucoperm kit (AbD Serotec) before Ab staining to identify intracellular binding. For fixation, cells are incubated with formaldehyde (Leucoperm reagent A) for 15 min. Fluorescence was measured by flow cytometry as described previously. Additionally, protein sulfamethoxazole adducts were identified using a previously described ELISA protocol (15). Briefly, dendritic cells were incubated with SMX (500 μ M), SMX-NO (10 μ M), or vehicle for either 0.1 or 24 h, washed, resuspended in distilled water, and lysed by repeated freeze-thaw cycles. The samples were spun to remove insoluble cell debris at 10,000 rpm for 5 min, the protein content of the supernatants was determined as above and standardized to 250 μ g/ml. One hundred-microliter aliquots were plated in duplicate on 96-well ELISA plates and left to adsorb for 16 h at 4°C. The wells were then washed with casein-Tris buffer (0.5% casein, 0.9% NaCl, 10 mM Tris-HCl, 0.01% thimerosal (pH 7.6)), blocked for 2 h with casein-Tris buffer, and incubated at 4°C with a rabbit anti-SMX Ab (1:500 in casein-Tris buffer) for 24 h. The wells were then washed with casein-Tris buffer and incubated with a goat anti-rabbit alkaline phosphatase-linked Ab (1:250) for 16 h. Finally, the wells were washed and incubated for 1 h with alkaline phosphatase yellow liquid substrate. The OD of the wells at 405 nm was then determined. For some experiments, 1 mM SMX was added to the primary Ab 1 h before addition to the wells to identify hapten inhibition of the Ab binding. The ELISA protocol does not require cells to be fixed before analysis and thus a possible interaction between SMX and formaldehyde can be excluded.

Quantitative real-time RT-PCR analysis of metabolic enzyme expression

Expression of a panel of metabolic enzymes (CYP1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, 4A11, MPO, cyclooxygenase-2 (COX-2)) and GAPDH was measured by quantitative real-time RT-PCR. First, total RNA was extracted from dendritic cells ($n = 10$), PBMCs ($n = 10$), and human hepatocytes ($n = 8$) using a Qiagen RNeasy 96 kit. DNA contamination was removed with DNase treatment with 0.5 U of DNase I in a thermocycler (Eppendorf), at 37°C for 10 min followed by 75°C for 5 min. Total RNA was quantified by fluorescent measurement with RiboGreen (excitation 480 nm/emission 520 nm). cDNA was synthesized with DNase-treated RNA as a template using Superscript II bulk mix according to the manufacturer's protocol. Quantitative RT-PCR was performed on the resulting cDNA in a 96-well optical reaction plate on an ABI Prism 7900 Sequence Detection System (PerkinElmer-Applied Biosystems). The reaction conditions were as follows: 2 min at 50°C, 15 min at 95°C, and 40 PCR cycles of 15 s at 95°C and 1 min at 60°C. Standard curves of human genomic DNA were set-up under identical conditions for

quantitative purposes and cycle threshold values used to derive absolute copy numbers in RNA samples.

In vitro metabolism of sulfamethoxazole

CYP-expressing supersomes (CYP1B1/2B6/2C8/2C9/2D6/3A4; 20 pM P450) were incubated in 50 mM Tris buffer (pH 7.4) with SMX (800 μ M), MgCl (3.3 mM), ascorbic acid (1 mM), and NADPH (1 mM) for 30 min. Alternatively, some incubations were set up with SMX (800 μ M), MPO (1.56 U/ml), and 1 mM ascorbic acid with H₂O₂ (200 μ M). In certain experiments, ABT (1 mM) was preincubated with the enzyme and cofactors for 1 h before addition of the drug and additional cofactors. Reactions were terminated and protein was precipitated by addition of 1 ml of ice-cold acetonitrile. The supernatant was analyzed for SMX-NHOH formation by HPLC analysis as described previously (12). The possibility of a direct interaction between SMX (metabolites) and ABT was excluded by liquid chromatography-mass spectrometry analysis scanning for molecular weights related to conjugate formation.

Time-dependent inhibition of enzyme activity with ABT

E. coli bacosomes expressing CYP enzymes (25 μ g protein; Cypex) were incubated with fluorescent substrates (ethoxyresorufin (50 μ M; CYP1A2), 7-methoxy-4-trifluoromethylcoumarin-3-acetic acid (12.5 mM; CYP2C9), 3-butyryl-7-methoxycoumarin (2.5 mM; CYP2C19), 4-methylaminomethyl-7-methoxycoumarin (2.5 mM; CYP2D6), 7-benzoquinoline (100 μ M; CYP3A4), and diethoxyfluorescein (2.5 mM; CYP3A4)) and ABT (1 mM). Drug-free incubations were used as negative controls. Plates were incubated at 37°C for 5 min and 25 μ l of an NADPH-generating system (5.53 mM glucose-6-phosphate; 0.44 mM NADP; 1.2 U/ml glucose-6-phosphate dehydrogenase) was added to initiate the reaction. Fluorescence was measured at 5-min intervals for 60 min on a Cytofluor Series 4000 Plate Reader running Cytofluor v4.2 software (Applied Biosystems). Initial blanks were subtracted from data, and inhibition was calculated as a percent decrease from drug-free CYP activity.

Determination of sulfamethoxazole metabolite immunogenicity in the mouse

BALB/C strain mice ($n = 4$ /group) were administered SMX-NO (1 mg/kg) by s.c. injection into the nape of the neck on days 0, 1, and 2. After 5 days, draining lymph node cells were harvested and incubated (1×10^6 ; total volume 200 μ l) with [³H]thymidine (2 μ Ci) for 24 h to measure proliferation. Control animals received vehicle alone. A further group of mice were administered an anti-CD40L blocking Ab (MR1; 175 μ g) via a single i.p. injection 1 h before SMX-NO administration and subsequent s.c. doses (25 μ g) with each dose of SMX-NO.

Induction of a primary T cell response in vitro with lymphocytes from sulfamethoxazole naive volunteers

Human naive T cells were primed with SMX-NO according to the technique of Engler et al. (48). Briefly, PBMCs were isolated from peripheral blood by density centrifugation, and were frozen in 1 ml of FBS with 10% DMSO. Cells were defrosted and incubated at 2.5×10^6 cells/ml in 2 ml of RPMI 1640 with 10% human AB⁺ serum, HEPES buffer (25 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), 50 μ M SMX-NO. Some wells contained either agonistic anti-CD40 Ab (82111) or antagonistic anti-CD40 Ab (82102). On day 3 of culture, rhIL-2 (10 U/ml) was added to the cultures, and on day 7 and weekly thereafter the cultures were stimulated with SMX-NO (50 μ M) and irradiated (4500 rad) autologous PBMCs from frozen stocks. Ag specificity was determined weekly by stimulation with SMX-NO and irradiated PBMC as APCs and measurement of proliferation by [³H]thymidine uptake, as described previously (48). Proliferation was recorded as stimulation index (SI: cpm in drug-treated cultures/cpm in cultures with DMSO alone).

Statistical analysis

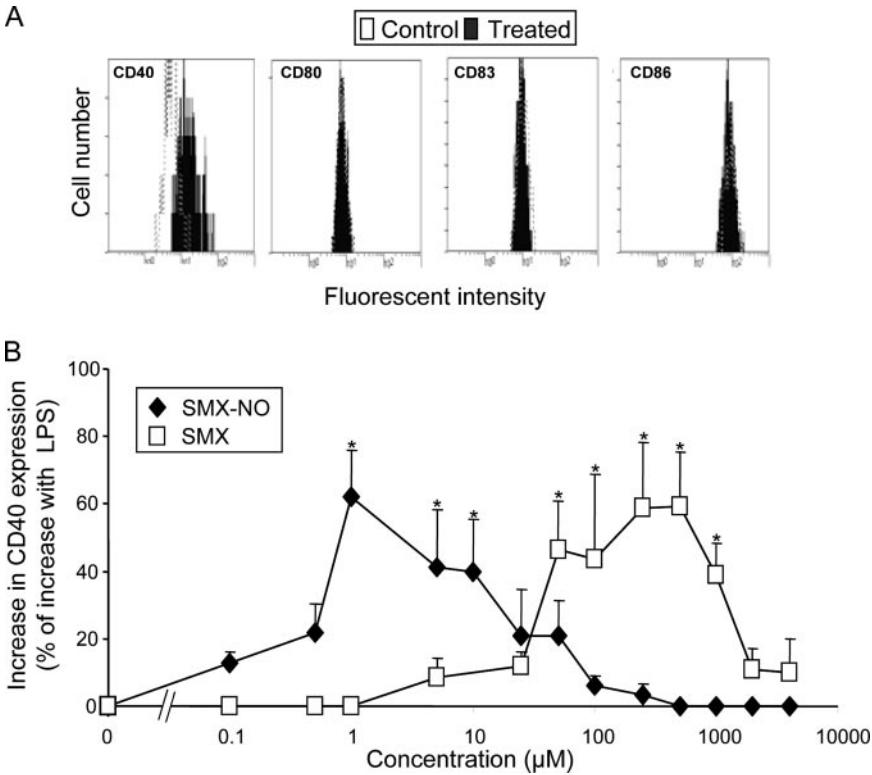
Results are presented as mean $\pm$ SEM. Shapiro-Wilks was used to test for normality. Results which were not normally distributed were compared using the Mann-Whitney *U* test.

Results

SMX and SMX-NO treatment is associated with a specific increase in dendritic cell surface CD40 expression

Dendritic cells exposed to SMX (maximal effect: 250–500 μ M) and SMX-NO (maximal effect: 1 μ M) consistently displayed a concentration-dependent increase in CD40 expression (Fig. 2), but

FIGURE 2. Selective up-regulation of cell surface CD40 by monocyte-derived dendritic cells in response to 24 h incubation with SMX or SMX-NO. *A*, Representative FACS traces showing CD40, but not CD80, 83, or 86 up-regulation following incubation with SMX-NO. *B*, Extent of up-regulation of CD40 in monocyte-derived dendritic cells, as measured by FACS analysis and recorded as the percentage of the increase with LPS over control, induced by varying concentrations of SMX ($n = 8$) and SMX-NO ($n = 6$) over a 24-h incubation period. Data are presented as mean $\pm$ SEM. *, Significantly different from untreated cells; $p < 0.05$.



no increase in CD80, CD83, or CD86 (Fig. 2A). In contrast, cells exposed to LPS showed an increase in expression of all the markers examined. Importantly, the response to SMX and SMX-NO was unaffected by preincubation with polymyxin B (20 μ g/ml), whereas the response to LPS was completely eliminated (data not shown). As polymyxin B readily binds to and inactivates LPS, these data show that the observed response was not due to LPS contamination of the stock compounds.

Dendritic cell death in response to SMX-NO is only seen at concentrations above 500 μ M

There was no significant cell death when dendritic cells were incubated with SMX at any concentration below 4 mM (data not shown), whereas significant quantities of necrotic cells were observed with SMX-NO only at 500 μ M (Fig. 3A). No increase in apoptotic cell death was observed at any concentration.

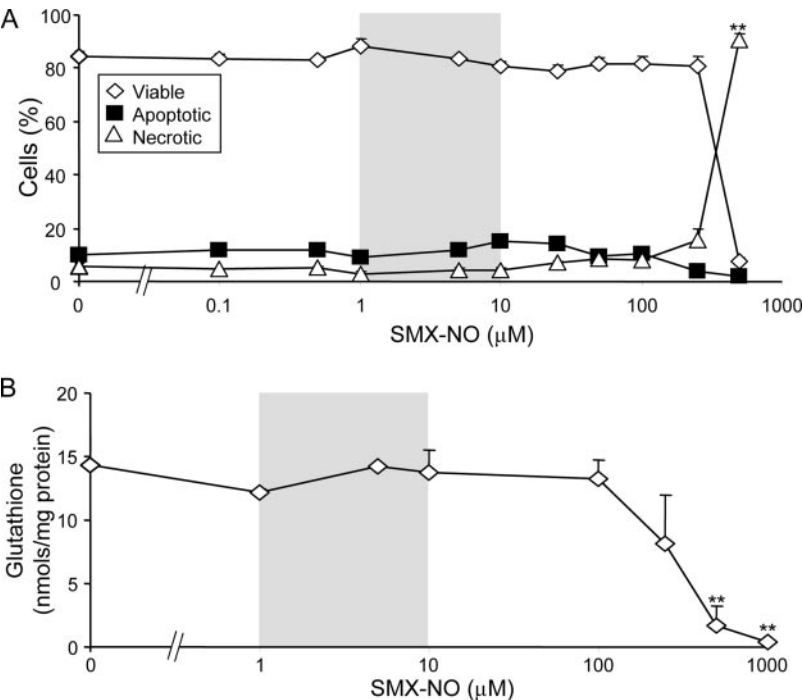


FIGURE 3. *A*, SMX-NO is not directly cytotoxic at concentrations associated with dendritic cell activation. Dendritic cells were incubated with varying concentrations of SMX-NO for 24 h, and proportions of cells viable, apoptotic and necrotic were determined by FACS analysis of annexin V and propidium iodide staining ($n = 3$). Gray region represents concentrations associated with CD40 up-regulation. Data are presented as mean $\pm$ SEM. **, Significantly different from untreated incubations; $p < 0.01$. *B*, SMX-NO does not deplete glutathione at concentrations associated with dendritic cell activation. Dendritic cells were incubated with varying concentrations of SMX-NO for 24 h, and intracellular glutathione levels were quantified using the method of Vandeputte et al. (47) ($n = 4$). Gray region represents concentrations of SMX-NO associated with increased CD40 expression. Data are presented as mean $\pm$ SEM. **, Significantly different from untreated incubations; $p < 0.01$.

Dendritic cell glutathione was depleted by SMX-NO only at concentrations of 500 μ M and above

Because oxidized glutathione levels were consistently at or below the level of detection, total glutathione has been used as a surrogate for GSH concentrations. No significant glutathione depletion was seen with SMX at any concentration studied (data not shown), while significant dendritic cell glutathione depletion was seen with SMX-NO only at concentrations of 500 μ M or higher (Fig. 3B).

Covalent binding of SMX metabolites to dendritic cell protein is seen at concentrations associated with CD40 up-regulation

Binding of SMX-NO and SMX-derived metabolites to dendritic cell protein was measured using immunofluorescence analysis following repeated washing steps to exclude the possibility that the anti-SMX Ab might detect unbound SMX. Incubation of SMX-NO (0.5–50 μ M), but not SMX (50–4000 μ M), with dendritic cells resulted in the formation of surface (Fig. 4A) adducts. However, SMX treatment of dendritic cells was associated with formation of intracellular SMX metabolite adducts at concentrations above 100 μ M (Fig. 4B). Additionally, protein-SMX metabolite adducts could be identified using an ELISA methodology following SMX-NO (100 μ M) incubation for 0.1 or 24 h and SMX (500 μ M) incubation for 24 h (Fig. 4C). Importantly, incubation of SMX (800 μ M) for 0.1 h did not lead to adduct formation (Fig. 4C), effectively ruling out the possibility that the parent compound is detected within dendritic cells following repeated washing.

The addition of 1 mM SMX to the primary Ab (to saturate Ab binding sites) before addition to the wells completely abrogated any binding to adsorbed protein, demonstrating that the binding was SMX metabolite specific (data not shown).

CYP2C9, 2C8, and MPO can bioactivate SMX

Of the metabolic enzymes investigated only MPO, CYP2C9, and CYP2C8 were capable of metabolizing SMX to the N4-hydroxylamine (Table I).

Dendritic cells express CYP1B1 and MPO, but low levels of other metabolic enzymes

RT-PCR analysis of dendritic cell mRNA from 10 healthy donors was performed to quantify the expression of several metabolic enzymes, and this was compared with expression in human hepatocytes and PBMCs (Fig. 5A; Table I). As expected, enzyme expression was higher in human hepatocytes than in both PBMCs and dendritic cells. High levels of both CYP1B1 and MPO were detected in dendritic cells compared with both PBMCs (fold differences: 50; 60) and hepatocytes (fold differences: 80; no MPO detected in hepatocytes), but expression of other metabolic enzymes was considerably lower in dendritic cells when compared with either PBMCs (average fold difference: 90) or hepatocytes (average fold difference: 7800). GAPDH expression was comparable between the cell types (dendritic cells: 84 copies/ng total RNA; PBMCs: 110.0 copies/ng total RNA; hepatocytes: 170 copies/ng total RNA).

Inhibition of drug metabolism enzyme activity prevents increased CD40 expression with SMX, but not with SMX-NO

Time-dependent inhibition studies over 1 h demonstrated that 1 mM ABT was a nonspecific inhibitor of all CYPs and peroxidases studied (inhibition after 1 h: 98.5–100%; Fig. 5B). Preincubation for 1 h with ABT (1 mM) completely inhibited SMX-NHOH formation by all enzymes studied (Table I). When ABT (1 mM) was preincubated with dendritic cells, it com-

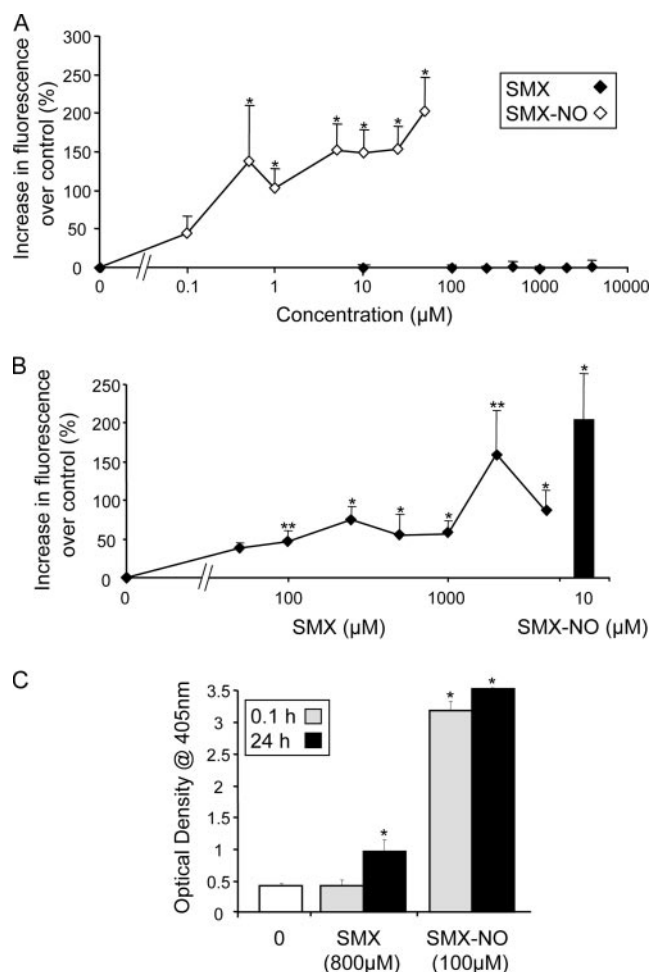


FIGURE 4. SMX adducts are detectable in dendritic cells following incubation with both SMX and SMX-NO. *A*, Cell surface covalent binding is seen with SMX-NO but not with SMX. Dendritic cells were incubated with SMX-NO (0.1–50 μ M; $n = 5$) or SMX (100–4000 μ M; $n = 7$) for 24 h, and cell surface covalent binding was determined by FACS using an anti-SMX rabbit Ab. Data are presented as mean $\pm$ SEM. *, Significantly different from untreated cells; $p < 0.05$. *B*, Intracellular covalent binding of SMX and SMX-NO is present at concentrations associated with dendritic cell activation. Dendritic cells were incubated with SMX or SMX-NO ($n = 4$) for 24 h and intracellular binding was determined by FACS using an anti-SMX rabbit Ab following fixation and permeabilization of the cells. Data are presented as mean $\pm$ SEM. **/***, Significantly different from untreated cells; $p < 0.05/0.01$. *C*, Protein SMX adducts were detected following incubation with 500 μ M SMX and 100 μ M SMX-NO ($n = 8$). Dendritic cells were incubated with drug for 0.1 or 24 h, protein was extracted, standardized, and adsorbed onto a 96-well ELISA plate, followed by addition of rabbit anti-SMX Ab for 24 h, alkaline-phosphatase-linked goat anti-rabbit IgG Ab for 16 h. The wells were then incubated with an alkaline phosphatase colorimetric substrate for 1 h and the OD at 405 nm was measured. Data are presented as mean $\pm$ SEM. *, Significantly different from untreated cells, $p < 0.05$.

pletely abrogated the CD40 up-regulation induced by SMX, but had no effect on the response to SMX-NO or the positive control LPS (Fig. 5C).

CD40L blockade inhibits nitroso sulfamethoxazole-induced T cell activation in the mouse

Exposure of BALB/C strain mice to SMX-NO was associated with a significant increase in lymph node cell proliferation when compared

Table I. Enzyme expression and SMX metabolism

Enzyme	Expression (copies/ng RNA)			SMX-NHOH Formation (nM/ml)	
	Dendritic cells	PBMCs	Hepatocytes	-ABT	+ABT
CYP1B1	591.1 ± 82 ^a	11.62 ± 1.7	10.19 ± 3.99	ND	ND
CYP2B6	0.04 ± 0.02	5.98 ± 0.83	67.23 ± 18.2	ND	ND
CYP2C8	0.39 ± 0.06	12.58 ± 1.8	2465 ± 381	0.308	ND
CYP2C9	0.30 ± 0.20	12.48 ± 1.2	7400 ± 1600	0.780	ND
CYP2D6	0.93 ± 0.11	6.79 ± 1.3	39.10 ± 13	ND	ND
CYP3A4	ND	0.45 ± 0.21	3367 ± 1060	ND	ND
MPO	9.73 ± 3.2 ^a	0.13 ± 0.12	ND	0.394	ND
COX-2	0.08 ± 0.03	23.11 ± 2.6	1.75 ± 1.28	ND	ND

^a Significantly different to expression in PBMCs/hepatocytes, *p* < 0.0001.

with mice exposed to vehicle alone. However, concurrent dosing with an antagonistic anti-CD40L Ab completely eliminated the increased lymph node cell proliferation associated with SMX-NO treatment (Fig. 6A).

CD40 receptor stimulation enhances the drug-specific response following primary induction of an SMX-NO-specific T cell response in vitro

PBMCs isolated from SMX-naïve volunteers were successfully sensitized in vitro to SMX-NO over the course of 5 wk. When PBMCs from the same volunteers were cocultured with an agonistic anti-CD40 Ab, an enhanced SMX-NO specific response was seen (total positive wells: 11/20 vs 3/20; average SI: 3.07

vs 1.26; Fig. 6B) after 4 wk. Furthermore, the addition of an antagonistic anti-CD40 Ab prevented the sensitization in all cases.

CD40 up-regulation was enhanced in an SMX-hypersensitive HIV⁺ patient when compared with SMX-tolerant HIV⁺ patients

Dendritic cells generated from an HIV⁺ patient with SMX-hypersensitivity tested acutely displayed greatly increased CD40 up-regulation in response to SMX-NO when compared with DCs generated from HIV⁺, SMX-tolerant patients (Fig. 6C). The response to LPS was not appreciably different between the two groups.

FIGURE 5. A, Dendritic cells express MPO and CYP1B1, although other metabolic enzymes are expressed at low levels. Expression of metabolic enzymes in dendritic cells (*n* = 10), PBMCs (*n* = 10), and human hepatocytes (*n* = 8) was determined by quantitative real-time RT-PCR using a cDNA library generated from RNA extracted from immature dendritic cells. The results are presented as mean copies per nanogram of total RNA. B, ABT acts as a potent inhibitor of a wide range of metabolic enzymes. Time-dependent inhibition of several metabolic enzymes (CYP1A2; CYP1B1; CYP2C9; CYP2C19; CYP2D6; CYP3A4; MPO) was determined by inhibition by 1 mM ABT of fluorogenic substrate turnover by expressed enzymes over 1 h. Results were calculated as the percent inhibition at various time points. C, ABT inhibits CD40 up-regulation with SMX, but not SMX-NO or LPS. Monocyte-derived dendritic cells were preincubated with 1 mM ABT or vehicle for 1 h, followed by incubation with SMX or SMX-NO for 24 h (*n* = 3). CD40 up-regulation was determined by FACS analysis, and is presented as the percent increase in median fluorescence over control. Data are presented as mean ± SEM. *, Significantly different from vehicle treated (*p* < 0.05).

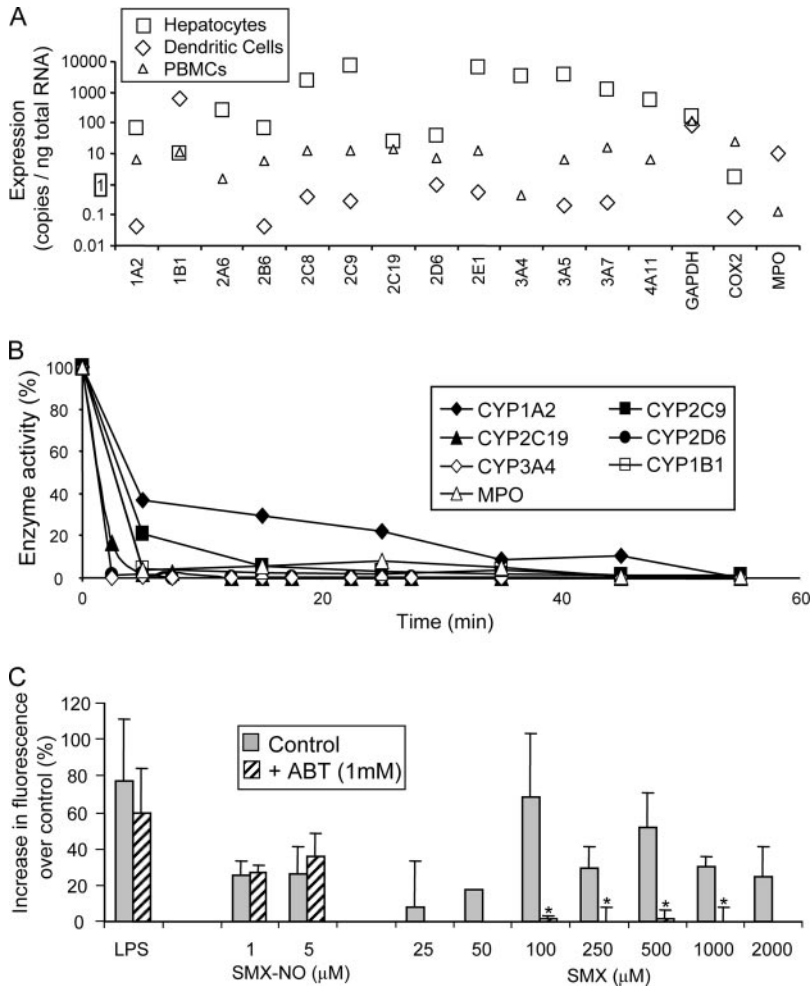
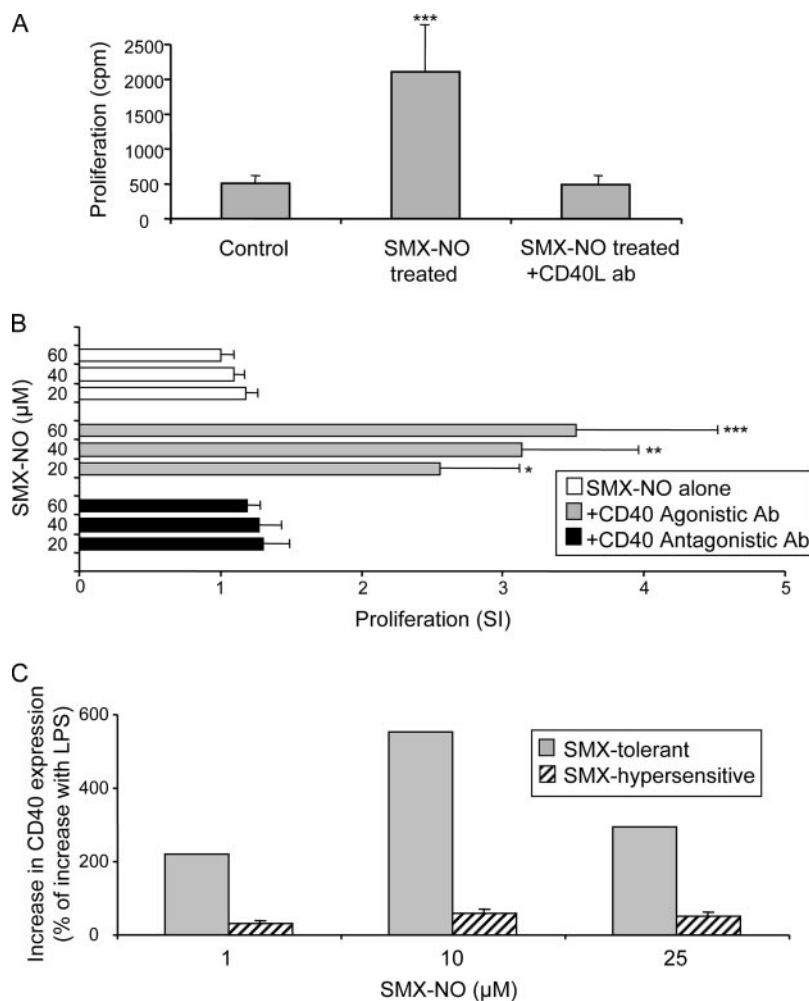


FIGURE 6. A, CD40 blockade inhibits in vivo activation of T cells in mice. Mice ($n = 4$) were dosed with SMX-NO $\pm$ an antagonistic anti-CD154 Ab s.c. daily for 5 days. Lymphocytes from draining lymph nodes isolated and proliferation was measured by [^{3} H]thymidine incorporation over 16 h. ***, Significantly different from both vehicle and Ab treated ($p < 0.005$). B, CD40 activation enhances in vitro primary stimulation of T cells to SMX-NO. PBMCs were isolated from healthy, non-SMX-exposed volunteers, and exposed to 10 U/ml IL-2, and 20 μ M SMX-NO alone, or with an agonistic or antagonistic anti-CD40 Ab for 3 wk ($n = 20$). SMX-NO specific T cell activation was then determined by rechallenge in fresh medium with 20–60 μ M SMX-NO for 48 h, with proliferation over the final 16 h determined by [^{3} H]thymidine incorporation. Data are presented as mean $\pm$ SEM of SI, equal to (cpm with SMX-NO)/(cpm without). */**/***, Significantly different from untreated incubations ($p < 0.05/0.01/0.001$). C, Dendritic cells generated from an SMX-hypersensitive HIV $^+$ patient ($n = 1$) show an increased up-regulation of CD40 in response to SMX-NO than those from SMX-exposed and tolerant HIV $^+$ patients ($n = 4$). Immature monocyte-derived dendritic cells were incubated with SMX-NO for 24 h and CD40 surface expression was determined by FACS analysis. Data are presented as the percentage of increase recorded with LPS, and are shown as mean $\pm$ SEM.



Discussion

The requirement for distinct costimulatory signals, in addition to the Ag, for T cell immunogenicity was first proposed by Matzinger (20). Since then, many studies have identified signals, which can generally be divided into pathogen-associated patterns, such as LPS (49, 50) and CpG oligodeoxynucleotides (51, 52), and indicators of cell toxicity (27), such as uric acid release (28) or thiol depletion (30, 33, 53). The effect of these signaling pathways is to switch a T cell response to a specific Ag from tolerance to full activation.

Dendritic cells respond to such signaling by increased cell surface expression of costimulatory receptors, particularly CD80/86, and/or cytokine secretion, which in turn provide additional signals for activation and phenotypic differentiation of T cells (54, 55). Costimulatory signaling is not unidirectional: the interaction of activated T cells with the dendritic cell, particularly through CD40 receptor ligation, causes further dendritic cell maturation and enhanced Ag-presenting capability (56, 57).

Little is known about whether drugs or drug metabolites may act to generate signals that lead to activation of dendritic cells. To address this, we studied the effects of SMX and SMX-NO on the expression of dendritic cell surface costimulatory receptors. Dendritic cells were generated from healthy human volunteers, incubated with SMX or SMX-NO, and surface marker expression was determined by flow cytometry. With both compounds an increase in surface CD40, but not CD80, 83, or 86, expression was seen after 24 h (Fig. 2). A specific effect on CD40 expression was also seen following a longer term drug exposure of up to 48 h (results

not shown). The only difference observed with the dendritic cell response to SMX and SMX-NO was that the concentration of SMX to induce maximal CD40 expression was 250-fold higher.

An interesting question for us to determine was whether metabolism by the dendritic cells was an obligatory step in the observed CD40 expression. It is possible that both the drug and its chemically reactive nitroso metabolite could stimulate dendritic cell maturation. For instance, the increased CD40 expression may due to direct interaction of SMX itself with receptors found on the surface of dendritic cells such as TLRs. A precedent for this already exists: the imidazoquinoline, imiquimod, has been shown to activate macrophages through TLR7 and thereby induce the production of cytokines (58). However, our evidence suggests that it is the intracellular metabolic activation of SMX in dendritic cells that is responsible for the increased CD40 expression. Using a highly specific anti-SMX Ab (4, 13, 15), we were able to demonstrate by both flow cytometry and ELISA haptenated intracellular proteins when SMX, at similar concentrations to those which stimulated increased CD40 expression, was incubated with dendritic cells (Fig. 4, B and C). Importantly, the formation of haptenated proteins was time dependent (Fig. 4C) and not observable immediately after incubation of SMX with cells. By contrast, SMX-NO bound rapidly to both intra- and extracellular proteins, consistent with previous work from several laboratories including ours (4, 5, 9, 13–15).

To explore further the role of metabolism in this process, expression of metabolic enzymes in dendritic cells was determined, and contrasted with those seen in other cell types (PBMCs and

hepatocytes). Dendritic cells expressed the majority of CYPs studied (Fig. 5A; Table I), albeit at a low level compared with both PBMCs and hepatocytes. The sole exception was CYP1B1, which was expressed at a significantly higher level than that seen in either alternative cell type. A previous study has looked at CYP expression in dendritic cells (59), but the data presented herein are the first which have both quantified the expression and compared this to other cell types of known metabolic capacity. CYP1B1 is a polymorphic enzyme that plays an important role in the activation of carcinogens. However, our data suggests that it does not catalyze SMX metabolism (Table I) and thus an association between CYP1B1 expression and SMX hypersensitivity seems unlikely. There is a degree of controversy surrounding the expression of MPO in dendritic cells (60, 61). In this study, it was apparent that dendritic cells generated from 10 donors expressed consistently high levels of MPO mRNA (Table I), and this was significantly higher than that in PBMCs (fold difference: 58.9; $p < 0.0001$).

CYP2C9 and MPO, consistent with previous literature (10, 11, 62), but also CYP2C8, were found to be capable of SMX-NHOH generation (Table I). All other enzymes studied were inactive. Based on this, and the CYP expression data, it seems plausible that MPO is the enzyme responsible for SMX metabolic activation in dendritic cells. Additionally, dendritic cells expressed low levels of COX-2; however, in contrast to early reports, COX-2 has recently been shown not to play a direct role in SMX oxidation (63). Because analytical techniques are not sufficiently sensitive to detect dendritic cell-mediated SMX metabolism directly, a series of enzyme inhibitor studies were performed to substantiate a causal relationship between SMX metabolism by dendritic cells and increased cell surface CD40 expression. ABT, a suicide inhibitor of hemoxygenases, which acts via alkylation of the enzyme-associated haem group (64), was shown to inhibit 1) activity of all metabolic enzymes studied over the course of 1 h (Fig. 5B); 2) metabolism of SMX to SMX-NHOH catalyzed by CYP2C8, 2C9 and MPO (Table I); and 3) SMX-mediated increased CD40 expression. By contrast, SMX-NO- and LPS-mediated CD40 expression was not affected by ABT preincubation (Fig. 5C). Taken together, our observations suggest that increased CD40 expression following exposure of dendritic cells to SMX is dependent on MPO-mediated oxidative drug metabolism. The site and nature of SMX metabolite protein modification, and indeed whether specific proteins are modified, has yet to be deciphered.

There are several possible hypotheses by which drugs and their metabolites could increase CD40 expression. First, release of cell contents from necrotic cells stimulates increased CD40 expression (65, 66). Indeed, in a recent study it was demonstrated that dendritic cell activation in response to contact sensitizers is dependent on low levels (~10–20%) of necrotic cell death (34). However, in the present work, there was no significant increase in necrotic or apoptotic cell death observed with SMX at any concentration tested, and for SMX-NO only at concentrations that were ~500-fold higher than required for increased CD40 expression. Second, depletion of glutathione has also been shown to be associated with dendritic cell maturation, particularly increased expression of costimulatory receptors (53, 67). However, depletion of glutathione was only seen at concentrations of SMX-NO ~200-fold higher than required for increased CD40 expression, and was not seen at all with SMX. Additionally, the level of oxidized glutathione was below the limit of detection, and no increase in oxidized glutathione was observed. These data reveal that covalent binding of sulfamethoxazole metabolites to viable dendritic cells is associated with increased CD40 expression. It is worth mentioning that the association between bioactivation and CD40 up-regulation is not necessarily due to covalent binding: auto-oxidative conversion of

SMX-NHOH to SMX-NO can generate reactive oxygen species and oxidative stress (68), both known inducers of dendritic cell maturation (69, 70).

The importance of CD40 signaling in drug hypersensitivity has not been considered previously. Differential regulation of CD40 and CD80/86 expression has been previously described and some of the upstream pathways are known. For instance, RelB-deficient dendritic cells fail to up-regulate CD40 in response to LPS, but up-regulate CD80 and CD86 normally, and are incapable of effective Ag-specific priming (71). So called partial dendritic cell maturation (i.e., increased expression of only certain costimulatory receptors or increased expression of costimulatory receptors but no cytokine secretion) has been associated with the induction of tolerogenic, rather than pathogenic, T cell responses (72, 73). In this respect, one must note that binding of the natural CD40L CD154 to dendritic cells expressing high levels of the CD40R stimulates further dendritic cell maturation and, as such, the observed highly specific effect of SMX metabolites on CD40 may, in a mixed cell population, ultimately result in fully matured dendritic cells. Moreover, activation of APCs through CD40 signaling is known to precipitate animal models of autoimmune disease (57), while CD40 blockade can prevent the progression of diseases such as systemic lupus erythematosus, cardiac allograft arteriopathy, and spontaneous autoimmune diabetes (74–76). We have used several experimental approaches to evaluate the role of CD40 signaling in SMX metabolite immunogenicity. First, pretreatment of mice with a blocking anti-CD40 Ab completely inhibited SMX-NO immunogenicity; second, treatment of human lymphocytes from healthy SMX naive volunteers with a stimulatory anti-CD40 Ab and SMX-NO enhanced the strength of the recall proliferative response with SMX-NO; and third, increased dendritic cell CD40 expression was 5-fold higher when cells from a patient in the acute phase of a SMX hypersensitivity reaction was compared with cells from four SMX-exposed healthy volunteers. Although this is only from one patient, it is consistent with diseases such as ulcerative colitis where the extent of disease activity has been found to be proportional to CD40 expression levels (77).

Collectively, our data show that exposure of dendritic cells to SMX and its reactive metabolite SMX-NO is associated with generation of costimulatory signals required to initiate a primary immune response. Furthermore, we have demonstrated for the first time that dendritic cells are capable of activating SMX to SMX-NO intracellularly, which is more reactive in both a chemical and immunological sense. We would argue that this is an immunologically important pathway that is highly relevant to hypersensitivity. Furthermore, the potential for cross-talk between drug metabolism and intracellular signaling in dendritic cells is an unexplored area that requires further investigation in the context of drug hypersensitivity.

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Disclosures

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