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Drug Antigenicity, Immunogenicity, and Costimulatory Signaling: Evidence for Formation of a Functional Antigen through Immune Cell Metabolism

Ayman Elsheikh,^{*,†,1} Sidonie N. Lavergne,^{*,2} J. Luis Castrejón,^{*} John Farrell,^{*} Haiyi Wang,^{*} Jean Sathish,^{*} Werner J. Pichler,[‡] B. Kevin Park,^{*} and Dean J. Naisbitt^{*}

Recognition of drugs by immune cells is usually explained by the hapten model, which states that endogenous metabolites bind irreversibly to protein to stimulate immune cells. Synthetic metabolites interact directly with protein-generating antigenic determinants for T cells; however, experimental evidence relating intracellular metabolism in immune cells and the generation of physiologically relevant Ags to functional immune responses is lacking. The aim of this study was to develop an integrated approach using animal and human experimental systems to characterize sulfamethoxazole (SMX) metabolism-derived antigenic protein adduct formation in immune cells and define the relationship among adduct formation, cell death, costimulatory signaling, and stimulation of a T cell response. Formation of SMX-derived adducts in APCs was dose and time dependent, detectable at nontoxic concentrations, and dependent on drug-metabolizing enzyme activity. Adduct formation above a threshold induced necrotic cell death, dendritic cell costimulatory molecule expression, and cytokine secretion. APCs cultured with SMX for 16 h, the time needed for drug metabolism, stimulated T cells from sensitized mice and lymphocytes and T cell clones from allergic patients. Enzyme inhibition decreased SMX-derived protein adduct formation and the T cell response. Dendritic cells cultured with SMX and adoptively transferred to recipient mice initiated an immune response; however, T cells were stimulated with adducts derived from SMX metabolism in APCs, not the parent drug. This study shows that APCs metabolize SMX; subsequent protein binding generates a functional T cell Ag. Adduct formation above a threshold stimulates cell death, which provides a maturation signal for dendritic cells. *The Journal of Immunology*, 2010, 185: 000–000.

Hypersensitivity represents one of the most feared adverse events in the drug-development process. The presence of drug-specific cytotoxic T cells in the peripheral circulation and target organs of hypersensitive patients, but not in drug-exposed controls, provides a robust case for their involvement in the pathogenesis of the reaction (1–4). To initiate an immune response, the drug Ag must be presented in the context of specific MHC molecules expressed on dendritic cells (DCs) to specific TCRs in a microenvironment rich in costimulatory signaling and cytokines, which are necessary for sustained T cell expansion (5, 6).

Our understanding of the chemical basis of drug-hypersensitivity reactions derives from the field of allergic contact dermatitis, in which Landsteiner and Jacobs (7) defined low m.w. chemical allergens as “incomplete Ags” because the compounds themselves were not directly antigenic (i.e., do not bind with high affinity to immunological receptors) and only gained immunogenic potential following conjugation with a protein carrier. The β -lactam antibiotics, which cause a high incidence of drug hypersensitivity reactions, also react spontaneously with protein (8, 9), and synthetically constructed penicillin protein adducts stimulate specific T cells following processing (10). On this basis, the “hapten concept” (hapten referring to any substance that modifies protein to induce an immune response) is the hypothesis most commonly used to describe the interaction of drugs with immune cells. The picture is further complicated because most drugs are not directly protein reactive. However, they may gain protein reactivity through normal metabolic processes, generating a hapten with the potential to modify specific amino acid residues on protein. Using the antibacterial agent sulfamethoxazole (SMX) as a model drug allergen, independent research groups showed that synthetic metabolites of SMX interact with MHC and TCRs with sufficient affinity to stimulate blood- and skin-derived T cells from hypersensitive patients (4, 11–14). These data confirm that hapten-specific T cells are present in hypersensitive patients and that T cell responses are directed against drug metabolite-conjugated protein. The current thinking is that hepatic metabolism first generates drug metabolites that form protein adducts that are then processed and presented by APCs. It is not known whether the APCs could also generate drug metabolites and subsequent haptened Ags. Thus, critical experiments showing a causal relationship between compound distribution, drug metabolism, and protein modification in APCs and the development of T cell responses have not been described. Detection of T cell responses

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Abbreviations used in this paper: ABT, 1-aminobenzotriazole; DC, dendritic cell; Meth, methimazole; ND, not detectable; PI, propidium iodide; SMX, sulfamethoxazole; SMX-NO, nitroso sulfamethoxazole.

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to protein adducts formed as a consequence of APC metabolism would signify that hepatic metabolism is not necessarily involved in drug hypersensitivity and that the propensity to form chemically reactive metabolites by liver enzymes is not a sensitive method for predicting drugs that cause hypersensitivity.

DCs perform an important role in determining the equilibrium between immune tolerance and immune reactivity through the provision of receptor ligand interactions (costimulatory signaling) and cytokine secretion (15). Drugs, including abacavir, amoxicillin, and SMX, were shown to partially stimulate human DC costimulatory signaling (16–18), which could potentially drive pathogenic immune responses in hypersensitive patients. DCs express multiple drug-metabolizing enzymes (16, 19–22) that can generate drug-derived protein adducts in increasing amounts when exposed to danger signals (22). Furthermore, SMX-mediated DC signaling was shown to be dependent on intracellular metabolism and protein-adduct formation (16). One pathway for DC activation is through exposure to products derived from dying cells (e.g., ATP, heat shock proteins, HMGB1, urate phosphatidyl serine). It is unclear whether DC activation in the context of drug hypersensitivity requires cytotoxicity; thus, the relationship between drug-specific DC signaling and cytotoxicity remains controversial. In particular, the relationship between bystander cell death following exposure to haptenic drugs and DC activation has not been studied.

In this study, we used the drug SMX to explore the functional consequences of metabolite formation in immune cells (Fig. 1). Data presented in this article show that T cells from animal models of SMX immunogenicity and SMX-hypersensitive patients are stimulated by drug metabolite–protein conjugates generated intracellularly as a consequence of metabolism in APCs. We also show that bystander cell death associated with exposure to high levels of drug metabolite is required for the provision of maturation signals to DCs. Our findings suggest that APCs alone are sufficient to generate drug metabolites and haptened Ags, as well as to induce the drug-specific T cell response.

Materials and Methods

Mice

Female BALB/c mice (6–10 wk of age) were purchased from Charles River (Margate, Kent, U.K.) and maintained under specific pathogen-free conditions. All experiments were carried out under the provisions of the United Kingdom Animals (Scientific Procedures) Act, 1986. Drug-naïve animals were used to obtain bone marrow-derived DCs, unless stated otherwise.

Immune cells

Bone marrow-derived mouse DCs. Bone marrow-derived mouse DCs were generated according to the procedure of Lutz et al. (23), with slight modifications. Harvested cells were filtered, suspended in culture medium (RPMI 1640 supplemented with heat-inactivated and filtered FBS [10% v/v], penicillin [100 U/ml], streptomycin [100 µg/ml], L-glutamine [2 mM], HEPES [25 mM], 2-ME [50 µM], and recombinant murine GM-CSF [20 ng/ml]) and aliquoted in sterile Petri dishes (10 ml, 2×10^5 cells/ml). Half of the medium was replaced with fresh complete medium on days 3, 6, and 8, and immature DCs were ready for use on day 9.

Mouse splenocytes

Spleens were removed using aseptic technique and homogenized. Splenocytes were filtered prior to the removal of RBCs by density centrifugation using Lymphoprep (Nycomed, Birmingham, U.K.). Splenocytes were suspended in culture medium (RPMI 1640 supplemented with heat-inactivated and filtered FBS [10% v/v], penicillin [100 U/ml], streptomycin [100 µg/ml], L-glutamine [2 mM], HEPES [25 mM]) prior to each experiment.

Human blood lymphocytes

Lymphocytes were isolated from heparinized blood of three healthy volunteers and from three SMX-hypersensitive patients via centrifugation through Lymphoprep. Approval for the study was obtained from the Liverpool local research ethics committee; informed written consent was ob-

tained from all donors. Clinical details of the hypersensitive patients were described previously (14). Cells were suspended in cell culture medium (RPMI 1640, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, 25 mM HEPES) prior to each experiment.

Human monocyte-derived DCs

Monocyte-derived DCs were generated using an established procedure (16). Adherent monocytes were cultured in 1 ml RPMI 1640 medium, supplemented with 10% FBS, HEPES buffer (25 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/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 DCs were ready for use on day 6. Purity of DC populations was determined by FACS analysis with an anti-human CD11c Ab. CD11c expression was consistently >95%.

EBV-transformed B cell lines

EBV-transformed B cell lines were generated from blood lymphocytes isolated from healthy volunteers ($n = 3$) and SMX-hypersensitive patients ($n = 3$) using supernatant from the EBV-producing cell line B9-58 (24).

T cell clones

SMX (metabolite)-stimulated lymphocytes from hypersensitive patients were cloned by serial dilution using previously described methodology (25). CD phenotype and monoclonality were determined by flow cytometry; a detailed account of the phenotypic data was described previously (14). Clones were maintained in IL-2-containing medium (RPMI 1640 supplemented with 10% heat-inactivated human blood type AB serum, HEPES buffer [25 mM], L-glutamine [2 mM], transferrin [25 µg/ml], streptomycin [100 µg/ml], and penicillin [100 U/ml]) and restimulated every 14 d with the mitogen PHA (5 µg/ml).

Reagents and chemicals

Human and murine GM-CSF and IL-4 were obtained from PeproTech (London, U.K.). FBS, mouse serum, and human AB serum were purchased from Innovative Research of America (Sarasota, FL). Lymphoprep was obtained from Axis-Shield PoC AA (Oslo, Norway). Multiplex kits for detection of secreted cytokines were purchased from Millipore (Herts, U.K.). [^3H]Thymidine was purchased from Moravsek Biochemicals (Brea, CA). The oxidative metabolites of SMX (SMX-hydroxylamine and nitroso SMX [SMX-NO]) were synthesized according to the method of Naisbitt et al. (26). The following mouse Abs were used for flow-cytometric characterization of DCR expression: PE-labeled CD11c (Caltag, Bucks, U.K.); FITC-labeled CD40, FITC-labeled CD86, and PE-labeled I-A/I-E (all from BD Biosciences Pharmingen, Oxford, U.K.); FITC-labeled CD11b; PE-labeled Gr-1 (Milenyi Biotec, Surrey, U.K.); and FITC-labeled neutrophil, FITC-labeled CD19, and FITC-labeled CD204 (all from AbD Serotec, Oxford, U.K.). All other reagents were purchased from Sigma-Aldrich (Gillingham, U.K.).

To generate an anti-SMX Ab for immunochemical detection of SMX protein adducts, SMX was conjugated to human serum albumin (for use as a positive control for the validation of the ELISA protocol), and to keyhole limpet hemocyanin for use as a soluble Ag for rabbit immunization, using a previously described procedure (27). Drug-protein conjugation was confirmed by spectrophotometry, as well as by reactivity with rabbit anti-SMX Ab, kindly provided by Dr. Michael Rieder (London, ON, Canada).

The rabbit immunization against SMX-keyhole limpet hemocyanin was approved by an Institutional Animal Care and Use Committee, under a U.S. Department of Agriculture license (#35-B-0097 and 36-R-0108) and a Public Health Service accreditation (#A4284-01) (Panigen, Branchardville, WI) (27). The reactivity of anti-sera against SMX-human serum albumin was confirmed by ELISA and immunoblotting.

Drug exposure

Cells (1×10^6 /ml; unless indicated otherwise) were incubated with SMX (0.05–2 mM) or SMX-NO (0.1–1000 µM; final DMSO concentration <0.5%) over various periods of time (5 min–6 d). Certain experiments were conducted in the presence of the enzyme inhibitors methimazole (an inhibitor of peroxidases and flavin-monooxygenases) or 1-amino-benzotriazole (a nonselective suicide inhibitor) at a concentration that blocks CYP2C9 and peroxidase-catalyzed SMX metabolism (both 1 mM). On completion of the incubation period, cells were washed three times with HBSS prior to processing for detection of SMX- and SMX metabolite-derived adduct formation (confocal microscopy and ELISA), cell viability, depletion of intracellular glutathione, DC function, and T cell activation.

Detection of DC surface-receptor expression and cytokine secretion

Bone marrow-derived DCs were incubated with SMX, SMX metabolites, or LPS (1 μ g/ml) for 24 h. On completion of the incubation period, washed cells were analyzed by flow cytometry, using Abs described above, on a Coulter Epics flow cytometer (Coulter Epics, XL software; Beckman Coulter, Fullerton, CA). Data files were further processed using WinMDI software (version 2.9; The Scripps Research Institute, La Jolla, CA).

Levels of secreted DC cytokines (IL-1 β , IL-4, IL-5, IL-6, IL-10, IL-12, IL-15, IFN- γ , and TNF- α) were measured simultaneously using a Millipore multiplex assay kit (Millipore) on a Bio-Plex Suspension Array System.

Detection of SMX metabolite protein adduct formation

Cells incubated with SMX or SMX metabolites were incubated with rabbit anti-SMX antisera (1:2000; overnight at 4°C), an alkaline phosphatase-conjugated anti-rabbit IgG (1:1000; 2 h at room temperature), and an alkaline phosphatase substrate (30 min at room temperature) to quantify drug-protein adducts. The OD was then measured at 405 nm, using a microplate reader (MRX; Dynatech Laboratories, Chantilly, VA). Results are expressed as Δ OD = sample OD – vehicle OD. To verify that the Ab binding truly reflects expression of SMX-derived adducts, hapten-inhibition experiments were performed using soluble SMX (2 mM) in the presence of the primary anti-SMX Ab. Detection of SMX-derived protein adducts was completely inhibited by soluble SMX (data not shown). To visualize SMX-derived protein adduct formation, fixed cells were incubated overnight with rabbit anti-SMX Ab (1:500) prior to incubation with FITC-labeled anti-rabbit IgG for 2 h. Slides were mounted in Vectashield H-1200 (Vector Laboratories, Peterborough, U.K.) prior to analysis by confocal microscopy.

Analysis of cell viability

Viability of mouse DCs and splenocytes incubated with SMX or SMX-NO was analyzed using the Annexin-V^{FITC}/propidium iodide (PI) double-staining method originally described by Vermees et al. (28) to detect apoptotic and necrotic cell death. The combination of these two characteristics permits simultaneous detection of viable (Annexin[−]/PI[−]), apoptotic (Annexin⁺/PI[−]), and necrotic cells (Annexin⁺/PI⁺). Cells incubated with formaldehyde or DMSO (10%) served as a positive control.

Analysis of intracellular glutathione content

Glutathione content in SMX- and SMX metabolite-treated bone marrow-derived mouse DCs was measured using a microtiter plate assay, according to the method of Vandeputte et al. (29).

Coculture of DCs with SMX-NO-treated splenocytes

Splenocytes were incubated with SMX-NO (10–500 μ M) for 16 h. After repeated washing to remove noncovalently bound drug, splenocytes (3×10^6 /ml) were cultured with mouse bone marrow-derived DCs (1×10^6 /ml) in 24-well cell-culture plates. After 16 h, DCs were assayed for cell viability, expression of cell-surface receptors, and cytokine secretion, using methods described above.

Immunization protocol for the analysis of SMX metabolite-specific T cell responses in the mouse

Female BALB-c strain mice were administered SMX-NO (5 mg/kg; $n = 4$) in DMSO (100 μ l) i.p. four times weekly for 2 wk, according to a previously described protocol (30). On completion of this dosing regimen, animals were killed, and the spleen was removed for analysis of splenocyte proliferation.

Spleens were homogenized in HBSS (10 ml) and filtered to liberate a single-cell suspension. RBCs were removed by centrifugation with Lymphoprep. Splenocytes were washed, suspended in culture medium at a concentration of 1.5×10^6 cells/ml, and placed in a round-bottom 96-well plate (1.5×10^5 /well) with SMX-NO (10–100 μ M) or SMX (100–1000 μ M) for 72 h (37°C, 5% CO₂). After 4 d, proliferation was measured by the addition of [³H]thymidine for 16 h. Cells were harvested, and incorporated radioactivity was measured (cpm) on a beta counter (PerkinElmer Life Sciences, Cambridge, U.K.). Data are presented as stimulation indices (cpm in drug-treated cultures/cpm in cultures containing solvent alone).

To explore the ability of protein conjugates, generated through direct conjugation of SMX-NO or through metabolism of SMX, to stimulate a proliferative response, spleen cells and DCs from naive mice were pulsed with SMX or SMX-NO for 1–16 h, washed repeatedly to remove unbound drug (metabolite), irradiated to prevent proliferation, and incubated with splenocytes from SMX-NO-sensitized mice. After 4 d, proliferation was measured by the addition of [³H]thymidine for 16 h.

Adoptive transfer of drug-treated DCs and analysis of SMX metabolite-specific T cell responses in the mouse

Bone marrow-derived DCs, cultured in medium supplemented with mouse serum to prevent T cell responses against FBS-derived Ags, were incubated for 16 h in culture medium with SMX (2 mM) or SMX-NO (100 μ M). For immunization, DCs were harvested, washed extensively, and injected i.v. in the lateral tail vein of BALB/c strain mice. Each mouse received one injection of 0.5×10^6 viable DCs in HBSS (200 μ l). Control mice received unmodified DCs or DCs treated with DMSO. After 21 d, mice were sacrificed, and splenocytes were isolated as described above. Splenocytes (1.5×10^6 /ml) were stimulated in 96-well U-bottom culture plates with SMX (0.25–2 mM), SMX-NO (10–50 μ M), or modified spleen cells. Proliferation was quantified by the addition of [³H]thymidine.

Analysis of the drug metabolite-specific proliferative response of lymphocytes and T cell clones from hypersensitive patients

Proliferation of lymphocytes (1.5×10^5 /well) from hypersensitive patients and SMX-exposed nonhypersensitive volunteers with SMX (250–2000 μ M) and SMX-NO (20–80 μ M) was quantified by measurement of [³H]thymidine incorporation after 6 d, as described previously (31). Proliferative responses were calculated as stimulation indices (cpm in drug-treated cultures/cpm in cultures with solvent alone).

CD4⁺ T cell clones (5×10^4 /well) were aliquoted in 96-well U-bottom cell-culture plates and incubated with irradiated autologous EBV-transformed B cells (1×10^4 ; 60 Gy), with or without SMX or SMX-NO. After 48 h, [³H]thymidine was added for the analysis of proliferation.

To evaluate the role of SMX metabolism by human APCs in drug-specific T cell proliferation, lymphocytes (used as additional APCs in the lymphocyte proliferation assay) or EBV-transformed B cells (used as APCs with T cell clones) were incubated with SMX or SMX-NO for 16 h, prior to repeated washing to remove unbound drug, irradiated to prevent proliferation, and cocultured with autologous lymphocytes or Ag-specific T cell clones. Proliferation was measured using [³H]thymidine incorporation, as described above.

Levels of secreted cytokines (IL-1 β , IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-17, IFN- γ , TNF- α , MCP-1, and GM-CSF) were measured from the supernatant of SMX- and SMX-NO-stimulated T cell clones using a Millipore multiplex assay kit, as described above.

Enzyme-inhibition experiments

To confirm that SMX-derived protein adduct formation and T cell proliferation are dependent on the enzymatic oxidation of SMX, certain experiments were conducted in the presence of various enzyme inhibitors. APCs were preincubated for 1 h with 1-aminobenzotriazole (a nonselective suicide inhibitor; 1 mM) (32) or methimazole (an inhibitor of peroxidases and flavin-monoxygenase; 1 mM) (33).

Statistical analysis

Values to be compared were analyzed for normality using the Shapiro-Wilks test. Normally distributed data were analyzed using a Student *t* test. Nonnormally distributed data were compared by the Mann-Whitney *U* test using SPSS 16.0 software (SPSS, Chicago, IL). In all cases, $p < 0.05$ was considered statistically significant.

Results

SMX, intracellular SMX metabolites, and SMX-NO do not activate mouse DCs

To study whether SMX or the metabolite SMX-NO activate bone marrow-derived DCs directly (Fig. 1), both compounds were cultured with DCs, prior to analyzing cell viability, depletion of intracellular glutathione, adduct formation, costimulatory receptor expression, and cytokine secretion. DC death and glutathione depletion were detected at SMX-NO concentrations ≥ 250 μ M, whereas there was no significant effect when DCs were incubated with SMX (Fig. 2B, 2C). Binding of SMX-NO to DCs was concentration dependent, with adducts detected at concentrations ≥ 50 μ M. SMX treatment of DCs was associated with intracellular adduct formation at a concentration of 500 μ M. Thus, direct binding of SMX-NO to DCs and binding following SMX metabolism were detectable on viable cells.

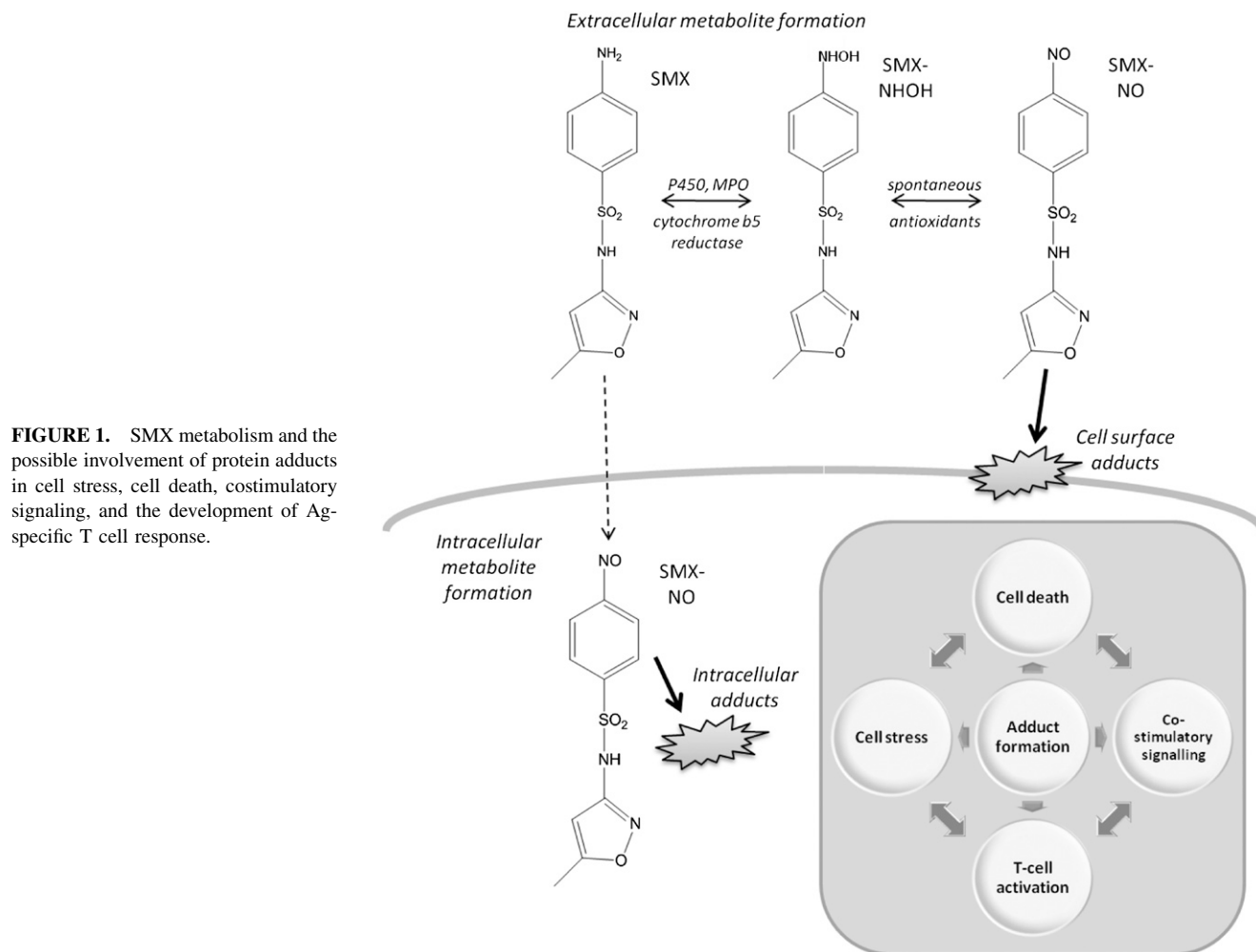


FIGURE 1. SMX metabolism and the possible involvement of protein adducts in cell stress, cell death, costimulatory signaling, and the development of Ag-specific T cell response.

DCs stimulated with LPS displayed an increase in cell-surface markers (CD40, CD86, and MHC class II) and cytokine secretion (TNF- α , IL-1 β , IL-6, and IL-12). However, SMX and SMX-NO exposure, at toxic and nontoxic concentrations, failed to stimulate DC activation (Fig. 2D–G).

Activation of DCs by necrotic cells modified with SMX-NO

To explore the relationship between SMX metabolite-mediated cell death and DC activation, SMX- and SMX-NO-treated splenocytes were cultured with DCs prior to analysis of DC activation. SMX-NO bound rapidly to splenocytes (within 1 h), and necrotic cell death was detectable at concentrations ≥ 50 μ M. Adduct formation in splenocytes cultured with SMX was time and concentration dependent and detectable by ELISA and confocal microscopy. However, adduct formation with SMX was not associated with an increase in apoptotic or necrotic cell death (Fig. 3A–C), indicating that the number of modified proteins did not exceed a threshold that must be surpassed prior to the development of proportionate increases in cell death (34).

The addition of untreated mouse splenocytes to DCs did not increase DC costimulatory receptor expression or cytokine secretion. In contrast, SMX-NO-treated splenocytes activated DCs in a concentration-dependent manner (costimulatory receptors, MHC class II, and cytokine release; Fig. 3D, 3E). The number of activated DCs was associated with increasing quantities of SMX-NO-modified necrotic splenocytes. DC activation was not detectable with nontoxic concentrations of SMX-NO.

SMX metabolism in APCs generates a functional Ag for T cells

Splenocytes from mice administered SMX-NO displayed a concentration-dependent proliferative response on in vitro stimulation with soluble synthetic SMX-NO (10–50 μ M; Fig. 4B). Splenocytes from sensitized mice were additionally stimulated with SMX-NO modified (10–100 μ M; 1 h), irradiated cells from naive syngeneic mice (Fig. 4C). Thus, SMX-NO bound to the surface of APCs stimulates T cells.

Splenocytes from SMX-NO sensitized mice were stimulated to proliferate when incubated with irradiated splenocytes and DCs pulsed with SMX (0.1–2 mM) for 16 h (Fig. 4C). To investigate the relationship between the dynamics of SMX metabolism and the generation of a functional Ag for spleen cells, APCs were pulsed with SMX or SMX-NO for 0.1–16 h in the presence or absence of the enzyme inhibitors methimazole (an inhibitor of peroxidases and flavin-monooxygenases) and 1-aminobenzotriazole (a nonselective suicide inhibitor), at a concentration that blocks CYP2C9 and peroxidase-catalyzed SMX metabolism (both 1 mM). The proliferative response of splenocytes against SMX-pulsed APCs (naive splenocytes and DCs) was time dependent; significant proliferation was detectable at 6–16 h (Fig. 4D, 4E), the time required for SMX metabolism and protein binding (Fig. 3B). In contrast, a 6-min pulse with SMX-NO was sufficient for adduct formation and T cell proliferation.

Importantly, methimazole and 1-aminobenzotriazole pretreatment of splenocytes blocked binding of SMX-derived metabolites (Fig. 4A) and eliminated the concentration-dependent T cell response

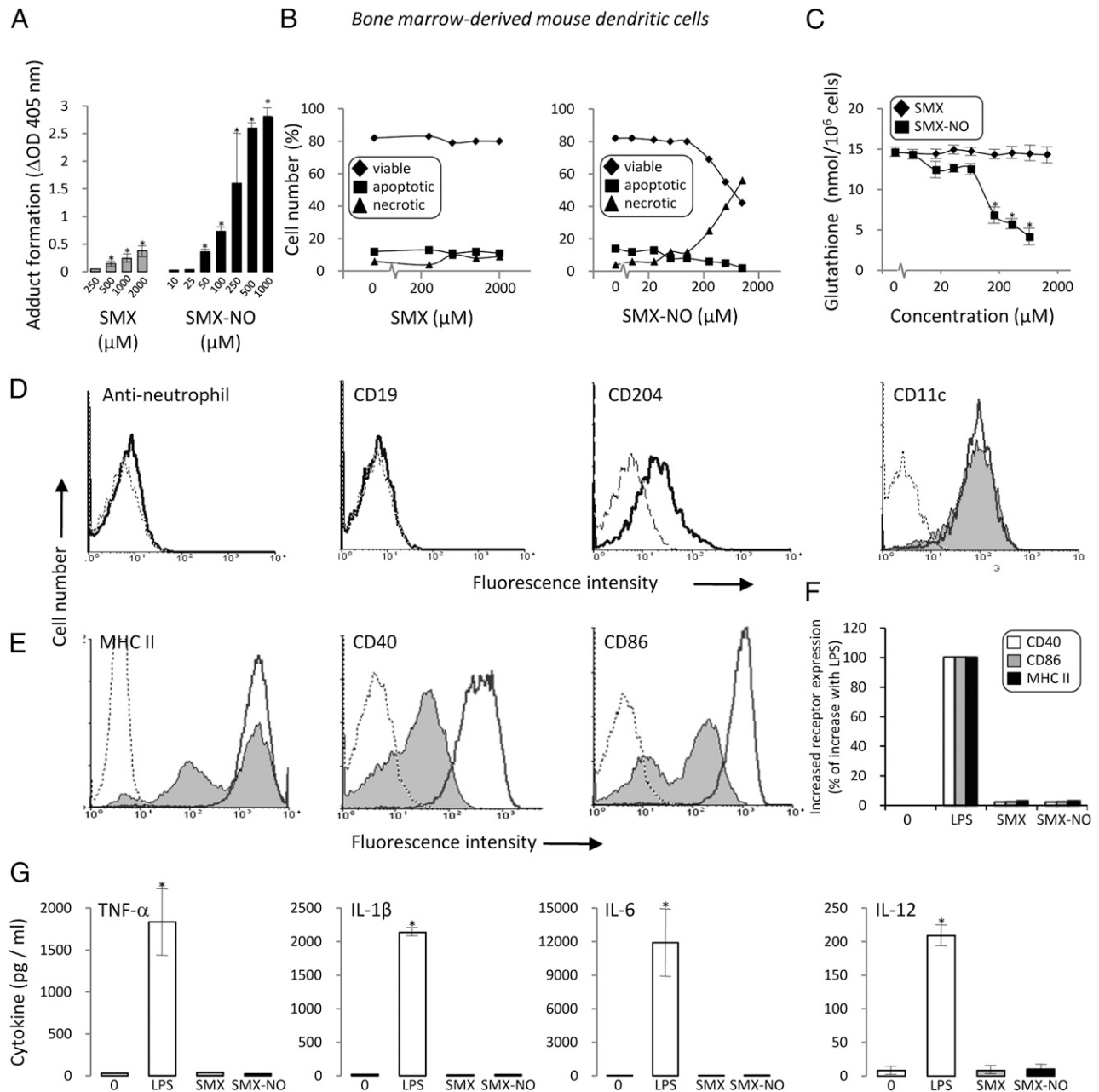


FIGURE 2. SMX metabolism and protein adduct formation in mouse bone marrow-derived DCs is not associated with costimulatory signaling and cytokine secretion. DCs were incubated with SMX (250–2000 μM) or SMX-NO (10–1000 μM) for 16 h. *A*, Concentration-dependent protein adduct formation in SMX- and SMX-NO-treated cells was quantified by ELISA using a specific anti-SMX Ab. *B*, Necrotic and apoptotic cell death was quantified by flow cytometry using Annexin V/PI staining. *C*, Intracellular glutathione levels were quantified using the method of Vandeputte et al. (29). *D*, Cells were stained with CD11c, CD204, CD19, and an anti-neutrophil Ab and subjected to flow-cytometric analysis to confirm the DC phenotype (dotted line: background fluorescence; solid line: Ab stained, LPS treated; shaded graph: Ab stained, untreated). *E*, Upregulation of CD40, CD86, and MHC class II expression in response to LPS (dotted line: background fluorescence; solid line: Ab stained, LPS treated; shaded graph: Ab stained, untreated). *F*, CD40, CD86, and MHC class II expression on DCs cultured with SMX or SMX-NO. Upregulation of cell-surface receptor expression is presented as the percentage of increase with LPS over control. *G*, LPS-, SMX-, and SMX-NO-mediated DC cytokine secretion measured using multiplex analysis. Data are presented as mean ± SD of at least three separate experiments. **p* < 0.05, significantly different from untreated cells. Flow-cytometry traces are representative of three experiments.

against APCs pulsed with SMX for 16 h (Fig. 4F). Adduct formation and T cell responses to the directly reactive SMX-NO were unchanged by enzyme inhibition (Fig. 4A, 4F).

To the best of our knowledge, these data show for the first time that drug metabolites formed locally in APCs bind irreversibly to intracellular protein, and the resultant protein adducts stimulate T cells.

DCs generate SMX-derived Ag and can induce an in vivo immune response against SMX-NO

To test whether protein adducts derived from SMX metabolism in APCs can sensitize mice, DCs incubated with SMX (and SMX-NO) for 16 h were administered to naive recipient mice via a single i.v. injection. SMX treatment of DCs was associated with adduct formation (Fig. 2A) and provoked an immune response

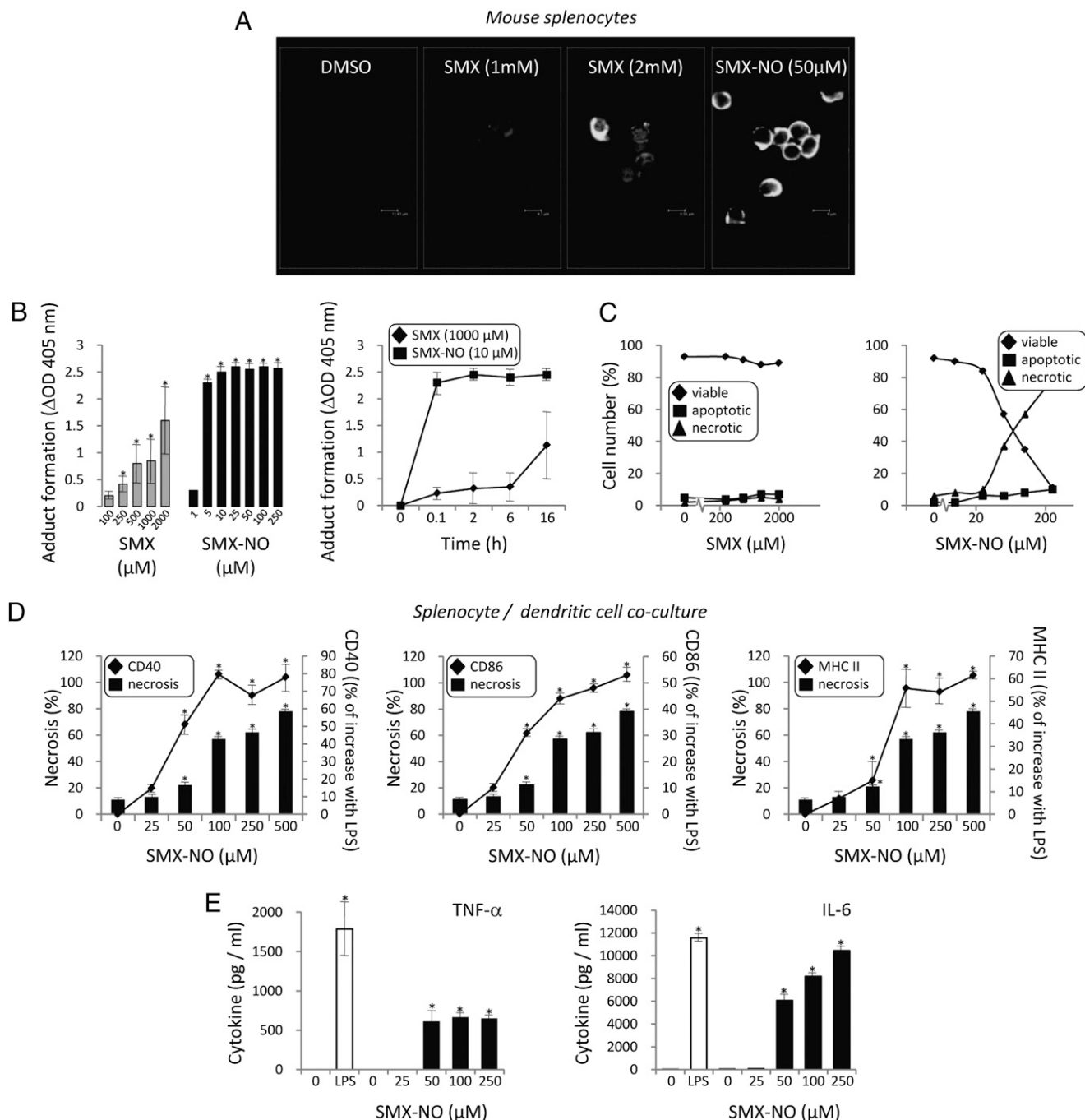


FIGURE 3. SMX metabolite-mediated necrotic cell death provides a potent signal for DC costimulatory signaling and cytokine secretion. SMX- and SMX-NO-treated mouse splenocyte were incubated in a coculture system with DCs prior to the analysis of costimulatory signaling and cytokine secretion. *A*, Confocal microscopy imaging showing the formation of protein adducts when splenocytes were exposed to SMX and the protein-reactive metabolite SMX-NO. Protein adducts were not detected in cells exposed to DMSO alone. Original magnification $\times 63$. *B*, Concentration- and time-dependent protein adduct formation in SMX- and SMX-NO-treated splenocytes quantified by ELISA using a specific anti-SMX Ab. *C*, Necrotic and apoptotic splenocyte death quantified by flow cytometry using Annexin V/PI staining. *D*, CD40, CD86, and MHC class II on DCs cultured with SMX- or SMX-NO-treated splenocytes. Upregulation of cell-surface receptor expression is presented as the percentage of increase with LPS over control. Graphical representations compare SMX-NO-mediated necrotic cell death with upregulation of costimulatory receptor expression. *E*, DC cytokine secretion measured using multiplex analysis following coculture with SMX-NO-modified splenocytes. Data are presented as mean $\pm$ SD of at least three separate experiments. $*p < 0.05$, significantly different from untreated cells.

(Fig. 5). Splenocytes isolated 21 d after the adoptive transfer were stimulated to proliferate ex vivo. The proliferative response was directed against SMX-NO-derived (Fig. 5A) and SMX-derived metabolites (Fig. 5B), but not the parent drug. The absence of soluble SMX after washing and prior to DC transfer was confirmed using ^{14}C -labeled SMX (data not shown).

Human APCs form intracellular SMX-derived protein adducts that stimulate T cells from drug-hypersensitive patients

Using ELISA, SMX-derived protein adducts were detected in human lymphocytes, monocyte-derived DCs, and EBV-transformed B cell lines. Confocal microscopy showed that although SMX-NO first binds to the membrane of human monocyte-derived DCs and

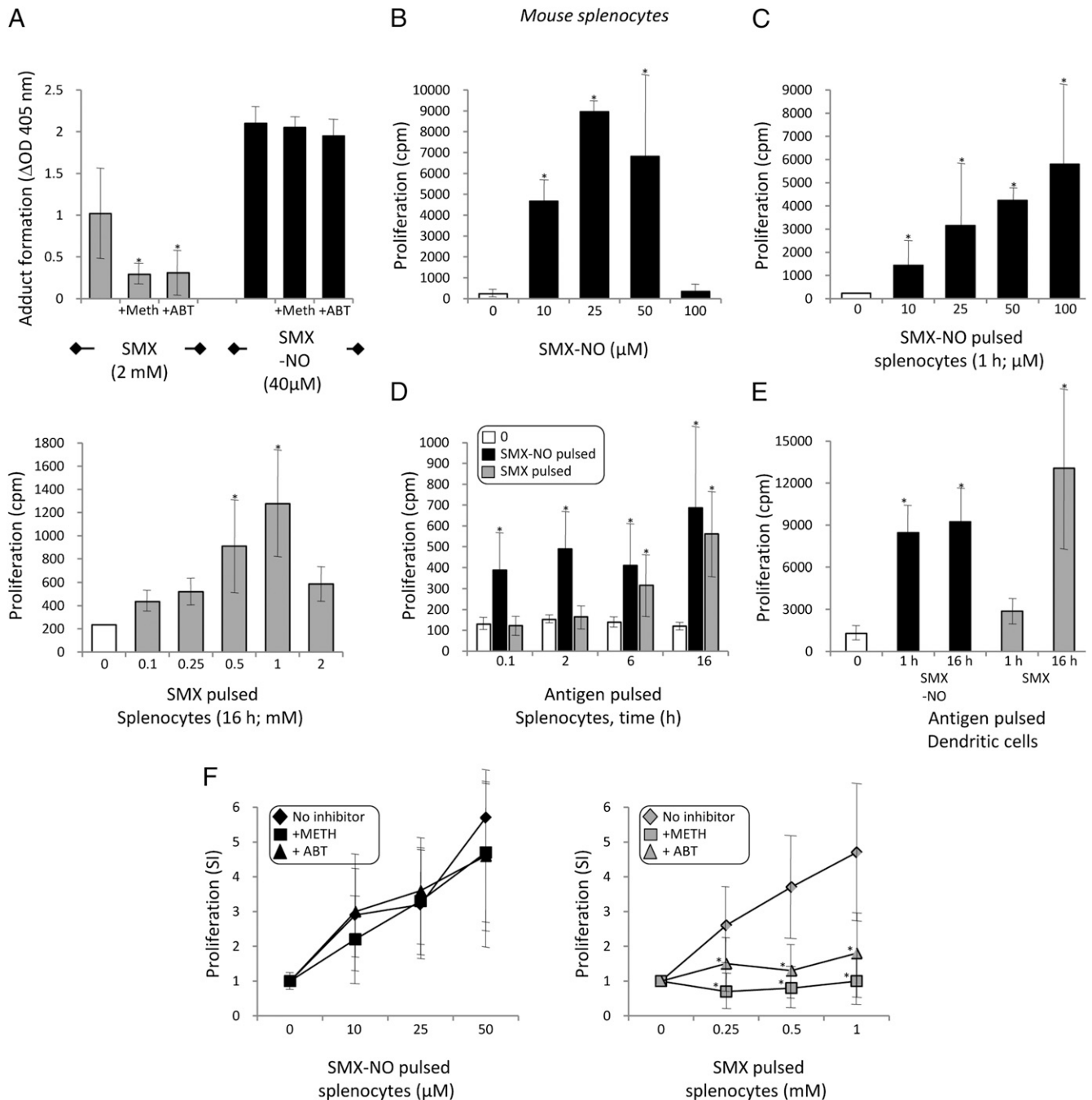


FIGURE 4. SMX-derived protein adduct formation in APCs represents a functional Ag for SMX-NO-sensitized mice. *A*, Inhibition of protein adduct formation in SMX- and SMX-NO-treated splenocytes quantified by ELISA using a specific anti-SMX Ab. The inhibitors 1-aminobenzotriazole (ABT) and methimazole (Meth) were incubated with cells for 1 h before the addition of SMX or SMX-NO. Dose-dependent proliferative response of splenocytes from SMX-NO-sensitized mice cultured with soluble SMX-NO (*B*) or syngeneic naive irradiated splenocytes pulsed with SMX-NO (1 h) or SMX (16 h) (*C*). After 72 h, proliferation was measured by incorporation of [^3H]thymidine over an additional 16 h. Time-dependent proliferative response of splenocytes from SMX-NO-sensitized mice cultured with syngeneic naive irradiated splenocytes pulsed with SMX-NO or SMX (0.1–16 h) (*D*) and bone marrow-derived DCs pulsed with SMX-NO or SMX (1 and 16 h) (*E*). In these pulsing experiments, sensitized splenocytes were not exposed to soluble drug. After 72 h, proliferation was measured by incorporation of [^3H]thymidine over an additional 16 h. *F*, 1-Aminobenzotriazole (ABT) and methimazole (Meth) inhibited the concentration-dependent proliferation of sensitized splenocytes cultured with naive irradiated splenocytes pulsed with SMX (250–1000 μM ; 16 h). Enzyme inhibition did not block the proliferation carried out in triplicate. Statistical analysis compares the proliferative response of sensitized splenocytes after the addition of drug Ag and solvent alone. * $p < 0.05$.

is then incorporated intracellularly, SMX forms intracellular protein adducts (Fig. 6A). Adduct formation with SMX was concentration and time dependent (Fig. 6B). Protein adducts were detected instantaneously with SMX-NO (data not shown).

EBV-transformed B cells from SMX-hypersensitive patients and volunteers displayed no discernable difference between the quantity of protein adducts formed when cultured with SMX for 16 h (Fig. 6B). Adducts were subsequently detectable for 3 d (Fig.

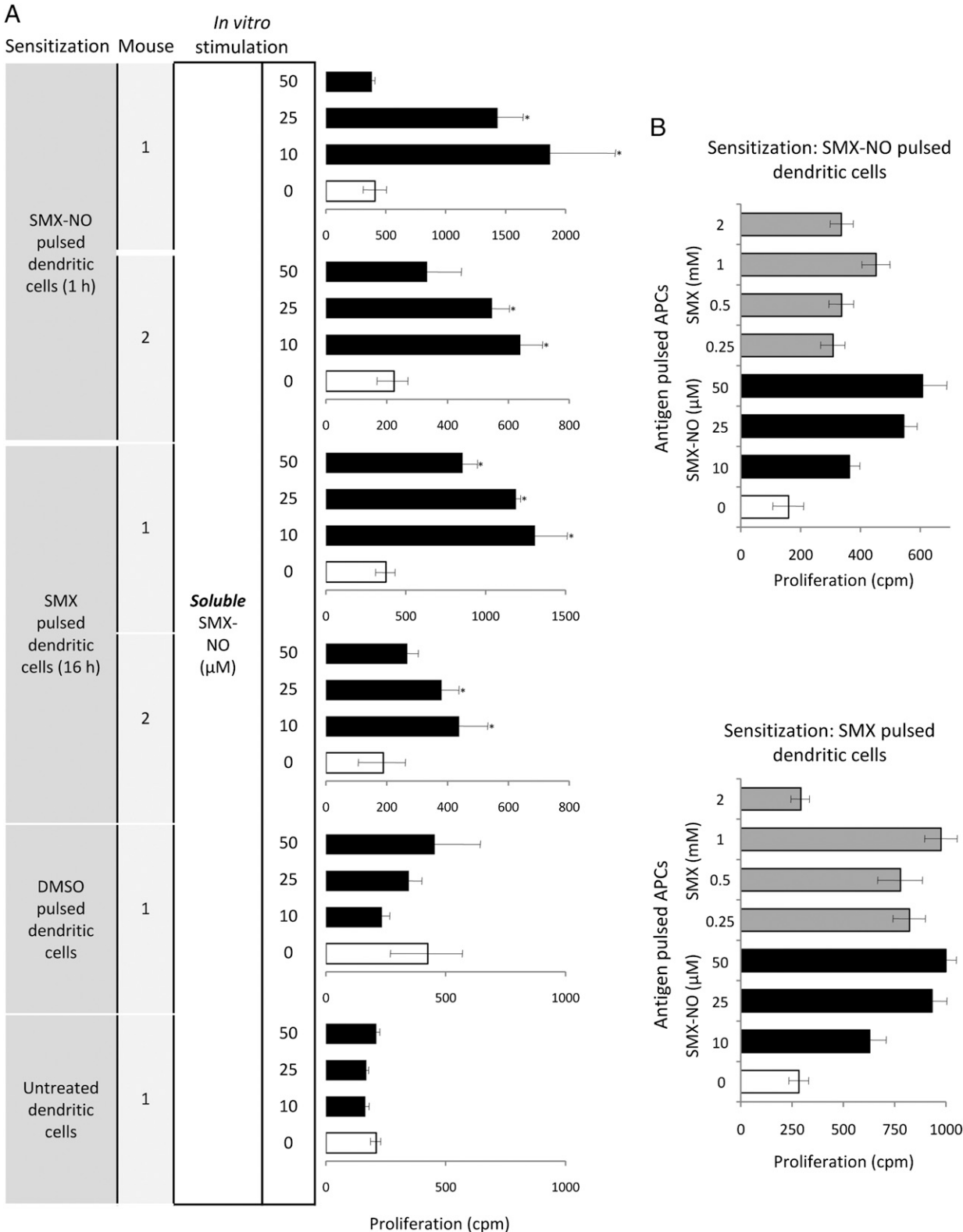
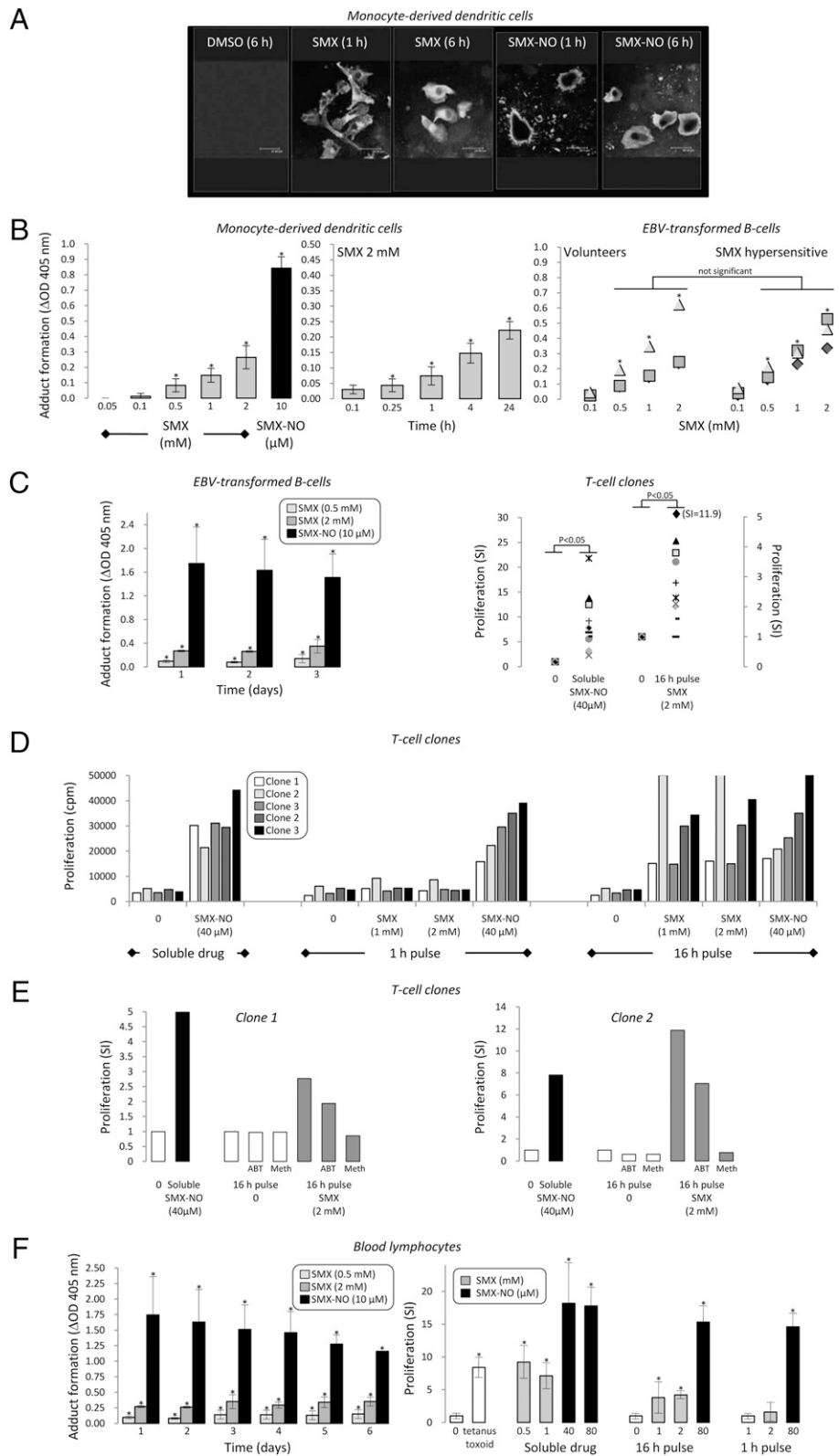


FIGURE 5. Stimulation of drug (metabolite)-specific T cells following adoptive transfer of SMX- and SMX-NO-treated DCs. SMX- and SMX-NO-derived DC protein adducts stimulated a cellular immune response in naive recipient mice. However, the response of splenocytes from mice immunized with SMX-treated DCs was directed against SMX-NO-derived (A) and SMX-derived (B) metabolites, not the parent drug. Bone marrow-derived DCs were incubated for 16 h in culture medium with SMX or SMX-NO. For immunization, DCs were washed extensively and administered i.v. in the lateral tail vein. Control mice received unmodified DCs or DCs treated with DMSO. After 21 d, mice were sacrificed. Splenocytes were stimulated *in vitro* with soluble Ag (A) and Ag-pulsed splenocytes (B). Proliferation was quantified by the addition of [^{3} H]thymidine. Data are presented as mean $\pm$ SD, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitized splenocytes after the addition of drug and solvent alone. * $p < 0.05$.

FIGURE 6. SMX-derived protein adduct formation in APCs represents a functional Ag for lymphocytes and T cell clones from hypersensitive human patients. **A**, Confocal microscopy images showing formation of cell membrane adducts when monocyte-derived DCs are incubated with SMX-NO and the parent drug. SMX-NO-derived adducts become internalized and are detected inside cells after 6 h. In contrast, intracellular cytoplasmic adducts are detected with DCs exposed to the parent drug. Protein adducts were not detected with DCs exposed to DMSO. Original magnification $\times 63$. **B**, Concentration- and time-dependent protein adduct formation in SMX- and SMX-NO-treated DCs and EBV-transformed B cells from hypersensitive patients and healthy volunteers, quantified by ELISA using a specific anti-SMX Ab. **C**, The quantity of SMX- and SMX-NO-derived protein adducts in EBV-transformed B cells from hypersensitive patients remains constant for 3 d, the duration of a proliferation assay with T cell clones. EBV-transformed B cells pulsed with SMX for 16 h stimulated 9 of 11 SMX-NO-responsive T cell clones. **D**, Stimulation of a second independent panel of T cell clones with EBV-transformed B cells pulsed with SMX-NO for 16 h. **E**, Enzyme inhibition with 1-aminobenzotriazole (ABT) and methimazole (Meth) diminished the proliferative response of drug-specific T cell clones to SMX-pulsed EBV-transformed B cells. **F**, SMX-pulsed lymphocytes generate a functional Ag that stimulates the proliferation of lymphocytes from hypersensitive patients. The quantity of SMX- and SMX-NO-derived protein adducts in lymphocytes from hypersensitive patients remains constant for 6 d, the duration of the lymphocyte-transformation test. Lymphocytes from hypersensitive patients proliferated in the presence of soluble SMX and SMX-NO and irradiated lymphocytes pulsed overnight with SMX-NO and the parent drug. In contrast, lymphocytes pulsed with SMX 1 h and used as a source of Ag did not stimulate a proliferative response.



6C), the duration of a proliferation assay. EBV-transformed B cells pulsed with SMX (1 and 2 mM) overnight stimulated 9 of 11 SMX-NO-specific T cell clones, with a stimulation index ranging from 2.0–11.9 (Fig. 6C).

To the best of our knowledge, these data provide the first evidence that T cells from allergic patients are stimulated with SMX-derived protein conjugates generated as a consequence of intracellular metabolism by APCs. To validate these results, a second

panel of clones was generated; 10 Ag-specific T cell clones were stimulated to proliferate with APCs pulsed with SMX for 16 h but not 1 h (Fig. 6D).

Adduct formation and the T cell response directed against SMX-derived protein adducts were decreased by preincubating EBV-transformed B cells with 1-aminobenzotriazole or methimazole (adduct formation: SMX 2 mM, OD $0.84 \pm 0.24\%$; + 1-aminobenzotriazole, OD $0.29 \pm 0.14\%$ [percentage inhibition 65.0%,

$p < 0.05$]; + methimazole, OD $0.24 \pm 0.13\%$ [percentage inhibition 71.4%, $p < 0.05$]; SMX-NO 50 μM , OD $2.65 \pm 0.27\%$; + 1-aminobenzotriazole, OD $2.64 \pm 0.21\%$ [percentage inhibition 0.4%, NS]; + methimazole, OD 2.60 ± 0.25 [percentage inhibition 1.9%, NS] (Fig. 6E). The low numbers of clones that responded exclusively to the parent drug SMX were not stimulated with SMX-pulsed EBV-transformed B cells.

Adduct formation in blood lymphocytes incubated with SMX was also time dependent for 16 h, whereas subsequently, the quantity of adducts formed remained relatively stable for 6 d, the duration of the lymphocyte-transformation test. Lymphocytes from hypersensitive patients were found to proliferate in the presence of soluble SMX and SMX-NO and SMX pulsed- (1 and 2 mM; 16 h) and irradiated autologous lymphocytes (Fig. 6F). In contrast, APCs pulsed with SMX for 1 h did not stimulate a proliferative response.

Lymphocytes from nonhypersensitive patients ($n = 3$) were not stimulated with SMX, synthetic SMX-NO, or SMX-derived protein conjugates (Supplemental Fig. 1).

Cytokine secretion from SMX- and SMX metabolite-stimulated T cell clones

When culture supernatants from incubations containing T cells, APCs, and SMX or SMX-NO were analyzed for cytokine expression, high levels of IL-5, IL-13, IFN- γ , and TNF- α were detected (Table I). Ag stimulation of certain clones was also associated with secretion of lower levels of IL-4, IL-6, IL-8, and IL-17.

Discussion

In this study, we embarked upon a series of experiments to address the hypothesis that APCs metabolize drugs in sufficient quantities to form protein adducts and haptenated Ags that stimulate DCs and T cells. The immune response may arise directly through drug-protein adduction of viable cells or indirectly through inducing necrotic bystander cell death and the subsequent release of endogenous Ag and possibly "danger signals" (35–39).

The oxidative metabolite of SMX (SMX-NO) stimulates skin- and blood-derived T cells from all SMX-hypersensitive patients to proliferate, secrete cytokines, and kill target cells (4, 11, 13, 14). These data indicate that T cells play a causal role in the development of clinical signs. However, the way in which drugs stimulate T cells remains an area of intense debate and is complicated by the fact that until now, it has been impossible to generate and

characterize physiologically relevant intracellular drug-protein adducts in biological test systems. In *in vitro* assays, synthetic drug metabolites (e.g., SMX-NO) generate cell-surface adducts that are unlikely to be formed in patients where metabolism occurs inside the cell. In this study, immunochemical methods to quantify SMX metabolite protein binding and enzyme inhibitors were used to relate drug metabolism in APCs to T cell activation. To the best of our knowledge, we show for the first time that APC drug metabolism and irreversible binding of the derived metabolites to intracellular protein result in the ability to stimulate T cell responses. We believe that these data are the first to show a prohapten mechanism of T cell activation that is active in hypersensitive patients and animal models of immunogenicity.

The detection of T cell responses against protein adducts formed as a consequence of APC drug metabolism have important ramifications for future drug development. The pharmaceutical industry tests molecules for their propensity to form chemically reactive metabolites in the liver and attempt to relate this to risk/hazard identification. Our data suggest that this approach may not be relevant for predicting/assessing the risk for drug hypersensitivity.

The distribution of SMX-NO in *in vitro* culture is complex. Auto-oxidation generates SMX-NO, self-conjugation generates azo and azoxy dimers, and reduction through enzymatic reactions or glutathione-dependent processes liberates a hydroxylamine and the parent drug (Fig. 1) (26, 34, 40–43). Although these reactions result in rapid (within 5 min) and complete degradation of SMX-NO, multiple meta-stable protein adducts that become internalized via a caveole-dependent mechanism (16, 44, 45) are readily detectable. In contrast, SMX is relatively stable, with most of the parent drug being recovered unchanged after 6 d in culture. Nevertheless, human monocyte-derived DCs (16) and DC-like cell lines (46) were shown to metabolize SMX to an unstable intermediate that forms intracellular protein adducts. Data presented in this article show the presence of T cell stimulatory intracellular protein adducts in viable mouse and human APCs (splenocytes, lymphocytes, EBV-transformed B cell lines, and DCs) incubated with the parent drug SMX (Figs. 2, 3). Importantly, metabolism and adduct formation was time and dose dependent and was not detectable immediately after SMX exposure. Consistent with these findings, T cell responses were only obtained with APCs pulsed with SMX for 6–16 h.

Table I. Cytokine secretion from SMX and SMX metabolite-stimulated T cell clones from hypersensitive patients

Cytokine (pg/ml) ^a										
ID	CD	IL-4	IL-5	IL-6	IL-8	IL-13	IL-17	IFN-γ	TNF-α	GM-CSF
SMX-stimulated clones										
1	8	–	–	–	–	426	32	544	358	271
2	4	137	28,190	–	25	16,157	62	33	149	273
3	8	136	373	13	59	18,204	71	1,202	1,967	991
4	4	–	–	56	1,408	7,375	57	8,004	3,346	1,676
5	8	–	14	–	–	726	36	108	34	108
SMX metabolite-stimulated clones										
1	8	55	1,791	323	56	>20,000	77	817	2,058	827
2	4	–	–	–	–	1,012	37	441	953	441
3	4	–	–	11	56	978	35	326	855	326
4	4	16	12	186	295	7,742	56	947	2,499	947

Data represent mean of duplicate cultures with cytokine levels (<10 pg/ml: IL-4, IL-5, IL-6, IL-8, and TNF- α ; <20 pg/ml: IFN- γ ; <50 pg/ml: IL-13, IL-17, and GM-CSF) in drug-free wells subtracted.

^aNo detectable drug-specific secretion of IL-1 β , IL-7, IL-10, or MCP-1.

–, not detectable.

Drug-protein adducts formed in human APCs exposed to the parent drug SMX were detectable over several days (Fig. 6). These data have implications regarding the use of drugs in biological tests to diagnose drug hypersensitivity. To fully define the sensitivity and specificity of the test, future validation studies must incorporate steps to relate the nature of the antigenic determinant(s) formed in situ to the biological readout. The development of sensitive mass spectrometers to characterize drug metabolites and protein adducts makes this approach feasible.

We previously reported that human monocyte-derived DCs expressed consistently high levels of myeloperoxidase, but only low levels of cytochrome P450 enzymes, leading us to propose that a peroxidase is responsible for SMX metabolism in DCs (16). More recently, we showed that pathogenic conditions that potentially activate peroxidase enzymes enhance the formation of SMX-derived protein adducts in immune cells (22). To substantiate a causal relationship between peroxidase-mediated SMX metabolism and T cell immunogenicity, enzyme inhibitor studies were performed with 1-aminobenzotriazole, a nonselective suicide inhibitor, and methimazole, an inhibitor of peroxidases and flavin-monooxygenases. Both inhibitors blocked SMX-derived adduct formation and the T cell response in mouse and human systems.

Detection of T cells with reactivity to functional intracellular Ags, arising as a consequence of metabolism and extracellular Ags generated through direct conjugation of synthetic SMX-NO to cell-surface proteins, implies that the T cell response is directed against multiple peptide sequences. The one common component is the necessity for the drug metabolite to irreversibly modify specific cysteine residues on protein (45). SMX-derived adduct formation by splenocytes was 3-fold higher compared with DCs (Figs. 2A, 3B). Despite the lower quantity of adduct formed, DCs stimulated a 10-fold stronger T cell response (Fig. 4C, 4E), which likely relates to their superior capacity to present endogenous Ags.

Previous studies using lymphocytes from SMX-hypersensitive patients and animal models of SMX immunogenicity demonstrated that Ag-stimulated T cells secrete high levels of the Th2 cytokine IL-5 and the Th1 cytokine IFN- γ (41, 47, 48). Data presented in the current study using multiplex methods and cloned T cells confirm these initial reports. Ag-stimulated T cell clones also secreted IL-4, IL-6, IL-8, IL-13, IL-17, and TNF- α . The profile of cytokine secretion did not differ when SMX, synthetic SMX-NO, or SMX-derived metabolites were used.

The role of metabolism in the development of a primary immune response is ill-defined, because an experimental approach to relate metabolism to immune activation is lacking. Ag-specific T cells from hypersensitive patients cannot be used to define antigenic determinants that prime T cells because it is impossible to differentiate between cross-reactive drug Ags. Thus, to evaluate whether protein adducts formed intracellularly in APCs drive T cell responses, a mouse adoptive-transfer system was developed. SMX and SMX-NO were cultured with bone marrow-derived DCs and administered to syngeneic mice following quantification of adduct formation. Splenocytes isolated from mice that received SMX-treated DCs were stimulated in vitro with SMX-NO and SMX-derived intracellular metabolites, but not the parent drug (Fig. 5). These data provide unequivocal evidence that protein adducts generated following SMX metabolism by DCs represent functional Ags that drive drug-specific immunity.

The Ag-presenting capacity of DCs is enhanced in response to TLR signaling (e.g., LPS, CpG oligodeoxynucleotides) (49, 50) and molecules released from necrotic (e.g., uric acid high-mobility group box 1 protein) (36, 51) or oxidatively stressed cells (52). DCs respond to these signals by secreting polarizing cytokines and increased expression of costimulatory receptors and MHC class II.

Previously, we showed that exposure of human monocyte-derived DCs to nontoxic concentrations of SMX selectively increased CD40 expression. Furthermore, CD40 expression was 5-fold higher on drug-treated cells from a hypersensitive patient compared with volunteers (16). Our experiments using mouse bone marrow-derived DCs did not reproduce these findings, indicating that drug-specific CD40 signaling might be restricted to human cells. Drug-protein adducts were detected on viable human and mouse DCs incubated with SMX-NO (50–100 μ M; Fig. 2A). In mouse cells, increasing the concentration of SMX-NO (250–1000 μ M) was associated with depletion of intracellular glutathione and increased necrotic cell death (Fig. 2). SMX-derived adducts were detected in DCs; however, glutathione depletion and necrotic cell death were not seen with SMX. Although no increase in costimulatory receptor expression or MHC class II or cytokine secretion was detected following the direct application of SMX or SMX-NO, in a coculture system consisting of SMX/SMX-NO-treated splenocytes as bystander cells and DCs, there was a strong association between SMX metabolite-mediated necrotic cell death and DC activation. Splenocytes incubated with SMX-NO (at concentrations ≥ 50 μ M) stimulated an increase in DC CD40, CD86, and MHC class II expression and cytokine secretion (Fig. 3D, 3E). Previously, we reported that SMX metabolite-derived adduct formation on splenocytes must surpass a critical threshold to induce necrosis (34). The cells that became intensely haptenated were the same as those that underwent necrotic cell death. Collectively, these data imply that high drug concentrations, which are observed clinically with many drugs associated with a high incidence of hypersensitivity reactions, may be needed to drive T cell responses to drugs.

The quantity of adduct formation in SMX-treated splenocytes presumably did not reach the threshold to induce necrosis and, therefore, DC activation. However, it is possible that this may occur in patients in whom environmental factors (bacterial endotoxins, flu viral proteins and cytokines) alter the oxidation state of cysteine residues on protein and increase SMX-derived protein adduct formation (22). These observations might explain the increased in vitro drug-specific cell death observed in hypersensitive patients (53–56), because patients administered SMX are carriers of such factors.

Previous studies exploring the importance of drug metabolism in the pathogenesis of hypersensitivity reactions focused on genetic polymorphisms and/or metabolism in liver (57, 58) and target organs, such as the skin (59, 60), and produced largely negative results. Using cells from animal models and hypersensitive human patients, the current study shows that metabolism in APCs generates functional Ag(s) for T cells. Given that environmental factors enhance metabolism-derived adduct formation, it is possible that, at the time of drug exposure, the levels of adduct formed in various cell types exceed the threshold needed to induce cell death and the activation of DCs, providing multiple costimulatory signals to initiate and drive the pathogenic immune response, resulting in hypersensitivity.

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Disclosures

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Supplemental figure: SMX, SMX-NO and SMX-derived protein adducts in antigen presenting cells do not stimulate lymphocytes from non-hypersensitive human patients.

Lymphocytes from 3 non-hypersensitive patients were incubated with soluble SMX, SMX-NO and drug-pulsed antigen presenting cells. Proliferation was measured after 6 days by incorporation of [³H] thymidine.

