The Development of In Vitro Culture Methods to Characterize Primary T-Cell Responses to Drugs

Lee Faulkner,* Klara Martinsson,*† Anahi Santoyo-Castelazo,* Karin Cederbrant,† Ina Schuppe-Koistinen,† Helen Powell,‡ Jonathan Tugwood,‡ Dean J. Naisbitt,*‡ and B. Kevin Park*†

*Centre for Drug Safety Science, Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, University of Liverpool, Liverpool L69 3GE, U.K.; †Molecular Toxicology, Global Safety Assessment, AstraZeneca R&D, S-151 85 Södertälje, Sweden; and ‡Molecular Toxicology, Global Safety Assessment, AstraZeneca R&D, Alderley Park, Macclesfield SK10 4TG, U.K.

1Present address: Molecular Toxicology, Global Safety Assessment, AstraZeneca R&D, S-151 85 Södertälje, Sweden.
2To whom correspondence should be addressed. Fax: +44-151-794-4450. E-mail: dean.naisbitt@liverpool.ac.uk.

Received October 14, 2011; accepted January 31, 2012

Adverse drug reactions represent a major stumbling block to drug development and those with an immune etiology are the most difficult to predict. We have developed an in vitro T-cell priming culture method using peripheral blood from healthy volunteers to assess the allergenic potential of drugs. The drug metabolite nitroso sulfamethoxazole (SMX-NO) was used as a model drug allergen to establish optimum assay conditions. Naive T cells were cocultured with monocyte-derived dendritic cells at a ratio of 25:1 in the presence of the drug for a period of 8 days, to expand the number of drug-responsive T cells. The T cells were then incubated with fresh dendritic cells, and drug and their antigen responsiveness analyzed using readouts for proliferation, cytokine secretion, and cell phenotype. All five volunteers showed dose-dependent proliferation as measured by 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester content and by 3H-thymidine uptake. CD4 T cells that had divided in the presence of SMX-NO had changed from a naive phenotype (CD45RA+) to a memory phenotype (CD45RO+). These memory T cells expressed the chemokine receptors CCR2, CCR4, and CXCR3 suggesting a mixture of TH1 and TH2 cells in the responding population, with a propensity for homing to the skin. Drug stimulation was also associated with the secretion of a mixture of TH1 cytokines (interferon γ) and TH2 cytokines (interleukin [IL]-5 and IL-13) as detected by ELISpot. We are currently developing this approach to investigate the allergenic potential of other drugs, including those where an association is suspected. We have developed an in vitro model drug allergen to establish optimum assay conditions. Castrejon et al., 2001; Castrejon et al., 2010; Elsheikh et al., 2003; Nassif et al., 2004; Schnyder et al., 2000). When not bound to proteins, most drugs/metabolites are too small to act as conventional antigens. However, in some cases the drug may bind directly to HLA molecules and/or TCRs to initiate a T-cell response. This binding interaction may involve the formation of covalent or noncovalent bonds between the drug and the two immune receptors (Gerber and Pichler, 2006).

Individual susceptibility is difficult to explain based on the chemical features of the drug. All patients are exposed to the same chemical entity, and differences in drug metabolism and enzyme expression are not thought to represent major susceptibility factors (Alfirevic et al., 2003, 2009). It is more likely that individual susceptibility is based on the interaction between the drug/metabolite and the immune receptors. Notably, recent genome-wide association studies have

Immunological drug reactions, which only become apparent when a drug has been approved for widespread use, represent a major obstacle in the drug development process. The reason why they represent such a problem is that they are difficult to predict and this is because the propensity for an individual to develop a reaction is a function of both the chemistry of the drug and the biology of the patient. T cells are thought to be involved in the pathogenesis of most forms of immunological drug reaction (Lopez et al., 2007; Pichler et al., 2011; Roujeau and Stern, 1994; Rozieres et al., 2009). During an immune response, antigens are processed and presented on the human leucocyte antigen (HLA) molecules of antigen presenting cells. These are then detected by the T-cell receptor (TCR) on the T cell. The specificity of the response is determined by the structural specificity of the antigen, the TCR, and the HLA molecule. Such structural specificity can be unique to an individual. Drugs (and/or their metabolites) may be presented to T cells after they become bound to carrier proteins and processed. The resulting peptide-drug conjugates have been shown to act as a pharmacophore, stimulating T cells from patients with different forms of immunological reaction (Burkhart et al., 2001; Castrejon et al., 2010; Elsheikh et al., 2010; Farrell et al., 2003; Nassif et al., 2004; Schnyder et al., 2000). When not bound to proteins, most drugs/metabolites are too small to act as conventional antigens. However, in some cases the drug may bind directly to HLA molecules and/or TCRs to initiate a T-cell response. This binding interaction may involve the formation of covalent or noncovalent bonds between the drug and the two immune receptors (Gerber and Pichler, 2006).

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identified HLA alleles as factors that relate susceptibility to specific forms of immunological drug reaction (Chung et al., 2004; Daly et al., 2009; Mallal et al., 2002). For example, the drug abacavir, is presented to T cells exclusively in the context of HLA-B*5701 (Chessman et al., 2008), indicating that the genetic association is directly involved in the disease pathogenesis.

Considerable effort has been devoted to developing assays that diagnose immunological drug reactions in drug hypersensitive patients. The lymphocyte transformation test for measurement of T-cell proliferation and ELISpot for cytokine release and cytotoxicity markers such as granzyme B display a high degree of sensitivity and specificity for certain patient groups (Porebski et al., 2011). Importantly, each of these assays measure the responses of drug-specific memory T cells and are not suitable for predicting the allergenic potential of a drug in nonhypersensitive individuals.

Thus, the objective of the current study was to develop a reproducible assay to detect the drug-specific stimulation of naive T cells in nonhypersensitive individuals. Generation of naive T-cell responses requires professional antigen presenting cells, so monocyte-derived dendritic cells (Mo-DC) were used. We focused our investigations on the model drug metabolite nitroso sulfamethoxazole (SMX-NO) for several reasons. First, SMX-NO has been shown to stimulate T cells and T-cell clones from patients with different forms of immunological reaction (Castrejon et al., 2010); second, administration of SMX-NO to experimental animals is associated with the development of a reproducible primary T-cell response (Naisbitt et al., 2001; Naisbitt et al., 2002); and finally, the synthetic metabolite is available for functional studies.

**MATERIALS AND METHODS**

**Tissue culture reagents and antibodies.** All cells were cultured in Roswell Park Memorial Institute 1640 medium, containing 10% AB serum, 100mM L-glutamine, 100 μg/ml penicillin, 100 μl streptomycin, 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 25 μg/ml transferin, except for assays using CD3 naive cells where penicillin and streptomycin were omitted. The AB serum was purchased from Innovative Research (Novi, MI), and all other reagents were purchased from Sigma-Aldrich (Poole, U.K.). Pan T isolation kit II, the Naive CD4 isolation kit, and CD4, CD14, CD25, and CD45RO antibodies were purchased from Miltenyi Biotec Ltd. (Bisley, U.K.). Antibodies used for flow cytometry staining purchased from BD Biosciences (Oxford, U.K.) were CD1a-fluorescein isothiocyanate (FITC) (clone HI 149), CD3-allophycocyanin (APC) (clone UCHT1), CD4-APC (clone RPA T4), CD4-phycocerythrin (PE) (clone RPA T4), CD8-FITC (clone HT8a), CD8-PE (clone HT8a), CD14-FITC (clone M5E2), CD45RA-FITC (clone HI 100), CD45RO-PerCP-Cy5.5 (clone UCHL1), CD86-FITC (clone GL1), CCR4-PE (clone 1G1), and HLA-class II-FITC (clone T239); from Serotec (Kidlington, U.K.) were CD40-FITC (clone LOB 7/6); from Miltenyi Biotec Ltd. were CD25-PE (clone 4E3); and from R&D Systems (Abingdon, U.K.) were CD83-FITC (clone HB15e), CCR2-PE (clone 48607), CCR5-FITC (clone C9C5), and CXCR3-APC (clone 49081). Tissue culture grade phytomenadiontin (PHA) and dimethyl sulfoxide (DMSO) and Hank’s balanced salt solution (HBSS) were purchased from Sigma-Aldrich. 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from eBioscience Ltd. (Hatfield, U.K.).

**Cell isolations.** Blood was taken from five healthy volunteers after informed consent. The protocols for this study were approved by the Local Research Ethics Committee. Peripheral blood mononuclear cells were isolated from fresh blood collected in lithium heparinized Vacutette tubes (Greiner Bio-One Ltd., Stonehouse, U.K.) using Lymphoprep density gradient separation media (Axis Shield, Dundee, U.K.). CD14+ cells were isolated by positive selection using CD14 microbeads, and CD4 T cells were isolated by positive selection using CD4 microbeads. CD4 naive T cells were isolated by negative selection using the Pan T isolation kit II followed by removal of CD25 and CD45RO positive cells by positive selection, leaving untouched CD3 naive cells. All bead isolations were carried out according to the manufacturer’s instructions (Miltenyi Biotec Ltd.). The phenotype of the isolated cells was determined by flow cytometry using CD14-FITC, CD3-APC, CD45RO-PerCP-Cy5.5, CD45RA-FITC, CD4-PE, CD8-FITC, and CD25-PE antibodies. Purity of the isolated T cells exceeded 97%. Naive CD3 cells contained 0.44% CD25 positive cells after isolation. Cells were resuspended in medium at 10–20 × 10^6 cells/ml, and an equal volume of 20% DMSO, 80% AB serum was added dropwise on ice. One-milliliter vials were left overnight in Mr Frosty tubs at −80°C and then transferred to −150°C for storage.

**Generation of Mo-DC.** Isolated CD14+ monocytes were differentiated for 7–8 days in medium containing 800 μl/ml granulocytemacrophage colony-stimulating factor (GM-CSF) and 800 μl/ml interleukin (IL)-4 (PeproTech EC Ltd., London, U.K.). Fresh or frozen cells were resuspended at 1–2 × 10^7 cells/ml and aliquoted at 3 ml per well in six-well plates. Cells were fed every 2 days with 5 ml per well fresh medium containing 800 μl/ml GM-CSF and 800 μl/ml IL-4. The phenotype of immature Mo-DC was determined by flow cytometry. Cells were CD14^hi, CD14^lo, CD80^lo, CD83^hi and CD40^lo, CD69^lo (data not shown).

**T-cell and Mo-DC coculture.** Immature Mo-DC were harvested 7 days after culture and aliquoted at 8 × 10^5 cells per well in 200 μl in 24-well plates. T cells were thawed quickly at 37°C, and medium added dropwise to 10× the initial volume. After washing, 2 × 10^5 T cells per well were added in 1 ml of medium. A stock solution of 50mM SMX-NO (Dalton Pharma Services, Toronto, Canada) was dissolved in DMSO and diluted to 200μM in medium. Five hundred microliter per well was added resulting in a final concentration of 50μM SMX-NO. Cultures were incubated for 7–8 days at 37°C in 5% CO₂.

**T-cell assays.** After the coculture period, the cultures were restimulated, and proliferation, cytokine release, and cell phenotype were assessed. All the assays were set up using the same cell concentrations and culture volumes. T-cell cultures were harvested and resuspended at 1 × 10^6 cells/ml and aliquoted at 100 μl per well in 96 U-bottomed plates or ELISpot plates. Immature Mo-DC were harvested 7–8 days after culture and resuspended at 8 × 10^5 cells/ml and aliquoted at 50 μl per well. PHA at 20 μg/ml and drugs at various concentrations was added at 50 μl per well. Abacavir (a kind gift from GlaxoSmithKline Ltd., Uxbridge, U.K.) and fluocoxacinil (sodium salt for injection; CP Pharmaceuticals Ltd., Wrexham, U.K.) were dissolved in DMSO and diluted to 200μM in medium. Five hundred microliter per well was added resulting in a final concentration of 50μM SMX-NO. Cultures were incubated for 7–8 days at 37°C in 5% CO₂.
CFSE labeling. CFSE labeling of T cells was carried out according to Quah et al. (2007). Briefly, 1–5 × 10^6 T cells were resuspended in 1 ml of 5% AB serum in HBSS. With the tube resting at an angle, a drop of PBS was placed near the neck of the tube and 0.5 μl of 5μM CFSE (eBioscience) mixed into this drop. The lid was put on and the tube rapidly inverted several times. After 5 min incubation at room temperature, in the dark, the cells were washed twice in 5% AB serum in HBSS and counted.

Cytokine analysis. Cytokine release was measured by ELISpot. The spot development for interferon (IFN)-γ, IL-5, and IL-13 ELISpots was carried out after 48 h of culture, according to the manufacturer’s instructions (Mabtech, Nacka Strand, Sweden).

Flow cytometry. Cells were stained with antibodies for 20 min on ice and washed once with 10% fetal calf serum in HBSS. Cells were acquired using a FACSCanto II (BD Biosciences) and data analyzed by Cyflogic. For CFSE and chemokine receptor analysis, a minimum of 50,000 lymphocytes were acquired using forward scatter/side scatter (FSC/SSC) characteristics.

Statistics. Student’s t-test (SigmaPlot 11.0) was used to analyze the proliferation and ELISpot data.

RESULTS

Measurement of Proliferation by 3H-Thymidine

Naive CD4, total CD4, and naive CD3 cells were cocultured with Mo-DC at a ratio of 25:1, in the presence of 50μM SMX-NO for 8 days. These cells were harvested and then restimulated with fresh Mo-DC, and three different drugs for 3 days and the proliferative responses assessed by 3H-thymidine. A dose-dependent response to SMX-NO was detected when CD4, CD4 naive, and CD3 naive T cells were used in the assay (Fig. 1). For donor 2, the level of proliferation was significantly higher when naive CD4 cells were used compared with total CD4 cells (Fig. 1). The response to SMX-NO was antigen specific because the CD3 T cells failed to respond to when restimulated with abacavir and flucloxacillin, after a coculture period in the presence of SMX-NO (Fig. 1).

Measurement of Proliferation by CFSE

Duplicate assays were set up to measure proliferation using CFSE-labeled naive T cells. The amount of CFSE in the cells and their phenotype was measured by flow cytometry. During the analysis, specific populations were identified by gating. The cells were first gated on FSC and SSC to identify the lymphocyte population. The lymphocyte population was then gated for CD3 or CD4 expression and the amount of CFSE plotted for this double-gated population. When a cell divides, the level of CFSE in the daughter cells is reduced and a small population of divided cells can be detected to the left of the undivided population. All the donors showed an increase in divided cells in the presence of SMX-NO (Fig. 2). Like the response measured by 3H-thymidine, the proliferative response measured by CFSE was dose dependent and varied between the donors (Fig. 2). Incubating the cells for a total of 6 days did not significantly increase the response for either the 3H-thymidine assay (data not shown) or for the CFSE assay (Figs. 2D and E). The response to SMX-NO was antigen specific because the CD3 T cells failed to respond to when restimulated with abacavir and flucloxacillin, after a coculture period in the presence of SMX-NO (Fig. 2B).

CD45RO Expression on Proliferating Cells

The memory phenotype of the CFSE-labeled cells was assessed by staining with a CD45RO antibody. Two histogram gates were set according to the amount of CFSE in the cells separating the cell population into nondivided cells (Fig. 3A, H1) and divided cells (Fig. 3A, H2). The expression of CD45RO in these two populations was then examined. The nondividing cells were CD45RO negative and the dividing cells were CD45RO positive (Fig. 3B). To check that the reverse was also the case, two histogram gates were set according to the CD45RO expression of the cells, separating the cell population into CD45RO negative (Fig. 3C, H3) and CD45RO positive (Fig. 3C, H4). The amount of CFSE in these two populations was then examined. CD45RO negative cells were CFSE high, i.e., undivided, whereas CD45RO positive cells were CFSE low, i.e., divided (Fig. 3D). Therefore, T cells, which are SMX-NO specific and have divided in the presence of the drug, have a memory phenotype and are CD45RO positive. Thus, the number of divided cells and CD45RO positive cells was the same for donors 1–4 (Figs. 3E and F).

Chemokine Receptor Expression

Duplicate assays were set up to assess the phenotype of the cells. Cells were stained with CD45RO and chemokine receptor antibodies after 6 days of culture with 50μM SMX-NO. Cells were gated according to their expression of CD45RO, and the expression of chemokine receptors on CD45RO positive and negative cells was compared. The chemokine receptor expression on T cells from donor 1 and donor 3 are shown in Figure 4. Expression of CCR2, CCR4, and CXCR3 was higher on SMX-NO responsive T cells (CD45RO positive cells) compared with nonresponding T cells for both donors. Expression of these receptors could be detected after 3 days (data not shown) but was more pronounced after 6 days as the cells matured.

Measurement of Cytokine Release by ELISpot

Duplicate assays were set up to assess cytokine release by the cells using the ELISpot assay. IFNγ was chosen to measure T111 responses, and IL-5 and IL-13 were chosen to measure T112 responses. The release of these cytokines by T cells from donors 1, 3, and 5 is shown in Figure 5. All three cytokines were released when CD4 cells from donors 1 and 3 were exposed to SMX-NO for 3 days. This response was dose dependent, although the dose at which the maximal response occurred varied between donors and the cytokine assayed. CD3 T cells from donors 5 released significantly more IFNγ and IL-13 in the presence of SMX-NO compared with medium alone (Fig. 5D). The response to SMX-NO was antigen specific because CD3 T cells failed to respond to when restimulated with abacavir and flucloxacillin, after a coculture period in the
presence of SMX-NO (Fig. 5D). No significant release of IL-5 was detected for donor 5 (data not shown).

DISCUSSION

Adverse drug reactions represent a major impediment to drug development. Reactions with an immunological etiology are one of the most feared as they show no simple dose-response relationship and they can affect almost any organ. Moreover, immunological drug reactions cannot be easily reproduced in animal models, and there are no established methods to predict the immunogenic potential of a drug. Thus, our aim was to develop a reproducible in vitro T-cell priming assay using peripheral blood from healthy volunteers. The drug
metabolite SMX-NO was used as a model drug allergen to establish optimum assay conditions. Although SMX-NO is unstable in cell culture conditions (Naisbitt et al., 2002), multiple adduction reactions with the cysteiny1 residues of cellular and serum proteins have been described (Callan et al., 2009; Manchanda et al., 2002; Naisbitt et al., 2001). Furthermore, SMX-NO–modified protein adducts have been shown to readily stimulate lymphocytes and T cell clones from hypersensitive patients (Callan et al., 2009; Castrejon et al., 2010; Farrell et al., 2003; Schnyder et al., 2000).

FIG. 2. Antigen-specific T-cell responses to SMX-NO measured by CFSE content. Naive CD3 and CD4 cells were cocultured with Mo-DC at a ratio of 25:1 in the presence of 50 μM SMX-NO for 8 days. These cultures were labeled with 2.5 μM CFSE, and 1 × 10^5 cells per well were restimulated with fresh Mo-DC at 4 × 10^3 cells per well and different drugs in 96 U-bottomed plates in a final volume of 0.2 ml. The cells were incubated for 3 or 6 days. Cells were labeled with anti-CD3-APC or anti-CD4-APC antibodies and analyzed by flow cytometry using a FACSCanto II. A minimum of 50,000 lymphocytes were acquired using FSC/SSC characteristics, and CD3 or CD4 positive cells were gated for CFSE analysis. CFSE profiles of CD4 cells for donors 1–4 (A); number of dividing CD3 cells for donor 5 (B); number of dividing CD4 cells for donors 1–4 (C); number of dividing cells on day 3 (black bars) and day 6 (gray bars) for donor 1 (D) and donor 3 (E).
In vitro T-cell assays attempt to recapitulate key elements of events that occur in vivo during elicitation of an allergic drug response by combining T cells, drug and dendritic cells together in culture. The conditions of the initial coculture, therefore, are critical in determining whether priming of the T cells occurs (Dietz et al., 2010; Ho et al., 2006; Moser et al., 2010). Thus, most of the factors considered during method development assessed the conditions of this coculture, namely: the DC:T cell ratio, the T-cell density, the drug concentration, addition of IL-2, and length of coculture. Maturation of the Mo-DC was considered in both the coculture and various readouts of antigen specificity. CD4 naïve T cells were used for the majority of the assays as previous work has shown that the majority of T cells responding to SMX-NO are CD4 positive (Burkhart et al., 2001; Castrejon et al., 2010; Schnyder et al., 2000).

The surface phenotype and the functional attributes of dendritic cells vary considerably according to their lineage origin, tissue origin, and their stage of differentiation or activation (Liu and Nussenzweig, 2010). Thus, the precise culture conditions used to generate dendritic cells can have an effect on the type of T-cell response generated (Duperriera et al., 2000; Duraisingham et al., 2009; Osugi et al., 2002). We used a standard 7- to 8-day culture of CD14+ cells in IL-4 and GM-CSF to generate Mo-DC, which were CD1a-low, CD14-low, CD80-low, CD83-low and CD40-high, CD86-high and class II-high. Overnight treatment with lipopolysaccharide and tumor necrosis factor α matured the cells and increased their CD40 and CD86 expression. Maturation of the Mo-DC, changing the
DC:T cell ratio from between 1:20 to 1:40, increasing the length of the coculture to 10 days, and addition of IL-2 did not significantly enhance or reduce the generation of SMX-NO–specific T cells. A cell density of $2 \times 10^6$ cells per well in a 24-well plate and a DC:T cell ratio of 1:25 gave a good cell recovery at the end of the coculture period (60–80%). This assay method does require some prior knowledge of an effective dose range of the drug because a single concentration is used during the coculture period and for the readout on cell phenotype. A suboptimal rather than a maximal drug concentration was used to reduce any toxicity.

For the successful priming of T cells against SMX-NO, naive T cells were cocultured with Mo-DC and the drug for a period of 8 days to expand the number of drug-responsive T cells prior to analysis of antigen responsiveness using readouts for proliferation, cytokine secretion, and cell phenotype. Proliferation was measured by CFSE content and $^3$H-thymidine uptake, cytokine release was measured by ELISpot, and the cell phenotype of responding cells was assessed using flow cytometry.

Increased proliferation in the presence of SMX-NO was detected in five donors using both $^3$H-thymidine uptake and CFSE content. A similar result was obtained irrespective of whether naive CD3 or naive CD4 cells were used during the culture method. Antigen specificity was demonstrated when CD3 T cells exposed to SMX-NO during the coculture period, responded to SMX-NO upon restimulation but failed to respond to when restimulated with abacavir and flucloxacillin. For three of the five donors, CFSE labeling increased the magnitude of the SMX-NO response compared with $^3$H-thymidine uptake. As has been observed previously with chemical sensitizers (Dietz et al., 2010; Vocanson et al., 2008), SMX-NO–induced proliferation was reduced when total CD4 cells were used rather than naive CD4 T cells. This may be due to the presence of naturally occurring FoxP3$^+$ T regulatory cells in the total CD4 population, which are removed during the selection process for naive CD4 cells.

The use of CFSE as a readout was as sensitive as $^3$H-thymidine for the measurement of proliferation and offered several advantages in that it directly measured the number of dividing cells and the number of cell divisions they had undergone. Furthermore, additional phenotyping of the cells, for chemokine receptor expression, for example, requires little additional effort.

To initiate an immune response to traditional peptide antigens, two signals must be present to activate the T cell, namely, the antigenic signal detected by specific TCRs and costimulatory signals provided by the dendritic cells. There is some controversy in the drug allergy field as to whether dendritic cells and dendritic cell signaling are needed for the development of an aberrant T-cell response and ultimately an adverse drug reaction. In fact, it has been proposed that drugs might bypass dendritic cells and simply stimulate previously primed effector and memory T cells (Adam et al., 2011).
However, because this theory derives largely from *ex vivo* studies using preprimed T cells from hypersensitive human subjects (Burkhart *et al.*, 2001; Castrejon *et al.*, 2010; Schnyder *et al.*, 2000), it has been very difficult to explore directly the role of dendritic cell signaling in T-cell priming and the nature of the primary antigenic determinant that stimulates T cells. We have used flow cytometry to define the specific T-cell populations stimulated with SMX-NO when the drug was presented by Mo-DC. The drug-specific T cells changed from a naive phenotype, at the start of the coculture period, to a memory phenotype by the time antigen specificity was measured. Thus, divided cells, identified by a reduced CFSE content, were CD45RO+. The expansion in drug-specific T cell numbers and the change in phenotype from naive to memory were not observed when Mo-DCs were omitted from the assay; thus, highlighting the importance of dendritic cells in the priming of a drug-specific T-cell response.

The phenotype of the divided cells was further investigated using chemokine receptor expression. We noted an upregulation of CCR2, CCR4, and CXCR3 on the surface of drug-specific CD4 cells compared with non-responding CD4 cells. CCR2 and CXCR3 are associated with Th1 polarization and IFNγ secretion, whereas CCR4 is associated with Th2 cells, skin-tropic CD4 cells, and IL-4 secretion (Bromley *et al.*, 2008). This is consistent with our cytokine ELISpot results, which show release of IFNγ and Th1 cytokines IL-5 and IL-13 upon SMX-NO stimulation. This mixed Th1/Th2 response may be due to the activation state of the Mo-DC and the DC:T cell ratio used in during culture method (Tanaka *et al.*, 2000). Secretion of IFNγ, IL-2, IL-5, and IL-13 has also been associated with the stimulation of T cells from SMX allergic patients (Lochmatter *et al.*, 2009). Lochmatter *et al.* (2009) screened supernatant from drug-stimulated lymphocyte cultures from allergic patients and tolerant controls for 17 cytokines and chemokines. The level of these four cytokines was significantly increased in patients when compared with controls. Furthermore, exposure of BALB/c strain mice to SMX-NO has been shown to stimulate IL-5 and IFNγ production by CD4+ T cells (Hopkins *et al.*, 2005).

Our findings clearly describe the cellular composition of an *in vitro* culture method that can be used reproducibly to identify the stimulation of naive T cells from healthy volunteers with the model drug allergen, SMX-NO. The assay is flexible in that the phenotype and function of T cells can be measured using a battery of readouts. Of particular importance was quantification of the change in phenotype to CD45RO+ on CFSE-labeled drug-stimulated dividing cells using flow cytometry as these cells can be subjected to further analysis. This culture method is undergoing further development to generate responses to other drugs and to develop responses in both CD4 and CD8 naive T cells. We hope this system will ultimately provide the cellular platform to investigate new drug safety issues, including those where an association between specific HLA alleles and susceptibility to an immunological reaction has been established.

**FUNDING**

This work was supported by a grant from AstraZeneca (grant number BXR10946) as part of the Medical Research Council Centre for Drug Safety Science (G0700654).

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