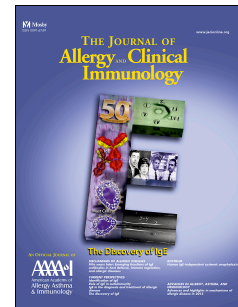


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Identification of drug- and drug-metabolite immune responses originating from both naïve and memory T-cells

Andrew Gibson, MRes, Lee Faulkner, PhD, Sally Wood, BSc, B. Kevin Park, PhD, Dean J. Naisbitt, PhD



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Title page: letter to the editor

Identification of drug- and drug-metabolite immune responses originating from both naïve and memory T-cells.

Andrew Gibson, MRes; Lee Faulkner, PhD; Sally Wood, BSc; B. Kevin Park, PhD; Dean J. Naisbitt, PhD

MRC Centre for Drug Safety Science, Department of Clinical and Molecular Pharmacology, Sherrington Building, Ashton Street, The University of Liverpool, Liverpool, L69 3GE, England

Corresponding author: Dr Dean J. Naisbitt, MRC Centre for Drug Safety Science, Department of Pharmacology, Sherrington Building, Ashton Street, The University of Liverpool, Liverpool, L69 3GE, England

Telephone: 0044 151 7945346; **Fax:** 0044 151 7945540; **e-mail:** dnes@liv.ac.uk

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Summary: Both naïve and memory T-cells from drug-naïve donors can be stimulated by parent drug and drug haptens. Thus, drug-derived antigens can stimulate naïve T-cells alongside pre-existing memory T-cells through proposed heterologous immunity with an as yet undetermined peptide antigen.

Keywords: T-cells, drug hypersensitivity, human

To the Editor: T-cell-mediated drug hypersensitivity remains a clinical problem as reactions are difficult to predict and diagnose. This is partly because mechanisms of T-cell activation involving drug-derived antigens have not been fully defined. Here, we focus on sulfamethoxazole (SMX), a sulphonamide antibiotic used for the treatment of opportunistic infections, to investigate the origin of drug and drug metabolite-specific T-cells.

T-cells isolated from blood of patients with SMX-hypersensitivity are generally CD4⁺ and secrete Th1 and Th2 cytokines in response to both SMX and the downstream metabolite nitroso sulfamethoxazole (SMX-NO). CD8⁺ T-cells that display cytotoxicity are detected in lower numbers.^{1,2} SMX also generates non-toxic acetylated metabolites which have been reported to stimulate a proportion of patient T-cell clones.³ While such stimulation likely stems from cross-reactivity to the core sulphonamide structure, acetylated SMX antigens represent a possible source for immune activation during cell culture with SMX. SMX is chemically stable and able to stimulate T-cells via (1) a processing-independent direct MHC-TCR interaction and (2) a processing-dependent pathway involving metabolism in antigen presenting cells (APCs). Meanwhile, SMX-NO binds directly to the cysteine residues of proteins to generate a number of potentially immune-activating neo-antigens.⁴ Animal models poorly reflect the human response as administration of SMX-NO to rats results in the formation of anti-SMX-hapten IgG antibodies and SMX-NO-specific T-cells, whilst no such effect was identified for SMX.⁵ Previous studies using PBMC or whole T-cell populations from healthy human donors have shown that SMX-NO activates T-cells from all individuals, while SMX stimulates T-cells in approximately 30% of individuals.⁶ In contrast, both drug and metabolite are able to promote the maturation of healthy donor-derived dendritic cells (DCs), a critical step in the efficient presentation of drug-antigens on APCs to naive T-cells.⁷ Despite intensive research, the origin of drug and drug metabolite-responsive T-cells has not

been defined. Moreover, it is not known whether previously identified SMX-NO-responsive T-cells in healthy donors originate from the naïve or memory T-cell compartment. Determining the presence of memory T-cells in drug-naïve donors that respond to SMX-derived antigens is crucial for understanding whether hypersensitivity is partially mediated by heterologous immunity, where drug-antigens cross-react with T-cells initially primed to non SMX-derived peptides. Thus, we sought to investigate the activation of naïve and memory T-cells from healthy donors to both SMX and SMX-NO. Furthermore, both SMX- and SMX-NO-specific T-cells were cloned to assess cross-reactivity to SMX-NO and SMX, respectively.

We utilised an established *in vitro* T-cell priming assay⁸ (study design and results summarised in figure E1) where PBMC were isolated from peripheral blood, before further isolation of CD14⁺ cells and naïve and memory T-cells by magnetic bead sorting. T-cells were then combined with autologous, fully matured monocyte-derived DCs, and drug or metabolite for one week. T-cells were harvested and cultured with a fresh batch of mature DCs and tested for antigen-specificity using [³H] thymidine incorporation and ELISpot to analyse proliferation and cytokine secretion, respectively. IL-13 was used as previous studies using drug-specific T-cells from hypersensitive patients shown that the cytokine is secreted in high levels. T-cell cloning was performed to generate SMX and SMX-NO-responsive clones and to explore cross-reactivity, cytokine release and MHC restriction. Detailed methods are available in the journals on-line repository.

Both SMX-NO-primed naïve T-cells and SMX-NO-exposed memory T-cells proliferated when re-stimulated with SMX-NO in all drug-naïve donors (n=11; Figure 1a). In contrast, the priming of naïve T-cells or the culture of memory T-cells with SMX failed to induce an

antigen-specific proliferative response in all 11 donors (Figure 1b). However, upon analysis of IL-13 secretion, both SMX-NO- and SMX-responsive T-cells were detected in 2/3 donors (Figures 1c and 1d, respectively). From one donor, we obtained SMX-responsive cells derived from both the naïve and memory T-cell cultures (Figure 1d), while another donor showed a marginal SMX-specific response after naïve T-cell priming. Both SMX and SMX-NO-primed cultures did not cross-react with the alternative antigen (supplementary figure 1). While it is difficult to determine the nature of the antigen presenting cell utilised during hypersensitivity reactions in patients, it is likely that DCs are responsible for naïve T-cell priming as they are the only APC capable of doing so *in vitro* (figure E2).

SMX-NO- and SMX-specific T-cell clones were generated from both the naïve and memory compartments of SMX-responsive donors (Figure 2a). Both SMX- and SMX-NO-responsive CD4+ and CD8+ clones generated from naïve T-cell priming were found to be highly specific and did not cross-react with SMX-NO or SMX, respectively (Figure 2b and 2c), mirroring the majority of T-cell clones isolated from hypersensitive patients (figure E3). Over 95% of SMX and SMX-NO-responsive clones from the memory T-cell compartment were CD4+, possibly because most memory CD8+ T-cells are tissue resident. 3/5 SMX-responsive T-cell clones from the memory compartment proliferated in the presence of SMX-NO, though these responses were much weaker than in the presence of SMX. Furthermore, 1 SMX-NO-responsive memory T-cell clone responded strongly to both SMX and SMX-NO. SMX and SMX-NO-specific T-cell clones secreted a similar panel of cytokines including IFN- γ , IL-13, IL-5. Interestingly, the cytolytic molecules granzyme B and perforin were also released (Figure 2d and 2e) thus reflecting the predominant cytotoxic phenotype of T-cells isolated from the blister fluid of SMX-hypersensitive patients. The secretion of IL-17 was not detected from

either drug- or metabolite-responsive clones. As expected, CD8⁺ and CD4⁺ SMX-responsive clones were MHC class I- and II-restricted, respectively (figure E4).

In summary, we have uncovered the origins of drug antigen-responsive T-cells. We have shown that SMX and SMX-NO activate both naïve and memory T-cells. While all donors responded to SMX-NO, SMX activated naïve and memory T-cells were detected in only two donors. The low levels of cross reactivity with naïve T-cells suggests that clones are activated with the parent drug and nitroso metabolite via different mechanisms; however, it is also possible that intracellular metabolism of the parent drug generates novel drug metabolite antigens. The inability of the thymidine proliferation assay to detect SMX-responsive cells from the initial priming cultures highlights a known lack of sensitivity in comparison to the ELISpot, but also that SMX is a less potent immunogen than SMX-NO in drug-naïve donors. It is likely that in individuals with specific but as yet undetermined predisposing genetic factors the frequency of T-cells responsive to SMX-derived antigens is enhanced, rising above a threshold for overwhelming immune activation and hypersensitivity. Similar to patients, SMX-responsive cells derived from drug-naïve donors were a mixture of CD4⁺ and CD8⁺ T-cells.¹⁻³

As SMX- and SMX-NO-responsive CD4⁺ T-cells deriving from the memory pool were detected in healthy donors, the data raise the possibility that peptide-specific T-cells are present within the general population, which are reactive against drug-associated peptides through some form of molecular mimicry within the MHC antigen-binding cleft. Indeed, heterologous immunity has been previously proposed to be the result of pathogen-primed T-cells as viral-specific T-cells have the propensity to cross-react with MHC-restricted drug-antigen.⁹ Furthermore, in comparison to T-cell clones derived from naïve T-cell priming

which were highly antigen-specific, those generated from SMX- and SMX-NO-exposed memory cultures displayed the ability to cross-react with SMX-NO and SMX, respectively. Thus, our data indicate that the cross-reactive T-cells observed in hypersensitive patients are likely derived from pre-existing memory T-cells.

While ongoing studies are required to provide data more representative of the generalised population, using an initial small cohort of donors we conclude that both the priming of naïve T-cells, but also the activation of pre-existing memory T-cells by SMX-derived antigens, may play a role in the onset of SMX-hypersensitivity. Therefore, determining the precise nature of the peptides responsible for the initial priming of naïve T-cells is crucial to understanding drug-induced hypersensitivity reactions and is thus key to the future safe development of drugs. More work will be needed to determine whether a detailed analysis of a patient's memory T-cell response could be utilised at a clinical level to evaluate allergic risk.

Andrew Gibson, MRes

Lee Faulkner, PhD

Sally Wood, BSc

B. Kevin Park, PhD

Dean J. Naisbitt, PhD

MRC Centre for Drug Safety Science, Department of Clinical and Molecular Pharmacology,
Sherrington Building, Ashton Street, The University of Liverpool, Liverpool, L69 3GE, England

E-mail: dnes@liv.ac.uk.

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Figure Legends

Figure 1. Naïve and memory T-cells were exposed to SMX-NO and SMX for 8 days and then recall responses tested. Proliferative responses to **(a)** SMX-NO and **(b)** SMX were measured by ^3H -thymidine incorporation (n=3). Error bars indicate standard deviation of triplicate wells (*p ≤ 0.05, **p ≤ 0.005, ***p < 0.001). IL-13 secretion to **(c)** SMX-NO and **(d)** SMX were measured by ELISpot (n=3).

Figure 2. T-cell clones were isolated from 2 donors following exposure of naïve and memory T-cells to SMX-NO and SMX. **(a)** Generation and phenotype of T-cell clones. **(b, c)** Proliferation (SMX: 2 from naïve and 5 from memory precursors; SMX-NO: 5 from naïve and 2 from memory precursors) and **(d, e)** secretion of cytokines/cytolytic molecules by T-cell clones isolated following SMX and SMX-NO exposure. Error bars indicate standard deviation of triplicate wells (*p ≤ 0.05, **p ≤ 0.005, ***p < 0.001).

Figure E1. Overview of study design and results. Delineation of naïve and memory T-cell responses to SMX-derived antigens in drug-naïve donors.

Figure E2. Comparative ability of dendritic cells and B-cells to induce naïve and memory T-cell responses to drug-antigen *in vitro*. Naïve and memory T-cells from drug-naïve donors were exposed to SMX-NO and SMX for 8 days in the presence of EBV-transformed B-cells **(a)** or dendritic cells **(b)**, and then recall responses tested by ^3H -thymidine incorporation. Error bars indicate standard deviation of triplicate wells.

Figure E3. Antigen cross-reactivity of T-cell clones derived from priming in drug-naïve donors and hypersensitive patients. Naïve and memory T-cells from drug naïve donors were exposed to SMX and SMX-NO for 8 days and then recall responses tested. Cross-reactivity to SMX-NO and SMX was measured by ³H-thymidine incorporation and/or ELISpot after exposure of naïve and memory T-cells to SMX-NO (**a**, n=3) and SMX (**b**, n=3), or exposure of representative SMX-NO- (**c**) and SMX-responsive (**d**) T-cell clones from hypersensitive patients. Error bars indicate standard deviation of triplicate wells (*p ≤0.05, **p ≤0.005, ***p <0.001).

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The authors declare that they have no relevant conflicts of interest.

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Figure 1

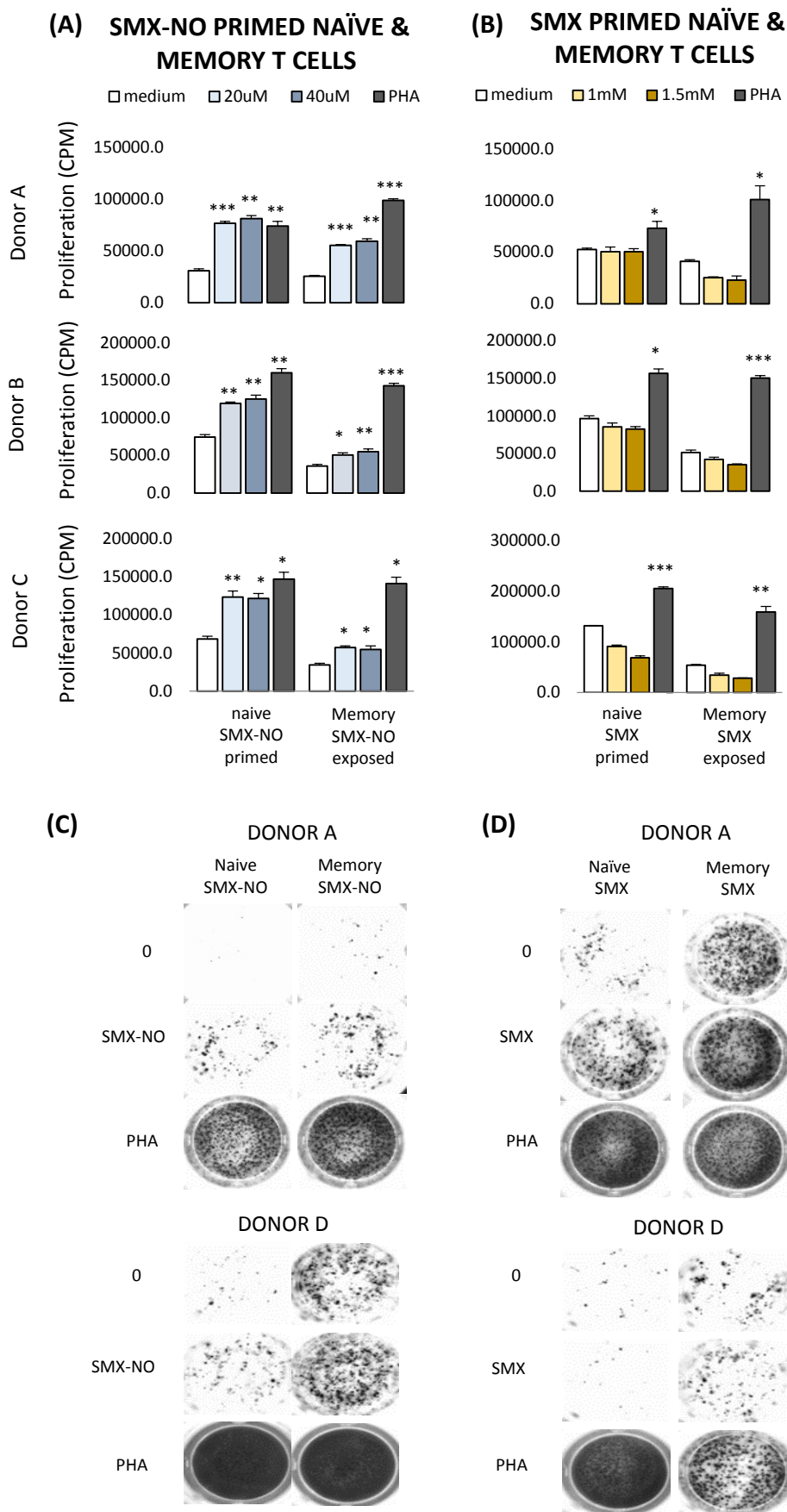
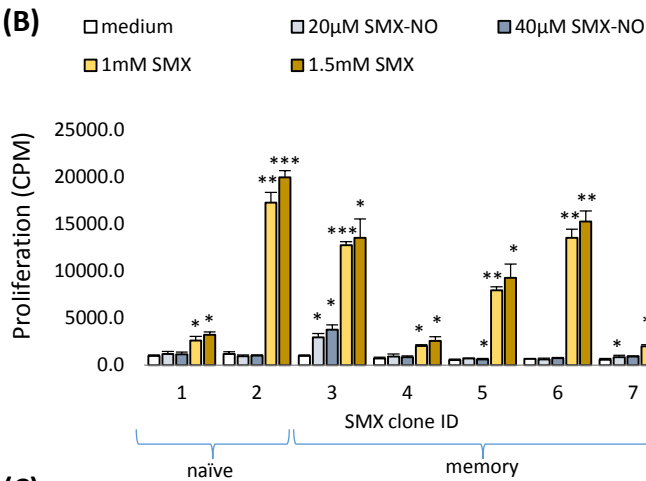


Figure 2

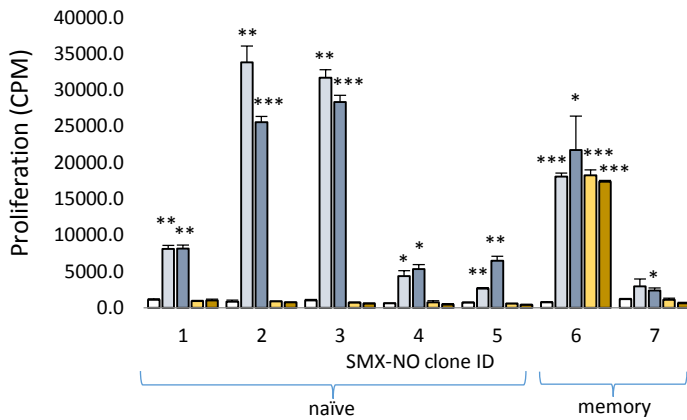
(A)

	DONOR A				DONOR D			
	Naïve SMX-NO	Memory SMX-NO	Naïve SMX	Memory SMX	Naïve SMX-NO	Memory SMX-NO	Naïve SMX	Memory SMX
Number of clones tested	144	144	120	168	144	144	114	144
Number of specific clones	10	7	5	7	11	6	11	6
Proliferation of specific clones (SI range)	1.7-36.8	2.0-14.3	1.7-6.3	1.7-54.2	1.7-22.5	1.6-4.8	1.7-35.2	1.7-2.6
Number of CD4 clones	5/9	6/7	1/5	6/7	5/11	6/6	7/11	4/6
Number of CD8 clones	3/9	0/7	3/5	0/7	4/11	0/6	4/11	1/6

(B)



(C)



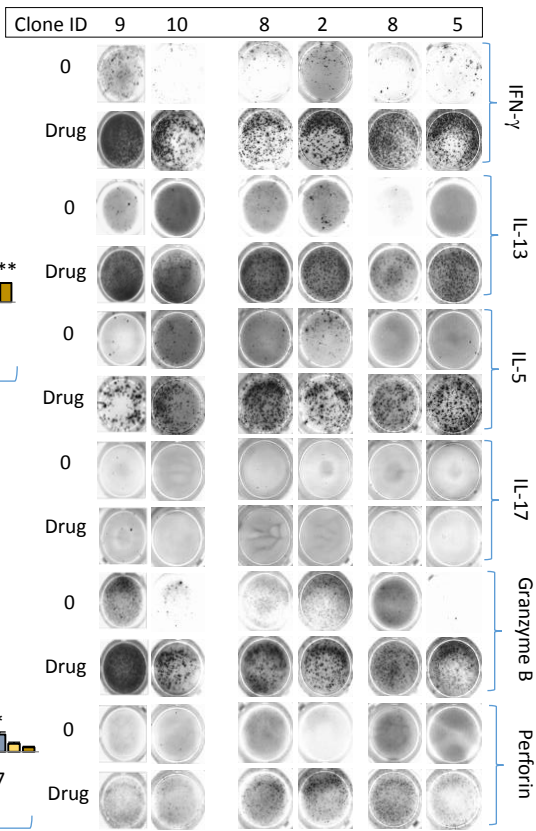
(D)

Naïve SMX-NO clones

(E)

Naïve SMX clones

Memory SMX clones



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Title page: letter to the editor

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MRC Centre for Drug Safety Science, Department of Clinical and Molecular Pharmacology, Sherrington Building, Ashton Street, The University of Liverpool, Liverpool, L69 3GE, England

Corresponding author: Dr Dean J. Naisbitt, MRC Centre for Drug Safety Science, Department of Pharmacology, Sherrington Building, Ashton Street, The University of Liverpool, Liverpool, L69 3GE, England

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Supplementary methods

Blood donation, PBMC extraction, and cell separation

Venous blood samples were taken from SMX hypersensitive patients and SMX-naïve healthy volunteers. Study approval was granted by the Liverpool local research ethics committee and all blood donors gave informed written consent.

Extraction of PBMC from whole blood was performed by density gradient separation using Lymphoprep (Axis-shield, Dundee). Magnetic bead separation columns were then employed to separate distinct cell populations as devised by the manufacturer (Miltenyi Biotec, Bisley, U.K.). First, positive selection was used to separate CD14⁺ monocytes from total PBMC. Second, a negative selection was performed on the non-CD14 population, which utilised an anti-T-cell antibody cocktail, to extract a CD3⁺ pan T-cell population. Lastly, two consecutive isolations were carried out on the pan T-cell population; positive selection of CD25⁺ T-cells to identify and remove T regulatory cells, followed by positive selection of CD45RO on the CD25⁻ population to separate naïve (CD45RO⁻) and memory (CD45RO⁺) T-cells. All cells were frozen in 10% DMSO and kept at -150°C prior to use.

Culture of antigen-presenting cells (APC)

Dendritic cells (DC) and Epstein-barr virus-transformed B-cells (EBV) were used as APC for in vitro priming and T-cell cloning assays, respectively. DC generation required an 8 day culture of CD14⁺ monocytes (3-5 x 10⁶/well; 6-well plate; total volume, 6 ml; 37°C/5% CO₂) in R9 medium (RPMI 1640, 100 µg/ml penicillin, 100 U/ml streptomycin, 25 µg/ml transferrin, 10% human AB serum [Innovative Research], 25 mM HEPES buffer, 2 mM L-glutamine) supplemented with GM-CSF- and IL-4 (800 U/ml) [PeproTech Ltd]. In order to produce

functionally mature DC, 25 ng/ml TNF- α [PeproTech Ltd] and 1 μ g/ml lipopolysaccharide [Sigma-Aldrich Ltd, *E.coli* 0111:B4] were added on the penultimate day of culture. Autologous EBV-transformed B-cells were generated as previously described¹.

***In vitro* priming assay**

Mature DCs (0.8×10^5 /well) were cultured with autologous naïve or memory T-cells (2.5×10^6 /well; 24 well plate; total volume, 2 ml) and either SMX (1 mM) or SMX-NO (50 μ M) for 8 days. To assess antigen-specific responses, drug antigen-primed naïve or memory T-cells were then harvested and re-plated (1×10^6 /well; 96 well plate; total volume, 200 μ l) with autologous mature DCs (4×10^3 /well) and either SMX (1-2 mM), SMX-NO (20-50 μ M), or phytohemagglutinin (PHA; 20 μ g/ml) as a positive control. Analysis of cytokine/cytolytic molecule secretion was performed using pre-coated ELISpot plates (duplicate cultures) and proliferative capacity by [^{3} H] thymidine incorporation (triplicate cultures) after a 48 hr incubation (37°C/5% CO₂).

The secretion of IL-13 was determined according to the manufacturer's ELISpot protocol (Mabtech, Nacka Strand, Sweden). [^{3} H] thymidine (0.5 μ Ci/well) was added to the proliferation plate and then incubated for an additional 16 hr prior to analysis of incorporated radioactivity using a Microbeta Trilux 1450 LSC beta counter (PerkinElmer, Cambridge, U.K.).

T-cell cloning

Both naïve and memory T-cell cultures were subject to well documented serial dilution and mitogen-driven expansion protocols^{2, 3} to generate T-cell clones. To assess functionality, T-cell clones (5×10^4 /well; 96 well plate; total volume, 200 μ l) were cultured with autologous

irradiated EBV-transformed B-cells (1×10^4 /well) in the presence or absence of SMX (1mM) or SMX-NO (40 μ M) for 48 hrs (37°C/5% CO₂). Antigen-specificity was validated by [³H] thymidine incorporation for an additional 16 hrs before analysis by scintillation counting to measure cellular proliferation. T-cell clones with a stimulation index (mean cpm drug-treated wells / mean cpm of control wells) of > 2 were expanded by repetitive stimulation with allogeneic PBMCs (5x10⁴/well; 96 well plate; total volume, 200 μ l) in R9 medium supplemented with PHA (5 μ g/ml) and IL-2. Further analyses were performed similarly, including the measurement of IFN- γ , IL-5, IL-13, IL-17, perforin, and granzyme B by ELISpot (duplicate cultures), and using [³H] thymidine incorporation assays to analyse MHC restriction by adding MHC I- (IgG₁, 10 μ g/ml) and MHC II-blocking (IgG_{2a}, 10 μ g/ml) antibodies to particular wells alongside respective isotype controls (triplicate cultures).

Flow cytometry was utilised to detail the phenotype of individual T-cell clones. A FACS Canto II flow cytometer was used to acquire T-cells based upon forward/side scatter characteristics. Collected data was analysed using associated FACS DIVA or Cyflogic software.

Statistics

Proliferation data from the *in vitro* priming of naïve and memory T-cells was performed in triplicate cultures and on 3 or more separate donors. Data is presented as the mean of the triplicate values with associated standard deviation. A paired t test was implemented for statistical analysis (Sigmaplot 13 software).

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Figure E3

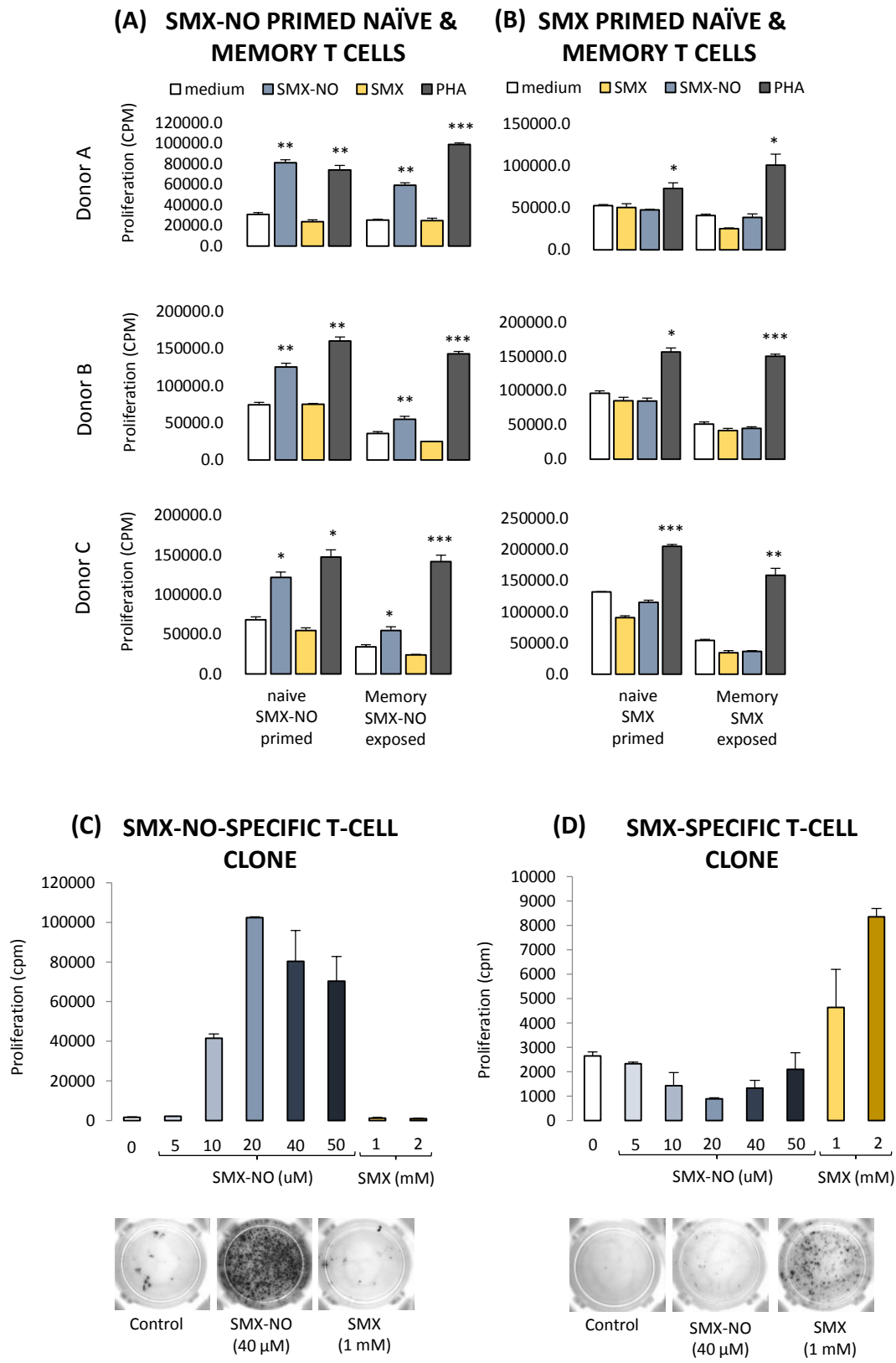


Figure E4

