**Title page: letter to the editor**

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

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**Summary:** Both naïve and memory T-cells from drug-naive 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.

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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 ofdendritic 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).

**Figure E4.** MHC I and II restriction of SMX-responsive T-cell clones derived from drug-naïve donors. Naïve or memory-derived SMX-responsive T-cell clones from drug-naïve donors were cultured with autologous EBV-transformed B-cells and SMX, with or without MHC I or II blocking antibodies for 48 hr. Proliferative responses were measured by 3H-thymidine incorporation for a further 16 hr. Error bars indicate standard deviation of triplicate wells (\*p ≤0.05, \*\*p ≤0.005, \*\*\*p <0.001).

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

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