**TITLE PAGE**

**Development of an improved T cell assay to assess the intrinsic immunogenicity of drugs**

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**Running title: T cell assay to assess drug immunogenicity**

**Abstract**

Drug hypersensitivity is a difficult to predict adverse event. *In vitro* T cell priming assays that assess immunogenicity have been developed; however, their application is limited due requirements for two batches of autologous dendritic cells (DC) and inconsistent results. Hence, we aimed to develop an improved, but simplified assay, termed the T cell multiple well assay (T-MWA), that permits assessment of whether drugs activate T cells, alongside analysis of the strength of the induced response and the number of cultures that respond. DC T cell priming was conducted in multiple cultures, for two weeks with model haptens (nitroso sulfamethoxazole [SMX-NO], Bandrowski’s base [BB] or piperacillin [PIP]). Cultures were re-challenged with hapten and T cell proliferation was measured using [3H]-thymidine incorporation. Priming of naïve T cells was observed with SMX-NO, with no requirement for DC during restimulation. Greater than 65% of cultures were activated with SMX-NO; 8.0, 30.8 and 27.2% displayed weak (stimulation index (SI)=1.5-1.9), good (SI=2-3.9) and strong responses (SI>4), respectively. The number of responding cultures and strength of the response was reproducible when separate blood donations were compared. Checkpoint blockade increased the strength of the proliferative response, but not the number of responding cultures. Good to strong priming responses were detected with BB, while PIP stimulated only a small number of cultures to proliferate weakly. Inducible CD4hiCD25hiFoxP3hiCD127low Tregs were generated during priming. To conclude, the T-MWA offers improvements over existing assays and with development it could be used to study multiple HLA-typed donors in a single plate format.

**Key words:** drug hypersensitivity, human, immune system

**Introduction**

In recent years, drug hypersensitivity reactions (DHR) have become increasingly problematic for patients, clinicians and the pharmaceutical industry. These reactions result in a high incidence of morbidity and mortality in patients. The financial burden of post-licensing drug withdrawal due to DHR, in-part, contributes to the high cost of drug development, estimated at over $2 billion. Hence, accurate prediction of DHR is of the upmost importance (Adams and Brantner, 2006; Morgan *et al.*, 1999).

Delayed type DHRs have an onset greater than one hour after drug administration, as the mechanism involves the activation of drug-specific T cells (Bousquet *et al.*, 2007). Genome-wide association studies have shown links between expression of certain HLA alleles and susceptibility to specific forms of delayed DHR (Daly *et al.*, 2009; Mallal *et al.*, 2002; Man *et al.*, 2007; McCormack *et al.*, 2011). Unwanted T cell activation leading to drug hypersensitivity arises from the drug derivative (drug or metabolite) forming a complex with a peptide, through either covalent or non-covalent bonds, and the protein encoded by the specific risk allele. Interestingly, the majority of carriers of HLA risk alleles do not present with clinically defined DHR following exposure to a suspect drug. This highlights that although HLA allele expression is an important feature for drug immunogenicity, other factors determine whether presentation of the drug derivative in an appropriate form will result in a pathogenic response and a DHR.

Immune inhibitory pathways signal to determine whether antigen exposure will lead to T cell activation. For example it has been shown that dysregulation of Tregs and checkpoint receptors can propagate autoimmunity, while blocking these pathways in cancer models significantly enhance functional T cell responses (Chuang *et al.*, 2005; Hodi *et al.*, 2010; Kouki *et al.*, 2000; Sakuishi *et al.*, 2010). With respect to DHRs, Takahashi et al. (Takahashi *et al.*, 2009) reported a reduced ability of Tregs to suppress effector T cell responses to drugs, while Wang et al. (Wang *et al.*, 2018) demonstrated that the success of TNF-α antagonist therapy in patients with toxic epidermal necrolysis was partly due to increased activity of Treg populations. The impact of immune checkpoint receptor signalling on the development or prevention of DHRs is only just beginning to be realized; however, several studies have reported an increased frequency of DHRs in patients with a history of previous exposure to immune checkpoint inhibitors (Ford *et al.*, 2018; Uhara *et al.*, 2018; Yamazaki *et al.*, 2015). Together, these data suggest that immune dysregulation may at least in part determine whether drug exposure will result in a health benefit or the development of a DHR.

Although prediction of DHR remains an inherent problem for Pharma, it is possible to study the intrinsic drug immunogenicity. Existing methodological approaches are adapted from studies in the field of contact dermatitis where low molecular weight chemicals have been shown to prime naïve human T cells *in vitro* (Dietz *et al.*, 2010; Martin *et al.*, 2010; Newman *et al.*, 1977; Rougier *et al.*, 1998; Vocanson *et al.*, 2008). Naïve T cell priming assays make use of isolated naïve T cells from healthy human donors, which are then exposed to a specific drug (or metabolite) over a 14 day period. Upon re-challenge with the specific drug a number of different readouts can be used to detect drug-specific T cell activation; these include T cell proliferation measured by 3[H] thymidine incorporation and cytokine release measured by ELIspot or flow cytometry (Faulkner *et al.*, 2016; Faulkner *et al.*, 2012). Similar to the work with chemical contact allergens (Vocanson, *et al.*, 2008), Tregs control whether effective drug-specific priming of naïve T cells is observed. Tregs are depleted from the naïve T cell population prior to drug exposure, while the addition of Tregs during the initial 14 day co-culture inhibits priming in a cell concentration-dependent manner (Gibson *et al.*, 2017a). This assay is malleable and can be used to investigate the role of the immune checkpoints (PD-1 and CTLA4) in the priming of naïve T cells to drugs. For example, priming of naïve T cells to the drug metabolite nitroso sulfamethoxazole (SMX-NO) is enhanced with PD-L1 and CTLA-4 block (Gibson, *et al.,* 2017a; Gibson *et al.*, 2014).

One limitation of standard naïve T cell priming assays is the requirement for two batches of dendritic cells (DC). The second batch of DC often leads to an unwanted increase in proliferation or cytokine release in control wells containing DC, primed T cells and medium alone. Furthermore, the assay is cumbersome and requires an experienced researcher to establish the priming culture. The assay also fails to take into account the precursor frequency and T cell repertoire for a specific antigen; instead assuming that all drugs will react in the same manner. However, due to the diversity of the T cell repertoire, naïve T cells specific for an HLA peptide complex are rare and can vary from drug to drug (Moon *et al.*, 2007). Thus, the objective of the current project was to further optimize the existing T cell priming assay to take into account all of the above, whilst successfully being able to determine the potential immunogenicity of drugs. We focused on three haptenic compounds as exemplar drug/chemical immunogens; SMX-NO, piperacillin (PIP) and Bandrowski’s base (BB). SMX-NO is a reactive metabolite of the antibiotic SMX, that binds covalently to multiple amino acids including cysteine and lysine (Callan *et al.*, 2009; Tailor *et al.*, 2019) to activate T cells from patients and healthy donors (Castrejon *et al.*, 2010; Gibson *et al.*, 2017a). PIP is a β-lactam antibiotic that binds directly to lysine residues on proteins to stimulate patient T cells (Meng *et al.*, 2017; Sullivan *et al.*, 2018). Finally, BB is a trimer of the oxidized form of the hair dye component *p-*phenylenediamine (Jahn *et al.*, 2012). BB binds covalently to cysteine residues and, similar to the other compounds, activates allergic patient T cells and primes naïve T cells from healthy human donors (Coulter *et al.*, 2008; Gibson *et al.*, 2015). The data presented herein indicate that the revised assay termed the T cell multi-well assay (T-MWA) can be used to investigate drug immunogenicity. Utilization of up to forty eight replicates per condition ensures that the precursor frequency of naïve T cells for each drug is taken into consideration alongside provision of a scaling system for immunogenicity assessment as opposed to the yes/no outcome of standard priming assays.

**Materials and methods**

**Human subjects**

One hundred and eight mL of venous blood was donated by drug-naive human donors, all of whom had given informed written consent to partake in this study approved by the Liverpool local research ethics committee. Blood was drawn into lithium heparin coated tubes.

**Isolation of peripheral blood mononuclear cells (PBMC) and immune cell separation using antibody-conjugated beads**

PBMC were isolated from whole blood (108 ml) using density centrifugation. Briefly, blood was layered in a 1:1 volume ratio on top of Lymphoprep (Axis-Shield PoC AA (Oslo, Norway) and centrifuged at 2000rpm, for 25min without brake. The PBMC buffy coat was collected using a Pasteur pipette and washed twice with HBSS buffer solution and then resuspended in appropriate volume of separation buffer before immune cell isolation. Initially, CD14+ monocytes were positively selected from total PBMC using CD14 microbeads (Miltenyi Biotec, Gadbach, Germany). This was followed by CD25 depletion and then negative selection for CD45RA+ naïve T cells. CD14+ monocytes used for DC generation were cultured in RPMI 1640 containing 100 mg/ml penicillin, 100 U/ml streptomycin, 25 mg/ml transferrin, 10% human AB serum (Innovative Research), 25 mM HEPES buffer, 2 mM L-glutamine, GM-CSF (800 U/ml) and IL-4 (800 U/ml).

**DC Generation**

CD14+ monocytes (2 x 106) were seeded in a 6 well plate in culture medium supplemented with GM-CSF (800U/mL) and IL-4 (800U/mL) for 6 days. On day 6, DC were matured with TNF-α (25ng/mL) LPS (1µg/mL) overnight.

**Established DC T cell priming assay**

DC (0.8×105/well) were cultured with naı̈ve CD3+ T cells (2.5×106/well) and SMX-NO for 14 days at 37°C, 5% CO2 (24-well plate; total volume 2 mL). Primed T cells were then harvested and prepared for restimulation. Briefly, T cells (1×105) were cultured with of DC (4×103) and titrated SMX-NO concentrations with triplicate wells per treatment condition. T cell activation was assessed after 48h using [3H]thymidine (0.5 µCi/well) to measure proliferation. After 16h, plates were harvested using TomTec Harvester 96 (Receptor Technologies) onto filter mats and sealed with scintillation wax added and then counted using a MicroBeta TriLux 1450 LSC β-counter (PerkinElmer).

**T cell multi-well assay (T-MWA)**

DC (8 x 103) were cultured with naïve T cells (1 x 105) with compound (SMX-NO [40µM], BB [5µM] and PIP [1mM]) for 14 days at 37°C, 5% CO2 (up to 96 cultures were established depending on availability of cells in 96-well plates; total volume 0.2 mL). Compound concentrations were selected according to previous studies characterizing the activation of T cells from individuals with allergic contact dermatitis or drug hypersensitivity (Castrejon *et al*., 2010; Coulter *et al.*, 2008; Sullivan, *et al.,* 2018). Immune checkpoint inhibitors (human targeted anti-PD-L1 [5µg/mL] and anti-CTL4 [10µg/mL]; BioLegend, London, UK) were added to certain experiments 30min prior to the addition of drugs to investigate the role of T cell regulatory pathways in naïve T cell priming. After 14 days, wells were washed 3 times with excess culture media being removed following centrifugation at 2200rpm for 4 minutes. Half of the primed cultures were restimulated with the same compound (SMX-NO [40µM], BB [5µM] or PIP [1mM]) with no requirement for a second batch of DC. 3 [H]-thymidine (0.5 µCi/well) was added for the final 16h to assess compound-induced T cell proliferation. Plates were harvested using TomTec Harvester 96 (Receptor Technologies) onto filter mats and sealed with scintillation wax added and then counted using a MicroBeta TriLux 1450 LSC β-counter (PerkinElmer).

Figure 1 illustrates the similarities and differences between the standard naïve T cell assay and the T-MWA.

**Characterization of Treg expression during the priming of naïve T-cells**

Induced Tregs may be generated from the naïve T cell population during priming. Thus, we investigated whether Tregs are generated in our T cell cultures primed against SMX-NO and PIP. An aliquot of cells was taken from the priming cultures at four different time points, with Tregs then being quantified by flow cytometry. T cells were stained with fluorochrome-conjugated antibodies for CD4 (SK3), CD25 (MA-251), CD127 (HIL-7R-M21) (all BD Biosciences, Berkshire, UK) and FOXP3 (236A/E7) (VWR International Ltd; Leicestershire, uk). For detection of intracellular FOXP3, cells were first fixed and permeabilized. Flow cytometry was conducted on a BD FACSCanto flow cytometer. The acquisition gates were restricted to lymphocyte gates based on morphological characteristics, and at least 50,000 T cells were acquired and analyzed. The results are expressed as a percentage of the total CD4+ T cell population.

**Statistics**

All experiments were prepared on three or more separate donors. Assays measuring cell proliferation from SMX-NO-responsive T cells in the standard priming assay were conducted in triplicate. Assays measuring cell proliferation from SMX-NO, BB- and PIP-responsive T cells in the T-MWA were conducted in varying replicate numbers (up to 48 per treatment condition). Data from the T-MWA are presented as dot plots that include the mean response of replicate values. Mann Whitney test was used for statistical analysis (GraphPad Prism software).

**Results**

**Comparison between the standard naïve T cell priming assay and the T-MWA**

In order to improve on the standard naïve T cell priming assay, it was important to compare the degree of priming to a model drug (i.e., SMX-NO), using existing methods and the newly developed T-MWA, with cells from the same donors.

After restimulation of SMX-NO primed T cells with DC and SMX-NO using the standard priming assay conditions, the T cells from all 3 donors exhibited and concentration-dependent proliferative response (Figure 2A-C). However, addition of the second batch of DC during restimulation increased background proliferation of the primed T cells even in the absence of SMX-NO, which limits the dynamic range of the assay and as such it is often difficult to discriminate between strong and weak responses. The T-MWA does not rely on a second round of DC generation as primed T cells remain in the same well with DC are restimulated with SMX-NO alone. The T-MWA evaluated SMX-NO-induced T cell proliferation at an optimal concentration of SMX-NO (40µM) across up to 48 test wells in a 96 well U-bottomed plate. Statistically significant SMX-NO-induced T cell proliferation was observed with the SMX-NO restimulated wells in all 3 donors (Figure 2 D-F). The level of unwanted DC induced proliferation in control wells tends to be much lower than that seen in the standard priming assay. The strength of T cell priming in each of well was classified according to the system described by Faulkner et al. (Faulkner *et al.,* 2016) as: negative, SI < 1.5; weak, SI 1.5-1.99; good, SI 2-3.99; and strong, SI>4). The percentage of responsive cultures per donor were 55%, 82.5% and 77% for donors 1, 2 and 3, respectively, with the majority of responding wells displaying good or strong responses (Figure 2 G-I).

**Assessment of intra-individual variability of T-MWA and T-MDA**

After comparing SMX-NO-specific T cell responses using the standard priming assay and the T-MWA, the next focus was to investigate potential intra-individual variations. To do this, two batches of blood (Experiment 1 and 2; Figure 3) were collected six months apart from 3 donors. PBMC were separated into individual cell components and subjected to SMX-NO priming using the T-MWA. The percentage positive priming of naïve T cell cultures and the strength of the induced response in individual culture wells in experiment 1 and 2 were similar in all 3 donors (percentage of positive priming wells: Donor 1, 77.5% & 77.5%; Donor 2, 57.5% & 55.0%; Donor 3, 66.6% & 70.0%) (Figure 3 A-C).

**The effect of immune checkpoint receptor blockade on naïve T cell activation using the T-MWA**

Our previous studies using the standard form of the T cell priming assay has shown that immune checkpoint receptor blockade with anti-PDL1 and anti-CTLA4 antibodies modulate the T cell activation threshold for SMX-NO, visualized as a stronger SMX-NO-specific recall response when the primed T cells are cultured with DC and the drug metabolite (Gibson *et al.,* 2017a; Gibson *et al.*, 2014). The same checkpoint inhibitor antibodies were added to the T-MWA and SMX-NO immunogenicity assessed. As described above, significant priming of naïve T cells to SMX-NO was observed in all three donors in the absence of immune checkpoint blockade (control condition in Figure 4 A, E and I). The majority of SMX-NO responding wells displayed good and strong responses (Figure 4B, F and J). The addition of PD-L1 and CTLA4 antibodies to co-cultures from donors 1-3 increased the strength of the proliferative response in many of the SMX-NO stimulated wells; a statistically significant increase in the strength of the response was observed with all three donors when SMX-NO restimulated wells with and without CTLA4 block were compared. However, blockade of immune checkpoints also increased the degree of proliferation in several of the media-treated control wells and this resulted in somewhat skewed profiles showing the number of responding wells according to the weak to very strong classification. Importantly, immune checkpoint blockade did not increase the number of cultures that responded to SMX-NO (Figure 4).

**Assessment of the immunogenicity of SMX-NO, BB and PIP using the T-MWA**

Three model haptenic compounds (SMX-NO, BB and PIP) displaying different protein binding characteristics were selected to begin to assess the applicability domain of the T-MWA. The assay was performed using cells from 3 donors. Statistically significant priming was observed to SMX-NO (2/3 donors, p < 0.0001), BB (3/3 donors, p < 0.0001) and PIP (1/3 donors, p < 0.05) (Figure 5 A, E and I). Approximately 60, 88 and 24% of compound primed wells were stimulated to proliferate with SMX-NO, BB and PIP, respectively. With SMX-NO, an assortment of weak, good and strong proliferative responses were observed (Figure 5 B, F and J). BB yielded mostly good and strong responses (Figure 5 C, G and K), while the majority of PIP responding wells gave weak or good responses (Figures 5 D, H and L).

In separate experiments, a slightly different format of the assay was used to assess the immunogenicity of the 3 haptens with PBMC from 8 donors. In this experiment, naïve T cells from each donor were co-cultured with DC and the test compounds in 6 wells of a 96 well plate and only three wells were restimulated with test compounds on day 14 prior to assessment of proliferation. Positive proliferative responses (SI > 1.5) were observed in all 8 donors with SMX-NO (100%), 4 donors BB (50%), and 2 donors with PIP (25%) (Figure 6 A-C).

**Generation of induced Tregs during immunogenicity assessment with SMX-NO and PIP**

Tregs are depleted from the naïve T cells prior to establishing priming co-cultures. However, induced Tregs may be generated from the naïve T cell population during priming, proliferating further and eliciting suppressive effects in the T-MWA. Thus, we investigated whether Tregs were generated in our priming co-cultures with SMX-NO and PIP. Expression of Tregs, determined as CD4+, CD25hi, CD127lo, FOXP3+ cells, was assessed at four different time points during the co-culture. In all 3 experiments, the number of Tregs (presented as a percentage of the total CD4+ T cell population) increased from day 0 to day 14 in response to treatment with both SMX-NO and PIP (Figure 7A-C). The number of Tregs generated was similar across the 3 donors and when the 2 haptens were compared.

**Discussion**

The development of a preclinical *in vitro* screening assay to predict the intrinsic T cell immunogenicity of drugs is becoming increasingly important for the drug discovery process. This is because drug withdrawal due to unwanted immunogenicity post-licensing leads to large monetary loss. Moreover, patients lose the benefit of useful and otherwise safe drugs. Any assay that screens for T cell drug immunogenicity must use primary human cells and encompass the variety of patient (e.g., HLA, T cell receptors and drug distribution) and environmental factors that influence the fine stimulatory/regulatory T cell triggering threshold.

Significant advances have been made in the development of *in vitro* assays which discriminate between chemicals that activate human T cells and induce allergic contact dermatitis, and those that do not (Martin *et al.*, 2010). Such assays focus on the naïve T cell compartment and utilize DC to present the chemical in an appropriate form (i.e., peptides derived from hapten-modified protein). Tregs are depleted prior to chemical exposure to obtain optimal assay conditions to identify a chemically-induced naïve T cell response. Thus, the output from the assay is an assessment of intrinsic compound immunogenicity. Modern day assays use proliferation and cytokine release as readouts to determine whether or not T cell activation has occurred after restimulation of the primed T cells with the chemical and a second batch of DC. Consistent T cell responses are detected, across multiple donors, to model probe substrates such as dinitrochrolobenzene when used directly or when conjugated to DC or model carrier proteins (Dietz *et al.*, 2010). However, other less well defined compounds have a tendency to yield inconsistent results.

We have recently used a similar DC T cell priming assay to explore the immunogenicity of direct covalent binding drugs, drugs that acquire reactivity through metabolism and drugs that modify HLA directly through reversible bonds (Faulkner *et al.,* 2016; Faulkner *et al.*, 2012; Gibson *et al.*, 2017b). The availability of a cell bank containing PBMC from 1200 HLA-typed, healthy drug-naïve donors permitted the assessment of the HLA-allele restricted forms of drug hypersensitivity. Drugs such as flucloxacillin, carbamazepine and allopurinol were found to interact preferentially with the proteins encoded by the HLA risk alleles to activate naïve T cells (Faulkner *et al.*, 2016; Usui *et al.*, 2018). In contrast, naïve T cell responses were either weaker or not detected in donors expressing other HLA alleles. Although the existing form of the DC T cell priming assay has been used effectively for retrospective mechanistic investigations and to study the potential immunogenicity of structurally-related compounds when a reaction has been detected in clinic, at present it is difficult to use to predict the likelihood that a novel compound in development will induce T cell responses. This is partly due to the complexity of the assay, in particular the requirement to restimulate primed T cells with additional DC. Furthermore, the precursor frequency of circulating naïve T cells that are subsequently activated by the drug is not taken into consideration and this can lead to negative or inconsistent results with PBMC from certain donors. Thus, this project focuses on development of the existing DC T cell priming assay into a simpler format not requiring a second batch of DC and with an expansive readout for proliferation that permits assessment of the number of responding T cells to a given drug alongside the strength of the induced response.

Method development work utilized SMX-NO, a haptenic drug metabolite that binds covalently to nucleophilic amino acids on proteins and/or HLA binding peptides to activate T cells from hypersensitive patients (Burkhart *et al.*, 2001; Castrejon *et al.*, 2010; Schnyder *et al.*, 2000). SMX-NO has also been shown to prime naïve T cells from almost 100% of healthy donors when Tregs are removed from the priming assay (Faulkner *et al.,* 2016). Since the primary objective of the T-MWA was to establish an assay that assesses the strength of a drug-specific T cell response induced across multiple cultures, DC and naïve T cells from the same donor were cultured with SMX-NO in up to 96 wells of a 96 well plate. Cultures were maintained for a period of 14 days to allow the primed T cells to return to a resting state. At this point, half of the wells were restimulated with SMX-NO before assessment of the level of proliferation in each individual well. Although not conducted herein, it is possible to estimate the SMX-NO-specific T cell precursor frequency using the data generated (Geiger *et al.*, 2009). An important step forward was the observation that a second batch of DC were not required at the restimulation step as the complete assay is conducted in the same culture plate. Statistically significant naïve T cell priming to SMX-NO was observed in all donors. The strength of the response induced with SMX-NO in individual wells was categorized as negative, weak, good and strong, according to the classification of Faulkner et al. (Faulkner *et al.,* 2016), with over 50% of the SMX-NO-primed wells displaying good or strong responses. To access the reproducibility of the T-MWA, T cell priming was performed with blood drawn 6 months apart from 3 donors. The number of wells responding to SMX-NO as well as the strength of the response induced in individual wells was strikingly similar.

Clinical studies are beginning to show that patients exposed to immune checkpoint inhibitors such as pembrolizumab (anti-PD-1) or ipilimumab (anti-CTLA4) develop more frequent hypersensitivity reactions to low molecular weight drugs. For example, Ford et al. (Ford *et al.,* 2018) described the development of sulfasalazine skin reactions in patients with pembrolizumab- or ipilimumab-treated metastatic melanoma. Furthermore, six out of seven patients that developed serious cutaneous reactions after exposure to the B-raf inhibitor vemurafenib had been exposed previously to anti-PD-1 antibody therapy (Uhara *et al.,* 2018). Finally, dacarbazine therapy was deemed intolerable when combined with ipilimumab due to serious liver injury (Yamazaki *et al.,* 2015). These date suggest that the immune checkpoint inhibitors influence the co-stimulatory/co-inhibitory balance, permitting the development of hypersensitivity in almost all patients. Our previous *in vitro* studies have shown that addition of anti-PD-L1 and anti-CTLA4 antibodies to the standard DC T cell priming assay resulted in significant increase in the strength of naive T cell priming. Addition of the same antibodies to the T-MWA had a tendency to increase the strength of the proliferative response in strongly responding wells. However, since the addition of immune checkpoint inhibitors (i) increased proliferation recordings in control wells and (ii) did not increase the number of wells that were primed to SMX-NO, they were not used in subsequent experiments.

To investigate the performance of the T-MWA with other haptenic compounds, we selected BB and PIP, chemical and drug haptens with known protein binding profiles (Jenkinson *et al.*, 2010; Meng *et al.,* 2017) and well-characterized T cell responses in patients with adverse reactions (Coulter *et al.,* 2008; Sullivan *et al.,* 2018; Whitaker *et al.*, 2011). Priming of naïve T cells was detected with both compounds using PBMC from three donors. The number of BB-responsive wells and the strength of the induced BB-specific T cell proliferative response was similar to, and in some cases stronger than that observed with SMX-NO. In contrast, PIP activated a smaller number of wells and the strength of the PIP-specific proliferative response was weak to good. The same three compounds were used in a variant of the T-MWA to assess T cell immunogenicity with PBMC from 8 donors in a single experiment. In this experiment 6 DC T cell co-cultures were primed to the test compounds and 3 were restimulated. Priming of naïve T cells was observed in all 8 donors with SMX-NO, 4 donors with BB and only 2 donors with PIP. These data clearly illustrate that an increased number of co-cultures needs to be established otherwise the number of compound-responsive donors will be underestimated using the T-MWA.

Tregs are known to control T cell responses to self and non-self protein antigens. Natural Tregs expressing CD4high, CD25high and CD127low and the FOXP3 transcription factor circulate in the periphery and are depleted from DC T cell co-culture assays to detect the priming of naïve T cells to drugs and chemicals (Faulkner *et al.,* 2012; Vocanson *et al.,* 2008). There is an increasing body of evidence to suggest that dysregulation of Tregs and therefore a reduction in the capacity to suppress effector T cell responses impacts on susceptibility to drug hypersensitivity reactions and possibly even the nature of the iatrogenic disease (Morito *et al.*, 2014; Takahashi *et al.,* 2009; Wang *et al.,* 2018). Naïve or inducible Tregs might also influence susceptibility to drug hypersensitivity by impacting on (i) the priming of naïve T cells or (ii) the strength of the recall response induced when memory T cells are stimulated. However, to date, little is known about whether drugs stimulate the expansion of naïve Tregs. To address this, Treg expression was measured during the priming of naïve T cells to SMX-NO and PIP. In each experiment, exposure to both drug haptens resulted in a time-dependent increase in the number of Tregs in the co-culture. Although the number of Tregs generated in the presence of SMX-NO and PIP did not differ, the ability (or not) of drugs to induce expression of Tregs may be an important, as yet under investigated, feature in determining the outcome of human drug exposure. Thus, future studies are planned to explore whether inducible Tregs are activated with drugs and if so to define pathways of effector T cell suppression.

In summary, the T-MWA represents an improved version of standard DC T cell priming assays. As we move forward we will begin to explore whether the assay can be used to study the priming of naïve T cells with drugs that interact directly with HLA. Further optimization might result in a screening assay for use by Pharma when an issue of immunogenicity is anticipated or when immunogenicity has been observed in clinical trials.

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**List of abbreviations**

Human leukocyte antigen, HLA; peripheral blood mononuclear cells, PBMC; dendritic cells, DC; stimulation index, SI; T cell multiple well assay, T-MWA; drug hypersensitivity reaction, DHR; nitroso sulfamethoxazole, SMX-NO; piperacillin, PIP; Bandrowski’s base, BB.

**Figure legends**

**Figure 1**

**Schematic representation showing the different components of the standard naïve T cell priming assay and the T-MWA.**

**Figure 2**

**Comparison of SMX-NO T cell priming using the standard naïve T cell priming assay and the T-MWA.**

Naïve T cells (2.5x106/well) obtained from healthy volunteers were co-cultured with autologous DC (8x104/well) and SMX-NO (40 µM) for 14 days in a 24-well plate for the standard priming assay. T cells were then harvested and plated in a 96-well plate in triplicate (1x105/well) with a second batch of autologous DC (4x103/well) and a range of SMX-NO concentrations (25-100µM) or media as negative control. After 48-hour incubation [3H]thymidine (0.5µCi/well; 16h) was added before plates were harvested and drug-specific T cell proliferation determined using a β-counter (counts per minute, cpm) **(A-C)**. In the T-MWA, naïve T cells (2 x 105/well) were plated in a U-bottomed 96-well plate with autologous DC (8 x103/well) with SMX-NO (40µM). After 14 days plates were centrifuged, and cells were washed to remove free drug from the media. Cells were gently re-suspended and re-challenged with SMX-NO (40µM); multiple media treated negative control wells were also established. After 48-hour incubation [3H]thymidine (0.5µCi/well; 16h) was added before plates were harvested and drug-specific T cell proliferation determined using a β-counter **(D-F)**. Stimulation index (SI) was calculated as mean cpm in drug treated wells / mean cpm in negative control wells. The degree of naïve T cell priming is represented as bar chats: negative response (SI <1.5 SI), weak response (SI = 1.5-1.99), good response (SI = 2-3.99), and strong response (>4 SI) **(G-I)**. Student’s T test was performed to determine statistical significance of T cell proliferation at different drug concentrations compared to no drug treatment. Statistical significance was determined as; \*p ≤ 0.05; \*\*p ≤ 0.005; \*\*\*p < 0.001.

**Figure 3**

**Assessment of intra-individual variability of T-MWA for SMX-NO T cell priming**

Naïve T cells (2x105/well) were plated in a 96-well plate with autologous DC (8 x103/well) and SMX-NO (40µM). After 14 days plates were centrifuged, and cells were carefully washed to remove free drug. Cells were gently re-suspended and re-challenged with SMX-NO (40µM) or media as a negative control; 8 wells of negative control, 40 drug-treated wells. After 48-hour incubation [3H]thymidine (0.5µCi/well; 16h) was added before plates were harvested and T cell proliferation determined using a beta-counter. Assays were repeated with the same donor, with blood taken 6 months apart (n = 3). SI was calculated as mean cpm in drug treated wells / mean cpm in negative control wells. The degree of naïve T cell priming is represented as bar chats: negative response (SI <1.5 SI), weak response (SI = 1.5-1.99), good response (SI = 2-3.99), and strong response (>4 SI). White bars show data from first experiment, while dark bars represent experiments performed 6 months later **(A-C)**.

**Figure 4**

**Effect of immune check point inhibition on SMX-NO T cell priming**

Naïve T cells (2x105/well) were plated in a U-bottomed 96-well plate with autologous DC (8x103/well) in the presence and absence of either PD-L1 or CTLA4 antibodies and SMX-NO (40µM). After 14 days plates were centrifuged, and cells were washed to remove free drug. Cells were gently suspended in media and re-challenged with SMX-NO (40µM); multiple media treated negative control wells were also established. After 48-hour incubation [3H]thymidine (0.5µCi /well; 16h) was added before plates were harvested and drug-specific T cell proliferation determined using a beta-counter (counts per minute, cpm) **(A, E and I)**. SI was calculated as mean cpm in drug treated wells / mean cpm in negative control wells. The degree of naïve T cell priming is represented as bar chats: negative response (SI <1.5 SI), weak response (SI = 1.5-1.99), good response (SI = 2-3.99), and strong response (>4 SI) **(B, F, J no inhibitor; C, G, K PD-L1 block; D, H, L CTLA4 block)**. Mann-Whitney test performed to determine statistical significance of T cell proliferation of drug-treated wells compared to no drug control. Statistical significance was determined as; \*p ≤ 0.05; \*\*p ≤ 0.005; \*\*\*p < 0.001.

**Figure 5**

**SMX-NO, PIP and BB T cell priming using the T-MWA.**

Naïve T cells (2x105/well) were plated in a U-bottomed 96-well plate with autologous DC (8x103/well) in the presence of SMX-NO (40µM), PIP (2mM) or BB (5µM). After 14 days plates were centrifuged, and cells were washed to remove free drug. Cells were gently suspended in media and re-challenged with SMX-NO (40µM), PIP (2mM) or BB (5µM); multiple media treated negative control wells were also established. After 48-hour incubation [3H]thymidine (0.5µCi /well; 16h) was added before plates were harvested and drug-specific T cell proliferation determined using a beta-counter (counts per minute, cpm) **(A, E and I)**. SI was calculated as mean cpm in drug treated wells / mean cpm in negative control wells. The degree of naïve T cell priming is represented as bar chats: negative response (SI <1.5 SI), weak response (SI = 1.5-1.99), good response (SI = 2-3.99), and strong response (>4 SI) **(B, F, J SMX-NO; C, G, K BB; D, H, L PIP)**. Mann-Whitney test performed to determine statistical significance of T cell proliferation of drug-treated wells compared to no drug control. Statistical significance was determined as; \*p ≤ 0.05; \*\*p ≤ 0.005; \*\*\*p < 0.001.

**Figure 6**

**SMX-NO, PIP and BB T cell priming with cells from eight donors using a minaturized T-MWA.**

Naïve T cells (2x105/well) were plated in *6 wells* of a U-bottomed 96-well plate with autologous DC (8x103/well) in the presence of SMX-NO (40µM), PIP (2mM) or BB (5µM). After 14 days plates were centrifuged, and cells were washed to remove free drug. Cells were gently suspended in media and re-challenged with SMX-NO (40µM), PIP (2mM) or BB (5µM) (3 wells per compound); 3 media treated negative control wells were also established. After 48-hour incubation [3H]thymidine (0.5µCi /well; 16h) was added before plates were harvested and drug-specific T cell proliferation determined using a beta-counter. SI was calculated as mean cpm in drug treated wells / mean cpm in negative control wells. **(A, SMX-NO; B, BB; C, PIP)**.

**Figure 7**

**Treg expression during SMX-NO and PIP T cell priming**

Priming cultures from 3 healthy donors were established through culture of naïve T cells (2.5x106/well) with autologous DC (8x104/well) and SMX-NO (40 µM) or PIP (2mM) for 14 days in a 24-well plate The number of Tregs (CD4hi, CD25hi, CD127low, FOXP3hi) were measured by flow cytometry as a percentage of CD4 cells on days 0, 1, 7 and 14.

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