**TITLE PAGE**

**Detection of primary T cell responses to drugs and chemicals in HLA-typed volunteers: implications for the prediction of drug immunogenicity**

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**Running title:** HLA alleles and drug hypersensitivity

**ABSTRACT**

A number of serious adverse drug reactions are caused by T-cells. An association with HLA alleles has been identified with certain reactions, which makes it difficult to develop standardized preclinical tests to predict chemical liability. We have recently developed aT cell priming assay using the drug metabolite nitroso sulfamethoxazole (SMX-NO). We now report on reproducibility of the assay, establishment of a biobank of PBMC from 1000 HLA-typed volunteers, and generation of antigen-specific responses to a panel of compounds. Forty T cell priming assays were performed with SMX-NO; 5 gave weak responses (1.5-1.9) and 34 showed good (SI 2.0-3.9) or strong responses (SI >4.0) using readouts for proliferation and cytokine release. Thus, SMX-NO can be used as a model reagent for *in vitro* T-cell activation. Good to strong responses were also generated to haptenic compounds (amoxicillin, piperacillin and Bandrowski’s base) that are not associated with an HLA risk allele. Furthermore, responses were detected to carbamazepine (in HLA-B\*15:02 donors), flucloxacillin (in one HLA-B\*57:01 donor) and oxypurinol (in HLA-B\*58:01 donors), which are associated with HLA-class I-restricted forms of hypersensitivity. In contrast, naïve T cell priming to ximelagatran, lumiracoxib and lapatinib (HLA-class II-restricted forms of hypersensitivity) yielded negative results. Abacavir, which activates memory T-cells in patients, did not activate naïve T-cells from HLA-B\*57:01 donors. This work shows that the priming assay can be used to assess primary T-cell responses to drugs and to study mechanisms T-cell priming for drugs that display HLA class I restriction. Additional studies are required to investigate HLA-class II-restricted reactions.

**Key words:** HLA, T cells, drug hypersensitivity

**INTRODUCTION**

The mechanisms involved in the etiology of adverse drug reactions (ADRs) are complex with both drug and patient-specific factors contributing towards susceptibility. Up to 20% of ADRs are classified as hypersensitivity reactions where the immune response causes an unexpected clinical reaction (White *et al* 2015). Hypersensitivity reactions frequently involve the skin but may also involve other organs such as the liver. Most chemicals, drugs and their metabolites are too small to act as conventional T cell antigens. However, they can become bound to carrier proteins which are large enough to be antigenic or in some instances they may bind directly to immune receptors resulting in cell activation (Adam *et al*. 2011). The role of T cells in the development of drug hypersensitivity has been studied for several drugs including abacavir, carbamazepine, sulfamethoxazole, piperacillin and allopurinol (Chessman *et al.* 2008, Ko *et al.* 2011, Naisbitt *et al*. 2001, El-Ghaiesh *et al.* 2012), but the critical factors determining which individuals among the treated population will suffer from drug hypersensitivity remains poorly understood.

Genome wide screens have identified an association with several different HLA alleles and drugs which cause both skin and liver reactions (Chung *et al.* 2004, Daly *et al.* 2009, Mallal *et al.* 2002). HLA molecules are key proteins that regulate T cell–mediated immunity. HLA class I molecules present antigens to CD8 T cells and HLA class II molecules present antigens to CD4 T cells. The relationship between HLA-restricted responses and the aetiology of drug hypersensitivity has been clearly demonstrated for HLA-B\*57:01 which is associated with abacavir and flucloxacillin hypersensitivity. Abacavir-specific CD8+ T cells respond only to antigen presented in the context of HLA-B\*57:01 (Chessman *et al.* 2008). In patients with drug-induced liver injury, flucloxacillin-protein adducts are presented to T cells by HLA-B\*57:01 via a hapten mechanism, whereas in normal volunteers when naïve T cells are primed *in vitro*, the primed T cells can respond to flucloxacillin presented on number of different alleles via a direct interaction (Monshi *et al.* 2013, Yaseen *et al.* 2015).

In order to investigate the role of specific HLA alleles in drug hypersensitivity we have established a biobank of peripheral blood mononuclear cells (PBMCs) isolated from HLA-typed volunteers (Alfirevic *et al.* 2012). We initially recruited 400 volunteers during 2009-2010. These volunteers are representative of the North-West population and consist of 64% female and 36% male volunteers with a mean age of 29 years (9+/-10 years, range 18-60 years). The ethnicity of the volunteers also reflects regional diversity which is primarily Caucasian (84%) with Asian Indians (6%), Chinese (4%) and Blacks (1%) as minor populations. Typing of HLA–A, B, C, DRB1 and DQB1 alleles showed that all major Caucasian haplotypes were represented (Alfirevic *et al*. 2012). We have now extended the biobank by an additional 600 volunteers, who were recruited during 2014 and here we report on the characteristics of these new volunteers.

Once the biobank was established, we developed a dendritic cell (DC) priming assay to detect drug-specific stimulation of naive T cells from the HLA typed volunteers (Faulkner *et al.* 2012). Initially we used nitroso-sulfamethoxazole (SMX-NO) to establish the assay. Naive T cells were incubated with SMX-NO and monocyte-derived dendritic cells (Mo-DC). Antigen-specific responses were then detected by proliferation and cytokine release and the cells phenotyped for chemokine receptors. Importantly, regulatory T-cells are removed prior to commencing experiments to reveal responses that would not be detectable if PBMC were cultured directly with drugs for a similar duration. In this study we report on the reproducibility of SMX-NO as a positive control and on the generation of drug-specific responses to 10 different chemicals/drugs: the beta lactam antibiotics amoxicillin, piperacillin and flucloxacillin, the anti-epileptic carbamazepine, the anticoagulant ximelagatran and its metabolite melagatran, the xanthine oxidase inhibitor oxypurinol (the active metabolite of allopurinol), the Cox-2 inhibitor lumiracoxib and 4 of its metabolites, the tyrosine kinase inhibitor lapatinib, the reverse transcriptase inhibitor abacavir and Bandrowski’s base, an oxidation product of the hair dye component *p*-phenylenediamine (Figure 1). T cell responses to SMX-NO, Bandrowski’s base, amoxicillin and piperacillin are not associated with a specific HLA allele whereas there is an HLA class I (B\*57:01 for flucloxacillin and abacavir (Daly *et al.*, 2009; Mallal *et al.*, 2008), B\*58:01 for oxypurinol (Hung *et al.*, 2005), B\*15:02 and A\*31:01 for carbamazepine (Chung *et al.*, 2004; McCormack *et al.*, 2011)) or class II (DRB1\*07:01;DQA1\*02:01 for ximelagatran and lapatinib (Kindmark *et al.*, 2008; Spraggs *et al.*, 2011), DRB1\*15:01; DQB1\*06:02 for lumiracoxib (Singer *et al.*, 2010)) association for the other drugs: Abacavir was included as reactions in patients are thought to occur following activation of pre-existing memory T cells (Lucas *et al.,* 2015). Collectively, our study shows that the established T-cell priming assay can be used as a test for non HLA-restricted forms of drug hypersensitivity. Furthermore, it is an effective tool to (1) study mechanisms of T-cell priming to drugs that display HLA class I restriction and (2) redesign drugs that cause HLA class I-restricted hypersensitivity in man.

**MATERIAL AND METHODS**

***HLA tissue bank.*** The study was approved by the North West of England Research Ethics Committee and all participants gave written informed consent. Recruitment of the volunteers was undertaken via advertisements in local newspapers and on the University of Liverpool website. Study eligibility and exclusion criteria have been published previously (Alfirevic *et al.* 2012). Healthy volunteers (n=600) were recruited from North West England following the protocol published previously and 100ml of blood was collected for DNA extraction and peripheral mononuclear cell isolation (Alfirevic *et al* 2012). PBMCs were frozen in aliquots of 10 million cells. We maintained volunteer confidentiality at all times by double coding DNA samples and cells and by restricting access to participant’s personal data to trained clinical personnel.

Genomic DNA was extracted from 10 ml venous blood using Chemagic magnetic separation (Chemagen, Baesweiler, Germany). High-resolution sequence-based HLA typing using next generation massively parallel sequencing platforms, was performed by Histogenetics laboratory (Histogenetics, New York, NY, USA) at the following six loci: HLA-A, -B, -C, -DRB1, -DQB1 and DQA1. Ambiguities were described by a suffix 'g'. Full sequencing data was obtained from 586 individuals. Individual lists of all HLA alleles are available on request. Allele, genotype and haplotype frequencies were calculated using the PyPop software (Lancaster *et al*. 2007).

***Tissue culture medium and reagents.*** All PBMCs were cultured in RPMI 1640 medium, containing 10% AB serum, 100 mM L-glutamine, 25 mM HEPES, 100 g/ml penicillin and 100 U/ml streptomycin and 25 g/ml transferrin. Penicillin and streptomycin were omitted when responses to amoxicillin, flucloxacillin and piperacillin were investigated. The AB serum was purchased from Innovative Research (Novi, MI, USA) and all other reagents were purchased from Sigma-Aldrich (Poole, UK). Magnetic beads for cell isolations were purchased from Miltenyi Biotec Ltd, (Bisley, UK). Antibodies used for flow cytometry were CD4-APC, CD8-PE, CD45RO-PerCP-Cy5.5, CLA-FITC (BD Biosciences, Oxford, UK) and CCR1-PE, CCR2-APC CCR3-FITC, CCR4-PE, CCR5-FITC, CCR8-FITC, CCR9-APC, CCR10-PE, CXCR3-APC, CXCR6-PE (R&D Systems, Abingdon, UK). Carbamazepine, piperacillin, tissue culture grade PHA-P, LPS (*E.coli* 0111:B4) and DMSO were purchased from Sigma-Aldrich. 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester, (CFSE) was purchased from eBioscience Ltd (Hatfield, UK). GM-CSF, IL-4 and TNF. were purchased from PeproTech (London, UK). Flucloxacillin (sodium salt for injection) was obtained from CP Pharmaceuticals Ltd (Wrexham, UK), nitroso-sulfamethoxazole (SMX-NO) from Dalton Pharma Services (Toronto, Canada), Bandrowski’s base from Apollo Scientific (Stockport, UK), amoxicillin, piperacillin, carbamazepine and oxypurinol were purchased from Sigma-Aldrich, abacavir and lapatinib were a gift from GlaxoSmithKline, Stevenage, UK) ximelagatran and melagatran were a gift from AstraZeneca R&D (Alderley Park, UK), and lumiracoxib and its metabolites were a gift from Novartis, (East Hanover, New Jersey, USA).

***Dendritic cell priming assay.*** The DC priming assay has previously been described in Faulkner *et al*. (2012). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from blood by density centrifugation and cell populations isolated by magnetic bead separation. CD14+ cells were isolated by positive selection and grown for 7-8 days in 800 U/ml GM-CSF and 800 U/ml IL-4. Monocyte-derived dendritic cells (Mo-DC) were matured overnight with 1 g/ml LPS and 50 ng/ml TNF before use.

Mo-DC at 8x104 cell/well and 2x106 cell/well naïve T cells, isolated by negative selection, were co-cultured for 8-14 days in the presence of drug in a 24 well plate at 2 ml/well. Flucloxacillin, piperacillin, amoxicillin and abacavir were dissolved in medium and all other drugs were dissolved in DMSO. A final concentration of 25-50 M SMX-NO, 50-100 M ximelagatran and melagatran, 100 g/ml carbamazepine, 5  Bandrowski’s base, 35 M abacavir, 50 M lumiracoxib and its metabolites, 50 M oxypurinol, 1-2 mM amoxicillin and piperacillin, 250-500 g/ml flucloxacillin (0.5-1 mM) and 2.5 M lapatinib were used for priming. Drugs and available metabolites were used in the DC priming assay at the highest non-toxic concentration, as assessed by measuring PHA-mediated PBMC proliferation in the presence of titrated concentrations of drug or its metabolites, or at concentrations that have previously been shown to activate T cells from hypersensitive patients.

T cell responses were then assessed by re-stimulation with fresh Mo-DC and drug. T cells were labelled with 2.5 M CFSE for 5 minutes. PHA at 5 g/ml was used as a positive control. T cell cultures were plated at 1x105 cells/well and Mo-DC at 8x104 cells/well (96 U plate or ELISpot plates, 200 l/well). Proliferation was measured after 3 days (3H-thymidine, CFSE staining). IFN, IL-13 and Granzyme B release were measured by ELISpot (Mabtech, Nacka Strand, Sweden) after 2 days. Chemokine receptor expression was measured after 6 days. A minimum of 20,000 cells were acquired by flow cytometry using a FACSCanto II (BD Biosciences, Oxford, UK) for CFSE analysis and a minimum of 50,000 events for chemokine receptor analysis. Data were analyzed by Cyflogic. The stimulation index is defined as the readout for drug divided by the readout for medium alone (cpm, % dividing cells, number of spots). The mean fluorescence index is defined as the mean fluorescence of unstained cells divided by the mean fluorescence of stained cells.

***Generation of T-cell clones*.** T cell cloning was performed on cells harvested from the initial co-cultures. Where an HLA Class I association with hypersensitivity has been shown then the CD4 and CD8 cells were separated using positive selection with CD8 microbeads according to the manufacturer’s instructions (Miltenyi Biotec Ltd, Bisley, UK). Serial dilution was performed with cells plated at 3 cells/well, 1 cell/well and 0.3 cells/well in the presence of 5 g/ml PHA, 250 U/ml IL-2 (PeproTech EC Ltd, London, UK) and irradiated allogeneic PBMCs at 5x104 cells/well as feeder cells (irradiated for 15 minutes with a dose of 50 Grays). T cell clones were then maintained in medium containing IL-2 and expanded by the addition of IL-2, PHA and irradiated allogeneic PBMC every 2 weeks.

Epstein-Barr virus (EBV) transformed B-cell lines were created from PBMC by transformation with supernatant from the virus-producing cell line B9.58. Lines were maintained in RPMI 1640 supplemented with 10% Foetal bovine serum (Invitrogen, Paisley, UK), 100 mM L-glutamine, 100 µg/ml penicillin, 100 U/ml streptomycin and used as a source of autologous antigen presenting cells.

***Statistics.*** Student’s T test and Mann Whitney Rank Sum test (SigmaPlot 12.5) was used to analyse the data.

**RESULTS**

***HLA typed tissue bank.*** The original HLA tissue bank, established in 2010, consisted approximately 80x106 PBMCs isolated from the blood of 400 normal volunteers. The volunteers were typed for HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 alleles (Alfirevic *et al.* 2012). Recently, we obtained ethical permission to extend this to 1000 volunteers. In 2014, over a period of 12 months, a total of 600 healthy unrelated individuals were recruited. Of these, 394 (66%) were female and 202 (34%) were male. If known, self-reported ethnicity was recorded for volunteers, their parents and paternal and maternal grandparents. A large proportion of volunteers (N=541) (90%) were white Europeans, of which 519 (96%) were UK born and 493 (91%) were White British. Only 23 (4%) of individuals self-reported mixed ethnic origin, 17 (3%) were from the Indian subcontinent, 9 (1.5%) Chinese and 7 (1%) were Black Africans. Age range was from 18 to 60 years (average 31.5 ± 10.6 years).

We generated HLA genotype data using sequence based typing for 586 individuals for the HLA-DQA1, in addition to the same five alleles as before. A large diversity of HLA alleles was found: 38 distinct HLA-A alleles, 66 HLA-B alleles, 29 HLA-C alleles, 40 HLA-DRB1 alleles, 20 HLA-DQB1 alleles, and 10 HLA-DQA1 alleles (n=203). The most common alleles in our cohort have similar frequencies as those reported for the UK population in the Allele frequency net database (http://www.allelefrequencies.net/). We estimated pairwise linkage disequilibrium (LD) and found the highest LD between the DRB1:DQA1 (D' 0.976), DRB1:DQB1 (D' 0.939), B:C (D' 0.921) and DQB1:DQA1 (D' 0.890) loci. In addition, we submitted our anonymised HLA-typing data in the subset of healthy volunteers from North West England to the Allele Frequency Net database (http://www.allelefrequencies.net/), which now contains information on adverse drug reactions associated with HLA alleles (Gonzalez-Galarza *et al*. 2015). The number of volunteers in our tissue bank with specific alleles relating to the drugs investigated in this study can be seen in Table 1.

***T cell priming to SMX-NO.*** Using SMX-NO as a model drug antigen, naive CD3 T cells were co-cultured with Mo-DC at a ratio of 25:1 in the presence of drug for 8 days. These cells were harvested and then re-stimulated with fresh Mo-DC and SMX-NO. IFN and IL-13 release was assessed by ELISpot and proliferation was assessed by 3H-thymidine incorporation and flow cytometry of CFSE labelled cells. Forty priming assays were performed where SMX-NO was used as the main drug of interest or included as a positive control. An example of an SMX-NO response is shown in Figure 2 where there is a dose dependent response to SMX-NO in all four readouts (3H-thymidine, CFSE, IFN and IL-13). When all 40 experiments were analysed, a significant response was observed in the presence of SMX-NO compared to medium alone in all four readouts (Figure 3). The strength of the response was then graded according to the stimulation index (SI). Only one of the 40 assays was negative (SI <1.49) and 5 assays gave weak responses (SI 1.5-1.99). The remaining assays showed good (SI 2.0-3.99) or strong responses (SI >4.0). Analysing the readouts separately gave between 78-92 % positivity (Table 2) where seven assays had a single readout (proliferation), nine assays had 2 readouts, eighteen assays had 3 readouts and six assays had 4 readouts.

***T cell priming to haptenic compounds with no HLA allele association:*** Having established the assay we were interested to see whether we could prime naive T cells to a variety of different drugs associated with drug hypersensitivity. We initially chose 3 common causes of skin hypersensitivity reactions where the role of T cells has already been established, but HLA allele associations have not been identified, namely the hair dye component Bandrowski’s base (Coulter *et al*. 2008, Jenkinson *et al*. 2010) and the -lactam antibiotics amoxicillin and piperacillin (El-Ghaiesh *et al.* 2012, Jenkins *et al.* 2013). For these 3 compounds, drug haptens have been detected in patients and are known to act as T cell antigens.

Amoxicillin and piperacillin-specific T cells have been detected in patients with cutaneous hypersensitivity reactions but not in non-hypersensitive patients or normal volunteers (El-Ghaiesh *et al.* 2012, Jenkins *et al.* 2013). Both drugs are β-lactam antibiotics that bind spontaneously to lysine residues on protein. Naïve T cells were activated by amoxicillin and piperacillin in the DC priming assay. Good or strong proliferative responses and IFNγ release were observed, from all 5 donors, when primed T cells were restimulated with piperacillin. With amoxicillin, good or strong proliferative responses and/or cytokine release were detected in 3 donors, weak responses were detected in 4 further donors and 2 donors were negative (Figure 4; Table 3).

Memory T cell responses to Bandrowski’s base can be detected in both patients hypersensitive to the hair dye component, *p*-phenylenediamine, and in normal volunteers (Coulter *et al*. 2008, Jenkinson *et al*. 2010). The DC priming assay was performed with PBMCs from normal volunteers to determine whether Bandrowski’s base also activates naïve T cells. All volunteers showed good or strong responses to Bandrowski’s base in at least one readout and at least half the volunteers showed good or strong responses in 2 readouts (Figure 4; Table 3).

***T cell priming to HLA-class I associated compounds.*** Allopurinol is one of the most common causes of severe cutaneous adverse reactions in Asia and Europe. There is a strong association between these reactions and expression of HLA-B\*58:01 in Han Chinese populations, in Japanese, Korean, Thai, and other Asian populations as well as European populations (Hung *et.al* 2005). T cell responses to the allopurinol metabolite, oxypurinol, can be detected in hypersensitive patients (Yun *et al.* 2013 & 2014, Lin *et al*. 2015). More importantly, the development of clinical symptoms and the presence of drug-specific T cells is restricted to HLA-B\*58:01 positive patients. Using the DC priming assay we attempted to generate T cell responses to oxypurinol from HLA-B\*58:01 positive and negative volunteers. Two out of three HLA-B\*58:01 volunteers showed good responses to oxypurinol by IFN ELISpot (Table 4), whereas all four HLA-B\*58:01 negative volunteers were negative in both readouts (data not shown).

Flucloxacillin-specific T cells can be isolated from patients with drug-induced liver injury (Monshi *et al.* 2013). Using the DC priming assay we attempted to generate T cell responses to flucloxacillin from 5 HLA-B\*57:01 positive and 4 HLA-B-57:01 negative volunteers. One donor had a good response and the remaining 4 volunteers showed only weak responses to flucloxacillin (Table 4). Four out of 5 volunteers only had a response in a single readout. For 3 of these volunteers chemokine receptor expression was obtained. All 3 volunteers showed higher expression of CCR2, CCR4, CCR9 and CXCR3 on CD45RO positive cells compared to CD45RO negative cells (Figure 5). Next we wanted to see whether we could prime T cells to flucloxacillin in 4 volunteers without the risk allele. Three volunteers were negative in at least 4 different readouts and one donor had a weak IL-13 response (data not shown).

Severe cutaneous hypersensitivity reactions to carbamazepine are associated with HLA-B\*15:02 in Chinese and Thai populations (Tasseneeyakul *et al*. 2010, Zhang *et al.* 2011) and with HLA-A\*31:01 in European populations (McCormack *et al*. 2011). Drug-specific CD4 and CD8 T cells have been isolated from these individuals (Wu *et al*. 2007, Ko *et al.* 2011, Wei *et al*. 2012, Lichtenfels *et al.* 2014). Using the DC priming assay we attempted to generate T cell responses to carbamazepine from 2 HLA-B\*15:02 volunteers and 2 HLA-A\*31:01 volunteers. Both HLA-B\*15:02 volunteers showed strong responses in at least 2 readouts, however, only weak responses were detected in the HLA-A\*31:01 volunteers (Table 4). The data for one of the HLA-B\*15:02 volunteers showing dose-dependent proliferation and cytokine release in the presence of carbamazepine is depicted in Figure 6. The CFSE data clearly shows a greater response to carbamazepine by CD8 rather than CD4 cells. Next we wanted to see whether we could prime T cells to carbamazepine in 3 volunteers without either risk allele. Two volunteers were negative in 4 different readouts and one donor had a weak IL-13 response to carbamazepine (data not shown).

Hypersensitivity reactions to abacavir are restricted to patients carrying the HLA-B57:01 allele (Mallal *et al.* 2002). Abacavir reactive T cells can be readily isolated from the memory compartment of treated patients and normal volunteers carrying the risk allele (Lucas *et al.*, 2015, Chessman *et al*. 2008, Adam *et al*. 2012, Illing *et al.*, 2012, Bell *et al*. 2013). Thus, we attempted to prime naïve T cells to abacavir using the DC assay. No positive responses were detected using a minimum of 2 different readouts in 4 different volunteers carrying HLA-B\*57:01 (Table 4).

***T cell priming to HLA-class II associated compounds.*** Lapatinib, ximelagatran and lumiracoxib are all associated with drug-induced liver injury (DILI) in a small number of patients. Genome wide screening of affected patients identified that the risk of DILI is associated with carriage of a specific HLA-DRB1 allele (Kindmark *et al*. 2008, Singer *et al.* 2010, Spraggs *et al.* 2011). A detailed analysis of the drug-specific T cell response has not been conducted. Despite, this we attempted to generate T cell responses to these drugs using the DC priming assay in 3-4 donors. Assessing 3-4 different readouts, no positive responses were detected for lapatinib, for the pro-drug ximelagatran (Table 4) or the active metabolite melagatran (data not shown). Assays using lumiracoxib and 4 stable metabolites showed a single weak IL-13 response for lumiracoxib (Table 4) and for one of the metabolites, 4’hydroxy-5-carboxyl lumiracoxib (data not shown). SMX-NO was included in every assay and each time gave a positive result.

***T cell cloning from the DC priming assay***. We have been successful in cloning drug-specific T cells from individuals with skin and liver reactions to a number of different drugs, even when assays such as the lymphocyte transformation test using PBMCs have been negative (El-Ghaiesh *et al.* 2012, Monshi *et al*. 2013, Lichtenfels *et al.* 2014). We have also been successful in cloning SMX-NO- and flucloxacillin-specific T cells from cultures set up during the DC priming assay (Monshi *et al*. 2013, Gibson *et al.* 2014). In the majority of cases cloning was successful when good or strong responses were detected in the DC priming assay (7/20), such as for Bandrowski’s base, piperacillin and carbamazepine (B\*15:02). However, we did have some success when only weak responses were detected in the DC assay for flucloxacillin and carbamazepine (A\*31:01) (Tables 3 & 4). When the DC priming assay was negative, such as for ximelagatran and lumiracoxib, then T cell cloning also failed. T cell cloning was performed on ximelagatran co-cultures and 288 T cell clones were selected for each of the 3 volunteers. However, none of these clones were drug-specific on testing. T cell cloning from lumiracoxib co-cultures resulted in isolation of 228 and 198 T cell clones from 2 different donors and cloning from the co-cultures of the lumiracoxib metabolites, from a single donor, resulted in selection of 153, 35, 160 and 190 T cell clones for 5-carboxy lumiracoxib, 4’hydroxy-5-carboxyl lumiracoxib, 4’hydroxy lumiracoxib and 5-hydroxy lumiracoxib, respectively. None of these clones were drug-specific on testing (data not shown).

**DISCUSSION**

Drug-responsive T-cells are detectable in skin and blood of patients with delayed-type cutaneous hypersensitivity reactions (Uetrecht and Naisbitt 2013). Cloned T cells from these patients secrete cytolytic molecules and kill autologous targets following drug exposure (El-Ghaiesh *et al.*, 2012; Kuechler *et al.*, 2004; Nassif *et al.*, 2004; Wu *et al.*, 2007), which indicates that they are directly involved in the disease pathogenesis. Recently, drug-specific T cells have also been cloned from patients with liver injury induced by flucloxacillin (Monshi *et al.*, 2013), co-amoxiclav (Kim *et al.* 2015) and isoniazid (unpublished data). This demonstrates that T cells can participate in hypersensitivity reactions targeting different organs. Genome-wide association studies have shown that some, but not all forms of hypersensitivity are associated with expression of specific HLA alleles, which suggests that the drug-derived antigen binds selectively to the protein encoded by the HLA allele to activate T cells (Phillips *et al.*, 2011). For a limited number of drug reactions associated with specific HLA class I alleles such as abacavir (Chessman *et al.*, 2008), oxypurinol (Yun *et al*., 2013), flucloxacillin (Monshi *et al.*, 2013a) and carbamazepine (Wei *et al.*, 2012), it has been possible to show a selective drug-specific and HLA allele-restricted activation of CD8 T cells. In contrast, it is not known whether the HLA class II associations relate to the drug-specific CD4 T cells.

*In vitro* assays for the prediction of whether a drug or chemical activates naive T cells are at a very early stage of development and have been designed using only 2 or 3 well-defined antigens i.e., dinitrohalobenzenes and SMX-NO (Dietz *et al.*, 2010; Faulkner *et al.*, 2012; Karton and Martin, 2012; Martin *et al.*, 2010). For effective priming, Tregs must be removed and the drug antigen needs to be presented by autologous DCs. One of the reasons why a test for detecting T cell responses to drugs has not been established is that cells from volunteers expressing specific HLA alleles are required. Thus, the purpose of the current study was to expand our cryopreserved HLA-typed volunteer PBMC bank to explore (1) reproducibility of the existing DC priming assay and (2) whether the assay is sufficiently sensitive to detect T cell responses to a variety of drug/chemical antigens (several associated with HLA class I or II risk alleles) that activate T cells via different pathways. Our tissue bank is primarily composed of PBMCs from white European/British individuals (90%) and therefore some alleles, especially those from Asian populations such as HLA-B\*15:02, are under-represented. This does limit our ability to investigate some HLA-specific T-cell responses and the ethnicity of our tissue bank must be taken into account when assessing our results on non-HLA specific responses.

SMX-NO-responsive T cells circulate in 100% of hypersensitive patients presenting with different forms of cutaneous hypersensitivity reaction (Castrejon *et al.*, 2010; Nassif *et al.,* 2004; Schnyder *et al.*, 2000). SMX-NO is also a potent T cell immunogen in rodents and rabbits (Farrell *et al.*, 2003; Naisbitt *et al.*, 2002) and it activates human volunteer PBMC following repeated rounds of stimulation (Engler *et al.*, 2004). Thus, SMX-NO represents the ideal compound to explore reproducibility of the DC priming assay. Forty priming assays were performed using SMX-NO and only one of these failed to result in any priming. Five assays gave weak responses and the remaining assays showed good or strong responses with a stimulation index greater than 2.0. Measuring proliferation resulted in a greater number of positive assays compared to cytokine release but the number of assays showing a good to strong response was similar in all 4 readouts.

Since SMX-NO-specific T cell responses were detected in the majority of volunteers then factors other than drug metabolite exposure must determine susceptibility to hypersensitivity. HLA alleles are not major predisposing factors for this drug (Alfirevic *et al.*, 2009; Vitezica *et al.*, 2008). An attractive alternative explanation is disruption of immune regulation, which might be both drug-specific and disease-specific. In a recent study we showed that blockade of PD1/PDL1 co-inhibition during DC priming to SMX-NO effectively enhances to magnitude of the T cell response (proliferation and cytokine release) (Gibson *et al.*, 2014), while addition of Tregs blocks priming (unpublished observation). Confirmation that disruption of co-stimulatory/co-inhibitory signalling is a determinant of susceptibility will require a large scale prospective study, with collection of PBMCs prior to and during drug exposure, to allow analysis of patients which do and do not develop hypersensitivity reactions.

To explore whether T cell responses against other drugs/chemicals is detectable using the DC priming assay we focused on 3 haptenic compounds not associated with a specific HLA-risk allele; namely, piperacillin, amoxicillin and Bandrowski’s base. Piperacillin and amoxicillin bind covalently to specific lysine residues on protein to generate adducts that activate T-cells. Bandrowski’s base is a trimer of the hair-dye component *p*-phenylenendiamine, which covalently modifies cysteine residues on protein (Jahn *et al.*, 2012). We were successful in priming naïve T cells from all the donors tested against all 3 compounds. Re-stimulation of the primed T cells with the relevant drug/chemical resulted in good or strong proliferative responses and IFN or IL-13 secretion. Amoxicillin- and piperacillin-responsive CD4 T cell clones generated from the primed volunteer cells had a similar phenotype to those isolated hypersensitive patients (El-Ghaiesh *et al.,* 2012). Bandrowski’s base-responsive T cells from the hypersensitive patients secrete high levels of Th2 cytokines following T cell receptor triggering (Coulter *et al.*, 2010; Coulter *et al.*, 2008; Jenkinson *et al.*, 2010). Naïve T cells from all 6 volunteers were activated with Bandrowski’s base and stimulation of the primed cells was associated with secretion of IL-13, but not secretion of IFN, which is consistent with a Th2 phenotype.

The next component of the study was to explore whether haptenic or non-haptenic drugs associated with HLA class I-restricted forms of skin or liver injury activate naïve T cells *in vitro.* To do this, our HLA-typed cell bank was expanded to 1000 volunteers. The population of volunteers is typical of the North West of England and all the common alleles were represented. The most common alleles in our cohort have similar frequencies as those reported for the UK population in the Allele frequency net database. The first drug selected was the β-lactam antibiotic flucloxacillin. Flucloxacillin-induced liver injury is strongly associated with expression of the HLA class I allele, B\*57:01 (Daly *et al.,* 2009). Cloned flucloxacillin-specific T cells from patients are predominantly CD8 and restricted to antigen presenting cells expressing the HLA risk allele (Monshi *et al.,* 2013; Yaseen *et al.* 2015). Priming of naïve T cells to flucloxacillin was attempted with 4 volunteers expressing HLA-B\*57:01 and 4 volunteers expressing other HLA-B alleles. Weak responses were detected with PBMC from all 4 HLA-B\*57:01 volunteers and isolation of CD8 flucloxacillin-responsive clones was successful when attempted (i.e., from 3 patients). The strength of the flucloxacillin-specific T cell response following DC priming was lower than that for piperacillin- and amoxicillin-responsive T cells, which likely relates to the difference types of T cell preferentially activated by these drugs. Flucloxacillin preferentially activates CD8 cells which generally have a lower proliferative capacity *in vitro* than the CD4 cells. CD8 T cell clones from patients with liver injury express high levels of the chemokine receptors CCR4, CCR9 and CXCR3 and migrate towards CCL17 and CCL25 (Monshi *et al.,* 2013). Analysis of chemokine receptor expression on flucloxacillin-primed T cells from volunteers also showed higher expression of CCR4, CCR9 and CXCR3 on CD45RO positive cells compared to CD45RO negative cells, indicating the same homing characteristics for flucloxacillin-specific T cells derived both volunteers and patients with liver injury. Wuillemin *et al*. (2013, 2014) have also been able to isolate flucloxacillin-specific CD8 T cell clones from HLA-B\*57:01 negative donors after repeated stimulation of PBMC for 4-5 weeks. Activation of naïve T cells was observed using the DC priming assay with PBMC from 1 out of 4 HLA-B\*57:01 negative volunteers, which suggests that it is more difficult to activate T cells with flucloxacillin in volunteers that do not express the risk allele.

Two non-haptenic drugs carbamazepine and oxypurinol (the active metabolite and T-cell stimulatory form of allopurinol) were selected next. Although some carbamazepine metabolites are capable of acting as haptens following bioactivation (Pearce *et al.*, 2005; Pearce *et al.*, 2002), T cells from hypersensitive patients have been shown to respond to the parent drug bound directly to HLA class I alleles, without the need for antigen processing, hence we have classified carbamazepine as non-haptenic. Carbamazepine and allopurinol hypersensitivity reactions are strongly associated with specific HLA class I alleles: B\*58:01 (Hung *et al.,* 2005) for allopurinol; B\*15:02 (Chung *et al.,* 2004) and A\*31:01 for carbamazepine (McCormack *et al.,* 2011). Memory T cell responses to the parent drugs are readily detectable in hypersensitive patients and/or healthy volunteers carrying the risk alleles (Yun *et al.,* 2013 & 2014; Ko *et al.*, 2011; Lichtenfels *et al.*, 2014; Wei *et al.,* 2012). Good to strong priming of naïve T-cells was observed with carbamazepine using volunteer PBMC expressing HLA-B\*15:02. Most carbamazepine-responsive clones generated after priming were CD8 T cells, which is in agreement with the strong genetic association with a class I allele. T cell priming was also observed with PBMC from HLA-A\*31:01 positive volunteers, but the strength of the response was much weaker than that observed with HLA-B\*15:02 positive volunteers. Nevertheless, carbamazepine-responsive CD8+ T-cell clones were generated from one of the volunteers following priming. Good or strong priming of naïve T cells was also detectable with oxypurinol in 2 out of 3 volunteers expressing HLA-B\*58:01..

In contrast to carbamazepine and oxypurinol, priming of naïve T cells in HLA-B\*57:01 volunteers was not observed with abacavir. This was somewhat surprising as it is simple to isolate abacavir-responsive CD8 T cell clones from PBMCs of HLA-B\*57:01 positive volunteers (Adam *et al.,* 2012; Adam *et al.*, 2014; Bell *et al.*, 2013). However, our data agrees with Lucas *et al.* (2015) who have shown that abacavir activates pre-existing memory T cells primed by earlier exposure to another antigen. These cells are depleted prior to DC priming.

The final experiments attempted to prime naïve T cells against three drugs that are associated with DILI. Ximelagatran and lumiracoxib were withdrawn from use because of an unacceptable incidence of liver injury. Lapatinib is approved for the treatment of HER2 over-expressing metastatic breast cancer, in combination with chemotherapy. However, the prescribing information for Tykerb/Tyverb has a FDA Black Box Warning for hepatotoxicity and an EMA special warnings and precautions for hepatotoxicity. In each case an HLA class II association has been identified (Kindmark *et al.,* 2008; Spraggs *et al.,* 2011; Singer *et al.,* 2010). In contrast to the drugs discussed above, the phenotype and function of T cells in patients with liver injury due to these drugs has not been defined. Priming of naïve T cells was not detected with ximelagatran, melagatran or lapatinib using PBMC from HLA-DRB1\*07:01 positive volunteers. Furthermore, weakly positive results were only recorded with 2 out of 52 experiments in the lumiracoxib (Table 4) and lumiracoxib metabolite priming experiments with PBMCs from HLA-DRB1\*15:01 positive volunteers. Drug(metabolite)-specific proliferative responses were not detected after testing over 1000 individual clones generated from the ximelagatran or lumiracoxib priming assays. The reason for the negative results is unclear. It is possible that the HLA association does not relate directly to the activation of drug-specific CD4+ T cells. Alternatively, the assay might require refinement in order to generate the appropriate drug antigen within the target tissue to activate T cells. In this respect, we are currently investigating whether drug-treated primary hepatocytes transfer signals to immune cells during priming altering the balance between DC co-stimulatory/co-inhibitory signalling.

In conclusion, we describe a test system for the prediction of non-HLA restricted drug-specific T-cell responses. The availability of a cell bank containing viable HLA-typed PBMC from 1000 volunteers provides direct access to appropriate cells to investigate hypersensitivity reactions associated with specific HLA alleles. We have successfully primed naïve T cells from healthy volunteers against a range of HLA class I-restricted drug antigens and as such the assay may be used to help redesign drugs that cause HLA class I associated hypersensitivity reactions. However, further work is needed to determine whether the increasing number of drug reactions associated with an HLA class II allele involve drug-specific CD4+ T cells.

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**FIGURE LEGENDS**

**Figure 1. Drugs used in the DC priming assay**.

**Figure 2. Priming of naïve T cells to SMX-NO.** Naive CD3 cells were co-cultured with Mo-DC at a ratio of 25:1 in the presence of SMX-NO for 8 days. These cultures were then re-stimulated with fresh Mo-DC and SMX-NO. Proliferation was measured by 3H-thymidine incorporation or CFSE staining after 3 days. Cytokine release was measured by ELISpot after 2 days. The data was analysed by Student’s t Test and \* denotes statistically significance p< 0.05.

**Figure 3. Priming of naïve T cells to SMX-NO.** Naive CD3 cells were co-cultured with Mo-DC at a ratio of 25:1 in the presence of SMX-NO for 8 days. These cultures were re-stimulated with fresh Mo-DC and SMX-NO. Proliferation was measured after 3 days and cytokine released was measured after 2 days. The data shows the maximal SMX-NO response (25 µM or 50 µM SMX-NO) from 40 assays. The box plot shows the median and 25th/75th percentiles, with error bars showing the 10th/90th percentiles. The data was analysed by the Mann Whitney Rank Sum Test.

**Figure 4. Priming of naïve T cells to haptenic compounds.** Naive CD3 cells were co-cultured with Mo-DC at a ratio of 25:1 in the presence of various drugs for 8-14 days. The cultures were then re-stimulated with fresh Mo-DC and drug. Proliferation was measured after 3 days by 3H-thymidine incorporation (amoxicillin, piperacillin, Bandrowski’s base [BB], SMX-NO). The highest stimulation index from the dose range for each drug was selected. The SMX-NO results shown are the positive controls included in the piperacillin assays.

**Figure 5. Chemokine receptor expression on flucloxacillin-primed T-cells.** Naive CD3 cells were co-cultured with Mo-DC at a ratio of 25:1 in the presence of flucloxacillin for 8 days. These cultures were re-stimulated with fresh Mo-DC and drug. Chemokine receptor expression was measured after 6 days by flow cytometry. Open symbols show expression on CD45RO negative cells and filled symbols show expression on CD45RO positive cells. Shapes show 3 individual donors.

**Figure 6. Priming of naïve T cells to carbamazepine.** Naive CD3 cells from donor HLA-164 were co-cultured with Mo-DC at a ratio of 25:1 in the presence of carbamazepine for 8 days. The cultures were then re-stimulated with fresh Mo-DC and drug. Proliferation was measured after 3 days by 3H-thymidine incorporation (A) and by CFSE incorporation (B). Cytokine release was measured after 2 days by ELISpot (C). The data was analysed by the Mann Whitney Rank Sum Test and was statistically significant (shown by \*) for proliferation measured by 3H-thymidine incorporation (p<0.001) and for IFNγ release and for IL-13 release (p<0.05).

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|  | **Table 1. HLA allele frequency in the HLA tissue bank** | | |
| **Drug** | **HLA alleles** | **Heterozygous** | **Homozygous** |
| Carbamazepine | A\*31:01 | 53 | 0 |
| B\*15:02 | 11 | 0 |
| Ticlopidine | A\*33:03 | 20 | 1 |
| Abacavir  Flucloxacillin | B\*57:01 | 71 | 3 |
| Allopurinol | B\*58:01 | 18 | 0 |
| Ximelagatran  Lapatinib | DRB1\*07:01/DQA1\*02:01 | 142 | 16 (both alleles) |
| Co-amoxiclav  Lumiracoxib | DRB1\*15:01/DQB1\*06:02 | 238 | 22 (both alleles) |

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| **Table 2. SMX-NO assays** | | | |
|  | | **Response** | **Number** |
| Proliferation | **Thymidine** | - | 2 |
| + | 5 |
| ++ | 13 |
| +++ | 2 |
| Total positive | 18/22 (81.8%) |
| **CFSE** | - | 2 |
| + | 5 |
| ++ | 13 |
| +++ | 6 |
| Total positive | 24/26 (92.3%) |
| ELISpot | **IFN** | - | 7 |
| + | 6 |
| ++ | 12 |
| +++ | 7 |
| Total positive | 25/32 (78.1%) |
| **IL-13** | - | 4 |
| + | 1 |
| ++ | 12 |
| +++ | 7 |
| Total positive | 20/24 (83.3%) |
| (-) no response with stimulation index <1.49  (+) weak response with stimulation index 1.5-1.99  (++) good response with stimulation index 2.0-3.99  (+++) strong response with stimulation index >4.0 | | | |

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| **Table 3. Priming of naïve T cells to compounds not associated with an HLA risk allele** | | | | | | |
|  |  |  | **Proliferation** | **ELISpot** | | **Cloning** |
| **Alleles** | **Drug** | **Donor** | **3H** | **IFN** | **IL-13** |  |
| None | Bandrowski’s Base  (Skin) | V01 | ++ | - | + |  |
| V02 | ++ |  | + | Yes |
| V03 | +++ | - | ++ |  |
| V04 | + |  | ++ |  |
| V05 | ++ | + | +++ |  |
| V06 | ++ | + | +++ |  |
| None | Piperacillin  (Skin) | V07 | ++ | +++ | + | Yes |
| V08 | ++ | ++ | - | Yes |
| V09 | ++ | + | - | Yes |
| V10 | +++ | +++ | - | Yes |
| V11 | ++ | - | - |  |
| None | Amoxicillin  (Skin) | H395 | ++ |  |  | Yes |
| H419 | + | - | - | No |
| H426 | +++ | ++ | +++ | No |
| H436 | - | + | + |  |
| H747 | + | - | - |  |
| H897 | - |  |  |  |
| V12 | - |  |  |  |
| V13 | + |  |  | Yes |
| V14 | ++ |  |  |  |
| (-) no response with stimulation index <1.49  (+) weak response with stimulation index 1.5-1.99  (++) good response with stimulation index 2.0-3.99  (+++) strong response with stimulation index >4.0 | | | | | | |

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| **Table 4. Priming of naïve T cells to drugs associated with an HLA risk allele** | | | | | | | | |
| ***HLA class I associated reactions*** | | | | | | | | |
|  | | | **Proliferation** | | **ELISpot** | | | **Cloning** |
| **Alleles** | **Drug** | **Donor** | **3H** | **CFSE** | **IFN** | **IL-13** | **Gran B** |  |
| B\*57:01 | Abacavir  (Skin) | H155 |  | - | - |  |  |  |
| H164 | - | - | - |  |  |  |
| H364 |  | - | - |  | - |  |
| H389 |  | - | - |  | - |  |
| B\*57:01 | Flucloxacillin  (Liver) | H027 | - | + | - | - | - | Yes |
| H202 |  | + | - |  | + |  |
| H307 | - |  | ++ |  |  | Yes |
| H364 |  | + | - |  | - | Yes |
| H389 | - |  | - |  | + |  |
| B\*58:01 | Oxypurinol  (Skin) | H710 | - |  | +++ |  |  |  |
| H747 | - |  | - |  |  |  |
| H749 | + |  | ++ |  |  |  |
| B\*15:02 | Carbamazepine  (Skin) | H164 | +++ | ++ | ++ | ++ |  | Yes |
| H295 | ++ | - | - |  | ++ |  |
| A\*31:01 | Carbamazepine  (Skin) | H015 | + |  | - | - | + | No |
| H142 | + | - | - | - | - | Yes |
| ***HLA class II associated reactions*** | | | | | | | | |
| DRB1\*0701  DQA1\*02:01 | Ximelagatran  (Liver) | H327 | - |  | - | - |  |  |
| H367 | - |  | - | - |  | No |
| H331 | - |  | - | - |  | No |
| H399 | - |  | - | - |  | No |
| DRB1\*07:01  DQA1\*02:01 | Lapatinib  (Liver) | H019 |  | - | - | - |  |  |
| H038 |  | - | - |  |  |  |
| H764 | - | - | - | - |  |  |
| H979 |  | - | - | - |  |  |
| DRB1\*15:01  DQB1\*06:02 | Lumiracoxib  (Liver) | H015 | - | - | - | + |  |  |
| H040 |  | - | - | - |  |  |
| H198 | - | - | - | - |  | No |
| H292 | - | - | - | - |  | No |
|  | (-) no response with stimulation index <1.49  (+) weak response with stimulation index 1.5-1.99  (++) good response with stimulation index 2.0-3.99  (+++) strong response with stimulation index >4.0  Gran B = Granzyme B | | | | | | | |