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Title page

Shedding light on drug-induced liver injury: activation of T-cells from drug naive human donors with tolvaptan and a hydroxybutyric acid metabolite

Sean Hammond,[*] Andrew Gibson,[*] Kanoot Jaruthamsophon,[*] Sharin Roth,[†] Merrie Mosedale,[‡] Dean J. Naisbitt[*]

[*]MRC Centre for Drug Safety Science, Department of Molecular and Clinical Pharmacology, University of Liverpool, Ashton Street, Liverpool L69 3GE, UK; [†]Otsuka Pharmaceutical Dev. & Comm., Inc., Research Blvd, Rockville, MD 20882, USA; [‡]Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, Chapel Hill, North Carolina 27599, USA

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

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

Running title: T-cells in drug-induced liver injury

Abstract

Exposure to tolvaptan is associated with a significant risk of liver injury in a small fraction of patients with autosomal dominant polycystic kidney disease. The observed delayed onset of liver injury of between 3 and 18 months after commencing tolvaptan treatment, along with rapid recurrence of symptoms following re-challenge is indicative of an adaptive immune attack. This study set out to assess the intrinsic immunogenicity of tolvaptan and pathways of drug-specific T-cell activation using *in vitro* cell culture platforms. Tolvaptan (n=7), as well as oxybutyric (DM-4103 n=1) and hydroxybutyric acid (DM-4107 n=18) metabolite-specific T-cell clones were generated from tolvaptan naive healthy donor peripheral blood mononuclear cells. Tolvaptan and DM-4103 T-cell clones could also be activated with DM-4107, while T-cell clones originally primed with DM-4107 were highly specific to this compound. A signature cytokine profile (IFN-γ, IL-13, granzyme B and perforin) for almost all T-cell clones was identified. Mechanistically, compound-specific T-cell clone activation was dependent on the presence of soluble drug and could occur within 4 hours of drug exposure, ruling out a classical hapten mechanism. However, antigen processing dependence drug presentation was indicated in many T-cell clones. Collectively these data show that tolvaptan-associated liver injury may be attributable to an adaptive immune attack upon the liver, with tolvaptan- and metabolite-specific T-cells identified as candidate effector cells in such aetiology.

Key words: Tolvaptan, drug-induced liver injury, T-lymphocytes, ADPKD, human.

Introduction

Drug-induced liver injury denotes a formidable iatrogenic disease which, depending on frequency and severity of manifestation, can result in impediment of a drugs clinical utility; ranging from contraindications, black box warnings and mandated routine liver chemistry screening through to outright withdrawal. Retrospective analysis of patients with drug-induced liver injury and population-matched controls has identified genetic risk factors for drug-induced liver injury reactions associated with several drugs that appear to be immune-mediated. In particular, specific expression of human leukocyte antigen (HLA) class I and II genes has exhibited considerable positive predictive value, with examples including flucloxacillin [HLA-B*5701] (Daly et al., 2009), ximelagatran [DRB1*07:01, DQA1*02:01] (Kindmark et al., 2008), and amoxicillin-clavulanic acid [A*02:01, A*30:02, B*18:01, DRB1*15:01-DQB1*0602 haplotype] (Lucena et al., 2011). Whilst not uniform across all drug-induced liver injury, such associations provide a potential tool for pre-emptive selection of alternative treatment pathways as epitomized with abacavir [HLA-B*5701] (Mallal et al., 2008), and provisional mechanistic insight, as proteins encoded by these genes are accountable for the presentation of drugs to T-lymphocytes. Indeed, *in vitro* platforms that explore T-cell responses in patients and healthy donors have provided tangible evidence for the role of the adaptive immune system for two of the aforementioned drug-induced liver injury -HLA associations (Monshi et al., 2013; Kim et al., 2015).

In recent studies we have identified of tolvaptan and tolvaptan metabolite-responsive T cells within the peripheral circulation of trial subjects with tolvaptan-associated liver injury (Hammond et al., manuscript under review). However, long-term culture and expansion of the drug-responsive T-cells was not possible and detailed characterization was not feasible. Thus, the objective of this study was to investigate the intrinsic antigenicity/immunogenicity of

tolvaptan through the use of *in vitro* peripheral blood mononuclear cell culture platforms generated from samples of healthy donors. This is possible because tolvaptan-associated liver injury is not associated with or dependent on expression of a single HLA genotype and the majority of individuals have a T-cell repertoire for drugs (e.g., abacavir; Schnyder et al., 2013), drug haptens (e.g., β-lactam antibiotics; Azoury et al., 2018) and drug metabolites (e.g., nitroso sulfamethoxazole; Faulkner et al., 2016). Furthermore, the assays used have been designed to detect intrinsic drug immunogenicity in a small number of donors through presentation of the drug by matured dendritic cells provided with optimal co-stimulatory signals for activation and depletion of memory and regulatory T-cells (Faulkner et al., 2012; Faulkner et al., 2016; Ogese et al., 2020). Tolvaptan is an effective drug for the treatment of autosomal dominant polycystic kidney disease (ADPKD). However tolvaptan-associated liver injury was first reported in two prospective, randomised phase 3 clinical trials: Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and Its Outcomes (TEMPO) 3:4 (NCT00428948), and the open label follow on trial TEMPO 4:4 (NCT01214421) (Watkins et al., 2015). The adverse events were observed between 3 and 18 months after commencing tolvaptan treatment. These observations, along with rapid recurrence of symptoms following re-challenge are indicative of an adaptive immune attack. Substantiating this claim is data from a genetically diverse mouse population and a hepatocyte study which suggest that tolvaptan may modulate the immune system (Mosedale et al., 2017; Mosedale et al., 2018). Previous studies with other hypersensitivity causing compounds have demonstrated the importance of stable and reactive metabolites in the determination of T-cell immunogenicity (Wu et al., 2006; Castrejon et al., 2010). The metabolism of tolvaptan is complex and extensive, with approximately 20 phase I metabolism-derived metabolites having been identified to date (Mazzarino et al., 2017). Therefore, the intrinsic immunogenic properties of two predominant metabolites of tolvaptan, putatively derived via azepine ring cleavage and subsequent

carboxylation, DM-4103 an oxybutyric acid derivative, and DM-4107, a hydroxybutyric acid derivative, were also evaluated in this study. The phenotypic and functional characteristics of the T-cells were explored alongside an evaluation of pathways of T-cell activation.

Materials and Methods

Human Subjects.

Blood was collected from 19 healthy donors (3 for T-cell priming and cloning experiments, 16 for generation of HLA-typed antigen presenting cells). The study was ethically authorized by the Liverpool local research ethics committee, and informed written consent was obtained from each donor. 108 mL of blood was collected for peripheral blood mononuclear cell and DNA isolation. Chemagic magnetic separation (Chemagen, Baesweiler, Germany) was used to extract genomic DNA, and high-resolution sequence-based HLA typing was performed by the Histogenetics laboratory (Histogenetics, Ossining, NY) at the following loci: HLA-A, -B, -C, -DRB1, -DQB1, and DQA1. The HLA type of the healthy donors used in cloning experiments are shown in Table 1; HLA typing data of additional antigen presenting cells are shown in the relevant figures.

Priming Naïve T-Cells from Drug-Inexperienced Healthy Donors

Healthy donor peripheral blood mononuclear cells were separated and utilized in an established *in vitro* priming assay as previously described (Faulkner et al., 2012). Co-cultures were set up using 8 x 10^4 mature dendritic cells, (2 x 10^5) naïve CD3 T-cells, and drug derivative; tolvaptan (10-30 μM), DM-4103 (20-40 μM), DM-4107 (125-250 μM), and SMX-NO (20-40 μM, positive control) per well (final volume 2 mL), and incubated for 12 days (37 ^{0}C; 5% CO_2). Interrogation of compound specificity was conducted using a re-constituted co-culture consisting of generated primed T-cell lines (1 x 10^5), fresh dendritic cells (4 x 10^3) and relevant compound at the respective priming concentration. Co-cultures were incubated for 48 hours after which compound specificity was assessed via specific proliferation ([^{3}H] tritiated thymidine incorporation) and IFN-γ release (ELISpot; Mabtech Inc, USA).

Generation of T-cell clones

Bulk cultures were generated through 14 day culture of the drug-inexperienced healthy donor peripheral blood mononuclear cells (2 x 10^6 cells/well; 1 mL) in the presence of tolvaptan (10-30 μM), DM-4103 (20-40 μM), and DM-4107 (125-250 μM), in R9 medium (RPMI 1640 supplemented with 10% human AB serum (Class A; Innovative Research Inc., Novi, MI), 25 mM of HEPES, 10 mM of L-glutamine, and 25 mg/mL of transferrin (Sigma-Aldrich, Gillingham, UK)). On days 6 and 9, cultures were supplemented with 200 IU/mL of recombinant human interleukin (IL)-2 (PeproTech, London, UK). T-cells were cloned from cell lines (cell lines generated from the priming assay or peripheral blood mononuclear cell bulks) via serial dilution, as described previously (Sullivan et al., 2018).

Epstein-Barr virus (EBV)-transformed B-cell lines were generated from peripheral blood mononuclear cells via transformation with supernatant from the EBV-producing cell line, B95.8. EBV-transformed B-cells were used thereafter as an immortalised autologous source of antigen presenting cells, and were maintained in RPMI1640 supplemented with 10% foetal bovine serum (Invitrogen, Paisley, UK), 100 mM L-glutamine, penicillin, and streptomycin.

Specificity of T-cell clones

T-cell clone specificity screening was conducted using a standard co-culture format of 5 x 10^4 T-cell clones and 1 x 10^4 autologous irradiated EBV-transformed B-cells per well (96-well U bottomed) incubated with relevant compound for 48 hours. Wells were then pulsed with [^{3}H]thymidine (0.5 μCi/well, 5 Ci/mmol; Morovek Biochemicals, Brea, CA) for an additional 16 hour culture period, allowing measurement of compound specific T-cell clone proliferation through ensuing incorporation. T-cell clones with stimulation indices (SIs) (proliferation in the presence of compound/proliferation in control wells) above 1.5 at this stage were deemed

compound-responsive and were subject to repetitive mitogen driven (PHA 5mg/mL) expansion in the presence of irradiated autologous peripheral blood mononuclear cells (5 x 10^4 cells/well) in R9 medium supplemented with IL-2 (200 IU/mL) in order to progress to extensive characterisation.

Dose-response and Cross-reactivity of T-cell clones

Extensive dose-response experiments were undertaken for specific T-cell clones using autologous irradiated EBV-transformed B-cells and tolvaptan (0.1-100 μM), DM-4103 (0.1-150 μM) or DM-4107 (0.1-500 μM). Cross-reactivity profiles were investigated for respective alternative compounds using a minimum of two different stimulatory concentrations.

Profiling of Cytokine and Cytolytic Molecule Release by T-cell clones

An ELISpot panel was utilised to profile compound specific cytokine (IFN-γ, IL-5, IL-13, IL-17, and IL-22) and cytolytic molecule (granzyme B, perforin) secretion from standard co-culture format assays with optimal stimulatory concentrations of tolvaptan or metabolite. Plates were pre-coated with appropriate capture antibody for 24 hours. After washing, cytokine-secreting cells were visualized according to the manufacturer's instructions (Mabtech) and spots were counted using an AID ELIspot reader (Oxford Biosystems Cadama, Oxfordshire, UK).

Phenotyping of T-cell clones

Cell phenotyping included CD4/8, chemokine receptor expression, and T-cell receptor Vβ usage. Flow cytometry was conducted on a FACSCanto II (BD Biosciences, USA) using CD4, CD8, CDR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR9, CCR10, CXCR6, E-CAD, CLA antibodies (BD Biosciences) and the IOtest Beta Mark TCR Vβ repertoire kit.

Delineating HLA-Restriction of T-cell clones.

HLA class I/II (3 μL; BD Biosciences, Oxford, UK) and class isoform (DR, DP, DQ) (1 μL; Abcam, Cambridge, UK) blocking antibodies were used sequentially to analyse HLA restriction. Allelic restriction was assessed in standard format assays (5 x 10^4 T-cell clones, 1 x 10^4 EBV-transformed B-cells) using panels of allogeneic genotyped EBV-transformed B-cells derived from healthy donors enrolled in the in-house genotyped biobank at the University of Liverpool. Activation of the T-cell clones was measured using [^{3}H]-thymidine after a 48 hour culture period.

Mechanistic Studies of Antigen Presentation

T-cell clones responsive towards dapsone nitroso, carbamazepine, and abacavir were used alongside the tolvaptan (metabolite)-responsive T-cell clones to control for different pathways of drug-specific T-cell activation. Autologous EBV-transformed B-cells were subject to glutaraldehyde fixation (0.05%; SigmaAldrich) prior to co-culture in order to terminate metabolic processes/intracellular processing. EBV-transformed B-cells were pulsed with drug for various time periods from 10 minutes to 24 hours before extensive washing to remove soluble drug prior to co-culture. Cultures were supplemented with glutathione (1 mM; pre-incubated with compound 1 hour), a soft nucleophile, in order to competitively quench covalent modification of biological macromolecules. CD3 downregulation was used to determine the kinetics of T-cell receptor internalization; drug-stimulated T-cell clones were stained with a fluorescein allophycocyanin–labelled anti-CD3 mAb after 15 minutes, 1 hour, 4 hours and 24 hours of culture, fixed in formaldehyde and analysed by flow cytometry.

Results

Generation of Tolvaptan and Metabolite-Responsive T-cell clones From Healthy Donors

Despite a lack of observed tolvaptan (metabolite)-specific proliferation in T-cell lines generated from priming cultures, tolvaptan-, DM-4103- and DM-4107-specific T-cell clones were detected following the serial dilution and cloning of these T-cell lines. Approximately 24,000 cultures were generated from 3 donors containing isolated precursor T-cell clones. These were subject to repetitive mitogen driven expansion, selected based on outgrowth, and then screened for compound-specific proliferation responses (determined through incorporation of [^{3}H] thymidine) in the presence of autologous EBV-transformed B-cells (**Figure 1**). A total of 26 tolvaptan or tolvaptan metabolite-responsive T-cell clones were identified during initial testing: 7, 1 and 18 T-cell clones derived from T-cell lines generated with tolvaptan, DM-4103 and DM-4107, respectively. No overt predilection was observed in CD4/CD8 phenotype of T-cell clones (**Figure 1**).

Dose-Response and Crossreactivity Profiles of Tolvaptan and Metabolite-Responsive T-cell clones

Extensive dose response and crossreactivity assays were performed for all T-cell clones primed to each compound (tolvaptan: 0.1-100 μM; DM-4103: 0.1-150 μM; DM-4107: 0.1-400 μM). Proliferation was dose-dependent with variable profiles exhibited across individual T-cell clones. Tolvaptan- and DM-4103-activated T-cell clones at concentrations ranging from 10-80 μM; higher concentration inhibited proliferation due to direct toxicity (**Figure 2**). DM-4107 stimulated T-cell clones to proliferate at 10-300 μM, the latter being the highest concentration tested. Crossreactivity assessment revealed differential profiles depending on the chemical entity the T-cell clones were purportedly primed to; tolvaptan and DM-4103 primed T-cell

clones often exhibited reactivity towards DM-4107 but not each other (**Figure 2**). In contrast, no cross-reactivity was observed with DM-4107-primed T-cell clones.

Phenotypic Characterization of T-cell clones

Heterogeneity of TCR Vβ usage was observed as from 15 T-cell clones tested, 7 distinct TCR Vβs were detected, with the remaining 8 TCR Vβs not discernible through the use of the IOtest Beta Mark TCR Vβ repertoire kit and thus likely to represent rare Vβs (**Figure 3a**). Chemokine analysis of four DM-4107-responsive T-cell clones revealed discernible expression of CCR2, CCR4, CCR6, CCR9, CCR10 and E-cadherin (data not shown).

Profile of Secreted Cytokines/Cytolytic Molecules Upon Compound-Specific Activation of T-cell clones

The secretory molecule profiles of 10 T-cell clones; 3 tolvaptan-, 1 DM-4103- and 6 DM-4107-derived, were interrogated through the use of ELISpot. Upon compound stimulation, secretion of IFN-γ, IL-13, granzyme B and perforin was universally observed. Meanwhile, heterogeneous secretory profiles of IL-10, IL-22 and IL-5 were identified with tolvaptan and DM-4107 responsive clones. IL-17 secretion was only detected in 2 T-cell clones responsive to tolvaptan (**Figure 3b**). Representative well images over the ELISpot panel for T-cell clones treated with media and optimal stimulatory concentrations of tolvaptan, DM-4103 are DM-4107 are provided (**Figure 3c**).

Tolvaptan and DM-4107 T-cell clones are Activated in HLA Allele-Restricted Manner

CD8+ and CD4+ T-cell clones were shown through the use of HLA specific blocking antibodies to be HLA class I and II restricted, respectively (**Figure 4a**). HLA class II isoform-specific blocks unveiled HLA-DR restriction of CD4+ DM-4107-responsive T-cell clones

(**Figure 4b**). Furthermore, antigen presenting cell mismatch assays demonstrated allelic preference of T-cell clones, with the most profound example being DM-4107 T-cell clone responses restricted by antigenic presentation on the class II allele HLA-DRB1*04:01 (as demonstrated by its unique expression in donor 853) (**Figure 4c** and **Figure 4d**). DM-4103 clones did not expand adequately for use in HLA restriction studies, thus the focus for the remainder of the project was centred on parent drug and the DM-4107 metabolite.

Pathway of Tolvaptan- and DM-4107-Specific Activation of T-cell clones

Antigen presenting cell pulsing experiments have customarily been utilised to differentiate between classical hapten mechanisms dependent on formation and processing of drug-protein adducts, and pharmacological interaction and altered peptide repertoire mechanisms reliant on soluble drug (Schnyder et al., 1997; Schnyder et al., 2000). Herein, nitroso dapsone-, carbamazepine-, and abacavir-responsive T-cell clones were used as controls for hapten, pharmacological interaction and altered peptide repertoire pathways of drug-specific T-cell activation, respectively. Nitroso dapsone-, carbamazepine-, and abacavir-responsive T-cell clones were all stimulated to proliferate when cultured with antigen presenting cells and soluble drug for the duration of the experiment. As expected, nitroso dapsone-responsive T-cell clones were activated with antigen presenting cells pulsed with the drug metabolite for 10 minutes-24 hours; while carbamazepine-responsive T-cell clones were not activated with drug-pulsed antigen presenting cells (**Figure 5a**) (Wu et al., 2007; Zhao et al., 2019). Antigen presenting cells pulsed with abacavir for 4-16 hours, the time required for antigen processing and the display of novel peptide sequences on the cell surface (Illing et al., 2012; Adam et al., 2012; Ostrov et al., 2012; Norcross et al., 2012; Bell et al., 2013), activated abacavir-responsive T-cell clones (**Figure 5a**). Glutaraldehyde fixation of antigen presenting cells, a technique commonly used to abrogate antigen processing, attenuated nitroso dapsone and abacavir-

specific proliferation, but had less of an effect on the activation of T-cell clones with carbamazepine (**Figure 5b**).

For tolvaptan and DM-4107-responsive CD4+ and CD8+ T-cell clones, T-cell proliferation was significantly reduced when antigen presenting cells were omitted from the assay. However, residual levels of proliferation were evident with high concentrations of the drug/metabolite (data not shown). Proliferative responses were absent for cultures containing tolvaptan or DM-4107-pulsed antigen presenting cells (pulse duration; 10 minutes-24 hours), whilst re-introduction of soluble drug reinstated significant proliferation (**Figure 6a**). Experiments involving the use of glutathione, which interacts with soft nucleophiles preventing formation of protein adducts (Naisbitt et al., 1996), failed to inhibit activation of T-cell clones with tolvaptan or DM-4107 (data not shown). Glutaraldehyde-attenuated proliferative responses were also seen for tolvaptan and DM-4107 T-cell clones (**Figure 6b**). The conservation of PHA responses in such experiments demonstrated T-cell viability was maintained within cultures following fixation (results not shown).

CD3 internalisation assays have been used to track kinetics of TCR internalisation in response to a number of compounds, with rapid kinetics of T-cell activation supporting the existence of pharmacological interaction pathway as demonstrated for carbamazepine and sulfamethoxazole (Zanni et al., 1998; Schnyder et al., 2000; Wu et al., 2006). However, in such assays performed on DM-4107 responsive T-cell clones stimulated with compound, downregulation generally was observed at 4-24 h into co-culture (**Figure 6c and d**). These kinetics of T-cell clone activation are incompatible with a direct pharmacological activation. Tolvaptan-responsive clones were not available for these experiments.

Discussion

The *in vitro* assay using naive T-lymphocytes derived from healthy donors with a priming and re-challenge format was not adequately sensitive to detect proliferative responses to tolvaptan and 2 key tolvaptan metabolites. Comparable outcomes observed in previous studies have been attributed to a function of T-cell pre-cursor frequency (Faulkner et al., 2016), with the re-challenge cultures possessing limited sensitivity in the detection of rare T-cells as their responses are effectively masked by the background "noise" present in the heterogeneous T-cell lines. To overcome this lack of sensitivity, T-cell cloning was proceeded to, in which progenitor clonal populations of T-cells are isolated and expanded, facilitating the investigation of T-cell responses at the unicellular level. T-cell clones with variation in CD4+/CD8+ phenotype and TCR Vβ expression displayed responses towards tolvaptan and DM-4107. One T-cell clone was generated that was responsive towards DM-4103. Crossreactivity series revealed differential drug specificity profiles; with tolvaptan- and DM-4103-primed T-cell clones displaying cross-reactivity for DM-4107 but not each other, whilst DM-4107-primed T-cell clones were responsive only towards DM-4107. It is intriguing that cross-reactivity between DM-4103 and DM-4107 T-cell clones is unidirectional, especially considering the similar molecular structures (the only difference being possession of oxybutyric acid and hydroxybutyric acid moieties respectively), however, this may be attributable to greater conformational flexibility afforded to DM-4107 by this structural discrepancy.

To confirm that the above results with peripheral blood mononuclear cells from healthy donors are truly attributable to the actions of tolvaptan and its metabolites on T-cells, it is important to consider the sensitivity and specificity or the in vitro assays employed. We have recently demonstrated that haptenic compounds and drugs that cause adverse events through binding to a single HLA molecule readily prime naïve T-cells (Faulknet et al., 2016; Ogese et al., 2020).

The reactive metabolite of acetaminophen (*N*-acetyl-*p*-benzoquinone imine [NAPQI]) has been included in the assay as a negative control since acetaminophen in overdose is thought to cause liver injury via a pathway not involving the adaptive immune system. Priming of naïve T-cells with NAPQI was negative and NAPQI-responsive clones were not detected (unpublished data). However these data should be regarded with the caveat that acetaminophen exposure is associated with a small, but significant incidence of severe skin reactions (Roujeau et al., 1995), and therefore the drug (and/or metabolite) likely does have the capacity to activate T-cells. Thus, to further address the sensitivity and specificity of the T-cell assays employed we have recently conceived and are conducting a detailed assessment of 15 drugs (including compounds with drug-induced liver injury liabilities ascribed to both adaptive-immune and non-adaptive-immune mechanisms as well as compounds with no drug-induced liver injury liabilities) blinded to researchers.

Since drug-specific responses elicited from CD8+ and CD4+ T-cell clones were shown to be HLA molecule class I and II restricted through the use of blocking antibodies, it can be concluded that the respective drug derivatives are presented in the context of HLA molecules. Moreover, further resolution for HLA class II restriction of CD4+ T-cell clones was provided through the use of isoform specific blocking antibodies, which facilitated the assessment of allelic restriction of antigen presentation through the use of antigen presenting cell mismatch experiments. Whilst the allele identified as critical for response of several DM-4107-responsive T-cell clones (DRB1*04:01) is likely inconsequential in terms of delineation of a population-based risk allele, it does unequivocally demonstrate that tolvaptan-associated antigen presentation can occur in an HLA-restricted manner. Detection of tolvaptan and DM-4107 T-cell clones in donors expressing different HLA-DR molecules and the absence of genomic

studies linking tolvaptan-associated liver injury to expression of a specific HLA allele suggests that tolvaptan can interact with a number of different HLA molecules to activate T-cells.

Drugs are known to activate T-cells through either hapten or direct HLA binding pathways. The molecular initiating event for haptenic drug or drug metabolite (e.g., β-lactam antibiotics, nitroso dapsone) reactions is the covalent modification of nucleophilic amino acid residues on cellular and/or serum proteins (Whitaker et al., 2011; Meng et al., 2017). Covalently-modified proteins are subjected to antigen processing within antigen presenting cells, a procedure that generates drug hapten-modified peptides that associate with HLA proteins (Waddington et al., 2020). Specific T-cell receptors are believed to be triggered when they come into contact with the hapten-modified peptides displayed by HLA proteins on the surface on antigen presenting cells. Drugs and metabolites additionally stimulate T-cells though a direct, readily reversible binding interaction with HLA proteins or peptides already associated with the HLA molecule (Zanni et al., 1998; Schnyder et al., 2000; Illing et al., 2012). The different pathways of drug presentation by antigen presenting cells can be differentiated using battery of established functional assays. Herein, these assays were utilized to explore T-cell activation with model compounds (nitroso dapsone [haptenic drug metabolite; Zhao et al., 2019], carbamazepine [drug that interacts with HLA peptide complexes via a direct reversible interaction; Wu et al., 2006] and abacavir [drug that binds to HLA proteins altering the natural display of peptides; Ostrov et al., 2012; Illing et al., 2012; Adam et al., 2012; Norcross et al., 2012) tolvaptan and DM-4107. Pulsing experiments yielded negative results for both tolvaptan and DM-4107, ruling out a classical hapten mechanism as seen with nitroso dapsone and indicating that the continuous presence of soluble drug was a prerequisite for a T-cell response. Aldehyde fixation experiments indicated dependence on antigen processing, as fixation of antigen presenting cells significantly diminished responses, which contradicts a direct HLA peptide binding interaction

as seen with carbamazepine. Collectively, these experiments suggest that the pathway by which tolvaptan and DM-4107 activate T-cells does not conventionally comply with classical hapten or pharmacological interaction concepts. TCR internalisation bolstered this notion, as downregulation of CD3 was only observed after 4-24 hours following exposure of T-cells to drug. This downregulation demonstrates a short latency period before T-cell activation, alluding to some degree of processing/signalling following exposure to the relevant drug derivatives. Tolvaptan and its metabolites may therefore confer antigenicity through a mechanism not classically described in hypersensitivity dogma, perhaps akin to the manner by which abacavir does, or alternatively through interruption of antigen processing machinery.

The exclusivity of liver injury to the ADPKD population indicates a predisposition of these individuals to such adverse events. Contributing factors pertaining to ADPKD aetiology may include the heightened inflammatory state reported in these individuals, with key inflammatory mediators such as monocyte chemoattractant protein-1 and tumour necrosis factor-α identified to date (Ta et al., 2013). Pharmacokinetic aspects of ADPKD may also contribute, with perhaps the most obvious disease-related perturbation of tolvaptan (and metabolite disposition) being the progressive renal impairment symptomatic of continuous cyst growth, which may have important ramifications for metabolites which are appreciably excreted renally, namely DM-4107 (Sorbera et al., 2002). Interestingly, a rodent model of ADPKD has been found to display alterations in hepatic transporter expression, with a 3-fold reduction in MRP2 expression (Bezencon et al., 2019). Quantitative systems toxicology modelling also reinforces the hypothesis that decreased efflux of tolvaptan metabolites increases susceptibility to tolvaptan-related liver injury with MRP2 dysfunction a being a key factor (Beaudoin et al., 2020). The extended window of onset for tolvaptan-associated drug-induced liver injury is much larger

(i.e. 3-18 months) than what is typically observed for idiosyncratic drug-induced liver injury (1-3 months), which suggests that disease progression can influence outcome.

DM-4103 and DM-4107 are derived from the parent drug via similar metabolic pathways and so would be likely to co-localise in a manner that potentially promotes synergistic accumulation and toxic sequela. Thus, a working hypothesis for the exclusivity of drug-induced liver injury within ADPKD which incorporates both direct toxicological mechanisms (Wu et al., 2015; Mosedale et al., 2017; Woodhead et al., 2017; Mosedale et al., 2018; Beaudoin et al., 2020) and the adaptive immune system could be that these individuals (through greater exposure to tolvaptan and metabolites or otherwise) exhibit enhanced toxicity through direct mechanisms, in a manner conducive to the initiation of an adaptive immune response directed against drug-related antigens for which an augmented epitope density may be available.

To conclude, tolvaptan has been approved in many countries and is the only treatment currently approved to slow kidney function decline in adults at risk of rapidly progressing ADPKD. However, in the TEMPO 3:4 trial in subjects with ADPKD and the open-label extension (TEMPO 4:4), tolvaptan caused liver injury within a small subset in a manner characteristic of an immune-medicated aetiology. Data presented herein using peripheral blood mononuclear cells from healthy human donors demonstrate that tolvaptan and its DM-4107 metabolite activate T-cell responses when regulatory T-cells have been removed and dendritic cells have been matured to provide co-stimulatory signals. DM-4107 additionally activated T-cell clones primed to the parent compound suggesting that metabolite-specific T-cells may be candidate effector cells in patients with tolvaptan-associated liver injury. DM-4107-specific T-cell clone activation was dependent on the presence of soluble drug, could occur within 4 hours of DM-

4107 exposure and was dependent on antigen processing ruling out classical hapten and pharmacological interactions pathways.

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Abbreviations: human leukocyte antigen, HLA; Epstein-Barr virus, EBV; autosomal dominant polycystic kidney disease, ADPKD; Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and Its Outcomes, TEMPO; Hepatic Adjudication Committee, HAC.

Figure legends

Figure 1. Isolation of tolvaptan- and tolvaptan metabolite-responsive CD4+ and CD8+ T-cell clones from healthy donor peripheral blood mononuclear cells. T-cell clones were generated from 3 healthy donor (HD) peripheral blood mononuclear cells primed to tolvaptan, DM-4103 and DM-4107 by serial dilution and repetitive mitogen stimulation. Each T-cell clone was tested for study compound responsiveness by culturing T-cell clones (0.5×10^5) with tolvaptan, DM-4103 or DM-4107 and autologous EBV-transformed B-cells (0.1×10^5) for 48 h at 37°C, 5% CO_2. [^{3}H]-thymidine (0.5μCi/well) was added for the last 16 h of incubation before plates were harvested and proliferation measured. Individual bars represent mean drug treated/mean media control proliferation responses. All T-cell clones with an SI of 1.5 or above as indicated by horizontal lines were expanded for further investigation. Table depicts the number of CD4+ and CD8+ T-cell clones that displayed proliferative responses in the presence of tolvaptan, DM-4103 or DM-4107.

Figure 2. Tolvaptan- and tolvaptan metabolite-responsive T-cell clones exhibit differential crossreactivity profiles. Dose-response curves and crossreactivity series for representative tolvaptan (TOL)-, DM-4103- and DM-4107-responsive T-cell clones. T-cell clones (0.5×10^5) were cultured with autologous EBV-transformed B-cells (0.1×10^5) and titrated concentrations of tolvaptan, DM-4103 or DM-4107 for 48 h at 37°C, 5% CO_2. [^{3}H]-thymidine (0.5μCi/well) was added for the last 16 h of incubation before plates were harvested and proliferation measured. *$P<0.05$; Mann-Whitney test.

Figure 3. Tolvaptan- and tolvaptan metabolite-responsive T-cell clones are derived from heterogeneous clonotypes but exhibit comparable secretory profiles upon stimulation with drug. (A) TCR VB usage of tolvaptan- and DM-4107-responsive T-cell clones as measured by flow cytometry with each distinct VB portrayed as a compartment. **(B)** Overview

of secretory molecule profiles of T-cell clones upon stimulation with tolvaptan (TOL), DM-4103 or DM-4107 and autologous EBV-transformed B-cells for 48 h at 37°C, 5% CO_2.. Cytokine secretion was measured by ELISpot. **(C)** ELISpot well images depicting secretory profiles of representative T-cell clones responsive towards tolvaptan (TOL), DM-4103, and DM-4107 upon stimulation with drug relative to control cultures.

Figure 4. Tolvaptan and DM-4107 are presented to and activate CD4+ and CD8+ T-cell clones in the context of MHC in an allele restricted manner. (A) CD4+ and CD8+ T-cell clones (0.5 x 10^5) were cultured with tolvaptan or DM-4107 and autologous EBV-transformed B-cells (APC; 0.1 x 10^5) for 48 h at 37°C, 5% CO_2 in the presence or absence of an MHC blocking antibodies and the isotype control (Iso). **(B)** Two DM-4107-responsive T-cell clones (0.5 x 10^5) were cultured with DM-4107 and autologous EBV-transformed B-cells (APC; 0.1 x 10^5) for 48 h at 37°C, 5% CO_2 in the presence or absence of an MHC class II blocking antibodies (HLA-DR, -DP and -DQ). **(C)** T-cell clones (0.5 x 10^5) were cultured with DM-4107 and either autologous (Aut) or heterologous EBV-transformed B-cells expressing various HLA-DRB1 and DQB1 alleles for 48 h at 37°C, 5% CO_2. Numbers refer to the id of the different heterologous blood donors. The HLA allele profile of the B-cell lines from these donors with the same ids are shown in **(D)**. [^{3}H]-thymidine (0.5μCi/well) was added for the last 16 h of incubation before plates were harvested and proliferation measured. All data bars represent mean proliferation values of triplicate cultures.

Figure 5. Nitroso dapsone, carbamazepine and abacavir presentation to T-cell clones follow hapten, pharmacological HLA interaction and altered self-peptide repertoire pathways. (A) T-cell clones (0.5 x 10^5) were cultured with EBV-transformed B-cells (0.1 x 10^5), pulsed with nitroso dapsone, carbamazepine or abacavir for 10 min - 24 h, for 48 h at

37°C, 5% CO_2. EBV-transformed B-cells were washed repeatedly to remove unbound drug prior to culturing with the T-cell clones. **(B)** T-cell clones (0.5 x 10^5) were cultured with soluble nitroso dapsone, carbamazepine or abacavir and glutaraldehyde-fixed autologous EBV-transformed B-cells (0.1 x 10^5) for 48 h at 37°C, 5% CO_2. EBV-transformed B-cells were fixed to prevent protein processing. [^{3}H]-thymidine (0.5μCi/well) was added for the last 16 h of incubation before plates were harvested and proliferation measured.

Figure 6. Tolvaptan and DM-4107 presentation to T-cell clones is dependent on the presence of soluble drug and occurs in a processing dependent manner. (A) T-cell clones (0.5 x 10^5) were cultured with EBV-transformed B-cells (0.1 x 10^5), pulsed with tolvaptan or DM-4107 for 10 min - 24 h, for 48 h at 37°C, 5% CO_2. EBV-transformed B-cells were washed repeatedly to remove unbound drug prior to culturing with the T-cell clones. Soluble tolvaptan or DM-4107 were added as a positive control. **(B)** T-cell clones (0.5 x 10^5) were cultured with soluble tolvaptan or DM-4107 in the absence of EBV-transformed B-cells and in the presence of glutaraldehyde-fixed or irradiated EBV-transformed B-cells (0.1 x 10^5) for 48 h at 37°C, 5% CO_2. EBV-transformed B-cells were fixed to prevent protein processing. [^{3}H]-thymidine (0.5μCi/well) was added for the last 16 h of incubation before plates were harvested and proliferation measured. **(C)** T-cell clones (0.5 x 10^5) were cultured with EBV-transformed B-cells (0.1 x 10^5) and DM-4107 for 1 - 24 h (at 37°C, 5% CO_2) and CD3 receptor expression was measured as a marker of T-cell receptor triggering by flow cytometry. **(D)** Overlay histograms depicting temporal relationship of drug-treated culture staining and relative media control staining for 2 representative T-cell clones included in (C).

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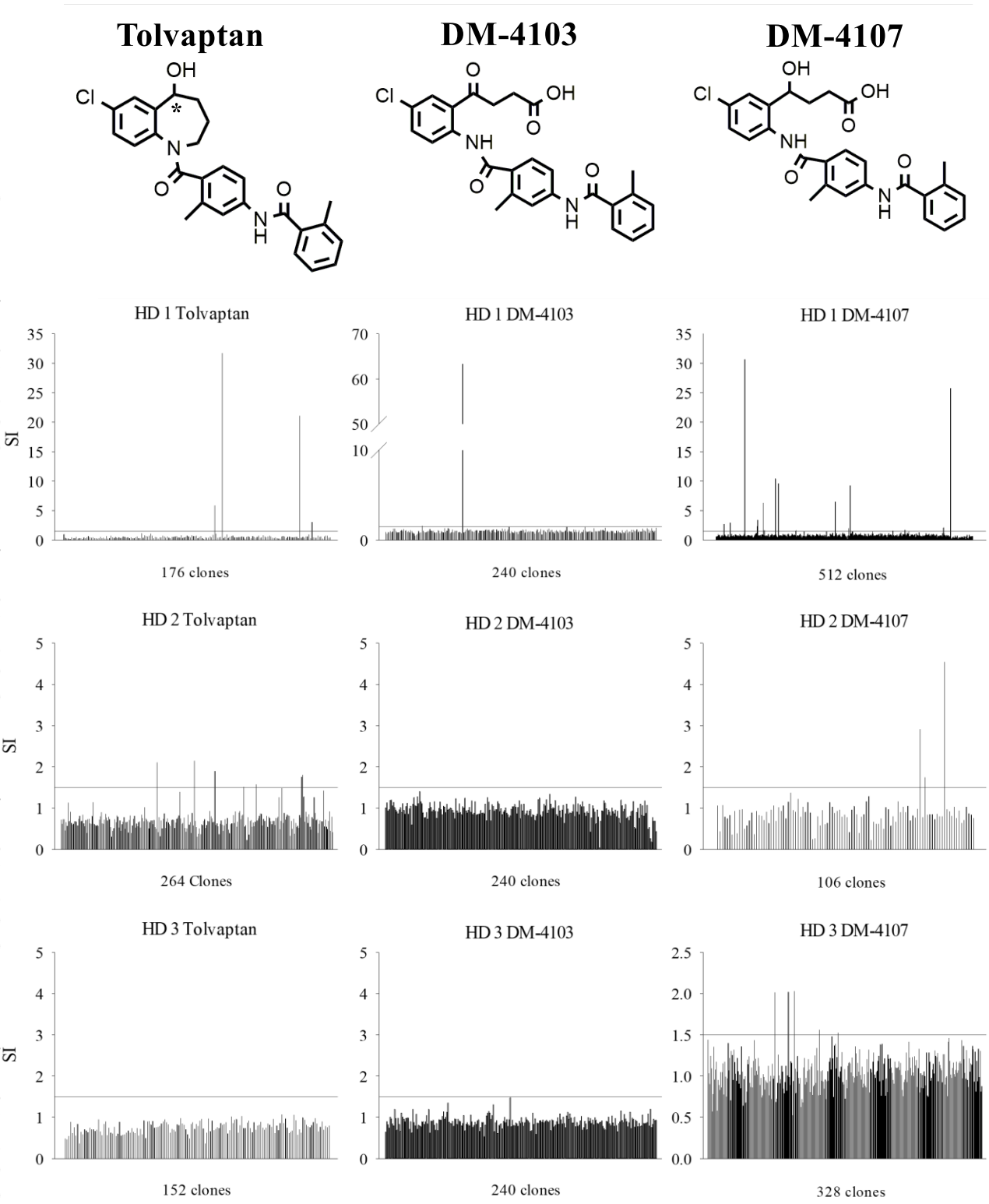
Table 1. HLA type of blood donors subjected to T-cell cloning

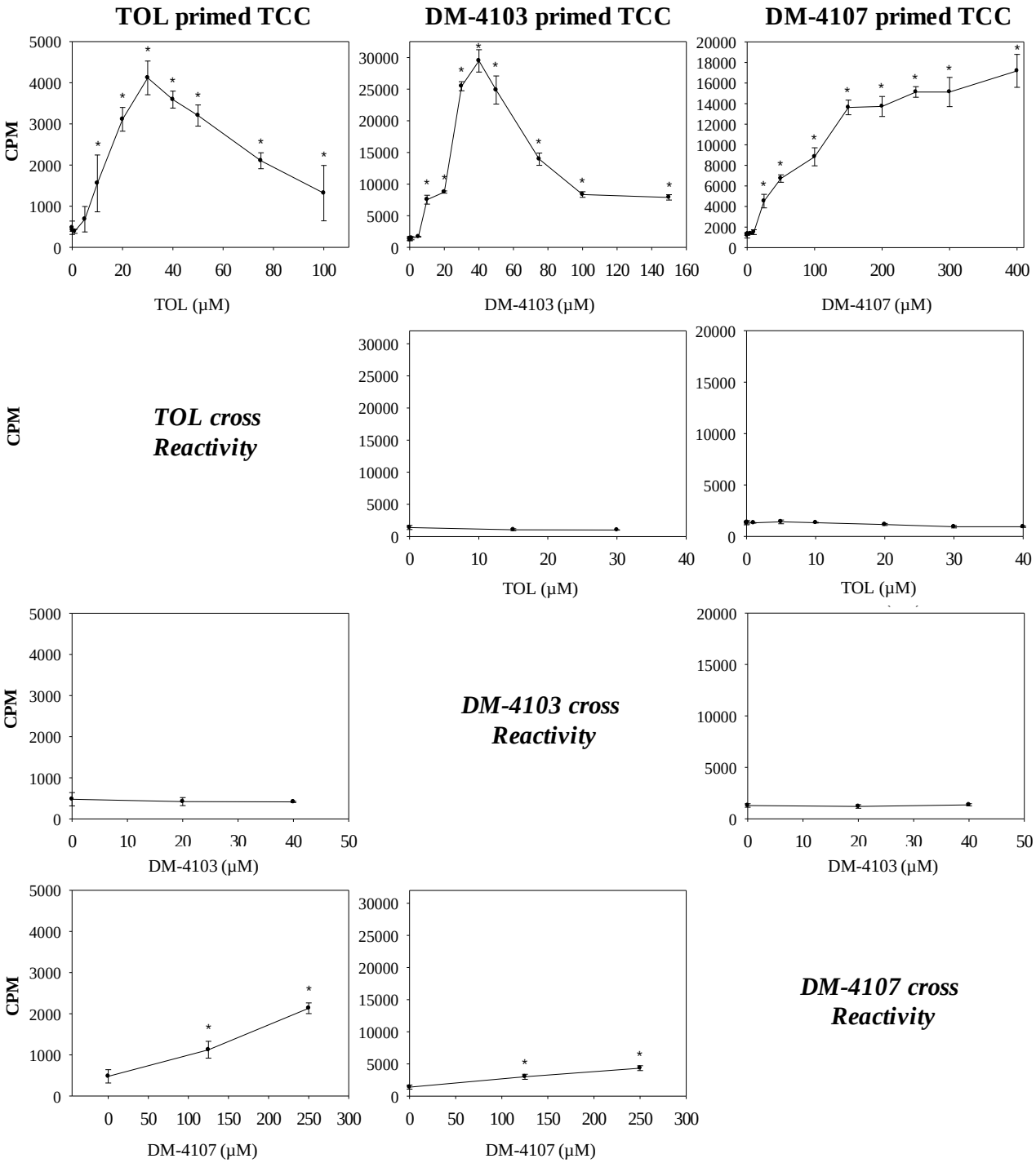
	HLA-A		HLA-B		HLA-C		HLA-DQRB1		HLA-DRQB1	
HD1[1]	01:01	01:01	08:01:	15:01	03:04	07:01	04:01	14:54	03:02	05:03
HD2	01:01	02:01	45:01	51:01	01:02	06:02	01:01	07:01	02:01	05:01
HD3	24:02	29:02	35:03	45:01	01:02	06:02	04:01	07:01	03:01	03:03

[1]TCC from HD1 are used in APC mismatch assay – figure 4C.



	# Tolvaptan Primed TCC		# DM-4103 primed TCC		# DM-4107 primed TCC	
	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+
Donor 1	-	2	-	1	8	3
Donor 2	3	2	-	-	-	2
Donor 3	-	-	-	-	-	5

Figure 2


TOL primed TCC
DM-4103 primed TCC
DM-4107 primed TCC
CPM
TOL (µM)
DM-4103 (µM)
DM-4107 (µM)
TOL cross Reactivity
DM-4103 cross Reactivity
DM-4107 cross Reactivity

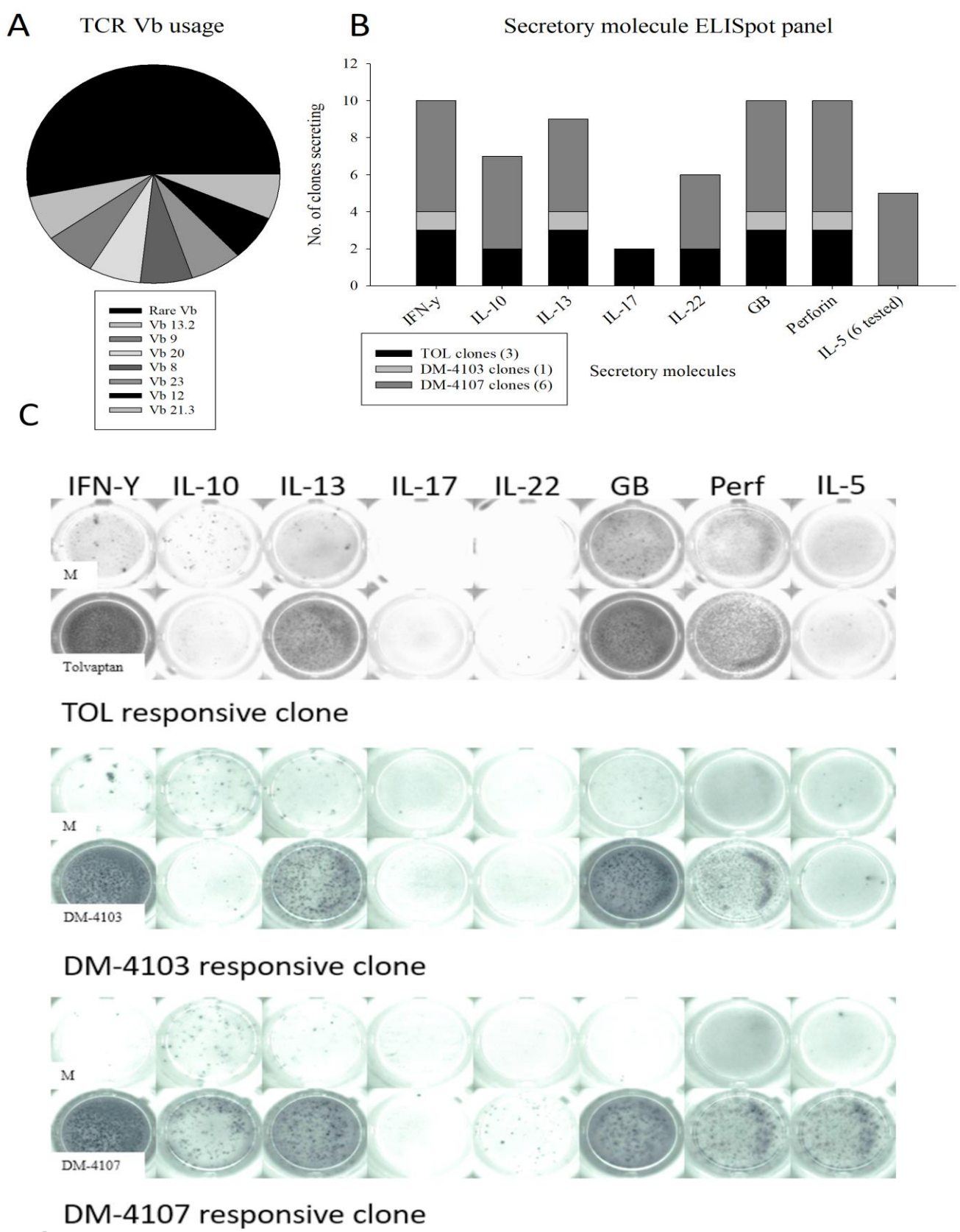
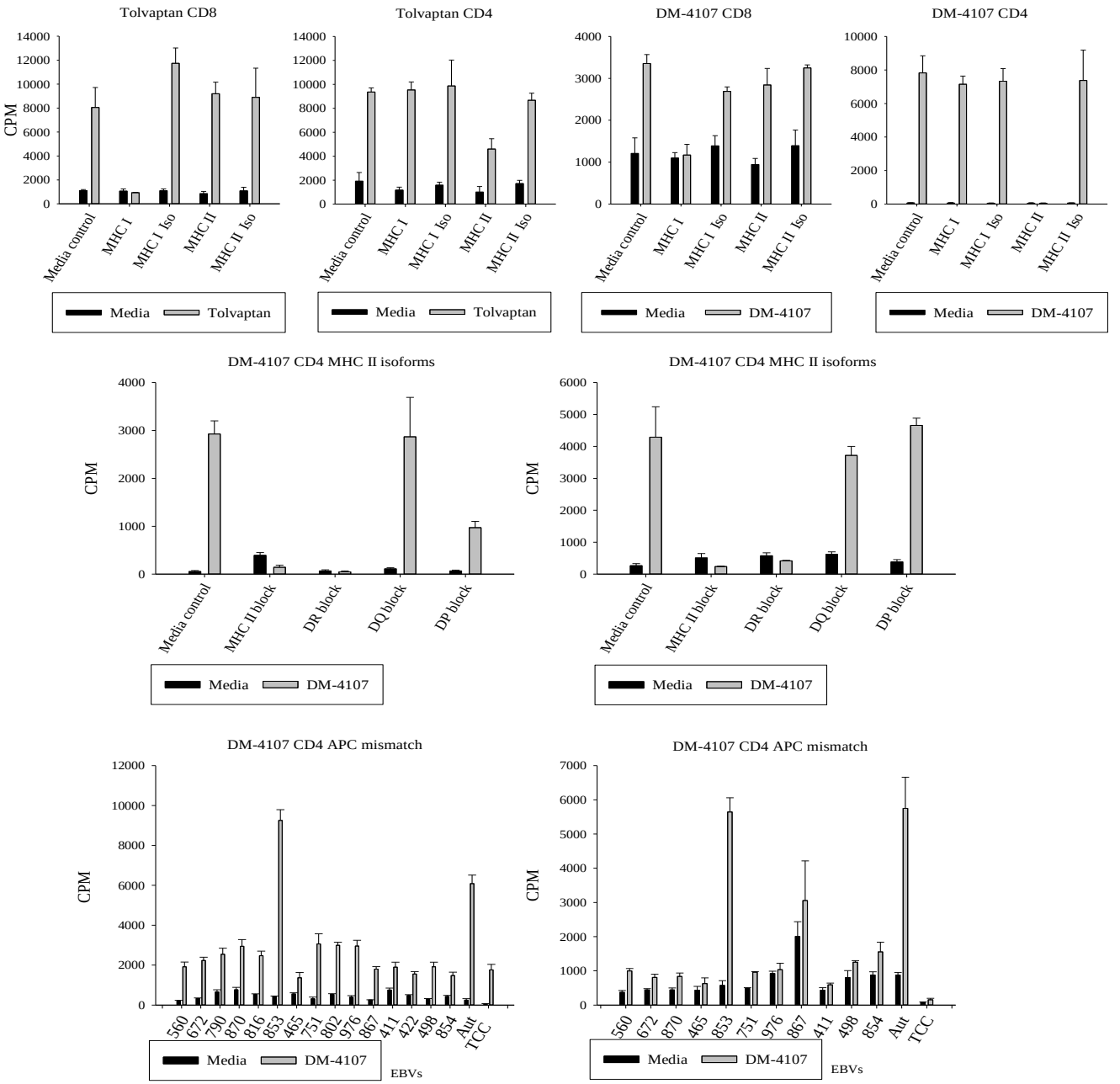

A
TCR Vb usage
Rare Vb
Vb 13.2
Vb 9
Vb 20
Vb 8
Vb 23
Vb 12
Vb 21.3
B
Secretory molecule ELISpot panel
No. of clones secreting
0
2
4
6
8
10
12
IFN-γ
IL-10
IL-13
IL-17
IL-22
GB
Perforin
IL-5 (6 tested)
TOL clones (3)
DM-4103 clones (1)
DM-4107 clones (6)
Secretory molecules
C
IFN-Y
IL-10
IL-13
IL-17
IL-22
GB
Perf
IL-5
M
Tolvaptan
TOL responsive clone
M
DM-4103
DM-4103 responsive clone
M
DM-4107
DM-4107 responsive clone

Figure 4



D

Donor ID	HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB		HLA-DQA	
560	01:01	02:01	45:01	51:01	01:02	06:02	01:01	07:01	02:01	05:01	01:01	02:01
672	03:01	29:02	45:01	51:01	02:02	06:02	01:01	13:01	05:01	06:03	01:03	01:01
790	02:60	03:01	07:02	45:01	06:02	07:02	04:05	15:01	03:02	06:02	01:02	03:01
870	02:01	68:01	07:02	45:01	06:02	07:02	07:01	15:01	02:01	06:02	01:02	02:01
465	01:01	24:02	07:02	44:03	03:03	07:01	07:01	15:01	02:01	06:02	01:02	02:01
816	02:01	29:02	40:01	45:01	03:04	06:02	03:01	04:04	02:01	03:02	03:01	05:01
853	24:02	29:02	35:03	45:01	01:02	06:02	04:01	07:01	03:01	03:03	02:01	03:01
751	01:01	33:01	14:02	44:05	02:02	08:02	01:02	07:01	03:03	05:01	01:01	02:01
802	03:01	33:03	27:05	44:03	02:02	07:01	01:01	07:01	02:01	05:01	01:01	02:01
976	02:01	03:01	44:03	44:02	05:01	16:01	07:01	15:01	02:01	06:02	01:02	02:01
867	01:01	02:01	13:02	44:02	05:01	06:02	11:04	12:01	03:01	06:02	01:02	05:01
411	02:01	31:01	15:01	44:02	03:03	05:01	07:01	10:01	02:01	05:01	01:01	02:01
422	01:01	31:01	07:02	52:01	07:02	12:02	15:01	15:02	06:01	06:02	01:03	01:02
498	11:01	31:01	40:02	40:01	03:03	03:04	13:01	14:01	05:03	06:03	01:03	01:01
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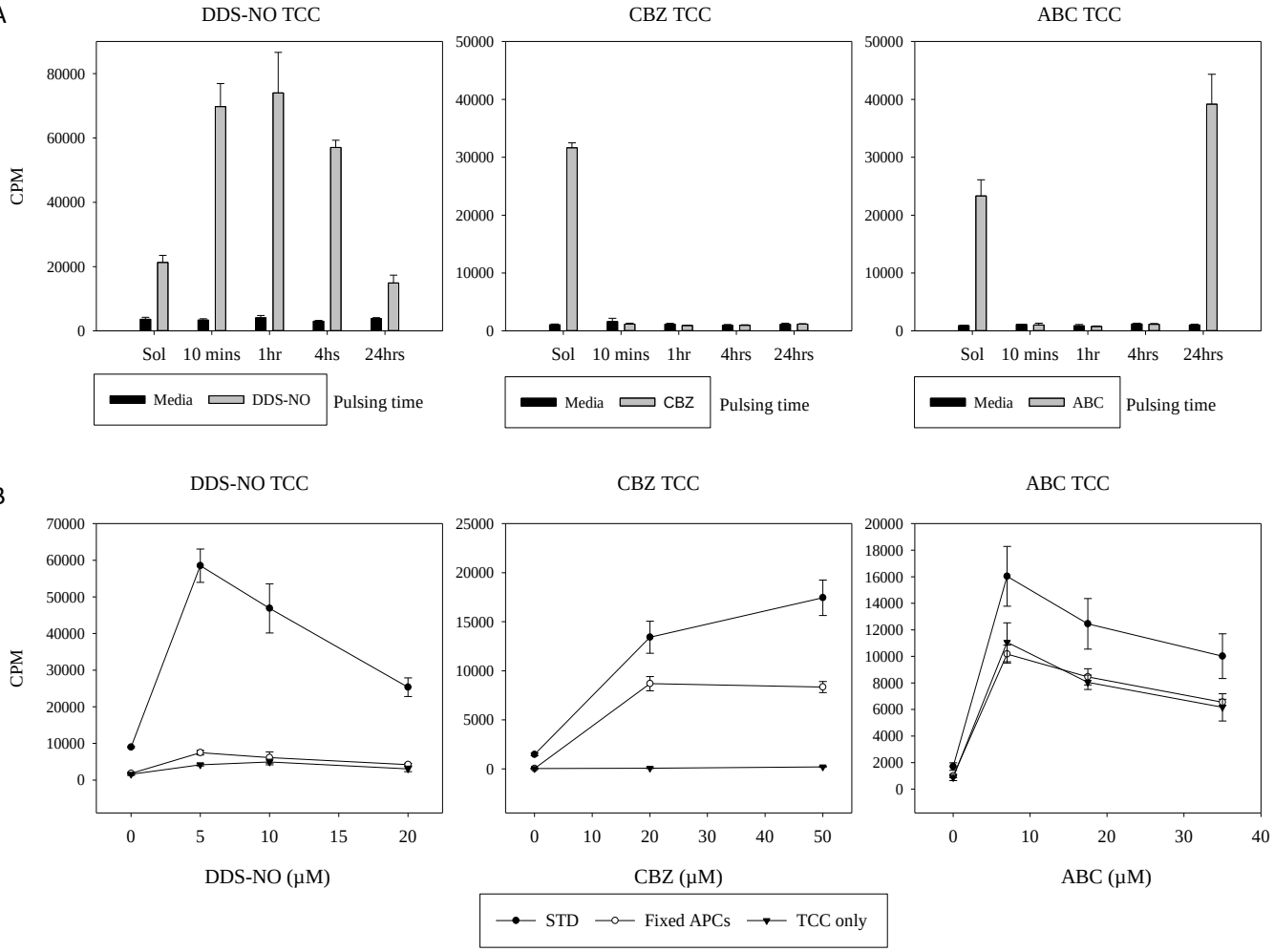
A
DDS-NO TCC
CBZ TCC
ABC TCC
CPM
Sol
10 mins
1hr
4hs
24hrs
Media
DDS-NO
CBZ
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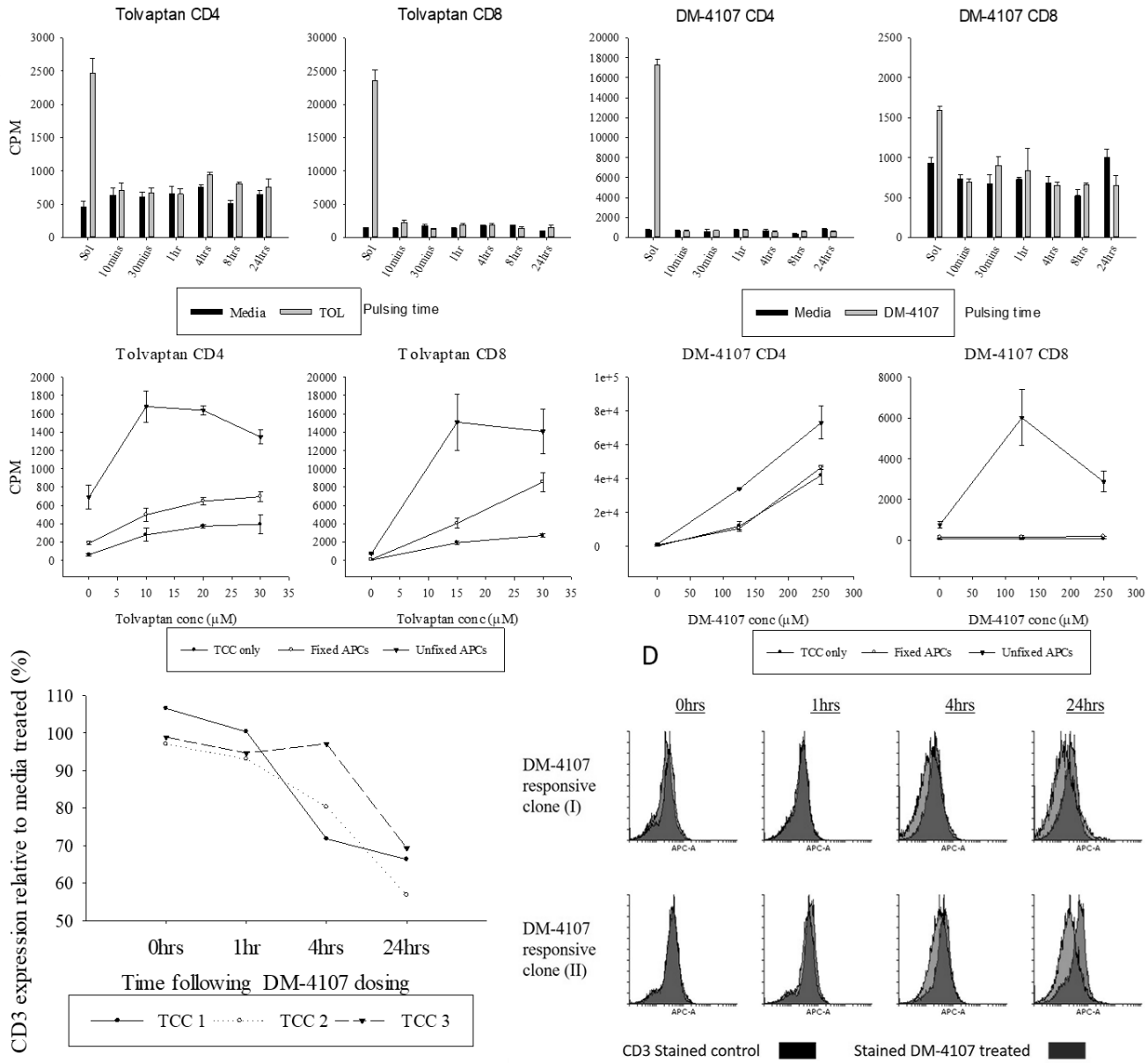
ABC (µM)
STD
Fixed APCs
TCC only

Figure 6


A
Tolvaptan CD4
Tolvaptan CD8
DM-4107 CD4
DM-4107 CD8
CPM
Sol
10mins
30mins
1hr
4hrs
8hrs
24hrs
Media
TOL
DM-4107
Pulsing time
B
Tolvaptan conc (μM)
DM-4107 conc (μM)
TCC only
Fixed APCs
Unfixed APCs
C
CD3 expression relative to media treated (%)
0hrs
1hr
4hrs
24hrs
Time following DM-4107 dosing
TCC 1
TCC 2
TCC 3
D
DM-4107 responsive clone (I)
DM-4107 responsive clone (II)
APC-A
CD3 Stained control
Stained DM-4107 treated