UNIVERSITY OF LIVERPOOL

DETERMINING THE IMMUNOGENICITY OF GLYCOPEPTIDE ANTIBIOTICS IMPLICATED IN DRUG HYPERSENSITIVITY REACTIONS

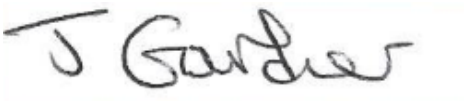
This thesis is submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

Joshua Gardner

September 2022

Declaration

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree.



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Acknowledgements

The last 4 years have been a challenging and hugely rewarding experience working as part of the immunopharmacology research group. There are many people who I have shared this never ending journey with and helped me along the way, with support and guidance that has been instrumental in my development not only as a scientist, but as a person. Firstly, I would like to thank my supervisor, Professor Dean Naisbitt, for his enthusiasm and encouragement when discussing new ideas alongside his open door policy, which I feel is hugely valuable for a progressive research team. I would also like extend thanks to my industrial partner, Dr Catherine Betts of AstraZeneca, for her valued input into my PhD project, abstracts and publications through various meetings over the past years. At this point I also feel it is important to acknowledge those present at the start of my academic journey that laid the foundation for my enthusiasm and interest in scientific research. From my undergraduate project supervisors Dr George Johnson and Dr Jatin Verma, to Dr Amy Chadwick and the rest of Team Bioenergetics for all the help, patience and encouragement during my MRes.

Now embarking on my 6th year in the 'cell lab' there are many people, both past and present, who have made an impact on my time here in Liverpool and throughout my PhD studies. Firstly, a special mention must go out to Dr Monday Ogese for helping me to become an independent lab scientist and whose dedication to the field of hypersensitivity was admired by all he crossed paths with. Thank you to Dr Sean Hammond for the all the guidance in the early stages of my PhD, you were a great mentor and pushed me to find my 'niche' within the group, as well as enlisting me in various side projects throughout the years when dead ends were encountered. I would also like to show my gratitude to Dr Paul Thom**p**son, who will most likely end up being 1 of only 4 people to read this thesis front to back, not forgetting accompanying me to SoT in San Diego for my one and only international conference (Covid!). I had the pleasure of starting my PhD while the DJN group was still populated by the 'old guard', specifically Andy, Arun, Paul, Adam and James who were all excellent role models and prepared me for life within the group. Notable mentions must also go to Kareena, Serat-E, Kanoot and Mubarak for making PhD life interesting and enjoyable. I would also like to recognise more recent additions to the DJN group. Liam, James, Sophie, Meg, Georgia, Samantha, Ted and Ghazi, I look forward to working with you all over the coming years and watching you go on to great things. A special mention must be extended to Liam, for his lack of will power meaning I could always rely on him to accompany me to the AJ after work on a Friday. I would also like to place on record my gratitude to PharmB's favourite healthy donor, Chrysa Koukorava, for her endless supply of venous blood, without which many experiments in this thesis would not have been possible.

Finally, I would like to thank my family for their unconditional support throughout my PhD, even though you still have no idea what I have been doing for the last 4 years. I will print you a copy of this thesis and maybe one day we can discuss, I can only apologise you won't find it more exciting! But seriously, thank you for always being there to talk when I've needed it and for offering help and guidance along the way, it has made the (long) journey bearable and all the more worthwhile.

Covid-19 impact statement

The COVID-19 pandemic and subsequent closure of laboratories had a significant impact on my PhD research. Crucial data rich experiments were terminated prior to the closure of laboratories in March 2020 that were the culmination of over 3 months intensive work. In addition to this, primary cells (drug-specific T-cell clones) that had been expanded for 6 weeks were unable to be studied in greater depth and instead required cryopreservation, due to laboratory closure.

The phased return to the lab from August 2020 was on a part-time basis due to the reduced capacity in order to comply with social distancing rules and therefore the level out output was reduced over these months. Almost all studies within this thesis were reliant on access to venous blood samples from healthy volunteers, organised between researchers and nurses from the Royal Liverpool Hospital. Due to the pandemic, access to fresh blood for PBMC isolation was only readily available for a combined total of 8 weeks within the 10 month period stretching from March 2020 – February 2021. To mitigate against this disruption, cryopreserved PBMC samples were initially used in an attempt to bypass this issue. However, we observed that frozen samples were not an appropriate substitute for the use of fresh blood (PBMC) as T-cell cultures were unable to expand and lost previously characterised drug-specificity. This directly impacted T-cell cloning experiments within Chapters 3 and 5, as many potential vancomycin-specific T-cell clones generated from healthy donor 32, patient 3, patient 5 and patient 6 had to cryopreserved at an early stage and full characterisation of phenotypic and functional properties could not be performed due to a loss of drug-specificity.

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Contents

Publications

Published articles

Joshua Gardner, Monday Ogese, Catherine Betts, Munir Pirmohamed D.J. Naisbitt (2022). Characterisation of teicoplanin-specific T-cells from drug naïve donors expressing HLA-A*32:01. *Chemical research in Toxicology* **35:199-202.**

Sean Hammond, Anna Olsson-Brown, Sophie Grice, **Joshua Gardner** et al. (2021) Checkpoint inhibition reduces the threshold for drug-specific T-cell priming and increases the incidence of sulfasalazine hypersensitivity. *Toxicological Sciences* **186:58-69.**

Sean Hammond, Anna Olsson-Brown, **Joshua Gardner** et al. (2019) T-cell mediated hypersensitivity to previously tolerated iodinated contrast media precipitated by introduction of atezolizumab. *Journal for immunotherapy of cancer* **9:5.**

Monday Ogese, **Joshua Gardner**, Adam Lister, Xiaoli Meng et al. (2021) Deciphering Adverse Drug Reactions: *In Vitro* Priming and characterisation of vancomycin-specific T-cells from healthy donors expressing HLA-A*32:01. *Toxicological Sciences* **183:139-153.**

As part of the **COVID-LIV Study Group, (1)** Prospective observational study of SARS-CoV-2 infection, transmission and immunity in a cohort of households in Liverpool City Region, UK (COVID-LIV): a study protocol. *BMJ* (2021).

(2) Investigation of SARS-CoV-2 faecal shedding in the community: a prospective household cohort study in the UK. *BMC Infectious Diseases* (2021).

Ane Ogbe, Barbara Kronsteiner, **Joshua Gardner** et al. (2020) T cell assays differentiate clinical and subclinical SARS-CoV-2 infections from cross-reactive antiviral responses. *Nature Communications* **12:2055.**

Manuscripts in preparation

Joshua Gardner, Sean Hammond, Catherine Betts, Amy Chadwick, D.J. Naisbitt. Glycolysis: an early marker for drug-specific T-cell activation.

Joshua Gardner, James Line, Monday Ogese, Catherine Betts, D.J. Naisbitt. Determining T-cell responses in vancomycin hypersensitive patients expressing HLA-A*32:01.

Joshua Gardner, Sean Hammond and D.J. Naisbitt. Chemical allergy and the relevance of new models. *Frontiers in Toxicology* (Review).

Abstracts and communications

BBSRC DTP annual conference, Durham, UK (2020) – Poster

Immunogenicity of glycopeptide antibiotics implicated in drug hypersensitivity.

20th European Allergy and Clinical Immunology Winter School (2022) – Talk

*Determining T-cell responses in vancomycin hypersensitive patients expressing HLA-A*32:01.*

Society of Toxicology 61st annual meeting - San Diego, USA (2022) – Poster

Glycolysis: an early maker for drug-specific T-cell activation.

Society of Toxicology 61st annual meeting - San Diego, USA (2022) - Poster

*Characterisation of teicoplanin-specific T-cells from drug naïve donors expressing HLA-A*32:01.*

British Pharmacological Society annual meeting (BPS), Liverpool, UK (2022) - Poster

Glycolysis: an early maker for drug-specific T-cell activation.

Abstract

Type IV drug hypersensitivity reactions are mediated by the adaptive immune response and often delayed in nature. Pharmacogenomic risk factors can influence predisposition and strong correlations between the carriage of specific human leukocyte antigen (HLA) alleles and therapeutic compounds have been identified following genome-wide association studies (GWAS). The activation of T-cells is known to play a fundamental role within hypersensitivity manifestation due to the severity of adverse drug reactions (ADRs) associated. Recently, vancomycin has been linked with the development of drug reaction with eosinophilia and systemic symptoms (DRESS). Vancomycin is a naturally occurring glycopeptide antibiotic typically used as a 'drug of last resort' to combat severe, life threatening bacterial infection and remains the gold standard treatment option. DRESS is archetypal of a delayed-type drug hypersensitivity reaction and studies have now shed light on an association between vancomycin-induced DRESS and HLA-A*32:01 expression, indicating involvement of the adaptive immune system within reaction pathogenesis and a potential role for the activation of T-cells. Hence, we aimed to assess the intrinsic immunogenic potential of vancomycin and structurally related glycopeptide antibiotics, teicoplanin and daptomycin, using *in vitro* cell culture platforms and healthy donor models to evaluate the phenotypic and functional characteristics of drug-specific T-cells.

I*n vitro* priming assays detected vancomycin-responsive T-cells within peripheral blood mononuclear cell (PBMC) cultures of 7/10 donors positive for HLA-A*32:01 and 1/10 healthy donors negative for HLA-A*32:01, indicating preferential interaction between vancomycin and the associated risk allele. Vancomycin-responsive T-cell clones (TCCs) exclusively expressed a CD8+ phenotype and were successfully generated in 3/3 healthy donors expressing HLA-A*32:01. Upon vancomycin rechallenge, TCCs secreted Th1 (IFN-γ) and Th2 cytokines (IL-13) alongside cytolytic molecules, granzyme B and perforin. Proliferative responses to vancomycin were inhibited following MHC class I blockade and unaffected following fixation of antigen presenting cells (APCs) with glutaraldehyde, suggesting interactions between vancomycin and T-cell receptor (TCR) hinge upon HLA class I presentation, with T-cell responses evoked by direct, non-covalent binding, consistent with the pharmacological interaction (p-i) concept. Certain vancomycin-responsive TCCs displayed cross-reactivity with teicoplanin, mirroring the pattern of contraindication observed within clinical settings between structurally similar glycopeptide compounds. Studies focusing on the phenotype and functionality of vancomycin-response T-cells in vancomycin hypersensitive patients demonstrated the utility of the lymphocyte transformation test within the diagnosis of vancomycin hypersensitivity. Furthermore, the isolation of CD4+ drug-responsive T-cells from hypersensitive patients further implicates immune modulation within vancomycin-induced DRESS. TCCs generated were functionally similar to those generated within healthy donor studies, although activation was observed to ensue in a HLA class II-restricted manner. Teicoplanin-specific TCCs were generated from healthy volunteers expressing HLA-A*32:01 and pathways of T-cell activation and HLA restriction were defined. Teicoplanin-reactive TCCs exclusively expressed a CD8+ phenotype and exhibited a processing independent HLA class I-restricted mechanism of activation. Cross-reactivity was demonstrated with the lipoglycopeptide daptomycin within proliferation assays and cytokine/cytolytic molecule release (IFN-γ, IL-5, IL-13, granzyme B, perforin and FasL) was observed. These data suggests teicoplanin, like vancomycin, is intrinsically immunogenic and capable of activating T-cells, which may play a role in the pathogenesis of teicoplanin-induced DRESS.

The study of immunometabolic pathways as an early maker for drug-specific T-cell activation revealed a flux towards a glycolytic state accommodates cellular energetic requirements for vancomycin-specific T-cell activation. Acute vancomycin exposure induced a dose-dependent and drug-specific glycolytic shift, with cross-reactivity between structurally similar glycopeptide antibiotics detected. TCCs with prior specificity for vancomycin were found to exhibit glycolytic switching after exposure to teicoplanin, for which T-cell responses have been implicated within the pathogenesis of immune-mediated ADRs. Notably, glycolytic activation of TCCs was observed to be HLA restricted, as exposure to HLA-DR blockade attenuated the glycolytic flux with TCCs remaining in a quiescent state. In summary, these studies demonstrate the utility of metabolic readouts within the functional characterisation of T-cells involved in hypersensitivity reactions to further elucidate patterns of reactivity alongside early drug/HLA mediated TCR triggering.

To conclude, this study implicates T-cell involvement in vancomycin-induced DRESS and a preferential interaction between vancomycin and HLA*32:01. Functional assays focusing on healthy donor responses to vancomycin and teicoplanin have eluded to the intrinsic immunogenic potential of both glycopeptide compounds, with complex patterns of T-cell cross-reactivity observed. Studies conducted using vancomycin hypersensitive patient PBMC suggest participation of drug-reactive CD4+ T-cells within vancomycin hypersensitivity, although further work will be required to fully determine the role of CD8+ T-cells within DRESS pathogenesis. Furthermore, energetic readouts in the form of glycolysis measurement have applications towards studying both the chemical and molecular basis of drug-specific T-cell activation and further assay development may provide a greater understanding of metabolic factors that influence the propensity to suffer from drug hypersensitivity reactions.

Abbreviations

AB serum	Antibody serum
ADE	Adverse drug event
ADP	Adenosine diphosphate
ADR	Adverse drug reaction
AGEP	Acute generalised exanthemous pustulosis
AHS	Abacavir hypersensitivity syndrome
ALP	Alkaline phosphatase
APC	Allophycocyanin
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BCIP-NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium dye
BCL6	B-cell lymphoma 6 protein
BSA	Bovine serum albumin
CBZ	Carbamazepine
CCR	Chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
Ci	Curie
CLA	Cutaneous leukocyte-associated antigen
CO_2	Carbon dioxide
CPT1a	Carnitine palmitoyl transferase
CSA	Cyclosporine A
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte Associated Protein-4

CYP	Cyctochrome P450
Da	Daltons
DAMP	Damage associated molecular pattern
DC	Dendritic cells
DDS	Dapsone
DDS-NO	Dapsone nitroso
DHS	Drug hypersensitivity syndrome
DILI	Drug induced liver injury
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus transformed B-cell
E-cad	E-cadherin
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme-linked immunospot
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FAO	Fatty acid oxidation
FasL	Fas Ligand
FBS	Foetal bovine serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FITC	Fluorescein isothiocyanat
FoxP3	Forkhead box P3
FSC	Forward scatter

GB	Granzyme B
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
h	Hours
HBSS	Hanks' balanced salt solution
HD	Healthy donor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHV-6	human herpes viruses 6
HHV-7	human herpes viruses 7
HIF	hypoxia inducible factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSA	Human serum albumin
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LAG-3	Lymphocyte activation gene 3
LPS	Lipopolysaccharide
LS	Lymphocyte selection
LTT	Lymphocyte transformation test
M	Molar
MACS	Magnetic activated cell sorting (
MAPK	Mitogen activated protein kinase
MFI	Mean fluorescence index

mg	Milligram
MHC	Major histocompatibility complex
Min	Minutes
mL	Millilitre
mM	Millimolar
Mo-DCs	Monocyte-derived dendritic cells
MPE	Maculopapular exanthema
MRSA	Methicillin-resistant Staphylococcus aureus
NF-KB	Nuclear factor kappa B
NK	Natural Killer
Omy	Oligomycin
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
PE	Phycoerythrin
PFA	Paraformaldehyde
pH	Power of hydrogen
PHA	Phytohaemagglutinin
P-i	Pharmacological interaction
PI3K	Phosphoinositide 3-kinase
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene difluoride

RMS	Red man syndrome
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SCAR	Severe cutaneous adverse reaction
SFU	Spot forming units
SI	Stimulation index
SJS	Stevens Johnson Syndrome
SLAM	Signalling lymphocytic activation molecules
SMX	Sulfamethoxazole
SMX-NHOH	Sulfamethoxazole-hydroxylamine
SMX-NO	Sulfamethoxazole nitroso
SSC	Side scatter
STAT	Signal Transducer and Activator of Transcription
TCA cycle	Tricarboxylic acid cycle
TCC	T-cell clone
TCR	T-cell receptor
TEN	Toxic epidermal necrolysis
T_Fh	T follicular helper cells
TGF-β	Transforming growth factor β
T_h	T helper cell
TIM-3	T-cell immunoglobulin mucin-3
T-MWA	T-cell multi-well assay
TNF-α	Adenosine diphosphate
Treg	T-regulatory cells

w/v	Weight/volume
WHO	World Health Organisation
°C	Degrees Celsius
µl	Microliter
µM	Micromolar
2-DG	2-deoxy-D-glucose

1. General Introduction.

1.1 The immune system.

The immune system incorporates complex physiological processes with the fundamental role of protecting the host from harmful foreign bodies such as bacteria, viruses and chemicals (Schultz and Grieder, 1987). A key feature of the immune system is the ability to constantly monitor for the presence of environmental agents within the body. This dynamic process is known as immunosurveillance and hinges on the hosts capability to recognise and discriminate 'self' from 'non-self' antigens with dysregulation leading to the development of autoimmune diseases such as rheumatoid arthritis and Systemic Lupus Erythematosus (Lorenz et al., 2001). This intricate system is comprised of innate and adaptive immunity with the response often coordinated in a synergistic manner.

White blood cells, essential for innate and adaptive immune responses, originate in either the thymus or bone marrow. The innate response acts as the first line of defence against foreign antigens and immediately transpires following infection incorporating both hematopoietic and non-hematopoietic derived cell types such as dendritic cells (DCs), natural killer (NK) cells, mast cells, eosinophils, macrophages and neutrophils (Chaplin, 2010, Balato et al., 2009, Jonsson and Yokoyama, 2009). Such responses typically present on the skin in addition to manifestations within respiratory and gastrointestinal tracts mediated by epithelial cells at the protective barrier of the host (Schleimer et al., 2007). Adaptive immunity, sometimes referred to as 'acquired' immunity, is a more recent evolutionary adaptation of the hosts defence system and is unique to higher vertebrates (Litman et al., 2010). Unlike innate immunity, the adaptive response is delayed and possesses greater complexity and plasticity. This highly specific response requires the activation of lymphocytes and can be defined, rather simplistically, as either cell-mediated (T-cells) or antibody-mediated (B-cells) (Clark and Kupper, 2005). This work will focus on the cellular components comprising the adaptive immune response.

1.1.2 Adaptive immunity.

The cellular components and systematic processes of both the innate and adaptive immune response are not mutually exclusive and instead possess the capacity for complementation to sustain effective immunological clearance (Turvey and Broide, 2010). However, distinct points of divergence do exist. In contrast to innate immunity, the acquired response is an antigen-dependent process and is activated when the innate response is unable to provide sufficient protection to the host against pathogenic invasion (Parish and O'Neill, 1997). The high degree of specificity associated with adaptive immunity contributes to the lag time observed between initial antigenic exposure and effector function (Bonilla and Oettgen, 2010). Cells of the adaptive response are proficient at distinguishing between “self” and “non-self” antigens in addition to possessing immunological memory function. This unique capacity for memory allows for a more rapid and enhanced response following re-exposure to the same antigen (Kaech et al., 2002). The cellular constituents implicated produce either a cell-mediated or antibody mediated (humoral) response to infection. Lymphocytes are the chief instigators of both cell-mediated and humoral immunity and can be classified as T lymphocytes or B lymphocytes although various sub-classes exist.

1.1.2.1 T lymphocytes.

The importance of the thymus for T-cell development and selection was initially proposed in 1969, in which optimal microenvironment conditions were identified for differentiation and maintenance of regulatory function (Nishizuka and Sakakura, 1969). Following development and selection towards specific groups of differentiated lymphocytes, T-cells are ideally suited to a prominent role within cell-mediated responses due to their ability to recognise specific antigen moieties displayed on antigen presenting cells (APCs) (Kaech et al., 2002). Stimulation is achieved following interactions between antigenic peptides, APCs and the T-cell receptor (TCR) leading to proliferation and expansion of effector and helper populations that

coordinate a highly specialised response against non-self-antigens. Cells expressing the CD4 co-receptor (CD4+) are commonly referred to as helper T-cells while cells expressing the CD8 co-receptor (CD8+) are known as cytotoxic T-cells (Ahmed et al., 1988, Cerottini et al., 1970). However, effector function of both major groups is not absolute as empirical evidence exists demonstrating the cytotoxic capabilities of CD4+ T-cells (van de Berg et al., 2008).

1.1.2.2 T helper cells.

T helper (T_h) cells express the CD4+ co-receptor and are a crucial cellular component of the hosts defence system. Function is ubiquitous throughout the adaptive immune response and they possess a high degree of versatility. This is evidenced by their ability to assume multiple functional roles such as promotion of antibody secretion and the activation of macrophages and cytotoxic T-cells (Vinuesa et al., 2005). The Activation of T_h cells involves the presentation of antigenic peptides on major histocompatibility complex (MHC) class II molecules, expressed on the surface of APCs, to the TCR (Doyle and Strominger, 1987). Following stimulation and activation, T_h cells are unique in their ability to polarize and differentiate into distinct T_h cell subsets to maintain effective cellular immunity.

Upon stimulation of antigen naïve T_h cells, commonly denoted as T_h0 cells, differentiation commences towards either a T_h1 or T_h2 subclass (Mosmann et al., 1986). These two subgroups are functionally dissimilar and possess many non-overlapping traits that contribute towards a CD4+ response (Swain, 1995). In terms of functional roles within the immune response the T_h1 subclass is most proficient in clearing both viruses and pathogenic material (Sacks and Noben-Trauth, 2002), with T_h2 cells being more involved within humoral immunity and orchestrating effector function against parasites such as helminth infections (Anthony et al., 2007).

The dichotomy between the T_h1 and T_h2 subclass becomes apparent when observing the conditions necessary for polarisation. Differentiation of uncommitted T_h cells to the T_h1 subset is dependent on polarising signals provided by IL-12 and IFN-γ (Hsieh et al., 1993). In contrast,

polarisation to Th_2 cells is primarily driven by IL-4 and IL-10 (Swain et al., 1990). Preceding the aforementioned conditions necessary for successful polarisation of T_h cells, a paradigm exists between T_h1 and T_h2 subsets in which the concept of detached lineages has been evidenced (Romagnani, 1997). 'Master' transcriptional factors have now been identified in the form of T-bet (Tbx21) and GATA-3 that both regulate and drive transcription towards a T_h1 or T_h2 lineage, respectively (Szabo et al., 2000, Zheng and Flavell, 1997). Lineage direction is dependent on initial cytokine-mediated signalling via the signal transducer and activator and transcription (STAT) pathway. It has been suggested that members of that STAT family, specifically STAT4 and STAT1, induce differentiation toward a T_h1 lineage (Afkarian et al., 2002, Thierfelder et al., 1996) whereas STAT6 induces T_h2 specific lineage differentiation (Kaplan et al., 1996). However, this may be viewed as an over simplistic narrative due to inherent plasticity between T_h subsets.

Functionally, T_h1 cells play a crucial role within immune responses against fungi, bacteria and protozoa aided by their ability to secrete macrophage activating IFN-γ in addition to pro-inflammatory cytokines such as IL-2, IL-6, IL-12 and tumour necrosis factor α (TNF-α) (Murphy and Reiner, 2002). Conversely, cells expressing a Th_2 phenotype are associated with the secretion of cytokines including IL-4, IL-5, IL-6, IL-9 and IL-13 to facilitate antibody-mediated immune responses against allergens, bacteria and other toxins (Mosmann and Coffman, 1989). Primordial studies proposed, somewhat controversially, the involvement of Th_1 cells within cytotoxic CD8+ responses in which priming occurs in the absence of inflammation (Bennett et al., 1998, Ridge et al., 1998, Schoenberger et al., 1998). These findings were initially viewed as paradoxical due to the functionally dissimilar nature of T_h cells in regard to downstream effector function. CD4+ T-cells have now been described as an essential precursor to CD8+ mediated effector memory function, even under conditions supportive of an independent CD8+ response following infection (Janssen et al., 2003, Shedlock and Shen, 2003). Interestingly, both positive and negative feedback exists within this paradigm of

polarisation. Indeed, the presence of T_h1 polarizing and effector cytokines will actively suppress functionality of the Th_2 phenotype and simultaneously drive differentiation further in the direction of the T_h1 subclass (Fiorentino et al., 1991). This phenomenon is also consistent with polarising signals associated with the T_h2 phenotype.

Th_{17} cells were first described by Harrington et al. in 2005 in which signature cytokines, such as IL-17A and IL-17F, were found to be associated with a specific CD4+ mediated T-cell response (Harrington et al., 2006). These findings gave credence to the recognition of Th_{17} cells as distinct subset and provided the initial challenge to the previously accepted T_h1/T_h2 paradigm proposed by Mosmann and Coffman (Mosmann et al., 1986). The activation of Th_{17} cells and associated effector cytokines is believed to be in response to extracellular bacterial or fungal infection for which both Th1 and Th2 responses are deemed inadequate (Weaver et al., 2006). In line with the generation of other T_h cell subsets, T_h17 development and differentiation is driven by a myriad of transcriptional factors. The case has been made for the involvement of the *Rorc* gene from which the transcriptional regulators RORα4 and RORγt are encoded. Studies have alluded to the synergic relationship between both RORα4 and RORγt in which co-expression drives IL-17 production (Yang et al., 2008, Ivanov et al., 2006). However, in isolation this is not sufficient for full development of the T_h17 program with other regulators of transcription such as hypoxia inducible factor α (HIF-1α) and STAT3 found to be involved (Dang et al., 2011, Durant et al., 2010). Interestingly study of these transcriptional regulators, specifically STAT3, has advanced understanding of the role of T_h17 within autoimmune responses. Aberrant STAT3 signalling and by extension increased T_h17 activity has now been found within the tissues of individuals presenting with both Crohn's disease and multiple sclerosis (Lovato et al., 2003, Frisullo et al., 2006).

More recently, T_h22 cells have been described. These cells are typically pro-inflammatory and were initially observed within peripheral blood monocular cells (PBMC) of healthy individuals and later found to be capable of skin infiltration (Eyerich et al., 2009). Indeed, recent work has characterised the involvement of skin-resident T_h22 cells within drug hypersensitivity, specifically relating to adverse events associated with β-lactam antibiotics (Sullivan et al., 2018). T_h22 cells can be defined by secretion of IL-22 in addition to TNF-α and unlike other T_h subsets these populations lack common co-secretion factors such as IFN-γ, IL-4 and IL-17 (Trifari et al., 2009). Whilst T_h17 cells are also capable of IL-22 secretion, the signature cytokine profile of T_h22 cells is unique in their capacity for exclusive IL-22 secretion in the absence of IL-17, a phenomenon not reciprocated (Duhen et al., 2009). In recent years alternative classification systems have been proposed, such as categorisation based on immune function and chemokine receptor profiles (Spits et al., 2013). It is perhaps understandable that unilateral agreement has not been achieved due to the inherent plasticity of these cell types ultimately making definitive classification problematic. A full summary of the polarising conditions necessary for T_h cell differentiation and associated effector cytokines for each T_h subset can be viewed in Figure 1.1.

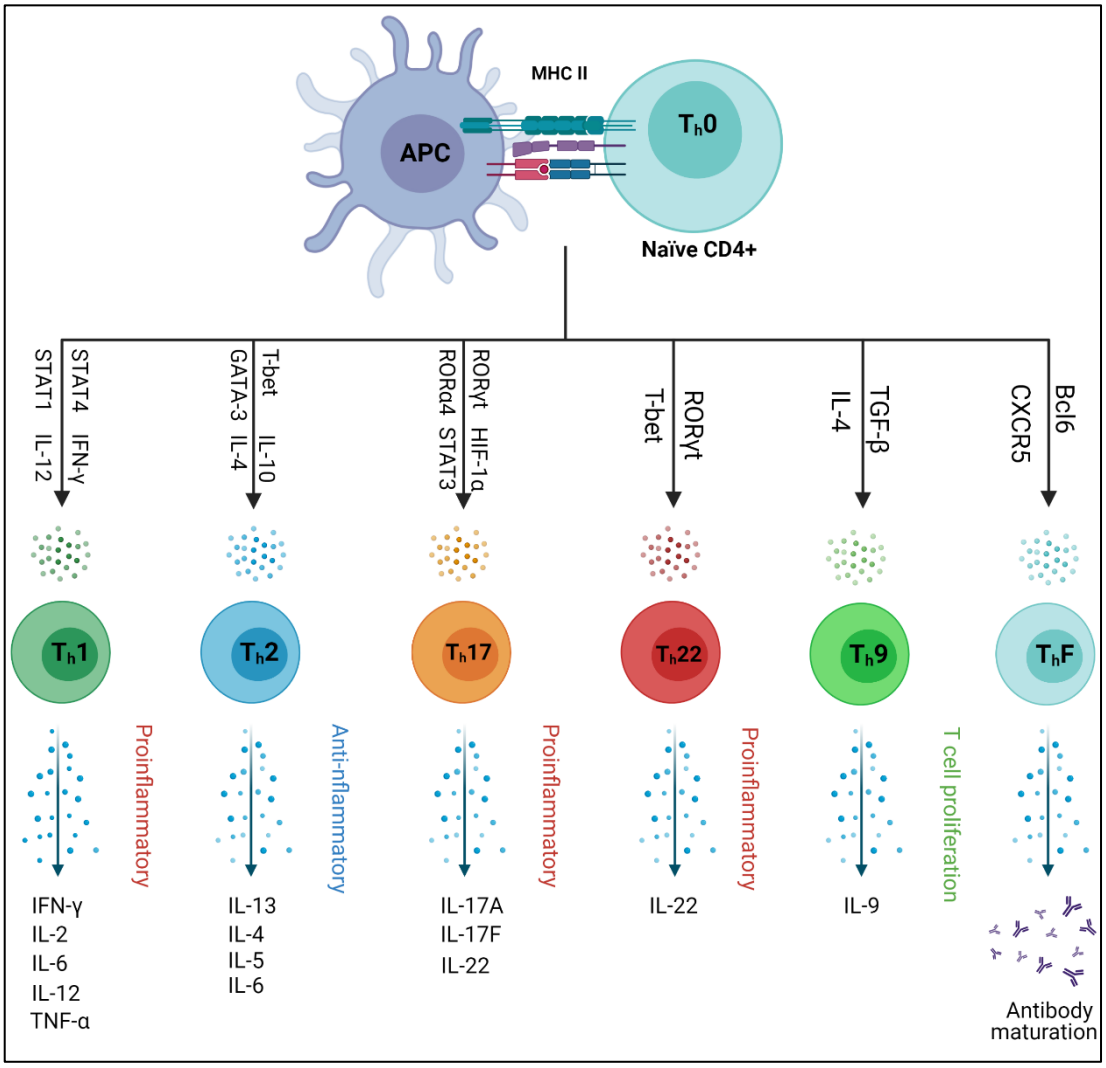


Figure 1.1. Summary of the polarising conditions necessary for naive CD4+ expressing T helper cell differentiation into helper cell subsets and effector cytokine secretion patterns of Th1, Th2, Th17, Th22, Th9 and ThF cells.

1.1.2.3 Regulatory T-cells.

Regulatory T-cells (Tregs) represent another branch of the CD4+ T-cell response. These heterogeneous populations may be natural or inducible and are both derived from activation of the Foxp3 transcriptional regulator (Hori et al., 2003). Tregs are traditionally characterised by the expression of CD25+ on the cellular surface in addition to co-expression of both CTLA-4 and CD127+ (Sakaguchi et al., 1995, Read et al., 2000, Seddiki et al., 2006). However, empirical evidence now suggests these surface markers are not entirety specific to Treg populations and may be transiently expressed on the surface of all activated T-cells.

Classically, Tregs are tasked with maintaining self-tolerance and they play an integral role in suppressing immune function arising from the activation of T_h cells (Sakaguchi et al., 1995). The suppressive activity of Tregs first requires TCR mediated activation. To this end, *in vivo* mouse studies have shown the immunosuppressive function of Tregs to be highly antigen-dependent (Thornton and Shevach, 1998). It is thought that optimal suppression occurs at the point both the Treg and the cognate supressed effector cell have a similar specificity for the same antigen, although evidence exists that T_h cell suppression may still occur when antigen avidity varies (Tang et al., 2004). An important characteristic of Tregs within tolerogenic pathways is their ability to recognise self from non-self-antigens. Interestingly, initial studies in mice reported reactivity to self-peptides (Takahashi et al., 1998), although more recent work analysing an extended pool of TCR repertoires involved in Treg activation found preferential recognition of self-peptides (Pacholczyk et al., 2007).

1.1.2.4 Cytotoxic T lymphocytes.

Cytotoxic T lymphocytes, commonly referred to as CTLs, are produced in the thymus and can be characterised by expression of the CD8+ co-receptor. These cells are predominantly recognised for their effector function following stimulation via MHC class I antigen presentation and play a leading role within cytotoxic responses to viruses, intracellular pathogens and bacteria and possess the capacity for tumour surveillance (Castelli et al., 2000). CD8+ expressing CTLs can exist as naïve, effector or memory T-cells with each physiological state dependent on both the degree of maturation and prior exposure to antigenic determinants. Cell surface markers, specifically co-stimulatory and chemokine receptors, have been identified and enable classification of CTLs based on phenotypic properties. This has proven particularly useful for the determination of naïve, effector and memory populations, arising from elevated expression of phenotypic markers such as CD45RA, CD45RO, CD28 and CCR7 (Sobao et al., 2001, Tomiyama et al., 2002, Champagne et al., 2001).

The elicitation of cytotoxic T-cell responses hinges upon the action of effector CD8+ expressing populations. Functionally speaking, CTLs are involved in the destruction of infected cells via apoptotic or granzyme B releasing pathways. Effector CD8+ T-cells can be categorised according to cytokine secretion and distinct subpopulations have now been described (Tc1 and Tc2). Both Tc1 and Tc2 cells have been found to predominantly favour the release of cytotoxic granules such as perforin which results in the formation of pores within the cell membrane, allowing serine proteases (granzymes) to flood infected cells and cleave proteins critical for viral expansion. This process, performed following the activation of procaspase-10 by granzyme B, culminates in cellular destruction via apoptosis of target cells (Sad et al., 1997, Brunner et al., 2003, Barry and Bleackley, 2002). Proteoglycans such as serglycin have been shown to aid the delivery of cytotoxic molecules into target cells (Figure 1.2), with studies clearly demonstrating that serglycin-deficient CTLs are associated with defective granzyme B storage and delivery (Grujic et al., 2005). CTLs are also known to exert their cytotoxic effects via direct activation of extrinsic apoptotic pathways in which the secretion of FasL by activated CTLs and subsequent interaction with FAS receptors results with initiation of the caspase cascade (Brunner et al., 2003).

The downstream cytotoxic action of CTL has now been implicated within the development of adverse drug reactions, and specifically within hypersensitive responses to therapeutic compounds. Indeed, blister fluid derived from patients presenting with Stevens-Johnson syndrome has eluded to the presence of CD8+ CTLs (Le Cleach et al., 2000). Furthermore, additional study within this area has now identified CD8+ drug-specific CTLs within blister fluid following exposure and subsequent hypersensitive responses to both carbamazepine and trimethoprim-sulfamethoxazole (Bigby, 2001).

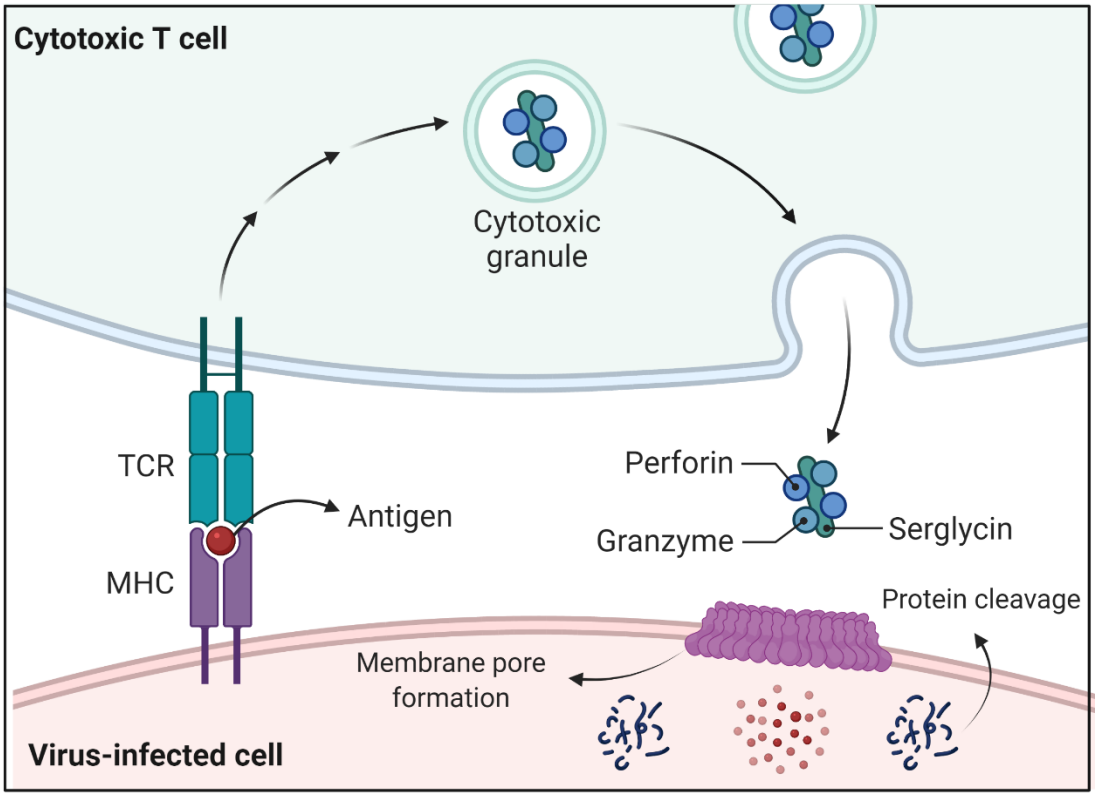


Figure 1.2. Schematic displaying the action of cytotoxic T-cells relating to the destruction of virus-infected cells, mediated by the release of cytolytic and cytotoxic molecules such a perforin and granzyme B.

1.1.2.4 Dendritic cells.

DCs belong to the mononuclear phagocyte system and possess antigen presentation capabilities (Guilliams et al., 2014). Mature DCs are present in tissues that surround protective barriers such as the skin, whereas in their immature state DCs can be found within the bloodstream. DCs are responsive against pathogen invasion and express multiple pattern recognition receptors (PRRs) such as TLR4, necessary for their innate immune function (Basset et al., 2003). However, they are widely considered to be professional APCs and possess a stellate morphology which promotes their ability for antigenic presentation to naïve T-cells via MHC molecules (Steinman and Cohn, 1973, Steinman and Witmer, 1978). Interestingly, DCs are unique in their ability to prime naïve T-cells to foreign antigens and do so in an extremely efficient manner. One particular study revealed that between 1000 and 3000 naïve T-cells could be successfully primed from just a single DC (Banchereau and Steinman, 1998).

Monocytes expressing CD14+ can undergo the reversible process of differentiation to DCs of an immature state in response to signalling from IL-4 and GM-CSF (Sallusto and Lanzavecchia, 1994). Immature DCs are typically activated in response to inflammation induced by cellular stress and necrotic signalling. For activation to occur, danger signals are required from pathogen-associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs), typically provided by molecules such as lipopolysaccharide (LPS) and TNF-α which are now widely deployed within *in vitro* assays to provide external stimuli and propagate conditions conducive to T-cell activation (Watts, 1997, Shi et al., 2003). Once mature, DCs are effective in antigen capture through pathways of micropinocytosis or receptor-mediated endocytosis (Sallusto et al., 1995). The migratory capacity and homing ability of DCs in response to innate immune signalling and prior inflammation is enhanced post antigen capture, enabling the selective targeting of tissues. In this way, DCs can assume the role of messengers between these two complex physiological systems and bridge the gap between innate and adaptive immunity. As a consequence, the activity of DCs are a pivotal component of immune responses to drugs, allergens and chemicals for which effector T-cell function can induce unwanted adverse effects, such as tissue damage, but is ultimately dictated by initial innate signalling and prior inflammatory cascades.

1.2. Adverse drug reactions.

1.2.1 Definition of an adverse drug reaction (ADR).

According to The World Health Organisation (WHO) an ADR is defined by “a response to a drug which is noxious and unintended, and which occurs at doses normally used in man for the prophylaxis, diagnosis, or therapy of disease, or for the modifications of physiological function” (WHO, 1969). This definition is often confused with an adverse drug event (ADE), an umbrella term describing any untoward occurrence that may present during treatment with a pharmaceutical product but does not necessarily have a causal relation to the treatment (WHO, 2005). These two definitions diverge when comparing the contributing factors to the adverse event or reaction. ADRs can manifest independently of correct prescription and dosage whereas ADEs may arise from the inappropriate application of a drug, independent of known pharmacological interactions.

1.2.2 Classifying adverse drug reactions.

The clinical manifestation of an ADR can be generally placed within the bounds of two distinct categories. Reactions intrinsically augmented by drug dosage (type A) are predictable and directly relate to the pharmacological characteristics of the compound (Royer, 1997). However, idiosyncratic or “bizarre” reactions (type B) are unpredictable in nature and are often unrelated to a drugs primary pharmacology (Edwards and Aronson, 2000). Other classifications include chronic (type C), delayed (type D), withdrawal (type E) and treatment failure due to drug interactions (type F) (Edwards and Aronson, 2000).

Type A reactions are referred to as “on-target” events and account for the vast majority of cases with over 70% of ADR related hospital admissions resulting from these predictable dose-dependent adverse effects (Einarson, 1993). Although these reactions are common, mortality rates are generally low and management strategies are simple and effective. Indeed, reducing dosage or withdrawing the culprit drug is often a successful approach along with consideration

for the impact of concomitant treatment options (Edwards and Aronson, 2000). The anticoagulant, Warfarin, is archetypal of a type A inducing compound due to the severity of ADRs suffered dependent on drug dosage. Warfarin administration is associated with severe ADRs such as bleeding and haemorrhage, with the drug cited as a major cause of ADR that leads to hospitalisation (Pirmohamed et al., 2004).

Drugs that induce type B reactions act in an "off-target" manner with adverse effects induced following the modulation of other targets. These reactions are rare and far more sporadic than type A reactions, only accounting for approximately 10-15% of all adverse reactions (Ju and Uetrecht, 2002). In contrast, mortality rates associated with type B reactions are relatively high in view of difficulties when predicting and preventing adverse effects (Lazarou et al., 1998). Due to their inherent unpredictability these reactions are termed idiosyncratic which refers to their specificity in susceptible individuals within clinically tolerated dose ranges (Uetrecht, 2007). An interesting aspect is the involvement of the adaptive immune response within these idiosyncratic reactions. ADRs facilitated by the host's adaptive immune system are described as drug hypersensitivity reactions or simply, drug allergies (Demoly and Hillaire-Buys, 2004). This response is multi-faceted and involves interactions between drug-specific T lymphocytes, antigenic complexes and IgE antibodies with genetic aetiologies contributing to predisposition also known to play a role (Riedl and Casillas, 2003). This work will predominantly focus on type B reactions, mediated by the adaptive immune response.

1.2.3 Drug hypersensitivity reactions.

Hypersensitivity reactions are categorised as either allergic or non-allergic responses to a drug. Non-allergic reactions (pseudoallergic idiosyncratic responses) are often referred to as anaphylactoid reactions and clinical features can be indistinguishable from allergic responses (Farnam et al., 2012). One popular hypothesis attributes these non-allergic reactions to the release of inflammatory cytokines from mast cells and basophils mediated by the innate

immune response (Abraham and Arock, 1998, Buonomo et al., 2010). A classic example of pseudoallergic hypersensitivity is the onset of red man syndrome in response to treatment with the glycopeptide antibiotic vancomycin (Sivagnanam and Deleu, 2003).

Allergic drug hypersensitivity reactions exhibit an immunological origin. These reactions are mediated by the adaptive response and are best defined by a "state of altered reactivity in which the body reacts with an exaggerated immune response to a foreign substance" (Roujeau, 2005). Immune-related idiosyncratic reactions can range from mild symptoms such as skin rash or mild elevation of liver enzymes (alanine transaminase) to potentially fatal conditions including Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) and acute liver failure (Carr and Pirmohamed, 2018). Drugs that induce hypersensitivity via this route interact with the adaptive immune system in a complex manner and patterns of activation are generally poorly understood despite considerable recent advancements. Therefore, it is of paramount importance to further expand our understanding of the underlying chemical and molecular mechanisms for immune-mediated drug interactions to decipher individual susceptibility.

1.2.4 Classifying drug hypersensitivity.

The traditional classification system for describing drug hypersensitivity reactions was originally proposed by Gel and Coombs in 1963. This pioneering system underpins much of the basis for our understanding of the pathophysiological mechanisms involved in the onset of immune-mediated hypersensitivity and incorporates four distinct categories of drug allergy (Figure 1.3). Type I allergic reactions are perhaps the most unambiguous in nature regarding the clear-cut link between the innate immune system and their immunopathological characteristics. These reactions primarily originate from the binding of immunoglobulin E (IgE) antibodies to mast cells and basophils. Resultant degranulation induces the release of the pro-inflammatory mediators, histamine and leukotriene, leading to the presentation of immediate

and delayed symptoms respectively. Certain β-lactam antibiotics are known to promote hypersensitivity via this route giving rise to clinical manifestations such as anaphylaxis (Torres et al., 2003).

Type II drug hypersensitivity reactions are synonymous with antigen-antibody interactions and mediated by the production of the chemoattractant, anaphylatoxin. The ensuing mobilization of polymorphonuclear leukocytes induces tissue injury via the release of neutrophilic enzymes. This cytotoxic reaction is dependent upon the interaction of IgM or IgG antibodies and can be described as a 'deleterious' reaction in which the host is protected from foreign antigens. The mechanisms that comprise Type II hypersensitivity are complicit in the development of several conditions affecting blood vessels, collectively known as vasculitis. In contrast to Type II reactions in which antigen-antibody interactions are tissue based, Type III hypersensitivity involves the formation of antigen-antibody complexes within the blood. These reactions are typically IgM mediated and antigen-antibody complexes accumulate inside glomerular and pulmonary membranes. Following complex deposition within the basement membrane, the cellular basis for hypersensitivity follows a similar pattern to that of Type II hypersensitivity in which the recruitment of polymorphonuclear leukocytes promotes tissue damage via activation of the complement cascade (Rajan, 2003).

Type IV hypersensitivity reactions are antibody independent and clinical manifestations are delayed in nature with symptoms typically presenting between 2-28 days following exposure to the culprit drug (Jacysyn et al., 2001). Several factors can affect the time-course of the reaction such as previous sensitisation to the drug, with adaptive immune responses heavily implicated. Indeed, the activation of T-cells is now known to play a fundamental role within the development of Type IV hypersensitivity. Reactions are provoked following presentation of drug antigens by APCs, stimulating a T lymphocyte response and resulting in the release of effector cytokines which can lead to contact dermatitis in the form of skin eruptions (Krasteva,

1993). Due to their abundance, drug-induced Type IV hypersensitivity reactions can be further divided into four subgroups, resulting from distinct variances in basic pathophysiology and the effector cells involved.

Although the Gel and Coombs classification system is still accepted within the majority of immunological fields, questions have surfaced regarding its validity when applied to specific compounds and physiological processes. Applied to hypersensitivity reactions mediated by the adaptive response the system can be viewed as an oversimplification due to the complex nature of antigenic interactions and T-cell involvement. This is evident in the case of the β-lactam antibiotics in which a degree of overlap can be observed in their ability to induce both anaphylaxis (Type I) and contact dermatitis (Type IV). Such constraints of the traditional system do not account for plasticity and crossover analogous with immunological function. Moving forward it will be necessary to improve upon the system, specifically within the context of Type IV reactions, to better define immune-mediated drug hypersensitivity and ultimately aid predictive strategies.

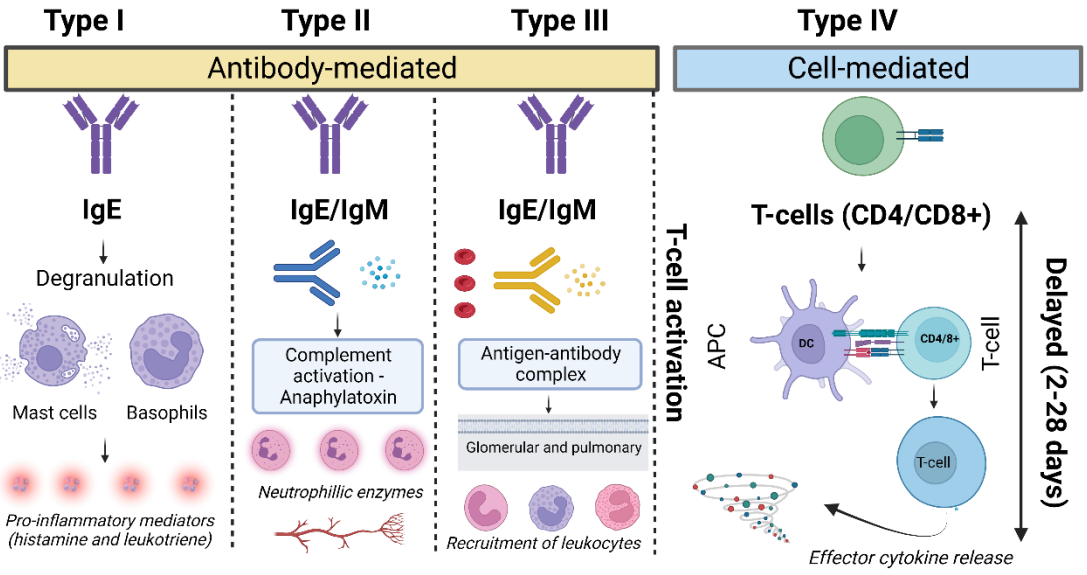


Figure 1.3. Overview of Type I –Type IV drug hypersensitivity reactions according to the Gel and Coombs classification system, as defined by reaction pathogenesis and effector molecules.

1.2.5 Immediate and delayed-type drug hypersensitivity.

The time course between drug treatment and the onset of clinical symptoms can provide mechanistic insight into the nature of hypersensitivity reaction experienced. Within a clinical setting, drug hypersensitivity reactions can be classified as either immediate or delayed-type responses to drug that have diverging pathophysiological characteristics (Demoly et al., 2014). Hypersensitivity that presents in an immediate fashion typically occurs within 1 hour following treatment with the culprit drug and are associated with the onset of conditions such as anaphylaxis, urticaria and angioedema (Schrijvers et al., 2015). In contrast, clinical symptoms typical of delayed-type drug hypersensitivity reactions commonly manifest over an exaggerated time frame of between days and weeks following drug administration (Hausmann et al., 2010). Disease states analogous with delayed-type hypersensitivity are known to target vital organs, primarily affecting the function of both the skin and liver. The manifestation of severe cutaneous adverse reactions (SCARs) and drug-induced liver injury (DILI) is consistent with the pathogenesis of delayed-type hypersensitivity and can contribute towards high mortality rates of between 20-30% in extreme cases (Husain et al., 2013). The clinical manifestations of both cutaneous drug reactions and DILI will be discussed at greater length in Section 1.2.8.

A myriad of mechanistic factors underpin the physiological differences observed between the onset of immediate and delayed-type drug hypersensitivity. The accelerated response shown with immediate type reactions is thought to be caused by the rapid sensitisation of mast cells and subsequent release of cytokines within minutes of drug exposure, facilitated by IgE antibodies produced by B-cells within the adaptive immune response (Galli and Tsai, 2012). Delayed-type hypersensitivity reactions are also mediated by the adaptive response and specifically by the activation of T-cells (Naisbitt et al., 2003a). The T-cell response comprises multiple cellular and molecular processes including priming, activation and proliferation that

leads to a delayed response (Zikherman and Au-Yeung, 2015). Indeed, the commitment of effector T-cells to a drug antigen takes place less than 1 hour following antigenic exposure, whereas a naïve T-cell requires 20 hours of continuous signalling for commitment (Iezzi et al., 1998). However, the process of T-cell priming, along with proliferation and differentiation can require approximately 5-7 days (Ortiz et al., 1997), with *in vitro* experiments suggesting a time period of 8 days for naïve to T-cell priming to model compounds such as nitroso-sulfamethoxazole (SMX-NO) (Faulkner et al., 2016b). On this basis, the rationale for the rapid onset of hypersensitivity following rechallenge with the culprit drug is clear. Conventional rate-limiting steps of the T-cell response can be bypassed, and the presence of drug-primed memory T-cells allow for a swifter response that may be of greater severity than the initial adverse reaction (Sousa-Pinto et al., 2016).

1.2.6 Epidemiology and risk factors of drug hypersensitivity.

There are several factors to consider when determining the risk to an individual such as those directly relating to the culprit drug itself and factors surrounding the specific characteristics of susceptible patients. Well characterised risk factors that are unilateral in their propensity for ADRs include advancing age and gender, with females displaying greater predisposition (Schöpf et al., 1991, Sharma et al., 2008, Gurwitz and Avorn, 1991). For this work, individual risk factors relating to type B drug hypersensitivity will be considered.

Correlation has been observed between the degree of drug exposure and prevalence of drug hypersensitivity reactions. In 2003 a noteworthy study found the topical application of the contact sensitizer, neomycin, exhibited a dose-dependent relationship with T-cell mediated hypersensitivity (type IV) inducing contact allergies in the form of skin allergies such as eczema (Jensen et al., 2003). Evidence also suggests that lower therapeutic doses (< 10 mg/day) are generally safer and reduce the risk of immune-mediated adverse reactions (Uetrecht, 1999). The route of administration has also been shown to impact predisposition. Orally administered

drugs, due to the absence of contact sensitization, are generally considered a safer alternative. Empirical evidence for this phenomenon exists in the case of penicillin, which was withdrawn from topical formulations due its contact sensitizing properties in favour of the orally administered route we are familiar with today (Idsoe et al., 1968).

Patients already presenting with disease states is another predisposing factor requiring careful consideration. The increased frequency of drug hypersensitivity reactions has been linked to several different patient populations suffering with specific disease states. Instances include the relationship between cystic fibrosis and hypersensitivity induced by piperacillin (Whitaker et al., 2012) with parallels also drawn between the use of drugs, such as carbamazepine and abacavir, for the treatment of epilepsy and human immunodeficiency virus (HIV) respectively. The aforementioned examples are indicative of disease-hypersensitivity reactions, and in the case of HIV pathogenesis relating to hypersensitivity is associated with aberrations within immunological regulation caused by the viral infection, leading to glutathione depletion and impaired drug detoxification (Phillips and Mallal, 2007).

A patients previous clinical history of hypersensitivity is often a prelude to further incidences of drug hypersensitivity reactions. This factor of predisposition is largely observed within the setting of polypharmacy in which drugs sharing structural similarities are used as secondary and tertiary treatment options, inducing further hypersensitivity due to unpredictable cross-reactivity between therapeutics (Romano et al., 2005). This has been shown in β-lactam antibiotics within the penicillin family, such as anaphylaxis, in which chemically-related side chains between cephalosporin and imipenem analogues display patterns of cross-reactivity (James and Gurk-Turner, 2001). Therefore, previously employed treatment strategies should be reviewed to minimise the risk of successive hypersensitive manifestations.

1.2.7 Pharmacogenomic risk factors.

A multitude of pharmacogenomic factors have recently been linked with an individuals propensity to present with drug hypersensitivity. Indeed, strong correlation of specific genotypic characteristics, in the form of human leukocyte antigen (HLA) associations, have been identified following genome-wide association studies (GWAS) in a plethora of drugs known to induce hypersensitivity (Pavlos et al., 2012). The genetic associations uncovered have assisted our ability to understand the nature of drug-HLA interactions and consequently aid efforts to predict with the aim of screening potentially susceptible individuals prior to treatment.

The HLA complex itself is the human equivalent of the MHC and encodes genes that determine allelic expression. This complex represents the most polymorphic genetic system in humans and expression frequency of specific HLA haplotypes can vary between different ethnic groups (Williams, 2001). In terms of function, proteins encoded by specific HLA alleles are capable of presenting peptides to T-cells for the elicitation of effector responses. The HLA regions that encode both HLA class I and II genes are tightly linked and are universally located on chromosome 6 (position 6p21) in humans (Shiina et al., 2009). HLA class I genes can be categorised as classical (HLA-A, HLA-B and HLA-C) or non-classical (HLA-E, HLA-F and HLA-G). Structurally, HLA class I complexes contain a peptide binding groove consisting of two α chain (α1 and α2) with additional interaction with β-2 microglobulin. Conversely, HLA class II genes and can be subdivided into HLA-DR, HLADP, and HLA-DQ genes and although the class II complexes encoded share the heterodimeric structure of its class I counterpart, these molecules can be structurally characterised by a non-covalent linkage of both of both α and β (α1 and β1) chains within the peptide binding cleft (Bodmer, 1987, Williams, 2001).

The nucleoside reverse transcriptase inhibitor abacavir, employed for the treatment of HIV, is a prime example of HLA association being utilised for pre-treatment genetic screening (Mallal

et al., 2008). Expression of the HLA-B*57:01 risk allele has been shown to be a key determining factor for predisposition to abacavir hypersensitivity syndrome (AHS), with one study reporting positive predictive values of 55% and negative predictive values of 100% (Mallal et al., 2002). The case of abacavir represents an important milestone for prediction centred on HLA association in terms of both sensitivity and specificity along with economic viability relating to the cost-effective nature of screening (Ruiz-Iruela et al., 2016). However, it is important to understand that while genetic associations can prove useful when determining susceptibly, a degree of caution must be applied when studying HLA interactions. This is in part due to the multifaceted nature of drug hypersensitivity from a genetic standpoint, reinforced by the fact that only a select few drug-HLA associations are currently screened for with the notable exceptions of abacavir, carbamazepine and allopurinol (Hung et al., 2005, Chung et al., 2004). A summary of the most extensively studied HLA-associated drug hypersensitivity reactions can be viewed in Table 1.1.

Table 1.1. Panel of immune-mediated drug-HLA associations and clinical manifestations elucidated from GWAS study.

Drug	Class	HLA allele	Reaction	Reference
Abacavir	Nuceloside reverse transciptase inhibitor	B*57:01	AHS	(Mallal et al., 2002)
Carbamazepine	Anticonvulsant	B*15:02 A*31:01	SJS/TEN HSR/MPE	(Chung et al., 2004, Hung et al., 2006)
Allopurinol	Xanthine oxidase inhibitor	B*58:01	SJS/TEN	(Hung et al., 2005)
Dapsone	Suflone	B*13:01	Dapsone hypersensitivity syndrome	(Zhang et al., 2013)
Vancomycin	Glycopeptide antibiotic	A*32:01	DRESS	(Konvinse et al., 2019)
Flucloxacillin	β-lactam	B*57:01	DILI	(Daly et al., 2009)
Amoxacillin-clavulante	β-lactam	DRB1*15:01	DILI	(Lucena et al., 2011)
Ticlopadine	Antiplatelet	A*33:03	DILI	(Hirata et al., 2008)
Lamotrigine	Phenytriazine	A*31:01 B*15:02	SJS/TEN	(McCormack et al., 2012)
Phenytoin	Anticonvulsant	B*15:02	SJS/TEN	(Man et al., 2007)

1.2.8 Clinical manifestations of drug hypersensitivity.

Drug hypersensitivity reactions can induce physiological aberrations in multiple organ systems yielding disease states of varying severity. Reactions are widely documented to induce disease states related to skin, heart, kidney, lungs, and liver function. The skin is the most commonly targeted organ for drug-induced hypersensitivity, accounting for over 95% of all hypersensitivity reactions presented in clinic (Thong and Tan, 2011). Approximately 2-3% of all ADRs observed in hospitalised patients are attributed to these cutaneous based hypersensitivity reactions with around 5% of cases leading to potentially life-threatening outcomes (Marzano et al., 2016). The skin is explicitly prone to reactions of this nature, arising from basic physiological factors that increase the likelihood of immune-mediated adverse events. Greater surface area and vasculature facilitates the interaction between immune cells of the adaptive response present within the blood (Ardern-Jones and Friedmann, 2011). This heightens the skins susceptibility to T-cell mediated events that commonly manifest as rashes or eruptions, for which symptom reporting is reliable and consistent within patients due to the visible nature of the reaction (Uetrecht and Naisbitt, 2013).

1.2.8.1 Maculopapular exanthema.

Maculopapular exanthema (MPE) is the most common clinical manifestation of hypersensitivity, accounting for over 95% of all cutaneous events (Bigby, 2001). MPE typically presents as either papular eruptions or erythematous macular eruptions on the trunk of hypersensitive patients. Other symptoms can include low-grade fever along with severe itching, known as pruritus (Ardern-Jones and Friedmann, 2011). The vast majority of MPE cases arise from drug-induced hypersensitivity and well-characterised culprit drugs include sulfonamides, anticonvulsants and penicillins (Ardern-Jones and Friedmann, 2011).

The time-course for symptom development is of delayed fashion and typically occurs between 7-14 days following initial exposure and can persist for up to 2 weeks after withdrawal of the

culprit drug (Yawalkar, 2005). The cellular basis for the pathophysiology of MPE involves mediation via a drug-specific CD4+ T-cell response (Hertl and Merk, 1995). The cytotoxic response of CD4+ T lymphocytes facilitates the secretion of cytolytic molecules such as IL-5, IFN-γ, perforin and granzyme B required for the activation of cell death pathways and the destruction of basal keratinocytes (Ye et al., 2017, Posadas et al., 2000, Pichler et al., 2002).

1.2.8.2 Acute generalized exanthematous pustulosis.

Morphologically, acute generalized exanthematous pustulosis (AGEP) is characterised by the presence of small, pinhead sized non-follicular pustules supported by an erythematous base that typically originates in flexural areas before becoming more widespread and reaching the trunk and extremities (Roujeau et al., 1991). In addition to the development of an acute rash, symptoms commonly include fever and pruritus which present between 24-48 hours after treatment (Sidoroff et al., 2007). Although considered more severe than MPE, the incidence on AGEP is less common and only affects 1-5 patients in every million each year (Sidoroff et al., 2001) with 90% of cases related to drug-induced reactions (Szatkowski and Schwartz, 2015).

In vitro study shows the pathophysiology of AGEP constitutes a T-cell mediated response and a clear Type IV allergic reaction. Culprit drugs include a number of antibiotics such as the sulfonamides, penicillins and antifungal agents (Feldmeyer et al., 2016). Drug-specific CD4+ and CD8+ T-cells are both implicated and facilitate the secretion of the IL-8 chemokine along with corresponding CXCL-8 ligands (Kabashima et al., 2011, Britschgi and Pichler, 2002). T-cell mediated chemokine secretion promotes an innate response and subsequent neutrophil recruitment gives rise to the classic histopathologic features of AGEP such as the formation of neutrophil rich sterile pustules on the epidermis (Schaerli et al., 2004).

1.2.8.3 Drug reaction with eosinophilia and systemic symptoms.

The term drug reaction with eosinophilia and systemic symptoms (DRESS) was first coined by Bocquet and colleagues in 1996 and can be described as a form of drug-induced hypersensitivity syndrome with idiosyncratic tendencies (Bocquet et al., 1996). DRESS encompasses a wide range of clinical features which are not solely limited to cutaneous based reactions and can be prevalent within multiples organ systems, often targeting the liver (Sullivan and Shear, 2001). Cutaneous manifestations of DRESS are typically characterised by both urticarial and maculopapular eruptions along with facial oedema and erythroderma (Eshki et al., 2009). DRESS can be differentiated from other cutaneous manifestations of hypersensitivity, such as MPE and AGEP, by the recruitment and involvement of eosinophils. Therefore it stipulates that eosinophilia is a distinguishing haematological trait of DRESS along with more generic associated symptoms such as fever, pruritus and leukocytosis (Cacoub et al., 2011). One particular study had speculated that cutaneous-based reactions are present in over 70% of cases (Peyrière et al., 2006), however it should be noted that acute liver failure accounts for the majority of deaths attributed to DRESS (Amante et al., 2009).

The incidence of DRESS can be difficult to accurately determine due to frequent misdiagnosis arising from the vast array of clinical manifestations and the degree of convergence with similar cutaneous based adverse events. Studies have approximated the incidence of DRESS to be somewhere between 1 in 1000 and 1 in 10,000 exposures and consequences can be life-threatening, illustrated by a relatively high mortality rate of 10% (Chen et al., 2010, Chiou et al., 2008). The reaction itself is consistent with delayed-type hypersensitivity with symptoms typically presenting anywhere between 2 - 8 weeks after exposure to the offending drug (Walsh and Creamer, 2011). The pathogenesis of onset from an immunological perspective is still relatively poorly understood although eosinophil recruitment and the ensuing propagation of inflammatory cascades has been linked to IL-5 secretion (Choquet-Kastylevsky

et al., 1998), mediated by the activation of drug-specific CD4+ and CD8+ T-cells of the adaptive immune system.

Classes of therapeutics frequently associated with DRESS include sulfonamides, anticonvulsants and antivirals such as nevirapine. The pathomechanisms of DRESS are multifaceted and genetic factors are known to contribute towards predisposition, specifically polymorphisms of HLA alleles that influence interactions at the immunological synapse (Pirmohamed et al., 2015). The anticonvulsant carbamazepine is a model example, for which expression of the HLA-B*15:02 and HLA-A*31:01 risk alleles increase susceptibility to DRESS (McCormack et al., 2011, Chung et al., 2004). However, these reactions are not unequivocally associated with DRESS alone, with overlap observed with other SCAR related disease states. More recently, the HLA-A*32:01 allele has been associated with vancomycin-induced DRESS (Konvinse et al., 2019). One interesting phenomenon implicated in the pathogenesis of DRESS is the possibility of viral reactivation. This has been evident for human herpes viruses 6 and 7 (HHV-6 and HHV-7) in addition to EBV and cytomegalovirus (Shiohara and Kano, 2007), with some postulating that the skin may be the major immunological active site for reactivation (Hashizume et al., 2013). Indeed, many cutaneous manifestations of DRESS appear to develop from the collegial interaction between both drug-specific CD4+ and virus-specific CD8+ T-cells (Niu et al., 2015).

1.2.8.4 Stevens-Johnson syndrome and toxic epidermal necrolysis.

SJS and TEN represent the most serious delayed-type hypersensitivity reactions, primarily targeting the skin. Clinical manifestations of SJS are considered milder in nature, however progression to TEN is often observed in the absence of intervention while overlap exists within simultaneous presentation of both SJS-TEN (Bastuji-Garin et al., 1993). The incidence of these life-threating cutaneous reactions is extremely rare, with recent figures estimating SJS and TEN

affect 8 and 2 patients per million each year respectively, while there are approximately 1.5 cases of SJS-TEN per million each year (Hsu et al., 2016).

Common clinical features include skin lesion in the form of erythematous and livid macules, accompanied by ocular mucosa in over 90% of cases (Revuz et al., 1987). The two disease states only differ in the degree of skin detachment. Indeed, the extent and severity of skin coverage can be used as a diagnostic tool for discrimination between SJS and TEN. The presentation of SJS can be characterised by less than 10% coverage of the skin with flat lesions on an erythematous base, whereas TEN comprises over 30% skin coverage and more critically, full detachment of epidermal sheets from the dermis (Roujeau, 1994). Logically, it follows that SJS-TEN overlap accounts for skin and body coverage between 10-30% (Bastuji-Garin et al., 1993). The degree of variance observed in clinical characteristics is reflected in patient outcomes, with SJS associated with 10% mortality (Pavlos et al., 2012, Gomes and Demoly, 2005) and TEN shown to be linked with a markedly high mortality rate of between 25-35% (Harr and French, 2010). The vast majority of SJS-TEN cases are drug-induced, with a myriad of causative agents implicated over the years. Classes of therapeutics routinely identified as culprit drugs include sulfonamides (cotrimoxazole), non-steroidal anti-inflammatory drugs (ibuprofen and naproxen), antibiotics (cephalosporins and penicillins), anticonvulsants (carbamazepine, phenytoin, lamotrigine) and xanthine oxidase inhibitors such as allopurinol (Kikuchi and Okazaki, 1978, Sharma et al., 2008, Lin et al., 2014, Roujeau et al., 1995, Halevy et al., 2008).

Genetic association between the expression specific HLA alleles and SJS-TEN development has been demonstrated in certain ethnic groups. A landmark study by Chung et al. found a strong association in Han Chinese populations between the HLA-B*15:02 risk allele and SJS following exposure to carbamazepine (Chung et al., 2004). This study, in which 100% of SJS patients were found to express the risk allele now forms the basis for FDA recommended genetic screening

of Han Chinese and other Asian populations prior to treatment (Ferrell and McLeod, 2008). Until now the mechanistic basis for the onset of SJS-TEN had been poorly defined. The pathogenesis, ultimately leading to epidermal necrolysis, is now understood to be associated with immune-mediated hypersensitivity. Immunopathogenesis is thought to be MHC-I restricted (Wei et al., 2012) with both Fas ligand (FasL) and perforin/granzyme B pathways implicated in keratinocyte cell death following T-cell activation (Abe et al., 2003). Presentation of the culprit drug on keratinocytes induces the secretion of pro-inflammatory cytokines, specifically IFN-γ, and cytolytic molecules such as granzyme B and perforin resulting in an augmented apoptotic response mediated the FasL pathway (Viard-Leveugle et al., 2013, Nassif et al., 2004). Programmed keratinocyte cell death and ensuing cytotoxic responses have been evidenced by elevated levels of FasL, IFN-γ, perforin/granzyme B and granulysin detected in the blister fluid of patients presenting with SJS-TEN (Posadas et al., 2002). The clinical manifestations associated with SCARs are illustrated below (Figure 1.4).

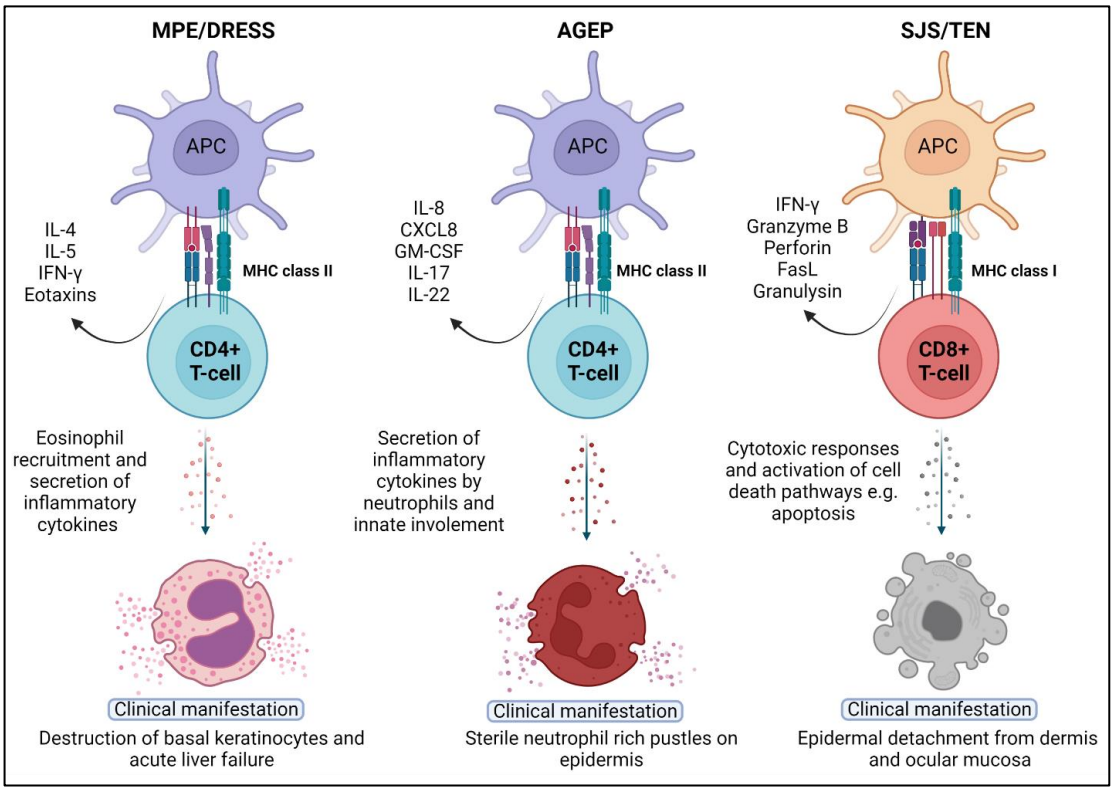


Figure 1.4. Illustration detailing the clinical manifestations and associated pathways of cytotoxic and cytolytic molecule release involved within the pathogenesis of Type IV drug hypersensitivity reactions (MPE, DRESS, AGEP and SJT/TEN).

1.2.8. Drug-induced liver injury.

DILI can concomitantly manifest alongside cutaneous reactions, such as MPE and DRESS, or exclusively perturb hepatocyte function (Lee et al., 2013). The prevalence of DILI i accounts for over 9% of all ADRs with many cases unreported due to sporadic diagnosis (Lazarou et al., 1998). Consequently, incidence can vary with estimates ranging from 1 in 10,000 to 100,000 cases following exposure (Pirmohamed et al., 2004). Manifestation is typically cholestatic or hepatocellular in nature and approximately 50% of all acute liver failures are attributed to DILI (Lee, 2013), prompting concern for drug developers resulting from idiosyncratic hepatic injury (Andrade et al., 2006).

The liver is the primary metabolic active site within the body and responsible for toxin removal, processes that render the organ vulnerable to hepatotoxicity (Remmer, 1970). Drug metabolizing enzymes present within the liver, specifically the cytochrome P450 (CYP450) family, facilitate phase-1 xenobiotic drug metabolism and the generation of hepatotoxic reactive metabolites (Boelsterli, 2002, Spahn-Langguth and Benet, 1992). Both CD4+ and CD8+ drug-responsive T-cells have been detected in the blood of patients presenting with DILI following treatment with flucloxacillin and isoniazid (Monshi et al., 2013, Usui et al., 2017), while *in vitro* priming of T-cells in healthy volunteers expressing specific HLA risk alleles has also proven successful (Kim et al., 2015, Yaseen et al., 2015). Although exact pathomechanisms of T-cell mediated hepatocyte destruction are poorly understood, the identification predisposing genetic factors may facilitate prediction. More recently, HLA associations between ticlopidine (HLA-A*33:03), flucloxacillin (B*57:01) and minocycline (HLA-B*35:02) have been linked to the development of idiosyncratic DILI (Hirata et al., 2008, Daly et al., 2009, Urban et al., 2017), however low predictive values negate the economic viability of screening programmes.

1.2.9 Management of ADRs.

The management of ADRs ideally comprises multiple components, such as correct and timely diagnosis, preventative screening and additional treatment regimens. In the case of mild and moderate SCAR related disease states, such as AGEP, reactions are not considered life-threatening and cessation of adverse events have been known to occur even after continued administration of the causative agent (Fernández et al., 2009). Similarly, when considering disease management of other mild reactions (MPE) adverse events have been known to spontaneously resolve after approximately 15 days following withdrawal of the offending drug (Szatkowski and Schwartz, 2015). Therefore, preferred treatment strategies for mild disease typically involve the immediate withdrawal of the offending drug, with prompt cessation drastically improving prognosis (Santiago et al., 2010). With regards to diagnosis, prompt identification of the culprit drug is critically important and due to the serious nature of more severe SJS-TEN reactions, gold standard diagnostic procedures such as systematic drug rechallenge are unsuitable. Methods such as skin patch testing show sufficient sensitivity (50%) for both MPE and AGEP (Balachandran et al., 2002), but when applied to SJS-TEN sensitivity is limited with studies suggesting positive results are observed in just 10% of SJS-TEN cases (Wolkenstein et al., 1996). Instead, *in vitro* assays and specifically lymphocyte transformation tests (LTTs) are deployed with greater success. Moreover, in the case of SJS-TEN induced by anticonvulsants, such as carbamazepine and phenytoin, 50% sensitivity has been reported (Karami et al., 2016). When applied to SCARs, therapeutic intervention often centres around symptom management, while corticosteroids are deployed on occasion with limited success (Roujeau and Stern, 1994).

1.3 Diagnostic approaches to determine drug hypersensitivity.

1.3.1 *In vivo* diagnosis.

Diagnosis of hypersensitivity and the identification of offending medications is of critical importance for patient safety and to ensure positive outcomes regarding treatment regimes. Although imperfect due to the complexity of immunologically mediated reactions, diagnostic tests play a central role in mitigating against re-exposure to culprit drugs and further hypersensitivity. Additionally, proper diagnosis can help to inform clinicians as to the source of undesirable adverse events in settings where polypharmacy is common, allowing for the recommencement of efficacious treatment. However, the diagnostic tools currently available and utilised by clinician's lack many essential ideological components such as proper standardisation across various drug classes and correct stratification of risk coupled to a patient's clinical history. Current *in vivo* diagnostic approaches for the detection of hypersensitivity, combined with need for improved procedures to predict or circumvent hypersensitivity at an earlier stage are explored below.

Although not adept at deciphering between immune and non-immune mediated hypersensitive responses, drug provocation remains the gold standard to identify offending treatments, in addition to determining an individuals tolerance (Aberer et al., 2003). Within the context of IgE mediated reactions, for which clinical manifestations are immediate, drug rechallenge possesses an exquisitely high negative predictive value of 100%, although sensitivity reduces when applied to more severe reactions of delayed aetiology after rechallenge with just a single dose (Copaescu et al., 2021, Hjortlund et al., 2012, Bousquet et al., 2008). Nevertheless, due to the clinical severity of some delayed, immune mediated adverse reactions such as DRESS and SJS/TEN this diagnostic procedure carries inherent risk to the patient. Obvious drawbacks become apparent when considering cytostatic agents for which approximately 30% of patients suffer severe reactions following provocation and this is

further exemplified in the case of the general anaesthetic halothane in which a staggering 51% mortality rate has been reported after rechallenge yielded a positive result (Madrigal-Burgaleta et al., 2019, Mushin et al., 1971). As such, drug provocation is often contraindicated when applied to potential life-threatening reactions and only deployed during situations in which the considerable risk is outweighed by benefit, typically during immunotherapy (Rive et al., 2013, Trubiano and Phillips, 2013). In summary, although exhibiting high levels of specificity for the isolation of adverse agents during polypharmacy the need for safer alternative diagnostic tools is demonstrably clear.

Although lacking the sensitivity and specificity of drug provocation, skin testing represents a more cautious diagnostic approach for the identification of immunogenic compounds and is associated with a reduced risk to patient safety. Patch testing is one such method and involves incubation with the suspected allergen at therapeutically relevant doses for between 24-28 hours on a small 'patch' of skin (Pichler and Tilch, 2004). *In vivo* patch testing is typically performed approximately 4-6 weeks after the cessation of delayed-type hypersensitive responses and can be deployed for the detection of MPE, DRESS, AGEP and SJS/TEN with some success and relatively high specificity (Phillips et al., 2019, Barbaud et al., 2001). Unfortunately, a negative patch test cannot rule of drug allergy and sensitivity can vary substantially with each drug class applied to each clinical manifestation, with antiepileptic's and β-lactam antibiotics found to provide greater utility in terms of detection (Johansen et al., 2015).

Additional *in vivo* diagnostics that target the skin, often supplementary to both drug provocation and patch testing, include skin prick and intradermal testing. Prick testing is the primary method for detecting immediate allergy mediated by IgE antibodies and is considered to be minimally invasive. Soluble allergen extracts are taken up by a needle and applied directly to the skin and 'pricking' ensures a small amount of the culprit allergen is introduced to the epidermis. Prick testing is limited to soluble drugs at high non-irritating concentrations for the

detection of immediate responses, with histamine typically used as a positive control (Phillips et al., 2019, Heinzerling et al., 2013). However, it has now been suggested that sensitivity can be improved for drugs such as vancomycin when concentrations are increased to threshold levels to induce initial irritation (Konvinse et al., 2016).

Intradermal testing is utilised as an auxiliary tool when previous skin-based testing yields a negative result and possesses increased relevance for the diagnosis of delayed-type hypersensitivity (Brockow et al., 2002). These tests are more specialised and performed on the volar surface of the forearm with the allergen in question injected intradermally to form a small bleb on the surface of the skin. Clinicians can interpret a positive result when erythema at the site of injection exceeds 5 mm when compared to baseline levels (Empedrad et al., 2003). As responses to the culprit allergen are often delayed, reading is performed up to 1 week after injection (Brockow et al., 2013). Although considered to be more sensitive than prick testing, intradermal testing possess greater risk to patient safety due to its more invasive nature and is associated with an increased risk of anaphylaxis and viewed by some as potentially harmful when applied to reactions carrying SCAR phenotypes such as SJS/TEN and DRESS (Makris et al., 2010, Watts, 2017). The balance between test sensitivity and patient risk is a common theme that runs throughout skin-based diagnostic tools, with issues surrounding dose standardisation across multiple drug classes, drug stability and dilution as well as the potential for serious adverse reactions thrown in for good measure. Consequently, due to the invasive nature and often inconclusive results given by *in vivo* tests it is imperative these tests are used in conjugation with a multitude of *in vitro* diagnostic assays complemented with a detailed clinical history to successfully diagnose compound hypersensitivity reactions.

1.3.2 *In vitro* assays.

1.3.2.1 Lymphocyte transformation test.

The LTT is the most widely used *in vitro* assay for the detection of both immediate and delayed-type hypersensitivity. This assay has been long established and is a sensitive indicator of immune activation, achieved by measuring the proliferation of T-cells after the stimulation of bulk cultures (Figure 1.5). On a technical level, an LTT involves the exposure of PBMC, isolated from the blood of patients with suspected hypersensitivity, with the compound of interest for a period of 6-7 days (Pichler and Tilch, 2004). The guiding principle of the assay is that T-cells previously sensitised to the culprit drug will proliferate upon re-exposure. This is most commonly measured by ^{3}[H]-thymidine incorporation assays in which the radioactive isotope (^{3}H) is taken up by dividing T-cells in a dose-dependent manner and can be used as a reliable indicator of cell proliferation (Luque et al., 2001, Pichler and Tilch, 2004). Other methods for the detection of T-cell sensitisation to relevant antigens include carboxyflourescein diacetate succinimidyl ester (CFSE) or surface marker upregulation e.g. CD69 and CD25, however such methods are more suited to the diagnosis of immediate reactions and therefore sensitivity in terms of a delayed response is often compromised (Koponen et al., 1986).

It has been shown that individuals who do not exhibit drug allergy will not provide proliferative T-cell responses after exposure and this information can be a useful control when testing the immunogenic potential of novel compounds within LTT assays (Naisbitt et al., 2003a). However, what constitutes a positive LTT result can vary and sometimes be controversial. Proliferative values obtained are universally reported as stimulation index (SI) given by the average proliferation of drug treated cultures/average proliferation of control cultures, for which an SI > 2 is generally considered as positive. Although this can differ depending on the drug of interest, for example positive responses to β-lactam antibiotics are classified as positive with a SI > 3 (Porebski et al., 2011). Interestingly, proliferation is often found to be

enhanced at just a single drug concentration, potentially related to the diverse mechanistics involved within drug/TCR binding and reinforces the need for dose titration to achieve the appropriate level of sensitivity (Pichler, 2002).

Due to the delayed nature of many hypersensitivity reactions LTT assays are generally performed after a minimum interval of 3 weeks after initial exposure and clinical manifestation in order to ensure the elimination of free, circulating culprit drugs from the blood (Porebski et al., 2011). Contradictory to this, patients presenting with SCARs such as SJS/TEN have been observed to produce heightened proliferative LTT responses after 1 week which then diminishes throughout the recovery phase, with optimal responses detected after the resolution phase (> 5 weeks) in DRESS reactions (Kano et al., 2007, Polak et al., 2013). Therefore, due to the heterogeneity of such reactions it can be concluded that the time lapse between symptomatic ADRs and proliferative measurement depend on the nature of clinical presentation and should be managed on case-by-case basis.

In terms of sensitivity, LTTs can vary with drug class and have a broad reported range of between 27% and 74% with specificities reported as high as 85% (Porebski et al., 2013, Nyfeler and Pichler, 1997). These *in vitro* assays are more suited to delayed-type reactions as opposed to immediate reactions which are more analogous with *in vivo* skin-based testing as previously described, with studies reporting a 10-15% reduction in terms of sensitivity when applied to *in vitro* based proliferative assays (Luque et al., 2001). In summary, LTT assays are well established and still possess enormous clinical relevance over 50 years from inception. Used in parallel with *in vivo* testing, the LTT can provide sensitive readouts to determine delayed-type hypersensitivity and identify culprit drugs during polypharmacy. Nevertheless, the assay would benefit from harmonisation between both protocols and concordant readout interpretation across the multitude of drugs implicated in causing hypersensitivity.

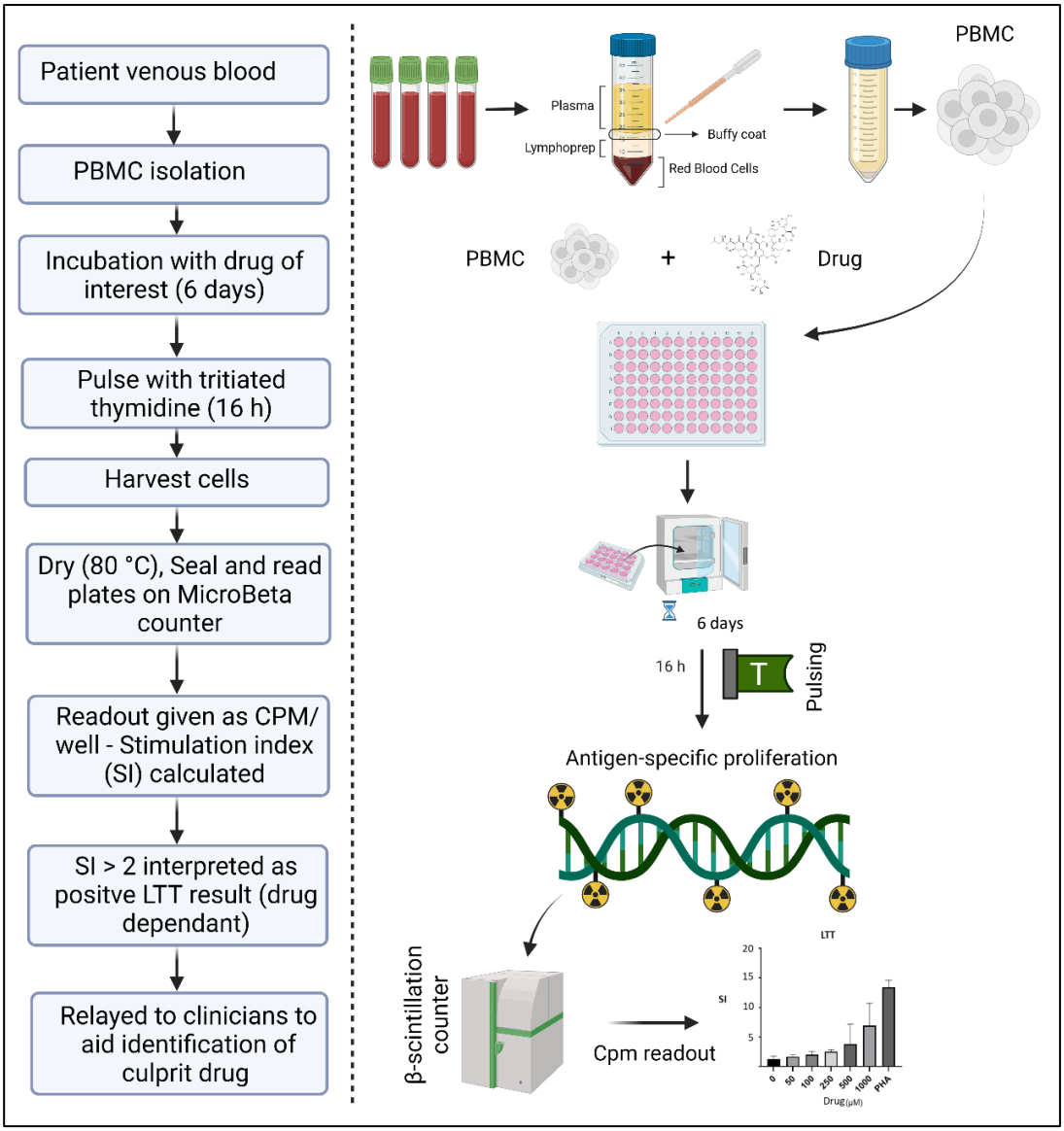


Figure 1.5. Visual representation of the LTT. PBMC from hypersensitive patients are exposed to the compound of interested for 6 days before proliferative measurement of lymphocyte transformation using thymidine incorporation assay.

1.3.2.2 Enzyme-linked immunospot assay.

Often used to compliment diagnosis given by the LTT, the enzyme-linked immunospot (ELISpot) assay is adept at quantitatively determining cytokine and cytotoxic molecule release from drug activated T-cells. This may be useful as both a marker of hypersensitivity to culprit drugs and for the identification of pathomechanisms involved in the reaction (Roujeau et al., 1985). Similarly to the LTT, ELISpot assays are performed using PBMC isolated from patients, incubated with the drug of interest for a shorter period of 48 h. Upon activation, T-cells are

known to secrete a plethora of drug and reaction-specific cytokines such as IFN-γ, IL-5, IL-13, IL-22 to name a few, in addition to granzyme B, perforin, granulysin and FasL as mediators of cytotoxic and cellular death pathways (Khalil et al., 2008, Sachs et al., 2002, Porebski et al., 2013). Technical aspects of the assay centre on the release and presence of cytokines within the cell supernatant and subsequent binding to polyvinylidene difluoride (PVDF) membranes pre-coated with the target cytokine. Detection antibodies conjugated to enzymes, typically alkaline phosphatase (ALP), produce spots representative of cytokine release following binding to an enzyme-activated substrate. Advantages of cytokine release assays include the ability to provide mechanistic insight into reactions within a short time frame, for example high levels of IL-5 secretion can be indicative of DRESS reactions due to its involvement in eosinophil recruitment (Choquet-Kastylevsky et al., 1998, Kouro and Takatsu, 2009). In terms of readout, the activation of drug-specific T-cells is given by the number of spot forming units (SFU) and a major drawback of the assay is the lack standardisation applied between clinicians and researchers as to the threshold for a positive response. Accepted levels of background secretion can also be problematic in addition to the significant cost implications and specialist expertise required for assay development.

1.3.2.3 *In vitro* T-cell assays.

To detect the immunogenic potential of low molecular weight compounds *in vitro* T-cell priming assays have proven to be a useful tool for the detection of *de novo* responses to compounds implicated in hypersensitivity reactions. These assays may be applicable to pre-clinical screening programmes in which novel therapeutics with potential immune liabilities can be identified at an early stage and validation of such assays would be invaluable to drug development and by extension, patient safety. Initial versions of the assay involved long-term PBMC culture (4 weeks) and repeated stimulation of cultures with drug, irradiated autologous APCs and IL-2 culminating in a proliferative readout given by ^{3}[H]-incorporation after drug

rechallenge for a period of 48 h, with SMX-NO deployed as a model immunogen (Engler et al., 2004). More recent iterations of priming-based techniques, initially theorised for applications within the detection of chemical allergens and contact hypersensitivity, incorporate monocyte-derived dendritic cells (Mo-DC) matured for 7-8 days in the presence of LPS and TNF-α. Following cytokine-induced maturation, Mo-DCs are co-cultured with naïve T-cells and the antigen of interest for a further 8-14 days before rechallenge with fresh Mo-DCs and drug antigen. This is followed by a final incubation of 48 h and *de novo* naïve T-cells responses measured by proliferative assays such as ^{3}[H]-incorporation or CFSE (Faulkner et al., 2012). These studies describe the cellular composition necessary for successful and reproducible priming of naïve T-cells to the model allergen, SMX-NO. Further work has validated the assay for studying the mechanistics of primary T-cell responses in genotyped healthy donors to compounds displaying HLA class I restriction such as carbamazepine, flucloxacillin and oxypurinol (Faulkner et al., 2016b).

Although adept at providing consistent readouts regarding primary T-cell responses to chemically reactive model stimulants (SMX-NO) and contact sensitizers such as paraphenylenediamine and Bandrowski's base, problems are encountered when considering many drug-specific responses, specifically pertaining to those sharing HLA class II genetic associations (Gibson et al., 2015, Faulkner et al., 2012). This reported lack of sensitivity could be attributed to a variety of factors, but perhaps most significantly due to the signal intensity of drug-specific T-cells essentially being masked by populations with a reduced avidity for the antigen, owing to the high diversity of TCR repertoires present within mixed polyclonal cultures (Nikolich-Zugich et al., 2004). Of late, progress has been made towards attempting to solve the lack of sensitivity associated with conventional priming assays in the context of primary drug-specific T-cell responses. The T-cell multi-well assay (T-MWA) allows for an increased number of experimental replicates and unlike the traditional priming assay, only requires a single stimulation with Mo-DCs and as a consequence may possess utility for high-

throughput immunogenic screening (Ogese et al., 2020). Although the T-MWA is susceptible to many of the limiting factors previously described, the assay has shown promise in detecting both primary responses to model immunogens and enhancement after checkpoint blockade (Ogese et al., 2020). Moreover, the assay has shown capacity for the detection of drug-specific naïve T-cell responses to glycopeptide antibiotic, vancomycin, for which causal HLA class II associations have been reported (Ogese et al., 2021, Nakkam et al., 2020). However, it must be said that a positive assay result should be interpreted with a degree of caution due to the inherent nature of the assay set up in which regulatory T-cells are often depleted and subsequent regulatory function, be it co-inhibitory or co-stimulatory, is impeded. Therefore, assays of this type serve to answer a singular question; does the compound possess intrinsic immunogenic potential? Further assay development would be required to adequately predict if such artificially provoked primary responses manifest *in vivo* under physiological conditions.

To circumvent the issue of background 'noise' resulting from the heterogeneity of mixed pre-cursor T-cell populations and to study the pathomechanisms of drug-specific responses it would be advantageous to elucidate these primary responses at the single cell level. T-cell cloning assays can be used to assess the immunogenic potential of suspect compounds, but also allow for the characterisation of any drug-specific T-cell responses identified. Cloning assays involve initial bulk priming of PBMC to the compound of interest followed by serial dilution of T-cells to achieve a seeding density of just a single cell per well. Upon mitogen driven expansion of drug-responsive T-cell clones, the presence of single, homogenous TCRs among clonal populations with identical repertoires for specific antigens enable further functional analysis to reveal unique features relating to the antigenic determinant and also the presence of cross-reactivity with related antigens (Beeler and Pichler, 2006).

Other important facets of functional T-cell based assays used for characterisation of a drug-specific T-cell responses include cytokine release assays (ELISpot), HLA blockade, phenotyping

in addition to assays to determine routes of TCR stimulation (Kim et al., 1985). Drug-specific T-cell clones have been detected in patients and healthy donors to a myriad of compounds with HLA class I (e.g. abacavir, vancomycin and carbamazepine) and HLA class II associations (e.g. isoniazid and coamoxiclav) in addition to drugs with no known genetic associations such as sulfamethoxazole (SMX) (Bell et al., 2013, Ogese et al., 2021, Naisbitt et al., 2003a, Usui et al., 2017, Ariza et al., 2020, Farrell et al., 2003). Importantly, these assays show increased sensitivity compared to other commonly used *in vitro* T-cell assays, as demonstrated in the case of atabecestat in which initial LTT assays performed on patient PBMC failed to detect the presence of drug-responsive precursor T-cells. Conversely drug-specific T-cell clones have been successfully generated and subsequent characterisation has implicated the compound in drug-hepatic injury (Thomson et al., 2021). Despite this increased sensitivity, T-cell cloning assays can be laborious, costly, and labour intensive, notwithstanding the heightened risks of infection and exhaustion of T-cell cultures due to repeated stimulation over a long period of time (> 2 months).

1.4. Antigenic stimulation: Tolerance or hypersensitivity?

The induction of elicitation or tolerogenic responses to antigens, was traditionally described via the self/non-self model of recognition. This rather simplistic model formed the basis for our understanding of both central and peripheral tolerance for over two decades, in which two distinct iterations explained the historical theories of antibody induction within models derived from the concept of antigen-sensitive cells. Lederberg and colleagues initially proposed that stem cells incapable of antigen recognition develop into mature antigen-sensitive inducible cells and that subsequent cellular accumulation with non-self specificity leads to discriminative function (Lederberg, 1959). Later, it was postulated that every antigen-sensitive cell possesses an inherent ability to become inducible and form antibodies, with this only occurring following interaction between antigen-sensitive cells and carrier antibodies in which a conformational change induces a second signal dictating the initiation of tolerance or an immune response (Bretscher and Cohn, 1970). More recently, a conceptual switch away from inducible antigen-sensitive cells and towards the theory of danger signalling has transformed our understanding of the complexities relating to tolerogenic pathways. First described by Polly Matzinger, the 'danger hypothesis' attempts to predict tolerance or elicitation by considering both the propagation and nature of primary, secondary and tertiary signals (Matzinger, 1994). The intricacies of each signal within the context of immune tolerance or ensuing hypersensitive/allergic responses to drugs and chemical allergens is explored in detail below.

1.4.1 Signal 1.

In this section we will consider danger signalling pertaining to T-cell stimulation that results in either activation or immune tolerance to antigens recognised as 'self'. Put simply, signal 1 refers to the recognition of cognate antigens by the TCR via interactions with major MHC complexes at the immune synapse (Grakoui et al., 1999). Within populations of resting T

lymphocytes, signal 1 is perpetually 'switched off' and although still fundamental to the activation of naïve precursor T-helper cells (T_h0) the importance of signal 1 as a determining factor *per ipsum* has been called into question (Guerder and Matzinger, 1992). Although not acceptable as the sole parameter for T-cell activation, genetic analysis and specifically the genetic diversity that exists among HLA alleles has been exploited to aid prediction of compound hypersensitivity. Indeed, for drugs such as abacavir and carbamazepine strong associations have been elucidated with carriage of HLA-B*57:01 and HLA-B*15:02 respectively (Mallal et al., 2002, Chung et al., 2004), with genetic screening now in place to combat the incidence of hypersensitive responses (Ruiz-Iruela et al., 2016).

Whilst allelic expression of specific HLA complexes can be a uniquely sensitive tool for prediction and provides an exceptionally high degree of negative predication in the case of abacavir (100%) (Mallal et al., 2008), the vast majority of individuals expressing a known a HLA 'risk' allele do not progress to a hypersensitive state when administered with a culprit drug. In short, while formation of the MHC/drug/TCR complex clearly plays a role within the specificity such responses, it does not exclusively account for the nature i.e. a tolerogenic response or the stimulation of immune receptors. This phenomena indirectly gives credence to need for additional signalling necessary for complete T-cell activation and downstream effector function.

1.4.2 Signal 2.

Contemporary models of naïve T-cell activation have widely established the need for a secondary signal that can determine tolerance or immune activation, coinciding with the presence of signal 1 (Lenschow et al., 1996, Cunningham and Lafferty, 1974). Signal 2 comprises a multitude of pleotropic co-signalling pathways and acts similarly to a rate-limiting step within tolerogenic pathways in which stimulation of any remaining autoreactive T-cells is tightly controlled to prevent autoimmune outcomes (Fife and Bluestone, 2008). The

interactions between ligands and cognate receptors present on the surface of APCs determines the threshold for positive or negative signalling exerted on T-cells, resulting in the activation of costimulatory or co-inhibitory pathways (Huppa and Davis, 2003). Co-inhibition acts to dampen immunological responses and tip the balance in favour of immune tolerance via stimulation of pathways such as programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte associated protein-4 (CTLA-4) and to a lesser extent T-cell immunoglobulin mucin-3 (TIM-3). Subsequent 'switching off' of signal 2, even proceeding initial interactions between drug antigens and MHC/TCR complexes results in T-cells remaining quiescent and entering an inactive state referred to as anergy (Sharpe and Abbas, 2006).

The activation of co-stimulatory molecules combined with the propensity of T-cells to reach thresholds necessary for elicitation centres on the CD28/B7 paradigm (Lenschow et al., 1996). First described by Linsley et al. the CD28/B7 system comprises the dominant co-stimulatory pathway and describes ligation of CD28 co-receptors. These molecules are constitutively expressed by naïve helper CD4+ and CD8+ cytotoxic T-cells by both B7-1 and B7-2 on the surface of immature and activated APCs (Linsley et al., 1990, Rudd et al., 2009). In this section, B7-1 and B7-2 will be referred to as CD80 and CD86 respectively. Other immune receptors that exist outside the classical CD28/B7 paradigm that contribute towards co-stimulation include interactions between CD2 and signalling lymphocytic activation molecules (SLAM) in addition to CD40L and lymphocyte activating 3 (LAG-3) (Schwartzberg et al., 2009, Guo et al., 1996, Sierro et al., 2011). Interestingly, recent work has eluded to the synergetic relationship between co-signalling molecules in order to fine tune responses to a specific antigen. For example, activation of CTLA-4 pathways may directly or indirectly lower B7 expression on APCs via mechanisms such as transendocytosis of CD80/CD86 within T-cells, thus reducing bioavailability of co-stimulatory molecules on the surface of APCs (Qureshi et al., 2011). Conversely, mechanisms of B7 down-modulation, mediated by CTLA-4, can act indirectly following the release of cytokines such as IL-10 or TGF-β (Wing et al., 2008). A schematic

overview of the co-signalling pathways that lead to both co-stimulatory and co-inhibitory outcomes, and by definition tolerance or triggering of an immune cascade, can be viewed in Figure 1.6.

Due to the physiological significance of signal 2 in terms of danger signal propagation and T-cell activation, therapeutics targeting specific co-inhibitory molecules have been developed. To this end, the application of immune-checkpoint inhibitors, specifically CTLA-4, PD-1 and PD-L1 have proven efficacious within oncotherapy treatment due to their inherent ability to effectively eliminate the regulatory capacity mediated by co-signalling pathways and incite T-cell responses with anti-tumour capabilities (Okazaki and Honjo, 2007, Lee et al., 2019). However, treatment with checkpoint inhibitors have recently been associated with immune-mediated adverse events due to the inherent immune liabilities possessed, attributed to a lowering of the threshold needed for elicitation of T-cell responses (Postow et al., 2018). This has been demonstrated in patients presenting with hypersensitivity to previously tolerated drugs such as iodinated contrast media following completion of immunotherapy with PD-L1 (Hammond et al., 2021). This is further complemented by previous *in vitro* work, in which blockade of inhibitory signalling pathways (PD-1/CTLA-4) was found to enhance T-cell priming to model stimulatory antigens such as SMX-NO (Gibson et al., 2017, Gibson et al., 2014).

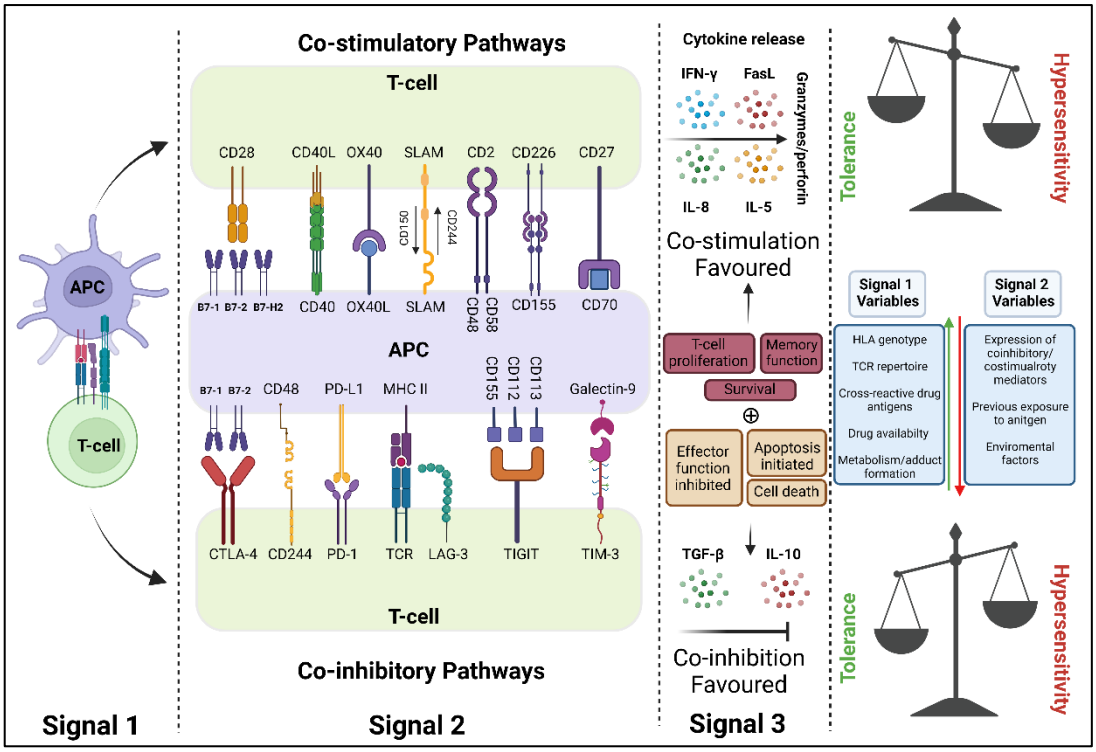


Figure 1.6. Pathways of co-stimulation and co-inhibition. Signal 1 can be characterised between MHC, antigen and TCR, and can determine the specificity of drug-specific T-cell responses, whereas signal 2 propagation ultimately determines nature of such response e.g. tolerogenic action or progression to hypersensitive disease states.

Additionally, danger signals can directly influence signal 2 propagation and induce responsive T-cell states originating from endogenous sources such as damage-associated molecular patterns (DAMPs), released from necrotic cells after drug interaction, or intracellular heat shock proteins (Tsan and Gao, 2004, Uetrecht, 1999). Exogenous danger signalling is often attributed to the release of LPS from invading bacteria, termed pathogen-associated molecular patterns (PAMPs) (Kumar et al., 2011).

1.4.3 Signal 3.

If tolerance is not imposed and co-stimulatory pathways are favoured following antigen recognition by TCRs an ensuing T-cell response occurs, mediated by the release of cytokines or soluble material such as heat shock proteins e.g. HSP60 (Curtsinger and Mescher, 2010, Moré et al., 2001). Such responses account for signal 3, and the secretion of polarising cytokines that determine effector T-cell differentiation and function contribute to many of the

clinical manifestations associated with delayed type IV hypersensitive disease states when danger signalling becomes excessive. For example, IL-5 secretion is associated with the onset of DRESS reactions due to its intrinsic property to mediate eosinophil recruitment (Choquet-Kastylevsky et al., 1998). In addition, T-cells releasing cytolytic molecules such as IL-8, perforin, granzyme B and FasL have been linked to the development of severe SJS/TEN (Viard-Leveugle et al., 2013, Nassif et al., 2004, Yoshikawa et al., 2020).

1.5 Pathways of T-cell activation.

1.5.1 The hapten hypothesis.

Sufficient danger signalling leads to signal 1 propagation and interaction at the immunological synapse between allergens, MHC complexes, peptides and the TCR. However, various routes of drug-specific signal 1 activation exist (hapten hypothesis, pharmacological interactions and altered peptide mechanisms) and it is important to consider each independent pathway within the context of specific immunogenic peptides or antigens implicated in hypersensitivity. For the purpose of this work MHC complexes will be referred to as HLA due to the focus on routes of immune stimulation after drug exposure in humans.

Low molecular weight compounds (< 1000 Da) were initially hypothesised to be inert due to their size. Such compounds were thought to be incapable of sensitising the immune system and activating immunological receptors. The original hypothesis for the development of antigenicity was proposed following an early landmark study by Landsteiner and Jacobs, who postulated that the formation of a hapten-carrier conjugate would enable immune-cell activation after exposure to small molecule therapeutics (Landsteiner and Jacobs, 1935). Although ground-breaking at the time, more evolved contemporary models now pinpoint the formation of HLA-peptide complexes, in which small molecules bind native HLA peptides in a covalent manner. For example, derivatives of penicillin have been shown to covalently modify lysine residues of serum albumin (Schneider and De Weck, 1965). Recognition of covalently

bound ‘hapetenised’ proteins by the TCR is essential for aberrant responses to drug-modified peptides now identified as foreign. For this to occur, drug antigens of low molecular weight have to be taken up, processed by MHC peptides and displayed on the surface of APCs (Aiba, 1998, Martin and Weltzien, 1994).

Examples of drugs that induce T-cell stimulation following the formation of HLA binding drug-modified peptides include β-lactam antibiotics such as flucloxacillin, amoxicillin and piperacillin arising from the inherent susceptibility of the β-lactam ring to nucleophilic attack at positions containing cyclic amides (Waddington et al., 2020, Meng et al., 2016, Meng et al., 2017). It has been suggested that β-lactam antibiotics preferentially bind to extracellular proteins such as human serum albumin (HSA) and recently this has been demonstrated with the discovery that piperacillin-HSA adducts possess the ability to activate T-cells isolated from the PBMC of patients presenting with piperacillin hypersensitivity (Brander et al., 1995).

Alternatively, some haptenic drugs have their chemical reactivity conferred through bioactivation, typically achieved through drug metabolism via cytochrome P450 (CYP) enzymes (Griem et al., 1998). This concept is commonly referred to as the pro-hapten hypothesis, for which the reactive metabolites of SMX provides a classical example. SMX itself remains chemically inert. However, upon intracellular metabolism via CYP2C9 present within the liver, SMX undergoes bioactivation to SMX-NO from sulfamethoxazole-hydroxylamine intermediates (Sanderson et al., 2007). The former is a highly reactive metabolite capable of covalent binding with MHC peptides and producing antigenic determinants involved in the elicitation of T-cell responses.

1.5.1 The pharmacological interaction concept.

More recently, additional models of T-cell stimulation through more direct routes that bypass innate involvement have been proposed, namely the pharmacological interaction with immune receptors (p-i) concept (Pichler, 2002, Pichler, 2005). The p-i concept represents a diversion away from the widely accepted theory that the immunogenic potential of drugs could be predicted solely by protein reactivity and ability to form protein-hapten carriers. According to this concept, drugs incapable of bioactivation or covalent interaction can still bind to HLA peptides and/or the TCR in a non-covalent fashion. Stimulation of T-cells via this route is reversible and can be enhanced by further interaction with the HLA-bound peptides, however it has been suggested that activation may occur independently from peptides presented on the surface of the HLA complex (Pichler, 2003).

Although the phenomena of direct, processing independent mechanisms of immune stimulation can be viewed as contradictory to the delayed nature of archetypical type IV hypersensitive responses, a plethora of *in vivo* and *in vitro* immunological evidence exists that is compatible with pharmacological interactions being superfluous to the time-consuming process of peptide binding. Firstly, it must be considered that memory T-cells possess an inherently reduced threshold for activation after interaction with cognate antigens. This reinforces the notion that hapten and prohapten antigens are associated with the sensitisation of naïve T-cells due to an increased threshold for stimulation and resistant properties, whereas memory T-cells are more susceptible to direct stimulation that can occur rapidly and thus rule out any involvement of peptide processing pathways (Pichler, 2005, Zanni et al., 1998).

Clinical findings have described hypersensitive responses to iodinated contrast media upon primary exposure, with initial sensitisation phases and additional re-exposure not required (Brockow et al., 2009). Furthermore, compound-specific HLA-dependent T-cell clone (TCCs)

have been identified in patients administered with chemically inert drugs, as shown with carbamazepine, lamotrigine and SMX (Naisbitt et al., 2003a, Naisbitt et al., 2003b, Pichler, 2002). *In vitro* study detailing the ability of drug-specific TCCs to proliferate after APC processing capabilities are inhibited following fixation with glutaraldehyde, combined with an abrogated response following aldehyde-fixed APC and drug removal, provides further evidence for processing independent pathways of T-cell activation (Schnyder et al., 1997, Zanni et al., 1998).

1.5.3 The altered peptide repertoire model.

Novel mechanisms of HLA-peptide mediated T-cell activation have now been elucidated, specifically the altered peptide repertoire or 'altered self' model. Within the context of aberrant T-cell stimulation and downstream susceptibility to hypersensitive responses, the altered peptide model refers to conformational alterations of self-peptides within the antigen binding pocket induced by drug-HLA interactions, and ultimately results in a revised peptide configuration presented by HLA molecules, now recognised as 'non-self' following immunosurveillance (Ostrov et al., 2012). The vast majority of experimental evidence that exists for this model is based on interactions between the nucleoside reverse transcriptase inhibitor, abacavir, and the HLA-B*57:01 allele in which predisposition to abacavir hypersensitivity syndrome is heavily implicated with a high degree of sensitivity with regards to prediction (Illing et al., 2012, Norcross et al., 2012, Mallal et al., 2008). As drug antigens bind to the HLA region in a non-covalent manner to induce a T-cell response this model can be viewed as an appendage to the p-i concept. In the well documented case of abacavir, non-covalent binding between unmodified drug and HLA-B*57:01 is confined to the F-pocket of the antigen-binding cleft, typically containing a carboxy-terminal tryptophan and phenylalanine (Illing et al., 2012). Highly specific and dose-dependent drug binding induces modifications within the arrangement of anchoring peptides, with isoleucine and leucine

residues now present within the HLA-B*57:01 peptide motif resulting in the generation of relevant immunogenic neo-antigen peptides capable of driving polyclonal CD8+ T-cell stimulation (Norcross et al., 2012).

Furthermore, recent *in vitro* work detailing T-cell responses to abacavir and multiple analogues in healthy donors expressing HLA-B*57:01 has suggested perturbation of native HLA peptides is essential for T-cell stimulation (Thomson et al., 2019). In addition, a dual mechanism of T-cell activation has been proposed in which abacavir can stimulate T-cells either by direct interactions with HLA molecules and subsequent induction of HLA: TCR interaction or by the alteration of HLA binding peptides after initial abacavir exposure (Bell et al., 2013). A summary of the 3 pathways of T-cell activation can is shown below (Figure 1.7).

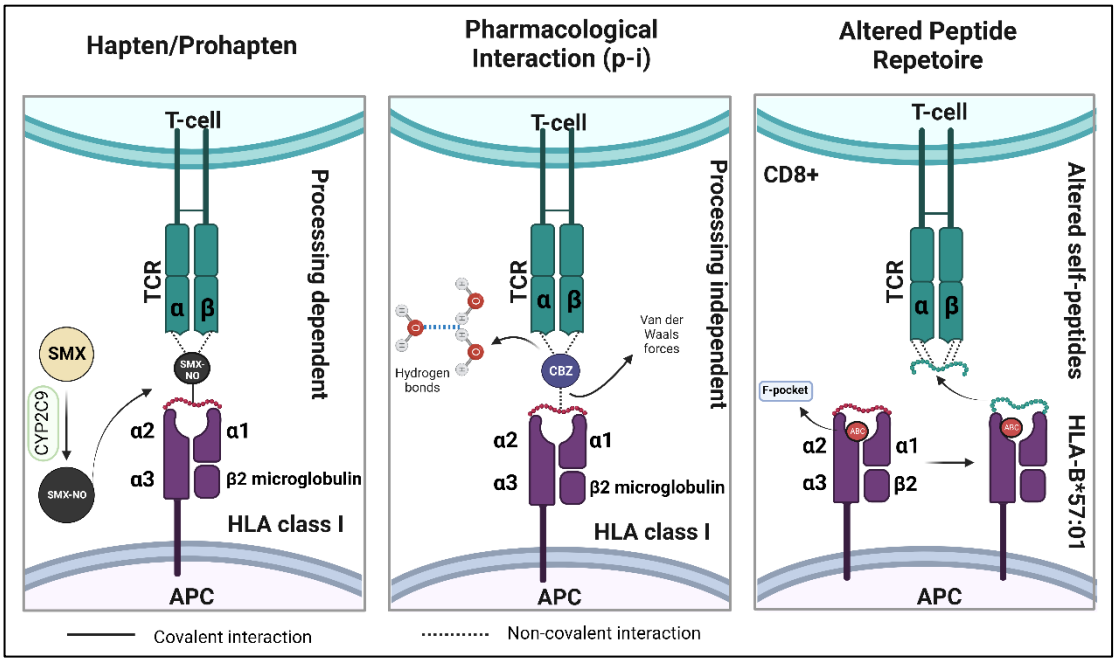


Figure 1.7. Recognised pathways of T-cell activation. After antigenic exposure, T-cells can be activated by 3 distinct mechanisms. (1) Covalent binding between low molecular weight compounds e.g. SMX-NO and MHC peptides. (2) Direct, non-covalent interaction between compounds e.g. carbamazepine (CBZ) and HLA molecules. Drugs can also directly stimulate via TCR interaction only. (3) Drug (e.g. abacavir) binding to HLA complexes can induce conformational changes within self-peptides found in antigen binding pockets, resulting in a revised peptide configuration presented by HLA molecules.

1.6 Metabolic routes of T-cell activation.

To maintain cellular function within immune populations, either in quiescent or activated states, adenosine 5'-triphosphate (ATP) production and subsequent energy availability is absolutely fundamental. The metabolic pathways that ultimately result in ATP production to quench cellular energetic requirements are hugely complex and comprise a myriad of aerobic and anaerobic pathways such as glycolysis, the tricarboxylic acid (TCA) cycle, oxidative phosphorylation and fatty acid β-oxidation (Pearce and Pearce, 2013). Adaptable and reprogrammable metabolic characteristics are a prerequisite to enable immune cells to meet energy demand under transient conditions and importantly, contribute towards regulatory function (Pearce et al., 2013). Interconnectivity and plasticity between ATP producing metabolic pathways is well documented and recent work has implicated and correlated metabolic changes within immune cells and functional modulation, leading to the development of an emerging new field, coined 'immunometabolism'.

1.6.1 Glycolysis.

In order to provide a constant supply of ATP under anaerobic conditions, glycolysis is the metabolic pathway of choice. Glycolytic pathways represent an immediate energy source for cellular components to perform essential metabolic function, which essentially involves the conversion of glucose to pyruvate. First described by Embden, Meyerhof and Parnas, glycolysis takes place within the cell cytoplasm following a series of enzyme-catalysed reactions and ultimately results in the transfer of inorganic phosphates between glycolytic intermediates and adenosine diphosphate (ADP) to form ATP. However in the context of cellular activation, considering carcinoma cells as a prime example, cells become prone to a metabolic shift away from the conventional route of anaerobic ATP production due to the large amounts of energy required for growth and differentiation, thus heightening demand for glucose uptake. Instead, the terminal glycolytic end product under anaerobic conditions, pyruvate, undergoes further

reduction to lactate in an aerobic process referred to as the Warburg effect (Warburg, 1925, Warburg et al., 1927). This phenomenon was later supplemented by studies describing how certain subsets of proliferating tumour cells utilise ATP generated solely from glycolysis, due to its immediacy, even accounting for oxygen abundance and the presence of functional mitochondria (Crabtree, 1929). This anaerobic process of ATP production, now widely known as the Crabtree effect, relies on fermentation instead of classical respiration and is far less efficient per molecule of glucose but is crucially associated with an increased rate of ATP production (Pfeiffer and Bonhoeffer, 2002). This physiological process has been exploited to study drug-induced mitochondrial dysfunction with liabilities towards DILI, for which the Crabtree effect can be circumvented in both HepG2 and HepaRG cell lines following substitution of glucose-based media for galactose, thus forcing reliance on oxidative phosphorylation (OXPHOS) and enabling detection of mitochondrial toxins (Marroquin et al., 2007, Kamalian et al., 2018).

Another exciting avenue is the measurement of glycolysis as an energetic determinant of immune activation, for example when studying T-cell responses to cognate antigens (van der Windt et al., 2016a). *In vitro* study of glycolytic parameters is possible by measurement of the extracellular acidification rate (ECAR) of cell culture medium containing activated T-cells, with proton release a useful marker of lactate excretion following pyruvate conversion (Wu et al., 2007). Interestingly, studies have begun to reveal the different metabolic characteristics associated with T-cell populations e.g. effector T-cells vs naïve T-cells, with energetic preferences often relating to their specific role within tolerogenic mechanisms or immune clearance.

1.6.2 Oxidative phosphorylation.

When considering OXPHOS in isolation, the abundant source of ATP needed for efficient cellular metabolism becomes clearly evident. Indeed, OXPHOS is responsible for the vast majority of cellular ATP production under quiescent conditions, accounting for approximately 24 – 28 molecules of ATP per molecule of glucose following progression through glycolysis and the TCA cycle. Unlike glycolysis, OXPHOS itself takes place fully within the confines of the mitochondria and is a highly aerobic process. Mechanistically, OXPHOS comprises of a series of redox reactions resulting in the transfer of electrons between protein complexes, collectively known as the electron transport chain.

Oxygen functions as the terminal electron acceptor in this process and gains a proton to produce a single molecule of water. In this way the electron transport chain and OXPHOS thereafter, possesses oxygen sensing capabilities and contributes towards both the generation of reactive oxygen species (ROS) and crucially, oxygen consumption (Chandel et al., 1998, Doege et al., 2005). This knowledge has been utilised in terms of assay development, specifically pertaining to measurement of the oxygen consumption rate (OCR) as an indicator of mitochondrial reparation using bioenergetic analysis and provides utility for the detection of drugs (e.g. leflunomide) and toxins (*Bacillus cereus*) able to induce mitochondrial dysfunction (Jones et al., 2021, Decleer et al., 2018). The energy requirement from OXPHOS is known to be imperative for the propagation of both innate and adaptive signalling pathways such as DAMPs and mitochondrial-derived molecules, specifically mitochondrial reactive oxygen species (Zhou et al., 2011). In fact, there is now accumulating evidence suggesting that mitochondria represent the 'hub' of the immune system and subsequent immunological signalling pertaining to a plethora of immune subsets. This has now been particularly well defined in the case of antigen-specific T-cell activation (Sena et al., 2013).

Revisiting immune-cell activation, for which glycolysis has been described as a critical determinant for proliferative responses, it can be useful to consider the energetic processes implicated within T-cell stimulation to highlight the involvement and synergy of metabolic pathways. In addition to glycolysis and the immediate supply of energy provided for proliferative function, OXPHOS and aerobic mitochondrial respiration has been identified as a requirement for optimal T-cell activation and ensuing expansion (Sena et al., 2013). This is in part due to OXPHOS providing an abundant source of ROS. This dependence for both pathways of metabolism is evident in the case of graft versus host disease when applied to bone marrow transplantation, during which alloreactive T-cells simultaneously exhibit elevated levels of OXPHOS and glycolysis, in contrast to the predominantly glycolytic nature of proliferating bone marrow cells (Gatza et al., 2011).

Additionally, other pathways have been implicated within immune-cell activation. In the case of T-cells, fatty acid oxidation and lipid metabolism has been shown to perform a critical function following the propagation of activation signalling within CD4+ and CD8+ T-cells (Michalek et al., 2011, Pearce et al., 2009). This is clearly evident during the transition between quiescent naïve T-cells and effector T-cells for which energy demand is increased to enable both rapid proliferation and cytokine secretion. Multiple phenotypic characteristics make this possible, namely expression of membrane transport proteins such as CD36 on the cell surface facilitating the entry of fatty acids into the T-cell membrane for β-oxidation within the mitochondria to support transient metabolic requirements (Couturier et al., 2019).

1.6.3 Naïve T-cell activation.

When quiescent, circulating naïve T-cells have a long lifespan and prior to antigenic stimulation energetic demand is reduced and basal metabolic needs are met via glucose and glutamine oxidation within the mitochondria to yield ATP in an OXPHOS-dependent manner (Bental and Deutsch, 1993). Metabolic precursors with the capacity for oxidation, such as essential

nutrients, are only consumed at minimal rates throughout the resting phase, to allow basic homeostatic function and general housekeeping (Jones and Thompson, 2007). Glucose has been shown to be particularly important for the synthesis of oligosaccharides. Furthermore, the oxidation of CO_2 for the production of ATP and allows naïve T-cells to maintain a greater amount of steady-state intermediates that are suitable precursors for macromolecule synthesis arising from lymphocyte transformation toward glycolytic pathways (Hume et al., 1978). Extracellular signalling provides chemical-based instructions for naïve T-cells to maintain and perpetuate this metabolic phenotype and the cessation of extrinsic signalling leaves cells unequipped for glucose uptake and ultimately results in apoptosis or cell atrophy (Rathmell et al., 2000). Cytokines are also involved in maintaining metabolic homeostasis within populations of resting naïve T-cells. Indeed, studies focusing on the role of IL-7 have shown that deletion results in both size reduction and a failure to sustain basal levels of glycolytic flux (Rathmell et al., 2001, Jacobs et al., 2010).

Metabolic differences between quiescent naïve T-cells expressing either CD4+ or CD8+ co-receptors have been described. This is particularly applicable when considering transcription factors implicated within energetic regulation. Resting, drug naïve T-cells expressing a CD8+ phenotype are dependent on sirtuin 1, an NAD dependent histone deacetylase responsible for maintaining the structural integrity of Fox01 and allows for sustained OXPHOS in resting CD8+ clonal populations (Jeng et al., 2018). Conversely, studies focusing on the activity of CD4+ expressing naïve T-cells have identified key modulatory nodes, such as Akt and STAT5, responsible for early metabolic programming in the form acute energetic switching and increased rates of both OXPHOS and glycolysis of following TCR stimulation (Jones et al., 2019).

The activation of naïve T-cells from their inherent resting and naïve state requires a form of metabolic reprogramming in which fuel sources are readily available to cope with the energetic demands of T-cell expansion. Mechanistically, T-cells exit the naïve phase following

engagement between cognate antigenic peptides, presented on the surface of APCs by MHC molecules, and the TCR, with additional costimulatory signalling provided by CD28 (Harding et al., 1992). Numerous signalling cascades are linked to T-cell progression from naïve to activated states following antigenic stimulation, with specific growth factors such as IL-2 heavily implicated in both signal propagation and transcriptional activation (Beadling et al., 1994). Indeed, IL-2 has been shown to promote signalling through a myriad of pathways involved in T-cell activation including PI3K-Akt, ERK/MAPK, NF-KB and mTOR pathways (Prasad et al., 1993, D'Souza et al., 2008).

The cumulative effect of these physiological processes results in the upregulation of aerobic glycolysis, reinforced by studies describing impaired proliferation under conditions limiting the activity of glucose transporters such as GLUT1 (Macintyre et al., 2014). Therefore, it can be said that increased glucose uptake and metabolism is the principle metabolic adaption of activated T-cells. Revisiting the co-stimulatory signalling necessary for full T-cell activation after initial peptide/MHC/TCR interaction, extrinsic signalling mediated by CD28 has been identified as the primary regulator of GLUT1 expression and by extension is critical for glucose import and metabolism (Frauwirth et al., 2002). Additional studies have revealed the significant role of the PI3K-Akt-mTOR axis for the rapid increase in GLUT1 expression in activated T-cells to aid glucose trafficking into T-cells to meet energetic demand and facilitate proliferation (Wieman et al., 2007). Although the activation of naïve T-cells is characterised by a fuel pathway switch to glycolysis, *de novo* ATP generation using mitochondrial OXPHOS has been suggested to be contributing source of cellular energy. This has been evidenced in studies evaluating the effect of the mitochondrial ATP synthase inhibitor, oligomycin, which have subsequently shown that blockade of early activation markers of T-cell expansion result in 'blunted' proliferation following TCR ligation with cognate antigens (Chang et al., 2013).

1.6.4 Effector T-cells.

After clonal expansion has been initiated and quiescence 'exit' has been achieved, T-cells undergo further metabolic adaptation to enable effector programming. The metabolic profiles involved correspond to downstream functional properties that are directly influenced by differentiation to specific effector T-cell subsets. Both CD4+ and CD8+ expressing T-cells can rapidly acquire effector functions and are heavily involved in steering immune responses towards destructive outcomes that typically result in the killing of infected cells, often mediated by the secretion of inflammatory and cytotoxic molecules (Kaech et al., 2002).

Effector cells perform overlapping functions and display high levels of plasticity, that when examined simplistically can be distinguished by phenotypic expression of either CD4+ or CD8+ co-receptors. In terms of immune function, CD8+ effector T-cells recognise antigens presented on all nucleated cells in the context of MHC class I and can be characterised by secretion of cytotoxic and cytolytic molecules. Conversely, CD4+ effector T-cells, referred to T_h cells, recognise antigens presented on the surface of professional APCs in the context of MHC class II and exhibit cytokine secretion patterns that have applications within regulation, memory and also provide signalling for the direction and activation of CD8+ mediated cytotoxic responses (Seder and Ahmed, 2003).

Although much focus has been placed on the energetic requirements for metabolic switching as T-cells exit the naïve state and become activated, metabolism is thought to not only support T-cell activation, but to also determine the specificity of T-cell responses (Wang and Green, 2012). As a result, metabolic reprogramming is now known to be vital prerequisite for the decision making that governs differentiation into specific effector T-cell subsets. Key nutrients involved in regulating the differentiation of activated T-cells include glucose and glutamine, with added input from mTOR signalling pathways. Firstly, glutamine assists with leucine retention in an indirect manner through the LAT1-CD98 heterodimer, prompting activated T-

cells to differentiate into either T_h1, T_h2, T_h17 or CD8+ expressing effector T-cells (Sinclair et al., 2013). In addition, both glutamine and leucine have routinely been shown to activate mTORC1 (Yang et al., 2013, Sinclair et al., 2013, Nakaya et al., 2014), so it is believed the nutrient availability can regulate differential and co-stimulatory function by facilitating metabolic fluxing to effector subtypes following exit from quiescence. Furthermore, glutaminolysis, the metabolic process by which T-cells can convert glutamine into intermediates for TCA cycle entry, has been shown play an important role within T_h differentiation, specifically targeting T_h17 cells (Johnson et al., 2018). Other studies have suggested that glutaminolysis, together with the ability of this metabolic process to generate α-ketoglutarate, plays a prominent part within the initial programming of T_h1 cells (Klysz et al., 2015).

Subclasses of effector T-cells possess different thresholds for activation and are associated with divergent physiological *in vivo* traits that are of relevance when studying the immunometabolic profiles for CD4+ and CD8+ T-cells. Proliferative potential, as well as the tendency for clonal expansion, in response to antigenic stimulation are perhaps the most prominent differences between CD4+ and CD8+ effectors. CD8+ expressing T-cells proliferate more rapidly upon initial activation and develop effector function after migration to the site of infection (Seder and Ahmed, 2003). These cells rely on glycolytic function under glucose rich conditions to keep pace with increased energy demand that is not only associated with regeneration of metabolic nutrients for oxidation but has also been shown to be supportive of cytokine synthesis (Delgoffe and Powell, 2015, Chang et al., 2013). Concordant with the metabolic pathways associated with the activation of naïve T-cells, effector T-cell function hinges around a switch to Warburg metabolism due to the energetically expensive nature of rapid proliferation and downstream effector function. This is illustrated by the reduced doubling time (< 8 h) of virus-specific CD8+ expressing T-cells in response to viral antigens during times of infection (De Boer et al., 2003). Recently, nicotinamide has been identified as

a potential therapeutic target for conditions associated with hyperactive T-cell responses. In an elegant study, nicotinamide was found to increase glycolysis while simultaneously inhibiting mTORC1 and the production of effector cytokines, thus uncoupling activation from effector function in CD8+ T-cells (Agliano et al., 2022).

1.6.5 Memory T-cells.

Following sufficient effector T-cell responses and removal of the offending antigen, the vast majority of expanded CD8+ expressing effectors die (90-95%). Any remaining cells are present at low, stable levels as memory CD8+ T-cells that do not require professional antigen presentation and can be recalled rapidly following antigen re-exposure (Murali-Krishna et al., 1998). Difficulties have been encountered when studying the function of memory T-cells expressing a CD4+ phenotype, owing to reduced proliferation and low precursor frequencies present in the blood after antigen exposure and immune clearance (Foulds et al., 2002). Instead, the memory activity of CD4+ T-cells have been traditionally studied within the context of a 'memory phenotype' as functional traits can be deduced by the expression of specific surface markers, such as CD62 and CD44 (Bingaman et al., 2005). Put simply, CD8+ memory T-cells are long lived and can be described as antigen experienced with a greater capacity for clonal expansion than CD4+, when considering the rapid nature of recall responses to a secondary antigenic encounter.

The immunometabolic profiles that govern the ability for swift proliferative responses of memory populations after secondary exposure have been widely studied in recent years and a number of physiological and mechanistic determinants have now been defined. Co-stimulatory molecules have been shown to 'prime' mitochondria for recall responses and studies focusing on the action of CD28 have observed elevated levels of spare respiratory capacity within bioenergetic profiles of activated cells (Klein Geltink et al., 2017). During times of quiescence, circulating antigen experienced T-cells possess the ability to quickly reacquire

effector function and re-engage with aerobic glycolysis by way of Warburg metabolism (Gubser et al., 2013). This transition is characterised by a rapid, more efficient uptake of glucose in addition to increased rates of mitochondrial fatty acid oxidation (FAO) by memory T-cells that are long-lived and quiescently programmed but sustain a primed state for antigen rechallenge (O'Sullivan et al., 2014).

When evaluating metabolic variations between CD4+ and CD8+ T-cells, different subsets exhibit different energetic signatures. For example, *in vitro* studies have used bioenergetic analysis to elucidate metabolic shifts within populations of antigen-specific T-cells. One particular study, performed by the Francis lab, demonstrated visible metabolic adaptations between CD4+ and CD8+ expressing peptide-specific T-cell clones (TCCs). Analysing ECAR as an indicator of glycolysis, both CD4+ and CD8+ T-cells displayed a glycolytic flux after stimulation with CD3 and CD28 activating antibodies (αCD3/28). Contrary to the physiological and metabolic characteristics discussed earlier, CD4+ T-cells were observed to possess greater glycolytic potential with CD8+ TCCs more dependent on mitochondrial OXPHOS for overall metabolism and cytokine secretion (Jones et al., 2017). Other markers of glycolytic activity, such as GLUT1 and Hexokinase II, have been analysed by immunoblot and indicated enhanced expression in populations of CD4+ T-cells following artificial stimulation (Macintyre et al., 2014, Marko et al., 2010, Jones et al., 2017). These studies utilise antigen-specific TCCs to measure energetic profiles after both artificial (αCD3/28) and peptide-induced stimulation but cannot be viewed as indicative of true memory T-cells responses due the absence of initial T-cell priming to the antigen of interest.

Much of the current literature relating to the metabolic conditions needed to permit memory T-cell expansion upon rechallenge with cognate antigens is almost exclusively limited to the activity of CD8+ expressing populations. A landmark study by Gubser et al. first described an immediate-early 'glycolytic switch' in CD8+ memory populations, with effector memory cells

additionally associated with rapid IFN-γ production after TCR mediated activation (Gubser et al., 2013). Importantly, studies of this type have phenotypically defined populations of CD8+ memory T-cells via expression of CD45RO+ surface markers and have also elucidated signalling pathways implicated within early metabolic shifts. During conditions supportive of rapid effector function, signalling through costimulatory CD28 molecules was found to be rapamycin insensitive, in contrast to the activation of both naïve and effector T-cells. Instead, these cells are more reliant upon the Akt signalling pathway and it has been suggested that this characteristic is imprinted on lymphocytes after differentiation and can determine the glycolytic potential of recall responses (Gubser et al., 2013). In terms of the machinery involved within regulatory function, some studies have pointed towards the activity of mTORC2-dependent signalling within regulatory mechanisms of CD8+ T-cell memory cell differentiation (Pollizzi et al., 2015, Zhang et al., 2016).

When accounting for the metabolic phenotype of regulatory pathways that govern CD8+ memory T-cell development, mitochondrial respiratory capacity has been deemed as a critical determinant. Studies in mice undertaken by van der Windt et al. first showed the requirement for a substantial spare respiratory capacity in CD8+ memory T-cells using mitochondrial stress-based assays. This was shown to be a key feature within oxidative metabolism and cytokines, such as IL-15, were identified to regulate spare respiratory capacity of CD8+ T-cells by promoting mitochondrial biogenesis (van der Windt et al., 2012). Furthermore, enzymatic expression of master regulators involved within FAO, specifically carnitine palmitoyl transferase (CPT1a), were also found to be of paramount importance when defining the spare respiratory capacity of CD8+ memory T-cells, thereby indicating the involvement of FAO for CD8+ mediated recall responses after antigenic rechallenge. More recently this theory has been challenged and importance of CPT1a and FAO for memory T-cell differentiation has been questioned. Genetic studies using etomoxir, which acts to inhibit the rate-limiting step of FAO, found that CPT1a mediated FAO was a dispensable process for the generation and function of

CD8+ memory T-cells and instead a greater emphasis on oxidative metabolism has now been proposed (Raud et al., 2018).

Differentiation of CD8+ T-cells to effector and memory function has universally been shown to be synonymous with an initial commitment to proliferation. When studying discrepancies between subsets various models have been proposed that rest on, firstly, the threshold for activation signalling and secondly, the accumulation of these signals. Indeed, CD8+ expressing effector memory cells have been associated with a reduced threshold for activation, although both CD4+ and CD8+ subsets have been shown to commit to proliferation after brief periods of stimulation (Kaech et al., 2002). This phenomenon may explain why CD8+ memory T-cells have a greater capacity for clonal expansion than CD4+. The idea that accumulative early signalling can lead to threshold activation and downstream effector function is pertinent for the development of therapeutics that target the immune system, such as vaccines, as acute exposure to immunotherapy agents can induce long-lasting T-cell signalling and activation even after cessation of treatment. This has been demonstrated when stimulating CD8+ T-cells for time-points as low as 2 hours. In these studies, naïve and memory CD8+ responses to cognate antigens were found to facilitate several cycles of cellular division indicating an early commitment to proliferation resulting from early signal transduction can be sustained for long periods (van Stipdonk et al., 2001, Kaech and Ahmed, 2001). Moreover, this has been further illustrated in the case of co-stimulatory molecules as the mere presence of CD28 or 4-1BB during activation has been found to modulate the metabolic profiles of memory T-cells after activation signalling had long been removed (Krause et al., 1998, Menk et al., 2018b). Work has also been undertaken to study the metabolic programme within populations of terminally exhausted CD8+ T-cells, with a recent *in vivo* study focusing of tissue-infiltrating lymphocytes in mice suggesting stimulation with IL-10 can promote OXPHOS and revitalise exhausted T-cells, restoring effector function in the process (Guo et al., 2021). Further understanding of the mechanisms that underpin sustained activation and the exact signalling pathways implicated

will be crucial to the efficacy of immunotherapy in addition to enhancing T-cell priming after vaccine administration. A summary of the metabolic pathways involved within the activation of naïve, effector and memory T-cells can is illustrated below (Figure 1.8).

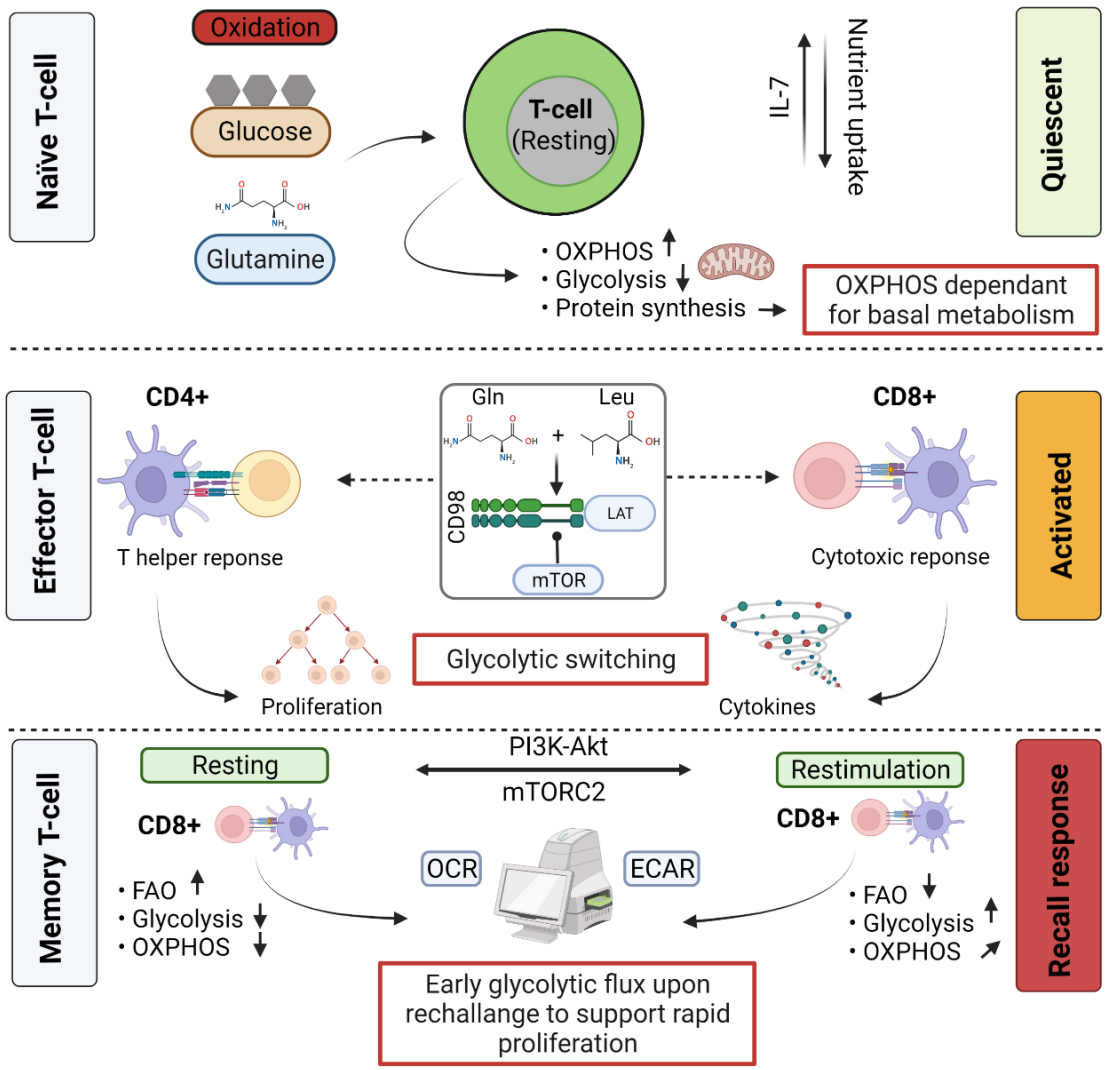


Figure 1.8. Metabolic pathways implicated in the activation of naïve, effector and memory T-cells following antigenic stimulation.

1.7 Glycopeptide antibiotics.

Glycopeptide antibiotics have been deployed for over 50 years to combat bacterial infection. Their discovery and clinical development represented a significant advancement for the treatment of infections caused by Gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* (Anstead et al., 2014, Venugopal and Johnson, 2012). The inception of glycopeptide antibiotics revolutionised the field of medicine and now enabled the treatment of bacterial infection that had since developed resistance to sulfonamides and penicillins that were initially developed in the 1930s and routinely used thereafter. Although highly efficacious, glycopeptides are reserved for severe, life-threatening infections. Therefore, these compounds are often referred to as a 'drug of last resort', with glycopeptides such as vancomycin ideally administered after all treatment regimens have been exhausted due to the plethora of contraindications associated and the elevated risk of adverse events.

This class of antibiotic are derived from actinomycetes, which are generally Gram-positive filamentous bacteria with aerobic function and are credited with the development of approximately 70% of all antibiotics developed for human use (Bérdy, 2005). Mechanistically, these drugs are hugely efficacious due to their ability to inhibit peptidoglycan synthesis which provides structural integrity for the formation of the bacterial cell wall. This mode of action is also highly specific and can be targeted to individual peptide sequences only found within the bacterial cell wall (L-aa-D-aa-D-aa) and therefore, in theory, glycopeptides are only selectively toxic (Reynolds, 1989). Drug interaction with unique peptide sequences takes place by way of hydrogen bonding and as a result it is now thought that glycopeptides are able to exert their inhibitive effects by way of steric hindrance. (Reynolds, 1989). The majority of glycopeptides follow a similar structural pattern which can be typically defined by the

presence of either a tricyclic or tetracyclic heptapeptide scaffold that can vary by the degree of glycosylation or presence of a lipophilic side chain comprising fatty acids (Butler et al., 2014).

1.7.1 Naturally occurring glycopeptide antibiotics.

First generation glycopeptide antibiotics are large, structurally complex drugs consisting entirely of natural products and have been developed using a wide variety of actinomycete-derived bacteria. Vancomycin and teicoplanin are the most widely used and prototypic of naturally occurring glycopeptides (Binda et al., 2014). Vancomycin, produced from *Amycolatopsis orientalis,* was initially developed and introduced into the clinic in 1958 by Eli Lilly. The drug has been recommended as a first-line treatment agent and remains the 'poster child' for glycopeptide use arising from its remarkably high efficacy for the treatment of Gram-positive infection, specifically MRSA (Watanakunakorn, 1982). Clinical studies have reported effectiveness of between 70-75% amongst critically ill MRSA patients at optimal intravenous loading doses of 10-20 mg/kg (Flannery et al., 2021, Mei et al., 2019, Huang et al., 2018).

Although conforming to the general structural pattern of most glycopeptides, vancomycin possess unique characteristics both within its chemical structure and mode of action. Vancomycin acts to prevent the second stage of bacterial cell wall synthesis by inhibiting the formation of the phosphodisaccharide-pentapeptide lipid complex (Anderson et al., 1965). It does so by direct, high affinity binding to bacterial peptides containing D-alanyl-D-alanine at the carboxyl terminus (Perkins, 1969). Ingeniously, by binding to bacterial peptide motifs and inducing mutations within target enzymes, vancomycin is able to eliminate common pathways of antibiotic resistance (Yim et al., 2014). According to the classification system initially proposed in 1999 by Nicolaou and colleagues, for which 5 distinct glycopeptide subclasses (Type I – Type V) are described based on structural traits, vancomycin is classified as a Type I glycopeptide, owing to the presence of valine, asparagine and glutamine residues surrounding its heptapeptide core (Figure 1.8) (Nicolaou et al., 1999).

Teicoplanin, produced from *Actinoplanes teichomyceticus,* was first discovered in 1978 but only introduced into European clinical settings in 1988, which in terms of the 'golden age' of antibiotic development represents a fairly contemporary addition (Binda et al., 2014). Teicoplanin is often described as vancomycin's 'sister' antibiotic due to its close structural alignment and similar mode of action with regards to peptidoglycan inhibition (Somma et al., 1984). The presence of a fatty acid residue in place of a sugar moiety (Figure 1.9) is a unique feature of teicoplanin that is not replicated in other non-synthetic glycopeptides and for this reason, teicoplanin has been classified as a Type IV glycopeptide (Nicolaou et al., 1999). Moreover, the addition of a fatty acyl side the molecule that exhibits hydrophobic chemical properties has been hypothesised to aid function and enhance activity of the drug by allowing stable anchorage at peptide target sites and facilitate strong intramolecular interactions (Allen and Nicas, 2003).

Like vancomycin, teicoplanin is naturally occurring and is associated with a similar spectrum of antibacterial activity, but crucially has been shown to display increased potency against certain bacterial isolates such as *Enterococcus, Streptococcus* and *Staphylococcus* (Van Bambeke, 2006, Jeya et al., 2011). Furthermore, some studies have eluded to an increased potency of between 2 – 8 fold against *Enterococci*, potentially arising from preferential interaction between teicoplanin and peptidoglycan precursors within the bacterial peptide motif (Cynamon and Granato, 1982, Fainstein et al., 1983). Large scale clinical trials and subsequent meta-analysis comparing the comparative efficacy and safety of vancomycin and teicoplanin revealed similar levels of efficacy, with an estimated 75% of patients in matched treatment groups displaying clinical responses to teicoplanin (Wood, 1996). When compared to vancomycin, the optimal dosage of teicoplanin is substantially lower, with between 6 – 12 mg/kg recommended (Wilson et al., 1994). Teicoplanin possesses greater versatility in terms of administration, with therapeutic intervention by either intravenous bolus or intramuscular injection suitable and effective. Crucially, teicoplanin has been found to be more tolerable

among patient groups and therefore, by extension, associated with a reduced incidence of ADR. Indeed, some studies have reported ADR frequencies of 10%, which still poses a significant risk to patient safety but is considerably lower than vancomycin, which has an estimated incidence of around 22% (Davey and Williams, 1991, Wood, 1996). Further examples of naturally occurring glycopeptides include β-avoparcin (Type II), ristocetin A (Type III), actaplanin A (Type III) and complestatin (Type V).

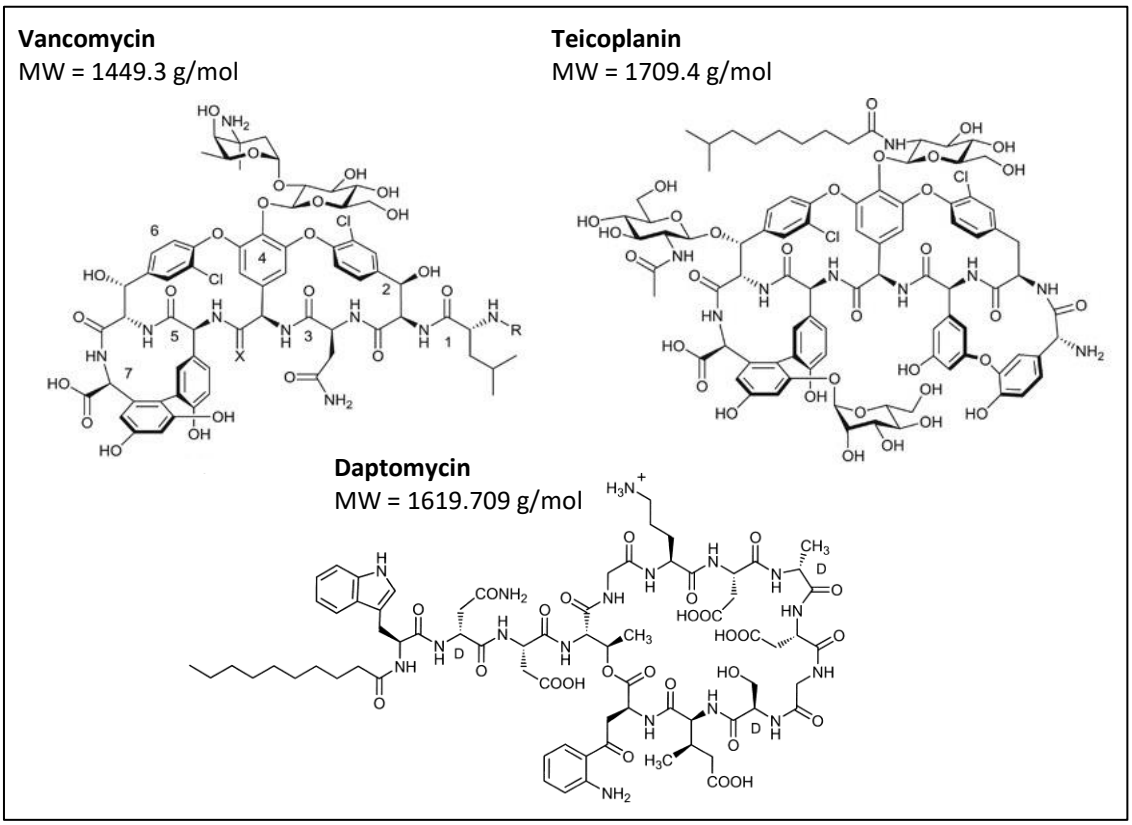


Figure 1.9. Chemical structure of widely used naturally occurring glycopeptide antibiotics (vancomycin and teicoplanin) and naturally occurring lipopeptide antibiotics (daptomycin) for the treatment of severe Gram-positive bacterial infection.

Alternative antibiotics have been developed for the treatment of Gram-positive bacterial infection that are closely related to the glycopeptide family. One such example is daptomycin, a naturally occurring cyclic lipopeptide with bactericidal activity produced by *Streptomyces roseosporus*. Daptomycin was introduced under the trade name cubicin in 2003 amid the rise of vancomycin and teicoplanin resistant strains of MRSA and differs from conventional glycopeptides by the absence of a heptapeptide core in addition to the presence of a lipophilic

chain (Streit et al., 2004). The addition of a lipophilic decanoyl side chain to the molecule (Figure 1.8) contributes to the drugs novel mode of action, during which daptomycin's lipid 'tail' inserts into the bacterial cell membrane, inducing depolarisation and membrane permeability in addition to the inhibition of bacterial RNA and DNA synthesis (Silverman et al., 2003).

Studies examining the *in vitro* potency of daptomycin have indicated exceptionally high proficiency when applied to MRSA and methicillin-susceptible clinical isolates, with certain groups reporting > 99.9% bacterial cell death after just 1 h exposure to clinically relevant doses of between 4 – 12 mg/kg (Fuchs et al., 2002). Consequently, daptomycin represents an efficacious and viable option, particularly for the treatment of *Streptococci* infection. Usage has been enhanced by the negligible levels of antibiotic resistance historically detected in both clinical and *in vitro* study, although more recently resistant clinical isolates have been identified arising from mutation within clinical MRSA strains (Silverman et al., 2001, Roch et al., 2017).

1.7.2 Adverse drug reactions associated with glycopeptide antibiotics.

As with many highly potent treatment agents utilised against severe infection, there is the potential for adverse outcomes following administration. These unwanted side effects can take place immediately after drug use or can be of a delayed nature, following mediation from the adaptive immune system and involvement of T lymphocytes. For this reason, in addition to the rapid rise of β-lactam antibiotic usage, many glycopeptides antibiotics have been consigned to 'drug of last resort' status. However, both naturally occurring and semi-synthetic glycopeptides still routinely deployed within clinical settings for severe, life threatening MRSA infection and also during specific cases in which hypersensitivity to β-lactam antibiotics has been observed.

1.7.2.1 Non-immune mediated reactions.

Perhaps the most common example of ADR induced by glycopeptide exposure is red man syndrome (RMS), predominantly associated with vancomycin and characterised by the degranulation of mast cells and basophils. RMS is typically infusion related and provides a classic example of pseudoallergic hypersensitivity as common clinical manifestations, such as an erythematous rash and pruritus, are indicative of symptoms often attributed to immune-mediated reactions (Davis et al., 1986). Estimates as to the incidence of RMS after vancomycin infusion wildly varies dependent on the infection status of the individual. Large cohort studies have suggested a prevalence of between 3.7% and 47% in patients suffering severe bacterial infection, with other analysis indicating a staggering 90% of individuals defined as healthy volunteers present with RMS after vancomycin exposure (Wazny and Daghigh, 2001, Sivagnanam and Deleu, 2003). One hypothesis that potentially explains this disparity is the innate release of histamine in infected individuals, which in turn reduces the impact of vancomycin on both mast cells and basophils (Wallace et al., 1991). As a result, the symptoms of RMS can often be relieved by a simple treatment course of antihistamines, with pre-treatment often the preferred course of action (Sahai et al., 1989). Fortunately, symptoms in the vast majority of patients are mild, to the extent many cases will go unreported via self-diagnosis.

This reaction itself is of an anaphylactoid nature, which simply refers to the involvement of non-IgE mediators. Symptoms manifest rapidly, appearing less than 1 hour after initial doses of vancomycin, with some reports indicating onset between 4 – 10 minutes post infusion (Wilson, 1998). Although, delayed onset has been noted in a small cohort of patients infused with vancomycin for longer periods in excess of 7 days, after previous toleration of the drug (Renz et al., 1999). In recent times, RMS has been reclassified as vancomycin flushing syndrome, although other glycopeptide antibiotics have since been postulated to be capable

of eliciting such reactions. Teicoplanin has been evaluated as a culprit drug for the onset of RMS, due to it structural similarities to vancomycin and inherent ability to aid degranulation of mast cells and basophils. However, clinical data is limited for the induction of RMS isolated to teicoplanin exposure, although the disease can be amplified during combination therapy after sequential administration of both drugs, indicating potential cross-reactivity (Khurana and de Belder, 1999). Other, less common, adverse events have been reported after glycopeptide exposure including nephrotoxicity, ototoxicity, neutropenia and thrombocytopenia, with these conditions not requiring a modulation of immune function or the presence of drug-reactive T-cells (Rybak et al., 1990, Traber and Levine, 1981, Gerstein et al., 2018).

1.7.2.2 Immune mediated reactions.

The second route of anaphylactoid-based hypersensitivity to glycopeptide antibiotics is from anaphylaxis. This condition is a serious, Type I immune reaction and mediated by IgE antibodies, with the bulk of supporting data that exists pertaining to vancomycin due to its perpetual usage over the past 50 years (Chopra et al., 2000). Anaphylaxis presentation after vancomycin infusion is rare and the exact incidence is not well established, although one particular study has suggested anaphylaxis directly contribute to an estimates 10% of all hypersensitivity reactions attributed to vancomycin (Minhas et al., 2016). Perhaps unsurprisingly for an immune-mediated reaction, the greatest risk factor for vancomycin-induced anaphylaxis is prior exposure to vancomycin itself, with true drug hypersensitivity reactions typically not manifesting after single culprit drug exposure due the requirement for drug-specific immune cells. Interestingly, in the case of anaphylaxis to vancomycin, case reports have actually identified disease onset and the presence of drug-specific IgE antibodies after first exposure to vancomycin either administered orally or via intravenous infusion (Baumgartner et al., 2017, Knudsen and Pedersen, 1992). Detectable clinical manifestations of

anaphylaxis occur rapidly, often within 2 minutes post vancomycin infusion, and a multitude of symptoms have been reported including angioedema, pruritus, hypertension and urticaria (Minhas et al., 2016, Chopra et al., 2000).

More recently, vancomycin has been associated with the onset of delayed-type IV immune mediated hypersensitivity reactions. DRESS represents a prominent example and has been strongly correlated with vancomycin exposure. DRESS is rare condition affecting anywhere between 1 in 1000 and 1 in 10000 people following drug exposure and is an archetypal of SCAR. Symptoms include skin rash, eosinophilia, and renal impairment in addition to multiple organ failure, with the lung, liver and kidneys often targeted (Webb and Al-Mohammad, 2016, Blumenthal et al., 2012). Due to its severe nature with regards to organ involvement, vancomycin-induced DRESS has been associated with a high mortality rate of up to 10%, although more contemporary analysis has estimated mortality to be as low as 2% (Kardaun et al., 2013). Nevertheless, vancomycin still poses a significant risk to patient safety and has been found to be responsible for the bulk of DRESS reactions attributed to antibiotic use, accounting for around 40% of all DRESS cases caused by antibiotic deployment (Wolfson et al., 2019).

The development of DRESS associated with vancomycin is a relatively new concept, with only 23 confirmed cases reported as of 2017 (Wilcox et al., 2017). However, this figure is now outdated, with clinically defined cases more prevalent arising from more extensive research focus. Indeed, recent studies have identified a genetic association between vancomycin administration and expression of HLA-A*32:01 within patient cohorts presenting with vancomycin-induced DRESS (Konvinse et al., 2019). Due to the widespread use of vancomycin since the 1950s it is clear that these reactions are not a new phenomenon, instead challenges with diagnosis due to the delayed nature of onset (2-6 weeks) and wide range of symptoms, have probably contributed to either misdiagnosis or no diagnosis altogether (Huang et al., 2020).

1.8 Thesis aims and objectives.

The primary aim of this thesis was to investigate the intrinsic immunogenicity of glycopeptide antibiotics implicated within drug hypersensitivity reactions. To achieve this, *in vitro* T-cell assays were deployed to study the immunological aetiology of hypersensitivity reactions induced by vancomycin, teicoplanin and daptomycin within healthy donor models and hypersensitive patients. Within this thesis, a focus was placed on the study of individuals positive for expression of the HLA-A*32:01 allele, recently associated with vancomycin-induced DRESS, with functional assays performed to define T-cell involvement. A secondary aim of this work was to develop a functional assay for the measurement of bioenergetic changes within T-cell populations following antigenic stimulation, with these assays subsequently utilised to further study the activity of vancomycin-responsive TCCs and elucidate potential routes of cross-reactivity. The specific aims of this thesis were as follows;

1) To assess preferential interaction between vancomycin and HLA-A*32:01 using *in vitro* T-cell priming assays and donors positive and negative for risk allele carriage.

2) To characterise the phenotype and function of vancomycin-specific TCCs generated from healthy donors expressing HLA-A*32:01.

3) To investigate the immunogenic potential of structurally similar glycopeptide antibiotics, such as teicoplanin and daptomycin, using diagnostic T-cell assays (LTT) and naïve T-cell priming assays.

4) To characterise the phenotype and function of teicoplanin-specific TCCs generated from drug naïve healthy donors positive for HLA-A*32:01 carriage.

5) To develop a functional bioenergetics assay capable of detecting metabolic changes within populations of activated T-cells and subsequently study glycolysis as an early maker for drug-specific T-cell activation.

2. Materials and methods.

2.1 Acquisition of reagents, drugs and chemicals.

PBMC isolation and T-cell separation

Lymphoprep; STEMCELL™ Technologies (Cambridge, UK).

Magnetic cell separation apparatus, columns and antibody-conjugated beads (CD8+, CD14+, pant-T, CD25+, CD45RO+, CD45RA+); Miltenyi Biotec (Surrey, UK).

Immune cell phenotyping

Conjugated fluorophores for use in flow cytometry (CD4 APC/FITC/PE, CD8 PE, CD3 APC); BD Biosciences (Oxford, UK).

Antibodies

Pan Class I purified mouse anti-human HLA-ABC (isotype, IgG2; clone, W6/32), LEAF™ purified isotype control (isotype, IgG1; clone, MOPC-21), Ultra-LEAF™ purified isotype control (isotype, IgG2; clone, MOPC-173); Biolegend (Cambridge, UK).

Pan Class II purified NA/LE mouse anti-human HLA-DR, DP, DQ (isotype, IgG2; clone, Tu39), Purified NA/LE mouse anti-human HLA-DR (isotype, IgG2; clone, G46-6); BD Biosciences (Oxford, UK).

Anti-human CD3 monoclonal functional grade antibody (clone, OKT3), Anti-human CD28 monoclonal functional grade antibody (clone, CD28.2); Thermo Fisher Scientific (Altrincham, UK).

Multiscreen® filter plates for ELISpot; Merck (Dorset, UK).

ELISpot kits containing coating and biotinylated antibodies (IFN-γ, IL-5, IL-10, IL-13, IL-17, IL-22, Granzyme B and perforin); Mabtech (Nacka Strand, Sweden).

Conjugated alkaline phosphatase (ALP) antibodies for ELISpot detection and 5-Bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium dye (BCIP-NBT) substrate solution; Mabtech (Nacka Strand, Sweden).

Fas ligand ELISpot kit (Coating and detection); Abcam (Cambridge, UK).

Cytokines and growth factors

Recombinant human IL-2, human IL-4, recombinant human GM-CSF, recombinant TNFα; Peprotech (London, UK).

Lipopolysaccharides (LPS) from Escherichia coli, Cyclosporine A (CSA); Merck (Dorset, UK).

Foetal Bovine Serum (FBS); Thermo Fisher Scientific (Altrincham, UK).

Human AB Serum; Merck (Dorset, UK).

Proliferation assays

Tritiated [^{3}H]-methyl thymidine (5 Ci/mmol), Moravek (California, USA).

Meltilex wax scintillator sheets, sample bags, and printed glass fibre filter mats; Perkin-Elmer (Waltham, USA).

Cell culture

Corning® T25 and T75 flasks; Merck (Dorset, UK).

Nunc™ 6, 12, 24, 48 and 96-well plates, Cell scrapers; Thermo Fisher Scientific (Altrincham, UK).

0.45 µM and 0.22 µM pore size membrane filters; Merck (Dorset, UK).

Drugs, stimulants and chemicals

Vancomycin hydrochloride from Streptomyces orientalis, teicoplanin, carbamazepine (CBZ), amoxicillin, piperacillin, phytohemagglutinin-L (PHA) from *Phaseolus vulgaris;* Merck (Dorset, UK).

Daptomycin; accord (NHS, UK).

Flucloxacillin; CP Pharmaceuticals (Wrexham, UK).

Roswell Park Memorial Institute (RPMI)-1640, human holo-transferrin, L-glutamine, Hanks balanced salt solution (HBSS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, bovine serum albumin (BSA) penicillin-streptomycin, ethylenediaminetetraacetic acid (EDTA), sodium azide, glutaraldehyde, glycine, and dimethyl sulfoxide (DMSO); Merck (Dorset, UK).

Bioenergetics

Seahorse 96-well cell culture plates, Seahorse xFe^{96} sensor cartridge, Seahorse XF calibrant solution, Seahorse XF base medium; Agilent (Cheshire, UK).

Mitochondrial inhibitors (oligomycin, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), antimycin A, rotenone); Merck (Dorset, UK).

Sodium pyruvate, L-glutamine, D-glucose, 2-deoxy-D-glucose (2-DG); Merck (Dorset, UK).

Corning® Cell-Tak™ cell and tissue adhesive; Merck (Dorset, UK).

2.2 Medium and buffers.

R9 - 1 L RPMI-1640, 100 µg/ml penicillin, 100 U/ml streptomycin, 12.5 mg/ml transferrin, 25 mM HEPES buffer, 2 mM L-glutamine, 10% human AB serum.

F1 – 1 L RPMI-1640, 100 µg/ml penicillin, 100 U/ml streptomycin, 10% FBS, 25 mM HEPES buffer, 2 mM L-glutamine.

HBSS - As purchased from Merck.

FACS buffer - 90% HBSS, 10% FBS, 0.2 mg/ml sodium azide.

MACS buffer - 500 ml HBSS, 2 mM EDTA, 5 µg/ml BSA.

Freezing medium - 80% FBS, 20% DMSO.

Seahorse assay medium – Glucose free Dulbecco's modified eagle medium (DMEM), 2 mM L-glutamine, 2 mM sodium pyruvate.

2.3 Isolation of peripheral blood mononuclear cells from venous blood.

Whole venous blood was collected using vacutainer heparinised tubes from healthy volunteer's naïve for drug exposure or patients presenting with hypersensitivity. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood using density centrifugation, during which whole blood was layered onto an equal volume of lymphoprep (1:1 ratio) and then centrifuged for 25 mins at 2000 rpm with no brake applied. The resulting 'buffy coat', comprising the lymphocyte layer, was carefully removed by gentle resuspension using a Pasteur pipette and transferred to a fresh 50 mL tube. The newly transferred lymphocyte layer was then washed in HBSS, following centrifugation at 1800 rpm for 18 mins. Supernatant was discarded and cell pellets were resuspended in 2 mL HBSS and then pooled into a single 50 mL tube. Resuspended cells were washed again in HBSS and centrifuged at 1500 rpm for 10 mins. Finally, cells were resuspended in 30 mL R9 cell culture medium and 10 µL of cell suspension was stained with trypan blue (0.2% w/v) using a 1:5 dilution and counted on a Leica DME microscope (Lecia Microsystems, Milton Keynes, UK) using a Neubauer haemocytometer. The overall cell viability was determined using trypan blue exclusion, in which uptake of trypan by cells indicated a permeabilized membrane and was indicative of cell death, with viabilities > 95% typically achieved.

Viable, freshly isolated PBMCs were then utilised in functional T-cells assays, described below, or cryopreserved for use at a later date. For optimal cryopreservation, $1x10^7$ cells/mL were suspended in equivalent volumes of R9 cell culture medium and freezing medium (80% FBS, 20% DMSO). The complete mixture of cells, R9 and freezing medium was then transferred in 1 mL aliquots ($1x10^7$ cells) into 1.8 mL cryovials before being placed inside isopropanol filled Mr frosty containers, which allow for a freezing rate of -1 °C/min when placed inside a -80 °C freezer for optimal cryopreservation. After 24 h, cells were removed from the Mr frosty container at -80 °C and transferred to either a -150 °C freezer, or in specific cases liquid

nitrogen was used as an alternative (-195 °C). Upon thawing, cryopreserved cells were washed by centrifugation (1500 rpm, 10 mins) in excess R9 medium to remove DMSO and then resuspended at the required volume for immediate use, with viability typically ranging anywhere between 60-90%.

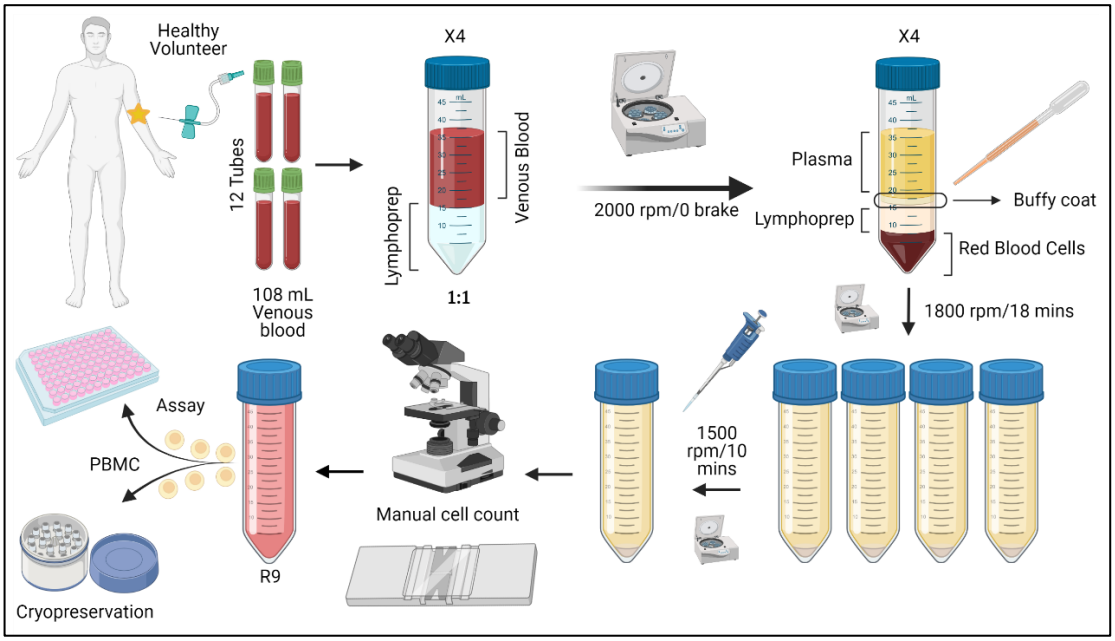


Figure 2.1. PBMC isolation from venous blood. Blood was collected from healthy volunteers or patients with confirmed hypersensitivity and PBMCs were isolated using a density centrifugation protocol. PBMCs were then quantified and either utilised within *in vitro* assays or cryopreserved for future use.

2.4 Positive and negative selection of T-cell subsets.

For many of the assays detailed in this section, whole PBMC represented a starting point for which separation into its fractional immune cell and T-cell components was necessary. This process was performed by magnetic activated cell sorting (MACS) with the combined use of antibody conjugated superparamagnetic microbeads, specific for T-cell markers, and a separator comprising a ferromagnetic sphere within an LS column. Briefly, heterogeneous immune cell populations were incubated with antibody conjugated microbeads specific for markers expressed by the desired T-cell population. After incubation, microbeads are washed off and tagged cells are then filtered through an LS column, and the desired population is

either retained by the column (positive selection) or expelled from the column after plunging (negative selection). The full separation methodology from whole PBMC to the subsets required for functional T-cell assays, such as memory and naïve cells, are described in detail below.

Whole PBMC were isolated from venous blood, as described in Section 2.3. Following resuspension in R9 medium, cells were centrifuged at 1500 rpm for 15 mins at room temperature (RT). Supernatant was discarded and PBMCs were resuspended in 800 µL MACS buffer per 10^8 cells and 200 µL CD14+ microbeads per 10^8 cells (300 µL maximum) , and then incubated for 15 mins at 4 °C. After incubation and subsequent tagging of cells expressing the CD14+ co-receptor, cells were washed in MACS buffer (15 mL/10^8 cells) and centrifuged for 8 mins at 4 °C. MACS buffer was then added to the LS column for the purpose of pre-washing prior to the introduction of tagged cells. After completion, cells were resuspended in MACS buffer (500 µL/10^8 cells) and added to the column and allowed to drain through, with cells tagged for CD14+ positively selected for remaining bound by magnetic force and subsequently plunged into a collection tube after the addition of 5 mL MACS buffer. Cells negatively selected for (CD14 -ve) flowed through the LS column, with both fractions counted and either cryopreserved, cultured or further separated into more distinct T-cell fractions.

The remaining fraction of CD14 -ve cells were then subjected to further MACS separation to deplete undesired cell types such as B-cells, natural killer cells, granulocytes and monocytes to produce a pure T-cell population. This was achieved with the use of Pan-T antibody cocktail which excludes non-relevant populations by exclusively containing biotin-conjugated antibodies specific to a panel expressed by non-T-cells, including CD11b, CD16, CD20, CD56, and CD66. In short, 40 µl MACS buffer was added per 10^7 CD14 -ve cells, in addition to a Pan-T biotin cocktail (40 µl/10^7 cells) and incubated for 10 mins at 4 °C. Following incubation, MACS buffer and anti-biotin microbeads were added to the cells (30 µl and 20 µl/10^7 cells

respectively) and incubated for a further 15 mins at 4 °C. Tagged cells were then centrifuged for 10 mins 4 °C after the addition of 1.5 mL MACS buffer per 10^7 cells. Following centrifugation and removal of unbound conjugated antibodies, tagged cells were resuspended in MACS buffer, and drained through a pre-washed LS column as previously described before being quantified. Cells that were positively selected for remained in the column (CD3 –ve/ non-T-cells) were cryopreserved due to their utility when generating B-cell lines, with negatively selected for cells passing through the column (CD3 +ve) subjected to further MACS separation.

In order to deplete regulatory T-cells, populations expressing CD25+ were removed by positive selection in conjunction with the use of CD25+ microbeads. Briefly, CD3 +ve populations were centrifuged at 1500 rpm for 10 mins at 4 °C and following the removal of supernatant, 70 µl MACS buffer and 10 µl of CD25+ microbeads were added to the cells and incubated for 15 mins at 4 °C. Cells were then washed with MACS buffer, resuspended and passed through the LS column as previously described and T-cells possessing regulatory function and expression of CD25+ were positively selected for and either discarded or cryopreserved. Cells negatively selected for (CD25 –ve) were then subject to further separation produce naïve or memory T-cell population. However, it is important to note that other T-cell subtypes will also express CD25+, so although the vast majority of regulatory T-cells are depleted, small but potentially significant populations of both resting and activated memory T-cells are also likely to removed.

Finally, acquisition of the remaining T-cell populations (CD45RA+ naïve and CD45RO+ memory) were facilitated by CD45RO microbeads, with remaining populations deemed to possess a naïve state by process of elimination. 20 µl CD45RO microbeads per 10^7 cells were added to the remaining population of CD25 –ve cells and incubated for 15 mins at 4 °C. The remaining steps in the positive selection process remain consistent with the methodology previously described, with CD45RO+ memory cells bound within the LS column and CD45RA+ naïve cells present as flow through after negative selection. Populations of both CD45RA+ naïve and

CD45RO+ memory cells were then counted and immediately deployed within T-cell assays or cryopreserved, as described in Section 2.3.

2.5 Differentiation and maturation of CD14+ monocytes to dendritic cells.

Dendritic cells (DCs) assume the role of professional antigen presentation and play a pivotal role within T-cell priming assays. Following the acquisition of CD14+ monocytes by MACS separation and the positive selection process, a 6 day culture protocol was initiated during which monocytes were exposed to cytokines and growth factors that facilitate both phenotypic and functional changes. CD14+ expressing monocytes, either fresh or from cryopreserved stocks, were cultured for 6 days (37 °C, 5% CO_2) in R9 cell culture medium (3 mL/well) supplemented with GM-CSF (800 U/ml) and IL-4 (800 U/ml) to drive differentiation to a DC phenotype. On days 2 and 4, cells were supplemented with a further 3 mL of medium containing GM-CSF and IL-4, at the concentrations previously stated. Finally, on day 6 DCs were exposed to maturation factors (LPS, 1 µg/ml; TNF-α, 25 ng/ml) via the removal of 3 mL cell culture medium containing GM-CSF and IL-4 and subsequent addition of 3 mL R9 supplemented with LPS and TNF-α maturation factors. Flow cytometry and staining for classical DC markers, such as CD40, CD80 and CD86 could be utilised at this point to validate successful DC maturation.

2.6 PBMC toxicity assays.

In order to determine maximal drug concentrations that would not inhibit mitogen driven proliferation, toxicity assays were undertaken on healthy donor PBMC. Toxicity assays were typically performed on the PBMC of 3-5 healthy donors to account for any inter-individual variably that may arise after exposure to the compound of interest. PBMC were isolated, as described in Section 2.3, resuspended in R9 medium, and transferred in triplicate cultures into a 96-well U-bottomed plate (100 µL/well) at a seeding density of $1.5x10^5$ cells/well. Graded concentrations of drug at twice the desired final concentration were added to the plate in triplicate, with the addition of R9 medium in isolation used to determine basal levels of proliferation for comparative reference. Drug treated cultures were incubated for 24 h (37 °C, 5% CO_2) before PHA (10 µg/mL) was added to every well of the assay plate in promote non-specific cell proliferation. Cells were incubated for a further 48 h in the presence of PHA before being pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Following the final incubation, plates were harvested onto printed fibreglass filter mats using a cell harvester (Harvester 96 Tomtec; Tomtec Imaging Systems GMbH, Unterschleissheim, Germany) and dried at 80 °C in an oven. Once dried, fibre glass mats were heat sealed inside Wallac sample bags with MeltiLex melt on scintillator sheets using a Wallac 1495-021 Microsealer (Perkin Elmer, Waltham, MA, USA). Radioactivity incorporated within the DNA of proliferating PBMC was detected and quantified using a MicroBeta 2450 microplate counter (Perkin Elmer, Waltham, MA, USA) and readout were given by counts per minute (cpm). It is important to note that this assay only provides indirect measure of drug toxicity within PBMC cultures and toxicity is assumed by a cells inability to proliferate. This format may prove problematic when attempting to determine toxic concentrations of compounds that exert their mechanisms of action by directly inhibiting cellular proliferation. In such cases, viability assays that measure cell death may be more applicable.

2.7 Lymphocyte transformation test.

PBMC from hypersensitive patients or healthy volunteers were isolated from fresh venous blood by density gradient centrifugation as previously described. PBMC were diluted in R9 cell culture medium to achieve a seeding density of $1.5x10^6$ cells/mL and a volume of 100 µL ($1.5x10^5$ cells) was transferred into a U-bottomed 96 well-plate in triplicate, cell numbers depending. Graded concentrations of drug (100 µL) were added to each well at twice the desired final concentration. The addition of R9 cell culture medium was used as a negative control and the inclusion of conditions in which PBMC were exposed to PHA (10 µg/mL) represented a positive control for the assay by way of detecting non-specific lymphocyte transformation. PBMC were incubated with the drug of interest, and both negative and positive controls, for a period of 6 days (37 °C, 5% CO_2). On day 6, [^{3}H]-thymidine was added to each assay condition at a concentration of 0.5 µCi/well, and incubated for a further 16 h (37 °C, 5% CO_2). On day 7, plates were harvested as previously described and readouts were given by cpm. Data was analysed by calculation of a stimulation index (SI) given by: (average cpm cultures dosed with drug) / (average cpm cultures treated with medium).

2.8 T-cell multi-well assay.

The T-cell multi-well assay (T-MWA) was developed from initial iterations of the T-cell priming assay, as described in Section 1.3.2.3. Traditional T-cell priming assays assess the intrinsic ability for a *de novo* immune response to a drug in healthy, compound-naïve individuals, with this assay format crucially requiring two separate batches of mature autologous DCs (Faulkner et al., 2012). Conversely, the T-MWA is a miniaturised version of the original T-cell priming assay and allows for a greater number of replicates owing to the requirement for a single batch of mature DCs. This assay interrogates the antigenicity of a compound and the propensity to elicit a naïve T-cell response whilst giving a sensitive indication of pre-cursor frequency (Ogese et al., 2020).

Following the differentiation and maturation of monocyte-derived DCs, performed according the protocol described in Section 2.5, mature, adherent DCs were harvested using a cell-scraper. Cells were then centrifuged at 1500 rpm for 5 mins to remove any remaining cytokine milieu, before resuspension in R9 medium and quantification. Naïve T-cells, separated according to the MACS protocol as previously described, were counted and co-cultured with DCs over multiple replicates in a 96-well U-bottomed plate, achieving a total volume of 200 µL/well. The composition of each well within the assay entailed $2x10^5$ naïve T-cells, $1x10^4$ DCs and the drug of interest. Plates were then cultured for 12 days (37 °C, 5% CO_2) to allow for sufficient naïve T-cell priming, facilitated by mature DCs, and expansion of subsequent drug-specific pre-cursors. After the 12 day priming period plates were thoroughly washed in R9 cell culture medium (3x) to exclude both cytokine milieu and more importantly, soluble drug. Following the completion of washing and drug removal, wells were rechallenged with either R9 medium for the purpose of a basal proliferative control, or rechallenged with drug to assess the degree of naïve T-cell priming via a proliferative response. Rechallenged plates were incubated for 48 h (37 °C, 5% CO_2) before being pulsed with tritiated [^{3}H]-thymidine (0.5

µCi/well) for a further 16 h. Plates were then harvested according to the protocol described in Section 2.6. The readout, interpreted via cpm values, was then analysed to investigate the propensity for *de novo* naïve T-cells responses to the culprit drug, in addition to the degree of naïve T-cell priming.

2.9 Long-term stimulation of PBMC cultures.

To assess a drugs ability to interact with immunological receptors and stimulate T-cells in non-sensitised individuals, repetitive stimulation of PBMC cultures was performed in order to detect primary responses to the antigen in question. The assay described was developed and optimised from the protocol first described by Engler et al. and has potential utility when investigating preferential drug interactions with specific HLA haplotypes. Cryopreserved PBMC isolated from HLA-typed healthy individuals were cultured in a 96-well plate at $2x10^5$ cells/well in R9 cell culture medium containing the relevant antigen and IL-2 (50 U/mL), achieving a total well volume of 200 µL. On day 3, cultures were supplemented with 100 µL fresh R9 medium containing IL-2 (50 U/mL) after removal of an equivalent volume. Restimulation of PBMC cultures was performed on day 7 and weekly thereafter, with cells exposed to a cocktail of irradiated autologous PBMC ($5x10^4$/well), drug and IL-2 (50 U/mL). After 3 weeks, PBMC cultures were washed in R9 cell culture medium (x5) to ensure the removal of soluble drug and then rechallenged with irradiated autologous PBMC (10^3/well) and either the drug of interest or R9 medium control to assess basal levels of stimulation. PBMC cultures were incubated with the restimulation cocktail for 48 h (37 °C, 5% CO_2) and pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for the final 16 h of incubation and harvested as previously described. Proliferation was given by cpm values and results were expressed as cpm in individual culture wells.

2.10 Generation of Epstein-Barr virus transformed B-cells.

To conduct functional T-cell assays assessing the degree of T-cell stimulation after drug exposure, access to sufficient numbers of autologous antigen presenting cells (APCs) that are both donor specific and possess the capacity for sustained growth and long term culture are an important prerequisite. To this end, autologous immortalised B-lymphoblastoid cell lines with the potential for antigen presentation were generated. These cells lines were generated via the transfection of B-cells from the donor of interest with the Epstein-Barr virus (EBV). In order to generate these EBV-transformed autologous B-cell lines, 10 mL (10^6 cells) of confluent B9-58 cells (primate cell line with the capacity for EBV production) were centrifuged at 1500 rpm for 10 mins and the resulting supernatant was passed through a 0.22 µM filter. Approximately 5 mL of filtered supernatant was then incubated overnight (37 °C, 5% CO_2) with $5x10^6$ PBMC or fractions of CD3 –ve populations, in addition to F1 medium supplemented with CSA (1 µg/mL). Following 24 h incubation, cells were centrifuged at 1500 rpm for 5 mins and after removal of supernatant resuspended in F1 medium further supplemented with CSA (1 µg/mL) and plated into a 24-well plate. From this point on, cells were supplemented with fresh F1 containing 1 µg/mL CSA on a bi-weekly basis for a period of 3 weeks. After 3 weeks, T-cells were successfully depleted due to the selective toxicity of CSA and EBV-transformed autologous B-cell cultures were maintained with fresh F1 medium only. Following rapid expansion of cultures, cells were transferred into T25 and then T75 flasks and maintained with F1 medium approximately twice per week or when confluency exceeded $1x10^6$ cells/mL.

2.11 Generation of drug-specific T-cell clones.

To study and characterise unique features and mechanistics of drug-specific T-cell responses in both healthy donors and hypersensitive patients, it was necessary to study the nature of such responses on a unicellular level by conducting T-cell cloning experiments generated from drug exposed bulk PBMC population. Drug-specific T-cell clones (TCCs) could be enriched in the PBMC of hypersensitive patients, or induced in the PBMC of healthy donors following incubation with the relevant compound. Bulk PBMC populations were seeded at $1x10^6$ PBMC/well with the drug of interest in a 48-well flat-bottomed plate and incubated (37 °C, 5% CO_2) for a 14 day period. On days 6 and 9, PBMC cultures were supplemented with fresh R9 medium (approximately 30% volume replaced) containing IL-2 (200 U/mL) in order to promote and facilitate the expansion drug-specific T-cells.

On day 14 and following completion of bulk cultures, T-cells were enriched for either CD4+ or CD8+ expressing populations by MACS separation with the use of CD8+ microbeads. MACS separation of bulk T-cell populations was performed via incubation with CD8+ microbeads (20 µL/10^7 cells) and MACS buffer (80 µL/10^7 cells) for 15 mins at 4 °C, with the remaining steps concordant with the MACS separation protocol previously described. For the generation of feeder cells to promote CD4+ and CD8+ T-cell growth, allogenic PBMC were isolated by density gradient centrifugation as previously described and irradiated for 20 mins to inhibit any proliferative potential, but importantly still allowing short term presentation of allogenic HLA peptides to aid stimulation. Irradiated allogenic PBMC ($5x10^5$ cells/mL), T-cells, IL-2 (200 U/mL) and PHA (5 µg/mL) were diluted in R9 medium to produce a restimulation 'master mix'. T-cells were serially diluted in 35 mL R9 medium to 3, 10 and 30 cells/mL. The combined stock solution was then plated into a 96-well U-bottomed plates in 100 µL volumes and therefore resulting in 0.3, 1 and 3 T-cells per well. This was done to achieve an average of 1 cell/well for the generation of pure monoclonal populations. Cultures were incubated for 5 days (37 °C, 5%

CO_2) and maintained with fresh R9 medium supplemented with IL-2 (200 U/mL) every 2 days thereafter for 14 days.

After the 14 day incubation period, cultures were further restimulated with freshly isolated allogenic PBMC ($5x10^5$ cells/mL) diluted in R9 medium supplemented with IL-2 (400 U/mL), and PHA (10 µg/mL) and 50 µL of complete restimulation mix was added to each well to assist mitogen driven expansion of T-cell cultures. Cultures were then maintained every 2 days as previously described and plates were visually inspected for clonal expansion, with well-growing cultures identified by the formation of large, yellow cell pellets transferred into 2 wells of a separate 96-well plate. After expansion, confluent TCCs were split once more into 4 wells and maintained twice per week with R9 medium supplemented with IL-2 (50 U/mL) for a 14 day period until a reduction in growth rate was observed. If necessary, clones occupying the original plate were repetitively restimulated up to 3 times as previously described, depending on the levels of clonal expansion.

After 14 days, volumes of 'picked' clones were reduced to 100 µL and 2 of the 4 wells were pooled and transferred in 50 µL aliquots (x4) to a new 96-well U-bottomed plate for the conduction of testing. Autologous EBV-transformed B-cells were irradiated for 20 min and resuspended in R9 to remove cells from F1 medium before being transferred to the assay plate in 50 µL aliquots ($2x10^5$ cells/mL). Testing was performed over 4 wells in duplicate conditions, with 100 µL R9 medium added to two wells (negative control) and 100 µL drug at twice the desired final concentration added to the other two wells of the assay plate. Test cultures were incubated for 48 h (37 °C, 5% CO_2) and pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for the final 16 h of incubation to allow incorporation into cellular DNA and harvested as previously described. SI vales were calculated according to the methodology detailed in

Section 2.7, and clones exhibiting an SI > 1.5 were subject to further mitogen driven expansion to enable functional study of compound-specific TCCs.

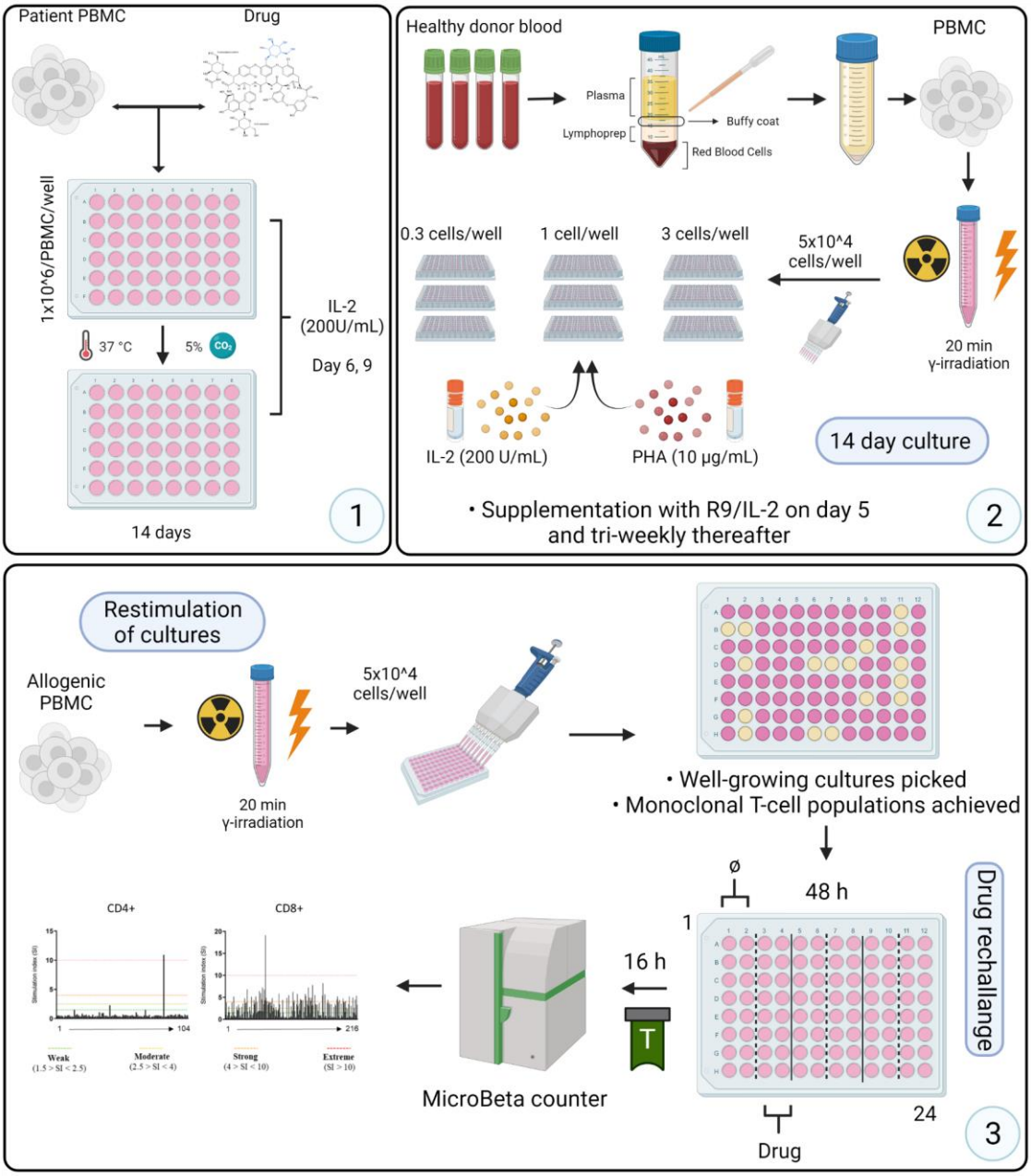


Figure 2.2. Generation of TCCs from bulk PBMC populations. Bulk PBMC cultures were seeded at $1x10^6$ PBMC/well with graded drug concentrations and incubated for 14 days. T-cells were enriched for CD4+ or CD8+ expressing populations by MACS separation and transferred to 96-well U-bottomed plates (1 T-cell/well average) with irradiated allogenic PBMC ($5x10^4$ cells/well) and R9 medium supplemented with IL-2 (200 U/mL) and PHA (5 µg/mL). After sufficient clonal expansion test cultures rechallenged with the compound of interest or R9 medium only, in the presence of irradiated autologous APCs ($1x10^4$ cells/well). Testing was performed over 4 wells in duplicate conditions and cultures were incubated for 48 h and pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for the final 16 h of incubation.

2.12 Confirmation of TCC specificity via dose-response analysis.

Once clones were deemed to show specificity during initial testing and sufficient mitogen driven expansion of clonal populations had taken place, it was necessary to confirm these TCCs as drug-responsive at graded concentrations of the target compound. This was to ensure both true specificity to the compound and also to verify the expansion of monoclonal T-cell populations, as expansion of polyclonal populations would often mask any detectable proliferative response. Drug-specific TCCs were washed in R9 medium, to exclude IL-2, and resuspended at $5x10^5$ cells/mL. Autologous EBV-transformed B-cells were irradiated as previously described and resuspended in R9 to exclude F1 medium, at $2x10^5$ cells/mL. Test cultures were set up within a 96-well U-bottomed plate in triplicate to allow for statistical analysis. Each condition consisted of TCCs ($5x10^4$ cells), EBV-transformed B-cells ($1x10^4$ cells) and graded concentrations of drug added in 50 µL aliquots, 4x the desired final concentration to assess dose dependency. PHA (10 µg/mL) was included to verify the viability and proliferative potential of cultures (positive control), with the addition of R9 medium in isolation providing an indication of basal proliferative levels. Drug-treated cultures were incubated for 48 h (37 °C, 5% CO_2) and subjected to [^{3}H]-thymidine incorporation (0.5 µCi/well) as previously described.

2.13 Fixation of antigen presenting cells.

To investigate the processing dependency of drug-specific TCCs, mechanisms of antigen presentation by APCs were assessed by fixation assays incorporating the use of glutaraldehyde. Glutaraldehyde is a powerful fixative agent and forms a protective covalent ring around the surface of APCs (Schnyder et al., 1997). This effectively inhibits the kinetic actions of the cell by preventing drug uptake and processing capabilities, whilst maintaining cellular structural integrity. First, autologous EBV-transformed B-cells ($2x10^6$/mL) were washed with HBSS to remove FBS from the cells and subsequently resuspended in 1 mL HBSS.

APCs were then pulsed with 25% glutaraldehyde for 30 seconds and thoroughly mixed to ensure sufficient distribution and cell fixation. After 30 seconds, the cell suspension containing glutaraldehyde was then quenched by the incorporation of 1 M glycine for a further 45 seconds, facilitating glutaraldehyde reduction and elimination from the suspension after binding has taken place. Quenched EBV-transformed B-cells were then thoroughly washed in R9 medium (3x) to exclude any residual glutaraldehyde and resuspended in R9 medium at $2x10^5$ cells/mL before being irradiated for 20 mins, alongside non-fixed autologous EBV-transformed B-cells. The assay was the performed with TCCs ($5x10^4$ cells) exposed to either fixed or non-fixed APCs ($1x10^4$ cells) in both the presence and absence of drug within 96-well U-bottomed plates. Testing of proliferative responses was then conducted by [3H]-thymidine incorporation as previously described.

2.14 APC pulsation assays.

Pulsation assays are typically performed in parallel with glutaraldehyde fixation to elucidate processing mechanisms associated with APCs after drug exposure. EBV-transformed B-cells ($1x10^6$ cells) were cultured in 24-well flat-bottomed plates and pulsed with optimal concentrations of drug antigen for multiple time-points, typically ranging between 10 mins and 24 h, although the exact number of conditions was often dependent upon the number of drug-specific TCCs available. After pulsing, APCs were thoroughly washed (5x) with HBSS to remove unbound soluble drug before resuspension in R9 medium and subsequent 20 min irradiation. Drug-specific TCCs ($5x10^5$ cells) were then co-cultured with irradiated pulsed autologous EBV-transformed B-cells ($1x10^4$ cells) in a 96-well U-bottomed plate, with non-pulsed EBV-transformed B-cells also included for consistency and treated with soluble drug to provide a positive control. Drug pulsed APC/TCC co-cultures were incubated for 48 h (37 °C, 5% CO_2) and subjected to [^{3}H]-thymidine incorporation (0.5 µCi/well) as previously described.

2.15 MHC blocking assays

Major histocompatibility complex (MHC) blocking antibodies were used to assess the dependence of drug-specific TCCs for specific human leukocyte antigen (HLA) complexes and can help elucidate HLA restricted T-cell activation by proliferative blockade. Drug-specific TCCs ($5x10^4$ cells) were co-cultured in a 96-well U-bottomed plate with irradiated (20 min) autologous EBV-transformed B-cells ($1x10^4$ cells) to achieve a total well volume of 100 µL. Cultures were then incubated with 50 µL HLA blocking antibodies (HLA-ABC, HLA-DR, DP, DQ, HLA-DR; 10 µg/mL) prior to drug exposure for 1 h to pre-emptively block T-cell self-presentation. After 1 h, R9 medium (negative control) or 50 µL soluble drug was added to the co-culture at 4x the final concentration and cells were incubated for 48 h (37 °C, 5% CO_2). Cultures were then pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h and proliferation after HLA blockade was determined by scintillation counting as previously described. Replicates without exposure to HLA blocking antibodies were also included to provide reference for antigen-specific proliferative responses and both IgG1 and IgG2 isotypes were also incorporated.

2.16 Enzyme-linked immunospot assay.

Antigen-specific cytokine release from drug-specific TCCs was assessed using an enzyme-linked immunospot (ELISpot) assay. This enabled the detection of T-cells secreting specific cytokines of interest, using a pre-determined panel of cytokines and cytolytic molecules. T-cells were washed in R9 medium to exclude any cytokine milieu and diluted to $5x10^5$ cells/mL after resuspension. Drug-specific TCCs ($5x10^4$ cells) were co-cultured in a 96-well U-bottomed plate, either in singlet or duplicate wells, with irradiated (20 min) autologous EBV-transformed B-cells ($1x10^4$ cells) to achieve a total well volume of 150 µL. The relevant compound was then added at 4x the desired concentration in 50 µL aliquots and drug exposed cultures were incubated for 24 h (37 °C, 5% CO_2). On the same day, Protein Binding Immobilon-P Membrane

96-well Multiscreen® filter plates were activated by the addition of 35% ethanol solution (60 secs) before being thoroughly washed (5x) with 200 µL distilled water. Activated Multiscreen® filter plates were then coated with 100 µL of capture antibody (IFN-γ, IL-5, IL-10, IL-13, IL-17A, IL-22, perforin, granzyme B and FasL) diluted in sterile HBSS according to the manufactures instructions (Mabtech, Nacka Strand, Sweden). Coated plates were then incubated for 24 h at 4 °C.

On day 1, ELISpot plates pre-coated for the cytokines of interest were washed thoroughly (5x) with 200 µL HBSS to remove any remaining capture antibodies, before being blocked at RT with 200 µL R9 cell culture medium containing 10% human AB serum for 2 h. After blocking, co-cultures of T-cells, EBV-transformed B-cells and drug were transferred (200 µL volume) into corresponding wells of the pre-coated ELISpot plate and incubated for a further 24 h (37 °C, 5% CO_2). On day 2, cells were discarded and ELISpot plates were washed (5x) with 200 µL HBSS. Detection antibodies, conjugated with biotin, corresponding to the cytokines of interest were diluted in HBSS supplemented with 0.5% FBS according to the manufactures instructions, which may vary between cytokines (e.g. 1/1000 dilution or 1/500 dilution). 100 µL of biotinylated antibody solution was added to each well of the assay plate and incubated for 2 h at RT. Following incubation, plates were washed (5x) with 200 µL HBSS and 100 µL of streptavidin conjugated alkaline phosphatase (strep-ALP) was added to each well and plates were incubated for 1 hr at RT. Sterile filtered (0.45 µM) BCIP-NBT substrate solution was added to each assay well following the removal of strep-ALP and further washing of the plate (5x; HBSS). During the development process, plates were incubated at RT for 15-20 mins in the dark to allow colorimetric reaction to occur. Throughout development, wells were visually inspected for spot formation at regular intervals to prevent saturation and subsequent overdevelopment. Following assay completion, cessation of the reaction was achieved by washing plates with an excess of cold water. Developed ELISpot plates were dried overnight

and spots were imaged and quantified the following day using an AID ELISpot reader (Cadima Madical, Stourbridge, UK).

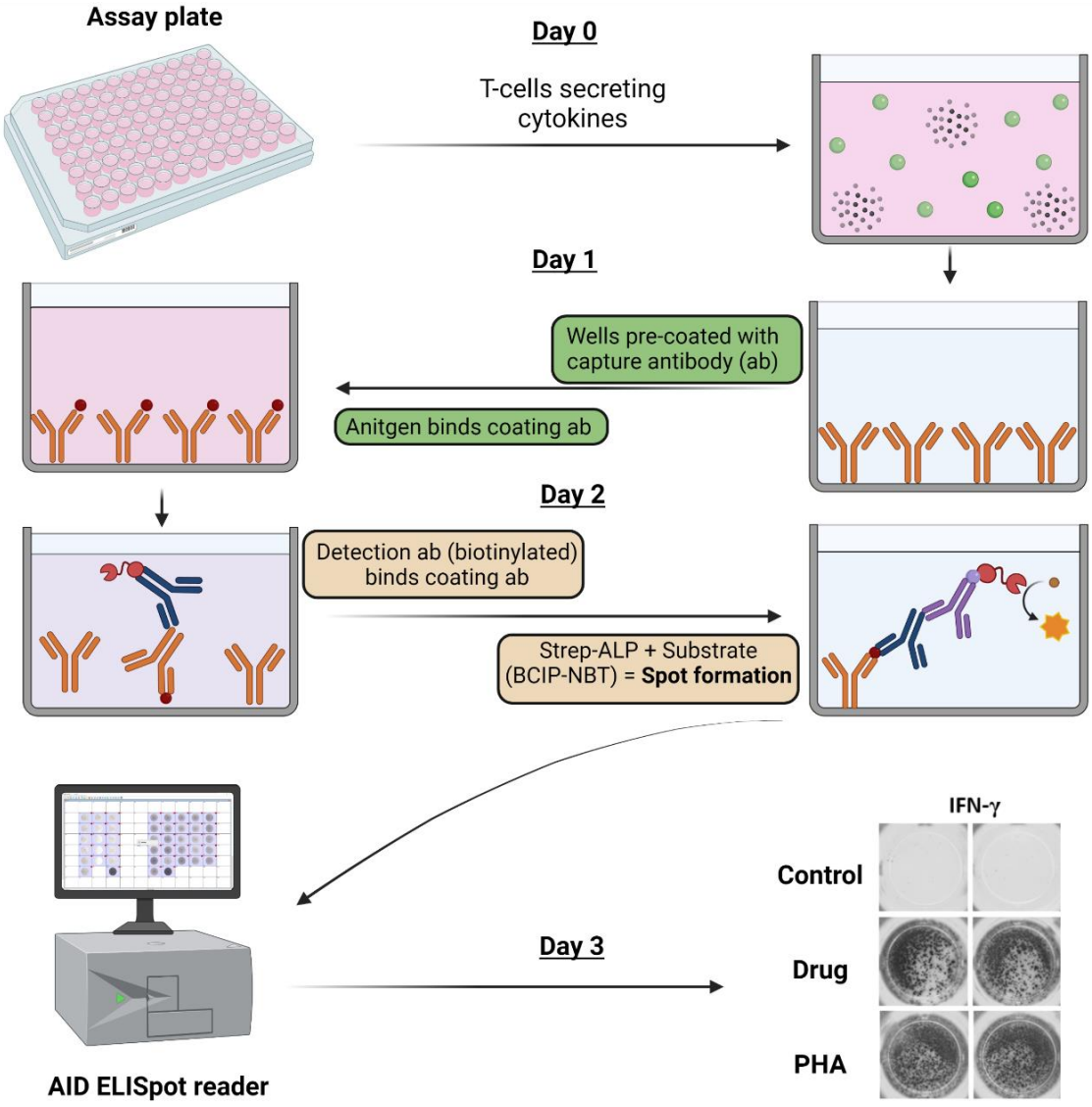


Figure 2.3. ELISpot assay for the detection and quantification of cytokine release. Cytokine release was determined after drug exposure for IFN-γ, IL-5, IL-10, IL-13, 1L-17A, 1L-22, granzyme B, perforin and FasL using activated Immobilon-P Membrane 96-well Multiscreen® filter plates pre-coated for cytokines of interest. Secretion profiles were studied after 48 h incubation (37 °C, 5% CO_2) with drug and ELISpot plates were developed according to the manufactures instructions. Quantitative analysis of cytokine release was determined by assessment of spot formation with the use of an AID ELISpot reader.

2.17 APC mismatch assays.

The expression of specific HLA alleles can be a critical determinant of susceptibility to hypersensitive T-cell mediated responses. To provide insight into the dependence of T-cell responses for HLA alleles of interest, APC mismatch assays were performed using panels of allogenic EBV-transformed B-cells previously genotyped for HLA expression. In this way, HLA alleles of interest could be incorporated into T-cell assays to determine the existence of HLA restricted drug-specific T-cell responses.

EBV-transformed B-cell lines were generated from genotyped PBMC cultures, as previously described in Section 2.10. Panels of allogenic EBV-transformed B-cells, typically from between 15-20 donors expressing a variety of HLA alleles, were centrifuged at 1500 rpm for 5 mins and resuspended in F1 medium to facilitate the removal of FBS. Cells were then counted, irradiated for 20 mins and diluted to achieve a concentration of $2x10^5$ cells/mL. Drug-responsive TCCs were removed from culture plates ($5x10^5$ cells) and co-cultured with a panel of allogenic EBV-transformed B-cells ($1x10^4$ cells) in a 96-well U-bottomed plate with optimal concentrations of the drug of interest for 48 h (37 °C, 5% CO_2). In cases where TCCs had been confirmed to have the capacity for self-presentation, sub optimal drug concentrations were applied. Cultures were then pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h and proliferation was determined by scintillation counting as previously described.

2.18 Flow cytometry.

Fluorescence-activated cell sorting (FACS) analysis was used to determine cellular phenotypes of drug-specific TCCs via analysis of distinct physical traits. These characteristics are distinguished by surface marker expression, which in turn could be elucidated with the application of stains consisting of fluorophores conjugated to specific antibodies of interest. Typically, combinations of fluorophores, and by extensions multiple variations of surface marker expression, were analysed for each T-cell clonal population. Consequently,

compensation controls were a pre-requisite in order to avoid emission spectra overlap. All FACS analysis described in this section was performed using a FACS-Canto II instrument, integrated with FACS DIVA operating software (BD Biosciences), with phenotypic analysis carried out using Flowing 2 software.

2.18.1 CD4+/CD8+ T-cell phenotyping.

Phenotyping of T-cells to determine CD4+/CD8+ expression was performed using fluorophore conjugated CD4/CD8 antibodies (BD Biosciences), and experiments were conducted according to the manufacturer's instructions. Briefly, 50-100 µL of cell suspension, confluency depending, were stained with conjugated fluorophores (FITC, 3 µL; APC, 3 µL; PE, 0.5 µL) for 20 mins at 4 °C. Following incubation, stained T-cells were washed with 2 mL FACS buffer and centrifuged at 1500 rpm for 10 min at 4 °C. Supernatant was discarded and cells were then resuspended in 100-200 µL of either FACS buffer or 4% paraformaldehyde (PFA) solution if cell fixation was required for analysis at a later date.

2.18.2 CD3 downregulation assays.

To explore the time points at which markers of drug-specific T-cell activation are detectable, extracellular CD3 expression was studied as a biomarker for TCR activation. Co-cultures of TCCs ($5x10^4$ cells/well) and autologous EBV-transformed B-cells ($1x10^5$ cells/well) were pulsed with drug for multiple time points (48 h, 24 h, 4 h, 2 h, 1 h, 30 min, 10 min, 5 min) in 96-well U-bottomed plates. Following incubation of the final time point, the drug exposed cell suspensions were transferred to FACS tubes and stained with 5 µL anti-CD3 antibody for 15 mins 4 °C. Stained cells were washed in FACS buffer to remove unbound antibody and resuspended in 4% PFA and stored at 4 °C. Both unstained and untreated cultures were set up in parallel for comparison and samples were analysed using a FACS-Canto II instrument, as previously described. Reduced CD3 (APC) fluorescence intensity, was indicative of downregulation and drug-specific T-cell activation.

2.18.3 Chemokine receptor analysis.

Chemokine receptor analysis (CRR) analysis of TCCs was performed using a standard panel of antibody conjugated fluorophores (CXCR3, CCR5, CCR1, CCR4, CCR9, CCR8, CCR6, CLA, CXCR6, E-cad, CXCR1, CCR2, and CD69). 50-100 µL of cell suspension were stained in separate FACS tubes with 3 µL of each antibody, with care taken to avoid emission spectra overlap (APC, PE, FITC). Incubation, washing and resuspension was performed in line with the methodology previously described. The resulting emission spectra was analysed using Flowing 2 software spectral shifts between stained and unstained controls were converted to produce an output of mean fluorescence index (MFI).

2.19 Mitochondrial Stress Test assay.

Mitochondrial stress-based assays were used determine specific parameters of oxidative phosphorylation (OXPHOS) and mitochondrial function of drug-specific TCCs after acute stimulation. These assays involve the sequential injection of mitochondrial toxins such as oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and combination of rotenone and antimycin-A. The aforementioned compounds act to target and inhibit complexes involved within OXPHOS and therefore isolate fuel pathway dependency for specific bioenergetic parameters, such as basal respiration, ATP-linked respiration, maximal and reserve capacities in addition to any respiration not facilitated by mitochondrial sources. All assays described were performed using the Seahorse XFe96 Analyzer, with analysis and visualization conducted using Wave® XF software.

One day prior to assay commencement, the Seahorse XFe96 cell culture microplate was coated with 25 µL Corning® Cell-Tak™ cell and tissue adhesive solution as a concentration of 22.4 µg/mL, diluted in sterile filtered $NaHCO_3$ (pH 8.0). Coated microplates were incubated at RT for 1 h before being washed with (x2) with sterile water and stored at 4 °C overnight. On the same day, the Seahorse xFe96 sensor cartridge was hydrated with 200 µL Seahorse XF

calibrant solution and incubated overnight at 37 °C. Autologous EBV-transformed B-cells for acute injection within the assay were irradiated for 20 min, resuspended in F1 cell culture medium and incubated in flasks overnight (37 °C, 5% CO_2).

On the day of the assay, the pH of Seahorse XF base medium was adjusted to 7.4 and the coated cell culture microplate was brought up to RT, prior to cellular addition. Seahorse XF base medium (pH 7.4) was supplemented with glucose (25 mM), L-glutamine (2 mM) and sodium pyruvate (1 mM). Mitochondrial inhibitors were then diluted to the desired concentrations (oligomycin, 2 µM; FCCP, 0.75 µM; antimycin A, 1 µM; rotenone, 1 µM) in supplemented Seahorse XF base medium, with serial dilution dependent upon relevant injection ports (Port A, x8, Port B, x9; Port C, x10; Port D, x11). Drug-specific TCCs were harvested from culture plates, counted and resuspended in Seahorse XF base medium. Cells were then transferred into coated Seahorse XFe96 cell culture microplates in 50 µL aliquots ($3x10^5$/cells/well) and centrifuged at 1500 rpm for 2 mins to facilitate adherence. Cell culture microplates containing adhered T-cell cultures were then incubated in the absence of CO_2 for 1 h at 37 °C. Irradiated EBV-transformed B-cells were counted, centrifuged and diluted in Seahorse XF base medium to $2x10^6$/mL. Diluted mitochondrial inhibitors were then added to the injection cartridge (25 µL per injection port) along with irradiated EBV-transformed B-cells ($5x10^4$ cells) and unadulterated Seahorse XF base medium for control purposes. Whilst T-cells were incubating, the injection cartridge was calibrated and well volumes of the assay plate were then increased to 175 µL. Following successful calibration, the assay plate was inserted into the Seahorse XFe96 Analyzer and energetic readouts, specifically the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), were taken from T-cell populations after exposure via acute injection to mitochondrial inhibitors. As T-cells were counted and plated on the same day of the assay, normalisation to protein was deemed unnecessary due to the inability of T-cells to proliferate within these time points. Energetic parameters were then analysed using the Seahorse XF Cell Mito Stress Test Report Generator.

2.20 Glycolysis Stress Test assay.

The Glycolysis Stress Test was deployed to determine the parameters of glycolytic function in monoclonal drug-specific T-cell populations after activation with cognate antigens. This assay represents the gold standard for measuring cellular glycolytic flux after acute stimulation and addresses key components of glycolysis, such as glycolytic capacity, glycolytic reserve, as well as acidification present form alternative non-glycolytic sources. The assay was performed using the Seahorse XFe96 instrument, via direct measurement of the acidification rate and subsequent interpretation to an 'extracellular acidification rate', referred to as the ECAR value. Analysis and visualization was undertaken using Wave® XF software and in depth study of the parameters of glycolytic flux was performed using the Seahorse XF Cell Glycolysis Stress Test Report Generator.

Seahorse XF base medium was supplemented with L-glutamine (2 mM), otherwise, assay preparation, EBV-transformed B-cell preparation and T-cell seeding within Seahorse 96-well cell culture microplates was performed according to the methodology previously described. Injection ports within the Seahorse XFe96 sensor cartridge were loaded with 25 mM D-glucose (Port A), $5x10^4$ EBV-transformed B-cells (Port B), the drug of interest (Port C) and 25 mM 2-DG (Port D) in 25 µL aliquots. Conventional Glycolysis Stress Test assays utilise the ATP synthase inhibitor, oligomycin, for which acute injection abrogates mitochondrial ATP production and shifts all energy production to glycolysis. The assay described in this section deviated from traditional methodology via the substitution oligomycin for soluble drug, thus allowing for the detection of any glycolytic flux directly imposed by relevant compounds. Additionally, a final injection of the glucose analogue, 2-deoxyglucose (2-DG), is a universal component of the assay and acts to competitively inhibit hexokinase II and induce glycolytic blockade, confirming ECAR shifts observed within the assay run are due to elevated levels of glycolysis.

2.21 HLA genotyping.

HLA genotyping of healthy donors or patients was performed using PBMC or EBV-transformed B-cell lines, if generated. DNA was purified from $5x10^6$ lymphocytes using a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufactures instructions and DNA quantification was performed using a NanoDrop. Full classical HLA typing (HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DRB345, HLA-DQA1, HLA-DQB1, HLA-DPA1, HLA-DPB1) at 3x resolution by the Histogenetics laboratory (Histogenetics, Ossining, NY) after shipment of purified DNA samples (200 µL) on dry ice (-109 °C).

2.22 Statistical analysis.

Statistical analysis was performed using GraphPad Prism 9.0 software. For the evaluation of T-cell responses for which data was normally distributed, such as dose response and toxicity assays, a student's *t*-test was performed. For data sets not normally distributed, comprising multiple donors and conditions, specifically T-cell priming assays (T-MWA) and functional T-cell assays, statistical analysis was performed using a Mann-Whitney U test. For both tests, *p< 0.05 was considered to be the universal threshold of significance, with **p< 0.01, ***p<0.001 and ****p<0.0001 also applied to determine greater degrees of significance.

2.23 Figure creation.

All chemical structures were produced using Chemdraw professional 16.0. Graph construction was performed exclusively using GraphPad Prism 9.0 software. Figures, diagrams and tables were generated using BioRender® software (Publication licence, FR23IE6C0F).

2.24 Funding sources.

This work was performed with funding from a BBSRC/GSK Industrial Partnership award (Grant Number BB/R008108/1). The project also received core funding from the MRC Centre for Drug Safety Science (Grant Number G0700654). The conduction of HLA genotyping on vancomycin

hypersensitive patients was funded by the National Institutes of Health (Grant Number P50GM115305).

3. *In vitro* priming and characterisation of vancomycin-specific T-cells from healthy donors expressing HLA-A*32:01.

Contents

3.1 Introduction.

Type IV hypersensitivity reactions can be mediated by the adaptive immune response, specifically influenced by the activation of T-cells (Naisbitt et al., 2003a). The T-cell response encompasses multiple cellular and molecular processes including priming, activation and proliferation that lead to aberrant immune function of a delayed nature (Zikherman and Au-Yeung, 2015). Accordingly, the clinical manifestations associated with such reactions typically present anywhere between 2-28 days following exposure to the culprit drug (Jacysyn et al., 2001, Justiz Vaillant et al., 2022). Drug reaction with eosinophilia and systemic symptoms (DRESS) is a classic example of delayed-type hypersensitivity. However, DRESS reactions are often associated with a longer period of onset (2-8 weeks) between drug administration and disease manifestation (Walsh and Creamer, 2011, Santiago et al., 2010). DRESS encompasses a wide range of features, with clinical signatures often surfacing within multiples organ systems such as the liver, in addition to more established cutaneous based reactions commonly associated with cell-mediated hypersensitivity (Sullivan and Shear, 2001). Although rare, DRESS reactions are linked with relatively high levels of mortality when compared to other drug-induced hypersensitive disease states, such as maculopapular exanthema and acute generalized exanthematous pustulosis, with a mortality rate of approximately 10% (Chen et al., 2010). Importantly, DRESS can be distinguished from other, more prevalent, cutaneous manifestations by the recruitment and involvement of eosinophils within reaction pathogenesis, with symptoms such as fever, pruritus and leukocytosis also reported (Cacoub et al., 2011). Unfortunately, it can be problematic to accurately determine exact incidence of DRESS arising from the broad range of clinical manifestations and the degree of convergence with near indistinguishable cutaneous based adverse events, further highlighting the importance of distinguishing haematological traits, such as eosinophilia, within diagnosis.

Disease pathogenesis in terms of immune modulation remains relatively poorly understood, with biomarkers of DRESS development limited to the emergence of IL-5 as a key phenotypic marker arising from its involvement within pro-inflammatory cascades and eosinophil recruitment (Choquet-Kastylevsky et al., 1998). Earlier studies have addressed the potential role of cytokine activity and immune involvement within DRESS, suggesting pathogenesis may be linked to activation of drug-specific CD4+ and CD8+ T-cells (Mauri-Hellweg et al., 1995). Viral reactivation has also been associated with DRESS pathogenesis. Indeed, the activation of CD8+ antiviral T-cells has been observed in DRESS patients following viral cascades involving human herpes viruses 6 and 7 (HHV-6 and HHV-7) and cytomegalovirus (Niu et al., 2015, Shiohara and Kano, 2007, Musette and Janela, 2017).

Vancomycin is a first generation glycopeptide antibiotic routinely used for the treatment of severe infection caused by Gram-positive bacteria. The drug is typically recommended as a first-line treatment agent and remains the gold standard for glycopeptide use, arising from its remarkably high efficacy for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* (Watanakunakorn, 1982). Whilst conforming to many chemical traits associated with other glycopeptide drug classes (described in Section 1.7.1), vancomycin possesses distinct characteristics, both within its chemical structure and mode of action. Functionally, the drug acts to block bacterial cell wall synthesis via the inhibition of phosphodisaccharide-pentapeptide lipid complex formation (Anderson et al., 1965). This is made possible by direct, high affinity binding to bacterial peptides containing D-alanyl-D-alanine at the carboxyl terminus (Perkins, 1969).

Extensive study into the link between vancomycin exposure and DRESS reactions has now shed light on a specific human leukocyte antigen (HLA) association, indicating involvement of the adaptive immune system and potential role for the activation of T-cells. DRESS reactions attributed to vancomycin exposure were initially postulated to result from variation within

HLA class I loci due to reaction severity, with cytotoxic CD8+ T-cell involvement likely implicated. Indeed, a genome wide associated study (GWAS) undertaken in 2019 revealed a genetic association between vancomycin-induced DRESS and carriage of HLA-A*32:01 (Konvinse et al., 2019). In this landmark study, 19/23 (82.6%) of individuals presenting with probable vancomycin-induced DRESS were positive for HLA-A*32:01 expression following genetic analysis in the form of HLA typing. Additionally, *in silico* molecular docking studies have suggested preferential binding in the absence of HLA peptides, indicating potential T-cell activation aligned with the pharmacological interaction (p-i) concept (Pichler, 2002). Although consisting of a relatively low sample size, these data suggest that in populations with European ancestry, for which carriage of HLA-A*32:01 equates to 6.8%, 1/5 individuals positive for risk allele expression will progress to DRESS onset following vancomycin exposure within a 4 week period. However, such studies fail to address the issue of low positive prediction (20%) thereby making genetic screening, such is the case with abacavir (55%), economically and clinically unviable (Mallal et al., 2008). Although the pharmacogenomic link seems promising, more extensive research is required to further define the pathophysiological mechanism of vancomycin-induced DRESS and role of T-cells in the context of a hypersensitive response.

In order to elucidate the role of T-cells within the pathogenesis of vancomycin induced-DRESS and investigate the nature of interactions between vancomycin and HLA-A*32:01, our study will utilize mechanistic and functional T-cell assays. Specifically, immunogenicity was determined via the study of *de novo* primed T-cell responses using the recently developed T-cell multi-well assay in conjugation with a peripheral blood mononuclear cell (PBMC) biobank of HLA-typed healthy donors for the delineation of preferential T-cell interaction with HLA-A*32:01 (Ogese et al., 2020, Alfirevic et al., 2012). Such assays provide insight regarding a compounds immunogenic potential and propensity for a *de novo* response under optimal culture conditions, replicating specific cellular compositions necessary for successful T-cell priming *in vivo* (Faulkner et al., 2012). Furthermore, T-cell cloning assays were conducted using

PBMC isolated from drug naïve healthy donors expressing HLA-A*32:01 to study vancomycin-specific T-cell responses at the unicellular level in order for functional, phenotypic and mechanistic assessment with an emphasis placed on the study of cross-reactivity with structurally-related glycopeptide antibiotics.

3.2 Aims.

1) To investigate the intrinsic immunogenicity of vancomycin in healthy donors positive and negative for HLA-A*32:01 expression via the assessment of *de novo* primed T-cell responses.

2) To generate vancomycin-specific T-cell clones (TCCs) from healthy donors with HLA-A*32:01 carriage, by serial dilution.

3) To elucidate and characterise phenotype and function of vancomycin-specific TCCs within the context of cytokine profiles, pathways of activation and cross-reactive potential.

3.3 Results.

3.3.1 PBMC proliferation assays reveal direct toxicity of vancomycin.

To determine the concentrations at which immune cell cultures display toxicity to vancomycin, toxicity assays were performed on PBMC isolated from 6 healthy donors assessing proliferative inhibition after [^{3}H]-thymidine incorporation and mitogen driven stimulation at graded concentrations of vancomycin (Figure 3.1). The toxicity profiles observed after exposure to vancomycin in PBMC cultures varied between donors with drug toleration observed up to and including 2 mM concentrations in 3/6 donors (Figure 3.1). PBMC cultures isolated from 2 PBMC donors (Donors 2 and 5) exhibited toxicity at 2 mM, with vancomycin tolerated at a 1 mM dosage after proliferative assessment following mitogen driven stimulation and drug exposure. Dose-dependent PBMC toxicity was observed in donor 6 at concentrations exceeding 100 µM.

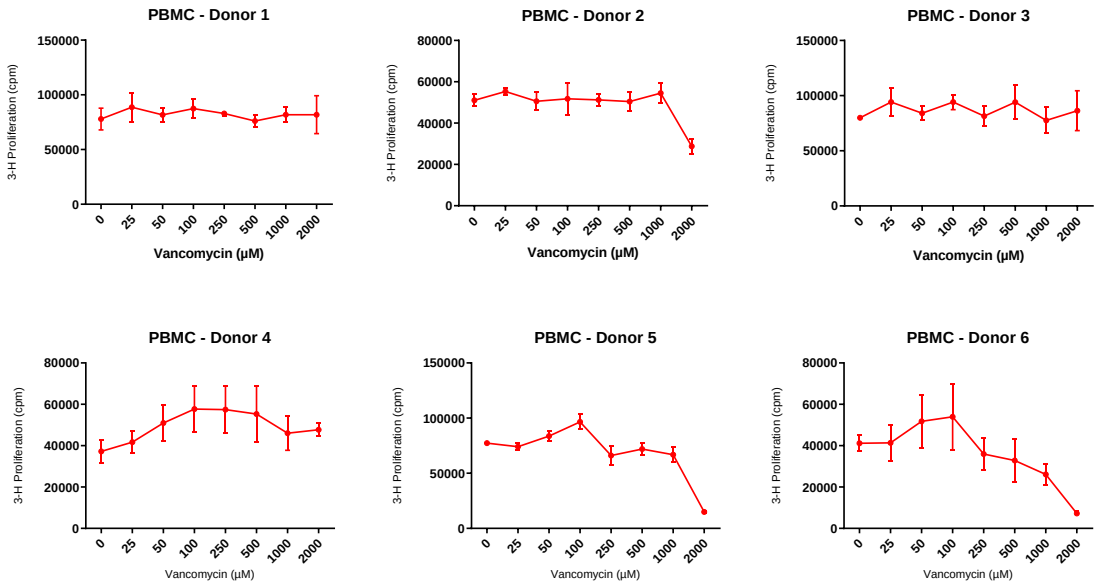


Figure 3.1. Thymidine incorporation assay to measure proliferative inhibition induced by vancomycin in mitogen stimulated PBMC cultures (n=6). Separate PBMC cultures ($1.5x10^5$ cells/well) were exposed, in triplicate, to graded concentrations of vancomycin (0-2000 µM) for 24 h (37 °C, 5% CO_2). Proliferation was then induced by mitogen driven stimulation following the addition of PHA (10 µg/mL) to every well of the assay plate. Cells were incubated for a further 48 h in the presence of PHA before being pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Proliferative responses were detected by way of radioactivity incorporation within cellular DNA and interpreted as counts per minute (cpm).

3.3.2 HLA haplotypes of individuals utilised within healthy donor studies for the assessment of glycopeptide antibiotic immunogenicity.

To assess the intrinsic immunogenicity of glycopeptide antibiotics, such as vancomycin, teicoplanin and daptomycin, *in vitro* T-cell assays were deployed using healthy donor models to study memory, *de novo* and drug-specific T-cell responses. For the conduction of healthy donor studies, a HLA-typed PBMC biobank was utilised to identify healthy individuals positive and negative for HLA*32:01 expression. In total, 33 healthy donors were studied (15 HLA*32:01 negative vs 18 HLA*32:01 positive) within the present experimental chapter and Chapter 4. Classical HLA genotypes, performed at 3x resolution (HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1) and the relevant *in vitro* T-cell assays for which each healthy donor used can be viewed in Tables 3.1 and 3.2, below.

Table 3.1. Classical HLA genotype of healthy donor PBMC used for immunogenicity studies of glycopeptide antibiotics (HLA-A*32:01 negative healthy donors). Classical HLA typing (HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1) is displayed at 3x resolution for HD-1 - HD-15 and was attained from a HLA-typed biobank of healthy volunteers. *In vitro* T-cell assays used for immunogenicity assessment are listed for each healthy donor studied.

Donor ID	*HLA class I*						*HLA class II*					
	HLA-A		*HLA-B*		*HLA-C*		*HLA-DRB1*		*HLA-DQB1*		*Assays*	
HD-1	*A*01:01*	*A*01:01*	*B*07:02*	*B*44:02*	*C*05:01*	*C*07:01*	*DRB1*13:01*	*DRB1*15:01*	*DQB1*06:02*	*DQB1*06:03*	LTT	-
HD-2	*A*02:01*	*A*66:01*	*B*41:02*	*B*44:02*	*C*05:01*	*C*17:01*	*DRB1*11:01*	*DRB1*13:01*	*DQB1*03:01*	*DQB1*06:03*	LTT	PBMC priming
HD-3	*A*02:01*	*A*24:02*	*B*44:02*	*B*57:01*	*C*05:01*	*C*06:02*	*DRB1*04:01*	*DRB1*07:01*	*DQB1*03:01*	*DQB1*03:03*	LTT	-
HD-4	*A*26:01*	*A*31:01*	*B*15:01*	*B*40:01*	*C*03:03*	*C*03:04*	*DRB1*01:01*	*DRB1*04:01*	*DQB1*03:02*	*DQB1*05:01*	LTT	PBMC priming
HD-5	*A*03:01*	*A*03:01*	*B*07:02*	*B*57:02*	*C*04:01*	*C*07:02*	*DRB1*07:01*	*DRB1*15:01*	*DQB1*02:01*	*DQB1*06:02*	LTT	PBMC priming
HD-6	*A*02:03*	*A*31:01*	*B*38:01*	*B*39:01*	*C*07:02*	*C*12:03*	*DRB1*13:01*	*DRB1*01:01*	*DQB1*05:01*	*DQB1*06:03*	Naïve T-cell priming	-
HD-7	-	-	-	-	-	-	-	-	-	-	Naïve T-cell priming	-
HD-8	*A*24:02*	*A*25:01*	*B*15:01*	*B*57:01*	*C*03:03*	*C*06:02*	*DRB1*04:04*	*DRB1*07:01*	*DQB1*03:02*	*DQB1*03:03*	Naïve T-cell priming	-
HD-9	*A*01:01*	*A*03:01*	*B*07:02*	*B*14:01*	*C*07:02*	*C*08:02*	*DRB1*07:01*	*DRB1*15:01*	*DQB1*02:01*	*DQB1*06:02*	PBMC priming	-
HD-10	*A*02:01*	*A*24:02*	*B*07:02*	*B*49:01*	*C*07:02*	*C*07:01*	*DRB1*01:01*	*DRB1*04:01*	*DQB1*03:01*	*DQB1*05:04*	PBMC priming	-
HD-11	*A*68:01*	*A*68:01*	*B*55:01*	*B*58:01*	*C*03:03*	*C*07:01*	*DRB1*14:01*	*DRB1*12:01*	*DQB1*03:01*	*DQB1*05:03*	PBMC priming	-
HD-12	*A*24:02*	*A*26:01*	*B*14:02*	*B*35:01*	*C*04:01*	*C*08:02*	*DRB1*01:03*	*DRB1*13:02*	*DQB1*05:01*	*DQB1*06:09*	PBMC priming	-
HD-13	*A*01:01*	*A*02:01*	*B*07:02*	*B*57:01*	*C*06:02*	*C*07:02*	*DRB1*07:01*	*DRB1*11:01*	*DQB1*03:01*	*DQB1*03:03*	PBMC priming	-
HD-14	*A*03:01*	*A*26:01*	*B*49:01*	*B*55:01*	*C*03:04*	*C*07:01*	*DRB1*13:02*	*DRB1*14:01*	*DQB1*05:03*	*DQB1*06:04*	PBMC priming	-
HD-15	*A*03:01*	*A*03:01*	*B*35:03*	*B*40:01*	*C*03:04*	*C*04:01*	*DRB1*04:03*	*DRB1*04:04*	*DQB1*03:02*	*DQB1*03:02*	PBMC priming	-

Table 3.2. Classical HLA genotype of healthy donor PBMC used for immunogenicity studies of glycopeptide antibiotics (HLA-A*32:01 positive healthy donors). Classical HLA typing (HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1) is displayed at 3x resolution for HD-16 – HD-33 and was attainted from a HLA-typed biobank of healthy volunteers. *In vitro* T-cell assays used for immunogenicity assessment are listed for each healthy donor studied.

Donor ID	*HLA class I*						*HLA class II*					
	HLA-A		*HLA-B*		*HLA-C*		*HLA-DRB1*		*HLA-DQB1*		*Assays*	
HD-16	*A*02:01*	***A*32:01***	*B*14:01*	*B*44:02*	*C*05:01*	*C*08:02*	*DRB1*13:01*	*DRB1*07:01*	*DQB1*02:01*	*DQB1*06:03*	LTT	Naïve T-cell priming
HD-17	*A*03:01*	***A*32:01***	*B*07:02*	*B*44:02*	*C*03:03*	*C*07:02*	*DRB1*13:01*	*DRB1*15:01*	*DQB1*06:02*	*DQB1*06:03*	LTT	-
HD-18	*A*03:01*	***A*32:01***	*B*07:02*	*B*51:01*	*C*07:02*	*C*15:02*	*DRB1*01:01*	*DRB1*11:04*	*DQB1*03:01*	*DQB1*05:01*	LTT	T-cell cloning
HD-19	*A*26:01*	***A*32:01***	*B*44:02*	*B*51:01*	*C*05:01*	*C*15:02*	*DRB1*11:01*	*DRB1*12:01*	*DQB1*03:01*	*DQB1*03:01*	LTT	PBMC priming
HD-20	*A*02:06*	***A*32:01***	*B*27:05*	*B*41:01*	*C*02:02*	*C*17:01*	*DRB1*04:03*	*DRB1*07:01*	*DQB1*02:01*	*DQB1*03:02*	LTT	-
HD-21	*A*01:01*	***A*32:01***	*B*44:02*	*B*57:01*	*C*05:01*	*C*06:02*	*DRB1*07:01*	*DRB1*12:01*	*DQB1*03:01*	*DQB1*03:03*	Naïve T-cell priming	-
HD-22	*A*01:01*	***A*32:01***	*B*08:01*	*B*14:01*	*C*07:01*	*C*08:02*	*DRB1*03:01*	*DRB1*11:01*	*DQB1*02:01*	*DQB1*03:01*	Naïve T-cell priming	-
HD-23	*A*03:01*	***A*32:01***	*B*07:02*	*B*35:01*	*C*04:01*	*C*07:02*	*DRB1*03:01*	*DRB1*14:01*	*DQB1*02:01*	*DQB1*05:03*	Naïve T-cell priming	-
HD-24	*A*26:01*	***A*32:01***	*B*13:02*	*B*44:02*	*C*05:01*	*C*07:01*	*DRB1*07:01*	*DRB1*12:01*	*DQB1*02:01*	*DQB1*03:01*	PBMC priming	-
HD-25	*A*31:01*	***A*32:01***	*B*27:05*	*B*44:02*	*C*03:04*	*C*05:01*	*DRB1*12:01*	*DRB1*13:01*	*DQB1*03:01*	*DQB1*06:03*	PBMC priming	-
HD-26	*A*24:02*	***A*32:01***	*B*15:01*	*B*40:01*	*C*03:03*	*C*03:04*	*DRB1*04:04*	*DRB1*13:01*	*DQB1*03:02*	*DQB1*06:03*	PBMC priming	-
HD-27	*A*11:01*	***A*32:01***	*B*35:01*	*B*44:02*	*C*02:02*	*C*04:01*	*DRB1*01:03*	*DRB1*13:01*	*DQB1*05:01*	*DQB1*06:03*	PBMC priming	-
HD-28	*A*01:01*	***A*32:01***	*B*08:01*	*B*27:05*	*C*01:02*	*C*07:01*	*DRB1*01:01*	*DRB1*03:01*	*DQB1*02:01*	*DQB1*05:01*	PBMC priming	-
HD-29	*A*03:01*	***A*32:01***	*B*35:01*	*B*44:03*	*C*04:01*	*C*16:01*	*DRB1*01:01*	*DRB1*07:01*	*DQB1*02:01*	*DQB1*05:01*	PBMC priming	-
HD-30	*A*03:02*	***A*32:01***	*B*35:01*	*B*44:02*	*C*04:01*	*C*05:01*	*DRB1*01:03*	*DRB1*15:01*	*DQB1*05:01*	*DQB1*06:02*	PBMC priming	-
HD-31	***A*32:01***	***A*32:01***	*B*40:02*	*B*44:02*	*C*02:02*	*C*05:01*	*DRB1*12:01*	*DRB1*13:01*	*DQB1*03:01*	*DQB1*06:03*	PBMC priming	-
HD-32	*A*31:01*	***A*32:01***	*B*40:02*	*B*44:02*	*C*02:02*	*C*07:04*	*DRB1*08:01*	*DRB1*13:01*	*DQB1*04:02*	*DQB1*06:03*	PBMC priming	Naïve T-cell priming
HD-33	*A*01:01*	***A*32:01***	*B*57:01*	*B*57:01*	*C*06:02*	*C*06:02*	*DRB1*07:01*	*DRB1*07:01*	*DQB1*03:03*	*DQB1*03:03*	T-cell cloning	Naïve T-cell priming

3.3.3 PBMC proliferation and cytokine-based assays reveal no immunogenic preference in favour of HLA-A*32:01 in healthy donors.

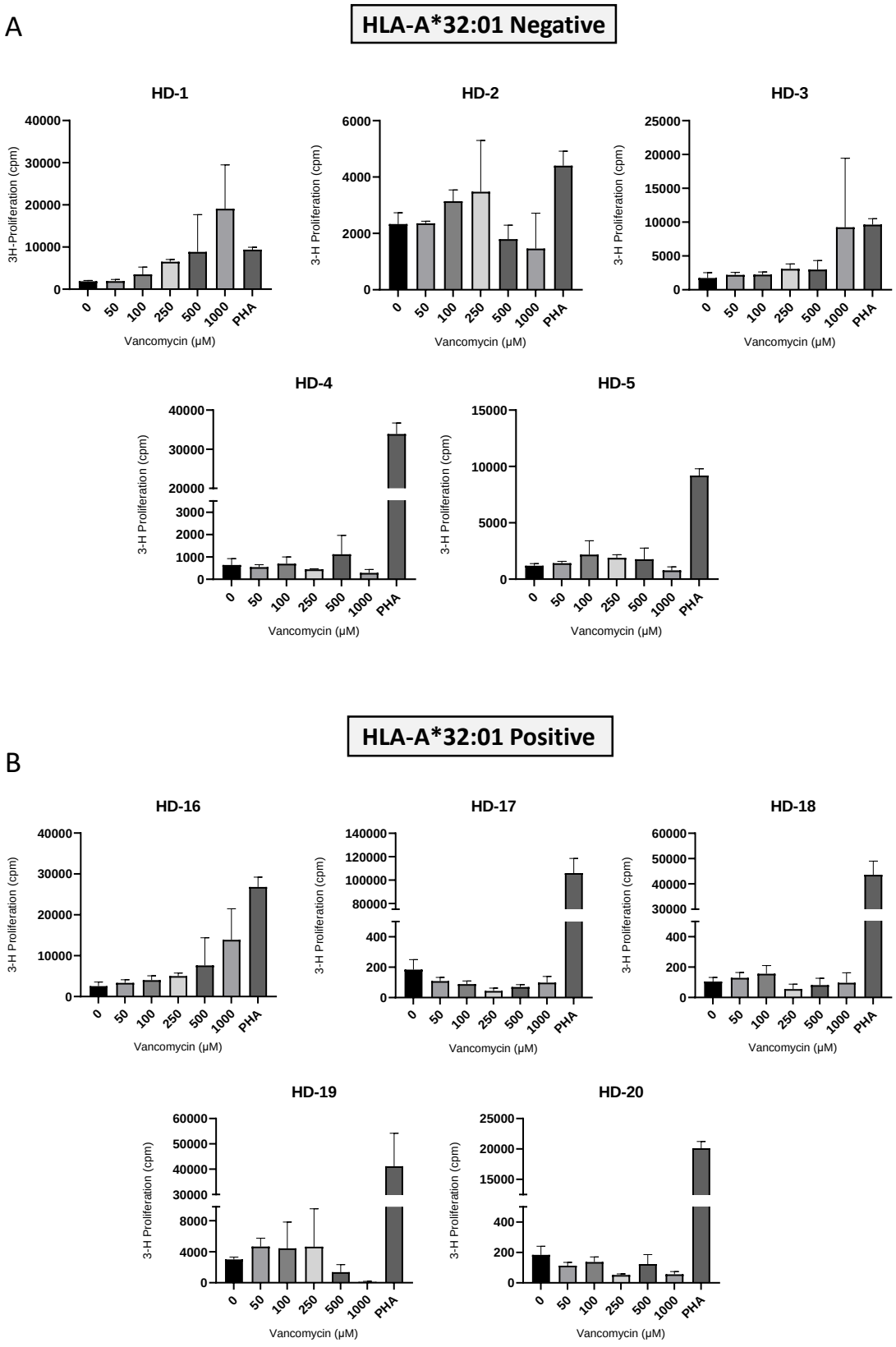


Figure 3.2. A) Lymphocyte transformation test (LTT) assessing proliferative responses to vancomycin in healthy donors negative for HLA-A*32:01 expression. PBMC from healthy volunteers negative for HLA-A*32:01 expression (n=5) were isolated by density gradient centrifugation and diluted in R9 cell culture medium to achieve a seeding density of $1.5x10^6$ cells/mL. PBMC were plated at $1.5x10^5$ cells/well in triplicate cultures and exposed to graded concentrations of vancomycin (0-1000 μM). The addition of PHA (10 μg/mL) represented a positive control for the assay for the detection of non-specific lymphocyte transformation. Cultures were incubated for 6 days (37 °C, 5% CO_2) before pulsation with tritiated [^{3}H]-thymidine (0.5 μCi/well) for an additional 16 h. Proliferative responses were detected by radioactivity incorporation and interpreted as cpm. **B) LTT assessing proliferative responses to vancomycin in healthy donors positive for HLA-A*32:01 expression.** Lymphocyte transformation was assessed via LTT assay and conduced on healthy donor PBMC expressing HLA-A*32:01. Proliferative responses to vancomycin were determined by [^{3}H]-thymidine incorporation as previously described.

It was important to study the specificity of vancomycin for the associated risk allele, achieved through the use of a HLA-typed PBMC biobank and assessment of PBMC proliferation and cytokine secretion following vancomycin exposure. Diagnostic LTT assays conducted on healthy donors with and without expression of HLA-A*32:01 revealed no discernible difference in lymphocyte proliferation after exposure to graded concentrations of vancomycin (Figure 3.2). 1/5 donors positive for HLA-A*32:01 expression exhibited a dose-dependent proliferative response to vancomycin (Figure 3.2B). However, identical results were observed within PBMC cultures negative for HLA-A*32:01, with 1/5 healthy donors displaying a dose-dependent proliferative response to vancomycin (Figure 3.2A). In all PBMC cultures (n=10) the addition of PHA induced non-specific lymphocyte proliferation.

Cytokine secretion was also studied in healthy donors both positive and negative for HLA-A*32:01 carriage. Analysis of IFN-γ release, detected with the use of Enzyme-linked immunospot (ELISpot) assays, revealed no preferential cytokine release within the PBMC cultures either with or without carriage of HLA-A*32:01 (Figure 3.3). Weakly positive IFN-γ release was detected within the PBMC of 2/5 healthy donors (HD-16, HD-19) known to express the HLA-A*32:01 risk allele. Similar results were obtained when studying IFN-γ secretion in PBMC cultures from donors negative for HLA-A*32:01, for which 2/5 healthy donors (HD-4, HD-5) were found to secrete IFN-γ after exposure to graded drug concentrations. All PBMC cultures (n=10) were positive for cytokine release after stimulation with PHA.

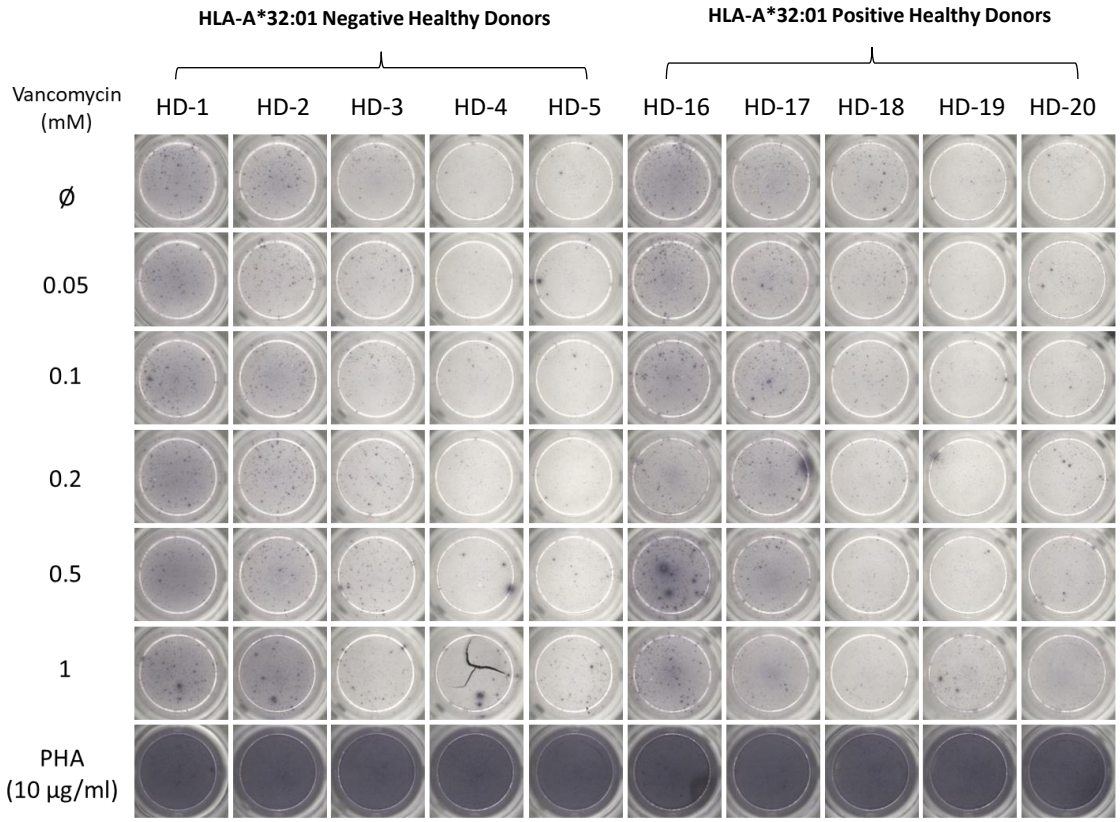


Figure 3.3. LTT assay assessing cytokine release after vancomycin exposure in healthy donor PBMC negative (n=5) and positive (n=5) for HLA-A*32:01 expression. HLA-typed PBMC from healthy volunteers positive and negative for HLA-A*32:01 expression (n=10) were isolated by density gradient centrifugation and diluted in R9 cell culture medium to achieve a seeding density of $5x10^6$ cells/mL. PBMCs were plated at $5x10^5$ cells/well in singlet cultures and exposed to graded concentrations of vancomycin (0-1000 µM) or PHA (10 µg/mL) for 48 h (37 °C, 5% CO_2). IFN-γ secretion was determined by ELISpot assay. Spot formation was indicative of cytokine release and developed ELISpot plates were imaged using an AID ELISpot reader.

3.3.4 *In vitro* priming assays suggest preferential interaction between vancomycin and HLA-A*32:01.

To investigate interactions between vancomycin and HLA-A*32:01, *in vitro* priming assays were deployed incorporating both naïve T-cells (Figure 3.4) and whole PBMC (Figure 3.5, 3.6). To investigate the intrinsic immunogenic potential of vancomycin in healthy donors positive and negative for HLA-A*32:01 expression, recently developed T-cell multi-well assays were performed. Additionally, the ability to prime HLA-typed PBMC cultures after repetitive drug simulation was assessed.

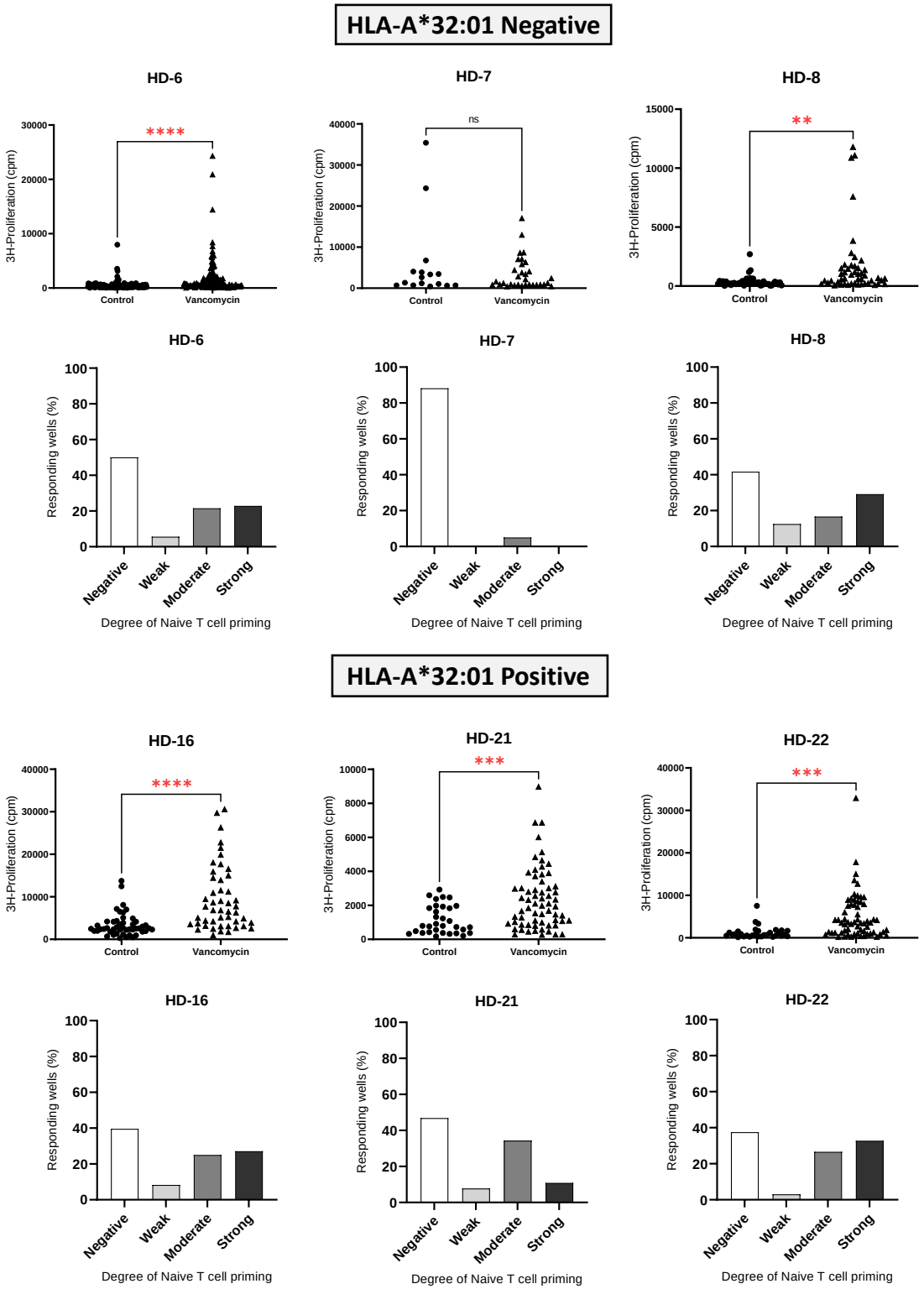


Figure 3.4. Assessment of naïve T-priming to vancomycin in HLA-A*32:01 negative and positive donors using the T-cell multiple well assay (T-MWA). Naïve T-cells (1x10^5) from 3 HLA-A*32:01 negative and 3 HLA-A*32:01 positive donors were incubated with 8x10^3 autologous dendritic cells and vancomycin (0.5 mM) for 14 days (37 °C, 5% CO_2). Free drug was removed by extensive washing with R9 medium and cells were rechallenged with either medium or vancomycin (0.5 mM) for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Proliferative responses were detected by radioactivity incorporation and interpreted as cpm values. Stimulatory index (SI) values were calculated as average cpm drug treated well / average cpm media treated wells. Degree of naïve T-cell priming to vancomycin was interpreted as; negative response (SI < 1.5), weak response (1.5 > SI < 2.5), moderate response (2.5 > SI < 4) or strong response (4 > SI < 10). Statistical significance was determined using a Mann-Whitney U test (**p< 0.01, ***p< 0.001, ****p<0.0001).

T-MWA analysis, incorporating drug naïve T-cells and autologous mature dendritic cells (DCs), was employed to study both the intrinsic immunogenic potential of vancomycin in addition to the extent of naïve T-cell priming in healthy donors positive and negative for HLA-A*32:01 expression (Figure 3.4). Vancomycin-specific T-cell activation was observed after proliferative study following drug rechallenge in 3/3 healthy volunteers positive for expression of the HLA-A*32:01 allele, when compared to rechallenge with R9 cell culture medium only (HD-16, HD-21 and HD-22). In these individuals, > 20% of responding wells, in which successful naïve T-cell priming was achieved, exhibited 'moderate' (SI = 2.5 - 4) proliferative responses after vancomycin rechallenge. Interestingly, in naïve T-cell cultures generated from healthy donor PBMC negative for HLA-A*32:01 expression, statistically significant naïve T-cell priming after drug rechallenge was observed in 2/3 healthy donors. When considering overall naïve T-cell priming in both groups, approximately 60% of naïve T-cells from donors positive for HLA-A*32:01 carriage were successfully primed to vancomycin compared with 34.6% of naïve T-cells in healthy donors without HLA-A*32:01 expression.

PBMC from HLA-A*32:01 positive (n=10) and negative (n=10) donors were assessed for *de novo* proliferative responses to vancomycin after long-term restimulation over a period of 4 week followed by drug rechallenge (Figure 3.5, 3.6). Vancomycin-responsive T-cells were detected within PBMC cultures in 7/10 donors positive for HLA-A*32:01 expression (HD-19, HD-25, HD-26, HD-27, HD-28, HD-29 and HD-31) and 1/10 healthy donors negative for HLA-A*32:01 (HD-2). From a statistical standpoint, 4/10 donors with risk allele carriage exhibited significant T-cell priming after repetitive stimulation, compared with 0/10 healthy donors negative for risk allele expression.

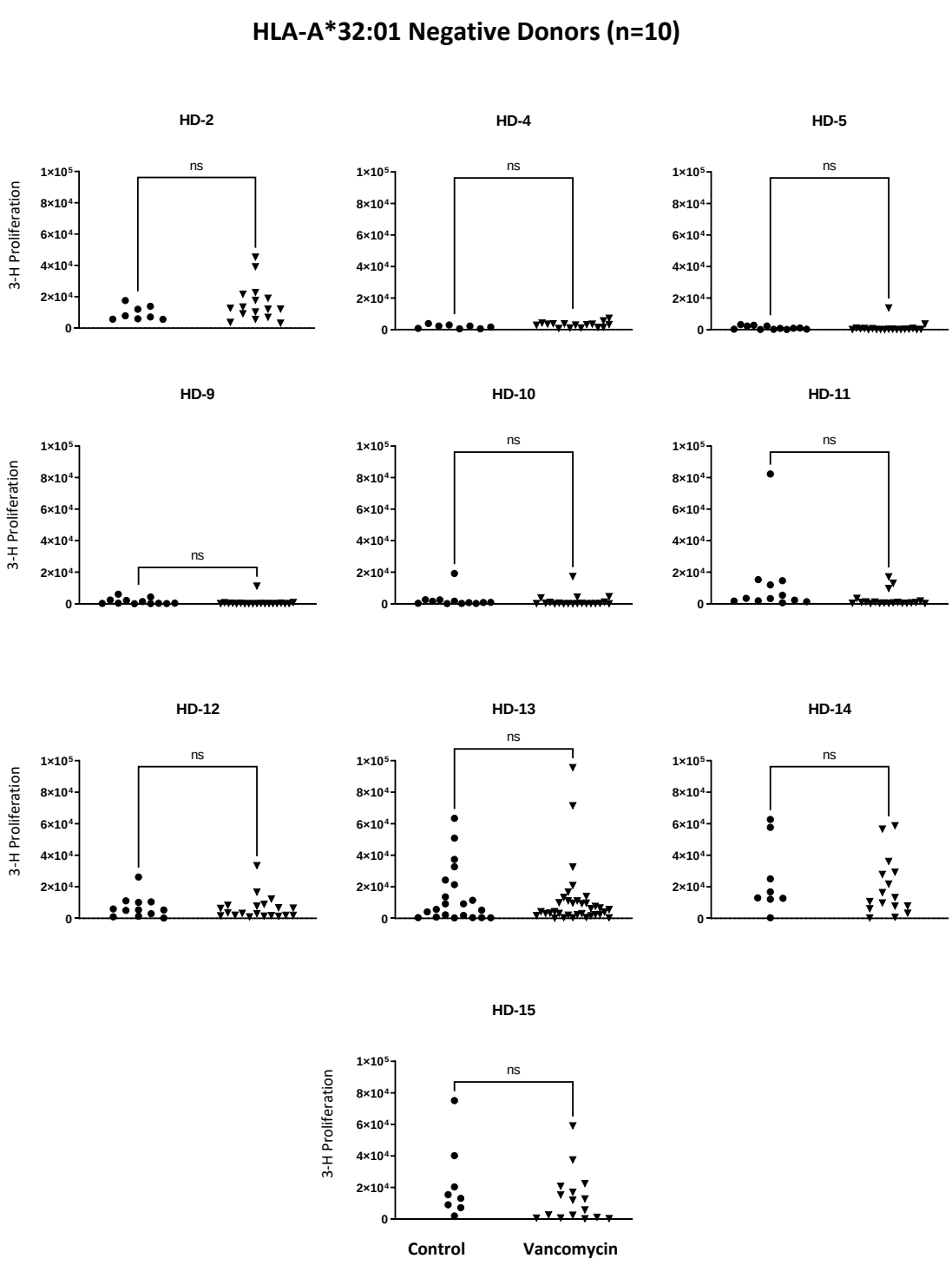


Figure 3.5. Long-term stimulation of PBMC cultures with vancomycin in healthy donors negative for HLA-A*32:01 expression (n=10). $2x10^5$ cryopreserved PBMC (HLA-typed) isolated from HLA-A*32:01 negative healthy donors were cultured in a 96-well plate with R9 cell culture medium containing vancomycin (0.5 mM) and IL-2 (50 U/mL). Following supplementation of cultures on day 3 with fresh medium containing IL-2, PBMC cultures were restimulated on day 7 and weekly thereafter during which each well of the assay plate was exposed to a cocktail containing irradiated autologous PBMC ($5x10^3$/well), vancomycin (0.5 mM) and fresh IL-2. After 4 weeks, restimulated PBMC cultures were washed 5 times in cell culture medium to remove free soluble drug and then rechallenged with irradiated autologous PBMC ($5x10^3$/well) in addition to either vancomycin (0.5 mM) or medium for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Proliferative responses were are expressed as cpm values in individual culture wells and statistical significance was determined using a Mann-Whitney U test.

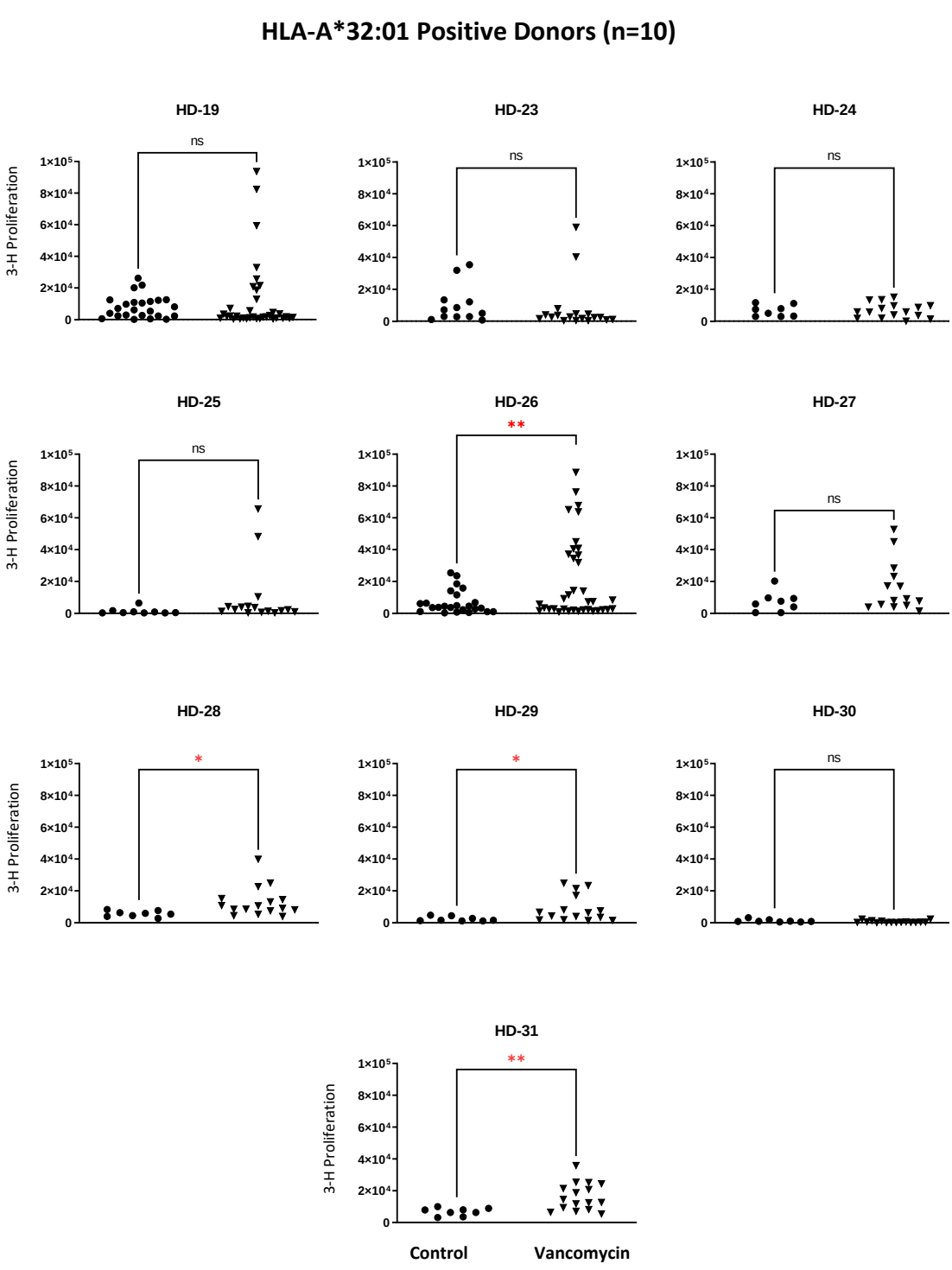


Figure 3.6. Long-term stimulation of PBMC cultures with vancomycin in healthy donors positive for HLA-A*32:01 expression (n=10). $2x10^5$ cryopreserved PBMC (HLA-typed) isolated from HLA-A*32:01 positive healthy donors were cultured in a 96-well plate with R9 cell culture medium containing vancomycin (0.5 mM) and IL-2 (50 U/mL). Following supplementation of cultures on day 3 with fresh medium containing IL-2, PBMC cultures were restimulated on day 7 and weekly thereafter during which each well of the assay plate was exposed to a cocktail containing irradiated autologous PBMC ($5x10^3$/well), vancomycin (0.5 mM) and fresh IL-2. After 4 weeks, restimulated PBMC cultures were washed 5 times in cell culture medium to remove free soluble drug and rechallenged with irradiated autologous PBMC ($5x10^3$/well) in addition to either vancomycin (0.5 mM) or medium for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Proliferative responses interpreted as cpm values for individual culture wells and expressed as cpm drug-treated and medium-treated wells. Statistical significance was determined using a Mann-Whitney U test (*p< 0.05, **p< 0.01).

3.3.5 Generation of vancomycin-specific TCCs from healthy donors expressing HLA-A*32:01.

For the generation of vancomycin-specific TCCs from healthy donor PBMC, 3 healthy donors positive for HLA-A*32:01 expression were selected for T-cell cloning studies and subsequent functional analysis. Classical HLA-typing, encompassing HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 was performed at 3x resolution and is displayed for each healthy donor utilised within this section (HD-18, HD-32 and HD-33). PBMC from HD-18, HD-32, and HD-33 were observed to be heterozygous for HLA-A*32:01 expression (Table 3.3). Carriage of other HLA class I alleles, including HLA-A*01:01, HLA-A*31:01 and HLA-A*03:01, respectively, were detected within the HLA-A region. Expression of additional HLA types of interest included homozygous expression of HLA-B*57:01, present within the PBMC of HD-33, and heterozygous expression of HLA-DRB*01:01 for HD-18.

Table 3.3. HLA expression from quantified DNA isolated from PBMC in 3 healthy donors. Classical HLA typing (HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1) was performed at 3x resolution by the Histogenetics laboratory after initial DNA isolation via QIAamp® DNA Mini Kit and quantification by NanoDrop.

Donor ID	*HLA class I*						*HLA class II*			
	HLA-A		*HLA-B*		*HLA-C*		*HLA-DRB1*		*HLA-DQB1*	
HD-18	*A*03:01*	***A*32:01***	*B*07:02*	*B*51:01*	*C*07:02*	*C*15:02*	*DRB1*01:01*	*DRB1*11:04*	*DQB1*03:01*	*DQB1*05:01*
HD-32	*A*31:01*	***A*32:01***	*B*40:02*	*B*44:02*	*C*02:02*	*C*07:04*	*DRB1*08:01*	*DRB1*13:01*	*DQB1*04:02*	*DQB1*06:03*
HD-33	*A*01:01*	***A*32:01***	*B*57:01*	*B*57:01*	*C*06:02*	*C*06:02*	*DRB1*07:01*	*DRB1*07:01*	*DQB1*03:03*	*DQB1*03:03*

Vancomycin-responsive TCCs were successfully generated in 3/3 healthy donors expressing HLA-A*32:01 following the analysis of initial monoclonal test cultures (Figure 3.7). Following CD8+ T-cell enrichment, large numbers of drug-specific TCCs were generated in 2 healthy donors (HD-32 and HD-18). Remarkably, a total of 103 vancomycin-specific TCCs were initially generated from a total of 216 test cultures within CD8+ enriched bulk populations generated from the PBMC of HD-18.

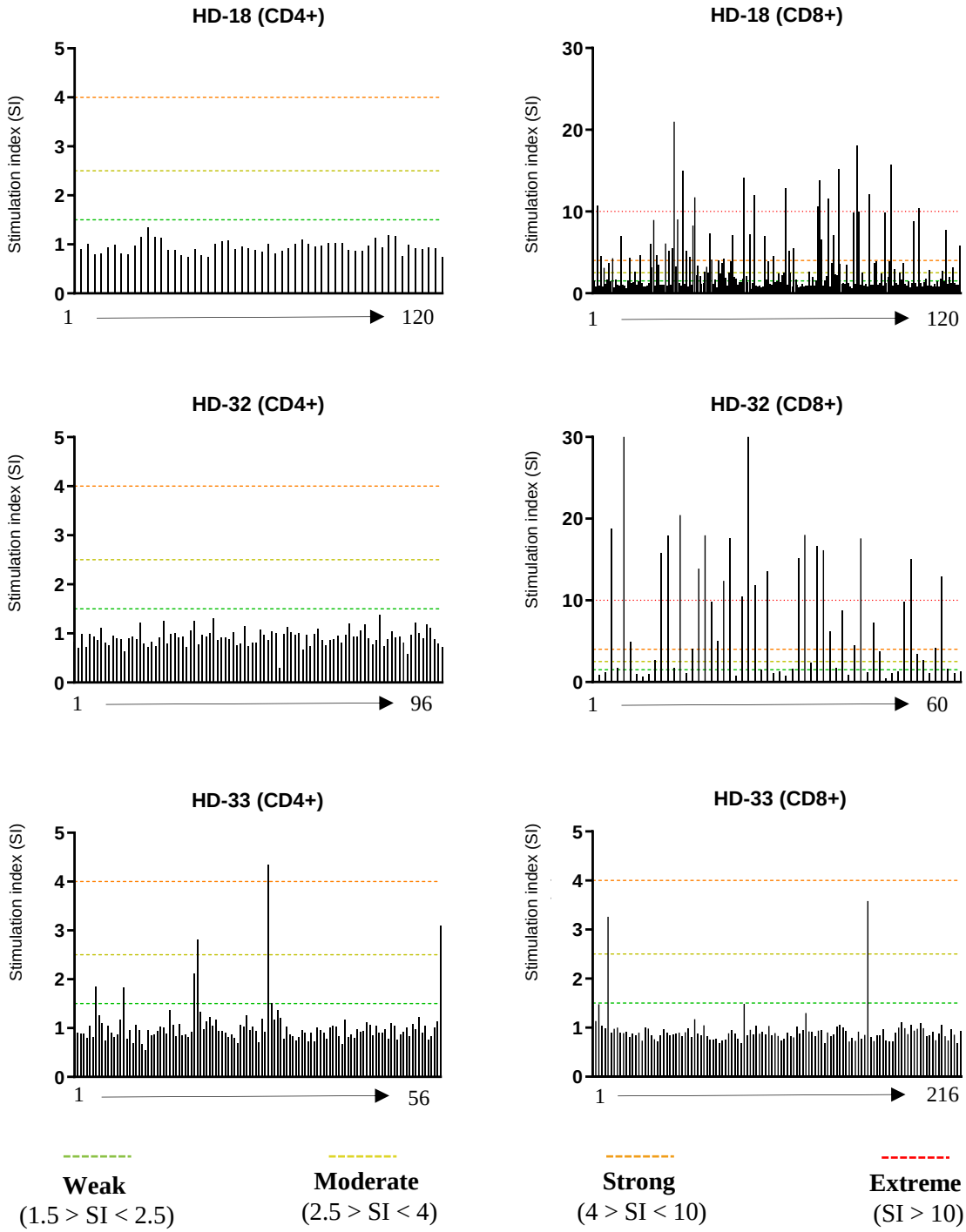


Figure 3.7. Generation of vancomycin-specific TCCs from PBMC enriched for CD4+ and CD8+ T-cells in healthy donors expressing HLA-A*32:01 by serial dilution. Bulk PBMC cultures were seeded at 1x10^6 PBMC/well with graded concentrations of vancomycin (0.1-0.5 mM) and incubated (37 °C, 5% CO_2) for 14 days. T-cells were enriched for CD4+ or CD8+ expressing populations by MACS separation and transferred to 96-well U-bottomed plates (1 T-cell/well average) with irradiated allogenic PBMC (5x10^4 cells/well) and R9 medium supplemented with IL-2 (200 U/mL) and PHA (5 µg/mL). Serial dilution protocol was performed as previously described, with test cultures rechallenged with vancomycin (0.5 mM) or R9 medium only, in the presence of irradiated autologous EBV-transformed B-cells (1x10^4 cells/well). Testing was performed over 4 wells in duplicate conditions and cultures were incubated for 48 h (37 °C, 5% CO_2) and pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for the final 16 h of incubation. Readouts were initially interpreted as cpm and converted SI values given by; [average drug treated wells / average control wells]. SI values for each test culture were interpreted as either weak (1.5 > SI < 2.5), moderate (2.5 > SI < 4), strong (4 > SI < 10) or extreme (SI > 10).

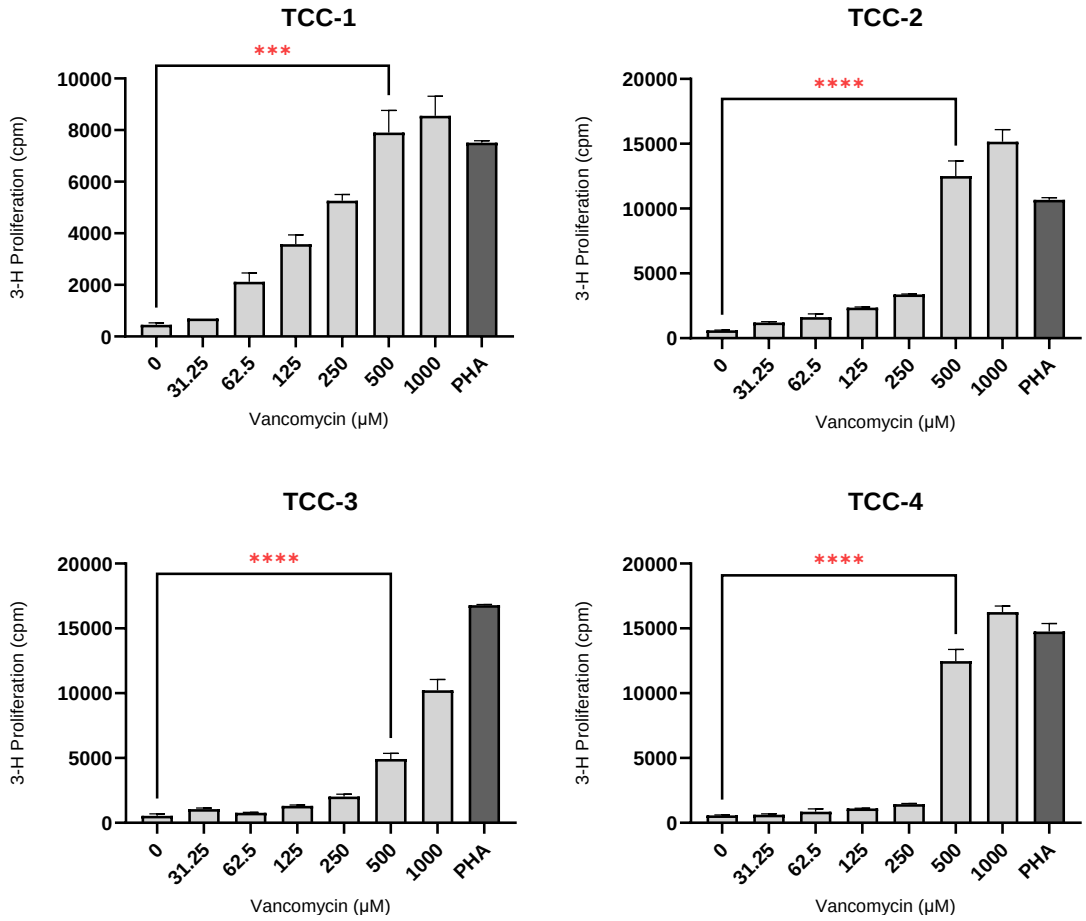


Figure 3.8. Proliferative response of vancomycin-specific TCCs at graded drug concentrations (n=4). TCCs ($5x10^4$ cells) were co-cultured with autologous EBV-transformed B-cells ($1x10^4$ cells) and either vancomycin (0-1 mM), R9 medium or PHA (10 µg/mL). Cells were incubated for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined using a student's *t*-test (***p<0.001, ****p<0.0001).

Initial test cultures, generated from the PBMC of HD-18 and displaying proliferative responses at singular vancomycin concentrations (0.5 mM) were expanded by mitogen driven stimulation for dose-response analysis. A panel of representative TCCs exhibited dose-dependent proliferative responses after exposure to graded drug concentrations (Figure 3.8). To further confirm drug specificity, proliferative responses were assessed following exposure to graded concentrations of carbamazepine (Figure 3.9). Treatment with the structurally unrelated compound produced no proliferative effect in vancomycin-specific TCCs. In both assays, the addition of PHA successfully induced non-specific proliferation of drug-specific TCCs.

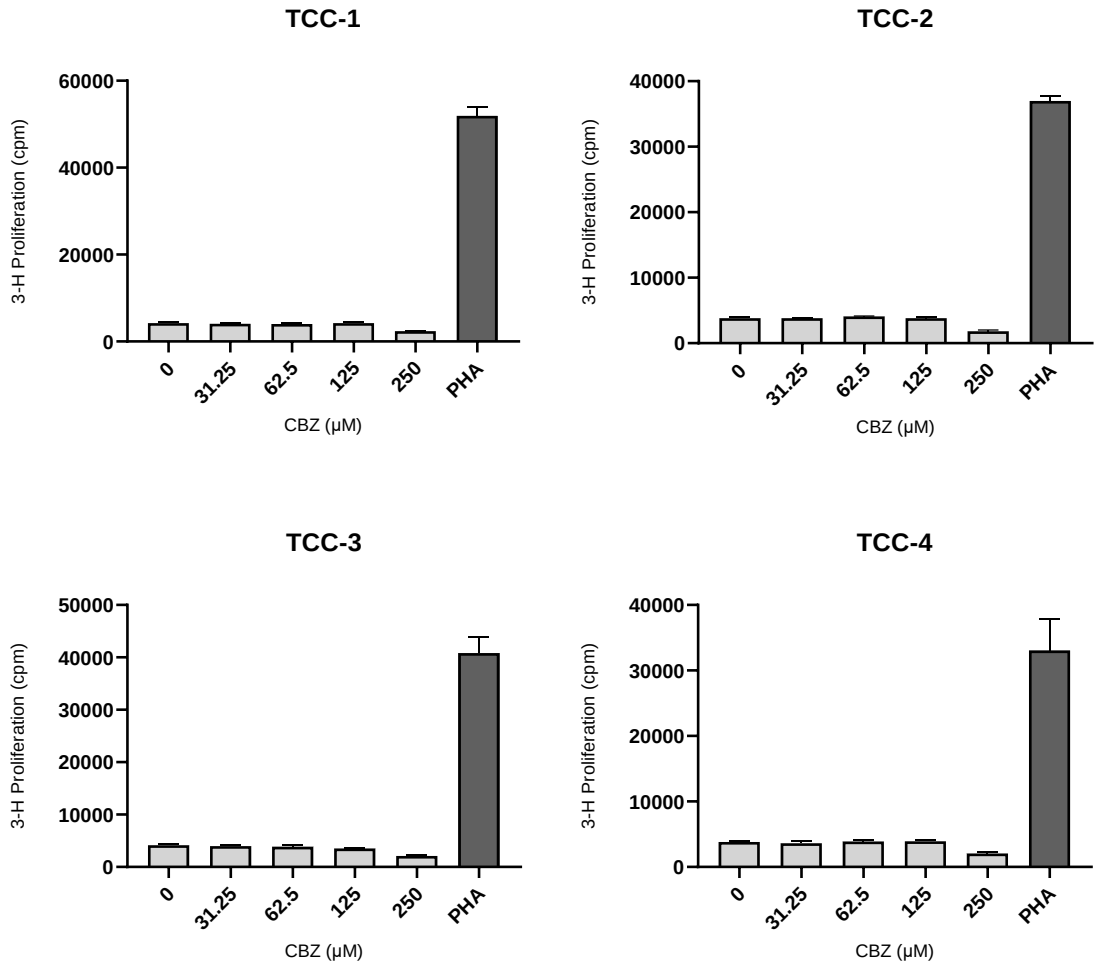
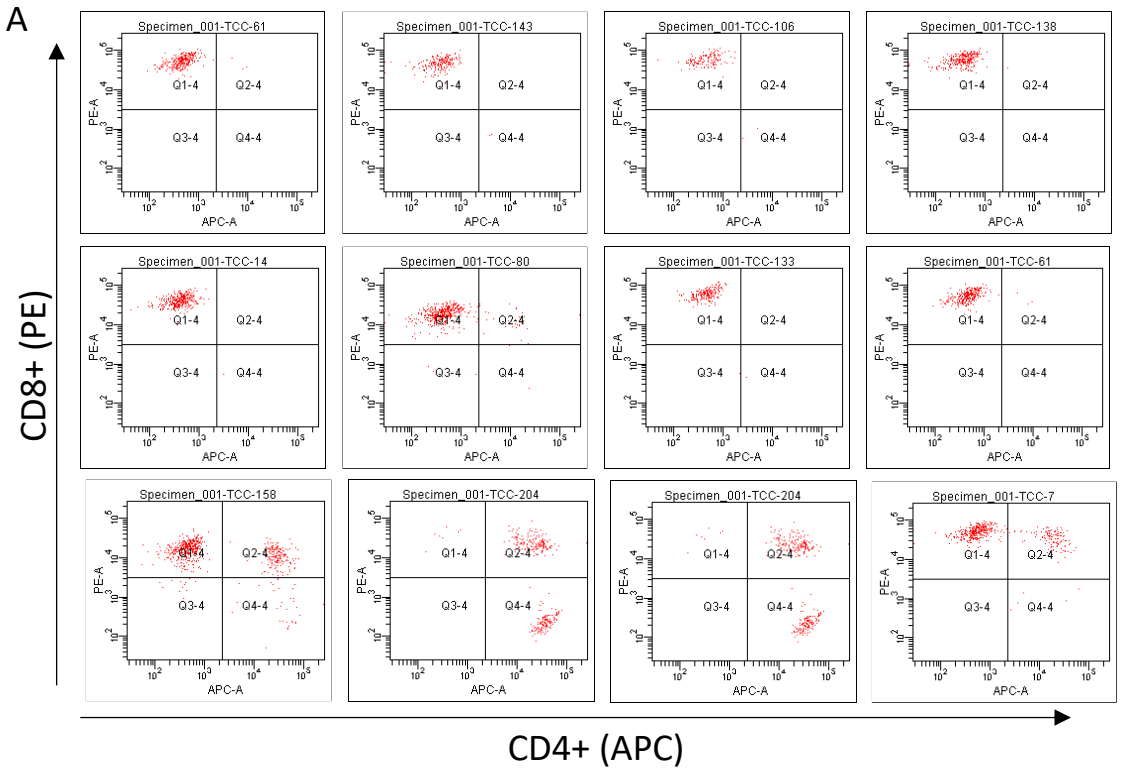


Figure 3.9. Proliferative response of vancomycin-specific TCCs at graded concentrations of carbamazepine (n=4). $5x10^4$ TCCs were co-cultured with autologous EBV-transformed B-cells ($1x10^4$ cells) and either carbamazepine (0-250 μM), R9 medium or PHA (10 μg/mL). Cells were incubated for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 μCi/well) for 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values.

3.3.6 Phenotypic, functional and cross-reactive characterisation of vancomycin-responsive TCCs generated from healthy donors expressing HLA-A*32:01.

Vancomycin-responsive TCCs generated from the PBMC of healthy donor HD-18 (HLA-A*32:01 positive) were assessed for phenotypic and functional characteristics. Clonal populations deemed positive (SI > 1.5) for drug-specificity following dose-response assays were further expanded by repetitive mitogen driven stimulation to produce confluent test cultures for viable functional study.



Total TCC Phenotyped	Pure CD8+ Population	Pure CD4+ Population	Mixed Population	N/A
94	78	0	24	2

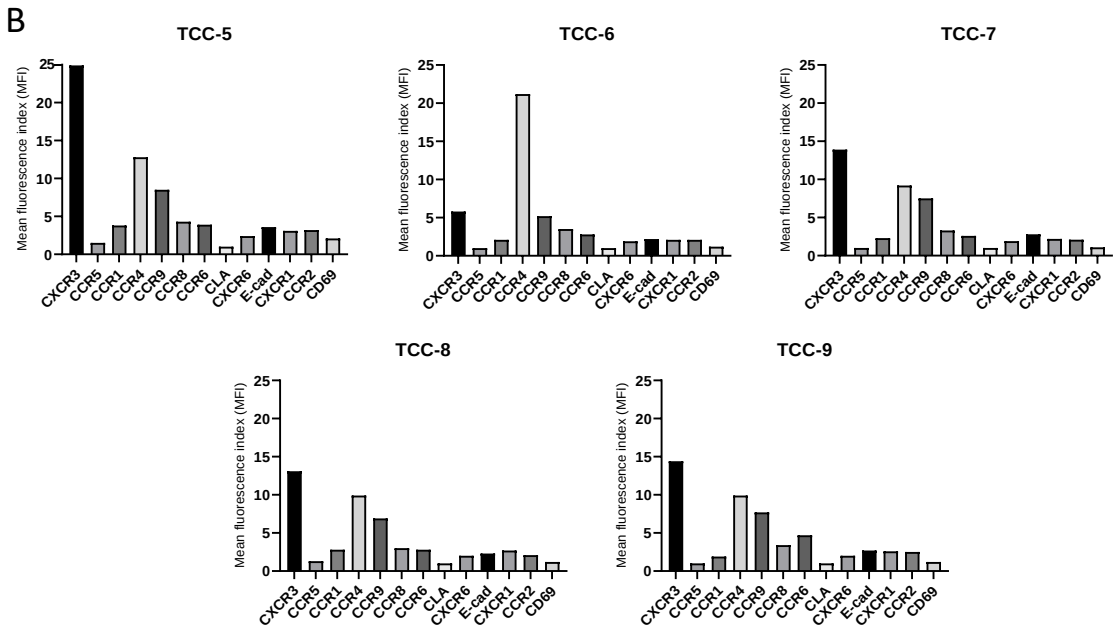


Figure 3.10. A) Phenotypic characterisation (CD4+/CD8+) using a panel of representative vancomycin-specific TCCs (n=12). T-cells with predetermined specificity for vancomycin were stained with 3 µL anti-CD4 (APC) and 0.5 µL of anti-CD8 (PE) for 15 mins at 4 °C. Stained cells were washed in FACS buffer to remove unbound antibody and resuspended in 4% PFA. Unstained cultures were set up in parallel for comparison and samples analysed using a FACS-Canto II instrument integrated with FACS DIVA operating software. Phenotypic analysis was carried out using Flowing 2 software. Gating of lymphocyte populations was performed on unstained cultures and 10^4 events were analysed. **B) Chemokine receptor expression analysis of CD8+ vancomycin-specific TCCs (n=5).** TCCs were stained with chemokine receptor conjugated antibodies for CXCR3-APC, CCR5-PE, CCR1-PE, CCR4-PE, CCR9-APC, CCR8-FITC, CCR6-APC, CLA-FITC, CXCR6-PE, E-Cad-PE, CXCR1, CCR2-APC and CD69-FITC. Surface marker expression was assessed as previously described and interpreted as mean fluorescence index (MFI).

Phenotypic analysis of vancomycin-responsive TCCs generated from HLA-A*32:01 positive healthy donor PBMC (HD-18) revealed an overwhelmingly dominant CD8+ expressing phenotype (Figure 3.10A). Of a total of 94 drug-specific TCCs studied for surface marker expression, 78 TCCs were pure clonal populations and positive for expression of the CD8 co-receptor. In total, 24 TCCs from CD8+ enriched T-cell cloning experiments displayed mixed populations, in reference to the presence of either dual CD8+ expressing populations or double positive CD4+ and CD8+ expressing populations. Pure CD8+ TCC populations were further utilised for functional T-cell assays described in greater detail below. No pure CD4+ populations were observed from the large panel of TCCs phenotyped. Chemokine receptor expression analysis of vancomycin-response TCCs was performed on 5 dose-responsive CD8+ expressing populations (Figure 3.10B). Universally, TCCs expressed high levels of CXCR3 and CCR4 following phenotypic study and MFI analysis. Furthermore, CCR1, CCR6, CCR8, CCR5, CCR2, CXCR1, CLA, E-Cad and CD69 were all expressed at reduced levels. Additionally, CCR9 was expressed at moderately high levels in 4/5 TCCs profiled for chemokine receptor expression analysis.

Representative vancomycin-specific TCCs (n=3) were profiled for secretion of both cytokines and cytolytic molecules by ELISpot, after exposure to graded drug concentrations (Figure 3.11A). Cytokine secretion profiles pertaining to TCC-1, TCC-2 and TCC-3 followed a similar, distinct pattern. Dose-dependent Th1 cytokine (IFN-γ) secretion was observed alongside the release of the Th2 cytokine (IL-13), with IL-5 production absent. Additionally, cytolytic molecules, such as granzyme B, perforin and FasL were also found to be released after vancomycin rechallenge. PHA successfully induced non-specific cytokine and cytolytic molecule secretion across the full panel of TCC assessed.

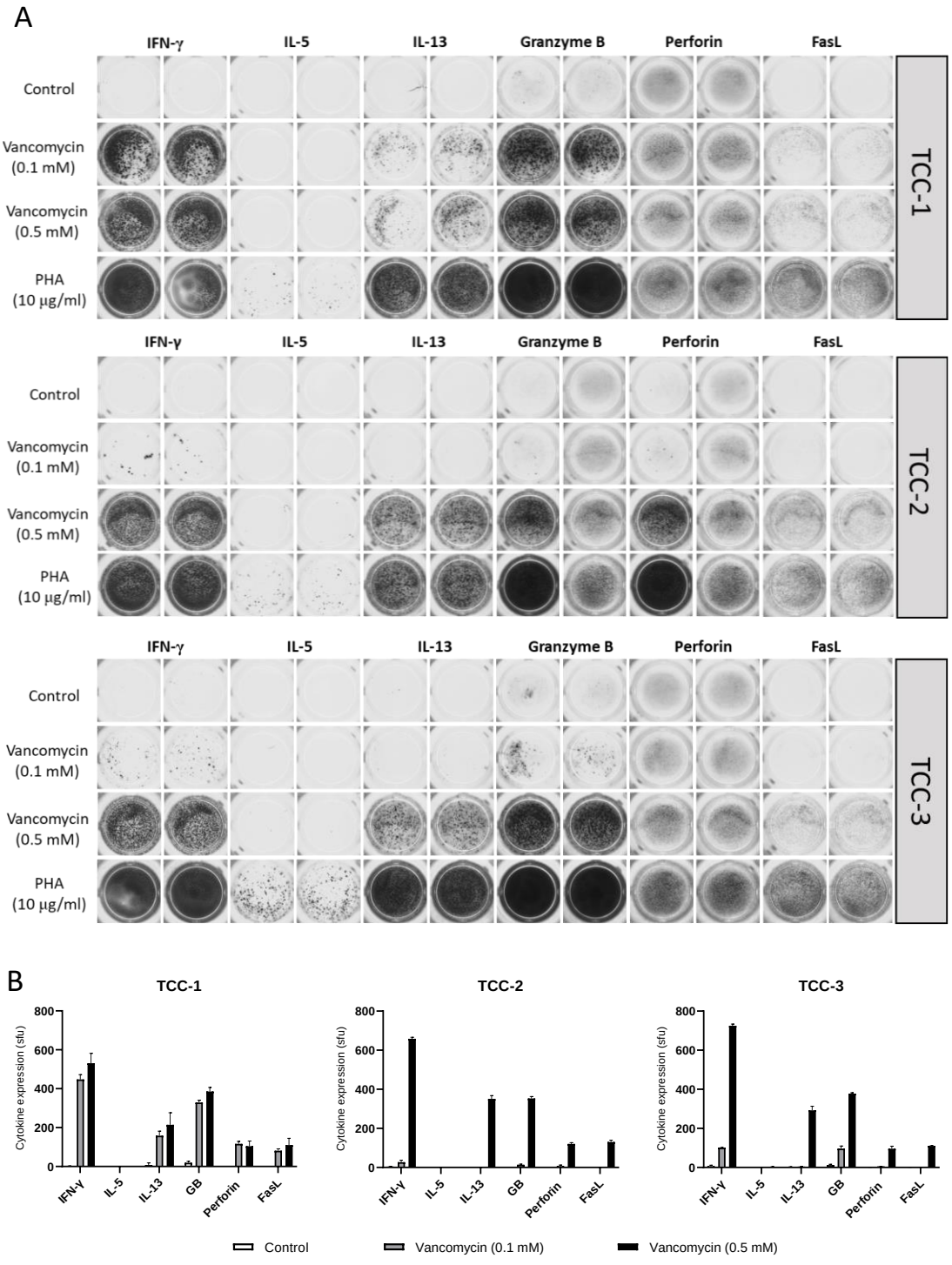


Figure 3.11. A) Cytokine secretion profile of vancomycin-specific TCCs (n=3) determined by ELISpot readout. TCCs ($5x10^4$ cells) were co-cultured with autologous EBV-transformed B-cells ($1x10^4$ cells) and either graded concentrations of vancomycin (0.1 and 0.5 mM), R9 medium or PHA (10 µg/mL). Cytokine release was determined for IFN-γ, IL-5, IL-13, granzyme B, perforin and FasL using activated Immobilon-P Membrane 96-well Multiscreen® filter plates pre-coated for cytokines of interest. Secretion profiles were studied after 48 h incubation (37 °C, 5% CO_2) with vancomycin and ELISpot plates were developed according to the manufactures instructions. **B) Quantitative analysis of cytokine release from vancomycin-responsive TCCs (n=3).** Spot formation was analysed using an AID ELISpot reader and cytokine secretion was interpreted as spot forming units (sfu).

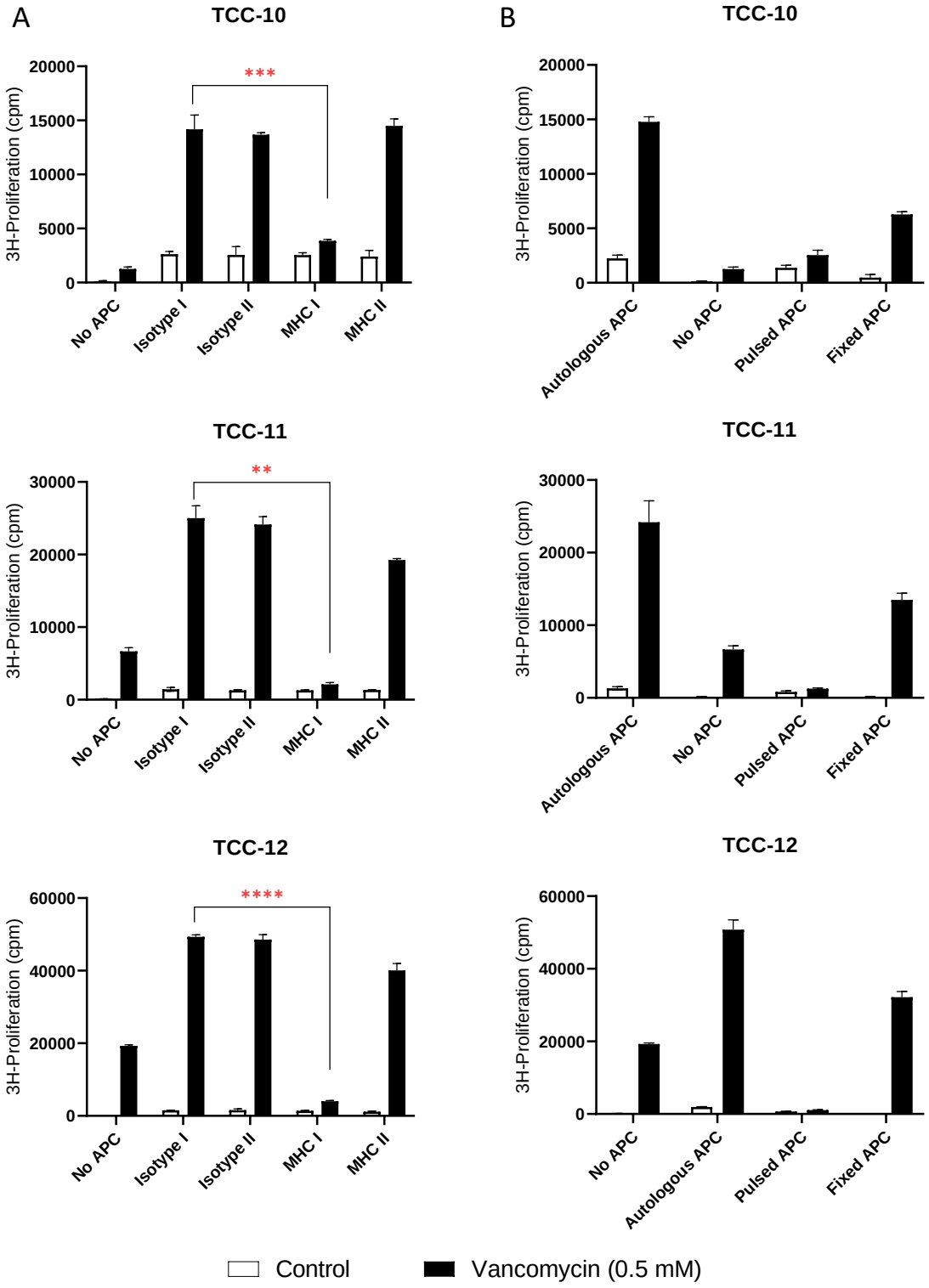


Figure 3.12. A) HLA restriction of vancomycin-specific TCCs (n=3). CD8+ expressing TCCs ($5x10^4$ cells/well) were co-cultured with autologous irradiated APCs in triplicate and either HLA Class I (10 µg/mL), HLA Class II (10 µg/mL) or corresponding IgG1 and IgG2 isotype controls (10 µg/mL) for 1 h (37 °C, 5% CO_2). Blocked cultures were treated with vancomycin (0.5 mM) or R9 cell culture medium for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined using a Mann-Whitney U test (*p< 0.05, **p< 0.01, ****p<0.0001). **B) Activation pathways of vancomycin-responsive CD8+ TCCs (n=3).** Autologous APCs ($2x10^6$) were pulsed with vancomycin (0.5 mM) for 16 h before washing to remove unbound drug or fixed with glutaraldehyde (25%, 1 µL) for 30 secs and quenching with glycine (1 M, 1 mL) for 45 secs. TCCs were incubated for 48 h (37 °C, 5% CO_2) with either fixed APCs and vancomycin (0.5 mM) or pulsed APCs ($1x10^4$). Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values after 16 h incubation (0.5 µCi/well).

The presence of HLA restricted T-cell activation was determined through HLA blockade (Figure 3.12A). Representative CD8+ TCCs generated from the PBMC of healthy donor HD-18 (TCC-10, TCC-11 and TCC-12) were treated with a panel of anti-HLA blocking antibodies. Proliferation of CD8+ expressing TCCs was uninfluenced following HLA class II blockade, when compared to basal proliferative responses to corresponding isotype controls after stimulation with optimal stimulatory concentrations of vancomycin. In each TCC assessed, proliferative responses to vancomycin were inhibited following MHC class I blockade, indicative of MHC class I restricted activation. Although to a reduced extent, drug-responsive TCCs were observed to still possess the capacity for proliferation in 2/3 TCCs after exposure to vancomycin in the absence of autologous antigen presenting cells (APCs). Following exposure of TCCs to pulsed APCs (16 h) no proliferative response was observed following co-culture with vancomycin-specific TCCs (Figure 3.12B). Conversely, after APC fixation and subsequent inhibition of peptide processing, vancomycin-responsive T-cells were capable of proliferation following treatment with a co-culture of glutaraldehyde-fixed APCs and vancomycin.

To assess the existence of preferential interactions between vancomycin-specific TCCs and HLA-A*32:01 within the context of a proliferative response following drug exposure, a panel of EBV-transformed B-cells (APCs) were utilised in T-cell assays (Figure 3.13). The APC panel deployed to study the importance of HLA-A*32:01 for vancomycin-specific T-cell proliferation consisted of 14 heterologous APCs expressing a range of HLA alleles (Table 3.4). Stimulation of TCCs was induced in the presence of heterologous APCs positive for heterozygous HLA-A*32:01 expression (Donors 1-9) and autologous APCs. Stimulation was also observed across multiple TCCs following exposure to vancomycin and heterologous APCs negative for HLA-A*32:01 expression (Donors 12, 13 and 14).

Table 3.4. Full classical HLA genotype of APCs used for HLA mismatch experiments in healthy donors positive and negative for HLA-A*32:01 expression. Classical HLA typing (HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1, and HLA-DQA1) was performed at 3x resolution by the Histogenetics laboratory after initial DNA isolation via QIAamp® DNA Mini Kit and quantification by NanoDrop. Autologous APCs were generated from the PBMC of HD-18.

Subject ID	***HLA class I***						***HLA class II***					
	HLA-A		***HLA-B***		***HLA-C***		***HLA-DRB1***		***HLA-DQB1***		***HLA-DQA1***	
Healthy donors												
Autologous ***(HD-18)***	*A*03:01*	*A*32:01*	*B*07:02*	*B*51:01*	*C*07:02*	*C*15:02*	*DRB1*01:01*	*DRB1*11:04*	*DQB1*03:01*	*DQB1*05:01*	*DQA1*01:01*	*DQA1*05:01*
Donor 1	*A*01:01*	*A*32:01*	*B*08:01*	*B*08:01*	*C*07:01*	*C*08:02*	*DRB1*03:01*	*DRB1*11:01*	*DQB1*02:01*	*DQB1*03:01*	*DQA1*05:01*	*DQA1*05:01*
Donor 2	*A*01:01*	*A*32:01*	*B*44:02*	*B*57:01*	*C*05:01*	*C*06:02*	*DRB1*07:01*	*DRB1*12:01*	*DQB1*03:01*	*DQB1*03:03*	*DQA1*02:01*	*DQA1*05:01*
Donor 3	*A*02:01*	*A*32:01*	*B*14:01*	*B*44:02*	*C*05:01*	*C*08:02*	*DRB1*07:01*	*DRB1*13:01*	*DQB1*02:01*	*DQB1*06:03*	*DQA1*01:03*	*DQA1*02:01*
Donor 4	*A*01:01*	*A*32:01*	*B*08:01*	*B*27:05*	*C*01:02*	*C*07:01*	*DRB1*01:01*	*DRB1*03:01*	*DQB1*02:01*	*DQB1*05:01*	*DQA1*01:01*	*DQA1*05:01*
Donor 5	*A*03:01*	*A*32:01*	*B*07:02*	*B*44:02*	*C*03:03*	*C*07:02*	*DRB1*13:01*	*DRB1*15:01*	*DQB1*06:02*	*DQB1*06:03*	*DQA1*01:03*	*DQA1*01:02*
Donor 6	*A*03:01*	*A*32:01*	*B*35:01*	*B*44:03*	*C*04:01*	*C*16:01*	*DRB1*01:01*	*DRB1*07:01*	*DQB1*02:01*	*DQB1*05:01*	*DQA1*01:01*	*DQA1*02:01*
Donor 7	*A*26:01*	*A*32:01*	*B*44:02*	*B*51:01*	*C*05:01*	*C*15:02*	*DRB1*11:01*	*DRB1*12:01*	*DQB1*03:01*	*DQB1*03:01*	*DQA1*05:01*	*DQA1*05:01*
Donor 8	*A*02:01*	*A*32:01*	*B*15:01*	*B*44:02*	*C*03:03*	*C*05:01*	*DRB1*04:01*	*DRB1*15:01*	*DQB1*03:02*	*DQB1*03:01*	*DQA1*03:01*	*DQA1*05:01*
Donor 9	*A*03:01*	*A*32:01*	*B*14:02*	*B*27:05*	*C*01:02*	*C*08:02*	*DRB1*01:01*	*DRB1*15:01*	*DQB1*05:01*	*DQB1*06:02*	*DQA1*01:02*	*DQA1*01:01*
Donor 10	*A*02:01*	*A*24:02*	*B*07:02*	*B*15:25*	*C*07:26*	*C*07:02*	*DRB1*15:01*	*DRB1*15:01*	*DQB1*06:01*	*DQB1*06:02*	-	-
Donor 11	*A*11:01*	*A*31:01*	*B*40:02*	*B*40:01*	*C*03:03*	*C*03:04*	*DRB1*13:01*	*DRB1*14:01*	*DQB1*05:03*	*DQB1*06:03*	*DQA1*01:03*	*DQA1*01:01*
Donor 12	*A*01:01*	*A*02:01*	*B*45:01*	*B*51:01*	*C*01:02*	*C*06:02*	*DRB1*01:01*	*DRB1*07:01*	*DQB1*02:01*	*DQB1*05:01*	*DQA1*01:01*	*DQA1*02:01*
Donor 13	*A*11:01*	*A*30:01*	*B*13:02*	*B*35:01*	*C*04:01*	*C*06:02*	*DRB1*15:01*	*DRB1*16:01*	*DQB1*05:02*	*DQB1*06:03*	*DQA1*01:02*	*DQA1*01:02*
Donor 14	*A*01:02*	*A*26:01*	*B*40:01*	*B*49:01*	*C*03:04*	*C*07:01*	*DRB1*04:04*	*DRB1*07:01*	*DQB1*02:01*	*DQB1*03:02*	*DQA1*02:01*	*DQA1*03:01*

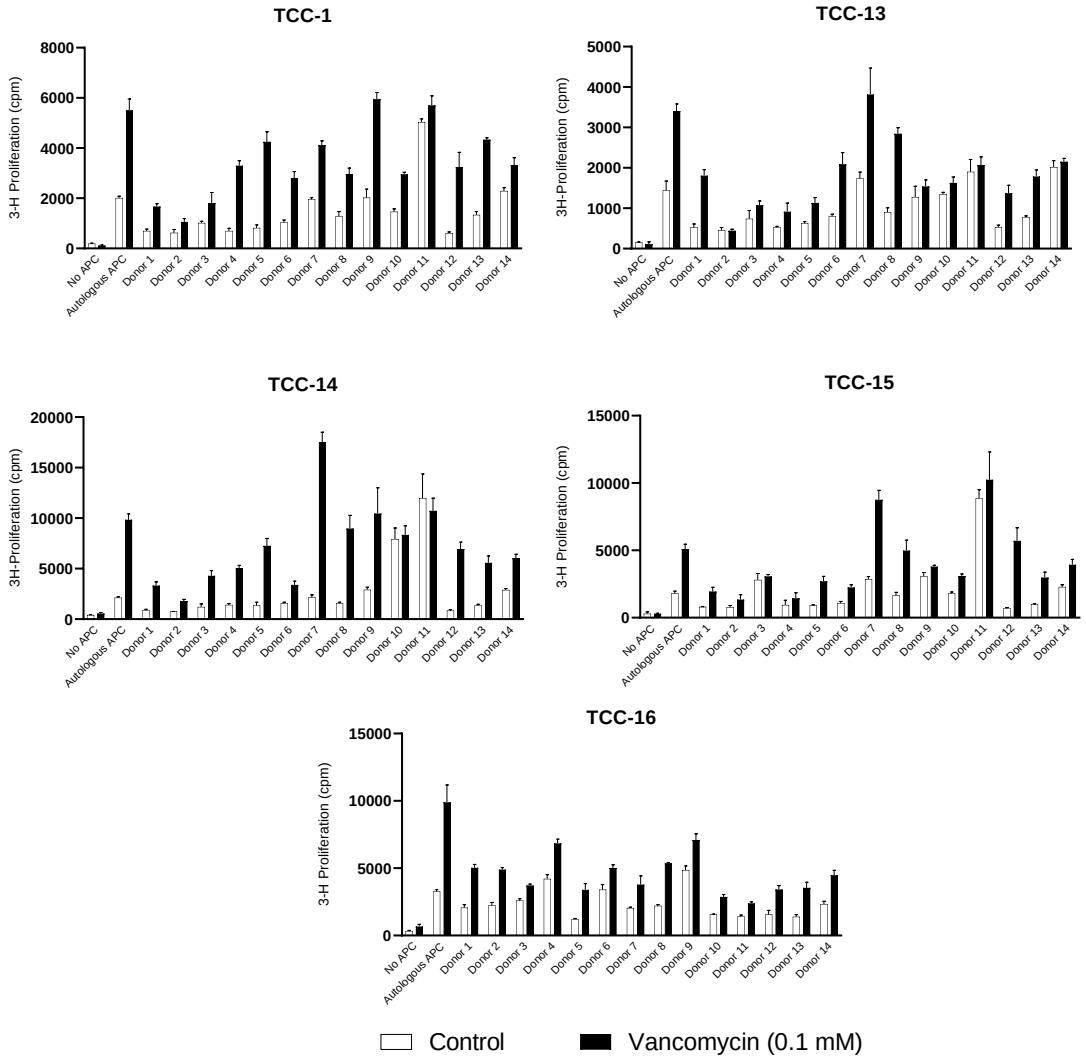


Figure 3.13. APC mismatch assay to determine interactions of vancomycin-specific TCCs with heterologous APCs positive and negative for HLA-A*32:01 (n=14). Vancomycin-responsive TCCs (n=5), generated from the PBMC of healthy donor HD-18 (HLA-A*32:01), were co-cultured with vancomycin (0.1 mM) and either irradiated autologous APCs or heterologous APCs expressing a range of similar and dissimilar HLA alleles. Exposure to R9 cell culture medium only in place of drug was used for negative control purposes and co-cultures were incubated for 48 h (37 °C, 5% CO_2) before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Proliferative responses were detected by radioactivity incorporation and interpreted as cpm values.

In order to investigate the potential for cross-reactive T-cell responses between CD8+ expressing vancomycin-reactive TCCs and structurally related glycopeptide antibiotics, proliferation after exposure to vancomycin, teicoplanin and daptomycin was assessed by radioactive thymidine incorporation (Figure 3.14). Surprisingly, only 3/6 TCCs (50%) that displayed initial specificity for vancomycin were found to be dose-responsive after additional exposure. 4/6 TCCs displayed additional reactivity with teicoplanin, with TCCs 17, 18 and 22

producing strong proliferative responses after exposure to teicoplanin. One TCC (TCC-20) was found to be cross-reactive with both teicoplanin and daptomycin, with moderately strong proliferative responses observed in each instance.

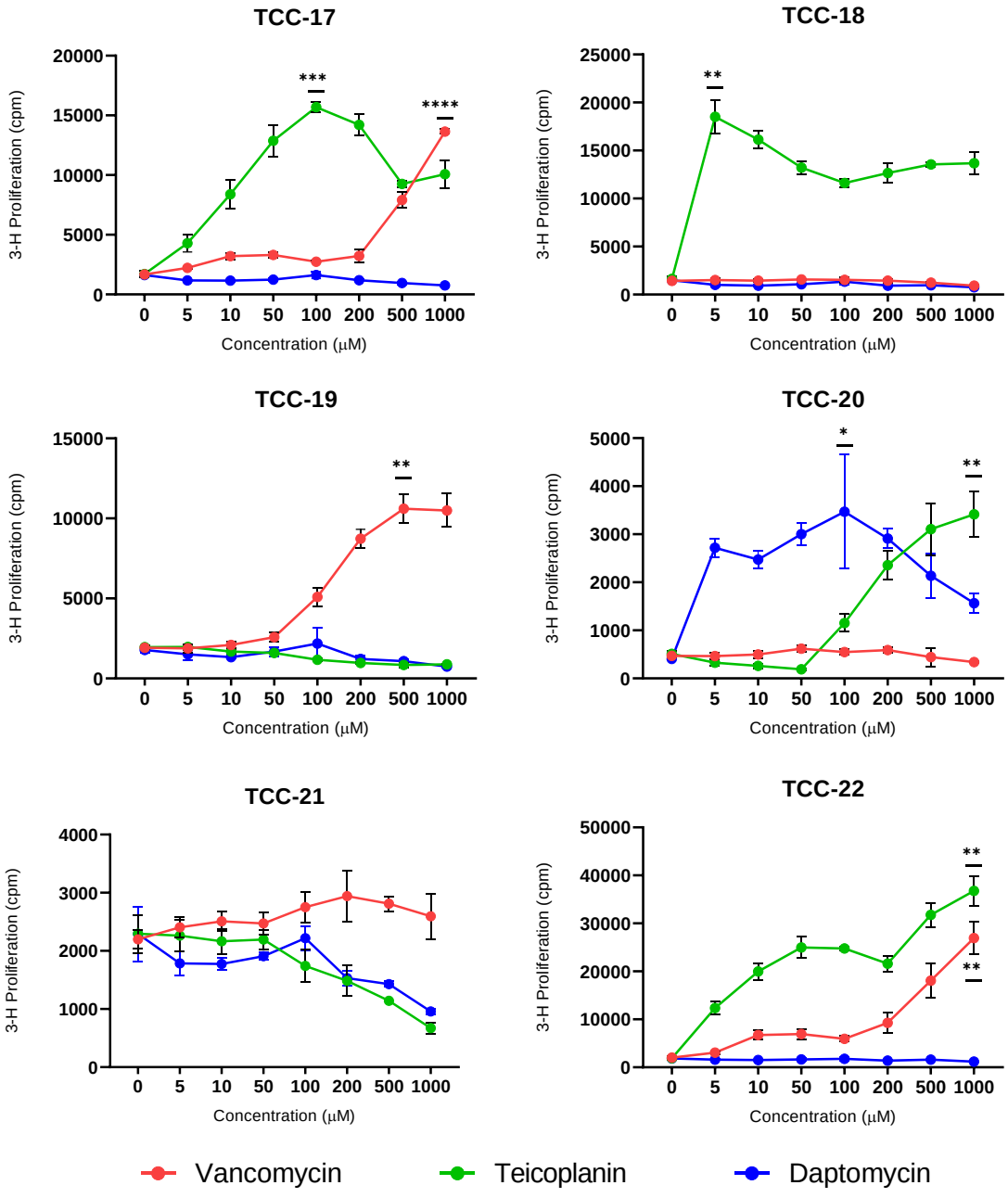


Figure 3.14. Proliferative cross-reactive study of CD8+ vancomycin-specific TCCs (n=6) generated from healthy donor PBMC positive for HLA-A*32:01 expression (HD-18) with structurally related glycopeptide antibiotics. TCCs ($5x10^4$ cells) were co-cultured with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells) and either vancomycin (0-1000 μM), teicoplanin (0-1000 μM), daptomycin (0-1000 μM) or R9 cell culture medium only. Cultures were incubated for 48 h (37 °C, 5% CO_2) before pulsation with tritiated [^{3}H]-thymidine (0.5 μCi/well) for a further 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined using a student's *t*-test (*p<0.5, **p<0.01, ***p< 0.001, ****p<0.0001).

3.4 Discussion.

Hypersensitive disease states to otherwise tolerable and efficacious therapeutics is a major cause for concern, relevant for drug development, clinicians and patients alike. The involvement of the adaptive response arising from immune modulation, and culminating in drug hypersensitivity targeting tissues (skin) and organs (liver) in small fractions of patients has long been established. As such, retrospective analysis in the form of GWAS studies have now elucidated the expression of specific HLA alleles as important contributing factors of susceptibility, with HLA implication further reinforcing the scope for involvement of the adaptive, cell-mediated immune response. However, prediction hinging solely on HLA association has proven problematic, with matters complicated by the sheer volume of allelic variants (>30,000) present within the human genome (Deshpande et al., 2022). Moreover, the multifaceted nature of T-cell mediated drug hypersensitivity is further illustrated by the simple fact that only a select few drug-HLA associations possess economic and physiological viability when applied to genetic screening prior to treatment, with the notable exceptions of abacavir, carbamazepine and allopurinol (Mallal et al., 2008, Chung et al., 2004, Hung et al., 2005).

The recent discovery of HLA-A*32:01 carriage as susceptibility factor within stratified patient groups presenting with vancomycin-induced DRESS further substantiates adaptive immune involvement within disease pathogenesis, arising from HLA implication in addition to the delayed nature of clinical manifestation post exposure (Konvinse et al., 2019). The high frequency of HLA-A*32:01 carriage within populations of European ancestry (6.8%) underpins the scale of issue, specifically when combined with high levels of vancomycin administration within clinical settings. Although pathomechanisms of immune activation were yet to be delineated within initial studies, it has been suggested that vancomycin may be capable of activating drug-specific T-cells either by direct or indirect interactions with HLA-A*32:01 encoded proteins. As aforementioned, further interrogation as to the nature of drug-HLA

interactions via *in silico* molecular docking study revealed that vancomycin may interact with the HLA peptide binding cleft with greater affinity in the absence of peptides present of the surface of HLA proteins (Konvinse et al., 2019). These preliminarily findings insinuate that vancomycin, due to its HLA class I association, may activate CD8+ T-cells, acting as a peptide mimetic in the process.

To investigate the existence of preferential binding between vancomycin and HLA-A*32:01 our study utilised PBMC from an established HLA-typed biobank in order to determine if allelic expression was a critical determinant for lymphocyte transformation, naïve T-cell priming and long-term PBMC stimulation. LTT assays conducted on HLA-typed PBMC from healthy donors positive and negative for HLA-A*32:01 carriage revealed no detectable variability in proliferative lymphocyte transformation after exposure to vancomycin, with each subgroup exhibiting a positive LTT result in 20% of individuals tested (Figure 3.2). LTT assays are the most widely used *in vitro* technique for the detection of delayed-type hypersensitive responses to culprit compounds in hypersensitive patients, typically deployed to elicit memory T-cell responses following concomitant therapy. These assays involve exposure of PBMC, isolated from the blood of patients with suspected hypersensitivity, to the compound of interest for 6-7 days (Pichler and Tilch, 2004). Therefore, it is perhaps unsurprising that these assays were inconclusive for the detection of preferential interaction between HLA-A*32:01 and vancomycin due to the use of PBMC from drug naïve healthy donors and not hypersensitive patients, for which circulating drug-specific T-cells would elicit memory responses after drug rechallenge. The short time-course of the assay, initially developed for the detection of memory T-cell responses, may be insufficient for the induction of *de novo* primary T-cell responses. This has been evidenced by studies suggesting a minimum time period of 7-14 days for successful T-cell priming culminating in proliferative function (Ortiz et al., 1997, Faulkner et al., 2016a).

For the assessment of naïve T-cell priming after vancomycin exposure, T-MWA experiments were conducted utilising co-cultures of naïve T-cells, autologous DCs and vancomycin (Figure 3.4). A more recent iteration of the conventional T-cell priming assay, the T-MWA allows for larger groups of experimental replicates and importantly only requires a single stimulation with autologous DCs, therefore possessing utility for high-throughput immunogenic screening (Ogese et al., 2020, Faulkner et al., 2012). Naïve T-cell priming was achieved in all 3 healthy donors positive for HLA-A*32:01 expression, with proliferative responses identified in > 50% of vancomycin exposed wells. Crucially, significant naïve T-cell priming was also achieved 2/3 donors without HLA-A*32:01 expression, indicating vancomycin may also be capable of stimulating T-cells following antigen presentation by other HLA complexes.

To further explore the drugs propensity for immune activation, PBMC from 10 HLA-A*32:01 positive and 10 HLA-A*32:01 negative healthy donors were cultured and repetitively restimulated with vancomycin, IL-2 and irradiated autologous PBMCs to facilitate antigen presentation, for a 4 week period. Here, we show that vancomycin may indeed have a binding preference for either HLA-A*32:01 or specific peptides present within the HLA-antigen binding cleft (Figure 3.5, 3.6). This assay format, adapted from Engler et al., may be associated with greater sensitivity for the detection of *de novo* T-cell responses due to the incorporation of bulk PBMC cultures containing all components of the immune milieu (Engler et al., 2004). Specifically, this may relate to the inclusion of regulatory T-cells for which utility has already been demonstrated when applied to priming assays utilising whole purified T-cell (CD3+) populations (Hammond et al., 2022).

Initial GWAS findings suggest an association between vancomycin-induced DRESS and HLA-A*32:01 and potentially implicate a CD8+ T-cell response within DRESS pathogenesis due to their HLA class I restricted nature (Konvinse et al., 2019). To further investigate this relationship, vancomycin-specific TCCs were generated from healthy donors positive for HLA-

A*32:01 expression in order to determine the exclusivity of vancomycin-mediated CD8+ T-cell activation and to determine the phenotypic and functional characteristics of vancomycin-responsive T-cells. To achieve this, pathways of activation and antigen presentation were assessed along with cross-reactivity using structurally similar glycopeptide antibiotics. Of the donors utilised for the conduction of T-cell cloning, HLA-typing revealed expression of additional HLA types of interest including homozygous expression of HLA-B*57:01 (HD-33) and heterozygous expression of HLA-DRB*01:01 (HD-18) for which genetic associations with abacavir and nevirapine-induced hypersensitivity have previously been identified (Mallal et al., 2002, Vitezica et al., 2008).

Drug-reactive T-cells were successfully generated from CD8+ enriched populations in 2/3 donors (HD-32 and HD-18) positive for risk allele expression (Figure 3.7). TCCs were confirmed as dose-responsive and vancomycin-specific following mitogen driven clonal expansion and exposure to graded concentrations of vancomycin and carbamazepine. Although vancomycin-responsive TCCs were detected in 2/3 donors, it remains unclear as to why drug-specific clonal populations were unable to be generated from HD-33. All donors were heterozygous for HLA-A*32:01 expression, however differences may exist between immunoregulatory components for which differential co-inhibitory expression may represent a plausible explanation.

A panel of TCCs from HD-18 were further assessed within phenotypic and functional T-cell assays after stimulation with vancomycin at graded concentrations only marginally surpassing physiological relevance. Indeed, proliferative responses to vancomycin were detected at concentrations < 100 μM, with several studies suggesting plasma concentrations between 10 - 20 μM are capable of eliciting pharmacological effects in patients treated with vancomycin (Tunkel et al., 2004, Wysocki et al., 2001). TCCs ubiquitously expressing a CD8+ phenotype additionally expressed high levels of CXCR3 (Figure 3.10), a chemokine receptor abundant on effector T-cells with functional roles within trafficking and migration to peripheral tissues and

the induction of Th1-type inflammation (Groom and Luster, 2011). Expression of CCR4+ was also detected. This chemokine receptor is typically associated with CD4+ memory T-cell function, specifically pertaining to the migration of Th2 cells. Although rare for CD8+ expressing T-cells, CCR4+ T-cells have previously been characterised for cytokine secretion (IFN-γ, IL-2 and IL-4) within populations of CD8+ memory T-cell subsets (Kondo and Takiguchi, 2009).

Analysis of cytokine profiles from a representative panel of vancomycin-responsive TCCs revealed secretion of both Th1 (IFN-γ) and Th2 (IL-13) related cytokines in addition to the release of cytolytic and cytotoxic molecules such as perforin, granzyme B and FasL (Figure 3.11), likely involved within disease pathogenesis (Yang et al., 2018). Surprisingly, within the limited panel of TCCs assessed, IL-5 secretion was not detected. IL-5 has been shown to be a key mediator of DRESS, due to its prominent role within eosinophil recruitment (Choquet-Kastylevsky et al., 1998). Within this study no drug-reactive TCCs expressing a CD4+ phenotype were generated, although previous work has suggested a potential role for vancomycin-responsive TCCs with cytokine secretion profiles also elucidated, however mechanistic and functional roles within antigen presentation have yet to be characterised (Ogese et al., 2021).

Vancomycin is a complex therapeutic compound, with a molecular weight (1449 kDa) notably larger than low molecular weight compounds (< 1000 kDa) typically associated with immunogenicity and the induction of hypersensitive disease states. As aforementioned, due to the substantial size of the drug, which may mirror the weight of certain HLA class I binding peptides, its possible vancomycin may displace HLA peptides. This has previously been suggested by molecular docking studies indicating preferential binding in the absence of HLA-bound peptides on the surface of APCs (Konvinse et al., 2019). Therefore, to uncover mechanisms of vancomycin-mediated T-cell activation it was important to determine

pathways of antigen presentation and processing and the requirement, if any, for covalent binding ultimately leading to T-cell stimulation and proliferative function.

Firstly, to elucidate mechanisms of antigen presentation, HLA blockade was studied after exposure to vancomycin and autologous APCs. Abrogated proliferative responses observed after HLA class I blockade suggest HLA class I restricted T-cell activation by vancomycin (Figure 3.12). This result neatly aligns with studies assessing T-cell responses to abacavir, for which HLA class I associations have also been identified and CD8+ T-cells activated following antigen presentation (Thomson et al., 2020). To determine pathways of antigen processing, TCCs were cultured with either drug pulsed or glutaraldehyde-fixed APCs. Following APC-drug pulsation, no proliferative response was detected after washing and removal of weakly bound drug from the HLA binding groove (Schnyder et al., 2000). However, after APC fixation with glutaraldehyde and inhibition of processing pathways and drug uptake, TCCs were capable of stimulation following vancomycin exposure (Figure 3.12). These data indicate that vancomycin activates CD8+ T-cells via HLA class I presentation in a processing independent and non-covalent manner, aligned with the p-i concept (Pichler, 2002). T-cell activation was also observed in the presence of heterologous APCs without HLA-A*32:01 expression. Indeed, vancomycin-responsive TCCs were found to proliferate when co-cultured with APCs expressing various HLA-A alleles, suggesting vancomycin is capable of complexing with multiple HLA molecules (Figure 3.13). To further investigate this possibility, it will be necessary to study vancomycin-specific T-cell responses using transfected B-cells, such as C1R cell lines, for which expression is limited to HLA-A*32:01 in isolation. Such studies, already characterised for HLA-B*57:01 expression with respect to abacavir-mediated T-cell activation (Chessman et al., 2008), would contribute to our understanding of any interactions of preference between vancomycin, HLA-A*32:01 and T-cell receptors.

Teicoplanin and daptomycin are glycopeptide antibiotics used for the treatment of Gram-positive bacterial infection and often deployed as auxiliary treatment options following initial adverse responses to vancomycin. In this study, we investigated potential cross-reactivity between vancomycin–responsive T-cells and structurally-related glycopeptides. Within the panel of TCCs assessed, 4 TCCs exhibited cross-reactivity with teicoplanin, while 1 TCC displayed cross-reactive proliferative responses with both teicoplanin and daptomycin (Figure 3.14). Surprisingly, loss of specificity to vancomycin in cross-reactive TCCs was also observed. Exact reasoning for this is unclear, although variations within activation thresholds somewhat provide rationale. Logically, due to the close-structural relation, specifically the presence of a heptapeptide scaffold, cross-reactivity is entirely plausible. This is amplified when considering the existence of numerous case studies reporting clinical cross-reactivity in patients sequentially administered with vancomycin and teicoplanin, ultimately manifesting in severe cutaneous adverse reaction (Kwon et al., 2006, Hsiao et al., 2010). To further elucidate the molecular basis for the observed cross-reactive responses between vancomycin, teicoplanin and daptomycin, modelling studies with a focus on shared TCR homology will be key to our understanding, as demonstrated with carbamazepine and its structural analogues in relation the development of Stevens-Johnson syndrome (Pan et al., 2019).

In summary, this study suggests that vancomycin preferentially primes both naïve T-cells and healthy donor PBMC positive for carriage of HLA-A*32:01, for which strong associations with vancomycin-induced DRESS have now been established. These interactions have now been characterised to hinge upon HLA class I presentation, with T-cell responses evoked by direct pharmacological interactions. Stimulation has been found to be associated with a plethora of cytokine and cytolytic molecule release potentially involved within DRESS pathogenesis, with clinical cross-reactivity now replicated within *in vitro* assays. Additional study assessing the intrinsic immunogenic potential of both teicoplanin and daptomycin will be required to further

understand patterns of cross-reactivity and interactions between glycopeptide antibiotics and HLA-A*32:01.

4. Investigating the immunogenic potential of teicoplanin and daptomycin in drug-naïve donors expressing HLA-A*32:01.

Contents

4.1 Introduction.

Glycopeptide antibiotics are routinely used for the treatment of serious Gram-positive bacterial infection and are often viewed as invaluable weapon in the armoury of clinicians when applied to therapeutic intervention involving β-lactam antibiotic resistant bacterial strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile* (Campoli-Richards et al., 1990). Since development, treatment outcomes have drastically improved for infections typically affiliated with the development of antibiotic resistance to compounds such as sulfonamides and penicillins. Most glycopeptide antibiotics are associated with a similar spectrum of activity, with potency demonstrated against specific 'hard to treat' bacterial isolates including *Enterococcus, Streptococcus and Staphylococcus* strains (Van Bambeke, 2006). Although referred to as a 'drug of last resort' and typically administered after exhaustion of alternative treatment regimens, glycopeptide antibiotics still remain the gold standard for treatment of severe infection. Moreover, both naturally occurring (vancomycin) and semi-synthetic glycopeptide compounds (teicoplanin and daptomycin) still lead the way as preferred treatment options with the vast majority of clinician's decades from inception.

The spread of resistance to vancomycin and other naturally occurring glycopeptides has established the need for a new, more chemically malleable compounds. As a consequence of this, teicoplanin, produced from *Actinoplanes teichomyceticus,* was developed for clinical use in 1984. Crucially, this compound formed the structural basis for the inception of more contemporary semi-synthetic glycopeptide antibiotics routinely used today. Teicoplanin is a first generation naturally occurring glycopeptide antibiotic that exerts its anti-bacterial properties in a similar fashion to vancomycin, during which peptidoglycan inhibition is achieved by direct high affinity binding of the drug to bacterial peptides at the carboxyl terminus (Perkins, 1969). However, disparities become apparent when considering the chemical traits of the drug, some of which have been hypothesised to aid function and

enhance anti-bacterial activity. Specifically, presence of a fatty acid residue in place of a sugar moiety is a unique feature of teicoplanin, allowing for stable anchorage at peptide target sites (Allen and Nicas, 2003, Nicolaou et al., 1999).

In addition to being associated with increased efficacy against some β-lactam resistant bacterial strains, teicoplanin has been found to be more tolerable among patient groups and as a consequence has been linked with a reduced incidence of adverse drug reaction (ADR) following drug administration (Davey and Williams, 1991). Although the incidence of ADR associated with teicoplanin use is considerably lower when compared with vancomycin (13.9% vs 21.9%), the drug still constitutes a significant risk to patient safety (Wood, 1996). When considering clinical presentation, teicoplanin administration has now been associated with both pseudoallergic responses of an immediate nature, such as red man syndrome, in addition to classical delayed-type hypersensitive disease states such as drug reaction with eosinophilia and systemic symptoms (DRESS) (Ben Romdhane et al., 2018, Khurana and de Belder, 1999). Case studies have emerged reporting incidences of cross-reactivity between vancomycin and teicoplanin within the context of hypersensitive responses to both compounds after sequential administration. Moreover, the phenomenon of clinical cross-reactivity observed between glycopeptide compounds specifically relates the onset of teicoplanin-induced DRESS following initial vancomycin hypersensitivity (Kwon et al., 2006, Hsiao et al., 2010).

Daptomycin, although not formally a member of the glycopeptide family, bares close structural relation and was developed from *Streptomyces roseosporus* for the treatment of Gram-positive bacterial infection. Daptomycin is a cyclic lipoglycopeptide, and was more recently developed to combat the rise of vancomycin and teicoplanin resistant strains of MRSA. Similarly to teicoplanin, daptomycin can be characterised by the presence of a lipophilic chain but crucially studies have shown the drug to be well tolerated among stratified patient groups with incidences of reported immune-mediated hypersensitivity limited to anaphylaxis

(Fowler et al., 2006). Furthermore, within the context of DRESS presentation daptomycin has yet to be formally implicated.

As outlined in greater detail within Chapter 3, a recent genome-wide association study (GWAS) has identified a genetic link with vancomycin-induced DRESS and expression of HLA-A*32:01 in European populations (Konvinse et al., 2016). Furthermore, adaptive immune involvement within the pathogenesis of vancomycin-induced DRESS has now been characterised for CD8+ T-cell function with preliminary studies modelling disease pathogenesis in healthy donors eluding to an MHC class I-restricted mechanism of activation hinging on non-covalent pharmacological interactions between drug and HLA proteins. Vancomycin may evoke T-cell activation by way of 'peptide mimicking', on account of its high molecular weight closely resembling that of HLA class I peptides. Additionally, *in vitro* T-cell studies assessing proliferative function have demonstrated cross-reactivity between teicoplanin and daptomycin with vancomycin-responsive T-cells generated from healthy donor peripheral blood mononuclear cells (PBMC) positive for HLA-A*32:01 expression (Ogese et al., 2021).

Following on from the complex clinical and *in vitro* cross-reactive patterns previously documented, the aim of the present study was to determine the intrinsic immunogenic potential of both teicoplanin and daptomycin in addition to further exploring patterns of cross-reactivity between structurally similar glycopeptide antibiotics. This was explored by the assessment of each drugs propensity to evoke T-cell responses in healthy donors positive for expression of HLA-A*32:01, associated with vancomycin-induced DRESS. To achieve this, *in vitro* T-cell priming assays were coalesced with T-cell cloning experiments, using PBMC isolated from drug naïve healthy donors expressing HLA-A*32:01. Glycopeptide-specific T-cell responses were studied at the unicellular level in order for functional, phenotypic and mechanistic assessment.

4.2 Aims.

1) To investigate the intrinsic immunogenicity of teicoplanin and daptomycin in healthy donors positive for HLA-A*32:01 expression using diagnostic T-cell assays in addition to the assessment of *de novo* primed T-cell responses.

2) To generate teicoplanin and daptomycin-specific T-cell clones (TCCs) from healthy donors with HLA-A*32:01 carriage, by serial dilution.

3) To determine phenotype and characterise functional traits pertaining to the activation of glycopeptide-responsive TCCs from healthy donors.

4) To further explore patterns of cross-reactivity between structurally-related glycopeptide antibiotics.

4.3 Results.

4.3.1 PBMC proliferation assays show direct toxicity of teicoplanin.

Firstly, to determine concentrations at which PBMC cultures exhibit toxicity to teicoplanin and daptomycin, toxicity assays were performed on healthy donor PBMC cultures (n=4) assessing proliferative potential after mitogen driven stimulation at graded drug concentrations of teicoplanin and daptomycin (Figure 4.1).

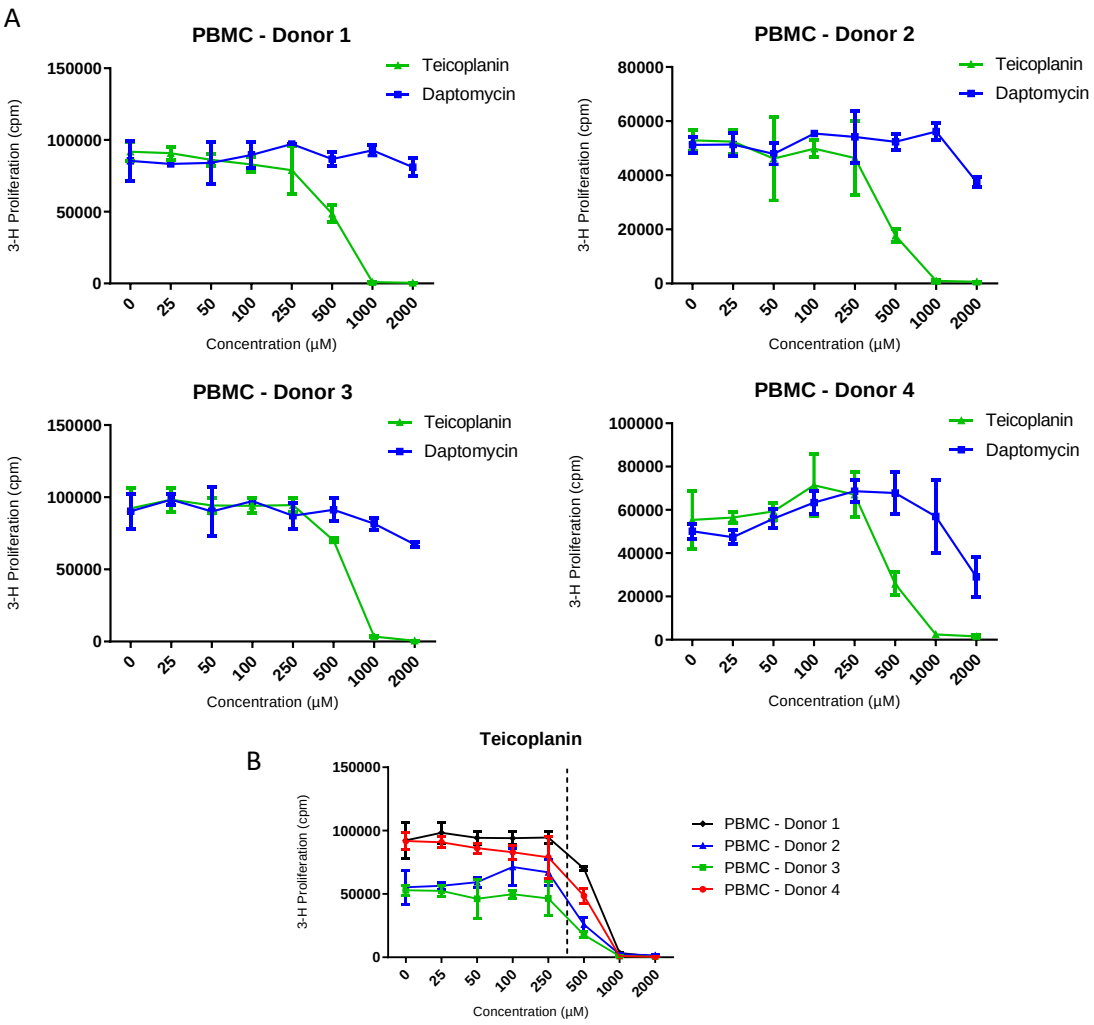


Figure 4.1. Thymidine incorporation assay to measure proliferative inhibition induced by teicoplanin and daptomycin in mitogen stimulated PBMC cultures isolated from healthy donors (n=4). Separate PBMC cultures ($1.5x10^5$ cells/well) were exposed, in triplicate, to graded concentrations of teicoplanin (0-2000 µM) or daptomycin (0-2000 µM) for 24 h (37 °C, 5% CO_2). Proliferation was then induced by mitogen driven stimulation following the addition of PHA (10 µg/mL) to every well of the assay plate. Cells were incubated for a further 48 h in the presence of PHA before being pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Proliferative responses were detected by radioactivity incorporation and interpreted as counts per minute (cpm). **B) Proliferative inhibition of PBMC after teicoplanin exposure (n=4).** Proliferative inhibition was assessed within PBMC cultures following mitogen driven stimulation as previously described. Overall trend across each donor after teicoplanin exposure at graded concentrations is displayed.

In each of the 4 donors for which PBMC toxicity was assessed, daptomycin was well tolerated at concentrations of 500 µM and below (Figure 4.1). At concentrations exceeding 1 mM toxicity was induced by daptomycin, specifically within PBMC cultures isolated from donors 2 and 4. Teicoplanin was observed to induce toxicity at concentrations exceeding 250 µM. This dose-dependent toxicity was universally observed within PBMC cultures isolated from all healthy donors (n=4), after proliferative assessment following mitogen driven stimulation and drug exposure.

4.3.2 PBMC proliferation assays reveal lymphocyte proliferation after teicoplanin exposure in HLA-A*32:01 healthy donors.

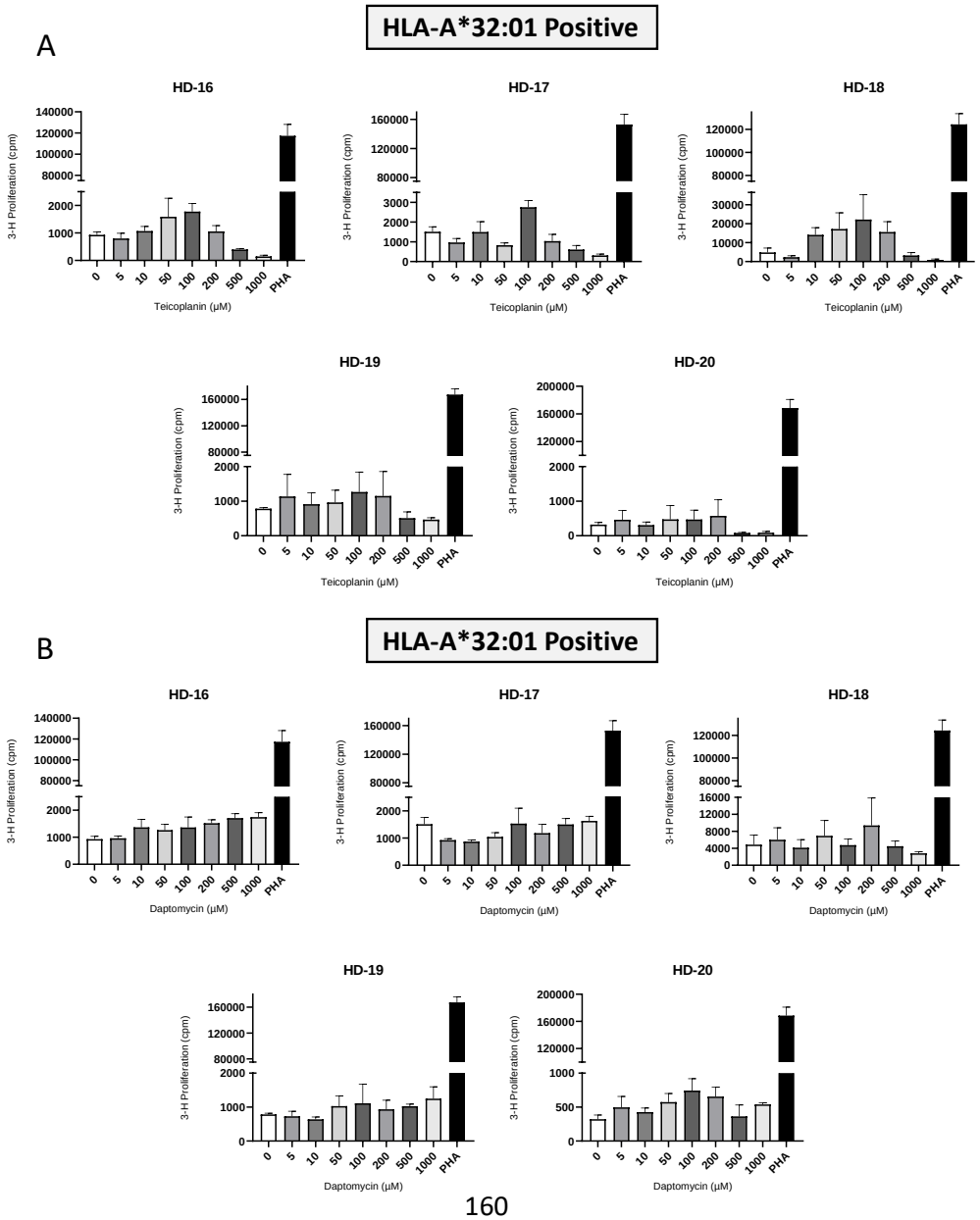


Figure 4.2. A) Lymphocyte transformation test (LTT) assessing proliferative responses to teicoplanin in healthy donor PBMC positive for HLA-A*32:01 expression (n=5). PBMC from healthy volunteers positive for HLA-A*32:01 expression (n=5) were isolated by density gradient centrifugation and seeded at $1.5x10^5$ cells/well in triplicate cultures and exposed to graded concentrations of teicoplanin (0-1000 µM). The addition of PHA (10 µg/mL) was used for a positive control for the assay for the detection of non-specific lymphocyte transformation. Cultures were incubated for 6 days (37 °C, 5% CO_2) before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Proliferative responses were detected by radioactivity incorporation and interpreted cpm. **B) LTT assessing proliferative responses to daptomycin in healthy donor PBMC positive for HLA-A*32:01 expression.** Lymphocyte proliferation was assessed via LTT assay and conducted on healthy donor PBMC expressing HLA-A*32:01. PBMC were seeded at $1.5x10^5$ cells/well in triplicate cultures and exposed to graded concentrations of daptomycin (0-1000 µM). Proliferative responses to vancomycin were determined by [^{3}H]-thymidine incorporation as previously described.

Immunogenicity assays conducted on HLA-typed PBMC taken from individuals positive for HLA-A*32:01 expression revealed detectable differences within lymphocyte proliferation after exposure to each compound for a 6 day period (Figure 4.2). 2/5 HLA-A*32:01 positive healthy donors exhibited a dose-dependent proliferative response to teicoplanin (HD-16 and HD-18), with elevated proliferation also observed at one concentration (100 µM) in HD-17 (Figure 4.2A). Although proliferative increases were observed it must be noted that statistical significance was not achieved. Conversely, after exposure to graded concentrations of daptomycin positive lymphocyte transformation was not observed. Moreover, 0/5 healthy donors displayed a proliferative effect after stimulation with daptomycin, with proliferation unchanged when compared to baseline values (Figure 4.2B). In all PBMC cultures (n=10) the addition of PHA successfully induced non-specific lymphocyte transformation.

4.3.3 Teicoplanin and daptomycin induce naïve T-cell priming in healthy donors with HLA-A*32:01 carriage.

To investigate the intrinsic immunogenic potential of both teicoplanin and daptomycin, in addition to the ability of glycopeptide compounds to interact with the HLA-A*32:01 allele, *in vitro* priming assays were deployed incorporating naïve T-cells and professional antigen presenting cells in the form of autologous dendritic cells (DCs). It was necessary to study the specificity of teicoplanin and daptomycin for a potentially relevant risk allele, achieved through the use of a HLA-typed PBMC biobank and assessment of naïve T-cell priming to investigate capacity for induced *de novo* T-cells responses utilising recently developed T-cell priming

assays allowing for multiple replicates. After proliferative study, following initial priming and drug rechallenge, teicoplanin was observed to successfully induce significant naïve T-cell priming in 1/5 (20%) healthy donors (HD-21) positive for expression of HLA-A*32:01, when compared to rechallenge with R9 cell culture medium only.

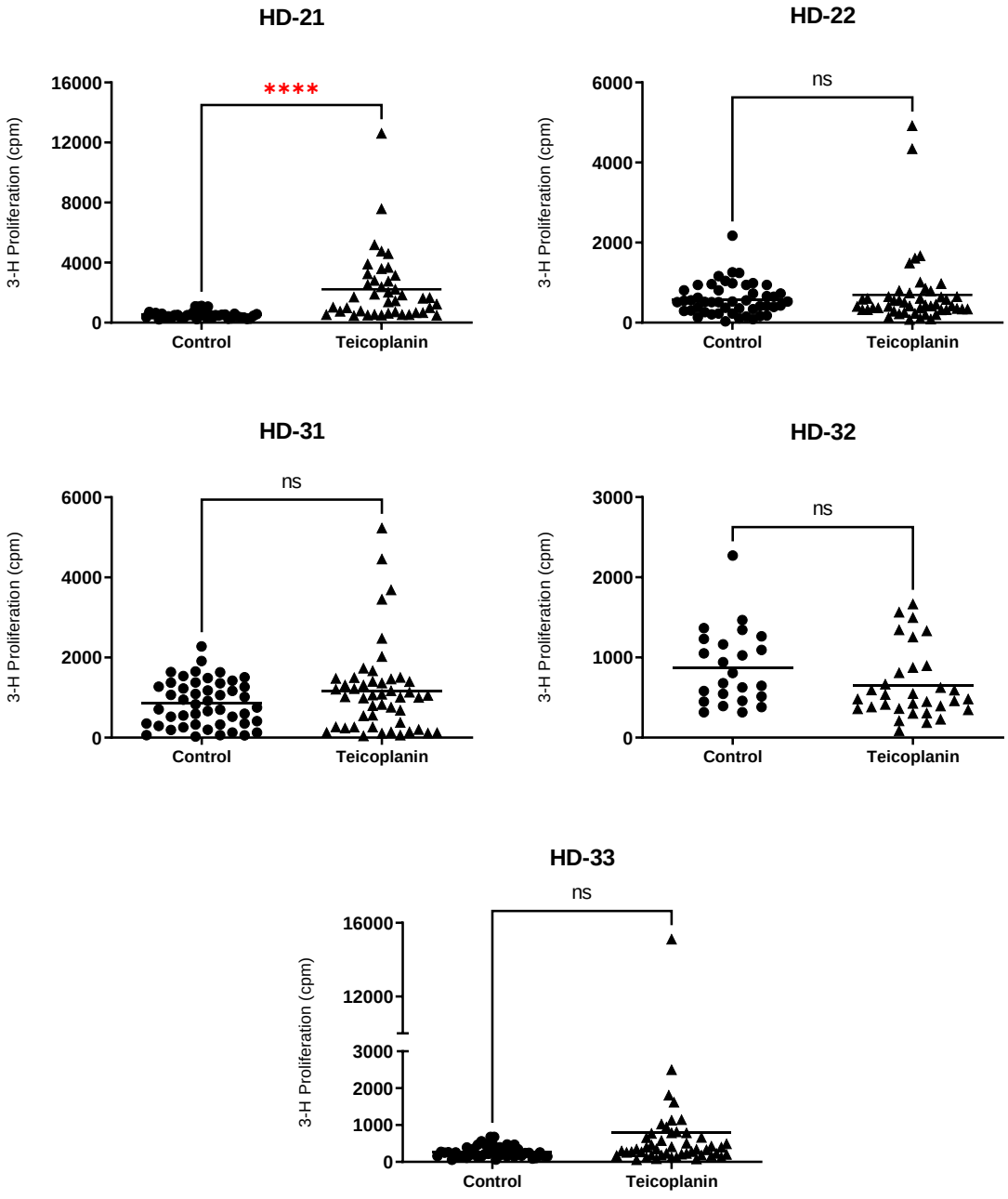


Figure 4.3. Assessment of naïve T-priming to teicoplanin in HLA-A*32:01 positive healthy donors (n=6) using the T-cell multiple well assay (T-MWA). Naïve T-cells ($1x10^5$) from 6 HLA-A*32:01 positive donors were isolated and incubated with $8x10^3$ autologous dendritic cells and teicoplanin (0.25 mM) for 14 days (37 °C, 5% CO_2). Free drug was removed from each well by extensive washing with R9 medium and cells were rechallenged with either medium or optimal stimulatory concentrations of teicoplanin (0.25 mM) for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Proliferative responses were detected by way of radioactivity incorporation and interpreted as cpm values. Statistical significance was determined using a Mann-Whitney U test (*p< 0.05, ****p<0.0001).

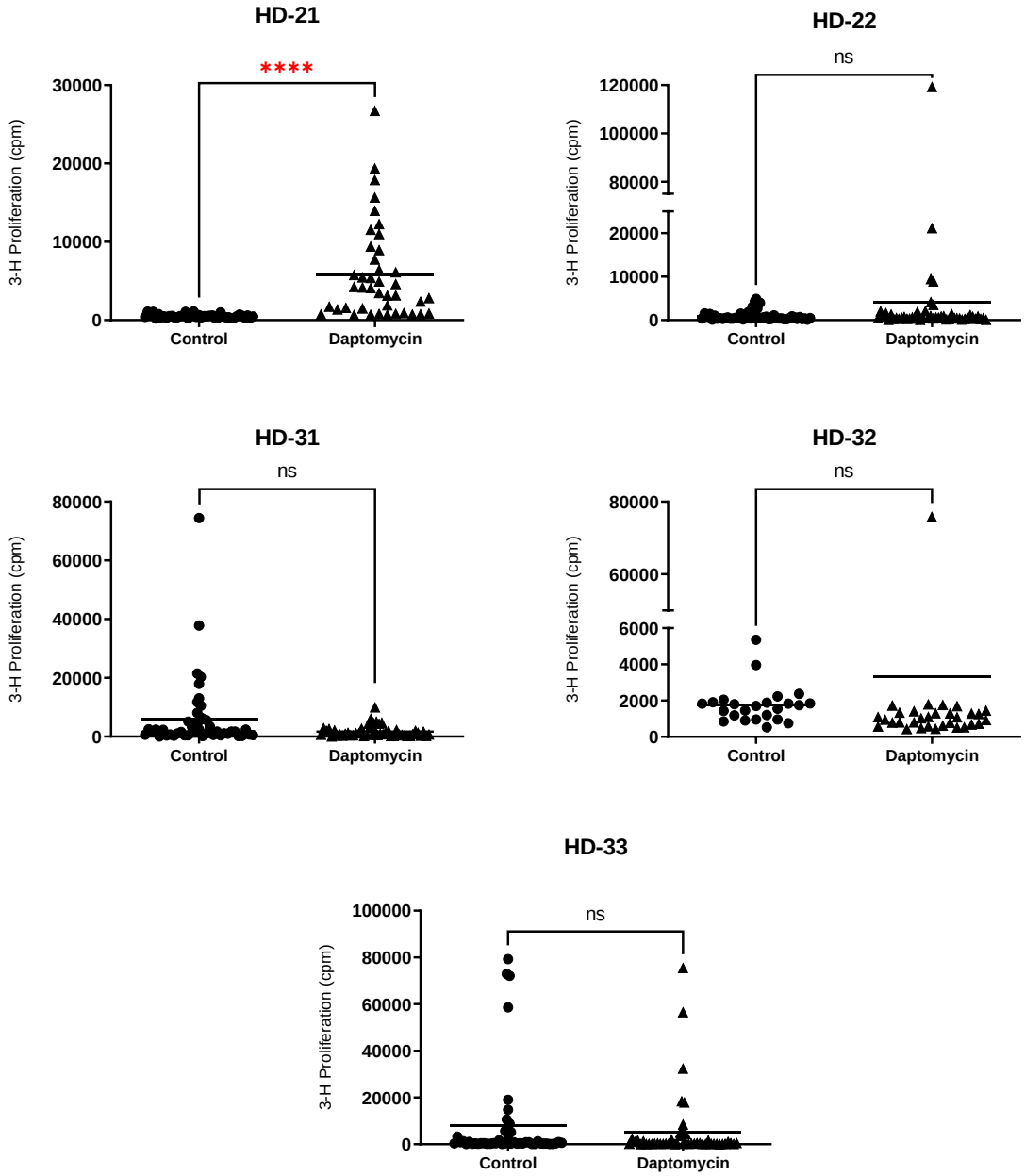


Figure 4.4. Assessment of naïve T-priming to daptomycin in HLA-A*32:01 positive healthy donors (n=6) using the T-MWA. Naïve T-cells ($1x10^5$) from 6 HLA-A*32:01 positive donors were isolated and incubated with $8x10^3$ autologous dendritic cells and daptomycin (0.5 mM) for 14 days (37 °C, 5% CO_2). Free drug was removed from each well by extensive washing with R9 medium and cells were rechallenged with either medium or daptomycin for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Proliferative responses were detected using radioactivity incorporation interpreted as cpm values. Statistical significance was determined using a Mann-Whitney U test (*p< 0.05, **p<0.01, ****p<0.0001).

When studying *de novo* naïve T-cell responses to daptomycin a similar pattern emerged (Figure 4.4). Statistically significant naïve T-cell priming was again observed in 1/5 (20%) healthy donors (HD-21) positive for expression of HLA-A*32:01 following drug rechallenge (0.5 mM).

4.3.4 Generation of teicoplanin and daptomycin-specific TCCs from healthy donors expressing HLA-A*32:01.

For the generation of teicoplanin and daptomycin-responsive TCCs, healthy donors utilised for T-cell cloning experiments were identical to donors studied within T-cell cloning experiments in Chapter 3 (HD-18, HD-32 and HD-33). This was for consistency when studying vancomycin, teicoplanin and daptomycin-specific T-cell responses for which full donor HLA genotype has previously been stated (Table 3.3).

Teicoplanin-responsive TCCs were generated in 3/3 donors expressing HLA-A*32:01 following analysis of initial monoclonal test cultures (Figure 4.5). Clonal populations generated from CD8+ enriched T-cells proliferated to a greater degree (HD-33, HD-32; SI > 40) and with greater frequency (HD-18; 118/216 TCCs SI > 1.5) than CD4+ enriched. Although initial test cultures performed using CD4+ enriched T-cells inferred specificity for teicoplanin, the presence of drug-specific T-cells displaying dose-dependent specificity to teicoplanin was restricted to monoclonal populations enriched for CD8+. After expansion of TCCs generated form CD4+ enriched populations, TCCs were found to be unresponsive to the drug in question following confirmatory dose-response tests. Teicoplanin-responsive TCCs, exclusively expressing a CD8+ phenotype, from donor HD-33, were further expanded by repetitive mitogen driven stimulation for additional functional characterisation. In contrast, T-cell cloning experiments to daptomycin yielded negative results (Figure 4.6). When studying T-cell responses to daptomycin in the same healthy volunteers positive for HLA-A*32:01 carriage, drug specific T-cells exhibiting dose-dependent proliferative responses to daptomycin were absent across all 3 donors.

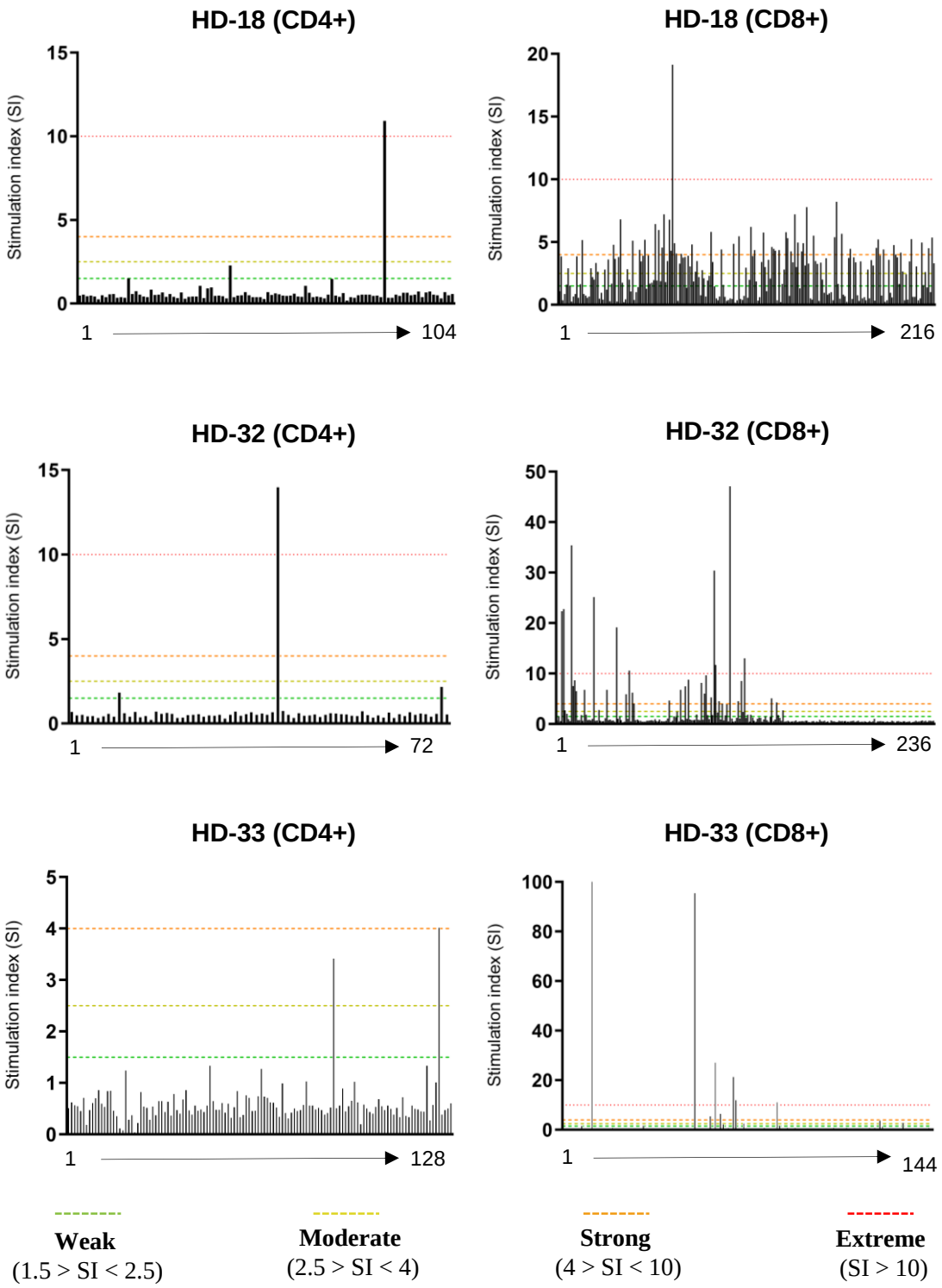


Figure 4.5. Generation of teicoplanin-specific TCCs from PBMC enriched for CD4+ and CD8+ T-cells in healthy donors (n=3) expressing HLA-A*32:01. Bulk PBMC cultures were seeded at $1x10^6$ PBMC/well with teicoplanin (0.25 mM) and incubated (37 °C, 5% CO_2) for 14 days. T-cells were enriched for CD4+ or CD8+ expressing populations by MACS separation and transferred to 96-well U-bottomed plates (1 T-cell/well average) with irradiated allogenic PBMC ($5x10^4$ cells/well) and R9 medium supplemented with IL-2 (200 U/mL) and PHA (5 µg/mL). Serial dilution protocol was performed as previously described, with test cultures rechallenged with teicoplanin (0.25 mM) or R9 medium only, in the presence of irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well). Testing was performed over 4 wells in duplicate conditions and cultures were incubated for 48 h (37 °C, 5% CO_2) and pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for the final 16 h of incubation. Readouts were initially interpreted as cpm and converted SI values given by; [average drug treated wells / average control wells]. SI values for each test culture were interpreted as either weak (1.5 > SI < 2.5), moderate (2.5 > SI < 4), strong (4 > SI < 10) or extreme (SI > 10).

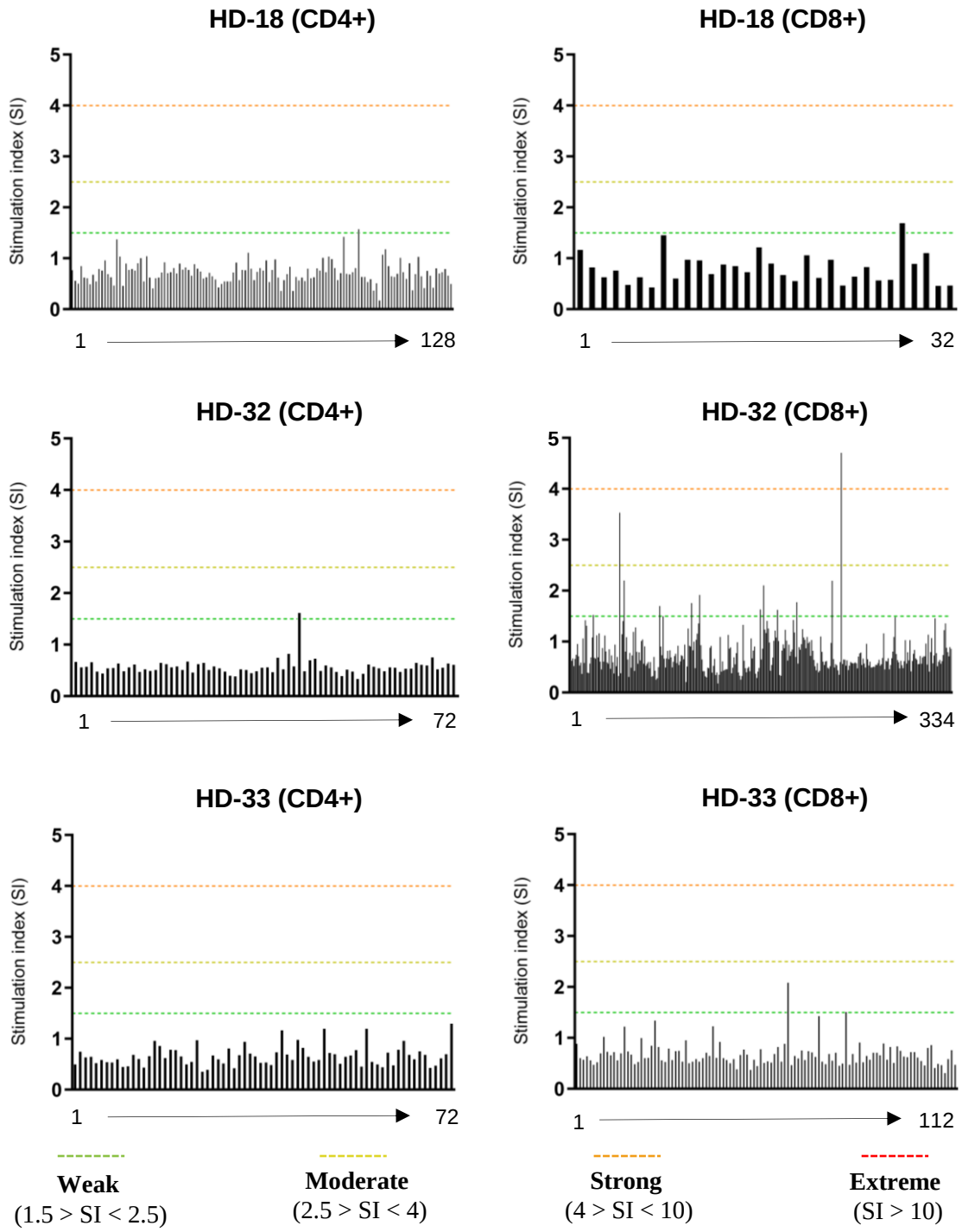


Figure 4.6. Generation of daptomycin-responsive TCCs from PBMC enriched for CD4+ and CD8+ T-cells in healthy donors (n=3) expressing HLA-A*32:01. PBMC cultures were seeded at $1x10^6$ PBMC/well with daptomycin (0.5 mM) and incubated (37 °C, 5% CO_2) for 14 days. T-cells were enriched for CD4+ or CD8+ expressing populations by MACS separation and transferred to 96-well U-bottomed plates (1 T-cell/well average) with irradiated allogenic PBMC ($5x10^4$ cells/well) and R9 medium supplemented with IL-2 (200 U/mL) and PHA (5 µg/mL). Serial dilution protocol was performed as previously described, with test cultures rechallenged with daptomycin (0.5 mM) or R9 medium only, in the presence of irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well). Testing was performed over 4 wells in duplicate conditions and cultures were incubated for 48 h and pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Readouts were interpreted as cpm and converted SI values given by; [average drug treated wells / average control wells]. SI values for each test culture were interpreted as weak (1.5 > SI < 2.5), moderate (2.5 > SI < 4), strong (4 > SI < 10) or extreme (SI > 10).

T-cell cloning experiments were also conducted using bulk PBMC cultures initially primed to carbamazepine (CBZ), for which alternative HLA association has been documented (Hung et al., 2006). These experiments were performed in parallel with cloning studies incorporating teicoplanin and daptomycin and were used to provide a negative control across the same panel (n=3) of healthy donors. Minimal numbers of weakly responsive (1.5 > SI < 2.5) of CBZ-specific TCCs were identified following initial specificity testing. Clonal populations were further tested for dose-dependent proliferative T-cell responses at graded drug concentrations, with no TCCs exhibiting dose-dependent or indeed any proliferative response to CBZ after further examination.

4.3.5 Phenotypic and functional characterisation of teicoplanin-responsive TCCs generated from healthy donors expressing HLA-A*32:01.

Teicoplanin-responsive TCCs generated from the PBMC of healthy donor HD-33 (HLA-A*32:01 positive) were assessed for phenotypic and functional characteristics. Functional analysis was performed on TCCs generated from this particular donor due to the magnitude of SI values associated and degree of clonal expansion following repetitive mitogen driven stimulation, enabling viable functional study resulting from confluent test cultures. Clonal populations deemed positive (SI > 1.5) for drug-specificity after initial proliferative assessment were further studied for drug-specific T-cell responses after exposure to graded concentrations of teicoplanin (Figure 4.7A). Additionally, phenotypic analysis via study of surface marker expression (CD4+/CD8+) was performed on a representative panel of TCCs positive for dose-dependent proliferative response to teicoplanin (Figure 4.7B).

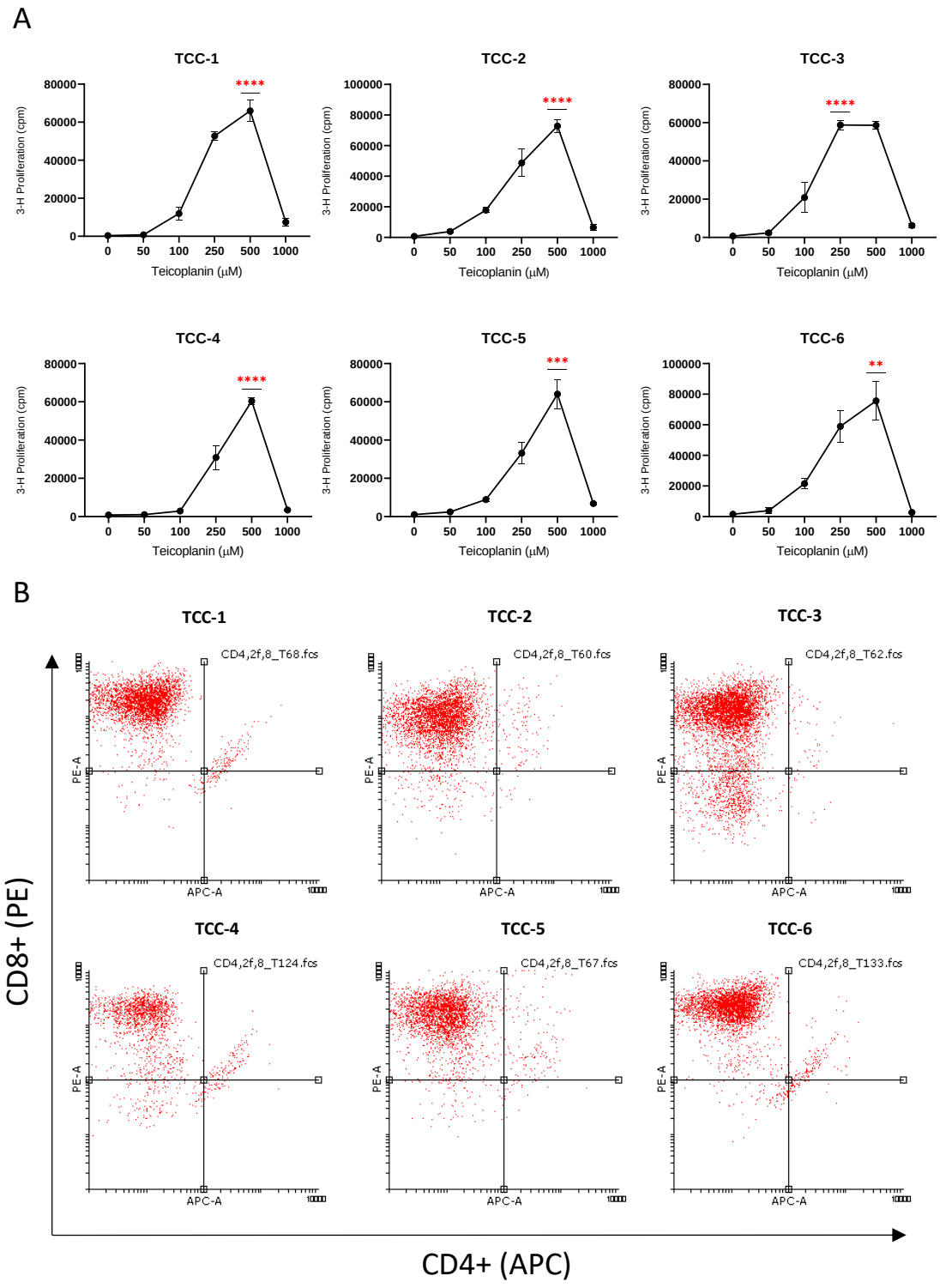


Figure 4.7. A) Proliferative response of TCCs generated from HD-33 (A*32:01 positive) graded concentrations of teicoplanin concentrations (n=6). TCCs (5x10^4 cells) were co-cultured with autologous EBV-transformed B-cells (1x10^4 cells) and teicoplanin (0-1000 μM) Cultures were incubated for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 μCi/well) for 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined using a student's *t*-test (**p<0.01, ***p< 0.001, ****p<0.0001**). B) Phenotypic characterisation (CD4+/CD8+) of representative teicoplanin-specific TCCs (n=6).** T-cells with predetermined specificity for vancomycin were stained with 3 μL anti-CD4 (APC) and 0.5 μL of anti-CD8 (PE) for 15 mins at 4 °C. Stained cells were resuspended in 4% PFA and samples analysed using a FACS-Canto II instrument integrated with FACS DIVA operating software. Phenotypic analysis was carried on 10^4 events using Flowing 2 software.

A representative panel of TCCs demonstrated strong dose-dependent proliferative responses after exposure to graded drug concentrations up to and including 500 µM, with strong toxicity subsequently observed at 1 mM concentrations (Figure 4.7A). This pattern was reproducible across all 6 TCCs assessed for dose-dependent proliferation. Phenotypic analysis of teicoplanin-responsive TCCs generated from HLA-A*32:01 positive healthy donor PBMC (HD-33) revealed a dominant CD8+ expressing phenotype (Figure 4.7B).

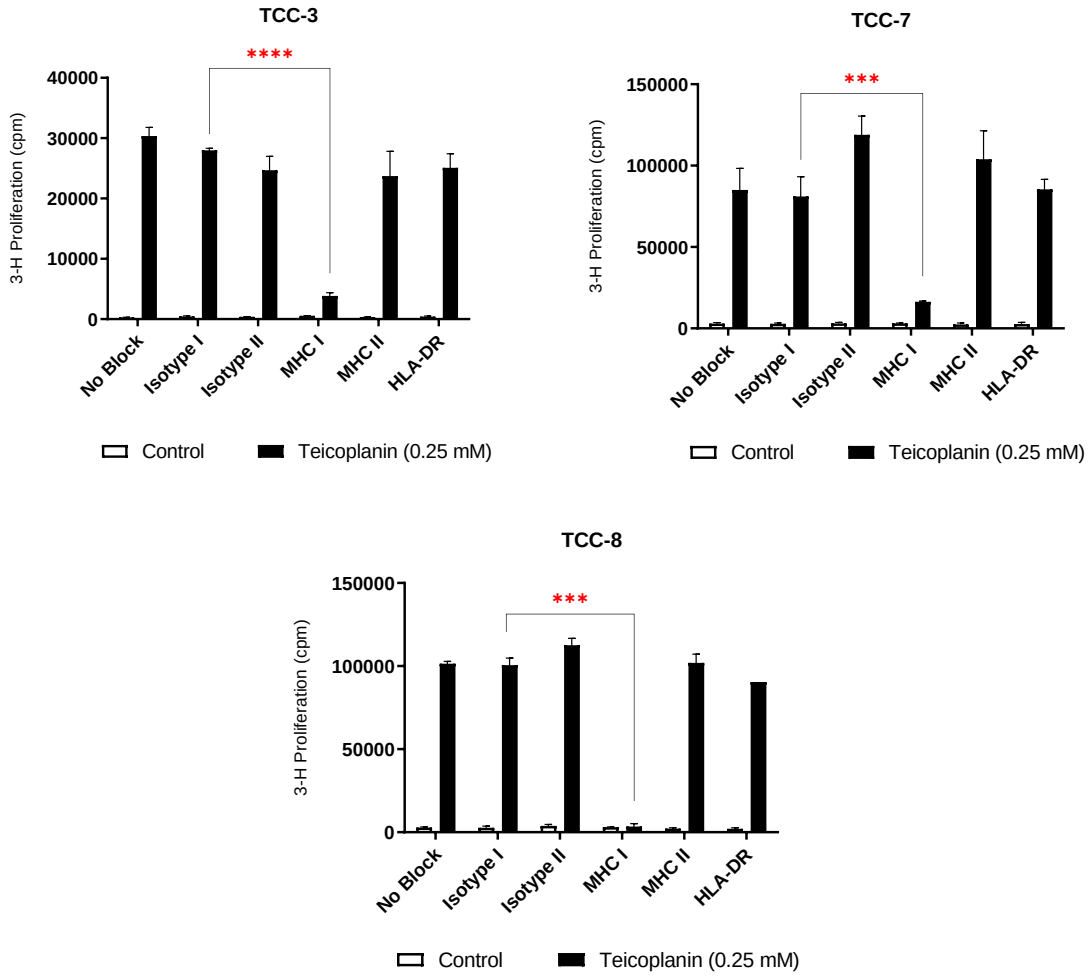


Figure 4.8. HLA restriction of teicoplanin-specific TCCs (n=3). CD8+ expressing TCCs ($5x10^4$ cells/well) were co-cultured with autologous irradiated EBV-transformed B-cells in triplicate and either HLA Class I (10 µg/mL), HLA Class II (10 µg/mL), HLA-DR (10 µg/mL) or corresponding IgG1 and IgG2 isotype controls (10 µg/mL) for 1 h (37 °C, 5% CO_2). Blocked cultures were treated with optimal stimulatory concentrations of teicoplanin (0.25 mM) or R9 cell culture medium for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined using a Mann-Whitney U test (***p< 0.01, ****p<0.0001).

After pre-treatment of both autologous EBV-transformed B-cells and teicoplanin-specific T-cell co-cultures with a panel of HLA blocking antibodies, proliferation following drug exposure was assessed in 3 representative TCCs expressing CD8+ phenotype (Figure 4.8). Proliferation of TCCs was unaffected following class II blockade (HLA-DP, HLA-DQ and HLA-DR) when compared with T-cell responses in the absence of blockade. However, proliferation was observed to be fully attenuated in the presence of MHC class I (HLA-A, HLA-B and HLA-C) blocking antibodies. Proliferative responses in line with basal responses after teicoplanin exposure were elicited after exposure to IgG1 and IgG2 isotype controls

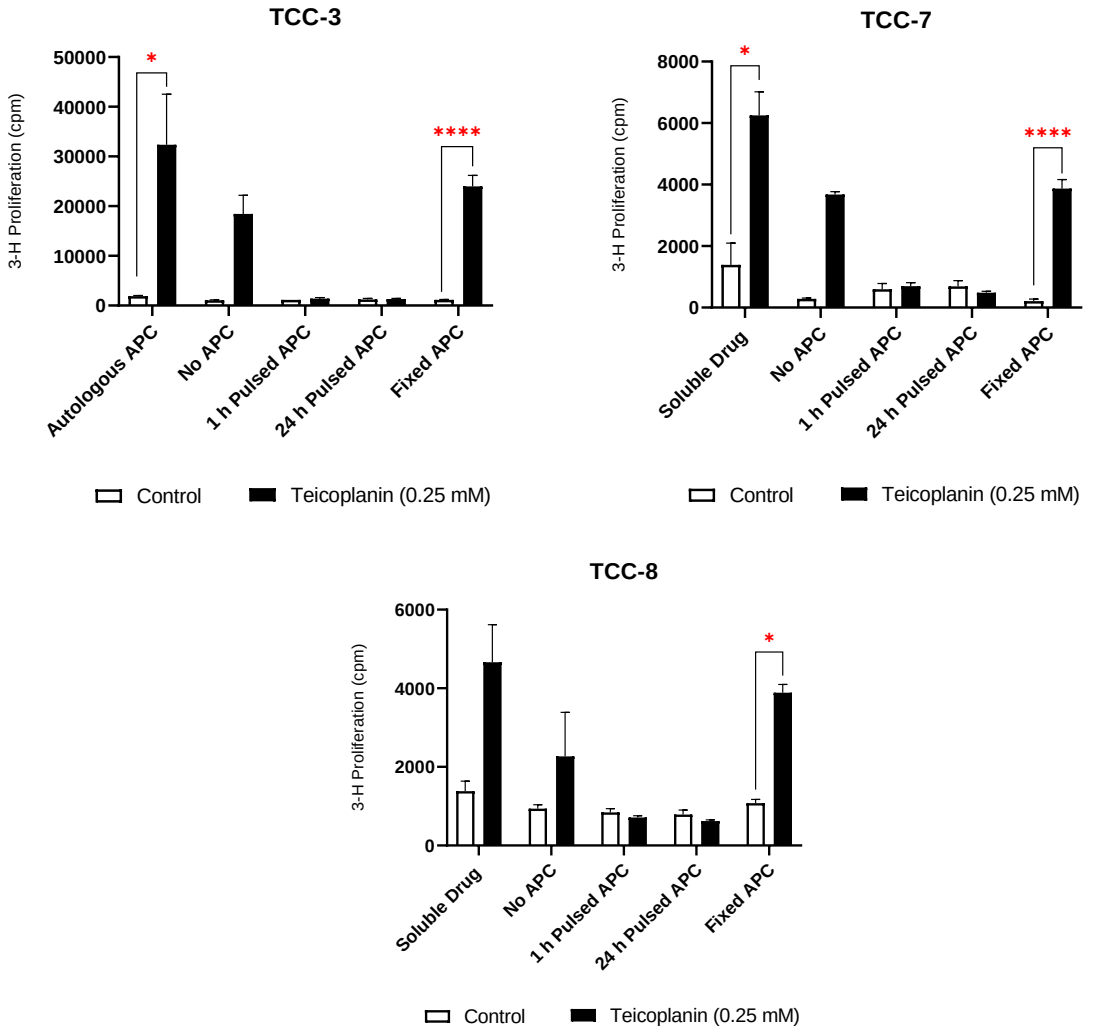


Figure 4.9. Activation pathways of teicoplanin-responsive CD8+ TCCs (n=3). Irradiated autologous EBV-transformed B-cells ($2x10^6$) were pulsed with teicoplanin (0.25 mM) for 1 h and 24 h before washing to remove unbound drug or fixed with glutaraldehyde (25%, 1 µL) for 30 secs before quenching with glycine (1 M, 1 mL) for 45 secs. TCCs were incubated for 48 h (37 °C, 5% CO_2) with both fixed APCs and teicoplanin or pulsed APCs ($1x10^4$ cells/well). Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values after 16 h incubation (0.5 µCi/well). Statistical significance was determined using a Mann-Whitney U test (*p< 0.01, ****p<0.0001).

Autologous EBV-transformed B-cells pulsed with teicoplanin at multiple time-points (1 h and 24 h) did not induce detectable proliferation after 48 h co-culture with teicoplanin-specific TCCs (Figure 4.9). After fixation of EBV-transformed B-cells with glutaraldehyde and blockade of antigen processing pathways, teicoplanin-responsive T-cells demonstrated proliferative capacity after treatment with a co-culture of fixed EBV-transformed B-cells and optimal stimulatory concentrations of teicoplanin. Interestingly, TCCs were still observed to proliferate above background levels without the presence of autologous EBV-transformed B-cells and antigen presentation, although the magnitude of proliferation was considerably lower when compared to proliferative readouts incorporating EBV-transformed B-cells together with soluble drug. Cytokine secretion analysis within the same panel of TCCs revealed IFN-γ release in 3/3 TCCs following EBV-transformed B-cell fixation and teicoplanin exposure (Figure 4.10).

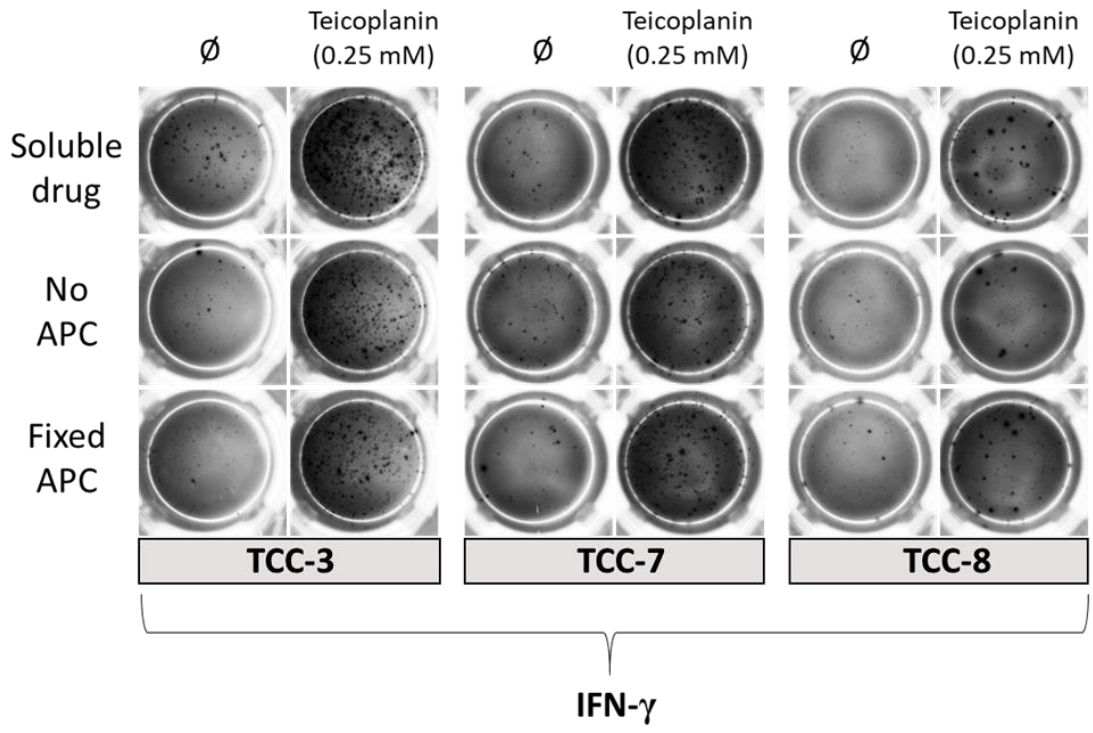


Figure 4.10. Enzyme-linked immunospot (ELISpot) profile after fixation of EBV-transformed B-cells (TCCs; n=3). Autologous EBV-transformed B-cells ($2x10^6$) were fixed with glutaraldehyde (25%, 1 µL) for 30 secs before quenching with glycine (1 M, 1 mL) for 45 secs. TCCs ($5x10^4$) were incubated for 48 h (37 °C, 5% CO_2) with fixed EBV-transformed B-cells and soluble drug or teicoplanin only. Cytokine release was determined for IFN-γ using activated Immobilon-P Membrane 96-well Multiscreen® filter plates pre-coated for the cytokine of interest and ELISpot plates were developed according to the manufactures instructions.

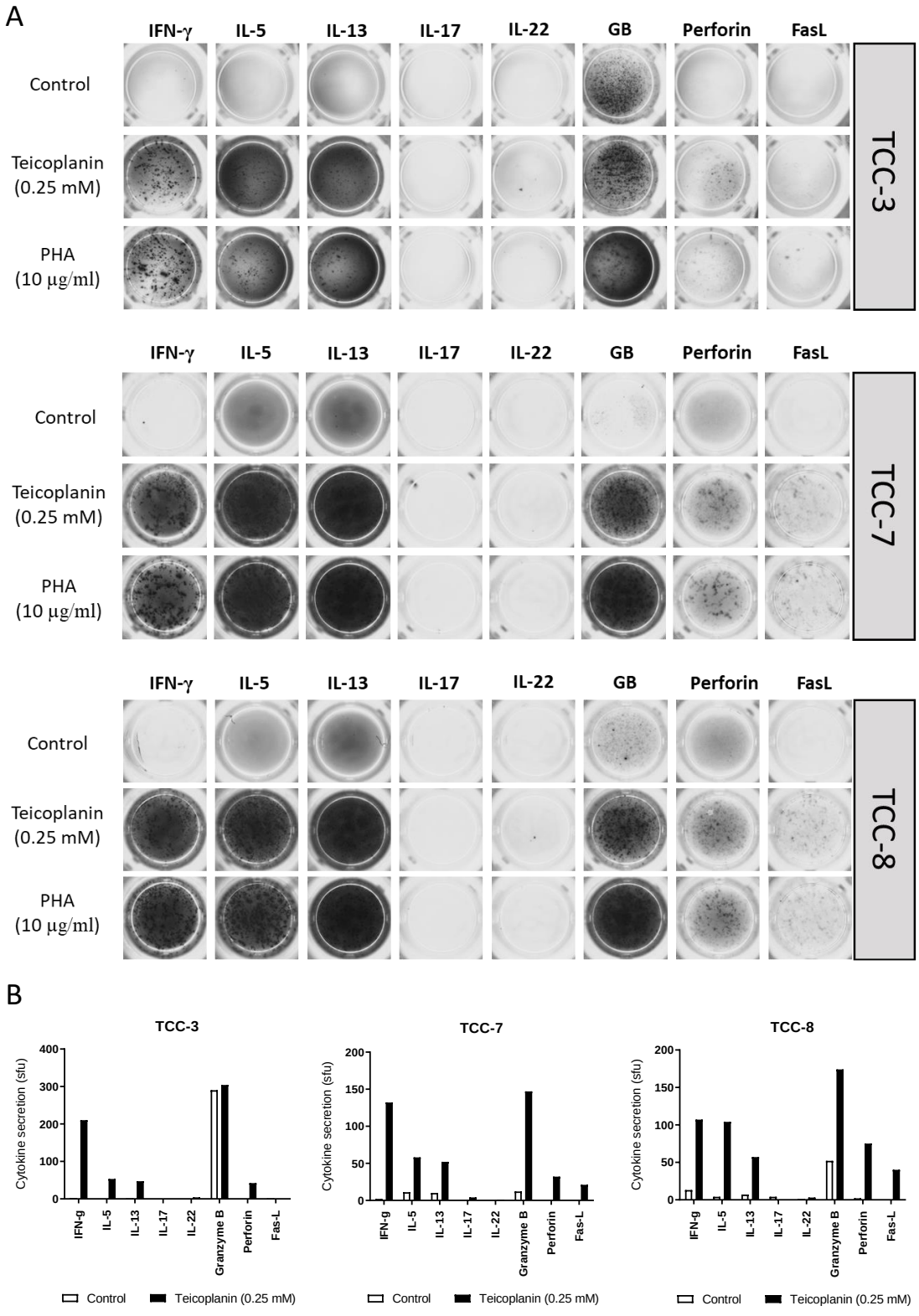


Figure 4.11. A) Cytokine secretion profile of teicoplanin-specific TCCs (n=3) determined by ELISpot readout. TCCs ($5x10^4$ cells) were co-cultured with autologous EBV-transformed B-cells ($1x10^4$ cells) and either teicoplanin (0.25 mM), R9 medium or PHA (10 μg/mL). Cytokine release was determined for IFN-γ, IL-5 IL-13, IL-17, IL-22, granzyme B (GB), perforin and FasL using activated Immobilon-P Membrane 96-well Multiscreen® filter plates pre-coated for cytokines of interest. Secretion profiles were studied after 48 h incubation (37 °C, 5% CO_2) with teicoplanin and ELISpot plates were developed according to the manufactures instructions. **B) Quantitative analysis of cytokine release from teicoplanin-responsive TCCs (n=3).** Spot formation was analysed using an AID ELISpot reader and cytokine secretion was interpreted as spot forming units (sfu).

Representative CD8+ teicoplanin-specific TCCs (n=3) were profiled for secretion of both cytokines and cytolytic molecules by ELISpot, after exposure to singular drug concentrations in addition to R9 medium and PHA (Figure 4.11). Cytokine secretion profiles pertaining to TCC-3, TCC-7 and TCC-8, for which phenotype and function have previously been characterised, followed a similar pattern. Th1 cytokine (IFN-γ) release was observed in addition to the secretion of Th2 cytokines (IL-13 and IL-5). Upon activation with teicoplanin, a complete absence of Th17 (IL-17) and Th22 (IL-22) related cytokines was observed. Additionally, cytolytic molecules, such as granzyme B, perforin and FasL were also found to be released after teicoplanin rechallenge, with granzyme B release detected at high levels in 3/3 TCCs (sfu > 100). PHA successfully induced non-specific cytokine and cytolytic molecule secretion across the full panel within each TCC assessed, with the exception of IL-17 and IL-22.

4.3.6 Teicoplanin-responsive TCCs generated from HLA-A*32:01 healthy donors display complex patterns of cross-reactivity with structurally-related glycopeptide antibiotics.

For the investigation of potential cross-reactivity between teicoplanin-responsive TCCs and structurally related glycopeptide compounds such as vancomycin and daptomycin, proliferation was assessed after drug rechallenge (Figure 4.12). This was conducted in a panel of TCCs generated from a single healthy donor with HLA-A*32:01 carriage with previous characterisation of phenotype and function (HD-33). As expected, memory T-cell responses to the culprit drug (teicoplanin) were associated with a high degree of proliferation. However, 3/3 TCCs displayed significant proliferative cross-reactivity with the lipoglycopeptide, daptomycin, at graded drug concentrations. No cross-reactive T-cells were detected after rechallenge with vancomycin. Statistical significance was determined for each compound at maximal stimulatory concentrations.

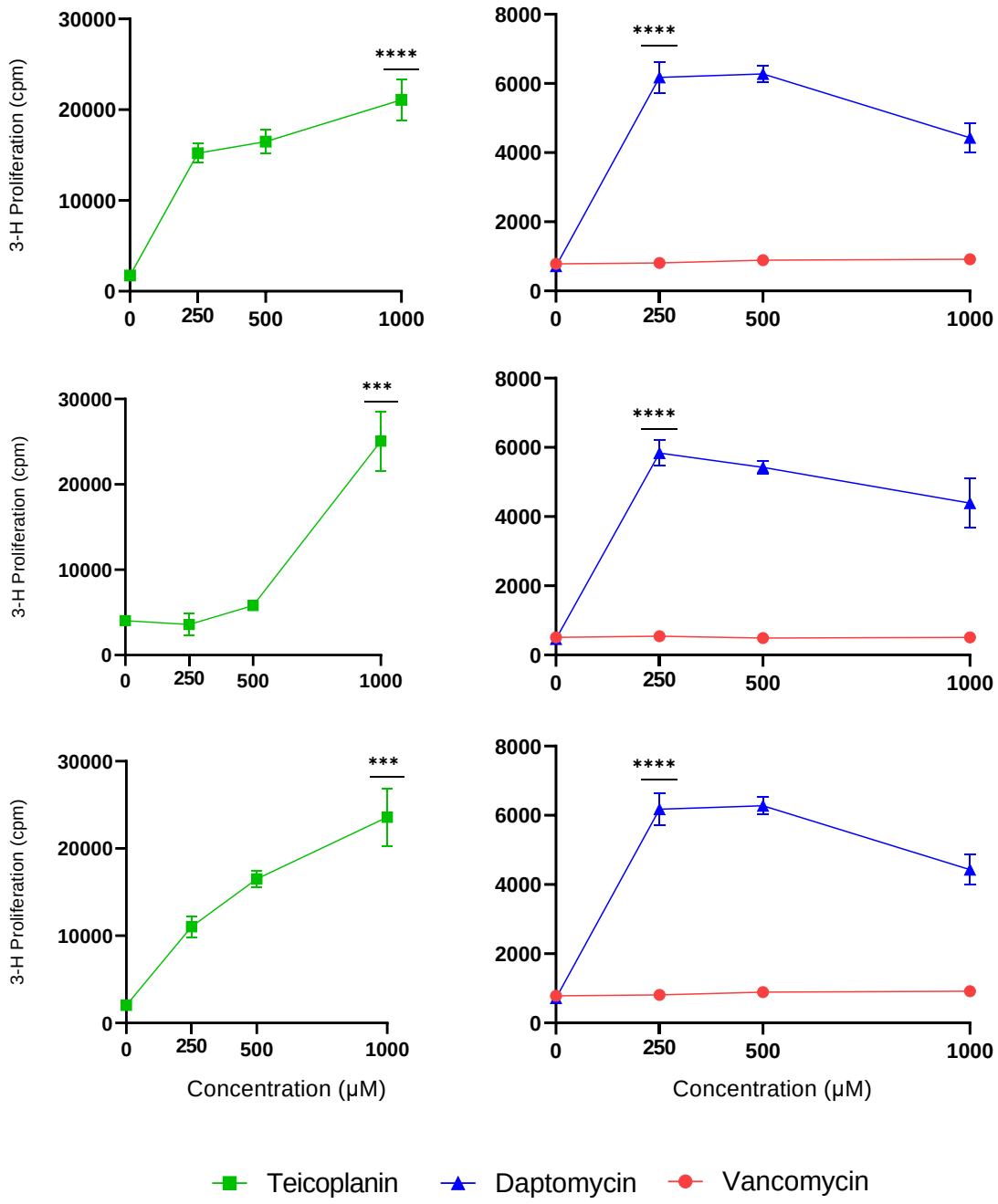


Figure 4.12. Proliferative cross-reactive study of CD8+ teicoplanin-specific TCCs (n=3) generated from healthy donor PBMC positive for HLA-A*32:01 expression (HD-33) with structurally related glycopeptide antibiotics. TCCs ($5x10^4$ cells) were co-cultured with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells) and either teicoplanin (250-1000 µM), vancomycin (250-1000 µM), daptomycin (250-1000 µM) or R9 cell culture medium only (0 µM). Cultures were incubated for 48 h (37 °C, 5% CO_2) before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for a further 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined using a student's *t*-test (***p< 0.001, ****p<0.0001).

4.4 Discussion

Over the past 50 years glycopeptide antibiotics have consistently demonstrated utility for the effective treatment of serious Gram-positive bacterial infections, with usage specifically pertinent for therapeutic intervention against β-lactam antibiotic resistant bacterial strains such as MRSA and *Clostridium difficile* (Campoli-Richards et al., 1990). As a direct result of the increased potency of glycopeptide compounds, the emergence of unwanted and sometimes severe side-effects in the shape of ADRs have become increasingly common, with some studies reporting that more traditionally used naturally occurring glycopeptide compounds such as vancomycin account for 40% of all DRESS cases arising from antibiotic usage (Blumenthal et al., 2019). Furthermore, although less well documented, studies have now implicated teicoplanin within DRESS pathogenesis (Ben Romdhane et al., 2018, Ebrahimpour et al., 2017). This may be aligned with prevalent and sustained administration by clinicians owing to the familiarity associated with such compounds when treatment regimens are initialised. However, efforts have more recently transitioned to the development of safer glycopeptide antibiotics with superior pharmacokinetic properties (Binda et al., 2014).

The new generation of glycopeptide compounds are typically semi-synthetic and encompass derivatives of teicoplanin, with the core structure of the molecule often incorporated due to its advantageous kinetic properties arising from the presence of lipophilic side chains (Ashford and Bew, 2012). However, vancomycin is still seen by many as the 'flagship' glycopeptide, with strong genetic associations now documented between incidences of vancomycin-induced DRESS and carriage of HLA-A*32:01 (Konvinse et al., 2019). Moreover, such associations point towards involvement of the adaptive immune response within DRESS pathogenesis, further reinforced by studies elucidating T-cell involvement (Chapter 3). Indeed, interactions between vancomycin and immunological receptors that induce stimulation have now been characterised to hinge upon HLA class I presentation, with CD8+ T-cell responses evoked by

direct pharmacological interactions (Ogese et al., 2021). Cross-reactive responses have now been identified between vancomycin and structurally-related glycopeptide antibiotics such as teicoplanin and daptomycin. Such cross-reactivity with teicoplanin has primarily been documented within isolated clinical case studies, with severe cross-reactive hypersensitive responses observed after previous vancomycin hypersensitivity and subsequent teicoplanin administration (Hsiao et al., 2010). Additionally, as detailed in Section 3.4, *in vitro* T-cell assays have recently elucidated complex patterns of proliferative cross-reactivity between vancomycin-reactive TCCs and both teicoplanin and daptomycin, even when initial drug specificity has been lost.

To investigate the intrinsic immunogenic potential of cross-reactive glycopeptide compounds in addition to the role of HLA-A*32:01 within glycopeptide-induced immune stimulation, a variety of PBMC and T-cell based assays were performed. To date, limited *in vitro* work has been carried out exploring the activity of glycopeptide antibiotics such as teicoplanin and daptomycin. PBMC toxicity assays assessing proliferative inhibition after drug exposure and mitogen stimulation revealed that daptomycin was well tolerated at 1 mM, with toxicity and proliferative inhibition detected for teicoplanin at dosage exceeding 250 µM (Figure 4.1). Such assays provide valuable information regarding the toxicity of test compounds and inform on suitable dosage, with the aim of evoking maximal T-cells responses before toxicity is induced. The optimal drug concentrations of teicoplanin (250 µM) and daptomycin (500 µM) used *in vitro* do not directly correlate with the concentrations found at therapeutic doses within the blood. Approximately 10-20 µM is typically required within blood plasma to exert on-target pharmacological effects in patients when applied to glycopeptide antibiotics (Tunkel et al., 2004, Wysocki et al., 2001). Although the optimal dosage regarding *in vitro* use of glycopeptide compounds in this study is approximately 10-20 fold higher than therapeutic concentrations, previous preliminary studies within the group have shown that glycopeptides are capable of activating T-cells at much lower, more therapeutically relevant doses of between 5-50 µM.

To evaluate the potential for glycopeptide-induced stimulation of PBMC cultures LTT assays were performed for which positive lymphocyte transformation was identified in 2/5 healthy donors positive for HLA-A*32:01 expression after exposure to teicoplanin (Figure 4.2). These data suggests, albeit weakly, potential involvement of HLA-A*32:01 for glycopeptide-induced immune stimulation. To further investigate the role of HLA-A*32:01 and existence of preferential allelic interaction within the context of diagnostic assays it would necessary to explore the induction of T-cell responses within the PBMC of healthy donors negative for HLA-A*32:01 carriage. The presence of positive lymphocyte transformation in healthy drug naïve donors potentially supports the notion that glycopeptide compounds, due their size, are capable of acting as peptide mimetics. It is entirely possible that the observed lymphocyte stimulation may manifest in a non-specific manner during which large complex glycopeptide compounds assume to role of superantigens with stimulatory potential and immunoreactive properties (Li et al., 1999).

T-cell priming assays incorporating naïve T-cells and professional antigen presenting cells in the form of autologous DCs were used to assess the inherent immunostimulatory potential of teicoplanin and daptomycin (Figure 4.3, 4.4). Studies were conducted in HLA-typed donors (HLA-A*32:01 positive), with significant priming observed in 1/5 donors after rechallenge with either teicoplanin or daptomycin. The detection of naïve T-cell responses after initial priming with teicoplanin or daptomycin suggests these compounds possesses immunogenic potential. Consequently, progression to T-cell cloning experiments was deemed necessary to investigate the nature of T-cell responses to both teicoplanin and daptomycin. To explore the possibility of preferential interaction of both teicoplanin and daptomycin with HLA-A*32:01 more in depth study will be required during which primed naïve T-cell responses are induced within individuals positive and negative for allelic expression. Additionally, repetitive PBMC stimulation and proliferative measure upon rechallenge would represent a logical next step

due to high throughout capabilities allowing for study of an extended panel of donors with and without HLA-A*32:01 carriage (Engler et al., 2004).

Teicoplanin-reactive TCCs were successfully generated from CD8+ enriched populations in 3/3 donors positive for HLA-A*32:01 expression and T-cells were confirmed as drug-responsive following exposure to graded drug concentrations (Figure 4.5). The ability to generate drug-specific T-cells with high avidity for teicoplanin upon drug rechallenge gives credence to T-cell involvement within teicoplanin-induced DRESS reactions, aligned with reports of severe clinical cross-reactivity with vancomycin for which T-cell involvement has already been determined (Miyazu et al., 2016, Ogese et al., 2021). Although initial specificity testing eluded to the presence of teicoplanin-specific TCCs from bulk populations enriched for CD4+ expressing T-cells, only TCCs positive for CD8+ expression with true teicoplanin specificity were generated in this study (Figure 4.7). To explore potential reasoning for this it is important to consider that proliferation data pertaining to 'CD4+' TCCs are from populations merely enriched for CD4+ T-cells, but not phenotyped at this early point in the study. TCCs are only phenotyped for expression of CD4+ or CD8+ co-receptors after a confirmatory dose-dependent response analysis yields a positive result in relation to the compound of interest (teicoplanin). Therefore, as 100% CD4+/CD8+ separation is not obtained using magnetic beads (positive selection), it is entirely plausible that cells proliferating within CD4+ enriched populations may actually display a CD8+ phenotype and vice versa. In addition to this, TCCs generated in populations enriched for CD8+ gave a substantially higher stimulatory index, indicative of true proliferative responses to drug. Nevertheless, dose-response tests performed on potential 'CD4+' teicoplanin-specific TCCs after mitogen driven expansion yielded a negative result before phenotyping was conducted.

The ability to generate teicoplanin-responsive TCCs from individuals negative for HLA-A*32:01 carriage will be crucial to our understanding of the relationship between teicoplanin and the HLA-A*32:01 haplotype, in terms of any preferential binding. To answer this question and to investigate the specificity of teicoplanin for HLA-A*32:01, further T-cell cloning experiments with a focus on healthy donors negative for HLA-A*32:01 carriage will need to be performed and ultimate comparison made between T-cell responses utilising TCCs acquired from individuals with and without allelic expression. However, at this point it should be stated that currently there is no genetic association between teicoplanin and HLA-A*32:01 expression. It should also be pointed out that when considering the physiological relevance of HLA expression, such allelic associations are not sole critical determinants of disease pathogenesis after drug exposure. In addition to prediction with regards to glycopeptides such as vancomycin (20% PPV), this has been clearly demonstrated in the case of abacavir for which approximately 50% of individuals with HLA-B*57:01 carriage will not progress to abacavir hypersensitivity reaction (Konvinse et al., 2019, Mallal et al., 2008). This phenomenon could be due a myriad of contributing factors, with the concept of immune dysregulation now a burgeoning avenue of interest currently being explored within the field of immunopharmacology (Hammond et al., 2022, Naisbitt et al., 2019). Additionally, ongoing work involving the recruitment of teicoplanin hypersensitive patients positive and negative for HLA-A*32:01 expression will be a focal point moving forward in order to delineate T-cell involvement within the pathogenesis of teicoplanin-induced DRESS.

Analysis of cytokine profiles from a representative panel of CD8+ expressing teicoplanin-reactive TCCs generated from HD-33, revealed secretion of both Th1 (IFN-γ) and Th2 (IL-13 and IL-5) related cytokines in addition to the release of cytolytic and cytotoxic molecules such as perforin, granzyme B and FasL (Figure 4.11). This is consistent with cytokine profiles relating to TCCs generated from HLA class I associated compounds, such as abacavir (Monshi et al., 2013). The release of cytokines with cytotoxic action have been linked with DRESS

pathogenesis, with immunohistochemical studies specifically implicating IFN-γ, IL-5, granzyme B and FasL with ceftriaxone-induced DRESS following skin biopsy examination (Hansel et al., 2017). Furthermore, IL-5 secretion has historically been used as a biomarker for DRESS onset due to its prominent role within eosinophil recruitment (Choquet-Kastylevsky et al., 1998). Mechanistic study of CD8+ expressing representative teicoplanin-responsive TCCs generated form HLA-A*32:01 donors suggest a processing independent mechanism of immune activation that hinges on drug presentation by direct interactions with HLA class I molecules (Figure 4.8, 4.9). The observed direct pharmacological interactions with MHC complexes is consistent with the p-i concept of T-cell activation (Pichler, 2002), with findings concordant with previous mechanistic studies relating to T-cell responses after vancomycin exposure for which large glycopeptide compounds have been postulated to possess the capacity to mimic and displace HLA binding peptides (Chapter 3.4).

TCCs exhibited cross-reactivity with the cyclic lipoglycopeptide, daptomycin, at graded drug concentrations (Figure 4.12). The observed proliferative T-cell cross-reactivity between CD8+ teicoplanin-specific TCCs generated from healthy drug naïve individuals (HLA-A*32:01 positive) to daptomycin emphasises the complex patterns of cross-reactivity demonstrated between glycopeptide antibiotics. Cross reactive T-cell responses may be explained by the structural resemblance between teicoplanin and daptomycin, specifically the integration of a hydrophobic lipid chain within their respective chemical structures, as illustrated in Chapter 1.7.1 (Nicolaou et al., 1999, Silverman et al., 2003). The detection of proliferative cross-reactive T-cell responses to daptomycin, observed across teicoplanin and vancomycin-responsive TCCs accentuates the issues encountered with daptomycin-specific TCC generation, as daptomycin is clearly capable of T-cell stimulation. Nevertheless, drug-reactive TCCs were consistently evasive following the conduction of large-scale multi-donor T-cell cloning experiments. Reciprocal cross-reactive T-cell responses have been reported in Chapter 3 between vancomycin-specific TCCs and teicoplanin. Interestingly, cross-reactive T-cell

responses were absent after exposure of teicoplanin-specific TCCs to vancomycin. From a structural standpoint vancomycin contains a heptapeptide chain that crucially comprises of a disaccharide, composed of vancosamine and glucose, substituted for the fatty acid lipid tail found on both teicoplanin and daptomycin molecules. This potentially explains why some teicoplanin-specific T-cells are capable of proliferative responses when exposed to daptomycin but not vancomycin.

One particular approach to explore the nature of glycopeptide-induced cross-reactive T-cell responses involves the study of metabolic parameters of immune activation, such as glycolysis, which may provide greater sensitivity for the determination of T-cell activation thresholds upon drug presentation and subsequent clonal activation. However, to determine the specificity of teicoplanin for HLA-A*32:01, further cloning experiments focusing on individuals negative for HLA-A*32:01 expression will need to be conducted in addition to *in silico* molecular docking studies and potential GWAS analysis with an emphasis on patients presenting with clinically defined teicoplanin-induced DRESS. Supplementary genetic study and functional T-cell investigation following HLA-glycopeptide binding will be a necessity to elucidate full pathways of glycopeptide cross-reactivity alongside the extent of preferential interactions with HLA-A*32:01. This line of enquiry will help to ultimately predict potential susceptibility to severe cross-reactive and life-threatening immune stimulation and enhance patient safety following glycopeptide antibiotic administration.

5. Characterisation of the phenotype and functionality of T-cells in patients presenting with vancomycin hypersensitivity.

Contents

5.1 Introduction.

Immune-mediated hypersensitivity to otherwise efficacious and well tolerated therapeutic compounds have been characterised to a multitude of drug classes. These include β-lactam antibiotics, sulfonamides, and anticonvulsants, with the involvement of adaptive responses and specifically T-cells well defined in healthy donors and patients alike (Alzahrani et al., 2017, Bell et al., 2013, Solensky, 2003, Naisbitt et al., 2003a, Naisbitt et al., 2001). Vancomycin, amongst other glycopeptide compounds has more recently entered the equation, providing cause for concern among clinicians and patients due to widespread deployment for severe Gram-Positive bacterial infection. The potential for adverse outcomes is often synonymous with exposure to highly potent treatment regimens against severe infection. These unwanted and often unpredictable adverse events can either occur immediately after drug administration or clinical manifestations can be delayed in nature following mediation from the adaptive immune system and involvement of T-cells (Pavlos et al., 2015). These compounds have been implicated within the pathogenesis of severe cutaneous adverse reactions (SCARs), with a growing body of evidence now suggesting a causal link between vancomycin administration and clinical manifestation of drug reaction with eosinophilia and systemic symptoms (DRESS) syndrome (Littlehales et al., 2018, Hewitson, 2019).

DRESS is archetypal of delayed-type hypersensitivity, with clinical presentation typically manifesting between 2-8 weeks post exposure to the offending drug (Bocquet et al., 1996). Disease progression can impact multiple organ systems such as the kidneys and liver, with clinical manifestations characterised by the development of eosinophilia often combined with symptoms including fever, pruritus and leukocytosis (Sullivan and Shear, 2001, Cacoub et al., 2011). Although the delayed nature of onset points towards immune modulation, disease pathogenesis remains relatively poorly understood from an immunological perspective. Thus far, adaptive involvement has been implicated with the emergence of IL-5 as a key phenotypic

marker of DRESS arising from involvement within both pro-inflammatory cascades and eosinophil recruitment (Choquet-Kastylevsky et al., 1998). Furthermore, it has been postulated that cytokine release and immune involvement within DRESS pathogenesis most likely arises following the activation of drug-specific CD4+ and CD8+ T-cells (Mauri-Hellweg et al., 1995). Viral reactivation and CD8+ T-cell dysregulation has also been implicated within DRESS pathogenesis (Musette and Janela, 2017). The involvement of viral-specific T-cells is now widely accepted as a potential cause of DRESS, with delayed antiviral CD8+ T-cell responses observed in DRESS patients infected with human herpes viruses 6 and 7 (HHV-6 and HHV-7) and cytomegalovirus (Descamps et al., 2001, Seishima et al., 2006). Culprit drugs implicated within DRESS are diverse, although disease onset arising from the administration of glycopeptide compounds now contribute to widespread prevalence. Indeed, vancomycin has been outlined as the culprit drug in 40% of DRESS cases arising from the use of antibiotics (Blumenthal et al., 2019).

Only recently has further investigation between vancomycin exposure and DRESS manifestation eluded to the possibility of genotypic involvement. In 2019, Konvinse and colleagues first reported a genetic association shared among individuals presenting with clinically diagnosed vancomycin-induced DRESS. More specifically, a genome-wide association study (GWAS) suggested a strong association between expression of the HLA-A*32:01 allele and vancomycin-induced DRESS in European populations (Konvinse et al., 2019). Although vancomycin-induced adverse events have previously been reported and can actually be a common occurrence, the vast majority of incidences can be characterised as ‘pseudo’ allergic reactions arsing immediately following rapid infusion resulting in mast cell degranulation and histamine release, typically manifesting as red man syndrome (Sivagnanam and Deleu, 2003). However, this study represents the first of its kind to suggest human leucocyte antigen (HLA) involvement and by extension implicate adaptive immune pathways and potential T-cell mediation.

Previous work has elucidated the role of T-cells within vancomycin-induced DRESS, although currently this has been limited to healthy donor models, as detailed in Chapter 3. Interactions between vancomycin and T-cells have now been characterised to hinge upon HLA class I presentation, with T-cell responses evoked by direct, non-covalent pharmacological interactions. Furthermore, stimulation was observed to be associated with a multitude of cytokine and cytolytic molecule secretion, with clinical cross-reactivity now replicated within *in vitro* assays (Ogese et al., 2021). Moving forward, an emphasis must be placed on characterising the immunopathomechanisms of vancomycin-mediated T-cell activation from a position of greater physiological relevance, achieved through isolation of drug-specific T-cells from patients presenting with confirmed vancomycin hypersensitivity.

To further determine the cross-reactive tendency of glycopeptide antibiotics within polypharmic clinical settings, translational approaches must be employed studying T-cell responses in vancomycin hypersensitive patients after exposure to other, widely used, glycopeptide antibiotics. To this end, studies performed by Nakkam et al. have attempted to profile cross-reactivity within *ex vivo* experiments using peripheral blood mononuclear cells (PBMC) isolated from patients with 'probable' vancomycin-induced DRESS. In this study, for which IFN-γ release was assessed as a marker of immunological cross-reactivity, cytokine secretion was observed in 13-27% of patients after exposure to teicoplanin and telavancin respectively, with culprit drug exposure expectedly inducing a dose-dependent response in all patients (Nakkam et al., 2020). Interestingly, HLA-typing among patients (HLA-A*32:01) displaying cross-reactive responses revealed shared expression of a HLA class II haplotype, with virtual docking analysis suggesting potential HLA-DQ interaction within immunological cross-reactivity (Nakkam et al., 2021).

In order to examine the role of T-cells within DRESS pathogenesis, our work will focus on the isolation of vancomycin-specific T-cells from the peripheral blood of vancomycin

hypersensitive patients, in addition to exploring immunological pathways of vancomycin-mediated T-cell activation. Initially, assessment will be made with regards to the utility of first line diagnostic assays, such as the lymphocyte transformation test (LTT), for the detection of immune stimulation and subsequent diagnosis of hypersensitivity attributed to vancomycin exposure. Additionally, following the conduction of T-cell cloning experiments it will be valuable to study pathways of T-cell activation and the presence of HLA-restriction to elucidate preferential interactions between drug and MHC, alongside the phenotypic and functional features of CD4+ and CD8+ T-cells within DRESS manifestation.

5.2 Aims.

1) To assess the sensitivity of diagnostic assays, evaluating lymphocyte proliferation (LTT) and cytokine secretion (IFN-γ), for the detection of vancomycin-mediated immune stimulation within PBMC cultures isolated from patients presenting with suspected vancomycin hypersensitivity.

2) To generate vancomycin-specific T-cell clones (TCCs) from vancomycin hypersensitive patients positive for HLA-A*32:01 carriage, by serial dilution.

3) To examine phenotypic and functional characteristics of vancomycin-responsive TCCs isolated from vancomycin hypersensitive patients for comparison with healthy donor models and association with DRESS pathogenesis.

4) To determine the cross-reactive potential of CD4+ and CD8+ vancomycin-responsive TCCs generated from hypersensitive patients expressing HLA-A*32:01.

5.3 Results.

5.3.1 Diagnostic LTT assays detected positive lymphocyte transformation in vancomycin hypersensitive patients following drug rechallenge.

PBMCs were isolated from the venous blood of vancomycin hypersensitive patients by density gradient centrifugation before cryopreservation, as described in Section 2.3. Diagnostic LTT assays were conducted using cryopreserved PBMC of 9 patients presenting with suspected vancomycin hypersensitivity. Proliferative lymphocyte transformation for each individual following vancomycin rechallenge is documented in Table 5.1.

Table 5.1. Maximal stimulatory index (SI) and average counts per minute (cpm) values following conduction of LTT assays in vancomycin hypersensitive patients (n=9). Diagnostic LTT assays were performed according to the protocol described in Section 2.7. PBMCs were exposed to graded vancomycin concentrations (0 – 1000 µM) and maximal SI values are given by [average maximal cpm drug treated wells / average cpm control wells]. SI > 1.5 were deemed to be positive and values for each test culture were interpreted as either weak (1.5 > SI < 2.5), moderate (2.5 > SI < 4), strong (4 > SI < 10) or extreme (SI > 10).

Patient No.	*Control (cpm)*	*Vanc (cpm)*	*Maximal SI*	*Dose (µM)*	*SI > 1.5*	*Intensity*
1	312	15410	49.40	500	Y	Extreme
2	278	174	0.63	N/A	N	No response
3	1186	4442	3.75	100	Y	Moderate
4	1041	1954	1.88	100	Y	Weak
5	4497	7191	1.60	500	Y	Weak
6	1549	2476	1.60	500	Y	Weak
7	2960	50118	16.93	500	Y	Extreme
8	445	16563	37.22	500	Y	Extreme
9	333	20119	60.36	1000	Y	Extreme

Diagnostic LTT assays conducted on the cryopreserved PBMC cultures of 9 vancomycin hypersensitive patients revealed positive proliferative lymphocyte transformation (SI > 1.5) in 8/9 individuals tested (Table 5.1). Additionally, extreme proliferative responses (SI > 10) were detected in 4/9 patients (1, 7, 8, and 9). Across the individuals assessed, 5/8 patients exhibiting positive lymphocyte transformation produced maximal SI values at vancomycin concentrations of 500 µM.

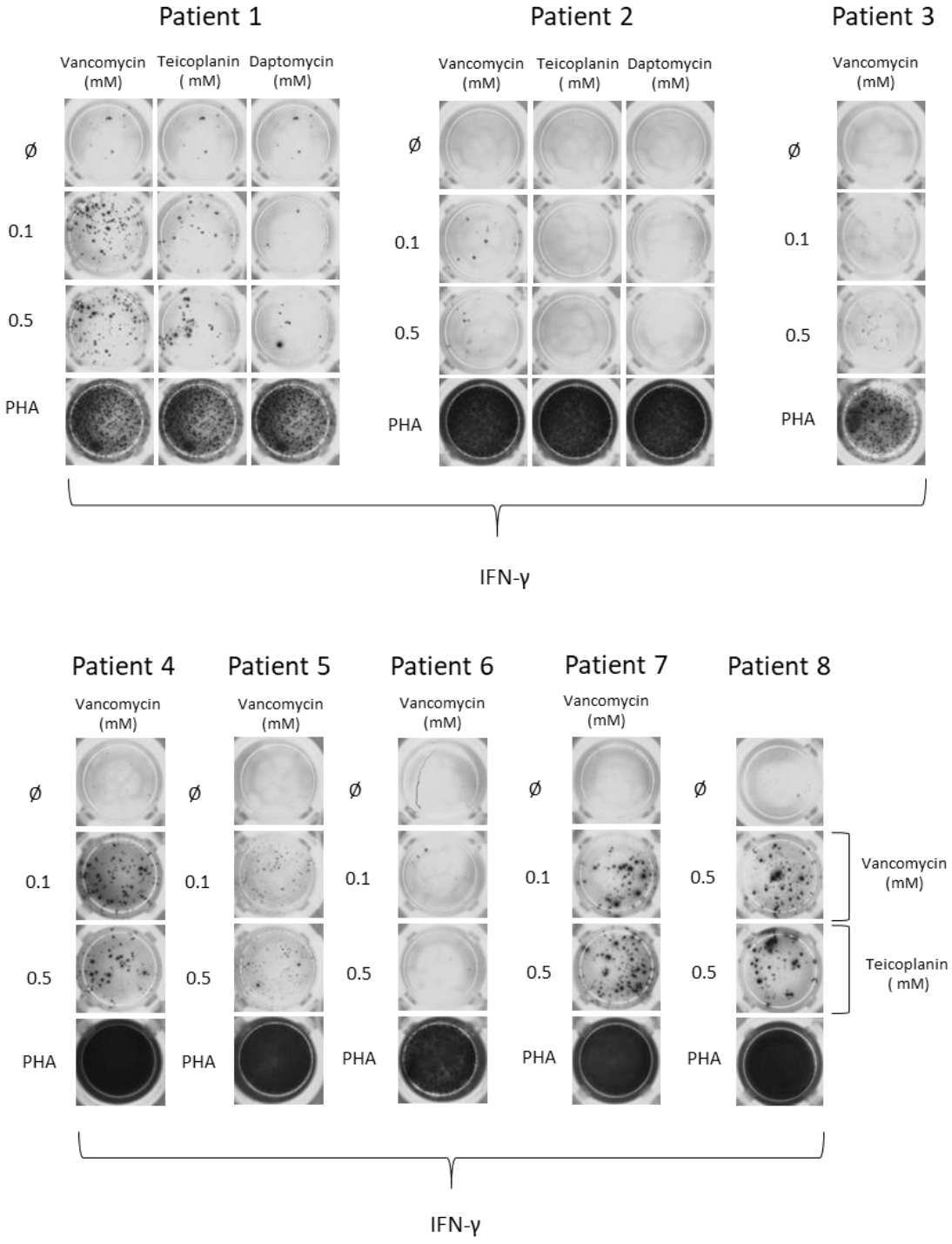


Figure 5.1. LTT assay profiling cytokine release after vancomycin, teicoplanin or daptomycin exposure in vancomycin hypersensitive patient PBMC (n=8). PBMC from vancomycin hypersensitive patients were isolated by density gradient centrifugation and diluted in R9 cell culture medium to achieve a seeding density of $5x10^6$ cells/mL. PBMC were plated at $5x10^5$ cells/well in singlet cultures and exposed to graded concentrations of vancomycin (0-500 µM), teicoplanin (0-500 µM), daptomycin (0-500 µM or PHA (10 µg/mL) for 48 h (37 °C, 5% CO_2). IFN-γ secretion was determined by enzyme-linked immunospot (ELISpot) assay. Spot formation was indicative of cytokine release and developed ELISpot plates were imaged using an AID ELISpot reader.

In addition to studying lymphocyte transformation after vancomycin exposure using proliferative readouts, cytokine secretion was also studied in a panel of hypersensitive patients (Figure 5.1). In total, 8/9 patients initially tested for proliferative responses after vancomycin rechallenge (Table 5.1) were further examined for IFN-γ release, due to low PBMC yields associated with patient 9. Cumulatively, IFN-γ secretion was detected in 7/8 patients presenting with vancomycin hypersensitivity after vancomycin rechallenge at concentrations of 100 and 500 µM. Due to limitations regarding cell numbers, arising from small volumes of venous blood received for the majority of patients, cross-reactivity to structurally similar glycopeptides was only studied in 3 patients (patients 1, 2 and 8). Cross-reactive IFN-γ responses were not observed across each donor tested following exposure to daptomycin. However, 2/3 patients tested for cross-reactivity with teicoplanin (patients 1 and 8) produced a positive result. In all PBMC cultures (n=8) the addition of PHA successfully induced non-specific lymphocyte transformation.

5.3.2 Vancomycin-responsive TCCs can be successfully generated from the PBMC of hypersensitive patients.

Certain vancomycin hypersensitive patients, for which diagnostic LTT assays had previously been performed (Figure 5.1), were further studied using T-cell cloning experiments. Individuals were selected for functional analysis according to inclusion criteria that was predominantly contingent on sample availability, specifically PBMC confluency and viability. As a result, a total of 6 vancomycin hypersensitive patients were identified for progression within T-cell cloning assays (Figure 5.2) due to the abundance of cryopreserved PBMC associated with each individual, sufficient for bulk PBMC culture and Epstein-Barr virus (EBV)-transformed B-cell generation.

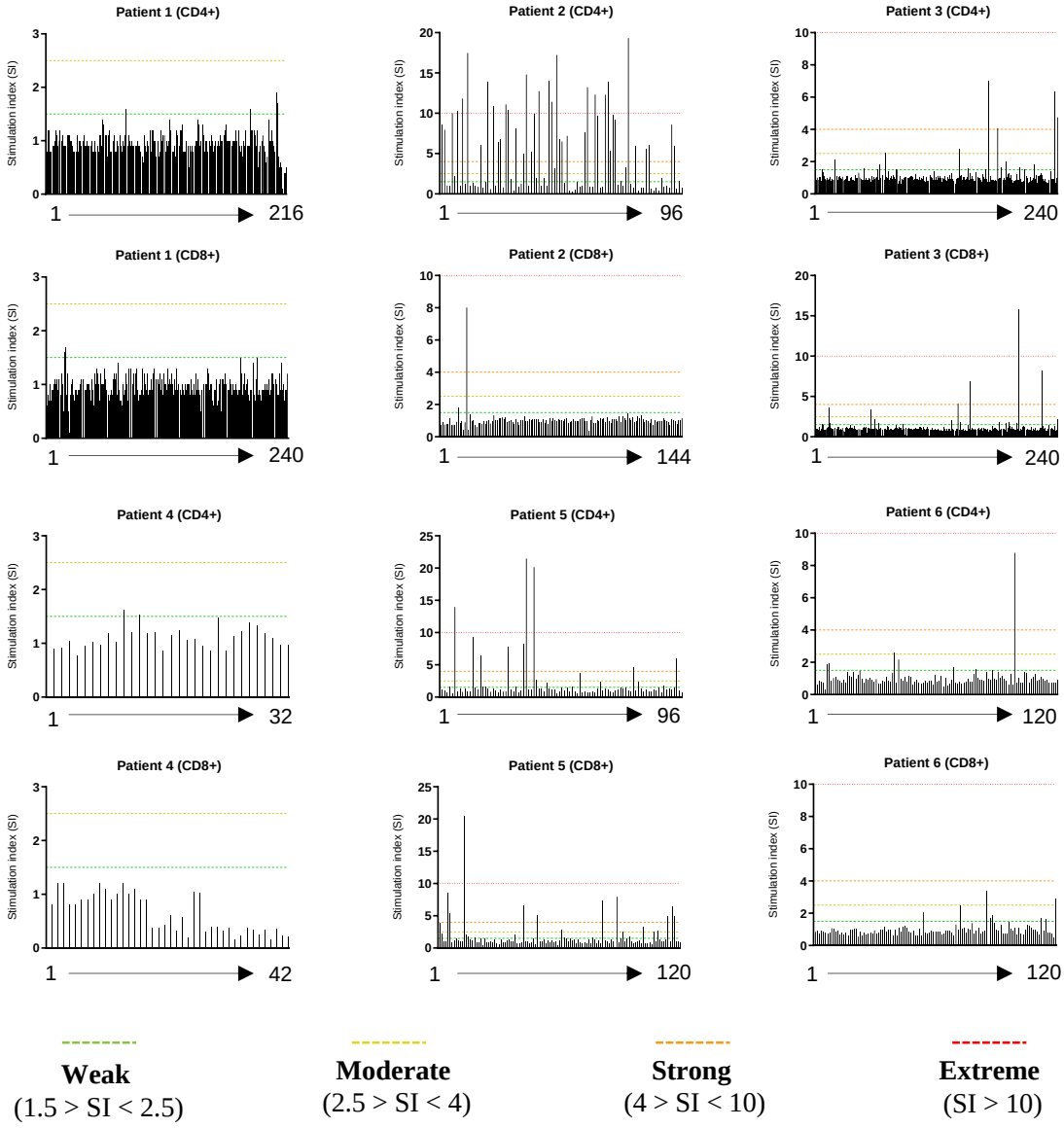


Figure 5.2. Generation of vancomycin-specific TCCs from PBMC enriched for CD4+ and CD8+ T-cells in vancomycin hypersensitive patients by serial dilution (n=6). Bulk PBMC cultures were seeded at $1x10^6$ PBMC/well with graded concentrations of vancomycin (0.1-0.5 mM) and incubated (37 °C, 5% CO_2) for 14 days. T-cells were enriched for CD4+ or CD8+ expressing populations by MACS separation and transferred to 96-well U-bottomed plates (1 T-cell/well average) with irradiated allogenic PBMC ($5x10^4$ cells/well) and R9 medium supplemented with IL-2 (200 U/mL) and PHA (5 µg/mL). Serial dilution protocol was performed as previously described, with test cultures rechallenged with vancomycin (0.5 mM) or R9 medium only, in the presence of irradiated autologous EBV-transformed B-cells ($1x10^4$ cells/well). Testing was performed over 4 wells in duplicate conditions and cultures were incubated for 48 h (37 °C, 5% CO_2) and pulsed with tritiated [^{3}H]-thymidine (0.5 µCi/well) for the final 16 h of incubation. Readouts were initially interpreted as cpm and converted SI values given by; [average drug treated wells / average control wells]. SI values for each test culture were interpreted as either weak (1.5 > SI < 2.5), moderate (2.5 > SI < 4), strong (4 > SI < 10) or extreme (SI > 10).

After testing monoclonal test cultures enriched for CD4+ or CD8+ T-cells for proliferative responses to vancomycin, potential drug-specific TCCs were identified in 4/6 patients (Figure 5.2). Following CD4+ T-cell enrichment, large numbers of drug-specific TCCs were generated

from patient 2, with > 50% of TCCs tested (96) exhibiting specificity for vancomycin after drug rechallenge. Furthermore, in the same patient approximately 20% of TCCs displaying initial specificity were categorised as 'extreme' responders (SI > 10). Additionally, considerable numbers of potential vancomycin-responsive were observed in both CD4+ and CD8+ enriched test cultures generated from patients 3, 5 and 6 (Figure 5.3). Following analysis of test cultures, a degree of variability can be observed within the number of TCCs generated for specificity testing. This is clearly evident for patient 4, for which low numbers of between 32-42 TCCs were examined for drug specificity, compared with more substantial test cultures associated with patients 1 and 3 (200+). Patient ID numbers directly correspond to patients assessed by LTT in Section 5.3.1.

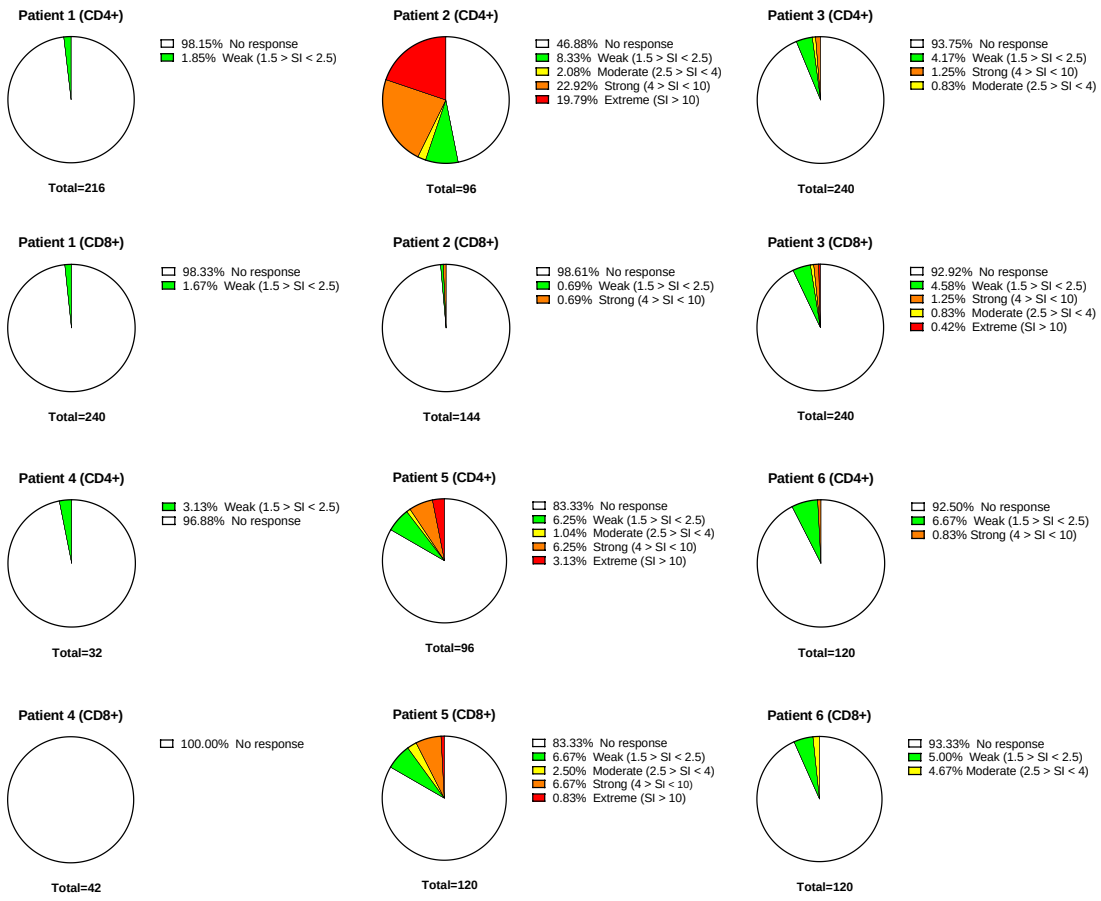


Figure 5.3. Index of stimulation analysis of initial TCC test cultures generated from vancomycin hypersensitive patients (n=6). SI values were given by [average cpm drug treated wells / average control wells]. Test cultures displaying SI values > 1.5 were deemed to be drug-specific TCCs. SI values for each test culture were interpreted and categorised as either no response (SI < 1.5), weak (1.5 > SI < 2.5), moderate (2.5 > SI < 4), strong (4 > SI < 10) or extreme (SI > 10).

Following the generation of vancomycin-specific TCCs from the PBMC of vancomycin hypersensitive patients it was essential to confirm HLA allele expression via genetic analysis in order to establish connections between disease pathogenesis and HLA carriage. Classical HLA-typing was performed at 3x resolution by the HistoGenetics laboratory (New York, USA) on DNA isolated from patient PBMC (patients 1-6). HLA typing was conducted on patient samples for which PBMC stocks were sufficient to facilitate T-cell cloning experiments.

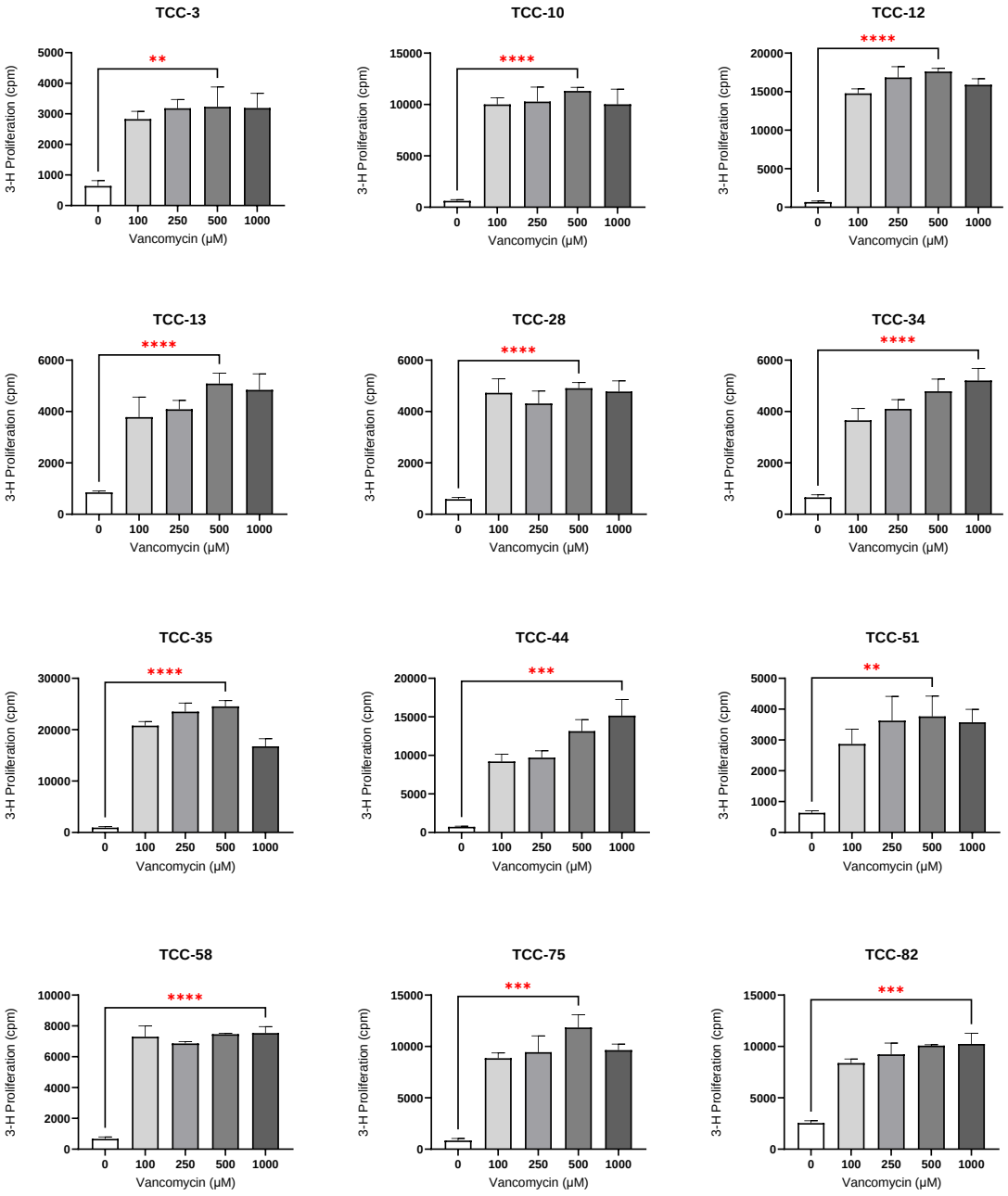
Table 5.2. HLA expression from quantified DNA isolated from PBMC in 6 vancomycin hypersensitive patients. Classical HLA typing (HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1, and HLA-DPB1) was performed at 3x resolution by the Histogenetics laboratory after initial DNA isolation via QIAamp® DNA Mini Kit and quantification by NanoDrop.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
HLA-A	*A*01:01*	*A*03:01*	*A*02:01*	*A*01:01*	*A*29:02*	*A*01:01*
	A*32:01	***A*32:01***	***A*32:01***	***A*32:01***	***A*32:01***	*A*24:02*
HLA-B	*B*08:01*	*B*07:01*	*B*14:01*	*B*44:02*	*B*15:01*	*B*08:01*
	*B*40:02*	*B*35:01*	*B*44:02*	*B*44:02*	*B*51:01*	*B*35:01*
HLA-C	*C*02:01*	-	*C*05:01*	*C*05:01*	*C*03:03*	*C*04:01*
	*C*07:01*	*C*07:02*	*C*08:02*	*C*05:01*	*C*15:02*	*C*07:01*
HLA-DRB1	*DRB1*03:01*	*DRB1*01:01*	*DRB1*04:01*	*DRB1*04:01*	*DRB1*04:01*	*DRB1*01:03*
	*DRB1*04:07*	*DRB1*15:01*	*DRB1*07:01*	*DRB1*07:01*	*DRB1*07:04*	*DRB1*03:01*
HLA-DQA1	*DQA1*03:03*	*DQA1*01:01*	*DQA1*02:01*	*DQA1*02:01*	*DQA1*03:01*	*DQA1*01:01*
	*DQA1*05:01*	*DQA1*01:02*	*DQA1*01:02*	*DQA1*03:01*	*DQA1*03:02*	*DQA1*05:01*
HLA-DPB1	*DPB1*01:03*	*DPB1*01:03*	*DPB1*02:01*	*DPB1*04:02*	*DPB1*02:01*	*DPB1*01:01*
	*DPB1*02:01*	*DPB1*04:01*	*DPB1*04:02*	*DPB1*13:01*	*DPB1*04:01*	*DPB1*02:01*

HLA genotyping analysis of 6 patients with confirmed vancomycin hypersensitivity, for which T-cell cloning experiments were conducted, revealed expression of the HLA-A*32:01 risk allele in 5/6 patients (Table 5.2). All patients with HLA-A*32:01 carriage were observed to be heterozygotes, with patient 6 representing the only individual without risk allele expression. Notably, HLA-DRB1*15:01 was found to expressed in patient 2, for which large numbers of responsive vancomycin TCCs have been generated.

A

Patient 2



B

Patient 3

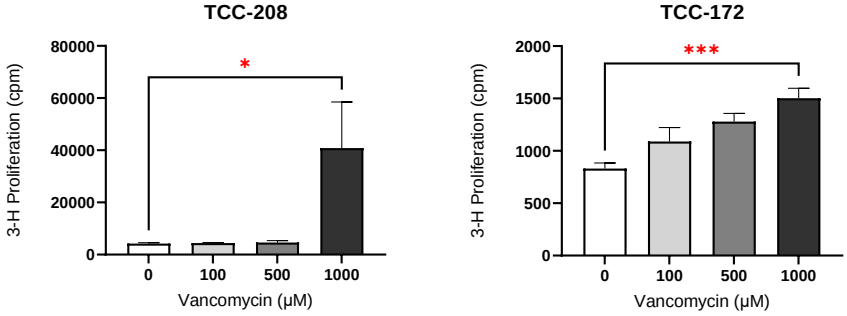


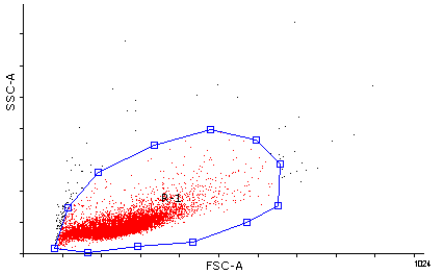
Figure 5.4. A) Proliferative response of vancomycin-specific TCCs generated from patient 2 at graded drug concentrations (n=12). TCCs ($5x10^4$ cells) were co-cultured with autologous EBV-transformed B-cells ($1x10^4$ cells), generated from the PBMC of patent 2, and either vancomycin (0-1000 µM) or R9 medium. Test cultures were incubated for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined using a student's *t*-test (**p<0.01, ***p<0.001, ****p<0.0001). **B) Proliferative response of vancomycin-specific TCCs generated from patient 3 at graded drug concentrations (n=2).** TCCs ($5x10^4$ cells) were co-cultured with autologous EBV-transformed B-cells ($1x10^4$ cells) and either vancomycin (0-1000 µM) or R9 medium. Cultures were incubated for 48 h before proliferative measurement, performed as previously described. Statistical significance was determined using a student's *t*-test (*p<0.05, ***p<0.001).

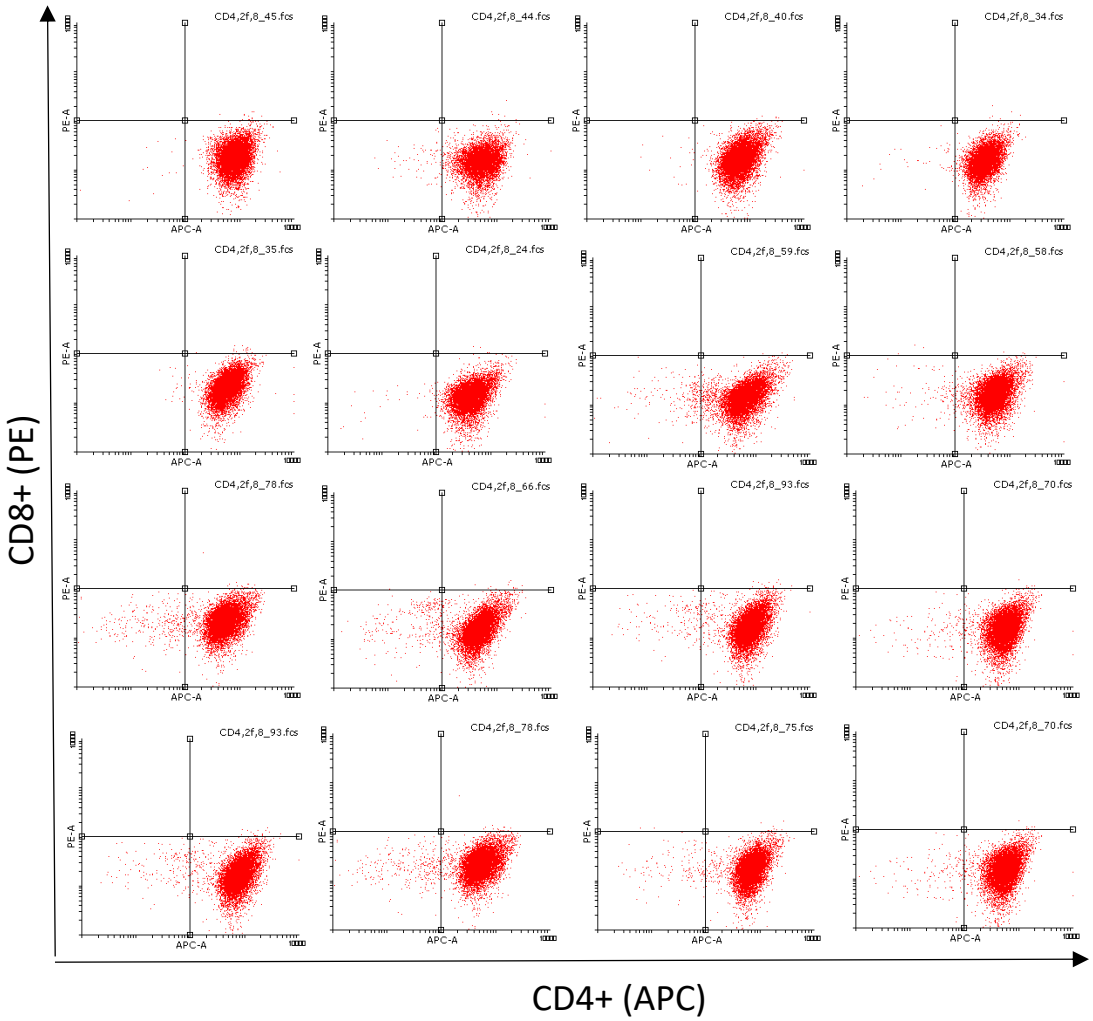
Initial test cultures, generated across 6 patients and displaying proliferative responses at singular vancomycin concentrations (0.5 mM) were expanded by mitogen driven stimulation for dose-response analysis. A panel of representative TCCs generated from patient 2 exhibited strong proliferative responses after exposure to graded vancomycin concentrations (Figure 5.4A). Statistical analysis was performed after exposure to optimal vancomycin dosage (500 µM), with all 12 TCCs assessed displaying statistically significant proliferative responses. Although, strong stimulation was achieved within each clonal population assessed at concentrations of 100 µM, indicating vancomycin may be capable of activating T-cells at low drug concentrations. Surprisingly, Only 2 TCCs generated from the PBMC of patient 3 produced dose-dependent proliferative responses to vancomycin, with a dosage of 1000 µM inducing statistically significant stimulation in each case (Figure 5.4B). Due to practical issues with clonal expansion (see Covid-19 impact statement), many TCCs exhibiting initial vancomycin specificity (Figure 5.2) could not be assessed for graded proliferative responses and therefore were excluded from the study.

5.3.3 Phenotypic and functional assessment of vancomycin-responsive TCCs generated from hypersensitive patients expressing HLA-A*32:01.

Patient 2

Unstained TCC





Total TCC Phenotyped	Pure CD8+ Population	Pure CD4+ Population	Mixed Population	N/A
23	0	23	0	0

Figure 5.5. Phenotypic characterisation (CD4+/CD8+) of a panel of representative vancomycin-responsive TCCs generated from patient 2 (n=16). T-cells with predetermined specificity for vancomycin were stained with 3 µL anti-CD4 (APC) and 0.5 µL of anti-CD8 (PE) for 15 mins at 4 °C. Stained cells were washed in FACS buffer to remove unbound antibody and resuspended in 4% PFA. Unstained cultures were set up in parallel for comparison and samples were analysed using a FACS-Canto II instrument integrated with FACS DIVA operating software. Phenotypic analysis was carried out using Flowing 2 software. Gating of lymphocyte populations was performed on unstained cultures and 10^4 events were analysed.

Phenotypic analysis of vancomycin-responsive TCCs generated from a HLA-A*32:01 positive hypersensitive patient (patient 2) revealed an overwhelmingly dominant CD4+ expressing phenotype (Figure 5.5). Of a total of 23 drug-specific TCCs studied for surface marker expression, all 23 TCCs were pure clonal populations and positive for expression of the CD4+ co-receptor. Pure CD4+ TCCs populations were further utilised in functional T-cell assays, described in greater detail in this section. No CD8+ expressing or mixed populations were observed in the panel of TCCs phenotyped from patient 2. Conversely, TCCs generated from patient 3 with confirmed vancomycin specificity expressed a CD8+ phenotype (Figure 5.6). Although, phenotypic analysis suggests that mixed T-cell populations may be present.

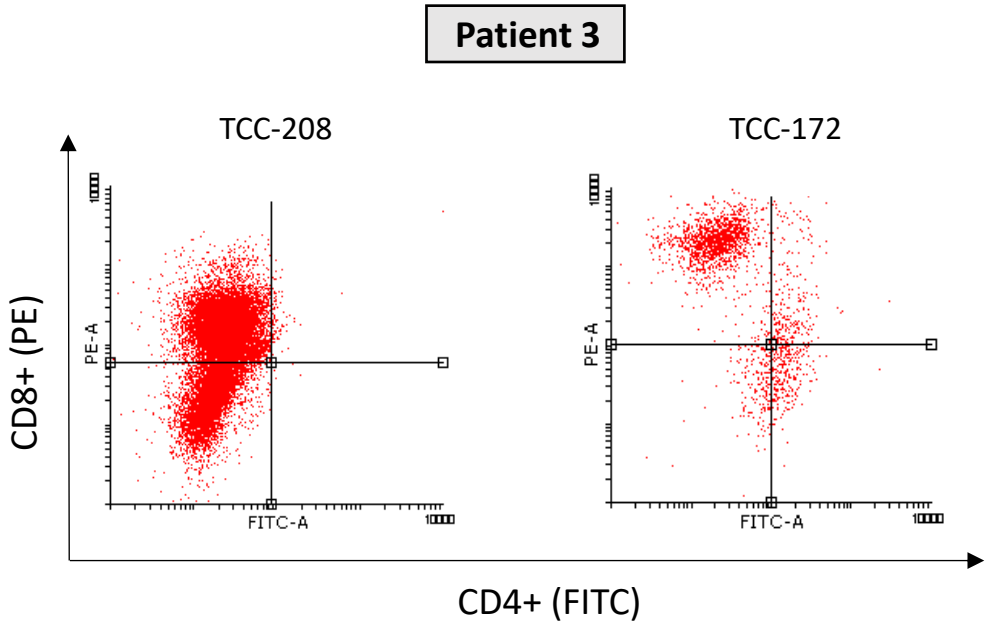


Figure 5.6. Phenotypic characterisation (CD4+/CD8+) of responsive TCCs generated from patient 3 (n=2). T-cells were stained with 3 µL anti-CD4 (FITC) and 0.5 µL of anti-CD8 (PE) for 15 mins at 4 °C. Stained cells were washed in FACS buffer and resuspended in 4% PFA. Samples were analysed using a FACS-Canto II instrument integrated with FACS DIVA operating software and phenotypic analysis was carried out using Flowing 2 software. Gating of lymphocyte populations was performed on unstained cultures and 10^4 events were analysed.

To aid functional T-cell study of clones generated from vancomycin hypersensitive patients, it was necessary to determine the drug concentrations at which vancomycin activates T-cells. To explore minimum thresholds relevant for activation, 3 representative TCCs generated from patient 2 were assessed for dose-dependent proliferative responses across an extended range of vancomycin concentrations (Figure 5.7). Statistically significant proliferative T-cell responses were observed across each clonal population after exposure to high drug concentrations. However, 3/3 TCCs were also found to be stimulated at vancomycin concentrations as low as 50 µM.

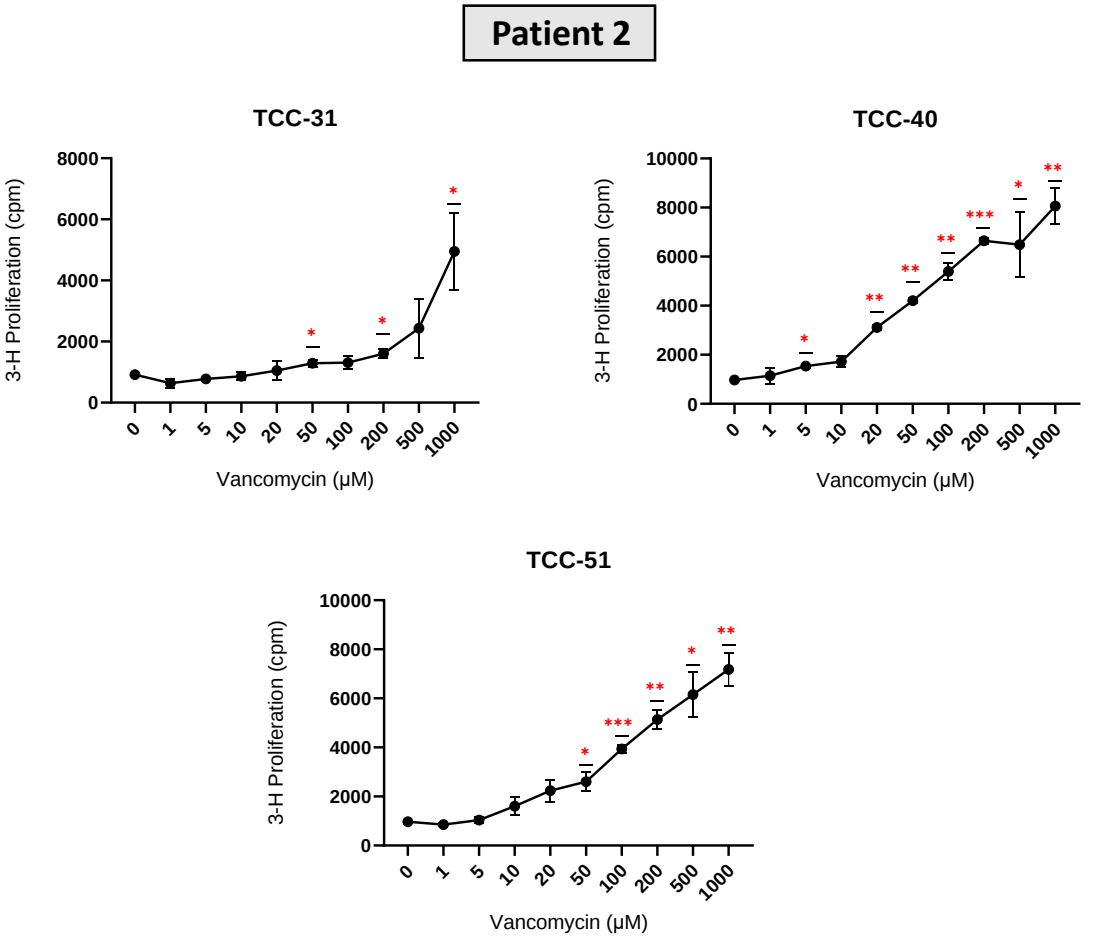


Figure 5.7. Proliferative dose-response analysis vancomycin-specific TCCs generated from patient 2 at extended drug concentrations (TCCS; n=3). TCCs ($5x10^4$ cells) were co-cultured with autologous EBV-transformed B-cells ($1x10^4$ cells) and vancomycin (0-1000 µM). Drug treated cultures were incubated for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined at each concentration using a student's *t*-test (*p<0.05, **p<0.01, ***p<0.001).

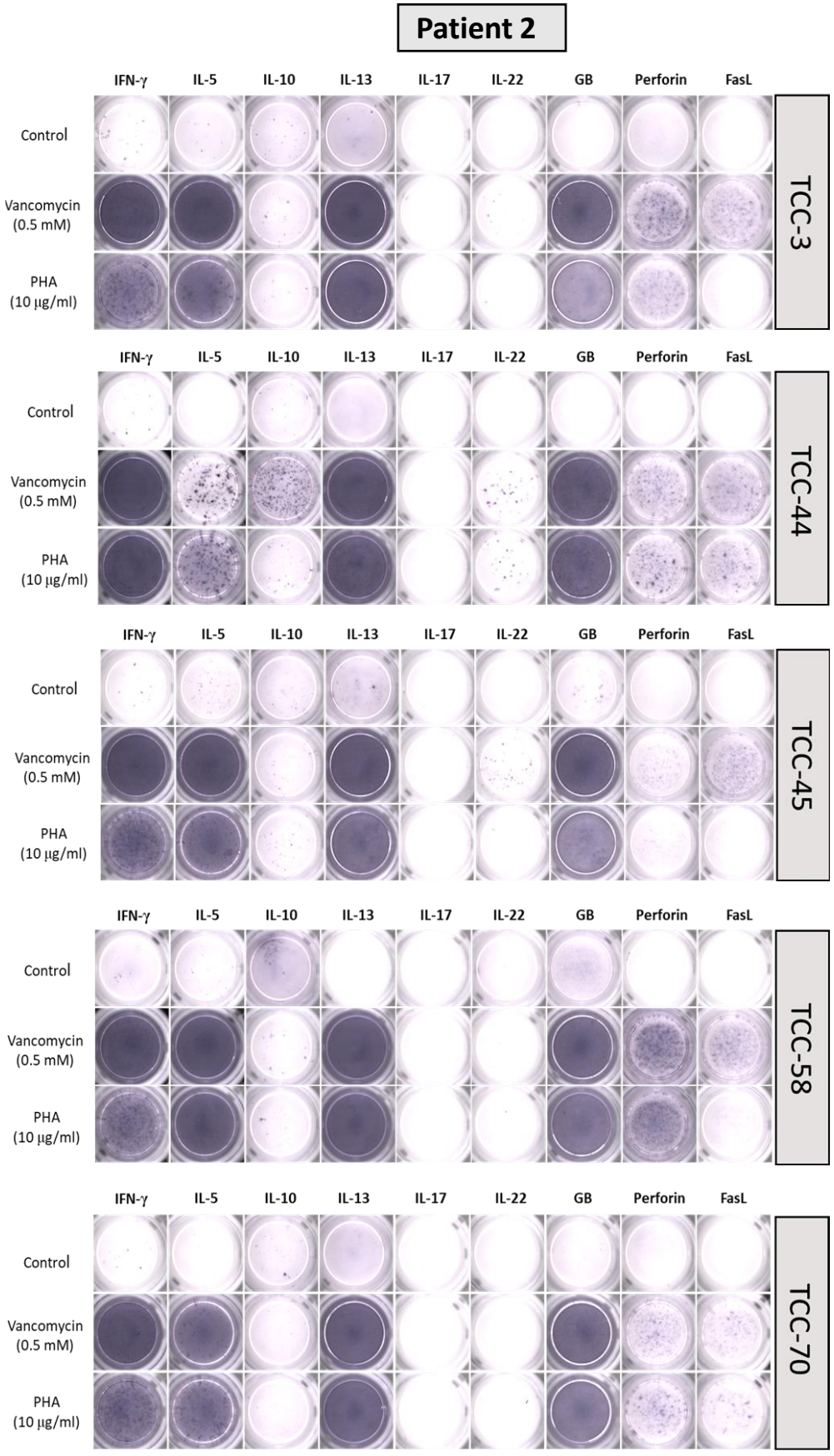


Figure 5.8. Cytokine secretion profile determined by ELISpot readout of vancomycin-specific TCCs (CD4+; n=5) generated from patient 2. TCCs ($5x10^4$ cells) were co-cultured with autologous EBV-transformed B-cells ($1x10^4$ cells) and vancomycin (0.5 mM), R9 medium or PHA (10 μg/mL). Cytokine release was for IFN-γ, IL-5, IL-10, IL-13, IL-17, IL-22, granzyme B (GB), perforin and FasL using activated Immobilon-P Membrane 96-well Multiscreen® filter plates pre-coated for cytokines of interest. Secretion profiles were studied after 48 h incubation with vancomycin (37 °C, 5% CO_2).

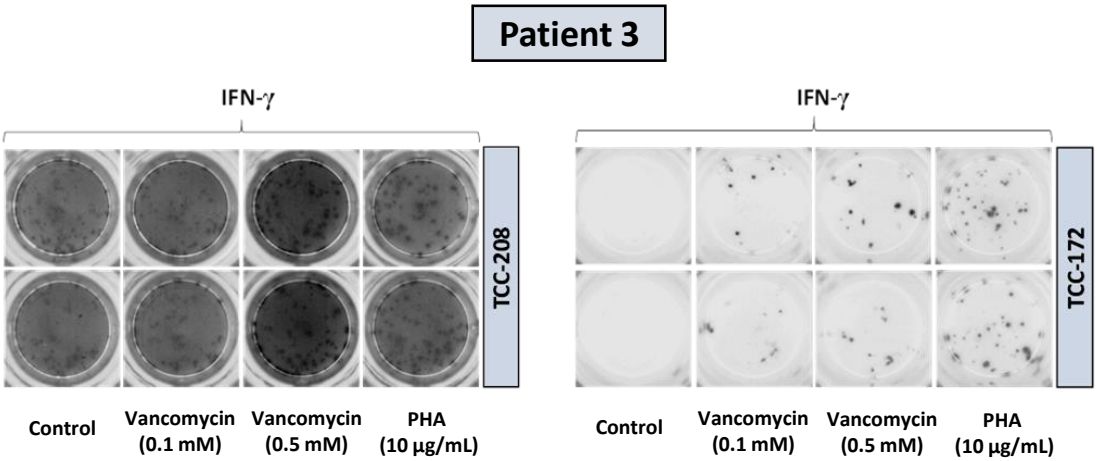


Figure 5.9. IFN-γ release determined by ELISpot readout of vancomycin-specific TCCs (CD8+; n=2) generated from patient 3. TCCs ($5x10^4$ cells) were co-cultured with autologous EBV-transformed B-cells ($1x10^4$ cells) and vancomycin (0.1 and 0.5 mM), R9 medium or PHA (10 µg/mL). Cytokine release was determined across duplicate wells for IFN-γ as previously described and according to the manufactures instructions.

5 representative CD4+ TCCs generated from patient 2 with confirmed vancomycin specificity were profiled for cytokine and cytolytic molecule release by ELISpot, after exposure to optimal (0.5 mM) drug concentrations (Figure 5.8). Interestingly, detection of CD4+ related cytokines (IFN-γ, IL-5, IL-10, IL-13 and IL-22) was simultaneously observed alongside the secretion of cytotoxic associated molecules such as granzyme B, perforin and FasL. PHA successfully induced non-specific cytokine and cytolytic molecule secretion across the full panel, with the exception of IL-17, within each TCC assessed. Due to severe limitations regarding the confluency of vancomycin-responsive T-cell cultures generated from patient 3, only IFN-γ secretion was assessed (Figure 5.9). TCCs (TCC-208 and TCC-172) expressing a CD8+ phenotype and exhibiting dose-dependent proliferative responses to vancomycin, were found to secrete IFN-γ after 48 h exposure to vancomycin at graded concentrations (0.1 and 0.5 mM). TCC-172 was associated with definitive dose-dependent IFN-γ secretion, although IFN-γ above background levels could was only visual at 0.5 mM concentrations in the case of TCC-208. Similarly, PHA was observed to induce non-specific cytokine release in both clonal populations assessed.

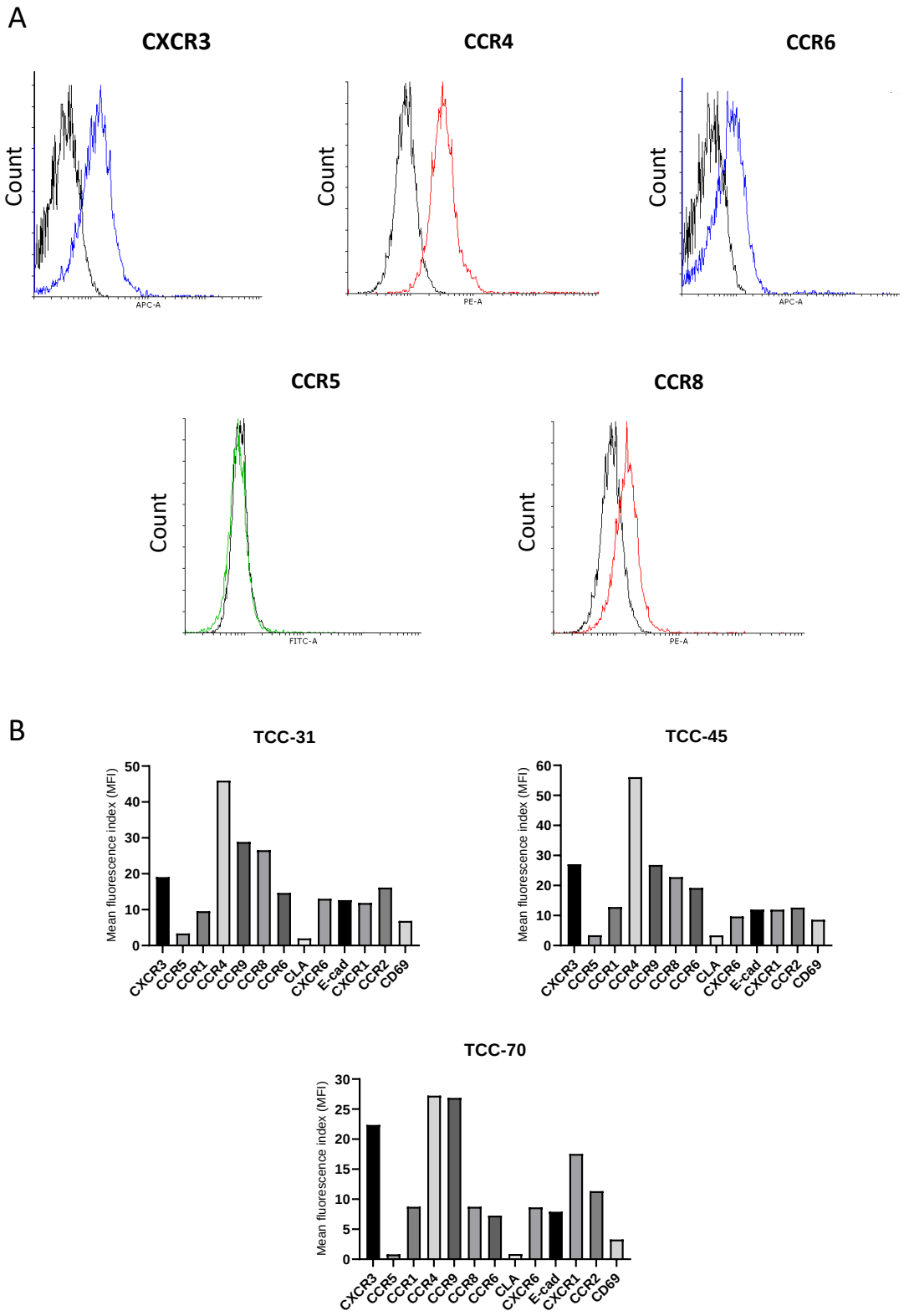


Figure 5.10. A, B) Chemokine receptor expression analysis of vancomycin-specific TCCs generated from patient 2 (TCCs; n=3). TCCs were stained with 3 µL chemokine receptor conjugated antibodies for CXCR3-APC, CCR5-PE, CCR1-PE, CCR4-PE, CCR9-APC, CCR8-FITC, CCR6-APC, CLA-FITC, CXCR6-PE, E-Cad-PE, CXCR1, CCR2-APC and CD69-FITC for 15 mins at 4 °C. Stained cells were washed in FACS buffer to remove unbound antibody and resuspended in 4% PFA. Unstained cultures were set up in parallel for comparison and samples analysed using a FACS-Canto II instrument integrated with FACS DIVA operating software and phenotypic analysis was carried out using Flowing 2 software. Gating of lymphocyte populations was performed on unstained cultures and 10^4 events were analysed. Surface marker expression interpreted as mean fluorescence index (MFI).

Chemokine receptor expression analysis of vancomycin-response TCCs isolated from patient 2 was performed on 3 CD8+ expressing populations (Figure 5.10). Mean fluorescence index (MFI) was given by the mean fluorescence of stained samples minus the mean fluorescence of unstained samples, visually represented for a panel of chemokine receptors in Figure 5.9B. Universally, MFI analysis revealed TCCs expressed high levels of CXCR3, CCR4 and CCR9. Additionally, across the 3 TCCs profiled CCR6 and CCR8 were found to be expressed at moderately high levels. Furthermore, CCR1, CCR2, CXCR1, CXCR6 and E-Cad were all expressed at reduced levels.

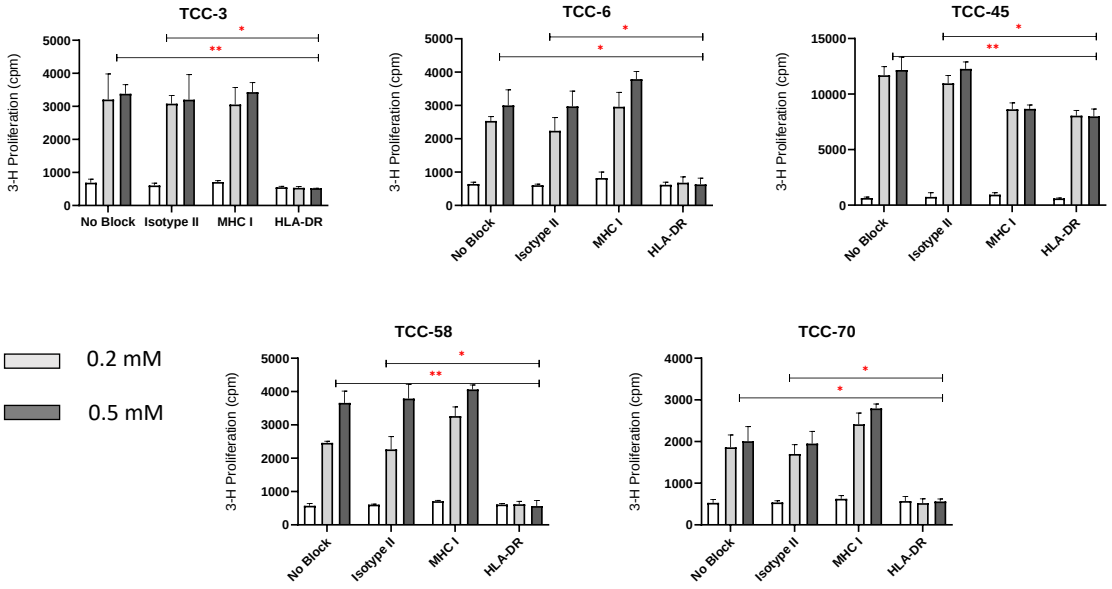


Figure 5.11. HLA restriction of vancomycin-specific TCCs (n=3) isolated from vancomycin hypersensitive patient 2. CD4+ expressing TCCs (5x10^4 cells) were co-cultured with autologous irradiated APCs in triplicate and either HLA Class I (10 µg/mL), HLA Class II (10 µg/mL), HLA-DR (10 µg/mL) or corresponding IgG2 isotype controls (10 µg/mL) for 1 h (37 °C, 5% CO_2). Blocked cultures were treated with graded vancomycin concentrations (0.2 mM and 0.5 mM) or R9 cell culture medium for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined using a Mann-Whitney U test (*p< 0.05, **p< 0.01).

HLA restricted T-cell activation was determined through HLA blockade (Figure 5.11). In 5/5 CD4+ TCCs assessed, proliferative responses to vancomycin were inhibited following HLA-DR blockade. T-cell proliferation was unaffected following HLA class I blockade when compared to proliferative responses following vancomycin exposure, in the absence of HLA blocking antibodies.

After exposure of vancomycin-specific TCCs to drug-pulsed antigen presenting cells (APCs), no proliferative response was observed (Figure 5.12). However, after APC fixation and blockade of antigen processing, vancomycin-responsive T-cells were capable of statistically significant proliferative responses after exposure to a co-culture of glutaraldehyde-fixed APCs and vancomycin. Additionally, across the panel of TCCs, T-cell stimulation was found to be hampered by the absence of autologous APCs. 3/3 TCCs displayed a strong proliferative response after treatment with vancomycin and autologous APCs.

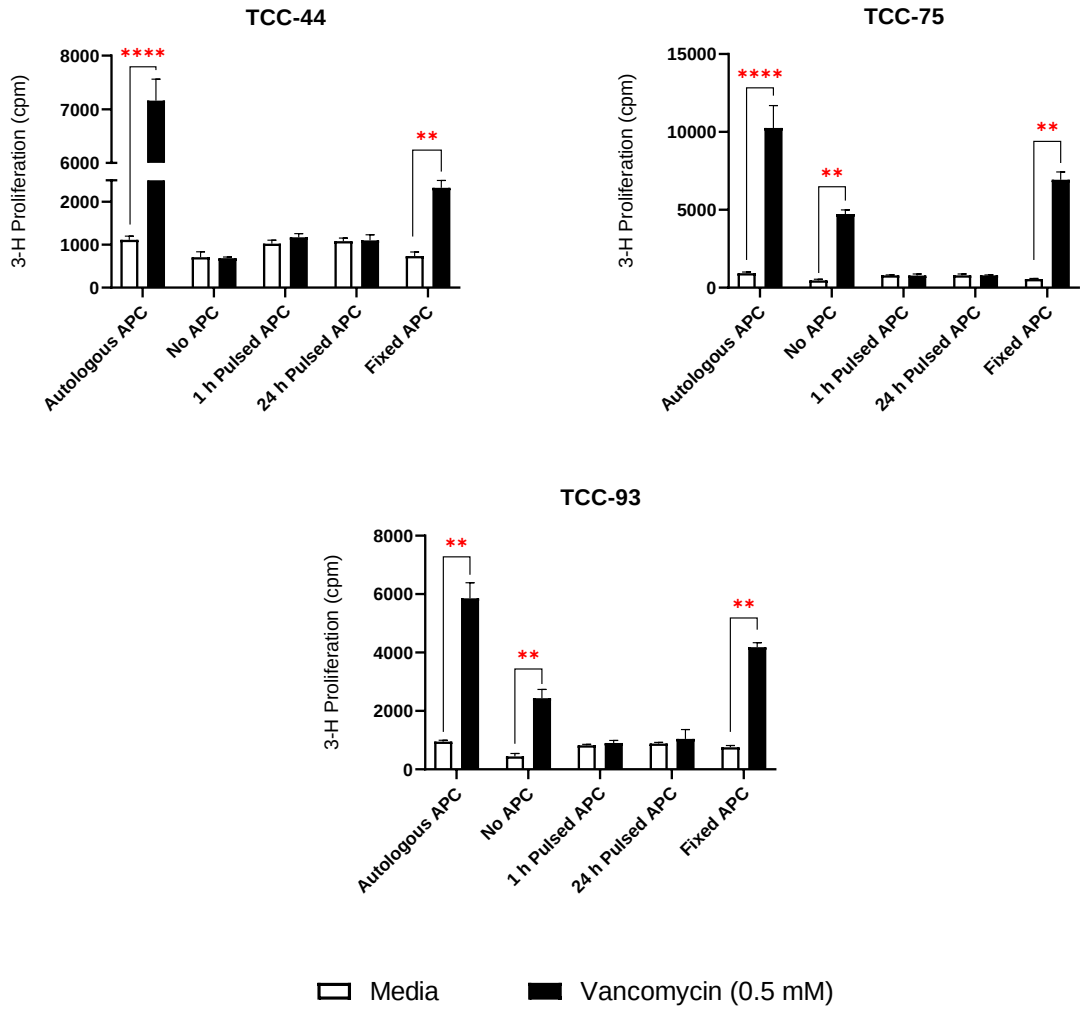
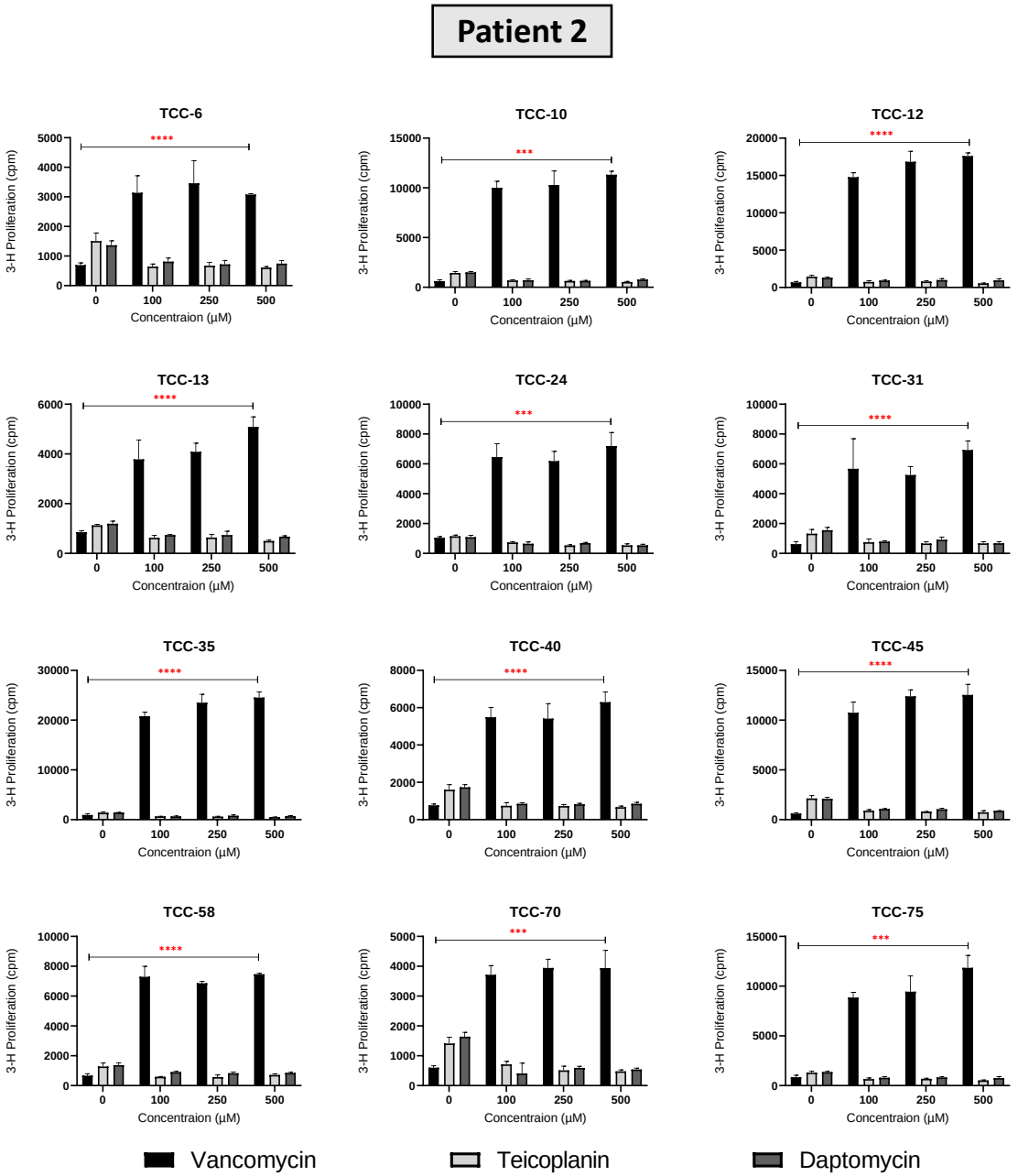


Figure 5.12. Activation pathways of vancomycin-responsive CD4+ TCCs (n=3) generated from patient 2. Autologous APCs ($2x10^6$ cells) were pulsed with vancomycin (0.5 mM) for 1 h and 24 h before washing to remove unbound drug or fixed with glutaraldehyde (25%, 1 µL) for 30 secs prior to quenching of the reaction with glycine (1 M, 1 mL) for 45 secs. TCCs were incubated for 48 h (37 °C, 5% CO_2) with either fixed APCs and vancomycin (0.5 mM) or pulsed APCs ($1x10^4$ cells). Parallel conditions were included for the determination of proliferative T-cell responses, using TCCs co-cultured with and without autologous APCs. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values after 16 h incubation (0.5 µCi/well). Statistical significance was determined using a Mann-Whitney U test (**p< 0.01, p****<0.0001).

5.3.4 Determining the cross-reactive potential of CD4+ and CD8+ vancomycin-responsive TCCs generated from hypersensitive patients expressing HLA-A*32:01.



TCC tested for cross-reactivity	Responsive to vancomycin	Cross-reactive with teicoplanin	Cross-reactive with daptomycin
16	16	0	0

Figure 5.13. Proliferative cross-reactive study of CD4+ vancomycin-specific TCCs (n=16) generated from hypersensitive patient 2 with structurally-related glycopeptide antibiotics. TCCs ($5x10^4$ cells) were co-cultured with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells) and either vancomycin (0-500 µM), teicoplanin (0-500 µM), daptomycin (0-500 µM) or R9 cell culture medium only. Test cultures were incubated for 48 h (37 °C, 5% CO_2) before to pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined using a student's *t*-test (***p< 0.001, ****p<0.0001).

To investigate the potential for cross-reactive T-cell responses between CD4+ and CD8+ expressing vancomycin-reactive TCCs generated from hypersensitive patients with structurally-related glycopeptide antibiotics, proliferation after exposure to vancomycin, teicoplanin and daptomycin was assessed by radioactive thymidine incorporation (Figure 5.13 and 5.14). No cross-reactivity with either teicoplanin or daptomycin was observed in a panel of 16 CD4+ expressing vancomycin-responsive TCCs isolated from patient 2 (12 representative TCCs shown). Although, each TCC assessed proliferated in the presence of vancomycin, with statistically significant proliferative responses detected at optimal stimulatory concentrations (Figure 5.13). Conversely, in the small panel of 2 CD8+ TCCs generated from patient 3 assessed for cross-reactivity T-cell responses, 1/2 TCCs (TCC-208) were found to be cross-reactive with teicoplanin at graded concentrations of between 100 µM and 500 µM (Figure 5.14). Cross-reactivity with daptomycin was not observed. Surprisingly, proliferative responses to vancomycin were not detected in each clonal population generated from patient 3.

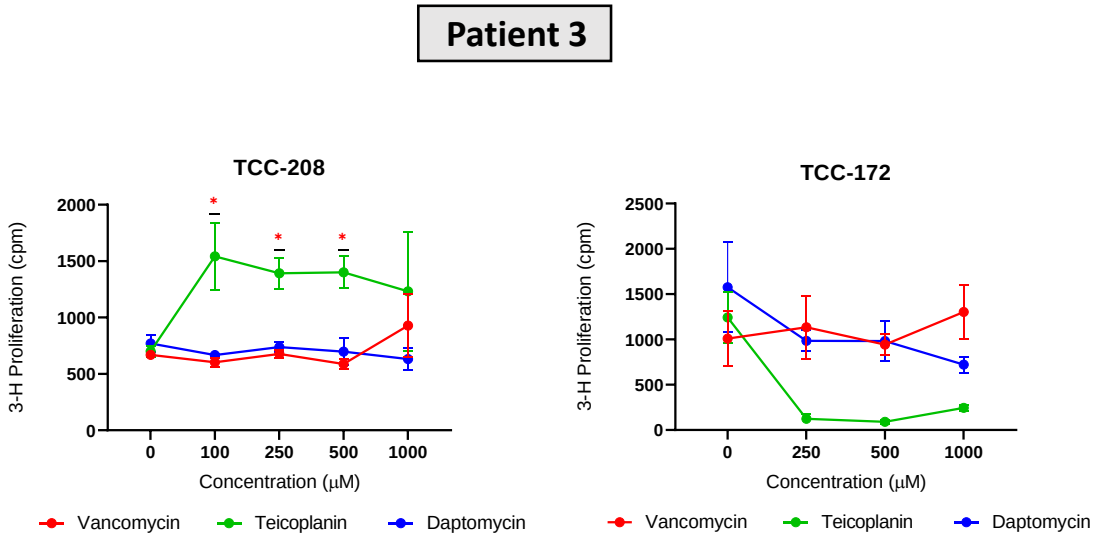


Figure 5.14. Proliferative cross-reactive study of CD8+ vancomycin-specific TCCs (n=2) generated from hypersensitive patient 3 with structurally-related glycopeptide antibiotics. TCCs ($5x10^4$ cells) were co-cultured with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells) and either vancomycin (0-1000 µM), teicoplanin (0-1000 µM), daptomycin (0-1000 µM) or R9 cell culture medium only. Test cultures were incubated for 48 h (37 °C, 5% CO_2) prior to pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for an additional 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined using a student's *t*-test (*p< 0.05).

5.4 Discussion.

In this chapter, vancomycin-specific TCCs were successfully generated from the PBMC of 2 hypersensitive patients positive for carriage of the HLA-A*32:01 risk allele, strongly associated with vancomycin-induced DRESS. Both CD4+ and CD8+ drug-specific TCCs displaying dose-dependent proliferative responses to vancomycin were isolated from hypersensitive patients and characterised for phenotypic and functional traits in order to further examine the role of T-cells within DRESS pathogenesis following vancomycin exposure. Previously, we have already eluded to the nature of interactions between vancomycin and CD8+ TCCs generated from healthy donors expressing HLA-A*32:01, for which activation was observed to hinge upon HLA class I presentation and T-cell responses induced by direct non-covalent pharmacological interactions (Ogese et al., 2021).

Although useful as a preliminary model for studying immune-mediated disease pathogenesis, studies of greater physiological relevance are required to confirm T-cell involvement in addition to uncovering pathomechanisms of immune stimulation. The use of PBMC isolated from patients with confirmed hypersensitivity to immunogenic compounds is widely considered to be the gold standard for mapping disease pathogenesis and isolating T-cell participation. This has been demonstrated for a range of compounds, such as those known to target the skin and liver through immune modulation. Indeed, studies characterising the role of drug-specific lymphocyte responses following exposure to β-lactam antibiotics and anticonvulsants provide classical examples for the use of PBMC isolated from hypersensitive patients. Consequently, effector T-cell functions have now been heavily implicated within the reaction pathogenesis of both carbamazepine and flucloxacillin-induced hypersensitive disease states, for which manifestations include maculopapular exanthema and drug-induced liver injury (Naisbitt et al., 2003a, Monshi et al., 2013).

Individuals with suspected vancomycin hypersensitivity were initially assessed via LTT assay to confirm culprit drug status of vancomycin. Proliferative diagnostic assays geared toward the detection of immune stimulation successfully identified vancomycin as a key contributor to hypersensitivity in 8/9 patients assessed (Table 5.1). Optimal dosage for the elicitation of T-cell responses to vancomycin were consistent with stimulatory concentrations necessary for the activation of vancomycin-specific T-cells generated from drug naïve healthy donors, as described in Chapter 3, with 500 µM sufficient for lymphocyte transformation in more than 60% of vancomycin hypersensitive patients. These data suggest alignment between activation thresholds required for vancomycin-mediated immune stimulation in T-cell populations isolated from both healthy donors and hypersensitive patients. LTT assays are typically used for initial diagnostic assessment of immunogenic compounds and are particularly useful during polypharmacy, with the detection of memory T-cell responses to offending compounds usually a sensitive indicator of culprit drug exposure (Pichler and Tilch, 2004). Therefore, this study further demonstrates the proficiency of the LTT for the measurement of memory responses applied to vancomycin-induced lymphocyte transformation in small cohort of hypersensitive patients.

Notably, in the limited number of patients studied for cross-reactive lymphocyte transformation, 2/3 patients presenting with vancomycin hypersensitivity were found to cross-react with teicoplanin following analysis of IFN-γ secretion (Figure 5.1). The use of IFN-γ as a maker for immune activation hinges upon its role as a cytotoxic differentiation signal and can be a more sensitive indicator of memory a T-cell responses than proliferative measurement (Maraskovsky et al., 1989). The presence of cross-reactivity between structurally related glycopeptide antibiotics is not new phenomenon, with numerous clinical case studies eluding to the development of teicoplanin-induced DRESS following initial vancomycin hypersensitivity (Kwon et al., 2006, Hsiao et al., 2010).

Due to limitations associated with studying hypersensitive patients, such as heavy reliance on sufficient volumes of venous blood for PBMC isolation, mechanistic study was unable to be performed on the full complement of patients, for which initial diagnostic LTT assays were conducted. A total of 6 hypersensitive patients were assessed for initial vancomycin specificity of TCCs generated by serial dilution (Figure 5.2). Drug-reactive T-cells with specificity at singular vancomycin concentrations were successfully identified in 4/6 patients. However, only TCCs generated from 2 patients were confirmed as dose-responsive and vancomycin-specific following mitogen driven clonal expansion and exposure to graded drug concentrations. It is interesting that considerable numbers of vancomycin-specific TCCs were generated from patient 2, for which LTT assays yielded a negative result. One possibility for this outcome may be due to a lower precursor frequency of drug-specific T-cells within the peripheral blood of this patient, potentially arising from an increased interval between hypersensitive manifestation and sample collection.

A myriad of factors could potentially explain the lack of true drug-specific TCCs generated from the majority of patients with confirmed hypersensitivity. Firstly, it is important to stress that preliminary work in the field has suggested that only 20% of individuals with HLA A*32:01 carriage will develop DRESS after vancomycin administration (Konvinse et al., 2019). Secondly, due to the laborious nature of T-cell cloning, serial dilution experiments were not conducted across each patient within a parallel time frame. IL-2 signalling induces T-cell exhaustion, therefore it is possible that variations within IL-2 activity with the cell culture system used between patients may have exhausted potential drug-specific T-cells and prevented proliferative measurement (Liu et al., 2021). Furthermore, the characterisation of drug-specific TCCs generated to a number of patients was significantly interrupted by the Covid-19 pandemic, as many potential vancomycin-reactive TCCs were cryopreserved and lost drug specificity upon restimulation.

To further examine HLA involvement within the pathogenesis of vancomycin-induced DRESS, it was important to correlate HLA expression in specific patients with the capacity for clonal generation. Importantly, HLA genotyping revealed all 4 patients for which vancomycin-responsive TCCs could be isolated were heterozygous for HLA-A*32:01 expression (Table 5.1). Delving further into the genotypic traits of patients assessed within the cohort, it is also worth noting that 8/9 patients presenting with vancomycin hypersensitivity were found to be positive for HLA-A*32:01 carriage. This finding, indicating that approximately 88% of vancomycin hypersensitive patients express a shared risk allele, further substantiate analysis performed by Konvinse et al. suggesting that 82.6% of patients presenting with vancomycin-induced DRESS express HLA-A*32:01 (Konvinse et al., 2019). Large numbers of vancomycin-responsive TCCs expressed a CD4+ phenotype (Figure 5.5). This is unusual for a reaction with strong HLA class I association, with interactions typically eliciting CD8+ T-cell responses during the development of systemic drug hypersensitivity reactions, such as DRESS (Chessman et al., 2008). The immunological nature of DRESS manifestation is thought to be predominantly mediated by CD8+ T-cells and is often accompanied by viral reactivation (60% of cases), typically by viruses of the herpes group (Tohyama et al., 2007). This has now been confirmed by the identification of CD8+ EBV-specific T-cells in the tissues of DRESS patients (Picard et al., 2010). The identification of CD8+ TCCs generated from a single patient (3) is more aligned with DRESS pathogenesis, for which reaction severity has been attributed to a 'spreading' of CD8+ dominant T-cell repertoires (Niu et al., 2015).

Due to insufficient clonal expansion, detailed cytokine profiling could not be performed on CD8+ expressing TCCs with vancomycin specificity. Instead, cytokine analysis was performed on a representative panel (n=5) of CD4+ vancomycin-specific TCCs (Figure 5.8) Interestingly, detection of CD4+ related cytokines (IFN-γ, IL-5, IL-10, IL-13 and IL-22) was simultaneously observed alongside the secretion of cytotoxic associated molecules such as granzyme B, perforin and FasL (Maraskovsky et al., 1989). Although CD4+ helper T-cells are traditionally

associated with the release of Th1 and Th2 cytokines, there is often a degree of plasticity between the cytokine secretion profiles of CD4+ and CD8+ T-cells. This phenomenon was initially described in murine models, for which new subsets of CD8+ T-cells were proposed (TC1 and TC2) with the capacity to secrete Th1-like cytokines (IFN-γ) and Th2-like cytokines such as IL-4, IL-5 and IL-10 (Sad et al., 1995). Furthermore, previous work characterising T-cell responses to vancomycin have reinforced this inherent plasticity, as CD8+ vancomycin-specific TCCs generated from healthy donors were observed to secrete both Th1-like cytokines and Th2-like cytokines such as IFN-γ, IL-5 and IL-13 (Ogese et al., 2021). Additional phenotypic analysis revealed chemokine receptor expression within TCCs isolated from hypersensitive patients was concordant healthy donor studies, with CXCR3 and CCR4 highly expressed (Figure 5.10), indicative of Th1-type inflammation following T-cell trafficking and migration to peripheral tissues (Groom and Luster, 2011).

To investigate mechanisms of antigen presentation and the conditions necessary to facilitate CD4+ T-cell activation after subjection to vancomycin, proliferative responses after HLA blockade were assessed (Figure 5.11). T-cell responses were universally nullified following HLA-DR blockade suggesting vancomycin is capable of activating CD4+ T-cells in a HLA class II-restricted manner. This result juxtaposes any pre-determined importance of HLA class I dependence, for which HLA-A*32:01 interaction has been deemed crucial within DRESS pathogenesis. Indeed, recent exploration of glycopeptide cross-reactivity have now suggested a common HLA class II haplotype amongst patients displaying cross-reactivity to teicoplanin, with alternate models of glycopeptide-induced T-cell activation being postulated, including antigen cross-presentation within the context of class II HLA molecules (Nakkam et al., 2021). Furthermore, mechanistic study of antigen processing implicated within the activation of CD4+ TCCs firmly suggests vancomycin activates CD4+ T-cells in a processing independent and non-covalent manner (Figure 5.12). This is consistent with the pharmacological interaction (p-i)

concept and previous mechanistic study involving TCCs generated from healthy donor PBMC positive for HLA-A*32:01 carriage (Pichler, 2002, Ogese et al., 2021).

Surprisingly, in the large panel of vancomycin-responsive TCCs (n=16) assessed for cross-reactivity with teicoplanin and daptomycin in terms of a proliferative readout, TCCs only displayed specificity for vancomycin (Figure 5.13). The lack of any apparent specificity to both daptomycin and teicoplanin in this patient does not necessarily confirm the absence of any interaction between these structurally similar glycopeptides. The immunological basis for the presence or absence of cross-reactive T-cell responses between immunogenic compounds can be complex. One logical explanation may involve inter-individual variability within the expression of co-inhibitory markers, therefore facilitating an elevated threshold required for T-cell activation (Muraille et al., 1995). It is also important to stress that clinical cross-reactivity and contraindication, specifically between vancomycin and teicoplanin, is still a rare occurrence and as such is not routinely observed within each patient. However, a single vancomycin-responsive TCC generated from patient 3 was found to cross-react with teicoplanin, even after initial vancomycin specificity was lost (Figure 5.14). Previous research into the patterns of reactivity between glycopeptide antibiotics within HLA-A*32:01 positive individuals presenting with vancomycin-induced DRESS has eluded to a shared HLA class II haplotype amongst cross-reactive patients following *ex vivo* study. Furthermore, *in silico* molecular docking analysis has suggested preferential binding to HLA-DQ alleles within individuals exhibiting cross-reactivity cytokine activity with teicoplanin (Nakkam et al., 2020). Therefore, it would be interesting to assess HLA restricted activation of cross-reactive TCCs to further elucidate any HLA-DQ involvement.

To summarise, this study demonstrates the utility of T-cell assays, such as the LTT, within the diagnosis of vancomycin hypersensitivity, both in terms of cytokine release and proliferative readouts. The isolation of drug-responsive T-cells from hypersensitive patients further

implicates immune modulation within vancomycin-induced DRESS, for which both CD4+ and CD8+ T-cells are involved within disease pathogenesis. Characterisation of CD4+ TCCs suggests HLA class II involvement with T-cell responses evoked by direct non-covalent pharmacological interactions, with the capacity for the secretion of cytokines (IL-5) and cytotoxic molecules (FasL) known to be linked with DRESS manifestation. Moving forward, it will be necessary to further study and characterise CD8+ T-cell responses to vancomycin within hypersensitive patients to aid understanding of possible of HLA class I-restricted T-cell activation. Once achieved, the overarching aim should be to stratify patients susceptible to aberrant cross-reactive immune responses following sequential therapy with structurally related glycopeptide antibiotics.

6. Glycolysis; an early marker of drug-specific T-cell activation.

Contents

6.1 Introduction.

The adaptive immune system has consistently been implicated within the pathogenesis of hypersensitivity reactions to a multitude of drug classes including β-lactam antibiotics sulfonamides, and anticonvulsants (Solensky, 2003, Naisbitt et al., 2003a, Naisbitt et al., 2001). T-cell activation has long been established as a key determinant within cell-mediated (Type IV) hypersensitive responses of a delayed nature. Ensuing effector functions, often resulting in the release of cytotoxic molecules and subsequent tissue damage, are known to depend on various signalling pathways that promote T-cell activation. Pleiotropic signalling pathways involved within the activation of T-cells can neatly align with the 'danger hypothesis', first described by Polly Matzinger in which a duo of conditions (signal 1 and signal 2) must be met for activation and initiation of effector T-cell responses in favour of tolerance (Matzinger, 1994). However, the metabolic phenotypes that underpin the initiation and propagation of danger signalling are poorly defined.

More recently, research focus has shifted towards characterising energetic pathways that underpin immune-cell activation, such as glycolysis and oxidative phosphorylation (OXPHOS), in an attempt to define the essential metabolic phenotypes necessary to support adenosine triphosphate (ATP) production, rapid cellular growth and proliferative function. Furthermore, the activation of T-cells from inherent resting and naïve states requires a form of metabolic reprogramming in which fuel sources are readily available to cope with energetic demands of clonal expansion. Antigen presentation evokes T-cell activation via interactions between drug, antigen presenting cells (APCs), displaying major histocompatibility complexes (MHC), and the T-cell receptor (TCR). Studies have now demonstrated that ensuing CD4+ and CD8+ T-cell effector functions are dependent on glycolysis (Jones et al., 2017, van der Windt et al., 2013). This form of metabolic reprogramming has been described as an immediate-early 'glycolytic switch' when applied to both *de novo* and memory T-cell responses (Gubser et al., 2013).

However, such studies have almost entirely focused on basal metabolic differences between CD4+ and CD8+ expressing T-cell populations. Additionally, such studies have profiled energetic changes after both artificial (αCD3/28) and peptide-induced stimulation and therefore cannot be interpreted as true memory T-cell responses to cognate antigens. Therefore, elucidation of the metabolic traits and fuel dependence that governs the activation of drug-specific T-cells will provide mechanistic insight into the conditions necessary for recall responses comprising a memory phenotype within the context of hypersensitive T-cell responses to immunogenic compounds.

The emerging field of immunometabolism, defined by the study of adaptable and reprogrammable metabolic characteristics, has led to development of numerous assays predominantly geared towards profiling energetic changes within the cellular components of immune populations (van der Windt et al., 2016a). To examine fuel pathway dependency, specifically pertaining to OXPHOS as a measure of mitochondrial respiration, stress-based assays utilising potent mitochondrial toxins have been deployed with some success for measuring the bioenergetic properties associated with T-cell activation (van der Windt et al., 2016a). The assays rely on mitochondrial complex inhibition, achieved by acute injection of oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and a combination of antimycin A/rotenone. The aforementioned mitochondrial toxins act to sequentially inhibit specific parameters of OXPHOS whilst shunting respiration towards available metabolic avenues, allowing for the isolation of the energetic parameters implicated within cellular development and activation (Gu et al., 2021). This is interpreted as a rate of oxygen consumption (OCR). Furthermore, stress-based assays have already identified specific bioenergetic features, such as mitochondrial respiratory capacity, as critical regulators of CD8+ T-cell development for which both OXPHOS and fatty acid oxidation have been recognised as preferential fuel pathways (van der Windt et al., 2012). Following recent advances in relation

to the immunometabolic profiles of artificially stimulated T-cells, fuel sources derived from glycolytic processes have been identified as a key marker of activation (Gubser et al., 2013). However, there is a current unmet requirement for the application of glycolysis stress-based assays for the study of early T-cell activation following acute drug stimulation, with particular relevance for the activation of drug-specific T-cells of a monoclonal nature when applied to the pathomechanisms of hypersensitive disease states.

The primary focus of this work was to develop an assay capable of detecting both non-specific and drug-specific markers of T-cell activation, through measurement of the glycolysis fuel pathway and with the use of the Seahorse XFe96 Analyzer. These assays enable measurement of the extracellular acidification rate (ECAR), which is subsequently interpreted as a function of glycolysis. In addition, access to a fully functional and optimised assay system would allow for in depth study of the metabolic signatures associated with the drug-specific activation process of vancomycin-reactive T-cell clones (TCCs) previously generated from healthy donors and patients expressing HLA-A*32:01. Moreover, due to the sensitive nature of fuel pathway analysis when compared to conventional proliferative and cytokine-based T-cell readouts, the delineation of cross-reactive potential between structurally related glycopeptides antibiotics, such as teicoplanin, will be assessed and form an integral part of the workflow. Relating back to hypersensitivity, development of such assays with the capacity for sensitive glycolytic measurements as an early marker of drug-specific T-cell activation would ultimately provide auxiliary support when screening for compounds with immunogenic potential.

6.2 Aims.

1) To optimise mitochondrial and glycolysis stress-based assays for use with drug-specific TCCs generated *in vitro* and incorporate the use of model stimulants for the detection of glycolytic switching.

2) To profile the energetic requirements necessary for the activation of vancomycin-specific TCCs following acute stimulation and to confirm responses are concordant with proliferative and cytokine-based readouts in terms of dose-responsiveness, drug-specificity and human leucocyte antigen (HLA) restriction.

3) Analysis of energetic profiles to determine the cross-reactive nature of structurally similar glycopeptides after acute stimulation of TCCs.

6.3 Results.

6.3.1 Optimisation of stress-based assays for use with mitochondrial inhibitors, non-adherent drug-specific TCCs and autologous antigen presenting cells.

In order to determine the concentrations at which immune cell populations display toxicity to specific mitochondrial inhibitors relevant for stress-based assays (oligomycin, FCCP, antimycin A and rotenone), toxicity assays were performed on peripheral blood mononuclear cells (PBMC) isolated from healthy donors (Figure 6.1).

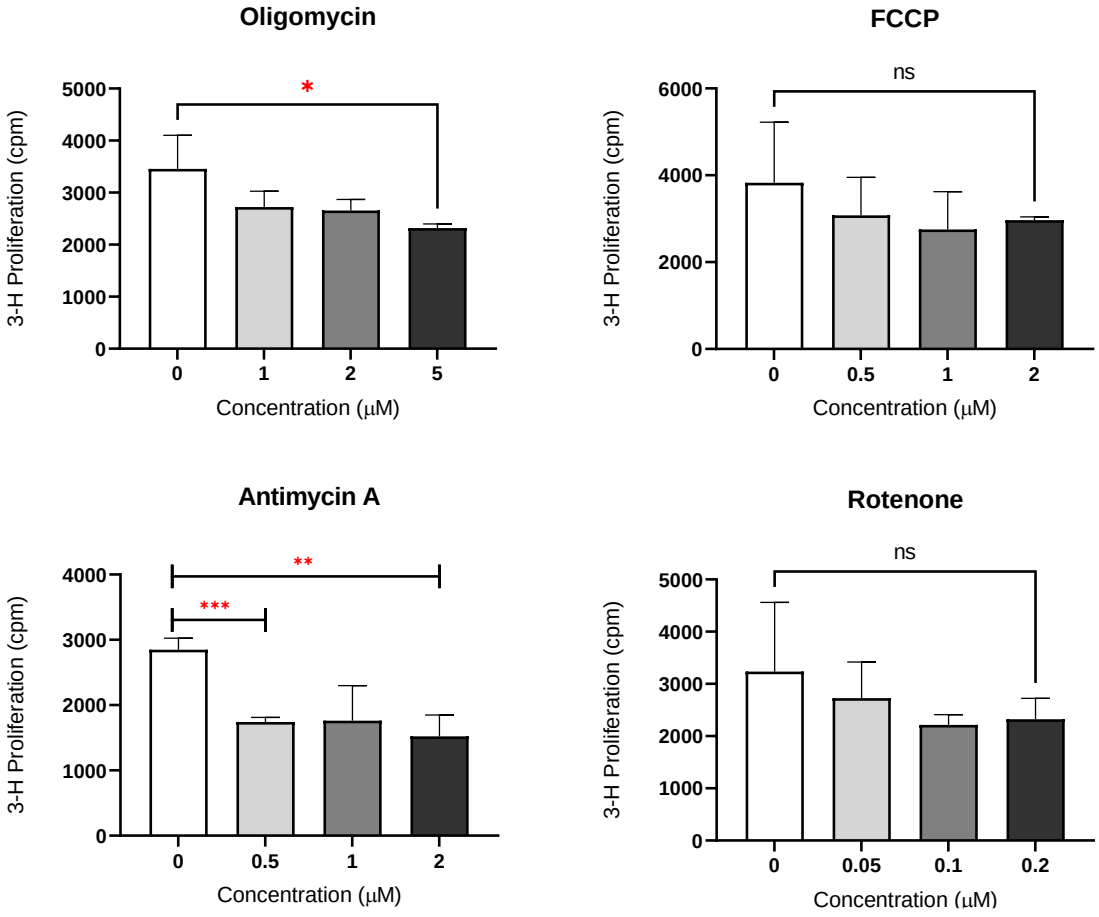


Figure 6.1. Thymidine incorporation assay to measure proliferative inhibition induced by mitochondrial inhibitors in PBMC cultures isolated from healthy donors. Separate PBMC cultures ($1.5x10^5$ cells/well) were exposed, in triplicate, to graded concentrations of oligomycin, FCCP, antimycin A and rotenone for 24 h (37 °C, 5% CO_2). Proliferation was then induced by mitogen driven stimulation following the addition of PHA (10 μg/mL) to every well of the assay plate. Cells were incubated for a further 48 h in the presence of PHA before being pulsed with tritiated [^{3}H]-thymidine (0.5 μCi/well) for an additional 16 h. Proliferative responses were detected by way of radioactivity incorporation and interpreted as counts per minute (cpm). Statistical significance was determined using a student's *t*-test (*P<0.05, **p< 0.01, ***p<0.001).

Toxicity profiles of mitochondrial inhibitors varied between each compound when assessed using PBMC from healthy donors. Concentration ranges were determined following extensive literature evaluation and graded dosage was employed for each compound to encompass both maximal and minimal concentrations of relevance for optimisation of mitochondrial stress test assays. Both FCCP (0 - 2 µM) and rotenone (0 - 0.2 µM) were tolerated by PBMC cultures and did not display detectable toxicity of any statistical significance. Conversely, oligomycin induced PBMC toxicity when cultures were exposed to high concentrations (5 µM) and toxicity was also detected after exposure to antimycin A at concentrations of 0.5 µM and 2 µM.

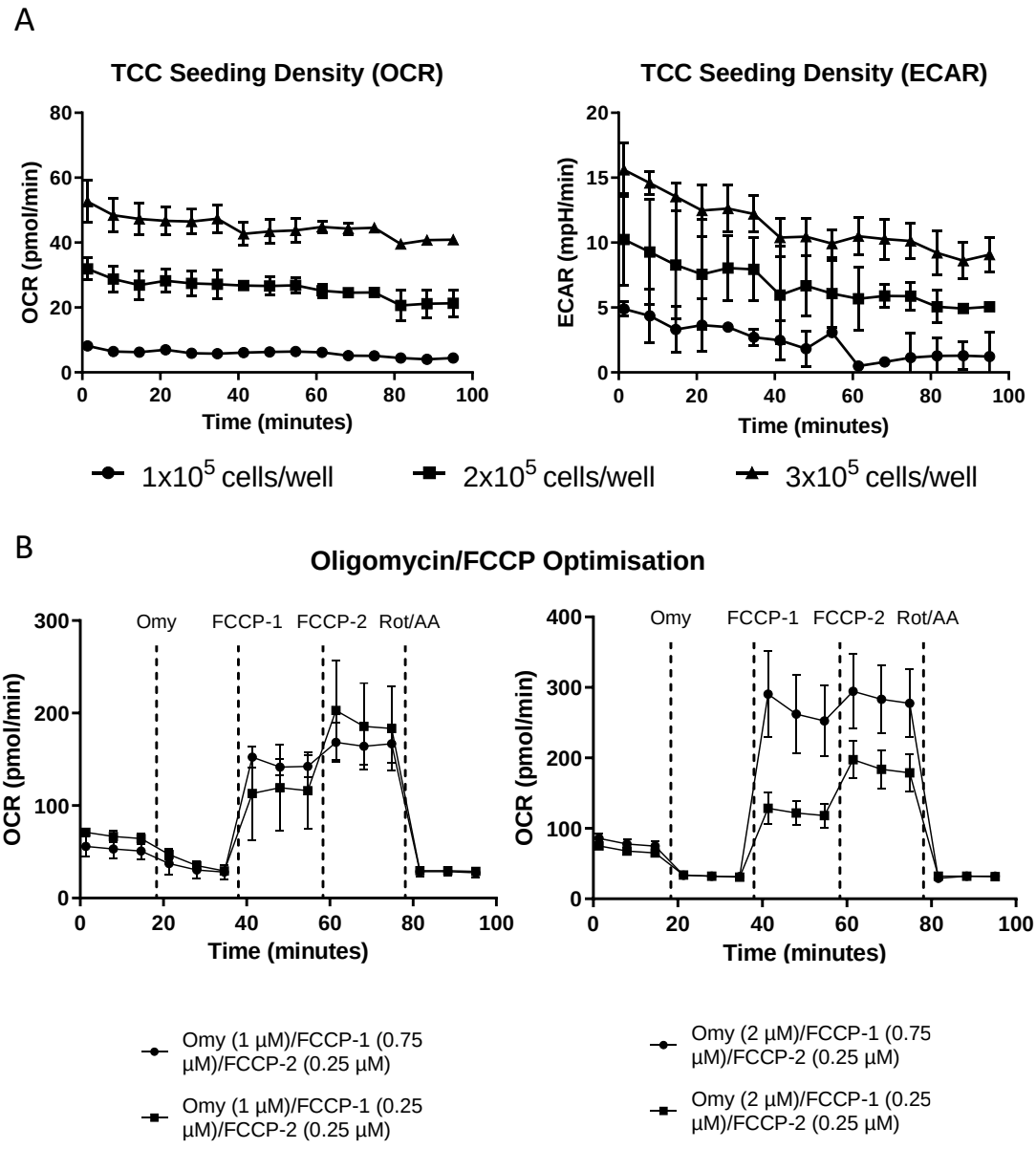


Figure 6.2. A) Basal OCR and ECAR measurements of TCCs. Drug-specific TCCs were harvested and resuspended in Seahorse XF base medium, diluted at $2x10^6$/mL, $4x10^6$/mL and $6x10^6$/mL. Cells were transferred to a Seahorse XFe96 cell culture microplate at seeding densities of at $1x10^5$, $2x10^5$ and $3x10^5$ T-cells per well. Basal OCR and ECAR readings were recorded by the Seahorse XFe96 Analyzer over a 96 min period without acute injection of stimulants or mitochondrial inhibitors. **B) Mitochondrial stress test with titrated concentrations of mitochondrial inhibitors.** T-cells were harvested and resuspended in Seahorse XF base medium and $3x10^5$ cells were transferred to a to Seahorse XFe96 cell culture microplate. Mitochondrial stress tests were performed with varying concentrations of inhibitors for optimisation purposes. Specifically, concentrations of oligomycin (omy) and FCCP were titrated between 1-2 µM and 0.25-0.75 µM respectively.

Basal OCR and ECAR measurements correlated with an increased seeding density of TCCs, with $3x10^5$ cells providing the highest and most viable energetic readout (Figure 6.2A). Acute injection of $3x10^5$ T-cells with oligomycin was shown to be optimal at 2 µM, while FCCP exhibited optimal activity at 0.75 µM (Figure 6.2B). A combination of rotenone (1 µM) and antimycin A (1 µM) successfully brought mitochondrial respiration to a halt. Acute injection of irradiated autologous APCs at titrated concentrations ($1x10^4$, $2.5x10^4$ and $5x10^4$) induced no alteration within basal OCR and ECAR readouts (Figure 6.3A). Additionally, at equivalent seeding densities no detectable OCR changes were observed in cultures of drug-specific TCCs ($3x10^5$ cells) after subjection to mitochondrial toxicants (Figure 6.3B).

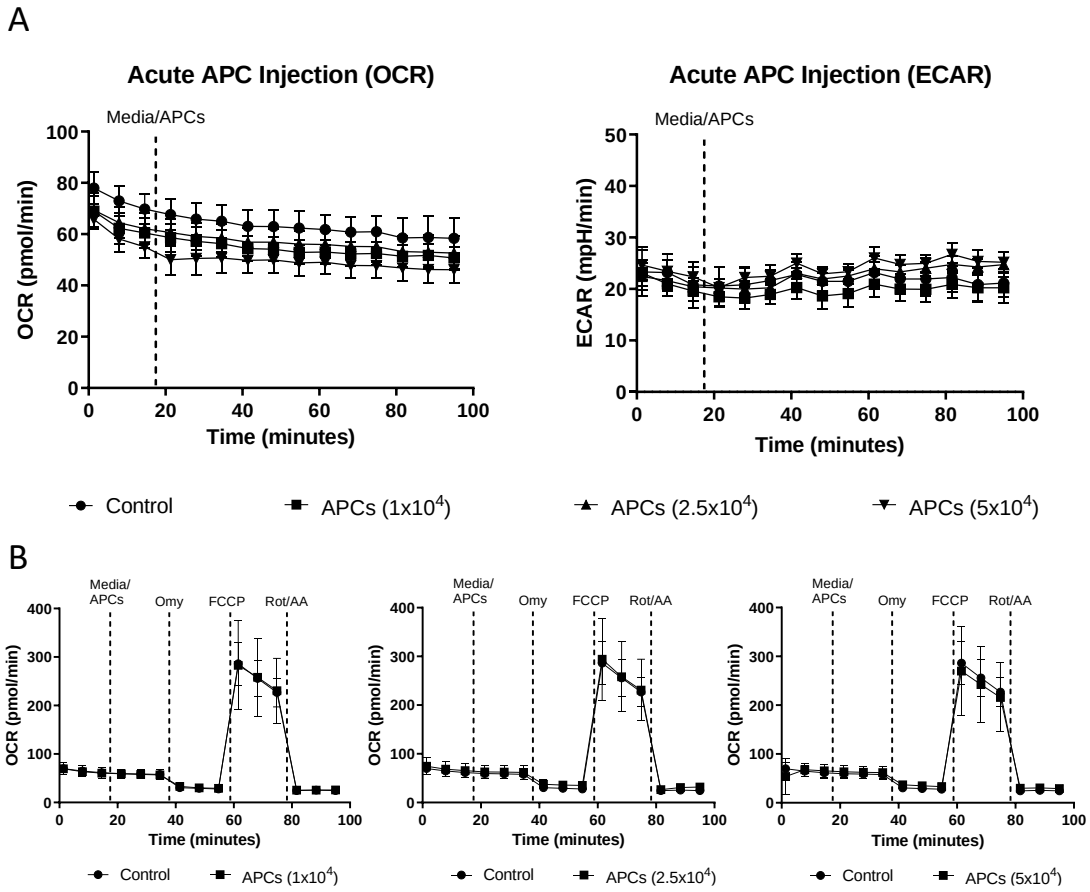


Figure 6.3. A) Basal OCR and ECAR measurements of TCCs after acute injection of irradiated autologous APCs. Drug-specific TCCs were harvested and resuspended in Seahorse XF base medium, diluted at $6x10^6$/mL and transferred to a Seahorse XFe96 cell culture microplate at a seeding density of $3x10^5$ T-cells per well. Basal OCR and ECAR readings were recorded by the Seahorse XFe96 Analyzer after acute injection of titrated APC concentrations ($1x10^4$, $2.5x10^4$ and $5x10^4$ cells). **Mitochondrial stress test with titrated concentrations of irradiated autologous APCs.** TCCs were resuspended in Seahorse XF base medium and $3x10^5$ cells were transferred to a to Seahorse XFe96 cell culture microplate. Mitochondrial stress tests were performed via acute injection with oligomycin (2 µM), FCCP (0.75 µM) and antimycin A/rotenone (1 µM), following an initial injection of APCs at titrated concentrations ($1x10^4$, $2.5x10^4$ and $5x10^4$ cells).

6.3.2 Phytohaemagglutinin (PHA) and CD3 activating antibodies are suitable model compounds and induce a glycolytic state in populations of drug-specific TCCs.

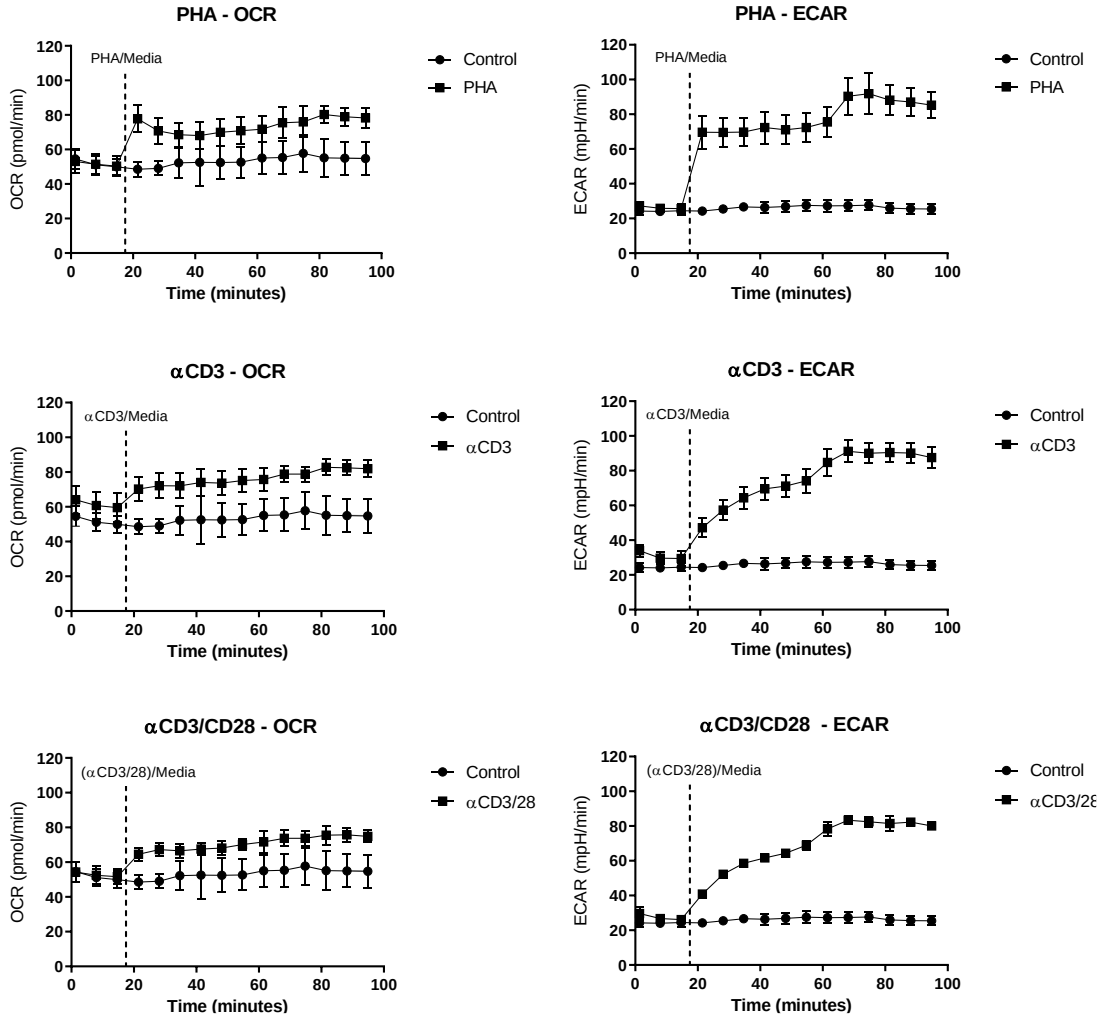


Figure 6.4. OCR and ECAR measurements of TCCs after acute injection of model stimulants. Drug-specific TCCs were resuspended in Seahorse XF base medium and transferred to a Seahorse XFe96 cell culture microplate in quadruplicate culture conditions at a seeding density of $3x10^5$ T-cells per well. Cells were then exposed to either PHA (10 µg/mL), αCD3 (10 µg/mL), αCD3/CD28 (10 µg/mL and 20 µg/mL) or Seahorse XF base medium only, via acute stimulation following the completion of 3 basal measurements. OCR (pmol/min) and ECAR (mpH/min) readouts were recorded by the Seahorse XFe96 Analyzer over a 96 min period.

Following acute simulation of TCCs, PHA induced non-specific T-cell activation characterised by immediate augmentation of ECAR by 70%. Although stimulation with PHA resulted in detectable increases within OCR values, this was limited to 30% of the initial basal readout. Similar results were also observed following direct TCR stimulation using anti-CD3 (αCD3). However, OCR increases, although detectable, were less pronounced and ECAR 'switching' was observed over an extrapolated time-period. The addition of anti-CD28 (αCD28) did not alter the energetic profile of TCCs compared with the use of αCD3 in isolation (Figure 6.4).

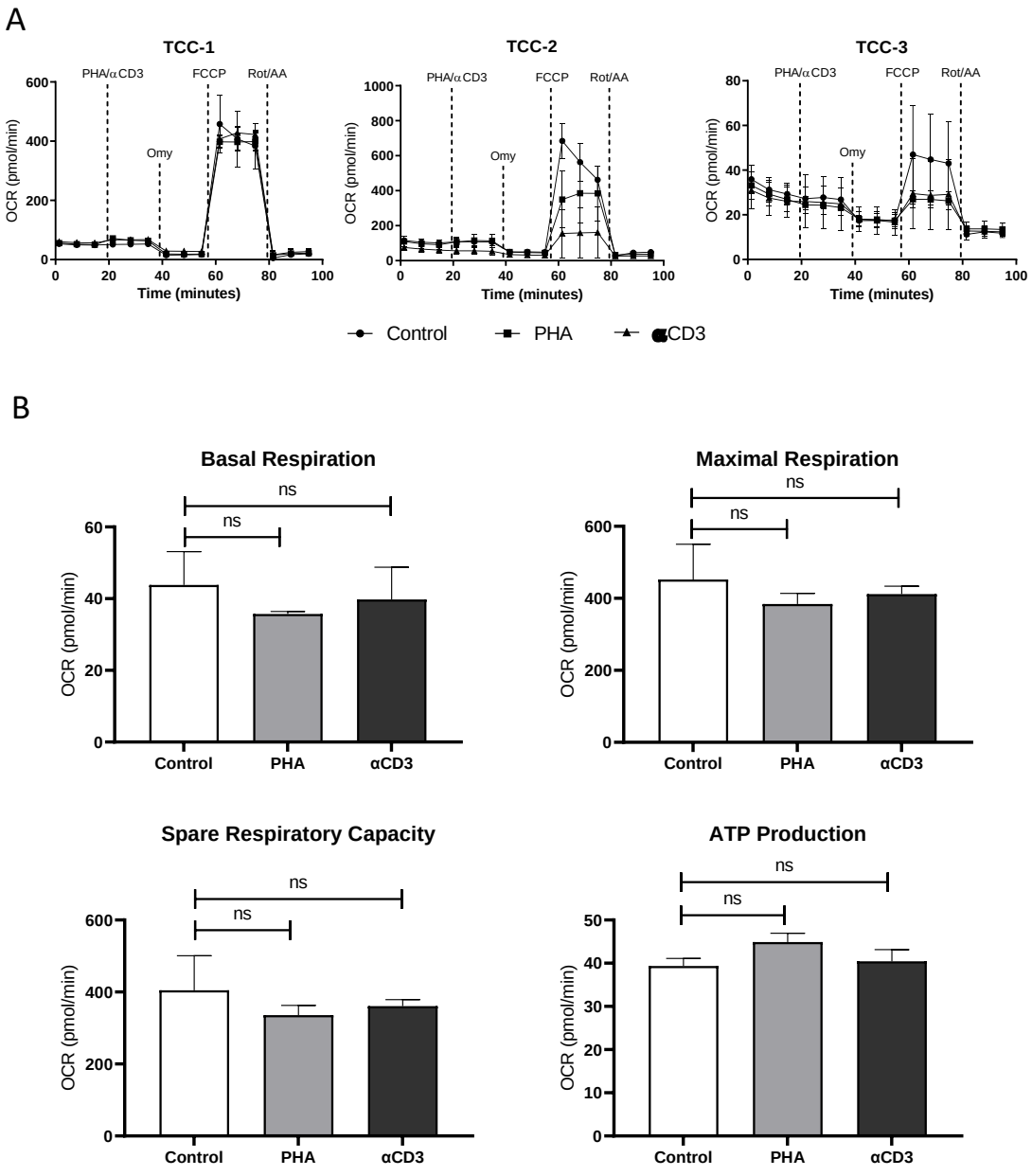


Figure 6.5. A) Mitochondrial stress test after acute injection of model stimulants (TCCs; n=3). Cells were resuspended in Seahorse XF base medium and $3x10^5$ cells were transferred to a to Seahorse XFe96 cell culture microplate. Mitochondrial stress tests were performed via acute injection with oligomycin (2 µM), FCCP (0.75 µM) and antimycin A/rotenone (1 µM), following an initial injection of either PHA (10 µg/mL), αCD3 (10 µg/mL) or Seahorse XF base medium (control). **B) Parameters of mitochondrial respiration.** Basal respiration, maximal respiration, spare respiratory capacity and ATP production were calculated using Wave™ Software and The Agilent Seahorse XF Cell Mito Stress Test report generator. Data shown is for a representative TCC (TCC-1) after acute stimulation with model compounds. Statistical significance was determined using a Mann-Whitney U test, in which a result of $P<0.05$ was deemed to be of statistical significance.

In each monoclonal drug-specific T-cell population, acute injection with PHA and αCD3 did not alter the mitochondrial stress profile in terms of augmentation, when compared to control conditions comprising of Seahorse XF base medium. Surprisingly, in 2/3 TCCs the addition of both PHA and αCD3 to the assay system resulted in reduction of maximal OCR values following sequential mitochondrial inhibition (Figure 6.5A). Further analysis of the functional parameters of mitochondrial respiration revealed that after acute stimulation with PHA and αCD3 there was no detectable or statistically significant changes within basal respiration, maximal respiration, spare respiratory capacity, or ATP production, when compared against the medium control (Figure 6.5B).

6.3.3 Model stimulants facilitate a transition towards glycolytic fuel pathways in glycolysis stress-based assays.

To assess the metabolic switching of T-cells to glycolytic fuel pathways, glycolysis stress assays were conducted. Such assays incorporate the glycolytic inhibitor, 2-deoxyglucose (2-DG), to verify glycolysis usage. Firstly, it was necessary to confirm the ability of 2-DG to inhibit immune stimulation (PBMC) and T-cell mediated cytokine release to validate glycolysis shutdown. Proliferative study of healthy donor PBMC cultures following exposure to graded concentrations of the 2-DG and mitogen driven stimulation resulted in potent inhibition of PBMC proliferation (Figure 6.6A). Proliferation of PBMC cultures from 2/3 healthy donors was found to be completely inhibited at concentrations of 2 mM, although in healthy donor 3 (HD-3) statistically significant proliferative blockade was not induced until exposure at 5 mM concentrations. ELISpot assays incorporating drug-specific TCCs (amoxicillin, dapsone and

flucloxacillin) revealed depleted IFN-γ secretion after exposure of TCCs to both drug and 2-DG (Figure 6.6B). Reduced cytokine secretion after 2-DG exposure was most prominent in populations of TCCs with specificity for dapsone.

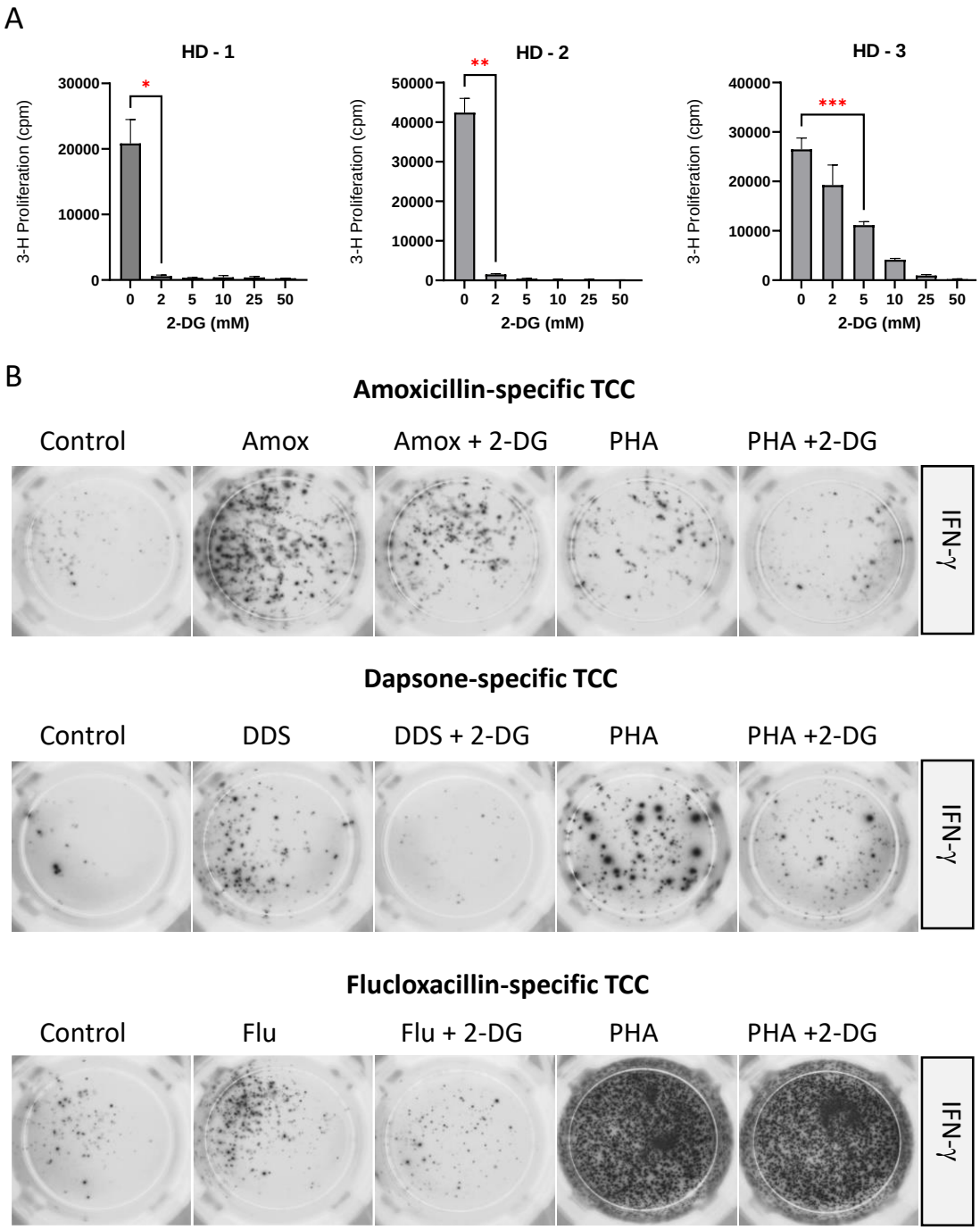


Figure 6.6. A) Thymidine incorporation to measure the effect of 2-DG on proliferation in PBMC cultures from healthy donors (n=3). Triplicate PBMC cultures ($1.5x10^5$ cells/well) were exposed to graded concentrations of 2-DG (0-50 mM) for 24 h (37 °C, 5% CO_2). Proliferation was induced by mitogen drive stimulation following the addition of PHA (10 µg/mL). PBMCs were incubated for a further 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for 16 h. Proliferative responses were interpreted as cpm and statistical significance was determined using a student's *t*-test (*P<0.05, **p< 0.01, ***p<0.001). **B) Cytokine release from drug-specific TCCs after exposure to 2-DG.** Drug-specific TCCs ($5x10^4$ cells) were co-cultured with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells), 2-DG (25 mM), cognate drug antigens [amoxicillin (amox); 2 mM, dapsone (DDS); 200 µM, flucloxacillin (flu); 1 mM) and either R9 medium or PHA (10 µg/mL)] for respective negative and positive controls. Cytokine secretion (IFN-γ) was measured after 48 h incubation (37 °C, 5% CO_2) by ELISpot.

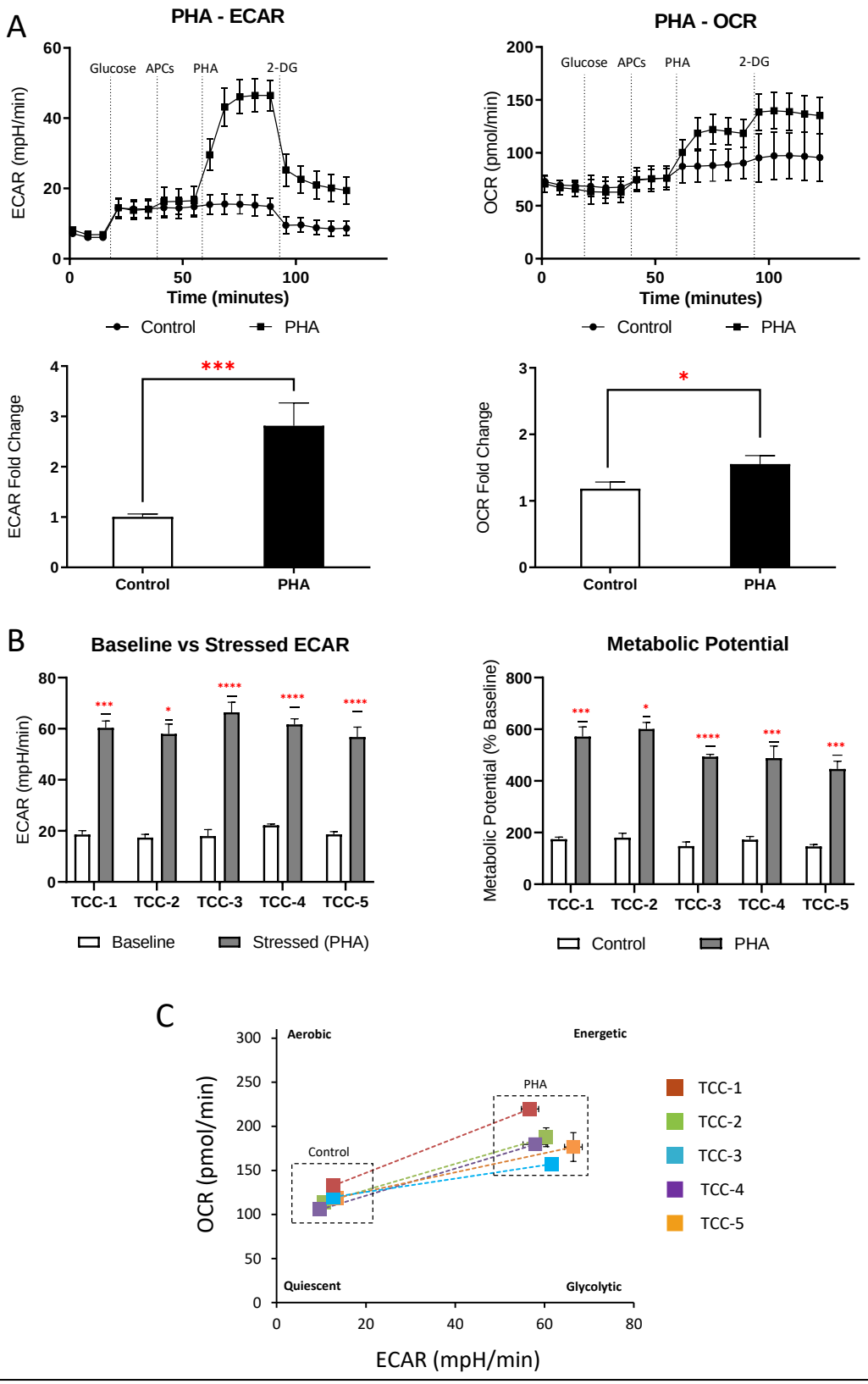


Figure 6.7. A) Glycolysis stress test incorporating acute exposure to PHA in drug-specific TCCs. 3x10^5 cells were transferred to a Seahorse XFe96 culture microplate. TCCs were sequentially exposed to D-glucose (25 mM), APCs (5x10^4), PHA (10 µg/mL) or Seahorse XF base medium followed by a final injection of 2-DG (25 mM) Data is shown for a representative TCC and statistical significance was determined using a student's *t*-test (*P<0.05, **p< 0.01, ***p<0.001, ****p<0.0001). **B, C) Glycolytic parameters and energetic shifting following acute injection of PHA.** Maximal ECAR values after PHA-induced stress and the metabolic potential of drug-specific TCCs were calculated using Wave™ Software and the Agilent Seahorse XF Cell Glycolysis Stress Test report generator. Data is shown for 5 representative TCCs.

Glucose injection successfully increased ECAR values to basal levels of glycolytic function and exposure to autologous irradiated APCs did not result in any detectable energetic changes. Glycolysis stress test assays assessing the utility of PHA for ECAR-dependent T-cell activation exhibited elevated ECAR (mpH/min) levels within 10 min post exposure in each of the 5 TCCs studied. PHA induced statistically significant increases in both ECAR and OCR levels, however the impact on ECAR was substantially higher with a 63% elevation detected compared to basal levels of glycolytic function (Figure 6.7A). These data were translatable in the 5 drug-specific clonal T-cell populations analysed when applied to increased ECAR levels following PHA-induced stress. Greater metabolic potentials were observed when compared with baseline values taken after glucose injection but prior to PHA exposure (Figure 6.7B). Metabolic phenotyping of 5 TCCs revealed a distinct shift from a quiescent state towards a more energetic and glycolytic state upon mitogen driven T-cell activation (Figure 6.7C).

Similar, but not identical, results were observed after stimulation of 3 drug-specific clonal populations with αCD3. ECAR dependence was again found to be favoured over OCR following acute stimulation with αCD3 antibodies in the glycolysis stress test assay system (Figure 6.8A). Once more, acute injection of D-glucose enabled TCCs to acquire optimal basal ECAR levels and rates of glycolytic function with exposure to APCs not inducing any detectable changes within the energetic profile. ECAR levels following αCD3-induced stress and the overall metabolic potential of TCCs were found to be increased compared to baseline and medium controls. However, further analysis only detected significance in 2/3 TCCs pertaining to αCD3-induced stress and 1/3 TCCs when considering overall metabolic potential (Figure 6.8B). Again, analysis of the metabolic phenotypes of these cellular populations via the amalgamation of both OCR and ECAR values before and after stimulation with αCD3 revealed a similar shift from quiescent and inactivated states towards a more energetic and glycolytic state upon TCR mediated activation (Figure 6.8C).

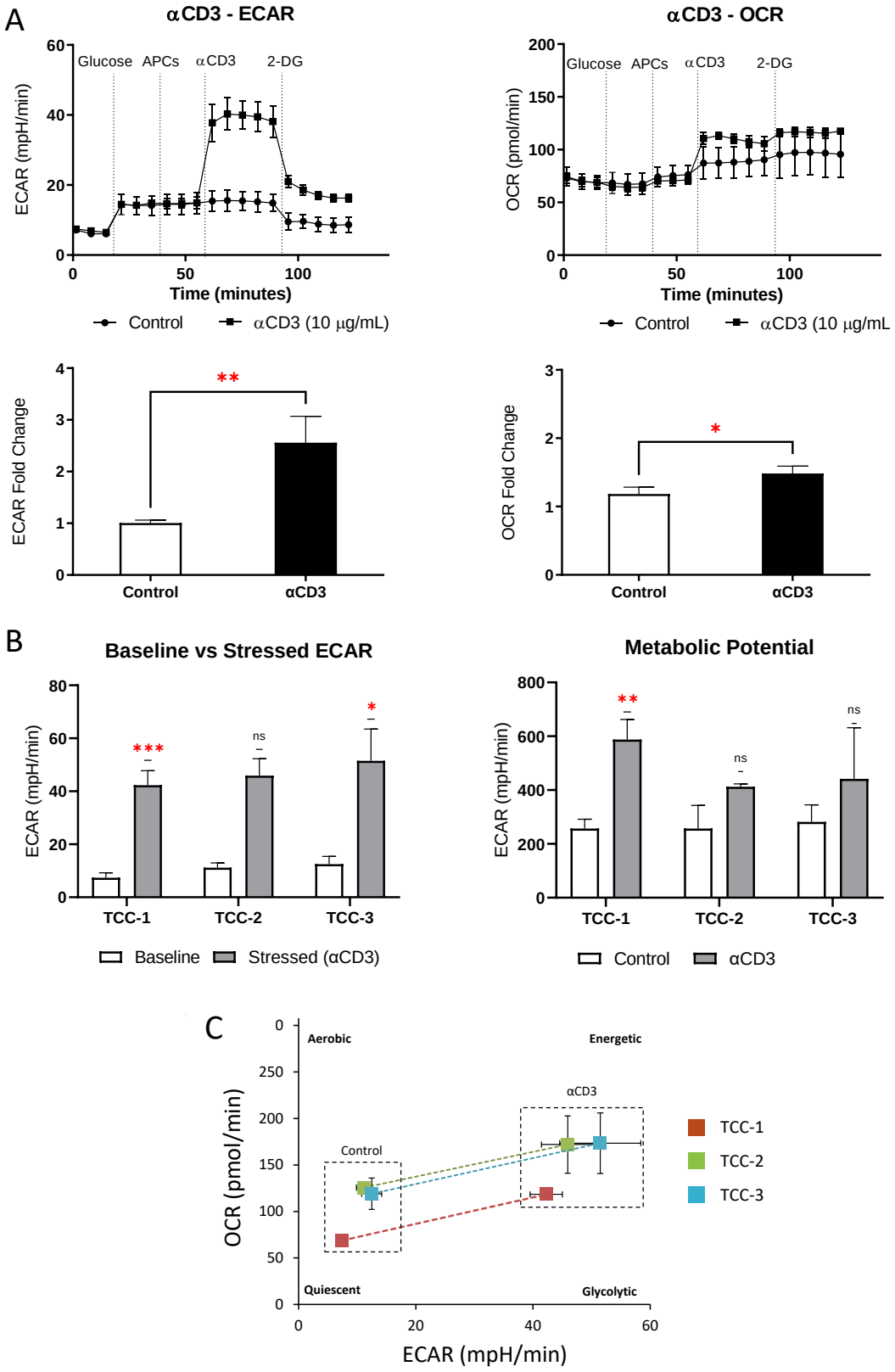


Figure 6.8. A) Glycolysis stress test incorporating acute exposure to αCD3 in drug-specific TCCs. 3x10^5 cells were transferred to a Seahorse XFe96 culture microplate. TCCs were sequentially exposed to D-glucose (25 mM), APCs (5x10^4), αCD3 (10 μg/mL) or Seahorse XF base medium followed by a final injection of 2-DG (25 mM) Data is shown for a representative TCC and statistical significance was determined using a student's *t*-test (*P<0.05, **p< 0.01, ***p<0.001). **B, C) Glycolytic parameters and energetic shift following acute injection of αCD3.** Maximal ECAR values after αCD3-induced stress and the metabolic potential of drug-specific TCCs were calculated using Wave™ Software and the Agilent Seahorse XF Cell Glycolysis Stress Test report generator. Data is shown for 3 representative TCCs.

6.3.4 Activation of vancomycin-reactive TCCs shows dependence for glycolysis in a dose-dependent and drug-specific manner.

Vancomycin-reactive TCCs generated via serial dilution from healthy donor PBMC (HLA-A*32:01 positive) were assessed for basal levels of cytokine secretion (IFN-γ) and proliferation after 48 h drug exposure (Figure 6.9). 3 representative CD8+ expressing TCCs were observed to release IFN-γ (Figure 6.9A) and proliferate in a dose-dependent manner after drug exposure at optimal stimulatory concentrations (Figure 6.9A). Within both assay systems, the addition of PHA was shown to induce strong, non-specific T-cell responses.

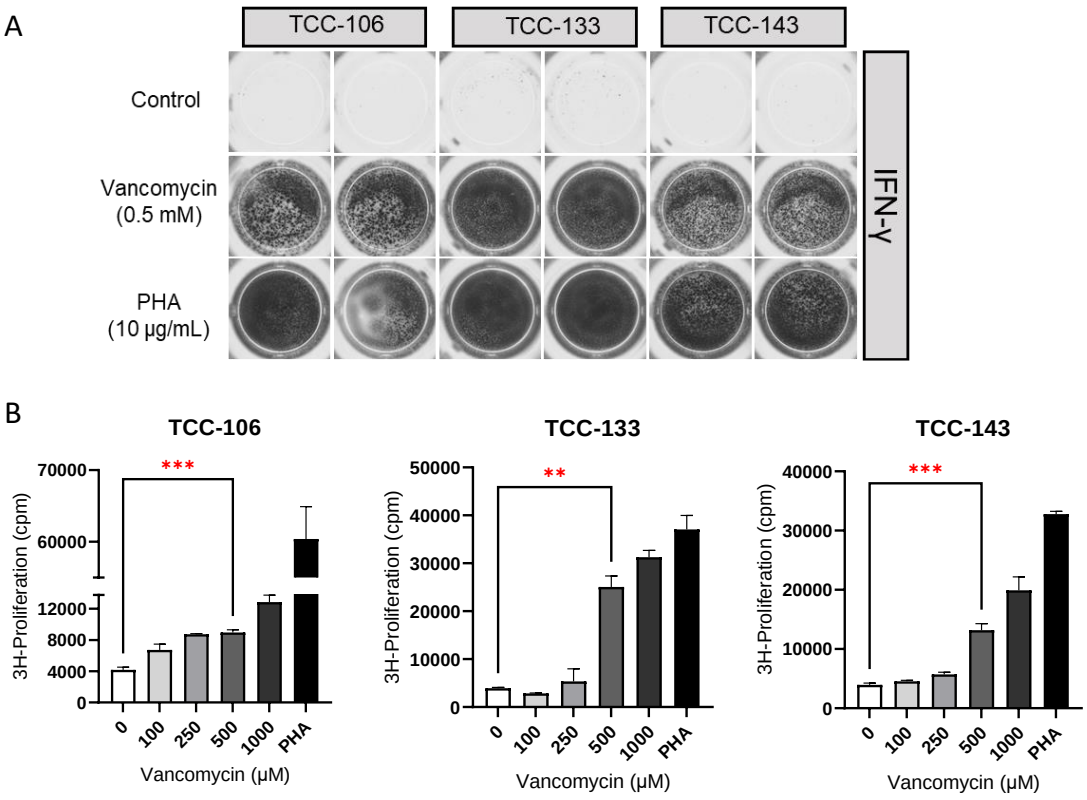


Figure 6.9. A) Cytokine release from vancomycin-specific TCCs. 5x10^4 TCCs were co-cultured with autologous EBV-transformed B-cells (1x10^4 cells) and either vancomycin (0.5 mM), R9 medium or PHA (10 µg/mL). Cytokine secretion (IFN-γ) was measured after 48 h incubation (37 °C, 5% CO_2) by ELISpot. **B) Proliferative response of vancomycin-specific TCCs.** 5x10^4 TCCs were co-cultured with autologous EBV-transformed B-cells (1x10^4 cells) and either vancomycin (0-1 mM), R9 medium or PHA (10 µg/mL). Cells were incubated for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Statistical significance was determined using a student's *t*-test (*P<0.05, **p< 0.01, ***p<0.001).

The activation of TCCs previously generated from healthy donors expressing the HLA-A*32:01 risk allele and with pre-determined specificity for vancomycin was observed to be a highly glycolytic process (Figure 6.10).

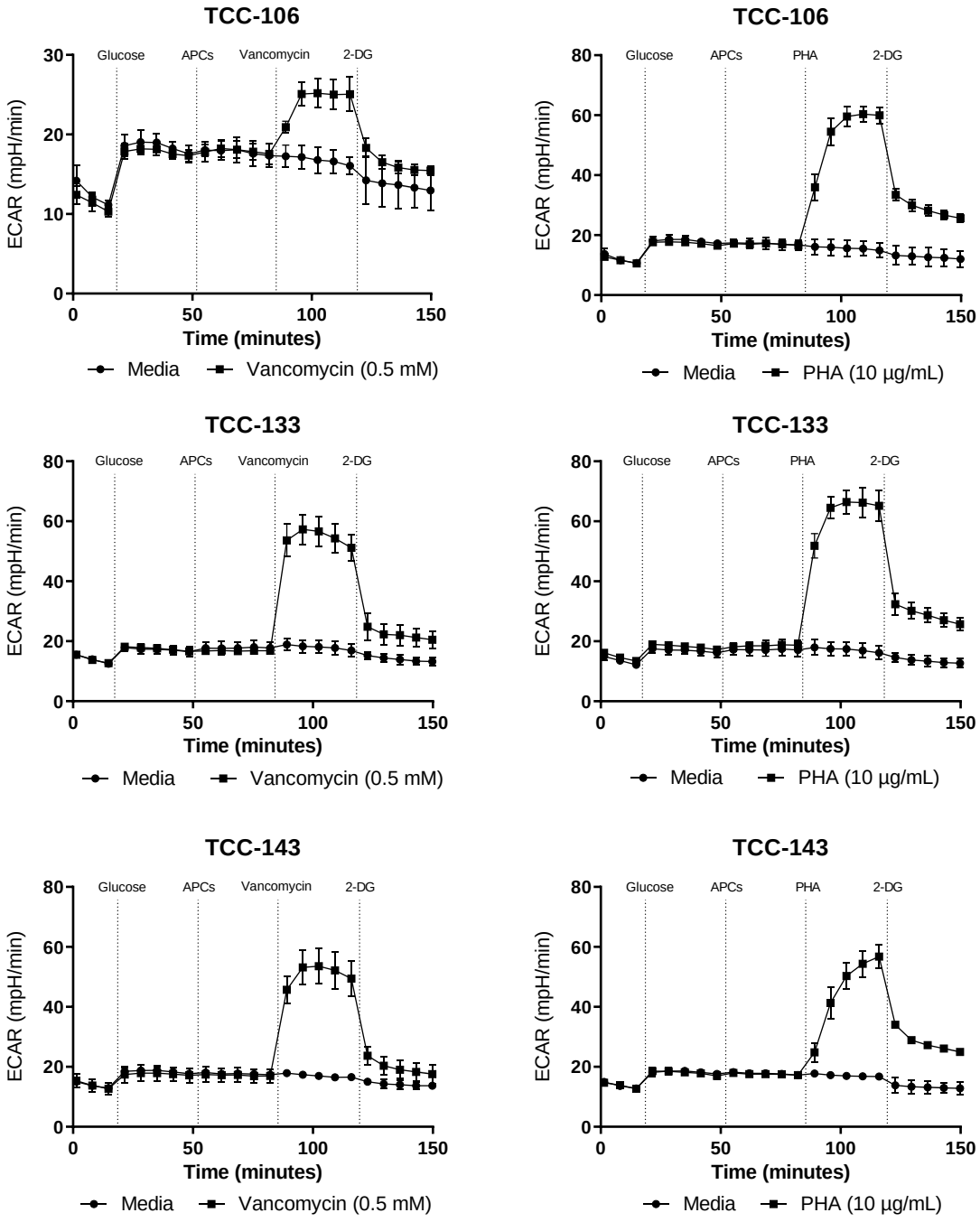


Figure 6.10. Glycolysis stress test incorporating acute exposure of PHA and vancomycin (TCCs; n=3). TCCs were sequentially exposed to D-glucose (25 mM), APCs ($5x10^4$), PHA (10 µg/mL) or vancomycin (0.5 mM) followed by a final injection of 2-DG (25 mM).

In glycolysis stress test assays assessing populations of vancomycin-reactive TCCs, PHA was found to induce elevated ECAR levels after acute injection, inferring transition to a glycolytic state after mitogen exposure and non-specific monoclonal activation (Figure 6.10). 3/3 drug-specific TCCs (TCC-106, TCC-133, TCC-143) exhibited glycolytic switching after acute exposure to autologous irradiated APCs and optimal concentrations of vancomycin (0.5 mM). Treatment with 2-DG successfully reversed the ECAR dependence observed, indicating that glycolysis was the primary metabolic pathway measured (Figure 6.10). These data were translatable as ECAR increases and glycolytic switching after vancomycin exposure was observed to be concordant with both IFN-γ secretion (Figure 6.9A) and proliferative responses (Figure 6.9B) within the same drug-specific TCC populations. Statistical analysis was performed at optimal vancomycin concentrations (0.5 mM) previously determined for use within *in vitro* assays.

Due to high levels of clonal expansion, one vancomycin-reactive TCC (TCC-143) was further assessed for the presence of both dose-dependent and drug-specific energetic responses to vancomycin and structurally unrelated compounds (Figure 6.11). Following D-glucose injection and activation of basal glycolytic function, vancomycin exposure resulted in a dose-dependent increase between 0.1 and 0.5 mM concentrations within the ECAR readout (Figure 6.11A). This response was found to be saturated at 1 mM, concordant with proliferative responses determined through [^{3}H]-incorporation assays after stimulation at graded concentrations of vancomycin up to a dosage of 1 mM, in addition to cytokine release assays (6.11C, D). Importantly, for the validity of the assay when assessing vancomycin-specific energetic responses, acute exposure to a structurally unrelated drug (CBZ) at optimal dosage induced no detectable change within the ECAR readout and produced no effect on the energetic profile (6.11C). Once more, these data neatly aligned with proliferative responses of vancomycin-specific TCCs when tested for cross-reactivity with CBZ at graded drug concentrations (6.11C).

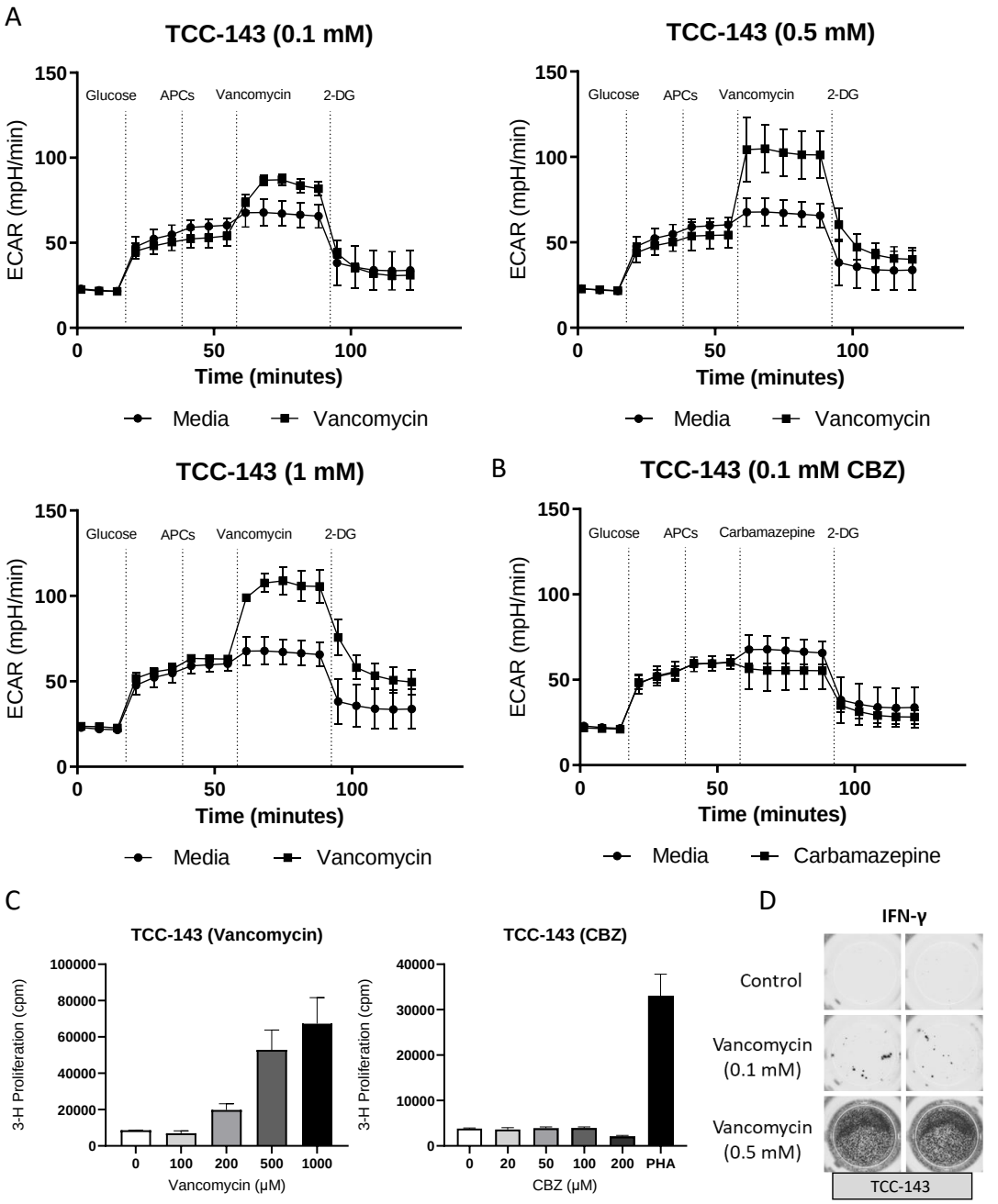


Figure 6.11. A) Glycolysis stress test at graded concentrations of vancomycin (TCC-143). 3x10^5 cells were transferred to a Seahorse XFe96 culture microplate. TCCs were sequentially treated with to D-glucose (25 mM), APCs (5x10^4) and either vancomycin (0.1, 0.5 and 1 mM) or Seahorse XF base medium followed by a final injection of 2-DG (25 mM). **B) Glycolysis stress test assessing energetic response to a structurally unrelated compound (TCC-43).** TCCs (3x10^5 cells) were consecutively exposed to D-glucose (25 mM), APCs (5x10^4) and either carbamazepine (100 µM) or Seahorse XF base medium before inhibition with 2-DG (25 mM). **C) Proliferative responses to vancomycin and CBZ (TCC-143).** 5x10^4 TCCs were co-cultured with irradiated autologous APCs (1x10^4 cells) and either vancomycin (0-1000 µM), CBZ (0-200 µM) or R9 medium. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values following 48 h incubation and 16 h pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well). **D) Cytokine release from vancomycin-specific TCCs at graded concentrations (TCC-143).** 5x10^4 TCCs were co-cultured with autologous EBV-transformed B-cells (1x10^4 cells) and either vancomycin (0.1 and 0.5 mM), R9 medium or PHA (10 µg/mL). IFN-γ release was detected after 48 h incubation by ELISpot.

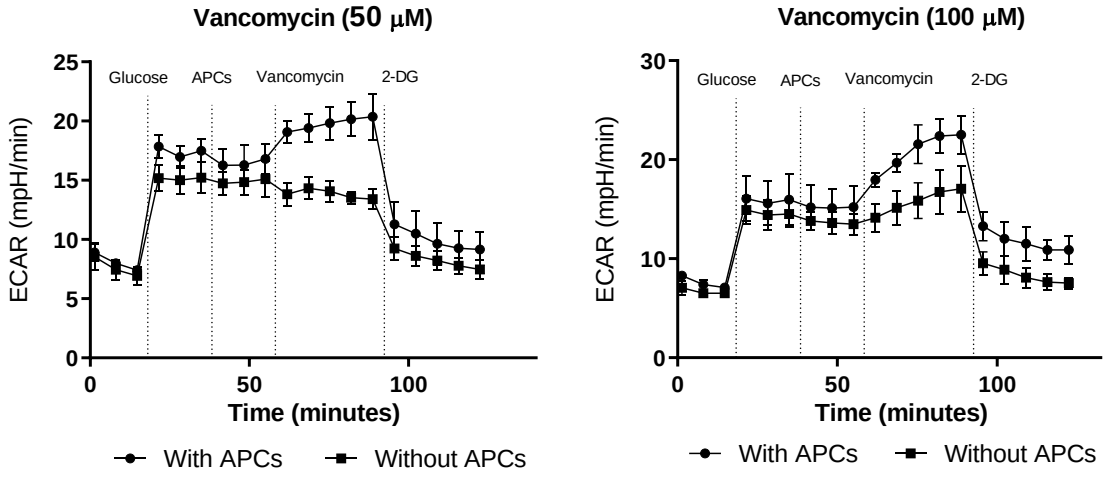


Figure 6.12. A) Glycolysis stress test to determine APC functionality within the activation of vancomycin-specific TCC (TCC-106). Drug-reactive TCCs generated from healthy donor PBMC (HLA-A*32:01 positive) with pre-determined specificity for vancomycin were harvested and transferred to a Seahorse XFe96 culture microplate ($3x10^5$ cells/well). TCCs were sequentially injected with to D-glucose (25 mM), APCs ($5x10^4$) or Seahorse XF base medium only. Following injection with D-glucose +/- EBV-transformed B-cells, TCC cultures were exposed to low, titrated concentrations of vancomycin (50 μM and 100 μM) or an alternative acute injection of Seahorse XF base medium. OCR (pmol/min) and ECAR (mpH/min) readouts were recorded by the Seahorse XFe96 Analyzer over a 122 min period. ECAR values shown for representative drug-specific TCC after stimulation with vancomycin.

To assess APC functionality, the ability of Epstein-Barr virus (EBV)-transformed B-cells to present drug antigens and subsequent elicitation of energetic responses were assessed within the glycolysis stress test assay system. TCCs (CD8+) generated from healthy donor PBMC positive for HLA-A*32:01 expression with previously characterised specificity for vancomycin (Chapter 3) were analysed after exposure to vancomycin at low dosage in the presence and absence of autologous irradiated APCs (Figure 6.12). Following acute stimulation with both 50 μM and 100 μM concentrations of vancomycin, T-cell activation was observed to possess greater glycolytic dependence denoted by elevated ECAR values immediately after acute drug injection in the presence of APCs, when compared to the Seahorse XF base medium control in place of APC subjection. At lower drug concentrations (50 μM), the departure from inactive states of basal glycolytic metabolism was more pronounced, to the extent composite stimulation with vancomycin and medium only (- APC) produced no detectable alteration within cellular metabolic states due to the absence of T-cell activation altogether.

6.3.5 TCCs with vancomycin specificity display HLA restricted activation and cross-reactive potential with structurally similar glycopeptides.

To corroborate the observed glycolytic responses of TCCs after vancomycin exposure with HLA dependency and to confirm the glycolytic stress test possesses utility when applied to the detection of responses requiring antigen presentation, HLA blockade within drug-specific populations was assessed (Figure 6.13).

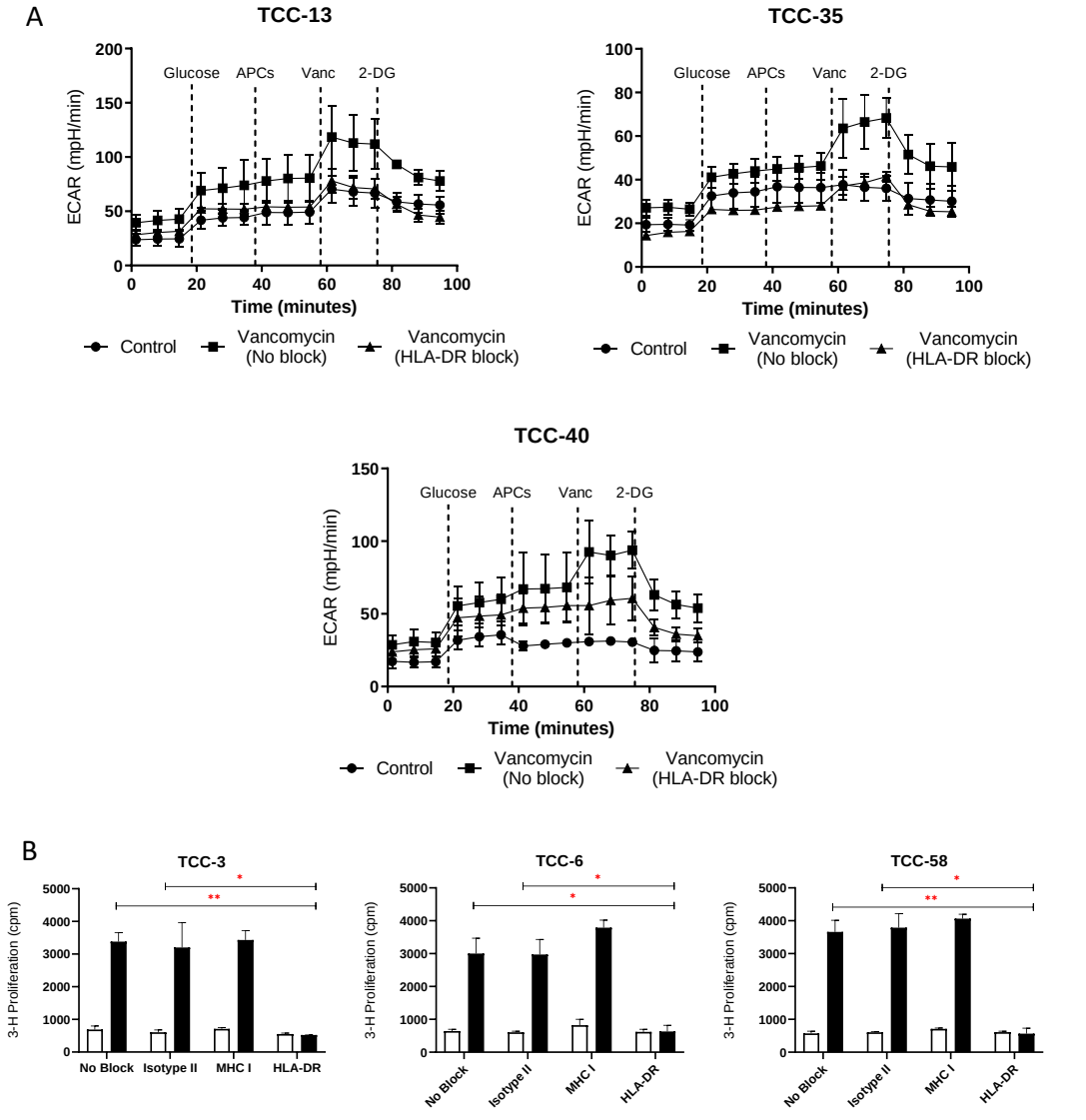


Figure 6.13. A) Glycolysis stress test in TCCs assessing energetic response to vancomycin after HLA-DR blockade. $3x10^5$ cells were transferred to a Seahorse XFe96 culture microplate and blocked with HLA-DR antibodies (10 µg/mL) for 1 h. TCCs were sequentially treated with to D-glucose (25 mM), APCs ($5x10^4$) and either vancomycin (0.5 mM) or medium followed by a final injection of 2-DG (25 mM). **B) Proliferative responses to vancomycin after HLA-DR blockade.** TCCs ($5x10^4$) were co-cultured with irradiated autologous EBVs ($1x10^4$) and either HLA Class I (10 µg/mL), HLA-DR (10 µg/mL) or corresponding IgG2 isotype controls (10 µg/mL) for 1 h. Blocked cultures were treated with vancomycin (0.5 mM) or medium for 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for 16 h. Proliferation interpreted as cpm values. Statistical significance was determined using a Mann-Whitney U test (*p< 0.05, **p< 0.01). Data is shown for representative vancomycin-specific TCCs (n=3) generated from autologous PBMC of a patient presenting with vancomycin-induced DRESS and expressing HLA-A*32:01.

3/3 CD4+ expressing TCCs generated from a vancomycin hypersensitive patient (illustrated within Chapter 5.3) displayed abrogated ECAR responses after HLA-DR blockade and vancomycin exposure, when compared to TCCs conventionally assessed in the absence of blockade (Figure 6.13A). These data, for which presentation via HLA-DR is suggested to be necessary for the induction of a glycolytic response, are concordant with HLA-DR restricted proliferative responses to vancomycin in a panel of TCCs generated from the same patient (Figure 6.13C).

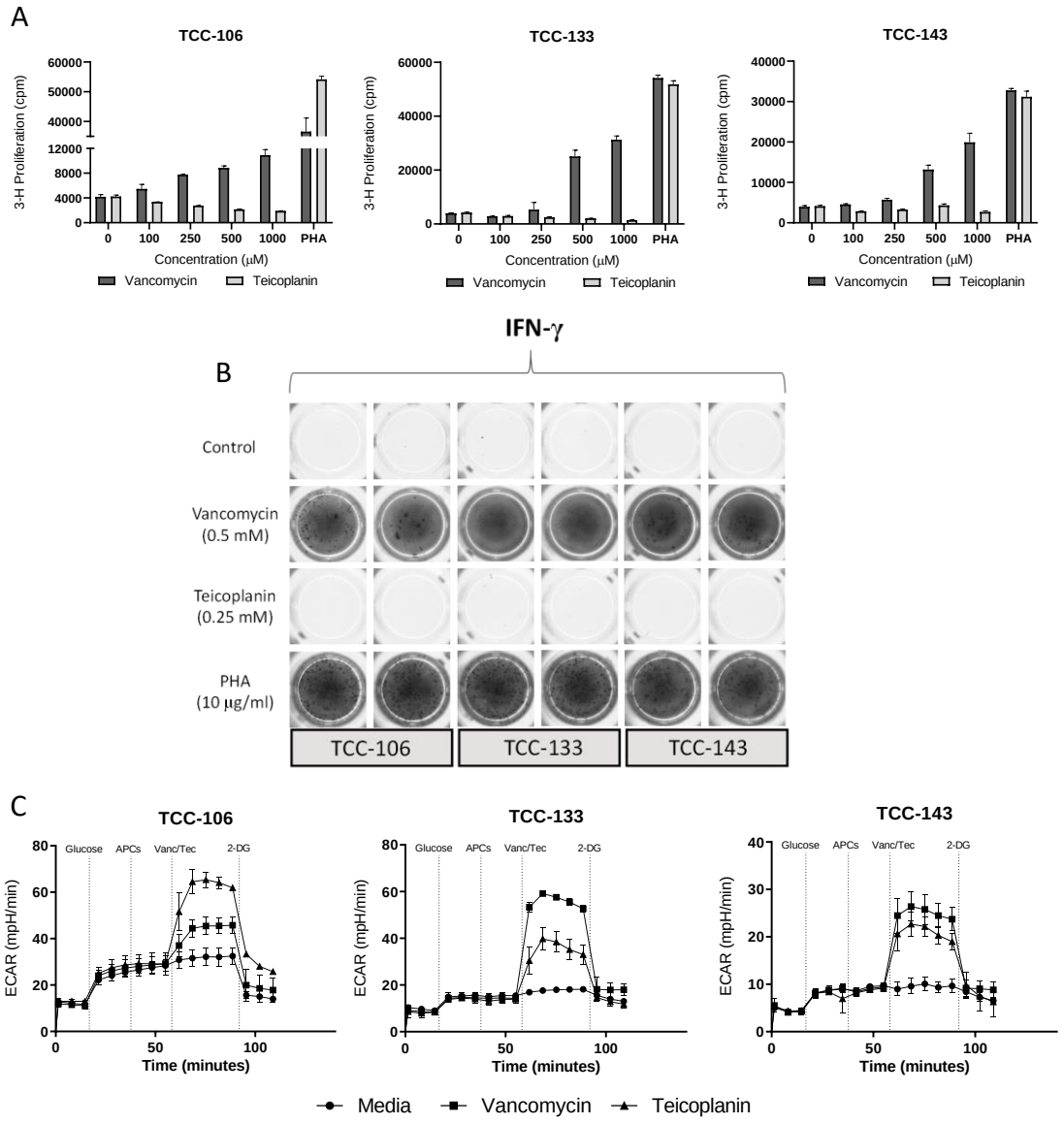


Figure 6.14. A) Proliferative cross-reactivity study of vancomycin-specific TCCs generated from healthy donor PBMC positive for HLA-A*32:01 expression (n=3). $5x10^4$ TCCs were co-cultured with irradiated autologous EBV-transformed B-cells ($1x10^4$ cells) and either vancomycin (0-1000 µM), teicoplanin (0-1000 µM) or PHA (10 µg/mL). Cultures were incubated for 48 h (37 °C, 5% CO_2) prior to pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for a further 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. **B) Assessment of glycopeptide cross-reactivity by cytokine release.** $5x10^4$ TCCs were co-cultured with autologous EBV-transformed B-cells ($1x10^4$ cells) and either vancomycin (0.25 and 0.5 mM), teicoplanin (0.25 and 0.5 mM), R9 medium or PHA (10 µg/mL) and IFN-γ release was detected after 48 h incubation by ELISpot. **C) Glycolysis stress test after acute injection of vancomycin or teicoplanin.** $3x10^5$ cells from each clonal population were harvested and transferred to a Seahorse XFe96 culture microplate. TCCs were sequentially treated with to D-glucose (25 mM), APCs ($5x10^4$) and either vancomycin (0.5 mM), teicoplanin (0.25 mM) or Seahorse XF base medium followed by a final injection of 2-DG (25 mM).

A representative panel of TCCs generated from healthy donor PBMC expressing HLA-A*32:01, initially primed and exhibiting dose-dependent specificity for vancomycin at graded drug concentrations (0-1000 µM), were tested for cross-reactivity with teicoplanin. Each clonal population (TCC-106, TCC-133 and TCC-143) expressed CD8+ surface markers (described in Chapter 3) and displayed graded proliferative responses to vancomycin but not teicoplanin, with PHA inducing a non-specific proliferative effect in all populations (Figure 6.14A). These findings were complemented following assessment of cytokine secretion for which IFN-γ secretion was detected after TCCs were exposed to optimal concentrations of both vancomycin and PHA, with no cytokine activity present after stimulation with teicoplanin (Figure 6.14B). Interestingly, cross-reactivity was detected when profiling the energetic parameters of T-cell activation after acute exposure to vancomycin and teicoplanin (Figure 6.14C). Immediate ECAR escalation was observed with vancomycin treatment but also following injection with teicoplanin in 3/3 TCCs profiled for cross-reactivity, with 2-DG inducing energetic blockade. Furthermore, activation of TCC-106 displayed a greater dependence for glycolysis after acute stimulation with teicoplanin, despite the absence of cross-reactive responses within conventional proliferative and cytokine-based assays.

6.3.6 Surface marker expression can delineate activation of vancomycin-specific TCCs at time points relevant for metabolic switching.

Vancomycin-reactive TCCs generated from the PBMC of patients positive for HLA-A*32:01 expression were assessed for CD3 downregulation after stimulation with vancomycin at time points correlating with glycolytic activation. Reduced CD3 (APC) fluorescence intensity was indicative of downregulation and drug-specific T-cell activation (Figure 6.15).

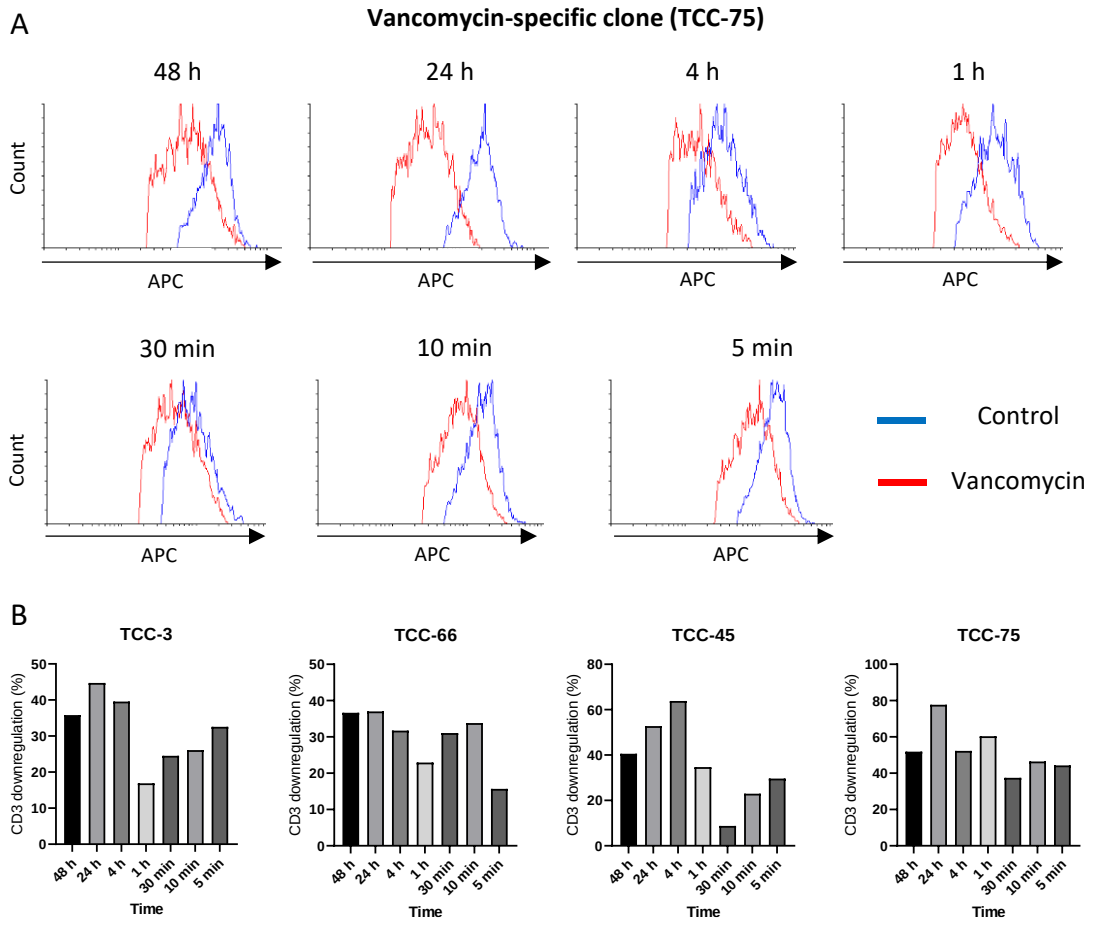


Figure 6.15. A) CD3 downregulation of drug-specific TCCs after vancomycin treatment. 5x10^4 T-cells with predetermined specificity for vancomycin were pulsed with optimal stimulatory concentrations (0.5 mM) at multiple time points (48 h, 24 h, 4 h, 1 h, 30 min, 10 min and 5 min) or R9 cell culture medium only. Activated cultures from a representative TCC (TCC-75) were then stained with 5 µL anti-CD3 (APC) antibody for 15 mins at 4 °C. Stained cells were washed in FACS buffer to remove unbound antibody and resuspended in 4% PFA. Samples were analysed using a FACS-Canto II instrument integrated with FACS DIVA operating software and phenotypic analysis was carried out using Flowing 2 software. **B) CD3 downregulation after vancomycin exposure (TCCs; n=4).** CD3 downregulation was interpreted as a % between control and drug exposed cultures. Unstained and untreated cultures were set up in parallel for comparison and samples were analysed using a FACS-Canto II instrument integrated with FACS DIVA operating software and phenotypic analysis was carried out using Flowing 2 software to calculate CD3 downregulation (%) of each TCC after pulsation with either vancomycin or medium for multiple time points.

After drug pulsation and staining, CD3 expression was found to be downregulated at each time point (Figure 6.15A). Within the panel of TCCs assessed (n=4), this trend was mirrored with varying degrees of vancomycin-induced CD3 downregulation observed (Figure 6.15B). Importantly, CD3 downregulation was detected at time points between 5-10 min post drug pulsation in each population. Assays studying the effect of transient vancomycin exposure on proliferation were conducted on drug-responsive TCCs generated from healthy donor PBMC (HLA-A*32:01). Similar results were across the panel of TCCs tested (n=3), with time points as low as 5 min capable of inducing a statistically significant proliferative effect, although T-cell responses were deemed to be optimal at time points between 2 h and 24 h (Figure 6.16).

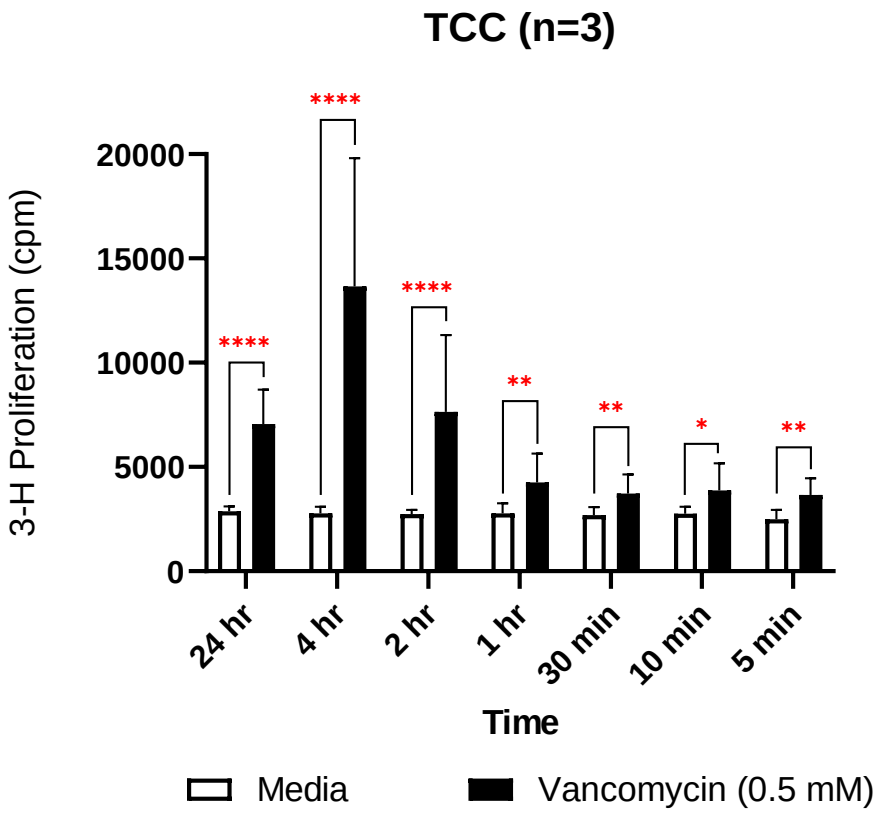


Figure 6.16. Proliferative responses to vancomycin after transient exposure (TCC; n=3). 5x10^4 TCCs were co-cultured with irradiated autologous EBV-transformed B-cells (1x10^4 cells) in triplicate cultures and pulsed with either vancomycin (0.5 mM) or R9 cell culture medium in parallel at multiple time points (48 h, 24 h, 4 h, 1 h, 2 h, 30 min, 10 min and 5 min) and incubated (37 °C, 5% CO_2). Pulsed TCC cultures were then thoroughly washed (5x) in R9 medium to exclude free drug from the assay and cultured for a further 48 h before pulsation with tritiated [^{3}H]-thymidine (0.5 µCi/well) for 16 h. Proliferation was determined by [^{3}H]-incorporation and interpreted as cpm values. Cpm values for 3 clonal populations were averaged for each time point studied. Statistical significance was determined using a student's *t*-test (*p<0.05, **p< 0.01, ****p<0.0001).

6.4 Discussion

Empirical evidence now exists detailing metabolic switching to both glycolysis and Warburg metabolism following the activation of resting, memory and naïve T-cells, with intrinsic differences now apparent between the metabolic activities of CD4+ and CD8+ expressing T-cells following TCR stimulation (Cao et al., 2014, Jones et al., 2017). This has been evidenced by a marked upregulation of glycolytic precursors within glucose metabolism as circulating lymphocytes exit quiescence following antigen exposure due to the immediate requirement of metabolic precursors to support rapid growth and clonal expansion (Frauwirth et al., 2002, Bauer et al., 2004). Applications towards utilising metabolic signatures, such as glycolytic switching, after stimulation as an early marker for drug-specific memory T-cell responses would aid detection of potentially immunogenic compounds. Indeed, utility has now been demonstrated when determining the presence of both memory and cross-reactive metabolic responses to glycopeptide antibiotics such as vancomycin and teicoplanin, for which drug-specific T-cells have successfully been generated (Ogese et al., 2021, Gardner et al., 2022).

In order to delineate the presence of metabolic adaptation that underpins the ability of drug-specific TCCs to enter a glycolytic-like state, it was necessary to optimise and develop assays capable of measuring the energetic parameters of T-cell activation following acute stimulation. Earlier studies attempting to address this issue have focussed on the development of basic mitochondrial stress test assays for application for T-cell bioenergetic measurement, with these assays providing initial starting points with regards to optimisation for T-cell seeding and mitochondrial inhibitor concentrations (van der Windt et al., 2016a). Oligomycin is a potent competitive inhibitor of the F_0 subunit that forms part of the ATP synthase complex implicated within electron transport and energy production through OXPHOS (Shchepina et al., 2002). In these studies, oligomycin was only found to induce toxicity within PBMC cultures at elevated dosage not deemed to be of physiological or experimental relevance within the context of T-

cell inhibition. Additionally, strong toxicity was detected in PBMC cultures after exposure to antimycin A followed by mitogen-induced proliferation (Figure 6.1). This is unsurprising, due to role of antimycin A within OXPHOS blockade and its ability to inhibit electron flow through Complex III combined with the known dependence of PBMCs for OXPHOS to support proliferative function (Ma et al., 2011, Nelson et al., 2021).

For the purpose of this study, a delicate balance had to be struck between sufficient T-cell seeding densities capable of detecting changes within immunometabolic profiles with a high degree of sensitivity, and exhausting drug-specific primary T-cell cultures. To this end, T-cell seeding densities of $3x10^5$ cells/well were deemed to provide near-optimal basal energetic readings (OCR and ECAR) whilst preventing depletion of test cultures, thus enabling thorough bioenergetic analysis of single, monoclonal populations (Figure 6.2). The use of immortalized cells, such as the HepG2 cell line, for bioenergetic assessment have been more extensively studied and optimised for use at vastly reduced cellular concentrations due to their intrinsically higher metabolic activity and adherent properties, although the application of such cell lines have predominantly been evaluated within the context of mitochondrial toxicity screening (Kamalian et al., 2018, Kamalian et al., 2015). EBV-transformed B-cells have been shown to be proficient at antigen presentation culminating in the elicitation of cytotoxic T-cell responses (Ramadan, 2008). As a result, incorporation of APCs within the assay system for the measurement of energetic parameters associated with T-cell activation represented a key component of the assay development process. APCs were exposed to gamma irradiation to inhibit proliferation whilst retaining functionality in terms of antigen presentation and acutely injected within the assay at similar ratios described in the conduction of conventional T-cell and functional based assays (Naisbitt et al., 2003a). Importantly, the addition of APCs to the assay systems induced no effect on either the basal energetic readout or mitochondrial stress profile (Figure 6.3). This indicated successful target cell adherence and the presence of an isolated T-cell energetic readout following direct contact with both oxygen and hydrogen

sensitive fluorophores, absent involvement or energetic contribution from acute APC exposure.

Building on the notion that an immediate-early 'glycolytic switch' facilities metabolic reprogramming when applied to both *de novo* and memory T-cell responses, we hypothesised that glycolytic measurement could be used as a sensitive indicator of drug-specific T-cell activation. Furthermore, it was anticipated that patterns of clonal reactivity could be elucidated using an energetic readout. Various assays systems exploring the existence of alternative activation markers have previously shown utility when detecting T-cell activation, such as the application of Ca^{2+} influx assays detailing T-cell reactivity with abacavir (Adam et al., 2012). To aid detection of drug-specific T-cell responses manifesting as a visible 'switch' between metabolic pathways, positive controls in the form of model compounds with well-defined stimulatory properties were introduced and optimised for using mitochondrial and glycolysis stress assays (Figure 6.4). Firstly, PHA isolated from *phaseolus vulgaris* was shown to induce a strong fluxing towards glycolytic dependence in preference to OXPHOS, as expected of the transition between T-cells in quiescent and activated states. Indeed, PHA activates human T-cells in a non-specific manner to induce a proliferation and is widely deployed within functional T-cell assays for validation purposes (Ceuppens et al., 1988).

To examine and model T-cell activation it was useful to assess energetic function after stimulation with compounds possessing greater physiological relevance. As a consequence, both CD3 and CD28 activating antibodies were studied in isolation and cooperatively to elucidate the impact of TCR mediated stimulation on the activation profile of drug-specific TCCs. CD3 and CD28 are typically amalgamated within functional assays attempting to induce polyclonal T-cell activation and cellular expansion. *In vitro* applications attempt to mimic both signal 1 and signal 2 interactions typically provided through interplay between the APC and TCR for which an activation threshold is determined (Trickett and Kwan, 2003). Interestingly,

integration of both CD3 and CD28 activating antibodies revealed no discernible energetic advantage when compared to the activity of anti-CD3 in isolation. From this it can be deduced that fuel pathway dependence, for which glycolysis was implicated, was influenced by the presence of signal 1 but not signal 2. In this way, glycolytic switching measured within the assay system was sensitive to interactions between antigen and TCR, mimicked by the addition of anti-CD3, but not to any co-stimulation provided by anti-CD28 for which T-cell survival would be promoted and a proliferative effect induced (Boise et al., 1995). Co-stimulation is necessary for proliferative output but signalling is not instantaneous, with some studies suggesting proliferative commitment of naïve T-cells requires a minimum of 6 hours (Kaech et al., 2002). Therefore, it is plausible that the current assay set up, for which energetic readouts are taken over a 1-2 hour timeframe is insufficient to capture the metabolic pathways involved within co-stimulatory signalling. This is further evidenced by the parallel proliferative study of TCCs after drug pulsation and subsequent removal (Figure 6.16), for which readouts suggest proliferative commitment and by extension sufficient co-signalling, is optimal at 2-4 hours. These findings neatly align with studies suggesting the duration of antigen: TCR stimulation is critical determinant of the co-stimulatory requirements of T-cells, for which a lowering of the threshold may manifest (Kündig et al., 1996).

For more detailed evaluation of the bioenergetic phenotypes induced by model stimulants and more importantly the activity of immunogenic compounds associated with hypersensitive disease states such as the glycopeptide antibiotics, glycolysis stress assays were developed. These assays deviated from traditional methodology via the substitution of oligomycin for soluble drug, thus allowing for the detection of any glycolytic flux directly imposed by relevant compounds. To assess glycolytic function, it was important to incorporate controls capable of confirming the induction of glycolysis. As a result, a final injection of the glucose analogue, 2-DG, was deployed following successful optimisation within both proliferative and cytokine-based assays for which inhibitive properties were demonstrated (Figure 6.6). 2-DG acts to

competitively inhibit hexokinase II and induce glycolytic blockade, confirming ECAR shifts observed within the assay are due to elevated levels of glycolysis (Pajak et al., 2019). After thorough assessment via glycolysis stress assays and detailed analysis of the metabolic parameters associated with the activation of drug-specific TCCs, both non-specific (PHA) and TCR mediated (αCD3) were deemed to be suitable model compounds with PHA possessing somewhat greater utility when applied to profiling T-cell responses following stimulation, perhaps due to its less targeted nature that may represent a 'scatter gun' approach to T-cell activation (Figure 6.7, 6.8).

To complement the existing body of work detailing fuel dependency after artificial stimulation, for which the metabolic pathways of glycolysis and OXPHOS have been consistently implicated, we wanted to determine if ECAR measurement, and by extension glycolytic induction, could be used as a metabolic signature of vancomycin-specific T-cell activation (van der Windt et al., 2012, Gubser et al., 2013). The drug itself has recently been associated with the onset of DRESS reactions in individuals expressing the HLA-A*32:01 allele and recent studies have elucidated the role of CD8+ T-cells within the pathogenesis of such reactions, perhaps unsurprising for a reaction associated with HLA Class I phenotype (Konvinse et al., 2019, Ogese et al., 2021). Vancomycin-reactive TCCs expressing CD8+ phenotypes generated from healthy donor PBMC (HLA-A*32:01 positive) with predetermined drug-specificity (detailed in Chapter 3) were found to exhibit metabolic switching accentuated by an increased dependence for glycolysis after acute vancomycin exposure (Figure 6.9). Furthermore, the energetic changes detected with stress-based assays were found to dose-dependent and drug-specific, concordant with assays performed in parallel detailing proliferative responses and cytokine release (Figure 6.11). This was important for validation of the assay and provided affirmation that; (1) the energetic readout is influenced by the number of interactions between vancomycin and the TCR, as proven by dose-dependency (2) the observed glycolytic response was vancomycin-specific and not caused by cellular stress, as indicated by the

absence of any detectable response following the addition of a structurally unrelated compound.

It was necessary to confirm the presence of functional antigen presentation within the glycolysis stress test to verify both physiological relevance and provide a degree of synchronization between metabolic readouts and *in vitro* T-cell assays. We have demonstrated that at low concentrations of vancomycin APCs are required for the activation of CD8+ expression T-cells (Figure 6.12). It is important to note that the functionality of APCs within the assay system was only detectable at drug concentrations situated around the minimum threshold of activation. This is likely due to expression of MHC Class I on the surface of vancomycin-specific TCCs facilitating self-presentation at higher drug concentrations, thus making the role of APCs redundant (Neefjes et al., 2011). Additionally, blockade of HLA-DR in vancomycin-specific TCCs generated from DRESS patients (HLA-A*32:01 positive) and expressing CD4+ surface markers resulted in abrogation of glycolytic dependence after vancomycin exposure (Figure 6.13). Therefore, antigen presentation capable of eliciting energetic responses to vancomycin is has been successfully modelled with HLA interactions clearly visible, adding to the functional relevance of the assay. However, to reinforce HLA involvement within the real-time measurement of bioenergetic parameters, further study will be required focusing on HLA Class I blockade in order to provide full alignment between the glycolytic dependence and association with HLA Class I and HLA Class II restricted T-cell activation. By extension, it will also be prudent to study any metabolic discrepancies that may exist between CD4+ and CD8+ expressing TCCs with drug-specificity, with the overarching aim connecting metabolic signatures to the unique phenotypic characteristics of both helper and cytotoxic T-cell responses implicated within hypersensitivity.

Most significantly, cross-reactivity between structurally similar glycopeptide antibiotics was detected when analysing energetic responses in populations of TCCs absent of any cross-

reactive potential when considering proliferative and cytokine readouts (Figure 6.14). In this case, TCCs with prior specificity for vancomycin were also found to exhibit glycolytic switching after exposure to teicoplanin, for which T-cell responses have also been implicated within the pathogenesis of teicoplanin-induced DRESS reactions (Gardner et al., 2022). The close structural relationship shared between compounds, specifically the presence of heptapeptide scaffold, potentially explains the cross-reactivity observed when focusing solely on activation measured by the induction of glycolysis (Cynamon and Granato, 1982). However, it is intriguing that this 'energetic' cross-reactivity is not translatable within conventional T-cell assays. This suggests that although interactions between drug, HLA molecules and the TCR (signal 1) are clearly visible in the form of metabolic readout, co-stimulatory function (signal 2) is lacking. Therefore, quiescence exit is not fully achieved and T-cells will not progress to a proliferative state following initial activation (Bretscher, 1999). These data again support the use of metabolic dependency as a measure of T-cell activation for which greater sensitivity can be acquired. Additionally, the presence of activation illustrated by a metabolic switch that does not progress to proliferative commitment is conformation of initial interaction between structurally related glycopeptides at the immune synapse and potential evidence for peptide mimetic properties.

Finally, it was important to corroborate the immediacy of the observed metabolic traits that signify T-cell activation with surface marker expression at time points relevant for the glycolysis stress test assay. CD3 was found to be downregulated after stimulation with vancomycin at time points correlating with glycolytic activation (Figure 6.15). The CD3 surface receptor, specifically when downregulated, can be used as a reliable maker of T-cell activation following drug: TCR ligation (Liu et al., 2000, Boyer et al., 1991). As a result, downregulation of such surface markers suggest T-cells are capable of activation after momentary drug exposure. Furthermore, transient exposure at energetically relevant time points to vancomycin followed by drug removal has been shown to induce a proliferative effect in populations of drug-specific

TCCs. This phenomenon has previously been illustrated when studying the impact of co-stimulation, for which the presence of either CD28 or 4-1BB during activation has been found to modulate the metabolic profiles of memory T-cells after activation signalling had long been removed (Krause et al., 1998, Menk et al., 2018a). Moving forward, due to the limitations associated with the generation and expansion of drug-specific T-cells this assay would most likely be optimised for the incorporation of PBMCs, with the objective of immunogenic screening using glycolysis as an early maker of immune-cell activation.

7. General discussion.

Contents

7.1 The escalating problem of adverse drug reactions.

Adverse drug reactions (ADRs) are typically defined by the development of aberrant and undesirable effects suffered post drug administration, with clinical presentation ranging from mild to severe. Although rare, the manifestation of severe ADRs are a major cause of both morbidity and mortality with the negative effect on individual patient outcomes universally understood. When extrapolated this contributes to a significant burden on healthcare systems throughout the world. Initially, it was thought that approximately 8% of all hospital admissions in the UK are attributed to ADRs accounting for up to 4% of hospital bed capacity in some regions (Chan et al., 2016, Pirmohamed et al., 2004). Updated prospective analysis now suggests the issue is growing as ADR prevalence now accounts for 16.5% of total admissions (Osanlou et al., 2022). This bestows a huge economic burden on healthcare services, with annual projections for the cost to the NHS at £466m (Pirmohamed et al., 2004). This figure may be substantially higher due to discrepancies when reporting ADRs combined with the rising number of clinically confirmed cases, with updated projections illustrating the magnitude of the issue at a cost to NHS England in excess of £2bn annually (Hazell and Shakir, 2006, Osanlou et al., 2022).

The vast majority of ADRs are potentially preventable and can inhibit the effectiveness of treatment strategies by limiting therapeutic intervention (Rommers et al., 2007). Various strategies have been deployed to combat the ever growing issue of ADR prevalence. Pharmacovigilance has been a valuable tool and involves the both recording and monitoring of potentially harmful drugs at the post-marketing stage and ultimately aims to identify and prevent instances of ADR (Aronson, 2017). This can lead to the withdrawal of licensed drugs from the commercial market, although the attrition of potentially efficacious drugs is an area of concern and can be problematic for patients, clinicians and drug developers. A recent systematic review identified 462 drugs withdrawn post-marketing between 1953 and 2013

with hepatotoxicity cited as the most common adverse effect (Onakpoya et al., 2016). Candidate drug attrition during clinical development leads to substantial financial implications. In 2001 the overall cost of drug discovery and development for a single drug was estimated at $804m, with more recent evaluations placing this figure closer to $1bn (Kola and Landis, 2004). This amplifies the need for a greater understanding of the underlying cellular and molecular mechanisms associated with ADRs, which will be imperative during the initial phases of drug development, in addition to continuous clinical assessment during post-marketing drug surveillance.

ADR manifestations can be predictable (Type A) and dose-dependent, with adverse events relating to a drugs known pharmacology. Conversely, Type B reactions are more problematic due to their idiosyncratic nature and off-target effects, with immune-modulation often cited as a route cause. Indeed, drug allergy has become a focal point for many research groups and immune-mediated drug hypersensitivity, encompassing T-cell involvement, has been isolated as a causal factor in numerous ADRs such as abacavir and carbamazepine-induced hypersensitivity syndromes (Chessman et al., 2008, Naisbitt et al., 2003a). This is further evidenced by the successful isolation of drug-specific T lymphocytes from both peripheral venous blood and blister fluid located at reaction epicentres within hypersensitive patients (Naisbitt et al., 2005, Nassif et al., 2002).

Over the years the field of drug hypersensitivity has been transformed by the discovery of certain genetic aetiologies shared by individuals presenting with a variety of clinical manifestations associated with immune-mediated ADRs. As previously discussed, abacavir represents the most prominent example with genetic screening for the identification of HLA-B*57:01 expression now common clinical practice as a direct result of exceptionally high positive prediction values (Mallal et al., 2002, Mallal et al., 2008). Furthermore, immunological mechanisms for the elicitation of HLA class I-restricted drug hypersensitivity have been

elucidated and can be defined by specific modes of T-cell activation, such as the pharmacological interaction concept (p-i), hapten/prohapten hypothesis and the altered peptide repertoire model (Pichler, 2002, Landsteiner and Jacobs, 1935, Illing et al., 2012). The association between shared HLA expression among hypersensitive cohorts is a dynamic area of study. Contemporary GWAS analysis has identified potential genetic-based causal risk factors on an almost yearly basis, as shown by the recent discovery of HLA associations between infliximab and green tea-induced liver injury and expression of HLA-B*39:01 and HLA-B*35:01 respectively (Bruno et al., 2020, Hoofnagle et al., 2021). Although HLA analysis can be a useful predictive tool for determining susceptibility, it is also clear that progression of hypersensitive disease states is multi-factorial, arising from contributing genetic, regulatory, environmental and drug-specific factors (Naisbitt et al., 2019).

7.2 The role of the adaptive immune system within DRESS reactions induced by glycopeptide antibiotics.

Recently, vancomycin administration has been linked to drug reaction with eosinophilia and systemic symptoms (DRESS) reactions in individuals expressing HLA-A*32:01, with positive and negative predictive values of 20% and 80% respectively (Konvinse et al., 2019). Although such findings provide a springboard for the detailed study of drug/HLA interactions and mechanistic insight into vancomycin-induced stimulation of immunological receptors, several questions remain unanswered. For example, why do only 20% of HLA-A*32:01 individuals exposed to vancomycin progress to DRESS and which underlying auxiliary immunological characteristics determine susceptibility? In this thesis, we successfully elucidated the mechanistic and functional roles of drug-specific T-cells within vancomycin and teicoplanin-induced DRESS using healthy donor and hypersensitive patient models, in addition to determining the specificity of vancomycin for HLA-A*32:01. One intriguing avenue encompassed within this study was the assessment of cellular energetic parameters, such as glycolysis, to explore cross-reactive T-cell responses via alternative metabolic routes. This arm of the study may provide

greater sensitivity for the determination of T-cell activation thresholds upon antigen presentation and enable further examination of the interplay between vancomycin and teicoplanin at the immunological synapse within the context of T-cell mediated DRESS pathogenesis.

Initial studies investigated vancomycin's propensity for immune stimulation by way of *de novo* responses following repetitive drug stimulation of PBMC cultures. This was achieved by utilising PBMCs from an established HLA-typed biobank (Alfirevic et al., 2012). Vancomycin preferentially interacted with HLA-A*32:01. Although possessing greater physiological relevance, these studies complement *in silico* molecular docking analysis performed by Konvinse and colleagues which predict vancomycin binding within antigen cleft residues of HLA-A*32:01 with greater affinity than closely related HLA types not associated with vancomycin-induced DRESS, such as HLA-A*29:02 (Konvinse et al., 2019). To further investigate the specificity of vancomycin for HLA-A*32:01, additional study prioritising x-ray crystallography and HLA peptide elution, proceeded by incorporation within functional T-cell assays, will be required.

Following the conduction of *in vitro* priming studies to assess preferential interaction between vancomycin and HLA-A*32:01, we strived to determine immunological pathways of vancomycin-mediated T-cell activation. Throughout this work, an emphasis was placed on studying how drug antigens can activate immunological receptors and stimulate downstream effector function. As described in Chapter 3, interactions between vancomycin and CD8+ T-cells have now been characterised to hinge upon HLA class I presentation, with T-cell responses evoked by direct non-covalent pharmacological interactions. Furthermore, stimulation has been found to be associated with a plethora of cytokine and cytolytic molecule release potentially involved within DRESS pathogenesis and clinical cross-reactivity with teicoplanin has been now replicated within *in vitro* assays (Choquet-Kastylevsky et al., 1998,

Hsiao et al., 2010). One way by which large, structurally complex glycopeptide antibiotics may differ from conventional modes of drug-induced T-cell activation is by their potential role as peptide mimetics, as suggested by preliminary studies indicating preferential HLA binding in the absence of native peptides (Konvinse et al., 2019). Within these studies, high affinity binding between vancomycin and HLA-A*32:01 was not predicted when native peptides, eluted from HLA-A*32:01, occupied the antigen-binding cleft. Indeed, due to its large molecular weight, peptide binding is potentially redundant as vancomycin has been shown to interact with HLA-A*32:01 with greater affinity in the absence of peptides present within the antigen binding cleft. By extension, it can be postulated that vancomycin may possess superantigenic properties in a similar way to some disease-causing bacterial proteins and subsequently activate T-cells in a non-specific manner, as previously demonstrated via the stimulation of naïve T-cells isolated from healthy donors without expression of HLA-A*32:01 (Li et al., 1999).

As previously stated, not all individuals carrying HLA-A*32:01 will progress to hypersensitive disease states, such as DRESS, following vancomycin exposure. This notion is amplified by T-cell cloning experiments demonstrating successful vancomycin-specific T-cell generation in a single healthy donor. Therefore, it is essential to revisit the overbearing question that has eluded pharmacologists regarding the interplay and importance of individual factors of susceptibility supplementary to classical HLA involvement. One emerging theme increasingly acknowledged in recent times lies within the dynamics of immunoregulation (Naisbitt et al., 2019). Crucially, intrinsic inter-individual differences exist between within pleiotropic signalling cascades, impacting the propensity for either co-stimulation or co-inhibition in response to compounds with immune liabilities. In hypersensitive individuals, co-stimulation will be favoured over tolerogenic mechanisms reducing the necessary threshold for T-cell stimulation (Matzinger, 2007). Conversely, hypersensitive progression can be influenced by regulatory breakdown of specific co-inhibitory ‘checkpoints’, such as PD-L1 and CTLA-4,

located at the interface between the CD28 and the B7 family (Wing et al., 2008, Wing and Sakaguchi, 2010, Teft et al., 2006). A pertinent example for the impact of immunoregulatory breakdown is the development of severe hypersensitivity after concomitant exposure to previously tolerated doses of amidotrizoate and the PD-L1 blocker, atezolizumab (Hammond et al., 2021). Such manifestations are almost certainly due to a 'removal of the brakes' within the context of aberrant T-cell activation and function, which may show strong utility within oncological settings due to enhanced T-cell antigenicity and anti-tumour activity. This further highlights the ongoing risk of drug hypersensitivity from uncontrolled T-cell responses following exposure to immunogenic compounds (Korman et al., 2006).

The observation of proliferative cross-reactivity between vancomycin specific T-cell clones (TCCs) generated from HLA-A*32:01 positive healthy donors and teicoplanin aligned with case-studies reporting severe hypersensitive responses within treated sequentially with both compounds (Kwon et al., 2006). Although teicoplanin is widely viewed a safer alternative to vancomycin, the drug is also implicated within the development of ADRs (Wood, 1996). However, the role of specific immunological receptors within reactions of a delayed nature are still poorly defined. Our *in vitro* studies explore the fundamental question regarding the intrinsic immunogenic potential of teicoplanin, specifically regarding the compounds ability to stimulate T-cells in a way affiliated with DRESS pathogenesis. Unsurprisingly, due to its close structural relation with vancomycin, teicoplanin was found to activate CD8+ TCCs in an identical manner to vancomycin, with T-cell responses elicited through a processing independent and MHC class I-restricted mechanism (Gardner et al., 2022). Furthermore, the role of T-cells within the pathogenesis of teicoplanin-induced DRESS syndrome was illustrated following the identification of cytotoxic and DRESS related cytokines, such as IL-5, following drug exposure. Indeed, the ubiquitous nature of IL-5 secretion within specific delayed hypersensitivity reactions, may provide justification for inclusion within diagnostic T-cell assays, such as the lymphocyte transformation test (LTT), as a DRESS specific marker. This

could be used in conjunction with an individual's clinical phenotype to achieve greater sensitivity and provide a more targeted approach for the identification of DRESS-associated compounds. However, it is important to note that LTT assays have the potential to indicate a false negative test, as hypersensitive manifestations may be associated with drug-specific T-cells localised to the skin (Pichler and Tilch, 2004). Moreover, some compounds, such as certain cystic fibrosis transmembrane conductance regulator (CFTR) modulators, need to be metabolised in the liver to before any immunogenic properties are observed (van der Meer et al., 2021). This can be problematic for the detection of memory T-cell responses to the culprit drug.

The observed cross-reactivity between teicoplanin-responsive TCCs generated from HLA-A*32:01 positive healthy donors and daptomycin, but not vancomycin, further highlights the complexities of interactions between glycopeptide antibiotics. Reciprocal cross-reactivity between structurally similar compounds is often observed, as illustrated between TCCs generated to both dapsone and its metabolite, nitroso-dapsone (Zhao et al., 2021). As such, it is surprising this trait is absent when studying glycopeptides, compounded with experiments consistently failing to generate drug-specific TCCs to daptomycin. As discussed in Section 4.4, a possible explanation for cross-reactivity with daptomycin may rest with the compounds increased structural alignment with teicoplanin, specifically the presence of a hydrophobic lipid chain (Reynolds, 1989). At this juncture, when assessing the potential involvement of HLA complexes regarding the observed cross-reactive responses, it is important to state that currently there is no genetic association between teicoplanin or daptomycin with HLA-A*32:01. Although highly preliminary, these data may warrant further consideration with respect to clinical ADR reporting after exposure to daptomycin following initial vancomycin or teicoplanin hypersensitivity. However, a greater body of *in vitro* and clinical evidence will be necessary to determine the scope for any potential GWAS analysis including teicoplanin or daptomycin.

In order to validate the functional role of T-cells within vancomycin-induced DRESS, illustrated with *in vitro* and allele positive healthy donor models, vancomycin hypersensitive patients were recruited to the study. Initial diagnostic assays (LTT) assessing memory T-cell responses to vancomycin in patients with suspected vancomycin-induced DRESS revealed positive lymphocyte proliferation in 8/9 individuals recruited. Based on our initial readouts for a cohort of vancomycin hypersensitive patients, LTT assays are deemed highly sensitive for the detection of memory responses applied to vancomycin-induced lymphocyte proliferation. Standardisation of the LTT for the detection of vancomycin-induced DRESS is a research goal shared by multiple groups. A preliminary study suggested LTT conduction alongside HLA typing and flow cytometry, for the detection of DRESS specific markers within activated PBMC cultures, may show promise for successfully diagnosing vancomycin hypersensitivity with a greater degree of sensitivity (Krummenacher, 2020).

The successful isolation of drug-responsive T-cells from vancomycin hypersensitive patients further implicates immune modulation within vancomycin-induced DRESS manifestations, for which both CD4+ and CD8+ T-cells were shown to be involved in disease pathogenesis. Our functional studies, predominantly focusing of the action of CD4+ TCCs, suggests HLA class II involvement with T-cell responses evoked by direct non-covalent pharmacological interactions, with the capacity for the secretion of cytokines (IL-5) and cytotoxic molecules (FasL) known to be linked with DRESS manifestation (Choquet-Kastylevsky et al., 1998). Detailed analysis of HLA genotyping pertaining to vancomycin hypersensitive patients, for which drug-specific TCCs were generated, seemingly reinforces genotypic involvement due to the shared expression of HLA-A*32:01 among hypersensitive individuals. The volume of CD4+ TCCs generated and HLA-DR restricted nature of clonal activation was a key finding in this study and is consistent with studies indicating DRESS onset hinges upon a Th2-mediated pathophysiology (Krummenacher, 2020). It is also poignant that large number of vancomycin-specific TCCs could be generated from a patient, in which expression of HLA-DRB*15:01 was

also detected. Significantly, haplotypes of HLA-DRB*15 alleles have recently been observed to be enriched in DRESS patients (n=94), further substantiating HLA involvement within drug-induced DRESS manifestations (Onuora, 2022). Furthermore, genetic analysis has now successfully identified numerous transcriptomic biomarkers implicated within the pathogenesis of DRESS reactions. Within these studies, RNA sequencing analysis of PBMC taken from DRESS patients has suggested a panel of biomarkers may provide high sensitivity (100%) and specificity (85-100%) for the identification of culprit drugs within cohorts presenting with antibiotic-associated DRESS. Specific biomarkers of interest include STAC, GPR183, CD40, CISH, CD4, and CCL8, with the study of these transcriptomic markers of potential relevance for the diagnosis of vancomycin-induced DRESS, alongside diagnostic T-cell assays (Teo et al., 2022).

Weak cross-reactivity was observed in a singular monoclonal T-cell population after teicoplanin exposure. However, it is plausible that cross-reactive T-cell responses may be more prevalent following continued proliferative study of a greater number of test cultures. Earlier analysis of cross-reactivity patterns within HLA-A*32:01 positive vancomycin-induced DRESS patient groups hinted at the possibility of a shared HLA-DQ haplotype among individuals susceptible teicoplanin hypersensitivity (Nakkam et al., 2020). These studies have shed light on potential modes of alternative HLA interaction beyond HLA-A*32:01 and have now specifically implicated both HLA-DQA1:01:01 and HLA-DQB1:05:03 within the induction of cross-reactive immunogenic responses to teicoplanin and other semi-synthetic glycopeptide antibiotics such as telavancin (Nakkam et al., 2021). To stratify patient susceptible to hypersensitive resurgence after rechallenge with structurally similar glycopeptide compounds, it would firstly be useful to determine the allelic frequency of both HLA-DQ alleles among various populations and ethnic groups, allowing for initial conclusions to be drawn regarding the likelihood of potential ‘risk’ allele status. Additionally, it is possible that detailed genetic analysis of larger patient cohorts could help identify specific risk alleles implicated within

cross-reactivity and aid the implementation of preventative measures, specifically relating to safer therapeutic options during treatment regimens targeted at clearing severe bacterial infection.

7.3 Glycolytic measurement can be used as an accurate marker for drug-specific T-cell activation.

Within the field of drug hypersensitivity there is an ever-increasing need to identify additional factors of susceptibility. The transition away from HLA as a singular focal point of prediction is required to achieve a more personalised approach to therapeutic intervention and drug safety. This is clearly evident based on a number of findings. Firstly, due to the limited number of compounds for which HLA screening is viable and secondly, because drugs with even the strongest HLA-based predictive models, such as abacavir, fail to explain why > 50% of individuals with risk allele carriage (HLA-B*57:01) fail to progress to hypersensitive disease states (Mallal et al., 2002, Mallal et al., 2008). In keeping with the theory that additional susceptibility factors must be elucidated, the present study aimed to explore T-cell energetics in response to immunogenic compounds to determine the potential involvement of metabolic phenotypes within drug-specific T-cell responses.

For the first time, this study has identified cellular dependency for specific metabolic pathways following stimulation with cognate drug antigens. CD8+ vancomycin-reactive TCCs generated from healthy donor PBMC (HLA-A*32:01 positive) were found to exhibit metabolic switching, evidenced by an increased dependence for glycolysis after acute vancomycin exposure. Additionally, blockade of HLA-DR in vancomycin-specific TCCs generated from vancomycin hypersensitive patients (HLA-A*32:01 positive) resulted in reduced glycolytic dependence after vancomycin exposure. As such, antigen presentation capable of eliciting energetic responses to vancomycin have been successfully modelled with clearly visible HLA interactions, further adding to the functional relevance of the assay system developed.

Significantly, the application of such assays towards the sensitive measure of drug/TCR interaction was validated by the detection of energetic cross-reactivity between structurally similar glycopeptide antibiotics in populations of TCCs absent of any cross-reactive potential when studying proliferative and cytokine readouts.

The importance of unique metabolic signatures, such as initial glycolytic reprogramming, for the activation and progression of T-cell responses have now been well defined (Gubser et al., 2013, van der Windt et al., 2013, van der Windt et al., 2016b, Vander Heiden et al., 2009). The actionable process of T-cell activation is energetically costly due to exponential demand arising from rapid proliferative function and relatively short doubling time, specifically in the case of virus-specific T-cells (De Boer et al., 2003). Empirical evidence firmly points towards elevated glucose uptake the induction of early-immediate glycolytic switch, necessary to keep pace with energetic demand of T-cell activation (MacDonald, 1977, MacDonald and Cerottini, 1979, Gubser et al., 2013). More recently, the role of glycolytic metabolism within T-cell activation has been further affirmed for a variety of cellular responses, most notably when studying graft-versus-host disease for which elevated and sustained levels of glycolysis have been associated with allogenic T-cell responses (Nguyen et al., 2014, Assmann et al., 2021) Simultaneously, it has long been recognised that immune activation and ensuing proliferative T-cell responses are heavily implicated within the pathogenesis of drug hypersensitivity reactions. Over the years this has been clearly demonstrated for a multitude of compounds with contrasting pathways of activation (altered peptide, p-i and hapten) such as abacavir, carbamazepine and nitroso-dapsone (Adam et al., 2014, Naisbitt et al., 2003a, Zhao et al., 2019). In each case, aberrant and overexuberant T-cell responses following the commencement of danger signalling combined with immunoregulatory breakdown typically leads to the release of effector cytokines and subsequent tissue damage, synonymous with clinical manifestation (Merk, 2005). With respect to fuel pathway dependence following immune stimulation, it follows that the cellular glycolytic potential of drug-reactive T-cells may govern the degree of

proliferative and functional response, if not ultimately the nature of such responses. Indeed, it is possible that this phenomenon may underpin cellular fate, either towards elicitation and hypersensitive progression or in favour of tolerance if fuel requirements for full activation are unmet.

Distinct genotypic characteristics may impact upon an individuals 'energetic' predisposition for which activation or tolerance may be favoured following formation of a stable immune synapse and appropriate co-signalling. These inter-individual differences, originating from fundamental transcriptomic variation have been suggested to account for discrepancies within human T-cell activation thresholds (Ye et al., 2014). Similarly, underlying genetic traits potentially favouring heightened expression of enzymes involved within glycolysis and associated with elevated energetic states, may also act as a checkpoint and dictate progression from states of early-activation to proliferation and subsequent effector function. A deeper understanding of the interplay between the molecular and energetic events that determine the fate of stimulated T-cells will be crucial to uncovering additional factors of susceptibility to hypersensitivity, for which HLA involvement is now known to be one of many potential contributing factors.

7.4 Alternative assay systems with potential relevance for the study of drug hypersensitivity reactions.

Currently, a host of assay systems are commercially available with direct application for glycolysis detection and measurement. These assays can be energetics-based (Seahorse XF assays), during which metabolic changes can be measured in real-time, or rely on the detection and quantification of an array of glycolytic pre-cursors and end products as an output of glycolytic function, specifically in relation to lactate production and glucose uptake. When deployed, such assays could provide greater insight towards defining fuel pathway dependency in addition to any metabolic plasticity involved within pathways of T-cell activation. Importantly within the context of T-cell mediated hypersensitivity, sensitive determination of the metabolic parameters that facilitate immune stimulation will shed light on specific activation thresholds, known to be a rate limiting step for T-cell responses after antigenic encounter (Adam et al., 2011).

When considering bioenergetic readouts pertaining to T-cell activation after acute stimulation, progression within the field has led to the development of numerous assays systems with application for real-time glycolysis measurement. Initial assays were heavily reliant on stress-based measurement and injection of mitochondrial toxins to elucidate glycolytic dependence, with an emphasis on extracellular acidification detection and indirect assessment of glycolytic function (Gubser et al., 2013). In recent times, these assays have been streamlined to solely focus on acute T-cell activation, namely the Agilent Seahorse XF Hu T Cell Activation Assay Kit. These more contemporary iterations importantly allow for calculation of a proton efflux rate, which can subsequently be correlated with rates of lactate production for more direct and physiologically relevant glycolytic readout (Brooks, 2018).

Additional assay systems have been developed that show utility for accurate lactate quantification and, by extension, glycolytic induction. The presence of extracellular lactate can

be reliable indicator of T-cell proliferation. Studies have now confirmed an intricate correlation between lactate production and proliferative T-cell expansion in response to *in vitro* stimulation with CD3/CD28 costimulatory molecules, found to be concordant with more traditional proliferative measures, such as [^{3}H] thymidine incorporation (Grist et al., 2018). Although assays exist with the ability to accurately detect extracellular lactate production for the purpose of glycolysis measurement, translational approaches to T-cell activation are still poorly defined. Commercially available colorimetric assay kits with the capacity for detecting end products of lactic acid fermentation rely on enzyme-mediated release of L-lactate to serve as gauge for glycolytic function (TeSlaa and Teitell, 2014). To a similar extent, assays deployed to directly measure glucose uptake as a precursor for glycolysis have proven effective for the measurement of glycolytic metabolism and elucidation as to the role of glucose transporters (GLUT), such as GLUT1 within metabolic pathways (Kido et al., 2020). To date, the application of such assays towards T-cell activation and function are limited, almost certainly due to the relative success of real-time energetic platforms with successful optimisation for a number of T-cell subsets now well-defined after artificial or peptide-induced stimulation (Jones et al., 2017, Menk et al., 2018a). Further ground will need to be broken within the field of immunometabolism for effective translatability of applications towards drug-specific T-cell activation, with an emphasis on correlating bioenergetically relevant thresholds of immune activation with expression of specific early activation markers, such as CD69 and CD25 (Shipkova and Wieland, 2012).

7.5 The future of immunometabolism for the detection of drug hypersensitivity.

The field of immunometabolism is still in its infancy. The bioenergetics of immune cells, both during quiescence and following stimulation, can provide insight into mechanisms underpinning physiological function and more importantly how metabolic signatures can be altered to support transient cellular requirements. Progress to this end has already identified fundamental metabolic differences between CD4+ and CD8+ expressing T-cells, specifically the presence of a glycolytic flux upon stimulation weighted in favour of T helper cells (Jones et al., 2017). This is certainly applicable to the field of drug hypersensitivity, for which the immunometabolic study of drug-specific T-cells may provide an alternative route of investigation for differential patterns of T-cell activation. There is clearly untapped potential regarding the incorporation of immunometabolic assays within diagnosis and prediction of drug hypersensitivity. To fully establish bioenergetic analysis with applications towards screening for potential susceptibility, a number of key events need to be determined. Firstly, following T-cell stimulation, it will be crucial to correlate the intensity of glycolytic indication with distinct, separate markers of co-stimulation and to further delineate glycolytic dependence with respect to both the initiation and propagation of danger signalling. Furthermore, due to the arduous nature of generating drug-specific T-cells and experimental limitations associated with primary cultures, an emphasis must now be placed on assay transformation, specifically focusing on the incorporation of PBMC cultures. Rigorous coordinated approaches will be necessary to the confirm physiological associations between activation signalling and metabolic output in greater depth, however once overcome will lay the groundwork for the development of next generation diagnostic screening with the aim of supplementing or even superseding more traditional, long established methods such as lymphocyte transformation.

7.6 Concluding remarks.

To conclude, our data implicates T-cell involvement in vancomycin-induced DRESS. In terms of functional studies, healthy donor work utilising PBMC with risk allele expression provided a suitable model for the elucidation of molecular interactions and downstream effector functionality of vancomycin-specific TCCs, with a degree of homology observed with parallel studies conducted following the recruitment of vancomycin hypersensitive patients. However, further work will be required to fully determine the role of CD8+ T-cells and associated mechanisms involved within DRESS pathogenesis. Energetic readouts represent a valuable addition, with applications towards studying both the chemical and molecular basis of drug-specific T-cell activation. Importantly, due to their heightened sensitivity, these assays may shed light upon an individuals propensity to suffer from adverse events and provide deeper understanding of the interplay and balance between co-signalling pathways, after which cellular fate assumes tolerance of elicitation. Finally, this may uncover hidden physiological traits of idiosyncrasy that could go some way to deciphering inter-individual differences, which has consistently proven to be a major hurdle for the diagnosis and prediction of drug hypersensitivity.

Bibliography

Abe R., Shimizu T., Shibaki A., Nakamura H., Watanabe H. and Shimizu H. (2003). Toxic epidermal necrolysis and stevens-johnson syndrome are induced by soluble fas ligand. *Am J Pathol:* 162; 1515-1520.

Aberer W., Bircher A., Romano A., Blanca M., Campi P., Fernandez J.*, et al.* (2003). Drug provocation testing in the diagnosis of drug hypersensitivity reactions: General considerations. *Allergy:* 58; 854-863.

Abraham S. N. and Arock M. (1998). Mast cells and basophils in innate immunity. *Semin Immunol:* 10; 373-381.

Adam J., Eriksson K. K., Schnyder B., Fontana S., Pichler W. J. and Yerly D. (2012). Avidity determines t-cell reactivity in abacavir hypersensitivity. *Eur J Immunol:* 42; 1706-1716.

Adam J., Pichler W. J. and Yerly D. (2011). Delayed drug hypersensitivity: Models of t-cell stimulation. *British journal of clinical pharmacology:* 71; 701-707.

Adam J., Wuillemin N., Watkins S., Jamin H., Eriksson K. K., Villiger P.*, et al.* (2014). Abacavir induced t cell reactivity from drug naïve individuals shares features of allo-immune responses. *PloS one:* 9; e95339-e95339.

Afkarian M., Sedy J. R., Yang J., Jacobson N. G., Cereb N., Yang S. Y.*, et al.* (2002). T-bet is a stat1-induced regulator of il-12r expression in naïve cd4+ t cells. *Nat Immunol:* 3; 549-557.

Agliano F., Karginov T. A., Ménoret A., Provatas A. and Vella A. T. (2022). Nicotinamide breaks effector cd8 t cell responses by targeting mtor signaling. *iScience:* 25; 103932.

Ahmed R., Butler L. D. and Bhatti L. (1988). T4+ t helper cell function in vivo: Differential requirement for induction of antiviral cytotoxic t-cell and antibody responses. *J Virol:* 62; 2102-2106.

Aiba S. (1998). Maturation of dendritic cells induced by cytokines and haptens. *Tohoku J Exp Med:* 184; 159-172.

Alfirevic A., Gonzalez-Galarza F., Bell C., Martinsson K., Platt V., Bretland G.*, et al.* (2012). In silico analysis of hla associations with drug-induced liver injury: Use of a hla-genotyped DNA archive from healthy volunteers. *Genome Med:* 4; 51.

Allen N. E. and Nicas T. I. (2003). Mechanism of action of oritavancin and related glycopeptide antibiotics. *FEMS Microbiology Reviews:* 26; 511-532.

Alzahrani A., Ogese M., Meng X., Waddington J. C., Tailor A., Farrell J.*, et al.* (2017). Dapsone and nitroso dapsone activation of naive t-cells from healthy donors. *Chem Res Toxicol:* 30; 2174-2186.

Amante M. F., Filippini A. V., Cejas N., Lendoire J., Imventarza O. and Parisi C. (2009). Dress syndrome and fulminant hepatic failure induced by lamotrigine. *Ann Hepatol:* 8; 75-77.

Anderson J. S., Matsuhashi M., Haskin M. A. and Strominger J. L. (1965). Lipid-phosphoacetylmuramyl-pentapeptide and lipid-phosphodisaccharide-pentapeptide:

Presumed membrane transport intermediates in cell wall synthesis. *Proc Natl Acad Sci U S A:* 53; 881-889.

Andrade R. J., Lucena M. I., Kaplowitz N., García-Muṇoz B., Borraz Y., Pachkoria K*., et al.* (2006). Outcome of acute idiosyncratic drug-induced liver injury: Long-term follow-up in a hepatotoxicity registry. *Hepatology:* 44; 1581-1588.

Anstead G. M., Cadena J. and Javeri H. (2014). Treatment of infections due to resistant staphylococcus aureus. *Methods Mol Biol:* 1085; 259-309.

Anthony R. M., Rutitzky L. I., Urban J. F., Jr., Stadecker M. J. and Gause W. C. (2007). Protective immune mechanisms in helminth infection. *Nat Rev Immunol:* 7; 975-987.

Ardern-Jones M. R. and Friedmann P. S. (2011). Skin manifestations of drug allergy. *Br J Clin Pharmacol:* 71; 672-683.

Ariza A., Fernández-Santamaría R., Meng X., Salas M., Ogese M. O., Tailor A*., et al.* (2020). Characterization of amoxicillin and clavulanic acid specific t-cell clones from patients with immediate drug hypersensitivity. *Allergy:* 75; 2562-2573.

Aronson J. K. (2017). Post-marketing drug withdrawals: Pharmacovigilance success, regulatory problems. *Therapie:* 72; 555-561.

Ashford P. A. and Bew S. P. (2012). Recent advances in the synthesis of new glycopeptide antibiotics. *Chem Soc Rev:* 41; 957-978.

Assmann J. C., Farthing D. E., Saito K., Maglakelidze N., Oliver B., Warrick K. A*., et al.* (2021). Glycolytic metabolism of pathogenic t cells enables early detection of gvhd by 13c-mri. *Blood:* 137; 126-137.

Balachandran C., Shenoi S. D., Sarkar D. and Ravikumar B. C. (2002). Patch tests in adverse cutaneous drug reaction. *Indian J Dermatol Venereol Leprol:* 68; 13-15.

Balato A., Unutmaz D. and Gaspari A. A. (2009). Natural killer t cells: An unconventional t-cell subset with diverse effector and regulatory functions. *J Invest Dermatol:* 129; 1628-1642.

Banchereau J. and Steinman R. M. (1998). Dendritic cells and the control of immunity. *Nature:* 392; 245-252.

Barbaud A., Gonçalo M., Bruynzeel D. and Bircher A. (2001). Guidelines for performing skin tests with drugs in the investigation of cutaneous adverse drug reactions. *Contact Dermatitis:* 45; 321-328.

Barry M. and Bleackley R. C. (2002). Cytotoxic t lymphocytes: All roads lead to death. *Nat Rev Immunol:* 2; 401-409.

Basset C., Holton J., O'mahony R. and Roitt I. (2003). Innate immunity and pathogen-host interaction. *Vaccine:* 21 Suppl 2; S12-23.

Bastuji-Garin S., Rzany B., Stern R. S., Shear N. H., Naldi L. and Roujeau J. C. (1993). Clinical classification of cases of toxic epidermal necrolysis, stevens-johnson syndrome, and erythema multiforme. *Arch Dermatol:* 129; 92-96.

Bauer D. E., Harris M. H., Plas D. R., Lum J. J., Hammerman P. S., Rathmell J. C.*, et al.* (2004). Cytokine stimulation of aerobic glycolysis in hematopoietic cells exceeds proliferative demand. *Faseb j:* 18; 1303-1305.

Baumgartner L. J., Brown L. and Geier C. (2017). Hypersensitivity reaction following administration of low-dose oral vancomycin for the treatment of clostridium difficile in a patient with normal renal function. *J Pharm Pract:* 30; 650-652.

Beadling C., Guschin D., Witthuhn B. A., Ziemiecki A., Ihle J. N., Kerr I. M.*, et al.* (1994). Activation of jak kinases and stat proteins by interleukin-2 and interferon alpha, but not the t cell antigen receptor, in human t lymphocytes. *Embo j:* 13; 5605-5615.

Beeler A. and Pichler W. J. (2006). In vitro tests of t cell-mediated drug hypersensitivity. *Expert Rev Clin Immunol:* 2; 887-900.

Bell C. C., Faulkner L., Martinsson K., Farrell J., Alfirevic A., Tugwood J.*, et al.* (2013). T-cells from hla-b*57:01+ human subjects are activated with abacavir through two independent pathways and induce cell death by multiple mechanisms. *Chem Res Toxicol:* 26; 759-766.

Ben Romdhane H., Chadli Z., Ben Fredj N., Chaabane A., Boughattas N. A. and Aouam K. (2018). Teicoplanin-induced dress syndrome: The importance of skin tests. *Med Mal Infect:* 48; 291-293.

Bénichou C. (1990). Criteria of drug-induced liver disorders. Report of an international consensus meeting. *J Hepatol:* 11; 272-276.

Bennett S. R., Carbone F. R., Karamalis F., Flavell R. A., Miller J. F. and Heath W. R. (1998). Help for cytotoxic-t-cell responses is mediated by cd40 signalling. *Nature:* 393; 478-480.

Bental M. and Deutsch C. (1993). Metabolic changes in activated t cells: An nmr study of human peripheral blood lymphocytes. *Magn Reson Med:* 29; 317-326.

Bérdy J. (2005). Bioactive microbial metabolites. *J Antibiot (Tokyo):* 58; 1-26.

Bigby M. (2001). Rates of cutaneous reactions to drugs. *Arch Dermatol:* 137; 765-770.

Binda E., Marinelli F. and Marcone G. L. (2014). Old and new glycopeptide antibiotics: Action and resistance. *Antibiotics (Basel):* 3; 572-594.

Bingaman A. W., Patke D. S., Mane V. R., Ahmadzadeh M., Ndejembi M., Bartlett S. T.*, et al.* (2005). Novel phenotypes and migratory properties distinguish memory cd4 t cell subsets in lymphoid and lung tissue. *Eur J Immunol:* 35; 3173-3186.

Black C. A. (1997). A brief history of the discovery of the immunoglobulins and the origin of the modern immunoglobulin nomenclature. *Immunol Cell Biol:* 75; 65-68.

Blumenthal K. G., Patil S. U. and Long A. A. (2012). The importance of vancomycin in drug rash with eosinophilia and systemic symptoms (dress) syndrome. *Allergy Asthma Proc:* 33; 165-171.

Blumenthal K. G., Peter J. G., Trubiano J. A. and Phillips E. J. (2019). Antibiotic allergy. *The Lancet:* 393; 183-198.

Bocquet H., Bagot M. and Roujeau J. C. (1996). Drug-induced pseudolymphoma and drug hypersensitivity syndrome (drug rash with eosinophilia and systemic symptoms: Dress). *Semin Cutan Med Surg:* 15; 250-257.

Bodmer W. F. (1987). The hla system: Structure and function. *J Clin Pathol:* 40; 948-958.

Boelsterli U. A. (2002). Mechanisms of nsaid-induced hepatotoxicity: Focus on nimesulide. *Drug Saf:* 25; 633-648.

Boise L. H., Minn A. J., Noel P. J., June C. H., Accavitti M. A., Lindsten T.*, et al.* (1995). Cd28 costimulation can promote t cell survival by enhancing the expression of bcl-xl. *Immunity:* 3; 87-98.

Bonilla F. A. and Oettgen H. C. (2010). Adaptive immunity. *J Allergy Clin Immunol:* 125; S33-40.

Bousquet P. J., Pipet A., Bousquet-Rouanet L. and Demoly P. (2008). Oral challenges are needed in the diagnosis of beta-lactam hypersensitivity. *Clin Exp Allergy:* 38; 185-190.

Boyer C., Auphan N., Luton F., Malburet J. M., Barad M., Bizozzero J. P.*, et al.* (1991). T cell receptor/cd3 complex internalization following activation of a cytolytic t cell clone: Evidence for a protein kinase c-independent staurosporine-sensitive step. *Eur J Immunol:* 21; 1623-1634.

Brander C., Mauri-Hellweg D., Bettens F., Rolli H., Goldman M. and Pichler W. J. (1995). Heterogeneous t cell responses to beta-lactam-modified self-structures are observed in penicillin-allergic individuals. *J Immunol:* 155; 2670-2678.

Bretscher P. and Cohn M. (1970). A theory of self-nonself discrimination. *Science:* 169; 1042-1049.

Bretscher P. A. (1999). A two-step, two-signal model for the primary activation of precursor helper t cells. *Proc Natl Acad Sci U S A:* 96; 185-190.

Britschgi M. and Pichler W. J. (2002). Acute generalized exanthematous pustulosis, a clue to neutrophil-mediated inflammatory processes orchestrated by t cells. *Curr Opin Allergy Clin Immunol:* 2; 325-331.

Brockow K., Garvey L. H., Aberer W., Atanaskovic-Markovic M., Barbaud A., Bilo M. B.*, et al.* (2013). Skin test concentrations for systemically administered drugs -- an enda/eaaci drug allergy interest group position paper. *Allergy:* 68; 702-712.

Brockow K., Romano A., Aberer W., Bircher A. J., Barbaud A., Bonadonna P.*, et al.* (2009). Skin testing in patients with hypersensitivity reactions to iodinated contrast media - a european multicenter study. *Allergy:* 64; 234-241.

Brockow K., Romano A., Blanca M., Ring J., Pichler W. and Demoly P. (2002). General considerations for skin test procedures in the diagnosis of drug hypersensitivity. *Allergy:* 57; 45-51.

Brooks G. A. (2018). The science and translation of lactate shuttle theory. *Cell Metab:* 27; 757-785.

Brunner T., Wasem C., Torgler R., Cima I., Jakob S. and Corazza N. (2003). Fas (cd95/apo-1) ligand regulation in t cell homeostasis, cell-mediated cytotoxicity and immune pathology. *Semin Immunol:* 15; 167-176.

Bruno C. D., Fremd B., Church R. J., Daly A. K., Aithal G. P., Björnsson E. S.*, et al.* (2020). Hla associations with infliximab-induced liver injury. *Pharmacogenomics J:* 20; 681-686.

Buonomo A., Altomonte G., De Pasquale T., Lombardo C., Pecora V., Sabato V.*, et al.* (2010). Allergic and non-allergic drug hypersensitivity reactions in children. *Int J Immunopathol Pharmacol:* 23; 881-890.

Butler M. S., Hansford K. A., Blaskovich M. a. T., Halai R. and Cooper M. A. (2014). Glycopeptide antibiotics: Back to the future. *The Journal of Antibiotics:* 67; 631-644.

Cacoub P., Musette P., Descamps V., Meyer O., Speirs C., Finzi L.*, et al.* (2011). The dress syndrome: A literature review. *Am J Med:* 124; 588-597.

Campoli-Richards D. M., Brogden R. N. and Faulds D. (1990). Teicoplanin. A review of its antibacterial activity, pharmacokinetic properties and therapeutic potential. *Drugs:* 40; 449-486.

Cao Y., Rathmell J. C. and Macintyre A. N. (2014). Metabolic reprogramming towards aerobic glycolysis correlates with greater proliferative ability and resistance to metabolic inhibition in cd8 versus cd4 t cells. *PloS one:* 9; e104104.

Carr D. F. and Pirmohamed M. (2018). Biomarkers of adverse drug reactions. *Experimental biology and medicine (Maywood, N.J.):* 243; 291-299.

Castelli C., Rivoltini L., Andreola G., Carrabba M., Renkvist N. and Parmiani G. (2000). T-cell recognition of melanoma-associated antigens. *J Cell Physiol:* 182; 323-331.

Cerottini J. C., Nordin A. A. and Brunner K. T. (1970). Specific in vitro cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. *Nature:* 228; 1308-1309.

Ceuppens J. L., Baroja M. L., Lorre K., Van Damme J. and Billiau A. (1988). Human t cell activation with phytohemagglutinin. The function of il-6 as an accessory signal. *J Immunol:* 141; 3868-3874.

Chalasani N., Fontana R. J., Bonkovsky H. L., Watkins P. B., Davern T., Serrano J.*, et al.* (2008). Causes, clinical features, and outcomes from a prospective study of drug-induced liver injury in the united states. *Gastroenterology:* 135; 1924-1934, 1934.e1921-1924.

Champagne P., Ogg G. S., King A. S., Knabenhans C., Ellefsen K., Nobile M.*, et al.* (2001). Skewed maturation of memory hiv-specific cd8 t lymphocytes. *Nature:* 410; 106-111.

Chan S. L., Ang X., Sani L. L., Ng H. Y., Winther M. D., Liu J. J.*, et al.* (2016). Prevalence and characteristics of adverse drug reactions at admission to hospital: A prospective observational study. *British journal of clinical pharmacology:* 82; 1636-1646.

Chandel N. S., Maltepe E., Goldwasser E., Mathieu C. E., Simon M. C. and Schumacker P. T. (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc Natl Acad Sci U S A:* 95; 11715-11720.

Chang C. H., Curtis J. D., Maggi L. B., Jr., Faubert B., Villarino A. V., O'sullivan D.*, et al.* (2013). Posttranscriptional control of t cell effector function by aerobic glycolysis. *Cell:* 153; 1239-1251.

Chaplin D. D. (2010). Overview of the immune response. *J Allergy Clin Immunol:* 125; S3-S23.

Chen Y. C., Chiu H. C. and Chu C. Y. (2010). Drug reaction with eosinophilia and systemic symptoms: A retrospective study of 60 cases. *Arch Dermatol:* 146; 1373-1379.

Chessman D., Kostenko L., Lethborg T., Purcell A. W., Williamson N. A., Chen Z.*, et al.* (2008). Human leukocyte antigen class i-restricted activation of cd8+ t cells provides the immunogenetic basis of a systemic drug hypersensitivity. *Immunity:* 28; 822-832.

Chiou C. C., Yang L. C., Hung S. I., Chang Y. C., Kuo T. T., Ho H. C.*, et al.* (2008). Clinicopathological features and prognosis of drug rash with eosinophilia and systemic symptoms: A study of 30 cases in taiwan. *J Eur Acad Dermatol Venereol:* 22; 1044-1049.

Chopra N., Oppenheimer J., Derimanov G. S. and Fine P. L. (2000). Vancomycin anaphylaxis and successful desensitization in a patient with end stage renal disease on hemodialysis by maintaining steady antibiotic levels. *Ann Allergy Asthma Immunol:* 84; 633-635.

Choquet-Kastylevsky G., Intrator L., Chenal C., Bocquet H., Revuz J. and Roujeau J. C. (1998). Increased levels of interleukin 5 are associated with the generation of eosinophilia in drug-induced hypersensitivity syndrome. *Br J Dermatol:* 139; 1026-1032.

Chung W. H., Hung S. I., Hong H. S., Hsih M. S., Yang L. C., Ho H. C.*, et al.* (2004). Medical genetics: A marker for stevens-johnson syndrome. *Nature:* 428; 486.

Clark R. and Kupper T. (2005). Old meets new: The interaction between innate and adaptive immunity. *J Invest Dermatol:* 125; 629-637.

Copaescu A., Gibson A., Li Y., Trubiano J. A. and Phillips E. J. (2021). An updated review of the diagnostic methods in delayed drug hypersensitivity. *Frontiers in Pharmacology:* 11;

Couturier J., Nuotio-Antar A. M., Agarwal N., Wilkerson G. K., Saha P., Kulkarni V.*, et al.* (2019). Lymphocytes upregulate cd36 in adipose tissue and liver. *Adipocyte:* 8; 154-163.

Crabtree H. G. (1929). Observations on the carbohydrate metabolism of tumours. *Biochem J:* 23; 536-545.

Cunningham A. J. and Lafferty K. J. (1974). Letter: Cellular proliferation can be an unreliable index of immune competence. *J Immunol:* 112; 436-437.

Curtsinger J. M. and Mescher M. F. (2010). Inflammatory cytokines as a third signal for t cell activation. *Curr Opin Immunol:* 22; 333-340.

Cynamon M. H. and Granato P. A. (1982). Comparison of the in vitro activities of teichomycin a2 and vancomycin against staphylococci and enterococci. *Antimicrob Agents Chemother:* 21; 504-505.

D'souza W. N., Chang C. F., Fischer A. M., Li M. and Hedrick S. M. (2008). The erk2 mapk regulates cd8 t cell proliferation and survival. *J Immunol:* 181; 7617-7629.

Daly A. K., Donaldson P. T., Bhatnagar P., Shen Y., Pe'er I., Floratos A.*, et al.* (2009). Hla-b*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nat Genet:* 41; 816-819.

Dang E. V., Barbi J., Yang H. Y., Jinasena D., Yu H., Zheng Y.*, et al.* (2011). Control of t(h)17/t(reg) balance by hypoxia-inducible factor 1. *Cell:* 146; 772-784.

Davey P. G. and Williams A. H. (1991). A review of the safety profile of teicoplanin. *J Antimicrob Chemother:* 27 Suppl B; 69-73.

Davis R. L., Smith A. L. and Koup J. R. (1986). The "red man's syndrome" and slow infusion of vancomycin. *Ann Intern Med:* 104; 285-286.

De Boer R. J., Homann D. and Perelson A. S. (2003). Different dynamics of cd4+ and cd8+ t cell responses during and after acute lymphocytic choriomeningitis virus infection. *J Immunol:* 171; 3928-3935.

Decleer M., Jovanovic J., Vakula A., Udovicki B., Agoua R. E. K., Madder A.*, et al.* (2018). Oxygen consumption rate analysis of mitochondrial dysfunction caused by bacillus cereus cereulide in caco-2 and hepg2 cells. *Toxins (Basel):* 10;

Delgoffe G. M. and Powell J. D. (2015). Sugar, fat, and protein: New insights into what t cells crave. *Curr Opin Immunol:* 33; 49-54.

Demoly P., Adkinson N. F., Brockow K., Castells M., Chiriac A. M., Greenberger P. A.*, et al.* (2014). International consensus on drug allergy. *Allergy:* 69; 420-437.

Demoly P. and Hillaire-Buys D. (2004). Classification and epidemiology of hypersensitivity drug reactions. *Immunol Allergy Clin North Am:* 24; 345-356, v.

Descamps V., Valance A., Edlinger C., Fillet A. M., Grossin M., Lebrun-Vignes B.*, et al.* (2001). Association of human herpesvirus 6 infection with drug reaction with eosinophilia and systemic symptoms. *Arch Dermatol:* 137; 301-304.

Deshpande P., Li Y., Thorne M., Palubinsky A. M., Phillips E. J. and Gibson A. (2022). Practical implementation of genetics: New concepts in immunogenomics to predict, prevent, and diagnose drug hypersensitivity. *J Allergy Clin Immunol Pract:*

Doege K., Heine S., Jensen I., Jelkmann W. and Metzen E. (2005). Inhibition of mitochondrial respiration elevates oxygen concentration but leaves regulation of hypoxia-inducible factor (hif) intact. *Blood:* 106; 2311-2317.

Doherty D. G., Melo A. M., Moreno-Olivera A. and Solomos A. C. (2018). Activation and regulation of b cell responses by invariant natural killer t cells. *Front Immunol:* 9; 1360.

Doyle C. and Strominger J. L. (1987). Interaction between cd4 and class ii mhc molecules mediates cell adhesion. *Nature:* 330; 256-259.

Duhen T., Geiger R., Jarrossay D., Lanzavecchia A. and Sallusto F. (2009). Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory t cells. *Nat Immunol:* 10; 857-863.

Durant L., Watford W. T., Ramos H. L., Laurence A., Vahedi G., Wei L.*, et al.* (2010). Diverse targets of the transcription factor stat3 contribute to t cell pathogenicity and homeostasis. *Immunity:* 32; 605-615.

Ebrahimpour S., Mohammadi M. and Gholami K. (2017). Drug reaction with eosinophilia and systemic symptoms (dress) with teicoplanin: A case report. *Drug Saf Case Rep:* 4; 1.

Edwards I. R. and Aronson J. K. (2000). Adverse drug reactions: Definitions, diagnosis, and management. *The Lancet:* 356; 1255-1259.

Einarson T. R. (1993). Drug-related hospital admissions. *Ann Pharmacother:* 27; 832-840.

Elyaman W. and Khoury S. J. (2017). Th9 cells in the pathogenesis of eae and multiple sclerosis. *Semin Immunopathol:* 39; 79-87.

Empedrad R., Darter A. L., Earl H. S. and Gruchalla R. S. (2003). Nonirritating intradermal skin test concentrations for commonly prescribed antibiotics. *J Allergy Clin Immunol:* 112; 629-630.

Engler O. B., Strasser I., Naisbitt D. J., Cerny A. and Pichler W. J. (2004). A chemically inert drug can stimulate t cells in vitro by their t cell receptor in non-sensitised individuals. *Toxicology:* 197; 47-56.

Eshki M., Allanore L., Musette P., Milpied B., Grange A., Guillaume J. C.*, et al.* (2009). Twelve-year analysis of severe cases of drug reaction with eosinophilia and systemic symptoms: A cause of unpredictable multiorgan failure. *Arch Dermatol:* 145; 67-72.

Eyerich S., Eyerich K., Pennino D., Carbone T., Nasorri F., Pallotta S.*, et al.* (2009). Th22 cells represent a distinct human t cell subset involved in epidermal immunity and remodeling. *J Clin Invest:* 119; 3573-3585.

Fainstein V., Leblanc B. and Bodey G. P. (1983). Comparative in vitro study of teichomycin a2. *Antimicrob Agents Chemother:* 23; 497-499.

Farnam K., Chang C., Teuber S. and Gershwin M. E. (2012). Nonallergic drug hypersensitivity reactions. *Int Arch Allergy Immunol:* 159; 327-345.

Farrell J., Naisbitt D. J., Drummond N. S., Depta J. P., Vilar F. J., Pirmohamed M.*, et al.* (2003). Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific t-cell responses in animals and humans. *The Journal of pharmacology and experimental therapeutics:* 306; 229-237.

Faulkner L., Gibson A., Sullivan A., Tailor A., Usui T., Alfirevic A.*, et al.* (2016a). Detection of primary t cell responses to drugs and chemicals in hla-typed volunteers: Implications for the prediction of drug immunogenicity. *Toxicological Sciences:* 154; 416-429.

Faulkner L., Gibson A., Sullivan A., Tailor A., Usui T., Alfirevic A.*, et al.* (2016b). Detection of primary t cell responses to drugs and chemicals in hla-typed volunteers: Implications for the prediction of drug immunogenicity. *Toxicol Sci:* 154; 416-429.

Faulkner L., Martinsson K., Santoyo-Castelazo A., Cederbrant K., Schuppe-Koistinen I., Powell H.*, et al.* (2012). The development of in vitro culture methods to characterize primary t-cell responses to drugs. *Toxicol Sci:* 127; 150-158.

Feldmeyer L., Heidemeyer K. and Yawalkar N. (2016). Acute generalized exanthematous pustulosis: Pathogenesis, genetic background, clinical variants and therapy. *Int J Mol Sci:* 17;

Fernández T. D., Canto G. and Blanca M. (2009). Molecular mechanisms of maculopapular exanthema. *Curr Opin Infect Dis:* 22; 272-278.

Ferrell P. B., Jr. and Mcleod H. L. (2008). Carbamazepine, hla-b*1502 and risk of stevens-johnson syndrome and toxic epidermal necrolysis: Us fda recommendations. *Pharmacogenomics:* 9; 1543-1546.

Fife B. T. and Bluestone J. A. (2008). Control of peripheral t-cell tolerance and autoimmunity via the ctla-4 and pd-1 pathways. *Immunol Rev:* 224; 166-182.

Fiorentino D. F., Zlotnik A., Vieira P., Mosmann T. R., Howard M., Moore K. W*., et al.* (1991). Il-10 acts on the antigen-presenting cell to inhibit cytokine production by th1 cells. *The Journal of Immunology:* 146; 3444-3451.

Flannery A. H., Wallace K. L., Rhudy C. N., Olmsted A. S., Minrath R. C., Pope S. M*., et al.* (2021). Efficacy and safety of vancomycin loading doses in critically ill patients with methicillin-resistant staphylococcus aureus infection. *Therapeutic Advances in Infectious Disease:* 8; 20499361211005965.

Foulds K. E., Zenewicz L. A., Shedlock D. J., Jiang J., Troy A. E. and Shen H. (2002). Cutting edge: Cd4 and cd8 t cells are intrinsically different in their proliferative responses. *J Immunol:* 168; 1528-1532.

Fowler V. G., Jr., Boucher H. W., Corey G. R., Abrutyn E., Karchmer A. W., Rupp M. E*., et al.* (2006). Daptomycin versus standard therapy for bacteremia and endocarditis caused by staphylococcus aureus. *N Engl J Med:* 355; 653-665.

Frauwirth K. A., Riley J. L., Harris M. H., Parry R. V., Rathmell J. C., Plas D. R*., et al.* (2002). The cd28 signaling pathway regulates glucose metabolism. *Immunity:* 16; 769-777.

Frisullo G., Angelucci F., Caggiula M., Nociti V., Iorio R., Patanella A. K*., et al.* (2006). Pstat1, pstat3, and t-bet expression in peripheral blood mononuclear cells from relapsing-remitting multiple sclerosis patients correlates with disease activity. *J Neurosci Res:* 84; 1027-1036.

Fuchs P. C., Barry A. L. and Brown S. D. (2002). In vitro bactericidal activity of daptomycin against staphylococci. *J Antimicrob Chemother:* 49; 467-470.

Galli S. J. and Tsai M. (2012). Ige and mast cells in allergic disease. *Nature medicine:* 18; 693-704.

Gardner J., Ogese M., Betts C. J., Pirmohamed M. and Naisbitt D. J. (2022). Characterization of teicoplanin-specific t-cells from drug naïve donors expressing hla-a*32:01. *Chem Res Toxicol:* 35; 199-202.

Gatza E., Wahl D. R., Opipari A. W., Sundberg T. B., Reddy P., Liu C*., et al.* (2011). Manipulating the bioenergetics of alloreactive t cells causes their selective apoptosis and arrests graft-versus-host disease. *Sci Transl Med:* 3; 67ra68.

Gerstein W., Colombo E. and Harji F. (2018). Documented vancomycin-induced severe immune-mediated thrombocytopaenia. *BMJ Case Rep:* 2018;

Gibson A., Faulkner L., Lichtenfels M., Ogese M., Al-Attar Z., Alfirevic A*., et al.* (2017). The effect of inhibitory signals on the priming of drug hapten-specific t cells that express distinct vbeta receptors. *J Immunol:* 199; 1223-1237.

Gibson A., Kim S. H., Faulkner L., Evely J., Pirmohamed M., Park K. B*., et al.* (2015). In vitro priming of naive t-cells with p-phenylenediamine and bandrowski's base. *Chem Res Toxicol:* 28; 2069-2077.

Gibson A., Ogese M., Sullivan A., Wang E., Saide K., Whitaker P*., et al.* (2014). Negative regulation by pd-l1 during drug-specific priming of il-22-secreting t cells and the influence of pd-1 on effector t cell function. *J Immunol:* 192; 2611-2621.

Gomes E. R. and Demoly P. (2005). Epidemiology of hypersensitivity drug reactions. *Curr Opin Allergy Clin Immunol:* 5; 309-316.

Grakoui A., Bromley S. K., Sumen C., Davis M. M., Shaw A. S., Allen P. M*., et al.* (1999). The immunological synapse: A molecular machine controlling t cell activation. *Science:* 285; 221-227.

Griem P., Wulferink M., Sachs B., González J. B. and Gleichmann E. (1998). Allergic and autoimmune reactions to xenobiotics: How do they arise? *Immunol Today:* 19; 133-141.

Grist J. T., Jarvis L. B., Georgieva Z., Thompson S., Kaur Sandhu H., Burling K*., et al.* (2018). Extracellular lactate: A novel measure of t cell proliferation. *J Immunol:* 200; 1220-1226.

Groom J. R. and Luster A. D. (2011). Cxcr3 in t cell function. *Exp Cell Res:* 317; 620-631.

Grujic M., Braga T., Lukinius A., Eloranta M. L., Knight S. D., Pejler G*., et al.* (2005). Serglycin-deficient cytotoxic t lymphocytes display defective secretory granule maturation and granzyme b storage. *J Biol Chem:* 280; 33411-33418.

Gu X., Ma Y., Liu Y. and Wan Q. (2021). Measurement of mitochondrial respiration in adherent cells by seahorse xf96 cell mito stress test. *STAR Protocols:* 2; 100245.

Gubser P. M., Bantug G. R., Razik L., Fischer M., Dimeloe S., Hoenger G*., et al.* (2013). Rapid effector function of memory cd8(+) t cells requires an immediate-early glycolytic switch. *Nature Immunology:* 14; 1064-+.

Guerder S. and Matzinger P. (1992). A fail-safe mechanism for maintaining self-tolerance. *J Exp Med:* 176; 553-564.

Guilliams M., Ginhoux F., Jakubzick C., Naik S. H., Onai N., Schraml B. U*., et al.* (2014). Dendritic cells, monocytes and macrophages: A unified nomenclature based on ontogeny. *Nat Rev Immunol:* 14; 571-578.

Guo Y., Wu Y., Shinde S., Sy M. S., Aruffo A. and Liu Y. (1996). Identification of a costimulatory molecule rapidly induced by cd40l as cd44h. *J Exp Med:* 184; 955-961.

Guo Y., Xie Y.-Q., Gao M., Zhao Y., Franco F., Wenes M*., et al.* (2021). Metabolic reprogramming of terminally exhausted cd8+ t cells by il-10 enhances anti-tumor immunity. *Nature Immunology:* 22; 746-756.

Gurwitz J. H. and Avorn J. (1991). The ambiguous relation between aging and adverse drug reactions. *Ann Intern Med:* 114; 956-966.

Hale J. S., Youngblood B., Latner D. R., Mohammed A. U., Ye L., Akondy R. S*., et al.* (2013). Distinct memory cd4+ t cells with commitment to t follicular helper- and t helper 1-cell lineages are generated after acute viral infection. *Immunity:* 38; 805-817.

Halevy S., Ghislain P. D., Mockenhaupt M., Fagot J. P., Bouwes Bavinck J. N., Sidoroff A*., et al.* (2008). Allopurinol is the most common cause of stevens-johnson syndrome and toxic epidermal necrolysis in europe and israel. *J Am Acad Dermatol:* 58; 25-32.

Hammond S., Olsson-Brown A., Gardner J., Thomson P., Ali S. E., Jolly C*., et al.* (2021). T cell mediated hypersensitivity to previously tolerated iodinated contrast media precipitated by introduction of atezolizumab. *J Immunother Cancer:* 9;

Hammond S., Olsson-Brown A., Grice S., Gibson A., Gardner J., Castrejón-Flores J. L*., et al.* (2022). Checkpoint inhibition reduces the threshold for drug-specific t-cell priming and increases the incidence of sulfasalazine hypersensitivity. *Toxicol Sci:* 186; 58-69.

Hansel K., Bellini V., Bianchi L., Brozzi J. and Stingeni L. (2017). Drug reaction with eosinophilia and systemic symptoms from ceftriaxone confirmed by positive patch test: An immunohistochemical study. *J Allergy Clin Immunol Pract:* 5; 808-810.

Harding F. A., Mcarthur J. G., Gross J. A., Raulet D. H. and Allison J. P. (1992). Cd28-mediated signalling co-stimulates murine t cells and prevents induction of anergy in t-cell clones. *Nature:* 356; 607-609.

Harr T. and French L. E. (2010). Toxic epidermal necrolysis and stevens-johnson syndrome. *Orphanet J Rare Dis:* 5; 39.

Harrington L. E., Mangan P. R. and Weaver C. T. (2006). Expanding the effector cd4 t-cell repertoire: The th17 lineage. *Curr Opin Immunol:* 18; 349-356.

Hashizume H., Fujiyama T., Kanebayashi J., Kito Y., Hata M. and Yagi H. (2013). Skin recruitment of monomyeloid precursors involves human herpesvirus-6 reactivation in drug allergy. *Allergy:* 68; 681-689.

Hausmann O., Schnyder B. and Pichler W. J. (2010). Drug hypersensitivity reactions involving skin. *Handb Exp Pharmacol:* 29-55.

Hazell L. and Shakir S. A. (2006). Under-reporting of adverse drug reactions : A systematic review. *Drug Saf:* 29; 385-396.

Heinzerling L., Mari A., Bergmann K. C., Bresciani M., Burbach G., Darsow U*., et al.* (2013). The skin prick test - european standards. *Clin Transl Allergy:* 3; 3.

Hertl M. and Merk H. F. (1995). Lymphocyte activation in cutaneous drug reactions. *J Invest Dermatol:* 105; 95s-98s.

Hewitson L. J. (2019). Vancomycin induced dress syndrome (drug reaction with eosinophilia and systemic symptoms) in a patient with tricuspid endocarditis. *BMJ Case Rep:* 12;

Hirata K., Takagi H., Yamamoto M., Matsumoto T., Nishiya T., Mori K.*, et al.* (2008). Ticlopidine-induced hepatotoxicity is associated with specific human leukocyte antigen genomic subtypes in japanese patients: A preliminary case-control study. *Pharmacogenomics J:* 8; 29-33.

Hjortlund J., Mortz C. G., Skov P. S., Eller E., Poulsen J. M., Borch J. E.*, et al.* (2012). One-week oral challenge with penicillin in diagnosis of penicillin allergy. *Acta Derm Venereol:* 92; 307-312.

Holt M. P. and Ju C. (2006). Mechanisms of drug-induced liver injury. *The AAPS journal:* 8; E48-E54.

Hoofnagle J. H., Bonkovsky H. L., Phillips E. J., Li Y. J., Ahmad J., Barnhart H.*, et al.* (2021). Hla-b*35:01 and green tea-induced liver injury. *Hepatology:* 73; 2484-2493.

Hori S., Nomura T. and Sakaguchi S. (2003). Control of regulatory t cell development by the transcription factor foxp3. *Science:* 299; 1057-1061.

Hsiao S. H., Chen H. H., Chou C. H., Lin W. L., Liu Yeh P. Y. and Wu T. J. (2010). Teicoplanin-induced hypersensitivity syndrome with a preceding vancomycin-induced neutropenia: A case report and literature review. *J Clin Pharm Ther:* 35; 729-732.

Hsieh C. S., Macatonia S. E., Tripp C. S., Wolf S. F., O'garra A. and Murphy K. M. (1993). Development of th1 cd4+ t cells through il-12 produced by listeria-induced macrophages. *Science:* 260; 547-549.

Hsu D. Y., Brieva J., Silverberg N. B. and Silverberg J. I. (2016). Morbidity and mortality of stevens-johnson syndrome and toxic epidermal necrolysis in united states adults. *J Invest Dermatol:* 136; 1387-1397.

Huang M., Wu H., Zhou J., Xu M. and Zhou S. (2018). Efficacy of vancomycin on gram-positive bacterial infection in elderly critical patients and risk factors associated with nephrotoxicity. *Arch Iran Med:* 21; 349-355.

Huang V., Clayton N. A. and Welker K. H. (2020). Glycopeptide hypersensitivity and adverse reactions. *Pharmacy (Basel):* 8;

Hume D. A., Radik J. L., Ferber E. and Weidemann M. J. (1978). Aerobic glycolysis and lymphocyte transformation. *Biochem J:* 174; 703-709.

Hung S. I., Chung W. H., Jee S. H., Chen W. C., Chang Y. T., Lee W. R.*, et al.* (2006). Genetic susceptibility to carbamazepine-induced cutaneous adverse drug reactions. *Pharmacogenet Genomics:* 16; 297-306.

Hung S. I., Chung W. H., Liou L. B., Chu C. C., Lin M., Huang H. P.*, et al.* (2005). Hla-b*5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. *Proc Natl Acad Sci U S A:* 102; 4134-4139.

Hunter E. B., Johnston P. E., Tanner G., Pinson C. W. and Awad J. A. (1999). Bromfenac (duract)-associated hepatic failure requiring liver transplantation. *Am J Gastroenterol:* 94; 2299-2301.

Huppa J. B. and Davis M. M. (2003). T-cell-antigen recognition and the immunological synapse. *Nat Rev Immunol:* 3; 973-983.

Husain Z., Reddy B. Y. and Schwartz R. A. (2013). Dress syndrome: Part ii. Management and therapeutics. *J Am Acad Dermatol:* 68; 709.e701-709; quiz 718-720.

Idsoe O., Guthe T., Willcox R. R. and De Weck A. L. (1968). Nature and extent of penicillin side-reactions, with particular reference to fatalities from anaphylactic shock. *Bull World Health Organ:* 38; 159-188.

Iezzi G., Karjalainen K. and Lanzavecchia A. (1998). The duration of antigenic stimulation determines the fate of naive and effector t cells. *Immunity:* 8; 89-95.

Illing P. T., Vivian J. P., Dudek N. L., Kostenko L., Chen Z., Bharadwaj M.*, et al.* (2012). Immune self-reactivity triggered by drug-modified hla-peptide repertoire. *Nature:* 486; 554-558.

Ishizaka K., Ishizaka T. and Hornbrook M. M. (1966). Physicochemical properties of reaginic antibody. V. Correlation of reaginic activity wth gamma-e-globulin antibody. *J Immunol:* 97; 840-853.

Ivanov, Ii, Mckenzie B. S., Zhou L., Tadokoro C. E., Lepelley A., Lafaille J. J.*, et al.* (2006). The orphan nuclear receptor rorgammat directs the differentiation program of proinflammatory il-17+ t helper cells. *Cell:* 126; 1121-1133.

Jacobs S. R., Michalek R. D. and Rathmell J. C. (2010). Il-7 is essential for homeostatic control of t cell metabolism in vivo. *J Immunol:* 184; 3461-3469.

Jacysyn J. F., Abrahamsohn I. A. and Macedo M. S. (2001). Modulation of delayed-type hypersensitivity during the time course of immune response to a protein antigen. *Immunology:* 102; 373-379.

James C. W. and Gurk-Turner C. (2001). Cross-reactivity of beta-lactam antibiotics. *Proceedings (Baylor University. Medical Center):* 14; 106-107.

Janssen E. M., Lemmens E. E., Wolfe T., Christen U., Von Herrath M. G. and Schoenberger S. P. (2003). Cd4+ t cells are required for secondary expansion and memory in cd8+ t lymphocytes. *Nature:* 421; 852-856.

Jeng M. Y., Hull P. A., Fei M., Kwon H. S., Tsou C. L., Kasler H.*, et al.* (2018). Metabolic reprogramming of human cd8(+) memory t cells through loss of sirt1. *J Exp Med:* 215; 51-62.

Jensen C. S., Menné T., Lisby S., Kristiansen J. and Veien N. K. (2003). Experimental systemic contact dermatitis from nickel: A dose-response study. *Contact Dermatitis:* 49; 124-132.

Jeya M., Moon H. J., Lee K. M., Kim I. W. and Lee J. K. (2011). Glycopeptide antibiotics and their novel semi-synthetic derivatives. *Curr Pharm Biotechnol:* 12; 1194-1204.

Johansen J. D., Aalto-Korte K., Agner T., Andersen K. E., Bircher A., Bruze M.*, et al.* (2015). European society of contact dermatitis guideline for diagnostic patch testing - recommendations on best practice. *Contact Dermatitis:* 73; 195-221.

Johnson M. O., Wolf M. M., Madden M. Z., Andrejeva G., Sugiura A., Contreras D. C.*, et al.* (2018). Distinct regulation of th17 and th1 cell differentiation by glutaminase-dependent metabolism. *Cell:* 175; 1780-1795.e1719.

Johnston R. J., Poholek A. C., Ditoro D., Yusuf I., Eto D., Barnett B.*, et al.* (2009). Bcl6 and blimp-1 are reciprocal and antagonistic regulators of t follicular helper cell differentiation. *Science:* 325; 1006-1010.

Jones N., Cronin J. G., Dolton G., Panetti S., Schauenburg A. J., Galloway S. A.*, et al.* (2017). Metabolic adaptation of human cd4+ and cd8+ t-cells to t-cell receptor-mediated stimulation. *Frontiers in immunology:* 8; 1516.

Jones N., Vincent E. E., Cronin J. G., Panetti S., Chambers M., Holm S. R.*, et al.* (2019). Akt and stat5 mediate naïve human cd4+ t-cell early metabolic response to tcr stimulation. *Nat Commun:* 10; 2042.

Jones R. G. and Thompson C. B. (2007). Revving the engine: Signal transduction fuels t cell activation. *Immunity:* 27; 173-178.

Jones S. W., Penman S. L., French N. S., Park B. K. and Chadwick A. E. (2021). Investigating dihydroorotate dehydrogenase inhibitor mediated mitochondrial dysfunction in hepatic in vitro models. *Toxicol In Vitro:* 72; 105096.

Jonsson A. H. and Yokoyama W. M. (2009). Natural killer cell tolerance licensing and other mechanisms. *Adv Immunol:* 101; 27-79.

Ju C. and Uetrecht J. P. (2002). Mechanism of idiosyncratic drug reactions: Reactive metabolite formation, protein binding and the regulation of the immune system. *Curr Drug Metab:* 3; 367-377.

Justiz Vaillant A. A., Zulfiqar H. and Ramphul K. 2022. Delayed hypersensitivity reactions. *Statpearls.* Treasure Island (FL): StatPearls Publishing

Kabashima R., Sugita K., Sawada Y., Hino R., Nakamura M. and Tokura Y. (2011). Increased circulating th17 frequencies and serum il-22 levels in patients with acute generalized exanthematous pustulosis. *J Eur Acad Dermatol Venereol:* 25; 485-488.

Kaech S. M. and Ahmed R. (2001). Memory cd8+ t cell differentiation: Initial antigen encounter triggers a developmental program in naïve cells. *Nat Immunol:* 2; 415-422.

Kaech S. M., Wherry E. J. and Ahmed R. (2002). Effector and memory t-cell differentiation: Implications for vaccine development. *Nat Rev Immunol:* 2; 251-262.

Kamalian L., Chadwick A. E., Bayliss M., French N. S., Monshouwer M., Snoeys J.*, et al.* (2015). The utility of hepg2 cells to identify direct mitochondrial dysfunction in the absence of cell death. *Toxicol In Vitro:* 29; 732-740.

Kamalian L., Douglas O., Jolly C. E., Snoeys J., Simic D., Monshouwer M.*, et al.* (2018). The utility of heparg cells for bioenergetic investigation and detection of drug-induced mitochondrial toxicity. *Toxicology in Vitro:* 53; 136-147.

Kano Y., Hirahara K., Mitsuyama Y., Takahashi R. and Shiohara T. (2007). Utility of the lymphocyte transformation test in the diagnosis of drug sensitivity: Dependence on its timing and the type of drug eruption. *Allergy:* 62; 1439-1444.

Kaplan M. H., Schindler U., Smiley S. T. and Grusby M. J. (1996). Stat6 is required for mediating responses to il-4 and for development of th2 cells. *Immunity:* 4; 313-319.

Karami Z., Mesdaghi M., Karimzadeh P., Mansouri M., Taghdiri M. M., Kayhanidoost Z*., et al.* (2016). Evaluation of lymphocyte transformation test results in patients with delayed hypersensitivity reactions following the use of anticonvulsant drugs. *Int Arch Allergy Immunol:* 170; 158-162.

Kardaun S. H., Sekula P., Valeyrie-Allanore L., Liss Y., Chu C. Y., Creamer D*., et al.* (2013). Drug reaction with eosinophilia and systemic symptoms (dress): An original multisystem adverse drug reaction. Results from the prospective regiscar study. *Br J Dermatol:* 169; 1071-1080.

Khalil G., El-Sabban M., Al-Ghadban S., Azzi S., Shamra S., Khalifé S*., et al.* (2008). Cytokine expression profile of sensitized human t lymphocytes following in vitro stimulation with amoxicillin. *Eur Cytokine Netw:* 19; 131-141.

Khurana C. and De Belder M. A. (1999). Red-man syndrome after vancomycin: Potential cross-reactivity with teicoplanin. *Postgraduate Medical Journal:* 75; 41-43.

Kido T., Murata H., Nishigaki A., Tsubokura H., Komiya S., Kida N*., et al.* (2020). Glucose transporter 1 is important for the glycolytic metabolism of human endometrial stromal cells in hypoxic environment. *Heliyon:* 6; e03985-e03985.

Kikuchi S. and Okazaki T. (1978). Stevens-johnson syndrome due to co-trimoxazole. *Lancet:* 2; 580.

Kim K. H., Solvay M. J. and Thomas D. W. (1985). Cellular requirements for antigen processing by antigen-presenting cells: Evidence for different pathways in forming the same antigenic determinants. *Cell Immunol:* 96; 267-276.

Kim S. H., Saide K., Farrell J., Faulkner L., Tailor A., Ogese M*., et al.* (2015). Characterization of amoxicillin- and clavulanic acid-specific t cells in patients with amoxicillin-clavulanate-induced liver injury. *Hepatology:* 62; 887-899.

Klein Geltink R. I., O'sullivan D., Corrado M., Bremser A., Buck M. D., Buescher J. M*., et al.* (2017). Mitochondrial priming by cd28. *Cell:* 171; 385-397.e311.

Klysz D., Tai X., Robert P. A., Craveiro M., Cretenet G., Oburoglu L*., et al.* (2015). Glutamine-dependent α-ketoglutarate production regulates the balance between t helper 1 cell and regulatory t cell generation. *Sci Signal:* 8; ra97.

Knudsen J. D. and Pedersen M. (1992). Ige-mediated reaction to vancomycin and teicoplanin after treatment with vancomycin. *Scand J Infect Dis:* 24; 395-396.

Kola I. and Landis J. (2004). Can the pharmaceutical industry reduce attrition rates? *Nature Reviews Drug Discovery:* 3; 711-716.

Kondo T. and Takiguchi M. (2009). Human memory ccr4+cd8+ t cell subset has the ability to produce multiple cytokines. *Int Immunol:* 21; 523-532.

Konvinse K. C., Phillips E. J., White K. D. and Trubiano J. A. (2016). Old dog begging for new tricks: Current practices and future directions in the diagnosis of delayed antimicrobial hypersensitivity. *Curr Opin Infect Dis:* 29; 561-576.

Konvinse K. C., Trubiano J. A., Pavlos R., James I., Shaffer C. M., Bejan C. A.*, et al.* (2019). Hla-a*32:01 is strongly associated with vancomycin-induced drug reaction with eosinophilia and systemic symptoms. *J Allergy Clin Immunol:* 144; 183-192.

Koponen M., Pichler W. J. and De Weck A. L. (1986). T cell reactivity to penicillin: Phenotypic analysis of in vitro activated cell subsets. *J Allergy Clin Immunol:* 78; 645-652.

Korman A. J., Peggs K. S. and Allison J. P. (2006). Checkpoint blockade in cancer immunotherapy. *Adv Immunol:* 90; 297-339.

Kouro T. and Takatsu K. (2009). Il-5- and eosinophil-mediated inflammation: From discovery to therapy. *Int Immunol:* 21; 1303-1309.

Krasteva M. (1993). Contact dermatitis. *Int J Dermatol:* 32; 547-560.

Krause A., Guo H. F., Latouche J. B., Tan C., Cheung N. K. and Sadelain M. (1998). Antigen-dependent cd28 signaling selectively enhances survival and proliferation in genetically modified activated human primary t lymphocytes. *J Exp Med:* 188; 619-626.

Krummenacher M. (2020). The lymphocyte transformation test, flow cytometry and hla typing in vancomycin induced dress: A case series from south australia. *Pathology:* 52;

Kumar H., Kawai T. and Akira S. (2011). Pathogen recognition by the innate immune system. *Int Rev Immunol:* 30; 16-34.

Kündig T. M., Shahinian A., Kawai K., Mittrücker H.-W., Sebzda E., Bachmann M. F.*, et al.* (1996). Duration of tcr stimulation determines costimulatory requirement of t cells. *Immunity:* 5; 41-52.

Kwon H. S., Chang Y. S., Jeong Y. Y., Lee S. M., Song W. J., Kim H. B.*, et al.* (2006). A case of hypersensitivity syndrome to both vancomycin and teicoplanin. *J Korean Med Sci:* 21; 1108-1110.

Landsteiner K. and Jacobs J. (1935). Studies on the sensitization of animals with simple chemical compounds. *J Exp Med:* 61; 643-656.

Lazarou J., Pomeranz B. H. and Corey P. N. (1998). Incidence of adverse drug reactions in hospitalized patients: A meta-analysis of prospective studies. *Jama:* 279; 1200-1205.

Le Cleach L., Delaire S., Boumsell L., Bagot M., Bourgault-Villada I., Bensussan A.*, et al.* (2000). Blister fluid t lymphocytes during toxic epidermal necrolysis are functional cytotoxic cells which express human natural killer (nk) inhibitory receptors. *Clin Exp Immunol:* 119; 225-230.

Lederberg J. (1959). Genes and antibodies. *Science:* 129; 1649-1653.

Lee H. T., Lee S. H. and Heo Y. S. (2019). Molecular interactions of antibody drugs targeting pd-1, pd-l1, and ctla-4 in immuno-oncology. *Molecules:* 24;

Lee T., Lee Y. S., Yoon S. Y., Kim S., Bae Y. J., Kwon H. S*., et al.* (2013). Characteristics of liver injury in drug-induced systemic hypersensitivity reactions. *J Am Acad Dermatol:* 69; 407-415.

Lee W. M. (2013). Drug-induced acute liver failure. *Clin Liver Dis:* 17; 575-586, viii.

Lenschow D. J., Walunas T. L. and Bluestone J. A. (1996). Cd28/b7 system of t cell costimulation. *Annu Rev Immunol:* 14; 233-258.

Li H., Llera A., Malchiodi E. L. and Mariuzza R. A. (1999). The structural basis of t cell activation by superantigens. *Annu Rev Immunol:* 17; 435-466.

Lin Y. F., Yang C. H., Sindy H., Lin J. Y., Rosaline Hui C. Y., Tsai Y. C*., et al.* (2014). Severe cutaneous adverse reactions related to systemic antibiotics. *Clin Infect Dis:* 58; 1377-1385.

Linsley P. S., Clark E. A. and Ledbetter J. A. (1990). T-cell antigen cd28 mediates adhesion with b cells by interacting with activation antigen b7/bb-1. *Proc Natl Acad Sci U S A:* 87; 5031-5035.

Litman G. W., Rast J. P. and Fugmann S. D. (2010). The origins of vertebrate adaptive immunity. *Nature reviews. Immunology:* 10; 543-553.

Littlehales E., Murray O. and Dunsmuir R. (2018). Vancomycin-induced dress syndrome: An important concern in orthopedic surgery. *Case Rep Orthop:* 2018; 1439073.

Liu H., Rhodes M., Wiest D. L. and Vignali D. a. A. (2000). On the dynamics of tcr:Cd3 complex cell surface expression and downmodulation. *Immunity:* 13; 665-675.

Liu Y., Zhou N., Zhou L., Wang J., Zhou Y., Zhang T*., et al.* (2021). Il-2 regulates tumor-reactive cd8+ t cell exhaustion by activating the aryl hydrocarbon receptor. *Nature Immunology:* 22; 358-369.

Locci M., Havenar-Daughton C., Landais E., Wu J., Kroenke M. A., Arlehamn C. L*., et al.* (2013). Human circulating pd-1+cxcr3-cxcr5+ memory tfh cells are highly functional and correlate with broadly neutralizing hiv antibody responses. *Immunity:* 39; 758-769.

Lorenz H. M., Herrmann M. and Kalden J. R. (2001). The pathogenesis of autoimmune diseases. *Scand J Clin Lab Invest Suppl:* 235; 16-26.

Lovato P., Brender C., Agnholt J., Kelsen J., Kaltoft K., Svejgaard A*., et al.* (2003). Constitutive stat3 activation in intestinal t cells from patients with crohn's disease. *J Biol Chem:* 278; 16777-16781.

Lucena M. I., Molokhia M., Shen Y., Urban T. J., Aithal G. P., Andrade R. J*., et al.* (2011). Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple hla class i and ii alleles. *Gastroenterology:* 141; 338-347.

Luque I., Leyva L., José Torres M., Rosal M., Mayorga C., Segura J. M*., et al.* (2001). In vitro t-cell responses to beta-lactam drugs in immediate and nonimmediate allergic reactions. *Allergy:* 56; 611-618.

Ma X., Jin M., Cai Y., Xia H., Long K., Liu J*., et al.* (2011). Mitochondrial electron transport chain complex iii is required for antimycin a to inhibit autophagy. *Chemistry & biology:* 18; 1474-1481.

Macdonald H. R. (1977). Energy metabolism and t-cell-mediated cytolysis. Ii. Selective inhibition of cytolysis by 2-deoxy-d-glucose. *J Exp Med:* 146; 710-719.

Macdonald H. R. and Cerottini J. C. (1979). Inhibition of t cell-mediated cytolysis by 2-deoxy-d-glucose:Dissociation of the inhibitory effect from glycoprotein synthesis. *Eur J Immunol:* 9; 466-470.

Macintyre A. N., Gerriets V. A., Nichols A. G., Michalek R. D., Rudolph M. C., Deoliveira D.*, et al.* (2014). The glucose transporter glut1 is selectively essential for cd4 t cell activation and effector function. *Cell Metab:* 20; 61-72.

Madrigal-Burgaleta R., Bernal-Rubio L., Berges-Gimeno M. P., Carpio-Escalona L. V., Gehlhaar P. and Alvarez-Cuesta E. (2019). A large single-hospital experience using drug provocation testing and rapid drug desensitization in hypersensitivity to antineoplastic and biological agents. *J Allergy Clin Immunol Pract:* 7; 618-632.

Makris M. P., Koulouris S. and Kalogeromitros D. (2010). Nonimmediate systemic hypersensitivity reaction to beta-lactam intradermal tests. *J Investig Allergol Clin Immunol:* 20; 630-631.

Mallal S., Nolan D., Witt C., Masel G., Martin A. M., Moore C.*, et al.* (2002). Association between presence of hla-b*5701, hla-dr7, and hla-dq3 and hypersensitivity to hiv-1 reverse-transcriptase inhibitor abacavir. *Lancet:* 359; 727-732.

Mallal S., Phillips E., Carosi G., Molina J. M., Workman C., Tomazic J.*, et al.* (2008). Hla-b*5701 screening for hypersensitivity to abacavir. *N Engl J Med:* 358; 568-579.

Man C. B., Kwan P., Baum L., Yu E., Lau K. M., Cheng A. S.*, et al.* (2007). Association between hla-b*1502 allele and antiepileptic drug-induced cutaneous reactions in han chinese. *Epilepsia:* 48; 1015-1018.

Maraskovsky E., Chen W. F. and Shortman K. (1989). Il-2 and ifn-gamma are two necessary lymphokines in the development of cytolytic t cells. *J Immunol:* 143; 1210-1214.

Marko A. J., Miller R. A., Kelman A. and Frauwirth K. A. (2010). Induction of glucose metabolism in stimulated t lymphocytes is regulated by mitogen-activated protein kinase signaling. *PloS one:* 5; e15425.

Marroquin L. D., Hynes J., Dykens J. A., Jamieson J. D. and Will Y. (2007). Circumventing the crabtree effect: Replacing media glucose with galactose increases susceptibility of hepg2 cells to mitochondrial toxicants. *Toxicol Sci:* 97; 539-547.

Marshall H. D., Chandele A., Jung Y. W., Meng H., Poholek A. C., Parish I. A.*, et al.* (2011). Differential expression of ly6c and t-bet distinguish effector and memory th1 cd4(+) cell properties during viral infection. *Immunity:* 35; 633-646.

Martin S. and Weltzien H. U. (1994). T cell recognition of haptens, a molecular view. *Int Arch Allergy Immunol:* 104; 10-16.

Marzano A. V., Borghi A. and Cugno M. (2016). Adverse drug reactions and organ damage: The skin. *Eur J Intern Med:* 28; 17-24.

Matzinger P. (1994). Tolerance, danger, and the extended family. *Annu Rev Immunol:* 12; 991-1045.

Matzinger P. (2007). Friendly and dangerous signals: Is the tissue in control? *Nat Immunol:* 8; 11-13.

Mauri-Hellweg D., Bettens F., Mauri D., Brander C., Hunziker T. and Pichler W. J. (1995). Activation of drug-specific cd4+ and cd8+ t cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. *J Immunol:* 155; 462-472.

Mccormack M., Alfirevic A., Bourgeois S., Farrell J. J., Kasperavičiūtė D., Carrington M.*, et al.* (2011). Hla-a*3101 and carbamazepine-induced hypersensitivity reactions in europeans. *N Engl J Med:* 364; 1134-1143.

Mccormack M., Urban T. J., Shianna K. V., Walley N., Pandolfo M., Depondt C.*, et al.* (2012). Genome-wide mapping for clinically relevant predictors of lamotrigine- and phenytoin-induced hypersensitivity reactions. *Pharmacogenomics:* 13; 399-405.

Mei H., Wang J., Che H., Wang R. and Cai Y. (2019). The clinical efficacy and safety of vancomycin loading dose: A systematic review and meta-analysis. *Medicine:* 98;

Meng X., Al-Attar Z., Yaseen F. S., Jenkins R., Earnshaw C., Whitaker P.*, et al.* (2017). Definition of the nature and hapten threshold of the beta-lactam antigen required for t cell activation in vitro and in patients. *J Immunol:* 198; 4217-4227.

Meng X., Earnshaw C. J., Tailor A., Jenkins R. E., Waddington J. C., Whitaker P.*, et al.* (2016). Amoxicillin and clavulanate form chemically and immunologically distinct multiple haptenic structures in patients. *Chem Res Toxicol:* 29; 1762-1772.

Menk A. V., Scharping N. E., Moreci R. S., Zeng X., Guy C., Salvatore S.*, et al.* (2018a). Early tcr signaling induces rapid aerobic glycolysis enabling distinct acute t cell effector functions. *Cell reports:* 22; 1509-1521.

Menk A. V., Scharping N. E., Rivadeneira D. B., Calderon M. J., Watson M. J., Dunstane D.*, et al.* (2018b). 4-1bb costimulation induces t cell mitochondrial function and biogenesis enabling cancer immunotherapeutic responses. *J Exp Med:* 215; 1091-1100.

Merk H. F. (2005). Diagnosis of drug hypersensitivity: Lymphocyte transformation test and cytokines. *Toxicology:* 209; 217-220.

Michalek R. D., Gerriets V. A., Jacobs S. R., Macintyre A. N., Maciver N. J., Mason E. F.*, et al.* (2011). Cutting edge: Distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory cd4+ t cell subsets. *J Immunol:* 186; 3299-3303.

Minhas J. S., Wickner P. G., Long A. A., Banerji A. and Blumenthal K. G. (2016). Immune-mediated reactions to vancomycin: A systematic case review and analysis. *Ann Allergy Asthma Immunol:* 116; 544-553.

Miyazu D., Kodama N., Yamashita D., Tanaka H., Inoue S., Imakyure O.*, et al.* (2016). Dress syndrome caused by cross-reactivity between vancomycin and subsequent teicoplanin administration: A case report. *Am J Case Rep:* 17; 625-631.

Monshi M. M., Faulkner L., Gibson A., Jenkins R. E., Farrell J., Earnshaw C. J.*, et al.* (2013). Human leukocyte antigen (hla)-b*57:01-restricted activation of drug-specific t cells provides the immunological basis for flucloxacillin-induced liver injury. *Hepatology:* 57; 727-739.

Moré S. H., Breloer M. and Von Bonin A. (2001). Eukaryotic heat shock proteins as molecular links in innate and adaptive immune responses: Hsp60-mediated activation of cytotoxic t cells. *International Immunology:* 13; 1121-1127.

Mosmann T. R., Cherwinski H., Bond M. W., Giedlin M. A. and Coffman R. L. (1986). Two types of murine helper t cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol:* 136; 2348-2357.

Mosmann T. R. and Coffman R. L. (1989). Th1 and th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol:* 7; 145-173.

Muraille E. M., De Becker G., Bakkus M., Thielemans K., Urbain J., Moser M.*, et al.* (1995). Co-stimulation lowers the threshold for activation of naive t cells by bacterial superantigens. *Int Immunol:* 7; 295-304.

Murali-Krishna K., Altman J. D., Suresh M., Sourdive D. J., Zajac A. J., Miller J. D.*, et al.* (1998). Counting antigen-specific cd8 t cells: A reevaluation of bystander activation during viral infection. *Immunity:* 8; 177-187.

Murphy K. M. and Reiner S. L. (2002). The lineage decisions of helper t cells. *Nat Rev Immunol:* 2; 933-944.

Musette P. and Janela B. (2017). New insights into drug reaction with eosinophilia and systemic symptoms pathophysiology. *Frontiers in Medicine:* 4;

Mushin W. W., Rosen M. and Jones E. V. (1971). Post-halothane jaundice in relation to previous administration of halothane. *Br Med J:* 3; 18-22.

Naisbitt D. J., Britschgi M., Wong G., Farrell J., Depta J. P., Chadwick D. W.*, et al.* (2003a). Hypersensitivity reactions to carbamazepine: Characterization of the specificity, phenotype, and cytokine profile of drug-specific t cell clones. *Mol Pharmacol:* 63; 732-741.

Naisbitt D. J., Farrell J., Chamberlain P. J., Hopkins J. E., Berry N. G., Pirmohamed M.*, et al.* (2005). Characterization of the t-cell response in a patient with phenindione hypersensitivity. *The Journal of pharmacology and experimental therapeutics:* 313; 1058-1065.

Naisbitt D. J., Farrell J., Wong G., Depta J. P., Dodd C. C., Hopkins J. E.*, et al.* (2003b). Characterization of drug-specific t cells in lamotrigine hypersensitivity. *J Allergy Clin Immunol:* 111; 1393-1403.

Naisbitt D. J., Gordon S. F., Pirmohamed M., Burkhart C., Cribb A. E., Pichler W. J.*, et al.* (2001). Antigenicity and immunogenicity of sulphamethoxazole: Demonstration of metabolism-dependent haptenation and t-cell proliferation in vivo. *British journal of pharmacology:* 133; 295-305.

Naisbitt D. J., Olsson-Brown A., Gibson A., Meng X., Ogese M. O., Tailor A.*, et al.* (2019). Immune dysregulation increases the incidence of delayed-type drug hypersensitivity reactions. *Allergy:*

Nakaya M., Xiao Y., Zhou X., Chang J. H., Chang M., Cheng X.*, et al.* (2014). Inflammatory t cell responses rely on amino acid transporter asct2 facilitation of glutamine uptake and mtorc1 kinase activation. *Immunity:* 40; 692-705.

Nakkam N., Gibson A., Mouhtouris E., Konvinse K., Holmes N., Chua K. Y.*, et al.* (2020). Cross-reactivity between vancomycin, teicoplanin and telavancin in hla-a*32:01 positive vancomycin dress patients sharing an hla-class ii haplotype. *J Allergy Clin Immunol:*

Nakkam N., Trubiano J., Gibson A. and Phillips E. J. (2021). Considerations for cross-reactivity between vancomycin and other glycopeptides. *J Allergy Clin Immunol Pract:* 9; 3233.

Nassif A., Bensussan A., Dorothée G., Mami-Chouaib F., Bachot N., Bagot M.*, et al.* (2002). Drug specific cytotoxic t-cells in the skin lesions of a patient with toxic epidermal necrolysis. *J Invest Dermatol:* 118; 728-733.

Nassif A., Moslehi H., Le Gouvello S., Bagot M., Lyonnet L., Michel L.*, et al.* (2004). Evaluation of the potential role of cytokines in toxic epidermal necrolysis. *J Invest Dermatol:* 123; 850-855.

Neefjes J., Jongsma M. L. M., Paul P. and Bakke O. (2011). Towards a systems understanding of mhc class i and mhc class ii antigen presentation. *Nature Reviews Immunology:* 11; 823-836.

Nelson M. A., Mclaughlin K. L., Hagen J. T., Coalson H. S., Schmidt C., Kassai M.*, et al.* (2021). Intrinsic oxphos limitations underlie cellular bioenergetics in leukemia. *eLife:* 10;

Nguyen H., Haarberg K. M., Wu Y., Fu J., Heinrichs J. L., Bastian D.*, et al.* (2014). Allogeneic t cells utilize glycolysis as the predominant metabolic pathway to induce acute graft-versus-host disease. *Blood:* 124; 2419-2419.

Nicolaou K. C., Boddy C. N., Bräse S. and Winssinger N. (1999). Chemistry, biology, and medicine of the glycopeptide antibiotics. *Angew Chem Int Ed Engl:* 38; 2096-2152.

Nikolich-Zugich J., Slifka M. K. and Messaoudi I. (2004). The many important facets of t-cell repertoire diversity. *Nat Rev Immunol:* 4; 123-132.

Nishizuka Y. and Sakakura T. (1969). Thymus and reproduction: Sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science:* 166; 753-755.

Niu J., Jia Q., Ni Q., Yang Y., Chen G., Yang X.*, et al.* (2015). Association of cd8(+) t lymphocyte repertoire spreading with the severity of dress syndrome. *Scientific Reports:* 5; 9913.

Norcross M. A., Luo S., Lu L., Boyne M. T., Gomarteli M., Rennels A. D.*, et al.* (2012). Abacavir induces loading of novel self-peptides into hla-b*57: 01: An autoimmune model for hla-associated drug hypersensitivity. *Aids:* 26; F21-29.

Nurieva R. I., Chung Y., Martinez G. J., Yang X. O., Tanaka S., Matskevitch T. D.*, et al.* (2009). Bcl6 mediates the development of t follicular helper cells. *Science (New York, N.Y.):* 325; 1001-1005.

Nyfeler B. and Pichler W. J. (1997). The lymphocyte transformation test for the diagnosis of drug allergy: Sensitivity and specificity. *Clin Exp Allergy:* 27; 175-181.

O'sullivan D., Van Der Windt G. J., Huang S. C., Curtis J. D., Chang C. H., Buck M. D.*, et al.* (2014). Memory cd8(+) t cells use cell-intrinsic lipolysis to support the metabolic programming necessary for development. *Immunity:* 41; 75-88.

Ogese M. O., Lister A., Gardner J., Meng X., Alfirevic A., Pirmohamed M.*, et al.* (2021). Deciphering adverse drug reactions: In vitro priming and characterization of vancomycin-specific t-cells from healthy donors expressing hla-a*32:01. *Toxicol Sci:*

Ogese M. O., Watkinson J., Lister A., Faulkner L., Gibson A., Hillegas A.*, et al.* (2020). Development of an improved t cell assay to assess the intrinsic immunogenicity of haptenic compounds. *Toxicol Sci:*

Okazaki T. and Honjo T. (2007). Pd-1 and pd-1 ligands: From discovery to clinical application. *Int Immunol:* 19; 813-824.

Onakpoya I. J., Heneghan C. J. and Aronson J. K. (2016). Post-marketing withdrawal of 462 medicinal products because of adverse drug reactions: A systematic review of the world literature. *BMC medicine:* 14; 10-10.

Onuora S. (2022). Dress linked to hla alleles. *Nature Reviews Rheumatology:* 18; 62-62.

Ortiz B. D., Nelson P. J. and Krensky A. M. (1997). Switching gears during t-cell maturation: Rantes and late transcription. *Immunol Today:* 18; 468-471.

Osanlou R., Walker L., Hughes D. A., Burnside G. and Pirmohamed M. (2022). Adverse drug reactions, multimorbidity and polypharmacy: A prospective analysis of 1 month of medical admissions. *BMJ Open:* 12; e055551.

Ostrov D. A., Grant B. J., Pompeu Y. A., Sidney J., Harndahl M., Southwood S.*, et al.* (2012). Drug hypersensitivity caused by alteration of the mhc-presented self-peptide repertoire. *Proc Natl Acad Sci U S A:* 109; 9959-9964.

Pacholczyk R., Kern J., Singh N., Iwashima M., Kraj P. and Ignatowicz L. (2007). Nonself-antigens are the cognate specificities of foxp3+ regulatory t cells. *Immunity:* 27; 493-504.

Pajak B., Siwiak E., Sołtyka M., Priebe A., Zieliński R., Fokt I.*, et al.* (2019). 2-deoxy-d-glucose and its analogs: From diagnostic to therapeutic agents. *Int J Mol Sci:* 21;

Pan R.-Y., Chu M.-T., Wang C.-W., Lee Y.-S., Lemonnier F., Michels A. W.*, et al.* (2019). Identification of drug-specific public tcr driving severe cutaneous adverse reactions. *Nature Communications:* 10; 3569.

Parish C. R. and O'neill E. R. (1997). Dependence of the adaptive immune response on innate immunity: Some questions answered but new paradoxes emerge. *Immunol Cell Biol:* 75; 523-527.

Pavlos R., Mallal S., Ostrov D., Buus S., Metushi I., Peters B.*, et al.* (2015). T cell-mediated hypersensitivity reactions to drugs. *Annual review of medicine:* 66; 439-454.

Pavlos R., Mallal S. and Phillips E. (2012). Hla and pharmacogenetics of drug hypersensitivity. *Pharmacogenomics:* 13; 1285-1306.

Pearce E. L. and Pearce E. J. (2013). Metabolic pathways in immune cell activation and quiescence. *Immunity:* 38; 633-643.

Pearce E. L., Poffenberger M. C., Chang C. H. and Jones R. G. (2013). Fueling immunity: Insights into metabolism and lymphocyte function. *Science:* 342; 1242454.

Pearce E. L., Walsh M. C., Cejas P. J., Harms G. M., Shen H., Wang L. S.*, et al.* (2009). Enhancing cd8 t-cell memory by modulating fatty acid metabolism. *Nature:* 460; 103-107.

Perkins H. R. (1969). Specificity of combination between mucopeptide precursors and vancomycin or ristocetin. *Biochem J:* 111; 195-205.

Peyrière H., Dereure O., Breton H., Demoly P., Cociglio M., Blayac J. P.*, et al.* (2006). Variability in the clinical pattern of cutaneous side-effects of drugs with systemic symptoms: Does a dress syndrome really exist? *Br J Dermatol:* 155; 422-428.

Pfeiffer T. and Bonhoeffer S. (2002). Evolutionary consequences of tradeoffs between yield and rate of atp production. 216; 51-51.

Phillips E. and Mallal S. (2007). Drug hypersensitivity in hiv. *Curr Opin Allergy Clin Immunol:* 7; 324-330.

Phillips E. J., Bigliardi P., Bircher A. J., Broyles A., Chang Y. S., Chung W. H.*, et al.* (2019). Controversies in drug allergy: Testing for delayed reactions. *J Allergy Clin Immunol:* 143; 66-73.

Picard D., Janela B., Descamps V., D'incan M., Courville P., Jacquot S.*, et al.* (2010). Drug reaction with eosinophilia and systemic symptoms (dress): A multiorgan antiviral t cell response. *Sci Transl Med:* 2; 46ra62.

Pichler W., Yawalkar N., Schmid S. and Helbling A. (2002). Pathogenesis of drug-induced exanthems. *Allergy:* 57; 884-893.

Pichler W. J. (2002). Pharmacological interaction of drugs with antigen-specific immune receptors: The p-i concept. *Curr Opin Allergy Clin Immunol:* 2; 301-305.

Pichler W. J. (2003). Delayed drug hypersensitivity reactions. *Ann Intern Med:* 139; 683-693.

Pichler W. J. (2005). Direct t-cell stimulations by drugs--bypassing the innate immune system. *Toxicology:* 209; 95-100.

Pichler W. J. and Tilch J. (2004). The lymphocyte transformation test in the diagnosis of drug hypersensitivity. *Allergy:* 59; 809-820.

Pirmohamed M., James S., Meakin S., Green C., Scott A. K., Walley T. J.*, et al.* (2004). Adverse drug reactions as cause of admission to hospital: Prospective analysis of 18 820 patients. *BMJ (Clinical research ed.):* 329; 15-19.

Pirmohamed M., Ostrov D. A. and Park B. K. (2015). New genetic findings lead the way to a better understanding of fundamental mechanisms of drug hypersensitivity. *J Allergy Clin Immunol:* 136; 236-244.

Polak M. E., Belgi G., Mcguire C., Pickard C., Healy E., Friedmann P. S.*, et al.* (2013). In vitro diagnostic assays are effective during the acute phase of delayed-type drug hypersensitivity reactions. *Br J Dermatol:* 168; 539-549.

Pollizzi K. N., Patel C. H., Sun I. H., Oh M. H., Waickman A. T., Wen J.*, et al.* (2015). Mtorc1 and mtorc2 selectively regulate cd8$^+$ t cell differentiation. *J Clin Invest:* 125; 2090-2108.

Porebski G., Gschwend-Zawodniak A. and Pichler W. J. (2011). In vitro diagnosis of t cell-mediated drug allergy. *Clin Exp Allergy:* 41; 461-470.

Porebski G., Pecaric-Petkovic T., Groux-Keller M., Bosak M., Kawabata T. T. and Pichler W. J. (2013). In vitro drug causality assessment in stevens-johnson syndrome - alternatives for lymphocyte transformation test. *Clin Exp Allergy:* 43; 1027-1037.

Posadas S. J., Leyva L., Torres M. J., Rodriguez J. L., Bravo I., Rosal M.*, et al.* (2000). Subjects with allergic reactions to drugs show in vivo polarized patterns of cytokine expression depending on the chronology of the clinical reaction. *J Allergy Clin Immunol:* 106; 769-776.

Posadas S. J., Padial A., Torres M. J., Mayorga C., Leyva L., Sanchez E.*, et al.* (2002). Delayed reactions to drugs show levels of perforin, granzyme b, and fas-l to be related to disease severity. *J Allergy Clin Immunol:* 109; 155-161.

Postow M. A., Sidlow R. and Hellmann M. D. (2018). Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med:* 378; 158-168.

Prasad K. V., Janssen O., Kapeller R., Raab M., Cantley L. C. and Rudd C. E. (1993). Src-homology 3 domain of protein kinase p59fyn mediates binding to phosphatidylinositol 3-kinase in t cells. *Proc Natl Acad Sci U S A:* 90; 7366-7370.

Qureshi O. S., Zheng Y., Nakamura K., Attridge K., Manzotti C., Schmidt E. M.*, et al.* (2011). Trans-endocytosis of cd80 and cd86: A molecular basis for the cell-extrinsic function of ctla-4. *Science:* 332; 600-603.

Rajan T. V. (2003). The gell–coombs classification of hypersensitivity reactions: A re-interpretation. *Trends in Immunology:* 24; 376-379.

Ramadan G. (2008). Epstein-barr virus-transformed b-cells as efficient antigen presenting cells to propagate aspergillus-specific cytotoxic t-lymphocytes. *Egypt J Immunol:* 15; 145-157.

Rathmell J. C., Farkash E. A., Gao W. and Thompson C. B. (2001). Il-7 enhances the survival and maintains the size of naive t cells. *J Immunol:* 167; 6869-6876.

Rathmell J. C., Vander Heiden M. G., Harris M. H., Frauwirth K. A. and Thompson C. B. (2000). In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability. *Mol Cell:* 6; 683-692.

Raud B., Roy D. G., Divakaruni A. S., Tarasenko T. N., Franke R., Ma E. H.*, et al.* (2018). Etomoxir actions on regulatory and memory t cells are independent of cpt1a-mediated fatty acid oxidation. *Cell Metab:* 28; 504-515.e507.

Read S., Malmström V. and Powrie F. (2000). Cytotoxic t lymphocyte-associated antigen 4 plays an essential role in the function of cd25(+)cd4(+) regulatory cells that control intestinal inflammation. *J Exp Med:* 192; 295-302.

Remmer H. (1970). The role of the liver in drug metabolism. *The American Journal of Medicine:* 49; 617-629.

Renz C. L., Thurn J. D., Finn H. A., Lynch J. P. and Moss J. (1999). Antihistamine prophylaxis permits rapid vancomycin infusion. *Crit Care Med:* 27; 1732-1737.

Reuben A., Koch D. G. and Lee W. M. (2010). Drug-induced acute liver failure: Results of a u.S. Multicenter, prospective study. *Hepatology:* 52; 2065-2076.

Revuz J., Penso D., Roujeau J. C., Guillaume J. C., Payne C. R., Wechsler J.*, et al.* (1987). Toxic epidermal necrolysis. Clinical findings and prognosis factors in 87 patients. *Arch Dermatol:* 123; 1160-1165.

Reynolds P. E. (1989). Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *Eur J Clin Microbiol Infect Dis:* 8; 943-950.

Ridge J. P., Di Rosa F. and Matzinger P. (1998). A conditioned dendritic cell can be a temporal bridge between a cd4+ t-helper and a t-killer cell. *Nature:* 393; 474-478.

Riedl M. A. and Casillas A. M. (2003). Adverse drug reactions: Types and treatment options. *Am Fam Physician:* 68; 1781-1790.

Rive C. M., Bourke J. and Phillips E. J. (2013). Testing for drug hypersensitivity syndromes. *Clin Biochem Rev:* 34; 15-38.

Roch M., Gagetti P., Davis J., Ceriana P., Errecalde L., Corso A.*, et al.* (2017). Daptomycin resistance in clinical mrsa strains is associated with a high biological fitness cost. *Frontiers in Microbiology:* 8;

Romagnani S. (1997). The th1/th2 paradigm. *Immunol Today:* 18; 263-266.

Romano A., Guéant-Rodriguez R. M., Viola M., Gaeta F., Caruso C. and Guéant J. L. (2005). Cross-reactivity among drugs: Clinical problems. *Toxicology:* 209; 169-179.

Rommers M. K., Teepe-Twiss I. M. and Guchelaar H. J. (2007). Preventing adverse drug events in hospital practice: An overview. *Pharmacoepidemiol Drug Saf:* 16; 1129-1135.

Roujeau J. C. (1994). The spectrum of stevens-johnson syndrome and toxic epidermal necrolysis: A clinical classification. *J Invest Dermatol:* 102; 28s-30s.

Roujeau J. C. (2005). Clinical heterogeneity of drug hypersensitivity. *Toxicology:* 209; 123-129.

Roujeau J. C., Albengres E., Moritz S., Piacentino A., Cuny M., Revuz J.*, et al.* (1985). Lymphocyte transformation test in drug-induced toxic epidermal necrolysis. *Int Arch Allergy Appl Immunol:* 78; 22-24.

Roujeau J. C., Bioulac-Sage P., Bourseau C., Guillaume J. C., Bernard P., Lok C.*, et al.* (1991). Acute generalized exanthematous pustulosis. Analysis of 63 cases. *Arch Dermatol:* 127; 1333-1338.

Roujeau J. C., Kelly J. P., Naldi L., Rzany B., Stern R. S., Anderson T.*, et al.* (1995). Medication use and the risk of stevens-johnson syndrome or toxic epidermal necrolysis. *N Engl J Med:* 333; 1600-1607.

Roujeau J. C. and Stern R. S. (1994). Severe adverse cutaneous reactions to drugs. *N Engl J Med:* 331; 1272-1285.

Royer R. J. (1997). Mechanism of action of adverse drug reactions: An overview. *Pharmacoepidemiol Drug Saf:* 6 Suppl 3; S43-50.

Rudd C. E., Taylor A. and Schneider H. (2009). Cd28 and ctla-4 co-receptor expression and signal transduction. *Immunol Rev:* 229; 12-26.

Ruiz-Iruela C., Padullés-Zamora N., Podzamczer-Palter D., Alonso-Pastor A., Candás-Estébanez B., Alía-Ramos P.*, et al.* (2016). Hla-b*57: 01 genotyping in the prevention of hypersensitivity to abacavir: 5 years of experience. *Pharmacogenet Genomics:* 26; 390-396.

Rybak M. J., Albrecht L. M., Boike S. C. and Chandrasekar P. H. (1990). Nephrotoxicity of vancomycin, alone and with an aminoglycoside. *J Antimicrob Chemother:* 25; 679-687.

Sachs B., Erdmann S., Malte Baron J., Neis M., Al Masaoudi T. and Merk H. F. (2002). Determination of interleukin-5 secretion from drug-specific activated ex vivo peripheral blood mononuclear cells as a test system for the in vitro detection of drug sensitization. *Clin Exp Allergy:* 32; 736-744.

Sacks D. and Noben-Trauth N. (2002). The immunology of susceptibility and resistance to leishmania major in mice. *Nat Rev Immunol:* 2; 845-858.

Sad S., Krishnan L., Bleackley R. C., Kägi D., Hengartner H. and Mosmann T. R. (1997). Cytotoxicity and weak cd40 ligand expression of cd8+ type 2 cytotoxic t cells restricts their potential b cell helper activity. *Eur J Immunol:* 27; 914-922.

Sad S., Marcotte R. and Mosmann T. R. (1995). Cytokine-induced differentiation of precursor mouse cd8+ t cells into cytotoxic cd8+ t cells secreting th1 or th2 cytokines. *Immunity:* 2; 271-279.

Sahai J., Healy D. P., Garris R., Berry A. and Polk R. E. (1989). Influence of antihistamine pretreatment on vancomycin-induced red-man syndrome. *J Infect Dis:* 160; 876-881.

Sakaguchi S., Sakaguchi N., Asano M., Itoh M. and Toda M. (1995). Immunologic self-tolerance maintained by activated t cells expressing il-2 receptor alpha-chains (cd25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol:* 155; 1151-1164.

Sallusto F., Cella M., Danieli C. and Lanzavecchia A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class ii compartment: Downregulation by cytokines and bacterial products. *J Exp Med:* 182; 389-400.

Sallusto F. and Lanzavecchia A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med:* 179; 1109-1118.

Sanderson J. P., Naisbitt D. J., Farrell J., Ashby C. A., Tucker M. J., Rieder M. J*., et al.* (2007). Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling. *J Immunol:* 178; 5533-5542.

Santiago F., Gonçalo M., Vieira R., Coelho S. and Figueiredo A. (2010). Epicutaneous patch testing in drug hypersensitivity syndrome (dress). *Contact Dermatitis:* 62; 47-53.

Schaerli P., Britschgi M., Keller M., Steiner U. C., Steinmann L. S., Moser B*., et al.* (2004). Characterization of human t cells that regulate neutrophilic skin inflammation. *J Immunol:* 173; 2151-2158.

Schleimer R. P., Kato A., Kern R., Kuperman D. and Avila P. C. (2007). Epithelium: At the interface of innate and adaptive immune responses. *J Allergy Clin Immunol:* 120; 1279-1284.

Schmitt E., Van Brandwijk R., Van Snick J., Siebold B. and Rüde E. (1989). Tcgf iii/p40 is produced by naive murine cd4+ t cells but is not a general t cell growth factor. *Eur J Immunol:* 19; 2167-2170.

Schneider C. H. and De Weck A. L. (1965). A new chemical spect of penicillin allergy: The direct reaction of penicillin with epsilon-amino-groups. *Nature:* 208; 57-59.

Schnyder B., Burkhart C., Schnyder-Frutig K., Von Greyerz S., Naisbitt D. J., Pirmohamed M*., et al.* (2000). Recognition of sulfamethoxazole and its reactive metabolites by drug-specific cd4+ t cells from allergic individuals. *J Immunol:* 164; 6647-6654.

Schnyder B., Mauri-Hellweg D., Zanni M., Bettens F. and Pichler W. J. (1997). Direct, mhc-dependent presentation of the drug sulfamethoxazole to human alphabeta t cell clones. *J Clin Invest:* 100; 136-141.

Schoenberger S. P., Toes R. E., Van Der Voort E. I., Offringa R. and Melief C. J. (1998). T-cell help for cytotoxic t lymphocytes is mediated by cd40-cd40l interactions. *Nature:* 393; 480-483.

Schöpf E., Stühmer A., Rzany B., Victor N., Zentgraf R. and Kapp J. F. (1991). Toxic epidermal necrolysis and stevens-johnson syndrome. An epidemiologic study from west germany. *Arch Dermatol:* 127; 839-842.

Schrijvers R., Gilissen L., Chiriac A. M. and Demoly P. (2015). Pathogenesis and diagnosis of delayed-type drug hypersensitivity reactions, from bedside to bench and back. *Clinical and Translational Allergy:* 5; 31.

Schultz K. T. and Grieder F. (1987). Structure and function of the immune system. *Toxicol Pathol:* 15; 262-264.

Schwartzberg P. L., Mueller K. L., Qi H. and Cannons J. L. (2009). Slam receptors and sap influence lymphocyte interactions, development and function. *Nat Rev Immunol:* 9; 39-46.

Seddiki N., Santner-Nanan B., Martinson J., Zaunders J., Sasson S., Landay A.*, et al.* (2006). Expression of interleukin (il)-2 and il-7 receptors discriminates between human regulatory and activated t cells. *J Exp Med:* 203; 1693-1700.

Seder R. A. and Ahmed R. (2003). Similarities and differences in cd4+ and cd8+ effector and memory t cell generation. *Nature Immunology:* 4; 835-842.

Seishima M., Yamanaka S., Fujisawa T., Tohyama M. and Hashimoto K. (2006). Reactivation of human herpesvirus (hhv) family members other than hhv-6 in drug-induced hypersensitivity syndrome. *Br J Dermatol:* 155; 344-349.

Sena L. A., Li S., Jairaman A., Prakriya M., Ezponda T., Hildeman D. A.*, et al.* (2013). Mitochondria are required for antigen-specific t cell activation through reactive oxygen species signaling. *Immunity:* 38; 225-236.

Sharma V. K., Sethuraman G. and Minz A. (2008). Stevens johnson syndrome, toxic epidermal necrolysis and sjs-ten overlap: A retrospective study of causative drugs and clinical outcome. *Indian J Dermatol Venereol Leprol:* 74; 238-240.

Sharpe A. H. and Abbas A. K. (2006). T-cell costimulation--biology, therapeutic potential, and challenges. *N Engl J Med:* 355; 973-975.

Shchepina L. A., Pletjushkina O. Y., Avetisyan A. V., Bakeeva L. E., Fetisova E. K., Izyumov D. S.*, et al.* (2002). Oligomycin, inhibitor of the f0 part of h+-atp-synthase, suppresses the tnf-induced apoptosis. *Oncogene:* 21; 8149-8157.

Shedlock D. J. and Shen H. (2003). Requirement for cd4 t cell help in generating functional cd8 t cell memory. *Science:* 300; 337-339.

Shi Y., Evans J. E. and Rock K. L. (2003). Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature:* 425; 516-521.

Shiina T., Hosomichi K., Inoko H. and Kulski J. K. (2009). The hla genomic loci map: Expression, interaction, diversity and disease. *Journal of Human Genetics:* 54; 15-39.

Shiohara T. and Kano Y. (2007). A complex interaction between drug allergy and viral infection. *Clin Rev Allergy Immunol:* 33; 124-133.

Shipkova M. and Wieland E. (2012). Surface markers of lymphocyte activation and markers of cell proliferation. *Clin Chim Acta:* 413; 1338-1349.

Sidoroff A., Dunant A., Viboud C., Halevy S., Bavinck J. N., Naldi L.*, et al.* (2007). Risk factors for acute generalized exanthematous pustulosis (agep)-results of a multinational case-control study (euroscar). *Br J Dermatol:* 157; 989-996.

Sidoroff A., Halevy S., Bavinck J. N., Vaillant L. and Roujeau J. C. (2001). Acute generalized exanthematous pustulosis (agep)--a clinical reaction pattern. *J Cutan Pathol:* 28; 113-119.

Sierro S., Romero P. and Speiser D. E. (2011). The cd4-like molecule lag-3, biology and therapeutic applications. *Expert Opin Ther Targets:* 15; 91-101.

Silverman J. A., Oliver N., Andrew T. and Li T. (2001). Resistance studies with daptomycin. *Antimicrob Agents Chemother:* 45; 1799-1802.

Silverman J. A., Perlmutter N. G. and Shapiro H. M. (2003). Correlation of daptomycin bactericidal activity and membrane depolarization in staphylococcus aureus. *Antimicrob Agents Chemother:* 47; 2538-2544.

Sinclair L. V., Rolf J., Emslie E., Shi Y. B., Taylor P. M. and Cantrell D. A. (2013). Control of amino-acid transport by antigen receptors coordinates the metabolic reprogramming essential for t cell differentiation. *Nat Immunol:* 14; 500-508.

Sivagnanam S. and Deleu D. (2003). Red man syndrome. *Critical care (London, England):* 7; 119-120.

Sobao Y., Tomiyama H., Nakamura S., Sekihara H., Tanaka K. and Takiguchi M. (2001). Visual demonstration of hepatitis c virus-specific memory cd8(+) t-cell expansion in patients with acute hepatitis c. *Hepatology:* 33; 287-294.

Solensky R. (2003). Hypersensitivity reactions to beta-lactam antibiotics. *Clin Rev Allergy Immunol:* 24; 201-220.

Somma S., Gastaldo L. and Corti A. (1984). Teicoplanin, a new antibiotic from actinoplanes teichomyceticus nov. Sp. *Antimicrob Agents Chemother:* 26; 917-923.

Sousa-Pinto B., Correia C., Gomes L., Gil-Mata S., Araújo L., Correia O*., et al.* (2016). Hla and delayed drug-induced hypersensitivity. *Int Arch Allergy Immunol:* 170; 163-179.

Spahn-Langguth H. and Benet L. Z. (1992). Acyl glucuronides revisited: Is the glucuronidation process a toxification as well as a detoxification mechanism? *Drug Metab Rev:* 24; 5-47.

Spits H., Artis D., Colonna M., Diefenbach A., Di Santo J. P., Eberl G*., et al.* (2013). Innate lymphoid cells--a proposal for uniform nomenclature. *Nat Rev Immunol:* 13; 145-149.

Steinman R. M. and Cohn Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med:* 137; 1142-1162.

Steinman R. M. and Witmer M. D. (1978). Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A:* 75; 5132-5136.

Streit J. M., Jones R. N. and Sader H. S. (2004). Daptomycin activity and spectrum: A worldwide sample of 6737 clinical gram-positive organisms. *J Antimicrob Chemother:* 53; 669-674.

Sullivan A., Wang E., Farrell J., Whitaker P., Faulkner L., Peckham D*., et al.* (2018). B-lactam hypersensitivity involves expansion of circulating and skin-resident t(h)22 cells. *J Allergy Clin Immunol:* 141; 235-249.e238.

Sullivan J. R. and Shear N. H. (2001). The drug hypersensitivity syndrome: What is the pathogenesis? *Arch Dermatol:* 137; 357-364.

Swain S. L. (1995). T-cell subsets: Who does the polarizing? *Current Biology:* 5; 849-851.

Swain S. L., Weinberg A. D., English M. and Huston G. (1990). Il-4 directs the development of th2-like helper effectors. *J Immunol:* 145; 3796-3806.

Szabo S. J., Kim S. T., Costa G. L., Zhang X., Fathman C. G. and Glimcher L. H. (2000). A novel transcription factor, t-bet, directs th1 lineage commitment. *Cell:* 100; 655-669.

Szatkowski J. and Schwartz R. A. (2015). Acute generalized exanthematous pustulosis (agep): A review and update. *J Am Acad Dermatol:* 73; 843-848.

Takahashi T., Kuniyasu Y., Toda M., Sakaguchi N., Itoh M., Iwata M.*, et al.* (1998). Immunologic self-tolerance maintained by cd25+cd4+ naturally anergic and suppressive t cells: Induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol:* 10; 1969-1980.

Tang Q., Henriksen K. J., Bi M., Finger E. B., Szot G., Ye J.*, et al.* (2004). In vitro-expanded antigen-specific regulatory t cells suppress autoimmune diabetes. *J Exp Med:* 199; 1455-1465.

Teft W. A., Kirchhof M. G. and Madrenas J. (2006). A molecular perspective of ctla-4 function. *Annu Rev Immunol:* 24; 65-97.

Teo Y. X., Haw W. Y., Vallejo A., Mcguire C., Woo J., Friedmann P. S.*, et al.* (2022). Potential biomarker identification by rna-seq analysis in antibiotic-related drug reaction with eosinophilia and systemic symptoms (dress): A pilot study. *Toxicol Sci:*

Teslaa T. and Teitell M. A. (2014). Techniques to monitor glycolysis. *Methods Enzymol:* 542; 91-114.

Thierfelder W. E., Van Deursen J. M., Yamamoto K., Tripp R. A., Sarawar S. R., Carson R. T.*, et al.* (1996). Requirement for stat4 in interleukin-12-mediated responses of natural killer and t cells. *Nature:* 382; 171-174.

Thomson P. J., Illing P. T., Farrell J., Alhaidari M., Bell C. C., Berry N.*, et al.* (2019). Modification of the cyclopropyl moiety of abacavir provides insight into the structure activity relationship between hla-b*57:01 binding and t-cell activation. *Allergy:*

Thomson P. J., Illing P. T., Farrell J., Alhaidari M., Bell C. C., Berry N.*, et al.* (2020). Modification of the cyclopropyl moiety of abacavir provides insight into the structure activity relationship between hla-b*57:01 binding and t-cell activation. *Allergy:* 75; 636-647.

Thomson P. J., Kafu L., Meng X., Snoeys J., De Bondt A., De Maeyer D.*, et al.* (2021). Drug-specific t-cell responses in patients with liver injury following treatment with the bace inhibitor atabecestat. *Allergy:* 76; 1825-1835.

Thong B. Y. and Tan T. C. (2011). Epidemiology and risk factors for drug allergy. *Br J Clin Pharmacol:* 71; 684-700.

Thornton A. M. and Shevach E. M. (1998). Cd4+cd25+ immunoregulatory t cells suppress polyclonal t cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med:* 188; 287-296.

Tohyama M., Hashimoto K., Yasukawa M., Kimura H., Horikawa T., Nakajima K.*, et al.* (2007). Association of human herpesvirus 6 reactivation with the flaring and severity of drug-induced hypersensitivity syndrome. *British Journal of Dermatology:* 157; 934-940.

Tomiyama H., Matsuda T. and Takiguchi M. (2002). Differentiation of human cd8(+) t cells from a memory to memory/effector phenotype. *J Immunol:* 168; 5538-5550.

Torres M. J., Blanca M., Fernandez J., Romano A., Weck A., Aberer W*., et al.* (2003). Diagnosis of immediate allergic reactions to beta-lactam antibiotics. *Allergy:* 58; 961-972.

Townsend J. M., Fallon G. P., Matthews J. D., Smith P., Jolin E. H. and Mckenzie N. A. (2000). Il-9-deficient mice establish fundamental roles for il-9 in pulmonary mastocytosis and goblet cell hyperplasia but not t cell development. *Immunity:* 13; 573-583.

Traber P. G. and Levine D. P. (1981). Vancomycin ototoxicity in patient with normal renal function. *Ann Intern Med:* 95; 458-460.

Trickett A. and Kwan Y. L. (2003). T cell stimulation and expansion using anti-cd3/cd28 beads. *J Immunol Methods:* 275; 251-255.

Trifari S., Kaplan C. D., Tran E. H., Crellin N. K. and Spits H. (2009). Identification of a human helper t cell population that has abundant production of interleukin 22 and is distinct from t(h)-17, t(h)1 and t(h)2 cells. *Nat Immunol:* 10; 864-871.

Trubiano J. and Phillips E. (2013). Antimicrobial stewardship's new weapon? A review of antibiotic allergy and pathways to 'de-labeling'. *Curr Opin Infect Dis:* 26; 526-537.

Tsan M. F. and Gao B. (2004). Endogenous ligands of toll-like receptors. *J Leukoc Biol:* 76; 514-519.

Tunkel A. R., Hartman B. J., Kaplan S. L., Kaufman B. A., Roos K. L., Scheld W. M*., et al.* (2004). Practice guidelines for the management of bacterial meningitis. *Clinical Infectious Diseases:* 39; 1267-1284.

Turvey S. E. and Broide D. H. (2010). Innate immunity. *J Allergy Clin Immunol:* 125; S24-32.

Uetrecht J. (2007). Idiosyncratic drug reactions: Current understanding. *Annu Rev Pharmacol Toxicol:* 47; 513-539.

Uetrecht J. and Naisbitt D. J. (2013). Idiosyncratic adverse drug reactions: Current concepts. *Pharmacol Rev:* 65; 779-808.

Uetrecht J. P. (1999). New concepts in immunology relevant to idiosyncratic drug reactions: The “danger hypothesis” and innate immune system. *Chemical Research in Toxicology:* 12; 387-395.

Urban T. J., Nicoletti P., Chalasani N., Serrano J., Stolz A., Daly A. K*., et al.* (2017). Minocycline hepatotoxicity: Clinical characterization and identification of hla-b∗35:02 as a risk factor. *J Hepatol:* 67; 137-144.

Usui T., Meng X., Saide K., Farrell J., Thomson P., Whitaker P*., et al.* (2017). From the cover: Characterization of isoniazid-specific t-cell clones in patients with anti-tuberculosis drug-related liver and skin injury. *Toxicol Sci:* 155; 420-431.

Van Bambeke F. (2006). Glycopeptides and glycodepsipeptides in clinical development: A comparative review of their antibacterial spectrum, pharmacokinetics and clinical efficacy. *Curr Opin Investig Drugs:* 7; 740-749.

Van De Berg P. J., Van Leeuwen E. M., Ten Berge I. J. and Van Lier R. (2008). Cytotoxic human cd4(+) t cells. *Curr Opin Immunol:* 20; 339-343.

Van Der Meer R., Wilms E. B. and Heijerman H. G. M. (2021). Cftr modulators: Does one dose fit all? *J Pers Med:* 11;

Van Der Windt G. J., Chang C. H. and Pearce E. L. (2016a). Measuring bioenergetics in t cells using a seahorse extracellular flux analyzer. *Curr Protoc Immunol:* 113; 3.16b.11-13.16b.14.

Van Der Windt G. J., O'sullivan D., Everts B., Huang S. C., Buck M. D., Curtis J. D.*, et al.* (2013). Cd8 memory t cells have a bioenergetic advantage that underlies their rapid recall ability. *Proc Natl Acad Sci U S A:* 110; 14336-14341.

Van Der Windt G. J. W., Chang C. H. and Pearce E. L. (2016b). Measuring bioenergetics in t cells using a seahorse extracellular flux analyzer. *Curr Protoc Immunol:* 113; 3.16b.11-13.16b.14.

Van Der Windt G. J. W., Everts B., Chang C. H., Curtis J. D., Freitas T. C., Amiel E.*, et al.* (2012). Mitochondrial respiratory capacity is a critical regulator of cd8(+) t cell memory development. *Immunity:* 36; 68-78.

Van Stipdonk M. J., Lemmens E. E. and Schoenberger S. P. (2001). Naïve ctls require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol:* 2; 423-429.

Vander Heiden M. G., Cantley L. C. and Thompson C. B. (2009). Understanding the warburg effect: The metabolic requirements of cell proliferation. *Science:* 324; 1029-1033.

Veldhoen M., Uyttenhove C., Van Snick J., Helmby H., Westendorf A., Buer J.*, et al.* (2008). Transforming growth factor-beta 'reprograms' the differentiation of t helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol:* 9; 1341-1346.

Venugopal A. A. and Johnson S. (2012). Current state of clostridium difficile treatment options. *Clin Infect Dis:* 55 Suppl 2; S71-76.

Viard-Leveugle I., Gaide O., Jankovic D., Feldmeyer L., Kerl K., Pickard C.*, et al.* (2013). Tnf-α and ifn-γ are potential inducers of fas-mediated keratinocyte apoptosis through activation of inducible nitric oxide synthase in toxic epidermal necrolysis. *J Invest Dermatol:* 133; 489-498.

Vinuesa C. G., Tangye S. G., Moser B. and Mackay C. R. (2005). Follicular b helper t cells in antibody responses and autoimmunity. *Nature reviews. Immunology:* 5; 853-865.

Vitezica Z. G., Milpied B., Lonjou C., Borot N., Ledger T. N., Lefebvre A.*, et al.* (2008). Hla-drb1*01 associated with cutaneous hypersensitivity induced by nevirapine and efavirenz. *Aids:* 22; 540-541.

Waddington J. C., Meng X., Illing P. T., Tailor A., Adair K., Whitaker P.*, et al.* (2020). Identification of flucloxacillin-haptenated hla-b*57:01 ligands: Evidence of antigen processing and presentation. *Toxicol Sci:* 177; 454-465.

Wallace M. R., Mascola J. R. and Oldfield E. C., 3rd (1991). Red man syndrome: Incidence, etiology, and prophylaxis. *J Infect Dis:* 164; 1180-1185.

Walsh S. A. and Creamer D. (2011). Drug reaction with eosinophilia and systemic symptoms (dress): A clinical update and review of current thinking. *Clinical and Experimental Dermatology:* 36; 6-11.

Wang R. and Green D. R. (2012). Metabolic checkpoints in activated t cells. *Nat Immunol:* 13; 907-915.

Warburg O. (1925). The metabolism of carcinoma cells. *The Journal of Cancer Research:* 9; 148-163.

Warburg O., Wind F. and Negelein E. (1927). The metabolism of tumors in the body. *J Gen Physiol:* 8; 519-530.

Watanakunakorn C. (1982). Treatment of infections due to methicillin-resistant staphylococcus aureus. *Ann Intern Med:* 97; 376-378.

Watts C. (1997). Capture and processing of exogenous antigens for presentation on mhc molecules. *Annu Rev Immunol:* 15; 821-850.

Watts T. J. (2017). Severe delayed-type hypersensitivity to chloramphenicol with systemic reactivation during intradermal testing. *Ann Allergy Asthma Immunol:* 118; 644-645.

Wazny L. D. and Daghigh B. (2001). Desensitization protocols for vancomycin hypersensitivity. *Ann Pharmacother:* 35; 1458-1464.

Weaver C. T., Harrington L. E., Mangan P. R., Gavrieli M. and Murphy K. M. (2006). Th17: An effector cd4 t cell lineage with regulatory t cell ties. *Immunity:* 24; 677-688.

Webb P. S. and Al-Mohammad A. (2016). Enigma: Infection or allergy? Vancomycin-induced dress syndrome with dialysis-dependent renal failure and cardiac arrest. *BMJ Case Rep:* 2016;

Wei C. Y., Chung W. H., Huang H. W., Chen Y. T. and Hung S. I. (2012). Direct interaction between hla-b and carbamazepine activates t cells in patients with stevens-johnson syndrome. *J Allergy Clin Immunol:* 129; 1562-1569.e1565.

Whitaker P., Naisbitt D. and Peckham D. (2012). Nonimmediate β-lactam reactions in patients with cystic fibrosis. *Curr Opin Allergy Clin Immunol:* 12; 369-375.

Wieman H. L., Wofford J. A. and Rathmell J. C. (2007). Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/akt regulation of glut1 activity and trafficking. *Mol Biol Cell:* 18; 1437-1446.

Wilcox O., Hassanein M., Armstrong J. and Kassis N. (2017). Case report: Atypical presentation of vancomycin induced dress syndrome: A case report and review of the literature. *BMC Pulm Med:* 17; 217.

Williams T. M. (2001). Human leukocyte antigen gene polymorphism and the histocompatibility laboratory. *J Mol Diagn:* 3; 98-104.

Wilson A. P. (1998). Comparative safety of teicoplanin and vancomycin. *Int J Antimicrob Agents:* 10; 143-152.

Wilson A. P., Grüneberg R. N. and Neu H. (1994). A critical review of the dosage of teicoplanin in europe and the USA. *Int J Antimicrob Agents:* 4 Suppl 1; 1-30.

Wing K., Onishi Y., Prieto-Martin P., Yamaguchi T., Miyara M., Fehervari Z*., et al.* (2008). Ctla-4 control over foxp3+ regulatory t cell function. *Science:* 322; 271-275.

Wing K. and Sakaguchi S. (2010). Regulatory t cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol:* 11; 7-13.

Wolfson A. R., Zhou L., Li Y., Phadke N. A., Chow O. A. and Blumenthal K. G. (2019). Drug reaction with eosinophilia and systemic symptoms (dress) syndrome identified in the electronic health record allergy module. *J Allergy Clin Immunol Pract:* 7; 633-640.

Wolkenstein P., Chosidow O., Fléchet M. L., Robbiola O., Paul M., Dumé L*., et al.* (1996). Patch testing in severe cutaneous adverse drug reactions, including stevens-johnson syndrome and toxic epidermal necrolysis. *Contact Dermatitis:* 35; 234-236.

Wood M. J. (1996). The comparative efficacy and safety of teicoplanin and vancomycin. *J Antimicrob Chemother:* 37; 209-222.

Wu M., Neilson A., Swift A. L., Moran R., Tamagnine J., Parslow D*., et al.* (2007). Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *Am J Physiol Cell Physiol:* 292; C125-136.

Wysocki M., Delatour F., Faurisson F., Rauss A., Pean Y., Misset B*., et al.* (2001). Continuous versus intermittent infusion of vancomycin in severe staphylococcal infections: Prospective multicenter randomized study. *Antimicrob Agents Chemother:* 45; 2460-2467.

Yang F., Chen S. A., Wu X., Zhu Q. and Luo X. (2018). Overexpression of cytotoxic proteins correlates with liver function impairment in patients with drug reaction with eosinophilia and systemic symptoms (dress). *Eur J Dermatol:* 28; 13-25.

Yang K., Shrestha S., Zeng H., Karmaus P. W., Neale G., Vogel P*., et al.* (2013). T cell exit from quiescence and differentiation into th2 cells depend on raptor-mtorc1-mediated metabolic reprogramming. *Immunity:* 39; 1043-1056.

Yang X. O., Pappu B. P., Nurieva R., Akimzhanov A., Kang H. S., Chung Y*., et al.* (2008). T helper 17 lineage differentiation is programmed by orphan nuclear receptors ror alpha and ror gamma. *Immunity:* 28; 29-39.

Yaseen F. S., Saide K., Kim S. H., Monshi M., Tailor A., Wood S*., et al.* (2015). Promiscuous t-cell responses to drugs and drug-haptens. *J Allergy Clin Immunol:* 136; 474-476.e478.

Yawalkar N. (2005). Drug-induced exanthems. *Toxicology:* 209; 131-134.

Ye C. J., Feng T., Kwon H.-K., Raj T., Wilson M. T., Asinovski N*., et al.* (2014). Intersection of population variation and autoimmunity genetics in human t cell activation. *Science (New York, N.Y.):* 345; 1254665-1254665.

Ye Y. M., Hur G. Y., Kim S. H., Ban G. Y., Jee Y. K., Naisbitt D. J*., et al.* (2017). Drug-specific cd4(+) t-cell immune responses are responsible for antituberculosis drug-induced

maculopapular exanthema and drug reaction with eosinophilia and systemic symptoms syndrome. *Br J Dermatol:* 176; 378-386.

Yim G., Thaker M. N., Koteva K. and Wright G. (2014). Glycopeptide antibiotic biosynthesis. *J Antibiot (Tokyo):* 67; 31-41.

Yoshikawa Y., Ueta M., Nishigaki H., Kinoshita S., Ikeda T. and Sotozono C. (2020). Predictive biomarkers for the progression of ocular complications in chronic stevens-johnson syndrome and toxic eeidermal necrolysis. *Scientific Reports:* 10; 18922.

Zanni M. P., Von Greyerz S., Schnyder B., Brander K. A., Frutig K., Hari Y*., et al.* (1998). Hla-restricted, processing- and metabolism-independent pathway of drug recognition by human alpha beta t lymphocytes. *J Clin Invest:* 102; 1591-1598.

Zhang F. R., Liu H., Irwanto A., Fu X. A., Li Y., Yu G. Q*., et al.* (2013). Hla-b*13:01 and the dapsone hypersensitivity syndrome. *N Engl J Med:* 369; 1620-1628.

Zhang L., Tschumi B. O., Lopez-Mejia I. C., Oberle S. G., Meyer M., Samson G*., et al.* (2016). Mammalian target of rapamycin complex 2 controls cd8 t cell memory differentiation in a foxo1-dependent manner. *Cell Rep:* 14; 1206-1217.

Zhao Q., Alhilali K., Alzahrani A., Almutairi M., Amjad J., Liu H*., et al.* (2019). Dapsone- and nitroso dapsone-specific activation of t cells from hypersensitive patients expressing the risk allele hla-b*13:01. *Allergy:* 74; 1533-1548.

Zhao Q., Almutairi M., Tailor A., Lister A., Harper N., Line J*., et al.* (2021). Hla class-ii–restricted cd8(+) t cells contribute to the promiscuous immune response in dapsone-hypersensitive patients. *J Invest Dermatol:*

Zheng W. and Flavell R. A. (1997). The transcription factor gata-3 is necessary and sufficient for th2 cytokine gene expression in cd4 t cells. *Cell:* 89; 587-596.

Zhou R., Yazdi A. S., Menu P. and Tschopp J. (2011). A role for mitochondria in nlrp3 inflammasome activation. *Nature:* 469; 221-225.

Zikherman J. and Au-Yeung B. (2015). The role of t cell receptor signaling thresholds in guiding t cell fate decisions. *Curr Opin Immunol:* 33; 43-48.