ANALYSIS OF ANTIGENIC DETERMINANTS IN HLA-B:13*01

DAPSONE HYPERSENSITIVE PATIENTS

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

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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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Abbreviation

AB	Antibody
ACN	Acetonitrile
ADCC	Antibody-Dependent Cellular Cytotoxicity
ADR	Adverse Drug Reaction
AGEP	Acute Generalized Exanthematous Pustulosis
ALP	Alkaline Phosphatase
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
AX	Amoxicillin
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BCR	B Cell Receptor
BP	Benzyl Penicillin
BSA	Bovine Serum Albumin
CBZ	Carbamazepine
CCR	Chemokine Receptor
CD	Cluster Of Differentiation
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CHS	Contact Hypersensitivity
CID	Collision-Induced Dissociation
CLA	Cutaneous Lymphocyte Antigen
CLIP	Class II-Associated Ii Peptide
CMV	Cytomegaloviruses
CPM	Counts Per Minute
CSA	Cyclosporine-A
DDA	Data-Dependent Acquisition
DDS	Dapsone
DDS-NHOH	Dapsone Hydroxylamine
DDS-NO	Dapsone Nitroso
DHR	Drug Hypersensitivity Reaction
DHS	Dapsone Hypersensitivity Syndrome
DIHS	Drug-Induced Hypersensitivity Syndrome
DILI	Drug-Induced Liver Injury
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Dioxyribonucleic Acid
DNFB	Dinitrofluorobenzene
DRESS	Drug Reaction with Eosinophilia and Systemic Symptoms
EBA	Epidermolysis Bullosa Acquisita
EBVs	Epstein-Barr Virus
EDN	Eosinophil-Derived Neurotoxin
EDTA	Ethylenediaminetetraacetic Acid
ELISA	The Enzyme-Linked Immunosorbent Assay
ELIspot	Enzyme Linked Immunospot Assay
EPI	Enhanced Product Ion
ER	Endoplasmic Reticulum

ERAD	Endoplasmic Reticulum Associated Degradation
ESI	Electrospray Ionization
FA	Formic Acid
FACS	Flurorescence Assisted Cell Sorting
FasL	Fas Ligand
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GB	Granzyme B
GC	Germinal Centre
GM-CSF	Granulocytes-Macrophage Colony Stimulating Factor
GPCR	G Protein-Coupled Receptors
GSH	Reduced Glutathione
GSTP	Glutathione S-Transferase Pi
GWAS	Genome-Wide Association Studies
HBSS	Hanks Balanced Salt Solution
HEPES	Hydroxyethyl Piperazineethanesulfonic Acid
HHS	Human Herpesvirus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPLC	High-Performance Liquid Chromatography
HSA	Human Serum Albumin
IAA	Iodoacetamide
IEC	Ion-Exchange Chromatography
IF	Immunofluorescence
IFN	Interferon
IL	Interleukin
ILC	Innate Lymphoid Cells
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-Linked
LC	Liquid Chromatography
LPS	Lipopolysaccharide
LTQ	Linear Trap Quadrupole
LTT	Lymphocyte Transformation Test
MACS	Magnetic-Activated Cell Sorting
MADDS	Monoacteyldapsone
MALDI	Matrix-Assisted Laser Desorption/Ionization
MBP	Major Basic Protein
MHC	Major Histocompatibility Complex
MIIC	MHC Class II Loading Compartment
MPE	Maculopapular Exanthema
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAPQI	N-Acetyl-P-Benzoquinone Imine
NBT	Nitro Blue Tetrazolium
NHS	National Health Service
NK	Natural Killer

NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PEP	Peptide
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PI	Pharmacological Interaction
pMHC	Peptide Major Histocompatability Complex
PMN	Polymorphonuclear Leukocytes
PRRs	Pattern Recognition Receptors
PTM	Post-Translational Modification
QTOF	Quadrupole Time of Flight
QTRAP	Quadropule Ion Trap
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RCF	Relative Centrifugal Force
RPMI	Roswell Park Memorial Institute
SCAR	Severe Cutaneous Adverse Reaction
SCX	Strong Cation Resin
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
SFU	Spot Forming Units
SI	Stimulation Index
SJS	Stevens-Johnson Syndrome
SMX	Sulfamethoxazole
SMX-NO	Sulfamethoxazole Nitroso
TAP	Transporter Associated with Antigen Processing
TCCs	T-Cell Clones
TCM	Central Memory T-Cells
TCR	T-Cell Receptor
TEM	Effector Memory T-Cells
TEMRA	T-Effector Memory Cells Re-Expressing CD45RA
TEN	Toxic Epidermal Necrolysis
TFA	Trifluoroacetic Acid
TGF	Transforming Growth Factor
TLR	Toll-Like Receptors
TMB	Tetramethylbenzidine
TNF	Tumour Necrosis Factor
TNP	Trinitrophenol
TOF	Time Of Flight
TQMS	Triple Quadrupole Mass Spectrometer
TT	Tetanus Toxoid
WHO	World Health Organisation

Publications

Zhao Q, Alhilali K, Alzahrani A, **Almutairi M**, Amjad J, Liu H, Sun Y, Sun L, Zhang H, Meng X, Gibson A, Ogese MO, Kevin Park B, Liu J, Ostrov DA, Zhang F, Naisbitt DJ. Dapsone- and nitroso dapsone-specific activation of T cells from hypersensitive patients expressing the risk allele HLA-B*13:01. Allergy. 2019 Aug;74(8):1533-1548.

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HLA-B*13:01-restricted activation of CD8+ T-cells from hypersensitive patients with designer nitroso dapsone-modified peptides.

Abstract

Dapsone (DDS) is used in combination with other drugs to treat infectious diseases such as leprosy and malaria. 0.5%-3.6% of treated patients develop a hypersensitivity syndrome characterised by fever, skin rash, and internal organ involvement, 4-6 weeks after treatment starts. It is still unclear if DDS or its metabolites triggers specific T-cell responses in these patients, thus more research is needed. HLA-B*13:01 has been shown as a risk allele for DDS hypersensitivity. Therefore, the objective of chapters 3 and 4 of this thesis was to explore the cellular basis of DDS hypersensitivity reactions and to determine the involvement of HLA-B*13:01 in T-cell activation using PBMC from DDS hypersensitive patients. *In vitro* activation of patient PBMC with DDS and nitroso dapsone (DDS-NO) was observed with all of the study participants. 395 DDS-responsive $CD4^+$ and $CD8^+$ T cell clones (TCCs) and 399 DDS-NO-responsive $CD4^+$ and $CD8^+$ TCCs were generated from the three patients. Responsive $CD4^+$ and $CD8^+$ TCCs were HLA class II and class I restricted, respectively, and they expressed an array of TCR Vβ receptors. Clones' reactivity was divided into three categories: compound-specific, moderately cross-reactive, and strongly cross-reactive. The results also indicate that the sulfone group and the position of the amine groups are essential for T-cell activation. Furthermore, DDS was found to activate T-cells in a processing-independent manner, likely through a direct interaction with HLA molecules. In contrast, DDS-NO-responsive T-cells were activated through a hapten mechanism in which DDS-NO bound covalently to cellular protein forming DDS-NO-protein adducts that require processing to generate antigenic peptides. Finally, HLA-B*13:01-restricted activation was observed with some, but not all DDS (metabolite)-responsive $CD8^+$ TCCs.

The hapten model of DDS hypersensitivity proposes that T-cells interact with a drug-modified peptide presented by HLA-B*13:01. However, this interaction has not yet been tested. Therefore, the objective of chapters 5 and 6 was to explore the immunogenicity of DDS-NO-modified peptides using autologous antigen presenting cells (APC) and APC transfected with the single HLA-B allele HLA-B*13:01. For this reason, we have designed and synthesised DDS-NO-modified HLA binding peptides to study the HLA-B*13:01-restricted T-cell response in greater detail and to explore the importance of the hapten moiety on the peptide backbone for T-cell activation. Three DDS-NO-modified peptides were synthesised in high purity. Each peptide contained 2 HLA-B*13:01 anchoring motifs and a nucleophilic cysteine residue for modification by DDS-NO. Peptides were fully characterised using mass spectrometry. DDS-NO-modified peptide responsive $CD8^+$ TCCs were generated from hypersensitive patients and characterised in terms of phenotype, function, and cross-reactivity. Autologous APC and C1R cells expressing HLA-B*13:01 were used to determine HLA restriction. Clones proliferated and secreted effector molecules with graded concentrations of DDS-NO-modified peptides. Clones displayed reactivity against DDS-NO, which forms adducts *in situ*, but not with the unmodified peptide or DDS. Cross-reactivity was observed between DDS-NO-modified peptides with the cysteine residue in different positions in the peptide

sequence. T-cell activation with DDS-NO-modified peptides was antigen presenting cell HLA-B*13:01 restricted.

Collectively, these studies have identified DDS-, DDS-NO-, and DDS-NO-modified peptide-responsive $CD8^+$ in patients with DDS hypersensitivity. Furthermore, DDS-NO-modified peptides were found to bind to HLA-B*13:01 to activate T-cells, which provides a framework for structural analysis of drug hapten HLA binding interactions.

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1.1 Immune System

The immune system is a network of cells and molecules that mediate the immune response against infectious and non-infectious foreign substances (e.g., Microbes) and the body's own damaged and malignant cells. The immune system detects and distinguishes various pathogens and cancer cells from healthy tissues. However, the definition of immune response is expanded to cover the abnormal autoimmune response against its healthy cells, as is observed in DHR (Abbas et al., 2021).

The immune system consists of various effector cells and molecules that protect the body in two forms of immunity: innate (also known as neutral or native) and adaptive (also known as specific or acquired) immunity. Innate immunity involves immunological processes that exist before an infection occurs and can defend against infectious agents within hours to days (Abbas et al., 2021). On the other hand, adaptive immunity develops in response to an infection by a particular pathogen that has already been encountered by the body leading to adaption and specific immune response to that infection (Murphy and Weaver, 2016).

Innate immunity is the older host defending mechanism. It protects the body against pathogens through several levels of defence, including physical and chemical barriers (e.g. epithelia and skin antimicrobial chemicals), recruitment of innate immune cells to sites of infection (e.g. neutrophils, macrophages, dendritic cells (DCs), mast cells, and natural killer cells (NKs)), releasing of blood proteins such as complement system proteins (Doan et al., 2015, Coico and Sunshine, 2015).

1.1.1 Pattern recognition receptors (PRRs)

PRRs are cellular protein receptors that play a critical role in the innate immune response. They recognise two groups of molecules: the microbial molecules

(pathogen-associated molecular patterns (PAMPs)) and the host's damaged cells molecules (damage-associated molecular patterns (DAMPs)). Various cells such as DCs, macrophages and neutrophils express PRRs to recognise various PAMPs such as bacterial LPS, viral DNA or RNA, and other microbial peptides and substances and identify different DAMPs molecules such as heat shock proteins and B-cell lymphoma 2 (Bcl-2) (Kumar et al., 2011, Doan et al., 2015). Based on PRRs localisation, they can be classified into membrane-bound PRRs such as Toll-like receptors (TLRs) and cytoplasmic PRRs such as NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). For instance, the bacterial LPS is recognised by TLR4, TLAR2 and TLR9, and TLR3 recognises the viral double-stranded RNA (dsRNA) (Takeuchi and Akira, 2010, Kawai and Akira, 2010, Kumar et al., 2009). Recognition of PAMPs or DAMPs by PRRs help in the induction of the innate immune response, stimulating interferons (IFNs), anti-inflammatory cytokines, and activating the cell-mediated immune response (Coico and Sunshine, 2015).

DAMPs, also known as danger signals, are intracellular danger molecules released from the infected, damaged cells or the traumatically damaged cells and help control infection and injury by delivering danger messages to the neighbouring cells such DCs via PRRS binding. DAMPs are localised in different sites within the cell and recognised with different PRRS receptors when they are released to the extracellular space. For instance, IL-1α is a nuclear cytokine that induces an inflammatory response once released and binds to the PRR IL-1R (Chen et al., 2007). Uric Acid and ATP are major cytoplasmic DAMPs recognised by NLPR3 and P2Y2, respectively (Zhang et al., 2017, Gasse et al., 2009, Roh and Sohn, 2018).

1.1.2 Cellular Components of Immune System

White blood cells (WBCs), also known as leukocytes, are the immune system cells derived from hematopoietic stem cells in the bone marrow and circulate throughout the body, including the lymphoid system and blood.

Leukocytes are nucleated, differentiating them from the other hematopoietic cells that do not have nuclei; red blood cells (RBCs) and platelets. Leukocytes can be classified based on their structure into granulocytes. Granulocytes such as neutrophils, mast cells, basophils, and eosinophils have granules in the cytoplasm. In contrast, agranulocytes such as monocytes and lymphocytes are characterised by the absence of granules in their cytoplasm. Based on the cell linage, Leukocytes can also be classified into myeloid cells or lymphoid cells.

Neutrophils are granulocytic and myeloid cells; they account for 50–70% of all circulating leukocytes. The neutrophils are phagocytic cells that represent the primary cellular arm in innate immunity by engulfing and killing pathogens. Neutrophils are the first line of protection against many pathogens, including bacteria, fungi, and protozoa (Edwards, 1994). Neutrophils are formed and differentiated in the bone marrow and are subsequently released into the circulatory system. One hundred billion neutrophils are released from the bone marrow to the circulation daily; however, they have a short lifespan in the bloodstream of around 6 hours and slightly longer in tissues (Scher et al., 2013, Edwards, 1994).

Once a pathogen has penetrated the physical and chemical barriers, neutrophils are the first innate immune cells that are immediately recruited from the circulation to sites of infection via a chemotaxis process. Neutrophils express a variety of

receptors, including pattern-recognition receptors (PRRs) and G protein-coupled receptors (GPCR), to detect the microbial molecules (Pathogen-associated molecular patterns (PAMPs)) and the inflammatory signals resulting from tissue damage (e.g., Chemoattractants and cytokines) and hence respond to stimuli and attack pathogens. Neutrophils destroy pathogens by three mechanisms: phagocytosis and secreting highly toxic reactive oxygen species (ROS), degranulation (release of antimicrobial mediators), and generation of neutrophil extracellular traps (NETs) (Teng et al., 2017, Kolaczkowska and Kubes, 2013).

Mast cells are granular leukocytes whose cytoplasm contains granules rich in sulphated proteoglycans (e.g., heparin and chondroitin sulphates). They are tissue-resident cells located mainly near blood vessels and at the epithelium. They have a significant role in the inflammatory response, and they are also involved in host defence against pathogens, wound healing, and autoimmune diseases. They are activated through antigen-mediated cross-linkage of surface-bound IgE. This leads to a process of degranulation and the release of chemical, lipid, and protein mediators (Stone et al., 2010).

Mast cells release histamine, prostaglandins, serine proteases (tryptase and chymase) and thromboxane A2 that interact with receptors to produce a very rapid response to antigen exposure. A broad range of cytokines, including IFN-γ, TNF-alpha, GM-CSF, IL-3, IL-6, IL-10, and IL-13 are also produced alongside various chemokines such as CXCL8 (IL-8) and CCL3 to bring about a second phase delayed response. Mast cells express FcεRI on their surface that bind to B lymphocytes generated IgE, with specificity for a given antigen. During the second exposure to the same antigen, the mast cells are activated once the antigen binds to and cross-

links pre-attached IgE molecules. (Stone et al., 2010, Adkinson Jr et al., 2013, Metcalfe, 2008, Metz et al., 2013).

Basophils are also granular leukocytes that have similarities with mast cells in many structural and functional features, primarily the expression of FcεRI and the release of granular histamine, production of Th2 cytokines, and their important role in clinical manifestations such as allergy. Basophils express larger granules, albeit in lower numbers than mast cells, and they circulate in the blood and are recruited to the tissue when needed. In contrast, mast cells seem to be tissue-resident cells for the most part (Sullivan and Locksley, 2009, Karasuyama et al., 2009).

Eosinophils are granular cells that contribute to the immune response against parasitic helminths. They also participate in allergic reactions and immunoregulatory responses. Eosinophils are characterised by their structurally unique cytoplasmic granules. These granules contain preformed cationic proteins, such as major basic protein (MBP), eosinophil peroxidase, and eosinophil-derived neurotoxin (EDN) (Hamann et al., 1991, Adkinson Jr et al., 2013). They also have a diversity of preformed cytokines and chemokines that are released on degranulation, including IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, GM-CSF, TGF- α, TNF-α, CCL5, and CCL11 (Lacy et al., 1999, Nakajima et al., 1998, Levi-Schaffer et al., 1995, Levi-Schaffer et al., 1996, Moqbel et al., 1995, Dubucquoi et al., 1994, Lacy et al., 1998, Spencer et al., 2009, Egesten et al., 1996, Davoine and Lacy, 2014, Stone et al., 2010).

Monocytes are phagocytic cells, and as macrophages and DCs discussed in detail below, they have a critical function in host antimicrobial defence and various inflammatory diseases. The circulatory monocyte produces pro-inflammatory

cytokines, including IL-1 and TNF, stimulating other leukocytes (Willis et al., 2003, Jakubzick et al., 2013). Based on the surface expression of CD14 and CD16, circulatory monocytes can be classified at least into two types; $CD14^{LOW}$ CD16+ non-classical patrolling monocytes and CD14++ CD16− migratory monocytes. The classical monocytes can move across the endothelium. In contrast, the non-classical monocytes are characterised by slow patrolling (where they adhere and migrate with or against the flow) of the endothelium, which allows a rapid response to local alarms (Auffray et al., 2007, Ingersoll et al., 2010, Randolph et al., 1998). it has become evident that the migrating monocytes in tissue can differentiate into macrophages, work as antigen-presenting cells to activate T lymphocytes, or stay as monocytes (Jakubzick et al., 2013, Kim and Braciale, 2009, Hohl et al., 2009).

Dendritic cells (DCs) are a type of leukocyte found in tissues, blood, and lymph nodes. Generally, DCs are known as the primary antigen-presenting cells that stimulate T lymphocytes against various infections. They process multiple intracellular and extracellular proteins to fragmented peptides and then present these peptides in the context of HLA molecules to prime naive T-cells. In addition, DCs play a role in innate immune responses when they identify and respond to different PAMPs and DAMPs, causing the acute inflammatory response (Collin and Bigley, 2018, Tang et al., 2012). During inflammation, monocytes differentiate into DCs under the influence of pro-inflammatory cytokines. Like most immune cells, DCs consist of subsets that differ in origin, function, or both. The four main types of DCs are identified as follows: conventional dendritic cells (cDCs), plasmacytoid dendritic cells (pDCs), monocyte-derived dendritic cells (moDCs), and Langerhans cells. Table 1.1 is shown the distinguishes features between cDCs, moDCs and pDCs,

including the toll-like receptors expressed and cytokines produced (Kadowaki et al., 2001, Jarrossay et al., 2001).

Table 1.1: Human dendritic cell subsets.

DCs Subset	Toll-like Receptors	Antigens	Important cytokines
cDCs	TLR 2, TLR 4	peptidoglycan and LPS	IL-12
moDCs			
pDCs	TLR 7, TLR 9	CpG DNA	IFN-I

Macrophages are phagocytic cells that play essential immunological roles in the phagocytosis of foreign pathogens and cancer cells; thus, they protect the body from infection and injury. Macrophages are present in all tissues with different names based on their location and phenotype, such as Kupffer cells (KCs) in the liver, Langerhans cells (LC) in the skin, alveolar macrophages (AMs) (Yona et al., 2013, Hoeffel et al., 2012). These tissue-resident macrophages eliminate foreign antigens and materials, help in tissue repair, and retain homeostasis (Gordon, 2002, Geissmann et al., 2010). In addition to their function in innate immune responses, they are an essential component in adaptive immunity as antigen-presenting cells for presenting antigenic material to lymphocytes. Tissue-resident macrophages can be either embryonic or monocyte-derived, and characterised by the particular expression of F4/80, CD64, MerTK, and CD14 (Gautier et al., 2012, Schulz et al., 2012, Tamoutounour et al., 2012, Gentek et al., 2014).

Macrophages can be activated to different phenotypic populations based on various functional responses to stimuli and signals encountered in the tissue. (Sica and Mantovani, 2012). However, the two most well-defined populations are M1 inflammatory (classical activated) and M2 anti-inflammatory (alternatively activated) macrophages. M1 macrophages are usually stimulated by

lipopolysaccharide (LPS), IFN-γ and TNF-α. Following activation, they release high levels of pro-inflammatory cytokines (e.g., IL-12) and low levels of IL-10 (Gordon, 2003, Mantovani et al., 2002, Sica and Mantovani, 2012, Shapouri-Moghaddam et al., 2018). The activated M1 macrophages eliminate pathogens through phagocytosis. They engulf pathogens, forming phagosomes that then fuse with lysosomes, forming a phagolysosome, leading to degradation by producing reactive oxygen species (ROS) (Verreck et al., 2004). On the other hand, M2 macrophages are activated by IL-4 and IL-13 and characterised by the production of high levels of IL-10, TGF-beta, and low levels of IL-12 (Stein et al., 1992, Mosser, 2003, Martinez et al., 2009, Shapouri-Moghaddam et al., 2018). M2 macrophages play a critical role in inhibiting the chronic inflammatory response and promoting wound healing and tissue repair (Kurowska-Stolarska et al., 2009, Jetten et al., 2014).

Macrophages, the primary antigen-presenting cells (APCs), are responsible for initiating and regulating the immune response. The macrophage's ability to capture, endocytose, and display both self and foreign antigens are critical to its role in innate and adaptive immune responses. After ingesting a pathogen, the macrophage processes the pathogenic proteins to fragmented peptides and present them as peptide HLA complexes on their surface. The macrophage then migrates to stimulate T-cells (Fujiwara and Kobayashi, 2005, Wynn et al., 2013).

Natural killer cells (NKs) are large granular cytotoxic lymphocytes belonging to innate lymphoid cells. They are an essential component of the innate immune system mediating immune response against viruses and tumours. In humans, they represent 5-20 % of all circulating lymphocytes (Langers et al., 2012). NKs express CD16 and CD56 molecules on their receptors, however; they do not express T or B

cell markers such as CD3, T-cell receptors (TCR), and B cell immunoglobulins (Ig) receptors (Lanier et al., 1989, Lanier et al., 1986, Abel et al., 2018). They were called natural killers because they can kill infected and cancer cells that often lack MHC I molecules without any priming or prior activation. NKs have an alternative mechanism to recognise and kill infected and cancer cells without targeting the normal healthy cells. This mechanism is based on a balance of signals from membrane receptors (activating receptors and inhibitory receptors). The activating receptors recognise molecules present on the infected and cancer cells surfaces, whereas the inhibitory receptors recognise self MHC I molecules. Once NKs are activated, they release their granular proteins, perforin and granzyme and induce cell lysis to the infected or cancer cells by the mechanism known as cell-mediated cytotoxicity (Lanier, 2008, Abel et al., 2018, Carrega and Ferlazzo, 2012). Cytokines such as IL-2, IL-15, IL-12, IL-18, and IL-21 play a crucial role in NK cell activation (McMichael et al., 2017, Tietje et al., 2017, Kim et al., 2017, Marçais et al., 2014).

1.1.2.1 The adaptive immune system

The adaptive immune response is activated when the infectious agent escapes the innate immune system. The adaptive immune system develops with infection to eliminate pathogens following a complex process of antigen recognition, procession, and presentation. The adaptive immune cells recognise specific "non-self" antigens then generate an immune response against the pathogen. Finally, once the immune response subsides, the adaptive immune system establishes immunological memory against a given pathogen to ensure that the individual does not become sick on second exposure to the same pathogen. The adaptive immune response takes several days to develop and establish itself after the first exposure

to the pathogen. It occurs in two broad forms, antibody-dependent and cell-mediated responses, mediated by B cells and T-cells, respectively (Murphy and Weaver, 2016).

1.1.2.1.1 B lymphocytes

B lymphocytes (or B cells) mature in the bone marrow and mediate humoral immune response by producing antigen-specific immunoglobulin (Ig) known as antibodies to recognise and neutralise pathogens. Antibodies form the same basic Y-shape consisting of two heavy chains and two light chains connected by disulphide bonds. These chains are divided into constant and variable regions (domains) that form the antigen-binding site. There are five main classes of antibodies called IgG, IgM, IgA, IgD and IgE (Murphy and Weaver, 2016) (Table 1.2). B cells can be activated through two main pathways: T-cell-independent activation and T-cell-dependent activation mechanisms. T-cell-independent antigens such as lipopolysaccharide (LPS), CpG DNA or viral dsRNA activate B cells without involvement of T-cells (Xu et al., 2008, Azulay-Debby et al., 2007, Hebeis et al., 2004). The T-cell-independent response is characterised by rapid but low antibody affinity, induced through toll-like receptors (TLRs) or extensive cross-linking of B cell receptors (BCRs) to specific an antigen epitope. T-cell-independent activated B cells differentiate into IgM secreting short-lived plasmablasts that die within a few weeks (Freer et al., 1994, Bachmann et al., 1993, Fehr et al., 1996, Szomolanyi-Tsuda and Welsh, 1998, Allman et al., 2019). T-cell-dependent activation is characterised by the delayed (within days) onset and the production of high-affinity antibodies. Antigens bind to the BCR and then are fragmented into peptides inside the B cells that end up being displayed on MHC II molecules on the cell surface to cognate

CD4+ T-cells. These T-cells via CD28 bind to the B7 receptor on the B cells expressed during the antigen fragmentation, leading to T-cell proliferation (Linsley et al., 1991). Furthermore, T-cells bind to CD40 on the B cell surface via CD40L, promoting immunoglobulin class switching, B cell proliferation, and releasing cytokines from both cells. The activated B cells differentiate into effector antibody-secreting cells or enter the B cell follicle in secondary lymphoid organs to form a germinal centre to generate long-lived plasma cells and memory B cells for persistent defence (Jawa et al., 2013, Parker, 1993, Armitage et al., 1992). Several autoimmune diseases such as systemic sclerosis, type 1 diabetes, and rheumatoid arthritis are associated with autoantibodies due to recognising self-antigen by the B cells (Bouaziz et al., 2007, Numajiri et al., 2021, Da Rosa et al., 2018, Bounia and Liossis, 2021).

Table 1.2: Antibody classes and function.

Antibody Class	Normal Serum Level	Biological Function
IgA	Up To 15%	Protection Against Pathogen
IgD	About 0.25%	Activate Basophils and Mast Cells to Release Antimicrobial Factors
IgE	About 0.05%	Activate Basophils and Mast Cells to Release Histamine, Anti-Parasitic Response, And Allergy
IgM	About 5%	1st Antibody in The Early Stage of B Cell Response
IgG	About 85%	Significant Components of Humoral Immunity in Controlling Infections

1.1.2.1.2 T lymphocytes

T lymphocytes (or T-cells) are important cellular components in adaptive immune systems. T-cells develop from hematopoietic stem cells in the bone marrow and migrate through the blood circulation to the thymus gland to mature. The initial T-cells that arrived in the thymus do not express CD4 and CD8 co-receptors;

therefore, they are called double-negative cells. In the thymus, $CD4^{-}CD8^{-}$ cells begin creating their functional, unique TCR that consists of alpha and beta chains and recognises a random range of antigenic fragments. Through a development process known as TCR beta rearrangement, the double negative cells create their functional TCR with the expression of both CD4 and CD8 co-receptor molecules. In the thymus cortex, the new double-positive cells ($CD4^{+}$and $CD8^{+}$), through positive selection, narrow to a specific cell with MHC affinity that can provide positive functions during a T-cell response. Generally, thymocytes will be tested whether their MHC molecules presented self-antigen on their surfaces. The thymocytes that successfully bind to MHC molecules will survive, and the other group will die. The cells that interact with the MHC II molecule will continue developing into single positive $CD4^{+}$ by downregulating the expression of the CD8 receptor, and the same process occurs with MHC I and $CD8^{+}$ cells. These cells then undergo negative selection in the boundary of the cortex and medulla in the thymus. Cells that exhibit a strong binding with self-antigen die by apoptosis. The other cells will represent the mature naïve T-cells in the thymus and circulation (Kondo et al., 2018, Starr et al., 2003, Singer et al., 2008, Klein et al., 2014).

The naïve T-cells differentiate into effector T-cells, including two major functional subsets, cytotoxic T-cells and T helper cells, or into memory T-cells.

1.1.2.1.2.1 Cytotoxic T-cells

Cytotoxic T-cells (or $CD8^{+}$ T-cells) are T lymphocytes that mediate adaptive immune response against tumour cells and intracellular pathogens, including viruses and bacteria. Antigen presentation is the primary mechanism for activating $CD8^{+}$ T-cells. Their TCR recognise specific antigens presented on MHC I molecules on the surface

of antigen-presenting cells (mainly DCs). Two signals induce naïve $CD8^+$ T-cell activation. First, the binding of TCR with the peptides presented on the MHC I molecule, while the binding between the costimulatory molecule CD28 expressed on the $CD8^+$ T-cells with the costimulatory molecule CD80 and CD86 of APC represents the second signal (Andersen et al., 2006, Slavik et al., 1999, Stockwin et al., 2000). Furthermore, cytokines may work as an additional signal in naïve $CD8^+$ T-cell activation, helping to polarise or direct a specific response. The activated $CD8^+$ T-cells undergo clonal expansion and proliferate in the presence of IL-2 (Andrus et al., 1984, Andersen et al., 2006). The activated $CD8^+$ T-cells differentiate into effector $CD8^+$ T-cells that migrate throughout the body to destroy target cells by secreting the pro-inflammatory cytokines IFN-γ and TNF-α and cytotoxic molecules, including granzymes, FasL, and perforin. Perforin, a pore-forming protein, binds and forms pores in the target cell's plasma membrane that facilitates the entry of the pro-apoptotic proteases (granzymes) inside the target cells. Granzymes activate apoptosis by cleaving intracellular proteins or by activating caspases. In addition, effector $CD8^+$ T-cells can kill the target cells through Fas-induced apoptosis. $CD8^+$ T-cells release Fas L that binds to the Fas receptor (TNF protein) that is found on the target cell surface, leading to activation of the caspase cascade and induction of cell death (Bolitho et al., 2007, Martínez-Lostao et al., 2015, Takeda et al., 2001, Anel et al., 1994, Andersen et al., 2006).

1.1.2.1.2.2 T helper cells

T helper cells (or $CD4^+$ cells) are another type of T lymphocyte that share features with $CD8^+$ T-cells in recognising antigens presented by APC. However, $CD4^+$ cells recognise only antigens presented on MHC II. The other difference between these

two populations is that the activated $CD4^+$ cells cannot effectively kill the target cell via cytotoxicity. They are referred to as" helpers" because they help in activating other immune cells that express MHC II molecules such as DCs, macrophage, and B cells (Louten, 2016).

Th1 and Th2 cells are the two significant subsets of T helper cells. Generally, Th1 cells are triggered by antigen in the presence of the polarising cytokines IL-12 and produce primarily IFN-γ that enhance the cellular response against pathogens by activating macrophages (Hsieh et al., 1993, Mosmann et al., 1986, Actor, 2012). The polarising cytokine IL-4 triggers Th2 cells, and when activated, they produce various cytokines such as IL-4, IL-5, IL-13 to stimulate B cells for proliferation and production of antibodies or to recruit eosinophils (Kopf et al., 1993, Coffman et al., 1989, Kuperman et al., 2002, Kaplan et al., 1996). Th17 cells, another subset of T helper cells that produce the pro-inflammatory IL-17 cytokine, help defend against extracellular pathogens and are associated with the progression of autoimmune diseases (Korn et al., 2009). The number of different populations that develop during antigen exposure continues to expand. Figure 1.1 shows these populations and lists the cytokines involved in T-cell differentiation (Zhu, 2018).

1.1.2.1.2.3 Memory T-cells

Memory T-cells differentiate from both $CD4^+$ and $CD8^+$ naïve T-cells. There are long-lived after the elimination of pathogens and can be activated to proliferate into many $CD4^+$ or $CD8^+$ effector cells during the re-exposure to the same antigen, leading to a rapid immune response to the repeated infection. Most memory T-cell subsets express the surface receptor CD45RO. The most common memory T-cells subsets are central memory T-cells (TCM cells), effector memory T-cells (TEM cells)

and effector memory cells re-expressing CD45RA (TEMRA cells) (Willinger et al., 2005).

1.1.2.1.2.4 Regulatory T-cells

Regulatory T-cells (or suppressor T-cells) are $CD4^+$ T-cells that express the CD25 (IL-2 receptor). Treg cells play an essential role in mediating the immunotolerance by suppressing the autoreactive T-cell that escapes during the negative selection in the thymus and controlling the effector immune response to prevent over-reactivity of the reactions against invading pathogens (Chen et al., 2003, Josefowicz et al., 2012). Treg cells develop in the thymus and are known as natural Treg cells or develop in the periphery, known as adaptive or induced Treg cells (Abbas et al., 2013). Both classes express Forkhead box protein P3 (FOXP3) that act as the master regulator during Treg cells development in which mutation in this gene results in autoimmune disorders such as Immunodysregulation polyendocrinopathy enteropathy X-linked (or IPEX) syndrome (Bacchetta et al., 2006, Chatila et al., 2000). Treg cells are thought to induce their regulatory functions through a variety of processes, mainly by secreting regulatory cytokines (such as IL-10, TGFβ, and IL-35) (Marie et al., 2005, Asseman et al., 1999, Green et al., 2003, Turnis et al., 2016). Other Treg cells develop during the immune response and do not express FOXP3, but they share the same suppressor function of $FOXP3^+$ Treg cells. For instance, Type 1 regulatory cells (Tr1) that produce a high level of IL-10 to regulate expression of inflammatory bowel diseases, T helper 3 cells (Th3) that secrete the anti-inflammatory cytokine transforming growth factor-beta (TGF-β), and T helper 17 cells (Th17) that have recently been reported as Treg cells (Tungland, 2018, Gagliani et al., 2015, Chen et al., 1994, Groux et al., 1997).

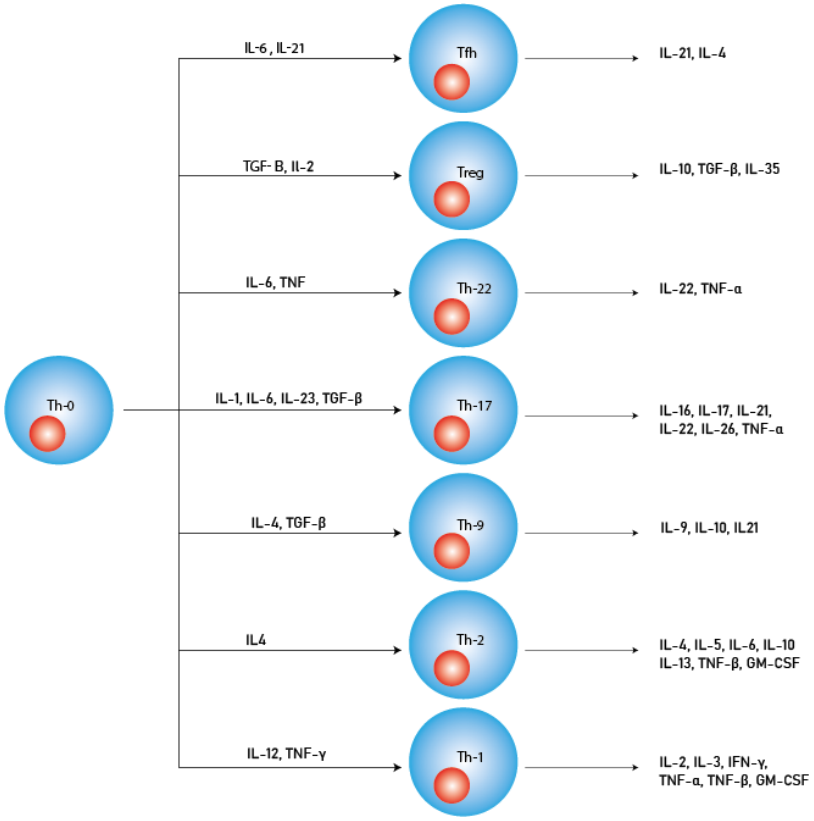

Figure 1.1: Models of naïve T-cell differentiation into T-cell subsets through cytokine signalling.

1.1.3 Major histocompatibility complex (MHC)

MHC is a set of genes that code for cell surface proteins essential for the immune system. Their main function is binding and presenting host self-antigens to avoid autoimmune responses and binding to non-self-antigens to eliminate pathogens by the appropriate activation of T-cells via antigen presentation (Holoshitz, 2013). MHC molecules are polygenic in which they are made up of multiple class I and class II

genes, and they are polymorphic in which each MHC gene is found with different alleles. The MHC molecules are known as Human leukocyte antigen (HLA) in humans. The human's MHC region is found on chromosome 6 and consists of a hundred genes, and spans about 3.6 megabase pairs of DNA. About 40% of humans' MHC genes are detected to have immunological functions. MHC I and MHC II are the two classes of MHC genes that code for cell surface proteins; MHC I and MHC II molecules are essential in antigen presentation to appropriate T-cells (Nature, 1999, Knapp, 2005).

MHC I molecules are cell surface proteins expressed on all body's nucleated cells. MHC I structurally consist of a single polypeptide α chain composed of three domains (α1, α2, and α3) that bind non-covalently with β2-microglobulin (B2M) via α3. B2M is a component of the MHC I molecule and encoded by the B2M gene on chromosome 15. The MHC I molecule is divided into classical and non-classical MHC molecules. The classical types include HLA-A, HLA-B, and HLA-C, and they are characterised by a polymorphic α chain in which they are made up of many alleles. α3 domain is a transmembrane attaching the MHC I molecule to the cell membrane and binding to the T-cell's co-receptor CD8 to keep the MHC molecule in a position of binding with the T-cell TCR via α1 and α2 domains. The folding structure of α1 and α2 domains form the peptide-binding groove where endogenous peptides of 8-10 amino acids are bound and presented to $CD8^+$ T-cells. However, recent studies reported that peptide sequences of 10 amino acids or greater could also bind to the MHC I molecule. The MHC I groove is closed, leading to the peptide's end residues attaching within the groove and serving as anchor residues (Figure 1.2). The non-classical HLA molecules, HLA-E, HLA-F, and HLA-G are less well understood. They

display limited polymorphic features, while their ability to present peptides is also limited (Mak et al., 2013, Mosaad, 2015, Wieczorek et al., 2017, Murphy and Weaver, 2016, Knapp, 2005).

MHC II molecules are also a group of cell surface proteins found mostly on antigen-presenting cells such as macrophages, B cells and DCs. MHC II molecules consist of two transmembrane polypeptide chains: α chain (α1 domain and α2 domain) and β-chain (β1 domain and β2 domain). The folded structure of α1 and β1 form the peptide binding site where exogenous peptides of 15-24 amino acids are bound and presented to CD4+ T-cells. The extracellular α2 and β2 domains form a membrane-proximal immunoglobulin-like domain (Figure 1.2). MHC II is open, which means peptides extend out of the groove, and terminal amino acids do not act as anchor residues. The HLA-D gene encodes MHC molecules, and the classical types include HLA-DP, HLA-DQ, and HLA-DR (Mak et al., 2013, Knapp, 2005, Mosaad, 2015, Wieczorek et al., 2017, Murphy and Weaver, 2016).

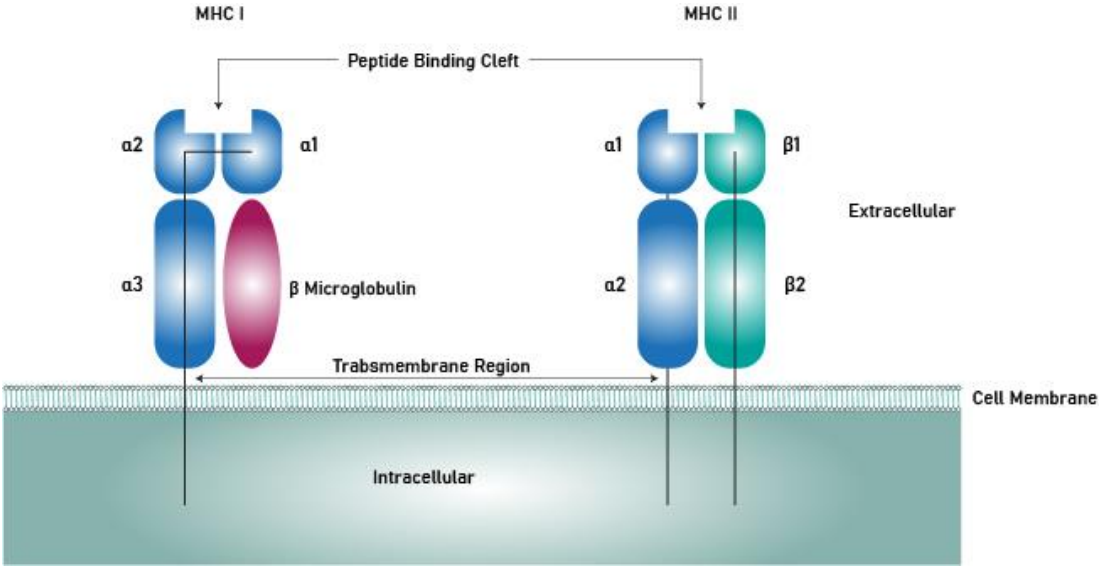

Figure 1.2: MHC I and MHC II structure.

1.1.3.1 MHC I antigen presentation

MHC I antigen presentation begins with the degradation of endogenous proteins of bacteria, viruses, or tumour cells into fragmented endogenous peptides through the proteasome (Rock and Goldberg, 1999, Schubert et al., 2000). The degraded peptides translocate from the cytoplasm into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP) (Reits et al., 2003, Androlewicz et al., 1993). TAP transports peptides ranging from 8 to 16 amino acids into the ER (Parcej and Tampé, 2010). Before attaching to MHC class I molecules, these peptides may need further trimming by ER aminopeptidase associated with antigen processing (ERAAP) (van Endert et al., 1994). MHC class I molecules pass through the ER quality control system that regulates the MHC class I molecules' folding and facilitates the optimal MHC class I peptide complex assembly. The quality control is mediated by the MHC I molecule peptide-loading complex, which consists of TAP, tapasin, calreticulin, Erp57, and calnexin (Peaper and Cresswell, 2008, Garbi et al., 2003, Dick et al., 2002, Wearsch et al., 2004). Once the peptide MHC I complex (pMHC) forms, it disassociates from the peptide loading complex and is transferred from the ER to the cell surface through the Golgi apparatus. Peptides that fail to bind with the MHC I molecule are removed from the ER via ER-associated protein degradation into the cytoplasm to be degraded (Hammond and Helenius, 1995) (Figure 1.3).

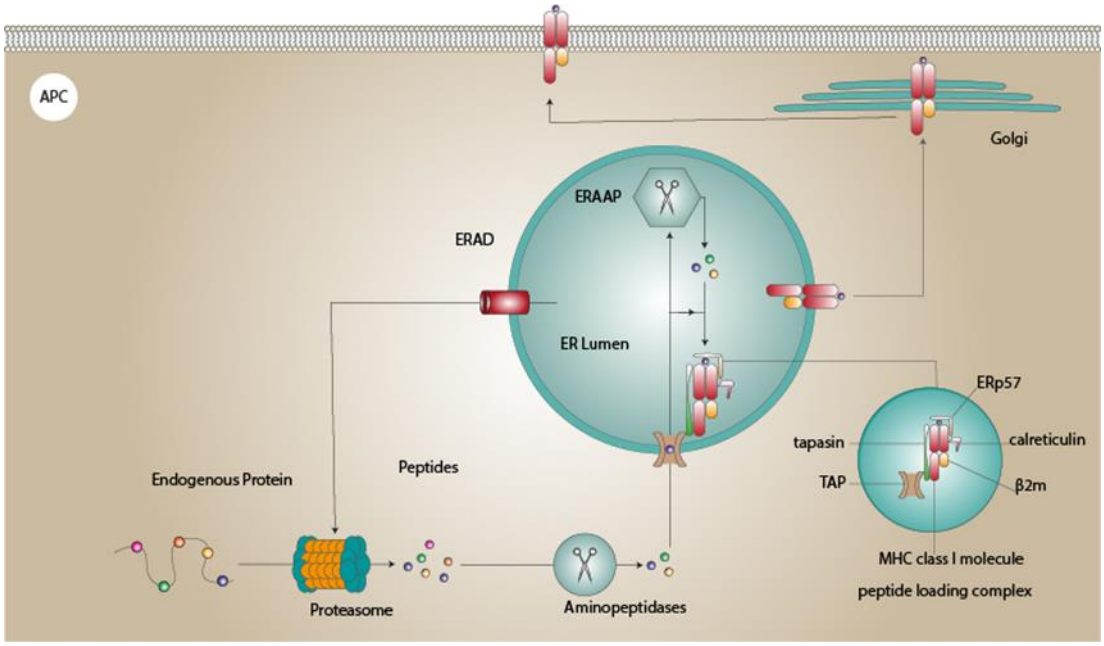

Figure 1.3: Antigen processing and presentation on MHC I molecules. Endogenous proteins are degraded in proteasome into peptides, transported into the endoplasmic reticulum through the TAP, and then loaded to the MHC I molecule by the peptide loading complex. pMHC I complexes are then transported via the Golgi apparatus to the cell surface for antigen presentation.

1.1.3.2 MHC II antigen presentation

MHC II is generally expressed on the surface of professional APCs such as DCs, macrophages and B cells. Those APCs usually eliminate pathogens by endocytosis, in which the exogenous proteins break down into many different peptides by lysosome-associated acidic enzymes (Murphy and Weaver, 2016). In the ER, MHC II α- and β-chains bind together and form a complex with a chaperone protein called invariant chain (Ii), which prevents the binding of the endogenous peptides to the MHC II molecule binding groove (Busch et al., 2000). The Ii–MHC class II complex is

then transported from ER and through Golgi to the endosomal MHC class II loading compartment (MIIC) in which both endocytosed peptides and Ii proteins are digested by proteases leaving only a tiny part of Ii, Class II-associated invariant chain peptide (CLIP), that binds to the peptide-binding groove (Lotteau et al., 1990). In the MIC, the clip is exchanged with the specific peptide antigen by the non-classical MHC-II (HLA-DM) (Lindstedt et al., 1995, Denzin and Cresswell, 1995, Kropshofer et al., 1996). Finally, the pMHC II is transported to the plasma membrane to present the antigenic peptide to $CD4^+$ T-cells (Neefjes et al., 2011, Tewari et al., 2005) (Figure 1.4).

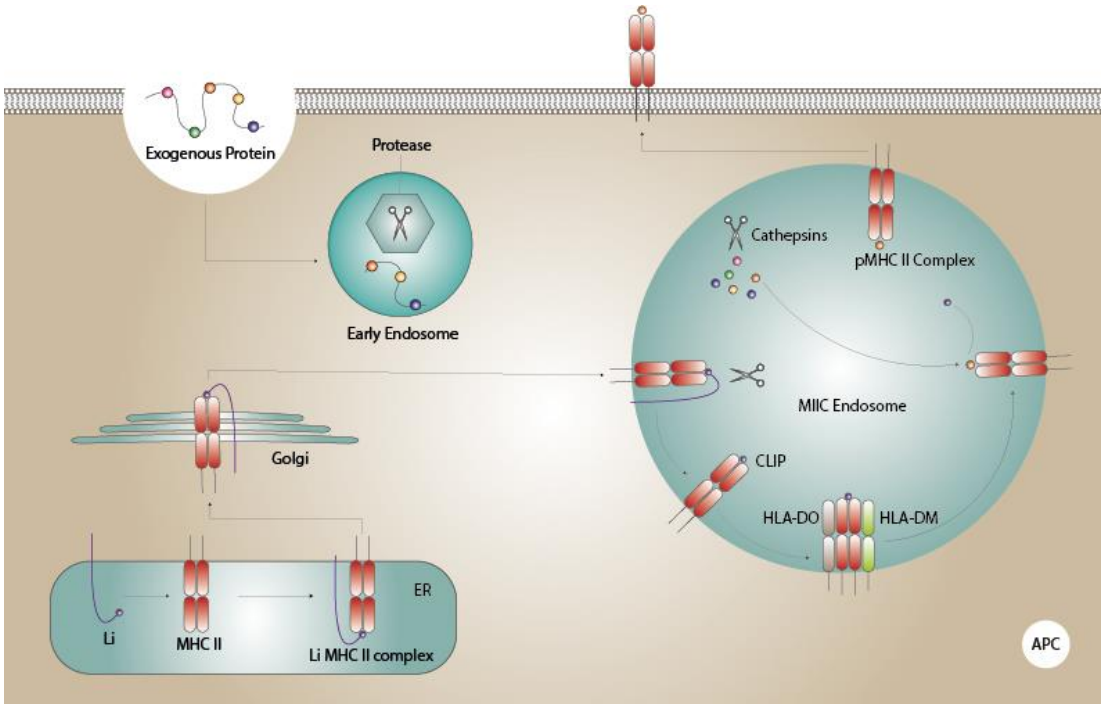

Figure 1.4: Antigen processing and presentation on MHC II molecules. Exogenous proteins are ingested by phagocytosis. The phagocytosed proteins are then cleaved by endosomal enzymes (proteases) into small peptides then transported into the MHC class II compartment (MIIC). MHC II molecule is synthesised and forms a complex with the invariant chain (Ii) in the endoplasmic reticulum. Ii–MHC II is then transported through the Golgi into MIIC. In MIIC, both Ii–MHC II and the small peptides undergo proteolytic

degradation. The final MHC II molecule is loaded with an antigenic peptide and then transported to the cell surface for antigen presentation.

1.1.4 T-cell Receptor

T-cell receptor (TCR) is an immunoglobulin-like protein complex receptor found on the surface of T-cells and has the function of recognising the fragmented peptide antigens presented on MHC molecules displayed by APC. Individual TCR can recognise many peptides with different affinities, allowing the T-cells to identify various pathogens (Birnbaum et al., 2014, Acuto et al., 1983). Antigen recognition results in the activation of T-cells and stimulation of proliferation and release of effector molecules that help destroy invading pathogens (SAMELSON and KLAUSNER, 1988).

In humans, T-cells expressing αβ TCR constitute the dominant T-cells found in the periphery. T-cells expressing γδ receptors which are composed of (γ/δ) chains, are found less commonly, however; they are contributed to many types of immune response and immunopathology (Vantourout and Hayday, 2013). TCR αβ is composed of two polypeptide chains, α and β, connected via a disulphide bond (Allison and Lanier, 1985). Both chains are divided into two constant domains, Cα and Cβ, and two variable domains Vα and Vβ, which are oriented toward the α1 and α2 of the pMHC. Cα and Cβ are followed by a transmembrane region and a short intracellular tail that does not seem to be involved in the signalling pathway. The TCRαβ bind non-covalently with the co-receptors CD3, including CD3γ, CD3δ, CD3ε, and CD3ζ chains (Figure 1.5). Each Vα and Vβ is divided into three complementarity-determining regions (CDRs). CDR3α and CDR3β are oriented toward the central position of the pMHC, thus mainly responsible for the antigen

recognition, whereas CDR1 and CDR2 have been shown to bind with amino acids of the α1 and α2 MHC helices (Garcia et al., 1999, Alcover et al., 2018, Rudolph et al., 2006).

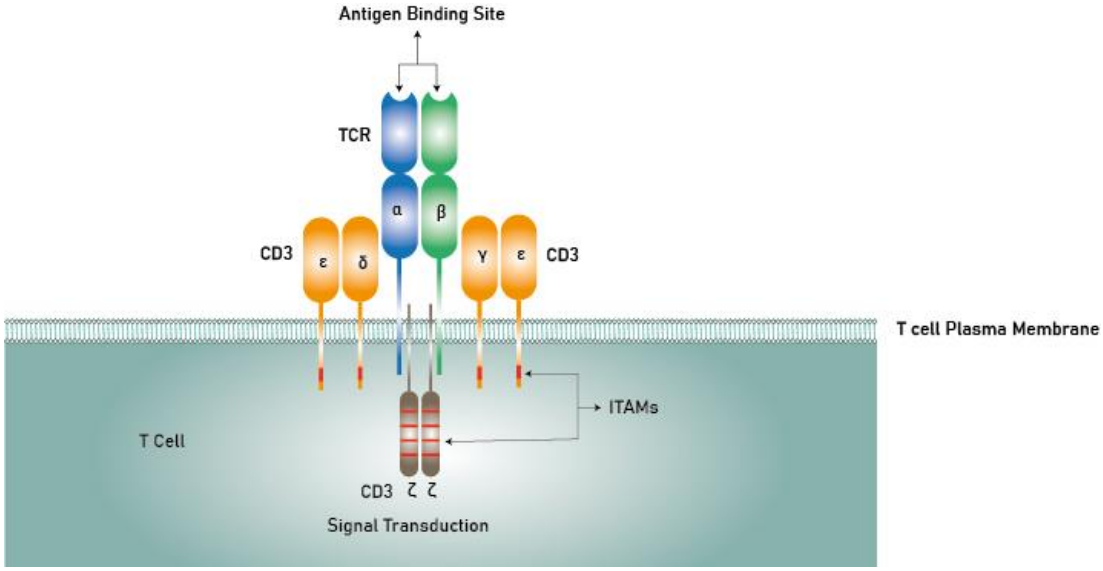

Figure 1.5: TCR structure.

1.2 Adverse Drug Reactions (ADRs)

According to the World Health Organisation (WHO), an ADR was defined in 1969 as "a response to a drug that is noxious, unintended and occurs at doses normally used in man for the prophylaxis, diagnosis or therapy of a disease, or for the modification of physiological function". Edwards and Anderson in 2000 criticised this definition because the use of the word 'noxious' did not include the not harmful inconvenient ADRs and the overdose related ADRs. They, therefore, defined ADRs as "an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product which predicts hazard from future administration and warrants prevention of specific treatment, alteration of the dosage regimen or withdrawal of the product (Edwards and Aronson, 2000).

ADRs have a significant clinical and financial challenge as they cause high morbidity and mortality rates. 6.5% of 18,820 hospital admissions were ADR cases, according to a prospective study in the UK, resulting in a financial burden of over £450 million on the National Health Service (NHS) (Pirmohamed et al., 2004).

In Europe, ADRs account for 197,000 deaths annually, with a financial burden of over € 70 billion (European Commission, 2008). Similarly, the annual death was recorded as 100,000 cases in the US (Lazarou et al., 1998). Following these reports, it has become clear to identify ADRs as a significant challenge for patients, the healthcare system, and the pharmaceutical industry.

1.2.1 Classification of ADRs

Type A ADRs (augmented, on target, pharmacological) make up most ADRs, approximately 80%, and can occur in any individual given a sufficient dose. They are also called "on target" ADRs in which reaction occurs at the intended target in the test system, and their mechanism is mainly known. Type A ADRs are predictable from the known pharmacologic properties of a drug with two forms, augmentation of the drug's therapeutic actions (primary) and action different from the drug's therapeutic actions (secondary). They are dose-dependent in which the reactions will immediately reverse once the dose is reduced or withdrawn (Pirmohamed et al., 1998, Coleman and Pontefract, 2016). Hypoglycaemia with oral hypoglycaemics and hypotension with anti-hypertensives are two examples of this type of reaction. Such reactions should be expected and may generally be avoided by lowering the dose (Park et al., 1992).

Type B ADRs (bizarre, off-target, idiosyncratic) account for approximately 10%-15% of ADRs. They are dose-independent, unpredictable, and often occur in a

susceptible group of patients. They are also referred to as "off-target" ADRs in which the reaction occurs at other targets in the test system. Type B ADRs represent drug hypersensitivity reactions (DHR) due to the involvement of the immune system (Pohl et al., 1988, Johansson et al., 2001). These reactions may involve drug-specific immunoglobulins, drug-specific T-cells, and other adaptive immunity components (Naisbitt et al., 2003). Type B ADRs are often more severe than type A and, in some cases, can result in death. Type B ADRs can occur in one organ or can be systemic, and typical examples of this type of reaction include drug-induced liver injury (DILI), Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN) and anaphylaxis (Wei et al., 2012b). Dapsone (DDS) hypersensitivity syndrome (DHS), which forms the basis of this thesis, is another example of a type B reactions.

There are four additional categories of ADR, named C-F ADRs. Type C (Chronic) ADRs occur due to long-term use and dose accumulation. Type D (Delayed) ADRs arise due to long term use of a drug that is not likely to accumulate. Type E (End of Treatment) ADRs occur due to the stopping of the drug, such as opiate withdrawal. Type F (Failure of Therapy) ADRs are referred to as "unexpected failure of therapy" and often occur with drug-drug interactions (Park et al., 1998).

Lately, the classification of ADRs was updated to involve additional criteria such as the severity and time course of reactions and the individual related susceptibility factors (the genetic, pathological, and other biological differences). This update resulted in a new classification system based on dose, timing, and susceptibility (DoTS) (Aronson and Ferner, 2003, Rawlins, 1981).

Various risk factors influence individuals' susceptibility to ADRs, including age; ADRs are more prevalent in older people as they are associated with polypharmacy (the

use of multiple drugs), causing disease-drug or drug-drug interactions (Greenblatt et al., 1981). Gender; females show a higher risk of developing ADRs with 1.5 to 1.7 fold more reactions observed when compared with males (Rademaker, 2001, Schwartz et al., 2001). Presence of susceptible specific HLA allele (Table 1.3); For example, patients positive for HLA-B*13:01 are more vulnerable to hypersensitivity reactions when exposed to DDS in China (Zhang et al., 2013a). Finally, the presence of viral infections or other diseases such as HIV and hepatic cirrhosis is associated with an increased frequency of hypersensitivity reaction (Hasselström et al., 1990, Coopman et al., 1993, Tohyama et al., 1998).

Table 1.3: Examples of HLA-restricted ADRs. Derived from (Phillips et al., 2011).

Drug	Allele	Ethnicity	Adverse Reaction
Carbamazepine	HLA-B*1502	Han Chinese	SJS/TEN
	HLA-B*1301	European	
Allopurinol	HLA-B*5801	Various Asians	SJS/TEN and DRESS
Abacavir	HLA-B*5701	Multiple ethnic groups	DRESS
Flucloxacillin	HLA-B*5701	Multiple ethnic groups	Liver injury

1.2.2 Drug Hypersensitivity Reaction

Drug hypersensitivity reaction (DHR) is a type B ADRs with immunological aetiology to a drug in a sensitised patient. Clinical classification of DHRs is based firstly on the time course of development of clinical symptoms, which is helpful for the diagnosis of DHR. DHR can have an immediate onset; alternatively, reactions may be delayed, with symptoms appearing days or weeks after initial drug exposure. Immediate reactions usually occur within the first hour of drug administration and are mediated by IgE (Blanca et al., 2005, Demoly and Hillaire-Buys, 2004). On the other hand, delayed-type reactions generally occur within one day to a few weeks due to the involvement of drug-responsive T-cell activation (Bousquet et al., 2007, Pichler,

2003). The T-cell activation pathway includes a T-cell commitment to an antigen - this occurs within 30 mins to an hour post-exposure to the antigen and is followed by T-cell proliferation and then T-cell differentiation within the first five days of the reaction. Activation of the differentiated T-cells can result in the release of cytotoxic molecules like perforin and granzyme-B that target tissue cells for destruction (Ortiz et al., 1997).

Based on the Gell and Coombs classification system, DHR immune mechanism can be categorised into the following four types (Riedl and Casillas, 2003) (Table 1.4).

1.2.2.1 Type I Hypersensitivity Reactions (IgE-Mediated)

In type 1 reactions, B cells are activated with an antigen to produce antigen-specific IgE that binds to FcεRI receptors on the surface of basophils and mast cells, causing them to be sensitised. Renewed contact with the same antigen cross-links the bound IgE on the cells leading to the immediate release of various mediators such as histamine, prostaglandin, and leukotriene. These molecules elicit peripheral vasodilation, vascular permeability, and smooth muscle contraction (Chapel et al., 2013). The clinical manifestation of type 1 DHR includes urticaria, angioedema, bronchospasm, pruritus, vomiting, diarrhoea, anaphylaxis (Riedl and Casillas, 2003, Mathelier-Fusade, 2006). Penicillin, cephalosporins, and neuromuscular blocking agents are some drugs involved in this type of DHR (Schnyder and Pichler, 2009, Hollander et al., 2011, Suzuki and Ra, 2009).

1.2.2.2 Type II Hypersensitivity Reactions (Cytotoxic)

Type II hypersensitivity is a cytotoxic reaction due to the direct binding of IgG or IgM to cell surface antigens (such as erythrocytes, leukocytes, platelets), resulting in cell lysis or damage through the mechanism of complement system activation or

antibody-dependent cell-mediated cytotoxicity. Haemolytic anaemia, neutropenia, thrombocytopenia are clinical forms of type II DHR (Delves et al., 2017). Methyldopa (haemolytic anaemia) (Schubothe et al., 1982), aminopyrine (leukopenia) (Pretty et al., 1965), and heparin (thrombocytopenia) (Arepally, 2017, Warkentin and Kelton, 2001) are examples of drugs that are involved in type II DHRs.

1.2.2.3 Type III Hypersensitivity (Immune Complex)

Type III hypersensitivity is characterised by tissue damage caused by the immune response because of an accumulation of uncleared antigen-antibody complexes (Kindt et al., 2007). A clinical manifestation of a type III DHR includes serum sickness with β-lactam antibiotics (Patterson-Fortin et al., 2016), drug-induced lupus erythematosus with quinidine or procainamide (McCormack and Barth, 1985, Uetrecht et al., 1981), and vasculitis with minocycline (Thaisetthawatkul et al., 2011)

1.2.2.4 Type IV Hypersensitivity (Delayed, Cell-Mediated)

Type IV hypersensitivity is also known as delayed-type hypersensitivity. It begins with a 1–2 week sensitization period following primary antigen exposure. T cells are activated and clonally expanded during this time by antigen presented with the MHC molecule on an appropriate antigen presenting cell. Subsequent exposure to the antigen activates effector T cells, producing a range of cytokines that recruit and activate macrophages and other inflammatory cells. The response does not usually appear until 24 hours after the second antigen exposure, and it peaks 48–72 hours later. Alternatively, the T lymphocytes may secrete mediators that induce tissue injury directly (Kindt et al., 2007). The clinical manifestation of type IV

hypersensitivity includes various cutaneous or systemic diseases, and drugs such as sulfonamides and β-lactam antibiotics are often involved in this type of reaction (Schnyder and Pichler, 2009, Cacoub et al., 2011, Batchelor et al., 1965, Naisbitt et al., 2001a, Choquet-Kastylevsky et al., 2001) (Table 1.5).

Table 1.4: Immunologic characteristics of hypersensitivity reactions.

	Type I (IgE-Mediated)	Type II (cytotoxic)	Type III (Immune complex)	Type IV (Delayed)
Immune mediators	IgE	IgG, IgM, and complement	IgG, complements and neutrophiles	T-cells
Immunological Reaction	mast cell activation and mediators release (ex. Histamine)	Complement mediated phagocytosis	Immune complexes-mediated inflammation	T-cells mediated inflammation and cytotoxicity
Onset	Within 1 hour	Hours to days	1 to 3 weeks	Days to weeks
Examples	Anaphylaxes and allergic asthma	Haemolytic anaemia and thrombocytopenia	Serum sickness and rheumatoid arthritis	AGEP and SJS/TEN

Table 1.5: Immunologic characteristics of subtypes of type IV hypersensitivity reactions.

subtype	T-cell Type	Effector Molecules	Mechanism	Examples
IVa	T helper 1	IFN-γ, TNFα, IL-1 and IL-2	Monocytic and Macrophage inflammation	Eczema
IVb	T helper 2	IL-4, IL-5, and IL-13	Eosinophilic inflammation	DRESS
IVc	Cytotoxic T-cells	Perforin, Granzyme B, and Fas L	CD4+/ CD8+ mediated T-cell killing	SJS/TEN
IVd	T-cells CD4+, CD8+	IL-8 and GM-CSF	Neutrophilic inflammation	AGEP

1.2.3 Clinical Manifestations of Cutaneous Drug Hypersensitivity

Although exposure to several drugs is associated with the development of systemic clinical manifestations, the skin remains the most common organ involved in DHR (Merk et al., 2007). The clinical presentation of cutaneous ADRs varies in severity from maculopapular exanthema (MPE) to severe cutaneous adverse reactions (SCAR) that are life-threatening. The different variants are discussed in more detail below.

MPE accounts for approximately 90% of cutaneous ADRs (Hunziker et al., 1997). It is characterised by a flat discoloured area (macule) with small pink or red bumps (papule) on the skin (Pinto Gouveia et al., 2016). This type of reaction is common in several diseases, including lymphoma (Fernández Fernández et al., 2007) or by various viruses such as human herpesvirus (HHV) (Wananukul et al., 2003). MPE has been shown with different types of drugs, such as quinolones and antibiotics (Schmid et al., 2006). MPE usually develops within 4-14 days after the drug administration and may continue for 1-2 days after drug termination (Valeyrie-Allanore et al., 2007). MPE is a T-cell-mediated reaction that results in cutaneous inflammation through the secretion of IFN-γ, TNF- α, IL-5, and specific chemokines (Posadas et al., 2002, Hari et al., 1999). MPE can be treated by withdrawal the drug with the administration of steroids and antihistamines in some severe cases.

SCAR is a group of different cutaneous drug reactions that may or may not involve internal organs damage. The most common types of SCAR disorders are acute generalised exanthematous pustulosis (AGEP), drug reaction with eosinophilia and systemic symptoms (DRESS), and Stevens-Johnson/toxic epidermal necrolysis overlap syndrome (SJS/TEN).

AGEP is a cutaneous reaction known as a pustular drug eruption or toxic pustuloderma. It is a type IV hypersensitivity reaction, particularly subtype IVd reaction. Drugs account for 90% of cases of AGEP (Beltraminelli et al., 2005), with an approximate incidence of 1–5 patients per million patients annually (Sidoroff et al., 2001). AGEP is characterised by the acute formation of non-follicular, sterile pustules on an erythematous base, with neutrophilia and fever (Trapp, 2016, Roujeau et al., 1991, Choi et al., 2010). Drugs involved in AGEP include aminopenicillins, sulfonamides, terbinafine, hydroxychloroquine, and diltiazem (Sidoroff et al., 2007). AGEP is mediated by T-cells like other drug-induced SCARs, but the time to onset is shorter, often as fast as one day (Roujeau et al., 1991).

DRESS is also a severe form of ADR with a fatality rate of approximately 10%. DRESS is also known as drug-induced hypersensitivity syndrome (DIHS), given that several researchers believe that drug exposure reactivates Herpes viral infections that participate in the development of clinical symptoms (Trapp, 2016, Suzuki et al., 1998, Descamps et al., 1997). DRESS is a type IV hypersensitivity reaction, particularly subtype IVb, and characterised by an acute onset of widespread skin rash, fever, and damage of some internal organs such as swollen lymph nodes, hepatitis, nephritis, pneumonitis, carditis, thyroiditis, and blood abnormalities such as abnormal increase of eosinophils, atypical lymphocytes, low number of platelets, and leukopenia (Peyrière et al., 2006, Um et al., 2010). However, some DRESS patients do not show skin rash. The onset of DRESS is 2-6 weeks after administration of the causative drug, and this is a distinguishing feature in diagnosis (Cacoub et al., 2011). During the development of DRESS, particular latent viruses become reactivated, such as Epstein–Barr virus, human herpesvirus 6 (HHS-6),

human herpesvirus 7 (HHS-7), and cytomegalovirus (Trapp, 2016). However, the nature of the relationship between DRESS and virus reactivation is poorly understood. The most common drugs involved in DRESS reactions include carbamazepine (Descamps et al., 2001) and other anticonvulsants, antivirals (e.g. abacavir) (Bonta et al., 2008) and sulfonamides (Mainra and Card, 2003). DHS is often referred to as DRESS; however, viral reactivation is rarely observed. Various susceptible specific HLA alleles show a clear relationship with DRESS, such as HLA-B*13:01 with DHS (Zhang et al., 2013a).

SJS, TEN, and SJS/TEN are three forms of the same severe skin reaction. These reactions are type IV hypersensitivity reactions, particularly subtype IVc. TEN is diagnosed with 30 % of skin detachment. It is the most severe skin rash induced by drugs, with a mortality rate of approximately 50%. SJS involves less than 10 % of skin detachment, and SJS/TEN crossover is diagnosed with an intermediate 10-30 % skin detachment (Sidoroff, 2009, Downey et al., 2012). Statistically, TEN has an annual incidence of one to six cases per million, while SJS has one in two cases per million (Rzany et al., 1996). SJS, SJS/TEN, and TEN are first characterised by fever, sore throat, cough, and burning on eyes and skin. Ulcers and other lesions develop in the oral, lips, vaginal, or anal mucosa (Maverakis et al., 2017). Classes of drugs that cause SJS/TEN include antibiotics (sulphonamides, tetracyclines, cephalosporins, macrolides, quinolones and penicillin), anticonvulsants (phenytoin, carbamazepine, phenobarbital, valproate, clobazam and lamotrigine), non-steroidal anti-inflammatory drugs, allopurinol, nevirapine, abacavir, NSAID and paracetamol (Mockenhaupt et al., 2008, Schwartz et al., 2013, Levi et al., 2009, Forman et al., 2002, Ferrandiz-Pulido and Garcia-Patos, 2013). Although the pathogenesis of TEN

remains poorly understood, it appears that drug-stimulated cytotoxic CD8+ T-cells are mainly responsible for keratinocyte apoptosis and skin detachment through the release of perforin, granzyme B, and granulysin, also, the Fas ligand cell death pathway appears to be also involved (Schwartz et al., 2013, PAUL et al., 1996, Nassif et al., 2004a, Chung et al., 2008).

1.3 Pathways of drug-specific T-cell activation

1.3.1 Hapten and Pro-hapten hypothesis

The initial discovery of the hapten hypothesis dates back to Karl Landsteiner and John Jacobs, who invented the term 'hapten' from the Latin word 'fasten'. They found that some low molecular weight compounds (haptens) can activate the immune system if conjugated with proteins. Briefly, they found that small haptenic chemicals such as dinitrofluorobenzene (DNFB) can modify protein's nucleophilic residues in vivo in experimental animals such as guinea pigs, and once bound, the hapten protein adducts stimulate allergic sensitisation (Landsteiner and Jacobs, 1935, Landsteiner and Jacobs, 1936).

The hapten hypothesis remains one of the molecular pathways that could lead to drug display by MHC molecules and thus begin to explain the pathogenesis of DHR. Low l molecular weight haptens (< 1000 D) such as drugs and drug metabolites could activate the immune system once they bind and modify specific proteins or peptides in the body. The hapten-protein complex could be processed by antigen-presenting cells and presented as antigenic hapten-modified peptides by MHC to stimulate T-cells (Lafaye and Lapresle, 1988, Pichler, 2008) (Figure 1.6). In addition, these hapten protein complexes can activate the B cells and stimulate them to release their antibodies (Manfredi et al., 2004).

Currently, the hapten hypothesis help in understanding the mechanism of many drug reactions; however, the hapten mechanism require drugs to be reactive molecules to modify proteins, and most drugs are not inherently protein-reactive. Many non-reactive drugs are thought to induce an immune response through metabolism to reactive metabolites that then conjugate with cellular proteins, forming modified proteins or peptides after processing (Uetrecht, 1992, Naisbitt et al., 2000). For instance, the antibacterial agent sulfamethoxazole (SMX) is associated with various cutaneous reactions. SMX is metabolised to sulfamethoxazole hydroxylamine (SMX-NHOH) in the liver, skin, and immune cells. SMX-NHOH is oxidised to the reactive metabolite sulfamethoxazole nitroso (SMX-NO) that can bind to the cysteine residues of cellular proteins (Naisbitt et al., 1999). In vitro, the parent drug SMX and its reactive metabolite SMX-NO stimulate circulatory T-cells and skin resident T-cells isolated from sulfamethoxazole hypersensitive patients. Furthermore, the SMX and SMX-NO specific T-cell clones were generated in vitro, and functional tests showed that SMX could activate the T-cells by binding directly to the HLA or TCR or both. In contrast, the SMX-NO were found to stimulate the T-cells by haptenation, primarily by binding covalently to the cellular proteins and forming SMX-NO modified peptides after processing or by modifying directly already HLA bound peptides displayed on the surface of antigen presenting cells (Pratoomwun et al., 2021, Elsheikh et al., 2011).

Piperacillin is a β-lactam antibiotic used widely in patients with cystic fibrosis that may induce a T-cell response in patients via the hapten hypothesis. Piperacillin specific T-cell clones were generated from piperacillin hypersensitive patients, and functional studies showed that the clones were activated with the drug bound to

albumin in the media at specific lysine residues. Furthermore, the T-cells exhibited cross-reactivity with a synthetic piperacillin-albumin conjugate via a processing-dependent manner (El-Ghaiesh et al., 2012, Meng et al., 2011).

1.3.2 Pharmacological Interactions (PI) Concept

Another pathway that could explain T-cell activation in patients with DHR is the PI concept. This concept states that drugs might also interact directly with MHC molecules with no requirement for a stable covalent bond to trigger TCRs. The PI concept has been shown to be the dominant pathway for T-cell activation for various non-reactive medicines and substances. These molecules directly induce immune responses without the requirement for processing of drug-proteins complexes (Pichler, 2019). It should be noted that T-cells from patients with several forms of hypersensitivity are activated by a PI pathway, but only in the presence of phase I metabolites formed in the liver or other organs (e.g., atabecestat, allopurinol) (Thomson et al., 2021, Lin et al., 2015). In vitro, the PI concept is supported by the finding that drug-related T-cell responses are only detectable in the presence of APC and soluble drug (i.e., if APC are cultured with drug, then that the drug is washed away, T-cells are not activated). This indicates that the formation of a covalently modified drug-protein adduct is not required for T-cell activation. Laboratory findings also found that the drug T-cells response is detectable even in the absence of protein processing. For example, the activation of T-cells with a drug is still detectable when APC are pre-treated with glutaraldehyde, which prevents processing. In addition, most drugs identified as PI T-cell activators are characterised by immediate T-cell responses (within seconds following drug

exposure), which is incompatible with a hapten pathway (Adam et al., 2011, Castrejon et al., 2010b, Schnyder et al., 2000).

The pharmacological interaction of drugs with immune receptors occurs in different forms named pi-TCR and pi-HLA (Pichler et al., 2015). In Pi-TCR, T-cell activation is dependent on the binding of a drug to TCR before the TCR pHLA complex interaction occurs. For example, the drug SMX may bind to the TCR outside the site of TCR pHLA interaction resulting in alteration of the TCR conformation and subsequently increased the affinity between the HLA peptide and the altered TCR (Watkins and Pichler, 2013). However, this concept currently lacks experimental evidence. pi-HLA may occur in two different models; first, a drug such as abacavir may bind to the F pocket of the peptide-binding groove of a specific allele (HLA B*57:01 with abacavir) in ER, resulting in alteration of the self-peptide repertoire and subsequently T-cell stimulation (Illing et al., 2012) (Figure 1.6). Second, a drug such as carbamazepine may fit in the middle of the peptide-binding site of HLA B*15:02 and be accessible to the TCR, resulting in increased affinity of the HLA and TCR (Ko et al., 2011, Wei et al., 2012a, Pichler, 2019).

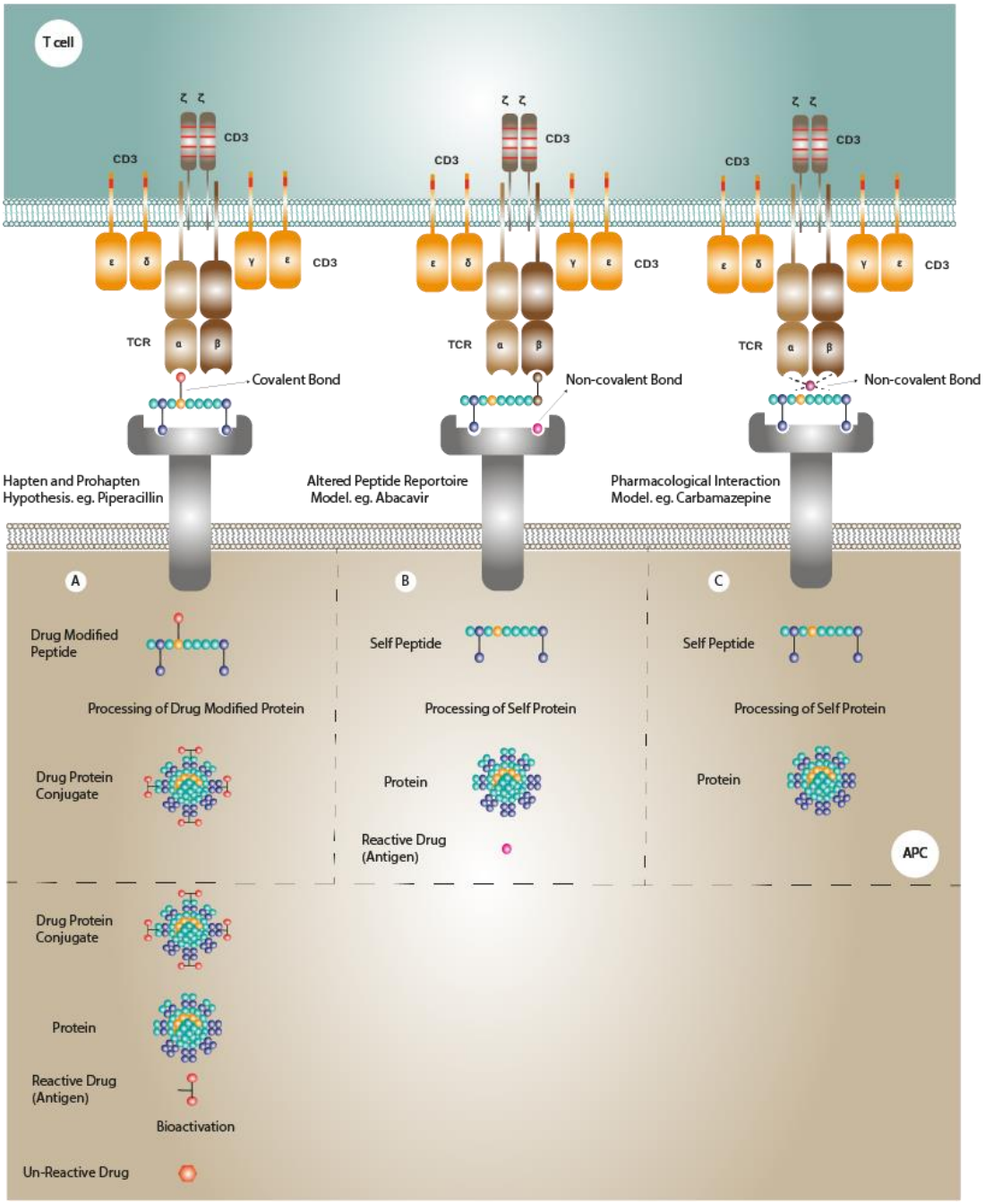

Figure 1.6: Pathways of drug-specific T-cell activation. A. In the hapten hypothesis, a drug like piperacillin covalently modifies protein. APC processes the protein adduct to be presented as drug modified peptide in MHC for T-cell activation. B. In the altered peptide repertoire model, the presence of drugs like abacavir within the peptide-binding pocket of the MHC molecule can alter the repertoire of peptides presented by the HLA to the TCR and then activate the T-cell. C. In the PI concept, a drug like carbamazepine binds non-covalently to pMHC complex, TCR, or both, activating T-cells.

1.3.3 Danger Signal

The danger hypothesis, initially proposed by Matzinger, is an alternative to the self-nonself hypothesis and has improved our understanding of how the immune system recognises foreign substances (Matzinger, 1994). it is based on the concept that foreign substances do not generate a significant immune response unless it causes cell damage (for example, stressed cells die by necrosis), releasing danger signals that stimulate an immune response. It generally states that when MHC-restricted antigen interacts with the T cell receptor (signal 1) without danger signals, tolerance develops, however when the danger signals secreted from stressed cells, costimulatory molecules on APCs and T cells are upregulated, resulting in a full immune response(Matzinger, 1994).

In terms of drug hypersensitivity, reactive metabolites could act as (1) haptens (signal 1) (Park et al., 2001), (2) can cause cell injury, which generates danger signals, leading to upregulation of costimulatory molecules (signal 2) (Uetrecht, 1999). it is possible that the reason why some viral infections increase the risk of drug hypersensitivity is the increased levels of cytokines and cell-surface markers that generate signals 2, thus, inducing a full immune response (Pirmohamed et al., 2002, Suzuki et al., 1998, Bayard et al., 1992).

The danger model suggests that damaged or necrotic cells release endogenous molecules called damage/danger-associated molecular patterns (DAMPs) that stimulate the immune system similarly to PAMPs, molecules released by microbes. As described before, PAMPs like bacteria LPS and DAMPs like ATP are recognised by various PRRs, such as TLRs, and stimulate the innate immune response to release various anti-inflammatory cytokines and to activate T and B cells (Valles et al.,

2014). Small molecular weight molecules can stimulate the innate immune response and release danger signals via binding with PRRs. For instance, the contact allergen nickel (Ni2+) that causes contact hypersensitivity has been shown to activate the epithelial cells via the Toll-like receptor 4 (TLR4)-MyD88–dependent signalling pathway (Schmidt et al., 2010). Furthermore, the antiviral imidazoquinoline derivatives imiquimod and R-848 has been shown to induce macrophages to release cytokines through TLR7 binding (Hemmi et al., 2002).

1.4 HLA Nomenclature

HLAs were initially discovered in 1958 by Jean Dausset, Jon van Rood and Rose Payne, who published in vitro studies showing that many but not all multi transfused tested individuals and multiparous tested women exhibited a serum reaction with leukocytes explained as serum antibodies that can recognise antigens on the leukocytes. HLA-A, HLA-B, and HLA-C loci were then detected before 1970, followed by discovering HLA II alleles by 1980 (Thorsby, 2009). Developments in genetics above serology methodology led to many advanced gene sequencing techniques that helped define the complete polymorphism of HLA genes. The WHO Nomenclature Committee was established in 1968 and regularly meets to discuss nomenclature issues, and it developed the HLA naming system in 2010 (Table 1.6).

Table 1.6: Nomenclature of HLA alleles (Robinson J, 2015).

HLA-A*02:101:01:02N as an Example	
HLA naming system	Explanation
HLA	refers to the human MHC gene
HLA-A	refers to a particular HLA allele within the MHC region
HLA-A*02	refers to a group of HLA alleles that encode the serological A02 antigen
HLA-A*02:101	refers to specific HLA protein
HLA-A*02:101:01	refers to the allele variant that differs by a synonymous substitution; thus, the produced protein is not modified
HLA-A*02:101:01:02	Refers to the allele with DNA mutation in a non-coding region
HLA-A*02:101:01:02 N	N: refers to null alleles that do not encode proteins L: refers to the alleles that express lower cell surface expression than normal cells S: refers to alleles that encode a soluble secreted protein but are not expressed on the cell surface Q: refers to a questionable expression of specific alleles that could affect the expression of others C: refers to alleles that encode cytoplasmic proteins but not cell surface. A: refers to alleles that have uncertain protein expression

1.5 Diagnosis of Drug Hypersensitivity Reaction

The diversity of drug hypersensitivity diseases and their complex disease mechanisms results in a difficulty for diagnosis; however, some effective in vivo and in vitro methods are currently in use and applied clinically or in specialised laboratories.

1.5.1 In-Vivo Skin Testing

The patch test is one of the diagnostic skin tests used to determine whether a tested individual is hypersensitive to a particular substance such as a drug. The drug must be tested before on non-allergic volunteers to find the optimal nontoxic concentration. The optimal quantity of drug is applied to the skin of the tested

individual for 24 to 48 hours, and in sensitised individuals, localised reactions including erythema with and without pustules are observed with a concomitant infiltration of T-cells (Barbaud et al., 2001, Pichler and Tilch, 2004). However, whether a sensitised individual will develop a positive patch test depends on various factors, including the presence of T-cells and the drug in the skin, the drug's ability to be presented and recognised by the T-cell, and the recruitment of effector cells into the skin. A positive result indicates the presence of drug sensitivity, and the positive diagnosis can be incredibly helpful in individuals exposed to multiple drugs at the time of the adverse event. In contrast, a negative result does not rule out a T-cell hypersensitivity reaction as it is possible that the drug is not absorbed sufficiently and/or is not presented locally in an appropriate form on the surface of APC (Pichler and Tilch, 2004).

1.5.2 Lymphocyte Transformation Test (LTT)

The LTT is an in vitro diagnostic method that relies on the proliferation of memory T-cells isolated from patient PBMCs upon stimulation with the suspected drug(s). The LTT has variable sensitivity and specificity depending on the test of interest (Nyfeler and Pichler, 1997). For instance, β-lactam antibiotics and anticonvulsants have an average of 60–70 % LTT sensitivity (positive response in hypersensitive patients) and 85–93 % LTT specificity (negative response in tolerant controls)(Pichler and Tilch, 2004). The type of adverse event also impacts the LTT sensitivity; for example, MPE, AGEP, and DRESS have been shown to have a higher LTT sensitivity than SJS/TEN. To conduct a LTT, PBMCs are isolated from the blood of hypersensitive patients and then cultured with the culprit drug for five days, ^{3}[H]-thymidine is then added, and the cells are cultured for a further 12-16 hours. The

cells are then harvested, and T-cell proliferation is calculated by generating a stimulation index (SI) that represents the proliferation ratio in the drug-treated cells and the negative control cells. A positive response is normally considered with a SI equal to or higher than 2; however, a SI cut-off of 3 has been proposed for β-lactam antibiotics.

1.5.3 Enzyme-linked immunosorbent assay (ELISpot)

ELISpot is a similar in vitro diagnostic test used to measure activation of hypersensitive patient PBMC after drug treatment; however, in the ELISpot assay, secreted cytokines are the readout for an antigen-specific T-cell response (Porebski et al., 2011). In the ELISpot assay, PBMCs or T-cell clones are cultured with culprit drug in a plate coated with cytokine-specific monoclonal antibodies (capture antibodies). Plated are then incubated for 48 hours. During this period, cytokines will be secreted from stimulated T-cells that bind to the capture antibodies. The plates are then washed to remove the cells and unbound substances. The captured cytokines are then detected using biotinylated cytokine specific detection antibodies. The streptavidin-enzyme conjugate is then added to create a strong affinity between the biotin and the streptavidin. A particular enzyme substrate solution is added based on the type of enzyme-conjugated with streptavidin. The catalytic reaction between the substrate and its enzyme will result in an insoluble precipitate that forms as spots on the membrane that can be counted by an ELISpot reader (Figure 1.7). Each formed spot represents a cytokine secreted by a single activated T-cell. Elispot is a widely used in vitro assay to measure several T-cells cytokines such as IFN-γ, TNFα, IL-4, IL-5, and IL-13, or cytotoxic molecules including FasL, Granzyme B, and perforin. The ELIspot assay has been used widely in the field

in recent years to measure the immune response that develops with Covid-19 infection and the response of individuals to Covid vaccination (Zuo et al., 2021, Tan et al., 2021).

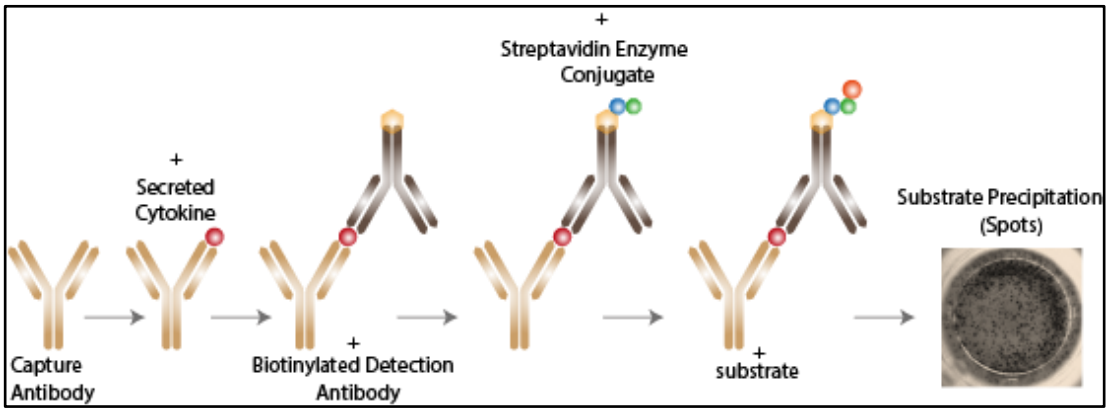

Figure 1.7: Diagram of the ELISpot assay for detecting cytokine secretion.

1.5.4 Flow cytometry

Flow cytometry is a common technique used to detect various cell surface and intracellular molecules in fields of immunology, including drug hypersensitivity (Martin et al., 2010). It can differentiate between the activated/resting and the naïve/memory T-cells by detecting the activation markers expressed before and after antigen exposure. For instance, CD69 is a cell surface biomarker that has been shown to be expressed on activated T-cells from hypersensitive patients after amoxicillin and carbamazepine treatment (Beeler et al., 2008). CD25 and HLA-DR have also been shown to be expressed on the surface of CD4+ and CD8+ T-cells stimulated with sulfamethoxazole, phenytoin, or carbamazepine (Mauri-Hellweg et al., 1995). Flow cytometry is also a widely used technique for identifying T-cell phenotype, chemokine expression, and assessing TCR repertoires. It is also an alternative diagnostic method to assess T-cell proliferation using the fluorescent cell staining dye (CFSE) capable of binding covalently and labelling the amino group in T-

cell intracellular molecules. After CSFE-labelled cells divide, each daughter cell will have half the level of CFSE and thus show a decrease in fluorescence that can reflect the level of T-cell proliferation.

1.5.5 T-cell cloning

T-cell cloning is effective in vitro method to generate single cell antigen-specific populations from the patient's PBMCs. Once the antigen-specific T-cell clones have been generated, they can be characterised to identify phenotype, cross-reactivity, MHC restriction, cytokine secretion and other functional characteristics. As such, clonal analysis has been used to define the nature of the drug-specific immune response induced in patients with different adverse event forms. T-cell cloning is achieved by setting up a serial dilution of the drug-treated PBMC cultures. The single cells are then stimulated repeatedly with mitogen to generate sufficient cells for testing in a proliferation or cytokine release assay. Clones displaying an antigen-specific response are then expanded further and characterised as detailed above.

1.5.6 Detection of Drug-Protein Adducts

Drug–protein adducts are distinct chemical structures that originate from the interaction of a protein and a drug or drug reactive metabolite. Protein adducts are formed when drugs or their metabolites covalently modify nucleophilic residues on proteins, leading to unexpected toxicities and severe drug responses through several mechanisms, including altering the target protein's activity or eliciting an immune response (Gan et al., 2016). It is challenging to characterise the protein adducts formed and the sites of covalent modification on specific proteins because drugs and their metabolites interact with many proteins at several locations (Yang and Bartlett, 2016). Drug–protein adducts are characterised using a variety of

approaches, and the type of sample determines which procedure is the most appropriate to use. HSA is one of the most abundant proteins in the body, making up 80 percent of serum protein content (Steel et al., 2003). HSA is a common target for many drugs since it is so abundant and expressed nucleophilic amino acid residues that create binding sites for different organic compounds (Elsadek and Kratz, 2012). For instance, β-lactam antibiotics have been shown to modify HSA through the covalent binding to lysine residues (Jenkins et al., 2013). Furthermore, in vitro model proteins like HSA and glutathione S-transferase pi (GSTP) have been used to increase our understanding of the chemistry of drug-protein adducts (Tailor et al., 2016). GSTP is important because it contains a highly reactive cysteine residue that is susceptible to drug binding. In recent years, proteomic methods have been used to characterise many drug-proteins adducts and identify the modified amino acids residues.

1.5.6.1 Electrophiles and neutrophils

Electrophiles (Lewis acids) are positively charged or neutral electron-deficient species that attract electron-rich species to form a covalent bond through addition and substitution reactions. Alternatively, nucleophiles (Lewis Bases) are negatively charged or charge neutral electron-rich species that donate electrons to form a covalent bond with electrophiles (Solomons et al., 2022).

Many drugs are metabolised into reactive intermediates that bind to proteins to form drug-protein adducts that may induce an immune response. Reactive metabolites including epoxides, quinones, nitroso compounds are well defined Phase I metabolites that act as electrophiles. They can cause cancer if they bind to DNA or a range of adverse events including drug hypersensitivity when they bind to

cellular proteins (Jurva et al., 2008, Naisbitt et al., 2001b). The nucleophilic sites in the cellular macromolecules proteins include the protein's N-terminus and amino acid side chains such as cysteine, histidine, and lysine (Lopachin et al., 2012).

The electron-deficient site of the electrophiles represents their reactivity site. Thus, the electrophiles are divided into charged neutrally soft electrophiles, and positively hard electrophiles based on how concentrated or diffuse their reactivity sites are (Attia, 2010). Hard electrophiles such as penicillins generally react with hard nucleophiles like lysine, whereas soft electrophiles such as Michael acceptors react with soft nucleophiles like cysteine and histidine (Lopachin et al., 2012, Doorn and Petersen, 2002).

For instance, carbamazepine (CBZ) 10, 11-epoxide is a major metabolite of CBZ, and it has been shown to form an adduct with glutathione S-transferase pi (hGSTP) at cys-47 (Yip et al., 2014). CBZ epoxide adducts have been displayed in the liver supersomes through cytochrome P450 3A4 (CYP3A4) biotransformation (Kang et al., 2008) (Figure 1.8). Furthermore, paracetamol (acetaminophen) is metabolised into toxic *N*-acetyl-*p*-benzoquinoneimine (NAPQI) that covalently binds to cysteinyl residues on proteins and causes hepatotoxicity (Pumford et al., 1990) (Figure 1.8).

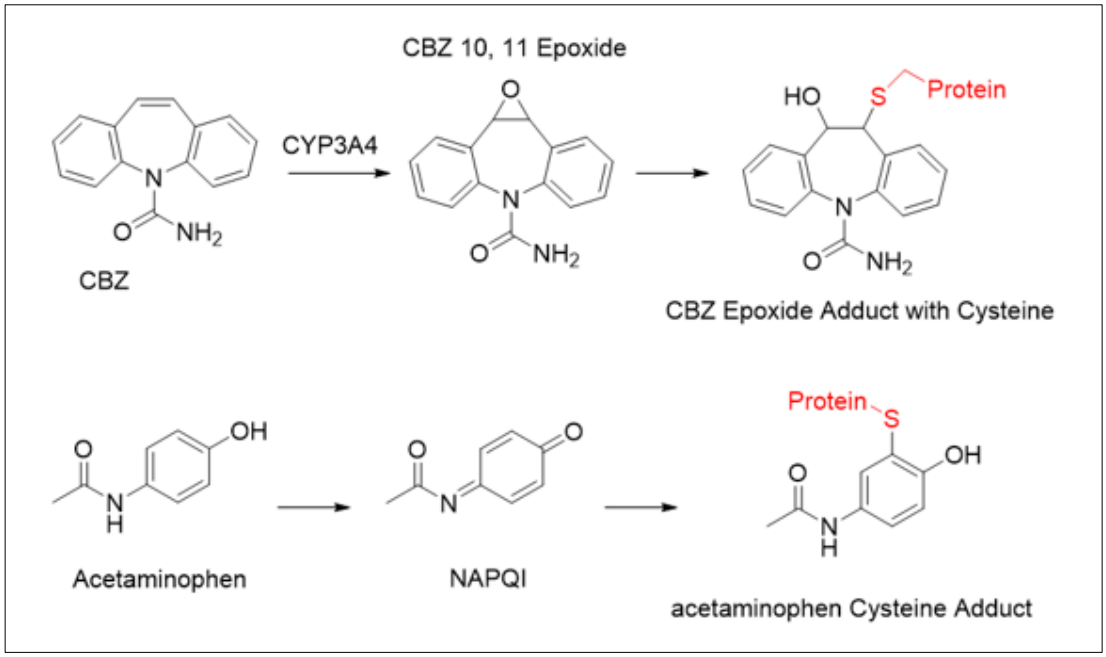

Figure 1.8: Scheme depicting the metabolism and adduct formation with carbamazepine and acetaminophen. Carbamazepine is metabolised by CYP3A4 to a 10,11 epoxide which can bind to cysteine. Acetaminophen is metabolised to N-acetyl-p-benzoquinone imine (NAPQI), forming adducts through cysteine binding.

1.5.6.2 Proteomics Techniques

Proteins are large biomolecules made up of smaller components known as amino acids linked together in long chains. Proteins play various roles in the organism, including structural, signalling, defence, transport, storage, and enzymatic functions. The term "proteome" refers to the whole set of proteins of a particular cell, tissue, or organism at any given time.

Proteomics is a broad term that encompasses wide-ranging experimental analysis of proteins and proteomes. It is the next phase in studying biological phenomena after genomics and transcriptomics. It is more complex than genomics because proteomes vary from cell to cell and across time, whereas genomes are somewhat consistent (Molnar, 2019).

Transcriptomics (mRNA expression) was previously used to investigate the protein expression; however, it was shown to not always correlate with protein levels because mRNA expression does not necessarily result in protein synthesis (Beynon, 2005). Additionally, many proteins are exposed to a wide range of post-translational modifications.

Proteins can be analysed using a variety of proteomics techniques, including antibody-based techniques (such as ELISA and western blotting), gel-based techniques (such as two-dimensional gel electrophoresis), chromatography-based techniques (such as ion-exchange chromatography (IEC), size exclusion chromatography (SEC) and affinity chromatography), protein microarrays, and finally mass spectrometry (MS)-based proteomics (Aslam et al., 2017).

1.5.6.2.1 MS-based proteomics

"Bottom-up proteomics" and "top-down proteomics" are the two most used techniques for analysing proteins using MS. In bottom-up proteomics, proteins are digested into peptides using proteolytic enzymes before MS analysis, whereas top-down proteomics analyses intact proteins. Bottom-up proteomics can be performed in two ways. First, by a gel-based method, the protein mixture can initially be separated by gel electrophoresis. The separated proteins are then extracted from the gel, digested into peptides, and analysed by MS. Second, by a gel-free method, where protein mixture can initially be digested into several peptides, separated by multidimensional separation methods before MS analysis (Figure 1.9). Bottom-up proteomics is the most established and commonly used method for identifying and characterising proteins, and it doesn't require complex instruments or specialised knowledge. It can also separate proteins with excellent resolution. However, this

approach has some disadvantages; for example, due to the sensitivity of detection, only a small portion of the protein sequence can be identified, leaving essential information about post-translational modification (PTM) or sequence variants undetected (Tim, 2006, Catherman et al., 2014, Fournier et al., 2007).

Top-down proteomics can characterise intact proteins, and it includes identifying proteins in complex mixtures without the need for protein digestion into peptides. Intact proteins are initially separated from complex biological samples using conventional separation techniques and then analysed using MS (Figure 1.9). The top-down proteomics is a time-saving approach and has two significant advantages: determining the entire protein sequence and the ability to detect and characterise PTMs. However, there are some limitations, such as the preferred instrumentation is costly to buy and run, and it is limited to analysing single proteins or simple mixtures (Tim, 2006, Catherman et al., 2014).

Figure 1.9: Flowchart of top-down and bottom-up proteomics.

Mass spectrometers are composed of three main parts: an ion source, a mass analyser, and a detector. Matrix-assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI) is the most common ionisation technique where the biomolecule sample is converted into gas-phase ions. The ions are then transported to the mass analyser and are separated according to their mass-to-charge ratio (m/z). There are many mass analysers; each type has advantages and disadvantages and varies in mass accuracy. However, quadrupole time-of-flight (QTOF), linear trap quadrupole (LTQ), and Orbitrap are the most common high-resolution mass analysers. The separated ions are then recorded by the detector (Gregorich et al., 2014).

Tandem MS, also known as MS/MS, refers to MS consisting of more than one type of mass analyser to increase specificity and sensitivity. It includes triple quadrupole MS (TQMS), quadrupole–time of flight (Q-TOF), and hybrid MS. For instance, TQMS is a tandem MS involving two quadrupole mass analysers (Q1 and Q3) with a collision cell (q2) between them. Q1 and Q3 scan over a mass range or monitor an ion of specific m/z value, while q2 is where fragmentation of the precursor ions isolated in Q1 occurs through collision with gas (Grebe and Singh, 2011, Pitt, 2009). There are various tandem scan modes, such as product ion scan and multiple reaction monitoring (MRM). In a product ion scan, a pre-defined precursor ion is selected in the Q1, fragmented in the q2, and then the entire m/z range is scanned in the Q3 and then detected. On the other hand, In MRM, both mass analysers (Q1 and Q3) are set to monitor a specific m/z value for precursor and product ions, resulting in the detection of a specific fragment ion from a particular precursor (Pitt, 2009, Grebe and Singh, 2011) (Figure 1.10).

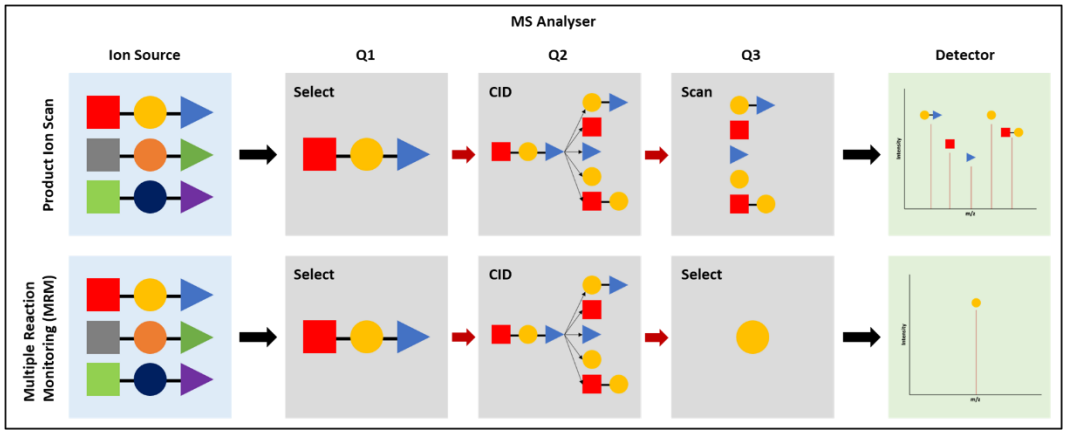

Figure 1.10: Two examples of TQMS operation modes: product ion scan and MRM. Q1 and Q3 mass analysers are either fixed (select) for a specific m/z value or scanned over a mass range. Q2 is a collision cell that induces fragmentation through collision-induced dissociation (CID).

1.5.6.2.1.1 De Novo Peptide Sequencing

Currently, Peptide sequencing is more commonly performed using data from a tandem MS experiment. In general, two methodologies are used: database search and de novo sequencing. In the database search, the MS/MS data is searched against a known database such as UniProtKB/Swiss-Prot with certain parameters including enzymatic digestion and PTMs to see whether there is a match with a previously identified peptide sequence. Peptide sequence with the highest matching score is selected for identification. Because it can only compare to pre-existing sequences in the database, this method does not identify novel peptides. In contrast, De novo peptide sequencing is a method used to determine the amino acid sequence of a peptide (including novel peptides) from its tandem mass spectrum (MS/MS). Six primary types of sequence ions are produced when the peptide backbone bonds cleave, including the N-terminal charged fragment ions (a, b, or c) and the C-terminal charged fragment ions (x, y, or z); however, a, b, and y ions are the most commonly seen fragment ions in the MS (Yan et al., 2017, Lu and Chen, 2004). The b and y ions appear to extend from the N-terminus (b1, b2, b3 etc.) and the C-terminus (y1, y2, y3 etc.), respectively. The fragments usually seen in a MS/MS spectrum represent the fragment ions formed in a collision cell of MS. The mass difference between these fragments determines the peptide sequence. In other words, the mass difference between two fragment ions reflects the monoisotopic mass of an amino acid residue on the pep-tide backbone (Hughes et al., 2010, Webb-Robertson and Cannon, 2007). For instance, the mass difference between the b_3 and b_4 ions in Figure 1.11 equals 71.037, the monoisotopic mass of

residue alanine (A). Similarly, the mass difference between b_4 and b_5 can identify the next nearby residue as cysteine (C).

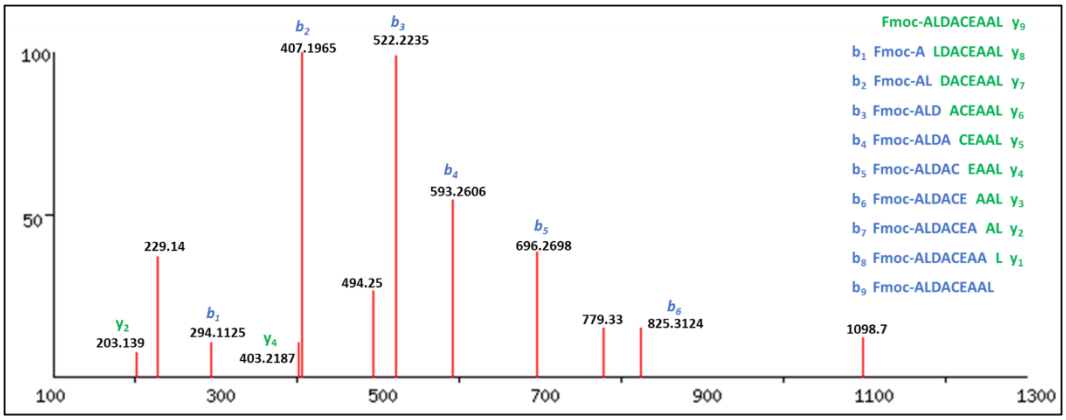

Figure 1.11: Example MS/MS spectrum of the peptide (Fmoc-ALDACEAAL) to explain the de novo peptide sequencing using b and y ions.

1.6 Dapsone (DDS)

DDS, also known as diaminodiphenyl sulfone (DDS), is an antibacterial and anti-inflammatory antibiotic synthesised in 1908 by Fromm and Wittmann. It is commonly used in various cutaneous diseases, including leprosy (Convit et al., 1970), and in combination with other drugs or the treatment or prophylaxis of P. falciparum malaria (Keuter et al., 1990). Chemically, DDS (4,4'-diamino-diphenyl sulfone) is an aniline derivative with 2 benzene rings attached via a sulfone group (Figure 1.12). DDS is entirely absorbed from the gastrointestinal tract with a bioavailability of more than 86 % after oral administration (Pieters and Zuidema, 1987), and peak plasma concentrations are reached within 2 to 8 hours. After absorption from the gut, DDS is transported to the liver through the circulation and metabolised by two different pathways: first, through *N*-acetylation by *N*-acetyltransferase to monoacteyldapsone (MADDS) (Ellard et al., 1972); and second, through *N*-hydroxylation by cytochrome P-450 enzymes to DDS hydroxylamine

(DDS-NOH) (Fleming et al., 1992). DDS can also be metabolized by polymorphonuclear leukocytes (PMN) and mononuclear cells (Uetrecht et al., 1988). DDS is metabolised into reactive DDS-NOH, which are thought to be responsible for the development of cutaneous drug reactions (Svensson, 2003). it has been demonstrated that DDS is bioactivated into DDS-NOH within normal human epidermal keratinocytes (NHEKs) (Reilly et al., 2000), resulting in the formation of a drug-protein adduct (Roychowdhury et al., 2005). Multiple cytochrome P450 enzymes, mainly CYP2C8/9, are involved in the N-hydroxylation of dapsone (Winter et al., 2000). However, previous studies have demonstrated that only flavin-containing monooxygenases (drug-metabolizing enzymes) appear to contribute to the bioactivation of DDS in NHEKs (Vyas et al., 2006b, Vyas et al., 2006a).

Like other sulfonamides, DDS is bacteriostatic and exerts a pharmacological effect by inhibiting the folic acid synthesis in a susceptible pathogen (Lang, 1979). In addition, DDS has anti-inflammatory actions by inhibiting the respiratory burst, mainly by inhibiting myeloperoxidase activity that has a function in transforming hydrogen peroxide (H_2O_2) into the most toxic oxidant hypochlorous acid (HOCl) (Wolf et al., 2002). DDS exposure is associated with various adverse effects, including pharmacologic reactions such as methemoglobinemia or idiosyncratic reactions such as dapsone hypersensitivity syndrome (Hindka et al., 2021, Zhang et al., 2013a). These adverse events will be explained in detail in the following chapters.

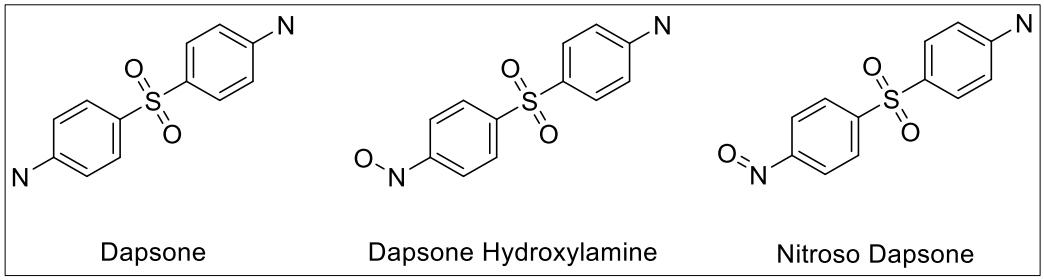

Figure 1.12: Chemical structure of DDS and its metabolites oxidative metabolites.

1.7 Thesis Aims

Using blood samples from DDS hypersensitive patients, we performed a set of immunological and proteomics experiments to investigate immunological factors that may be involved in DDS-specific T-cell activation.

Thus, the specific aims of the thesis were

- Study T-lymphocyte activation with DDS and nitroso dapsone (DDS-NO) using human blood samples from DDS hypersensitive patients.
- Study the relationship between HLA-B*13:01 expression on antigen presenting cells and the activation of T-cells with DDS and DDS-NO.
- Design a synthetic peptide approach to assess hypersensitive patient HLA-restricted T-cell activation with DDS-NO.

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2.1 Human Subjects

Up to 120 mL of venous blood was collected from clinically diagnosed dapsone (DDS) hypersensitive patients (patch-test positive). The Liverpool local research Ethics Committee approved this research, and informed consent was taken from all participating blood donors.

2.2 MEDIA & BUFFERS

Table 2.1: Media, Buffers, Reagents, chemicals, and drugs. Table showing the different Media and supplements used in the cell culture and tests. The tests' buffers, reagents, and drugs with suppliers are also listed.

Media and Buffers			
Name	Used for	Contents	Supplier
R9 medium	culture of PBMC and T-cell cloning	500 ml Roswell Park Memorial Institute (RPMI) 1064	Sigma-Aldrich (Dorset, UK)
		100 µg/ml penicillin	
		25 µg/ml transferrin	
		100 U/ml streptomycin	
		25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer	
		2 mM L-glutamine	
		10% (v/v) human AB serum	Innovative Research (Michigan, USA)
F1 medium	culture of immortalised EBVs derived B-cell lines	500 ml RPMI 1064	Sigma-Aldrich (Dorset, UK)
		100 µg/ml penicillin	
		100 U/ml streptomycin	
		2 mM L-glutamine	
		25 mM HEPES buffer	
		10% (v/v) Foetal Bovine Serum (FBS)	Invitrogen (Paisley, UK)
FACS Buffer	Cell preparation for flow cytometry	500 mL Hanks balanced salt solution (HBSS)	Sigma-Aldrich (Dorset, UK)
		sodium azide (0.02%)	
		10% (v/v) FBS	Invitrogen (Paisley, UK)
MACS Buffer (X	Cell separation	50 ml HBSS	Sigma-Aldrich (Dorset, UK)
		50 µg/ml bovine serum	

10)		albumin (BSA)	
		20 mM ethylenediaminetetraacetic acid (EDTA)	
Reagents, chemicals, and drugs			
Reagent		Supplier	
Dimethyl sulfoxide (DMSO)		Sigma-Aldrich (Dorset, UK)	
Lymphoprep		Axis-Shield (Dundee, UK)	
Magnets		Miltenyi Biotec (Surrey, UK)	
magnetic columns			
antibody-conjugated magnetic bead kits			
an anti-$CD4^+$ antibody, anti-$CD8^+$ antibody, anti-CD3 antibody [using mouse monoclonal FITC, PE, APC conjugate]		BD Biosciences (Oxford, UK)	
HLA blocking antibody and isotype control [anti-human HLA-ABC, mouse anti-human HLA-DR/DP/DQ, mouse anti-human HLA-DR anti-human HLA-DQ]			
mouse anti-human HLA-DP		Serotec (Kidlington, UK)	
Elispot 96 wells filter plates		Millipore (Watford, UK)	
Elispot reagent kits [Capture mAb, Detection mAb, biotin, Streptavidin-ALP/HRP,]		mabtech (Nacka Strand, Sweden)	
BCIP/NBT Plus liquid substrate solution			
Fas ligand Elispot reagent kits		Abcam (Cambridge, UK)	
interleukin 2		Peprotech (London, UK)	
GM-CSF			
interleukin 4			
[3H]-thymidine (5 Ci/mmol)		Moravek (California, USA)	
filter mats, sample bags, and Meltilex scintillator sheets		Perkin-Elmer (Waltham, USA)	
Cyclosporine A		Fluka Analytical (Dorset, UK)	
tetanus toxoid (TT)		Statens seruminstitut (Copenhagen, Denmark)	
Carboxyfluorescein diacetate succinimidyl ester (CFSE)		eBioscience (San Diego, USA)	
Cell culture flasks and Nunc plates		Thermo Scientific (UK)	
DDS and Dapsone hydroxylamine (DDS-NOH)		Sigma (UK)	
Leica DME microscope		Lecia Microsystems (Milton Keynes, UK)	
cryovials		Alpha Laboratories (Hampshire, UK)	

MultiSort kits	Miltenyi Biotec (Surrey UK)
Acetonitrile	Fischer Scientific (Loughborough, UK)
formic acid	VWR (Radnor, PA, USA)
phytohemagglutinin (PHA)	Sigma (UK)
TCR Vβ Repertoire Kit	PerkinElmer (Llantrisant, UK)

2.2.1 Synthesis of Nitroso Dapsone

DDS-NO was synthesized from DDS hydroxylamine using iron (III) chloride according to the method of Naisbitt et al. (1996). Briefly, DDS hydroxylamine (60 mg, 0.227 mmol) was dissolved in absolute ethanol (10 mL) and added to a stirred solution of iron (III) chloride hexahydrate (0.5g, 1.85 mmol) in distilled water (10 mL) over a period of 5-10 min. The reaction mixture was stirred at room temperature for 5 min and a yellow precipitate was formed. The mixture was filtered under vacuum and the yellow solid product was analysed for purity using LC-MS. A solution for analysis was prepared immediately by ten-fold dilution with acetonitrile. The LC-MS equipment was a 1260 Infinity LC system (Agilent Technologies, Waldbronn, Germany) connected to a 400 Qtrap (Sciex, Warrington, United Kingdom). An aliquot (3 µL) was eluted from an Agilent Eclipse XDB-C18 column (3.5-µm; 150 × 4.6 mm) at 0.5 mL/min with ACN-formic acid (0.1%, v/v) in formic acid (0.1%, v/v): 25% to 75% over 10 min; 75% for 5 min; 75% to 25% over 0.1 min. Full scanning positive-ion mass spectra were acquired over 5 s between m/z 100-1,000. The ionspray voltage was 5.5 kV; desolvation potential, 120 V; source temperature, 500 °C.

2.3 T-cell Methodology

2.3.1 Isolation of PBMCs from venous blood

PBMCs were isolated from patient peripheral blood collected in heparinised vacutainer tubes. Using 50 ml falcon tubes, the blood (25 mL) was carefully layered on top of 25 mL of lymphoprep using a 50 ml syringe and centrifuged for 25 min at 1610 rcf at 25°C with acceleration set to 4 and no brake. The PBMC layer (buffy coat layer) was carefully collected to a new 50 ml tube using a 10 ml Pasteur pipette. The PBMCs were washed twice with 30-40 ml of HBSS and centrifuged for 15 min at 1449 rcf at 25°C with acceleration and brake set to 9 to remove any remaining lymphoprep. The PBMCs were finally washed with 30-40 ml of HBSS and centrifuged for 10 min at 1208 rcf at 25°C with acceleration and brake set to 9. The pellet was resuspended in R9 medium before counting. The PBMCs were counted using a Neubauer hemacytometer under a Leica DME microscope after diluting 10µL of the cell suspension with 10 µl of 0.4% trypan blue. Cell viability was calculated as the live cell count / total cell count (the live and dead cell count) * 100.

Cells also can be frozen in 1.7 ml cryovials by resuspending them at 10-20 x 10^6 cells/ ml R9. In the cryovial, 0.5 ml of freeze mix (80% pooled human AB serum/20% DMSO) was added as drops to the same volume of cells on ice. Cryovials were frozen in Mr Frosty containers at -80°C for 24 hrs before being moved to -150°C for longer-term storage and later use.

2.3.2 Optimal Drug Concentrations For T-Cell Assays

The optimal concentration of drugs was determined using a PBMC toxicity assay. The objective of the assay was to detect the maximum non-toxic concentration of the drug (i.e., the concentration that did not inhibit PBMC proliferation). Graded

concentrations of drug were incubated with PBMCs ($1.5x10^5$) isolated from healthy volunteers for five days with the addition of PHA (5 µg/ml) for the final 24 hrs to provide a non-specific stimulation of cells. [3H]-thymidine was then added for the final 16 hrs of the incubation. Cells were then harvested, and the proliferative response of PBMC was analysed using a beta counter. The maximum concentrations for each compound used in T-cell assays were selected based on the highest concentration with no toxicity seen.

2.3.3 Lymphocyte Transformation Test (LTT)

LTT was performed to determine the presence of antigen-specific PBMCs in the peripheral blood of hypersensitive patients. PBMCs (100µl, 150,000 cells/well) were plated in triplicate into Nunc U-bottomed 96-well plates alongside 100 µl of 2 X the optimal concentrations of the drug. Tetanus toxoid (5 µg/mL) was used as a positive control and R9 medium as a negative control. The plates were incubated for six days at 37^{o}C, 5% CO^2. [3H]-thymidine was added to each well (0.5 µCi/well) for the final 16 hrs of the incubation. Cells were harvested onto paper filter mats using a Tomtec harvester 96 (Receptor Technologies). The filter mats were dried and sealed with MeltiLex scintillator sheets using a Wallac 1495-021 Microsealer (Perkin-Elmer, UK). The sealed filter mats were analysed using a Microbeta Trilux 1450 LSC (Perkin-Elmer, UK). The data generated is shown as radioactive counts per minute (CPM) representing the amount of [3H]-thymidine incorporated into the cells' DNA that provides a quantitative measure of cell division and proliferation. Alternatively, a stimulation index (SI) (Mean CPM of drug-treated cells / mean CPM of control cells) will be used to determine the positive or negative proliferative response, and an SI value of ≥2 is considered positive (Pichler and Tilch, 2004).

2.3.4 T-cell Cloning

Drug responsive T-cells are often present in the memory population of hypersensitive patients. This T-cell population can be used to investigate phenotypical and functional properties of T cells at the single cell level (T-cells cloning) (Figure 2.2).

2.3.4.1 Generation of EBV-Derived B-Cell Lines

Autologous Epstein-Barr virus-derived B-cells were used as functional APCs in several T-cell assays because they constantly divide as EBV incorporates into the DNA of B -cells within the donor PBMCs. EBVs were generated from a healthy donor or patient PBMCs using the following procedure. 7 ml of the B95.8 cell line (previously obtained from the European Cell Culture Collection) supernatant were collected to isolate the Epstein-Barr virus. The supernatant was filtered through a 0.22 µm filter to remove any remaining B95.8 cells. 5 ml of the filtered supernatant was then used to resuspend 5 x 10^6 donor or patient PBMCs. Cells were incubated for 24 hours at 37^{o}C, 5% CO_2 to generate transformed B-cells, and Cyclosporine A (CSA) (1 µg/ml) was added to prevent T-cell and un-transformed B-cell growth. Cells were then centrifuged, resuspended in 2 ml of F1 medium, and plated in a 24-well flat-bottomed plate at different cell concentrations. Cells were fed with 1 ml F1 medium supplemented with CSA (1 µg/ml) and incubated for three weeks with F1 CSA (1 µg/ml) feeding every three days. After two weeks, CSA was removed from the feeding medium to enhance B cell expansion. After sufficient growth of EBVs-transformed B-cells, the cells were transferred to cell culture flasks and fed twice a week with F1 medium.

2.3.4.2 Generation of T-cells Bulk Culture

Patient PBMCs were cultured in 48 well plates (500µl R9 medium; 1 x 10^6 cells/well) for 14 days, at 37°C, and 5% CO_2 with drug or drug metabolite (500µl; 2X of the optimal concentrations). Cells were fed with 330 µl R9 medium supplemented with interleukin 2 (IL2; 10 U/ml) on days 6 and 9 and split if required.

2.3.4.3 Serial Dilution of Bulks

On day 14, $CD4^+$ and $CD8^+$ T-cells were separated using MultiSort kit and serially diluted to generate T-cell clones (TCCs) (Figure 2.1). Each $CD4^+$ and $CD8^+$ bulk PBMC culture was counted and resuspended in R9 medium at a density of 1 x 10^4/ml. In addition to the bulk, serial dilution needs a restimulation cocktail that constitutes R9 medium supplemented with IL2 (10 U/ml), phytohemagglutinin (PHA) (5 µg/mL), and donor irradiated allogeneic PBMCs (5x10^5 cell/mL). The donor PBMCs were irradiated for 15 mins (45 Gy) to avoid cell proliferation while preserving cell surface antigen expression. Three tubes (35 ml) of restimulation cocktail were used for each drug condition, and 10 µl, 35 µl, and 105 µl of bulk suspension were added to each tube to obtain three 96 well plates (100 µl) with 0.3 cell/well, three with 1 cell/well, and three with 3 cell/well). The plates were incubated for 14 days at 37 °C and 5% CO_2 and fed with R9 medium (25 µl) supplemented with IL2 (10 U/ml) on day five and then every two days.

The well-growing TCCs displayed a clear cell pellet with a yellow medium. These TCCs were picked and transferred into two wells (half volume each) in a new 96-well plate. The new plates were incubated at 37 °C and 5% CO_2 and were fed with R9 medium (25 µl) supplemented with IL2 (10 U/ml) and were split into additional wells once the cell pellet expanded to end with four wells for each picked TCC.

After multiple feedings for the initial serial dilution plates, the volume of each well was reduced to 80 µl and restimulated with 50 µl of fresh restimulation cocktail. The plates were incubated at 37 °C and 5% CO_2 for another 14 days with continuous feeding with R9 medium (25 µl) supplemented with IL2 (10 U/ml) every two days. The picking process was repeated using these plates until all growing clones were established in 4 wells of a 96 well plate.

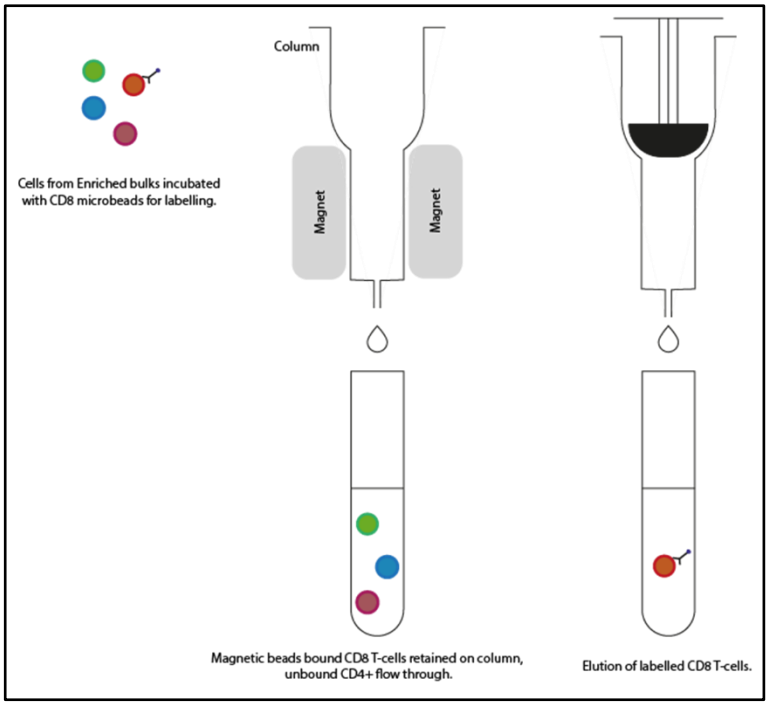

Figure 2.1: Diagram of the positive selection of $CD8^+$ T-cells using magnetic microbeads.

2.3.5 Antigen Specificity Test Via T-cell Proliferation Assay

The proliferation of drug-specific TCCs was performed for the picked TCCs. Two of the four wells were used to perform the test. The volume of the two wells was reduced to 100 µl, mixed, and transferred to 4 wells in a new 96-well plate (50 µl each). Cells were incubated with 50 µl of $2x10^5$ cells/ml irradiated autologous EBV-transformed B-cells (20 mins irradiation (60 Gy)). Two wells were used as a negative control with 100µl R9 medium, and 100µl of the diluted drug was added to the other two wells with 2X of the optimal concentration. The plates were incubated at

37 °C and 5% CO_2 for 48 hours. Proliferation was measured using [3H]-thymidine incorporation, as explained previously.

TCCs with a proliferative SI ≥2 were considered antigen-specific and picked and expanded in Nunc 48 well plates using the same stimulation cocktail outlined above containing irradiated allogenic PBMCs ($5x10^5$ cells/well), IL-2 (10 U/ml) and PHA (10 µg/mL). TCCs were cultured for 14 days before use in functional tests.

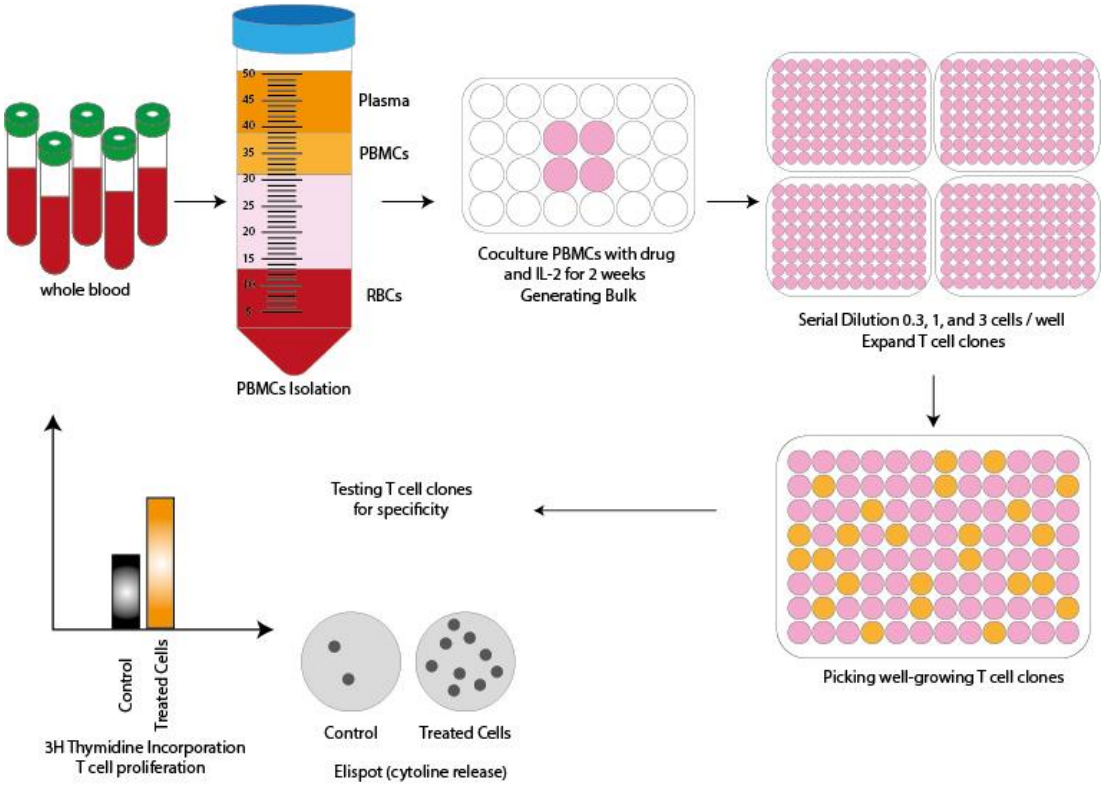

Figure 2.2: Generation of drug-specific TCCs from the blood of hypersensitive patients or healthy volunteers.

2.3.6 Elispot Assay

ELISpot was performed to measure the cytokines and cytolytic molecules released from stimulated TCCs. Elispot plates were wetted with 15µL of 35% ethanol (EtOH) and washed immediately five times with distilled water (dH_2O) (200µl/well). The plates were then coated with diluted capture antibody in HBSS (200µl/well) and

were incubated for 24 hours at 4^{o}C. The next day, the plates were washed five times with HBSS and blocked with R9 medium (100µl/well) for 30 mins at room temperature to prevent non-specific binding. The blocking medium was discarded, and the 50 µl of TCCs (5 x 10^4 cells/well) were incubated with 50 µl irradiated autologous EBV-transformed B-cells (1 x 10^4 cells/well), in the presence of drug (100 µl) or absence of drug (control wells, 100 µl R9 medium). The plates then were incubated for 48 hours at 37 °C and 5% CO_2. The plates were then washed five times with HBSS, and the biotin-labelled detection antibody was diluted in HBSS containing 0.5% FBS and was added to the plates for 2 hours (100 µl/well) at room temperature. The plates then were washed five times with HBSS and incubated with 100 µl Streptavidin-ALP diluted in HBSS containing 0.5% FBS for one hour at room temperature. The plates then were washed five times with HBSS and incubated in the darkroom at room temperature with 100 µl of filtered BCIP/NBT substrate for 15-30 mins. The plates were then washed under tap water to stop the reaction. The plates then were left overnight to dry at room temperature. The spots in the wells were counted using an AID ELISpot reader (Cadima Medical, Stourbridge, UK).

2.3.7 T-cells Phenotyping Using Flow Cytometry ($CD4^+$/$CD8^+$, Chemokines, and TCR Vβ)

Flow cytometry was used to detect the $CD4^+$ and the $CD8^+$ markers on the surface of the TCCs that could be classified into different phenotypes based on their marker expression. The flow cytometry analysis was performed using the following procedure. 50 µl of a well-growing TCC was transferred to the FACS tube. 1 µl of mouse monoclonal PE-conjugated anti-$CD8^+$ antibody and 3 µl of mouse monoclonal APC conjugated anti-$CD4^+$ antibody were added to each tube in the

dark and incubated for 20 mins on ice. Unstained T-cells (no antibody added) were also used as a control. 0.5 ml FACS buffer was then added to each tube and centrifuged (1208 rcf, full acceleration, and activated brake) for 5 mins at 4^{o}C. The supernatant was discarded, and the cells were resuspended with 200 µl FACS buffer. The cells were finally analysed for phenotyping using BD FACS CANTO II (BD Biosciences, Oxford, UK). 4% paraformaldehyde (PFA) was used in the final step instead of FACS buffer for non-immediate analysis. In some tests, mouse monoclonal FITC conjugated anti-CD4^{+} was used instead of APC conjugated anti-CD4^{+}.

Likewise, we also analysed the chemokine receptors expressed by TCCs using flow cytometry. A well-growing TCC was transferred to 5 FACS tubes (50 µl each). Groups of three antibodies with APC, FITC, and PE-conjugated fluorophores were added to each tube in the dark to end with 15 different markers for each TCC. The tubes underwent sample preparation and flow cytometry analysis as described above.

Similarly, we analysed TCR Vβ expression of the TCCs using the IOTest® beta mark (Beckman Coulter, Indianapolis, USA). The TCR Vβ kit consists of 8 antibodies (A-H). Each antibody can detect three different TCR Vβ (FITC, PE, and FITC/PE). A well-growing TCC was transferred to 8 FACS tubes (50 µl each) and labelled A-H. In the dark and at room temperature, the antibodies were added to their corresponding tube. The tubes underwent sample preparation and flow cytometry analysis as described.

Antibodies used for flow cytometry staining purchased from BD Biosciences (Oxford, UK) were CD4^{+}-APC (clone RPA T4), CD8^{+}-PE (clone HIT8a), CCR4-PE (clone 1G1), CLA-FITC (clone HECA-452); and from R&D Systems (Abingdon, UK) were

CCR1-Alex Fluor 488 (clone 53504), CCR2-PE (clone 48607), CCR3-FITC (clone 61828), CCR5-FITC (clone CTC5), CCR6-APC (Clone 53103), CCR8-PE (clone 191704), CCR9-APC (clone 248621), CCR10-PE (clone 314305), CXCR1-FITC (clone 42705), CXCR3-APC (clone 49801), CXCR6-PE (clone 56811) and E cadherin-Alexa Fluor 488 (clone 180224).

2.3.8 Dose-Response and Cross-Reactivity Studies

TCCs' responses were tested with different drug doses around the optimal concentration, and the response was analysed using the proliferation assay as described before or by ELISpot. In addition, TCCs also were tested with different doses of structurally related compounds or metabolites.

2.3.9 HLA Restriction Analysis

2.3.9.1 APC Dependency of the Drug-Specific T-cell Response

This test was always performed with the same protocols for dose-response and cross-reactivity tests and analysed via assessment of proliferation as described before. Briefly, TCCs (5 x 10^4 cells/well) were incubated without APC in the absence of drug (as a control) and with drug (as treated cells) to study the importance of APC for T-cell activation. The TCCs that proliferated in the absence of APC are referred to as 'Self Presenting'.

2.3.9.2 MHC Blocking

MHC blocking assay was performed to analyse the TCCs restriction to MHC I or MHC II. Irradiated autologous EBVs (1 x 10^4 cells/well) were incubated in 96 well plates with either MHC I or MHC II blocking antibodies at a concentration of 5 µg/mL and their isotypes for 30 mins. TCCs (5 x 10^4 cells/well) were then added to the plate in the presence or absence of the drug. Unblocked EBVs were used as a control, and

the plates were incubated for 48 hours at 37 °C and 5% CO_2. The T-cell proliferation was tested using [3H]-thymidine incorporation as described above to assess T-cell activation. IFN-γ Elispot was also used to analyse the T-cell cytokine release. A reduction in the T-cell response with either MHC I or MHC II indicates that this TCC is restricted to MHC (I or II).

2.3.9.3 Mismatch Assay (Allogeneic APC Assay)

After analysing the TCCs restriction to either MHC I or MHC II, they were tested to assess their restriction to specific HLA alleles using an allogeneic APC assay. The TCCs (5 x 10^4 cells/well) were incubated with irradiated autologous EBVs (1 x 10^4 cells/well) and with irradiated allogenic EBVs (1 x 10^4 cells/well) in presence or absence of drug. These allogenic EBVs consists of HLA's of interest- (positive or negative) donors. The plates were tested for T-cell activation using a standard proliferation assay.

2.3.10 Antigen Processing Analysis

2.3.10.1 Pulsing Assay

To assess pathways of drug presentation to TCCs, the T-cell proliferation was analysed with drug-pulsed APC. Autologous EBV-transformed B-cells (2 x 10^5 cells/ml) were centrifuged, resuspended in R9 medium, and treated with the optimal concentrations of the drug. The EBV-transformed B-cells were incubated for different time points (30mins – 24 hours) at 37°C and 5% CO_2. The cells were then washed 3-5 times to remove the non-covalently bound drug. These EBV-transformed B-cells, now referred to as 'Pulsed EBVs' were irradiated and incubated (1 x 10^4 cells/well) with TCCs (5 x 10^4 cells/well) in the absence of soluble drug. The plates were incubated for 48 hours at 37 °C and 5% CO2. Irradiated un-pulsed

autologous EBV-transformed B-cells were used in this test as a control and incubated with TCCs in the presence or absence of drugs. T-cell proliferation was analysed using [3H]-thymidine incorporation as described above. The T-cell response can be either dependent on the formation of drug-protein adducts where the T-cell response is detected with pulsed EBV-transformed B-cells, or a direct pharmacological interaction of a drug with MHC where the response only occurs with EBV-transformed B-cells and soluble drug.

2.3.10.2 Fixation Assay

To investigate whether TCCs proliferate in an antigen processing dependent or independent manner, TCCs were tested with fixed EBV-transformed B-cells to block the antigen uptake. Autologous EBV-transformed B-cells (2 x 10^6 cells/ml) were fixed with glutaraldehyde (1 µL/mL, 25%) for 30 seconds at room temperature. Glycine (0.2M) was then added to stop the reaction for 45 seconds. The fixed EBV-transformed B-cells were then washed three times, irradiated, and incubated with TCCs (5 x 10^4 cells/well) in the presence or absence of drugs. The irradiated un-fixed autologous EBV-transformed B-cells were also used in this test alongside fixed cells as a control. The plates were incubated for 48 hours at 37 °C and 5% CO_2. T-cell proliferation was analysed using [3H]-thymidine incorporation as described above. TCCs that proliferate only with unfixed EBVs indicates an antigen processing dependent pathway of drug presentation.

2.3.11 Glutathione (GSH) Inhibition Assay

The addition of excess glutathione to T-cell assays prevents the covalent binding of nitroso compounds to protein and the activation of drug metabolite-specific T-cell clones via a hapten mechanism. Hence, it is possible to explore whether T-cells are

activated with drug metabolite-modified protein adducts. TCCs were cultured with DDS or nitroso dapsone (DDS-NO) and EBV-transformed B-cells or drug-pulsed EBV-transformed B-cells in the presence or absence of glutathione (1mM). The stability of DDS and DDS-NO and the formation of DDS glutathione adducts during the culture period were measured by mass spectrometry (MS) as described below.

2.4 Proteomics And Immunochemical Methodology

2.4.1 Sample Preparation Using ZipTip

ZipTip is an ideal application for concentrating and purifying peptides, and they were used in this project to prepare a sample (digested proteins or peptides) for MS analysis.

SCX ZipTip

Samples were prepared in 20% acetonitrile (ACN)/ 10% Trifluoroacetic acid (TFA). SCX ZipTip (Merck Millipore, Burlington, MA, USA) was equilibrated three times with 10 µl 0.1% TFA. The sample was then pipetted slowly using the SCX ZipTip 15-20 times. The SCX ZipTip was then washed five times with 10 µl 0.1% TFA. Subsequently, the sample was eluted by pipetting up and down 15-20 times in 10 µl of the elution buffer (5% Ammonium Hydroxide (NH_4OH) / 30% Methanol (MeOH) / 0.1% TFA). The sample was then dried using nitrogen and was resuspended in 50 µl 0.1%FA / 2% ACN. 5 µl of the sample was used for MS analysis.

C18 ZipTip

C18 ZipTip (Merck Millipore, Burlington, MA, USA) was equilibrated three times with 10 µl ACN. The C18 ZipTip was then washed five times with 0.1% FA. The sample was then pipetted slowly using the C18 ZipTip 15-20 times. The C18 ZipTip has then rewashed five times with 0.1% FA. The sample was then eluted by

pipetting up and down 15-20 times in 10 µl of the elution buffer (70%ACN/ 0.1% FA). The sample was dried for 30 mins using a speed vac concentrator (Eppendorf, Hamburg, Germany) and was then resuspended in 60µl 2% ACN/ 0.1% FA for MS analysis.

2.4.2 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is an immunological tool that detects and measures different substances such as proteins, cytokines, and antibodies. It was used in this project to optimise some anti-drug antibodies. A 96 well plate was coated with 100 µl of the sample (antigen) solution in phosphate-buffered solution (PBS) (1 µg/ml) and was stored overnight at 4^{o}C. The plate was then washed five times with PBS / 0.1% tween and blocked with bovine serum albumin (BSA) (1 mg/ml in PBS) for two hours at room temperature. The blocking buffer was then removed, and the primary antibody was added with a range of concentrations in the blocking buffer and incubated for two hours at room temperature. The plate was then washed five times with PBS / 0.1% tween. The horseradish peroxidase secondary anti-rabbit antibody (Daco Scientific, Reading, UK) was added (1 µl in 2ml blocking buffer) for two hours at room temperature. The plate was then washed five times with PBS / 0.1% tween, followed by two washes with PBS. 100 µl/well of the diluted Avidin-HRP was added for 30 mins at room temperature and washed five times with PBS / 0.1% tween. The plate was then developed using tetramethylbenzidine (TMB) (100 µl/well), and 50 µl/well of 1M H2SO4 was then used to stop the reaction. Finally, the absorbance was detected at 405nm using a Dynex MRXe spectrophotometer (Magellan Biosciences) (Figure 2.3).

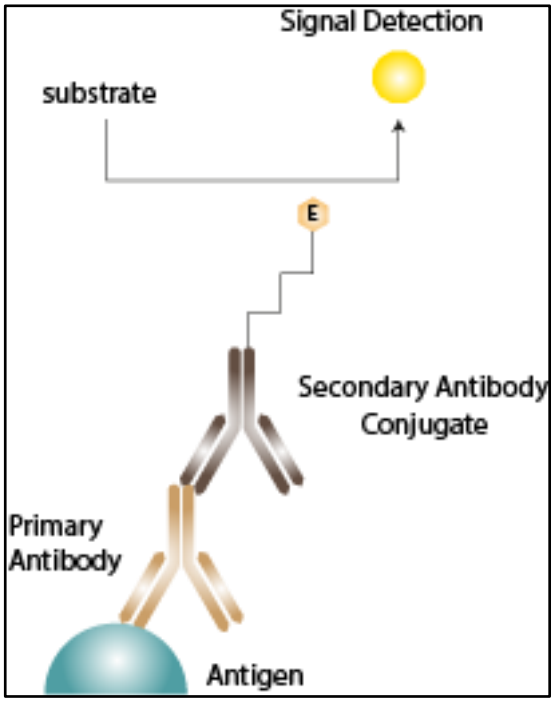

Figure 2.3: Diagram of the indirect ELISA.

2.4.3 Immunofluorescence (IF)

IF is a widely used biological method that visualises and localises various antigens within the cells and tissues. In this project, we used IF for the detection of drug and drug antibody binding. Patients autologous EBV-transformed B-cells (non-adherent cells) were cultured for 24 hours in the presence or absence of the drug. The cells were then washed and plated on a glass coverslip in 48 well plates using Cell-Tak (Corning). The cells were then centrifuged to attach to the surface. The cells were then washed and fixed for 30 mins at 4^{o}C using 4% PFA. The cells were then washed with PBS, permeabilised (0.004% Tween 20, 0.025% Triton-X-100, PBS) for 30 minutes at 4^{o}C. The cells were then blocked with BSA (5% in permeabilisation buffer) for 1 hour at room temperature, followed by the addition of a primary antibody in the blocking buffer overnight at 4^{o}C. The plate was sealed to avoid buffer evaporation. The next day, the cells were washed twice with the permeabilisation buffer for 15 mins, followed by the addition of secondary anti-

rabbit Alexa fluor 488 antibodies (Thermo Scientific, Waltham, MA, USA). Plates were incubated for 1 hour at room temperature. The cells were then washed with PBS and were stained for 20 mins with a mixture of Hoescht Alexafluor 352 (Dapi, 1 in 5,000 dilutions) and Phalloiden Alexafluor 568 (1 in 250 dilutions) (Thermo Scientific, Waltham, MA, USA). The cells were then washed twice with PBS and were mounted onto glass slides with Pro-Long Gold (Thermo Scientific, MA, USA) and sealed using nail varnish. Zeiss AxioObserver Z1 Microscope was used for image visualisation.

2.4.4 The Bradford Protein Assay

Bradford assay is a widely used technique to quantify proteins. 5 µl samples (diluted if required) and BSA standards (0.2 mg/ml to 1.4 mg/ml) were added to a 96 well flat-bottomed plate in triplicate. 200 µl of the Bradford reagent was added to each well (Sigma Aldrich, St. Louis, MO, USA), and the plate was incubated for 10 mins at room temperature for development. The absorbance was detected at 405 nm using a Dynex MRXe spectrophotometer (Magellan Biosciences).

2.4.5 Western Blotting

Drug-modified proteins were visualised using Coomassie blue staining, and their epitopes were detected using western blot. Ten µg of the protein samples were separated on a 10% SDS-PAGE gel (National Diagnostics, Atlanta, GA, USA) by electrophoresis (300 V, 60 mA, 1 hour). The separated proteins were then transferred onto a protran nitrocellulose membrane (GE Healthcare, Amersham, UK) by electroblotting (300 V, 250 mA, and 1 hour). The blotted membrane was then washed for 3 minutes with water and then stained with Ponceau S for 3 minutes to visualise the total protein. The blotted membrane was then blocked with

2% skimmed milk in Tris/saline/Tween (TST) buffer (150 mM NaCl, 10mM Tris-HCl, pH 8 0.05% Tween-20) for 2 hours at room temperature (or overnight at four ^{o}C). The blotted membrane was then incubated overnight at 4^{o}C with the optimal concentration of the primary antibody prepared in 2% skimmed milk. The blotted membrane was then washed with TST (4 times 4 minutes each) followed by the incubation of horseradish peroxidase-conjugated anti-rabbit IgG antibody (Daco Scientific, Reading, UK) prepared in 2% skimmed milk for 1 hour. The membranes were then washed with TST (4 times 4 minutes each), and signals were detected using Chemiluminescent blotting substrates (Western Lighting, PerkinElmer, Boston, USA), and were documented with autoradiography film (Amersham Plc, Amersham, UK) or ChemiDoc™ Imaging Systems (Bio-Rad) (Figure 2.4).

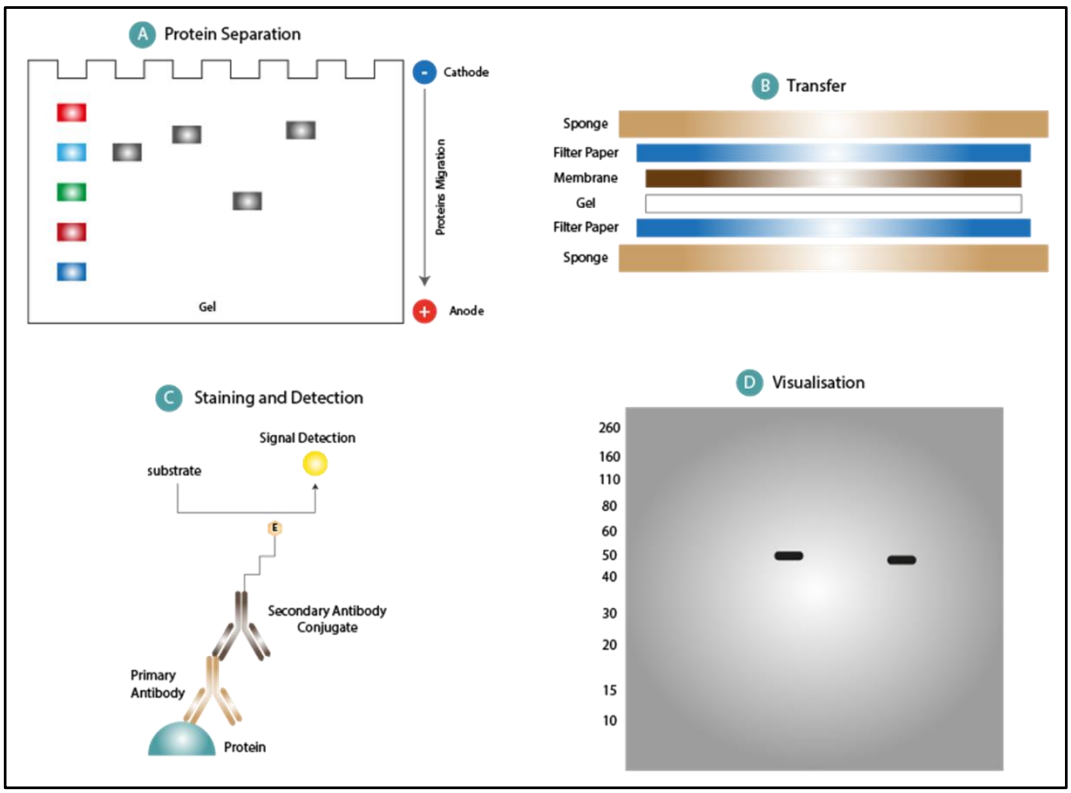

Figure 2.4: Diagram of the western blot.

2.4.6 Identification Of Un-Characterised Peptides Using TripleTOF MS

AB-Sciex Triple TOF 6600 and 5600 MS were used to analyse uncharacterised drug-modified peptides. Samples were loaded into the MS using automated in-line Reversed-phase liquid chromatography an Eksigent NanoUltra cHiPLC System (ABSciex) installed with a microfluidic trap and a ChromXP C18-CL column. Samples were loaded onto the microfluidic trap and washed for 10 min at two µL/min with 0.1% FA before analysis on the C18 column. The MS was coupled with a NanoSpray III source installed with a 10µm inner diameter PicoTip emitter (New Objective, Woburn, MA). The samples were run through the column using the following gradient method (2–50% ACN/0.1% FA, 300 nL/min, 90 mins). MS spectra were acquired using Data-dependent acquisition (DDA) mode operated by Analyst TF 1.5.1 software, and they were acquired in positive ion mode with a mass range of 400–1600 amu followed by MS/MS with a mass range of 100–1400 amu. Up to 25 MS/MS spectra were acquired per cycle (approx. 10 Hz) using a threshold of 100 counts per second, with the dynamic exclusion for 12 s and rolling collision energy. The PeakView 1.2.0.3 software (AB Sciex) was used to evaluate the spectral quality.

2.4.7 Sample Quantification Using Quadrupole Ion Trap (Q-Trap) MS

Samples were loaded into the QTRAP 5500 MS installed with a NanoSpray II source using automated in-line liquid chromatography (Eksigent NanoLC 400 system, Sciex; NanoAcquity 5 µm C18 180 µm x 20 mm C18 trap and 1.7 µm, 75 µm X 250 mm analytical column, Waters) via a 10um inner diameter PicoTip (New Objective, Woburn, MA).

Multiple reaction monitoring (MRM) is a very specific and sensitive method for quantifying targeted peptides or proteins. MRM transitions for the target peptides

(precursor ions) were created with their corresponding products. Based on this, the precursor ions will be selected in the first quadrupole (Q1) of the MS and transmitted to the second quadrupole (Q2) for additional fragmentation. The product ions will be detected from among the fragmented ions in the third quadrupole (Q3).

2.5 Statistical Analysis

Microsoft Excel was used to calculate mean values and standard deviations, and statistical analysis was performed using one way ANOVA, two way ANOVA and unpaired T-tests (GraphPad Prism 9 software).

3 Chapter 3: Characterization of the specificity and functionality of dapsone- and dapsone nitroso- specific T-cells isolated from dapsone hyper-sensitive patients expressing risk alleles HLA-B*13:01

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3.1 Introduction

Drug-specific T-lymphocytes have been identified in several forms of drug hypersensitivity reaction, including dapsone (DDS) hypersensitivity syndrome (DHS). However, the nature of drug antigen and pathway of drug presentation that result in the activation of T-cells remains unclear. Hapten theory is one of the predominant pathways that has been studied to describe how a drug molecule interacts with protein to activate T-cells. Drugs bind to the cellular proteins and form drug-protein adducts that can be processed by antigen presenting cells (APC), with liberated peptides subsequently being presented on surface MHC molecules. It is hypothesised that drug haptens are bound to the MHC associated peptide and provide fine specificity for the T-cell response; however, formal proof remains elusive, despite almost a century of research. According to the hapten hypothesis, drugs might also covalently modify MHC molecules or their presented peptides when displayed on the surface of APC, avoiding the requirement for processing of drug-protein adducts. In addition to this, drugs have been shown to bind directly to MHC molecule, TCR, or both (direct pharmacological interaction of drugs with immune receptors [PI] concept). Many drugs have been shown to activate T-cells through the PI mechanism; however, similar to the hapten concept, the exact nature of the binding interaction that leads to T-cell activation is still unclear (Pichler, 2008).

Furthermore, abacavir hypersensitivity represents an example of another drug-specific T-cell activation pathway, specifically referred to as the altered peptide repertoire model. Abacavir binds deep to the F-pocket of the peptide-binding groove of a specific HLA molecule (HLA-B*57:01) before peptide loading. Abacavir

binding alters the peptide-binding cleft conformation and results in the presentation of novel peptide ligands that could stimulate an immune response (Illing et al., 2012).

The involvement of stable and protein reactive drug metabolites is another debated subject in the field of drug hypersensitivity. Most drugs can be metabolised into one or more metabolite that activates T-cells through the pathways described above. For instance, *in vitro*, a sulfamethoxazole reactive metabolite, sulfamethoxazole nitroso, has been shown to activate T-cells through binding with cysteine, via a hapten mechanism, causing sulfamethoxazole-induced hypersensitivity reactions (Schnyder et al., 2000). Furthermore, the stable and pharmacologically active metabolite of allopurinol (oxypurinol) binds directly to HLA-B*58:01, resulting in T-cell activation and allopurinol-induced hypersensitivity (Yun et al., 2013). Therefore, the availability of synthetic drug metabolites for *in vitro* functional studies is essential in understanding drug metabolite T-cell responses, even though the disconnect between *in vitro* and *in vivo* findings is a complicated issue.

DDS is a sulfone antibiotic, and it acts as an anti-bacterial and anti-inflammatory drug and has been used successfully as a treatment for leprosy. DDS undergoes reversible acetylation to produce stable mono and diacetylated derivatives, while DDS *N*-hydroxylation by cytochrome P-450 produces the toxic metabolite dapsone hydroxylamine (DDS-NHOH) that can auto-oxidise to form nitroso dapsone (DDS-NO) (Zuidema et al., 1986, Ellard et al., 1974, Swain et al., 1983, Uetrecht et al., 1988). This DDS-NO has been shown to bind covalently to protein at cysteine residues (Alzahrani et al., 2017).

DDS is used in combination with other drugs to treat infectious diseases such as leprosy and malaria. 0.5%-3.6% of treated patients develop a hypersensitivity syndrome characterised by fever, skin rash, and internal organ involvement 4-6 weeks after treatment starts (Rao and Lakshmi, 2001). HLA-B*13:01 has been shown as a risk allele for DHS in Chinese leprosy patients (Zhang et al., 2013a). DDS-specific T-cells have been identified in HLA-B*13:01 positive patients from Taiwan and Malaysia with DHS (Chen et al., 2018). Furthermore, Alzahrani et al. found that DDS and DDS-NO-specific T-cell clones (TCCs) were successfully generated from two healthy donors expressing HLA-B*13:01. The data showed that DDS-NO-responsive TCCs were activated via a hapten pathway. At the same time, DDS-responsive TCCs were structurally specific and activated via a direct binding between the drug and HLA-B*13:01 (Alzahrani et al., 2017). These exciting findings suggest that DDS-specific T-cells have an essential role in DDS hypersensitivity. However, the activation of T-cells with DDS and DDS-NO has not been studied using DDS hypersensitive patients' samples.

3.2 Aims:

- Generation of DDS- and DDS-NO-specific TCCs from DDS hypersensitive patients.
- To investigate the pathways of DDS and DDS-NO-specific T-cell activation.
- To investigate the release of the cytokines, cross-reactivity, and phenotypic characteristics of DDS and DDS-NO-specific TCCs.

3.3 Materials and Methods

3.3.1 Patient Samples

Seven DDS hypersensitive patients classified according to the diagnostic criteria proposed by Richardus and Smith (1989) were included in the study. 100 ml of blood was collected from the patients, and DNA and peripheral blood mononuclear cells (PBMC) were isolated from the blood. Basic demographics of the cohort and results of HLA typing are depicted in Table 3.1 and Table 3.2. Patch testing was conducted on the back of patients with DDS (0.1-25%); the patch was removed after 48h. The results were recorded after a further 24h to exclude any false positive responses resulting from the patch tape. To measure PBMC proliferation *in vitro*, PBMC ($1.5x10^5$ cell/well) were incubated with DDS (125-500µM), DDS-NO (5-20µM) and tetanus toxoid (5 µg/mL) or PHA (1 µg/mL), as positive controls, in culture medium for five days. [3H]-Thymidine was added for the final 16h of the experiment. The patients provided informed consent, and the Ethical Committee of the Shandong Provincial Institute of Dermatology and Venereology approved the study. A material transfer agreement was signed before the shipment of PBMC to the University of Liverpool.

Table 3.1: Demographics of patients and summary of diagnostic testing.

Patient ID	Gender	Medication history	Time of reaction	The onset of symptoms (days)	Clinical presentation	Skin Patch Test	LTT	
							DDS	DDS-NO
P5	M	DDS, rifampin, clofazimine	Not known	30	Fever	(+)	(+)	(+)
P6	M	DDS, rifampin, clofazimine	Not known	48	Fever, rash, lymphadenopathy	(+)	(+)	(+)
P8	F	DDS, rifampin, clofazimine	04.2015	17	Fever, abnormal liver function tests	(+)	(+)	(+)
P9	F	DDS, rifampin, clofazimine	04.2015	29	Fever, rash, abnormal liver function tests, lymphadenopathy	(+)	(+)	(+)
P10	M	DDS, rifampin, clofazimine	04.2015	22	Fever, rash, abnormal liver function tests	(-)	(+)	(+)
P14	M	DDS, rifampin, clofazimine	08.2013	21	Fever, rash, abnormal liver function tests	(+)	(+)	(+)
P17	F	DDS, rifampin, clofazimine	08.2013	14	Fever, rash, abnormal liver function tests, lymphadenopathy	(+)	(+)	(+)

Table 3.2: HLA typing of hypersensitive patients.

	HLA-A		HLA-B		HLA-C		HLA-DQB1		HLA-DRB1	
P5	02:07	11:01	*13:01*	40:01	03:04	07:02	06:01	06:01	08:03	15:01
P6	02:07	11:01	*13:01*	46:01	01:02	03:04	06:01	06:10	15:01	15:01
P8	11:01	24:02	*13:01*	15:25	03:04	04:03	03:01	05:02	12:02	15:01
P9	11:01	24:02	*13:01*	40:01	03:04	07:02	03:03	06:01	09:01	15:01
P10	02:07	11:01	*13:01*	46:01	01:02	03:04	03:03	06:01	09:01	15:01
P14	11:01	11:01	*13:01*	38:02	03:04	07:02	05:01	06:01	15:01	15:02
P17	11:02	24:02	*13:01*	27:04	03:04	12:02	05:02	05:03	14:54	15:02

3.3.2 Media, Chemicals and Reagents

DDS-NO, the reactive metabolite of DDS, was synthesised from DDS-NHOH according to the method described by Alzahrani et al. (2017). Other materials are listed in Chapter 2.

3.3.3 Generation DDS- and DDS-NO-Specific $CD4^+$ and $CD8^+$ TCCs

Drug-specific $CD4^+$ and $CD8^+$ TCCs were generated from 3 lymphocyte transformation test positive hypersensitive patients as described in chapter 2 using DDS (125 – 500 µM) or DDS-NO (5 - 20 µM) to enrich the drug-specific T-cell population prior to establishing serial dilution cultures.

3.3.4 Characterisation of DDS- and DDS-NO-Specific $CD4^+$ and $CD8^+$ TCCs

Dose-dependent proliferative responses and the profile of secreted cytokines and cytolytic molecules (IFN-γ, IL-5, IL-13, granzyme B, Fas L, perforin, IL-17 and IL22) from DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ TCCs were measured using [3H]-thymidine and ELISpot, respectively. DDS- and DDS-NO-Specific $CD4^+$ and $CD8^+$ TCCs were also tested for cross-reactivity with non-toxic concentrations of structurally-related compounds (sulfamethoxazole, sulfamerazine, sulfadiazine,

sulfachlorpyridazine, sulfadoxine, sulphanilamide, 4,4-thiodianiline, 4,4-oxyaniline, 3,3-sulfonyldianiline and mono and diacetylated forms of DDS).

These methods were discussed in detail in chapter 2.

3.3.5 Pathways of Drug Presentation to T-cells

To explore the pathway of drug presentation to the TCCs, TCCs were subjected to specific tests, mentioned in detail in chapter 2, including analysing T-cell response in presence and absence of APC, MHC blocking, pulsing assay, fixation assay, and glutathione (GSH) inhibition assay.

3.4 Results

Six out of seven HLA-B*13:01 positive hypersensitive patients produced a positive patch test with DDS (Table 3.1). PBMC from all seven patients were stimulated to proliferate in the presence of DDS and DDS-NO (SI 2 or above; Figure 3.1). PBMC from 3 patch test-positive patients (P5, P6, and P8) were tested with rifampicin and clofazimine co-medications, and no activation was observed (Figure 3.1). PBMC were also stimulated to proliferate with the positive control phytohemagglutinin (results not shown).

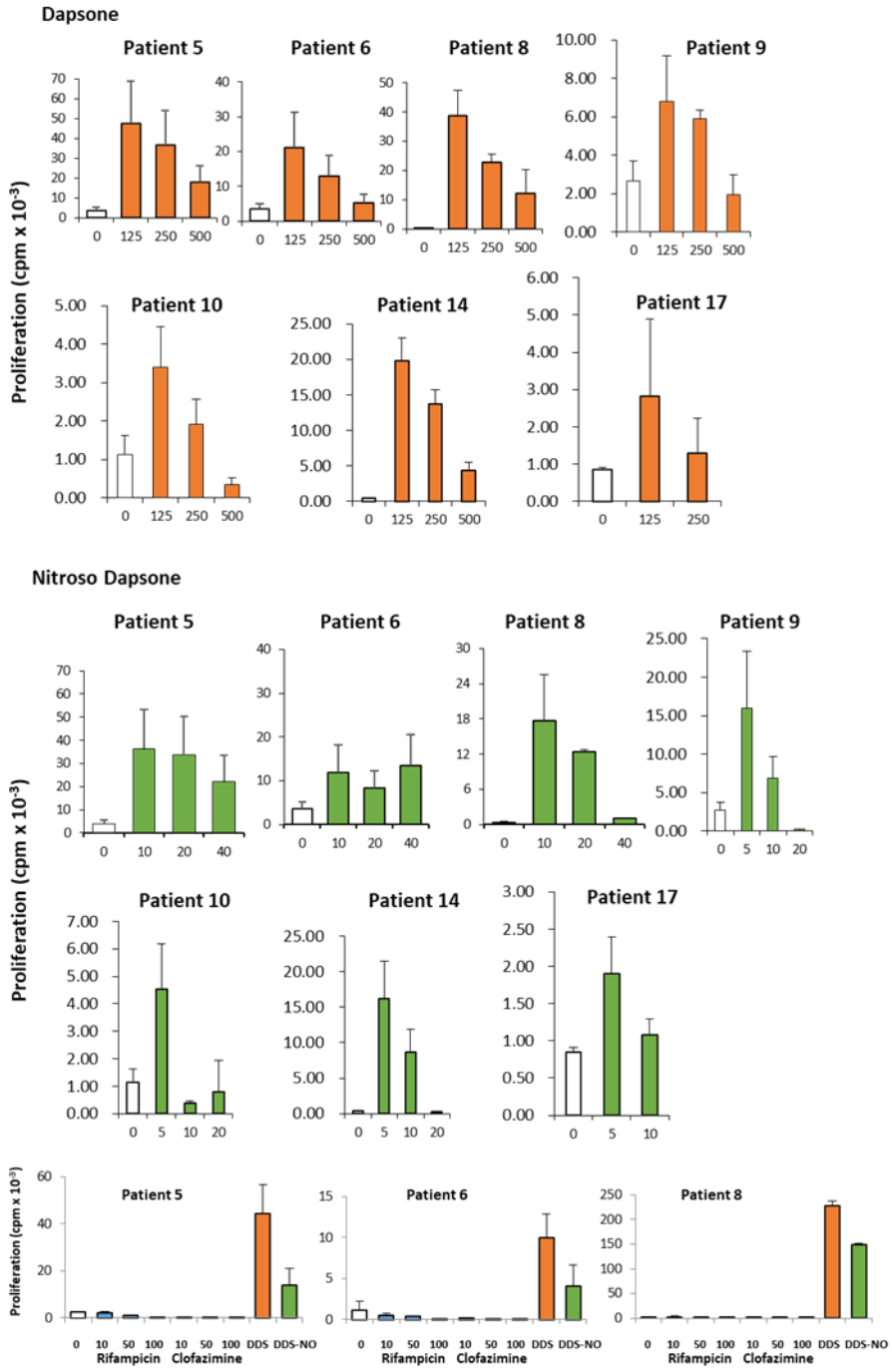
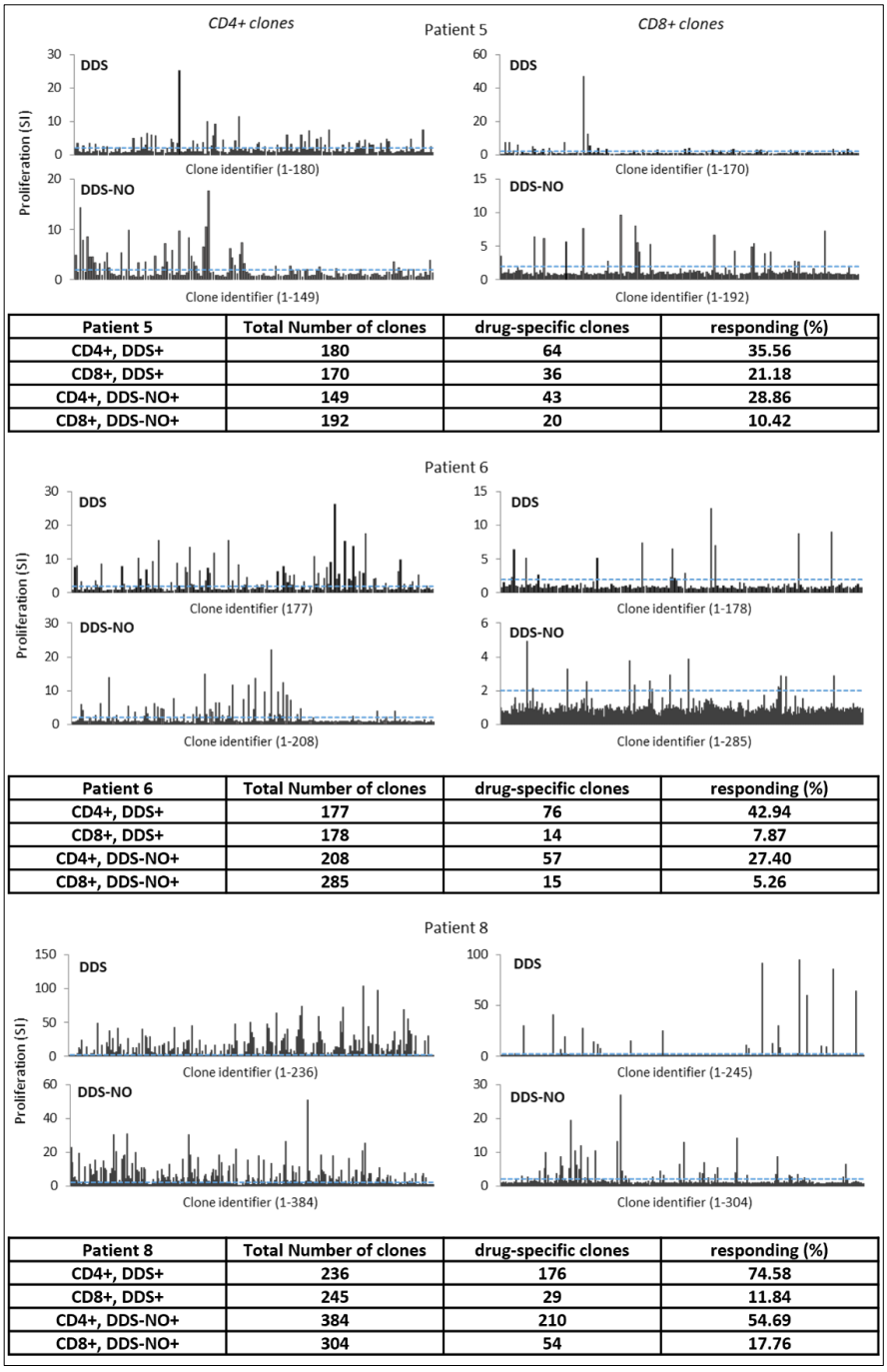


Figure 3.1: Lymphocyte transformation test data. PBMC from patients were exposed to graded concentrations of DDS (125-500 µM) and DDS-NO (5-40 µM), rifampicin (10-100 µM) and clofazimine (10-100 µM). The proliferation was measured after six days by the addition of [3H]-thymidine. Results are expressed as mean CPM of triplicate cultures, and a doubling of CPM in drug-treated cultures over vehicle control is considered positive.

3.4.1 DDS and DDS-NO Activate $CD4^+$ and $CD8^+$ Clones

PBMC from 3 patients (5, 6 and 8) were cultured with DDS or DDS-NO to generate drug-enriched T-cell lines. $CD4^+$ and $CD8^+$ T-cells were isolated by magnetic bead sorting before serial dilution to generate TCCs. Over 2728 TCCs were expanded and tested for responsiveness towards DDS or DDS-NO. 395 and 399 TCCs were stimulated to proliferate with DDS and DDS-NO, respectively (Figure 3.2). TCCs were phenotyped for $CD4^+$ and $CD8^+$ expression, and 80% and 78 % of the DDS and DDS-NO clones were $CD4^+$, respectively. 102 well-growing clones were selected for further analysis. TCCs were stimulated to proliferate with three well-tolerated concentrations of DDS (125-500 µM) and DDS-NO (5-20 µM) (Figure 3.3). $CD4^+$ and $CD8^+$ TCCs were stimulated to proliferate to a similar extent and secreted a similar panel of cytokines and cytolytic molecules, including IFN-y, IL-5, IL-13, IL-22, granzyme B, perforin, and Fas-L (Figure 3.4).



Patient 5	Total Number of clones	drug-specific clones	responding (%)
CD4+, DDS+	180	64	35.56
CD8+, DDS+	170	36	21.18
CD4+, DDS-NO+	149	43	28.86
CD8+, DDS-NO+	192	20	10.42

Patient 6	Total Number of clones	drug-specific clones	responding (%)
CD4+, DDS+	177	76	42.94
CD8+, DDS+	178	14	7.87
CD4+, DDS-NO+	208	57	27.40
CD8+, DDS-NO+	285	15	5.26

Patient 8	Total Number of clones	drug-specific clones	responding (%)
CD4+, DDS+	236	176	74.58
CD8+, DDS+	245	29	11.84
CD4+, DDS-NO+	384	210	54.69
CD8+, DDS-NO+	304	54	17.76

Figure 3.2: Generation of DDS- and DDS-NO-responsive CD4$^+$ and CD8$^+$ TCCs. TCCs were generated from PBMC of 3 patients by serial dilution and repetitive mitogen stimulation. TCCs were then incubated with autologous EBV-transformed B cells and either DDS or DDS-NO in duplicate cultures for 48h. Proliferative responses were measured by the addition of [3H]-thymidine. Clones with a stimulation index (SI) of 2 or above were expanded for further analysis. Phenotype and drug specificity of TCCs are displayed in the tables.

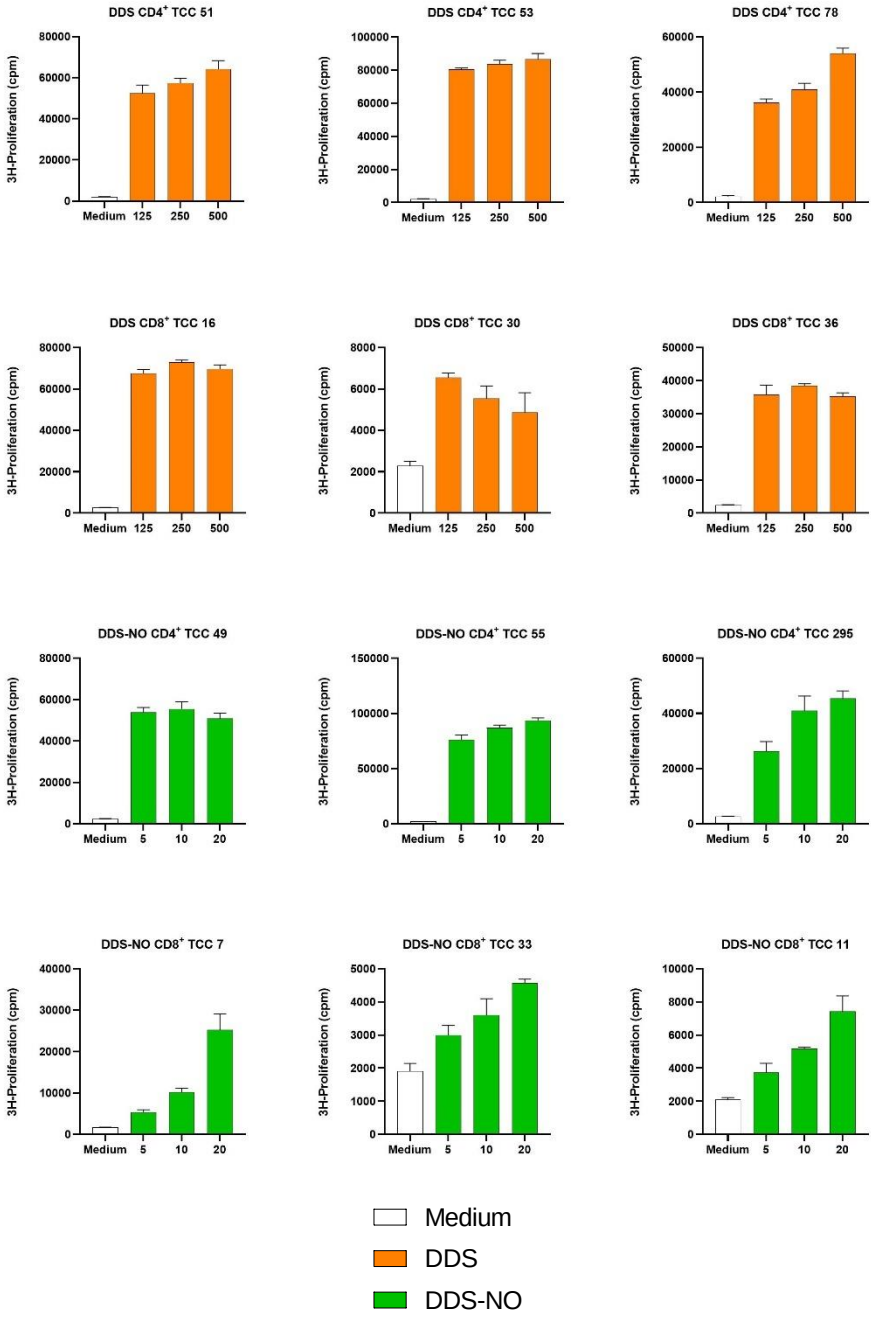
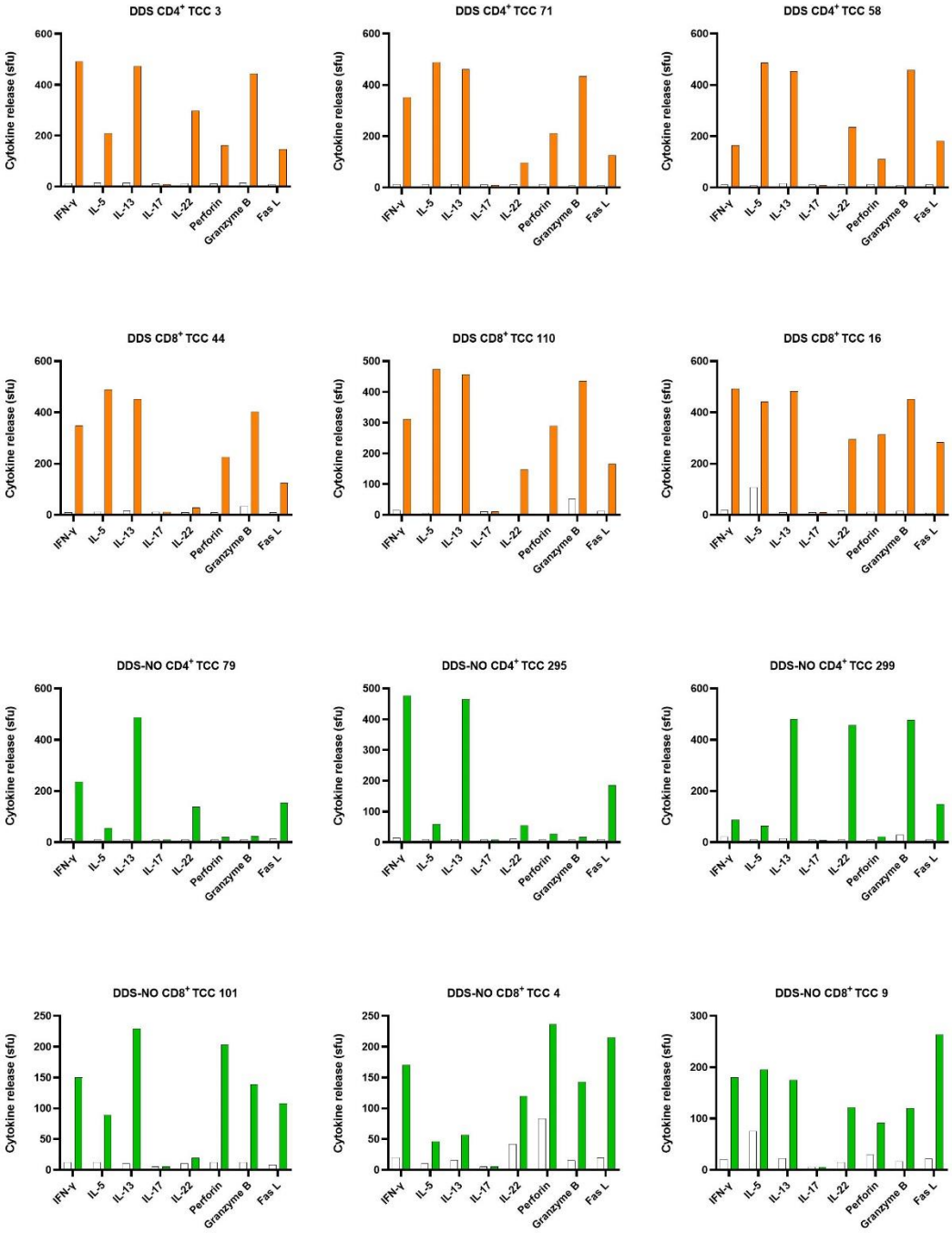


Figure 3.3: Dose-dependent proliferative responses of DDS- and DDS-NO-responsive CD4$^+$ and CD8$^+$ TCCs. TCCs were cultured with irradiated autologous EBV-transformed B cells and DDS (125-500 µM) or DDS-NO (5-20 µM) in triplicate cultures for 48 h.

A


DDS CD4+ TCC 3
DDS CD4+ TCC 71
DDS CD4+ TCC 58
DDS CD8+ TCC 44
DDS CD8+ TCC 110
DDS CD8+ TCC 16
DDS-NO CD4+ TCC 79
DDS-NO CD4+ TCC 295
DDS-NO CD4+ TCC 299
DDS-NO CD8+ TCC 101
DDS-NO CD8+ TCC 4
DDS-NO CD8+ TCC 9
Cytokine release (sfu)
IFN-γ
IL-5
IL-13
IL-17
IL-22
Perforin
Granzyme B
Fas L

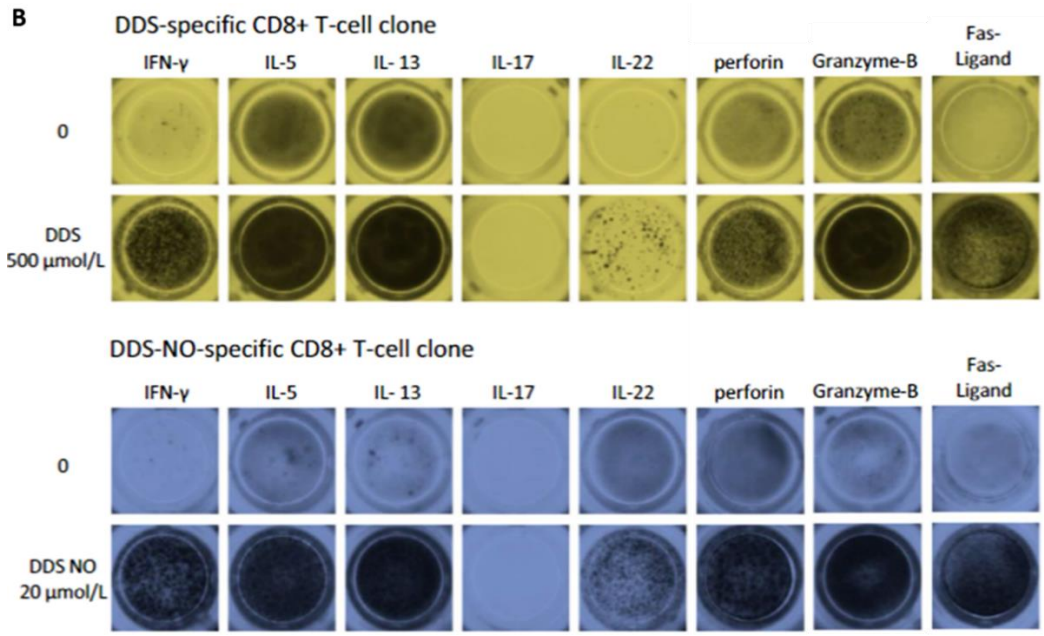


Figure 3.4: Comparison of cytokines release. (A) Detection of IFN-γ, IL-5, IL-13, IL-17, IL-22, perforin, granzyme B, and Fas-ligand secretion by DDS- and DDS-NO-responsive CD4+ and CD8+ TCCs. TCCs were incubated with autologous EBV-transformed B cells and either DDS or DDS-NO. The cytokine release was visualised by ELISpot. (B) Representative ELISpot images.

3.4.2 DDS- and DDS-NO-Responsive $CD4^+$ and $CD8^+$ TCCs Show Three Different Forms of Cross-Reactivity.

A cross-reactivity test was performed for sixty-three DDS- and ninety-eight DDS-NO-responsive $CD4^+$ and $CD8^+$ TCCs. Both DDS- and DDS-NO-responsive clones displayed three cross-reactivity patterns when tested with DDS and DDS-NO, involving compound-specific, weakly, or strongly cross-reactive. Almost 90% of the DDS-responsive clones were DDS-specific or weakly cross-reactive. In contrast, nearly 90% of the DDS-NO-responsive clones were DDS-NO-specific or highly cross-reactive (Figure 3.5).

Six strong and weakly cross-reactive clones were then selected to find the minimum stimulatory concentrations of DDS and DDS-NO. Clones were cultured with DDS and DDS-NO at graded concentrations of 0.1-100 μM. Clones were stimulated to proliferate with similar concentrations of DDS and DDS-NO (Figure 3.6).

Most DDS- and DDS-NO-responsive clones showed a cross-reactivity profile with DDS-NHOH. However, the clones didn't respond with closely related DDS analogues and other structurally different sulfonamides except for 3DDS, which showed low response with few clones. (Figure 3.7).

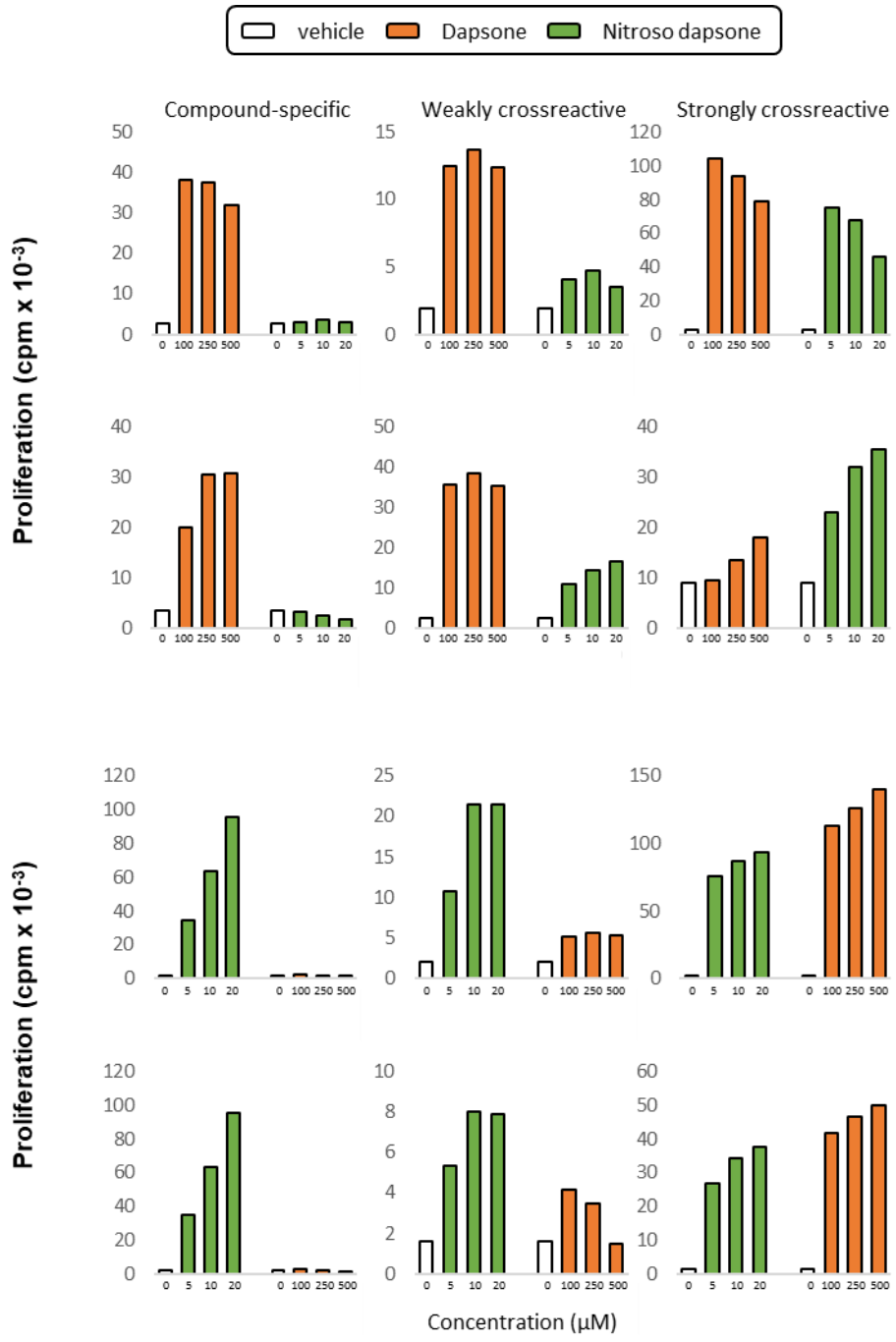


Figure 3.5: Cross-reactivity of DDS- and DDS-NO-responsive TCCs. DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ TCCs were cultured with irradiated autologous EBV-transformed B-cells and either DDS (100-500 µM) or the nitroso metabolite (5-20 µM) in triplicate cultures for 48h. (Representative clones show the three distinct cross-reactivity patterns).

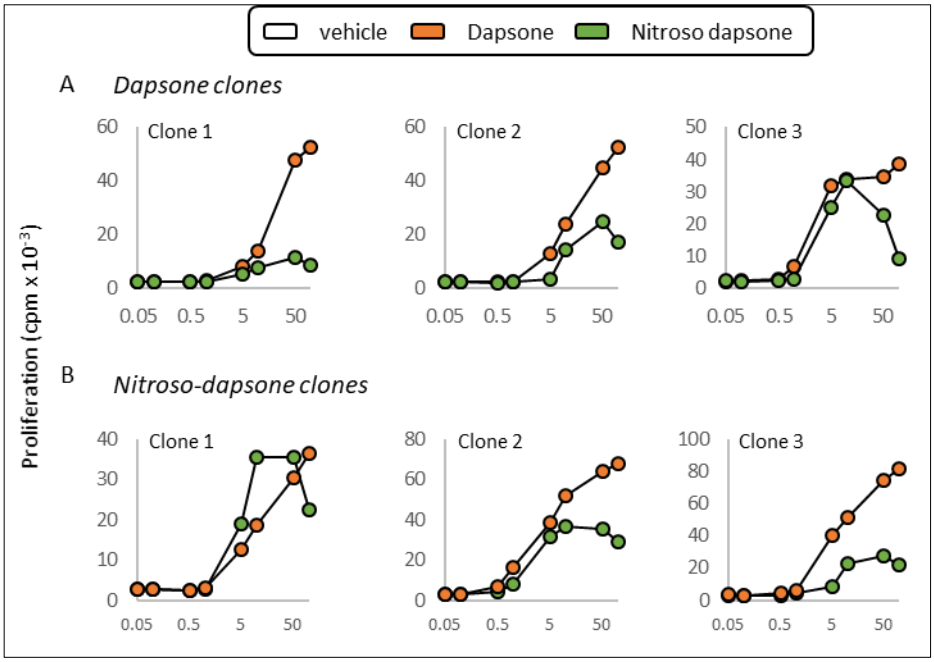
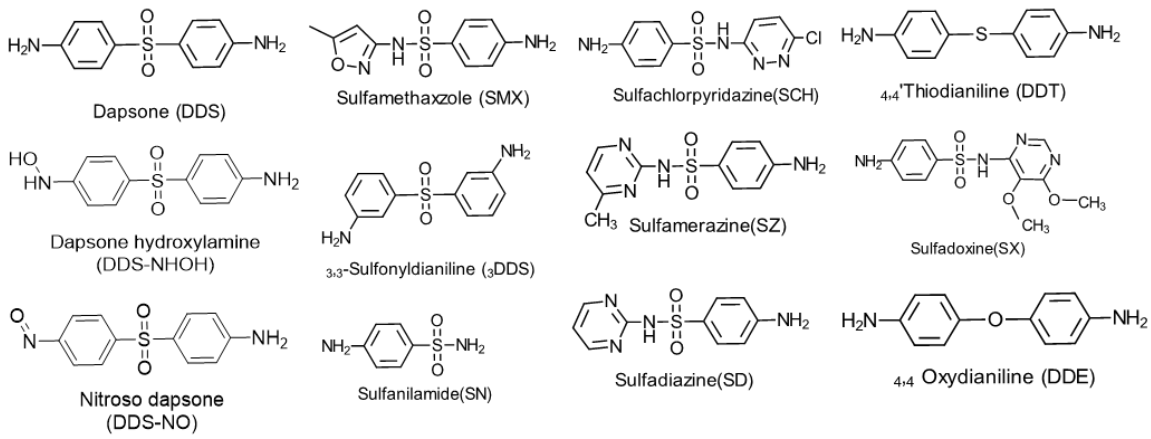
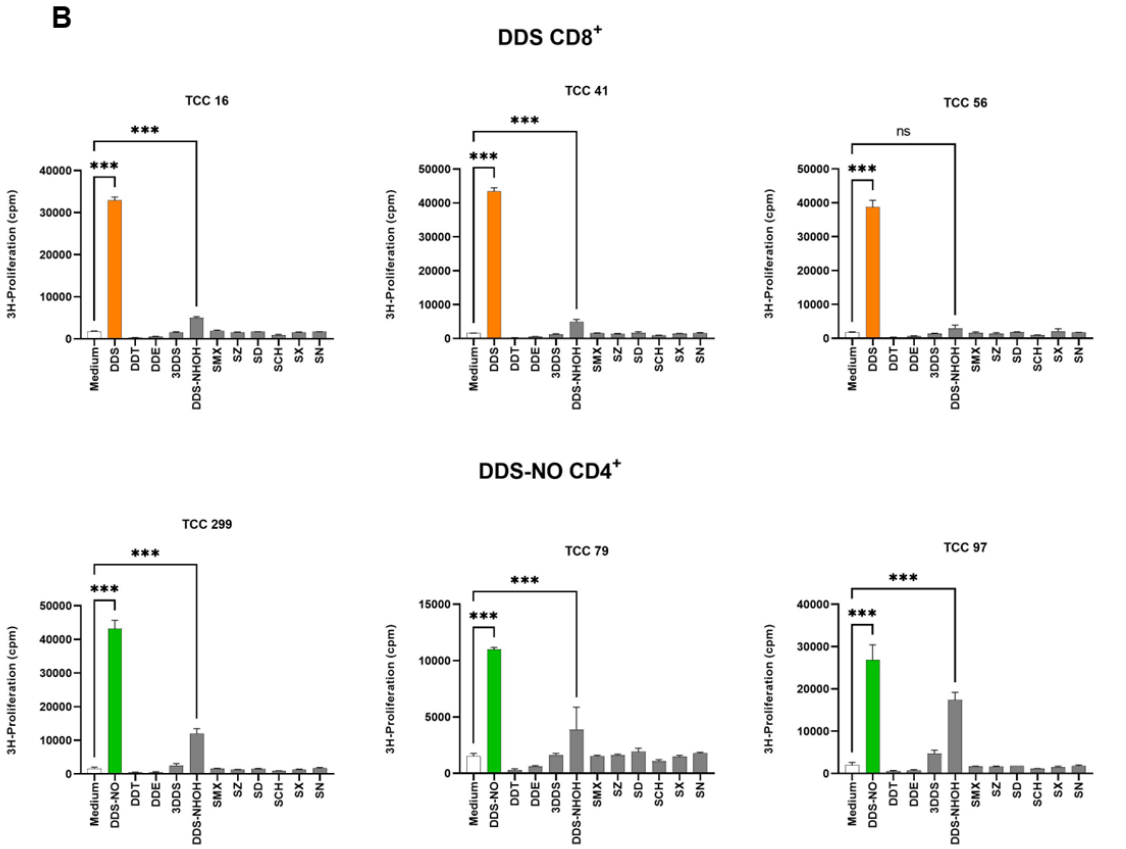


Figure 3.6: Cross-reactivity and dose-response of TCCs with similar drug and metabolite concentrations. DDS- and DDS-NO-responsive clones were cultured with irradiated autologous EBV-transformed B cells and DDS (0.1-100 µM) or DDS-NO (0.1-100 µM) in triplicate cultures for 48 h. Proliferative responses were measured by the addition of [3H]-thymidine.

A


Dapsone (DDS)
Sulfamethaxzole (SMX)
Sulfachlorpyridazine(SCH)
4,4'Thiodianiline (DDT)
Dapsone hydroxylamine (DDS-NHOH)
3,3-Sulfonyldianiline (3DDS)
Sulfamerazine(SZ)
Sulfadoxine(SX)
Nitroso dapsone (DDS-NO)
Sulfanilamide(SN)
Sulfadiazine(SD)
4,4 Oxydianiline (DDE)

B


DDS CD8+
TCC 16
TCC 41
TCC 56
DDS-NO CD4+
TCC 299
TCC 79
TCC 97
3H-Proliferation (cpm)
Medium
DDS
DDS-NO
DDT
DDE
3DDS
DDS-NHOH
SMX
SZ
SD
SCH
SX
SN
***
ns

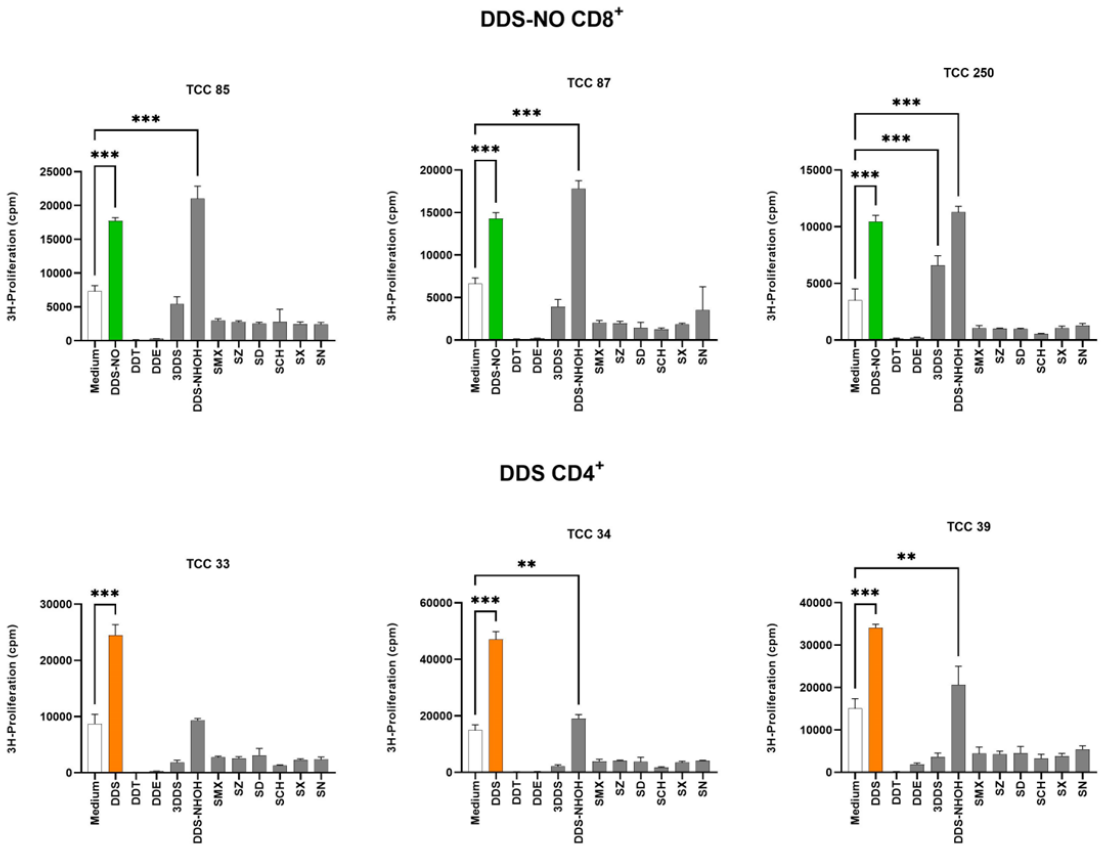


Figure 3.7: Cross-reactivity of DDS- and DDS-NO-responsive CD4⁺ and CD8⁺ TCCs with closely related analogues, sulphonamide, and DDS-NHOH. (A) Compound structures and (B) clones were incubated with irradiated autologous EBV-transformed B-cells and DDS or DDS-NO in triplicate cultures for 48 hrs. T-cell activation was then determined using [3H] thymidine incorporation. Statistical analysis was performed via a one way ANOVA, compared to the control media well. (NS [Not significant] = $P > 0.05$, $P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***).

3.4.3 The Expression of Chemokine Receptors and TCR Vβ Chains

The chemokine receptors CXCR3 and CCR4 were expressed at a high level by the CD4+ TCCs. In contrast, The CD8+ TCCs expressed more chemokine receptors, including CXCR3, CCR4, CCR10, CCR9 and CCR6 (Figure 3.8A). The data also showed that DDS- and DDS-NO-responsive CD4+ and CD8+ TCCs expressed single but variable TCR Vβ chains with no apparent differences between CD4+ and CD8+ or the two compounds' clones (Figure 3.8B).

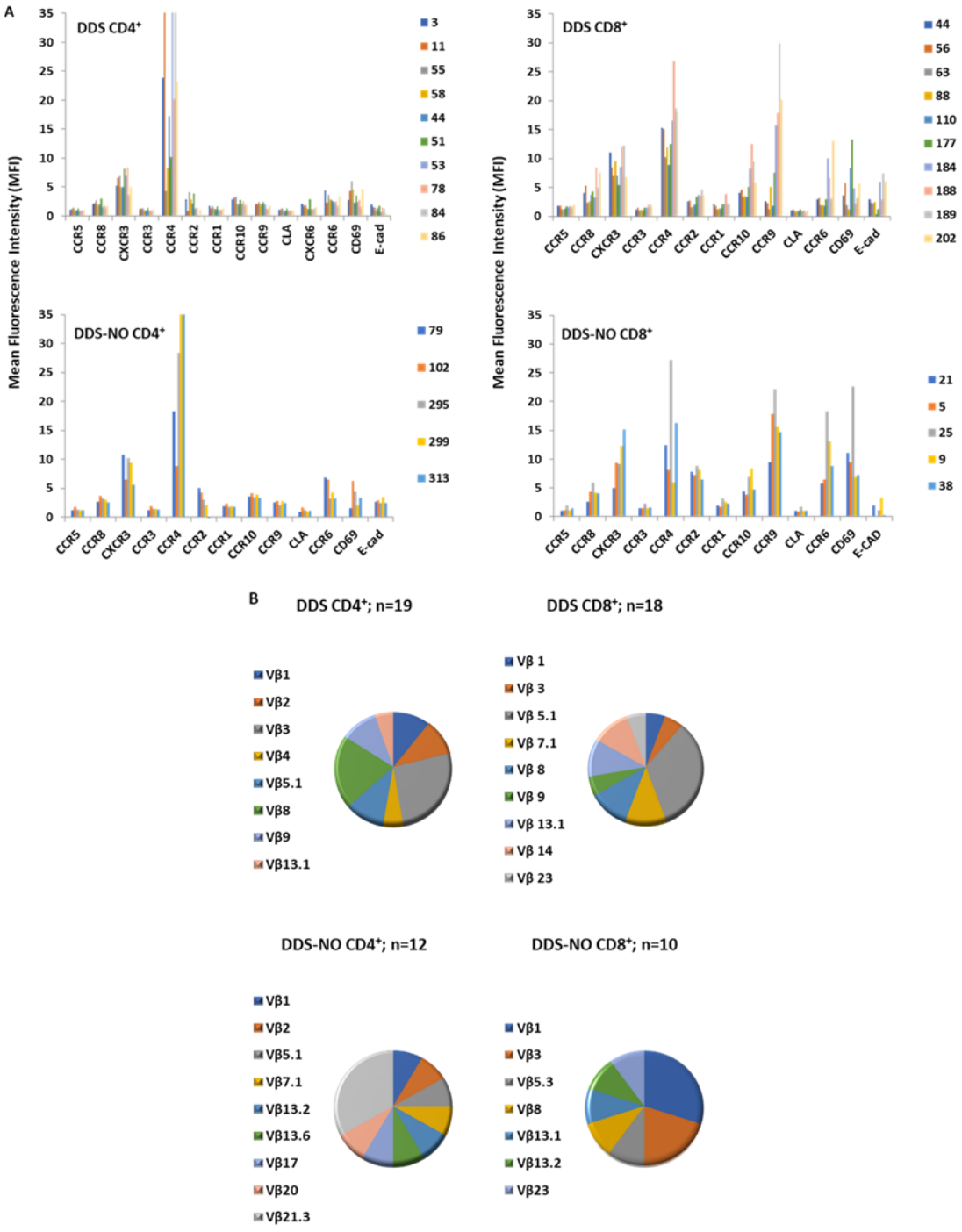


Figure 3.8: Chemokine receptors and T-cell receptor V β chains expressed on DDS- and DDS-NO-responsive CD4+ and CD8+ TCCs. (A) Chemokine receptors were measured on resting clones by flow cytometry. The expression is presented as mean fluorescence intensity of the whole population of each clone. (B) TCR Vβ expression was measured with

the IOTest® Beta Mark, TCR Vβ Repertoire Kit, which covers over 80% of the available TCRs, by flow cytometry.

3.4.4 HLA -Restricted Activation of DDS- and DDS-NO-Responsive $CD4^+$ and $CD8^+$ TCCs

The proliferation of the DDS- and DDS-NO-responsive TCCs was dependent on the presence of irradiated autologous EBV-transformed B-cells that function as antigen-presenting cells. The drug- and metabolite-specific proliferative response of $CD4^+$ clones was inhibited with an anti-HLA class-II blocking antibody, while the $CD8^+$ clone's proliferation was inhibited with an anti-HLA class-I blocking antibody. This suggests that the $CD4^+$ and $CD8^+$ proliferative responses were HLA class II and I restricted, respectively (Figure 3.9).

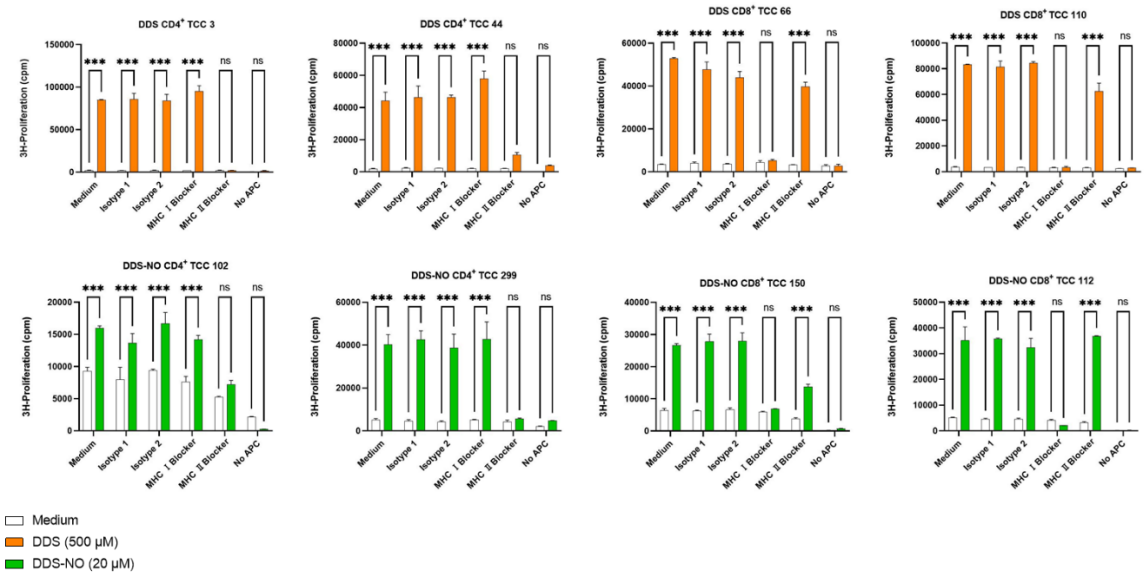


Figure 3.9: Assessment of HLA restriction. DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ TCCs were cultured with EBV-transformed B cells and DDS or DDS-NO in triplicate cultures for 48 h either in the presence or absence of anti-HLA class I and II blocking antibodies (and their respective isotype controls), and in the absence of APC (EBV-transformed B cells). Proliferative responses were measured by the addition of [3H]-thymidine. Results are expressed as mean±SD CPM. Statistical analysis was performed via a two way ANOVA, compared to the control media well. (NS [Not significant] = $P > 0.05$, $P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***).

3.4.5 Assessment of The Activation Pathway of DDS- and DDS-NO-Responsive $CD4^+$ and $CD8^+$ TCCs

Our data showed that fixation of EBV-transformed B cells with glutaraldehyde, which inhibits antigen processing (Schnyder et al., 1997), didn't block the $CD4^+$ or $CD8^+$ TCCs' activation with DDS. In contrast, approximately half of the $CD4^+$ and $CD8^+$ TCCs exhibited a reduced proliferation with DDS-NO in the presence of fixed EBV-transformed B cells (Figure 3.10).

DDS-NO-responsive $CD8^+$ and most $CD4^+$ TCCs were stimulated to proliferate with EBV-transformed B cells pulsed with DDS-NO for 0.5-2 h, while the DDS-responsive TCCs were not activated with EBV-transformed B cells pulsed with DDS (Figure 3.11).

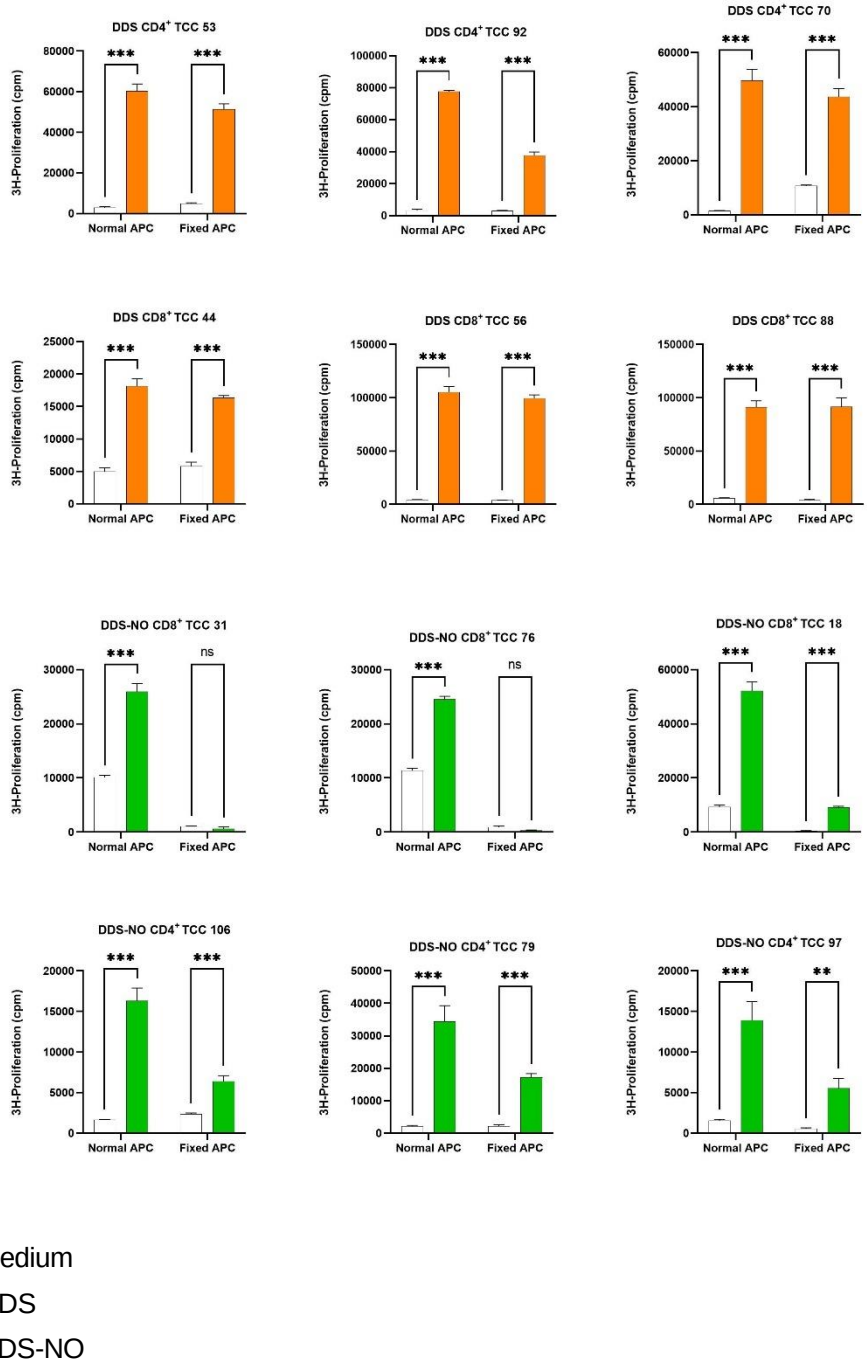


Figure 3.10: The pathway of DDS and DDS-NO T-cell activation (Fixation Assay). Activation of DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ TCCs with DDS and DDS-NO in the presence of irradiated and glutaraldehyde-fixed autologous EBV-transformed B cells. Proliferative responses were measured after 48 h by the addition of [3H]-thymidine. Results are expressed as mean±SD CPM. Statistical analysis was performed via a two way ANOVA,

compared to the control media well. (NS [Not significant] = P > 0.05, P ≤ 0.05 *, P ≤ 0.01 **, P ≤ 0.001 ***).

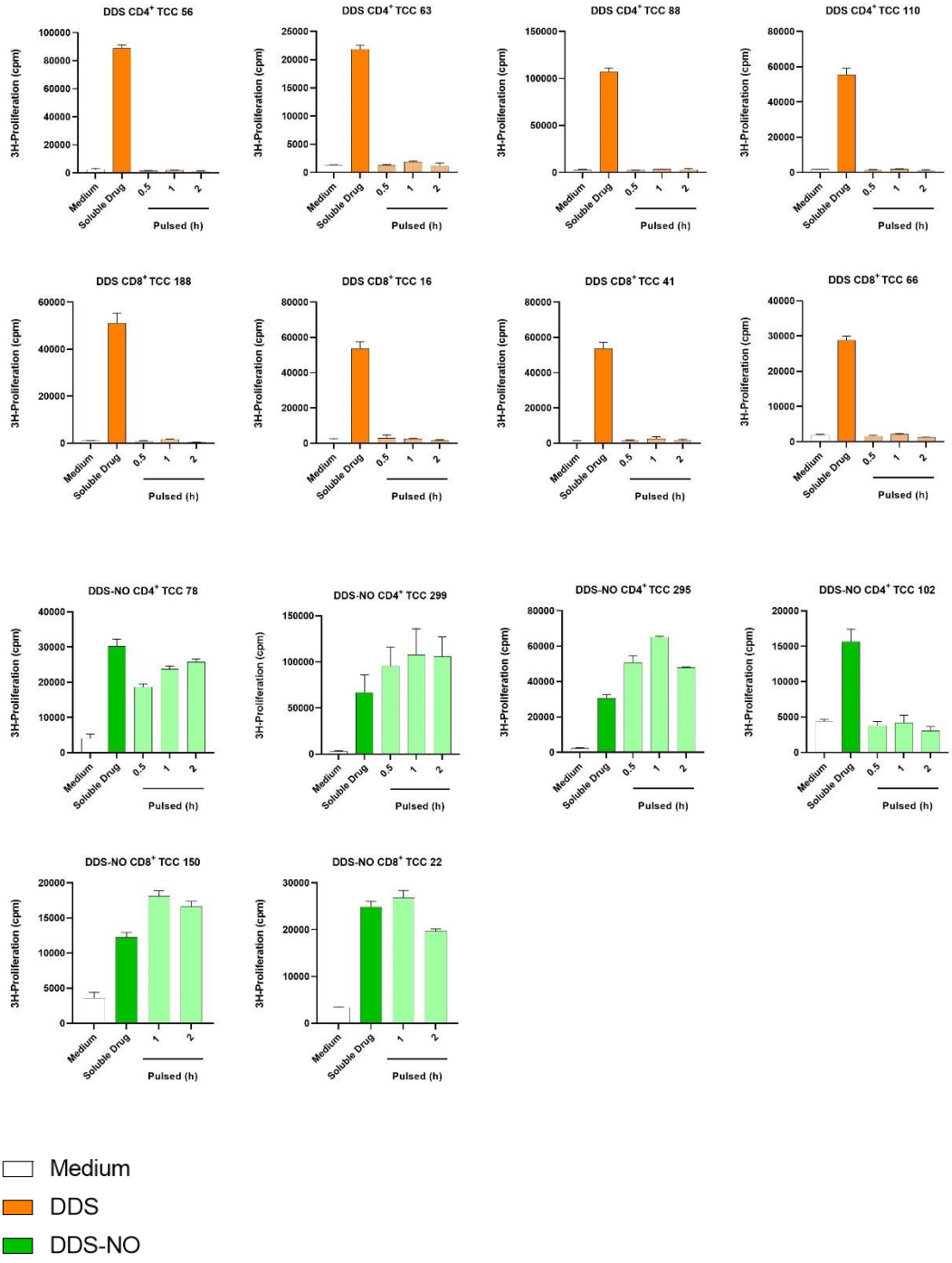


Figure 3.11: The pathway of DDS and DDS-NO T-cell activation (Pulsing Assay). Activation of DDS- and DDS-NO-responsive CD4+ and CD8+ TCCs with soluble compounds or EBV-transformed B cells pulsed with either DDS or DDS-NO for 0.5-2 h. Proliferative responses

were measured after 48 h by the addition of [3H]-thymidine. Results are expressed as mean±SD CPM.

GSH was added to the proliferation assay in two different procedures: (1) in a culture containing DDS-NO-responsive clones, EBV-transformed B cells and soluble DDS-NO, and (2) in a culture containing DDS-NO-responsive clones, EBV-transformed B cells pulsed with DDS-NO for 2 h. DDS-NO GSH adducts were formed rapidly within 2 h (Figure 3.12A). Furthermore, GSH decreased the strength of the proliferative response of the TCCs with soluble DDS-NO and completely inhibited the proliferative response with DDS-NO-pulsed EBV-transformed B cells (Figure 3.12B).

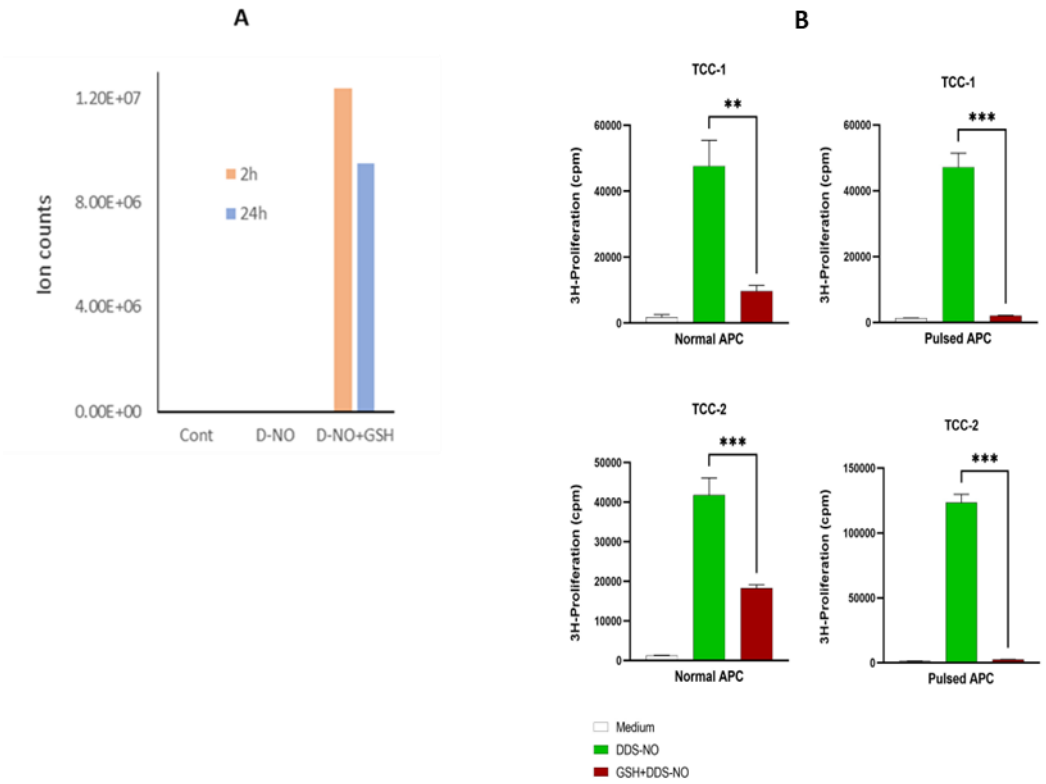


Figure 3.12: Effect of GSH DDS-NO adduct formation on T-cell activation. (A) Semi-quantitative analysis of DDS-NO GSH adduct levels in three experimental conditions containing EBV-transformed B-cells: (i) medium alone (Cont), (ii) medium containing DDS-NO (D-NO) and (iii) DDS-NO and GSH 1mM (D-NO+GSH). (B) the proliferative responses of DDS-NO responsive clones with (i) soluble DDS-NO and normal irradiated APC, or (2) APC pulsed with DDS-NO for 2 h. Both tests were performed in the presence and absence of GSH in tripli-cate cultures for 48hrs. GSH was added to cells before DDS-NO in both assays. Proliferative responses were measured by the addition of [3H]-thymidine. Results are expressed as mean±SD CPM. Statistical analysis was performed via unpaired T tests. (NS [Not significant] = $P > 0.05$, $P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***).

3.5 Discussion

A significant challenge in understanding the pathogenesis of HLA-associated drug hypersensitivity reactions is knowledge of the role of protein-reactive metabolites in the generation of epitopes that activate T-cells. A clear example linking the role of protein-reactive metabolites to the development of hypersensitivity is the observation that sulfamethoxazole reactive metabolites activate T-cells via the hapten pathway (Nassif et al., 2004b, Castrejon et al., 2010a). Recently, studies have focused on the interaction between the parent drug and the HLA molecule because of the availability of the parent drug for *in vitro* cell culture studies. For this reason, the current study aimed to explore the immunogenicity of the DDS and its reactive metabolite (DDS-NO) in DDS hypersensitive patients. DDS was used as a model study drug as hypersensitivity reactions in Chinese patients with leprosy administrated with DDS are strongly associated with the expression of HLA-B*13:01 (Zhang et al., 2013a). DDS-NO, the reactive metabolite of DDS, was synthesised from DDS-NHOH according to the method described previously (Alzahrani et al., 2017), and PBMC from HLA-B*13:01+ patients were available for mechanistic studies.

To explore DDS and DDS-NO immunogenicity, PBMC from seven HLA-B*13:01 positive hypersensitive patients receiving DDS in combination with rifampicin and clofazimine were stimulated to proliferate with DDS and DDS-NO. All patients displayed a positive patch test response to DDS except patient 10. *In vitro* activation of patient PBMC with DDS and DDS-NO was observed with all of the study participants. DDS and DDS-NO responsive $CD4^+$ and $CD8^+$ TCCs were isolated and expanded from the PBMC of patients 5, 6, and 8. $CD4^+$ and $CD8^+$ subpopulations

were separated before the T-cell cloning procedure. 395 DDS-responsive TCCs and 399 DDS-NO-responsive TCCs were generated from the three patients at the following $CD4^+$ and $CD8^+$ ratio: 4-5:1 in patients 6 and 8 and 2:1 in patient 5, demonstrating that drug antigens preferentially stimulate $CD4^+$ T-cell responses, but that $CD8^+$ T-cell responses are also detected in all patients. These data contrast with abacavir where the drug was found to only stimulate $CD8^+$ T-cells (Bell et al., 2013, Adam et al., 2012). Responsive $CD4^+$ and $CD8^+$ TCCs were HLA class II and class I restricted, respectively, and they expressed an array of TCR Vβ receptors.

The availability of $CD4^+$ and $CD8^+$ TCCs allowed for the assessment of DDS and DDS-NO cross-reactivity and activation with DDS analogues and a panel of sulphonamides. TCCs were not stimulated to proliferate with DDS analogues that don't carry the sulfone group, DDS analogues with amine groups in different aromatic rings positions (except 3DDS with few clones), and structurally different sulphonamides. These findings indicate that the sulfone group and the position of the amine groups are essential for T-cell activation.

Cross-reactivity studies also showed that 25%-50% of $CD4^+$ and $CD8^+$ clones are highly specific. Their activation was observed with only one compound (DDS or DDS-NO), indicating that T-cells recognise and respond selectively to a particular drug antigen. Although DDS and DDS-NO share similar structures in that two aromatic amines are connected to the sulfone group, the presence of the drug- or drug metabolite-specific T-cells opens the question into the precise nature of the antigenic determinant that activates T-cells.

DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cells showed high and low levels of cross-reactivity with the optimum concentrations of DDS (100-500 μmol/L) and

DDS-NO (5-20 μmol/L). The majority of DDS-responsive, cross-reactive clones showed low cross-reactivity levels with DDS-NO. Analysis of stability of DDS-NO in culture using quantitative mass spectrometry showed that the DDS-NO is converted to DDS in the second-day proliferation assay. Consequently, 30 μmol/L DDS-NO was exposed to 2.5-15 μmol/L of DDS, activating suboptimal T-cell proliferation. In contrast, most DDS-NO-responsive, cross-reactive clones showed high cross-reactivity with high concentrations of DDS (100-500 μmol/L). This suggests that the same clone may recognise and respond to both the parent drug and metabolite via different pathways (discussed in more detail below).

A series of functional assays, previously utilised to define the pathways of T-cell activation with drugs such as sulfamethoxazole and carbamazepine (Farrell et al., 2003, Castrejon et al., 2010a, Wu et al., 2007), were used to explore the way in which DDS and DDS-NO stimulated $CD4^+$ and $CD8^+$ clones. The strong activation of $CD4^+$ and $CD8^+$ clones with both DDS and DDS-NO was dependent on the presence of antigen-presenting cells. To investigate whether protein processing into antigenic peptide fragments participates in the T-cell activation, glutaraldehyde-fixed antigen-presenting cells were used in the proliferation assay. DDS activated TCCs with fixed and irradiated antigen-presenting cells. In contrast, T-cell response was reduced but not completely inhibited when approximately half of the T-cells were incubated with DDS-NO and fixed APC, and this low response could result from the conversion of DDS-NO into DDS in culture, as I have explained above. These data indicate that DDS activates T-cells in a processing-independent manner, likely through a direct interaction with HLA molecules. In contrast, DDS-NO-responsive T-cells were activated through a hapten mechanism in which DDS-NO binds covalently to cellular

protein forming DDS-NO-protein adducts that require processing to generate antigenic peptides. The APC pulsing assay was used to further confirm this conclusion. DDS-responsive TCCs were not activated with DDS-pulsed antigen-presenting cells. In contrast, the majority of DDS-NO-responsive clones were activated with DDS-NO-pulsed antigen-presenting cells with the same response strength seen with soluble DDS-NO.

GSH is a tripeptide antioxidant found in most cells. It protects cells from the toxicity of aromatic nitroso compounds by neutralising (reducing) reactions or direct conjugation (Naisbitt et al., 1996, Ellis et al., 1992). GSH was used in the TCCs proliferation assay to investigate the effect of GSH-metabolite conjugation on T-cells activation with DDS-NO. GSH was incubated with DDS-NO, antigen-presenting cells, and T-cells. The mass spectrometry data showed that GSH-DDS-NO adducts were formed rapidly in cultures. The proliferation assay with GSH showed that TCCs proliferative responses were reduced with soluble DDS-NO and inhibited with DDS-NO-pulsed antigen-presenting cells. This suggests that DDS-NO needs to bind covalently to cellular proteins and forms DDS-NO-modified protein adducts to activate T-cells.

In summary, our data indicate that DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cells are present in hypersensitive patients' blood. The parent drug and the drug metabolite selectively activated T-cells via direct HLA binding and a hapten pathway, respectively. HLA-B*13:01 is a risk allele for DDS hypersensitivity reaction. Therefore, in the next chapter, PBMC from DDS hypersensitive patients carrying HLA-B*13:01 were used to investigate the nature of the drug-specific T-cell

response and whether DDS and/or DDS-NO bind preferentially to HLA-B*13:01 to selectively activate $CD8^+$ T-cells.

4 Chapter 4: Role of HLA-B*13:01 In Dapsone- and Dapsone Nitroso-Responsive T-cell Activation

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4.1 Introduction

Since the first study in 2002 when abacavir hypersensitivity was substantially linked to HLA-B*57:01 (Mallal et al., 2002), several human leukocyte antigen (HLA) alleles have been found to be significant factors influencing susceptibility to various forms of drug hypersensitivity. The genetic correlations between abacavir hypersensitivity, carbamazepine-induced Stevens-Johnson syndrome (HLA-B*15:02; (Chung et al., 2004)) and allopurinol hypersensitivity (HLA-B*58:01;(Hung et al., 2005)) and HLA alleles suggest that HLA class-I alleles represent the most robust genetic associations. Mechanistic studies show that $CD8^+$ T-cells are activated when the three medications mentioned above selectively bind with the protein expressed by the risk HLA alleles (Yun et al., 2014). This points to HLA allele-restricted drug-specific T-cell responses as a critical factor in disease pathogenesis.

All nucleated cells express the HLA class-I proteins. Intracellular protein processing generates 9-12 amino acid peptides that interact with HLA class-I proteins before being transported to the cell surface and presented to $CD8^+$ T lymphocytes. On the other hand, exogenous protein-processed peptides are longer and are often presented to $CD4^+$ T-cells by HLA class-II proteins found only on professional antigen-presenting cells (Neefjes et al., 2011).

Fever, papular or exfoliative rash, hepatitis, and widespread lymphadenopathy are symptoms of dapsone (DDS) hypersensitivity syndrome (DHS), which Lowe initially described in 1949 (Lowe and Smith, 1949). The DHS affects 1.4 per cent of the patients treated with DDS, with 9.9 per cent of cases resulting in mortality (Lorenz et al., 2012). In the Chinese population, HLA-B*13:01 has been identified as a risk factor for DDS hypersensitivity (Zhang et al., 2013a). Similar association studies in

Taiwanese and Thai population followed (Chen et al., 2018, Tempark et al., 2017). Healthy donors' $CD4^+$ and $CD8^+$ T lymphocytes have been shown to be activated with DDS and the protein-reactive nitroso metabolite (DDS-NO) (Alzahrani et al., 2017, Chen et al., 2018). In addition, our findings in the preceding chapter showed that $CD4^+$ and $CD8^+$ T lymphocytes isolated from HLA-B*13:01 positive patients with hypersensitivity respond to DDS and DDS-NO. As a result, this chapter aimed to determine the involvement of HLA-B*13:01 in the activation of DDS- and DDS-NO-$CD8^+$ T-cell clones (TCCs) utilising autologous APCs, heterologous APC expressing HLA-B*13:01 allele and APC transduced with a single HLA-B allele (HLA-B*13:01 C1R cell line).

4.2 Materials and Methods

Methods that have been used in this chapter are described in detail in the general methods chapter (chapter 2).

4.2.1 Generation of a HMy2.C1R-HLA-B*13:01-P2A-B2M Cell Line

The pLJM1-EGFP plasmid (a gift from David Sabatini Addgene plasmid # 19319) was modified by replacing the EGFP with a Multiple Cloning Site, adding an Eμ enhancer (pLJM1- Eμ-SFFV-NewMCS) and a P2A sequence. *B2M* was PCR amplified from cDNA from a volunteer from our HLA biobank and ligated into the distal end of the P2A sequence using restriction sites Age1/SnaB1. HLA-B*13:01 was PCR amplified from the HLA-B*13:01 pcDNA3.1(+) plasmid, a kind gift from Prof. Ostrov, and ligated into the proximal end of the P2A sequence using restriction sites PME1 and EcoR1 to create the pJLM1-HLA-B*13:01-P2A-B2M plasmid.

The pJLM1-HLA-B*13:01-P2A-B2M plasmid was transfected into competent *Escherichia coli* and colonies grown on agarose plates under carbenicillin selection overnight at 37^{o}C. Positive colonies were identified by colony PCR and grown up overnight in a shaking incubator at 37^{o}C. Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). Correct integration was confirmed by Sanger sequencing (Source Bioscience Sequencing Oxford UK).

The HEK293 cell line was used as the machinery to generate 2nd generation lentivirus. The HEK293 cells were seeded at 350 x 10^5 in 1.5 ml of DMEM (Sigma, UK) and incubated overnight at 37 °C in a cell culture incubator. 100 µl NaCl (150 mM) was added to each 1.5 ml Eppendorf tube along with the desired amount of plasmid DNA and vortexed. 100 µl stock mix (100 µl NaCl + 4 µl polyethylenimine (PEI)) was added to each Eppendorf tube containing plasmid DNA and then

incubated at room temperature for 20 minutes. 200 µl of the plasmid PEI solution was added dropwise to the cells and then incubated for 24 hours at 37°C in a cell culture incubator. HLA-B*13:01-P2A-B2M lentivirus was collected from the supernatant 96 h post-transfection. HMy2.C1R cells were transduced with the HLA-B*13:01-P2A-B2M lentivirus supernatant and selected for positive transduction with puromycin. Protein expression was confirmed by western blot and flow cytometry (

Figure 4.1).

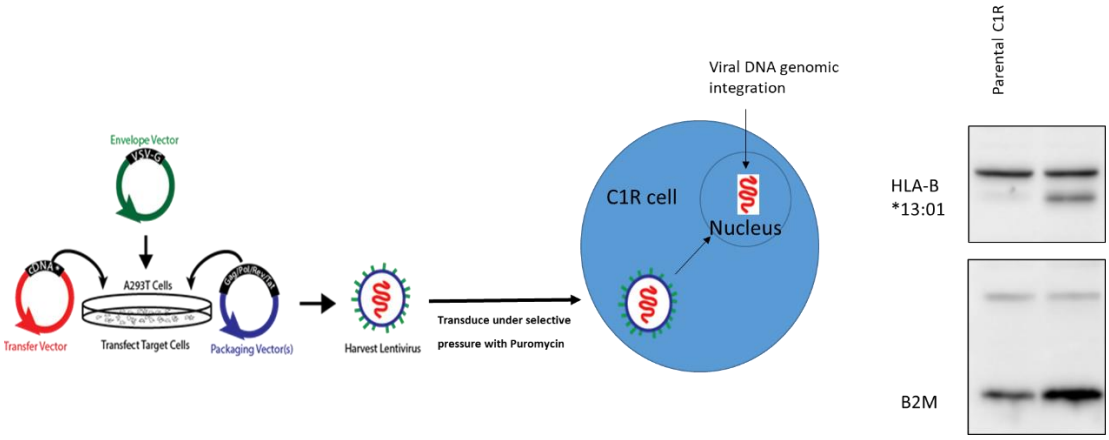


Figure 4.1: Generation of a HMy2.C1R-HLA-B*13:01-P2A-B2M Cell Line

4.3 Results

4.3.1 Generation and Characterisation DDS- and DDS-NO-Responsive $CD8^+$ TCCs From Patient 8 and 14

PBMCs from two DDS hypersensitive patients (8 and 14) were cultured with DDS or DDS-NO to produce drug-enriched T-cell lines. Magnetic bead sorting was used to isolate $CD8^+$ T-cells, then the cells were serially diluted to generate TCCs. Almost 750 TCCs were expanded and assessed for DDS or DDS-NO response. 280 TCCs were activated with DDS, while 103 were stimulated to proliferate in the presence of DDS-NO. More than 55% of the drug-treated TCCs had a SI of 10 or higher. TCCs were analysed for $CD4^+$ and $CD8^+$ phenotyping, and only those with $CD8^+$ expression were chosen for further testing (i.e., $CD4^+$ and $CD4^+CD8^+$ TCCs were not included in the study). DDS-responsive, DDS-NO-responsive, and cross-reactive TCCs were identified, as previously described in chapter 3. TCCs were activated in a concentration-dependent fashion with identical concentrations of DDS and DDS-NO (Figure 4.2).

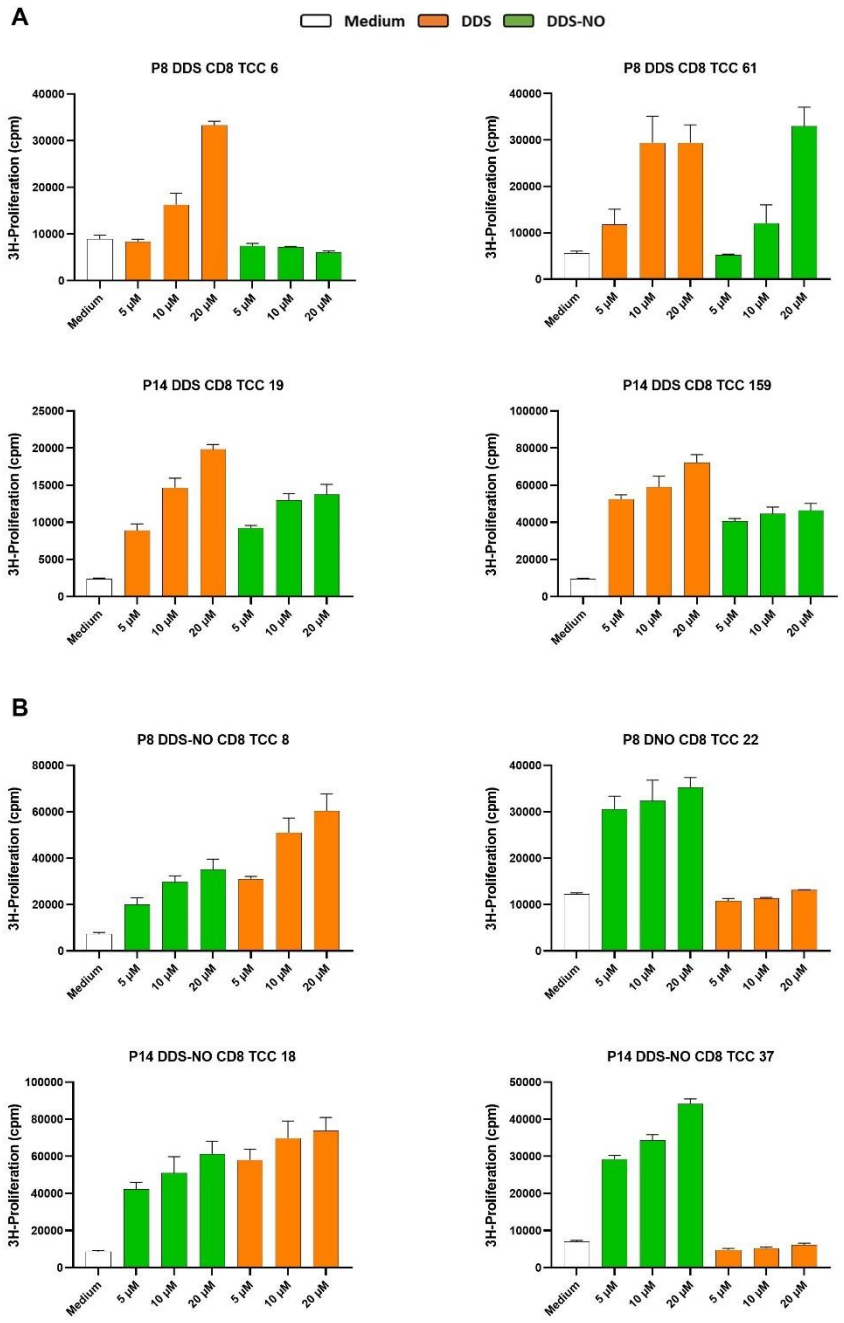


Figure 4.2: Dose-dependent proliferative responses of DDS- and DDS-NO-responsive $CD8^+$ TCCs. Dose-dependent proliferative response of (A) DDS- and (B) DDS-NO-responsive $CD8^+$ TCCs. TCCs were incubated in triplicate cultures with irradiated autologous EBV-transformed B cells and DDS (5-20 µM) or DDS-NO (5-20 µM) for 48 hours. The addition of [3H]-thymidine was used to assess proliferative responses. The results are given as mean±SD CPM.

Eight DDS-responsive TCCs and five DDS-NO-responsive TCCs were chosen to investigate the HLA molecules that have a role in drug presentation, and all were HLA class-I restricted (Figure 4.3).

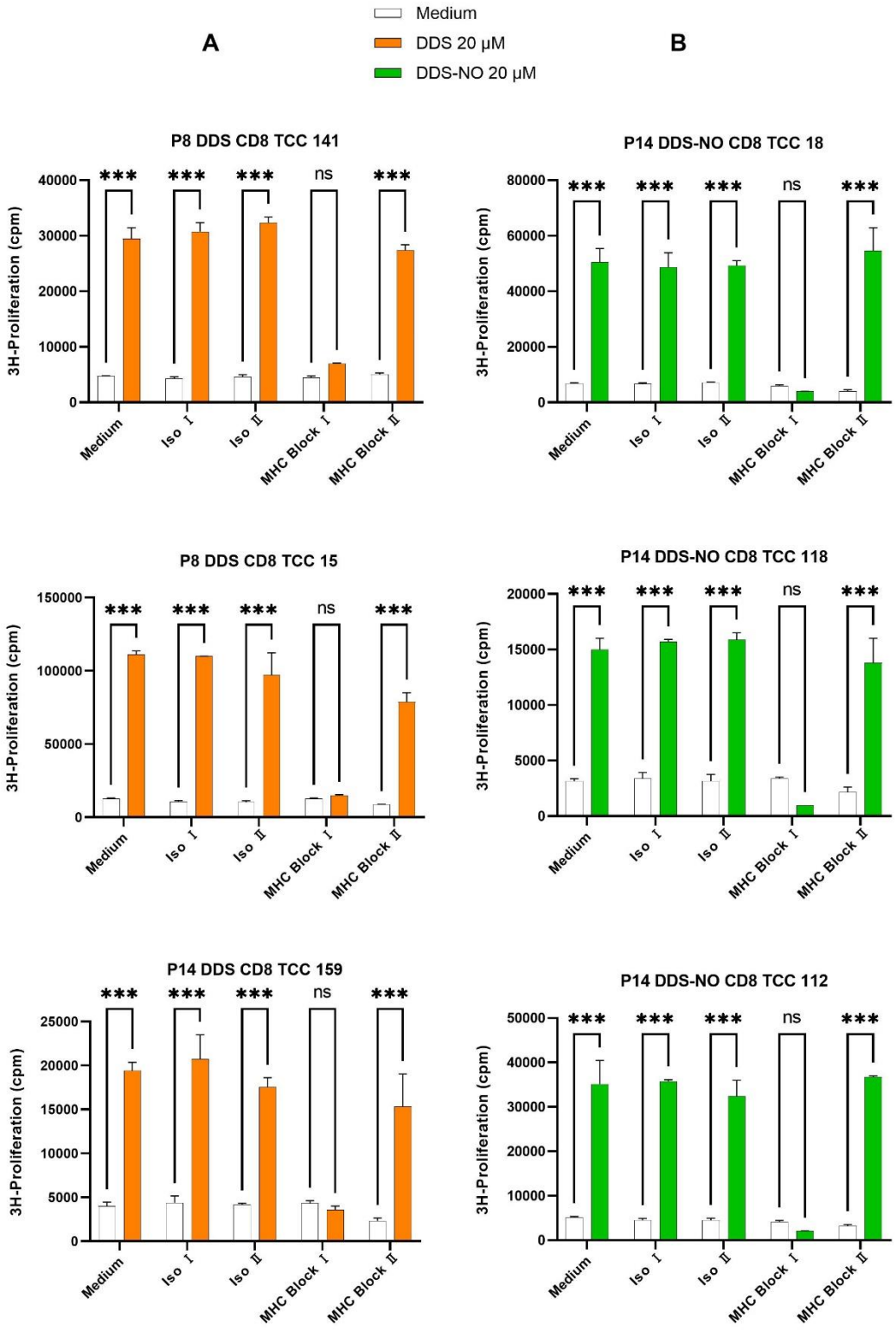
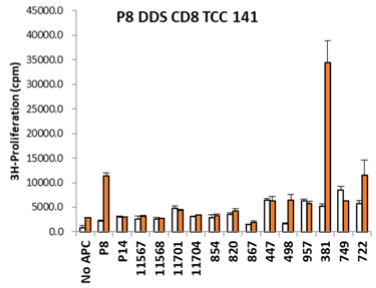


Figure 4.3: Assessment of HLA restriction of DDS- and DDS-NO-responsive CD8⁺ TCCs. (A) DDS- and (B) DDS-NO-responsive CD8⁺ TCCs were cultured with EBV-transformed B cells and DDS or DDS-NO in triplicate cultures for 48 h either in the presence or absence of anti-HLA class I and II blocking antibodies (and their respective isotype controls). The addition of [3H]-thymidine was used to assess proliferative responses. The results are given as mean±SD CPM. Statistical analysis was performed via a two way ANOVA, compared to the control media well. (NS [Not significant] = $P > 0.05$, $P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***).

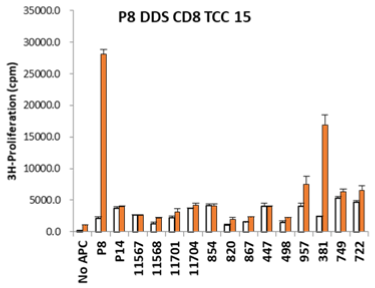
4.3.2 HLA Allele-Restricted Activation Of DDS- and DDS-NO-Responsive CD8⁺ TCCs

The same HLA class I-restricted CD8⁺ TCCs were tested with DDS or DDS-NO and HLA-typed EBV-transformed B cells from 15 human donors to investigate the HLA class-I alleles that have a role in drug presentation. Fifty percent of the responsive TCCs were self-presenting and were stimulated to proliferate in the absence of EBV-transformed B cells; therefore, they were excluded from the study. The HLA class-I-restricted TCCs that exhibit little or no self-presentation were activated with DDS and DDS-NO interacting with HLA-B alleles, but not always HLA-B*13:01. As illustrated in Figure 4.4A and B, TCCs from patients 8 and 14 were stimulated to proliferate with the drug (DDS or DDS-NO) interacting with HLA-B*15:25 or HLA-A*24:02 and HLA-B*38:02, respectively.

(A) HLA-B*15:25 or HLA-A*24:02

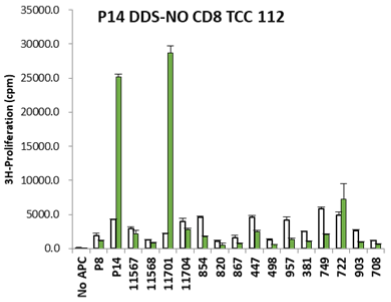


APC	SI
P8	5.3
P14	1.0
11567	1.2
11568	1.0
11701	0.9
11704	1.1
854	1.2
820	1.2
867	1.3
447	1.0
498	4.0
957	0.9
381	6.7
749	0.7
722	2.0

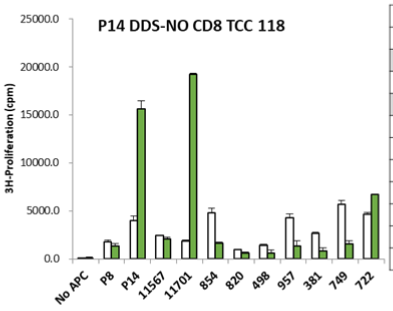


APC	SI
P8	13.4
P14	1.1
11567	1.0
11568	1.7
11701	1.4
11704	1.1
854	1.0
820	1.9
867	1.5
447	1.0
498	1.5
957	1.8
381	7.0
749	1.2
722	1.4

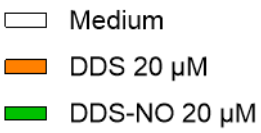
(B) HLA-B*38:02 Restricted CD8⁺ TCC



APC	SI
P8	0.6
P14	5.9
11567	0.7
11568	0.7
11701	13.1
11704	0.7
854	0.4
820	0.5
867	0.5
447	0.5
498	0.5
957	0.3
381	0.4
749	0.4
722	1.5
903	0.3
708	0.5



APC	SI
P8	0.8
P14	3.9
11567	0.8
11701	10.2
854	0.3
820	0.6
498	0.4
957	0.3
381	0.3
749	0.3
722	1.4



(C) HLA type of APCs

Number	HLA-A		HLA-B		HLA-C		HLA-DQB1		HLA-DRB1	
P 8	11:01	24:02	13:01	15:25	03:04	04:03	03:01	05:02	12:02	15:01
P 14	11:01	11:01	13:01	38:02	03:04	07:02	05:01	06:01	15:01	15:02
11567			13:01[1]	44:03						
11568			13:01[1]	40:01						
11701			13:01[1]	38:02						
11704			13:01[1]	44:03						
854	11:01	24:07	13:02	15:02	04:01	08:01	02:01	04:02	04:05	07:01
820	11:01	30:01	13:02	35:01	04:01	06:02	05:02	06:03	15:01	16:01
867	01:01	02:01	13:02	44:02	05:01	06:02	03:01	06:02	11:04	12:01
447	02:01	30:01	13:02	57:01	06:02	07:01	02:01	03:03	07:01	07:01
498	11:01	31:01	40:02	40:01	03:03	03:04	05:03	06:03	13:01	14:01
957	02:01	11:01	07:05	57:01	06:27	07:02	06:01	06:01	15:01	15:02
381	02:01	24:02	07:02	15:25	07:26	07:02	06:01	06:02	15:01	15:01
749	02:10	33:03	40:06	58:01	03:02	08:01	02:01	06:02	03:01	15:01
722	26:01	33:01	14:02	38:01	08:02	12:03	02:01	05:01	01:02	03:01
903	24:02	26:01	07:02	35:03	04:01	07:02	02:01	06:02	07:01	15:01
867	01:01	02:01	13:02	44:02	05:01	06:02	03:01	06:02	11:04	12:01
Bac-02	11:01	68:01	13:01	52:01	03:04	12:02			15:01	15:01
Bac-12	11:01	33:03	13:01	58:01	03:02	03:04			03:01	16:02

[1] Donors only genotyped for HLA-B alleles

Figure 4.4: Assessment of HLA allele restriction of DDS- and DDS-NO-responsive CD8$^+$ TCCs. (A) DDS- and (B) DDS-NO-responsive CD8$^+$ TCCs were cultured with drug or drug metabolite and irradiated EBV-transformed B cells from 15 donors expressing different HLA alleles in triplicate cultures for 48 h. The complete donors' HLA typing is shown in (C). The addition of [3H]-thymidine was used to assess proliferative responses. The results are given as mean±SD CPM.

To see if HLA-B*13:01-restricted TCCs could be identified in hypersensitive patients PBMCs, the serial dilution test was rerun with PBMCs from patients 8 and 14. Expanded TCCs were tested for proliferative responses with both P8 and P14 EBVs as APCs expressing HLA-B*13:01. There was only a minor HLA class-I overlap between these two individuals; as a result, this provided a helpful technique for rapid determination of which TCCs were likely to be HLA-B*13:01-restricted. Only TCCs with proliferative responses to DDS or DDS-NO, in the presence of both APCs,

were chosen for further testing. In response to DDS and DDS-NO, 45/111 and 29/146 TCCs were activated, respectively (Figure 4.5).

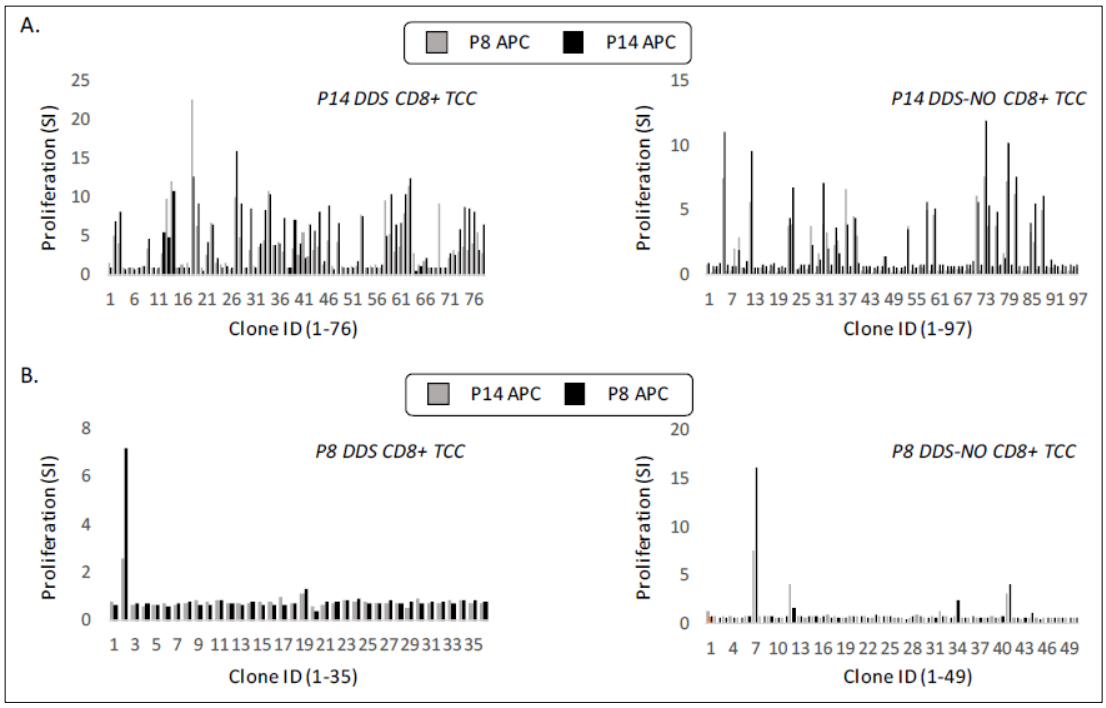


Figure 4.5: Assessment of HLA-B*13:01 restriction of DDS- and DDS-NO-responsive CD8$^+$ TCCs. TCCs were generated from PBMC of 2 patients, (A) patient 14 and (B) patient 8, by serial dilution and repetitive mitogen stimulation. TCCs were then incubated with EBV-transformed B cells from two donors expressing HLA-B*13:01 and either DDS or DDS-NO in duplicate cultures for 48h. Proliferative responses were measured by the addition of [3H]-thymidine. Results are expressed as SI.

Once the clones expanded, the DDS- and DDS-NO responsive TCCs were shown to be activated with EBV-transformed B cells expressing HLA-B*13:01, but not other HLA class-I alleles, including HLA-B*15:25 and HLA-B*38:02 (Figure 4.6).

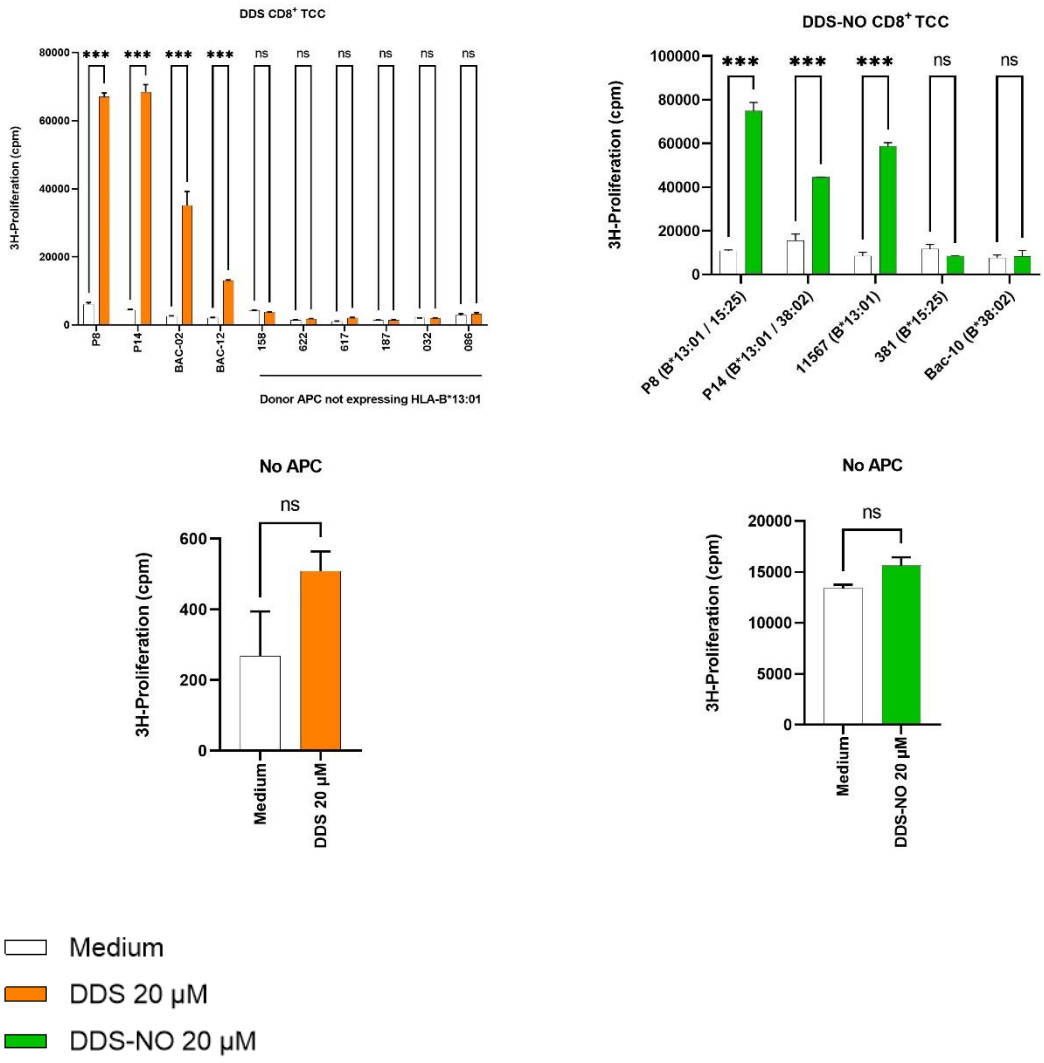


Figure 4.6: Assessment of HLA-B*13:01 restriction of DDS- and DDS-NO-responsive CD8⁺ TCCs using donors' APCs. DDS- and DDS-NO-responsive CD8⁺ TCCs were cultured with drug or drug metabolite and irradiated EBV-transformed B cells from donors expressing different HLA alleles in triplicate cultures for 48 h (only patient 8 and 14 cells expressed HLA-B*13:01). The addition of [3H]-thymidine was used to assess proliferative responses. The results are given as mean±SD CPM. Two representative TCCs are shown. Statistical analysis was performed via a two way ANOVA (Top) and unpaired T tests (Bottom), compared to the control media well. (NS [Not significant] = $P > 0.05$, $P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***).

The importance of the interaction of DDS and DDS-NO with HLA-B*13:01 in the activation of TCCs was next confirmed using C1R cells transduced with HLA-B*13:01. Because C1R cells are resistant to irradiation (necessary for APCs in T-cell proliferation studies), an IFN-ELISpot assay was employed to evaluate T-cell activation. TCCs were induced to release IFN-γ with DDS or DDS-NO in the presence of autologous EBV-transformed B cells and C1R B*13:01 cells, and showed low response with C1R parental cells, but not in the absence of APCs (Figure 4.7).

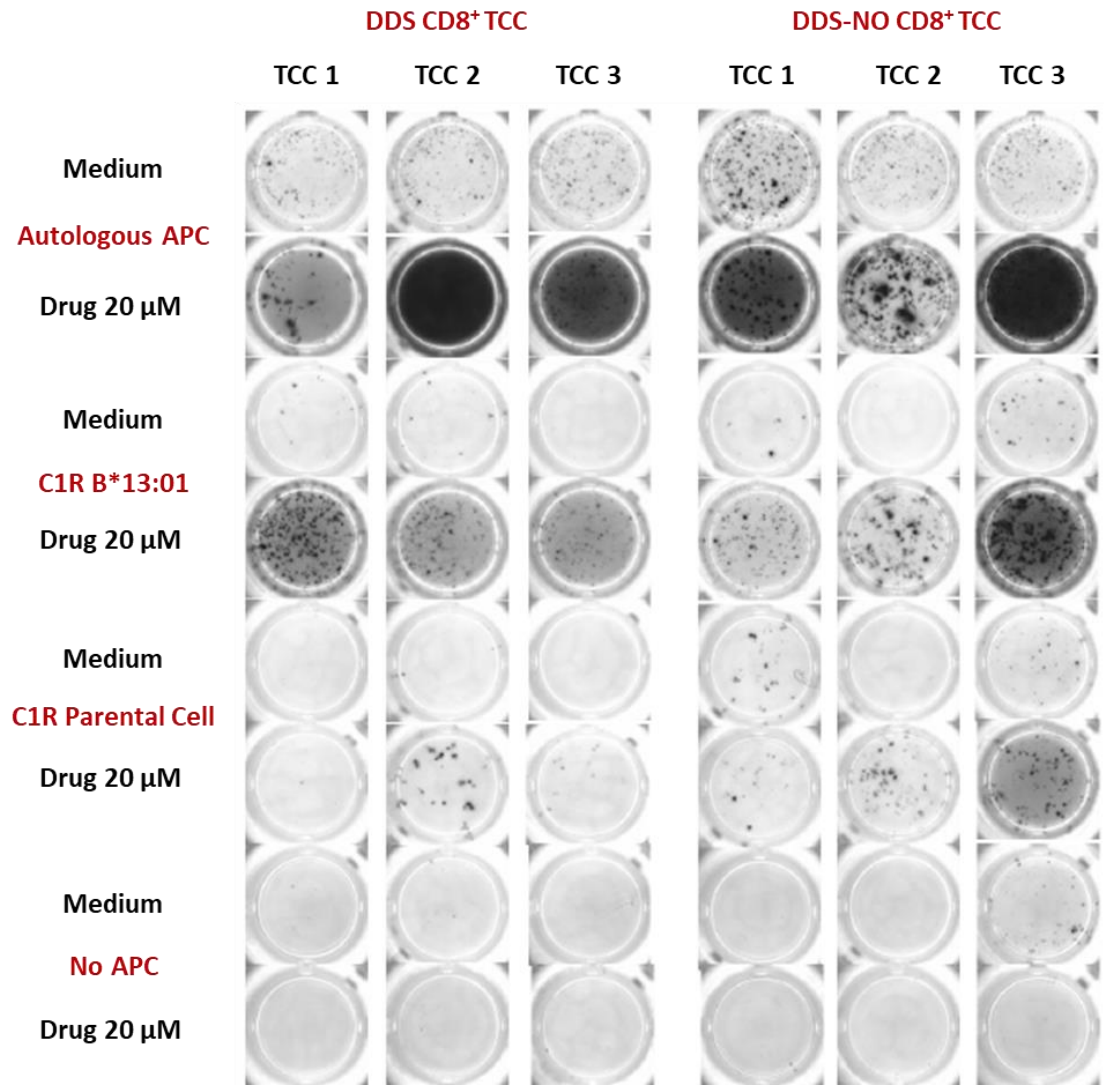


Figure 4.7: Assessment of HLA-B*13:01 restriction of DDS- and DDS-NO-responsive CD8+ TCCs using C1R B*13:01. Detection of IFN-γ secretion by six representative DDS- and DDS-

NO-responsive $CD8^+$ TCCs. TCCs were incubated with autologous APCs, C1R B*13:01 transduced or C1R parental cells and either DDS or DDS-NO. The cytokine release was visualised by ELISpot. TCCs cultured with DDS or DDS-NO in the absence of APCs served as a negative control.

4.4 Discussion

Drug hypersensitivity syndromes, also known as DRESS or DIHS, are T-cell-mediated adverse reactions caused by drugs. The extended duration to onset (mean 24 days) distinguishes it from other T-cell-mediated adverse events (17 days for Stevens-Johnson syndrome and 11 days for maculopapular exanthema) (Chen et al., 2015). Furthermore, even after the offending drug has been removed, the reaction may develop. Reactivation of the human herpesvirus has been found in some patients, with viral reactivation occurring later and being associated with more severe/prolonged disease (Descamps et al., 2001, Shiohara et al., 2006, Shiohara et al., 2007). Picard et al. found that circulating skin-homing herpesvirus-specific $CD8^+$ T-cells were activated in 76 per cent of patients, suggesting that both the drug and the virus have a role in disease pathogenesis, presumably through the activation of $CD4^+$ and $CD8^+$ T-cells (Picard et al., 2010). DDS and its nitroso metabolite have recently been demonstrated to stimulate $CD4^+$ and $CD8^+$ T lymphocytes from hypersensitive patients expressing HLA-B*13:01. Thus, this study aimed to investigate the phenotype and function of DDS- and DDS-NO-specific $CD8^+$ T-cell subsets, as well as the HLA alleles implicated in antigen presentation.

DDS is an aromatic amine that forms reversible ionic bindings (sulfone group), van der Waals forces (aromatic ring), hydrogen bonds (amine group), and hydrophobic interactions with protein including HLA molecules, but does not form covalent adducts (Chaitanya et al., 2015). DDS interacts with multiple hydrophobic amino acid residues in a drug sub-pocket around the F-pocket of the peptide-binding cleft, according to molecular modelling of DDS HLA-B*13:01 binding (Watanabe et al., 2017). The model provides a possible explanation for why HLA-B*13:01 expression

is highly linked to DDS hypersensitivity; nevertheless, the data should be interpreted with caution because it lacks the relevant binding peptide that would complete the antigen complex for T-cell recognition.

On the other hand, DDS-NO is electrophilic and produces irreversible adducts with nucleophilic amino acids (such as cysteine and lysine) in proteins, just like other aromatic nitroso compounds (Alzahrani et al., 2017, Vyas et al., 2006a, Vyas et al., 2006b, Callan et al., 2009b, Naisbitt et al., 1996, Tailor et al., 2019). Protein adducts are likely processed by antigen presenting cells into drug-modified peptides that are ultimately displayed on the cell surface by HLA molecules; however, the nature of the presented peptides is unknown.

PBMCs from 2 hypersensitive patients expressing HLA-B*13:01 were stimulated to proliferate with DDS and DDS-NO (chapter 3), and DDS- and DDS-NO-responsive $CD8^+$ TCCs were isolated and expanded. Although it is hard to estimate the significance of a link between the number of TCCs generated and the extent of disease, the frequency of DDS and DDS-NO responding T-cells generated from patients 8 and 14, as well as the degree of the induced proliferative response in the presence of the drug or metabolite, were higher than those that were previously described in many drugs (Kim et al., 2015, Monshi et al., 2013, Whitaker et al., 2011, Elsheikh et al., 2010). HLA blocking antibody experiments revealed that a drug and drug metabolite interaction with HLA class-I molecules was required to activate all DDS- and more than half of the DDS-NO-responsive $CD8^+$ TCCs as three clones were HLA class-II-restricted. As demonstrated by APC HLA mismatching tests, the drug/metabolite did not bind to HLA-B*13:01 to stimulate the TCCs. TCCs from patients 8 and 14 showed proliferative responses restricted to HLA-B*15:25 or HLA-

A*24:02 and HLA-B*38:02, respectively. To determine whether DDS and DDS-NO also activate TCCs by interacting with HLA-B*13:01, the serial dilution tests with patients 8 and 14 PBMCs were repeated. Only $CD8^+$ TCCs that responded to the drug or drug metabolite and APCs from the two patients were expanded and characterised further. There were more than 70 DDS- or DDS-NO responsive TCCs analysed. These TCCs were stimulated to proliferate with DDS or DDS-NO and EBV-transformed B cells expressing HLA-B*13:01, but not in the presence of EBV-transformed B cells expressing other HLA-B alleles. CIR-HLA-deficient cells were transduced with HLA-B*13:01 and utilised as APCs in the TCC tests to confirm that the TCCs were indeed activated through a drug-HLA-B*13:01 interaction. DDS- and DDS-NO-responsive TCCs were highly activated in the presence of the HLA-B*13:01 transfects compared to the low response with the parental cell line.

Taken together, these findings contrast with those obtained with abacavir, which binds to a single HLA molecule, HLA-B*57:01, to activate T-cells (Thomson et al., 2020a, Faridi et al., 2020, Redwood et al., 2019, Illing et al., 2012, Ostrov et al., 2012, Adam et al., 2012, Norcross et al., 2012). This is because abacavir interacts with HLA-B*57:01 with a high degree of specificity, modifying the shape of the peptide-binding groove and the peptides that bind to the HLA molecule. Our findings are considerably more consistent with oxypurinol and carbamazepine-specific T-cell responses, in that the drugs use HLA molecules discovered through GWAS research (HLA-B*58:01 oxypurinol (Hung et al., 2005); HLA-B*15:02 (Chung et al., 2004)to activate T-cells preferentially, but not exclusively. (Wu et al., 2007, Yun et al., 2014, Ko et al., 2011, Wei et al., 2012a, Chung et al., 2015).

DDS-NO-responsive clones were activated via a hapten mechanism, involving formation of DDS-NO-modified peptides through protein processing by APCs (chapter 3). Thousands of DDS-NO-modified HLA binding peptide sequences would be liberated during natural protein processing; however, only a few of them would be required to activate the T-cells reported here and elsewhere. DDS also interacts directly with numerous HLA molecules, most likely reversibly binding the outer surface of HLA peptide complexes, with T-cells receiving stimulatory signals from both the drug and the HLA bound peptide.

In conclusion, our findings demonstrate that DDS and DDS-NO interact with HLA-B*13:01 to activate $CD8^+$ TCCs in hypersensitive patients. However, more structural research is needed to determine the nature of the drug's interaction with the HLA peptide complex. Therefore, in subsequent chapters, we synthesised designer DDS-NO-modified HLA-B*13:01 binding peptides and conducted structural analyses and T-cell studies to define more precisely the nature of the HLA peptide drug T-cell receptor binding interaction.

5 Chapter 5: Generation of DDS-NO-Modified Designer Peptides

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5.1 Introduction

Previous research has identified HLA-B*13:01-restricted DDS-NO-specific $CD8^+$ T cells that reacted in an antigen processing dependent fashion, suggesting the involvement of a hapten mechanism. In addition, DDS-NO forms sulfonamide and *N*-hydroxyl-sulfonamide adducts with the thiol group of glutathione and human and mouse albumin (Alzahrani et al., 2017). However, to date, drug hapten interactions with T-cells have remained problematic and difficult to define due to the lack of precise epitopes to be included in T-cell assays. This challenge, however, can be solved by using a designer peptide model investigating possible antigenic epitopes involved in the T-cells stimulation. Incorporating individual peptides into the T-cell assay reduces antigenic diversity, which occurs when the whole proteins are degraded within the cell, resulting in many antigenic epitopes being displayed by MHC molecules. The methodology of designing these hypothetical antigens will allow to investigate the HLA restricted hapten specific T-cell response.

Only a few studies have used a single peptide approach to investigate the precise antigenic epitopes involved in drug response. For instance, Padovan et al. investigated the hapten interactions with T-cells using benzylpenicillin (BP)-reactive T cell clones generated from benzylpenicillin hypersensitive patients carrying HLA-DRB1*04:01. Three peptides: PEP-1 (EAYAKAASAAAA), PEP-2 (EAYAAAKSAAAA), and PEP-3 (EAYAAAASAKAA) were designed and used as a theoretical hapten carrier for benzylpenicillin. The tyrosine in position three and the serine in position eight were used as anchor residues to bind to HLA-DRB1*04:01. On the other hand, the lysine residue was inserted in three distinct places, 5, 7, and 10, to see if T-cell activation

was dependent on the site of drug modification. The remaining positions of these peptides were filled by alanine, a comparatively inert amino acid. Glutamic acid is also added to increase the solubility of the peptides. According to this study, benzylpenicillin-responsive T cells cross-reacted with BP-modified peptides; however, no cross-reactivity was observed between each positional drug-modified peptide reactive T cell clone and other modified peptides. This suggests that the T cell activation is dependent on the lysine location within the peptide sequence.

Furthermore, some of these T cell clones were activated with a benzylpenicillin-modified natural HLA-binding peptide, suggesting that activation of these T cell clones is carrier independent (Padovan et al., 1997). In another study, benzylpenicillin-haptenated peptides containing lysine were synthesised. Benzylpenicillin-haptenated peptide-specific $CD4^+$ were generated, and the T cell activation was observed with BP-haptenated peptides with multiple HLA class II molecule restrictions (Azoury et al., 2018). Tailor et al. (2020) have also utilised the designer peptide model to study the role of amoxicillin-modified peptides in idiosyncratic drug-induced liver injury (DILI). T cell clones were generated from patients with amoxicillin-clavulanate-induced liver injury and healthy donors who were all carriers of HLA-DRB1*15:01 and HLA-DQB1*06:02. Four peptides were generated: KP2 (EAALKAFAAIAE), KP3 (EAALAKFAAIAE), KP5 (EAALAAFKAIAE), KP6 (EAALAAFAKIAE). Anchor residues included leucine in position four, Phenylalanine in position seven, and Isoleucine in position 10 to ensure the peptides will dock into HLA-DRB1*15:01 and HLA-DQB1*06:02. Lysine residue that could bind to amoxicillin was placed in positions five, six, eight, nine, respectively. The remaining amino acids in the peptide backbone were alanine, a somewhat inert amino acid, or glutamic

acid to enhance peptide solubility. Amoxicillin-modified KP2-specific $CD4^+$ T cell clones and amoxicillin-modified KP3-specific $CD4^+$ T cell clones were generated. Amoxicillin -KP2 and amoxicillin -KP3 clones were activated with amoxicillin-modified-KP2 or -KP3, but they did not cross-react with the other peptides, proteins, or free amoxicillin. The lack of cross-reactivity between amoxicillin -KP2 and amoxicillin -KP3 reactive clones suggests that T-cell activation is highly specific or drug location dependent within the peptide backbone (Tailor et al., 2020).

This chapter aimed to design HLA-B*13:01 binding peptides that contain a reactive cysteine residue and generate DDS-NO-modified peptides to investigate their immunogenicity in T-cell culture assays with PBMC from hypersensitive patients.

5.2 Methods

5.2.1 Synthesis of Drug Modified Peptides

Three 9mer peptides were identified as potential HLA-B*13:01 high-affinity binders using the MHC binding prediction tool obtainable at www.iedb.org (Reynisson et al., 2020). Previous studies have shown that DDS-NO binds covalently to cysteine residues (Alzahrani et al., 2017), so peptides were designed to contain a single cysteine residue at positions distal from the HLA-B*13:01 binding motifs. Three positional derivatives of the peptide were designed by inserting cysteine in three different places within the binding motif. Poly-alanine was chosen as peptide backbone to minimize the interaction between peptides and TCR. Glutamic acid was also added to increase peptide solubility. All three peptides showed favourable binding to HLA-B*13:01 (a percentile rank of < 1 as predicted by NetMHC, Immune Epitope Database). To avoid a non-specific interaction between DDS-NO and primary amine, peptides were synthesised with a Fmoc protective group. Figure 5.1 summarises the peptide design method and predicted HLA-B*13:01 binding affinity. Peptide 1 (Pep1, F-MOC-AQDCEAAAL), Peptide 2 (Pep2, F-MOC-AQDACEAAL), and Peptide 4 (Pep4, F-MOC-AQDAEACAL) were purchased from SynPeptide Co Ltd (SynPeptide CO., LTD, Shanghai, China). HPLC and MS were used to confirm the purity and identity of the peptide. Fmoc-peptides were incubated with DDS-NO at a 4:1 molar ratio in 70% ACN/30% H_2O for 16-24 hrs at 37°C. DDS-NO-modified Fmoc-peptides were then analysed and purified by HPLC with a Gemini® 5 µM NX-C18 110 Å, LC Column 250 x 4.6 mm (Phenomenex, Macclesfield, Cheshire, United Kingdom) connected to an Agilent 1260 Infinity Quaternary LC (Agilent Technologies,

Stockport, Cheshire, United Kingdom). The following gradient using buffer A; H_2O with 0.1 % formic acid, and buffer B; ACN with 0.1 % formic acid was used to purify the synthetic DDS-NO modified peptides (Table 5.1).

Table 5.1: Gradient HPLC method.

Time (min)	Buffer A (%)	Buffer B (%)
0	95	5
2	95	5
32	5	95
37	5	95
37.1	95	5
50	95	5

As demonstrated in Figure 5.2, the HPLC peaks of DDS-NO, unmodified peptide, and DDS-NO-modified peptide were identified at various retention times. Using a TripleTOF 6600 (Sciex) mass spectrometer, the fractions containing modified peptides were subsequently analysed. Fmoc was removed from the purified conjugated peptides by adding piperidine at a 10:1 molar ratio in 70% ACN/30% H_2O for 3 hrs. The DDS-NO-modified peptides were then subjected to another HPLC analysis to ensure that the Fmoc protecting group had been removed. The fractions were collected and analysed by MS.

5.2.2 Mass Spectrometric Characterisation of DDS-NO Modified Peptides

DDS-NO-modified peptides were characterized using previously described methods on a TripleTOF 6600 (Sciex) mass spectrometer (16). Briefly, samples were delivered into the mass spectrometer by automated in-line reversed-phase liquid chromatography, using an Eksigent NanoLC 400 System (AB Sciex) mounted with a trap and analytical column (15 cm X 75 µm). A NanoSpray III source was fitted with a 10 µm inner diameter PicoTip emitter (New Objective). A gradient of 2–50% (v/v)

acetonitrile/0.1% (v/v) formic acid over 90 min was applied to the column at a 300 nl/min flow rate. Spectra were acquired automatically in positive ion mode using information-dependent acquisition, using mass ranges of 400–1600 Da in MS and 100–1400 Da in MS/MS. Up to 25 MS/MS spectra were acquired per cycle (approximately 10 Hz) using a threshold of 100 counts per s, with a dynamic exclusion for 12 s and rolling collision energy.

5.2.3 Quantification of Drug Molecules Remaining in The Peptide Fractions

Free DDS/DDS-NO remaining in the drug-modified peptide fractions were quantified by mass spectrometry. Calibration standards were prepared at the following concentrations (5-500 nM). Before analysis, all samples were diluted in 0.1% formic acid and spiked with the internal standard sulfamethoxazole (250 nM). Samples and standards were analysed immediately using a QTRAP5500 mass spectrometer (Sciex) coupled with an Ultimate 3000 LC system (Dionex Corporation, Sunnyvale, California). The multiple reaction monitoring transitions for each analyte were as follows: DDS 249.1/156.1, DDS-NO 263.1/156.1, and the internal standard 254.1/156.1. Other mass spectrometric parameters, such as voltage potential and collision energy, were optimized to achieve maximal sensitivity. Data acquisition and quantification were performed using Analyst 1.5 software.

5.2.4 Optimal Peptide Concentrations For T-Cell Assays

The optimal concentration of unmodified and drug-modified peptides was determined using a PBMC toxicity assay. The objective of the assay was to detect the maximum non-toxic concentration of the DDS-NO-modified peptides (i.e., the concentration that did not inhibit PBMC proliferation). Graded concentrations of unmodified and modified peptides were incubated with PBMCs ($1.5x10^5$) isolated

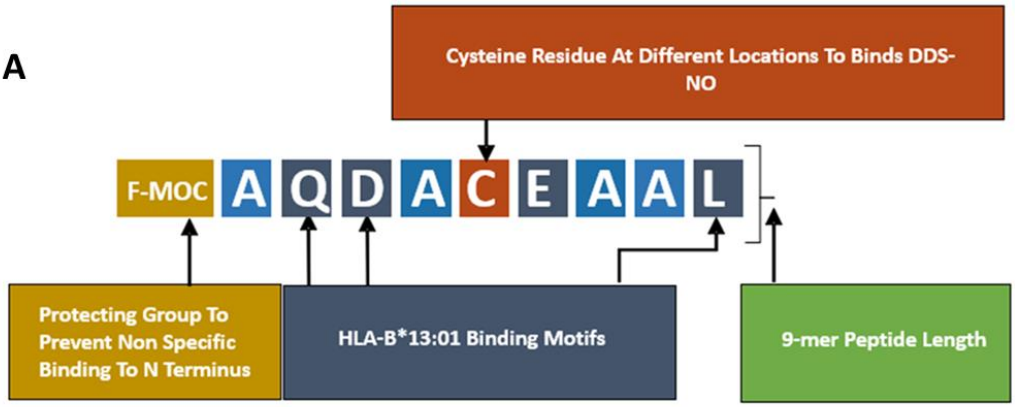
from healthy volunteers for five days with the addition of PHA (5 μg/ml) for the final 24 hrs to provide a non-specific stimulation of cells. [3H]-thymidine was then added for the final 16 hrs of the incubation. Cells were then harvested, and the proliferative response of PBMC was analysed using a beta counter. The maximum concentrations for each compound used in T-cell assays were selected based on the highest concentration with no toxicity seen.

5.3 Results

5.3.1 Synthesis Of DDS-NO-Modified Peptides

HLA-B*13:01 is a HLA allele associated with an increase frequency of DDS hypersensitivity (Zhang et al., 2013a). HLA-B*13:01 designer peptides were created by incorporating HLA-B*13:01 anchor residues and a cysteine residue for DDS-NO binding. Peptides were designed with a length of nine amino acids. Glutamine, aspartic acid, and leucine were chosen and placed in positions 2,3 and 9, respectively. These are anchor residues for the HLA-B* 13:01 allele revealed in a previous study from Zhang et al. (Zhang et al., 2013b). Cysteine was positioned in three different locations to generate three HLA-B*13:01 binding peptides for the investigation of fine structural specificity of TCR. Glutamic acid was also added to improve the peptide's solubility, and alanine was chosen to fill the remaining positions because it is an inert amino acid. To avoid a non-specific interaction between DDS-NO and a primary amine, peptides were synthesised with a Fmoc protective group. The designed peptides are predicted as strong binders using the MHC binding prediction tool (Figure 5.1C). These modified peptides were then prepared from the conjugation of N-terminal Fmoc protected peptides using DDS-NO at a 4:1 molar ratio in 70% ACN/30% H_2O for 16-24 hrs at 37°C.

A



B

C

Allele	Length	Peptide	Score	Rank
HLA-B*13:01	9	AQDCEAAAL	0.241605	0.67
HLA-B*13:01	9	AQDACEAAL	0.235073	0.69
HLA-B*13:01	9	AQDAEACAL	0.3651	0.29

Figure 5.1: Strategy to synthesize DDS-NO-modified peptides. (A) Schematic diagram illustrating the approach for designing DDS-NO-modified HLA binding peptides; (B) Sequences of three synthetic peptides with DDS-NO in position 4, 5, and 7 (C) all three peptides showed favourable binding to HLA-B*13:01 (a percentile rank of < 1 as predicted by NetMHC, Immune Epitope Database).

5.3.2 Purification of DDS-NO-Modified Peptides

To use DDS-NO-modified peptides in T-cell assays, they must be isolated from other impurities such as the unmodified peptide, free DDS-NO, and their degradation products. A series of HPLC analyses were performed to improve the purity of DDS-NO modified peptides (Figure 5.2). The peak retention time (t_R) of the unmodified

peptide and DD-NO was determined first. DDS-NO began to dimerize almost immediately. Peptide modification was detected by analysing peak shifts over time in a series of time-course incubations. Fmoc was deprotected by adding piperidine at a 10:1 molar ratio in 70% ACN/30% H_2O for 3 hrs, followed by HPLC purification. When the final DDS-NO-modified peptide peaks were separated, they shifted slightly; however, the MS data confirmed their identity. The final product was essentially free of soluble DDS, DDS-NO or unmodified peptide based on HPLC analysis (Figure 5.2F). Fractions from numerous HPLC runs were combined and dried before MS analysis.

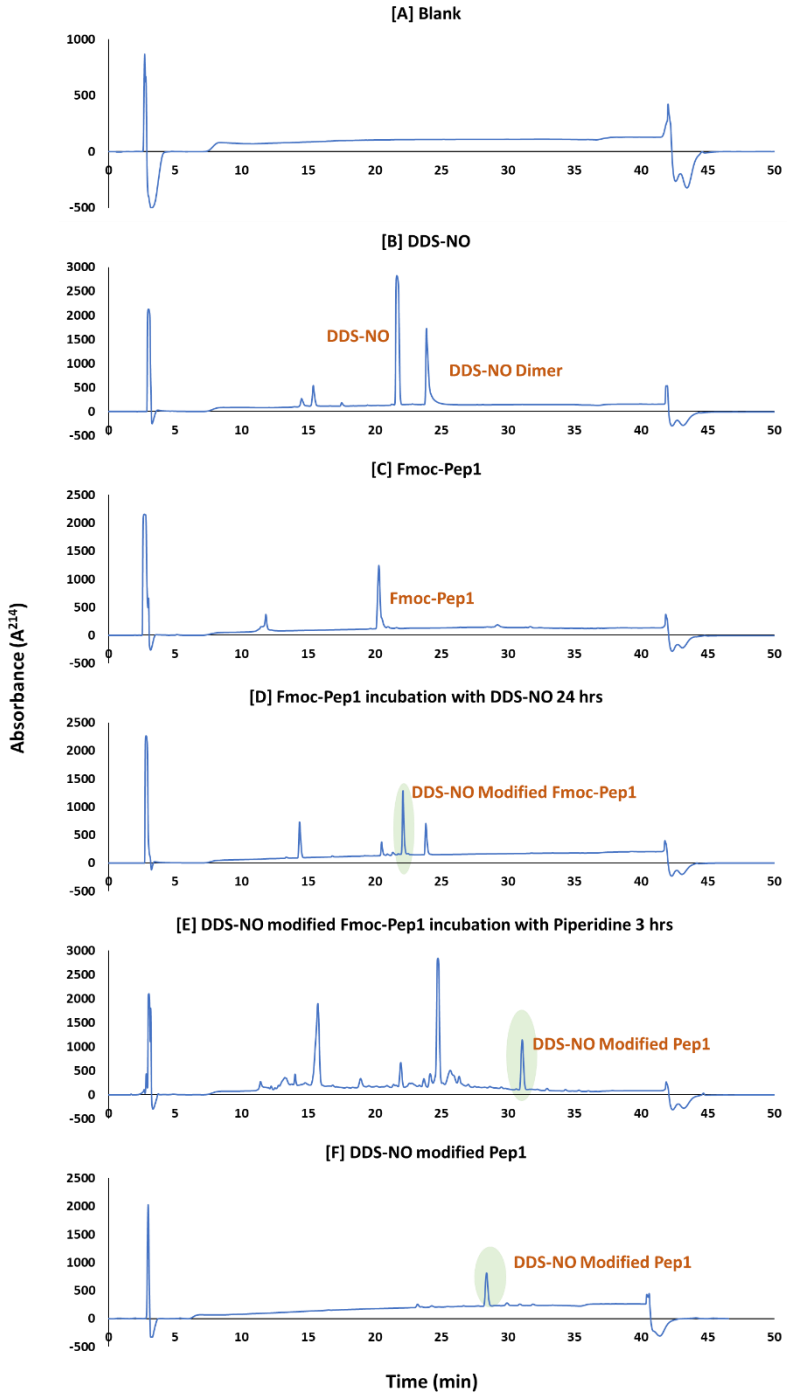


Figure 5.2: Purification of DDS-NO-modified HLA-B*13:01 binding peptides. HPLC trace shows the blank control (A), the retention time of DDS-NO (B) and unmodified Fmoc-Pep1 (C) before incubation; after 24 hrs incubation in 70% ACN/ H_2O (v/v), a new peak appeared representing DDS-NO modified Fmoc-Pep1 (highlighted in green, D); incubation of DDS-NO modified Fmoc-Pep 1 with piperidine at a 10:1 molar ratio in 70% ACN/ H_2O (v/v) for 3 hrs

resulted in the final product, DDS-NO Pep 1 (highlighted in green, E); HPLC trace shows DDS-NO Pep 1 generated in high purity (F).

5.3.3 MS Analysis of DDS-NO-Modified Peptides

Fractions containing DDS-NO-modified peptides were analysed using an AB SCIEX 5600 mass spectrometer after they were cleaned up using C18 ZipTips. Peptide sequence and DDS-NO haptenic structure were determined using MS/MS analysis. The unmodified peptides' MS/MS spectra revealed singly charged ions at m/z 891.3. MS/MS spectra of the final purified DDS-NO-modified peptides revealed doubly charged ions at m/z 585.235, corresponding to the peptides with a sulfonamide adduct (Δm=278). The peptide sequence was confirmed by the presence of a series of y and b ions. The modification site was confirmed by a series of adducted b ions, all with a mass addition of 278 amu, giving evidence of modification at cysteine residue (Figure 5.3, Figure 5.4).

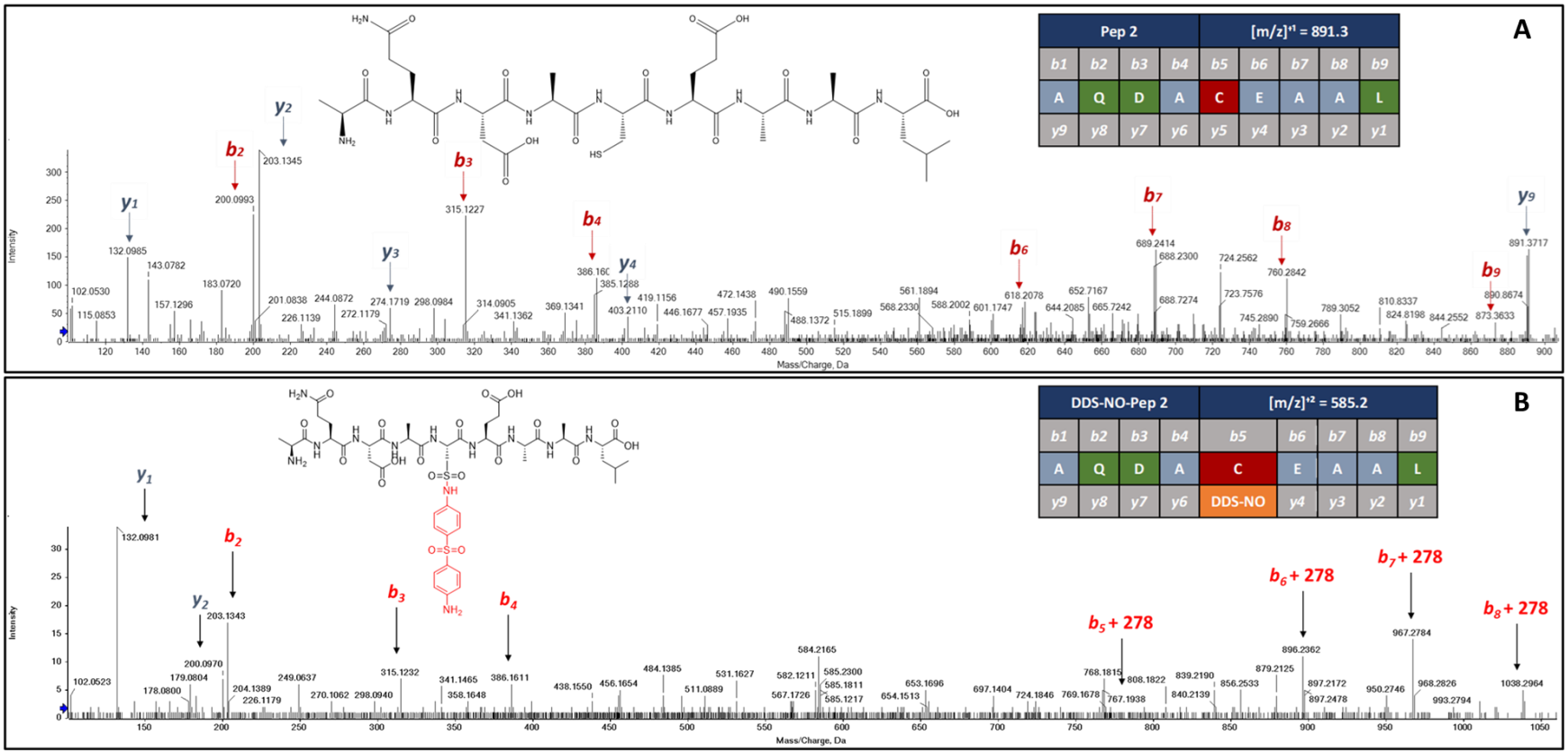


Figure 5.3: MS/MS for adduction of Pep2 (AQDACEAAL) by DDS-NO. AB Sciex TripleTOF 6600 MS/MS spectra of (A) unmodified Pep2 [m/z] +1 891.3 (B) DDS-NO-modified Pep2 [m/z] +2 585.2. Signals for b and y series fragment ions are shown. Characteristic peptide fragment ions with mass addition of 278 amu confirm the site of DDS-NO modification.

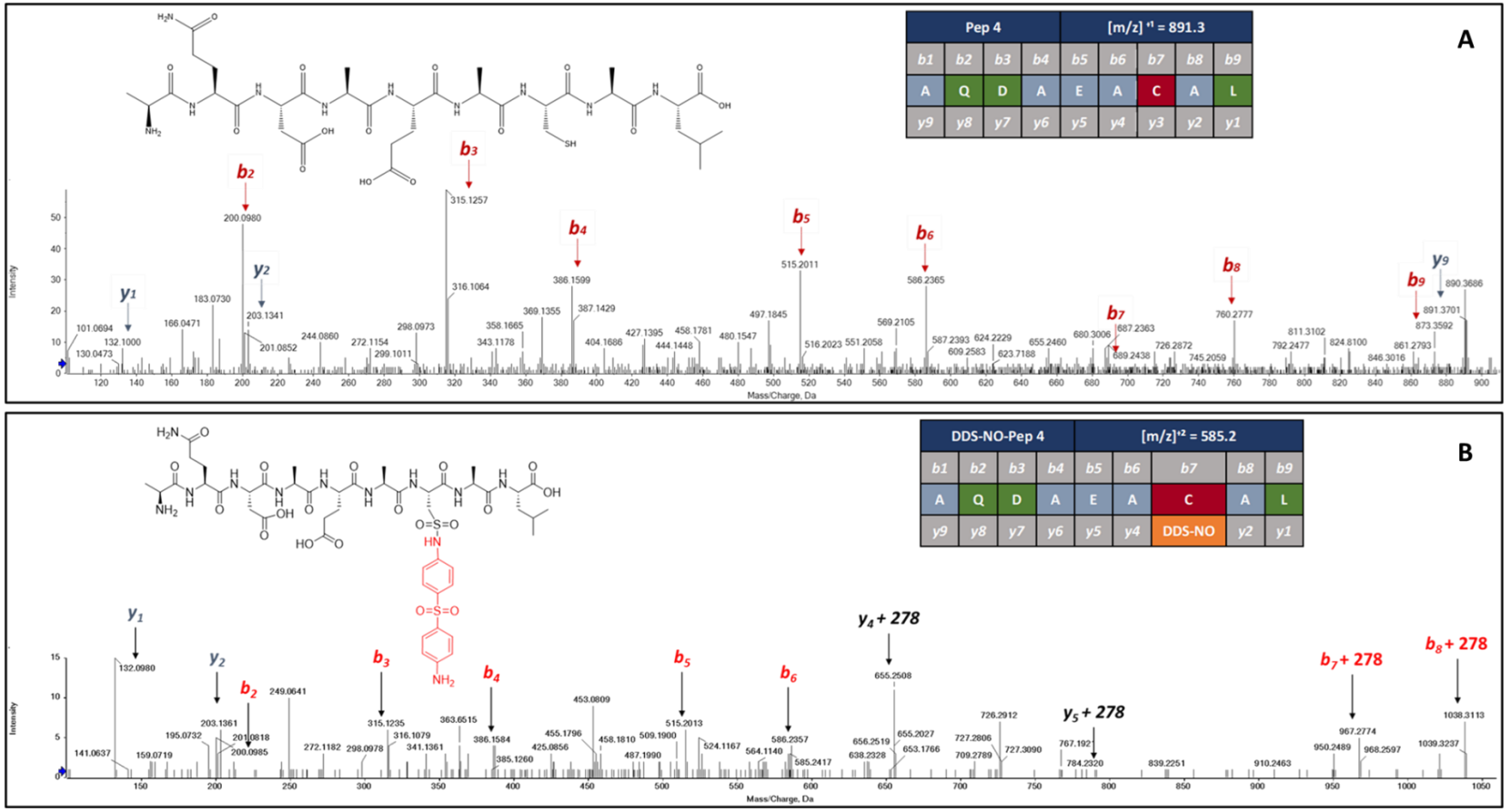


Figure 5.4: MS/MS for adduction of Pep4 (AQDAEACAL) by DDS-NO. AB Sciex TripleTOF 6600 MS/MS spectra of (A) unmodified Pep4 [m/z] +1 891.3 (B) DDS-NO-modified Pep4 [m/z] +2 585.2. Signals for b and y series fragment ions are shown. Characteristic peptide fragment ions with mass addition of 278 amu confirm the site of DDS-NO modification.

5.3.4 Quantification of Free DDS Remaining in DDS-NO-Modified Peptides

Free DDS remaining in the drug-modified peptide fractions were quantified by MS. After HPLC analysis, 40 nM free DDS was found in a 10 µM modified peptide solution (Figure 5.5), indicating that a 50 µM DDS-NO modified peptide (the most often utilised concentration) contains 0.2 µM free DDS. This was considerably lower than the concentration of DDS that has previously been found to activate T-cells (5 µM). 50 µM was selected as the highest concentrations for both unmodified and modified peptides for T-cell assays based on toxicity results (Figure 5.6).

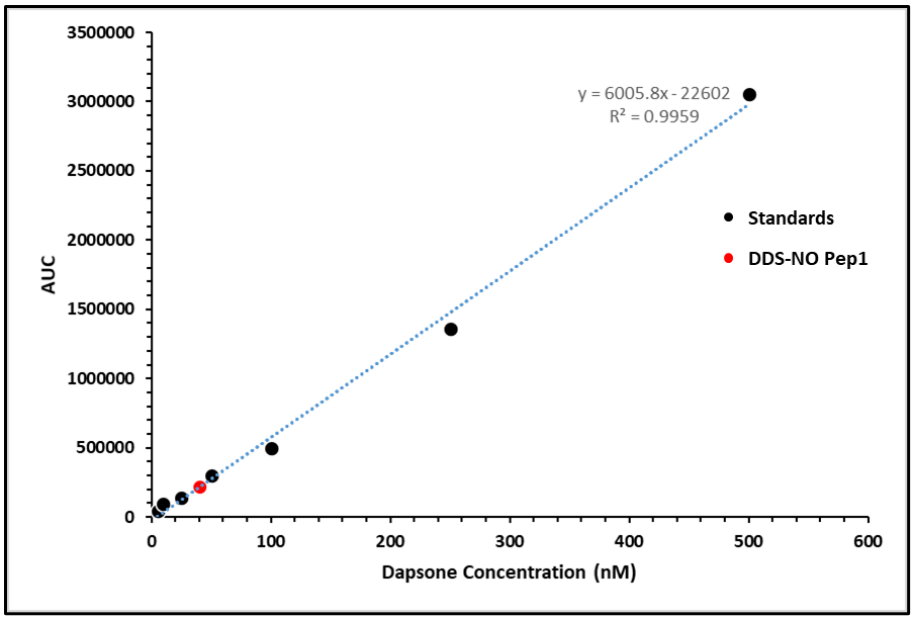


Figure 5.5: Relative Quantification of free DDS in a sample of 10 µM DDS-NO modified Pep1 post-HPLC purification using AB Sciex QTRAP 5500 and MRM method. A standard curve of DDS was prepared between 5 nM and 500 nM.

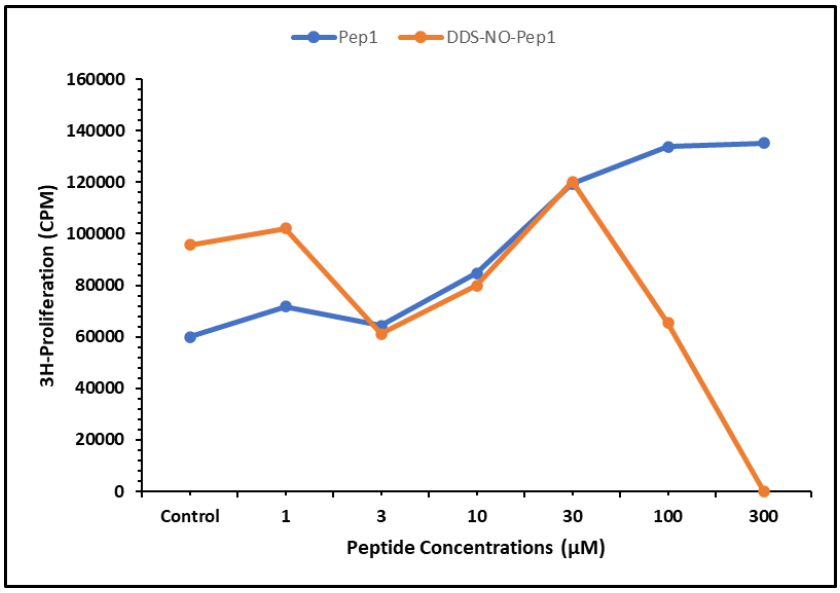


Figure 5.6: Toxicity of Pep1 and DDS-NO-Pep1. Healthy donor's isolated PBMCs ($1.5x10^5$) were incubated with a graded concentration of unmodified Pep1 (1-300 µM), and DDS-NO modified Pep1 (1-300 µM) for five days with the addition of PHA (5 µg/ml) for the final 24 hrs to stimulate cells. The addition of [3H]-thymidine was used to assess proliferative responses.

5.4 Discussion

Dapsone (DDS) is known to cause dapsone hypersensitivity syndrome (DHS) in 0.5 to 3.6% of exposed individuals. The dapsone metabolite (DDS-NO) is electrophilic and produces irreversible adducts with nucleophilic amino acids (such as cysteine and lysine) in proteins, just like other aromatic nitroso compounds (Alzahrani et al., 2017, Vyas et al., 2006a, Vyas et al., 2006b, Callan et al., 2009b, Naisbitt et al., 1996, Tailor et al., 2019). Our previous work showed that DDS-NO-responsive $CD8^+$ T-cells were activated via a hapten mechanism. The T-cell response was highly polymorphic; the drug metabolite interacted with several class I alleles, including HLA-B*13:01, to stimulate proliferation and cytokine release. Despite these findings, the nature of the binding interaction between DDS-NO and T cells has not been defined. This issue can be resolved by implementing a designer peptide methodology to investigate potential determinants of DDS-NO specific T-cells. Our study aimed to (i) design HLA-B*13:01 binding peptides that contain a reactive cysteine residue, (ii) generate DDS-NO modified peptides that are free of nitroso metabolite, (iii) explore immunogenicity of the peptides using autologous antigen-presenting cells (APC), and APC transfected with single HLA-B alleles (the latter is the subject of Chapter 6).

Three DDS-NO-modified peptides were synthesized in high purity. Each peptide contained 3 HLA-B*13:01 anchoring motifs (glutamine at P2, Aspartic acid at P3 and leucine at P9, the MHCI binding predictions were made using the IEDB analysis resource NetMHCpan 4.1 tool), an alanine backbone (previous studies show that non-anchoring amino acids, for the most part, do not define the specificity of the T-cell response (Ortmann et al., 1992, Martin et al., 1992) and a nucleophilic cysteine

residue for modification by DDS-NO. A Fmoc protecting group was used to prevent DDS-NO *N*-terminal binding, which was subsequently removed after DDS-NO was covalently bound to peptides. High yields of DDS-NO-modified Pep1 and Pep4 were obtained after HPLC purification; Pep2 was generated in a lower quantity and, as such, only used in T-cell cross-reactivity studies. Pep1 contains a cysteine residue in the fourth position, which is an important trinitrophenol hapten binding site for the generation of immunodominant $CD8^+$ + peptide epitopes. In contrast, trinitrophenol modification at more distal positions (e.g., position 7 in Pep4) generates qualitatively different determinants that tend to activate a lower frequency of T-cells (Martin et al., 1993).

HPLC and TripleTOF 6600 (AB Sciex) mass spectrometers were used to purify and characterise drug modified peptides. However, this work was affected by significant difficulties, particularly in the peptide purification process. These challenges include: (i) the need to achieve a high yield of modification; thus a 1:4 ratio between the starting unmodified peptide and DDS-NO was used, which is costly; (ii) DDS-NO dimerization due to the free NH_2 group, a product that is formed during the incubation with high concentrations of DDS-NO; and (iii) difficulties to identify HPLC fractions containing the DDS-NO modified peptides using MS. Alternative techniques for synthesising modified peptides could have been used. For instance, the method used by Professor Marc Pallardy group. They synthesised a penicillin-modified lysine monomer and incorporated it into a peptide sequence using Fmoc solid-phase synthesis (Azoury et al., 2018). Even while this method improves the yield of produced peptides, it does not address the problem of drug dimerization since the NH_2 group on the drug requires a protective group. Several amino

protecting group could overcome the challenges; however, removal of these protecting group require extra steps and could potentially reduce the yield of final products. Finally, another issue we encountered throughout the peptide generation was the loss of peptides during purification, leading to low yield of final products, which was around 10 % of the original starting material. Despite all of these issues, the final product was essentially free of soluble DDS, DDS-NO or unmodified peptide based on HPLC analysis. In addition, the quantitative MS allowed us to determine the amount of residual free DDS. in the modified peptide solution, which is below the level required to activate DDS-responsive T-cells. Furthermore, as shown in Chapter 6, the clones used in the immune investigations were not activated with DDS even at mM concentrations. Finally, although it was not possible to generate a standard curve and quantify free DDS-NO levels in the modified peptides the any remaining DDS-NO could be quenched through the addition of glutathione. Data shown in chapter 6 shows that glutathione binds covalently with DDS-NO and blocks DDS-NO-specific T-cell activation.

To conclude, the data presented herein show the generation of purified HLA-B*13:01 binding DDS-NO-modified peptides. These peptides were then used to explore the activation of DDS-NO-specific T-cell clones from hypersensitive patients.

6 Chapter 6: Activation of CD8+ T-cells from dapsone hypersensitive patients with nitroso dapsone-modified HLA-B*13:01-binding peptides

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6.1 Introduction

Dapsone (DDS) is a widely used antibacterial agent and is frequently prescribed for the treatment of leprosy. However, exposure to DDS is associated with the development of a hypersensitivity syndrome, characterised by fever, skin rash, hepatitis, and generalized lymphadenopathy, in 0.5-3.6 % of treated patients (Allday and Barnes, 1951, Liu et al., 2019). Recent studies have shown that expression of HLA-B*13:01 is associated with increased risk of DDS hypersensitivity among Asian populations (Wang et al., 2013, Zhang et al., 2013a, Satapornpong et al., 2021, Park et al., 2020).

DDS contains aromatic amine groups that are susceptible to acetylation by *N*-acetyltransferase enzymes, a process of detoxification that limits the lifespan of the drug. The aromatic amine groups also undergo cytochrome P450-mediated hydroxylation yielding DDS hydroxylamine (Gill et al., 1995). Nitroso dapsone (DDS-NO) is generated via auto-oxidation of the hydroxylamine metabolite. DDS-NO is protein-reactive and covalently modifies cysteine residues on serum and cellular proteins (Reilly et al., 2000, Roychowdhury et al., 2005, Vyas et al., 2006b, Alzahrani et al., 2017).

Peripheral blood mononuclear cells (PBMC) from hypersensitive patients expressing HLA-B*13:01 are stimulated to proliferate with both DDS and its nitroso metabolite (Chen et al., 2018, Zhao et al., 2019). Through the generation of T-cell clones HLA class II-restricted CD4+ and HLA-class I- and II-restricted CD8+ T-cells were shown to be stimulated with DDS and DDS-NO via different pathways (Zhao et al., 2021, Zhao et al., 2019). DDS interacts directly with HLA and/or HLA binding peptides to trigger T-cell receptors, whereas DDS-NO triggers T-cell receptors though a pathway

dependent on protein processing within antigen presenting cells presumably through the formation of protein adducts. If one focuses on the CD8+ T-cell response, both DDS and DDS-NO interact with multiple HLA class I proteins to activate T-cells (Chapter 4), which makes it challenging to focus research on the drug HLA-B*13:01 interaction. Thus, it is difficult to define the nature of binding between HLA-B*13:01, DDS-NO and specific T-cell receptors. The aim of this work was to explore immunogenicity of DDS-NO-modified peptides using autologous APC and APC transfected with the single HLA-B allele HLA-B*13:01.

6.2 Material and Methods

6.2.1 Anti-DDS antibody production

Ovalbumin-DDS conjugates were prepared by the reaction of DDS diazonium salts with ovalbumin using methods previously described (Manchanda et al., 2002). Antibody production was performed by Kaneka Eurogentec S. A. (Belgium) using a speedy 28-polyclonal package. Detailed information is available online (eurogentec.com).

6.2.2 Human subjects

Venous blood samples (50 ml) were taken from 2 DDS hypersensitive patients with positive (i) DDS patch test and (ii) DDS and DDS-NO lymphocyte transformation test as reported previously (Zhao et al., 2021). Patient 8 (female, 28 years old at time of adverse event) displayed fever and abnormal liver function tests following 17-day exposure to DDS. Patient 14 (male, 24 years old at time of adverse event) displayed fever, skin rash (erythema) and abnormal liver function tests following 21-day exposure to DDS. Both individuals expressed HLA-B*13:01; the full patient HLA profiles are available in Chapter 4. Approval for the study was acquired from Shandong Provincial Institute of Dermatology and Venereology and informed written consent was obtained. A material transfer agreement was signed prior to transport of PBMC to Liverpool.

6.2.3 Generation of DDS-NO-modified peptide-responsive T-cell clones

PBMC (1 x 10^6/well) were cultured in a 48-well plate with DDS-NO-modified Pep1 (patient 8 and 14) and Pep4 (patient 14 only due to limitations on the availability of the modified peptide) for 14 days to enrich the number of responsive T-cells prior to serial dilution. Peptide concentrations (10-50 µM) were selected based on a lack

of intrinsic toxicity and no inhibition of phytohemagglutinin (PHA)-treated healthy donor PBMC proliferation (see Chapter 5). T-cell clones were generated by serial dilution and repetitive mitogen-driven expansion. Briefly, irradiated allogenic PBMC (5×10^4 cells/well) in medium containing PHA and IL-2 were added to 96 well U bottom plates. T-cells were then diluted and added to the PBMC mixture at 0.3, 1 and 3 cell/well. Cultures were incubated for 14 days (37°C / 5% CO_2) and medium was supplemented with IL-2 every 2 days. On day 14, the growing clones were restimulated with PHA and irradiated allogenic PBMC (5×10^4 cells/well) in IL-2 containing medium and expanded for a further 14 days prior to testing for peptide specificity.

6.2.4 Characterization of drug-modified peptide-responsive T-cell clones

T-cell clones were phenotyped using flow cytometry for the CD4+ (CD4-APC (clone RPA T4)) and CD8+ surface receptors (CD8-PE (clone HIT8a)). T-cell clones (5×10^4 cells/well) were tested in dose-response studies for cross-reactivity with irradiated antigen presenting cells (1×10^4 cells/well) and DDS-NO-modified peptides (Pep1, 2 and 4; 1 to 100 µM) in triplicate cultures. Unmodified peptides subjected to the same culture conditions and purification steps were used as a negative control (1 to 100 µM). Clones were also cultured with soluble DDS-NO (1-40 µM). Proliferation of the clones was measured by addition of [^{3}H]-thymidine for the final 16 hrs of the experiment.

The secretion of IFN-γ from the clones was assessed using ELISpot. T-cell clones were incubated with antigen presenting cells in the presence and absence of DDS-NO-modified peptides and other study compounds in IFN-γ antibody-coated ELISpot plates (37°C, 5% CO_2) for 48 hrs. Plates were then developed according to the

manufacturer's instructions (Mabtech, Stockholm) and spots were counted using an AID ELISpot reader.

In order to determine whether the detected activation of clones with DDS-NO-modified peptides was due to residual DDS or DDS-NO or degradation of the peptides and liberation of free DDS, clones (5×10^4 cells/well) were cultured with irradiated antigen presenting cells (1×10^4 cells/well) (i) in the presence of soluble DDS (125-500 μM) and proliferation was measured by addition of [^{3}H]thymidine; and (ii) in the presence of DDS-NO-modified Pep1 or soluble DDS-NO and glutathione (1 mM), which binds covalently to the nitroso metabolite preventing protein binding (Alzahrani et al., 2017), and proliferation was measured by addition of [^{3}H]thymidine. The irreversible binding of DDS-NO to antigen presenting cells was measured in the presence and absence of glutathione (1 mM) using immunofluorescence staining with an anti-DDS antibody.

To explore the importance of HLA proteins in T-cell activation, T-cell clones (5×10^4 cells/well) were cultured with Pep1 or Pep4 (i) in the absence of antigen presenting cells; (ii) in the presence of C1R-B*13:01 or C1R-parental antigen presenting cells (1×10^4 cells/well); and (iii) in the presence of C1R-B*13:01 antigen presenting cells pre-treated with either isotype (IgG1) or HLA class-I (DX17) or HLA class-II (Tu39) blocking antibodies for 30 min. T-cell proliferation or IFN-γ release were measured using [^{3}H]thymidine or ELISpot, respectively.

6.3 Results

6.3.1 Generation and phenotypic assessment of DDS-NO-modified Pep1 and Pep4 T-cell clones

Initial testing of almost 400 T-cell clones derived from DDS-NO-modified Pep1 or Pep4-treated PBMC involved culture of T-cells with antigen presenting cells and the peptides or medium (as a negative control) in duplicate culture and comparison of proliferation. Almost 50% of the clones generated displayed reactivity against either DDS-NO-modified Pep1 (n=124) or Pep4 (n=48) and the strength of the proliferative response varied from a stimulation index (proliferation in test incubations with antigen / proliferation in control incubations with medium) of 2 to above 30 (Figure 6.1). These T-cell clones were expanded and analysed for CD phenotype. The majority of clones expressed the CD8+ receptor (Figure 6.2) and these were used in the experiments described below.

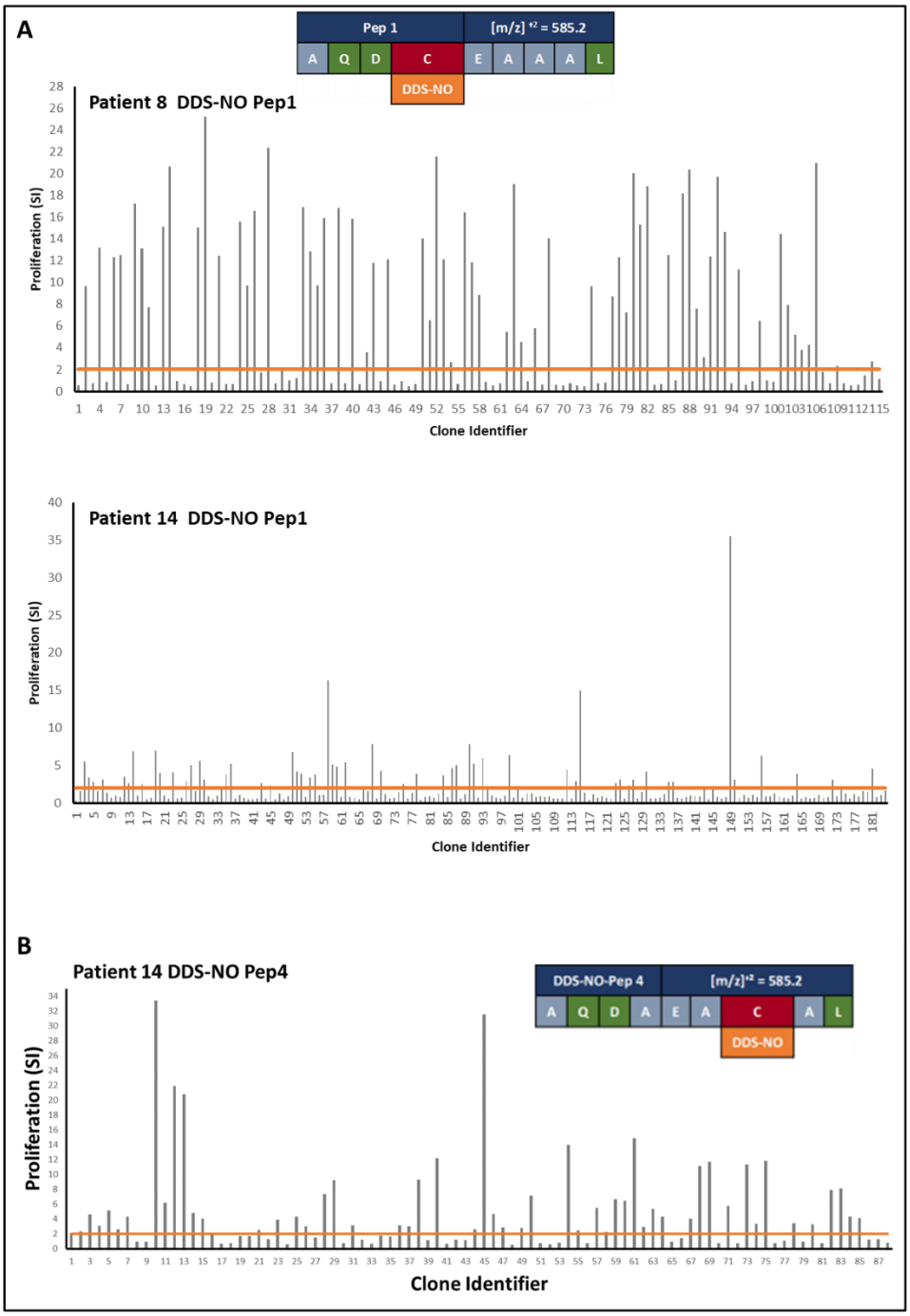


Figure 6.1: Generation of DDS-NO-modified Pep1- and Pep4-responsive T-cell clones from HLA-B*13:01+ DDS hypersensitive patients. T-cell clones were generated from patient 8 or patient 14 PBMC cultures by serial dilution and repetitive mitogen stimulation. Expanded clones (5 × 10^4 cells/well; 200 µl) were incubated with irradiated autologous antigen presenting cells (EBV-transformed B-cells; 1 × 10^4 cells/well) and DDS-NO-modified (A) Pep1 or (B) Pep4 in duplicate for 48 hrs. Proliferation was measured using [3H]-thymidine (0.5

µCi/well). T-cell clones with a stimulation index (SI) of > 2 were expanded for further experimental studies. Structures of the DDS-NO-modified peptides are also shown.

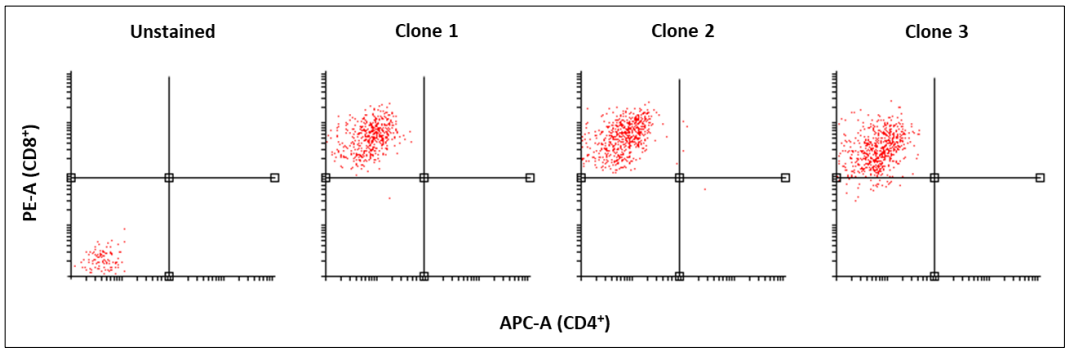
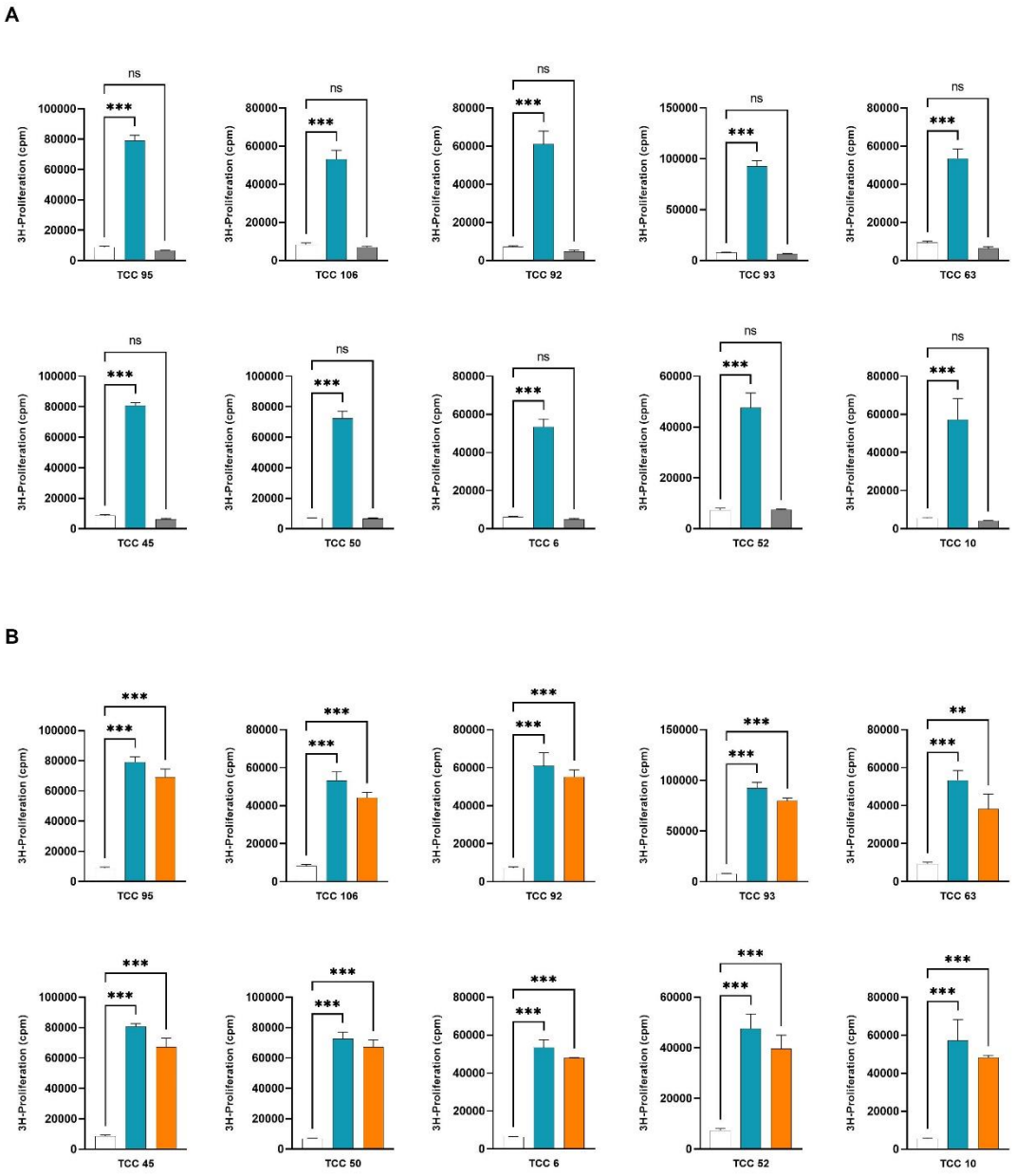


Figure 6.2: CD4$^+$/CD8 $^+$ phenotyping of three representative DDS-NO modified peptides specific TCCs. TCCS were labelled with anti-CD4$^+$ and anti-CD8$^+$ antibodies and analysed by flow cytometry.

6.3.2 Functional characteristics of DDS-NO-modified Pep1- and Pep4-responsive CD8+ T-cell clones

A panel of up to 30 T-cell clones were utilized to assess cross-reactivity. All clones were stimulated to proliferate with DDS-NO-modified Pep1 (Figure 6.3A) or Pep4 (results not shown); however, proliferative responses were not detected when the clones were cultured with unmodified peptides. All clones were also activated with soluble DDS-NO (Figure 6.3B), which forms adducts with protein in the cell culture assay (Figure 6.6B), and the strength of the maximal response induced was similar with optimal concentrations of DDS-NO and DDS-NO-modified peptide. Clones were activated with the modified peptide and soluble drug metabolite in a dose -dependent manner (Figure 6.4). Lowest stimulatory concentrations of DDS-NO varied from 0.25 – 10 µM. Concentrations of the modified peptides required to activate clones was similar. Clones were stimulated to proliferate (Figure 6.3C) and

secrete IFN-γ (Figure 6.5) with DDS-NO-modified Pep1, Pep2 and Pep4, with no discernible difference between the strength of the induced response observed.



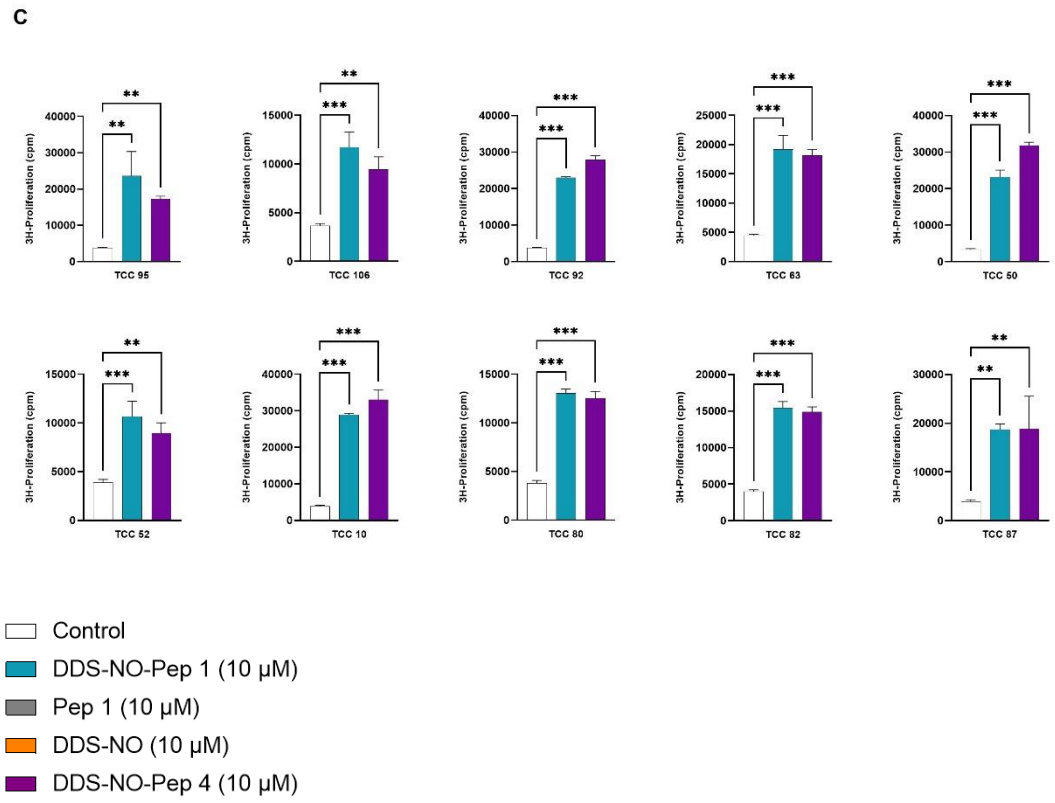


Figure 6.3: Cross-reactivity of DDS-NO-modified peptide-responsive CD8+ T-cell clones. 10 representative DDS-NO-modified peptide-responsive $CD8^+$ from a panel of up to 30 clones were used to explore cross-reactivity with unmodified peptide, soluble DDS-NO and modified peptides with cysteine located in different positions in the peptide sequence. Clones (5×10^4 cells/well; 200 µl) were incubated with irradiated autologous antigen presenting cells (EBV-transformed B-cells; 1×10^4 cells/well) and (A) DDS-NO-modified Pep1 or unmodified Pep1; (B) DDS-NO-modified Pep1 or soluble DDS-NO; or (C) DDS-NO-modified Pep1 or DDS-NO-modified Pep4 in triplicate for 48 hrs. Proliferation was measured using [3H]-thymidine (0.5 mCi/well). Results are expressed as mean±SD CPM. Statistical analysis was performed via a one way ANOVA, compared to the control media well. (NS [Not significant] = $P > 0.05$, $P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***).

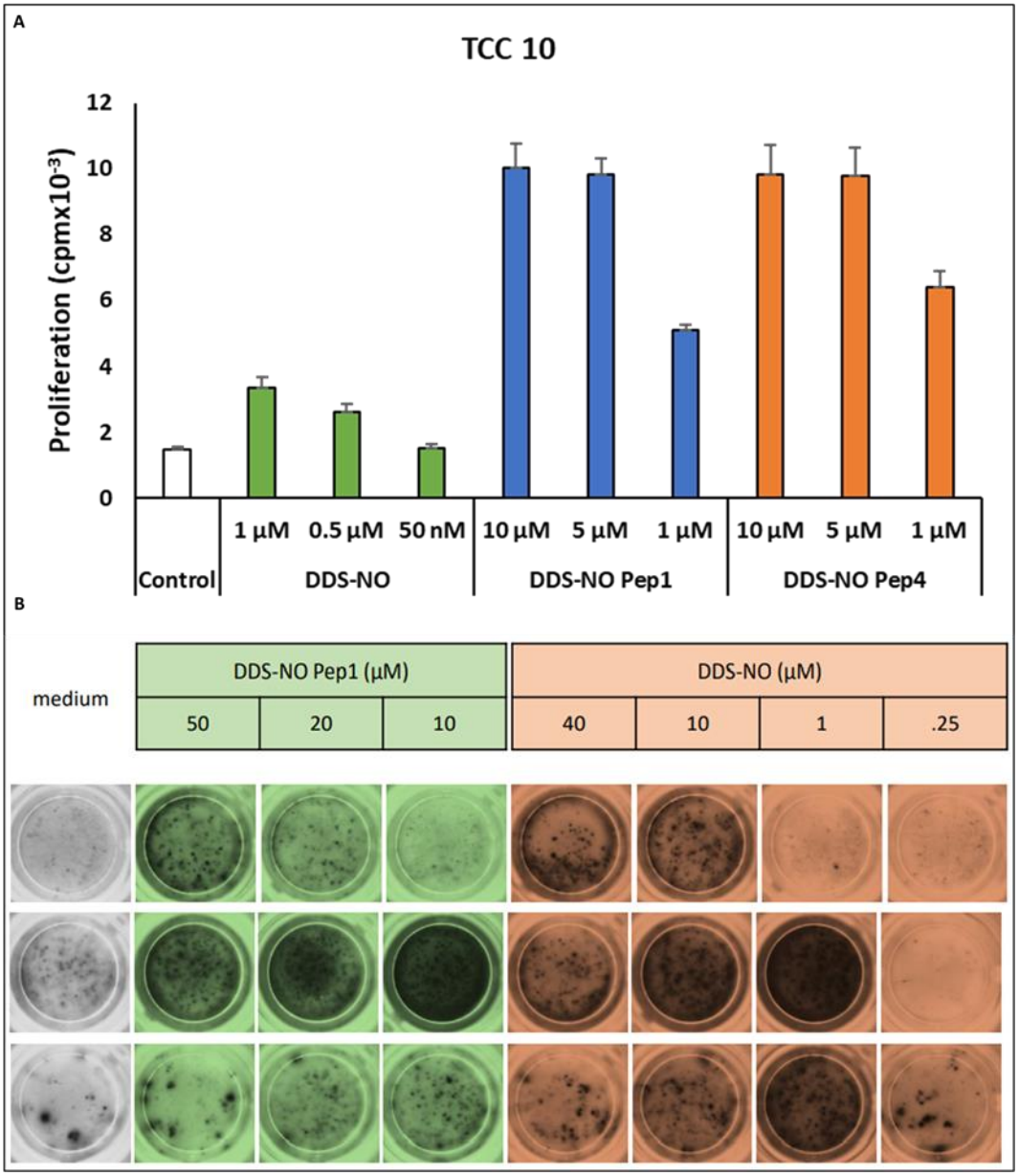


Figure 6.4: Dose-dependent activation of DDS-NO-modified peptide-responsive CD8+ T-cell clones. Clones (5 × 10^4 cells/well; 200 µl) were incubated with irradiated autologous antigen presenting cells (EBV-transformed B-cells; 1 × 10^4 cells/well) and either DDS-NO-modified Pep1 or Pep4 or soluble DDS-NO for 48 hrs. (A) Proliferation was measured by addition of [3H] thymidine; one representative clone is shown; (B) IFN-γ release was measured by ELISpot; 3 representative clones are shown.

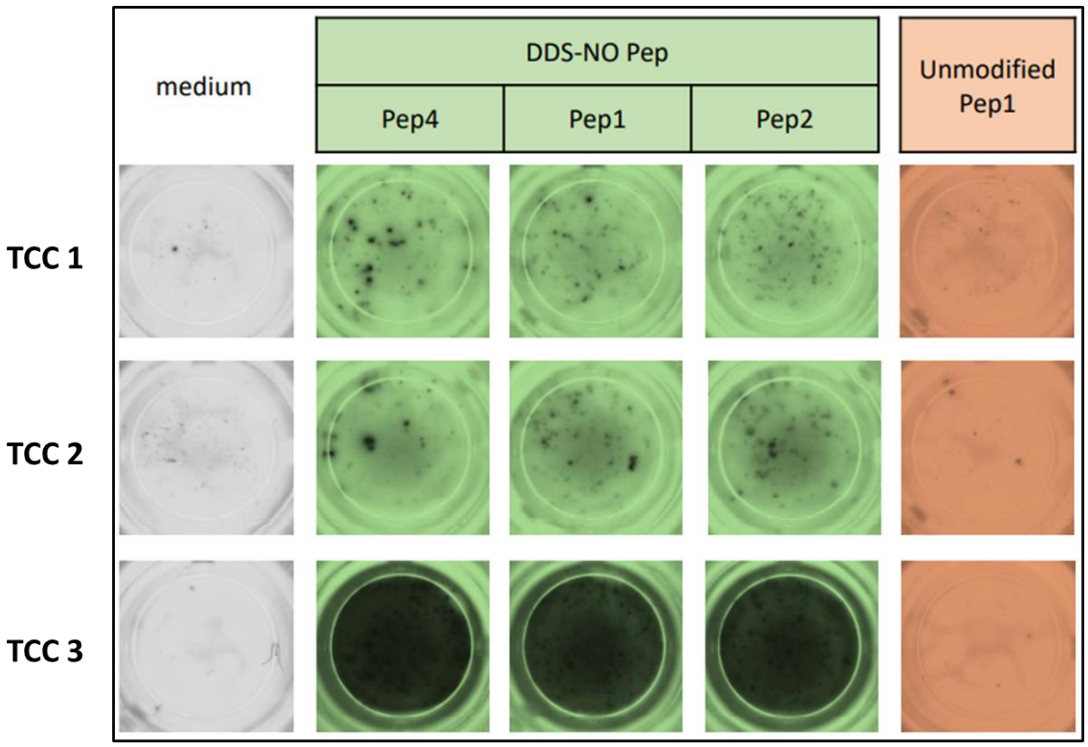
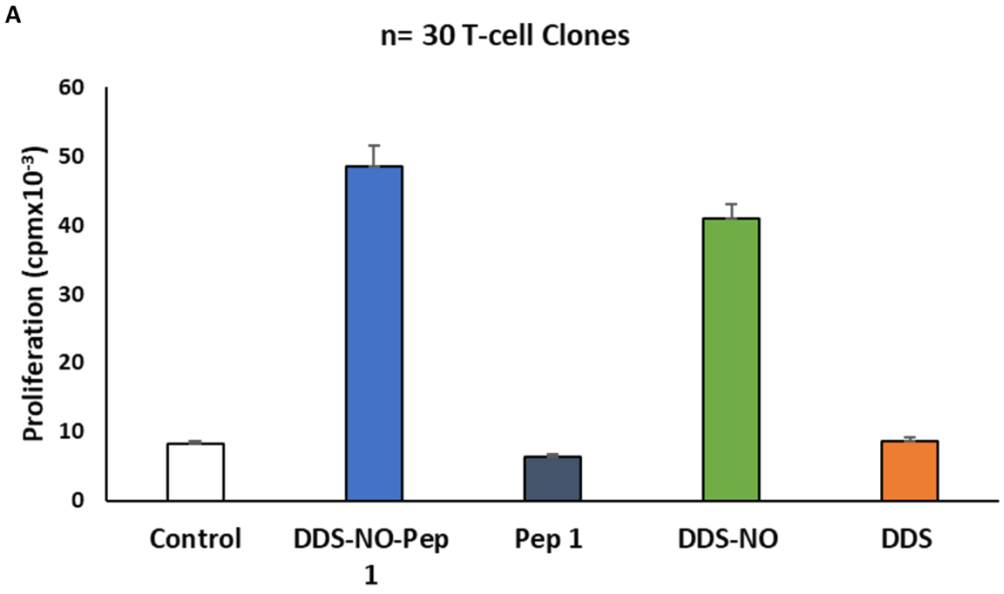


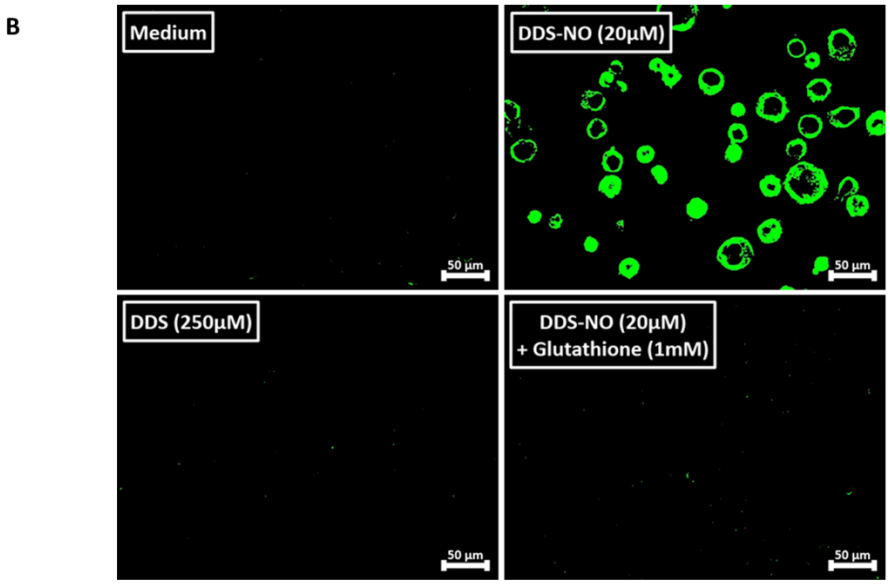
Figure 6.5: Activation of CD8+ T-cell clones with DDS-NO-modified Pep1, Pep2 and Pep4. DDS-NO-modified peptide-responsive CD8+ clones (Pep1 and Pep4) (5 × 10^4 cells/well; 200 µl) were incubated with irradiated autologous antigen presenting cells (EBV-transformed B-cells; 1 × 10^4 cells/well) and DDS-NO-modified Pep1, Pep2 or Pep4 for 48 hrs. IFN-γ release was measured by ELISpot.

6.3.3 Confirmation that CD8+ T-cell clones are not activated with residual DDS or DDS-NO in the conjugated peptides

To confirm that clones were not activated with residual DDS, DDS-NO-modified Pep1 and Pep4 clones were cultured with an optimal concentration of DDS. Clones were stimulated to proliferate with DDS-NO-modified peptide and soluble DDS-NO; however, proliferative responses were not detected with DDS itself (Figure 6.6A). Glutathione was used to differentiate between DDS-NO-modified peptide and soluble DDS-NO T-cell proliferative responses. The addition of glutathione to cell culture medium inhibits the covalent binding of DDS-NO to antigen presenting cells

(EBV-transformed B-cells) (Figure 6.6B) and the activation of clones with soluble DDS-NO. In contrast, glutathione did not alter the activation of clones with DDS-NO-modified peptides, where the nitroso moiety is already bound covalently to the cysteine residue in the peptide sequence (Figure 6.6C).


A
n= 30 T-cell Clones
Proliferation (cpmx10-3)
60
50
40
30
20
10
0
Control
DDS-NO-Pep 1
Pep 1
DDS-NO
DDS


B
Medium
DDS-NO (20µM)
DDS (250µM)
DDS-NO (20µM) + Glutathione (1mM)
50 µm

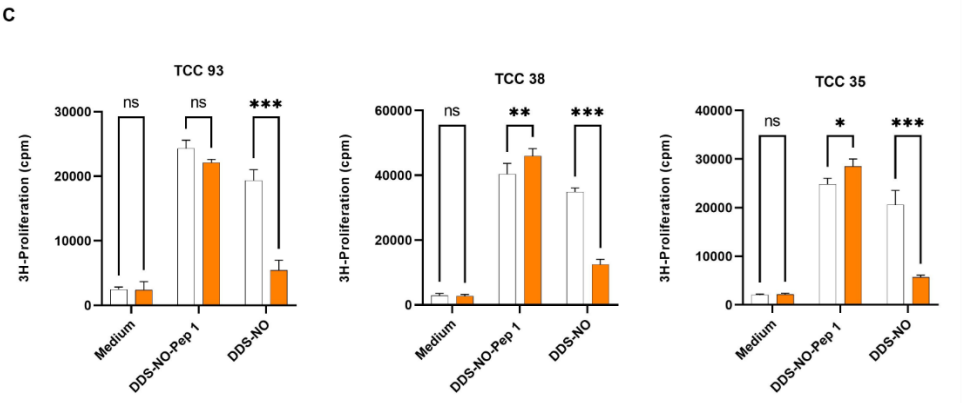
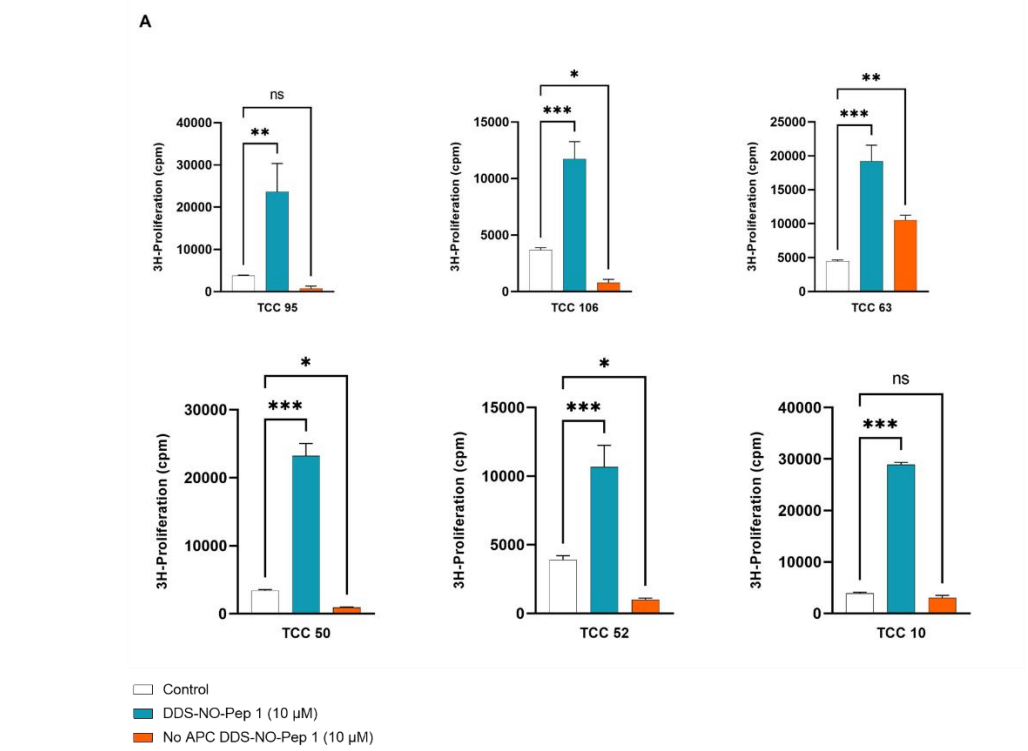

C
TCC 93
TCC 38
TCC 35
3H-Proliferation (cpm)
ns
**
***
*
Medium
DDS-NO-Pep 1
DDS-NO
without GSH
with GSH

Figure 6.6: Activation of DDS-NO-modified peptide-responsive CD8+ T-cell clones was not due to the presence of residual DDS or DDS-NO or degradation of the peptides and liberation of free DDS. (A) To explore whether DDS activates DDS-NO-modified peptide-responsive CD8+ T-cell clones, a panel of 30 clones (5 × 10^4 cells/well; 200 µl) were incubated with irradiated autologous antigen presenting cells (EBV-transformed B-cells; 1 × 10^4 cells/well) and DDS at previously defined optimal concentration (100 µM) for 48 hrs. DDS-NO-modified Pep1, soluble DDS-NO and unmodified Pep1 were added as controls. Proliferation was measured through addition of [3H]-thymidine (0.5 µCi/well) for the final 16 hrs of the culture period. (B) DDS and DDS-NO (in the presence and absence of glutathione) were cultured with EBV-transformed B-cells and formation of covalently-modified adducts was visualised using an anti-DDS antibody. (C) Clones (5 × 10^4 cells/well; 200 µl) were cultured with irradiated autologous antigen presenting cells (EBV-transformed B-cells; 1 × 10^4 cells/well) and DDS-NO modified Pep1 or soluble DDS in the presence of absence of glutathione (1 mM). As shown in (B) glutathione blocks the protein reactivity of DDS-NO. Proliferation was measured through addition of [3H]-thymidine (0.5 µCi/well) for the final 16 hrs of the culture period. Results are expressed as mean±SD CPM. Statistical analysis was performed via a two way ANOVA, compared to the control media well. (NS [Not significant] = $P > 0.05$, $P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***).

6.3.4 Activation of CD8+ T-cell clones with DDS-NO-modified peptides is HLA-B*13:01-restricted

In in vitro culture conditions, soluble DDS-NO interacts with multiple HLA proteins to activate CD4+ and CD8+ clones from hypersensitive patients (Zhao et al., 2021). The optimized culture conditions with extensive covalent modification of cellular protein potentially overrides the exquisite HLA restriction observed in patients. Thus, a stepwise approach was used to explore the restriction of the DDS-NO-

modified peptide-specific T-cell response. First, with the exception of a small number of self-presenting clones, T-cells were not stimulated to proliferate with DDS-NO-modified Pep1 when antigen presenting cells (EBV-transformed B-cells) were excluded from the assay (Figure 6.7A). Second, C1R-B13:01 antigen presenting cells, expressing HLA-B*13:01, but not the other HLA class I alleles expressed by the patients were used and antigen presenting cells in the place of autologous EBV-transformed B-cells. Clones were activated and secreted IFN-γ when cultured with either DDS-NO-modified peptides or soluble DDS-NO and C1R-B*13:01 cells (Figure 6.7B). Third, IFN-γ secretion above control levels was not detected when the experiment was repeated with DDS-NO-modified Pep1 or Pep4 and C1R-parental cells (Figure 6.8A). Finally, pre-treatment of C1R-B*13:01 antigen presenting cells with an anti-HLA class I blocking antibody inhibited peptide-induced IFN-γ secretion, whereas an anti-HLA class II blocking antibody had no effect (Figure 6.8B).



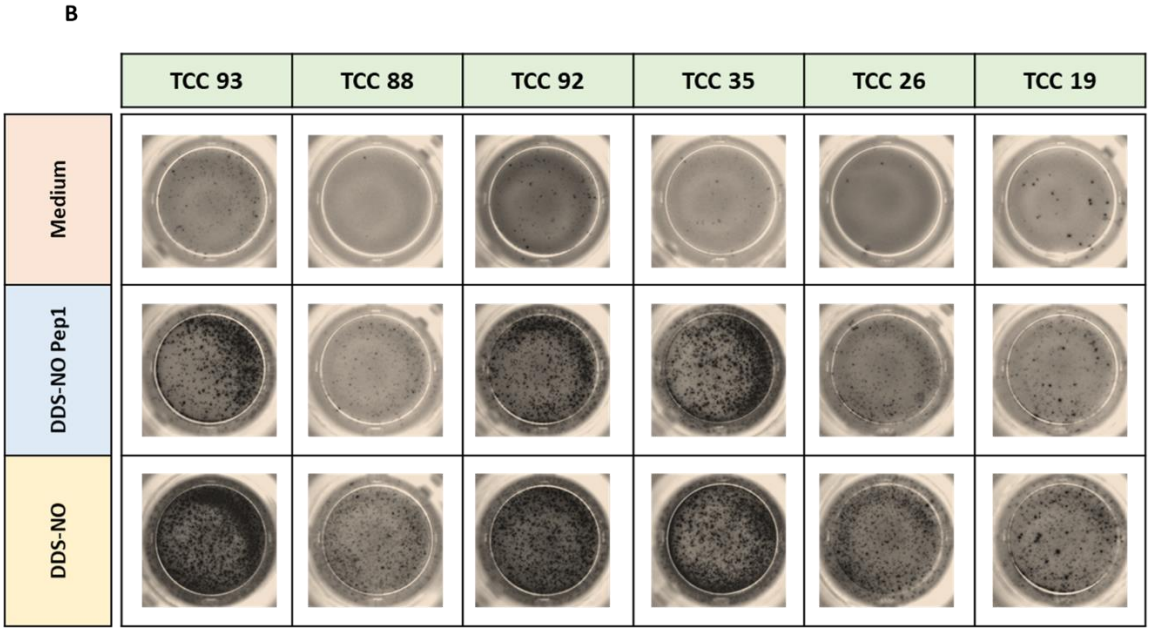


Figure 6.7: Assessment of HLA-B*13:01 restriction of DDS-NO-modified Pep1 and Pep4-responsive CD8+ TCC. (A) Clones (5 × 10^4 cells/well; 200 µl) were cultured with DDS-NO-modified Pep1 in the presence and absence of irradiated autologous antigen presenting (EBV-transformed B-cells; 1 × 10^4 cells/well) in triplicate cultures for 48 h. Proliferation was measured through addition of [3H]-thymidine (0.5 µCi/well) for the final 16 hrs of the culture period. Results are expressed as mean±SD CPM. Statistical analysis was performed

via a one way ANOVA, compared to the control media well. (NS [Not significant] = P > 0.05, P ≤ 0.05 *, P ≤ 0.01 **, P ≤ 0.001 ***). (B) Clones (5 × 10^4 cells/well; 200 µl) were incubated with C1R-B*13:01 transduced antigen presenting cells (1 × 10^4 cells/well) and either DDS-NO-modified Pep1 or DDS-NO for 48 hrs. IFN-γ release was visualized by ELISpot.

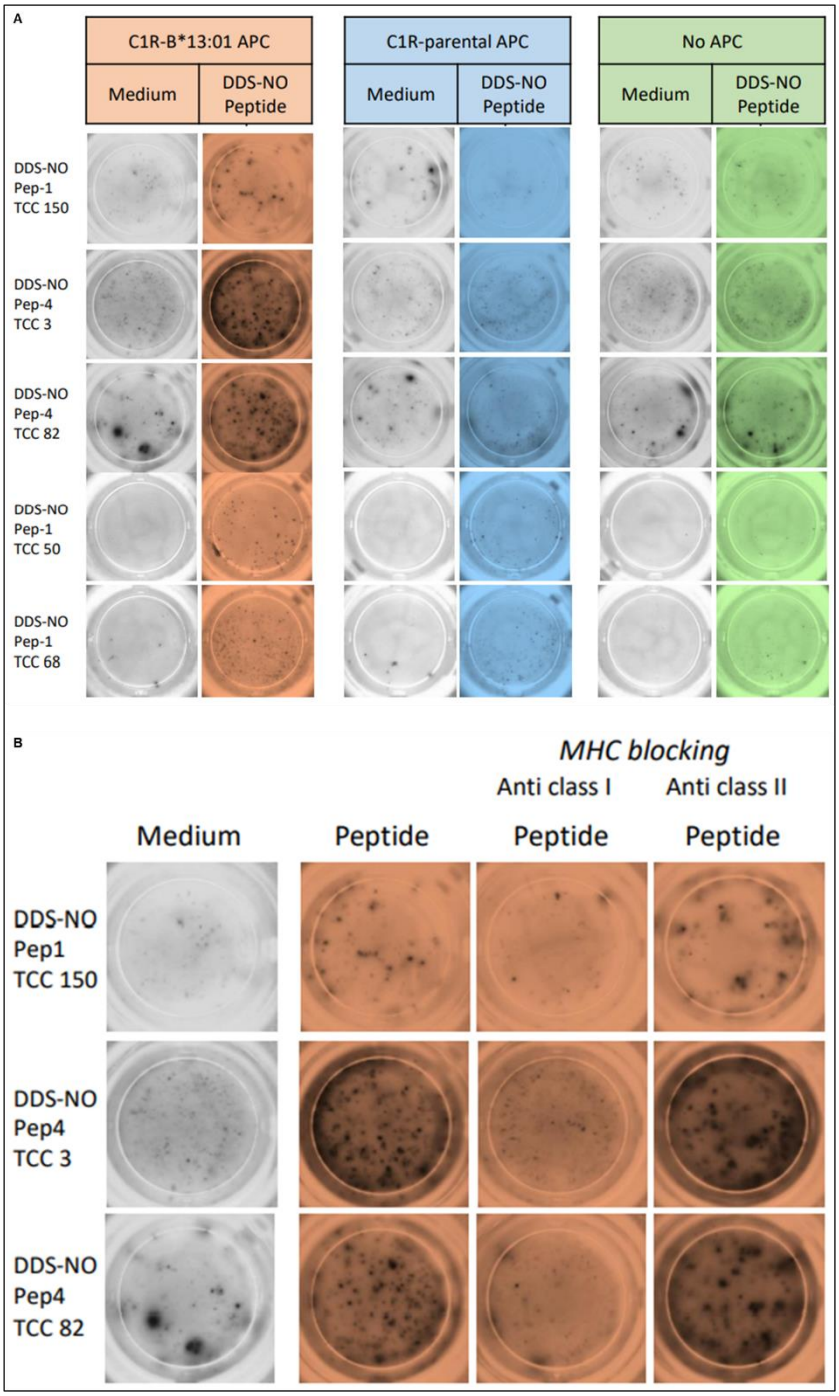


Figure 6.8: Assessment the importance of antigen presenting cells in the activation of DDS-NO-responsive CD8+ T-cell clones and of HLA restriction. (A) DDS-NO-modified Pep1 or Pep4 clones were cultured DDS-NO-modified peptide and either C1R-B*13:01 or C1R-parental antigen presenting cells. Clones cultured with DDS-NO-modified Pep1 or Pep4 in the absence of antigen presenting cells served as a negative control. (B) DDS-NO-modified peptide-responsive CD8+ clones (Pep1 and Pep4) (5×10^4 cells/well; 200 µl) were incubated with C1R-B*13:01 antigen presenting cells (1×10^4 cells/well) and DDS-NO-modified Pep1 or Pep4 in the presence or absence of anti-HLA class I and II blocking antibodies for 48 hrs. IFN-γ release was measured by ELISpot.

6.4 Discussion

Delayed-typed drug hypersensitivity reactions are a serious form of adverse event and represent a challenge to healthcare professionals attempting to delineate patient susceptibility and researchers in the Pharmaceutical industry attempting to identify structural liabilities within their molecules. Drug-responsive T-cells are believed to be the primary effector cells involved in the iatrogenic disease, with drug (metabolite) protein or peptide binding believed to be the molecular initiating event. This interaction may involve the drug molecule binding covalently to cellular or serum proteins, as is the case for β-lactam antibiotics such as flucloxacillin (Puig et al., 2020, Monshi et al., 2013, Waddington et al., 2020). The resultant adducts are processed by antigen presenting cells into peptide fragments that associate with HLA proteins for presentation to T-cells. At the opposite end of the spectrum, drugs such as carbamazepine form labile binding interactions with HLA proteins or peptides within the HLA antigen binding cleft to stimulate a similar effector T-cell response (Wei et al., 2012a, Naisbitt et al., 2003, Wu et al., 2006). It should be noted that although the pathways that lead drug display by HLA proteins differ, the chemical composition and 3D arrangement of molecules at the immunological synapse may be similar with the only difference being the nature of the drug peptide binding interaction.

Different forms of drug hypersensitivity reaction are strongly associated with expression of specific HLA class I alleles (Deshpande et al., 2021, Jaruthamsophon et al., 2022, Nicoletti et al., 2017). This suggests that a derivative of the drug may interact with exquisite selectivity with the protein encoded by the HLA allele to activate the T-cells that instigate the hypersensitivity reaction. Indeed for the

archetypal association between HLA-B*57:01 and abacavir hypersensitivity (Mallal et al., 2008, Mallal et al., 2002, Hetherington et al., 2002), the drug has been shown to adhere deep within the peptide binding cleft of HLA-B*57:01, altering the structure and the peptides that are displayed by the HLA protein to CD8+ T-cells (Illing et al., 2012, Ostrov et al., 2012, Norcross et al., 2012). A similar binding interaction is not observed with closely-related HLA proteins. Regrettably, the picture is not so clear for other forms of HLA class I allele-restricted forms of drug hypersensitivity reaction. Even with exemplars such as carbamazepine (HLA-B15:02 (Chung et al., 2004), HLA-A*31:01 (McCormack et al., 2011)) and flucloxacillin (HLA-B*57:01 (Daly et al., 2009)) the parent drug, drug metabolites and/or peptide adducts interact with multiple HLA class I and class II proteins to stimulate CD4+ and CD8+ T-cells in hypersensitive patients (Monshi et al., 2013, Yaseen et al., 2015, Wuillemin et al., 2013, Lichtenfels et al., 2014, Ko et al., 2011, Wu et al., 2007). In recent years research has focused on DDS hypersensitivity to further define pathways of T-cell activation as (i) the metabolism and protein reactivity of DDS is well defined (Roychowdhury et al., 2005, Reilly et al., 2000), (ii) stable and reactive metabolites of DDS have been synthesized and are available for functional studies (Alzahrani et al., 2017), and (iii) DDS hypersensitivity is strongly associated with HLA-B*13:01 expression (Zhang et al., 2013a). DDS and DDS-NO have been shown to activate CD4+ and CD8+ T-cells in all studied hypersensitive patients via different pathways, pharmacological HLA binding and hapten binding, respectively (see Chapters 3 and 4). A number of T-cells display DDS and DDS-NO cross-reactivity; however, others are highly selective in that they are stimulated with one molecule and not the other. Thus, exposure of susceptible patients to parent drug and

metabolite results in the development of divergent T-cell responses that act together produce the adverse event. HLA-B*13:01-restricted DDS- and DDS-NO-responsive CD8+ T-cells are detectable in assays utilizing antigen presenting from donors expressing matching and mismatched HLA-B alleles. Nevertheless, in the in vitro culture assay, the parent drug and metabolite interact with a variety of HLA-B alleles to trigger CD8+ T-cells. For this reason, we have designed and synthesized DDS-NO-modified HLA binding peptides to study the HLA-B*13:01-restricted T-cell response in greater detail and to explore the importance of the hapten moiety on the peptide backbone for T-cell activation. Three DDS-NO-modified peptides were synthesized in high purity (Chapter 5). Each peptide contained HLA-B*13:01 anchoring motifs (the MHCI binding predictions were made using the IEDB analysis resource NetMHCpan 4.1 tool), an alanine backbone (previous studies show that non-anchoring amino acids for the most part do not define the specificity of the T-cell response (Ortmann et al., 1992, Martin et al., 1992)) and a nucleophilic cysteine residue for modification by DDS-NO. As described in Chapter 5, high yields of DDS-NO-modified Pep1 and Pep4 were obtained after HPLC purification; Pep2 was generated in a lower quantity and as such only used in T-cell cross-reactivity studies.

Two DDS and DDS-NO lymphocyte transformation test positive HLA-B*13:01 positive hypersensitive patients (described in (Zhao et al., 2021)) were used to generate DDS-NO-modified peptide responsive T-cell clones. Almost 50% of clones generated from 14 day DDS-NO-modified peptide PBMC cultures were stimulated to proliferate in the presence of either modified Pep1 or Pep4. Clones expressed a CD8+ phenotype and were stimulated with the modified peptides in a dose-

dependent manner. Clones displayed 100% cross-reactivity between positional derivatives (i.e., Pep1, Pep2 and Pep4) and with soluble DDS-NO. DDS-NO extensively modified the surface of antigen presenting cells, which likely includes binding to cysteine-containing peptides already displayed by HLA class I on the cell surface; hence, the observed cross-reactivity was somewhat expected. In contrast to our cross-reactivity data with DDS-NO-modified peptides, cross-reactivity between HLA class II binding β-lactam-modified peptide positional derivatives was not observed (Padovan et al., 1997, Tailor et al., 2020). However, a system utilized by Honda et al (Honda et al., 2001), where CD8+ T-cell receptor α and β chains were alternately fixed prior to assessment of trinitrophenol-modified peptide positional derivative T-cell responses explains how a single T-cell receptor is capable of recognizing and responding to hapten structures in different positions. They demonstrated that hapten addition to HLA class I binding peptides (at positions 4 and 6) can cause substantial adjustments to the CD8+ T-cell receptor structure. Specifically, the β chain could adjust to interact with the hapten structure irrespective of whether it was at position 4 or 6.

It is theoretically possible that CD8+ T-cell activation with DDS-NO-modified peptides may be due to residual soluble DDS-NO or degradation of the adduct in culture and liberation of DDS. This was excluded however through (i) demonstrating that the clones were not activated with the parent compound, and (ii) neutralizing soluble DDS-NO-specific, but not the DDS-NO-modified peptide-specific T-cell response with glutathione. Glutathione contains a reactive cysteine group and when in excess binds to DDS-NO, preventing to formation of protein adducts.

To explore the importance of HLA proteins in T-cell activation, antigen presenting cells were firstly excluded from T-cell proliferation assays. The vast majority of clones were not activated with the DDS-NO-modified peptides in the absence of antigen presenting cells. Next, the HLA-A, B negative mutant C1R cell line was transduced with HLA-B*13:01 and used as antigen presenting cells in the place of autologous EBV-transformed B-cells. DDS-NO-modified Pep1 and Pep4, and soluble DDS-NO stimulated the clones to secrete IFN-γ in the presence of C1R-B*13:01 cells and the response was inhibited with an HLA class I blocking antibody. Similar activation of the T-cell clones was not observed when using the C1R parental cell line as antigen presenting cells.

Collectively, our study highlights the importance of drug metabolism, drug hapten binding and most importantly the formation of drug metabolite-modified HLA-B*13:01 binding peptides in the activation of CD8+ T-cells from DDS hypersensitive patients. The availability of T-cell stimulatory DDS-NO-modified HLA-B*13:01 binding peptides and C1R-B*13:01 cells offer the opportunity to study the structural elements of the drug hapten HLA peptide binding interaction.

7 Chapter 7: General Discussion

Adverse drug reactions increase hospital admissions and cause a significant financial burden on the world's health care system. They are prevalent and can be caused by a wide variety of drugs of different chemical classes. Many reactions can be controlled by stopping the medicine or reducing the dose. However, a small percentage a serious and maybe fatal even with prior drug withdrawal. Adverse drug reactions can cause various symptoms, ranging from relatively moderate nausea to life-threatening anaphylaxis, liver injury or toxic epidermal necrolysis. Even though type A reactions account for nearly 80% of ADR-related hospitalisations, type B reactions are the leading cause of death (Routledge et al., 2004). Numerous ADRs that affect the skin, liver, or blood are referred to as hypersensitivity because the pathogenesis is thought to involve an inadvertent, drug-specific, activation of the individual's adaptive immune system. Furthermore, such reactions have been linked to the presence of a specific HLA allele. The association of a specific adverse event with the expression of an individual HLA allele indicates that the culprit drug may interact selectively with the protein encoded by the HLA allele to stimulate the unwanted immune response. However, for most HLA-associated drug hypersensitivity reactions, it's still unclear how drugs interact with the HLA protein and activate immune cells, and what factors increase a person's susceptibility to such medications. Therefore, industrial tests to predict the immunogenicity of new medicines cannot be developed, and clinicians experience difficulty in managing patients with reactions as the disease pathogenesis is not fully defined. As a result, more research into patient

susceptibility factors and the pathways of drug-specific immune activation is needed.

Examples of HLA-linked drug hypersensitivity reactions include HLA-B*57:01 with abacavir (Mallal et al., 2002), HLA-B*5801 with allopurinol (Hung et al., 2005), HLA–B*1502 and HLA-A*31:01 with carbamazepine (Chung et al., 2004, Ozeki et al., 2011, McCormack et al., 2011), and HLA-A*13:01 with dapsone (DDS) (Zhang et al., 2013a). Drugs interact with HLA proteins to activate T-cells via different pathways. These include the pharmacological interaction concept, the hapten hypothesis, and the altered self-repertoire model. For instance, carbamazepine is believed to bind directly to the associated HLA molecules or peptide in the peptide binding groove expressed on the surface of APCs via a reversible pharmacological interaction to a stimulate T cell response (Wei et al., 2012a). The altered self-peptide repertoire model has been used to describe the pathogenesis of abacavir hypersensitivity syndrome, which is associated with HLA-B*57:01. Abacavir binds intracellularly within the peptide-binding cleft of HLA-B*57:01, altering its structure and causing a range of new self-peptides to be displayed and presented to TCRs (Bharadwaj et al., 2012, Chessman et al., 2008). It is widely believed that these novel peptide sequences displayed by cells expressing HLA-B*57:01 activate abacavir-responsive CD8+ T-cells and contribute to the development of abacavir hypersensitivity (Illing et al., 2012). This pathway to date has only been described for abacavir, and it may represent an exceptionally unique drug response as abacavir is the only drug that selectively activates CD8+ T-cells (Adam et al., 2012, Illing et al., 2012, Adam et al., 2014). Drugs activating T-cells via hapten or PI pathways often display a skewed CD4+ or CD8+ T-cell response in patients, which tends to link to known HLA class II

and I associations, respectively. However, in every hypersensitive patient studied to date, drug-specific CD4+ and CD8+ T-cells are activated simultaneously. Other drugs do not bind directly to HLA molecules, but they induce immune response via the hapten pathway. Drugs such as penicillin and SMX can bind covalently to lysine and the cysteine residues on the cellular proteins, respectively. These drug modified proteins undergo processing within antigen presenting cells to generate drug-modified peptides that produce neo-antigenic signals for specific T cells (El-Ghaiesh et al., 2012, Callan et al., 2009a).

DDS, a diaminophenyl sulphone, has long been used successfully to treat leprosy. DDS hypersensitivity syndrome (DHS), an uncommon but deadly idiosyncratic drug hypersensitivity reaction, can affect DDS therapy by causing fever, skin rash, eosinophilia, lymphadenopathy, and other systemic symptoms (Vinod et al., 2013). Genome-wide association studies showed that DDS hypersensitivity in Chinese and Thai patients is associated with carriage of HLA-B*13:01 allele (Zhang et al., 2013b, Tempark et al., 2017). A molecular docking model indicated that DDS might accommodate within the peptide-binding site of HLA-B*13:01 (Watanabe et al., 2017); however, this model failed to add a peptide to the MHC peptide binding groove and did not consider the potential interaction of DDS with the binding peptide. Previous studies have shown that DDS and nitroso DDS (the primary oxidative metabolite of DDS)-responsive T cell clones are generated from naïve T-cells of healthy donors expressing HLA-B*13:01 when the drug moieties were presented by dendritic cells. The data indicated that DDS and DDS-NO activate T cells via direct binding to MHC molecules and a hapten pathway, respectively (Alzahrani et al., 2017). The T-cell activation pathway has not been studied in

patients expressing HLA-B*13:01. Thus, this thesis had four primary objectives: (1) to determine whether DDS or DDS-NO activate hypersensitive patients' T cells; (2) to characterise the phenotypic and function of drug-responsive T cells; (3) to investigate whether HLA-B*13:01 plays a role in the drug-specific T-cell response; and (4) to design and synthesise DDS-NO-modified HLA-B*13:01 binding peptides to explore their immunogenicity.

DDS is bioactivated to DDS hydroxylamine metabolites, which undergo auto-oxidation to nitroso species. It has long been hypothesised that the nitroso derivative of DDS may be the ultimate mediator of hypersensitivity reaction (Svensson, 2003). The DDS-NO had already been synthesised in a stable form in our lab (Alzahrani et al., 2017). Thus, DDS-NO was cultured with hypersensitive patient PBMC, and drug-responsive T-cell clones were generated and characterised. Experiments were conducted in collaboration with clinical researchers in China who discovered the association between HLA-B*13:01 and DDS hypersensitivity syndrome (Zhang et al., 2013a); they provided us PBMC samples from HLA-B*13:01+ patients with DDS hypersensitivity syndrome. T cell proliferation *in vitro* was assessed using the lymphocyte transformation test (LTT) before conducting the T-cell cloning experiments. PBMC were found to proliferate in the presence of both DDS and DDS-NO.

To confirm the LTT findings, T cell clones were generated and characterised in terms of drug-responsiveness and assessed their activation pathways. Many $CD4^+$ and $CD8^+$ clones were generated and displayed reactivity against DDS or DDS-NO. There were three different forms of cross-reactivity among these clones: compound-specific (T cells activation occurred with only DDS or DDS-NO), weakly and strongly

cross-reactive with DDS and DDS-NO. DDS- and DDS-NO-responsive clones were also activated with DDS hydroxylamine. Next, we evaluated the reactivity of these clones with DDS analogues and structurally distinct sulfonamides. It was found that the location of DDSs amine groups on its aromatic rings was crucial for activating T-cell clones, as was the presence of a sulfone group. In addition, DDSs aromatic amines were essential for T-cell activation because a panel of sulfonamides with only one aromatic amine group could not activate clones. $CD4^+$ and $CD8^+$ clones produced comparable levels of cytokines and cytolytic molecules, including IFNγ, IL-5, IL-13, and IL-22, alongside the cytolytic molecules perforin, granzyme B, and FasL. In addition, these responsive clones expressed a range of chemokines, including CXCR3, CCR4, CCR6, CCR9, and CCR10. These cytokines/chemokines drive T cells' migration, proliferation, regulation, or activation. DDS-responsive T cell clones were HLA-restricted and were activated through the direct interaction of DDS with the HLA molecule. Conversely, DDS-NO activated T cells via a hapten pathway, mainly through protein processing of drug-protein adducts within antigen-presenting cells. Collectively these data indicate that T-cells are activated with DDS via different pathways within the same hypersensitive patients. Our findings illustrate that it is important to consider all metabolites of a drug when exploring whether an adverse reaction involves activation of an individual's T-cells. My data demonstrate that T-cells are activated with parent drug and a protein-reactive metabolite. Other studies exploring drug HLA binding and T-cell activation with allopurinol and atabecestat have shown that stable drug metabolites (oxypurinol in allopurinol reactions and a DIAT metabolite in atabecestat reactions) might also be the moiety primarily driving drug-specific T-cell activation (Thomson et al., 2021, Yun et al.,

2013). These data have important implications for clinicians diagnosing drug hypersensitivity reactions and managing patients with adverse events. If skin tests and *in vitro* testing yields negative results, this should not be interpreted as the patient did not develop a hypersensitivity reaction to the drug. It might be that the patient was exposed to an irrelevant form of the drug for T-cell activation. Similarly, our data adds an extra layer of complication for the prediction of intrinsic immunogenicity of new chemical entities in the pharmaceutical industry. Assays have been developed to study the drug-specific priming of naïve T-cells from healthy donors, and these assays are being used by industry to assess their applicability domain; however, if an irrelevant form of the drug is added to the assays, the generated results will be difficult to interpret.

HLA mismatching tests revealed that the DDS and DDS-NO-derived antigens interact with HLA B molecules such as HLA-B*15:25 and HLA-B*38:02, in addition to HLA-B*13:01 identified in genome-wide association studies. HLA-B*13:01-restricted T-cell activation was then confirmed using C1R cells transduced with HLA-B*13:01 as antigen-presenting cells. These results show the importance of HLA-B*13:01 in triggering DDS hypersensitivity syndrome, and indeed the interaction of DDS and DDS-NO with HLA-B*13:01 might be preferred over other HLA molecules. However, both DDS and DDS-NO do interact with and activate T-cells when associated with other HLA-B alleles. These data differ from that observed with abacavir, which only stimulates $CD8^+$ T-cells when bound to HLA-B*57:01(Illing et al., 2012, Thomson et al., 2020b, Adam et al., 2014, Adam et al., 2012); however, this scenario seems to be the exception rather than the rule. For example, carbamazepine exposure is associated with a high incidence of hypersensitivity reaction, and HLA risk markers

have been identified in different populations; however, in these patients, T-cells that are activated with the drug are $CD4^+$ and $CD8^+$ and restricted by HLA class I and II molecules (Wu et al., 2007, Wu et al., 2006). Furthermore, several patients that develop carbamazepine hypersensitivity do not express HLA risk alleles, thus the drug and or metabolites must interact with different HLA molecules. These data are important to consider when researchers use *in sillico* modelling to explore drug HLA binding interactions and also for researchers attempting to develop chips containing multiple HLA molecules to screen for drug binding. These studies are likely to identify a range of binding interactions, and it will be difficult to assess their relevance.

T-cell assays using parent drugs or drug metabolites do not reveal the exact antigenic determinant that interacts within the HLA peptide binding groove and is presented to T-cells. It might also be possible to use model drug protein adducts to explore the hapten mechanism in more detail, as has been demonstrated with β-lactam antibiotics (Meng et al., 2017). However, a drug-modified protein will still generate numerous antigenic determinants when it goes through proteasomal protein processing within antigen presenting cells. Consequently, current *in vitro* techniques do not identify whether a particular antigenic determinant is responsible for activating drug-responsive T cells. Elution of drug-modified peptides from the HLA of drug-treated cells is a helpful approach for determining the nature of drug-HLA interactions, as has recently been described with flucloxacillin (Waddington et al., 2020). However, the peptide elution procedure is time-consuming, expensive, and requires high-sensitivity MS. Furthermore, once these drug-modified peptides have been characterised, they still need to be synthesised and assessed in

functional assays with patient PBMC. Thus, we used an alternative method to explore the antigenic determinants needed for activation of T cells from patients with DDS hypersensitivity. This method included designing and generating drug-modified peptides restricted to HLA-B*13:01. DDS-NO was attached to nucleophilic cysteine residues located in different positions of the peptide backbone. Earlier studies, such as those done by Padovan et al., provided the framework for this method. Synthesis of designer benzylpenicillin-modified peptides containing a reactive lysine residue and anchor residues that allow interactions with specific HLA II alleles showed that the T-cells isolated from penicillin-hypersensitive individuals were stimulated by drug-peptide adducts, but the position of the lysine residue within the peptide determined whether or not the T-cells would respond (Padovan et al., 1997).

Using DDS-NO-responsive, HLA-B*13:01 restricted $CD8^+$ T-cells from hypersensitive patients, we were able to demonstrate that they were activated via a hapten pathway. Through the synthesis and detailed characterisation of three peptides containing defined anchor residues for HLA-B*13:01 and carrying a cysteine-bound DDS-NO adduct in three different positions (three positional derivatives), we were able to show that (1) DDS-NO responsive T-cells were activated with peptide adducts bound to HLA-B*13:01, and (2) cross-reactivity between the three positional derivatives was a common feature. These data contrast with studies exploring amoxicillin hypersensitivity where no cross-reactivity between positional derivatives of HLA class II binding β-lactam-modified peptides was observed (Tailor et al., 2020, Padovan et al., 1997). However, Honda et al. conducted elegant studies that might explain our observation of hapten location independence. They found

that T cells generated from TCR α-chain Transgenic (Tg α) mice displayed cross-reactivity between TNP-modified peptides (lysine in position 4 and position 6). In T cells generated from Tg α mice, the β-chain is thought to interact with the TNP group. The cross-reactivity demonstrates that the β-chain can adapt to interact with the TNP group to some extent, regardless of whether the TNP group is located at different positions in the peptide sequence. Furthermore, mobility of the TNP group may facilitate the cross-reactivity (Honda et al., 2001).

To confirm the specificity of the interaction of DDS-NO-modified peptides with HLA-B*13:01, C1R-B*13:01 cells were generated (antigen presenting cells that express HLA-B*13:01 and not other B alleles), and T-cell responses use these cells and the parental cell line were compared. The detection of and DDS-NO stimulated T-cell IFN-γ secretion only in the presence of HLA-B*13:01 C1R cells confirmed that the response was HLA class I restricted, with the peptide adduct binding specifically to HLA-B*13:01.

Collectively, our findings show that the formation of drug metabolite-modified HLA-B*13:01 binding peptides is important for activating $CD8^+$ T-cells in DDS hypersensitive patients. To conclude, the designer peptide model was a helpful approach for assessing drug antigen epitopes, even though peptides were not generated from natural proteins. Together with our collaborators, we are currently working to determine the structure of DDS-NO-modified peptides complexed with HLA-B*13:01 by X-ray crystallography, and also designing tetramers that will allow us to quantify drug-peptide conjugate-responsive T-cells in hypersensitive and tolerant patients and even in healthy donors expressing HLA-B*13:01 before drug exposure.

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