UTILISATION OF AN *IN VITRO* T-CELL PRIMING ASSAY TO CHARACTERISE THE EFFECTS OF CO-INHIBITORY SIGNALLING ON THE ACTIVATION OF ANTIGEN-SPECIFIC T-CELLS.

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

Andrew Gibson

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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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Contents

Abbreviations

aa	amino acids
ABC	Abacavir
ACD	Allergic Contact Dermatitis
ADAP	Adhesion and degranulation promoting adaptor protein
ADR	Adverse drug reaction
AGEP	Acute generalised exanthematous pustulosis
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APC	Antigen presenting cell
APC*	Allophycocyanin
BB	Bandrowki's Base
Bcl-10	B-cell lymphoma protein-10
BSA	Bovine serum albumin
BTLA	B and T lymphocyte attenuator
BTN	Butyrolphilin
CBZ	Carbamazepine
CCR	Chemokine receptor
CD	cluster of differentiation
CDR	Complementarity determining region
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLA	Cutaneous leukocyte-associated antigen
COX	Cyclooxygenase
CPM	Counts per minute
CSA	Cyclosporine A
CTLA4	Cytotoxic T-lymphocyte Associated Protein-4
CYP450	Cytochrome P450 enzyme
DAG	Diacyl glycerol

DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DHS	Drug hypersensitivity syndrome
DIHS	Drug-induced systemic hypersensitivity
DILI	Drug-induced liver injury
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNCB	Dinitrochlorobenzene
DoTS	Dose, time, and susceptibility
DRESS	Drug reaction with eosinophilia and systemic symptoms
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme-linked Immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorter
FasL	Fas Ligand
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
FSC	Forward scatter
GITR	Glucocorticoid-induced TNFR family related gene
GM-CSF	Granulocyte macrophage colony-stimulating factor
GTP	Guanosine triphosphate
HBSS	Hanks balanced salt solution
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HLA	Human Leukocyte Antigen
HMGB1	High mobility group box 1
HRP	Horseradish peroxidase

hrs	hours
HSA	Human serum albumin
HVEM	Herpesvirus-entry mediator
ICAM	Intracellular adhesion molecule
ICOS	Inducible costimulator
ICOSL	Inducible costimulator ligand
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
IL	Interleukin
iNKT	Invariant Natural Killer T-cells
IP3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
Itk	Tec-family tyrosine kinase
IVIG	Intravenous immunoglobulins
LAG-3	lymphocyte activation gene-3
LAIR	leukocyte-associated inhibitory receptor
LAT	Linker for activation of T-cells protein
LC	Langerhans cell
Lck	lymphocyte-specific protein tyrosine kinase
LEAF	Low endotoxin and azide-free
LFA	Lymphocyte function associated antigen
LPS	Lipopolysaccharide
LTT	Lymphocyte transformation test
MAPKK	mitogen-activated kinase kinase
MEK	Mitogen-activated protein kinase kinase
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility Complex
mins	minutes

MPE	Maculopapular exanthema
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NADHR	Nicotinamide adenine dinucleotide-dependent hydroxylamine reductase
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	*N*-acetyl transferase
NF-κβ	Nuclear factor kappa beta
NK	Natural Killer
NKT	Natural Killer T-cell
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered solution
PD-1	Programmed Death-1 receptor
PDK1	Phosphoinositide-dependant kinase 1
PD-L1/2	Programmed Death ligand 1/2
PE	Phycoerythrin
PHA	Phytohemaglutinin
PI	Pharmacological interaction
PI3K	Phosphoinositide-3-kinase
PLA2	Phospholipase A2
PIP2	Phosphatidylinositol-bisphosphate
PKC	Protein Kinase C
PLC	Peptide loading complex
PLCγ1	Phospholipase C gamma 1
PPD	*p*-phenylenediamine
RANTES	Regulated upon activation, normal T-cell expressed and secreted
Rap-1	Ras-related protein-1
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SCF	Stem cell factor

SD	Standard deviation
SFU	Spot forming units
SI	Stimulation Index
SJS	Stevens Johnson Syndrome
SKAP55	Sarcoma-kinase associated phosphoprotein of 55kDa
SLAM-SAP	Signalling lymphocyte activation molecule associated protein
SMX	Sulfamethoxazole
SMX-NO	Sulfamethoxazole-nitroso
SSC	Side scatter
STAT	Signal Transducer and Activator of Transcription
TAP	Transporter associated with antigen processing
T-bet	T-box transcription factor
TCR	T-cell receptor
TGF-β	Transforming growth factor-beta
Th	T-helper cell
TEN	Toxic epidermal necrolysis
TIM	T-cell immunoglobulin- and mucin-domain protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNFRSF	Tumour necrosis factor receptor superfamily
Treg	T-regulatory cell
TT	Tetanus toxoid
UDP	Uridine diphosphate
ULBP	Up-regulation of UL16 binding protein
v/v	volume/volume
WHO	World Health Organisation
w/v	weight/volume
Zap70	zeta chain-associated protein kinase-70
β2m	Beta 2 microglobulin

Publications

Published papers

Andrew Gibson, Seung-Hyun Kim, Lee Faulkner, Jane Evely, Munir Pirmohamed, Kevin B. Park, Dean J. Naisbitt. *In Vitro* Priming of Naive T-cells with *p*-phenylenediamine and Bandrowski's Base. Chem Res Toxicol. 2015, 28 (10), 2069-2077.

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Lee Faulkner, Andrew Gibson, B. Kevin Park, Dean J. Naisbitt. Detection of primary T-cell responses to drugs in volunteers with specific HLA alleles.

Abstracts

Andrew Gibson, Monday Ogese, Andrew Sullivan, Eryi Wang, Katy Saide, Paul Whitaker, Daniel Peckham, Lee Faulkner, B. Kevin Park, Dean J. Naisbitt. Negative regulation by PD-L1 during drug-specific priming of T-cells and the influence of PD-1 on effector T-cell function.
Oral presentation, 6th Drug Hypersensitivity Meeting in Bern, Switzerland (April, 2014)

Andrew Gibson, Seung-Hyun Kim, Lee Faulkner, Jane Evely, Munir Pirmohamed, Kevin B. Park, Dean J. Naisbitt. Characterization of Primary Human T-cell Responses to *p*-Phenylenediamine and Bandrowski's Base.
Poster presentation, 6th Drug Hypersensitivity Meeting in Bern, Switzerland (April, 2014)

Andrew Gibson, Seung-Hyun Kim, Lee Faulkner, Jane Evely, Munir Pirmohamed, Kevin B. Park, Dean J. Naisbitt. Activation of naïve T-cells from healthy human donors with Bandrowski's base, but not *p*-phenylenediamine, in comparison to allergic patient T-cell responses.
Poster presentation, EAACI winter school in Les Arc 1800, France (February, 2015)

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Abstract

Hypersensitivity denotes a form of immune-mediated adverse reaction associated with a high degree of morbidity and mortality. Due to a lack of animal models, research has focussed on patient lymphocytes *ex vivo*. However, such studies bypass investigation of naïve T-cell activation. Subsequently, *in vitro* assays to assess the priming of healthy donor naïve T-cells have been developed to facilitate the discrete analysis of primary and secondary T-cell responses. While these assays have been used to assess the association of specific HLA alleles with hypersensitivity, for most drugs, the majority of individuals who are positive for an HLA risk allele do not develop a reaction. Thus, predisposition to hypersensitivity is likely mediated by other parameters.

As polymorphisms in co-inhibitory pathways are associated with dysregulated immune responses, we first investigated the role of these pathways during the drug antigen (SMX-NO)-specific activation of T-cells. Antibody-mediated blockade of PD-L1 enhanced the activation of SMX-NO-primed naïve, but not memory T-cells. In comparison, inclusion of PD-L2 block had no effect, but in combination with PD-L1 block effectively dampened the enhanced T-cell activation seen with PD-L1-block alone. In comparison, inclusion of CTLA4 block enhanced the proliferative response of antigen-stimulated naïve, but also memory T-cells, suggesting a greater regulatory role for CTLA4 than PD-1 during secondary T-cell responses. Blockade of TIM-3 had no effect on T-cell activation. Further investigation focussed on the kinetics of receptor expression. While all receptors were upregulated on naïve and memory T-cells during the 3 week culture after antigen exposure, PD-1 was upregulated at earlier time points than CTLA4 and TIM-3 indicating a differential role for these receptors during early and late stage T-cell activation. Moreover, CTLA4 expression was induced in response to antigen far less on $CD4^+$ T-cells suggesting that CTLA4 has a greater regulatory role during the drug antigen-specific activation of $CD8^+$ T-cells. High expression of individual co-inhibitory receptors including PD-1 has previously been associated with exhausted T-cells, while other studies indicate that these cells are highly functional. To address this, we compared receptor expression with T-cell clone function. To assess function, we investigated the role of the newly identified Th17 and Th22 subsets in these responses. A range of Th1/Th2 cytokines were secreted in response to SMX-NO including IFN-γ, IL-13, and IL-5. While no IL-17 was detected, 50% of clones secreted IL-22. Further analysis uncovered two distinct antigen-responsive T-cell subsets that secrete Fas-L/IL-22 or granzyme B, the presence of which was confirmed utilising cells from SMX-hypersensitive patients. This is the first data to show production of IL-22 alongside IFN-γ by antigen-specific T-cells from drug-hypersensitive patients. Analysis of the level of individual co-inhibitory receptor expression found no correlation with T-cell proliferative capacity or secretion of cytokines/cytolytic molecules.

Both SMX and SMX-NO, stimulate T-cells from hypersensitive patients. 9/10 healthy donors respond to SMX-NO, while only 30% respond to SMX *in vitro*. These observations were made using a whole lymphocyte population and thus we utilised an *in vitro* T-cell priming assay, whereby naïve or memory T-cells from healthy donors are stimulated with drug-antigen using mature dendritic cells, to assess the T-cell origins of antigen-responsive cells. Both naïve and memory T-cells were activated by SMX-NO in all donors. Although SMX failed to induce proliferative responses, 2/3 donors displayed SMX-induced IL-13 secretion. SMX and SMX-NO-responsive clones were subsequently generated from these donors from naïve and memory T-cell cultures indicating that the priming of naïve T-cells and the re-activation of memory T-cells has a role in the onset of SMX-induced hypersensitivity. As these responses were detected using cells from drug-naïve donors, the memory T-cell responses are likely a result of cross-reactivity with T-cells primed to an undetermined peptide antigen. Our data are the first to show that both hapten and parent drug can stimulate pre-existing memory T-cells from drug-naïve donors.

PPD, a compound found in dyes used for hair colouring, is associated with ACD. Both PPD and a downstream oxidation product, BB, activate T-cells in allergic patients. Thus we used an *in vitro* T-cell priming assay to assess the propensity of PPD and BB to activate naïve and memory T-cells from healthy donors, and compared responses to those characterised from allergic patients. 105 PPD- and 122 BB-responsive T-cell clones were generated from five allergic patients. More than 90% of patient BB-clones were $CD4^+$, and all lacked cross-reactivity. In contrast, certain PPD-clones cross-reacted with BB, and 37.5% expressed CD8 promoting PPD as the central driving force behind the allergic reaction. However, upon utilising cells from healthy donors, neither naïve nor memory T-cells were activated by PPD suggesting the lack of important susceptibility factors. In comparison, both naïve and memory

T-cells were activated by BB, which similarly to patients displayed a lack of cross-reactivity and secreted a similar panel of cytokines including IFN-γ, IL-13, and IL-22.

In conclusion, the similar findings between drug and chemical antigen-responsive T-cells generated using an *in vitro* T-cell priming assay and those from patients highlight the promising use of this assay in the investigation of these responses. Indeed, the future definition and inclusion of (a) susceptibility factors and (b) the peptide-antigens responsible for naïve T-cell priming will form a promising platform for the safe screening of drugs.

Chapter 1: General Introduction

Contents

1.1 The Immune System

The role of the immune system is to perform homeostatic immune surveillance and protect the host from noxious stimuli upon encounter, including infections and viruses. This complex system is made up of two arms; innate and adaptive immunity. Whilst the innate response is rapid, inflammatory, non-antigen-specific, and deals with everyday infections such as the rhinovirus, the adaptive arm is antigen-specific, acquired (meaning that an individual is not born with this immunity to a particular stimulus), and has a slower onset. Despite a less rapid onset, the adaptive response is longer lasting, with a memory component that exists even long after antigen clearance to ensure future protection. The adaptive arm is often induced when the innate system is unable to rid the host of the threat for any reason.

A range of defences are utilised to form the innate pathway. These include natural physical barriers to infection such as the skin, a cellular barrier consisting of numerous cell types including phagocytes and Natural Killer (NK) cells which will be defined later in this chapter, and numerous chemical mediators including lysosomes and a cascade of hepatic-derived soluble proteins known as complement which functions to promote bacterial phagocytosis. Complement can in fact lyse infected cells through the formation of the membrane attack complex (MAC) and induce further inflammation. However, the backbones of both innate and adaptive immunity are cellular, with a range of cell types monitoring both tissues and blood for infectious agents. Cells of the innate system include NK cells, phagocytes, and monocytes, while cells of the adaptive system include T-and B-cells. All of these cells are derived from pluripotent hematopoietic stem cells and are described in detail below.

1.1.1 Lymphocytes.

Lymphocytes are a collection of cells formed from lymphoid progenitor cells, and while each member of this group is morphologically similar, they vastly differ in function. Three types of cell are categorised as lymphocytes; T-cells, B-cells, and NK cells. Whilst T- and B-cells form the major cellular component of the adaptive immune system, NK cells belong to the innate arm. Mature B-and T-cells circulate in both the blood and the lymphatic system, and are the only blood cells to do so. Both of these cell types also have a memory component, by which long-lived cells are produced so that the immune system remains responsive to previously encountered antigens.

1.1.1.1 B-cells.

B-cells are easily identifiable due to their unique expression of CD19, and are able to protect against pathogens through a diversity of mechanisms, all of which are mediated by antibodies. This antibody mediated response is also referred to as a humoral response. Antigen recognition occurs via surface bound immunoglobulin (Ig) proteins which are able to detect a structural range of antigens from peptides, to nucleic acids and polysaccharides. The humoral response is initiated immediately after antigen exposure partly due to IgM antibodies which are low affinity, but present within B-cells for instantaneous defence. During this early response, the B-cell undergoes antibody class switching. This is a process by which multiple other forms of immunoglobulins may be produced which differ in effector function, however all have the same antigen-specificity. While IgG are generated to form the predominant responding antibodies within the blood and lymph as they have a stable half-life, IgA is primarily located to urogenital, digestive, and respiratory tract secretions due to a heightened resistance to

enzyme mediated degradation, and IgE is formed in order to aid in further defence against parasites (Stavnezer, 1996). Secreted antibodies may aid pathogen opsonisation, activate complement, or simply neutralise the pathogens by binding to their surfaces. Activated B-cells establish a zone called the germinal centre within secondary lymphoid organs where they undergo affinity maturation, a process whereby higher affinity B-cells are produced using somatic hypermutation to produce either long-lived plasma cells or affinity-matured B-cells. The long-lived "memory" plasma cells are unable to divide but are fully differentiated. In comparison, the affinity-matured B-cells are not fully differentiated but are able to divide rapidly upon subsequent antigen exposure (LaRosa and Orange, 2008). Other B-cells may differentiate into rapidly responding plasma cells which function for up to 3 weeks to give an immediate response to antigen. Although for most of our lives B-cells are formed in the bone marrow, development in early life occurs in the foetal liver.

1.1.1.2 Natural killer cells.

NK cells are highly dependent on Interleukin (IL)-15 for their normal development and maintenance (Ma *et al.*, 2006). They are generated in the bone marrow and primarily function in a surveillance role, predominantly to identify viruses, but also able to respond to protozoa, bacteria, and even tumour cells (Yokoyama *et al.*, 2004). These cells are rapid responders, do not require previous sensitisation, and are easily identifiable by their unique expression of CD56. NK cells are a minority cell type, accounting for a maximum of 18% of human blood lymphocytes, but are widely distributed and are found in numerous locations including the blood, bone marrow, spleen, lymph nodes, liver, and lungs (Grégoire *et al.*, 2007; Vivier *et al.*, 2008).

NK cells are developmentally more simplistic than other lymphocytes as they don't undergo rearrangement of their antigen-receptor. In fact NK cells do not express a distinct antigen recognition receptor and as such are antigen non-specific (Yokoyama *et al.*, 2004). However, while T-cells interact with major histocompatibility (MHC) class I or II during antigen presentation (see section 1.5), NK cells target MHC class I-deficient cells. MHC class I is expressed on healthy cells and is only down regulated during periods of stress allowing NK cells to identify these cells. They do this through the expression of inhibitory receptors known as human killer cell immunoglobulin-like receptors (KIR) which effectively assess the expression of self-MHC on the surface of passing cells. When self-MHC is down regulated or absent, the inhibitory signals are lost and the NK cell becomes activated (Vivier *et al.*, 2008). Alternatively, activation can occur upon recognition of stress-induced MHC class I-related chain A or B (MICA, MICB) proteins (Chavez-Galan *et al.*, 2009). NK cells express a variety of other receptors capable of recognising stress-related ligands including the CD94/NKG2 receptor which responds to upregulation of UL16-binding protein (ULBP) and MIC, and CD16 which recognises antibody-coated cells targeted for cell death (Vivier *et al.*, 2008). The CD94/NKG2 receptor is used to identify human leukocyte antigen (HLA)-E expression in humans, a non-classical MHC molecule (Lanier, 2005). Upon recognition of HLA-E, these cells impose their cytotoxicity by the release of cytotoxic granules containing perforin and granzyme B. These cytotoxic mediators are present in circulating NK cells and so upon activation an immediate response is initiated, bypassing the time required for gene transcription (Lanier, 2005). NK cells also express numerous Toll-like receptors (TLRs) which upon activation leads to interferon gamma (IFN-γ) production (Vivier *et al.*, 2008). This provides an essential link between the innate and adaptive responses, as IFN leads to the activation of T-box transcription factor (T-bet) which is involved in T-cell development (LaRosa and Orange, 2008).

1.1.1.3 T-cells

1.1.1.3.1 Introduction to T-cells.

T-cells form an effective line of defence against a range of antigens, including peptide-based antigens, and are therefore able to defend the host from both bacterial and helminthic infections, as well as intracellular viruses. Stimulation occurs when an antigenic moiety, which may be presented on the surface of antigen presenting cells (APCs), binds to the cell surface T-cell receptor (TCR) with a high degree of specificity, prompting the development and expansion of that T-cell. These T-cells are either effector or helper (Th) T-cells, which kill intracellular viruses and bacteria, or promote the activity of other immune cells, respectively.

T-cell development occurs in the thymus. Initially, upon initial generation of the complete TCR complex on developing T-cells, T-cells express both CD4 and CD8 coreceptors, whose function is to promote TCR-MHC interactions at the immunological synapse. As such these cells are termed double positive thymocytes. These primitive T-cells are subject to a stringent elimination process before they are released into the circulation. Many double positive cells are not capable of engaging self-MHC, an interaction required for T-cell activation (see section 1.7), and therefore have no use and are subsequently destroyed by a process known as positive selection (Bosselut, 2004; Singer *et al.*, 2008). Those that survive are then subject to negative selection, whereby potentially damaging T-cells expressing TCRs with high affinity for self-peptides are removed from the circulation by induced apoptosis to prevent autoimmunity. After selection, T-cells are separated into two main groups classified by the expression of either the CD4 or CD8 coreceptor. $CD4^+$ cells are referred to as helper T-cells, while $CD8^+$ cells are cytotoxic T-cells. However, there is now evidence that $CD4^+$ T-cells may have cytotoxic functions too so blurring this distinction (van de Berg *et al.*, 2008).

There are also other subclasses of T-cell which are much rarer. Invariant NK T-cells (iNKT) use CD1d, a non-classical MHC, to recognise and respond to glycolipid antigens including those produced by bacteria. They release a number of cytokines including IL-4 and IL-13, or IFN-γ and tumour necrosis factor (TNF). The presence of the signalling lymphocyte activation molecule associated protein (SLAM-SAP) protein is required for iNKT generation. Additionally, while the majority of T-cells have a TCR composed of α and β chains, a minority have TCRs made of γ and δ chains, and are therefore known as γδ T-cells. These T-cells use non-classical MHC molecules such as CD1c. They are able to recognise an array of antigens both in the peripheral blood but also the epidermis. It is thought that they protect against a number of microbes by producing IFN-γ and TNF and inducing cytotoxicity (LaRosa and Orange, 2008).

An added layer of complexity to the adaptive immune system is that if it responds to, and effectively clears a novel antigen, a memory T-cell pool remains to counter any subsequent exposure to this antigen. There are two memory T-cell phenotypes; central memory T-cells (Tcm) defined by their confinement to lymphoid tissues and expression of CD45RO, CCR7, and CD62L, and effector memory T-cells (Tem) which are able to circulate in the periphery and do not express either CCR7 or CD62L. The effector memory component circulates and is able to mount a rapid response against an encountered antigen, but they are limited in their ability to proliferate. If a larger response is required then a central memory response occurs. Although unable to mount a response themselves, they are rapid proliferators and differentiate into effector T-cells (LaRosa and Orange, 2008).

1.1.1.3.2 T helper cell subsets.

It was work by Mosmann and colleagues that identified two distinct $CD4^+$ T-cell subsets that could be generated from the naïve $CD4^+$ (Th0) population which could be defined by distinct cytokine secretion patterns. A subset, termed T-helper (Th) 1 and considered pro-inflammatory, was defined by the ability to secrete IFN-γ and IL-2, whilst Th2, an anti-inflammatory population secreted IL-4 and IL-5. Subsequent work has seen many more cytokines be associated to each subset, with IL-10 and IL-13 associated with Th2, and IL-12 with Th1. Cytokines are also critical in determining which subset of T-cells is generated following T-cell priming. It is now known that priming and generation of Th-1 T-cells requires IL-12, while IL-4 is essential for Th2 T-cell generation (Liu *et al.*, 1998).

Th1 cell development is driven by the transcription factors T-bet and Signal Transducer and Activator of Transcription (STAT)-4 (Romagnani, 1997). They can activate NK cells and macrophages, and expand cytotoxic $CD8^+$ T-cells to protect against intracellular viruses and bacteria largely through the secretion of IFN-type cytokines. Th2 T-cell cells on the other hand control pathogenic infections such as those from helminths, but also bacterial and viral infections, by secreting IL-4, IL-5, IL-10, and IL-13 to promote an eosinophilic and IgE-mediated response (Romagnani, 1997). The development of these Th2 cells is promoted by the transcription factors GATA3 and STAT5 (Pulendran *et al.*, 2010).

In recent years, other Th subsets have been identified, including Th17 and Th22. Th17 cells are thought to play an important role in the effective elimination of particular pathogens, including fungal and gram-negative bacterial infections, which are ineffectively cleared by either the Th1 or Th2 subsets (Bettelli *et al.*, 2007). Indeed, the likelihood of infection with Candida fungus is increased in IL-17 receptor knockout mice models (Huang *et al.*, 2004). The development of Th17 cells is known to involve the

transcription factors STAT3, RORγt and RORα, and relies on the presence of transforming growth factor (TGF)-β, and IL-6 or IL-21. These cells are associated with multiple sclerosis and psoriasis and can potently induce a tissue-based inflammatory response (Korn *et al.*, 2009). The transcription factors associated with the development of either Th1 or Th2 responses (T-bet, STAT1/4/6) are not required for differentiation into Th17 cells. However, TGF-β may be an important for the formation of Th17 cells as it has previously been shown to signal through STAT3 and SMAD proteins to generate anti-inflammatory Th17 cells. Despite this, other studies discredit the necessity for the presence of TGF-β during Th17 differentiation (Ghoreschi *et al.*, 2010; Mangan *et al.*, 2006).

Th22 cells can be found in the skin and due to their enhanced presence in patients with psoriasis, atopic eczema, and allergic contact dermatitis (ACD), are implicated in the pathogenesis of skin disease (Eyerich *et al.*, 2009). Indeed they are known to be pro-inflammatory, with their development reliant on an array of Th1 and Th17 subset associated genes (Duhen *et al.*, 2009; Kagami *et al.*, 2010; Trifari *et al.*, 2009). These cells are characterised by the secretion of IL-22, but also TNF-α, along with the absence of secretion of IFN-γ and IL-4. Th17 cells also secrete IL-22 so it has been suggested that Th17 and Th22 might be better categorised as a single subset. However, one fact argues against their inclusion in a single subset, namely that Th22 cells lack the ability to secrete IL-17. Furthermore, the expression of the transcription factor RORC is greatly reduced in Th22 cells compared to Th17 (Eyerich *et al.*, 2009).

Both Th17 and Th22 are implicated in hypersensitivity reactions. While Th17 are reportedly upregulated in patients suffering from both DIHS and acute generalised exanthematous pustulosis (AGEP; see section 1.3.4.2) (Tokura *et al.*, 2010), the release of both IL-17 and IL-22 during hypersensitivity reactions has been assessed in perhaps the most detail for contact hypersensitivity (CHS), where both cytokines are implicated

in the pathomechanism. This can be seen in murine models, where psoriasis-like lesions can be created through the induction of Th17 cells by IL-23. This response was further assessed to be mediated by both IL-17 and IL-22 (Zheng *et al.*, 2007). Similar responses have been detected in humans, where the analysis of IL-17/IL-22 has primarily involved the assessment of nickel-induced CHS; traditionally seen as a mixed Th1/Th2 response (Minang *et al.*, 2005). The role of IL-17 was initially identified when both skin samples and T-cells from nickel-allergic patients were found to contain IL-17 messenger ribonucleic acid (mRNA), and further stimulation led to the secretion of IL-17. Additionally, murine models of contact allergy show that IL-17 knockout produces a significantly reduced ear swelling, the effects of which were mediated solely by $CD4^+$ T-cells (Albanesi *et al.*, 1999; Nakae *et al.*, 2002). Another study identified both IL-17 and IL-22 positive cells in patients with ACD. However, this study, like many others, failed to costain these cells and thus were unable to define the Th17 or Th22 origins of these cytokines. Indeed in light of this, it has been suggested that costaining of IL-17 and IL-22 should be routinely used to fully elucidate the origins of individual reactions (Cavani *et al.*, 2012; Peiser, 2013).

Other lesser known Th subsets include T follicular helper cells (Tfh), and Th9. Tfh T-cell differentiation is believed to be reliant on B-cell lymphoma protein-6 (Bcl-6), although early differentiation can also be stimulated by increased CXCR5 expression and STAT3 signalling induced via IL-6/21. Their primary role appears to be in aiding antibody class switching and the secretion of antibodies with high affinity (Johnston *et al.*, 2009; Liu *et al.*, 2014; Sun and Zhang, 2014; Vogelzang *et al.*, 2008). Th9 cells are so called because of their profound IL-9 secretion and have been reported to aid autoimmune and allergic disease development. Stimuli such as programme death-ligand 2 (PD-L2), jagged2, cyclooxygenase (COX)-2, IL-4/1, and TGF-β among others are known to be involved in

Th9 differentiation (Dardalhon *et al.*, 2008; Schmitt *et al.*, 1994; Sun and Zhang, 2014; Veldhoen *et al.*, 2008).

1.1.1.3.3 T regulatory cells.

It has long been known that self-reactive T-cells exist due to thymic inefficiencies during the clonal deletion stage of T-cell development. To counteract this potentially lethal problem, humans also have immune-inhibitory T-cells that are able to control the development of autoimmunity. Analysis of these T-cells identified CD25 as a marker of this population. The role of $CD25^+$ T-cells is demonstrated by the development of autoimmune diseases in athymic nude mice after $CD25^+$ T-cell depletion, indicating that these cells control the magnitude of the T-cell response (Sakaguchi *et al.*, 1995). Since these early experiments it has become increasingly clear how important $CD25^+$ T-cell regulation is to our everyday health and survival, with both microbial and tumour immunity significantly amplified by $CD25^+$ T-cell depletion, and this T-cell subpopulation aiding the establishment of feto-maternal tolerance. Due to their vital role in the regulation of the immune system, $CD25^+$ $CD4^+$ T-cells were termed T regulatory cells, more often referred to as T regulatory cells (Tregs). It is now estimated that 5-10% of all circulating $CD4^+$ T-cells are $CD4^+$ $CD25^+$ (Cavani, 2008).

A major transcription factor for Treg development is forkhead box P3 (FoxP3). The scurfy mouse model, which lacks this transcription factor, identifies its importance as a key immune regulator as these mice present with $CD4^+$ T-cell hyper activation (Brunkow *et al.*, 2001). Within the $CD4^+$ thymocyte population, those expressing FoxP3 account for just 5%. Upon leaving the thymus after their generation, Tregs are unique to all other T-cells in that they have been primed and are considered to be functionally mature.

However, it is important to note that as well as natural Tregs (nTregs) which are those derived from the thymus, induced Tregs (iTregs) can be produced through the action of TFGβ on naïve $CD4^+$ T-cells. Both nTregs and iTregs are potently suppressive of other T-cells and express similar surface markers including CD25 (Francisco *et al.*, 2009). iTregs are also $FoxP3^+$ and while they act similarly to nTreg to promote self-tolerance, they may also have specialist roles within the human body. Indeed they may promote oral tolerance by converting naïve T-cells to Tregs. This is based upon the fact that dendritic cells (DCs) which produce retinoic acid (RA) are present in the digestive tract and RA is known to facilitate the conversion of naïve T-cells to Tregs (Benson *et al.*, 2007; Sakaguchi *et al.*, 2008).

While the role of Tregs in the development of cutaneous reactions are yet to be fully explored, Treg-mediated control of effector T-cell responses is of therapeutic interest during reactions such as drug reaction with eosinophilia and systemic symptoms (DRESS; see section 1.3.4.3). A Japanese group have reported the expansion of Tregs during the acute stage of drug-induced hypersensitivity syndrome (DIHS) onset and were plentiful in associated skin lesions. Such rapid expansion is thought to limit effector T-cell-mediated collateral damage in the tissue and may account for the reduced B-cell population seen during the development of this response (Kano *et al.*, 2004; Takahashi *et al.*, 2009). Moreover, it was found that this expanded Treg population became functionally deficient after DIHS was resolved and may be responsible for the development of autoimmunity associated with the resolution of DIHS. Specifically, autoimmune thyroiditis and type 1 diabetes mellitus have previously been reported as follow-up reactions after DIHS (Kano *et al.*, 2007; Sekine *et al.*, 2001). It has been suggested that limiting the effector T-cell response and thus the severity of skin lesions subsequently allows for the associated viral reactivation. Furthermore, reactivated human herpes virus (HHV) is able to bind TLR2 expressed on the surface of Tregs,

preventing the expression of FoxP3 and thus maintains the suppression of Treg function (Sutmuller *et al.*, 2006).

However, the role of Tregs appears to be different during DIHS in comparison to most other cutaneous hypersensitivity reactions. In particular, Treg function during toxic epidermal necrolysis (TEN; see section 1.3.4.4) was found to be highly suppressed and so may account for the extensive tissue damage associated with this type of response. Additionally, Tregs lacked the ability to migrate to the cutaneous sites despite the identification of normal quantities of Tregs in peripheral circulation. Models of TEN have previously found that Tregs can prevent TEN-associated epidermal injury, thus these data highlight that susceptibility in patients may be linked to a qualitative, but not quantitative, effect on Tregs (Azukizawa *et al.*, 2005; Takahashi *et al.*, 2009). These studies suggest that therapies to enhance the functional response of Tregs may aid in the treatment of cutaneous hypersensitivity reactions. As the Treg response varies between reactions, therapies would have to be tailored for specific manifestations, and may not be effective for all reactions as Pichler's group have previously reported no abnormal Treg function in patients with multiple drug hypersensitivity (Daubner *et al.*, 2012).

The suppressive effects of Tregs are induced by much lower antigen concentrations than those required to stimulate naïve T-cells, which provides a mechanism by which Tregs can readily supress any T-cells able to promote autoimmunity (Takahashi *et al.*, 1998). This lower activation threshold also allows the innate immune system to respond to minor infections without the initiation of a full scale T-cell response, which can later come into play if the innate system fails to deal with the initial threat and antigen concentration is increased. How Tregs actually supress other T-cells is complex, mostly due to the vast number of mechanisms available to them to perform this function. This

includes multiple pathways where the effect produced by the Treg acts directly on the effector T-cells, but also many indirect pathways which involve signalling through APCs.

1.1.1.3.3.1 Direct Treg Suppression mechanisms

Tregs constitutively express CD25, part of the high affinity IL-2 receptor, whilst activated effector T-cells only do so after TCR engagement. Thus Tregs may initially dampen a T-cell response by consuming exogenous IL-2 (Barthlott *et al.*, 2005). Indeed the competitive uptake of IL-2 reduces exogenous IL-2 available for use by other T-cells thus limiting their activation. In some instances, a lack of IL-2 may induce T-cell to undergo apoptosis via activation of Bim, a proapoptotic factor. Whilst evidence for this is limited (Pandiyan *et al.*, 2007), many studies have shown that Tregs can supress IL-2 mRNA expression in these responder T-cells. This suppression was found to be cell-cell contact mediated and proposed to be caused by blocking co-stimulatory signal delivery to the responder T-cell (Thornton and Shevach, 1998).

Another directly suppressive mechanism used by Tregs is the secretion of specific cytokines. IL-10 and TGF-β are considered key to the induction and suppressive function of Tregs, however upon their neutralisation, Tregs are still able to supress effector T-cells via the other suppressive mechanisms. There is much debate about the true role of both of these cytokines in Treg function due to differences in results between *in vivo* and *in vitro* models. However, this is likely due to different modes of suppression being more vital when T-cells are exposed to different conditions. For instance, in a colitis model, IL-10 was found to be inessential for supressing colitis induced by naïve T-cells, but was vital in a similar disease state induced by memory T-cells. This implies that the modes of Treg suppression employed vary depending on whether a T-cell has previously

encountered antigen. Furthermore, this has implications for modes of suppression based upon Treg localisation, as naïve T-cell regulation will occur in the lymph nodes whereas regulation of memory T-cells occurs at the site of inflammation, most often in peripheral tissues (Asseman *et al.*, 2003). One type of Treg, the Tr1 cell, is known to exert its function primarily through secretion of IL-10 and leads to inhibition of DC and macrophage antigen presentation. IL-10 mediated inhibition of APCs is thought to be especially important during a response to contact allergens, as in a mouse model, IL-10 prevented the onset of CHS (Cavani, 2008).

These cytokines are also important for the generation of Tregs which can be portrayed using Eso2 and Pan02 murine cancer cell lines, which produce low and high TGF-β, respectively. Treg production from naïve $CD4^+$ $CD25^-$ $FoxP3^-$ T-cells occurred only in the Pan02 cells thus largely implicating TGF-β in this process (Moo-Young *et al.*, 2009). The mechanisms by which TGF-β exerts its immune suppressive properties are still undefined but it was initially presumed that TGF-β would exert a suppressive function through secretion into the extracellular environment. A latent form of TGF-β has since been proven to exist in a membrane-bound state on TCR-activated, but not resting Tregs and so is believed to function in a cell-cell contact dependent manner (Andersson *et al.*, 2008). Other cytokines are also secreted by Tregs and have been shown to aid suppressive function. These include IL-35, an immunosuppressive IL-12 family cytokine (Collison *et al.*, 2009).

Other directly inhibitory mechanisms include inducing cytotoxic cell death. Here, Tregs produce granzyme A subsequent to CD3 stimulation which they then secrete to kill responder T-cells. Both granzyme B and perforin-dependent T-cell killing pathways have been observed in Treg populations, with reports from tumour models that up to 30% of Tregs contain granzyme B (Cao *et al.*, 2007). The cell cycle may also be halted by binding

of galectin-1 expressed by Tregs to a range of ligands including CD45. It is still unknown whether galectin-1 functions in a cell contact dependent manner or is secreted by Tregs for this role. Nonetheless, galectin-1 binding prevents pro-inflammatory cytokine production and stimulates T-cell cycle arrest. In both murine and human models, Treg suppressive ability was reduced after the binding of galectin-1 was blocked, and galectin-1 deficient mice were less able to prevent effector T-cell IL-2 production (Garín *et al.*, 2007).

1.1.1.3.3.2 Indirect Treg suppression mechanisms.

Tregs can indirectly affect T-cell activation by interacting with APCs rather than targeting the responder T-cells directly. A number of these include the co-stimulatory and co-inhibitory pathways. These pathways have a fundamental role in Treg homeostasis as they regulate both the development and maintenance of these cells. For instance, there is a well-established critical role for the stimulatory pathway inducible costimulator (ICOS) in aiding Treg maintenance. Not only are fewer Tregs found in ICOS knockout mice (Burmeister *et al.*, 2008), but differential expression of surface ICOS may be indicative of individual Treg subsets. Indeed, Tregs with high ICOS expression were found to produce more IL-10 than $ICOS^{low}$ Tregs, but were found to have less membrane bound TGF-β1 (Ito *et al.*, 2008). Preferential expression of ICOSL on plasmacytoid DCs rather than myeloid DCs led to the conclusion that plasmacytoid DCs selectively aid $ICOS^{+}$ Treg activation, and so the development of $ICOS^{hi}$ or low Treg populations is dependent on different DC phenotypes. Co-stimulation from CD28, alongside medium to high affinity self-antigen induced activation of the TCR, is also seen as critical for the intrathymic development of Tregs. Using murine models, it was shown that knockout of CD28 leads to autoimmune

disease specifically mediated by defective Treg activity, and this can be reversed by further supplementation with $CD28^+$ Tregs (Salomon *et al.*, 2000).

Multiple other co-inhibitory pathways are employed by Tregs, which are critical to their development and function. This is exemplified by Treg expression of both programmed death-1 (PD-1) and programmed death ligand (PD-L)-1. The PD-1-PD-L1 interaction is able to block naïve $CD4^+$ T-cell protein kinase B (Akt) signalling to promote the differentiation of these cells into iTregs, even in the absence of TGF-β. Furthermore, PD-1 signalling is able to promote long-term FoxP3 expression (Francisco *et al.*, 2009). Despite this apparent ability to promote Treg development and function, the story is far more complicated. Intrahepatic Tregs from patients chronically infected with hepatitis C virus display enhanced PD-1 expression which has been associated with reduced Treg development and function (Franceschini *et al.*, 2009). The authors in this instance remind us of the potential for these receptors to have different roles in different disease settings. In this case, the PD-1 pathway allows for the continued presence and function of T effector cells to fight off the chronic infection by limiting Treg function. However, when the balance shifts from limiting to promoting Treg is unknown. Much more information of the differential functions of PD-1-PD-L1 between individual Treg subsets is needed.

Cytotoxic T-lymphocyte associated protein-4 (CTLA4) is also present on Tregs where it is constitutively expressed under the influence of FoxP3 (Sakaguchi *et al.*, 2008). The mechanisms by which CTLA4 suppress effector T-cell responses are summarised briefly below. CTLA4 provides an effective pathway to target the differentiation and priming of T-cells as its ligands, CD80 and CD86, are expressed on professional APCs. This may provide a conventional inhibitory signal directly to effector T-cells by sequestering these ligands that CTLA4 shares with CD28. Additionally, this interaction appears to signal back

to APCs, in a process called reverse signalling, to promote the expansion of Tregs by inducing APC expression of indoleamine 2,3-dioxygenase (IDO). CTLA4 has also been suggested to disrupt DC-T-cell immunological synapse formation, potentially through modulation of cell adhesion pathways such as lymphocyte function associated antigen-1 (LFA-1) (Schneider *et al.*, 2005).

Other indirect mechanisms of T-cell inhibition mediated by Tregs include the expression of CD39. Specifically, adenosine triphosphate (ATP) release from damaged cells may act as a danger signal to stimulate DCs to enter a mature state. Half of all Tregs express the enzyme CD39, which is required for full hydrolysis of ATP to the final end product adenosine monophosphate (AMP), thus removing this danger signal and preventing inflammatory immune activation (Borsellino *et al.*, 2007). Additionally, Tregs which contain cAMP may be able to transfer this via gap junctions to T-cells, where it prevents proliferation by inhibiting the activation of the transcription factor nuclear factor kappa beta (NF-κβ) (Bodor *et al.*, 2000; Minguet *et al.*, 2005). One very effective mode of suppression is through the expression of Neuropillin-1 (Nrp-1), a vascular endothelial growth factor receptor. Expression of Nrp-1 is induced by FoxP3 and as such is primarily found on Tregs. It has been suggested that Nrp-1, which increases the length of contact time between Tregs and DCs, may represent a method by which Tregs can dominate over effector T-cell responses during sub-optimal antigen exposure. Indeed, Treg suppression is completely blocked during sub-optimal antigen exposure to effector T-cells when Nrp-1 is inhibited (Sarris *et al.*, 2008). Some Tregs also have the ability to directly kill T-cells using mechanisms more associated with effector T-cell-mediated killing (Cao *et al.*, 2007).

1.1.1.3.4 T-cell plasticity.

The apparent divergence in cytokine secretion by differing Th subsets provides individual subsets with a distinct "cytokine signature". However, it is now understood that a cytokine, which historically had been associated with a particular T-cell subset, may be secreted by another subset. IL-10 is a good example of this as it is now associated with Th1, Th2, Tregs, and a range of cells of the innate immune system, but was originally classified as Th2-specific. Additionally, the supposed Tf-helper signature cytokine IL-21 is also secreted by Th1 and Th17 subsets (Nakayamada *et al.*, 2011; Nakayamada *et al.*, 2012; Suto *et al.*, 2008). Another term, "master regulator", which referred to a specific transcription factor that promoted the differentiation of naïve $CD4^+$ T-cells into a specific lineage, has also become confused by the discovery of subset plasticity. This plasticity alludes to the ability of Th subsets to change into other subsets, a process which is not well understood. It does however remind us that *in vivo* conditions are not fixed and may constantly change thus requiring adaptation of the responding T-cell subsets to suit the changing environment. This is best shown by reports that cells initially phenotyped as Th2 and GATA3 positive may be adapted to become IFN-γ secreting T-bet positive T-cells, while still being GATA3 positive IL-4 secretors after culture with a Th1-type promoting virus. Expression of both T-bet and GATA3 was stable and so the authors referred to these cells as $Th1^+Th2^+$ cells (Hegazy *et al.*, 2010). Similarly other lineages have become less distinct, with the traditionally Th1-associated cytokine IFN-γ seen to be secreted from Th17 cells (Lee *et al.*, 2009). Although this plasticity confuses the T-cell subset differentiation schematic, many of the changes make sense. For instance, during Th1-induced inflammatory responses, Tregs have been shown to upregulate the Th1-associated transcription factor T-bet. This promotes the expression of the chemokine receptor CXCR3 on Tregs allowing for the migration of these cells to the Th1-medaited

inflammatory site to aid in the effective dampening of a response once the antigen has been eradicated (Koch *et al.*, 2009). Treg plasticity may also involve the additional expression of other transcription factors including GATA3.

1.1.2 Granulocytes (phagocytes).

Granulocytes, a cell categorisation which includes eosinophils, neutrophils, and basophils, are all phagocytes produced from the myeloid progenitor cells which have the ability to engulf and then destroy microorganisms. These cells are so named due to their cytoplasmic granule contents which have the capacity to promote inflammation and protect against both fungal and bacterial infections. They are also called polymorphonuclear leukocytes as they have nuclei that are irregular in shape and have up to five lobes. Phagocyte recognition of antigen is dependent on the presence of pattern recognition receptors, which detect and bind to specific pathogenic sequences termed PAMPs (pathogen-associated molecular patterns) that aren't seen in mammalian cells. There are many forms of these receptors, a commonly researched example of which are TLRs. TLRs are able to identify a broad range of pathogenic sequences; TLR2 responds to peptidoglycan in the cell wall of gram positive bacteria, TLR4 is well known to recognise bacterial lipopolysaccharide, a structurally exposed component of gram negative bacteria, TLR3 to viral double stranded RNA, and TLR5 to bacterial flagellin, to name a few (Takeda *et al.*, 2003). The major phagocyte in the human body is the neutrophil, followed by eosinophils, with basophils being the least abundant.

1.1.2.1 Eosinophils.

Eosinophils are found at sites where external pathogens gain entry to the body including the lungs, gastrointestinal tract, and skin (Dahl, 2009). They have a broad range of functions including the regulation of vascular permeability, enhancement of cell adhesion properties, induction of cell damage, mucus secretion, smooth muscle constriction, and the ability to act as APCs. Indeed upon activation, eosinophils express MHC class II on their surface as well as the necessary co-stimulatory molecules for the induction of an immune response such as CD86 (Celestin *et al.*, 2001).They induce local inflammation by releasing an array of cytokines including TGF, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16 and IL-18, some of which are also involved in Th1 or Th2 polarisation and the activation of T-cell responses (Rothenberg and Hogan, 2006). Indeed, $CD4^+$ T-cell STAT6 transcription, and therefore Th2 differentiation, can be initiated by eosinophils when cultured with TNFα and IL-4. Alternatively, culture with TNFα and IFN-γ induces Th1 differentiation by induction of STAT1 transcription. T-cell polarisation may also be induced by eosinophils through the release of eosinophil-derived neurotoxin, a component of eosinophil granules, which is able to both mature and activate DCs to stimulate T-cells in a Th2-dependent manner (Yang *et al.*, 2004; Yang *et al.*, 2008).

1.1.2.2 Basophils.

Paul Erlich first described basophils in 1879 after noting similar but distinct characteristics between these cells and mast cells (Falcone *et al.*, 2000). Specifically, both cell types express a high affinity IgE receptor, which when cross-linked activates these cells to release lipid mediators, cytokines, leukotriene C4, and histamine (Wedemeyer *et al.*, 2000). However, whilst basophils are limited to the secretion of just a few cytokines

such as IL-4 and IL-13, mast cells have the ability to secrete a much wider range including IL-1, IL-3, IL-4, IL-5, IL-6, Il-8, IL-10, IL-13, IL-16, TNF, and TGF-β among others, which have subsequent effects on tissue remodelling, immunity, and inflammation.. The early development and differentiation of basophils, which constitute < 1% of peripheral blood cells, is heavily regulated by the haemopoietic growth factor IL-3. Indeed, the generation of basophils *in vitro* from blood-derived precursor cells is dependent on the presence of IL-3. In contrast, mast cell differentiation is thought to rely on the use of stem cell factor (SCF) (Falcone *et al.*, 2000; Iemura *et al.*, 1994; Valent *et al.*, 1989). Furthermore, while the differentiation of mast cells occurs in the tissues and affects early allergic reactions mediated by IgE, mature basophils are found in the blood but migrate to tissues during an immune response and are thought to regulate late-phase allergic reactions and helminthic infections alongside eosinophils and Th2 lymphocytes. Moreover, basophil migration to inflammatory sites is instigated by Th2-associated cytokines. While their precise role in these reactions is still not fully defined, they are proposed to promote $CD4^+$ helper T-cell subset differentiation and facilitate antibody class switching in B-cells (Sokol *et al.*, 2008; Yamaguchi *et al.*, 1992; Yanagihara *et al.*, 1998).

1.1.2.3 Neutrophils.

Neutrophils contain multiple different types of granule categorised by their contents. Primary granules, also known as azurophil granules, contain MPO whereas the secondary granules do not. Other granules are known for their high gelatinase content (Borregaard *et al.*, 2007). One way in which neutrophils defend against infection is to utilise nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to produce metabolites so harmful that neutrophils only assemble NADPH oxidase upon activation. The most utilised of NAPDH oxidase products is superoxide (O_2^-) which, through the activity of

superoxide dismutase, is converted to hydrogen peroxide. Subsequent conversion by MPO produces the highly bactericidal oxidant hypochlorous acid. These products are formed within the phagocytic vacuole to minimise their inadvertent release into the circulation. Other notable components of neutrophil granules are defensins which increase membrane permeability on target cells, metalloproteinases which are thought to aid neutrophil migration, the highly and directly cytotoxic "bactericidal increasing protein", proteinase 3 which enhances inflammation, the potent bactericidal phospholipase A2 (PLA2), and elastase to break down a gram-negative bacterial protein in the outer membrane (Burg and Pillinger, 2001).

1.1.3 Monocytes.

As with the granulocyte population, monocytes also represent downstream cells of myeloid cell progenitors but have a single indented nucleus, and are generally much larger. Out of the total leukocytes in human peripheral blood, approximately 5-10% are monocytes (Gordon and Taylor, 2005), which can be subdivided into three main cell types; the precursor monocytes found only in the blood, and two distinct monocyte-derived lineages; DCs and macrophages. The differentiation of these cells involves a complex pathway originating from myeloid progenitor cells. Initially a general precursor for monocytes is produced before subsequent formation of three independent groups which form two different arms to monocyte differentiation; common DC precursors, and two monocyte precursors (MPs) differentiated by their expression of Ly-6C. Along the first arm, both of the MPs enter the circulation from the bone marrow. Ly-$6C^-$ MPs differentiate into lung resident, alveolar macrophages that function to maintain homeostasis. In contrast, Ly-$6C^+$ MPs form a subset of DCs defined by the expression of CX_3CR1 which patrol the non-lymphoid tissues. In response to inflammation, Ly-$6C^+$ cells

form both inflammatory macrophages and a subset of DCs known as TipDCs which produce nitric oxide and TNF. Additionally, Ly-6C$^+$ cells can lead to the generation of myeloid-derived suppressor cells which are known to target tumours. On the second arm, common DC precursors form both pre-classical and plasmacytoid DCs. If deployed in lymphoid tissues, pre-classical DCs fully differentiate into classical DCs, but upon migration to non-lymphoid tissues, they form a subset of DCs defined by the expression of CD103. In contrast, plasmacytoid DCs simply enter the blood and tissues and begin patrolling for antigen (see (Geissmann *et al.*, 2010) for a detailed review of monocyte differentiation).

The differentiation of monocytes into either macrophages or DCs is dependent on the local cytokine milieu. Macrophage colony-stimulating factor (M-CSF) is a particularly important cytokine for macrophage survival. Indeed, IL-6 stimulates expression of the M-CSF receptor which promotes macrophage differentiation, whilst TNF promotes DC generation by limiting M-CSF cell surface expression. The overall function of these cells is the efficient phagocytosis of not only microorganisms but also in clearing the remnants of dead cells during steady-state homeostasis. Monocyte migration into tissues during infection is due to their expression of a range of chemokine and adhesion receptors (Chomarat *et al.*, 2003).

1.1.3.1 Macrophages.

Macrophages, due to their tissue-resident nature, form a major component of steady-state immune homeostasis. They are often the first cell to detect a threat and signal through the release of cytokines to other cells, including neutrophils, that a response is necessary. Specifically, macrophages aid tissue homeostasis by producing growth factors

and clearing dead or dying cells, including tumour cells. Macrophages have a range of receptors e.g. TLRs, which allow them to detect molecular patterns produced by pathogenic organisms and damaged cells (Parham, 2009).

Macrophages can be divided in to two groups, M1 and M2, which are pro- and anti-inflammatory, respectively. M1 macrophages are characterised by a IL-12^{high}, IL-23^{high}, Il-10^{low} phenotype, and are activated in response to microbial products and inflammatory cytokines such as IFN-γ. In direct contrast, the general phenotype of an M2 macrophage is IL-12^{low}, IL-23^{low}, IL-10^{high}, and these cells can be further subdivided into three groups. Those induced by IL-4 or IL-13 are defined as M2a, macrophages activated by IL-1R or TLR agonists as well as immune complexes are M2b, whilst M2c are activated by glucocorticoid hormones or IL-10. The typical effector mechanisms employed by M1 macrophages involve the production of inflammatory cytokines such as TNF, IL-6, and IL-1β, but also the production of reactive oxygen species (ROS). This promotes a Th1 response. Opposing this, M2 Macrophages promote tissue repair and remodelling but primarily aid Th2 type responses leading to parasite phagocytosis (Mantovani *et al.*, 2005; Mantovani *et al.*, 2004).

1.1.3.2 Dendritic cells.

DCs represent another differentiation pathway for monocytes. Morphologically DCs can be separated from other monocytes in their mature state due to their long dendrites which give them their name. DCs express both CD11c and high MHC class II, whilst lacking haematopoietic lineage markers (Hashimoto *et al.*, 2011). Similar to macrophages, DCs reside in the tissues, however their primary function is to present antigens for naïve T-cell priming. When DCs recognise an antigen they further upregulate their expression of

CD80/CD86 co-stimulatory receptor and MHC molecule surface expression is greatly increased. CCR7 is also expressed in order for the DCs to migrate to the lymph nodes, and lysosomal pathways are activated to process antigens into distinct peptides which are then presented at the cell surface bound to the MHC (Jiang *et al.*, 2007). Mature DCs migrate to the draining lymph nodes where they present antigens to passing T-cells to promote the induction of an adaptive immune response (Parham, 2009). In fact, DCs are professional APCs and the only cell capable of naïve T-cell priming and thus are the driving force behind the establishment of immunological memory. For this reason, DCs are often considered to be of high importance in the balance between antigen clearance versus antigen tolerance as immature DC will promote tolerance due to a lack of co-stimulatory signalling, but mature DCs with the correct co-stimulatory signalling will lead to an immune response.

Another DC specialist task is antigen cross-presentation. This refers to the ability of MHC class I on the surface of DCs, which is normally used for the presentation of intracellular antigens, to present extracellular antigens. This enables a cytotoxic $CD8^+$ T-cell to mount a response against modified or infected cells that display modified-self proteins or foreign antigens, and is described in further detail in section 1.5.2. This process is aided by the highly phagocytic capabilities of immature DC (Joffre *et al.*, 2012).

There are two main types of DC: classical and plasmacytoid. Classical DCs migrate between tissues and lymphoid organs but are short lived. Plasmacytoid DCs however are longer lived, and are found in all peripheral organs and the bone marrow, with activation associated with a much larger secretion of IFN type I than classical DCs, especially for those involved in reactions to viral infections (Geissmann *et al.*, 2010). The activation of DCs leads to a plethora of antigen-specific responses including the activation of NK cells, non-antigen-specific macrophages, B-cells, and cytotoxic $CD8^+$ T-cells (Banchereau *et al.*,

2000). It is also clear that DCs do not just stimulate a T-cell response but also direct it. A combination of IL-23 and IL-1β secretion from DC has been reported to aid Th17 differentiation, while IL-12 release polarises T-cells towards a Th1 phenotype, and IL-10 towards a Th2 phenotype (Liu *et al.*, 1998; Macatonia *et al.*, 1995; Sutton *et al.*, 2009).

While the primary function of such professional APCs is to present harmful antigens to passing T-cells, similar responses may occur in response to antigens that pose no threat such as drugs. Responses of this nature are categorised among the generalised term of adverse drug reactions (ADRs).

1.2 Adverse Drug Reactions

1.2.1 Defining an adverse drug reaction.

In 1969, the World Health Organisation (WHO) defined an ADR as "a response to a drug that is noxious, unintended or undesired, occurring at doses normally used for the prophylaxis, diagnosis or treatment of disease, or for modification of physiological function"(Edwards and Aronson, 2000).

Despite this definition being unchanged for the last 45 years, specific adaptations have been suggested for several reasons. First, this definition is restricted and does not apply to reactions elicited by inactive excipients or herbal medicines. Second, the word noxious applies through its very definition only to reactions which are harmful or hurtful to the patient, however many ADRs are not harmful but perhaps irritating such as the dry, tickling cough sometimes associated with angiotensin-converting enzyme (ACE) inhibitors. Third, it does not account for medication errors in dosing (Edwards and Aronson, 2000; Fogari *et al.*, 2011). Independent of the exact ADR definition, these

reactions are often severe and subsequently represent a significant burden on clinicians, pharmaceutical companies, and most importantly patients.

1.2.2 Adverse drug reaction reporting, prevalence, and health burden.

Pharmacovigilance, the observation and subsequent recording of potential ADRs, plays a large role in identifying problematic drugs post-marketing. Many countries operate spontaneous reporting systems whereby reports of a drug-specific ADR can be monitored, with overly frequent ADRs from a particular drug leading to the drug being flagged as a potential issue. In the UK, a yellow card system has allowed clinicians to monitor and report ADRs since 1964, which was recently updated in 2005 to allow patients themselves to submit ADR reports (McLernon *et al.*, 2010). If it is deemed that a substantial risk is posed to patients then either a black flag warning can be imposed on that drug, or to prevent any further ADRs, the drug may be withdrawn from the market altogether. A total of 22 drugs were withdrawn from use in patients during a ten year period from early 1990 to late 1999 due to the potential to cause an ADR, the majority of which were due to cardiac or hepatic toxicity (Arnaiz *et al.*, 2001).

In the US alone, it is estimated that 100,000 deaths occur annually due to serious ADRs (Wilke *et al.*, 2007). In a 1995, Boston based drug surveillance program, 6.1% of hospitalised patients developed an ADR and 1.2% of these reactions resulted in patient death (Bates *et al.*, 1995). Substantially higher hospitalised patient ADR incidence rates have been reported such as the 15.1% recorded by Lazarou and colleagues (Lazarou *et al.*, 1998). While primarily of concern as they are detrimental to patient health, this prevalence of ADRs additionally means that hospitals have more in-patients. Estimates have put the number of extra hospital bed days for in-patients due to ADRs at 2000 at

any one time. When combined with total bed-days for patients in hospital because of an ADR, the authors nicely illustrate this as the equivalent of ten 800-bed hospitals (Davies *et al.*, 2009). This strain on hospital beds translates into financial burden, with ADRs approximated to cost an extra £5000 per bed per year. This, along with other figures estimating the financial cost of hospital admissions due to ADRs at 5-9% of total inpatient costs, equates to a total annual financial burden of ADRs on the National Health Service (NHS) of approximately £637 million for England alone (Davies *et al.*, 2009; Kongkaew *et al.*, 2008). However, the actual figure could be much higher due to the under reporting of ADRs as a 2001 nationwide survey in the Netherlands found that only 1% of ADRs were reported (van der Hooft *et al.*, 2006).

1.2.3 General adverse drug reaction risk factors.

Although the term ADR describes a wide variety of responses, of differing degrees of severity, to an abundance of drugs, there are several generalised factors which are linked to their onset. Some of these relate directly to the drug. These include an increased potential for ADRs when drugs are administered repeatedly, perhaps with sporadic intervals in contrast to a continuous dosing regimen, but also the route of administration, with orally administered drugs deemed less sensitising to those given via a parenteral route (Thong and Tan, 2011).

There are also a number of "patient factors" which have partly been observed thanks to the detailed reporting of ADRs alongside statistical analysis allowing for the identification of specific demographic groups which are more susceptible to ADRs. In hospitalised patients, 5.3% of the general population will develop an ADR, but this prevalence increases with age, with elderly patients having more than double the admission rate

(10.7%) for ADRs than children (4.1%)(Kongkaew *et al.*, 2008). However, this value varies from one report to the next, an explanation for which is at least partly down to differing methods to detect and report ADRs. Women are also more likely than men to develop an ADR, which was verified in 2008 using a multivariate regressional analysis (OR: 1.596, p= < 0.0001). There are a number of reasons for this inter-gender differentiation such as differences in organ blood flow and function, menstruation, and metabolism among others (Zopf *et al.*, 2008).

Indeed drug metabolism can be classified as a risk factor by itself, and although this may partly be a drug-related risk factor as particular metabolic routes are more vulnerable to ADRs than others, it is perhaps better classified as a patient-factor as the development of a reaction may reflect an individual patients metabolic genotype. The issue here is raised by the presence of multiple variants of particular metabolising enzymes within a population caused by small genetic mutations known as single nucleotide polymorphisms (SNPs). This may mean that one variant has a faster or slower metabolic rate than another, perhaps so much so that a variant may be considered a functional knock-out. Perhaps the best example of the extent of this variation is seen within the cytochrome (CYP)-450 family of enzymes involved in the metabolism of numerous drugs. One of these is CYP2D6 which is highly utilised in drug metabolism. This particular isoform has > 550 associated SNPs of which, 341 are non-coding and have no functional relevance, while 134 are non-synonymous variants with some having enhanced, and others reduced, metabolic capacities (Zanger *et al.*, 2014).

The final major predisposing factor for ADR development is patient disease state. A simple example of this is where a patient with pre-existing liver disease develops a liver-based reaction which can be pinned to the diseased liver's incompetent detoxification and removal of drug (Uetrecht, 2007). Another important example of the influence of

patient disease state is viral infection. In patients with human immunodeficiency virus (HIV), drug hypersensitivity reactions have been reported to be up to 100 times more prevalent (Coopman *et al.*, 1993). This is thought to be partly related to an already disrupted immune system due to HIV virus, glutathione depletion which is normally required for drug detoxification, and increased immune signalling due to concomitant bacterial infection which is experienced by HIV positive patients (Phillips and Mallal, 2007). This is also the case for other viruses such as the herpes virus family, which includes human herpes virus (HHV) 6 and 7, cytomegalovirus, and Epstein-Barr virus (EBV) (Thong and Tan, 2011).

1.2.4 Adverse drug reaction classification system.

Despite recent extension of the ADR classification system, reactions are most commonly categorised as type A (augmented) or type B (bizarre).

Type A (on-target) reactions are pharmacologically predictable and account for the majority of ADRs. They may relate to the desired pharmacological effect but at a site other than that at which the drug was intended for, a larger response than was anticipated, or a secondary pharmacological effect (Gomes and Demoly, 2005; Lee, 2005). These reactions often occur due to incorrect dosing but may also be susceptible to metabolism and drug interactions if the patient is undergoing multiple drug therapy.

Type B (off-target) reactions are responsible for 10-15% of ADRs with many now linked with immunological and genetic aetiologies. Although they are rarer than type A reactions, they are more likely to cause serious illness or death due to their pharmacologically unpredictable nature. Due to this unpredictability they are also called idiosyncratic reactions (Gomes and Demoly, 2005; Lee, 2005). The concern surrounding

these reactions is evident in that black box warnings or complete drug withdrawals were issued to 10% of newly approved drugs in the US between 1975 and 2000 due to idiosyncratic reactions alone (Uetrecht, 2007).

There are also four more classifications; type C, D, E, and F reactions. Type C (chronic) reactions are dose and time-related, while type D (delayed) are purely time-dependent. Type E (end of use) reactions are associated with cessation of drug administration such as withdrawal syndrome after stopping opiate use. Finally, type F (failure) relates to failed therapy which may be caused by drug-drug interactions.

More recently, an extended classification of these reactions was drafted to include additional parameters of a reaction such as timing, severity, and individual susceptibility of the patient. This extended version is known as DoTS for dose, timing, and susceptibility and has been introduced as some reactions were dose dependent but not related to the pharmacology of the drug (Aronson and Ferner, 2003).

1.3 Hypersensitivity.

1.3.1 Definition of hypersensitivity.

Drug hypersensitivity is a form of ADR and so the ADR definition of “a response to a drug that is noxious, unintended or undesired” occurring at normal doses holds true. However, in hypersensitivity (also referred to as allergy), these particular reactions have an immunological basis and so can be better defined as reactions by which there is “a state of altered reactivity in which the body reacts with an exaggerated immune response to a foreign substance”(Roujeau, 2005). These reactions are categorised as type B ADRs due to their idiosyncratic nature. Despite extensive research in this field and

significant gains in knowledge, our understanding of who will develop hypersensitivity to a particular drug is still not fully understood.

1.3.2 Hypersensitivity reaction classification.

Within hypersensitivity reactions, there is great diversity in the classes of drugs which can promote these reactions, the mechanisms by which these reactions occur, and the severity of the responses that follow. However, it was not until 1968 that these reactions were grouped and a classification system was created within the hypersensitivity reaction spectrum. Gell and Coombs categorised drug hypersensitivity reactions based on time to onset and clinical features of the reaction. The original categorisation still forms the basis for the current day classification system despite recent modifications.

Type I are also known as anaphylactic or allergic reactions, but also immediate hypersensitivity, with examples being allergic asthma and hay fever. These reactions are propagated by the interaction of an antigen with mast cell-bound IgE and subsequent degranulation. This results in an immediate reaction due to histamine release, followed by a delayed reaction caused by an array of other mediators including prostaglandins, leukotrienes, and cytokines. These reactions are also associated with increased peristalsis, thickened mucus secretions, and vasodilation. The most observed manifestation of type I reactions are uticaria and angioedema, which can occur after just a few minutes in previously sensitised individuals. Although not direct effectors, T-cells may aid antibody class-switching in these reactions through the secretion of signalling cytokines.

Type II reactions are rare, tissue-based, antigen-antibody interactions mediated by production of the neutrophil chemoattractant anaphylotoxin, polymorphonuclear

leukocyte recruitment, and autolysis of neutrophils with subsequent release of hydrolytic enzymes and tissue damage. These cytotoxic responses are most often associated with IgM or IgG antibody production. Cell damage occurs through Fc-receptor mediated activation of neutrophils, eosinophils and macrophages, or through activation of the complement cascade.

Type III reactions are similar to type II in that they are rare and mediated by antigen-antibody interactions. However, type III reactions occur in the blood and lead to the formation of antigen-antibody complexes, usually IgM, which is observed in the basement membrane of the pulmonary and glomerular systems. These complexes are not associated with anaphylotoxin release (Rajan, 2003) but do cause local inflammation and complement activation which leads to the recruitment of macrophages and neutrophils.

Type IV reactions occur in response to environmental chemicals and cosmetics, as well as drugs. Here the antigen is presented by APCs to passing T-cells which are the effector cells of these reactions. Type IV reactions are now sub classified to form type IV reactions a-d. Type IVa reactions result in macrophage activation and thus are pro-inflammatory and arise due to Th1-cell mediated IFN-γ secretion. Type IVb are associated with eosinophil inflammation in response to IL-4, IL-13, and IL-5 secretion from Th2 cells. Type IVc are cluster of differentiation (CD) 4 and CD8 T-cell mediated cytotoxicity reactions, and type IVd are associated with neutrophil induced inflammation, increased levels of granulocyte macrophage colony-stimulating factor (GM-CSF) and CXCL8, and the presence of IL-8 secreting T-cells (Adam *et al.*, 2011). However, the above summary of type IV reactions is somewhat traditional, as there are several newly identified T-helper subsets which have yet to be placed among these classifications. For instance Th17 and Th22 T-cells, which secrete IL-17 and IL-22 or IL-17 but not IL-22, respectively, have both

pro- and anti-inflammatory functions, and are thought to play important roles in skin inflammation (Cavani *et al.*, 2012). Furthermore, $CD8^+$ T-cells may also be able to secrete these inflammatory cytokines (Hijnen *et al.*, 2013). Therefore, a great deal more work is required to more accurately define antigen-responsive T-cells in hypersensitive patients to establish an improved and up to date classification system.

1.3.3 Hypersensitivity reaction time course.

Hypersensitivity reactions can be grouped according to their reaction onset, which defines the time between drug exposure and the onset of the initial symptoms of hypersensitivity. Simply, these are immediate and delayed-type reactions. In general, immediate reactions are characterised as occurring within 1 hour of drug administration, while a delayed type reaction is any reaction taking longer than 1 hour to occur (Bousquet *et al.*, 2007). The rapid response seen with immediate reactions is due to the response being mast cell-mediated. These cells are able to rapidly produce effector molecules which mediate these reactions following IgE binding. The reason behind the longer time to onset for delayed-type responses is because these are T-cell mediated. The initial interaction between drug and naïve T-cells is more protracted with the generation of a response requiring T-cell priming, activation, and proliferation (Fife *et al.*, 2009). T-cell commitment to an antigen occurs within the first hour of antigen being displayed on MHC molecules expressed on APCs, with T-cell proliferation occurring between days 1-5, and differentiation overlapping this period by taking place from day 3 onwards. The cells in these latter stages of T-cell development which are antigen-specific show enhanced cytotoxic granule protein expression and an enhanced production of RANTES, a cytokine tasked with monocyte and eosinophil attraction to the inflammatory site (Ortiz *et al.*, 1997).

1.3.4 Hypersensitivity reactions.

Whilst hypersensitivity reactions can affect a number of organs, it has been reported that 30% of all ADRs affect the skin (Naldi *et al.*, 1999). Moreover, this figure rises to 95.7% if specifically looking at cases of drug hypersensitivity (Thong *et al.*, 2003). These cutaneous reactions have a wide spectrum of severity, however less than 10% are categorised as severe and the vast majority are classified as self-limiting drug rashes. Depending on the type of reaction, these rashes may only appear after withdrawal of the causative drug. For those that occur during drug treatment, non-severe reactions such as maculopapular rashes are initially found on the patient's trunk or extremities between days 4-14 after initial drug administration, before subsequently spreading over the rest of the body (Roujeau, 2005). As severe rashes have overlapping features, the term severe cutaneous adverse reactions (SCAR) is used to refer to the entire spectrum. Within this generalisation, these rashes can be divided into four main reactions which are outlined below.

1.3.4.1 Maculopapular exanthemas and fixed drug eruptions.

Maculopapular simply refers to the generalised morphology of a rash formed of macules or papules, which are flat or raised skin lesions of up to 1 cm in diameter, respectively. This lends itself to the description of these reactions as exanthematous in direct reference to these eruptions. Further commonly used descriptions include the term morbilliform eruption, alluding to similarities to the eruptions seen with measles infection. Indeed maculopapular exanthema (MPE) may be caused by certain diseases such as lymphoma and by viruses like cytomegalovirus and HHV, however it is most often induced by drugs (Fernández *et al.*, 2009). There is still some confusion surrounding the

exact mechanism of drug-induced MPE but it may be that there are two; one being a Th2 response with subsequent eosinophil recruitment and the involvement of IL-4 and IL-5, the other a joint CD4/CD8 cytotoxic response involving various cell death pathways including FasL-Fas, perforin, and granzyme B (Pichler, 2003). Other mild and generalised reactions may not affect the entire body, but just one area. Fixed drug eruptions (FDE) are characterised by recurring skin lesions, on the same area of the skin, in response to repeated exposure to an antigenic drug. FDE are often associated with accompanying nausea, vomiting, and fever. The affected areas of skin in patients with these reactions have infiltrating lymphocytes, neutrophils, and eosinophils (Svensson *et al.*, 2001). Upon recognition of FDE, the drug is usually withdrawn and topical steroids are administered (Segal *et al.*, 2007). There are, however, a number of more defined hypersensitivity reactions, which are detailed below.

1.3.4.2 Acute generalised exanthematous pustulosis

AGEP is a rare cutaneous reaction which is estimated to affect a maximum of just 5 cases per million per year (Sidoroff *et al.*, 2001), and, with 5% mortality, is classified as more serious than MPE which is essentially non-lethal (Roujeau, 2005). This reaction is often observed less than 48 hours after initial drug administration but spontaneously resolves by around 2 weeks. Due to this rapid resolution, patients may be treated with corticosteroids but the vast majority only require supportive care (Halevy, 2009). Morphologically, AGEP defines a rash formed of numerous pustules atop an inflamed, reddened section of skin and is strongly linked to simultaneous development of fever, as are to a lesser extent symptoms such as blisters and facial oedema. In approximately a fifth of cases there is involvement of the mucus membrane, particularly that of the mouth (Halevy, 2009). A wide variety of drugs have been associated with AGEP including

many anti-infectives such as the sulphonamide containing medication co-trimoxazole, but also much more common over-the-counter drugs such as paracetamol (de Coninck *et al.*, 1996; MacDonald *et al.*, 1986). While both $CD4^+$ and $CD8^+$ T-cells are implicated in these reactions, more defining is the fact that these responsive T-cells are able to recruit and induce neutrophils through the production of IL-8. In fact, it is this large influx of neutrophils that forms the sterile pustular eruptions associated with AGEP (Schaerli *et al.*, 2004).

1.3.4.3 Drug reaction with eosinophilia and systemic symptoms.

DRESS, also referred to as drug hypersensitivity syndrome (DHS), is considered a severe cutaneous adverse reaction due to a mortality rate of 10%, double that of AGEP (Roujeau, 2005). The key distinguishing features of this reaction are, as its name implies, eosinophil recruitment and the involvement of this reaction within a number of systems other than the skin, including the liver and hematologic system (Sullivan and Shear, 2001). DRESS has a reported incidence as rare as 1 in 10,000 exposures and has a delayed onset of 2-6 weeks. Associated symptoms are fever, eosinophilia, lymphadenopathy, severe skin eruption, and the inability of drug withdrawal to reduce symptoms (Cacoub *et al.*, 2011; Walsh and Creamer, 2011). Other features may also be present such as facial oedema, involvement of the liver, kidneys, lungs, and/or heart, with liver failure being the major cause of death for DRESS patients. Both $CD4^+$ and $CD8^+$ T-cells are thought to be involved which produce IL-5 prior to eosinophil recruitment (Choquet-Kastylevsky *et al.*, 1998). Recently, most interest in DRESS surrounds the potential role of viral reactivation in the induction of this reaction. Reactivation of cytomegalovirus, EBV, and HHV6/7 has been linked with DRESS, which is thought to arise due to a hypogammaglobulinaemic environment induced by the culprit drug. For this scenario it

is the viral reactivation that is then responsible for the development of DRESS and accounts for the lengthy time to onset due to the time required for viral expansion (Aihara *et al.*, 2003; Walsh and Creamer, 2011). However, it is also possible that it is the activation of T-cells that reactivates the virus. Identifying which comes first, T-cell or viral activation, is hindered by the fact that viral deoxyribonucleic acid (DNA) and virus-specific IgE, which are standardly used to assess viral activity, are only able to be measured many weeks after onset of a reaction (Shiohara *et al.*, 2006). Oral corticosteroids and drug withdrawal are used to treat DRESS, as is the immunosuppressive agent cyclosporine. Although at least 50 drugs are known to cause DRESS including sulfamethoxazole (SMX), abacavir (ABC), and more commonly used medications such as aspirin and ibuprofen, allopurinol and carbamazepine (CBZ) are the most common inducers (Cacoub *et al.*, 2011).

1.3.4.4 Stevens-Johnson syndrome and toxic epidermal necrolysis.

The most serious cutaneous reactions, due to their high mortality rates, are Stevens Johnson syndrome (SJS) and TEN. These two reactions are in fact the same reaction but of differing severity categorised by the extent of skin detachment, with TEN being the more debilitating. These reactions are caused by a myriad of drugs such as NSAIDs, antibiotics, anticonvulsants, sulphonamides, and allopurinol. SJS/TEN has a reaction onset time of between 1-3 weeks. Both of these reactions are associated with characteristic blistered lesions that are described as painful with a burning sensation (French, 2006; Svensson *et al.*, 2001). In fact, the treatment of SJS/TEN has many similarities to that of a severe burn, and indeed the specialist care of a burns unit is often required for SJS/TEN patients. Although considered a life threatening cutaneous reaction, if the drug is withdrawn and the reaction dampened, the skin of patients with

SJS/TEN recovers far quicker than that of burns patients as the reaction is limited to the epidermis (Ghislain and Roujeau, 2002).

SJS involves a skin reaction with 10% body coverage with 1-5% mortality, while TEN classification requires 30% body coverage and is associated with 30-50% mortality (Pavlos *et al.*, 2012). The initial damage during SJS/TEN is actually keratinocyte apoptosis, with epidermal necrolysis occurring later in the reaction causing the characteristic skin detachment as the epidermis and dermis separate (French, 2006). SJS occurs more frequently than TEN with incidences of 1-2 cases and 0.4-1.2 cases per million person years respectively (Rzany *et al.*, 1996). In both SJS and TEN, lesions have been examined and were found to contain natural killer T-cells (NKT) and cytotoxic T-cells that are the effector cells for the associated keratinocyte death (Wang *et al.*, 2013). The homing receptor cutaneous leukocyte-associated antigen (CLA) appears to be particularly important in recruitment of these T-cells to the skin as patient T-cells obtained from blister fluid have four fold higher CLA expression than peripheral blood mononuclear cells (PBMCs). The T-cell response, which involves both $CD4^+$ and $CD8^+$ T-cells, leads to the secretion of IFN-γ, which through inducing secretion of TNF-α, as well as expression of HLA-DR and Fas on keratinocytes, leads to the death of epidermal cells (Leyva *et al.*, 2000). Indeed blister fluid, as well as the epidermis, has been reported to contain cytokines such as IFN-γ, TNF-α, and FasL (French, 2006). The role of FasL is of particular interest as groups have been able to show effective Fas-FasL inhibition of skin detachment using both synthetic blocking antibodies as well as natural anti-Fas antibodies found in intravenous immunoglobulins (IVIG). Initial studies have highlighted that IVIG therapy is highly effective, with reduced skin detachment in all ten patients tested (French, 2006). However, while FasL represents a possible therapeutic approach, the major cytotoxic effector during SJS-TEN is granulysin which therefore has great therapeutic and diagnostic potential. Specifically, granulysin concentrations in the blister

fluid of patients has been shown to be 2-4 fold higher than other cytolytic mediators including FasL, but also granzyme B and perforin. This high expression translates to a significant functional effect, as mice exposed to granulysin from the blister fluid of patients developed SJS-TEN-like features, without the addition of any other factors (Chung *et al.*, 2008).

1.3.4.5 Allergic contact dermatitis.

Aside from the above mentioned cutaneous reactions, most of which have been referred to in the context of drug hypersensitivity, ACD, also known as contact hypersensitivity (CHS), refers to a similar T-cell based cutaneous inflammatory reaction, but to chemicals which are applied directly to the skin. The most extensively researched of these topical allergens include hair dyes, fragrances, and nickel. Eczema, which allows easier barrier penetration due to damaged skin, is a risk factor for ACD alongside the more generalised risk factors of gender, age, ethnicity, as well as certain genetic traits (Peiser *et al.*, 2012).

As this is a T-cell mediated reaction it can be classified as delayed type hypersensitivity, however innate immune cells such as NKT-cells have been suggested to play a role (Gober *et al.*, 2007). Importantly, onset of these reactions require multiple exposures to the antigen in question, whereby the primary encounter is referred to as the sensitisation phase, and a subsequent encounter known as the elicitation phase. The initial sensitisation is asymptomatic, with the hapten being recognised and processed by skin-resident DCs, a form of APC. Up to four different DCs can be found in human skin in order to provide enough diversity for these cells to respond to the wide array of antigens encountered in this organ. These include the classical skin-resident DCs called Langerhans cells (LC), but also $CD1c^+$ DCs, $CD14^+$ DCs, and $CD141^+$ DCs. While $CD14^+$ DCs

are monocyte derived, and $CD1c^+$ DCs are derived from pre-DCs, both can be sub-classified together as $CD11b^+$. The more recently discovered $CD141^+$ DCs have an important role in antigen cross-presentation and as such are effective activators of $CD8^+$ T-cells (Haniffa *et al.*, 2012; Heath and Carbone, 2009; Shortman and Heath, 2010). However while these cells reside in the epidermis, LCs are the only DC to reside in the dermis, sending their dendrites upwards, through tight junctions toward the cornified epithelial layer where they patrol for antigens without compromising the integrity of the skins outermost layer. It is due to this close proximity with the skins surface why LCs are most associated with contact hypersensitivity as many such antigens may fail to penetrate further than the dermis (Kubo *et al.*, 2009; Romani *et al.*, 2012). This antigen processing induces the maturation of these cells and promotes their migration to the lymph nodes where they are then able to induce naïve T-cell priming (Watanabe *et al.*, 2002). The process of LC migration is aided by the release of TNF-α from exposed keratinocytes as well as IL-1β produced by the LCs themselves. The latter of these two cytokines also functions to permit LC migration out of the dermis alongside the type IV collagenase, matrix metalloproteinase-9 (Kobayashi, 1997; Watanabe *et al.*, 2002).

During the elicitation phase, T-cells are able to reach the cutaneous site of exposure through chemokine signals which allow for cell extravasation (Watanabe *et al.*, 2002). A symptomatic response subsequently ensues, which is predominantly a Th1 T-cell response with a varied time to onset of pruritic dermatitis of between 2-96 hours (Fonacier and Sher, 2014; Kimber *et al.*, 2002). Indeed, for the well-characterised contact allergen nickel, both allergic and non-allergic individuals have been found to possess nickel specific memory $CD4^+$ T-cell populations; however, only those who are allergic were found to have nickel-specific $CD8^+$ T-cells (Cavani *et al.*, 1998). Despite the apparent dominance of Th1-secreting T-cells, effector responses involve both the production CHS-inducing IFN-γ secreting $CD8^+$ T-cells, but also reaction regulating IL-10-secreting $CD4^+$ T-

cells. Although cutaneous damage has already occurred, this ultimately means that CHS is a self-limiting response as it induces the expansion of an immune-inhibitory, regulatory T-cell population to dampen immune activation after the initial insult by the Th1 population (Xu *et al.*, 1996). It would therefore be logical to assume that without these regulatory $CD4^+$ T-cells, these reactions would develop further and be more severe. However, this seems to be dependent on the nature of the sensitising compound as it has been reported within an IL-4 knockout mouse model that oxazolone-induced ACD occurs as per normal, but development of 2,4-dinitrochlorobenzene (DNCB)-induced ACD is impaired (Traidl *et al.*, 1999). It is also worth noting that while a Th1 delayed type response is seen for a large number of these reactions, immediate reactions can also be detected for ACD-associated antigens. For instance, a type 2 specific response was detected in a study using fluorescein isothiocyanate, allergy to which comes in two waves, immediate and delayed. While the immediate response was confirmed as Ig-E-mediated, the delayed type response involved Th2 $CD4^+$ T-cells (Dearman and Kimber, 2000). The true balance of cells implicated in these reactions therefore remains to be completely defined. Furthermore, most mechanistic studies have been conducted in experimental animals. As such, it is largely unknown how these findings translate into reactions in human patients.

1.3.4.6 Reactions targeting organs other than skin.

Although the skin is commonly affected by drug hypersensitivity reactions, a range of other organs can also be targeted, with roughly one fifth of patients with severe MPE experiencing concurrent liver damage. These reactions that target both the skin and at least one other organ are characterised as drug-induced systemic hypersensitivity (DIHS) reactions. This far-reaching response is thought to arise from the presence of activated

immune cells present in the circulation of these patients (Hari *et al.*, 2001; Pichler, 2003). Indeed for reactions such as DRESS, multiple organs are affected simultaneously including the skin, lungs, heart, liver, and kidneys. Much rarer but nonetheless reported side effects include respiratory distress syndrome due to reduced pulmonary capacity, neurological cases of meningitis and encephalitis which lead to comas and seizures, gastrointestinal problems associated with pancreatitis and colitis, and musculoskeletal effects such as arthritis (Klimas *et al.*, 2015). Despite the wide range of adverse effects, the liver represents the most frequently targeted internal organ due to its primary role in the metabolism of xenobiotics such as drugs, and its subsequent exposure to a range of allergic moieties. This hepatic damage is most commonly referred to as drug-induced liver injury (DILI) and accounts for > 9% of ADRs (Lazarou *et al.*, 1998). DILI may be cholestatic or hepatocellular in nature, and diagnosis is most often determined by significant elevations in levels of the liver enzymes serum alkaline phosphatase (ALP) and serum alanine aminotransferase (ALT) (Farmer and Brind, 2011). These patients may experience jaundice and in life-threatening instances, a liver transplant has to be performed. In comparison, renal effects are usually mild, and are clinically diagnosed by elevated levels of blood urea nitrogen and serum creatinine (Klimas *et al.*, 2015).

DIHS is most commonly induced by drugs such as phenytoin, allopurinol, dapsone, and CBZ, among others (Shiohara *et al.*, 2006). The effects on internal organs such as the liver are often delayed in comparison to the associated skin reactions, taking between 2-6 weeks to develop (Yaylacı *et al.*, 2012). Diagnosis of internal organ damage is difficult as the damage is often outwardly asymptomatic. Even upon identification of damage, there is no direct test to relate this to drug exposure, and therefore clinicians are limited in their diagnosis, and must first exclude all other options. However, as our knowledge of these reactions improves, diagnostic scoring systems such as the Roussel-Uclaf causality

assessment method (RUCAM) to diagnose DILI are being introduced which work to diagnose the cause of damage by combining a number of well-defined criteria.

1.4 Diagnosis of Hypersensitivity.

1.4.1 Assays to diagnose drug hypersensitivity reactions and assess the phenotype of antigen-specific immune cells.

There are various methods by which it is possible to diagnose whether a person is hypersensitive to a particular substance. Patch testing is one of these, and requires an incubation period of 24-48 hours of the allergen in question on a small area of the skin of an individual, using a non-toxic concentration determined by prior non-allergic volunteer testing. A positive reaction is one in which the exposed skin develops a reaction which presents as erythema. This test does however depend on quite a few factors such as T-cell presence in the skin, ability of the drug to penetrate the skin, and the ability of T-cells to present the drug, among others. Although a negative patch test does not rule out hypersensitivity, a positive patch test is seen as a very reliable indication that an individual is hypersensitivity to a particular substance (Pichler and Tilch, 2004).

As well as the patch test, there are two other skin-based tests; prick and intradermal. The prick test is carried out by placing a needle through a solution containing the allergen before pricking the skin. This test is commonly used as it is the safest of the skin tests, but it is associated with low sensitivity. Usually an intradermal test is a result of a failure to determine hypersensitivity following a prick test. The intradermal test has increased sensitivity but is deemed less safe with the potential to induce a false positive reaction (Brockow *et al.*, 2002). If the administered concentration is low or the allergen is unable

to efficiently enter the skin, these skin tests can be associated with false-negative results leaving a potentially hypersensitive patient at risk of being exposed to a compound to which they are allergic.

In the event of unsuccessful skin testing, drug provocation tests (DPT) may be performed where an individual is simply given the drug. These tests are always carried out under medical care due to the implications of a reaction being positive. This occurs whether the test is done with the culprit drug or a structurally related moiety. There are obvious dangers and ethical implications for use of the DPT, which may require hospitalisation of an individual for the duration of the test. However, due to its ability not only to produce allergic symptoms but also to produce other manifestations associated with a reaction, the DPT is known as a gold standard test to diagnose hypersensitivity (Aberer *et al.*, 2003).

Re-administration of a drug to a hypersensitive patient is not always harmful and can be used to treat the patient through a process known as desensitisation. Drug desensitisation allows for the use of a compound associated with hypersensitivity by inducing temporary tolerance, which can be particularly beneficial when a drug is essential for the successful treatment of a patient. In practice this requires that the drug is given to a patient over a brief time-frame in small, but increasing doses, until the patient has been exposed to, and tolerated, a therapeutic dose. For drugs which are associated with the onset of hypersensitivity within 24 hours, a rapid dosing regimen to achieve optimal dose within 4-12 hours is often used. It is this continuous exposure of the individual to the drug that maintains the state of tolerance (Cernadas *et al.*, 2010). While the mechanisms of desensitisation are not well defined, protocols for this procedure are well characterised for the use of drugs associated with immediate reactions (Castells *et al.*, 2008). In contrast, the role of desensitisation to drugs

associated with delayed-type reactions is less established, but what data has been published suggests that this method is both safe and effective for these drugs too (Whitaker *et al.*, 2011b).

All of the above tests including skin tests and drug desensitisation have one shared major disadvantage, the potential to induce a harmful reaction in humans. To avoid this, *in vitro* tests are currently being developed and/or optimised which may allow us to better understand the phenotype and function of the immune cells implicated in hypersensitivity reactions. For patients who suffer from type IV T-cell mediated reactions it is possible to isolate T-cells from a peripheral blood sample, or to collect T-cells from the blister fluid or skin samples. This allows a direct T-cell phenotype to be established *ex vivo* utilising cells obtained from the site of reaction (Brockow *et al.*, 2002; Yawalkar *et al.*, 2000).

1.4.2 *In vitro* diagnosis of hypersensitivity.

1.4.2.1 Lymphocyte transformation test.

A commonly used *in vitro* T-cell test is the lymphocyte transformation test (LTT). It has a reported specificity of 85% and a sensitivity of 74%, a 12% increase in comparison to skin tests (Nyfeler and Pichler, 1997; Romano *et al.*, 2004). It involves a blood sample from a patient from which PBMCs are cultured with the culprit drug for five days before measurement of cell proliferation. This test relies on the presence of sensitised memory T-cells within the PBMC population to respond to the drug (Luque *et al.*, 2001). The disadvantages of the LTT include the requirement for radioactive isotopes for the measurement of proliferation, relatively lengthy test duration, and that it only provides a positive or negative reaction result without mechanistic insight into the aetiology of a

reaction. However, as this assay is based on the the presence and proliferation of T-cells and PBMCs, the LTT can be used to detect both immediate and delayed-type reactions. In delayed-type reactions the LTT is much more sensitive than skin tests, however the sensitivity for immediate reactions is just 64.5% compared to 77.4% for skin tests (Luque *et al.*, 2001).

1.4.2.2 Enzyme-linked immunosorbent assay.

An alternative *in vitro* diagnostic test is the enzyme linked immunosorbent assay (ELISPOT). As with the LTT, this can be performed using PBMCs from patient blood, but can also be integrated into assays looking at the activation of specific T-cell populations. The basis for this assay is the culture of PBMCs or T-cells with antigen on a membrane coated with a capture antibody for a particular cytokine of interest. Activated cells may release this cytokine which subsequently binds to the pre-coated antibodies. The removal of cells and addition of a detection antibody conjugated to an enzyme means that on the final step, addition of an enzyme-activated colour substrate, coloured spots appear in each well as a measure of cytokine release. By utilising antibodies with different binding-specificities the assay can be utilised to measure the release of an array of cytotoxic mediators such as FasL, Granzyme B, and perforin, as well as pro-inflammatory Th1 (IFN-γ, TNFα, IL-12) and anti-inflammatory Th2 (IL-4, IL-5, IL-13) cytokines (Bowen *et al.*, 2008; Olleros *et al.*, 2008). Thus a major advantage of this assay is that it does provide some mechanistic insight to the reaction at hand.

1.4.2.3 Flow cytometry.

Flow cytometry allows the detection of specific cell surface markers by staining the cells with antibody-conjugated fluorescent, each specific for a particular cell surface marker. This can be used to correctly ascertain cell phenotype, such as CD45Ra and CD45Ro for naïve and memory T-cells, respectively, or to look at T-cell activation, which is associated with increased expression of markers such as HLA-DR, CD40L, CD25, CD69, and CD71 (Beeler and Pichler, 2006). It has been shown that resting T-cells as well as those cells exposed to drug from non-sensitised individuals do not up regulate these activation markers and so flow cytometry can be a useful tool to look at the exact phenotype of drug-specific T-cells. Previous studies have also utilised flow cytometry to assess the ability of a compound to activate professional APCs, with both CD40 and MHC class II molecule expression found to be increased subsequent to exposure to a well-known sensitising agent (Coulter *et al.*, 2007a).

5, 6-Carboxyfluorescein diacetate succinimidyl ester (CFSE) staining can be a useful accompaniment to flow cytometry. CFSE is able to measure cellular proliferation, and can directly measure the number of rounds of cell division a cell has undergone as the cell CFSE content halves during each division (Beeler and Pichler, 2006). CFSE fluorescence has minimal variability within a stained population making it particularly easy to gate for proliferating cell populations (Quah and Parish, 2010). This can be particularly useful in combination with antibodies for other specific markers as the user can identify differences in marker expression between dividing cell populations. While the depletion of CFSE-intensity with each cell division forms the basis of this assay, this represents a major limitation as this leaves a low precursor frequency and thus limits detection to only the strongest responding cells (Lerch *et al.*, 2007).

1.4.2.4 T-cell cloning.

For defining the characteristics of a specific response it is useful to look at the single cell level. T-cell cloning requires a two week incubation with drug before the placement of a single cell per well in approximately twenty 96-well plates. Expansion of these T-cell clones allows the phenotype and function of a single cell that is responsive to a specific antigen to be analysed. The presence of a single TCR is useful for determining the cross reactivity of related antigens and therefore helps to define the important characteristics of the antigen (Beeler and Pichler, 2006). Clones are able to be restimulated every two weeks and can be kept alive for long periods of time but they are, however, susceptible to infection and exhaustion. Proliferation, cytokine release, and cell phenotype data can then be compiled for each clone using the assays listed previously. Indeed, it was data from clones deriving from patients with different forms of cutaneous hypersensitivity that helped to establish the expanded Gell and Coombs classification.

The successful implementation of any of these *in vitro* assays requires the identification of the susceptibility factors that make certain individuals develop hypersensitivity reactions while others, often the vast majority, remain tolerant. Two such factors which have been described in detail in relation to these reactions in recent years are the role of the HLA complex and drug metabolism.

1.5 Human Leukocyte Antigen Complex.

1.5.1 Introduction to HLA – location and structure.

The proteins encoded by HLA alleles play a major role in presenting antigenic peptides on the surface of APCs to passing T-cells. Such presentation allows for the recognition of

an antigen by a corresponding TCR and is a requirement for the formation of a stable immunological synapse and T-cell activation. Human chromosome 6 houses the HLA gene complex. The human genomic region associated with HLA genes comprises approximately $3x10^6$ base pairs (Bodmer, 1987) and consists of three separate gene classes; HLA classes I, II, and III.

The subdivision into classes I and II, although actually defined by structural similarities, can be distinguished between by the type of antigen that each presents; MHC class I is expressed on all nucleated cells and presents intracellular antigenic peptides to $CD8^+$ T-cells, MHC class II expression is mainly restricted to APCs and presents peptides derived from extracellular proteins to $CD4^+$ T-cells. These classes are further subdivided with HLA class I consisting of HLA-A, HLA-B, and HLA-C genes as well as the much lesser expressed non-classical HLA-E, HLA-F, and HLA-G, whilst HLA class II is subdivided into HLA-DR, HLA-DP, and HLA-DQ genes (Williams, 2001). The HLA locus is the most polymorphic in the human body leading to a broad range of genetic diversity.

The structure of HLA class I and II proteins are different, although both are heterodimeric (Figure 1.1). HLA class I consists of an alpha chain encoded by the HLA class I gene which makes up the peptide binding groove which interacts with β-2 microglobulin. In contrast, class II proteins are composed of an alpha and a beta chain which are non-covalently linked and encoded by individual HLA class II A and B gene loci, respectively. The peptide binding cleft is formed from the top of both chains (Williams, 2001). There are also non-classical HLA class III genes but these do not play a direct role in antigen presentation. Of the 57-60 HLA class III structural genes in humans, some encode proteins for complement such as sections of the C3 and C5 convertases, as well as TNF and heat shock proteins (Yung Yu *et al.*, 2000).

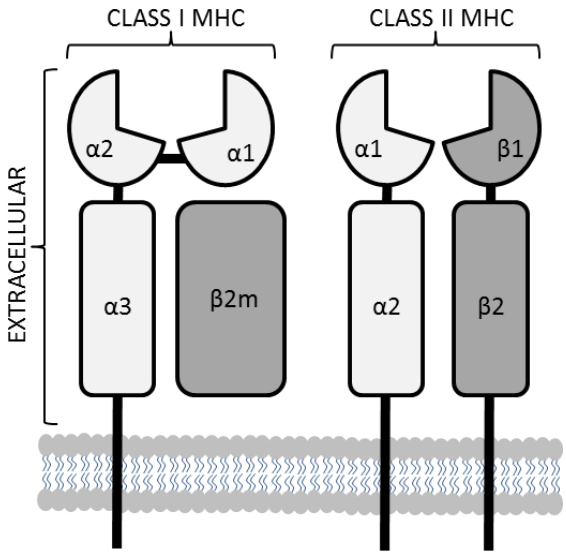


Figure 1.1. The structure of MHC class I and II molecules.

1.5.2 Intracellular routes for antigenic loading onto HLA molecules.

As HLA class I and II proteins present intracellular and extracellular antigens, respectively, their mechanisms for antigenic loading into the MHC peptide grooves differ. Endogenous antigens, mostly peptide remnants formed after proteasome-mediated ubiquitinated protein degradation, are processed to be displayed on HLA class I molecules. The peptide is first transported into the endoplasmic reticulum (ER) by protein transporters such as transport associated protein (TAP), before being loaded onto HLA class I by the chaperones calnexin, calreticulin, and tapasin. These chaperones act to fold the HLA structure to increase stability during peptide loading and as such, the combination of TAP and the chaperone proteins is called the peptide-loading complex (PLC). The HLA-peptide complex is then transported through the Golgi apparatus to the plasma membrane where between 10,000-50,000 HLA class I molecules may be presented on the surface of each cell (Neefjes *et al.*, 2011).

The mechanism is somewhat different for peptide loading onto MHC class II. Class II dimers are generated in the ER, where three of these dimers join to three invariant (Ii) chains. This complex leaves the ER, travels through the Golgi apparatus, and is internalised by lysosomes or endosomes which are protease rich and acidic. This environment degrades the Ii chain enabling the dimers to become fully functional HLA dimers that can bind to antigens. The loading of antigens onto MHC class II is aided by the presence of other class II molecules, HLA-DM and HLA-DO, after which the MHC class II-peptide complex is translocated to the cell surface. The loading of antigens into the MHC class II pathway occurs within the endocytic pathway where antigens are derived from internalised proteins, thus, the MHC class II molecules mainly present peptides derived from exogenous antigens (Guermonprez *et al.*, 2002). Both MHC class I and II pathways are depicted in figure 1.2.

These seemingly separate pathways do however show some overlap which is termed cross-presentation. In this process, HLA class I may present exogenous antigens. Cross presentation is a process that allows a cytotoxic T-cell response to be mounted against tumours and viruses, and may occur via two pathways. This process relies on the initial phagocytosis of antigen before the antigen enters the proteasome and thus the classical MHC I pathway through the ER. Alternatively, the antigen may join the pathway at a later stage by phagosomal degradation, and enter the cytoplasmic environment, before direct loading onto MHC class I molecules. Similarly, endogenous antigens may be presented on HLA class II when they are produced under specific circumstances such as autophagy (Joffre *et al.*, 2012; Nierkens *et al.*, 2013).

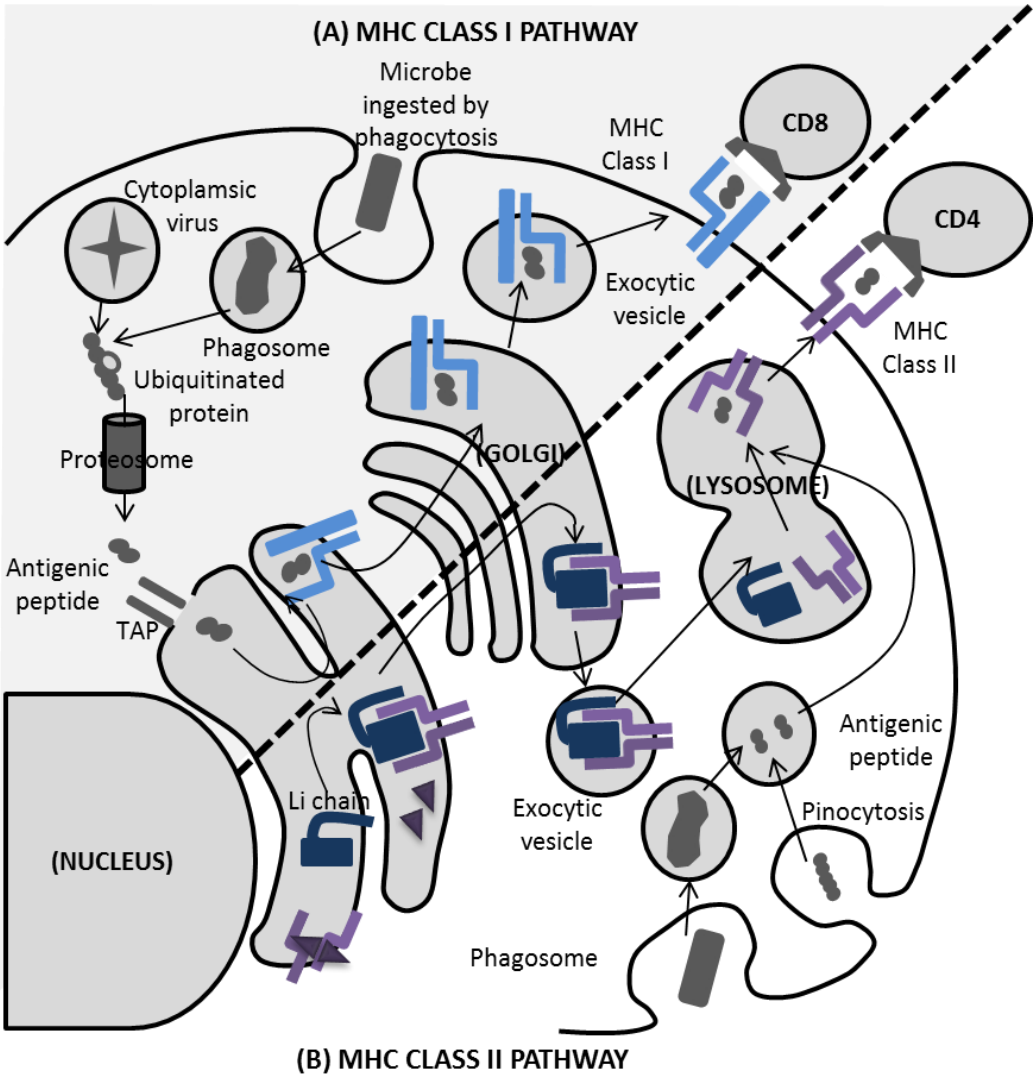


Figure 1.2 MHC class I and MHC class II antigen-processing pathways. (A) Endogenous proteins (including ingested microbes, or cytoplasmic viruses) may be ubiquitinated and processed into peptide fragments by the proteasome. Peptides are transported into the ER by TAP and loaded onto MHC class I molecules. The MHC class I-peptide complex is transported through the Golgi, and into an exocytic vesicle for expression on the cell surface. **(B)** Exogenous antigens are phagocytosed/pinocytosed and processed into peptides within phagosomes or after the formation of lysosomes which contain MHC class II molecules. MHC class II produced in the ER bind to stabilising invariant Li chains allowing transport to the Golgi apparatus. Here, the MHC class II-invariant chain complex is internalised by protease-rich lysosomes which degrade the Li chain. Upon loading of peptide fragments onto MHC class II, the complex is transported to the cell surface. Figure adapted from (Brigl and Brenner, 2004).

1.5.3 CD1.

DCs also contain another HLA molecule capable of antigen presentation called CD1 (Guermonprez *et al.*, 2002). Humans express five different CD1 genes named a-e, which

have varied antigen binding capacities in terms of antigenic structure (Brigl and Brenner, 2004). Unlike HLA class I and II which bind to protein antigens, CD1 binds to glycolipid antigens mainly present in endosomal compartments, but can also to a lesser extent bind to those at the DC surface (Guermonprez *et al.*, 2002). Another distinction from the classical MHC is that CD1 genes have an extremely limited allelic diversity, and although a few polymorphisms in CD1 genes have been identified, a number of these have been found to be silent polymorphisms or fall outside of the antigen binding cleft (Oteo *et al.*, 2001; Oteo *et al.*, 1999). Similar to HLA class I, CD1 forms a dimer with β-2 microglobulin. After formation of this heterodimer in the ER and binding to self-lipids, CD1 proteins are transported to the cell surface in a similar manner to MHC class I proteins. However, upon interaction at the cell surface with AP2, an adaptor protein, CD1 is internalised into sorting endosomes, at which point the different CD1 variants are trafficked back to the cell surface via different pathways (Brigl and Brenner, 2004).

1.5.4 HLA type and hypersensitivity.

The HLA locus is the most polymorphic in the human body leading to a broad range of genetic diversity. Due to the idiosyncratic nature of many hypersensitivity reactions it was conceivable that these reactions may be at least in part regulated by a particular genetic component. However, there was no strong basis for this theory until a pair of monozygotic twins developed CBZ-induced hypersensitivity which postulated some familial genetic predisposition (Edwards *et al.*, 1999). Multiple HLA allele associations with drug-induced hypersensitivities of both the skin and liver have now been discovered, with the most well documented being that of the reverse transcriptase inhibitor ABC and HLA-B*57:01 in 2002 (Mallal *et al.*, 2002). This association has a positive predictive value of approximately 50% but, more importantly, a negative

predictive value of > 99%. This has led to the introduction of patient HLA genotyping before administration of ABC. Only if a patient is HLA-B*57:01 negative will they be given ABC therapy (Martin *et al.*, 2012).

The only other drug for which administration requires the patient to be HLA-typed is CBZ, a commonly prescribed antiepileptic medication. CBZ is known to cause SJS/TEN in certain individuals and was first associated the HLA-B*15:02 allele in 2004 (Chung *et al.*, 2004). Both the HLA-B*15:02 allele and CBZ-induced hypersensitivity are more prevalent within the Han Chinese population than in Caucasians. Whilst no metabolic association was detected, HLA-B*15:02, although present in 3% of tolerant individuals, was found in all of the hypersensitive patients. HLA associations are often described in terms of sensitivity, which identifies the proportion of individuals who are positive for a particular risk allele who go on to develop hypersensitivity (a higher percentage indicates that HLA risk allele-positive individuals were more likely to develop a reaction), and specificity, which refers to the proportion of individuals without the risk allele who developed hypersensitivity (a higher percentage indicates that HLA risk allele-negative individuals were less likely to develop a reaction). Subsequently, a HLA-B*15:02 genetic test for potential hypersensitivity to CBZ was found to have a 100% sensitivity, and a specificity of 97% (Chung *et al.*, 2004).

Apart from ABC and CBZ-induced reactions, most HLA associations with drug hypersensitivities have much lower prognostic values meaning that patient genotyping for HLA type is not reason enough to withhold a potentially lifesaving drug. For instance, the same allele as for ABC-hypersensitivity, HLA-B*57:01, is associated with flucloxacillin-induced DILI; however just 1 in 500-1000 HLA-B*57:01 positive individuals who receive flucloxacillin will develop a hypersensitivity reaction (Andrews *et al.*, 2010). Moreover, further studies investigating flucloxacillin have identified a less stringent association of

hypersensitivity with this particular allele. Specifically, this refers to the differential binding capabilities of flucloxacillin to closely related alleles of the B17 serological family, of which HLA-B*57:01 is a member. Some of these alleles, including HLA-B*58:01, HLA-B*58:02, HLA-B*57:02, and HLA-B*57:03, share > 90% sequence homology to HLA-B*57:01 and as such have peptide-binding repertoires which overlap. Using $CD8^+$ T-cell clones from patients with flucloxacillin-induced liver injury, it has been shown that T-cell responses to flucloxacillin are mainly restricted by HLA-B*57:01, but also HLA-B*58:01 (Monshi *et al.*, 2013). In comparison, ABC-specific T-cell responses are stringently HLA-B*57:01 restricted, with no restriction by HLA-B*58:01, which differs in sequence by just one amino acid (aa) residue (Chessman *et al.*, 2008). Other HLA associations with DILI include lumiracoxib and HLA-DRB1*15:01, ximelagatran and HLA-DRB1*07:01 (Monshi *et al.*, 2013), nevirapine and efavirenz with HLA-DRB1*01, and lapatanib and HLA-DQA1*02:01 among others (Chen *et al.*, 2011; Locharernkul *et al.*, 2008; Spraggs *et al.*, 2011; Tassaneeyakul *et al.*, 2009; Vitezica *et al.*, 2008). It is important to bear in mind that linkage disequilibrium exists between genes in these loci and so an apparent association may be in truth due to another allele or haplotype such as the common 57.1 haplotype which contains HLA-B*57:01 alongside C4A6, HLA-DRB1*07:01, and HLA-DQB1*03:03 (Bharadwaj *et al.*, 2012; Mallal *et al.*, 2002; Pavlos *et al.*, 2012).

1.6 Drug metabolism.

1.6.1 Introduction to metabolism.

Metabolism is a natural process that occurs to ensure the efficient excretion of compounds from the body. Drugs, which are often lipophilic in order to cross the cell plasma membrane, are metabolised to prevent accumulation in the body and potential

toxicity. This complex process can be simply defined as making a lipophilic drug more water soluble for urinary excretion. Metabolism is understandably a major area for consideration in understanding the aetiology of drug hypersensitivity; a wide range of potentially toxic or reactive metabolites can be formed from a multitude of drugs, perhaps in varying quantities from one person to the next, depending on the degree of enzyme expression, or the presence of enzyme variants. Indeed, those drugs that are linked with the highest prevalence's of hypersensitivity commonly form downstream chemically reactive metabolites (CRMs). It is therefore key to assess the metabolic potential of a drug when considering the mechanism of associated hypersensitivity. Metabolism is a process divided into two key parts: phase I and phase II.

1.6.2 Phase I reactions.

Phase I reactions involve the addition of a functional group to the drug with the most common being carboxyl (-COOH), hydroxyl (-OH), amino (-NH2) or sulfhydryl (-SH) groups, a process which is often termed functionalization as it is these additional groups which permit latter metabolic pathways to commence.

Most often, phase I involves oxidation by a common enzyme family, cytochrome (CYP) 450. CYP450 is a superfamily of enzymes that are not only present in humans, but also other animals, plants, and bacteria. In total, up to 80% of clinically used drugs are metabolised by CYP450 enzymes (Ingelman-Sundberg, 2004). Many of the enzymes in this family have polymorphic variants, with CYP2C9, CYP2C19, and CYP2D6 being classed as highly polymorphic (Johansson and Ingelman-Sundberg, 2010). CYP enzymes within the superfamily are divided into clusters known as families and subfamilies based on sequence homology with limits of 40% and 55% similarity, respectively (Anzenbacher

and Anzenbacherová, 2001). The oxidising function of these enzymes is due to the presence of a heme group iron which is able to bind to dioxygen. CYPs are largely found in the liver, but can also be found throughout the body as exemplified by CYP3A4 which is broadly distributed with expression in the brain, liver, kidney, placenta, lymphocytes, endothelium, and gastrointestinal tract (Anzenbacher and Anzenbacherová, 2001).

Other phase I enzymes are also present in the skin such as Flavin monoxygenases (FMOs) and peroxidases. Like CYP450 enzymes, peroxidases such as myeloperoxidase (MPO) also contain an oxidising heme group. Peroxidases have been implicated as the cause of free radical formation from both phenytoin and CBZ intermediates and have thus been linked to the development of hypersensitivity (Lu and Uetrecht, 2008). FMO 1,3,4, and 5 are all known to be expressed in the skin, with FMO3 being responsible for the metabolism of dapsone and SMX to aryl hydroxylamines. These metabolites may then produce free radicals and undergo protein haptenation (Sharma and Uetrecht, 2014). FMOs function due to the presence of a stable hydroperoxyflavin, which oxidises soft nucleophiles in the presence of oxygen with the aid of NADPH as an energy donor. Other skin-based phase I metabolising enzymes include cyclooxygenases, alcohol dehydrogenase, and epoxide hydrolase.

1.6.3 Phase II reactions.

Phase II reactions occur subsequent to phase I metabolism, and generally produce more polar species. This process, often referred to as detoxification, forms a product that is easier to excrete. However, it is also possible that the products of these reactions may be able to induce toxicity through becoming protein reactive, such as the acyl glucuronides produced by non-steroidal anti-inflammatory drug (NSAID) metabolism

(Pumford *et al.*, 1993). If metabolic products are unstable they are termed CRMs. There are a number of phase II reactions; methylation, sulphation, acetylation, glucuronidation, and conjugation of either glutathione or glycine.

Many of these reactions involve the presence of an energy-rich donor to facilitate the high energy dependency of conjugation reactions as well as enzymes to promote the reaction. For instance, methylation involves methyltransferase and the energy donor S-adenosyl-L-methionine, acetylation requires N-acetyltransferases alongside the donor acetyl coenzyme A, and glucuronidation uses uridine diphosphate (UDP) glucuronyl transferase with the energy donor UDP-glucuronic acid.

One of the most commonly utilised phase II pathways, glutathione conjugation, is a component of the detoxification pathway found in all mammalian tissues, especially the liver. Glutathione is a nucleophilic tripeptide. One of the peptides within glutathione, a cysteine, is able to donate an electron with the aid of glutathione-S-transferase and bind a nucleophilic entity, which protects against cell damage from hydroperoxides produced during normal metabolism (Perricone *et al.*, 2009). Although glutathione conjugation does not produce reactive metabolites, in some cases such as for paracetamol, glutathione stores can become depleted. This reduced ability to detoxify drugs subsequently promotes the formation of damaging CRMs.

Another major phase II pathway of particular interest for hypersensitivity-inducing chemicals and drugs, such as *p*-Phenylenediamine (PPD) and SMX, is *N*-acetylation. This pathway is also involved in the metabolism of numerous other drugs such as isoniazid, and involves the conjugation of an acetyl group, taken from acetyl coenzyme A, to the substrate. This reaction is catalysed by a catalytic tripeptide within *N*-acetyl transferase (NAT) formed of histidine, aspartate, and cysteine. There are two isoforms, NAT1 and NAT2, with only NAT1 expression seen in the skin. This enzyme is highly polymorphic

with different variants often having different rates of metabolism (Blömeke *et al.*, 2009; Sim *et al.*, 2008).

1.6.4 Sites of metabolism.

One of the primary functions of the liver is to metabolise compounds in the body, and major drugs associated with hypersensitivity reactions such as ABC, SMX, and CBZ are no exception and all undergo extensive hepatic metabolism. The instability of many drug metabolites leaves the liver in direct contact with potentially damaging CRMs. However, the liver has high levels of glutathione, and can activate protective transcription pathways including NF-κβ, to minimise the risk. Additionally, T-cell activation in the liver leads to antigenic tolerance and not a damaging immune response due to enhanced FasL expression which prevents T-cell activation by inducing apoptosis. As such the liver, along with sites such as the eye and placenta, is considered to be an immunologically privileged organ (Sanderson *et al.*, 2006).

Despite the livers interaction with a substantial concentration of metabolites, the major organ affected by hypersensitivity reactions is the skin. Keratinocytes and fibroblasts, among other skin cells, express CYP450 enzyme mRNA and therefore have direct metabolic capabilities (Sanderson *et al.*, 2006). In fact, it has been shown in both neonatal and adult cells that SMX can be metabolised to the hydroxylamine by keratinocytes (Reilly *et al.*, 2000), and these cells are able to present MHC class I bound SMX-protein adducts (Roychowdhury *et al.*, 2005). While skin cells may be a more obvious place to look for metabolic competence during a cutaneous reaction, immune cells which are the effector cells of the reaction but also present in the skin, are also metabolically active. Indeed CYP1B1 is highly expressed in immune cells compared to

other cell types and has been shown to have xenobiotic metabolic potential (Shimada *et al.*, 1997). They also contain other highly expressed enzymes that can metabolise xenobiotics, such as MPO which is present in both monocytes and neutrophils (Sanderson *et al.*, 2006). It is therefore important when using *in vitro* cell systems to bear in mind the extent of metabolic similarity between cells *in vitro* and those *in vivo*.

1.6.5 The role of metabolic polymorphisms in hypersensitivity.

In most cases (with the exception of ABC and CBZ) individuals who express HLA risk alleles for the majority of drugs rarely develop a reaction to the culprit drug. This implicates the presence of other major risk factors. As drug metabolites are implicated in a vast number of drug-induced hypersensitivity reactions, it is arguable that the expression of polymorphic metabolising enzymes may expose individuals within a population to varied quantities of antigenic moieties. Indeed, this may affect both phase I and II metabolism pathways, whereby an individual may be more susceptible to the formation of active products, but also be less susceptible to their subsequent detoxification. Despite this, metabolism can rarely be classified as on or off, but instead metabolic rate may be on a sliding scale of fast to slow. Therefore, individuals are still exposed to potentially immunogenic metabolites independent of metabolic rate and thus it is unclear how metabolic variation translates to a predisposition to hypersensitivity. Another factor to consider is 'danger signalling' to the immune system. Certain individuals may be exposed to higher and thus more toxic concentrations of certain compounds, and would therefore be subject to enhanced danger signalling and thus an enhanced likelihood of T-cell activation. One of the most extensively studied drugs with regards to metabolic-influence on hypersensitivity is the sulfonamide antibiotic SMX, associated with idiosyncratic cutaneous reactions. SMX is subject to

direct detoxification by *N*-acetylation by both NAT1 and NAT2 enzymes. This process reduces the *in vivo* availability of SMX, and the downstream metabolite sulfamethoxazole-nitroso (SMX-NO), both of which can stimulate T-cells from allergic patients. Thus, the exposure to SMX-derived immunogenic antigens in a given individual is determined by the respective rates of function between detoxification and metabolic pathways. As exposure of keratinocytes to SMX is associated with cell death and the release of danger signals (Reilly *et al.*, 2000; Shi *et al.*, 2000), a higher SMX concentration due to slower detoxification has the propensity to increase the likelihood of T-cell activation. Indeed, it has been found that patients with hypersensitivity more frequently express the NAT2 slow acetylator phenotype than tolerant individuals. However, when Pirmohamed and colleagues addressed the role of acetylator phenotype on predisposition to SMX-hypersensitivity by investigating enzyme polymorphisms in HIV-positive patients with and without hypersensitivity, no significant associations were found with NAT or indeed with other enzymes associated with SMX metabolism such as CYP2C9 (Pirmohamed *et al.*, 2000; Rieder *et al.*, 1991). Therefore metabolism is not a major susceptibility factor for SMX-hypersensitivity. Extensive research has also been performed to investigate whether slow or fast acetylator status affects a person's susceptibility to PPD-induced contact dermatitis. As acetylation in this case is a detoxification pathway, it was suspected that slow acetylator phenotypes may not detoxify harmful compounds rapidly enough allowing for a build-up of toxic metabolites and the induction of hypersensitivity. Indeed, a potential inverse association with the rapid acetylator NAT1*10 with PPD-allergy has been reported (Blömeke *et al.*, 2009; Sim *et al.*, 2008).

There are other cases where metabolism is associated with the development of hypersensitivity. For instance, a deficiency in CYP2D6 has been shown to account for the hepatotoxicity associated with the anti-anginal medication perhexiline (Morgan *et al.*,

1984). Furthermore, P450 enzymes such as CYP2C9 have been reported to have allelic variants that predispose individuals to DILI, however other groups have been unable to replicate these findings (Aithal *et al.*, 2000; Morin *et al.*, 2001). In fact overall, although there are some polymorphic phase I metabolising enzymes associated with DILI, they do not seem to have a major role in inter-individual susceptibility to idiosyncratic reactions (Chalasani and Björnsson, 2010). Phase II metabolising enzymes are also associated with certain idiosyncratic drug reactions, including glutathione-S-transferase with multiple drugs (Lucena *et al.*, 2008), and diclofenac hypersensitivity with UGT-mediated glucuronidation (Daly *et al.*, 2007).

As metabolism seldom represents a one-step process, it is important to consider the implications of metabolism and detoxification pathways downstream of the parent compound, particularly in cases where metabolites are capable of activating T-cells. Indeed, the interplay of complex metabolism pathways is thought to account for the lack of association between isoniazid hepatotoxicity and acetylator phenotype (Weber *et al.*, 1983).

1.7 Antigenic stimulation of the immune system.

1.7.1 Signal 1: T-cell receptor stimulation by drugs.

T-cell activation consists of three signals. For T-cell stimulation to occur, an immunological synapse must form. This describes the stable binding of an MHC to a relevant TCR after binding of an immunogenic peptide. The MHC-TCR interaction is referred to as signal 1. Signal 2 refers to an array of co-signalling pathways which manipulate the T-cell activation threshold. For T-cell activation to occur, costimulatory pathway activation must outweigh the influence of co-inhibitory signals. Signal 3 relates

to the selective release of cytokines from APCs which drives the specific differentiation of T-cells. The process through which an antigen can induce signal 1 is complex and can occur via multiple routes depending on the specific characteristics of the antigen in question (summarised in Figure 1.3).

1.7.1.1 Hapten and pro-hapten hypotheses.

For the immune system to be responsible for hypersensitivity reactions, cells of the immune system must be able to recognise and respond to both drug and chemical antigens. Historically however, this was a confusing concept as molecules of less than 1000 Da, which most drugs are, are too small to be deemed immunogenic (Adam *et al.*, 2011). The hapten hypothesis provided an explanation for how such antigens overcame this apparent obstacle to induce immune-mediated reactions (Figure 1.3a). This proposed that immune stimulation occurred through the binding of a drug/chemical-derived antigen to protein to form a hapten-protein complex. Indeed a relationship of sensitisation potential to potential to bind protein was observed and reported by the landmark paper of Landsteiner and Jacobs in 1935 (Landsteiner and Jacobs, 1935). This complex, now capable of entering the antigen processing pathway, is taken up and processed by APCs. The peptide products of processing are subsequently presented on the cell surface by MHC molecules, which can be recognised by TCRs on passing T-cells (Adam *et al.*, 2011). A slightly different mechanism is needed for drugs and chemicals which lack chemical reactivity but are metabolised to protein reactive products. This, termed the pro-hapten theory, is similar to that of the hapten hypothesis but it is the metabolite that is recognised by APCs and not the parent compound (Figure 1.3b).

1.7.1.2 Pharmacological Interaction with immune receptors concept.

Protein unreactive parent drugs and chemicals can also directly activate T-cells by a non-covalent, reversible interaction directly with the TCR or MHC. This theory, the "pharmacological Interaction" or PI concept, states that a drug may directly bind to an antigen receptor, whether this be the TCR, MHC, or both to stimulate a T-cell response independent of antigen uptake and processing by DCs (Figure 1.3c). Drugs such as SMX are able to stimulate a TCR via this direct interaction as assessed by positive T-cell stimulation in the presence of fixed APCs so abolishing any antigen processing but allowing MHC-antigen binding (Elsheikh *et al.*, 2011). Further proof lies in the recognition that the stimulation of T-cells by some drug- or chemical-derived antigens occurs within seconds, which is too quick for an antigen to have been engulfed, processed, and externally displayed. It is generally thought that hapten and prohapten associated antigens stimulate a novel response and as such naïve T-cells are implicated, however memory T-cells are associated with the PI-concept (Adam *et al.*, 2011) as it is likely that these reactions only occur on T-cells with low thresholds for activation that have previously been activated (Pichler *et al.*, 2011). While this rapid activation of T-cells may at first appear contradictory to the delayed nature of T-cell hypersensitivity reactions, it is important to bear in mind even the first contact between DCs and T-cells occurs 3-15 hours after administration *in vivo*. Moreover, the activation of naïve $CD4^+$ T-cells *in vivo* has been reported to require a minimal stimulation period of 6 hours before subsequent clonal expansion, with a sustained $CD4^+$ response being dependant on additional signals produced by DC-T-cell contacts which occur more than 24 hours after the initial exposure (Breart and Bousso, 2006; Fife *et al.*, 2009; Obst *et al.*, 2005). Furthermore, these events only represent the beginning of T-cell responses which requires time consuming events such as gene activation and the production of cytokines. Indeed, these late signals

appear to be important for distinct functions of the response such as the generation of IFN-γ (Celli *et al.*, 2005; Celli *et al.*, 2007; Fife *et al.*, 2009; Obst *et al.*, 2005).

1.7.1.3 Altered peptide concept.

A more recently established theory for T-cell stimulation by drugs is the altered peptide model (Figure 1.3d). Much of the evidence for the existence of this model is based on the interaction of the drug ABC with HLA-B*57:01 and subsequent predisposition to hypersensitivity (Illing *et al.*, 2012; Norcross *et al.*, 2012). In this instance it is hypothesised that a drug antigen non-covalently binds to a region of the MHC thus changing the repertoire of self-peptides that are able to bind this region for presentation to circulating T-cells. Alternatively, the drug antigen may bind directly to a self-peptide presented on the MHC thus forming a neo-antigen seen by the TCR as non-self. For this reason this theory is often referred to as the "altered self". Even though these peptides are still able to bind to the HLA without the presence of drug, it is thought that the drug acts to stabilise the epitope allowing increased access for the peptides to the binding site (Norcross *et al.*, 2012). However at this time, this method of hypersensitivity induction has only been proven for ABC, along with some evidence for the antiepileptic drug CBZ (Illing *et al.*, 2012).

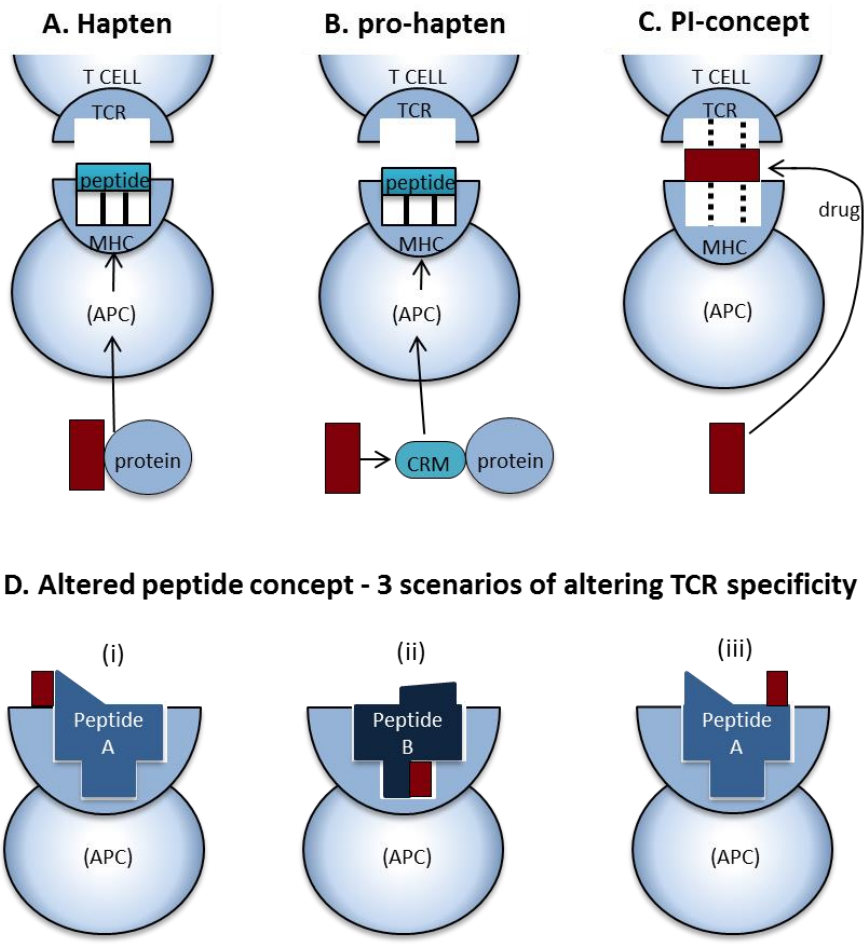


Figure 1.3 Models of TCR activation by drug or chemical antigens (signal 1). (A) The hapten hypothesis states that a drug binds to protein to form a hapten and become recognisably immunogenic. The hapten is then internalised and processed by APCs to form antigenic peptide fragments which are subsequently loaded onto MHC molecules (covalent binding) and presented at the cell surface to passing T-cells. (B) The pro-hapten theory is similar to the hapten hypothesis but it is a CRM that binds to protein to form a hapten. (C) The PI-concept is that chemically inert parent drugs or chemicals can interact (non-covalently) directly with the MHC/TCR without the need for protein-binding or antigen processing. (D) The altered peptide concept states that a drug may bind to the MHC-peptide complex in such a way that self-peptides represent an antigenic signal. This may refer to binding of drug (i) to HLA outside the peptide-binding groove, (ii) to HLA in the peptide-binding groove, or (iii) directly to the self-presented peptide. Figure adapted from (Ostrov *et al.*, 2012).

1.7.2 TCR triggering.

Antigenic stimulation which leads to an internal T-cell signalling cascade is termed TCR triggering. However, the precise details of how antigen binding mediates this process is still unclear. Several models have been suggested, which are categorised as aggregation,

segregation, and conformational alteration, all of which are summarised by figure 1.4 (van der Merwe and Dushek, 2011).

When considering TCR triggering models it is important to remember that TCR recognition of ligand differs somewhat to other ligand-receptor interactions. One of the most fundamental of these is that a TCR must be able to bind a wide range of ligands including those which do not originate from humans. This function can be performed by TCRs due to the non-conserved binding residue profile of TCRs which provides a great range of binding diversity. Specifically, while the interface on the MHC has the ability to form many contacts, only a few residues are required to bind in order to activate a TCR and the interaction of peptide with TCR is therefore by no means an exact fit (Tynan *et al.*, 2005).

The TCR also has to be highly sensitive to its corresponding ligand as a non-self-peptide is often only present at very low concentrations, bound to a subset of MHC molecules on APCs. The chance of TCR-antigen binding is therefore low and requires high affinity binding to ensure peptide detection. This low abundance of non-self-antigen presents another problem, discrimination between this and the much more abundantly presented self-peptides which are often referred to as “noise”. To account for this, it is thought that there are co-operative effects that aid this distinction. This may involve the interaction of numerous TCR-CD3 complexes which allow for internal signal amplification from self-peptides, or alternatively, a process known as signal spreading. Signal spreading is based upon the fact that non-self-peptide TCR affinity is greater than that of self-peptides. The high affinity binding overcomes a threshold whereby the signalling cascade is initiated. Extracellular signal-regulated kinase (ERK) is activated to phosphorylate, and therefore activates lymphocyte-specific protein tyrosine kinase (Lck), while self-peptides provide a suboptimal response only great enough to trigger a negative feedback loop whereby the

tyrosine phosphatase SHP-1 is recruited to inactivate Lck (Schamel *et al.*, 2006; Štefanová *et al.*, 2003; van der Merwe and Dushek, 2011).

1.7.2.1 Aggregation model.

Two models of TCR triggering are grouped into a category based on aggregation (Figure 1.4a). The first is the coreceptor heterodimerisation model in which binding of the MHC-TCR is accompanied by binding of either the CD4 or CD8 coreceptor to the MHC. If both interactions occur then Lck located on the intracellular portion of the coreceptor is brought into close proximity with the immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR CD3 chains and stimulates a phosphorylation cascade that leads to T-cell activation. However, the majority of studies have found that MHC monomers bound to peptide are unable to fully activate the TCR which lead to the proposal of multiple MHC-TCR interactions being required for T-cell activation. This adapted aggregation model is termed the pseudodimer model, as it relies on not only binding of non-self-peptide with TCR and MHC, but also a secondary interaction between an adjacent TCR and MHC molecules which may contain self-peptide. The coreceptor in this instance is bound to the self-peptide-MHC complex but due to the dimer formation of the TCR, the associated Lck is also able to phosphorylate the non-self-peptide TCR. While both monomeric and polymeric TCR complexes have been identified, it is thought that antigenic signals promote the dimerisation and clustering of the TCR complexes. In support of this polymeric model, it has been shown that self-peptide MHC complex formation allows for better recognition of non-self-peptide (van der Merwe and Dushek, 2011). It appears however that neither aggregation model can account for all cases of T-cell activation. Whilst for $CD8^+$ T-cell activation, binding of the coreceptor appears crucial

for TCR activity, for $CD4^+$ T-cells, those exposed to higher densities of an antigen can be activated in the absence of coreceptor binding (Irvine *et al.*, 2002; Purbhoo *et al.*, 2004).

1.7.2.2 Conformational change model.

An alternative model of TCR triggering is based upon the observation that the TCR undergoes a conformational change during T-cell activation (Figure 1.4b). It has been shown that the presence of lipid vesicles, mimicking the lipid structure of the plasma membrane, was able to promote the folding of the CD3ζ chain into a state where it was unable to be phosphorylated by Lck, therefore blocking zeta chain-associated protein kinase (Zap70) recruitment and T-cell activation (Aivazian and Stern, 2000). It is well established that the TCR undergoes conformational change upon binding to an antigen but this is primarily in and around the antigen binding region and not within the T-cell. Indeed, studies have reported altered TCR constant domain structures, but are generally reliant on the use of low resolution crystallographic techniques (Beddoe *et al.*, 2009). It is now thought that these extracellular conformational changes are translated through the plasma membrane to the intracellular portion of the TCR where the CD3 chains themselves then undergo a conformational change. Using a molecular dynamics model, Martinez-Martin *et al* were able to identify a structural alteration in CD3ε subsequent to ligand binding resulting in a more rigid structure (Martínez-Martín *et al.*, 2009) thought to expose ITAMs which can then be phosphorylated. More thorough analysis of this alteration showed that by introducing mutations TCR activation could be prevented. Furthermore, it was concluded that TCRs may form clusters within which TCRs are able to signal to one another as signalling in this model could not be restored by addition of non-mutated CD3ε.

1.7.2.3 Segregation model.

The final TCR triggering model is termed the segregation model, often referred to as the redistribution model (Figure 1.4c). This refers to the separation of the TCR complex from inhibitory proteins such as CD45, a receptor tyrosine phosphatase known to prevent T-cell activation. Indeed it is activation of cell surface proteins such as CD45 which prevent Lck from constitutively proceeding with the TCR signalling cascade. Although no more Lck is activated after TCR ligation, the constitutively active Lck is then free to phosphorylate downstream proteins (Nika *et al.*, 2010). The kinetic segregation model excludes TCR inhibitory phosphatases based on their longer length and maintains an area of close contact between T-cells and APCs using short adhesion molecules (Davis and van der Merwe, 2006). Both lengthening of the MHC complex, and the use of shortened CD45, can prevent TCR activation thus supporting this theory (Choudhuri *et al.*, 2005; Irles *et al.*, 2003). A slight variation to this model, states that TCR activation requires the presence of lipid rafts which are associated with the absence of inhibitory proteins such as CD45 but express others such as the T-cell activation cascade initiating kinase, Lck. While the presence of these rafts during T-cell activation is questionable due to the lack of markers identifying the co-localisation with TCR clusters, the theory is quite plausible as lipid rafts are known to be vital for translocating important proteins to the membrane (Hashimoto-Tane *et al.*, 2010).

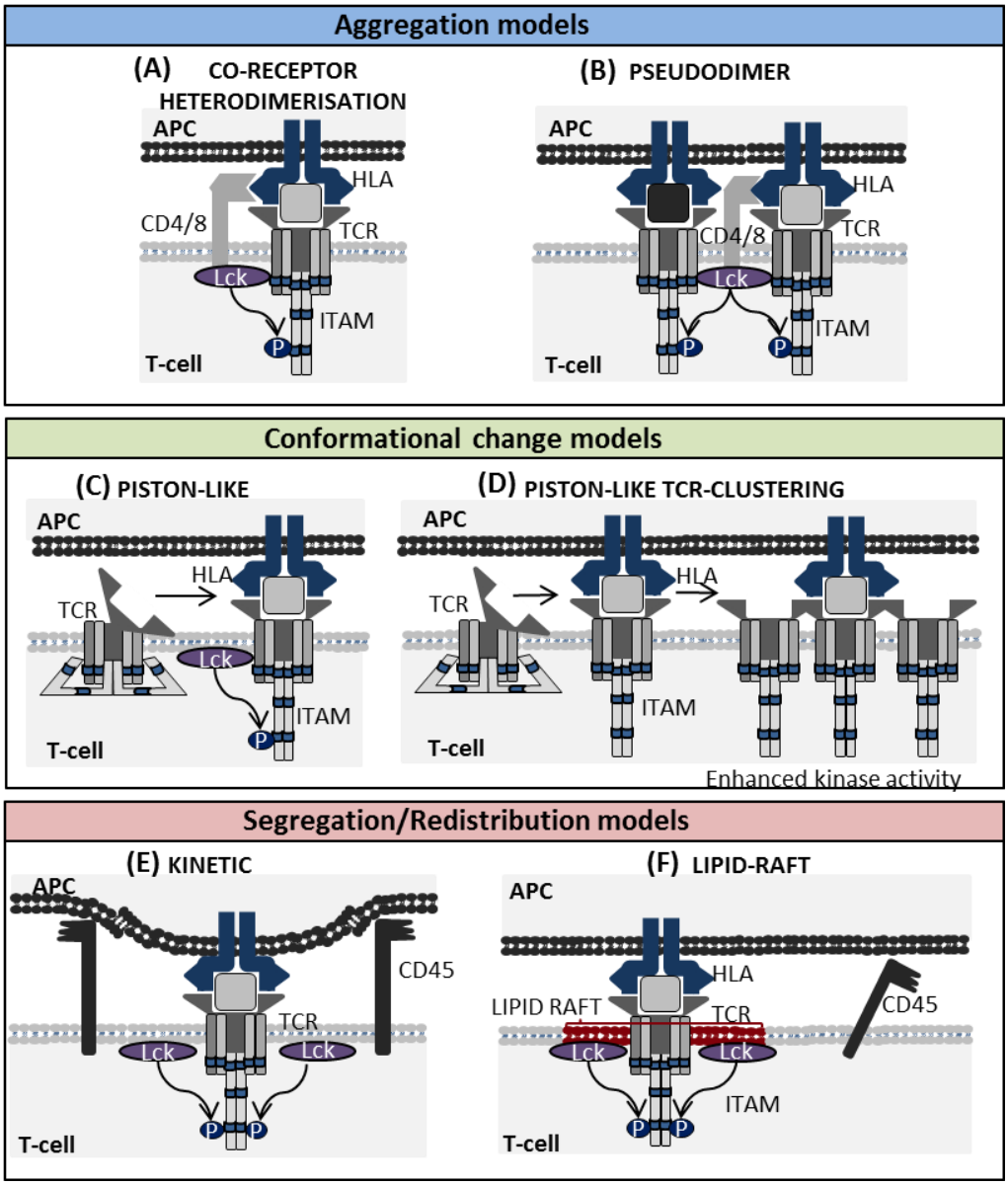


Figure 1.4 T-cell receptor triggering models. Aggregation model – **(A)** the co-receptor heterodimerisation model states that the TCR-MHC interaction is accompanied by binding of the CD4/8 coreceptor. Lck, a tyrosine kinase bound to the coreceptor is brought into close proximity with the ITAMs on the TCR CD3 chains to begin a phosphorylation cascade. **(B)** is similar and termed the pseudodimer model, but requires the additional presence of a second TCR-MHC interaction presenting self-peptide to boost downstream signalling. Conformational change model – **(C)** The piston-like model states that interaction of the TCR with a peptide-MHC complex initiates an extracellular conformational change that translates to the intracellular portion of the TCR to 'open' the folded conformation of the CD3 chains thus exposing ITAMs which can subsequently be phosphorylated/activated. **(D)** The piston-like clustering model is an adaption of this whereby piston-like movement occurs and results in TCR clustering and amplification of downstream activation pathways. Segregation/redistribution models – **(E)** The kinetic model identifies that cell surface proteins such as the tyrosine phosphatase CD45 prevent T-cell activation. Exclusion of these long inhibitory molecules from sites of TCR-MHC interaction would allow T-cell activation to proceed and thus may occur in areas of close contact between APCs and T-cells. **(F)** The lipid-raft model states that lipid rafts containing T-cell activation molecules such as Lck, but excluding the presence of inhibitory proteins (CD45) may associate with TCRs and allow for T-cell activation. Figure adapted from (van der Merwe and Dushek, 2011).

1.7.3 TCR signalling cascade.

Binding of an antigen to a TCR is the primary event in T-cell activation and allows for clonal T-cell expansion specifically directed to the antigen in question. The TCR itself is composed of a variable αβ heterodimer alongside one of numerous available CD3 protein dimers (ζζ, δε, γε). One of the major goals of the TCR signalling cascade is the activation of transcription factors which can enter the nucleus of the T-cell and promote the transcription of required genes. This occurs through a complex process, the main events of which are detailed below and summarised in figure 1.5.

Subsequent to TCR ligation, ITAMs located on the cytoplasmic tails of the CD3 chains are phosphorylated via activation of the Sarcoma (SRC) tyrosine kinases, Lck and Fyn. This promotes Zap70 recruitment to CD3, which is then also subject to Lck-mediated phosphorylation. Phosphorylated Zap70 is then able to phosphorylate the linker for activation of T-cells protein (LAT) and once this happens, a range of proteins are recruited to LAT to form a structure known as the LAT signalosome. This protein complex includes SRC homology-2 (SH2) domain-containing leukocyte phosphoprotein of 76Da (SLP76), phospholipase C-γ1 (PLCγ1), as well as growth factor receptor bound protein 2 (GRB2) and the associated adaptor protein (Gads) (Smith-Garvin *et al.*, 2009).

Phosphorylation activates SLP-76, promoting the recruitment of the Tec-family tyrosine kinase (Itk), which is then able to phosphorylate and further activate PLC-γ1. This forms an important part of the T-cell induction cascade as PLC-γ1 leads to the production of inositol triphosphate (IP3) and diacyl glycerol (DAG) through membrane hydrolysis of phosphatidylinositol-bisphosphate (PIP2). Both of these products of lipid hydrolysis operate distinct pathways. DAG launches a signalling cascade through the guanine nucleotide binding protein Ras to induce mitogen-activated protein kinase kinase (MEK)/ERK signalling which promotes activation of the transcription factor Fos. It is also

the action of Ras that causes the increased CD69 surface expression associated with activated T-cells. IP3 on the other hand leads to the release of ER stored calcium into the cytoplasm. To replenish these calcium stores the cell opens calcium release activated channels (CRAC) to bring in extracellular calcium. This influx activates calmodulin (CaM) which initiates two different pathways of downstream calcium-dependent transcription factors. One path activates CaM-dependant protein kinase IV, which phosphorylates the transcription factor CREB causing subsequent induction of the c-Fos gene. The other activates calcineurin leading to activation of the transcription factor nuclear factor of activated T-cells (NFAT). One of the major outcomes of this is the production of IL-2, a major T-cell survival cytokine (Ma *et al.*, 2014; Malissen *et al.*, 2014; Smith-Garvin *et al.*, 2009). PIP2 is also implicated in a different signalling pathway induced by TCR activation. The TCR associated kinase Lck is able to activate phosphoinositide-3-kinase (PI3K) which in turn produces PIP2. PIP2, binds to Akt, and aided by phosphorylation by phosphoinositide-dependant kinase 1 (PDK1), is able to activate a wide range of downstream targets associated with cell growth. One major target includes the mechanistic target of rapamycin complex 1, which is functions to promote insulin signalling, lipid metabolism, and protein synthesis pathways (Hung *et al.*, 2012; Vanhaesebroeck *et al.*, 2012). DAG-mediated activation of protein-kinase C (PKC)-θ via either pathway is required for activation of the transcription factor nuclear factor κβ (NK-κβ) (Chuang *et al.*, 2011). Furthermore, PKC-θ formed through the PI3K pathway is then involved in the recruitment of mitogen-activated kinase kinase 7 (MAPKK7), which along with additional proteins including B-cell lymphoma protein (Bcl)-10 forms the PKC-θ signalosome. The primary function of this signalosome is to activate the transcription factor c-Jun required for expression of cyclin-dependant kinase 2 that allows cells to enter the cell cycle (Srivastava *et al.*, 2012).

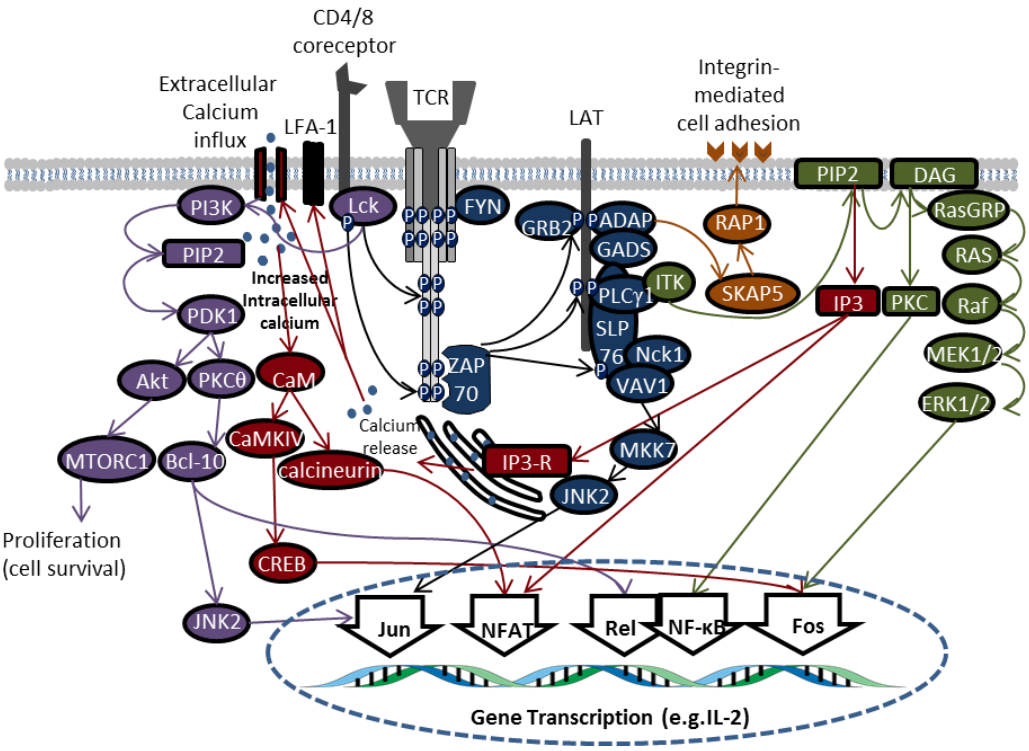


Figure 1.5 T-cell signalling pathways activated by TCR engagement. Successful activation of the TCR requires simultaneous binding of CD4/CD8 coreceptors which brings an associated tyrosine kinase, Lck, into close proximity with ITAMs on the TCR CD3 chains. Phosphorylation recruits Zap70 which is itself phosphorylated, and is then able to phosphorylate LAT. LAT recruits a range of molecules including GRB2, SLP-76, ADAP, GADS), PLC-γ1 to form the LAT-signalosome. Phosphorylated ADAP associates with SKAP55 to recruit RAP1 which induces integrin expression on the cell surface to aid stable immunological synapse formation. Phosphorylated SLP-76 recruits Itk to further phosphorylate and activate PLCγ1 which subsequently hydrolyses membrane bound PIP2 to IP3 and DAG. DAG activates PKC and induces the activation of the nuclear factor NF-κβ transcription factor, and also signals through Ras-GRP, RAS, Raf, and finally MEK/ERK to induce the Fos transcription factor. IP3 directly induces the transcription factor NFAT, but also activates the IP3-receptor on the endoplasmic reticulum to release intracellular calcium stores. To replenish these stores, CRAC channels open causing extracellular calcium influx. This further enhances expression of LFA-1 at the cell surface and activates CaM. CaM activates calcineurin, which induces the transcription of NFAT. Calcineurin also activates CaMKIV which phosphorylates CREB to induce the transcription factor Fos. PIP2 is also produced directly by Lck-mediated phosphorylation of PI3K. PIP2 is phosphorylated by PDK1 to activate multiple targets including Akt and PKC-θ. While Akt activates MTORC1 to promote protein synthesis and lipid metabolism, PKC-θ is phosphorylated and forms the PKC-θ signalosome with Bcl-10 which activates the transcription of both Jun and Rel transcription factors. Figure adapted from (Brownlie and Zamoyska, 2013).

TCR activation is also able to enhance the cell adhesion capability of the T-cell via increased activation of cell surface integrins such as LFA-1. This can occur via signalling out of the LAT signalosome, whereby the adhesion and degranulation promoting adaptor protein (ADAP) associates with the Sarcoma-kinase associated phosphoprotein of 55kDa

(SKAP55) to form a complex able to recruit the small guanosine triphosphate (GTP)ase Ras-related protein-1 (Rap-1). It has been shown that activation of integrins by this pathway aids optimal T-cell activation by promoting a long-lasting stable connection between the T-cell and the APC (Kliche *et al.*, 2006; Mitchell *et al.*, 2013).

1.7.4 Signal 2: T-cell co-signalling.

The immune system requires T-cells to respond to non-self-antigens whilst tolerating self-antigens. While the majority of self-reacting T-cells are selected out in the thymus during development of the cell, this process is not 100% dependable requiring peripheral mechanisms to control circulating auto reactive cells (Fife and Bluestone, 2008). Imbalance of T-cell activation one way or another has potentially lethal results; it can either promote non-self-antigen survival and potential serious infection, or activation of the immune system against self-antigens so promoting autoimmunity. The balance is therefore tightly controlled by multiple layers of regulation that act as fail-safes in the event that one of these regulatory mechanisms isn't working correctly.

The most widely accepted T-cell stimulation model stipulates that T-cells require two signals from APCs such as DCs, the first being the peptide-HLA complex interaction with a corresponding TCR which is discussed in detail above. This interaction provides the response with the parameter of specificity and identifies the peptide as foreign.

Signal 2 relates to a plethora of co-stimulatory and co-inhibitory pathways, collectively termed co-signalling pathways, which modulate the threshold of T-cell activation. If signal 1 occurs alongside a predominantly co-stimulatory signal 2, the result is T-cell activation. However, if signal 1 occurs but is not followed by signal 2, or a predominantly co-inhibitory signal 2, immune tolerance and an unresponsive state known as anergy

ensues (Sharpe and Abbas, 2006). The concept of co-signalling is therefore critical to understanding the balance between a state of responsiveness or tolerance in the T-cell.

Both sides of this delicate balancing act are fortified with numerous pathways to fine-tune a response. The most well defined cells that instigate these pathways are APCs; however, many other cell types express the ligands for these pathways including the endothelial cells of blood vessels and organs such as the placenta. Individually, the signalling properties of many of these pathways are well studied, but how this complex network of pathways interacts with one another, or how they function during hypersensitivity reactions is not. Indeed the potential for one pathway to compensate when another pathway is unresponsive would render signal 2 a formidable drug target to exploit.

It is now also known that these interactions not only modify T-cell activity, but can also promote or hinder T-cell subset differentiation. For instance, Th2 differentiation can be induced by the co-stimulatory molecule T-cell immunoglobulin- and mucin-domain (TIM)-4 expression, which simultaneously prevents Th1 and Th17 differentiation (Cao *et al.*, 2011; Kane, 2010; Mizui *et al.*, 2008). Others aid in the development of different cell types, such as the co-stimulatory molecule ICOS and the peripheral maintenance of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells. As T-cells play a role in a diverse number of disease states such as cancer, atherosclerosis, infection, and allergy, manipulation of co-stimulation has evolved as a promising target for drugs to either increase or decrease T-cell activation. It is also conceivable that inter-individual differences in expression of some of these pathways, perhaps due to polymorphic variants which have been identified for a number of these receptors (Chae *et al.*, 2004; Chang *et al.*, 2007; Donner *et al.*, 1997a), may manipulate the threshold of T-cell activation to such an extent that it is at least partly responsible for increased susceptibility to hypersensitivity reactions.

1.7.4.1 Signal 2 receptor families.

Based upon structural conformations, the majority of co-inhibitory and co-stimulatory pathways can be subdivided into two main superfamilies; the tumour necrosis factor receptor superfamily (TNFRSF), or the immunoglobulin superfamily (IgSF). Within both of these superfamilies, receptors are further subdivided. Families within the TNFRSF include type-V, type-L, and type-S. Type-V interactions are all co-stimulatory and include the receptors OX-40 and glucocorticoid-induced TNFR family related gene (GITR). Type-L pathways include a mixture of co-stimulatory pathways such as herpesvirus-entry mediator (HVEM) whose ligand is B and T lymphocyte attenuator (BTLA), but also those that lead directly to cell apoptosis including Fas. While there are a total of four families within the TNFRSF, the IgSF has eight namely CD28, B7, CD226, TIM, SLAM, Butyrolphilin (BTN), leukocyte-associated inhibitory receptor (LAIR), as well as two orphan receptors, lymphocyte activation gene-3 (LAG-3) and CD160. The CD28-B7 receptor pathways are perhaps the most well studied and are the most relevant to the data presented in this thesis (Chen and Flies, 2013).

1.7.4.2 The CD28 co-signalling family.

The CD28 family consists of six members, which includes CD28 itself (stimulatory), ICOS (stimulatory), CTLA4 (inhibitory), BTLA (inhibitory) and PD-1 (inhibitory). Despite being characterised as being in the same family, BTLA and PD-1 are somewhat different to the rest. Whilst the genes for CD28, ICOS and CTLA4 are found in close proximity to one another on chromosome 2q33, PD-1 and BTLA are located on chromosomes 2q37 and 3q13, respectively. Both PD-1 and BTLA are also found as cell surface monomers due to the absence of an unpaired cysteine residue whose presence in the other family

members allows the formation of dimers, and have two tyrosine motifs in their cytoplasmic tails, an immunoreceptor tyrosine based switch motif (ITSM) and an immunoreceptor tyrosine based inhibitory motif (ITIM) (Riley and June, 2005b).

Some of the earliest work on CD28 confirmed its co-stimulatory properties by utilising anti-CD28 antibodies to induce anergy in T-cells (Harding *et al.*, 1992). However, just half of $CD8^+$ T-cells express CD28 compared to 100% of $CD4^+$ T-cells (Riley and June, 2005a). CD28 has two co-stimulatory ligands, B7-1 (CD80) and B7-2 (CD86). It was discovered that the stimulatory function of this receptor was due to two sequences found in the cytoplasmic tail; YNMN and PYAP. Both sequences are involved in the recruitment and activation of PKC, as well as the binding of GRB2, allowing for translocation of NFAT to the nucleus whereby it promotes transcription of IL-2 (Chen and Flies, 2013). ICOS is found on activated T-cells, where it can bind to its ligand ICOS-L which is expressed on DCs, but also macrophages and B-cells (Bauquet *et al.*, 2009). ICOS is known to reduce IL-17 production from both Th17 and Tfh cells, a reported consequence of ICOS-mediated c-Maf induction (Bauquet *et al.*, 2009).

Although these co-stimulatory pathways are often vital to the development of T-cell responses, our focus lies within the co-inhibitory pathways, specifically at the CD28 family members PD-1 and CTLA4, but also the TIM-family member, TIM-3, and thus warrant a more thorough introduction.

1.7.4.3 Programmed Death-1.

PD-1, discovered on apoptotic cells in 1992, has been the focus of many immune regulation investigations as it is considered a major checkpoint for T-cell inhibition. Its role as an inhibitor of T-cell activation was first proposed after the observation that the

likelihood of autoimmune disease development was increased in murine PD-1-knock out models (Nishimura *et al.*, 1999). The critical immune regulatory role of PD-1, whose expression is at least partly regulated by the transcription factor NFATc1, is epitomised by its widespread expression not only on immune cells such as T-cells, B-cells, NKT-cells, and monocytes, but throughout the human body including the thymus and at major sites of immune privilege such as the placenta (Brown *et al.*, 2003; Keir *et al.*, 2007). PD-1 expression on inactive cells is generally low to non-existent, and it is only after activation of $CD4/8^{+}$ T-cells, monocytes and B-cells that expression of PD-1 is induced on the cell surface. PD-1 activation requires ligation by one of two known ligands; PD-L1 or PD-L2 (Keir *et al.*, 2007). Whilst the expression of PD-L2 is limited to macrophages, DC, and bone-marrow derived mast cells, PD-L1 expression is found on a much more diverse range of cells including all professional APCs, Tregs, B-cells, keratinocytes, and endothelial cells (Keir *et al.*, 2007). In a physiological setting, IFN-γ, GM-CSF, and IL-4 can induce PD-L2 expression, whereas PD-L1 is induced by type I and II IFNs (Keir *et al.*, 2007). Binding of either of these ligands to PD-1 is proposed to result in the recruitment of SHP-1 or SHP-2 proteins which subsequently mediate the downstream dephosphorylation of Zap70, PI3K, and Akt thus preventing T-cell stimulation (Fife *et al.*, 2009). For this to occur, the ligand interacting with PD-1 must be expressed on the same cell which expresses the MHC molecule that is interacting with the activated TCR inferring that close proximity of PD-1 and the TCR is important to this process (Keir *et al.*, 2007).

The basis for the inhibitory nature of PD-1 has been elucidated through structural studies. The binding of PD-1 to its ligands relies on a singular IgV-like domain at the N-terminus of PD-1, the complementarity determining region (CDR) 3 of which lacks conserved binding residues and is loosely ordered. Attached to this is a transmembrane domain, and importantly for signal transduction, a long cytoplasmic tail of around 95 residues which houses two tyrosine based motifs, the ITIM and ITSM. Utilising tyrosine

to phenylalanine point mutations within the ITIM or ITSM, Chemnitz *et al* were able to show that the ITSM, but not the ITIM, is responsible for the Akt inhibition observed after PD-1 activation (Chemnitz *et al.*, 2004). Further structural analysis of the PD-1 ligands has revealed that both ligands are type I transmembrane proteins and contain both IgV and IgC-like domains. Additionally, PD-L1 and PD-L2 are encoded by the adjacent CD274 and Pdcd1lg2 genes, respectively, on chromosome 9 in humans. Whilst the extracellular chains are required for binding to PD-1, most of which is dependent on the IgV-like domain, the intracellular chains of both ligands have no known associated signalling pathways (Keir *et al.*, 2007; Riley, 2009). However, PD-1 signalling may be bi-directional as signalling induced by PD-1-ligand interaction back into DCs has been reported. Indeed soluble PD-1 is able to enhance IL-10 production to reduce the activity of DCs, and this can be prevented by using anti-PD-1 antibodies (Dong *et al.*, 2003).

Due to the effective dampening of the T-cell response seen with PD-1, this pathway is considered a critical checkpoint and regulator of T-cell activation, which can be attributed to the employment of multiple methods of inhibition. Firstly, PD-1 activation directly hinders immunological synapse formation between a T-cell and a DC. This is demonstrated by studies which show that blocking PD-1 increases T-cell-DC contact time considerably and dramatically increased the number of cells that made contact from 8% to 75% (Fife *et al.*, 2009). This is thought to be a consequence of inhibited integrin-mediated adhesion, as adhesion to intracellular adhesion molecule-1 (ICAM-1) is reduced by PD-1 ligation during anti-CD3 stimulation. ICAM-1 is the ligand for the integrin LFA-1 which is normally activated during TCR stimulation (Saunders *et al.*, 2005). Secondly, a number of steps within the cell cycle are regulated by PD-1, thus PD-1 activation can prevent cell proliferation. One such step is the inhibition of Akt activation which subsequently impedes PI3K-Akt and MEK-ERK signalling pathways. The downstream consequences of this prevent the expression of the transcription factor S-

phase kinase-associated protein 2 (SKP2), which is normally required for the formation of the ubiquitin ligase SCF^{SKP2} so that a T-cell can pass the G1 phase checkpoint. Additionally, E2F gene expression is suppressed by PD-1 activity due to signalling which prevents retinoblastoma protein phosphorylation, whilst Smad3 activity is increased, leading to both a reduction in Cdc25A, a cell cycle promoting phosphatase, and increased production of p15INK4, a cell cycle inhibitor (Patsoukis *et al.*, 2012). In combination, these methods of T-cell inhibition by PD-1 activation are reported to reduce T-cell IL-2 production by up to 70% (Carter *et al.*, 2002; Chikuma *et al.*, 2009), indicating that PD-1 is an extremely effective co-inhibitory pathway.

Although our bodies naturally use PD-1 to dampen T-cell responses, this pathway is utilised during the development of multiple cancers. Cancer cells may evade immune recognition by expressing PD-1 to dampen any anti-tumour T-cell responses. This immune-recognition loop hole is also used by some parasites, such as Schistosoma Mansoni, which have evolved to evade immune recognition within the human body by increasing PD-L1 expression on the macrophages in which they inhabit (Smith *et al.*, 2004). The role of PD-1 is not however limited to regulating the immune system, but also in its efficient development. Negative selection in the thymus during early T-cell development relies in part on the expression of PD-1 on double negative thymocytes (Jin *et al.*, 2011).

Pharmaceutical companies are aware of the potential for therapeutic treatment that PD-1-immunotherapy holds. Nivolumab (Bristol-Myers Squibb), an anti-PD-1 biological, has proven to hold great potential for the treatment of melanoma during phase III trials by displaying an enhanced efficacy in comparison to other treatments in use at that time. For instance, while the use of chemotherapy to treat metastatic melanoma was associated with a disease free 1 year survival rate of 42.1%, the survival rate upon use of

nivolumab was far greater at 72.9%. Moreover, nivolumab treatment was successful in those patients whose melanoma had progressed after treatment with alternative therapies (Robert *et al.*, 2015; Weber *et al.*, 2015). Another PD-1 targeting therapy, pembrolizumab (Merck, lambrolizumab), was found to similarly enhance progression free-survival in patients with advanced melanoma (Hamid *et al.*, 2013b). Indeed, the food and drug administration approved the use of Nivolumab and pembrolizumab for the treatment of BRAF-mutated melanoma and metastatic melanoma in 2014. While phase II trials are currently underway for the use of both Nivolumab and pembrolizumab for other cancers, other biologicals are also in the development pipeline which have generally displayed a well-tolerated toxicity profile and shown to treat a range of cancer types including pidilizumab (Curetech, CT-011) which also targets PD-1 (Armand *et al.*, 2013). There are also at least two further agents directed against PD-L1, MEDI-4736 (AstraZeneca) and MPDL3280A (Roche), which are both in clinical trials and are well-tolerated and effective (Emens *et al.*, 2015; Mullard, 2013; Philips and Atkins, 2015). Although the listed indications for these therapies point only to the treatment cancer, the successful treatment of tumours using these therapies epitomises the importance of this pathway in regulating the immune system.

1.7.4.4 Cytotoxic T-Lymphocyte Associated Protein-4.

CTLA4 is a 233aa long, transmembrane glycoprotein which shares its ligands with the co-stimulatory receptor CD28, despite just 30% homology between the two receptors (Carreno and Collins, 2002). Whilst CD28 binds to its ligands CD80 and CD86 to promote T-cell activation, CTLA4 binds to prevent immune activation. The competition to bind CD80 and CD86 between CD28 and CTLA4, alongside the fact that up to 50 fold fewer CTLA4 molecules are present on the cell surface in comparison to CD28, appears to

favour stimulation of the immune system. However, this is counterbalanced by the much greater affinity of CTLA4 for its ligands than CD28, with dissociation constants for CD86 of 2.6 μM and 20 μM, respectively. This difference in affinity has been attributed to monomeric binding by CD28, but dimeric binding by CTLA4 (Collins *et al.*, 2002; Stamper *et al.*, 2001). The balance between stimulation and inhibition may simply relate to the dominant use of particular ligand. Indeed, based on differing expression patterns, some experts consider CD80 is principally co-inhibitory as its expression is only induced after activation and takes much longer to reach peak surface expression than CD86, which has been suggested to be predominantly co-stimulatory due to its constitutive APC expression and rapid up-regulation subsequent to activation (Teft *et al.*, 2006).

CTLA4 contains an Ig-V-like extracellular domain. This region is responsible for ligand binding to CD80 and CD86, which unlike the CDR3 binding domain of PD-1, contains conserved binding residues dominated by a MYPPPY motif (Keir *et al.*, 2007). This is attached to a stalk that connects to a transmembrane domain which contains three points vital to homodimerisation; a position 122 cysteine, and positions 78 and 110 which are sites of *N*-glycosylation. Finally, there is a 36aa long cytoplasmic tail. Like PD-1, CTLA4 expression is likely induced by the transcription factor NFAT, as the use of cyclosporine A (CSA), a potent NFAT inhibitor, reduces CTLA4 expression. Whilst Tregs constitutively express CTLA4, which increases further after activation, and memory T-cells have intracellular stores at their disposal, generally CTLA4 is expressed on T-cells two days after activation (Linsley *et al.*, 1992). In fact while studies suggest that the mRNA of CTLA4 can be detected as little as 1 hour after TCR stimulation, plasma membrane protein expression is not seen until 24-48 hours. (Finn *et al.*, 1997; Teft *et al.*, 2006). Only a small amount of total cell CTLA4 is expressed on the surface at any one time due to continuous endocytosis into clathrin-coated pits occurring even during periods of immune stimulation.

Similar to PD-1, CTLA4 is a reasonably well-studied receptor and is too considered to be a critical immune checkpoint. The importance of CTLA4 as a regulatory checkpoint can be seen clearly upon the development of CTLA4-deficient mice, which die around 4 weeks of age following lymphocyte infiltration into multiple organs with consequent destruction of tissue (Tivol *et al.*, 1995). This may in part be due to a loss of function of Tregs, which use CTLA4 as a mechanism of immune dampening, as the loss of CTLA4 on Tregs alone can lead to fatal autoimmune disease development through unregulated expression of CD80 and CD86 on DCs (Wing *et al.*, 2008). However, a range of other mechanisms are also involved in the effective dampening of the immune responses by CTLA4.

CTLA4-induced T-cell inhibition is partly mediated by ligand independent mechanisms. These were initially confirmed when the use of splice variants, without the ability to bind CD80 or CD86, still resulted in an apparent inhibitory signal. Furthermore, in CTLA4-deficient mice, induced expression of a CTLA4 variant unable to bind to its ligands was able to delay the onset of lethal lymhoproliferation. Exactly how this signal works remains undefined but it is dependent on the formation of the TCR-MHC complex (Chikuma *et al.*, 2005).

There are also various T-cell intrinsic mechanisms of inhibition reliant on ligand binding, which include simple competitive binding of CD80/CD86 to prevent CD28 stimulation of T-cell activation pathways, particularly GRB2 and PI3K (Alegre *et al.*, 2001). Co-ligation of CTLA4 alongside the TCR-MHC is able to prevent the clustering of Zap70 and lipid raft formation required for downstream TCR signalling. CTLA4 also modulates the adhesion properties of T-cells. Specifically, CTLA4 activation can increase LFA-1 clustering and ligand binding leading to enhanced cellular adhesion. This process would appear likely to enhance formation of a stable immunological synapse and thus promote T-cell

activation. Instead it is suggested that this increased stability may increase competition for APCs between T-cells and Tregs. The effect of CTLA4 on the strength of LFA-1 adhesion may be more complex than this as it appears to depend on CTLA4 signal strength. Similar to PD-1, and seemingly contradictory to the CTLA4-mediated up-regulation of LFA-1, CTLA4 hyper-activation can reduce T-cell-APC contact time and so prevent or limit immunological synapse formation (Schneider *et al.*, 2006). This is explained by the reverse stop signal model in which CTLA4 is able to adjust the LFA-1-ICAM-1 interaction affinity and avidity to such an extent that motility is promoted, which is due to the expression of ICAM-1 by blood vessel epithelial cells. This adhesion interaction is vital to the migration of T-cells and dominates DC-T-cell binding during high CTLA4 signalling. The CTLA4-ICAM-1 interaction is therefore a balance between the mobilisation of T-cells for migration, and the immobilisation of cells for the formation of the immunological synapse (Rudd, 2008; Schneider *et al.*, 2005).

As well as the intrinsic models of CTLA4 function, there are also extrinsic functions. Specifically, activation of CTLA4 is able to induce reverse signalling back into the APC which activates IDO, an enzyme involved in the degradation and possible depletion of tryptophan, which could inhibit T-cell activity. CTLA4 binding is also known to induce the production of the inhibitory cytokine TGF-β to block T-cell activation. Furthermore, not only can CTLA4 outcompete CD28 for its ligands, but it can also prevent CD28 ligation by inducing trans-endocytosis of its ligands to limit availability (Walker and Sansom, 2011).

There have been two main anti-CTLA4 monoclonal antibodies that have been through phase I and II clinical trials, namely tremelimumab and ipilimumab, which are IgG_2 and IgG_1 antibodies, respectively. Notably, ipilimumab is able to induce lymphocyte infiltration in cancer patients leading to the enhanced necrosis of tumour cells. However,

it should be noted that anti-CTLA4 therapy has been associated with the loss of immunological tolerance and the activation of autoimmune T-cells (Hodi *et al.*, 2003).

1.7.4.5 T-cell immunoglobulin- and mucin-domain protein-3.

Discovered in 2002, TIM-3 is a TIM family member, and so part of the immunoglobulin superfamily of co-signalling molecules. Whilst the expression of PD-1 and CTLA4 is found on a wide range of activated T-cells, TIM-3 is predominantly expressed on fully differentiated Th1 type T-cells, but is also expressed on DCs and monocytes (Lee *et al.*, 2010). Here it is available to bind to its ligand, galectin-9, expressed in several tissues but also on APCs (Wada and Kanwar, 1997).

The TIM-3-galectin-9 interaction is co-inhibitory in nature, the first indication of which was the association of rheumatoid arthritis with polymorphisms in the TIM-3 gene coding and promoter regions (Chae *et al.*, 2004). Following this, use of a TIM-3-Ig fusion protein to block this pathway led to Th1 cell hyper proliferation thus confirming that normal receptor-ligand interaction acts to supress T-cell activation (Sabatos *et al.*, 2003). Similar to PD-1 and CTLA4, TIM-3 appears to be of particular physiological relevance during early T-cell development, due to the kinetics of galectin-9 expression in the lymph nodes and spleen. In a naïve immune system, galectin-9 is highly expressed, but is down regulated shortly after immune activation to allow Th1 population generation (Zhu *et al.*, 2005).

Since these early studies, the regulatory role of TIM-3 has been extensively studied, although less so than CTLA4 and PD-1. Despite the lack of a current comprehensive definition of how TIM-3 signalling leads to immunosuppression, the structural interactions of the receptor-ligand interaction and potential downstream targets of the

pathway have been elucidated. Human TIM-3 is classified as a type I transmembrane glycoprotein of 302aa, with an analogue in mice sharing 63% homology. Disulphide-linked anti-parallel β sheets form an IgV domain, the oligosaccharide chains of which provide a binding platform for galectin-9 (Zhu *et al.*, 2011). Although no ITSM or ITIM motifs have been found as in PD-1, a sequence within TIM-3 that contains tyrosine residues can be found in the cytoplasmic tail which are phosphorylated subsequent to TIM-3 ligation. It was discovered that this phosphorylation is induced by Itk and occurs in a specific, highly conserved TIM-3 cytoplasmic tyrosine residue, Y265. As Y265 is situated within an SH2 binding domain it is suggested that this may lead to downstream cell death-related pathways through the initial recruitment of SH2-containing adaptor proteins (van de Weyer *et al.*, 2006).

Further investigations have focussed on identifying the mechanism of TIM-3 immunosuppression. These studies revealed that the interaction of TIM-3 with galectin-9 leads to the characteristic Th1-type T-cell suppression through the promotion of Th1 cell death, rather than the inhibition of intracellular processes. Cell death is induced rapidly, reported to peak just four hours after receptor activation, and occurs by intracellular calcium influx which promotes the activation of the calcium-calpain-caspase-1 pathway. Both apoptotic and necrotic cell death pathways are induced leading to the drastically reduced number of cells capable of producing IFN-γ associated with this pathway (Zhu *et al.*, 2005). In normal physiological circumstances, this dampening of the immune response and associated inflammation is actually induced partly by the Th1 response itself. IFN-γ, released by T-cells during a Th1 response, functions as part of a negative feedback loop to prevent a runaway immune response, which involves the IFN-γ-mediated up-regulation of galectin-9 (Zhu *et al.*, 2005).

A recent addition to this story is that TIM-3 ligation leads to expansion of a population of cells with similar characteristics to granulocytic myeloid-derived suppressor cells (GMDSCs). These cells are reported to be $CD11b^{+}Ly\text{-}6G^{+}$ cells and are found at much higher frequencies in TIM-3 transgenic mice than wildtype mice, and also in conditions of galectin-9 overexpression. GMDCs are well known to supress T-cell responses through, in part, an IFN-γ dependant mechanism, but may also rely on the production of reactive oxygen species and enhanced arginine metabolism for their suppressive effects (Dardalhon *et al.*, 2010).

Additionally to the suppressive effects on Th1 T-cells, TIM-3 has other roles within the immune cell. For instance, TIM-3 can play a role in the cross presentation of antigens. Indeed DCs which constitutively express TIM-3 respond to phosphatidyl serine from apoptotic cells by binding to this lipid and increasing the phagocytosis of these dying cells (Nakayama *et al.*, 2009). In addition, Th17 may be reduced by the TIM-3-galectin-9 interaction. This is due to the limited, but still detectable expression of TIM-3 on Th17 cells and points towards a potential therapy to treat inflammatory disorders of the skin.

As with many other co-signalling pathways, TIM-3 has potential therapeutic use. Firstly, in the field on cancer medicine, TIM-3 may be of therapeutic relevance as it is was found to be up-regulated alongside PD-1 in a murine cancer cell model, and the majority of cells infiltrating the tumour were $PD\text{-}1^{+}$ $TIM\text{-}3^{+}$ $CD8^{+}$ T-cells (Sakuishi *et al.*, 2010). It is therefore conceivable that blockade of both of these receptors in unison could dampen the immune-inhibitory signals from tumour cells allowing T-cells the potential to fight back. Secondly, it has been noted that T-cell TIM-3 expression is enhanced during chronic periods of infection leading to reduced Th1 cytokine production. Such low Th1 cytokine release was able to be reversed through the use of a TIM-3 blocking antibody (Golden-Mason *et al.*, 2009). Therefore, therapeutic blockade of TIM-3 may enhance Th1

immunity to aid in the clearance of stubborn infections. Thirdly, manipulation of TIM-3 expression may also be of interest for the treatment of an array of autoimmune diseases which have been associated with abnormal TIM-3 expression. Specifically, reduced TIM-3 expression is linked with multiple sclerosis (Koguchi *et al.*, 2006). Furthermore, in experimental autoimmune encephalomyelitis (EAE), the disease state was worsened by blocking the TIM-3 receptor (Monney *et al.*, 2002), whilst promotion of TIM-3 ligation using galectin-9 improved EAE (Zhu *et al.*, 2005), and in a psoriatic murine model, soluble galectin-9 administration ameliorated contact hypersensitivity by reducing T-cell cytokine secretion (Niwa *et al.*, 2009). Additionally, the TIM gene locus as a whole is associated with autoimmune diabetes (Grattan *et al.*, 2002).

Despite the well documented role of TIM-3 as a co-inhibitory receptor which acts to reduce inflammatory responses within the adaptive immune system, it is important to note that within the innate immune system this pathway has pro-inflammatory effects (Anderson *et al.*, 2007). When the immune system is in a 'resting state' the most abundant expression of TIM-3 in on DCs. Upon antigen encounter, TIM-3 stimulation on DCs acts alongside stimulated TLRs to promote TNF-α release thus inducing an inflammatory response. After the shift from innate to adaptive immunity, the numbers of fully differentiated Th1 T-cells outweighs that of the DCs. Thus, Th1 expression of TIM-3 outweighs that on APC populations which shifts the role of TIM-3 from pro-inflammatory to anti-inflammatory. The major factor controlling this change is the production of vast amounts of IFN-γ from the Th1 population, which then induces galectin-9 expression. Galectin-9 then binds to TIM-3, the majority of which is still expressed on activated Th1 T-cells, resulting in the termination of the Th1 response by the induction of Th1-cell death.

1.7.4.6 The danger hypothesis.

In 1994, Matzinger and colleagues proposed that a T-cell is tipped towards a responsive state if (in the case of DHS) a drug has caused cellular damage. It was thought that this signal was responsible for enhancing costimulatory molecule expression and thus the maturation of DCs, and so was the stimulus for the induction of signal 2. This was termed the danger hypothesis (Matzinger, 1994). In the context of drug hypersensitivity, if a drug is directly toxic to cells it will induce cell damage and potentially cell death. APCs consequently up regulate co stimulatory molecule expression on their cell surface in response to stimuli from these dead cells which renders these cells ready for T-cell stimulation (Li and Uetrecht, 2010).

Endogenous danger signals from dead cells are effectively the spilled contents of the cells and are known as DAMPs (damage associated molecular patterns), and include HMGB1 (high mobility group box-1 protein), S100 proteins, and heat shock proteins (Li and Uetrecht, 2010). In comparison this does not happen in the case of natural cell turnover where dying cells are effectively scavenged long before they disintegrate. There is now a large amount of experimental data that supports a role for danger signalling in hypersensitivity reactions such as that expression profiles of genes associated with oxidative stress change shortly after administration of tielinic acid, which is linked with hepatotoxicity (Pacitto *et al.*, 2007), and that DC expression of CD40 is enhanced by the metabolite of SMX (Sanderson *et al.*, 2007). One particular DAMP which has received considerable attention is uric acid, a product of homeostatic nucleic acid turnover. When an individual develops hyperuricemia, monosodium urate (MSU) crystals are deposited, particularly in the joints. These crystals are able to stimulate the NLRP3 inflammasome to produce the effector cytokine, IL-1β. The associated inflammatory response is known as gout. It appears that uric acid is a strong, and therefore important danger signal as its

depletion in mice can significantly dampen the immune response to induced cell death. In contrast, a similar effect on cell death was not detected in response to other inflammatory inducers, such as sterile irritants or microbes, indicating that certain DAMPs are selective for specific inflammatory responses (Kono *et al.*, 2010; Rock *et al.*, 2013). Release of HMGB1 can occur in response to both infectious and non-infectious stimuli. Although HMGB1 can act as a danger signal it has also been seen to aid tissue repair and to restore damaged tissue (Klune *et al.*, 2008).

Other danger signals may arise from exogenous sources such as bacterial lipopolysaccharide (LPS) or viral RNA and are known as pathogen-associated molecular patterns (PAMPs) (Kumar *et al.*, 2011). Infection is a well-known danger signal for developing hypersensitivity as exemplified by the enhanced susceptibility to isoniazid hepatotoxicity with concomitant chronic hepatitis and HIV infection (Kaplowitz, 2005). These viral danger signals instigate myeloid DC production of CD1d mRNA leading to the activation of NKT-cells.

1.7.6 Signal 3: T-cell subset polarising cytokines derived from APCs.

The fact that just 3 days after peripheral immunisation, Th1 or Th2 cytokines can be identified in the lymph nodes indicates that T-cell differentiation occurs rapidly after T-cell priming (Kaliński *et al.*, 1999). Interestingly, the differentiation pathway is related to the nature of the antigen to which the response is mounted, even though the naïve Th cells do not likely interact with the native antigen or the tissue where it is located. Therefore, it appeared likely that such T-cell polarising signals must originate from the responding DCs, which have migrated from the site of antigen exposure (Kaliński *et al.*,

1999). The cytokines controlling the differentiation of specific T-cell subsets have been previously discussed in section 1.4.2.

1.8 Experimental compounds in hypersensitivity.

1.8.1 Beta-lactams.

Beta-lactam antibiotics represent an ideal drug class to study hypersensitivity as they induce a range of responses including immediate, accelerated, and delayed-type allergic reactions. This means that these drugs can be associated with a broad range of adverse cutaneous reactions from uticaria, to AGEP and SJS-TEN, but also internal organ toxicity including hepatitis and vasculitis (Romano *et al.*, 2004). Indeed, It was work on beta lactams in the mid-1960's that first identified that drug-antigens may activate T-cells by the hapten mechanism, via the identification of antibodies specific for benzylpenicilloyl-modified protein structures in the sera of allergic patients (Levine and Price, 1964; Siegel and Levine, 1964). However, it was not until the mid-1990's that drug-protein adducts were shown to specifically activate T-cells in beta lactam-allergic patients (Brander *et al.*, 1995; Padovan *et al.*, 1996). A beta-lactam of particular interest is piperacillin, which is commonly used to treat patients with cystic fibrosis (CF) as these patients are particularly susceptible to recurrent infection. While piperacillin hypersensitivity occurs in up to 10% of the general population, up to half of all CF patients experience piperacillin hypersensitivity (Koch *et al.*, 1991; Parmar and Nasser, 2005; Pleasants *et al.*, 1994; Whitaker *et al.*, 2011c). Recent research has been able to define the distinct binding sites of drug-derived antigens to proteins in human serum, and designer HLA-binding drug-peptide adducts have been utilised to successfully stimulate T-cells, thus providing us with a more detailed understanding of the peptide antigens that instigate these

reactions (Weltzien and Padovan, 1998; Whitaker *et al.*, 2011a). While hypersensitivity to piperacillin is not thought to be associated with a specific HLA risk allele, individuals who are HLA-B*57:01 positive have an increased predisposition for the development of hypersensitivity to an alternative beta lactam, flucloxacillin. Despite this, it is clear that other factors are required for an allergic T-cell response as just 1 in 500-1000 HLA-B*57:01 individuals will develop flucloxacillin hypersensitivity (Daly *et al.*, 2009). Responses to individual beta lactam antibiotics can be quite diverse, so these drugs are ideal for the detailed investigation of drug-induced hypersensitivity reactions.

1.8.2 Sulfamethoxazole.

SMX is available in its therapeutic form as a combination therapy with trimethoprim (co-trimoxazole), and since its first use in the US in 1973, has been used in the treatment of opportunistic infections in patients with HIV as wells as those with CF suffering from recurrent respiratory infections (Bell *et al.*, 2010). The antibiotic activity of SMX comes from preventing tetrahydrofolic acid generation, which is required for bacterial DNA synthesis, through the inhibition of dihydrofolate reductase. However, SMX is associated with the development of hypersensitivity reactions in 3-8% of the general population, the most life threatening manifestation of which is TEN (Alfirevic *et al.*, 2009). Within the HIV positive patient group the incidence is even greater, as due to viral-induced immune system failures and danger signalling, low-dose prophylactic use of SMX is associated with 30% of patients developing a hypersensitivity reaction. This figure rises further to 50% in those given SMX for treatment of an active infection (Farrell *et al.*, 2003).

T-cells isolated from SMX-hypersensitive patients, either from the blood or SMX-reaction induced blisters, respond to SMX and also to the hapten-based metabolite SMX-NO. As

SMX is unreactive and does not bind to protein, while SMX-NO does, both the hapten hypothesis and the PI concept are implicated in T-cell activation and SMX-hypersensitivity (Farrell *et al.*, 2003). The first step in the formation of SMX-NO is metabolism of SMX by CYP2C9 enzymes in the liver to SMX-hydroxylamine, which is also unreactive with protein. Autoxidation transforms this hydroxylamine to SMX-NO, which can induce the formation of neo-antigens by binding to protein cysteine residues (Castrejon *et al.*, 2010). Despite the obvious reaction site for a primarily hepatically-metabolised drug being the liver, the liver is an uncommon target for SMX reactions. In contrast the skin is frequently subject to SMX-induced reactions. Indeed, metabolism of SMX can occur in the skin itself, and immune cells present in the skin (Faulkner *et al.*, 2014; Reilly *et al.*, 2000; Spielberg, 1996). It is thought that the livers abundance of glutathione, as well as other antioxidants, may protect this organ before a harmful reaction can occur (Adam *et al.*, 2011; Pichler *et al.*, 2011).

While the metabolism of SMX produces potentially immunogenic moieties, SMX is also subject to direct detoxification by *N*-acetylation. Both NAT1 and NAT2 enzymes are able to acetylate SMX, and therefore decrease the *in vivo* availability of SMX and SMX-NO. Thus, the exposure to SMX-derived immunogenic antigens in a given individual is determined by the respective rates of function between detoxification and metabolic pathways. Indeed, it has been found that patients with hypersensitivity more frequently express the NAT2 slow acetylator phenotype than tolerant individuals. However, overall acetylation status represents a poor marker of risk based on the high proportion of slow acetylators in the general population (Spielberg, 1996). Indeed, when Pirmohamed and colleagues addressed the role of acetylator phenotype on predisposition to SMX-hypersensitivity by investigating enzyme polymorphisms in HIV-positive patients, with and without hypersensitivity, no significant associations were found (Pirmohamed *et al.*, 2000). Other detoxification pathways have also been proposed to play a role in the

activation of T-cells to SMX-derived antigens, which occur downstream of SMX. One of these regards the microsomal NADH-dependent hydroxylamine reductase (NADHR)-mediated detoxification of SMX-hydroxylamine, as it has previously been identified that individuals who are less able to detoxify SMX-hydroxylamine are more likely to be hypersensitive (Shear *et al.*, 1986). Furthermore, promoting the formation of SMX-hydroxylamine and SMX-NO by manipulating redox cycling has been proposed to partly mediate SMX-NO cytotoxicity. Of interest, the anti-oxidants glutathione and ascorbate aid the formation of SMX-hydroxylamine by promoting the reduction of SMX-NO (Lavergne *et al.*, 2006). However, similarly to NAT expression, genetic association analysis has ruled out a role for glutathione-S-transferase enzymes in the predisposition on an individual to these SMX-induced hypersensitivity reactions (Pirmohamed *et al.*, 2000).

T-cells from SMX-hypersensitive patients have a complex response pattern; a minority are directly SMX-responsive (14%), but many more are either solely SMX-NO responsive or responsive to both parent and metabolite (44% and 43%, respectively) (Castrejon *et al.*, 2010). This forms an important distinction between those who are hypersensitive and those who are tolerant, as while PBMCs in 9/10 tolerant individuals will respond to SMX-NO, PBMCs from just 3/10 individuals respond to SMX (Engler *et al.*, 2004).

Both parent drug and metabolite enhance co-stimulatory CD40 expression on DCs and thus promote the initiation of a T-cell response to these compounds (Sanderson *et al.*, 2007). However, it is not known what predisposes only certain individuals to SMX hypersensitivity, associations with HLA alleles and potential SMX metabolising enzyme polymorphisms have been ruled out as major factors of predisposition by genetic association studies (Alfirevic *et al.*, 2009; Pirmohamed *et al.*, 2000). A combination of other factors which present within just a minority of individuals may be responsible for

this predisposition, and may include minor HLA associations or potentially the differential regulation of T-cell co-inhibitory pathways previously discussed.

Overall SMX represents an ideal model for the investigation of drug-induced hypersensitivity as it has a well-defined chemistry, a reactive metabolite (SMX-NO) which is stable enough to be utilised during *in vitro* culture, its immune aetiology involves both PI and hapten-mediated T-cell activation, and SMX-NO has been previously shown to successfully and reproducibly prime naïve T-cells from healthy donors (Faulkner *et al.*, 2012).

1.8.3 *p*-Phenylenediamine.

It is not only therapeutic drugs that can activate the immune system, but also chemicals such as PPD, a dye used for its dark colour and durability in henna tattoos and hair dyes. Over the past quarter century, there have been 14 reports of rare immediate-type allergic reactions induced by PPD, however the vast majority of cases are of delayed-type ACD which is the focus of our investigations (Rothe *et al.*, 2011). Due to its use in the cosmetics industry, as well as in a range of industrial processes including printing and plastics, PPD-induced ACD is a prominent source of occupational disability and morbidity (Jenkinson *et al.*, 2009b; Zhang *et al.*, 2009). Within hair dye formulations, 2% is the highest PPD concentration allowed by European law; although up to 15.7% has been reported for some henna tattoo dyes (Kind *et al.*, 2012). With PPD-containing products saturating 70% of the hair dye market, a market worth an estimated 16 billion euros in Europe alone in 2007, combined with the wide scale use of PPD in dyeing clothes, it is likely that the vast majority of the population is unknowingly and asymptomatically sensitised to PPD (Bonefeld *et al.*, 2010).

PPD undergoes spontaneous oxidation to an unstable quinonediimine intermediate which can trimerise to form the compound Bandrowki's Base (BB) within 16 hours (Faulkner *et al.*, 2014; Jenkinson *et al.*, 2009b). The "developer" which is combined with the colouring agent in hair dye formulations is an oxidant, thereby promoting a more rapid formation of BB within this setting (Bonefeld *et al.*, 2010). However, alternative oxidised forms of PPD may also be formed and have been shown to induce a local lymph node assay (LLNA) response and activate DCs (Aeby *et al.*, 2008). Metabolic detoxification of PPD can occur in epidermal keratinocytes due to the presence of NAT1, where acetylation products from PPD result in a negative LLNA (Aeby *et al.*, 2008). In allergic patients, both PPD and BB are known to stimulate T-cells, whereas T-cells in tolerant individuals only respond to BB; the aetiology of which remains undefined. It is clear that these BB responses are a result of sensitisation as proven by a lack of response when BB is cultured with cells from cord blood, indicating that BB is not just a simple mitogen (Coulter *et al.*, 2007b).

Previous studies show that after a short PPD exposure period (4hrs), PPD produced no T-cell responses, but after a longer period (16hrs) a response could be detected. Maximum irreversible PPD-protein binding takes 24 hours, as does antigen processing revealing that covalent binding of PPD to protein may be an important antigenic determinant (Coulter *et al.*, 2007b). Indeed in subsequent studies it was shown that PPD has the ability to stimulate T-cell clones from allergic individuals using both protein- and processing-dependant and independent routes (Jenkinson *et al.*, 2009a). As yet, no inter-individual susceptibility factors for PPD-induced allergyhave been described with any certainty.

Overall PPD represents an ideal model for the investigation of chemical-induced allergy as it has a well-defined chemistry, an available downstream product (BB) which is stable

enough to be utilised during *in vitro* culture, and its immune aetiology involves both PI and hapten-mediated T-cell activation.

1.9 Requirement for the development of *in vitro* assays.

Preclinical testing of drugs is a legal necessity in modern pharmaceutical practice. Testing of many of these agents was first carried out on suitable animal models however in recent years, due to ethical and moral obligations, use of animals is restricted for drug testing and the assessment of cosmetic sensitisation potential (7th amendment of the EU cosmetics directive [March 2009]).

In 2005, of the 12 million animals used in research, 8% were for proving toxicological safety (Martin *et al.*, 2010). In recent years there is a global effort within science as a whole to minimise the number of animals used in research however this requires there to be a suitable alternative. This has led to the development of *in vitro* assays that have the ability to assess the potential of drugs/chemicals and their metabolites to prime naïve T-cells.

1.10 Thesis aims and objectives.

The primary objective of this thesis was to explore the immunological aetiology of both drug- and chemical-induced hypersensitivity by utilising an *in vitro* human T-cell assay to explore antigen-specific T-cell responses from healthy donors. Of particular interest, we wanted to assess the role of co-inhibitory signalling on the regulation of these T-cell mediated reactions. To perform these investigations, we utilised a battery of biological

and functional assays on T-cells derived from both healthy donor and clinically diagnosed hypersensitive patient peripheral blood samples. Our specific aims are detailed below;

(1) To explore the role of PD-1/PD-ligand signalling on the regulation of drug antigen-specific T-cell responses.
(2) To analyse the potential for antigen-specific T-cells to secrete IL-17 and IL-22 and thus identify the role of the newly discovered T-cell subsets, Th17 and Th22, in the aetiology of hypersensitivity.
(3) To assess the comparative function and expression of the co-inhibitory CTLA4 and TIM-3 receptor pathways on drug antigen-specific T-cell responses.
(4) To characterise the activation of naïve T-cells from healthy donors by both SMX- and PPD-derived antigens.
(5) To investigate the existence and subsequent characteristics of pre-existing memory T-cells in healthy donors which are responsive to SMX- and PPD-derived antigens.

Chapter 2: Materials and Methods.

Contents

2.1 Reagents, drugs and chemicals.

Lymphoprep was purchased from Axis-Shield (Dundee, UK). Magnets, magnetic columns, and anti-body-conjugated magnetic bead kits for the isolation of cell subsets from PBMCs including $CD14^+$, pan-T, $CD25^+$, or $CD45RO^+$ populations were obtained from Miltenyi Biotec (Surrey, UK). A range of CD4 and CD8 conjugated fluorochromes for use in flow cytometry, along with CD1a-fluorescein isothiocyanate (FITC), CD11a-FITC, CD11c-phycoerythrin (PE), CD14-FITC, CD40-FITC, MHC class I-PE, MHC class II-FITC, PD-L1-PE, CD45RO-PerCP-Cy5, and the anti-mouse Ig / negative control compensation particles set were purchased from BD Biosciences (Oxford, UK). Additionally, PD-1-PE, CTLA4-allophycocyanin (APC*), TIM-3-PE, CD80-FITC, CD1a-FITC, and CD86-FITC were bought from Serotec (Kidlington, UK), CD83-PE was obtained from R&D systems (Minneapolis, USA), and galectin-9-APC* was bought from Biolegend (Cambridge, UK).

For their use in blocking studies mouse anti-human HLA-ABC (isotype, IgG_1; clone DX17), mouse anti-human HLA-DR/DP/DQ (isotype, IgG_{2a}; clone, Tu39), mouse anti-human HLA-DR (isotype, IgG_{2a}; clone G46-6), and mouse anti-human HLA-DQ (isotype, IgG_{2a}; clone, Tu169) monoclonal blocking antibodies and their respective isotype controls were obtained from BD Biosciences (Oxford, UK). Anti-human HLA-DP blocking antibodies were bought from Serotec (Kidlington, UK). LEAF purified (< 0.1 EU/µg endotoxin) mouse anti-human CD274 (PD-L1; isotype, IgG2b; clone, 29E.2A3), LEAF purified mouse anti-human CD273 (PD-L2; isotype, IgG1; clone, MIH18), LEAF purified mouse anti-human CD152 (CTLA4; isotype, IgG1; clone, L3D10), and LEAF purified mouse anti-human CD366 (TIM-3; isotype, IgG1; clone, F38-2E2) were azide-free and purchased from Biolegend (Cambridge, UK).

ELISpot multiscreen filter plates were bought from Millipore (Watford, UK), while the ELISpot kits containing coating and detection antibodies along with BCIP/NBT Plus liquid

substrate solution for IFN-γ, IL-13, IL-17, IL-22, IL-5, Granzyme B, and Perforin were obtained from Mabtech (Nacka Strand, Sweden). The ELISpot kit for Fas ligand was bought from Abcam (Cambridge, UK).

Recombinant human GM-CSF, recombinant human interleukin-2 (rhIL-2), and human IL-4 were purchased from Peprotech (London, UK). Foetal Bovine Serum (FBS) and Human AB Serum were bought from Invitrogen (Paisley, UK) and Innovative Research (Michigan, USA), respectively. Moravek (California, USA) supplied the tritiated [^{3}H]-methyl thymidine (5 Ci/mmol) while Meltilex scintillator sheets, sample bags, and printed glass fibre filter mats for thymidine analysis were obtained from Perkin-Elmer (Waltham, USA). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was bought from eBioscience (San Diego, USA). Cell culture flasks and nunc 6-, 24-, 48-, and 96-well plates were purchased from Thermo Scientific (UK). Purified tetanus toxoid (TT) was obtained from Statens seruminstitut (Copenhagen, Denmark). DNA-selective magnetic beads, protease, associated wash buffers and the Magnetic Separation Module-1 for the isolation of DNA came from Chemagen (Baesweiler, Germany). Cyclosporine-A was purchased from Fluka Analytical (Dorset, UK). Dimethyl sulfoxide (DMSO), Roswell Park Memorial Institute (RPMI)-1640, penicillin and streptomycin, transferrin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer), L-glutamine, Hanks balanced salt solution (HBSS), bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), sodium azide, and all other unlisted reagents were purchased from Sigma-Aldrich (Dorset, UK).

Sulfamethoxazole (SMX), carbamazepine (CBZ) and *p*-phenylenediamine (PPD) were purchased from Sigma-Aldrich (Dorset, UK), sulfamethoxazole-nitroso (SMX-NO) was bought from Dalton Pharma Services (Toronto, Canada), and Bandrowki's Base (BB) was

obtained from Apollo Scientific (Manchester, UK). Flucloxacillin was purchased from CP Pharmaceuticals (Wrexham, UK).

2.2 Media and Buffers.

R9 medium – 500 ml RPMI 1640, 100 µg/ml penicillin, 100 U/ml streptomycin, 25 µg/ml transferrin, 10% (v/v) human AB serum, 25 mM HEPES buffer, 2 mM L-glutamine.

F1 medium – 500 ml RPMI 1640, 100 µg/ml penicillin, 100 U/ml streptomycin, 10% (v/v) FBS, 25 mM HEPES buffer, 2mM L-glutamine.

FACS buffer – 500 ml HBSS, 10% (v/v) FBS, 0.2 mg/ml sodium azide.

MACS buffer (X 10) – 50 ml HBSS, 50 µg/ml BSA, 20 mM EDTA.

Phosphate buffered saline (PBS; X 10) - NaCl (80g), Na_2HPO^4 (11.6g), KH_2PO^4 (2g), KCl (2g). Dilute to 1 litre with distilled water, further 1:10 dilution required before use, final pH: 7.0.

2.3 Human Subjects.

Up to 120ml venous blood was collected from healthy drug-naïve donors, and from clinically diagnosed, patch-test confirmed SMX or PPD-allergic patients. Approval for the study was obtained from the Liverpool local Research Ethics Committee, and informed written consent was acquired from all participating blood donors.

2.4 Isolation of peripheral blood mononuclear cells from venous blood.

Whole blood was collected in vacutainer heparinised blood collection tubes. PBMCs were then isolated from both patient and healthy donor peripheral blood by density gradient separation using lymphoprep (Axis-shield; Dundee). Specifically, 25 ml blood was gently layered using a syringe on to 25 ml lymphoprep in a 50 ml tube (total of 4 x 50 ml tubes per average blood donation of 100 ml), and centrifuged at 2000 rpm for 25 mins with low acceleration and no brake to minimise disruption to the density layers formed during this process. From top to bottom the layers are easily identifiable by their individual colours; plasma (yellow), lymphocyte layer (cloudy white/grey), lymphoprep (transparent), erythrocyte layer (red). The lymphocyte layer was removed to a fresh tube using a sterile Pasteur pipette. The transferred lymphocyte suspension was then washed by diluting 10 ml of cell suspension with 40 ml HBSS. Tubes were centrifuged at 1800 rpm for 15 mins, after which the supernatant was discarded and the cells suspended in HBSS. Resuspended cell suspensions from two tubes were pooled and washed in HBSS. This process is depicted in Figure 2.1a-c. Cells were resuspended and the contents of the final two tubes were pooled and diluted to 40 ml in HBSS to be counted. To count the number of cells within a sample, a 10 µl aliquot was combined with a 10 µl aliquot of

trypan blue (0.2% w/v). After mixing, a 10 µl sample was analysed under a Leica DME microscope (Lecia Microsystems, Milton Keynes, UK) on a Neubauer haemocytometer. For PBMCs, cell viability was typically > 95%. Cell counts for all cell types were performed similarly.

Cells were then used in functional assays described in due course, or frozen for later use by resuspending them in R9 medium between 10-20 x 10^6 cells/ml and further diluting them 1:1 with "freezing mix" (80% human AB serum, 20% DMSO) in 1.8ml cryovials (total volume, 1 ml; maximum cell concentration per vial, 5 x 10^6/ml). Cryovials were placed in Mr. Frosty tubs and stored at -80°C for 24-48 hrs before being transferred to -150°C for longer term storage.

2.5 Lymphocyte Transformation Test.

To determine the presence of antigen-specific PBMCs in the peripheral circulation of allergic patients and healthy donors, we performed a lymphocyte transformation test (LTT; Figure 2.1d) as previously described (Castrejon *et al.*, 2010). Patient or healthy donor PBMCs were diluted to 1.5 x 10^6 cells/ml. 100 µl of cells (0.15 x 10^6) were pipetted to each well in a 96-well U-bottomed plate alongside 100 µl of drug (final well concentrations; SMX, 0.5-2 mM; SMX-NO, 5-50 µM; PPD or BB, 2-10 µM), R9 medium as a negative control, or tetanus toxoid (TT; 10 µg/ml) to non-specifically activate T-cells and thus act as a positive control. After a five day incubation of these cultures in a humidified atmosphere of 95% air / 5% CO_2 at 37°C, each well was pulsed with [^{3}H] thymidine (0.5 µCi/well). Following a further 16-hour incubation as described previously (Burkhart *et al.*, 2001; Castrejon *et al.*, 2010), cells were transferred on to filter mats and scintillation sheets melted over them. Analysis of incorporated radioactivity was

subsequently read using a Microbeta Trilux 1450 LSC (Perkin-Elmer, UK) which interpreted the data as radioactive counts per minute (cpm). A positive antigen-induced proliferative response was determined by calculating the stimulation index (SI; average cpm of drug-treated wells / average cpm of control wells), whereby an SI ≥ 1.6 was accepted as representative of drug-induced proliferation. Although an SI > 2 has been traditionally considered the boundary for T-cell activation, we have recently shown that T-cells where the SI equals 1.6 still respond to antigen in a similar albeit weaker fashion and remain antigen-responsive. Furthermore, this has been replicated for numerous drug antigens (manuscript in preparation; Faulkner, 2016).

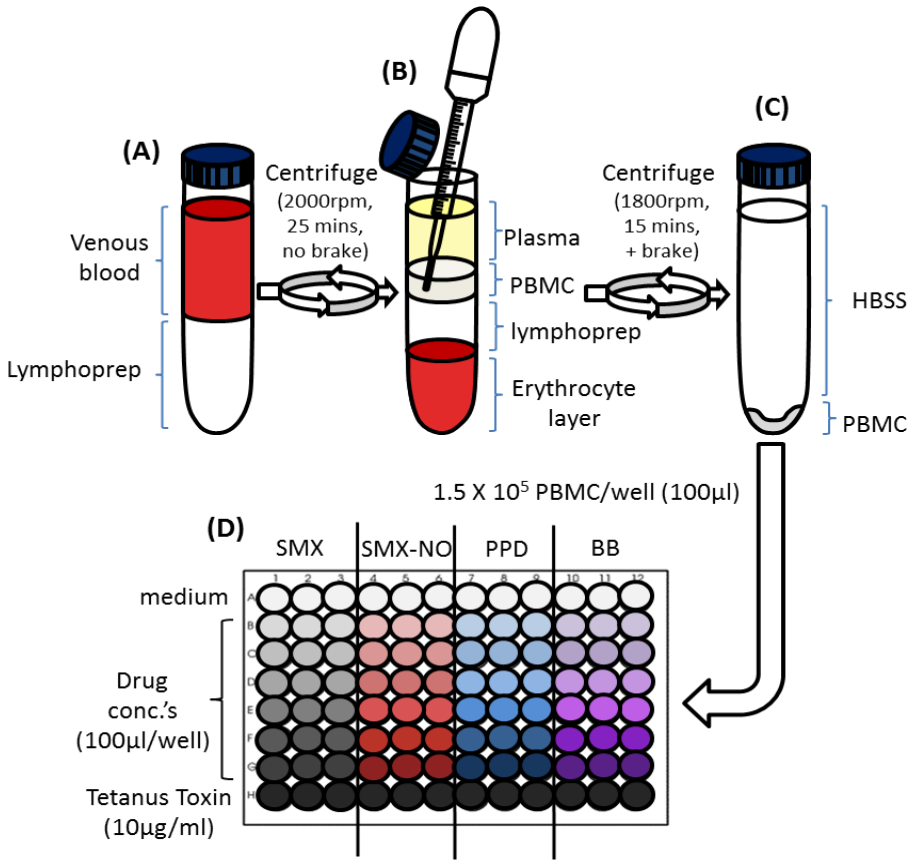


Figure 2.1 Isolation of PBMCs for use in a LTT. **(A)** Human venous blood was layered onto lymphoprep and centrifuged. **(B)** The cloudy middle layer containing PBMCs was transferred to a fresh tube, washed with HBSS, and centrifuged. **(C)** PBMCs were washed in HBSS. **(D)** PBMCs were resuspended in R9 medium at 1.5 x 10^6/ml and plated 100 µl/well in a 96-well U-bottomed plate. R9 medium (negative control), drug, or tetanus toxoid (positive control) were then added at 100 µl/well.

2.6 Isolation of distinct cell subsets from whole PBMCs using successive MACS magnetic bead separation columns.

For some assays, pure populations of $CD14^+$ monocytes, $CD45RO^-$ $CD45RA^+$ (naïve) T-cells, and $CD45RO^+$ $CD45RA^-$ (memory) T-cells were required. These different cell populations were separated from total PBMCs using magnetic beads and columns in a step by step process (Figure 2.2), according to the manufacturer's instructions (Miltenyi Biotec, Bisley, UK). For positive selection, antibodies specific for a required cell surface marker are bound to 50nm diameter, non-toxic, biodegradable, superparamagnetic particles to create MACS microbeads, which are subsequently able to bind to cells in the suspension which express that marker. Importantly, microbeads do not saturate the epitope on each cell thus allowing cells to maintain their functionality. The microbead-exposed cells are then passed through a column placed on a magnet, inside of which is a matrix of ferromagnetic spheres. The result is that cells bound to the magnetic beads via conjugated antibodies are retained within the column. The spheres are designed to enhance the magnetic field and thus ensure the efficient retention of cells which may only be minimally labelled within the column. Due to the magnetic properties of the beads and the lack of an efficient method to remove them after each successive isolation in a way that does not hinder cell functionality, cells which have been positively selected for cannot be subject to any further positive bead isolations. Negative selection refers to the use of antibodies which are selective for markers not found on the cell population of interest. Selections persistently achieved population purities of > 97% (Faulkner *et al.*, 2012). Therefore, there may be some contamination of memory T-cells in the naïve T-cell population, however the frequency is so low that false positive responses due to the presence of these cells would not likely be detected during *in vitro* priming cultures.

2.6.1 Positive selection of CD14$^+$ monocytes.

The first step in the process is the isolation of CD14$^+$ cells (Figure 2.2a). Total PBMCs were centrifuged at 1500 rpm for 10 mins and resuspended in MACS buffer (800 µl per 10^8 PBMCs) and CD14-specific microbeads (200 µl per 10^8 cells), before incubation at 10^{o}C for 15 mins. The cell suspension was then further diluted in MACS buffer (additional 15 ml per 10^8 cells) and centrifuged at 1500 rpm for 10 mins at 4^{o}C. The supernatant was discarded and the cells resuspended in MACS buffer (500 µl per 10^8 cells). A MACS LS column capable of binding up to 100 million cells was placed on to the magnet and prewashed with 3 ml MACS buffer, which passed through the column into the collection tube below. The cell suspension was then passed through the column before 3 x 3 ml MACS buffer washes were used to ensure all unlabelled cells had passed the length of the column and been eluted. The collection tube thus contained the CD14$^-$ cell population, and the column was removed from the magnet. The CD14$^+$ population was then extracted from the column by plunging 5 ml MACS buffer through the column into a new collection tube. Both CD14$^+$ and CD14$^-$ populations were counted as in section 2.4 before being centrifuged. CD14$^+$ cells were resuspended in R9 medium at 5-10 x 10^6 cells/cryovials in a 1:1 mixture with “freezing mix”. Cryovials were placed in Mr. Frosty tubs and stored at -80°C for 24-48 hrs before being transferred to -150°C for longer term storage.

2.6.2 Negative selection of CD3$^+$ T-cells.

CD14$^-$ cells were subject to further isolation for the isolation of the total T-cell population (Figure 2.2b), and so were resuspended in MACS buffer (400 µl per 10^8 cells) and pan T-cell antibody cocktail (100 µl per 10^8 cells). This cocktail consists of multiple antibodies

designed to positively select for cell surface markers not found on T-cells to allow for later positive selection within the T-cell population. Cells identified for selection by this cocktail include B-cells, stem cells, erythroid cells, DCs, monocytes, neutrophils, eosinophils, granulocytes, and natural killer (NK) cells, and uses a mixture of biotinylated antibodies: CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123, and CD235a. The cells were incubated for 10 mins at 10^{o}C in the presence of the antibody cocktail before the further addition of MACS buffer (300 µl per 10^8 cells) and anti-biotin microbeads (200 µl per 10^8 cells). This was followed by a second incubation at 10^{o}C for 15 mins. Cells were then further diluted in MACS buffer (1.5 ml per 10^7 cells), centrifuged, resuspended in MACS buffer (500 µl per 10^8 cells), and passed through a new LS column as previously described. The T-cells pass directly through the column while the non-T-cells are retained and are eluted by force using the plunger. Again, both populations are counted before centrifugation. Non-T-cells were frozen and stored as previously described in section 2.6.1.

2.6.3 Positive selection of CD25$^+$ (regulatory) and CD45RO$^+$ (memory) T-cell populations.

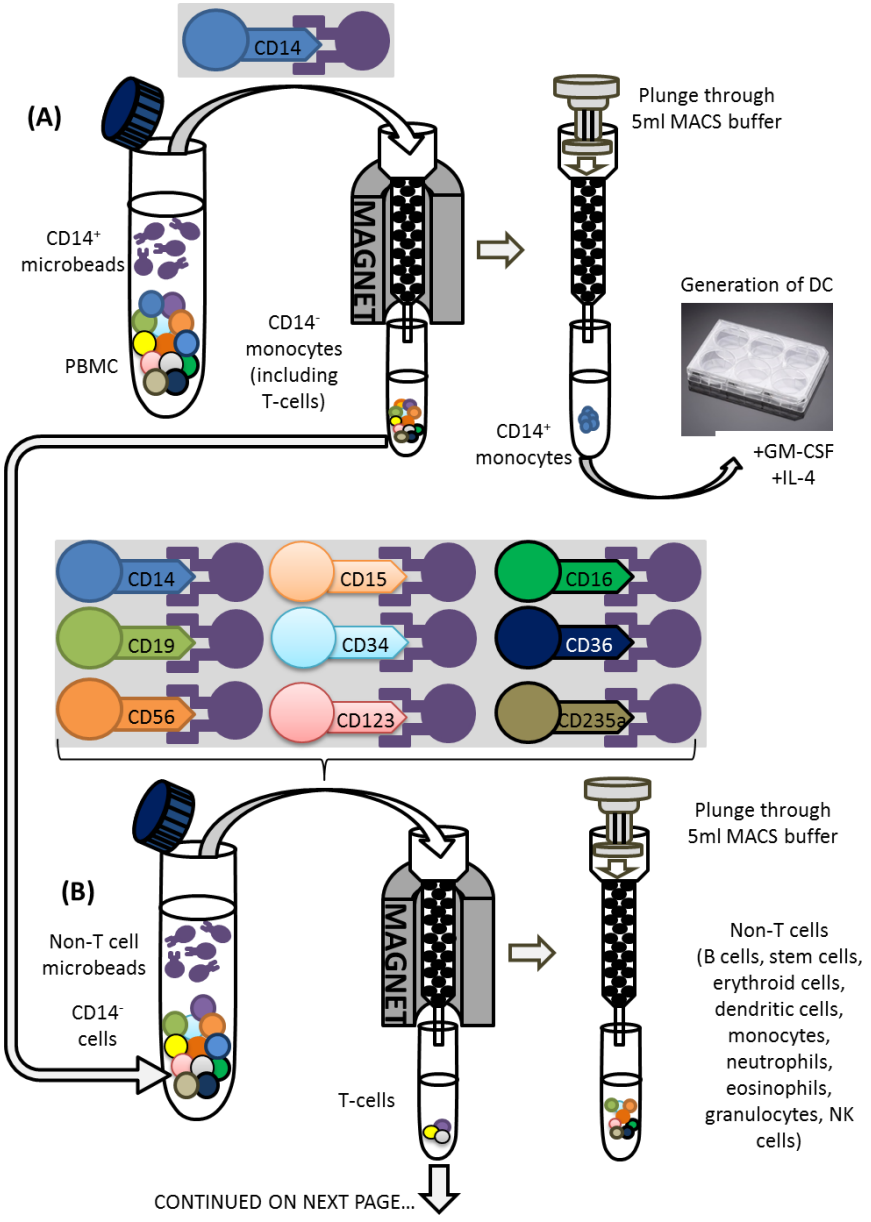
The T-cell population (CD3$^+$) was then subject to two final positive selection processes. First, T-cells were resuspended in MACS buffer (900 µl per 10^8 cells) and CD25$^+$ microbeads (100 µl per 10^8 cells) and incubated for 15 mins at 10^{o}C. Cells were then further diluted in MACS buffer (15 ml per 10^8 cells), centrifuged, resuspended in MACS buffer (500 µl per 10^8 cells), and passed through a new LS column as previously described. The CD25$^-$ T-cells pass directly through the column while the CD25$^+$ T-cells are retained and are eluted by force using the plunger (Figure 2.2c). Second, CD25$^-$ T-cells were resuspended in MACS buffer (800 µl per 10^8 cells) and CD45RO microbeads (200 µl

per 10^8 cells) and incubated for 15 mins at 10°C. Cells were then further diluted in MACS buffer (15 ml per 10^8 cells), centrifuged, resuspended in MACS buffer (500 µl per 10^8 cells), and passed through a new LS column as previously described. The $CD45RO^-$ T-cells pass directly through the column while the $CD45RO^+$ T-cells are retained and are eluted by force using the plunger (Figure 2.2d). Finally, all T-cell populations ($CD25^+$ T regulatory cells, $CD45RO^+$ memory T-cells, $CD45RO^-$ naïve T-cells) were counted and centrifuged before being frozen at 5-10 x 10^6 cells per cryovial in a 1:1 mixture with "freezing mix". All cells were frozen and stored as previously described in section 2.6.1.

2.7 Culture of dendritic cells from $CD14^+$ monocytes.

DCs are professional APCs and considered to be the only cell capable of the effective priming of naïve T-cells (Guermonprez *et al.*, 2002). To culture these cells for use *in vitro*, two vials of frozen $CD14^+$ monocytes were first thawed in a water bath (day 1, temperature < 30°C) and transferred to a fresh tube where R9 medium was added to dilute the DMSO from the "freezing mix" about 1:10. Cells were centrifuged at 1500 rpm for 10 mins and resuspended in R9 medium for counting. Cells were then further diluted with R9 medium to obtain cell concentrations of 1-2 x 10^6 cell/ml. To promote the differentiation of these cells into DCs, 800 U/ml GM-CSF and 800 U/ml IL-4 were added to the cell suspension as described in previous studies (Faulkner *et al.*, 2012), before plating 3 ml/well in a 6-well flat-bottomed plate. The DC culture was then incubated overnight in a humidified atmosphere of 95% air / 5% CO_2 at 37°C. The following day (day 2), each well was topped up with an additional 3 ml R9 medium containing 800 U/ml GM-CSF and 800 U/ml IL-4. Cells were fed on day 4 by carefully withdrawing and discarding 3 ml/well medium and replacing this with 3 ml/well R9 medium supplemented with 800 U/ml GM-CSF and 800 U/ml IL-4. On the penultimate day before use (day 6),

wells were fed as on day 4 but with the addition of the maturation factors LPS (1 µg/ml; bacterial source reference, Escherichia Coli 0111:B4) and TNF-α (25 ng/ml) before incubation overnight. The generation of DCs was assessed by identifying a panel of characteristic phenotypic markers (CD1a, CD11a, CD11c, CD14, CD40, CD80, CD83, CD86, CD274, MHC class I, MHC class II) as measured by flow cytometry, a technique described in detail later in section 2.12.



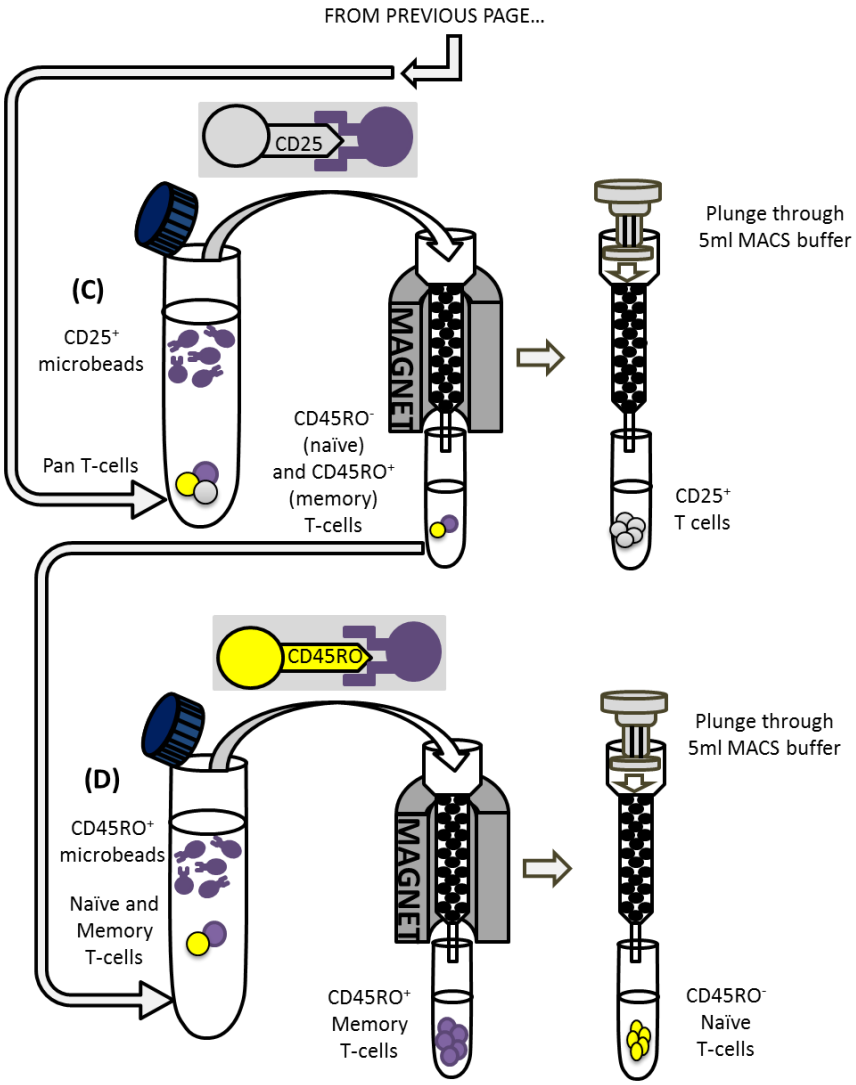


Figure 2.2. The stepwise magnetic bead-mediated isolation of distinct cell subsets from healthy donor PBMCs. **(A)** PBMCs labelled with CD14 microbeads are added to a column placed on a magnet. $CD14^+$ cells labelled with magnetic beads are retained on the column while $CD14^-$ cells are washed through. The column is removed from the magnet and the $CD14^+$ cells are forcibly eluted. **(B)** $CD14^-$ cells are incubated with a biotinylated antibody cocktail that binds to a range of cell surface receptors not associated with T-cells. Anti-biotin microbeads then bind to these antibodies. The cells are added to a column and T-cells are washed through. The non-T-cells are then forcibly eluted from the column. Two further separations are then performed on the T-cells. **(C)** The first involves positive selection of $CD25^+$ cells (Treg) and **(D)** the second involves positive selection of $CD45RO^+$ cells from the $CD25^-$ cells. Naïve ($CD45RO^-$) T-cells are washed through the column and $CD45RO^+$ (memory) T-cells are then forcibly eluted.

2.8 *In vitro* activation of naïve and memory T-cells from healthy donors using drug- or chemical-derived antigens.

In order to investigate both primary and secondary T-cell responses using cells derived from healthy donors, we assessed the discrete activation of naïve and memory T-cells in response to antigens (Figure 2.3). Specifically (day 7), frozen vials of naïve and memory T-cells were thawed using R9 medium, centrifuged at 1500 rpm for 10 mins and counted. Autologous mature DCs were harvested using a cell scraper due to the tendency for these cells to adhere to plastic, centrifuged and counted similarly. Mature DC were plated (0.8 x 10^5 per well) and cultured with naïve or memory $CD3^+$ T-cells (2.5 x 10^6 per well; 24-well flat-bottomed plate; total well volume, 2 ml) and drug. Most drug, metabolite, or chemical-antigens were weighed and dissolved in DMSO (SMX-NO, 50 mM; SMX, 39.5 mM; PPD or BB, 40 mM; CBZ, 40 mM). The stock solutions were then diluted in R9 medium to the required assay concentration, ensuring that the final DMSO concentration was < 0.5% and thus considered non-toxic. Flucloxacillin was diluted directly in R9 medium.

Where indicated, low endotoxin and azide-free (LEAF) purified anti-human PD-L1 or LEAF purified anti-human PD-L2 (5 µg/ml), LEAF purified anti-human CTLA4 (10 µg/ml), or LEAF purified anti-human TIM-3 antibodies (7.5 µg/ml) were added to certain wells for the assessment of immune-inhibitory pathway function. LEAF purification is associated with endotoxin < 0.01 EU/µg. The antibodies were added to specific wells along with T-cells and mature DC and incubated for ≥ 30 mins in a humidified atmosphere of 95% air / 5% CO_2 at 37°C to allow for antibody binding prior to addition of drug. Antibody concentrations were optimised in dose ranging studies around the concentration suggested by the supplier.

Drug- or chemical-derived antigen was then added (final well concentration; SMX, 1 mM; SMX-NO, 40 µM; PPD, 1-30 µM; BB, 5 µM; flucloxacillin, 1 mM). Any culture using flucloxacillin was performed using penicillin and streptomycin-free R9 medium to prevent cross-reactivity. Cultures were then incubated in a humidified atmosphere of 95% air / 5% CO_2 at 37°C for 8 days. All experiments were performed at least three times using cells from different blood donors with no previous history of sulfonamide or flucloxacillin exposure, or in the case of PPD and BB, no known allergic reaction to PPD-containing products. During the priming culture, a new batch of mature DCs was generated (from day 7 to day 15) from frozen autologous $CD14^+$ monocytes as described previously in section 2.7. Cell culture concentrations, well densities, and culture lengths had been previously optimised (Faulkner *et al.*, 2012).

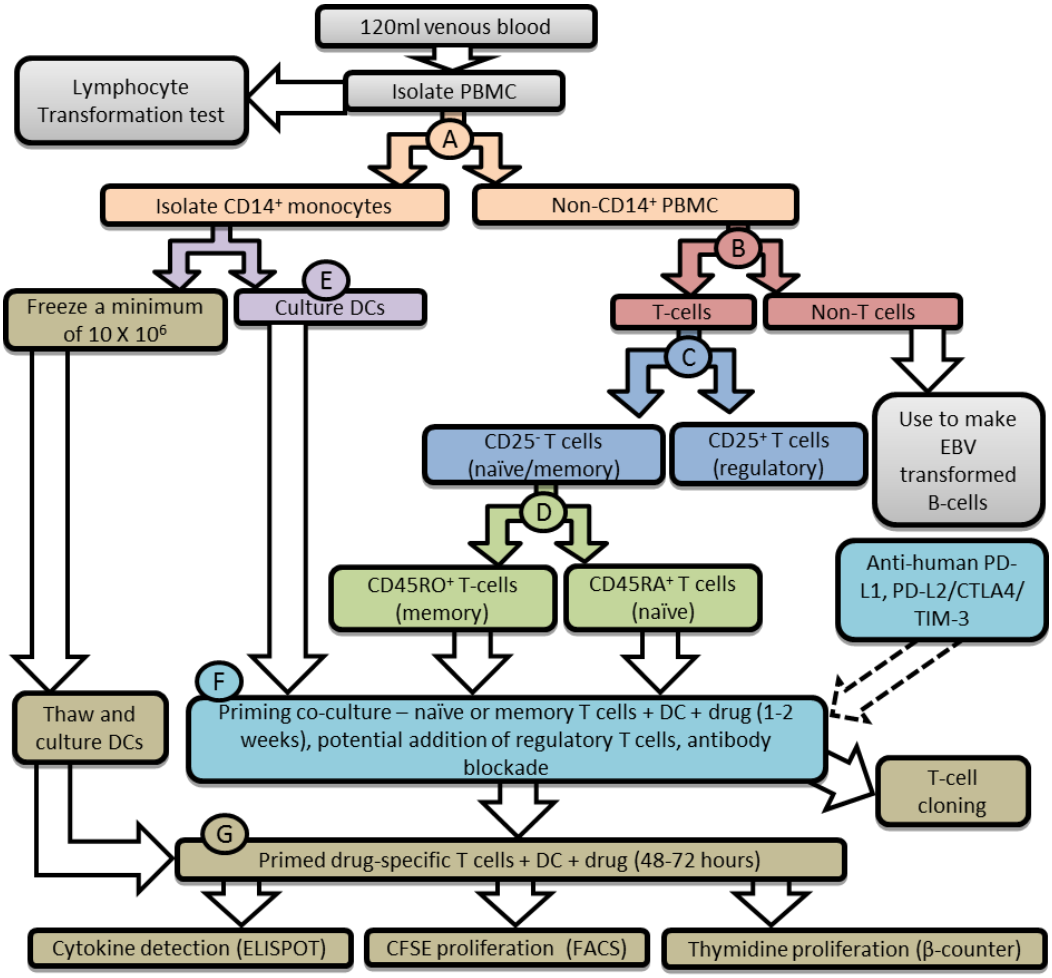


Figure 2.3 The *in vitro* T-cell activation assay. PBMCs are isolated from venous blood (Figure 1). **A:** $CD14^+$ cells are isolated by positive selection; **B:** $CD3^+$ T cells are isolated by negative selection from $CD14^-$ population; **C:** $CD25^+$ cells (Tregs) are removed by positive selection from the T cell population; **D:** $CD45RO^+$ cells (memory) are removed from the $CD25^-$ T cell population resulting in $CD45RO^-$ T-cells (naïve) and $CD45RO^+$ T-cells (memory). **E:** $CD14^+$ monocytes are differentiated into DC and then matured. **F:** Naïve or memory T-cells are cultured with mature DC and drug. Anti-coinhibitory receptor antibodies may be added to the T-cell / DC culture for 30 mins before the addition of drug. **G:** T-cells are harvested after 1 week and used for T-cell cloning, or combined with fresh mature DC and drug in a range of functional readouts.

2.9 [^{3}H] Thymidine incorporation to measure antigen-specific T-cell proliferation from *in vitro* activated healthy donor naïve and memory T-cells.

In order to detect antigen-specific T-cell responses from naïve and memory T-cell cultures, we measured the extent of T-cell proliferation in response to antigen exposure. Priming cultures were harvested (day 15), with each condition kept separate in a

different tube i.e. with and without PD-L1 block. The cultures were centrifuged at 1500 rpm for 10 mins, resuspended in R9 medium and counted. Primed naïve or memory T-cells were diluted in R9 medium to 1 x 10^6/ml. The cultured mature DCs were harvested using a cell scraper, centrifuged at 1500 rpm for 10 mins, resuspended in R9 medium, counted, and diluted to 0.8 x 10^5/ml.

1 x 10^5 T-cells were then plated per well (triplicate cultures per condition) in a 96-well plate (100 µl) along with 4 x 10^3 mature DCs (50 µl). Finally, 50 µl of R9 medium or Phytohemaglutinin (PHA; 20 µg/ml) were added as negative and positive control wells, respectively. To the remaining wells, 50 µl of drug was added (SMX-NO, 5-50 µM; SMX, 0.5-2 mM; PPD, 1-30 µM; BB, 2-10 µM; flucloxacillin, 0.5-2 mM; CBZ, 40-200 µM) before incubation for 48 hrs in a humidified atmosphere of 95% air / 5% CO_2 at 37°C. On day 17, each well was then pulsed with [^{3}H] thymidine (0.5 µCi/well). Following a further 16-hour incubation, cells were transferred on to filter mats and scintillation sheets melted over them (day 18). Analysis of incorporated radioactivity was subsequently read using a β-counter which interpreted the data as cpm. A positive antigen-induced proliferative response was determined by calculating the stimulation index (SI; average cpm of drug-treated wells / average cpm of control wells), whereby an SI ≥ 1.6 was accepted as representative of drug-induced proliferation.

2.10 Detection of IFN-γ, IL-13, IL-17, and IL-22 secretion from healthy donor naïve and memory T-cells using ELISpot.

On the day prior to setting up the T-cell readout assays (day 14), 96-well ELISpot plates were pre-incubated with a coating-antibody (Figure 2.4a); an antibody that binds specifically to the surface membrane at the bottom of each well and which is specific for the molecule in question. Firstly, the membrane at the bottom of each well was pre-

wetted with 35% ethanol (20 µl) which was subsequently washed out after 2 mins by five 200 µl distilled water washes. The antibodies were then diluted from their stock concentrations (IFN-γ, diluted from 1 mg/ml to 15 µg/ml; IL-13, IL-22, IL-17, diluted from 0.5 mg/ml to 10 µg/ml) in HBSS before 100 µl of coating antibody was then pipetted into each well and incubated overnight at 4^{o}C.

The following day (day 15, the same day as setting up the proliferation assay in section 2.9), both primed T-cells and mature DCs were harvested, counted, and diluted to stock concentrations as described in section 2.8. The pre-coated ELISpot wells were then subject to five 200 µl washes with HBSS to remove any unbound coating antibody. After each wash the washing buffer was discarded. After this, 200 µl R9 medium was added to each well for > 30 mins to block any remaining space on the well membrane to prevent non-specific binding. After ≥ 30 mins, the R9 medium was discarded from each well and replaced with 1 x 10^5 T-cells (100 µl) and 4 x 10^3 mature DCs (50 µl). 50µl of R9 medium or PHA (20 µg/ml) were added to negative and positive control wells, respectively. To the remaining wells, 50 µl of drug was added (SMX-NO, 5-50 µM; SMX, 0.5-2 mM; PPD, 1-30 µM; BB, 2-10 µM; flucloxacillin, 0.5-2 mM) before incubation for 48 hrs in a humidified atmosphere of 95% air / 5% CO_2 at 37°C (Figure 2.4b). Each condition was performed in duplicate.

On day 17, the ELISpot plates were washed five times with 200 µl HBSS. After each wash the washing buffer was discarded. A biotin-conjugated detection antibody was then added which specifically adhered to the cytokine-bound coating antibody (Figure 2.4c). Specifically, the detection antibodies were diluted from their stock concentrations in HBSS-0.5% FBS (IFN-γ, IL-17, IL-13, diluted from 0.5 mg/ml to 1 µg/ml; IL-22, diluted from 0.5 mg/ml to 0.5 µg/ml). 100 µl of detection antibody was then added per well and incubated at room temperature for 2 hrs.

The plates were then washed five times with 200 µl HBSS. After each wash, the washing buffer was discarded. For wells dedicated to the detection of IFN-γ, IL-13, and IL-17, streptavidin-conjugated to ALP was diluted 1:1000 in HBSS-0.5% FBS. The streptavidin binds to the biotinylated part of the detection antibody while the ALP provides a functional enzyme for transformation of a colour substrate added during a later stage of the process. For IL-22 detection, streptavidin-horseradish peroxidase (HRP) conjugate was used which also required a 1:1000 dilution with HBSS-0.5% FBS prior to use. 100 µl Streptavidin-ALP or -HRP was added to each well and incubated at room temperature for 1 hr (Figure 2.4d).

Wells were once again washed with 200 µl HBSS, with each wash being discarded to remove excess conjugate from the previous step. The colour substrate (TMB for IL-22, BCIP-NBT+ for IFN-γ, IL-13, and IL-17) was sterile filtered (0.45 µm) before being added 100 µl per well (Figure 2.4e). Plates were immediately incubated at room temperature in the dark for 10-15 mins. Upon spot formation (Figure 2.4f), plates were washed with tap water and air dried overnight. The following day, spots were counted using an AID ELISpot reader (Cadima Madical, Stourbridge, UK).

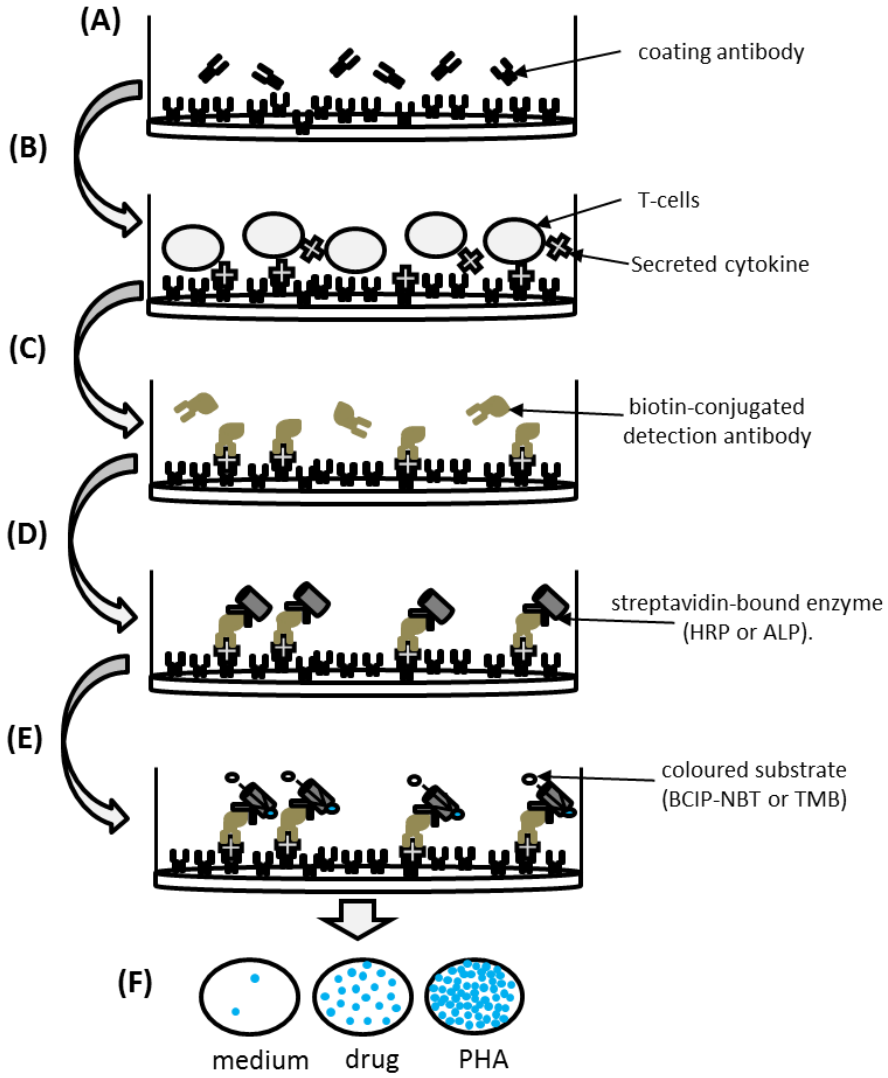


Figure 2.4. Detection of cytokine secretion using ELISpot (A) Cytokine-specific coating antibody is added and incubated overnight at 4°C, **(B)** The plate is washed and the membrane blocked with R9 medium. T-cells, APCs and drug are added and incubated for 48 hrs at 37°C, **(C)** The plate is washed, and biotin-conjugated detection antibody is added and incubated for 2 hrs at room temperature. **(D)** The plate is washed. Streptavidin-bound enzyme (HRP or ALP) is added and incubated for 1 hr at room temperature. **(E)** The plate is washed and substrate added (BCIP-NBT or TMB). Spot development is monitored for ~15 mins (in dark). **(F)** The plate is washed and left to dry overnight.

2.11 Carboxyfluorescein diacetate succinimidyl ester staining to measure of antigen-specific T-cell proliferation.

CFSE can passively enter cells. Once inside, the fluorescent carboxyfluorescein molecule is generated by cleaving the acetate groups of CFSE. The amount of fluorescence per cell

is halved with each cell division and thus CFSE can be used as an alternative method by which to assess antigen-specific T-cell proliferation. Fluorescence intensity is measured using flow cytometry, a technique outlined in figure 2.6.

Priming cultures were harvested (day 15), with each condition kept separate in a different tube i.e. with and without PD-L1 block. The cultures were centrifuged at 1500 rpm for 10 mins, and resuspended in HBSS-5%-FCS to achieve a cell concentration of 1 x 10^6/ml. 200 µl was removed and plated into two wells (100 µl/well) of a 96-well plate to form a CFSE-unstained control. The remaining cells were then subject to CFSE staining. Each tube was laid on its side with the end slightly elevated to prevent cell spillage, and a 50 µl droplet of HBSS was placed on the dry, upper-end of the tube. 5 mM CFSE (0.5 µl) was then pipetted into the HBSS droplet and the tube carefully recapped. The tubes were immediately inverted several times and vortexed to ensure rapid and efficient dispersion of CFSE to all cells. All tubes were then incubated at room temperature in the dark for 5 mins before each tube was subject to two washes with 5 ml HBSS-5%-FCS to remove any free CFSE. The supernatant was discarded after each wash cycle. The cells were resuspended in R9 medium, counted, and once again diluted in R9 medium to 1 x 10^6/ml.

The cells were then set up in duplicate as described in section 2.9 and incubated for 72 hrs in a humidified atmosphere of 95% air / 5% CO_2 at 37°C (Figure 2.5). Two additional wells of CFSE labelled cells were used as controls.

Duplicated wells per condition were then reduced to half volume and combined (day 18). Plates were centrifuged at 2500 rpm for 3 mins at 4^{o}C. After discarding the supernatant, cells were resuspended by gently tapping the edges of the plate and adding 3 µl CD4-APC* and 3 µl CD8-PE. Plates were incubated on ice in the dark for ≥ 20 mins to allow for antibody-bound fluorochromes to bind to corresponding cell surface markers. To each

well, 200 µl FACS buffer was added and the plates were again centrifuged at 2500 rpm for 3 mins at 4°C. The supernatant was discarded and each well was resuspended in 100 µl FACS buffer and transferred to individual FACS tubes pre-containing 200 µl FACS buffer (total volume 300 µl). Tubes were stored at 4°C for no more than a few hours before use.

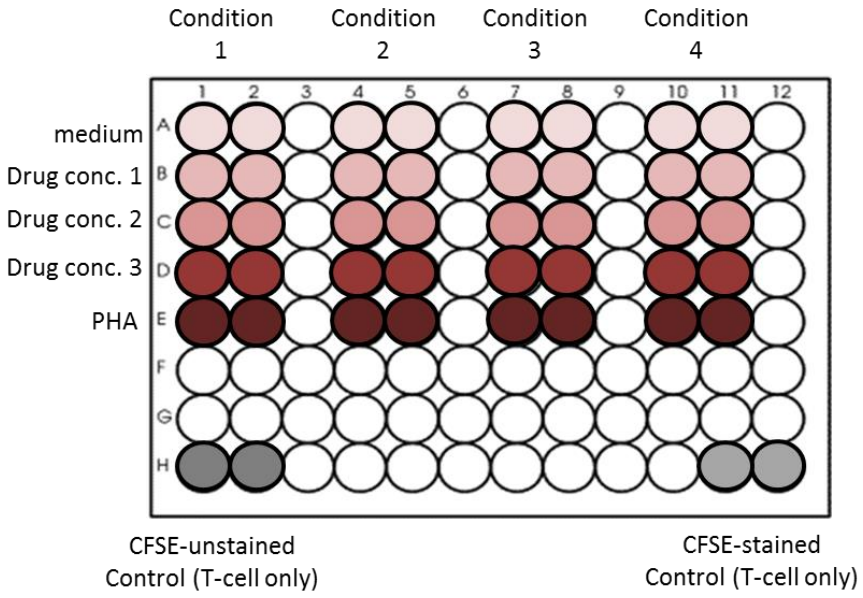


Figure 2.5 Plate Map for CFSE analysis. Each condition is set up in duplicate. After 72 hrs of culture, the contents of duplicate wells are combined and the cells stained with fluorescently labelled antibodies specific for CD4 and CD8 cell surface markers. Both CFSE-stained and unstained control wells are plated apart to prevent CFSE contamination in the negative control.

2.12. Flow cytometry.

Flow cytometry allows for the detection of specific markers on the surface of cells using antibodies specific for the marker in question which are conjugated to fluorochromes. If multiple markers are used within a single sample, the individual fluorochromes must be associated with different light wavelengths (colours) in order to distinguish between them (Figure 2.6).

2.12.1 Flow cytometry for the analysis of CFSE incorporation.

Due to the combination of fluorochromes in each well (CD45RO-PerCP-Cy5, CD4-APC*, CD8-PE, CFSE-FITC), to run flow cytometric analysis we needed to set up compensation samples to prevent fluorescence overlap which could provide false positive spectra.

One drop of anti-mouse Ig and one drop of negative control compensation particles were added to tubes each filled with 200 µl FACS buffer. 3 µl of each antibody were added to individual tubes and a final tube was left unstained to act as a non-fluorescent bead control. CFSE stained cells were used for the FITC compensation control (section 2.11). All tubes were incubated at 4^{o}C before use. Flow cytometry was performed using a FACS-Canto II machine with associated FACS-DIVA software (BD Biosciences). In some cases, antibodies specific for other cell surface markers were added e.g. PD-1-PE for independent analysis on dividing and non-dividing cells. To perform this analysis, T-cells, and then dividing and non-dividing subsets had to be gated for as in figure 2.7, before choosing to analyse PD-1 expression (analysis using cyflogic software, CyFlo Ltd, Finland).

2.12.2 Assessment of DC and T-cell phenotype using flow cytometry.

The T-cell surface expression of a range of other markers was also determined during priming and following restimulation. Staining was carried out using 3 µl of CD4-APC*, CD8-PE, PD-1-PE (CD279), CTLA4-APC* (CD152), and/or TIM-3-PE (CD366) antibodies to 5-10 x 10^3 cells in FACS tubes. Likewise, we also analysed the phenotype of our monocyte-derived mature DCs by staining 5-10 x 10^3 cells with CD1a-FITC, CD11a-FITC, CD11c-PE, CD14-FITC, CD40-FITC, CD80-FITC, CD83-PE, CD86-FITC, MHC class I-PE, MHC class II-FITC, PD-L1-PE, and/or galectin-9-APC* antibodies. All samples were incubated

on ice in the dark for ≥ 20 mins. Cells were then washed with 1 ml FACS buffer and centrifuged at 1500 rpm for 10 mins at 4°C. The supernatant was discarded, and cells resuspended in 200 µl FACS buffer. All tubes were incubated at 4°C for no more than a few hours before use.

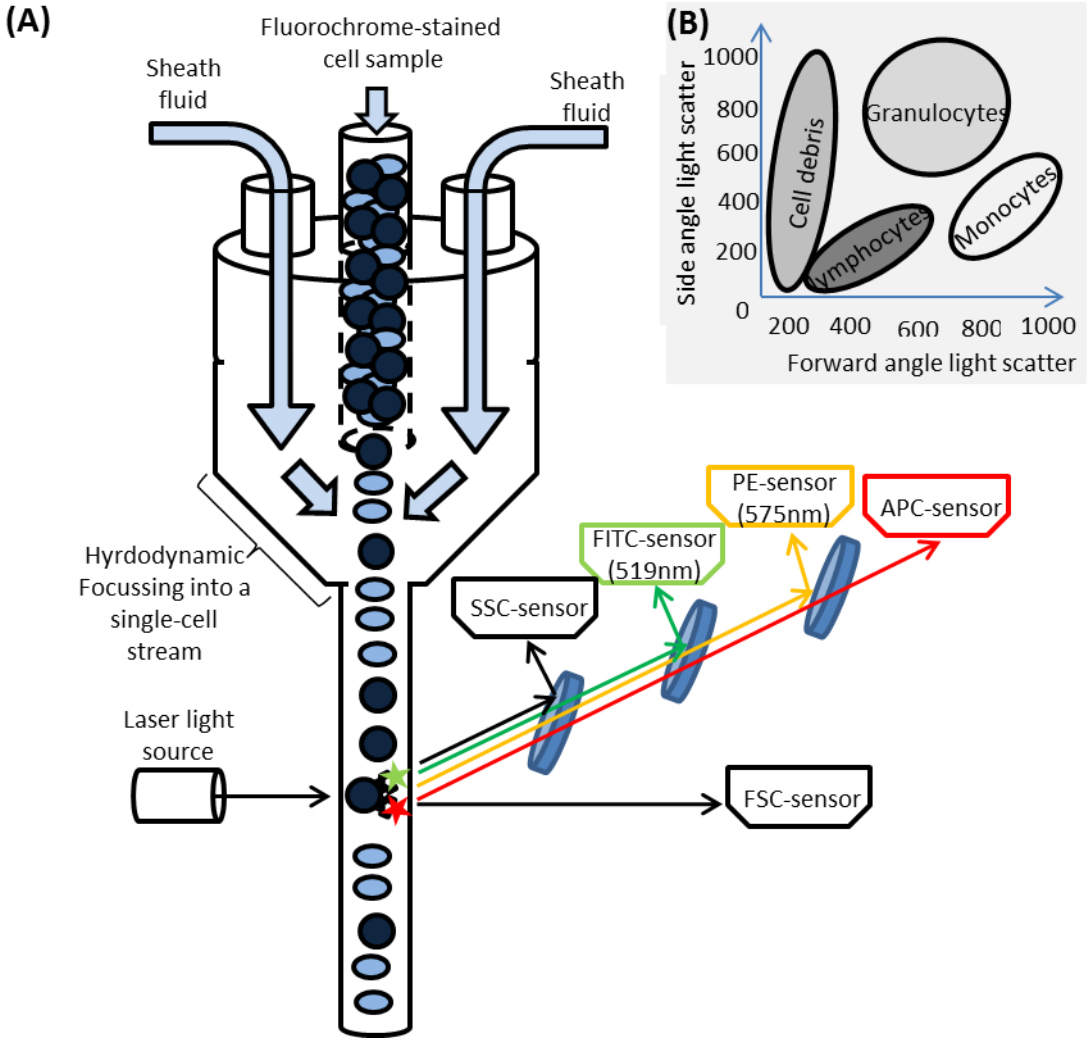


Figure 2.6. Cell phenotyping using flow cytometry. The cell population is stained with fluorochrome-bound antibodies. If multiple markers are used within a single sample, the individual fluorochromes must be associated with different light wavelengths in order to distinguish between them**. (A)** Cells pass through the flow cell as a single cell stream in fast flowing sheath fluid (hydrodynamic focussing). As each cell passes through the path of the laser light, fluorochromes bound to markers on the cell surface emit light at their respective wavelengths, which is subsequently detected by a range of sensors specific for different wave-bands. **(B)** Additional information based on the differential refraction of light based upon cell size and granularity is detected by the side scatter (SSC)- and forward scatter (FSC)-sensors to provide information indicative of particular cell populations.

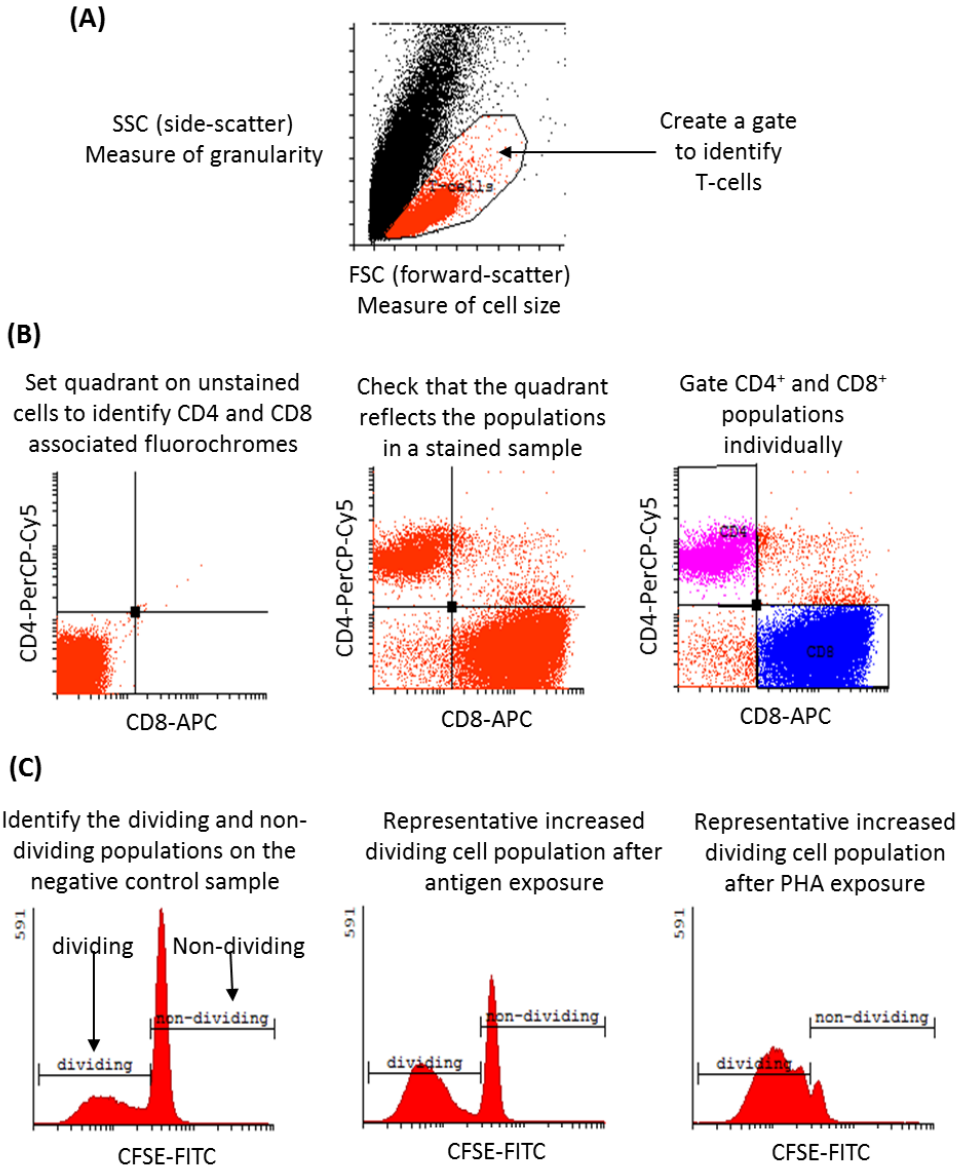


Figure 2.7. Representative analysis of CFSE flow cytometry data using Cyflogic software. **(A)** The T-cell population is gated on a dot plot of FSC and SSC. **(B)** This gated population is analysed in a new dot plot for CD4 and CD8 expression using quadrant analysis set using an unstained sample. The $CD4^+$ and $CD8^+$ T cells are then identified and gated using a stained sample. **(C)** The amount of CFSE (FITC channel) can be analysed in whole T-cells, $CD4^+$ or $CD8^+$ T-cells separately using histogram analysis. As the CFSE content of a cell is split when it divides, the peak representing high CFSE expression is gated as 'non-dividing' and those peaks with lower CFSE content are gated as 'dividing'. An antigen specific response can be seen when the dividing cell population increases in response to antigen exposure. PHA can be used as a positive control when the majority of the cells divide.

2.13 T-cell cloning.

2.13.1 Generation of Epstein-Barr virus-transformed B-cell lines.

Due to the long-term culture of T-cell clones and requirement for multiple assays, we used autologous EBV-transformed B-cells as functional APCs which replicate indefinitely. To generate these cell lines for an individual patient or donor, the supernatant of EBV-producing B9.58 cells (5 ml) was first filtered (0.22 µm) to remove any B9.58 cells, and then used to suspend PBMCs (5 x 10^6). This supernatant contains EBV virus DNA, which is able to enter, and incorporate into the DNA of B-cells within the donor PBMC suspension to generate transformed B-cells capable of continuous replication (Tosato and Cohen, 2001). Cells were incubated at 37°C in an atmosphere of 95% air / 5% CO_2 for 24 hrs in the presence of CSA (1 µg/ml). CSA, whilst widely toxic, acts to predominantly induce T-cell death and so promotes the survival of a purely B-cell derived population. Cells were subsequently washed, suspended in F1 medium and plated in a 24-well flat-bottomed plate in CSA-supplemented F1 medium for 3 weeks which was renewed every 3 days. From week 3, CSA was withdrawn from culture medium to allow for enhanced B-cell expansion, upon which time cells were transferred to tissue culture flasks and fed bi-weekly.

2.13.2 Generation of antigen-responsive bulk T-cell cultures prior to T-cell cloning from patient PBMCs.

To expand the number of antigen-responsive PBMCs, patient T-cells were cultured with a specific antigen for 2 weeks. Firstly, PBMCs were counted and diluted in R9 medium to 3 x 10^6/ml and plated 1 x10^6/well (330 µl) in a 48-well plate. Secondly, drug- or chemical-derived antigen concentrations were prepared (SMX, 2 mM; SMX-NO, 80 µM; PPD and

BB, 10 µM) and 330 µl of a specific antigen was added to each well giving final antigen concentrations of 1 mM SMX, 40 µM SMX-NO, 5 µM BB, and 5 µM PPD. T-cell clones derived from hypersensitive patients almost certainly are derived from the memory compartment. Thirdly, cultures were incubated in a humidified atmosphere of 95% air / 5% CO_2 at 37°C for 14 days. During this period, 330 µl IL-2 supplemented (200 U/ml) R9 medium was added on days 6 and 9 to increase the number drug-antigen specific T-cells.

2.13.3 Serial dilution of bulk cultures to generate T-cell clones.

T-cells from the priming assay (i.e. from drug naïve donors), or day 14 bulks from drug-hypersensitive patients, were cloned directly by serial dilution and repetitive mitogen-driven expansion using previously described methods (Mauri-Hellweg *et al.*, 1995). Bulks were counted and diluted in R9 medium to 1 x 10^4/ml.

PBMCs were isolated from the venous blood of an allogeneic donor as in section 2.4. PBMCs were resuspended in R9 medium, and irradiated for 15 mins to prevent cell proliferation while maintaining cell integrity. Irradiated PBMCs were then diluted to 5 x 10^5/ml and supplemented with IL-2 (200 IU/ml) and PHA (5 µg/ml), which was then termed the “restimulation cocktail”. For each condition 3 x 35 ml tubes of restimulation cocktail were used. 10 µl, 35 µl, and 105 µl of diluted bulk culture was then added to each tube so that upon plating at 100 µl/well the following cell concentrations were achieved: 0.3 cell/well, 1 cell/well, and 3 cells/well, respectively. This approach to plating allows for minor errors during counting. The major disadvantage of this technique is that it permits the potential for a well to be seeded with more than 1 cell and the development of a T-cell line rather than a clone. While this can often be determined at a later date by scrutinising the function and phenotype of ach potential clone, this

provides a potential reason for the functional blockade of both MHC class I and II on what was determined a $CD4^+$ T-cell clone in Chapter 5. Each 35 ml tube was plated into 3 x 96-well plates (100 µl/well) giving a total of 9 plates per bulk culture condition. Cultures were then incubated in a humidified atmosphere of 95% air / 5% CO_2 at 37°C for 14 days. Each well was fed on day 5 and then every subsequent 2 days with IL-2-supplmented (200 IU/ml) R9 medium.

2.13.4 Restimulation and growth of T-cell clones.

Serial dilution well volumes were reduced to 80 µl. PBMCs from an allogeneic donor were then isolated from venous blood as in section 2.4, and resuspended in R9 medium at 5 x 10^5 cells/ml. PBMCs were subject to irradiation for 20 mins, to prevent their proliferation while maintaining cell surface marker expression, before the medium was supplemented with IL-2 (400 IU/ml) and PHA (10 µg/ml) to form the "restimulation cocktail". 50 µl restimulation cocktail was added to each well and incubated in a humidified atmosphere of 95% air / 5% CO_2 at 37°C for a further 14 days, feeding every 2 days with IL-2 (200 IU/ml)-supplemented R9 medium.

Growth of a T-cell clone resulted in the formation of a visible cell pellet in the bottom of a well. These cells were then transferred and split between 2 wells in a new 96-well plate and are referred to as "picked clone" plates. These plates were also fed every 2 days with IL-2 (200 IU/ml)-supplemented R9 medium. When a large visible pellet was seen in the picked clone plates, the wells were split into a total of 4 wells total per clone. Continued expansion of the clones in the 96-well plate was supported by restimulation every 2-3 weeks using the protocol mentioned above.

2.13.5 Initial testing of T-cell clones for antigen specificity.

Once a clone had been expanded to at least 4 wells in a "picked clones" 96-well plate, it was tested for antigen specificity. The volume of 2 wells was reduced to 100 µl and the 2 wells combined. Then the cells were transferred into 4 wells (50 µl/well) in a new 96-well plate for testing. Autologous EBV-transformed B-cells were transferred to a 50ml tube, centrifuged, and resuspended in R9 medium. Following counting, the EBV cells were irradiated for 20 mins to prevent cell proliferation but maintain the ability of these cells to process and present antigen. EBV-transformed B-cells were diluted to 0.2 x 10^6 cells/ml and plated 1 x 10^4 per well (50 µl) into wells containing the clones to be tested. Two of the wells then received 50 µl R9 medium as a negative control, while the other two received 50 µl drug (final well concentration; SMX, 1 mM; SMX-NO, 40 µM; PPD and BB, 5 µM). Test plates were incubated for 48 hrs in a humidified atmosphere of 95% air / 5% CO_2 at 37°C. Each well was then pulsed with [^{3}H] thymidine (0.5 µCi/well). Following a further 16-hour incubation, analysis of incorporated thymidine was performed as described in section 2.5.

The remaining 2 wells in the 96-well "picked clones" plate, for clones deemed antigen-specific, were transferred to sterile 3 ml tubes and centrifuged at 1500 rpm for 10 mins at room temperature. Each tube was resuspended in 330 µl R9 medium and transferred to a single well in a 48-well plate to be restimulated. The restimulation cocktail for a 48-well plate is formed of irradiated allogeneic PBMCs which have been diluted to 1.5 x 10^6/ml in R9 medium supplemented with IL-2 (400 IU/ml) and PHA (10 µg/ml). 330 µl restimulation cocktail is then added to each well and incubated in a humidified atmosphere of 95% air / 5% CO_2 at 37°C. The next day, and every two days thereafter, each well was fed with 330 µl R9 medium supplemented with IL-2 (200 IU/ml). Clones were restimulated using this protocol every 2-3 weeks. During this time, expanding

clones which turned the medium yellow were split into new wells to maintain growth. During this expansion period (about 2 weeks) functional assays could not be performed as this non-drug-specific stimulation leads to high background in the assays. A flow diagram of the cloning procedure can be seen in figure 2.8.

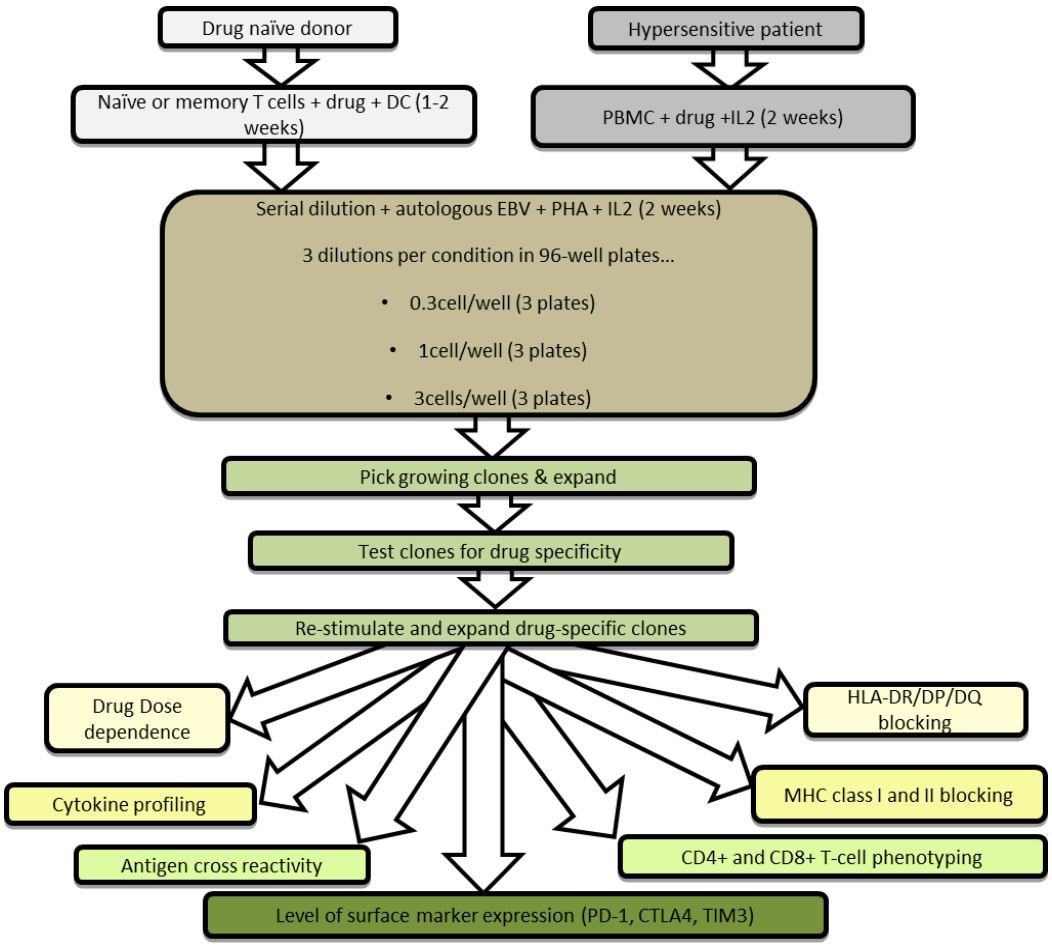


Figure 2.8. T-cell cloning from patients and drug-naive donors. T-cells from drug-naïve were primed to a specific antigen using the DC priming assay (see Figure 2.2). PBMCs from patients were cultured with an antigen and IL-2 for 2 weeks. The cells were harvested and plated at 0.3, 1.0, and 3.0 cell/well in a 96-well plate. After 2 weeks, cells were restimulated with allogeneic irradiated PBMCs, PHA, and IL-2. Growing clones were selected and expanded. The cells were tested for antigen specificity and the drug-specific clones were then restimulated for further phenotyping and functional assays.

2.13.6 T-cell clone functional proliferative assays determined by [^{3}H] thymidine incorporation.

Functional studies for T-cell clones included antigen dose-response, antigen cross-reactivity, assessing the role of co-inhibitory molecules during T-cell activation using antibody-mediated blockade, and the role of MHC class I and II in antigen presentation using antibody-mediated blockade.

Each clone was harvested and transferred to a sterile 50 ml tube before centrifugation at 1500 rpm for 10 mins at room temperature. The supernatant was discarded and the clones resuspended in R9 medium, counted, and subsequently diluted to 5 x 10^5 cells/ml. Autologous EBV-transformed B-cells were also harvested and centrifuged similarly. EBVs were resuspended in R9 medium, counted, irradiated, and diluted to 2 x 10^5 cells/ml. 5 x 10^4 T-cell clones (100 µl) and 1 X 10^4 irradiated EBV-transformed B-cells (50 µl) were pipetted into a 96-well plate. If the assay warranted inclusion of an antibody block, this was added to the corresponding well at this stage and incubated in a humidified atmosphere of 95% air / 5% CO_2 at 37°C for ≥ 30 mins for optimal binding before the addition of drug. Azide-free mouse anti-human HLA-ABC (IgG_1) and HLA-DR/DP/DQ (IgG_{2a}) blocking antibodies and their respective isotype controls were diluted in R9 medium and 10 µl added per well (well volume, 200 µl; final antibody concentration, 10 µg/ml). Mouse anti-human HLA-DR, DP, and DQ blocking antibodies were also used when required (final concentration, 10 µg/ml). Mouse anti-human PD-L1, CTLA4, and TIM-3 were used at similar final concentrations as determined for use in priming cultures; 5 µg/ml, 10 µg/ml, and 7.5 µg/ml, respectively.

50 µl of R9 medium or PHA was then added to the negative and positive control wells respectively, before preparation and addition of 50 µl drug or chemical-derived antigen to the antigen-treated wells (final well concentration; SMX, 0.5-2 mM; SMX-NO, 5-50

µM; PPD, BB, 2-10 µM and 50 µg/ml PHA). Cultures were then incubated in a humidified atmosphere of 95% air / 5% CO_2 at 37°C for 48 hrs. Each well was then pulsed with [^{3}H] thymidine (0.5 µCi/well). Following a further 16-hr incubation, testing and analysis of incorporated thymidine was performed as described in section 2.5.

2.13.7 Quantifying the secretion of IFN-γ, IL-5, IL-13, IL-17, IL-22, perforin, and granzyme B antigen-stimulated T-cell clones using ELISpot.

ELISpot was performed to assess the secretion of a range of cytokines and cytolytic molecules from T-cell clones. The procedure for pre-wetting, capture-antibody coating (IFN-γ, IL-5, granzyme B, diluted from 1 mg/ml to 15 µg/ml; IL-13, IL-22, IL-17, diluted from 0.5 mg/ml to 10 µg/ml; perforin, diluted from 1 mg/ml to 30 µg/ml) and time-scale of the ELISpot mirrors that described in section 2.10. However, the cell concentrations used during culture differ.

Clones were diluted to 5 x 10^5 cells/ml, while autologous EBV-transformed B-cells were diluted to 2 x 10^5 cells/ml and irradiated. 5 x 10^4 T-cell clones (100 µl) and 1 x 10^4 EBV-transformed B-cells (50 µl) were pipetted into a 96-well plate. If the assay warranted inclusion of an antibody block, this was added to the corresponding well at this stage. Mouse anti-human PD-L1, CTLA4, and TIM-3 were used at similar final concentrations as determined for use in priming cultures; 5 µg/ml, 10 µg/ml, and 7.5 µg/ml, respectively. Cultures were then incubated in a humidified atmosphere of 95% air / 5% CO_2 at 37°C for ≥ 30 mins for optimal antibody binding before the addition of drug. 50 µl of R9 medium or PHA was then added to the negative and positive control wells, respectively, before preparation and addition of 50 µl drug to the drug-treated wells (final

concentration; SMX, 0.5-2 mM; SMX-NO, 5-50 µM; PPD or BB, 2-10 µM). Cultures were then incubated for 48 hrs in a humidified atmosphere of 95% air / 5% CO_2 at 37°C.

The ELISpot wells were then subject to washing, exposure to diluted detection antibody (IFN-γ, IL-5, granzyme B, perforin, diluted from 1 mg/ml to 1 µg/ml; IL-17, IL-13, diluted from 0.5 mg/ml to 1 µg/ml; IL-22, diluted from 0.5 mg/ml to 0.5 µg/ml), streptavidin-ALP (IFN-γ IL-5, IL-13, IL-17, perforin, granzyme B, dilute) or streptavidin-HRP (IL-22), and colour substrate (IFN-γ, IL-5, IL-13, IL-17, perforin, granzyme B, BCIP-NBT; IL22, TMB) as described in section 2.10.

2.13.8 Detection of FasL secretion from activated T-cells using ELISpot.

Firstly, the membrane at the bottom of each well was pre-wetted with 35% ethanol (20µl) which was subsequently washed out after 2 mins by five 200µl distilled water washes. The FasL capture antibody was then diluted from the stock concentration (diluted 100µl in 10ml HBSS) before 100µl of coating antibody was then pipetted into each well and incubated overnight at 4^{o}C.

The following day, the pre-coated ELISpot well were subject to five 200µl washes with HBSS to remove any unbound coating antibody. After each wash, the washing buffer was discarded. 200µl of 2% skimmed milk was added to each well for ≥ 30 mins to block any remaining space on the well membrane to prevent non-specific binding. The plate was then subject to 1 X HBSS wash. T-cells and mature DCs were plated alongside R9, drug, or PHA as described in section 2.8.

After a 48 hr incubation, the plates were washed five times with 200 µl HBSS. After each wash the washing buffer was discarded. 100 µl of HBSS-0.1% Tween-20 was added to

each well and incubated at 4°C for 10 mins, before the plates were washed 3 times with HBSS-0.1% Tween-20. 100 µl of reconstituted detection antibody was diluted in 10 ml of 1% BSA-HBSS, added to each well (100 µl), and incubated at 37°C for 1 hr 30 mins.

The plate was then subject to 3 x 200 µl/well washes with HBSS-0.1% Tween-20. Streptavidin-conjugated to ALP was diluted 1:1000 in HBSS-1% BSA before 100 µl Streptavidin-ALP was added to each well and incubated at room temperature for 1 hr. The plate was again subject to 3 x 200 µl/well washes with HBSS-0.1% Tween-20. The colour substrate (BCIP-NBT+) was then sterile filtered (0.45 µm) and added 100 µl per well. Plates were immediately incubated at room temperature in the dark for 10-15 mins. Upon spot formation, plates were washed with tap water and air dried overnight. The following day, spots were counted using an AID ELISpot reader.

2.13.9 Phenotyping of cell surface markers on T-cell clones.

Shortly after the generation of T-cell clones they were phenotyped for CD4 or CD8 coreceptor expression. Clones were harvested, centrifuged (1500 rpm, 10 mins, room temperature), and resuspended for counting. 1 x 10^5 cells were transferred to two FACS tubes and centrifuged. Per clone, one tube was resuspended in 3 µl CD4-APC* and 3µl CD8-PE before incubation on ice in the dark for ≥ 20 mins. The other tube was not incubated with antibody-bound fluorochromes and was used as an unstained control. 1 ml FACS buffer was then added to each tube, which were centrifuged once again and resuspended in 200 µl FACS buffer. As two fluorochromes were combined in each tube, compensation samples were also required and were set up according to the protocol previously described in section 2.12.1. Samples were then analysed using a FACS Canto II machine for identification as either $CD4^+$ or $CD8^+$ T-cells.

Some clones were additionally phenotyped for their surface expression of PD-1, CTLA4, or TIM-3. Clones were harvested, and stained using the same method for CD4/8 expression but different antibody-bound fluorochromes were used, namely: 3 µl PD-1-PE, CTLA4-APC*, or TIM-3-PE. The analysis of these cell surface markers was recorded as the percentage of cells expressing the particular marker, or the mean fluorescence intensity (MFI) which is a measures the level of surface expression of that particular marker. MFI of PD-1, CTLA4, and TIM-3 were also recorded over time, with and without exposure to SMX-NO. This involved the culture of clones similar to that in section 2.13.6, whereby 1 x 10^5 T-cell clones (100 µl) and 4 x 10^3 autologous irradiated EBV-transformed B-cells (50 µl) were plated in each well of a 96-well plate. These wells were then either subject to the addition of 50 µl R9 medium, or SMX-NO (40 µM), and incubated in a humidified atmosphere of 95% air / 5% CO_2 at 37°C for the duration of the assay. At specific time-points, phenotyping of the three markers (PD-1, CTLA4, and TIM-3) was performed as previously stated.

2.14 Patient and Healthy donor PBMC genotyping.

To ascertain the HLA-type of both healthy donors and patients, we first needed to extract DNA from blood samples, for which we used the automated Chemagen magnetic bead isolation module. This employs the use of M-PVA magnetic beads (nanometer sized beads in a matrix of polyvinyl alcohol) selective for DNA markers to bind to, and isolate DNA. The process involves the addition of multiple buffers in 8 racks which must be prepared prior to starting the separation procedure. Each rack can hold up to 12 tubes which are placed on an automated delivery system, and thus 12 samples can be isolated during each run. Rack one was loaded with disposable tips, which were attached to magnetic rods within the magnetic separation module (MSM1) which can be magnetised

and demagnetised, while racks 2-8 were filled with 50 ml tubes. The tubes in each rack were then filled with separate buffers; the addition of 7.5 ml lysis buffer to tubes in rack 2, 10 ml wash buffer 3 or wash buffer 4 to those in racks 3 or 4, respectively, 2 ml or 10 ml of wash buffer 6 in racks 6 and 7, respectively, and 0.5 ml elution buffer in the tubes in rack 8. To begin the process we added one 5 ml blood sample from an individual and 20 µl protease per tube, before selecting the 1st automatic setting on the Magnetic Separation Module-1 (MSM1) where the rods are in a demagnetised state and are used to effectively mix the contents of the tubes in rack 2 for 10 mins, thus ensuring the release of DNA into the extracellular environment. Once this stops, we pipetted 19.5 ml binding buffer 2 to each tube along with 500 µl DNA-selective magnetic beads, and then returned each tube to its original position within rack 2 on the automated delivery system. We then started the 2nd automatic setting on the MSM1. This firstly involves insertion of the magnetic rods into the tubes in rack 2 and their subsequent magnetisation. This causes the attraction of the DNA-bound magnetic beads to rods which can then be lifted out of the tube and placed into successive tubes of wash buffers by a similar mechanism. In these tubes, the rod is demagnetised and rotated to disperse and mix the DNA-bound microbeads with wash buffer to remove impurities, before re-magnetisation and transfer to the next tube. In the final tube, elution buffer removes the microbeads from the DNA, the rods are magnetised and thus the microbeads removed. The full process is depicted in figure 2.9. The DNA sample was subsequently collected from rack 8 and the concentration and quality of the DNA determined with spectrophotometry using a Nanodrop machine. Samples were then diluted to 10 pg/ml in Chemagen elution buffer and frozen at -80°C before shipping to HistoGenetics (Ossoning, NY, USA).

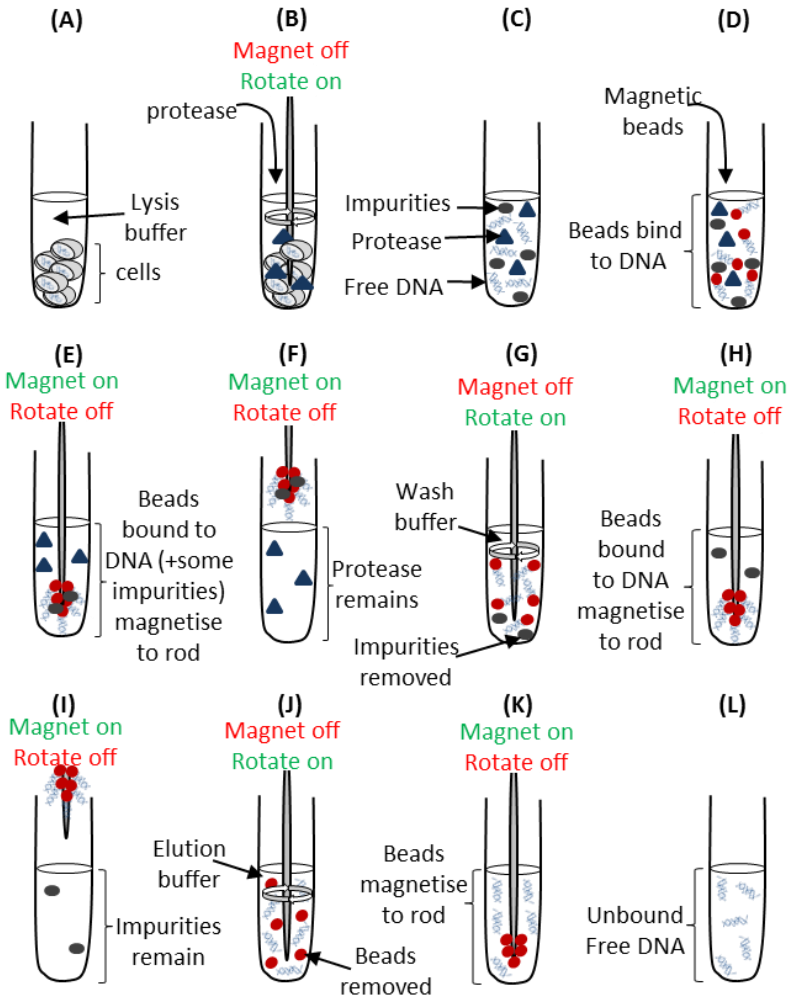


Figure 2.9. DNA isolation using the Chemagen Magnetic Separation Module (MSM1). (A) Whole blood was combined with lysis buffer, **(B)** protease was added **(C)** to free DNA from the cell, **(D)** DNA-binding magnetic beads were added, **(E)** rod is placed in the tube and magnetised causing the binding of DNA-microbeads, **(F)** magnetised rod is removed and transferred to a tube containing wash buffer, **(G)** rod is demagnetised and set to rotate which vortexes the contents to remove impurities (this wash process is repeated several times), **(H)** rod stops rotating and is re-magnetised thus attracting DNA-bound microbeads, **(I)** Rod is removed, leaving impurities behind, then transferred to a tube containing elution buffer, **(J)** rod is demagnetised and rotated causing the separation of DNA and microbeads, **(K)** rod stops rotating and is re-magnetised which attracts microbeads only, **(L)** rod is removed leaving a tube containing unbound free DNA only.

2.15 Statistics.

For *in vitro* priming of naïve T-cells, all experimental data shows the mean of three triplicate incubations. All priming experiments were conducted on at least three

separate occasions. To characterise cytokine signatures with clones, multiple clones (up to 17 per experiment) from different donors were analysed. Experiments were conducted in duplicate or triplicate, depending on the availability of cells.

Mean values and standard deviations were calculated, and statistical analysis was performed using paired t test (Sigmaplot 12 software).

For all data $p \leq 0.05$ was taken as significant. Differing levels of significance are indicated by the number of asterisks adorning each data set. *$p \leq 0.05$, **$p \leq 0.005$, ***$p < 0.001$.

Chapter 3: Negative Regulation by PD-L1 during Drug Antigen-Specific Priming of IL-22-Secreting T-cells

Contents

3.1 Introduction.

In recent years, genome-wide association studies have identified specific HLA alleles as important susceptibility factors for certain hypersensitivity reactions (Daly, 2012; Phillips *et al.*, 2011). For a limited number of drugs, the drug-derived antigen has been shown to interact specifically with the protein encoded by the HLA risk allele leading to the activation of T-cells. However, with the exception of ABC hypersensitivity, the majority of individuals who carry known HLA risk alleles do not develop clinically relevant immunological reactions when exposed to a culprit drug. For some drugs such as SMX, studies have screened for a range of relevant MHC gene associations, and despite the acknowledgement that that the onset of these reactions may be subject to minor HLA-specific influences, no significant HLA associations have been detected (Alfirevic *et al.*, 2009).

Other potential major influences on individual susceptibility to drug hypersensitivity may include drug metabolising enzyme variants, however few such genetic predisposing metabolic elements have ever been identified. Pirmohamed and colleagues addressed this by investigating differential distribution of SMX metabolising enzyme polymorphisms in HIV positive patients, both with and without hypersensitivity. No significant associations were found, including for NAT2, an enzyme involved in the detoxification of SMX by *N*-acetylation. The authors do elude to the fact that NAT2 slow acetylator phenotype is much lower in HIV negative patients than in those who were HIV positive, but overall it was concluded that this corresponds to a poor marker of risk (Pirmohamed *et al.*, 2000). Infection, especially reactivation of the herpes virus family (Descamps *et al.*, 2001; Picard *et al.*, 2010), has been put forward as an additional risk factor, whereby T-cells activate the virus, or the virus directly mediates hypersensitivity (Aihara *et al.*, 2003; Walsh and Creamer, 2011). Viral infection alone, however, does not fully explain the unpredictable nature of drug hypersensitivity as many patients who develop hypersensitivity show no signs of viral

reactivation (Tohyama *et al.*, 2007). Thus, there is a need to characterize the immunological parameters that are superimposed on HLA-restricted T-cell activation to determine why particular individuals develop drug hypersensitivity.

The identification of such parameters can be performed using an *in vitro* naïve T-cell priming assay, whereby naïve T-cells are isolated from healthy donors and primed to a specific antigen. Of note, this assay can also be used to simultaneously assess the response in memory T-cells. Subsequent addition of the drug-derived antigen in a panel of assays can be used to detect antigen-specific cytokine secretion or T-cell proliferation. Furthermore, if antigen-specific T-cells are present, T-cell cloning can be performed to look at the response at a single cell level for a more detailed investigation of cellular phenotype and function, including CD4/CD8 phenotyping, dose response and cross-reactivity to related compounds or associated metabolites, and HLA restriction analysis. However, this assay does have its limitations; it takes between 3-4 weeks to prime and assess the presence of antigen-specific T-cells and a further 2-3 months for T-cell cloning, *in vitro* conditions limit the quantity of cells recovered during independent stages of the assay, and high background activation can hinder the assessment of an antigen-specific response. Therefore this assay remains somewhat under development, but nonetheless presents us with a functioning *in vitro* assay whereby we can assess the activation of naïve and memory T-cells from healthy donors to fully characterise antigen-specific primary and secondary T-cell responses.

T-cell activation not only requires MHC-TCR interaction, termed signal 1, but also a multitude of other signals, termed signal 2. Signal 2 is composed of both stimulatory and inhibitory pathways, collectively termed co-signalling pathways, many that signal at the same time to regulate T-cell activation in a complex balancing act between tolerance and antigenic clearance. By stripping away these layers of regulation through blocking individual pathways, we can explore their discrete influence on drug-derived antigen-specific T-cell responses. In

this chapter we focussed on the T-cell co-inhibitory receptor PD-1. PD-1 has recently sparked interest due to its effective therapeutic targeting in cancers, which may activate this pathway to evade immune recognition by T-cells. Indeed, recent studies have identified PD-1 as a major checkpoint regulating the activation of T-cells, as epitomised by PD-1 pathway blocking studies in cancer patients, where individuals whose tumours highly express a PD-1 ligand are 4.5-fold more likely to die than those with comparatively low ligand expression (Thompson *et al.*, 2004).

Activation of the PD-1 receptor, which is transiently expressed on activated T-cells but also a wide range of immune cells and tissues (Keir *et al.*, 2007; Okazaki and Honjo, 2007), leads to clustering between TCRs and the phosphatase SHP2, the subsequent dephosphorylation of TCR signalling, and the suppression of antigen-specific T-cell responses (Yokosuka *et al.*, 2012). PD-1 has two ligands, PD-L1 (CD274) and PD-L2 (CD273). In contrast to PD-L1 which is expressed on a variety of immune cells, PD-L2 expression is limited to DCs, bone marrow-derived mast cells, and activated macrophages. The PD-1 pathway has previously been shown to regulate autoimmunity in several experimental models. Furthermore, genome-wide association studies have identified SNPs in the human PD-1 gene that are associated with a higher risk of developing autoimmune disease (Gianchecchi *et al.*, 2013), highlighting the potential for variants of PD-1 to influence the onset of T-cell responses in relation to drug hypersensitivity. While high PD-1 expression has been classified and used as a marker of T-cell exhaustion (Dyavar Shetty *et al.*, 2012; Zinselmeyer *et al.*, 2013), recent studies from independent laboratories describe an alternative perspective. Duraiswamy *et al* (Duraiswamy *et al.*, 2011) showed that most PD-1^{high} human $CD8^{+}$ T-cells are effector memory cells rather than exhausted cells. Zelinskyy *et al* (Zelinskyy *et al.*, 2011) showed that, although virus-specific $CD8^{+}$ T-cells upregulate PD-1 expression during acute infection, the majority of PD-1^{high} cells were highly cytotoxic and controlled virus replication. Finally, Reiley

et al (Reiley *et al.*, 2010) showed that PD-1^{high} CD4^{+} T-cells were highly proliferative and appeared to maintain effector T-cell responses during chronic infection.

Previous immunohistological studies from SMX-hypersensitive patients have noted the presence of both CD4^{+} and CD8^{+} SMX (metabolite)-specific T-cells capable of secreting a mixed panel of Th1/Th2 cytokines (Castrejon *et al.*, 2010; Elsheikh *et al.*, 2011; Pichler, 2003; Pichler *et al.*, 2002; Schnyder *et al.*, 2000; Schnyder *et al.*, 1998). However, the discovery of new T-cell populations (e.g., Th9, Th17, Th22) renders this classification out of date. As IL-22, which is secreted from both Th17 and Th22 subsets, is associated with skin-related immunological reactions (Akdis *et al.*, 2012; Cavani *et al.*, 2012; Eyerich *et al.*, 2010), it is vital that these classifications be updated for cutaneous hypersensitivity reactions. It is therefore important to characterise the cytokine signature(s) of drug antigen-specific T-cells and to study whether PD-1 expression/signalling governs the differentiation of T-cells into effector/helper subsets.

To investigate the potential influence of the PD-1 pathway on drug-derived antigen-specific T-cell activation we utilised two model drug haptens, SMX-NO and flucloxacillin. Both compounds have previously been shown to activate CD4^{+} and CD8^{+} T-cells isolated from patients presenting with drug-induced tissue injury (SMX-NO, skin injury; flucloxacillin, liver injury) (Castrejon *et al.*, 2010; Daly *et al.*, 2009; Engler *et al.*, 2004; Faulkner *et al.*, 2012; Monshi *et al.*, 2013; Nassif *et al.*, 2004; Schnyder *et al.*, 2000). SMX-NO is a cysteine-reactive metabolite of SMX that binds extensively to cellular protein, whereas flucloxacillin binds directly to lysine residues of serum proteins. This very different chemistry of antigen formation obviates compound-specific effects; as such, any regulation of T-cell priming must involve signalling pathways downstream of the drug interaction with protein.

3.2 Chapter aims.

1) To investigate the individual roles of PD-L1 and PD-L2 in the drug-derived antigen-specific priming of naïve T-cells.
2) To analyse how the surface expression of PD-1 changes in response to drug-derived antigen-exposure; during antigen-specific priming and after subsequent re-stimulation.
3) To explore the comparative expression and role of PD-1 on primary and secondary T-cell responses using drug-derived antigen-specific memory T-cells and T-cell clones.
4) To determine whether PD-1 expression correlates with an exhausted T-cell phenotype.
5) To assess the cytokine profile of PD-1 regulated drug-derived antigen-primed T-cells, including the secretion of IL-17 and IL-22.

3.3 Results.

3.3.1 PD-L1 block, but not PD-L2 block, enhances the priming of naive T-cells against SMX-NO.

For *in vitro* priming, naive $CD3^{+}$ T-cells from drug-naive donors were co-cultured with autologous mature DCs in the presence of SMX-NO (± PD-L1/PD-L2 block) for 1 week. DCs were routinely stained for costimulatory receptors and characterized as $CD1a^{negative}$ $CD11a^{high}$ $CD11c^{high}$ $CD14^{negative}$ $CD40^{high}$ $CD80^{mid}$ $CD83^{low}$ $CD86^{high}$ MHC class I^{high} MHC class II^{high} and $PD\text{-}L1^{high}$ (Figure 3.1). Previously, LPS addition has been identified to upregulate specific DC maturation markers more so than SMX-NO and therefore our assay bypasses the requirement for danger signalling (Sanderson *et al.*, 2007). Upon re-stimulation, dose-

dependent antigen-specific proliferation was clearly detectable (Figure 3.2; $p \leq 0.05$; SMX-NO, 12.5-50 µM). Inclusion of PD-L1 blockade markedly enhanced the proliferative response ($p \leq 0.05$; at each SMX-NO concentration). PD-L2 block, however, gave proliferative responses comparable to those without PD-ligand block (Figure 3.2). T-cells which were subject to simultaneous blockade of PD-L1 and PD-L2 elicited a somewhat enhanced proliferative response in comparison to those cultured without block ($p \leq 0.05$; SMX-NO, 12.5-50 µM), but this increase was far less than that induced by the addition of PD-L1 blocking antibodies alone in 2/3 donors (Figure 3.2). All assays, irrespective of the addition of blocking antibodies, responded significantly in the presence of SMX-NO.

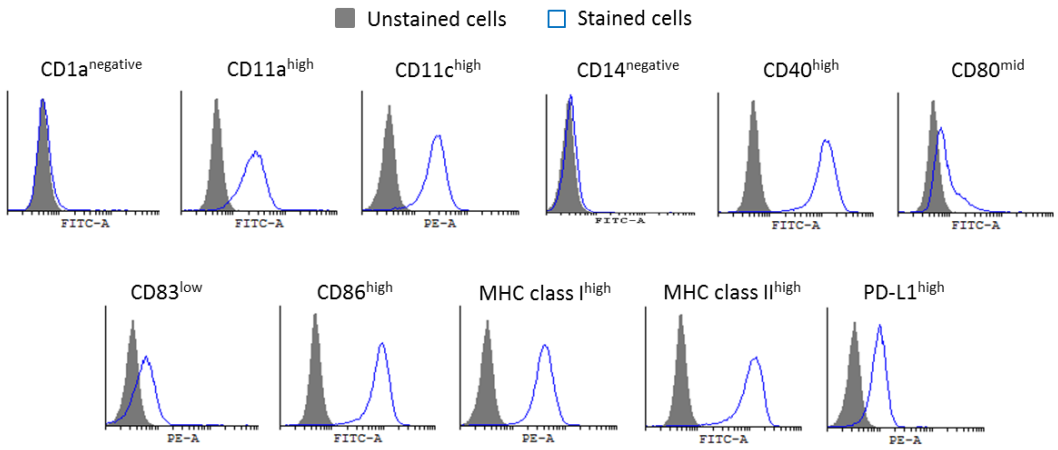


Figure 3.1. **Phenotype of mature monocyte-derived DCs utilised during the *in vitro* antigen-stimulation of naïve and memory T-cells.** DCs were generated through *in vitro* culture of $CD14^+$ monocytes with GM-CSF- and IL-4 (800 U/ml)-supplemented R9 medium for 7 days. On the penultimate day of culture, DCs were matured using LPS (1 µg/ml) and TNF-α (25 ng/ml) and phenotyped 24 hrs later. Each clone was pipetted into 11 tubes ($5x10^4$ cells/tube) and resuspended in 3 ul of antibody-bound fluorochrome specific for a particular marker ($CD1a^-$, $CD11a^+$, $CD11c^+$, $CD14^-$, $CD40^+$, $CD80^+$, $CD83^+$, $CD86^+$, MHC class I^+, MHC class II^+, and $PD\text{-}L1^+$). Cells were incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and analysed on a FACS Canto II machine using cyflogic software. Grey histogram represents unstained cells, blue histogram represents expression on stained cells.

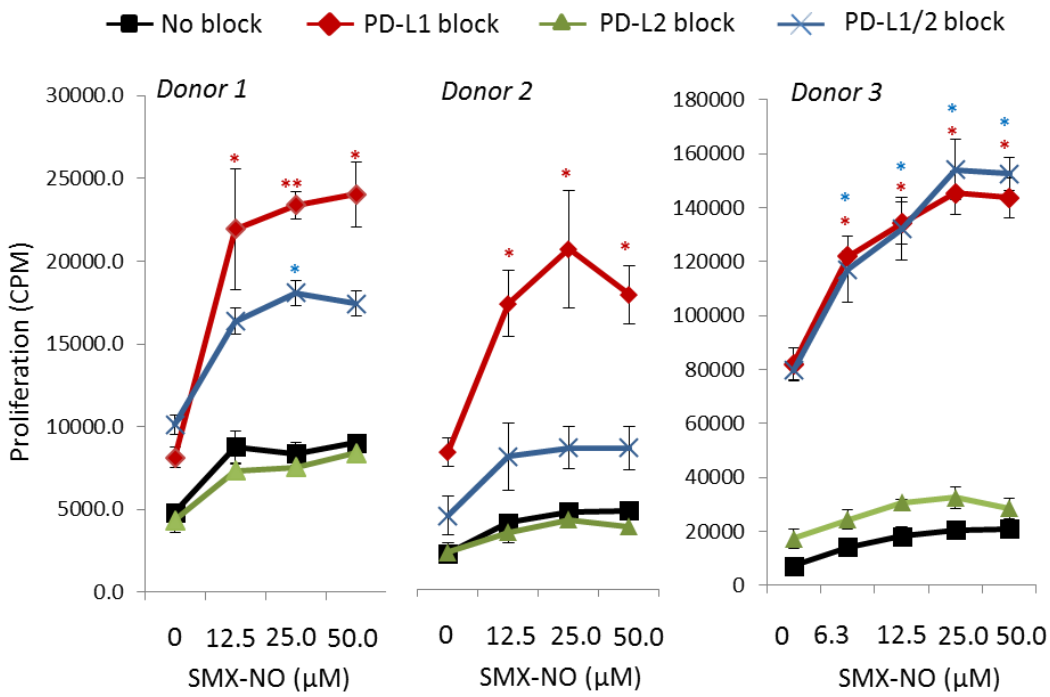


Figure 3.2. **SMX-NO-specific priming of naive T-cells from three healthy donors in the presence and absence of PD-L1/PD-L2 blocking antibodies.** Naïve T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from healthy donors were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) and SMX-NO (50 µM) for one 1 week, ± PD-L1 or PD-L2 blocking antibodies (5 µg/ml) in an atmosphere of 95% air / 5% CO_2 / 37°C. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (6.3-50 µM) for 48 hrs in similar conditions as before. [^{3}H]-thymidine (0.5 µCi/well) was added and incubated for a further 16 hrs. Data from three representative donors shows the mean ± SD of triplicate cultures. Statistical significance denotes a significant increase in proliferative response at a specific drug concentration compared to 'no block' treated wells after normalisation of all data values to account for differing basal stimulation (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

To determine whether similar trends apply regarding the respective roles of PD-L1 and PD-L2 for other functional aspects of T-cell activation, we assessed the secretion of IFN-γ and granzyme B. IFN-γ (Figure 3.3a) and granzyme B (Figure 3.3b) were released from SMX-NO-primed naïve T-cells following re-stimulation, and similar trends were observed as to those of the proliferative response. Specifically, PD-L1 blocking antibodies significantly enhanced cytokine secretion from SMX-NO-responsive T-cells as did the simultaneous block of both PD-ligands despite not showing an increase as large as for the addition of PD-L1 blocking antibodies alone. The enhanced secretion of both IFN-γ and granzyme B was reproducible in

two other donors, where an increase in secretion could be detected for both low (12.5µM, 25.0 µM) and high concentrations (50.0 µM) of SMX-NO (figure 3.4). To confirm antigen specificity, T-cell clones generated from SMX-NO-primed naïve T-cell cultures were cultured with alternative drugs prior to analysis of proliferation and/or cytokine secretion. SMX-NO-primed T-cells were not activated with flucloxacillin or CBZ (Figure 3.5).

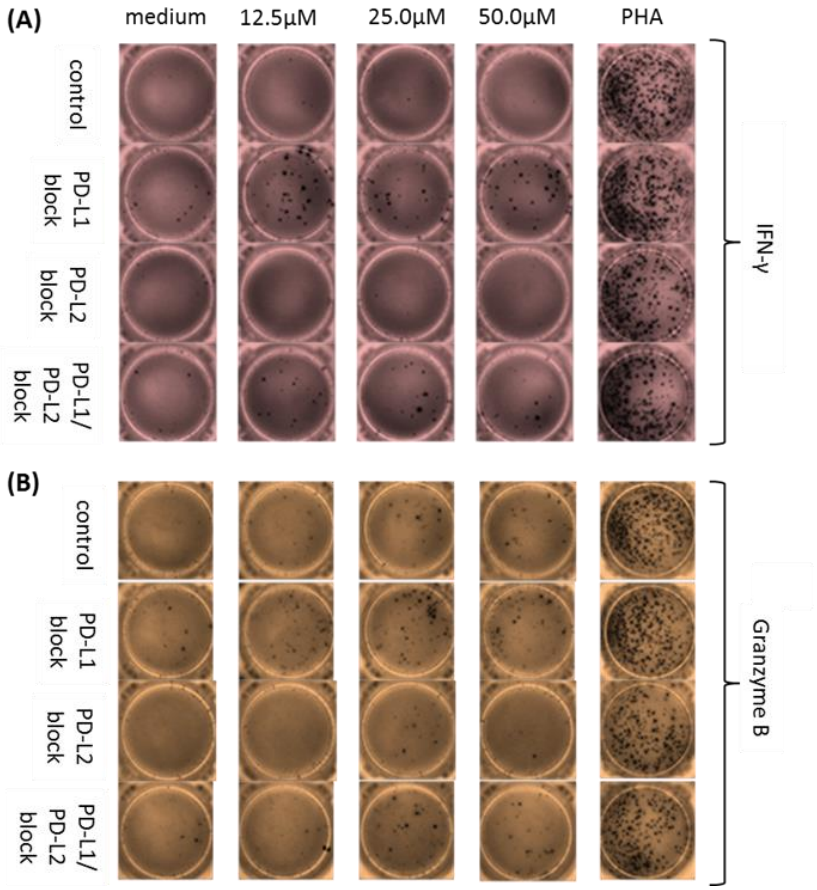


Figure 3.3. **Antigen–induced secretion of IFN-γ and granzyme B from healthy donor naive T-cells primed to SMX-NO ± PD-L1 or PD-L2 blocking antibodies**. Healthy donor naïve T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) and SMX-NO (50 µM), ± PD-L1 or PD-L2 blocking antibodies (5 µg/ml). Cells were cultured for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. In accordance with manufacturer's instructions, ELISpot plates were coated with **(A)** IFN-γ or **(B)** granzyme B capture antibody and incubated at 4°C overnight. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well ELISpot plate) with fresh monocyte-derived DCs ($4x10^3$/well) and SMX-NO (12.5-50 µM). The cells were incubated for 48 hrs in a similar atmosphere as before. The ELISpot plates were then washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader.

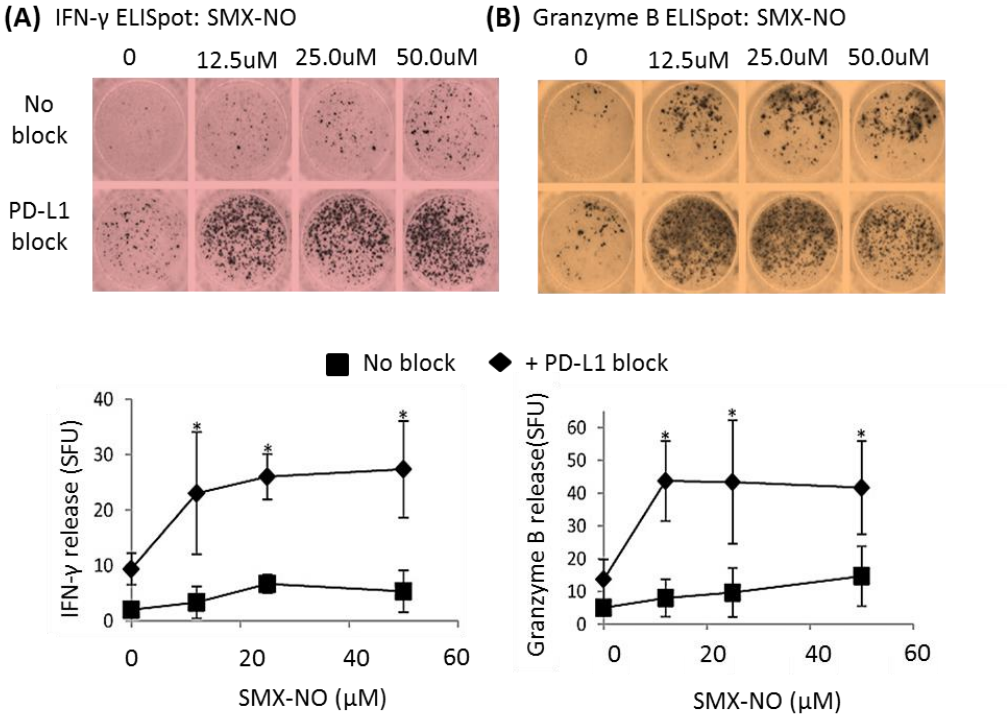


Figure 3.4. **Increased secretion of IFN-γ/granzyme B after naive T-cell priming to SMX-NO in the presence of PD-L1 blocking antibodies using cells from two further healthy donors**. Healthy donor naïve T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM), ± PD-L1 or PD-L2 blocking antibodies (5µg/ml). Cells were cultured for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. In accordance with manufacturer's instructions, ELISpot plates were coated with **(A)** IFN-γ or **(B)** granzyme B capture antibody and incubated at 4°C overnight. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well ELISpot plate) with fresh monocyte-derived DCs ($4x10^3$/well) and SMX-NO (12.5-50 µM). The cells were incubated for 48 hrs in a similar atmosphere as before. The ELISpot plates were then washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader.

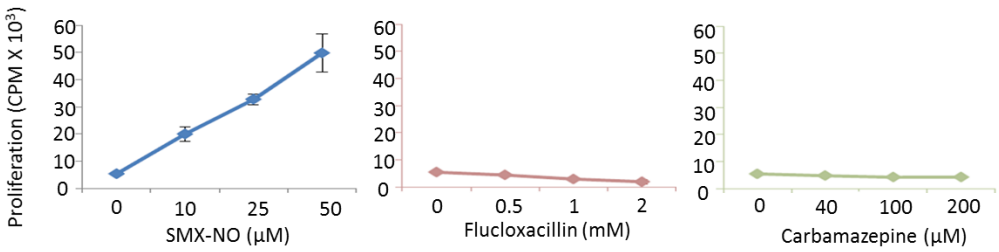


Figure 3.5. **No activation of SMX-NO-responsive T-cell clones with structurally-unrelated drugs.** T-cell clones derived from healthy donor SMX-NO-primed naïve T-cells ($5x10^4$/well; total volume, 200 µl, 96-well U-bottomed plate) were stimulated with SMX-NO (10-50 µM), flucloxacillin (0.5-2 mM), or CBZ (40-200 µM) using autologous irradiated EBV-transformed B-cells ($1x10^4$/well). After incubation in an atmosphere of 95% air / 5% CO_2 / 37°C for 48 hrs, proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a further 16 hrs. Data are presented as the mean ± SD; experiments were conducted in triplicate.

3.3.2 PD-L1 block enhances the priming of naive T-cells against other drug-derived antigens.

To explore whether PD-L1 regulates priming against other drug antigens, naive T-cells from HLA-B*57:01 positive donors were co-cultured with autologous mature DCs and flucloxacillin (2 mM; ± PD-L1/ PD-L2 block). In initial experiments, activation of naïve T-cells with flucloxacillin was only detected with PD-L1 block (Figure 3.6a); IFN-γ and granzyme B release above that of background was discernible with flucloxacillin. The granzyme B ELISpot was then repeated on two further occasions with naive T-cells from different donors (using triplicate samples per culture condition). Low levels of granzyme B secretion were detectable when T-cells primed in the absence of PD-L1 block were stimulated with flucloxacillin. Granzyme B secretion was significantly enhanced using T-cells primed in the presence of PD-L1 blocking antibodies (Figure 3.6b).

3.3.3 PD-L1 block has a greater effect on the regulation of primary, rather than secondary, T-cell responses.

To explore the comparative effect of PD-L1/PD-1 pathway activation on secondary T-cell responses opposed to primary, naïve T-cell responses, we performed a similar assay to that

of naïve T-cell priming but with memory T-cells from healthy donors. We first cultured memory T-cells with SMX-NO in the presence of absence of PD-L1 block, before re-stimulation, and assessed the degree of T-cell proliferation in response to SMX-NO. Initial analysis of data implied that there was a variation between the proliferative capacity of T-cells primed with and without PD-L1 block. However, upon normalisation to account for different baseline proliferation, and in stark comparison to SMX-NO-primed naïve T-cell responses (Figure 3.7a) which are significantly increased after culture with PD-L1 blocking antibodies, it was found that memory T-cell responses were not increased by blocking PD-L1 (figure 3.7b).

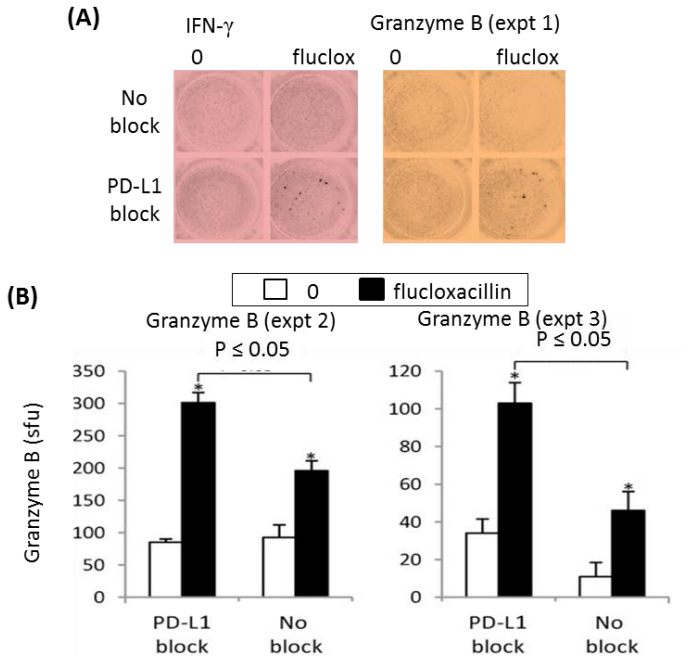


Figure 3.6. Increased secretion of IFN-γ/granzyme B after naïve T-cell priming to flucloxacillin in the presence of PD-L1 blocking antibodies using cells from three HLA-B*57:01 positive healthy donors. **(A)** HLA-B*57:01 positive healthy donor naïve T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of flucloxacillin (1 mM), ± PD-L1 blocking antibodies (5 µg/ml). Cells were cultured for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. In accordance with manufacturer's instructions, ELISpot plates were coated with IFN-γ or granzyme B capture antibody and incubated at 4°C overnight. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well ELISpot plate) with fresh monocyte-derived DCs ($4x10^3$/well) and flucloxacillin (1 mM). The cells were incubated for 48 hrs in a similar atmosphere as before. The ELISpot plates were then washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader. **(B)** Line graphs show mean ± SD of two experiments conducted in triplicate (*p ≤ 0.05). Statistical significance denotes a significant increase in proliferative response compared to 'medium only' treated wells.

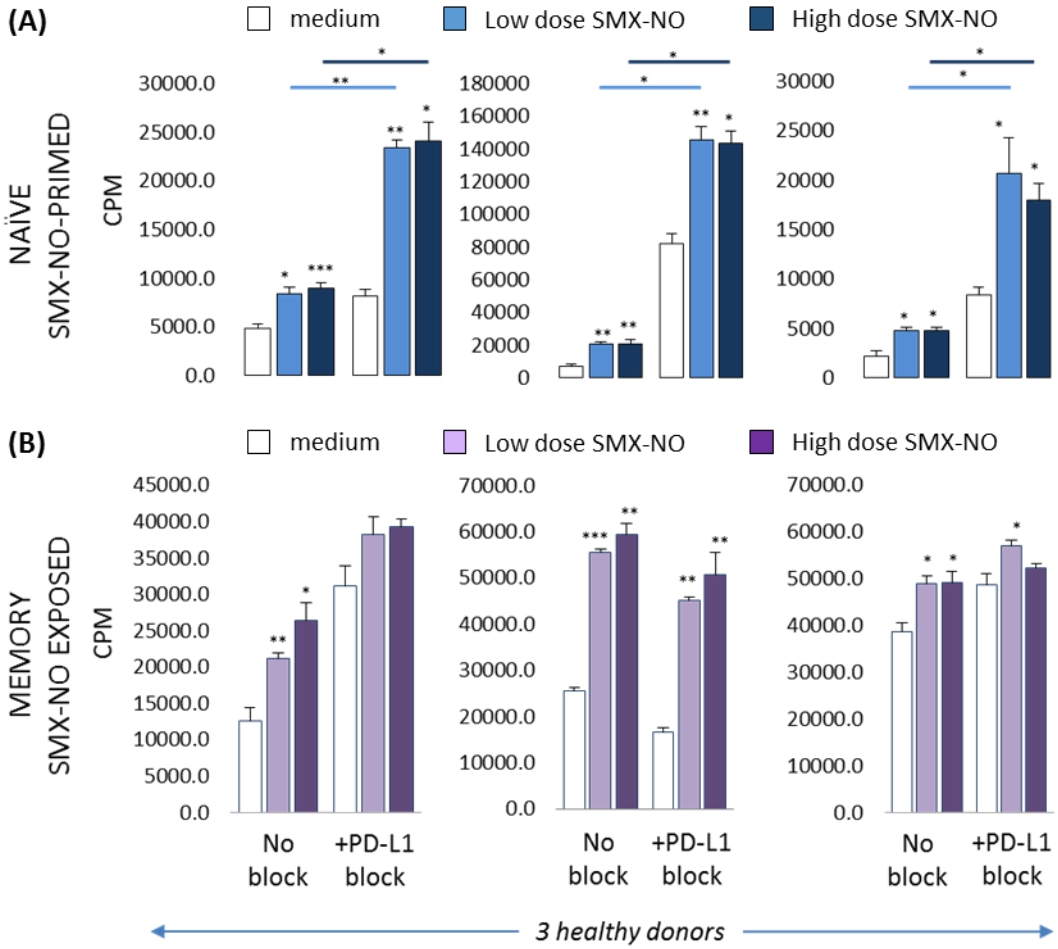


Figure 3.7. Comparative antigen-specific activation of SMX-NO-primed naive and memory T-cells from healthy donors in the presence and absence of PD-L1 blocking antibodies. (A) Naïve or **(B)** memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from healthy donors were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) for one 1 week, ± PD-L1 blocking antibodies (5 µg/ml), in an atmosphere of 95% air / 5% CO_2 / 37°C. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and either a low (20-30 µM) or high concentration (40-50 µM) of SMX-NO for 48 hrs in similar conditions as before. [^{3}H]-thymidine (0.5 µCi/well) was added and incubated for a further 16 hrs. Data from three representative donors shows the mean ± SD of triplicate cultures. Statistical significance above a bar denotes a significant increase in proliferative response compared to 'medium only' treated wells within that condition (i.e PD-L1 blocked cells only). Statistical significance between conditions denotes a significant increase in proliferative response compared to 'no block' treated wells at a particular drug concentration after normalisation of all data values to account for differing basal stimulation (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

3.3.4 PD-1 expression on T-cells increases early after initial exposure to drug-derived antigen and is present on dividing T-cells.

CFSE staining allows a more in-depth analysis of cell proliferation by specifically quantifying the number of dividing cells and distinguishing between different cell populations. The expression of CD45RO, a marker depicting memory T-cell status, was measured on dividing and non-dividing cells to characterise cell phenotype. As expected, the non-dividing cells were CD45RO negative (naïve), whereas the dividing cells stained CD45RO positive (memory) (Figure 3.8).

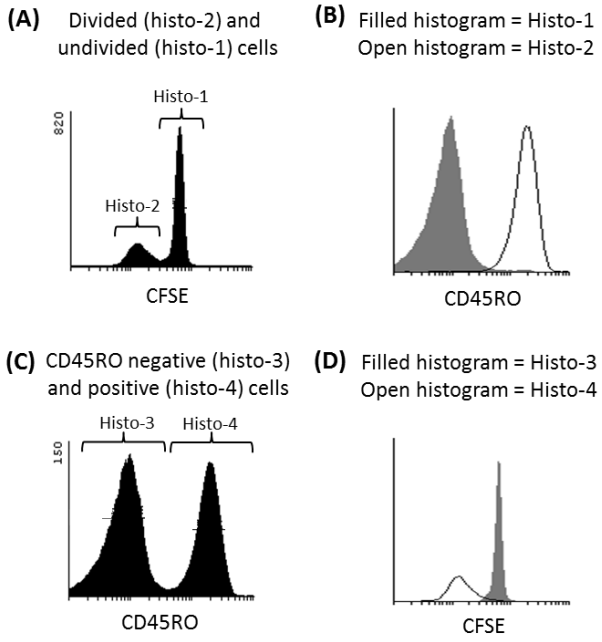


Figure 3.8. The dividing cell fraction of SMX-NO-primed naïve T-cells are CD45RO^{+}, indicating a memory phenotype. Naïve T-cells (2.5x10^{6}/well; total volume, 2 ml; flat-bottomed 24-well plates) from healthy donors were cultured with mature autologous monocyte-derived DCs (8x10^{4}/well) in the presence of SMX-NO (50 µM) for one 1 week in an atmosphere of 95% air / 5% CO_2 / 37°C. T-cells were harvested and a sample were stained with CFSE. CFSE stained and unstained cells were then re-plated (1x10^{5}/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs (4x10^{3}/well) and SMX-NO (50 µM) for 72 hrs in similar conditions as before. CFSE stained and unstained cells were transferred to FACS tubes and resuspended in 3ul of CD45RO-PerCP-Cy5 and incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and were analysed on a FACS Canto II machine using cyflogic software. **(A)** CFSE content of T-cells highlighting dividing (histo-2) and non-dividing populations (histo-1); **(B)** Expression of CD45RO on dividing and non-dividing SMX-NO stimulated T-cells; **(C)** Expression of CD45RO, Histo-3 represents CD45RO^{negative} cells, Histo-4 represents CD45RO^{positive} cells; **(D)** CFSE expression in dividing and non-dividing SMX-NO-stimulated T-cells (a minimum of 50,000 cells were acquired per test).

CFSE staining was used to measure PD-1 on dividing and non-dividing T-cells. Upon analysis of the $CD3^+$ T-cell population 48 hrs after SMX-NO re-stimulation, PD-1 was found to be more highly expressed on dividing T-cells than on non-dividing T-cells. Similar results were obtained by performing the more discrete analysis of the $CD4^+$ and $CD8^+$ populations individually (Figure 3.9). PD-1 expression was also measured during SMX-NO-specific naïve T-cell priming and for 72 hrs after re-stimulation (Figure 3.10). Little or no expression of PD-1 was detected on naive $CD4^+$ and $CD8^+$ T-cells. After priming, a small population of PD-1–positive cells was seen on day 7, both in the presence and absence of PD-L1 block. After re-stimulation with SMX-NO (day 9), PD-1 expression was rapidly upregulated on 20–40% of $CD4^+$ and $CD8^+$ T-cells in a transient fashion (Fig. 3.10, columns 1 and 3). PD-1 reverted back to pre-restimulation levels within 48-72 hrs. Based on the intensity of PD-1 staining detected by flow cytometry, it would seem that activated $CD4^+$ T-cells expressed higher levels of PD-1 when $CD4^+$ and $CD8^+$ T-cells were compared. Furthermore, $CD4^+$ T-cells were more responsive than $CD8^+$ T-cells and displayed enhanced proliferation in the presence of PD-L1 blocking antibodies. Collectively, these data show that $CD4^+$ and $CD8^+$ T-cells are activated during drug-derived antigen-specific priming, and that activated cells express high levels of PD-1 in a transient fashion. In the presence of PD-L1 block, the increase in PD-1 expression was sustained (Fig. 3.10, columns 2 and 4). Greater than 30% of $CD4^+$ cells stained positive for PD-1 72 hrs after restimulation.

In contrast to naïve T-cells, a large number of unstimulated memory T-cells expressed PD-1, with > 30% of $CD4^+$, and > 58% of $CD8^+$ T-cells expressing the receptor (Figure 3.11). The percentage of PD-1^+ memory T-cells increased significantly after restimulation, peaking at 24-48 hrs after re-exposure to SMX-NO. The number of $CD4^+$ memory T-cells expressing PD-1 increased in response to antigen by a maximum of 1.6-fold to > 50% of cells, while up to 74% of memory $CD8^+$ T-cells expressed PD-1 (fig 3.11, columns 1 and 3). Moreover, the

inclusion of PD-L1 blocking antibodies was found to enhance the percentage of both $CD4^+$ and $CD8^+$ T-cells expressing PD-1 (fig. 3.11, columns 2 and 4).

Although reduced cell viability has the potential to produce altered fluorescence traces we did not record the detailed viability of cells utilised to measure ligand expression during the time course or for similar experiments. However, (1) all cell samples were subject to counting in the presence of trypan blue prior to use and were deemed viable by visual inspection, (2) specific gating protocols for lymphocytes allowed the exclusion of the cell fragments of dying/dead cells during flow cytometric analysis, and (3) samples were obtained from the same populations used to determine proliferative and cytokine secretion responses which indicated that cells were functional.

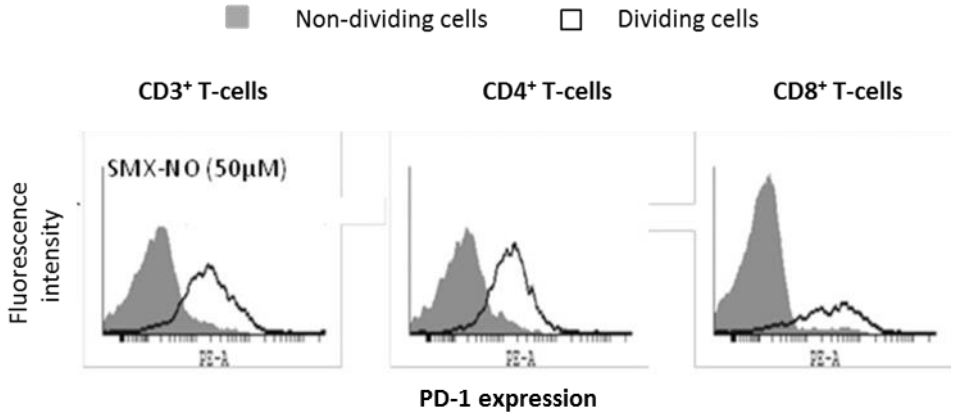


Figure 3.9. Comparative expression of PD-1 on dividing and non-dividing healthy donor-derived SMX-NO-primed naïve T-cells. Naïve T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from healthy donors were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) for one 1 week in an atmosphere of 95% air / 5% CO_2 / 37°C. T-cells were harvested and a sample were stained with CFSE. CFSE stained and unstained cells were then re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (50 µM) for 72 hrs in similar conditions as before. CFSE stained and unstained cells were transferred to FACS tubes and resuspended in 3ul of PD-1-PE and 3 ul of either CD4-APC* or CD8-APC*. Cells were then incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and analysed on a FACS Canto II machine using cyflogic software. Figures show PD-1 expression on $CD3^+$, $CD4^+$, or $CD8^+$ T-cell populations separately. Grey histograms represent PD-1 expression on dividing T-cells, black line represents PD-1 expression on non-dividing T-cells.

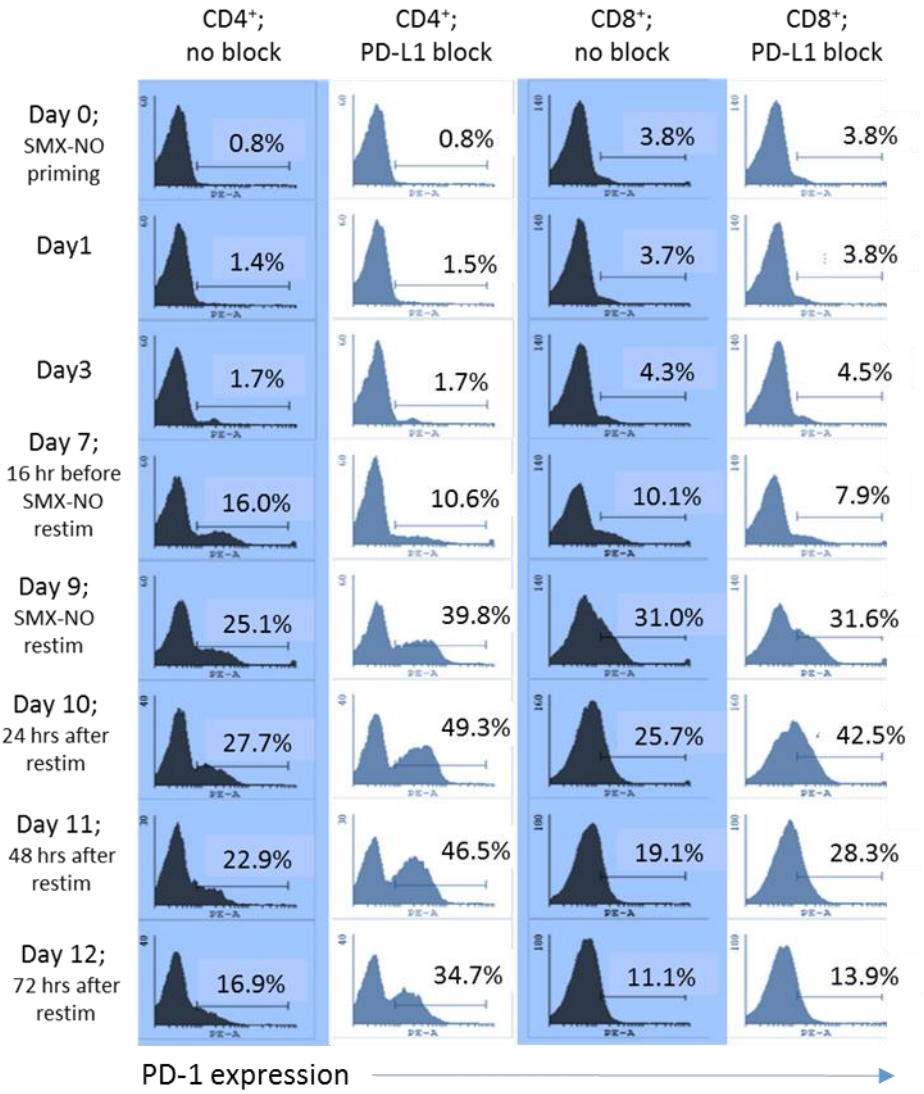


Figure 3.10. PD-1 expression during the SMX-NO-specific priming of $CD4^+$ and $CD8^+$ naïve T-cells from a healthy donor, ± PD-L1 blocking antibodies. Naïve T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from a healthy donor were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) for one 1 week in an atmosphere of 95% air / 5% CO_2 / 37°C, ± PD-L1 blocking antibodies (5 µg/ml). T-cells were harvested and then re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (50 µM) for 72 hrs in similar conditions as before. During the course of the priming assay, samples of T-cells ($5x10^4$) were harvested at various time-points and labelled with 3µl of CD4, CD8, and PD-1 fluorochrome-bound antibodies. Cells were then incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and analysed on a FACS Canto II machine using cyflogic software. Percentages indicate the proportion of cells which stain positive for PD-1.

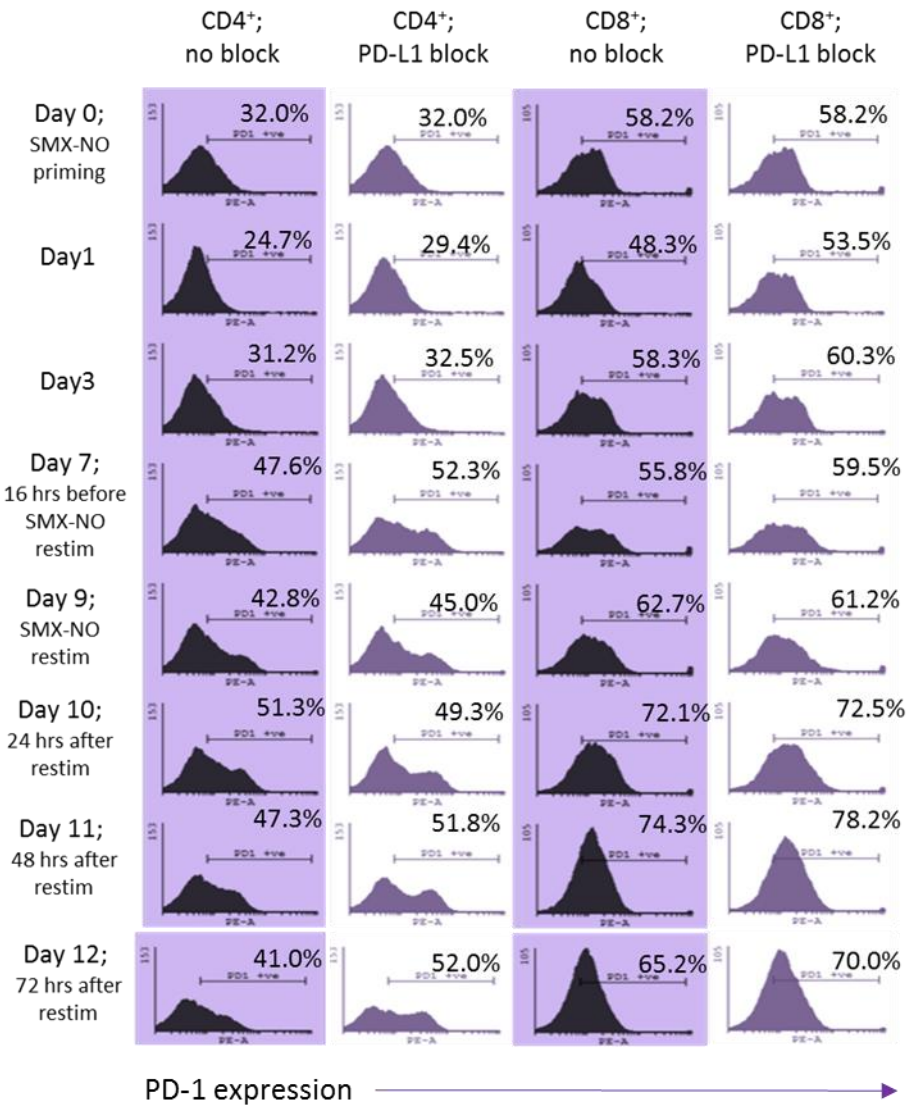


Figure 3.11. PD-1 expression during the SMX-NO-specific activation of CD4$^+$ and CD8$^+$ memory T-cells from a healthy donor, ± PD-L1 blocking antibodies. Memory T-cells (2.5x10^6/well; total volume, 2 ml; flat-bottomed 24-well plates) from a healthy donor were cultured with mature autologous monocyte-derived DCs (8x10^4/well) in the presence of SMX-NO (50 µM) for one 1 week in an atmosphere of 95% air / 5% CO_2 / 37°C, ± PD-L1 blocking antibodies (5 µg/ml). T-cells were harvested and then re-plated (1x10^5/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs (4x10^3/well) and SMX-NO (50 µM) for 72 hrs in similar conditions as before. During the course of the priming assay, samples of T-cells (5x10^4) were harvested at various time-points and labelled with 3µl of CD4, CD8, and PD-1 fluorochrome-bound antibodies. Cells were then incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and analysed on a FACS Canto II machine using cyflogic software. Percentages indicate the proportion of cells which stain positive for PD-1.

3.3.5 IL-22 secretion from SMX-derived antigen-specific T-cells.

Cutaneous reactions to drugs have been previously classified according to the phenotype and function of antigen-specific T-cells. However, the discovery of new T-cell subsets (e.g., Th17, Th22 cells) may render this classification somewhat obsolete. Thus, to study whether PD-1 expression/signalling governs the function of effector/helper T-cell subsets, it was important to characterise the cytokine signatures of antigen-specific T-cells.

SMX-NO-primed naïve T-cells secreted IFN-γ, IL-13, and IL-22 in response to restimulation with antigen. However, IL-17 release was not detected (Figure 3.12) despite the secretion of all four cytokines in the PHA positive control wells. Both hypersensitive patient clones and clones isolated from *in vitro* priming from healthy donors secreted similar Th1 and Th2 cytokines. We therefore utilised a combined patient/donor panel of 17 clones (from multiple individuals) with a strong growth pattern to study the secretion of a broader array of cytokines, including IL-17 and IL-22. Approximately 50% of clones were found to secrete IL-22 following exposure to SMXNO. In contrast, IL-17 secretion was only detected with one clone (Figure 3.13a), but nonetheless confirmed the functionality of the IL-17 ELISpot kit and validated the lack of IL-17 secretion from all other clones. SMX-NO responsive T-cell clones were also found to secrete an array of cytokines, with the majority of clones found to secrete IFN-γ, IL-5, and IL-13 in vastly differing quantities from one clone to the next. IL-22-secreting clones were subsequently isolated from hypersensitive patient PBMCs and following *in vitro* priming (Figure 3.13b, 3.13c). Importantly, the isolation of SMX-NO-responsive, IL-22-secreting T-cell clones from the priming assay was not dependent on the presence of Th22-polarizing cytokines, and clones were not maintained under Th22-polarizing conditions. ELISpot was also used to study secretion of the cytolytic molecules perforin, granzyme B, and

FasL. Interestingly, the clones were found to secrete either FasL or granzyme B but not perforin. The IL-22^{high} clones belonged exclusively to the FasL-producing subset.

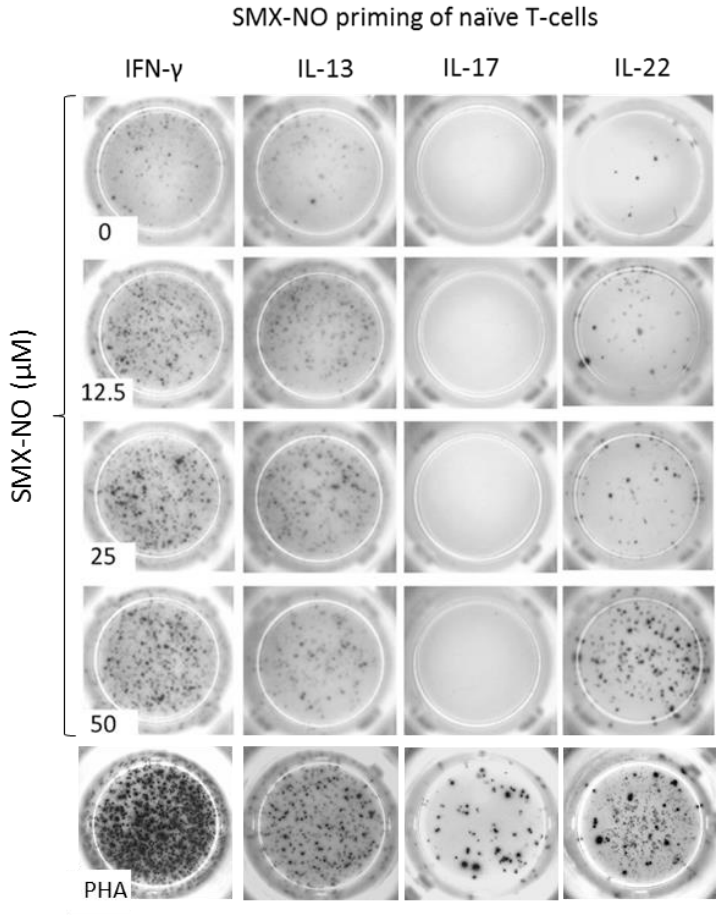


Figure 3.12. IL-22, but not IL-17, secretion by healthy donor-derived SMX-NO-primed naïve T-cells. Healthy donor naïve T-cells (2.5x10^6/well; total volume, 2 ml; flat-bottomed 24-well plates) were cultured with mature autologous monocyte-derived DCs (8x10^4/well) in the presence of SMX-NO (50 μM). Cells were cultured for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. In accordance with manufacturer's instructions, ELISpot plates were coated with IFN-γ, IL-13, IL-17, or IL-22 capture antibody and incubated at 4°C overnight. T-cells were harvested and re-plated (1x10^5/well; total volume, 200 μl; 96-well ELISpot plate) with fresh monocyte-derived DCs (4x10^3/well) and SMX-NO (12.5-50 μM). The cells were incubated for 48 hrs in a similar atmosphere as before. The ELISpot plates were then washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader. Figure shows representative data from one of three experiments.

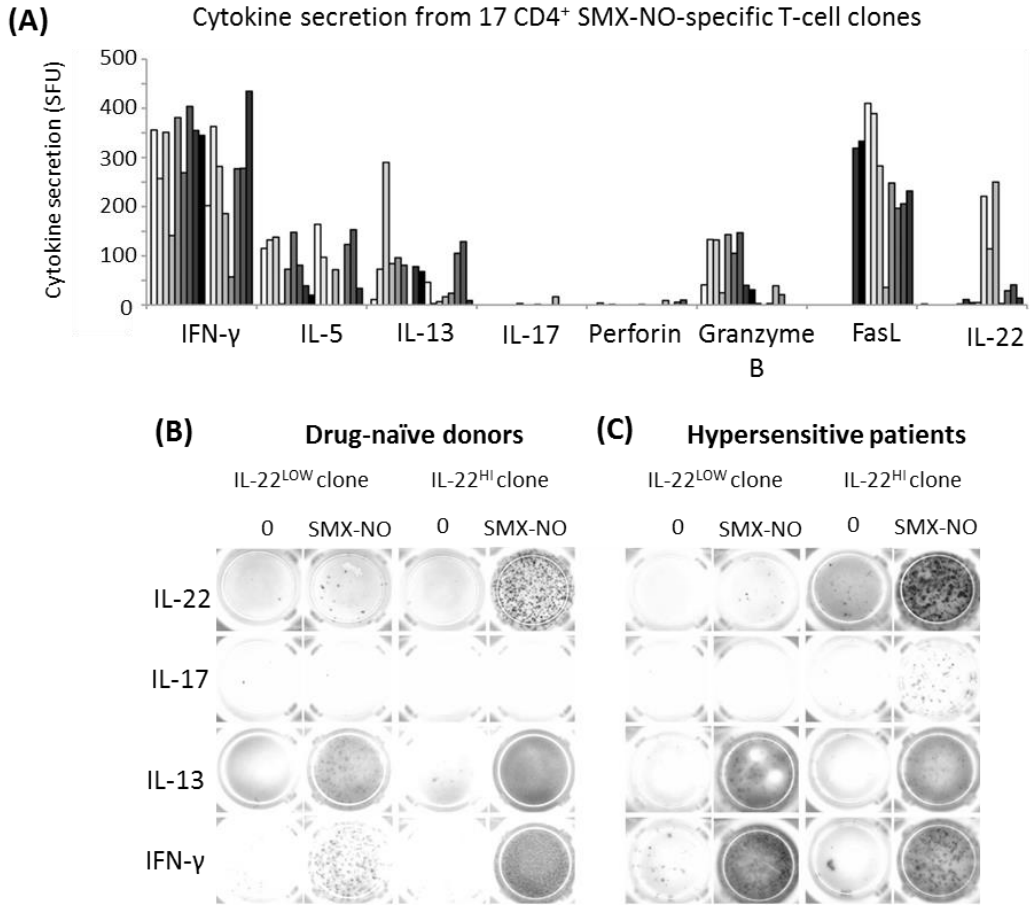


Figure 3.13. IL-22, but not IL-17, is secreted by CD4$^+$ SMX-NO–responsive T-cell clones derived from both healthy donors and hypersensitive patients. In accordance with manufacturer's instructions, ELISpot plates were coated with IFN-γ, IL-5, IL-13, IL-17, perforin, granzyme B, FasL, and IL-22 capture antibody and incubated at 4°C overnight. **(A)** 17 SMX-NO-responsive T-cell clones (5x10^5/well; total volume, 200 µl; 96-well ELISpot plate) were cultured with SMX-NO (40 µM) and autologous irradiated EBV-transformed B-cells (1x10^4/well) in an atmosphere of 95% air / 5% CO_2 / 37°C for 48 hrs. The ELISpot plates were washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader. **(B)** Similar analysis as above comparing SMX-NO-responsive T cell clones from healthy donors and hypersensitive patients identified distinctly separate IL-22^{high} – and IL-22^{low} –secreting clones and the secretion of IL-22 alongside IFN-γ.

3.3.6 PD-L1 signalling does not regulate the functionality of antigen-specific T-cells.

Although PD-1 is most commonly described as a marker of cell exhaustion, it has also been reported that PD-1^{high} cells are highly cytotoxic and/or proliferative (22, 23). Thus, our SMX-NO-specific T-cell clones were used to (1) measure PD-1 expression on individual cells, (2) explore the relationship between PD-1 expression and effector function, and (3) analyse whether PD-L1 block alters the levels or profile of cytokines secreted following antigen stimulation. Flow cytometric analysis of PD-1 on 40 clones revealed a 4-fold difference in expression (Figure 3.14a). PD-1 was stably expressed on the surface of T-cell clones; Figure 3.14b shows PD-1 staining on two representative clones maintained in culture for 10 days (± SMX-NO stimulation). PD-1 expression did not correlate with the strength of the drug-derived antigen-specific proliferative response or secretion of IFN-γ, IL-5, IL-13, IL-17, IL-22, perforin, granzyme B, or FasL (r2, 0.2 for all parameters tested; results documented in M. Ogese PhD thesis, 2014, The University of Liverpool). While blocking PD-L1 was not able to enhance the proliferative response of drug-derived antigen-specific T-cell clones (Figure 3.15a), PD-L1 blocking antibodies resulted in a modest increase in IFN-γ, IL-13, and granzyme B secretion when clones were stimulated with SMX-NO (Figure 3.15b).

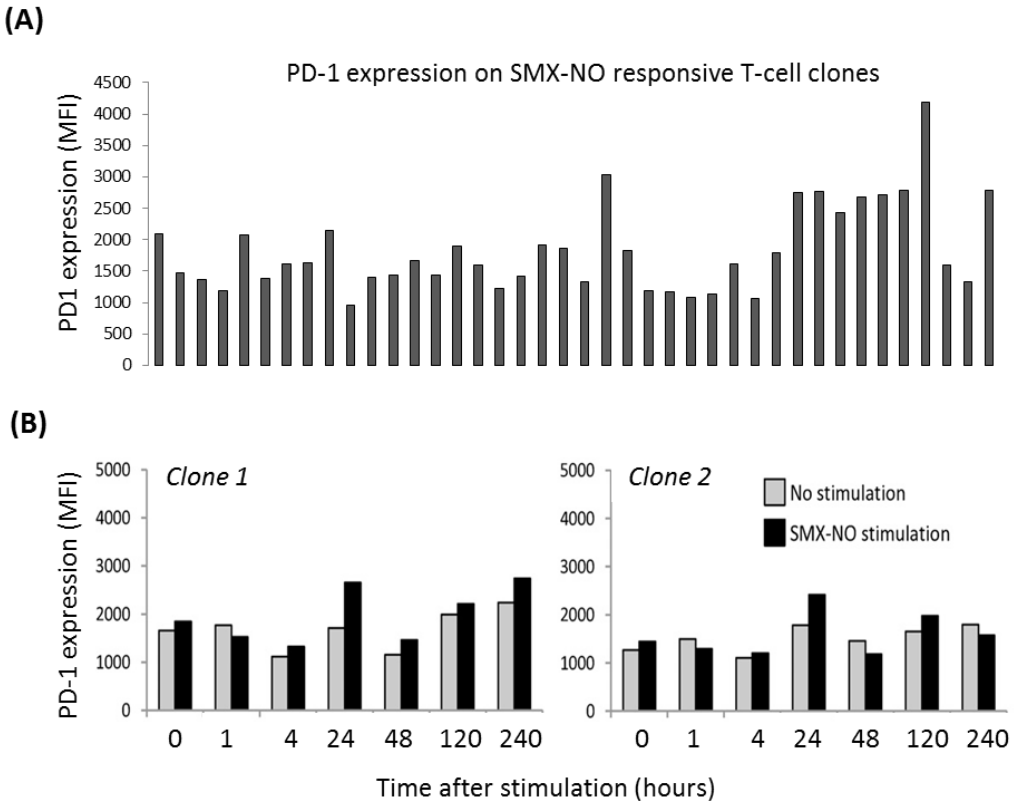


Figure 3.14. PD-1 expression on SMX-NO-specific T-cell clones. (A) PD-1 expression on 40 SMX-NO–specific T-cell clones. A sample of each clone ($5x10^4$ cells) was removed, resuspended in 3µl PD-1-PE and then incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in 300µl FACS buffer and analysed. **(B)** PD-1 expression on antigen stimulated and unstimulated SMX-NO-responsive T-cell clones. T-cell clones ($5x10^4$/well; total volume, 200 µl, 96-well U-bottomed plate) were stimulated with or without SMX-NO (40 µM) using autologous irradiated EBV-transformed B-cells ($1x10^4$/well). After incubation in an atmosphere of 95% air / 5% CO_2 / 37°C for 240 hrs. A sample of each clone ($5x10^4$ cells) was removed at various time points and stained for PD-1 expression as above. All flow cytometry was performed using a FACS Canto II machine and FACS DIVA software. PD-1 expression is presented as a measure of MFI.

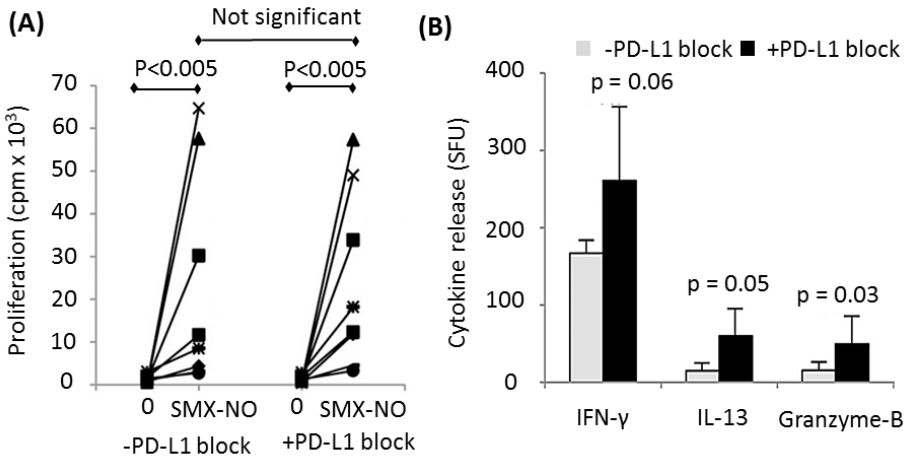


Figure 3.15. Proliferation and cytokine/cytolytic molecule secretion from antigen stimulated SMX-NO-responsive T-cell clones, ± PD-L1 blocking antibodies. (A) T-cell clones ($5x10^4$/well; total volume, 200 µl, 96-well U-bottomed plate) derived from healthy donor SMX-NO-primed naïve T-cells were stimulated with SMX-NO (40 µM) using autologous irradiated EBV-transformed B-cells ($1x10^4$/well), with and without inclusion of PD-L1 blocking antibodies (5 µg/ml). After incubation in an atmosphere of 95% air / 5% CO_2 / 37°C for 48 hrs, proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a further 16 hrs. Data are presented as the mean of duplicate wells. **(B)** In accordance with manufacturer's instructions, ELISpot plates were coated with IFN-γ, IL-13, or granzyme B antibody and incubated at 4°C overnight. Wells were then washed and blocked with R9 medium. T-cell clones were then cultured as above in triplicate. The ELISpot plates were washed and developed in concordance with the manufacturer's instructions. SFU counts were analysed from dry wells using an ELISpot reader.

3.4 Discussion.

We have focused on the regulation of drug-derived antigen-specific T-cell priming by the PD-1/PD-L1 pathway, the way in which PD-1 signalling influences effector T-cell responses, and the functionality of antigen-responsive T-cell clones generated through *in vitro* priming. The inhibitory function of PD-1 relies on the presence of an ITSM. Upon activation, this switch becomes phosphorylated and subsequently recruits the protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase 2. This causes the inhibition of downstream pathways through the dephosphorylation of proteins such as CD3 and Zap70 (Okazaki and Honjo, 2007; Riley, 2009), preventing further T-cell stimulation.

To assess the effect of PD-ligand blockade, and in particular whether this could be used as an immunogenic boost to enhance drug antigen-specific stimulation of naive T-cells, we utilised an *in vitro* T-cell priming assay and the model drug haptens SMX-NO and flucloxacillin. In agreement with a previous study from our group (Faulkner *et al.*, 2012), an 8-day culture period was sufficient to activate naive $CD3^+$ T-cells, and SMX-NO-specific T-cell responses were readily detectable following antigen recall using readouts for proliferation and IFN-γ or granzyme B secretion. CFSE staining revealed that naive $CD4^+$ and $CD8^+$ T-cells were activated during priming. The dividing cells were $CD45RO^+$, indicating a change in phenotype from naive to memory. PD-1 expression was induced on dividing T-cells during priming and following antigen recall. An increase in the magnitude of the drug-derived antigen-specific proliferative response and level of IFN-γ/granzyme B secretion was seen when naive T-cells primed to SMX-NO were exposed to PD-L1 blocking antibodies. In contrast, blocking PD-L2 had no effect and even hindered the increased activation of T-cells produced from PD-L1 blockade in 2/3 donors. We were also able to show that in the few donors who do not respond positively to SMX-NO initially, that the addition of PD-L1 block, but not PD-L2 block, can lead to detectable secretion of IFN-γ and granzyme B from these donors. Previous studies have shown that PD-1/PD-L2 signalling inhibits TCR-mediated triggering of proliferation and cytokine release. Thus, it is not clear why blocking PD-L2 did not enhance the priming of naive T-cells against SMX-NO. One potential explanation is that B7.1 (CD80), a CD28 costimulatory ligand, is known to interact with PD-L1, but not PD-L2 (Butte *et al.*, 2007). This, however, requires further investigation. Interestingly, CFSE staining suggested that the $CD4^+$ T-cells might be more sensitive to the effects of blocking PD-L1 than $CD8^+$ T-cells.

Next, we sought to establish whether PD-L1/PD-1 signalling negatively regulates the priming of T-cells against other drug-derived antigens. To do this, we focused on the beta-lactam

antibiotic flucloxacillin. In contrast to SMX-NO (Callan *et al.*, 2009; Naisbitt *et al.*, 1999; Rieder *et al.*, 1988), which forms antigenic determinants through the irreversible modification of cysteine, the beta-lactam ring of flucloxacillin is targeted by nucleophilic lysine residues (Jenkins *et al.*, 2009; Monshi *et al.*, 2013). Our research group have recently characterised drug-responsive $CD4^+$ and $CD8^+$ T-cells that express the gut-homing receptors CCR4 and CCR9 from patients with liver injury, but not tolerant controls (Monshi *et al.*, 2013). The HLA-B*57:01 genotype is a major determinant of flucloxacillin-induced liver injury, and, through the priming of naive T-cells from blood donors carrying the HLA risk allele, it has been possible to link the genetic association to the disease pathogenesis (Daly *et al.*, 2009; Monshi *et al.*, 2013; Wuillemin *et al.*, 2013). Importantly, priming naive T-cells against flucloxacillin generally leads to weak and inconsistent results. Confirmation of priming is often only obtained when flucloxacillin-responsive T-cells are cloned. In this study, we clearly show the enhanced priming of flucloxacillin-specific T-cells after blocking PD-L1 using IFN-γ and granzyme B ELISpot as readouts.

Whether PD-1 signalling regulates the activation of antigen-specific memory T-cell responses has yet to be fully defined. Therefore utilising our *in vitro* T-cell assay, on initial exposure of memory T-cells from healthy donors to SMX-NO, we simultaneously exposed a subset of these cells to PD-L1 blocking antibodies. A high number of unstimulated memory T-cells expressed PD-1, which was further enhanced in response to restimulation with SMX-NO. However, while $CD4^+$ and $CD8^+$ T-cells displayed a similar maximal increase in the percentage of PD-1 expressing cells in memory, as well as naïve subsets (increase in percentage PD-1 expression upon antigen exposure: naïve $CD4^+$, 26.9%; naïve $CD8^+$, 27.2%; memory $CD4^+$, 19.3%; memory $CD8^+$, 16.1%), this increase was much greater in naïve T-cells. Furthermore, unlike naïve T-cells where blocking PD-L1 significantly enhances drug-derived antigen-specific T-cell responses, no such increase was detected for memory T-cells exposed to PD-

L1 blocking antibodies. This suggests, despite the much higher basal expression of PD-1 on unstimulated memory T-cells, that the regulation of secondary T-cell responses is much less dependent on the PD-1 pathway that those of primary, naïve T-cell responses. This will be discussed in further detail in chapter 4.

Previous studies show that PD-1^{high} cells can be highly cytotoxic and that PD-1 expression might be a marker of effector memory function, which seems counterintuitive (Duraiswamy *et al.*, 2011; Zelinskyy *et al.*, 2011). Thus, using SMX-NO-responsive T-cell clones generated from healthy donors through priming and isolated directly from hypersensitive patient PBMCs (whose T-cells were primed at the time of the adverse reaction (Castrejon *et al.*, 2010; Nassif *et al.*, 2002; Schnyder *et al.*, 2000)), we assessed whether PD-1 expression correlates with the strength of the antigen-specific proliferative response and/or secretion of cytokines/cytolytic molecules. Furthermore, PD-L1/PD-L2 blocking antibodies were used to assess whether PD-1 signalling regulates the activation of antigen-specific memory T-cells. Detailed analysis of 40 clones revealed a 4-fold variation in PD-1 expression on T-cell clones, that PD-1 was stably expressed for up to 10 days after antigen stimulation, and there was no correlation between PD-1 expression and the magnitude of the SMX-NO-specific proliferative response or secretion of cytokines. Nevertheless, subtle increases in IFN-γ, IL-13, and granzyme B secretion were observed when clones were stimulated with SMX-NO in the presence of PD-L1 block. Blocking PD-L2 had no significant effect, and in fact acted to limit the regulatory response provided by PD-L1. Clearly, our data may relate in part to the manipulations involved in the long-term T-cell culture; however, at present there are no readily available alternatives to investigate these effects in humans.

Immunohistological studies characterizing the phenotype of T-cells infiltrating the inflamed skin of patients with maculopapular skin rashes describe the presence of large numbers of

$CD4^+$ T-cells and lower numbers of $CD8^+$ T-cells (Pichler, 2003; Pichler *et al.*, 2002). Those focusing on the SMX-specific T-cell response show that $CD4^+$ and $CD8^+$ T-cells can be activated by the drug to secrete cytolytic molecules; however, keratinocytes are specifically killed by $CD4^+$ T-cells (Schnyder *et al.*, 1998). In agreement with these findings, more recent studies show that most SMX (metabolite)-specific T-cells isolated from hypersensitive patients are $CD4^+$ and secrete a mixed panel of Th1/Th2 cytokines, including IFN-γ, IL-5, and IL-13 (Castrejon *et al.*, 2010; Elsheikh *et al.*, 2011; Schnyder *et al.*, 2000). However, the discovery of new T-cell populations (e.g. Th17, Th22) renders this classification out of date. We were able to generate SMX-NO-specific $CD4^+$ T-cell clones, isolated from the priming assay, which secreted IFN-γ, IL-5, and IL-13, but not IL-17. However, IL-22 secretion was detected from ∼50% of the T-cell clones. A similar pattern of cytokine secretion was seen with clones (IFN-γ^{high} IL-5^{high} IL-13^{high} IL-22^{low} and IFN-γ^{high} IL-5^{high} IL-13^{high} IL-22^{high}) isolated from SMX-hypersensitive patients. IL-22 is a cytokine that modulates tissue responses as expression of IL-22R1 is restricted to non-hematopoietic cells. In skin, the IL-22 receptor is expressed at high levels on keratinocytes, and IL-22 has been found to enhance keratinocyte proliferation and inhibit terminal differentiation (Boniface *et al.*, 2005). Furthermore, IL-22 has been shown to mediate inflammatory responses in patients with psoriasis, and IL-22-secreting cells have been identified in patients with ACD (Akdis *et al.*, 2012; Cavani *et al.*, 2012; Eyerich *et al.*, 2010). To our knowledge, these data are, however, the first to show the production of IL-22 alongside IFN-γ by antigen-specific T-cells from drug-hypersensitive patients. Given the heterogeneous secretion of IL-22 by individual $CD4^+$ clones, the release of cytolytic molecules (perforin, granzyme B, and FasL) were also measured using ELISpot. These studies clearly show that SMX-NO–responsive $CD4^+$ T-cells release cytolytic molecules when activated through their TCR. Two subsets of drug antigen-specific clones were identified and classified according to the production of either granzyme B or FasL. Importantly, the IL-22–secreting clones produced FasL following antigen stimulation. Indeed,

these separate FasL- and IL-22–secreting clones may be crucial mediators of the immunological reaction.

In conclusion, our *in vitro* study found that PD-L1/PD-1 signalling negatively regulates the priming of drug-derived antigen-specific T-cells that secrete a heterogeneous pattern of cytokines including IL-22, but not IL-17. In contrast, PD-L2 has a limiting effect on the regulation imposed by PD-L1. PD-L1 was unable to influence the proliferative capacity of secondary T-cell responses, but was able to minimally modulate cytokine secretion. While numerous previous studies have used PD-1 as a marker of T-cell exhaustion, we show that PD-1 is highly expressed on dividing T-cells. Although we have shown the propensity for this pathway to regulate the activation of drug-specific T-cells, we would urge caution in utilising anti-PD-1 pathway antibodies in diagnostic assays. As we remove more and more layers of immune regulation, we are likely to see more and more enhanced T-cell activation, however the point at which this translates into a predisposition has yet to be elucidated. Indeed, minimal immune activation may not be sufficient for a full-blown hypersensitivity reaction. Furthermore, although PD-1 pathway antagonists have been successfully utilised for the treatment of cancer, by blocking the immune system such therapies probably albeit inadvertently increase the risk of drug hypersensitivities and autoimmunity. This represents a tricky balancing act of enhancing immune responses against problematic antigens while maintaining tolerance against those that are non-harmful. Recently, Goldinger *et al* reported that anti-PD-1 cancer therapeutics are associated with adverse cutaneous eruptions including those that result in epidermal detachment. In this study, 22% of patients receiving anti-PD-1 therapy for stage IV melanoma developed cutaneous reactions, all of which were deemed TEN-like reactions based upon gene induction profiles (Goldinger *et al.*, 2016). They concluded that the PD-1 pathway is likely important for the maintenance of epidermal integrity during such inflammatory reactions. However, due to the success of anti-PD-1

therapies to treat cancers, the future use of anti-PD-1 therapeutics may be decided on whether a clinician decides there are no other alternatives and the advantages simply outweigh the disadvantages. Nonetheless, our data provide a foundation to explore PD-L1/PD-1 expression and activity in prospective studies of drug immunogenicity.

Chapter 4: The individual roles of the co-inhibitory CTLA4 and TIM-3 receptors on drug antigen-specific T-cell responses.

Contents

4.1 Introduction.

The management of T-cell activation via co-signalling pathways is a complex balance between an array of co-stimulatory, and co-inhibitory pathways that lower or increase the T-cell activation threshold, respectively. Over recent years, it is becoming increasingly apparent that the cumulative output from these pathways plays a significant role in the activation of T-cells. Indeed, the manipulation of these pathways is receiving considerable attention as a promising therapeutic strategy to treat a range of diseases with an immunological mechanism, with the main agents being developed in relation to the treatment of cancers (Hamid *et al.*, 2013a; Ngiow *et al.*, 2011; Yang *et al.*, 2007). Whilst the influence of these pathways in the control of cancer is well-researched, their respective roles in drug-derived antigen-specific T-cell activation remains largely unexplored.

While hypersensitivity to a variety of drugs has been recently associated with particular HLA-alleles, for the majority of drugs these associations are unlikely to be the only predisposing factor. It may be that inter-individual discrepancies in co-inhibitory receptor expression/activity control the balance between an antigenic response and tolerance. In fact, a synergistic interaction of HLA-allele association and polymorphic variants of the co-inhibitory receptor CTLA4 has already been proposed to lead to the predisposition for the T-cell mediated immunological disorder Grave's Disease (Takahashi and Kimura, 2010). These co-inhibitory pathways are therefore of great interest in the development of other T-cell-mediated disorders, including drug hypersensitivity reactions. While there is a diverse array of co-inhibitory pathways to explore, two are of particular interest in relation to PD-1 (discussed in chapter 4); CTLA4 and TIM-3.

CTLA4 has been identified as a major immune-regulatory checkpoint, alongside PD-1. This was made evident using early animal models where mice died within 3-4 weeks after birth with CTLA4 knockdown, and within a couple of months for PD-1 knockdown. In both cases,

death was related to the onset of lymphoproliferative disorders. The time difference between deaths for these two receptors has been attributed to differential roles within peripheral and central tolerance for the two pathways, as CTLA4 deficient mice die from a multi-organ lymphoproliferative disorder, whereas PD-1 deficient mice experience a targeted organ-specific response. Whilst PD-1 is found on a wide number of cells involved in immune responses, CTLA4 expression is more restricted and is a well-described checkpoint for the specific activation of T-cells. Despite this, CTLA4 commands a high level of control, which is perhaps unsurprising as it shares both of its ligands (CD80 and CD86) with the CD28 co-stimulatory pathway. Both of these ligands are presented on the surface of APCs, including DCs. Whilst far less CTLA4 is presented on the surface of T-cells in comparison to CD28, the affinity of both ligands is much greater for CTLA4 allowing the inhibitory pathway to dominate under certain conditions (Figure 4.1) (Teft *et al.*, 2006). It may be that during physiological T-cell responses, the balance between T-cell activation and inactivation is governed by differential ligand expression during early and late stage T-cell responses. Indeed, it has been proposed that CD86 is predominantly co-inhibitory as its expression is only induced after activation, while constitutive expression of CD80 on APCs may pinpoint a predominantly co-stimulatory function (Teft *et al.*, 2006). However, CTLA4 also acts to regulate T-cells through a variety of different mechanisms, including increasing T-cell binding competition between APCs and Tregs through induction of adhesion molecules, thus hindering the formation of a viable immunological synapse (Rudd, 2008; Schneider *et al.*, 2006). While it is often presumed that these pathways are similar between models and diseases, a recent gene association study revealed that this is not always the case. Specifically, they identified a polymorphism in the CTLA4 gene which results in a $CTLA4^{high}$ phenotype that is associated with the development of the T-cell mediated autoimmune disease myasthenia gravis (Chuang *et al.*, 2005). A variant of the same gene which produces a $CTLA4^{low}$ phenotype has also been associated with the onset of autoimmune disease (Ueda

et al., 2003). While a large proportion of previous studies have focussed on mouse models in reference to tumour or viral-specific T-cells, extrapolation to human drug-derived antigen-specific T-cells must be done so with caution; thus further investigations are warranted.

TIM-3 is a less well-defined co-inhibitory receptor than both PD-1 and CTLA4, perhaps due to its discovery being less than 15 years ago. In contrast to PD-1 and CTLA4, TIM-3 offers a narrower spectrum of T-cell influence as its expression is thought to be predominantly found on Th1-specific T-cells. While other pathways, including PD-1 and CTLA4, act to dampen T-cell responses through the manipulation of cell activation pathways such as those allowing for progression through the cell cycle, TIM-3 is thought to regulate T-cell responses by inducing cell death (Figure 4.1) (Zhu *et al.*, 2005). This different method of T-cell regulation renders TIM-3 an interesting regulatory pathway to investigate. However, TIM-3 is of particular interest as it is upregulated alongside the major checkpoint PD-1 on tumour-infiltrating T-cells in cancer models (Sakuishi *et al.*, 2010). In a murine cancer model, not only did all TIM-3^+ T-cells which were infiltrating the tumour co-express PD-1, these PD-1^+ TIM-3^+ T-cells represented the majority of infiltrating T-cells (Sakuishi *et al.*, 2010). TIM-3 is known to mediate its inhibitory effect through binding to galectin-9, a lectin found on a range of tissues and cells including APCs (Lee *et al.*, 2010; Wada and Kanwar, 1997). In patients with certain T-cell mediated immunological disorders, such as multiple sclerosis, enhanced expression of pro-inflammatory cytokines directly correlates with the expression of TIM-3, emphasising the role of this pathway in the activation of Th1 T-cells (Khademi *et al.*, 2004). The inhibitory role of this pathway was confirmed using a TIM-3-Ig fusion protein to block the receptor-ligand interaction, which resulted in T-cell hyper-proliferation (Sabatos *et al.*, 2003). Further studies identified that TIM-3 mediates its inhibitory effects through the induction of cell death, at least in part through activation of the calcium-calpain-caspase-1 pathway (Zhu *et al.*, 2005). Interestingly, Th1 cell-mediated release of IFN-γ induces galectin-9 expression, leading to activation of Th1-cell death, thus the TIM-3-galectin-9 pathway acts

as part of a negative feedback loop to prevent an overwhelming Th1-response (Zhu *et al.*, 2005). This may well provide the link to PD-1 expression as it is well documented that IFN-γ is able to induce PD-ligand expression on monocytes (Freeman *et al.*, 2000; Latchman *et al.*, 2001), and may provide the mechanism for the emerging anti-inflammatory role of IFN-γ seen in protection against certain autoimmune disorders, including arthritis and EAE (Billiau *et al.*, 1988; Vermeire *et al.*, 1997).

In addition to regulating autoimmunity in several experimental models, both CTLA4 and TIM-3 gene polymorphisms are associated with the onset of a range of autoimmune diseases (Chae *et al.*, 2004; Chang *et al.*, 2007; Donner *et al.*, 1997a; Donner *et al.*, 1997b; Gonzalez-Escribano *et al.*, 1999; Kouki *et al.*, 2000; Monney *et al.*, 2002). Therefore we wanted to investigate the independent influence that these pathways have on the regulation of T-cell responses to drug-derived antigens by utilising the model drug hapten SMX-NO.

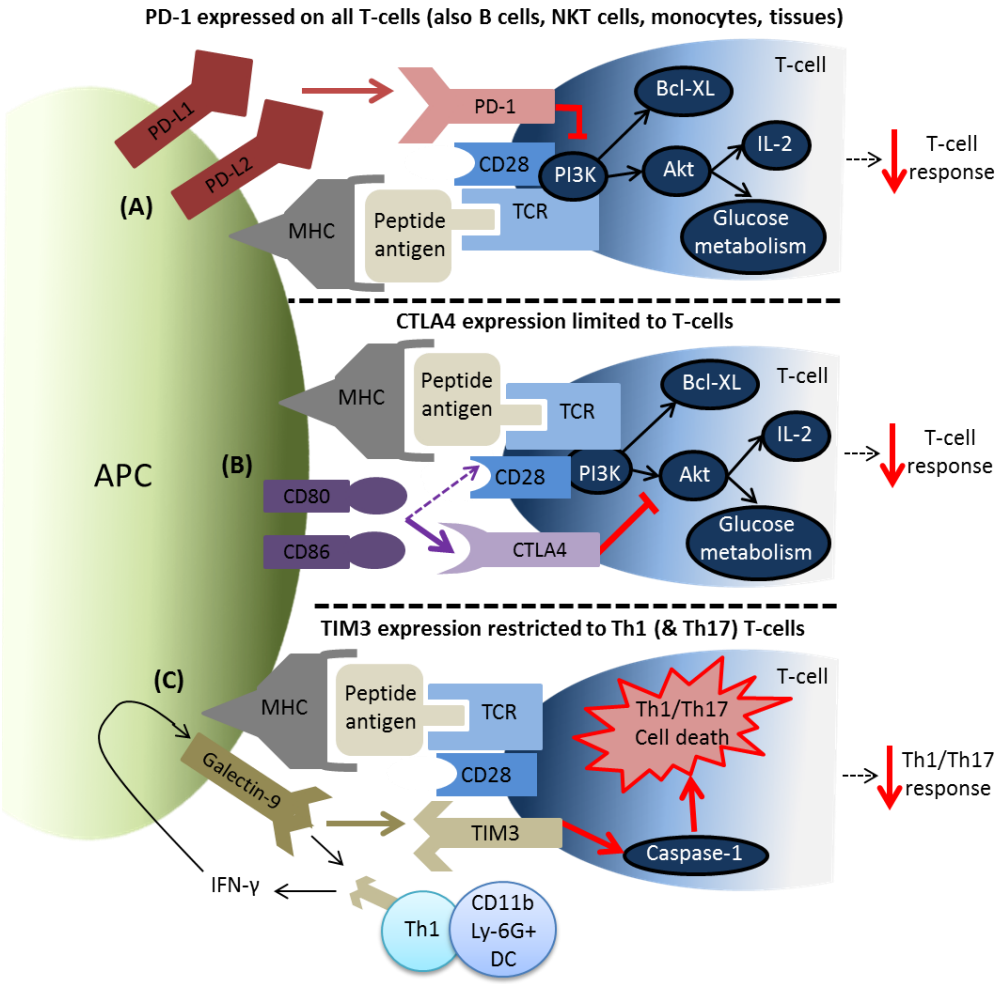


Figure 4.1. The interaction between the PD-1, CTLA4, and TIM-3 co-inhibitory receptors and their respective ligands during T-cell stimulation. **(A)** PD-1 is expressed on all T-cells and has two ligands expressed on APCs, PD-L1 and PD-L2. PD-1 activation blocks TCR-induced PI3K activation, thus dampening the T-cell response. **(B)** CTLA4 is expressed on all T-cells, and binds to CD80 and CD86 on APCs with higher binding affinity than that of the co-stimulatory receptor CD28. CTLA4 blocks T-cell signalling downstream of PI3K. **(C)** TIM-3 is mainly expressed on Th1, but also Th17 T-cells. It binds to galectin-9 on APCs which activates a caspase-1 pathway leading to cell death. TIM-3-galectin-9 interaction may also produce a subset of myeloid suppressor cells ($CD11b^{+}$ $Ly\text{-}6G^{+}$) which, along with Th1 cells themselves, secrete IFN-γ which acts as a positive feedback loop that promotes further galectin-9 expression. Adapted from Parry *et al* (Parry *et al.*, 2005).

4.2 Chapter aims.

1) Investigate the individual regulatory roles of CTLA4 and TIM-3 on the antigen-specific priming of naïve T-cells to SMX-NO.

2) To analyse how secondary T-cell responses are regulated by both CTLA4 and TIM-3 using memory SMX-NO-responsive T-cells from healthy donors.

3) To monitor the kinetics of CTLA4 and TIM-3 expression during the SMX-NO-specific activation of memory and naïve T-cells from healthy donors.

4) To characterise the function and surface expression of CTLA4 and TIM-3 on SMX-NO-specific T-cell clones.

4.3 Results.

4.3.1 CTLA4 negatively regulates the priming of naive T-cells against drug-derived antigens.

For *in vitro* priming, naive $CD3^+$ T-cells from healthy donors were co-cultured with autologous mature DCs in the presence of SMX-NO (± CTLA4 block). DCs were routinely stained for costimulatory receptors and characterised as previously detailed in chapter 3. Importantly, ligand expression was detected on APCs and EBV-transformed B-cells for both receptors (Figure 4.2). CTLA4 ligand expression on DCs were analysed as $CD80^{mid}$ $CD86^{high}$ (Figure 4.2b). Upon restimulation, dose-dependent antigen-specific proliferation was clearly detectable from SMX-NO-primed naïve T-cells, which was enhanced by the addition of CTLA4-blocking antibodies (Figure 4.3a; three representative donors; SMX-NO, 20 µM; $p \leq 0.05$). In total, inclusion of CTLA4 blockade significantly enhanced the proliferative response of SMX-NO-primed naïve T-cells using cells from 5/8 donors ($p \leq 0.05$). Despite this enhancement being generally lower than when blocking PD-L1, one donor displayed a > 6-fold increase with the addition of CTLA4 block ($p = 0.006$). An increase in SMX-NO-specific T-cell responses in the presence of CTLA4 blockade in the remaining 3 donors was not detected. The fold increase in naïve T-cell responses to SMX-NO, and associated significance, in the presence of CTLA4 blocking antibodies are detailed in table 1.

CTLA4 blockade was also found to enhance the proliferative response of SMX-NO-exposed memory T-cells using cells from 3/6 donors (Figure 4.3b; SMX-NO, 20 μM; $p \leq 0.05$), with again more than a 6-fold increase in SMX-NO-specific proliferation for one donor ($p = 0.006$). An increase in SMX-NO-specific memory T-cell response in the presence of CTLA4 blockade in the remaining 3 donors was not detected. For each donor, the fold increase in proliferative memory T-cell response to SMX-NO, and associated significance with the addition of CTLA4 block is detailed in table 2.

4.3.2 Blocking the TIM-3 pathway had no effect on the activation of healthy donor naïve or memory T-cells with SMX-NO.

In vitro priming was performed as described in section 4.3.1 but in the absence or presence of TIM-3 block instead of CTLA4. Expression of the TIM-3-ligand galectin-9 was detected on DCs and EBV-transformed B-cells (Figure 4.2c). Upon restimulation, dose-dependent antigen-specific proliferation was clearly detectable from SMX-NO-primed naïve T-cells, but was not enhanced by the addition of TIM3-blocking antibodies (Figure 4.4a; three representative donors; SMX-NO, 20 μM; $p \leq 0.05$), as was the response from SMX-NO-exposed memory T-cells (Figure 4.4b; three representative donors; SMX-NO, 20 μM; $p \leq 0.05$). In total, blocking TIM-3 was not found to enhance the proliferative response of SMX-NO-primed naïve T-cells or SMX-NO-exposed memory T-cells from any donor. For each donor, the fold increase in proliferative naïve or memory T-cell response to SMX-NO, and associated significance with the addition of CTLA4 block is detailed in tables 1 and 2, respectively.

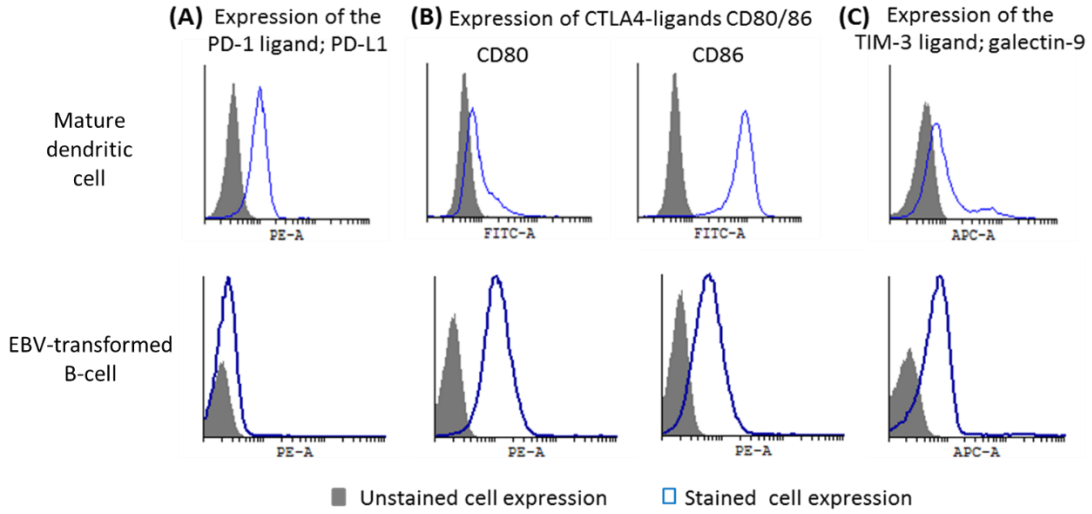


Figure 4.2. Co-signalling ligand expression on mature monocyte-derived DCs and on EBV-transformed B-cells. The expression of **(A)** the PD-1-ligand PD-L1, **(B)** the CTLA4-ligands CD80 and CD86, and **(C)** the TIM-3-ligand galectin-9 on mature DCs, utilised during the *in vitro* stimulation of naïve and memory T-cells, and on EBV-transformed B-cells, utilised during T-cell cloning. Grey histogram represents unstained cells, blue histograms represent stained cell expression. DCs were generated through *in vitro* culture of $CD14^{+}$ monocytes with GM-CSF- and IL-4 (800 U/ml)-supplemented R9 medium for 7 days. On the penultimate day, DCs were matured using LPS (1 µg/ml) and TNF-α (25 ng/ml). 24 hrs later, each clone was pipetted into 4 tubes ($5x10^{4}$ cells/tube) and resuspended in 3 ul of fluorochrome-bound antibody. Cells were incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and analysed on a FACS Canto II machine using cyflogic software.

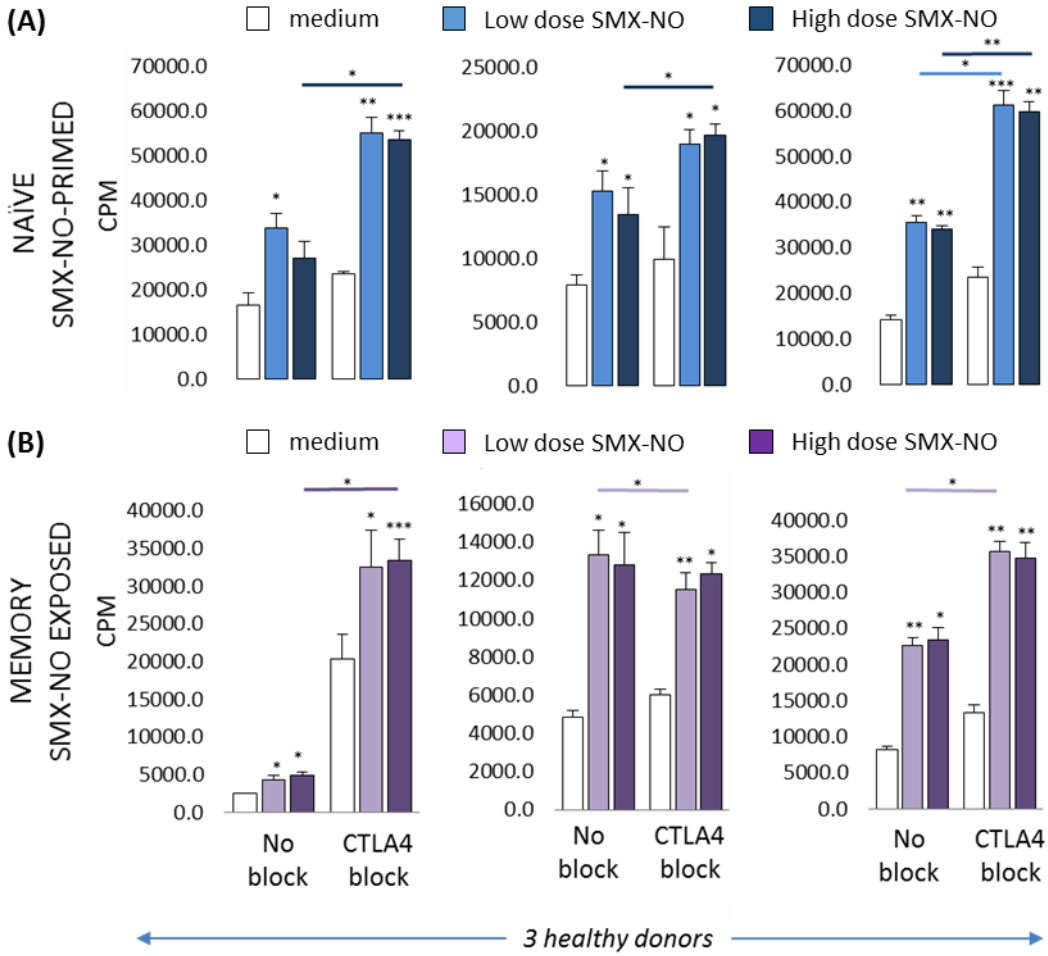


Figure 4.3. Comparative antigen-specific activation of SMX-NO-primed naive and memory T-cells in the presence and absence of CTLA4 blocking antibodies. (A) Naïve or **(B)** memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from healthy donors were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) for one 1 week, ± CTLA4 blocking antibodies (10 µg/ml), in an atmosphere of 95% air / 5% CO_2 / 37°C. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and either a low (20-30 µM) or high concentration (40-50 µM) of SMX-NO for 48 hrs in similar conditions as before. [^{3}H]-thymidine (0.5 µCi/well) was added and incubated for a further 16 hrs. Data from three representative donors shows the mean ± SD of triplicate cultures. Statistical significance above a bar denotes a significant increase in proliferative response compared to 'medium only' treated wells within that condition (i.e. CTLA4 blocked cells only). Statistical significance between conditions denotes a significant increase in proliferative response compared to 'no block' treated wells at a particular drug concentration after normalisation of all data values to account for differing basal stimulation (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

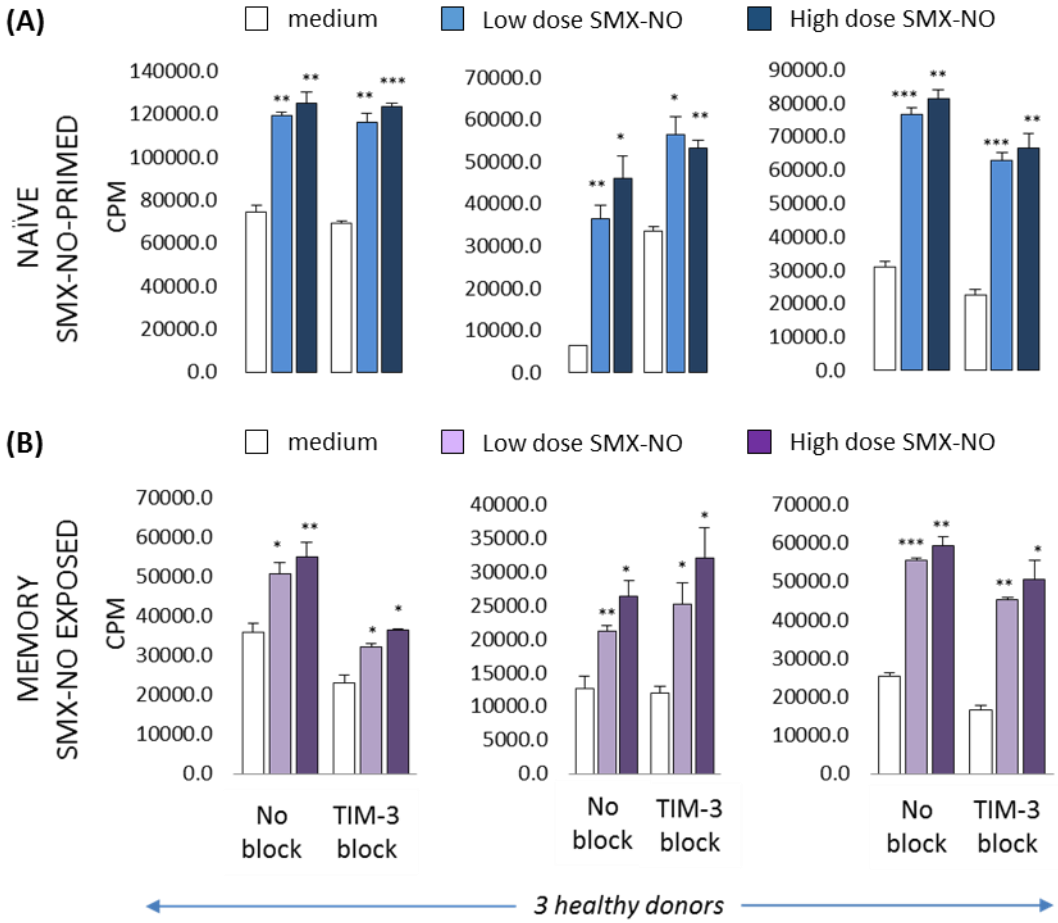


Figure 4.4. Comparative antigen-specific activation of SMX-NO-primed naive and memory T-cells in the presence and absence of TIM-3 blocking antibodies. (A) Naïve or **(B)** memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from healthy donors were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) for one 1 week, ± TIM-3 blocking antibodies (10µg/ml), in an atmosphere of 95% air / 5% CO_2 / 37°C. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and either a low (20-30 µM) or high concentration (40-50 µM) of SMX-NO for 48 hrs in similar conditions as before. [^{3}H]-thymidine (0.5 µCi/well) was added and incubated for a further 16 hrs. Data from three representative donors shows the mean ± SD of triplicate cultures. Statistical significance above a bar denotes a significant increase in proliferative response compared to 'medium only' treated wells within that condition (i.e. TIM-3 blocked cells only). Statistical significance between conditions denotes a significant increase in proliferative response compared to 'no block' treated wells at a particular drug concentration after normalisation of all data values to account for differing basal stimulation (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

Table 4.1. Summary of the comparative antigen-specific activation of SMX-NO-primed naive T-cells in the presence and absence of co-inhibitory pathway blocking antibodies. Naïve T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from healthy donors were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 μM) for one 1 week, ± PD-L1/CTLA4/TIM-3 blocking antibodies, in an atmosphere of 95% air / 5% CO_2 / 37°C. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 μl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (20-50 μM) for 48 hrs in similar conditions as before. [^{3}H]-thymidine (0.5 μCi/well) was added and incubated for a further 16 hrs. The maximal responding drug concentration was selected from each donor for the purpose of analysis in the table. Strength of response after restimulation with SMX-NO is shown as CPM above background; weak (< 15000), good (15000-30000), strong (30000-45000), very strong (> 45000). An enhanced response is shown by significant fold increase with block compared to without (*$p \leq 0.05$).

Block used	Donor number	- block drug Response (CPM)	+ block drug Response (CPM)	SI of comparative response with addition of block
PD-L1	Donor 1	weak (3549.0)	good (15214.0)	↑ 4.3 (p=0.002)
	Donor 2	weak (2543.3)	weak (12360.6)	↑ 4.9 (p=0.038)
	Donor 3	weak (13318.3)	very strong (63258.3)	↑ 4.7 (p=0.012)
	Donor 4	weak (7689.0)	good (29107.6)	↑ 3.8 (p=0.020)
	Donor 5	very strong (51009.6)	good (29463.6)	No increase
CTLA4	Donor 6	weak (5475.3)	weak (9750.3)	↑ 1.8 (p=0.043)
	Donor 7	good (19496.6)	strong (32843.3)	↑ 1.7 (p=0.027)
	Donor 8	good (15668.3)	strong (36262.0)	↑ 2.3 (p=0.023)
	Donor 9	weak (8050.0)	very strong (49669.3)	↑ 6.2 (p=0.006)
	Donor 10	very strong (50829.6)	very strong (68964.3)	↑ 1.4 (p=0.017)
	Donor 5	good (19659.0)	strong (36279.6)	↑ 1.8 (p=0.010)
	Donor 11	good (25933.6)	weak (14800.6)	No increase
	Donor 12	weak (8802.6)	weak (7135.6)	No increase
	Donor 4	strong (39129.3)	strong (38245.0)	No increase
TIM-3	Donor 13	very strong (50698.0)	very strong (54427.0)	No increase
	Donor 14	strong (43080.3)	good (23292.0)	No increase
	Donor 5	very strong (50357.3)	strong (44196.0)	No increase

Table 4.2. Summary of the comparative antigen-specific activation of SMX-NO-exposed memory T-cells in the presence and absence of co-inhibitory pathway blocking antibodies. Memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from healthy donors were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 μM) for one 1 week, ± PD-L1/CTLA4/TIM-3 blocking antibodies, in an atmosphere of 95% air / 5% CO_2 / 37°C. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 μl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (20-50 μM) for 48 hrs in similar conditions as before. [^{3}H]-thymidine (0.5 μCi/well) was added and incubated for a further 16 hrs. The maximal responding drug concentration was selected from each donor for analysis in the table. Strength of response after restimulation with SMX-NO is shown as CPM above background; weak (< 15000), good (15000-30000), strong (30000-45000), very strong (> 45000). An enhanced response is shown by significant fold increase with block compared to without (*$p \leq 0.05$).

Block used	Donor number	- block drug Response (CPM)	+ block drug Response (CPM)	SI of comparative response with addition of block
PD-L1	Donor 15	weak (13750.6)	weak (8158.0)	No increase
	Donor 5	strong (32780.0)	good (24932.3)	No increase
CTLA4	Donor 5	weak (14443.6)	good (22310.3)	↑ 1.5 (p=0.022)
	Donor 9	weak (7470.3)	weak (9981.6)	↑ 1.3 (p=0.009)
	Donor 8	weak (981.0)	weak (5666.6)	↑ 6.6 (p=0.002)
	Donor 11	weak (8032.6)	weak (6903.3)	No increase
	Donor 6	weak (8500.3)	weak (5548.0)	No increase
	Donor 10	very strong (47951.3)	good (29999.6)	No increase
TIM-3	Donor 5	strong (33874.0)	strong (34144.3)	No increase
	Donor 15	weak (13750.6)	good (19919.3)	No increase
	Donor 13	good (17515.3)	weak (13202.3)	No increase

4.3.3 Cytokine secretion from drug-derived antigen-primed naïve and memory T-cells, with and without CTLA4- or TIM-3-blockade.

As an alternative measure of T-cell activation following priming of naïve T-cells to SMX-NO, or memory T-cell exposure to SMX-NO, secretion of IL-13 was assessed using ELISpot. IL-13

secretion was assessed as a reliable and reproducible readout during initial SMX-NO-priming cultures. However, as TIM-3 is known to be predominantly expressed on Th1 T-cells, we also assessed the effect of TIM-3 blockade on the secretion of the Th1-associated cytokine, IFN-γ.

Both naïve and memory T-cell subsets were assessed for an increase in SMX-NO-specific IL-13 secretion following addition of either CTLA4 or TIM-3 blocking antibodies; two donors were assessed per inhibitory block. For the majority of cultures, little or no increase in IL-13 secretion could be detected after blockade of either CTLA4 (Figure 4.5) or TIM-3 (Figure 4.6) in comparison to non-inhibitory block-treated cultures when re-stimulated with SMX-NO. An increase in IL-13 secretion was however detected from SMX-NO primed-naïve T-cells in one donor treated with and without CTLA4 block (Figure 4.5; donor 4). Specifically, a 1.2-fold SMX-NO-specific increase in IL-13 secretion was detected without CTLA4 block (average IL-13 spot forming units: medium, 131.3 ± 19.0; 20 µM SMX-NO, 155.0 ± 19.7), rising to 1.6-fold in cultures where CTLA4 was blocked (average IL-13 spot forming units: medium, 101.3 ± 2.9; 20 µM SMX-NO, 157.3 ± 9.0) (Figure 4.5a). In a similar fashion, the memory SMX-NO-specific T-cell response was enhanced with the addition of TIM-3 block in one donor (Figure. 4.6; donor 10). A 1.4-fold SMX-NO-specific increase in IL-13 secretion was detected from non-TIM-3 block treated cultures (average IL-13 spot forming units: medium, 111.0 ± 48.1; 20 µM SMX-NO, 150.0 ± 7.1), rising to 1.6-fold in cultures where CTLA4 was blocked (average IL-13 spot forming units: medium, 132.5 ± 30.4; 20 µM SMX-NO, 238.5 ± 26.2).

The secretion of IFN-γ (Figure 4.7a) was compared to the secretion of IL-13 (Figure 4.7b) with and without TIM-3 block using SMX-NO cultures from a single individual. The secretion of IFN-γ was dramatically enhanced by the addition of CTLA4 blocking antibodies in memory cultures for both SMX-NO concentrations assessed (Figure 4.7a). In the presence of 30 µM

SMX-NO without block, IFN-γ secretion was 1.3-fold greater than medium alone, rising to 2.2-fold with the inclusion of TIM-3 blocking antibodies. This difference ± TIM-3 block increased further at 50 μM SMX-NO where standard cultures showed no increase in IFN-γ secretion in the presence of SMX-NO, whereas in cultures were TIM-3 was blocked, secretion was 5.9-fold greater (IFN-γ spot counts; memory –block: 0 μM, 348.5 ± 286.4; 30 μM, 444.5 ± 135.1; 50 μM, 352.5 ± 30.4 / memory + TIM-3 block: 0 μM, 89.5 ± 12.0; 30 μM, 194.0 ± 0.0; 50 μM, 532.5 ± 16.3). While IFN-γ secretion ± TIM-3 blocking antibodies showed a similar 1.3-fold increase in the presence of 30 μM SMX-NO, at 50 μM SMX-NO, no increase in secretion was detected for cultures without block, but a 2.1-fold increase in those exposed to TIM-3 blocking antibodies (IFN-γ spot counts; naive – block: 0 μM, 365.5 ± 3.5; 30 μM, 508.0 ± 22.6; 50 μM, 272.5 ± 34.6 / naive + TIM-3 block: 0 μM, 266.0 ± 90.5; 30 μM, 361.0 ± 141.4; 50 μM, 557.5 ± 33.2).

In comparison, the secretion of IL-13 was only slightly enhanced by the inclusion of TIM-3 blocking antibodies for both naïve and memory cultures (fold increase from 0-50 μM: naïve -TIM-3 block, 1.1-fold; naïve + TIM-3 block, 1.2-fold; memory - TIM-3 block, 1.3-fold; memory + TIM-3 block, 1.4-fold). Although this analysis is only based upon a 1.1-1.2 fold increase for naïve T-cells activated with SMX-NO due to high background spot counts, there remains no obvious visual difference between “no block” and “TIM-3 block” treated wells.

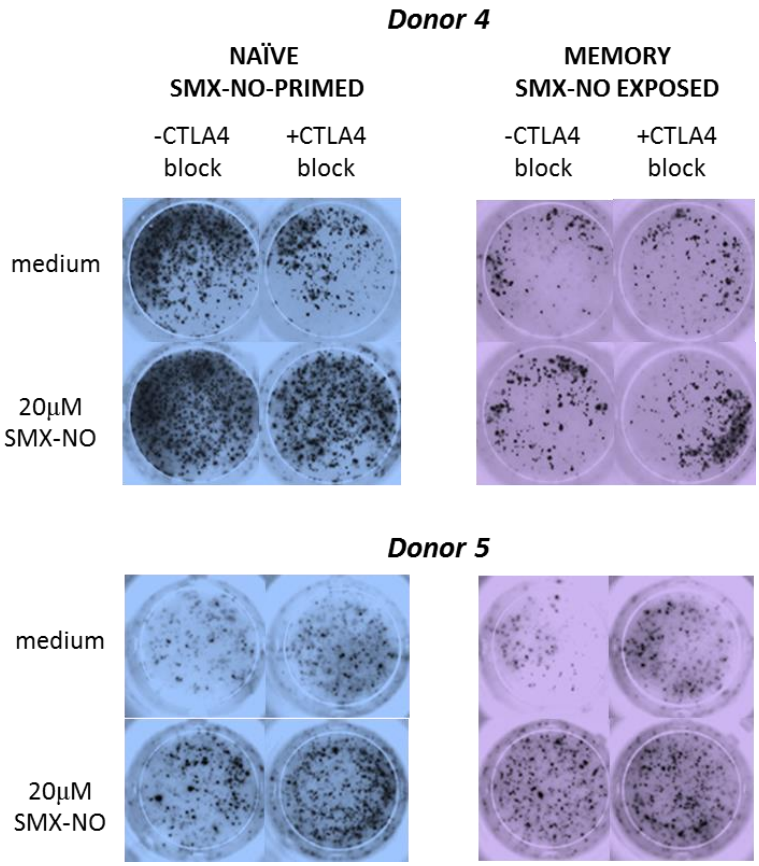


Figure 4.5. **The secretion of IL-13 after priming naïve and memory T-cell from healthy donors to SMX-NO, ± CTLA4 blocking antibodies**. Naïve and memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from two healthy donors were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 μM), ± CTLA4 blocking antibodies (10 μg/ml). Cells were cultured for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. In accordance with manufacturer's instructions, ELISpot plates were coated with IL-13 capture antibody and incubated at 4°C overnight. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 μl; 96-well ELISpot plate) with fresh monocyte-derived DCs ($4x10^3$/well) and SMX-NO (20 μM). The cells were incubated for 48 hrs in a similar atmosphere as before. The ELISpot plates were then washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader.

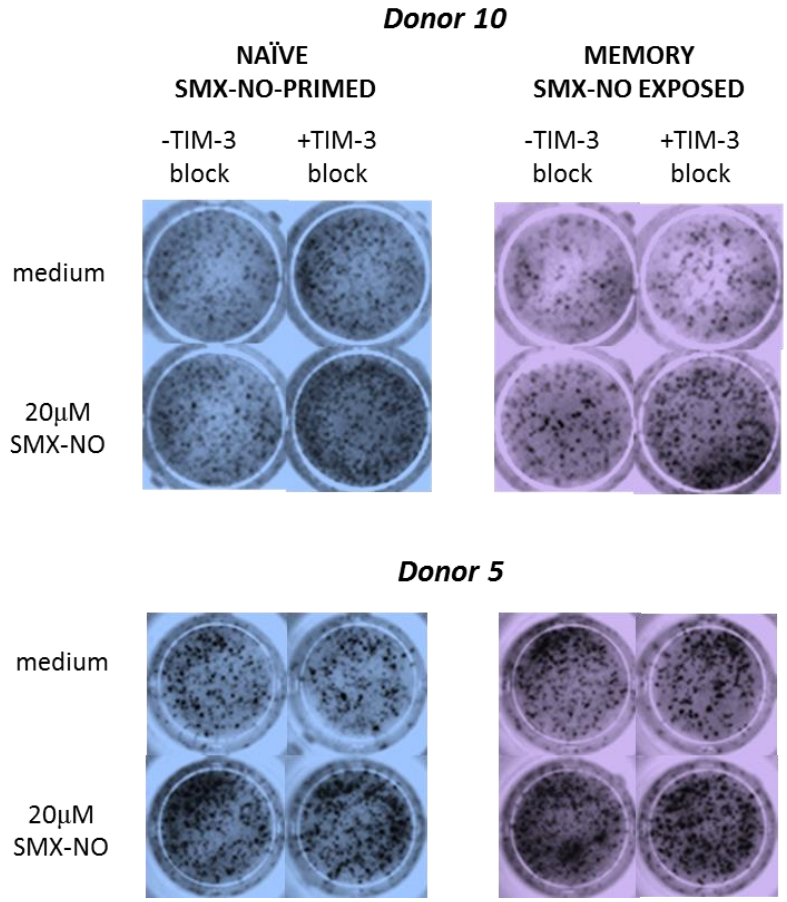


Figure 4.6. **The secretion of IL-13 after priming naïve and memory T-cell from healthy donors to SMX-NO, ± TIM-3 blocking antibodies**. Naïve and memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from two healthy donors were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM), ± TIM-3 blocking antibodies (7.5 µg/ml). Cells were cultured for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. In accordance with manufacturer's instructions, ELISpot plates were coated with IL-13 capture antibody and incubated at 4°C overnight. Wells were then washed and blocked with R9 medium. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed ELISpot plate) with fresh monocyte-derived DCs ($4x10^3$/well) and SMX-NO (20 µM). The cells were incubated for 48 hrs in a similar atmosphere as before. The ELISpot plates were then washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader.

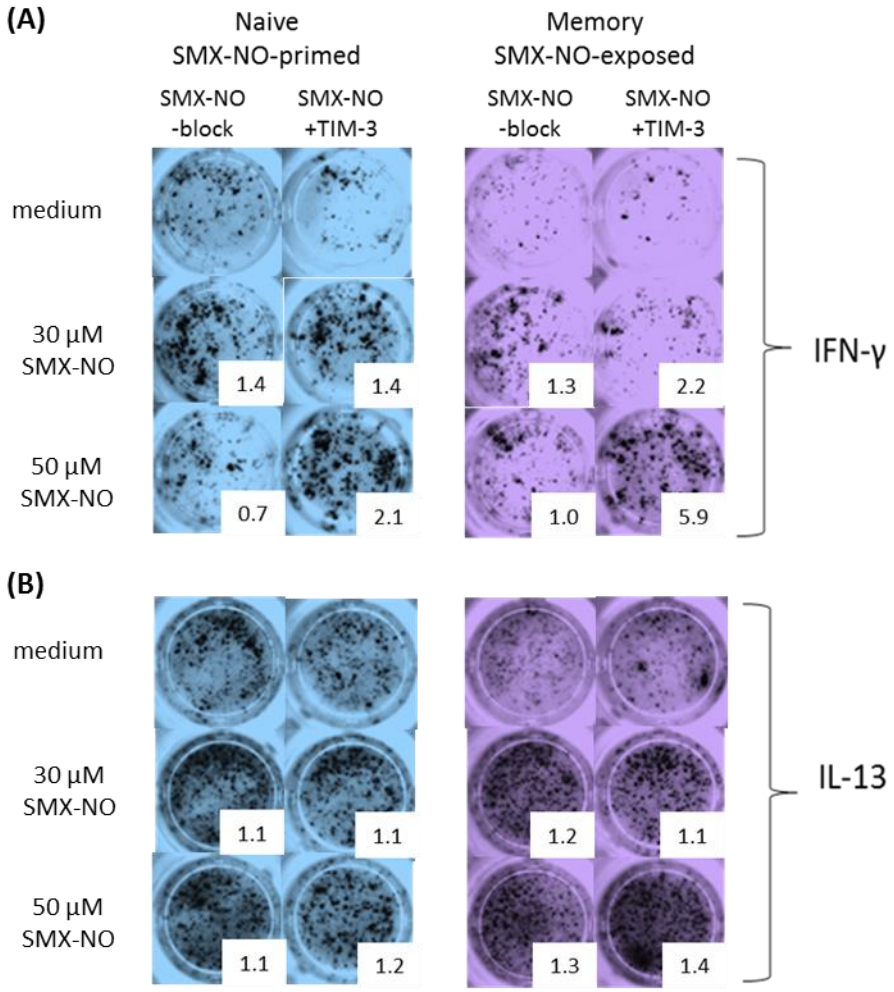


Figure 4.7. **The secretion of IFN-γ and IL-13 after priming naïve and memory T-cell from healthy donors to SMX-NO, ± TIM-3 blocking antibodies**. Naïve and memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from two healthy donors were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM), with or without TIM-3 blocking antibodies (7.5 µg/ml). Cells were cultured for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. In accordance with manufacturer's instructions, ELISpot plates were coated with IFN-γ or IL-13 capture antibody and incubated at 4°C overnight. Wells were then washed and blocked with R9 medium. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed ELISpot plate) with fresh monocyte-derived DCs ($4x10^3$/well) and SMX-NO (20 µM). The cells were incubated for 48 hrs in a similar atmosphere as before. The ELISpot plates were then washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader. The stimulation index (SFU of drug-treated wells / SFU of 'medium-only' treated wells) relating to drug-treated wells is shown.

4.3.4 The comparative expression of CTLA4 and TIM-3 on dividing versus non-dividing T-cells primed to SMX-NO.

During naïve T-cell priming to SMX-NO the T-cell surface expression of both CTLA4 and TIM-3 were measured. Here, we assessed how the expression of these receptors changes throughout this process, which we compared to the expression of PD-1 under similar conditions detailed in chapter 4. Expression was measured separately for both $CD4^+$ and $CD8^+$ T-cells.

As the high expression of individual co-inhibitory receptors has previously been associated with T-cell exhaustion we wanted to define the comparative expression of CTLA4 and TIM-3 on dividing versus non-dividing T-cell clones. Detailed analysis of cell proliferation was performed by co-staining cells with CFSE similar to that shown previously for PD-1 (Figure 4.8a). CTLA4 was found to be expressed on a much higher proportion of $CD4^+$ and $CD8^+$ dividing cells than non-dividing T-cells (Figure 4.8b; percentage of $CD4^+$ T-cells expressing CTLA4; non-dividing, 4.0%; dividing, 14.3%)(percentage of $CD8^+$ T-cells expressing CTLA4; non-dividing, 3.6%; dividing, 12.6%). Meanwhile, TIM-3 expression was found on a proportional 6-fold more dividing $CD8^+$ T-cells than those non-dividing (Figure 4.8c; percentage of $CD8^+$ T-cells expressing TIM-3; non-dividing, 20.4%; dividing, 84.4%), and on more than 9 times the number of dividing $CD4^+$ T-cells than those in a quiescent state (percentage of $CD4^+$ T-cells expressing TIM-3; non-dividing, 13.2%; dividing, 83.8%).

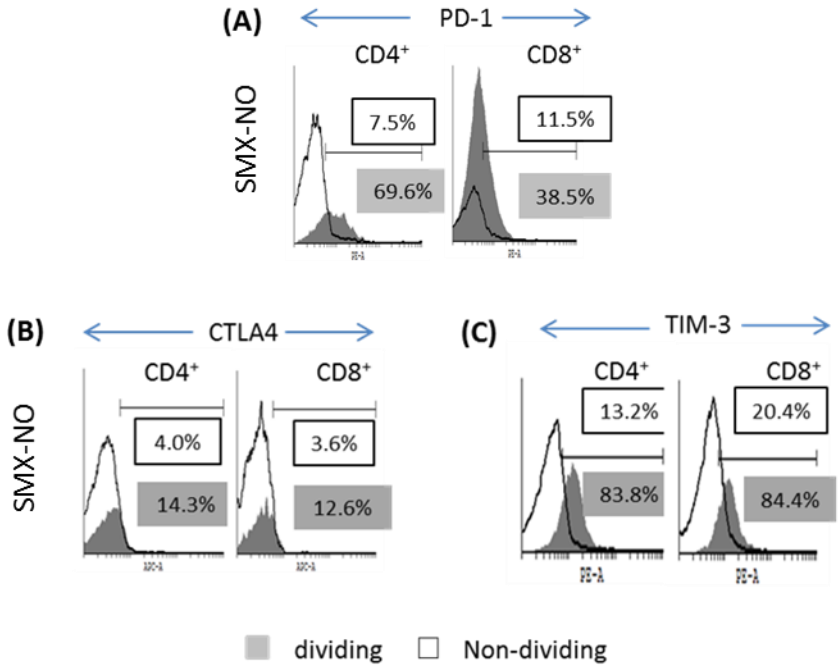


Figure 4.8. Comparative expression of PD-1, CTLA4, and TIM-3 on dividing and non-dividing healthy donor-derived SMX-NO-primed naïve T-cells. Naïve T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from healthy donors were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) for one 1 week in an atmosphere of 95% air / 5% CO_2 / 37°C. T-cells were harvested and a sample were stained with CFSE. CFSE stained and unstained cells were then re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (50 µM) for 72 hrs in similar conditions as before. CFSE stained and unstained cells were transferred to FACS tubes and resuspended in 3 ul of PD-1-PE, CTLA4-APC*, or TIM-3-PE, and 3ul of CD4- and CD8-specific fluorochromes. Cells were then incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and analysed on a FACS Canto II machine using cyflogic software. Figures show the expression of **(A)** PD-1, **(B)** CTLA4, and **(C)** TIM-3 on $CD3^+$, $CD4^+$, or $CD8^+$ T-cell populations separately. Grey histograms represent expression on dividing T-cells, black line represents expression on non-dividing T-cells. Percentages indicate the percentage of each population gated positive for each co-inhibitory receptor.

4.3.5 The expression profile of CTLA4 on naïve and memory T-cells after antigen exposure.

Subsequent analysis of CTLA4 and TIM-3 expression focussed on their expression during the SMX-NO-specific activation of naïve and memory T-cells. This was assessed by analysing CTLA4 expression of $CD4^+$ and $CD8^+$ T-cell subsets individually. It was found that around approximately 8% of naïve $CD4^+$ T-cells expressed CTLA4, which fell to around 4% after initial

naive T-cell priming to SMX-NO (Figure 4.9). However, the percentage of CTLA4$^+$ cells increased back to pre-stimulation levels by 48 hrs post SMX-NO re-stimulation and continued to increase at 72 hrs post re-stimulation (~10%). In naïve CD4$^+$ T-cell cultures where CTLA4-block was added, initial priming to SMX-NO again showed a reduction in cells expressing CTLA4 (naïve T-cell expression ~8%, 14 days post priming ~4%). The number of naïve CTLA4$^+$ cells increased on cells exposed to CTLA4 block, reaching ~11% by 72 hrs post restimulation. A similar trend was seen upon looking at CTLA4 expression on CD8$^+$ naïve T-cells during priming, with the percentage of CTLA4-expressing cells post priming generally falling whether in the absence or presence of CTLA4 block, before rising after re-stimulation with SMX-NO. Once again, the rate of expression increased faster in cells cultured with CTLA4 block (~11% expression, 24 hrs after restimulation) compared to without (~10% expression, 48 hrs after restimulation). The percentage of CTLA4 expressing cells for both CTLA4 blocking antibody treated and untreated CD8$^+$ T-cells was still increasing 72 hrs after re-exposure to SMX-NO (72 hr time-point; CD8$^+$ T-cells without block, 11.3%, CD8$^+$ T-cells + CTLA4 block, 11.4%). Overall, whereas SMX-NO-specific activation of CD4$^+$ naïve T-cells only increased the percentage of CTLA4-expressing T-cells from 8% to 10%, 2.1-fold more (5.3% to 11.3%) CD8$^+$ naïve T-cells expressed CTLA4 after restimulation with antigen.

In a similar fashion, the proportion of memory T-cells expressing CTLA4 increased after antigen stimulation, with peak expression for both CD4$^+$ and CD8$^+$ T-cells occurring 24-48 hrs after restimulation with antigen (Figure 4.10). Indeed, maximal expression of CTLA4 after antigen exposure on CD4$^+$ and CD8$^+$ memory T-cells was 2-fold and 4-fold higher than on unstimulated cells, respectively (memory CD4$^+$, 7.7% to 15.5%; memory CD8$^+$, 3.1% to 12.3%). In contrast to naïve T-cell priming, the inclusion of CTLA4 blocking antibodies during memory T-cell activation was not associated with an enhanced number of CTLA4$^+$ T-cells.

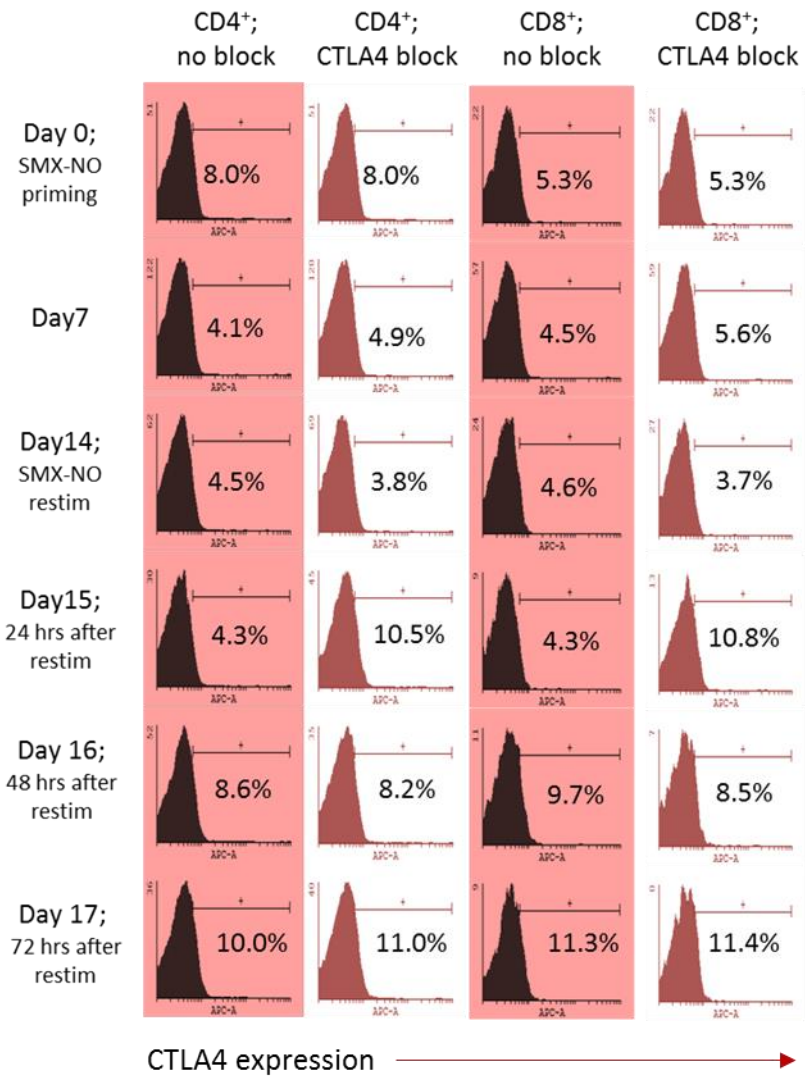


Figure 4.9. CTLA4 expression during the SMX-NO-specific priming of $CD4^+$ and $CD8^+$ naïve T-cells from a healthy donor, ± CTLA4 blocking antibodies. Naïve T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from a healthy donor were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) for one 1 week in an atmosphere of 95% air / 5% CO_2 / 37°C, ± CTLA4 blocking antibodies (10 µg/ml). T-cells were harvested and then re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (50 µM) for 72 hrs in similar conditions as before. During the course of the priming assay, samples of T-cells ($5x10^4$) were harvested at various time-points and labelled with 3 µl of CD4, CD8, and CTLA4 fluorochrome-bound antibodies. Cells were then incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and analysed on a FACS Canto II machine using cyflogic software. Percentages indicate the proportion of cells which stain positive for CTLA4.

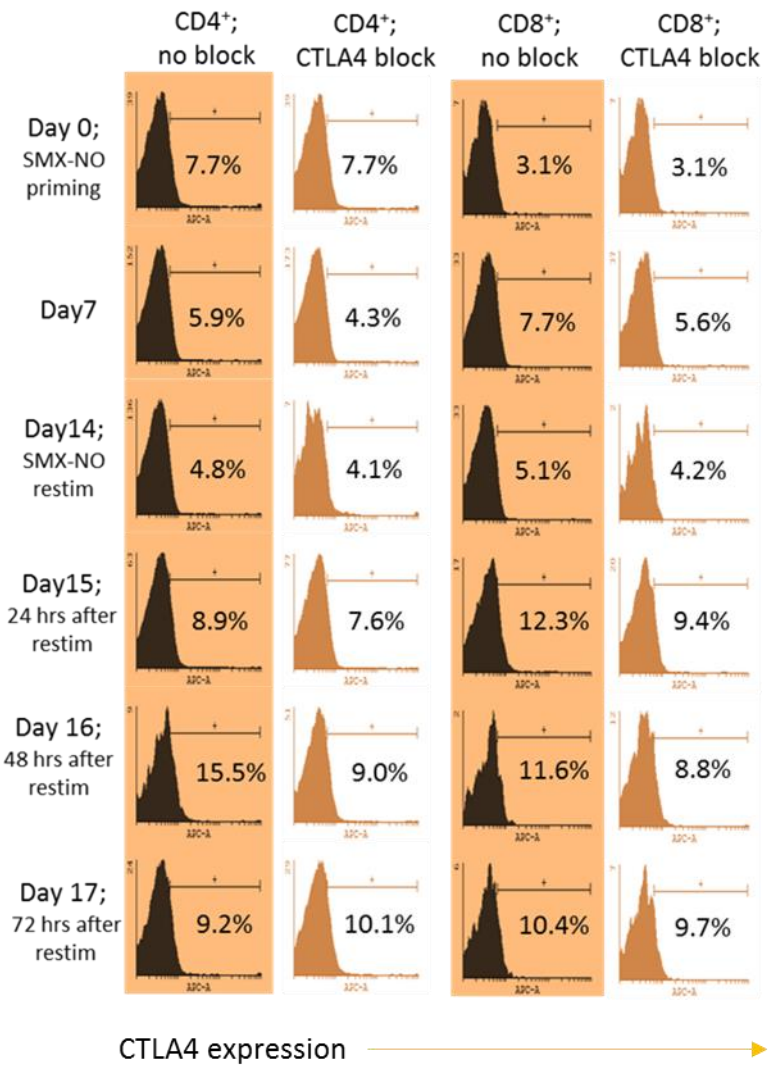


Figure 4.10. CTLA4 expression during the SMX-NO-specific activation of $CD4^+$ and $CD8^+$ memory T-cells from a healthy donor, ± CTLA4 blocking antibodies. Memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from a healthy donor were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) for one 1 week in an atmosphere of 95% air / 5% CO_2 / 37°C, ± CTLA4 blocking antibodies (10 µg/ml). T-cells were harvested and then re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (50 µM) for 72 hrs in similar conditions as before. During the course of the priming assay, samples of T-cells ($5x10^4$) were harvested at various time-points and labelled with 3 µl of CD4, CD8, and CTLA4 fluorochrome-bound antibodies. Cells were then incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and analysed on a FACS Canto II machine using cyflogic software. Percentages indicate the proportion of cells which stain positive for CTLA4.

4.3.6 The expression profile of TIM-3 on naïve and memory T-cells after antigen exposure.

The expression of TIM-3 throughout the priming process was similarly assessed. TIM-3 was expressed on 5.2% of naïve $CD4^+$ T-cells (Figure 4.11). In contrast to CTLA4 expression, the percentage of TIM-3 expressing $CD4^+$ T-cells dramatically increased by day 14 (day 14 TIM-3 expression with no block, 31.0%; with TIM-3 block, 19.3%). After re-exposure with SMX-NO, the number of TIM-3^+ cells fluctuated before again dramatically rising by 72 hrs post re-stimulation so that > 45% of all naïve-derived T-cells expressed TIM-3 (72 hrs post restimulation TIM-3 expression with no block, 58.5%; with TIM-3 block, 54.4%). The maximal increase in TIM-3 expression was similar for both $CD4^+$ and $CD8^+$ T-cells, with a 42.4%, and 47.9% rise in comparison to unstimulated naïve $CD4^+$ and $CD8^+$ T-cells, respectively. Although the number of cells expressing TIM-3 was enhanced for both $CD4^+$ and $CD8^+$ naïve T-cells in the presence of TIM-3-blocking antibodies 24 hrs post-restimulation, for all other time-points the culture of cells with the inclusion of TIM-3 block did not appear to drastically affect the extent of TIM-3 expression.

We further examined the expression of TIM-3 on memory T-cells (Figure 4.12). In a similar pattern, an increased number of TIM-3-expressing memory T-cells were present after activation with SMX-NO, and blocking antibodies were not observed to increase TIM-3 expression. Once again, TIM-3 expression was low directly after antigen-exposure but increased towards the end of both antigen culture periods, with maximal TIM-3 expression on $CD4^+$ and $CD8^+$ memory T-cells occurring 72 hrs after restimulation. The increase in TIM-3 expression for both $CD4^+$ and $CD8^+$ memory T-cells when stimulated with antigen was similar, with 3.6-fold (18.6% to 66.9%) and 3.3-fold more (21.9% to 71.4%) TIM-3^+ cells compared to unstimulated $CD4^+$ and $CD8^+$ memory T-cells, respectively.

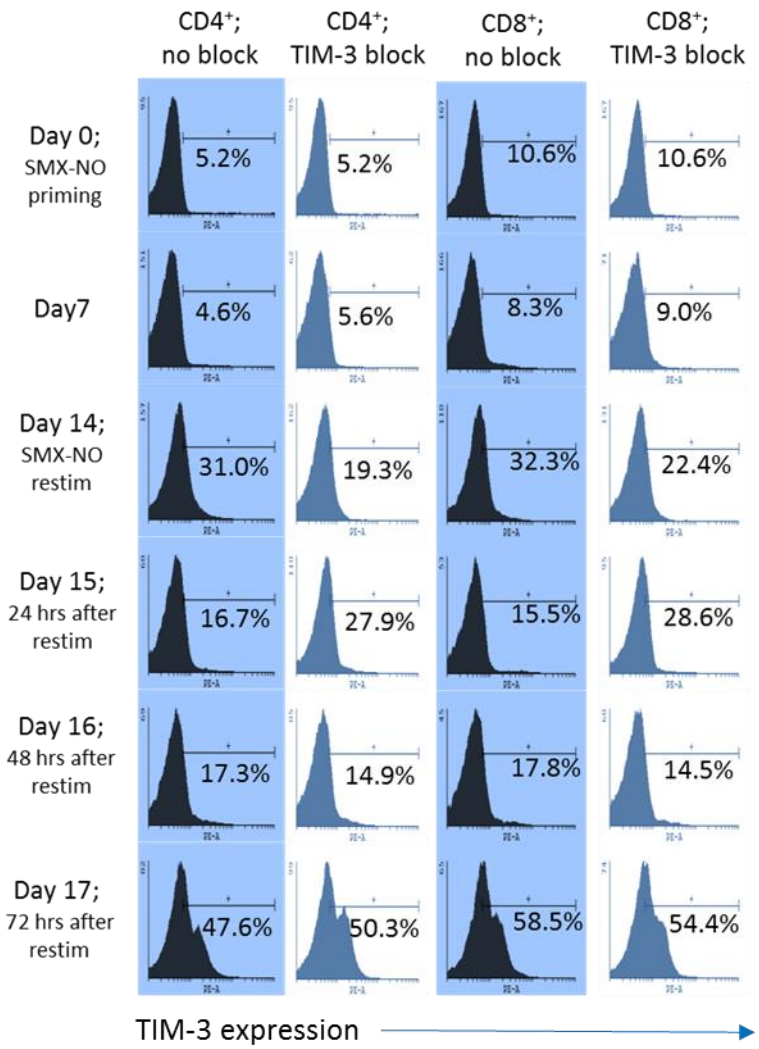


Figure 4.11. TIM-3 expression during the SMX-NO-specific priming of $CD4^+$ and $CD8^+$ naïve T-cells from a healthy donor, ± TIM-3 blocking antibodies. Naïve T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from a healthy donor were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) for one 1 week in an atmosphere of 95% air / 5% CO_2 / 37°C, ± TIM-3 blocking antibodies (7.5 µg/ml). T-cells were harvested and then re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (50 µM) for 72 hrs in similar conditions as before. During the course of the priming assay, samples of T-cells ($5x10^4$) were harvested at various time-points and labelled with 3 µl of CD4, CD8, and TIM-3 fluorochrome-bound antibodies. Cells were then incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and analysed on a FACS Canto II machine using cyflogic software. Percentages indicate the proportion of cells which stain positive for TIM-3.

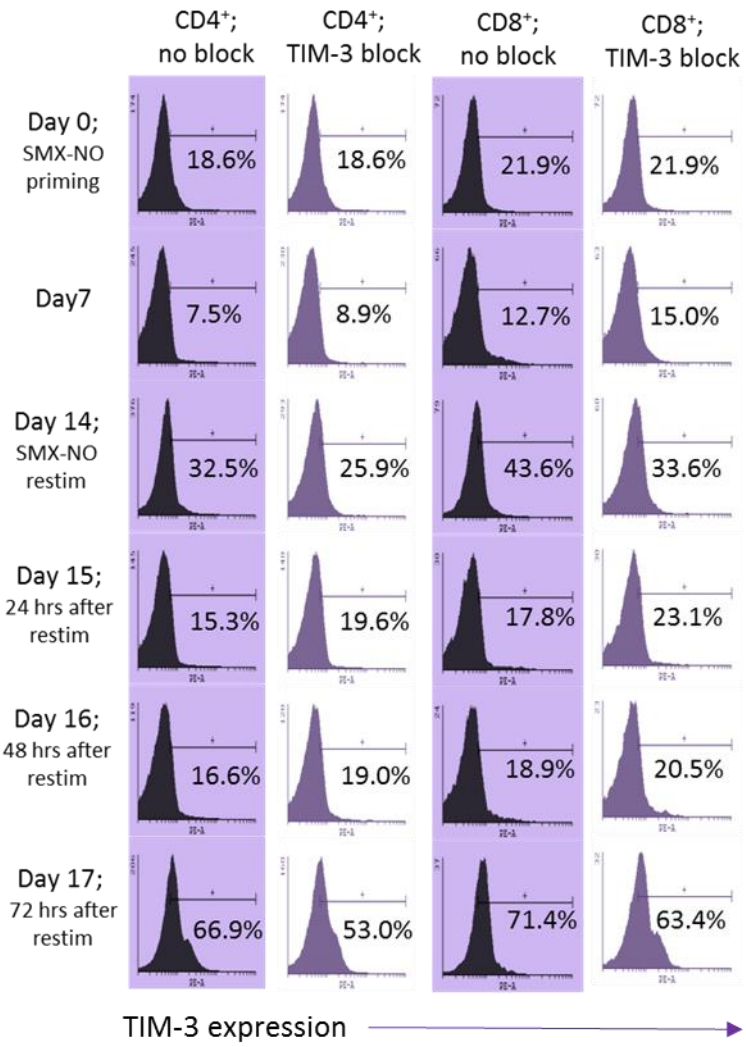


Figure 4.12. TIM-3 expression during the SMX-NO-specific activation of $CD4^+$ and $CD8^+$ memory T-cells from a healthy donor, ± TIM-3 blocking antibodies. Memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from a healthy donor were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) for one 1 week in an atmosphere of 95% air / 5% CO_2 / 37°C, ± TIM-3 blocking antibodies (7.5 µg/ml). T-cells were harvested and then re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (50 µM) for 72 hrs in similar conditions as before. During the course of the priming assay, samples of T-cells ($5x10^4$) were harvested at various time-points and labelled with 3 µl of CD4, CD8, and TIM-3 fluorochrome-bound antibodies. Cells were then incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and analysed on a FACS Canto II machine using cyflogic software. Percentages indicate the proportion of cells which stain positive for TIM-3.

4.3.7 The effect of CTLA4- and TIM-3-blockade on the activation of SMX-NO-specific T-cell clones, in comparison to blocking PD-L1.

To further assess the role of regulatory pathways in the regulation of secondary T-cell responses, we explored the role of CTLA4 and TIM-3 on SMX-NO-specific T-cell clones generated from healthy donor primed naïve T-cells. Both the proliferative capacity and ability to secrete cytokines and cytolytic molecules in the presence of SMX-NO was assessed on 6 representative T-cell clones, with and without PD-L1, CTLA4, or TIM-3 blockade. All clones significantly proliferated in the presence of SMX-NO ($p < 0.005$), and the addition of PD-L1- (Figure 4.13a), CTLA4- (Figure 4.13b), or TIM-3 blocking antibodies (Figure 4.13c) did not significantly increase the proliferative response to SMX-NO for any of the 6 clones.

Previously we identified that the addition of PD-L1-block produced a modest increase in IL-13, and granzyme-B secretion (Figures 4.14a). In contrast, the use of CTLA4- (Figures 4.14b) or TIM-3-blocking antibodies (Figures 4.14c) failed to enhance the secretion of these molecules from SMX-NO-specific T-cell clones.

4.3.8 Varied expression profiles for CTLA4 and TIM-3 on T-cell clones does not correlate with T-cell activity.

In comparison to the modest 4-fold difference in PD-1 expression between T-cell clones (figure 4.15a), flow cytometric analysis on 40 representative SMX-NO-specific T-cell clones revealed more than a 27-fold difference in CTLA4 expression (figure 4.15b), and more than a 10-fold difference in TIM-3 surface expression (figure 4.15c). Previously, the degree of PD-1 expression was found to have no effect on T-cell clone activity. Although there was a much greater variation in expression of CTLA4 and TIM-3 between T-cell clones, the level of expression of these co-inhibitory receptors was not found to relate to the proliferative ability

of each clone (Figure 4.16, 4 representative clones shown). While the clone with the highest TIM-3 expression (clone 49) was also the clone with the highest stimulation index, the previous 3 clones showed no correlation with TIM-3 expression. For example, clone 71 had a similar SI to that of clone 97 but only one third of the TIM-3 surface expression.

The surface expression of CTLA4 and TIM-3 were then measured over a period of 10 days, with and without SMX-NO stimulation. While staining over a similar time-period had shown relatively stable PD-1 expression (figure 4.17a), the expression profile over time for CTLA4 (Figure 4.17b) and TIM-3 (Figure 4.17c) was somewhat more varied. The expression of both receptors in the absence of SMX-NO generally remained constant over a period of 240 hrs. However, in the presence of SMX-NO, the expression of CTLA4 and TIM-3 increased above that of basal levels (Figure 4.17). In particular, clone 3 (CTLA4), and clones 5 and 6 (TIM-3) showed increased co-inhibitory receptor expression. This increased expression appears in some clones to be time-dependent reaching maximum expression for both receptors between 48-120 hrs, before tailing off between 120-240 hrs after SMX-NO stimulation. For clone 3, maximal CTLA4 expression was observed at 120 hrs, at which point expression on SMX-NO stimulated wells was > 2.5 fold higher than on cells prior to SMX-NO exposure (0 hr MFI: 136, 120 hr MFI: 358), and > 3-fold greater than the expression at a similar time-point on non-drug antigen treated cells (non-drug antigen-treated cells 120 hr MFI: 115). Both clones analysed for TIM-3 expression showed maximal expression at 48 hrs post SMX-NO exposure. At this time-point, expression of TIM-3 on SMX-NO stimulated cells was > 10-fold and > 2-fold higher than on cells prior to SMX-NO exposure for clone 5 and 6, respectively (clone 5: 0 hr MFI, 262; 48 hr MFI, 2626; clone 6: 0 hr MFI, 458; 48 hr MFI, 936), and > 13.5-fold and > 5.5-fold greater than the expression at a similar time-point of non-antigen treated cells (clone 5: non-SMX-NO-treated cells 120 hr MFI, 192; clone 6: 168).

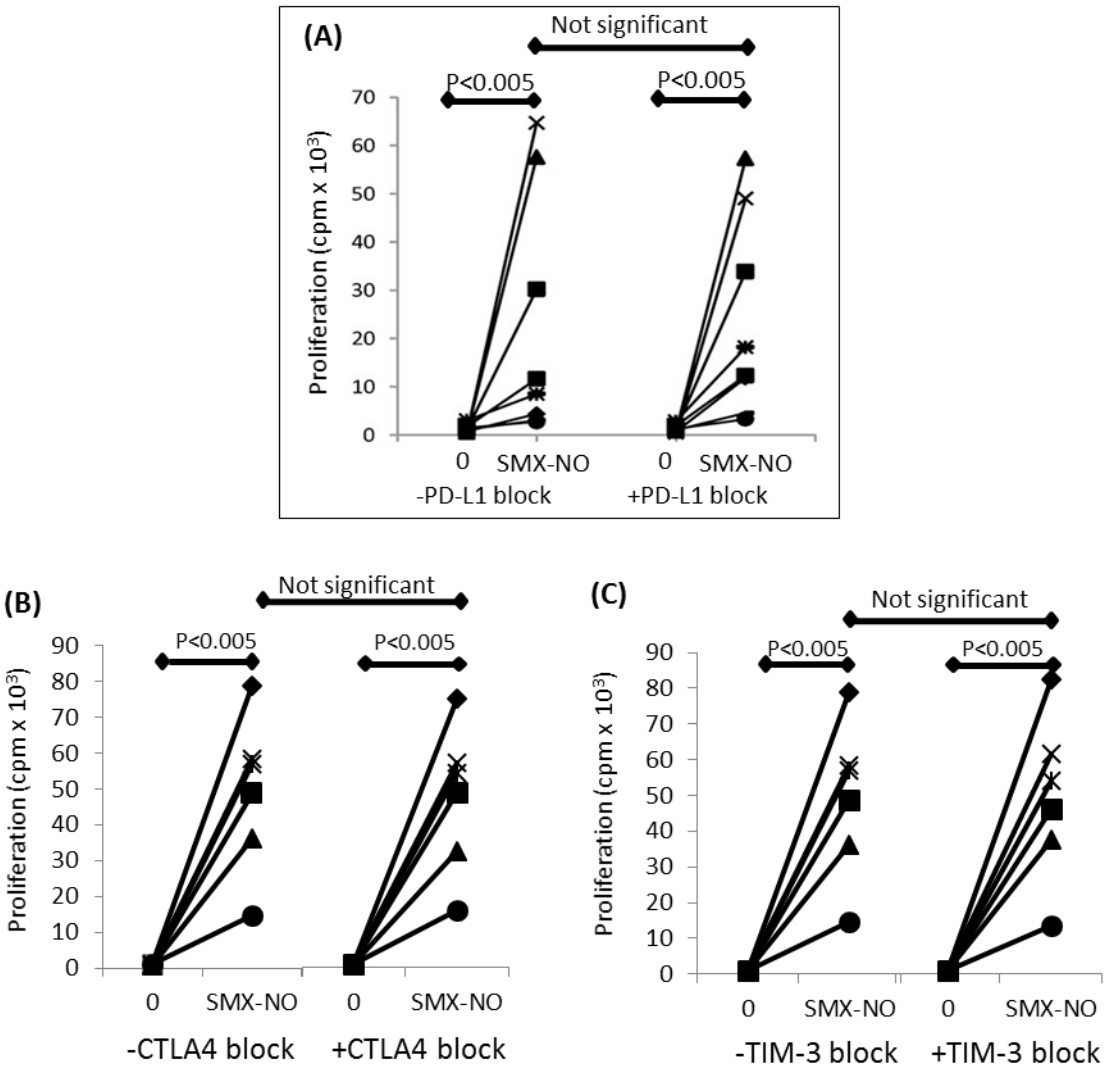


Figure 4.13. The proliferative response from antigen-stimulated SMX-NO-responsive T-cell clones, ± PD-L1/CTLA4/TIM-3 blocking antibodies. T-cell clones ($5x10^4$/well; total volume, 200 µl, 96-well U-bottomed plate) derived from healthy donor SMX-NO-primed naïve T-cells were stimulated with SMX-NO (40 µM) using autologous irradiated EBV-transformed B-cells ($1x10^4$/well), with and without inclusion of **(A)** PD-L1 blocking antibodies (5 µg/ml), **(B)** CTLA4 blocking antibodies (10 µg/ml), or **(C)** TIM-3 blocking antibodies (7.5 µg/ml). After incubation in an atmosphere of 95% air / 5% CO_2 / 37°C for 48 hours, proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a further 16 hrs. Data are presented as the mean of duplicate wells.

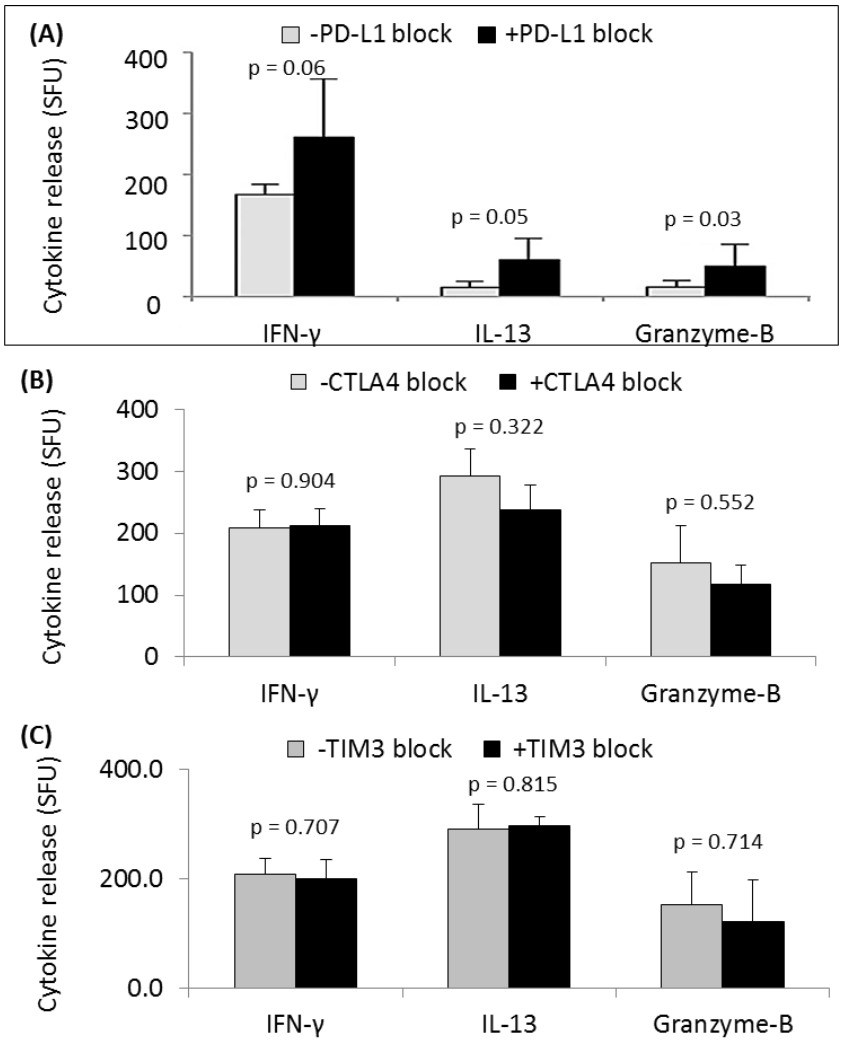


Figure 4.14. Secretion of cytokines/cytolytic molecules from antigen stimulated SMX-NO-responsive T-cell clones, ± PD-L1/CTLA4/TIM-3 blocking antibodies. In accordance with manufacturer's instructions, ELISpot plates were coated with IFN-γ, IL-13, or granzyme B capture antibody and incubated at 4°C overnight. Wells were then washed and blocked with R9 medium. T-cell clones ($5x10^4$/well; total volume, 200 µl, 96-well U-bottomed ELISpot plate) derived from healthy donor SMX-NO-primed naïve T-cells were cultured with SMX-NO (40 µM) and autologous irradiated EBV-transformed B-cells ($1x10^4$/well), ± **(A)** PD-L1 blocking antibodies (5 µg/ml), **(B)** CTLA4 blocking antibodies (10 µg/ml), or **(C)** TIM-3 blocking antibodies (7.5 µg/ml). After a 48 hr incubation, the plates were washed and developed in concordance with the manufacturer's instructions. SFU counts were analysed from dry wells using an ELISpot reader. Data is presented as mean ± SD of experiments performed in triplicate. Statistical significance denotes a significant increase in secretion with the inclusion of antibody blockade compared to 'no block' treated wells.

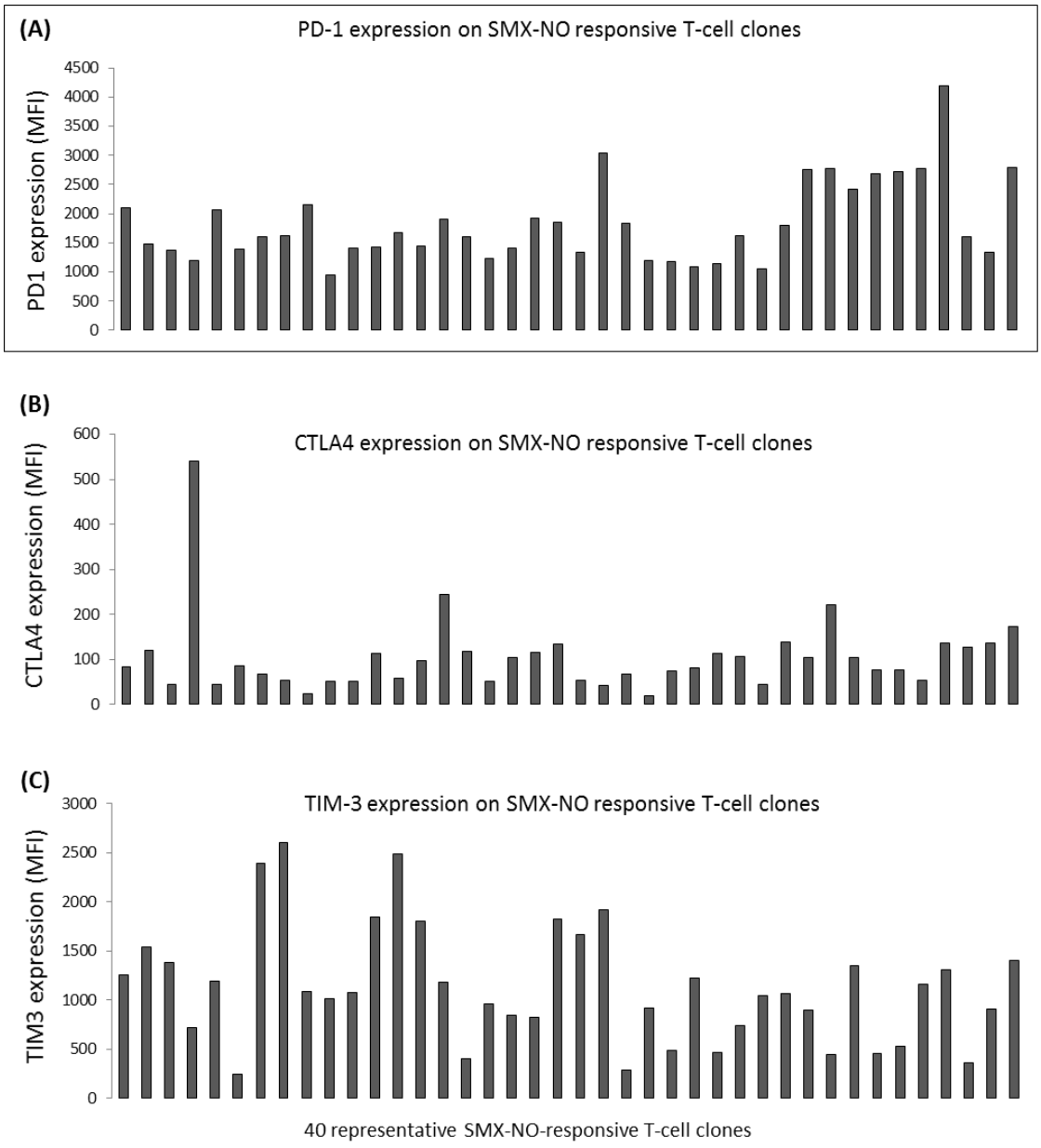


Figure 4.15. Analysis of PD-1/CTLA4/TIM-3 expression on SMX-NO-specific T-cell clones. Expression of **(A)** PD-1, **(B)** CTLA4, and **(C)** TIM-3 was assessed on 40 SMX-NO–specific T-cell clones. A sample of each clone ($5x10^4$ cells) was removed, resuspended in 3 µl PD-1-PE, CTLA4-APC*, or TIM-3-PE and then incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in 300 µl FACS buffer and analysed. Flow cytometry was performed using a FACS Canto II machine and FACS DIVA software. Co-inhibitory receptor expression is presented as a measure of MFI.

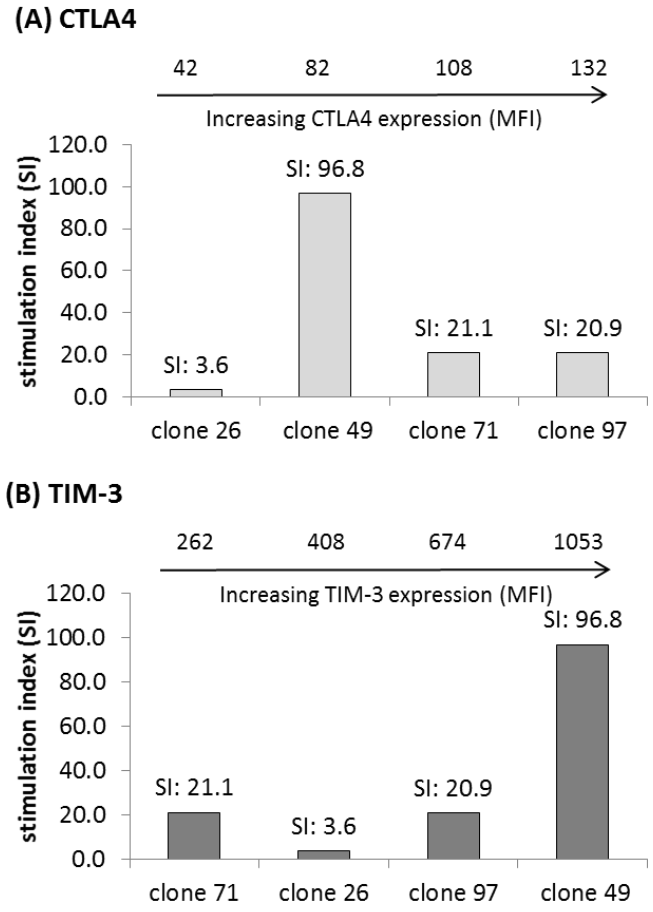


Figure 4.16. Correlation of CTLA4 or TIM-3 expression with T-cell clone proliferative activity in response to antigen. A sample of each clone ($5x10^4$ cells) was removed, resuspended in **(A)** 3 µl CTLA4-APC* or **(B)** TIM-3-PE and then incubated in the dark and on ice for 20 mins before being washed with FACS buffer. Cells were resuspended in FACS buffer and analysed using a FACS Canto II machine and FACS DIVA software. CTLA4 and TIM-3 expression is presented as a measure of MFI. Additionally, T-cell clones ($5x10^4$/well; total volume, 200 µl, 96-well U-bottomed plate) were cultured with autologous irradiated EBV-transformed B-cells ($1x10^4$/well), ± SMX-NO (40 µM). After incubation in an atmosphere of 95% air / 5% CO_2 / 37°C for 48 hrs, proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a further 16 hrs. Data are presented as the stimulation index (proliferation in response to antigen / proliferation in response to medium only), and is written above each clone.

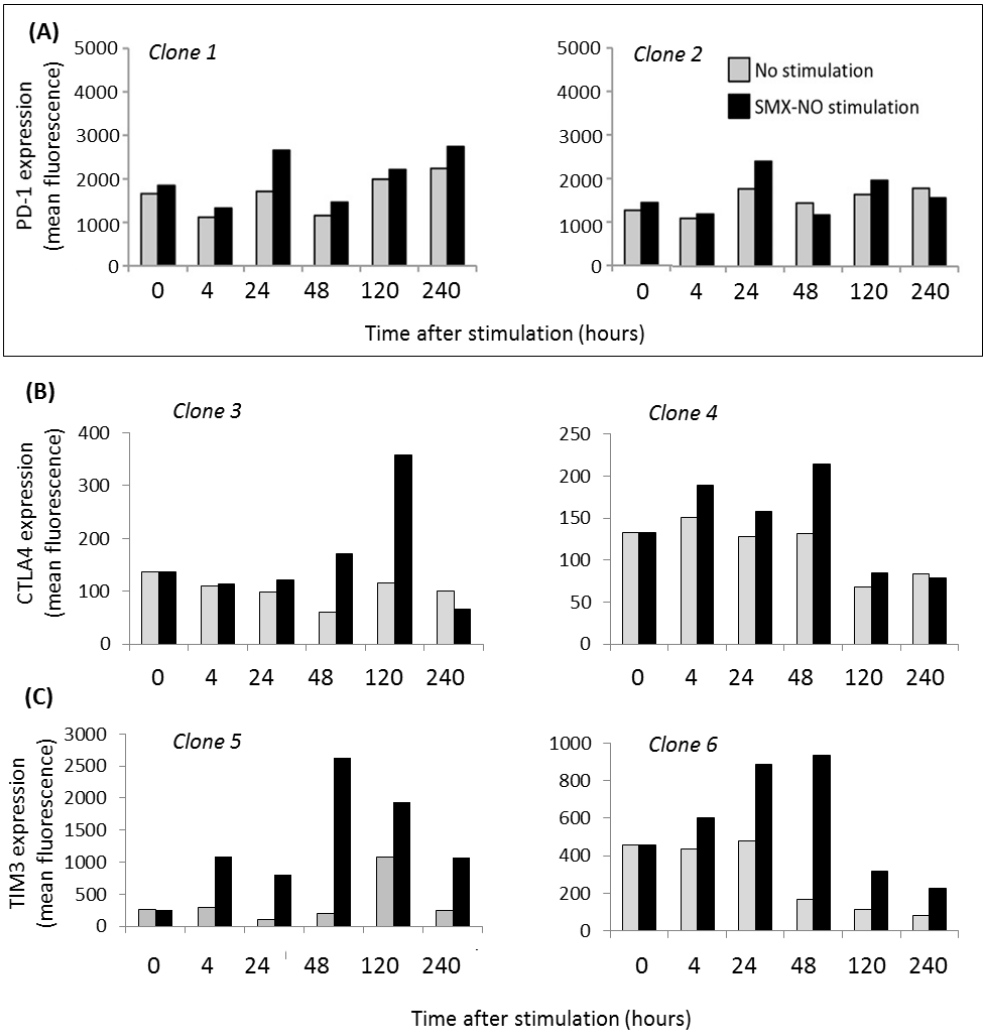


Figure 4.17. PD-1, CTLA4, and TIM-3 expression on SMX-NO-specific T-cell clones over ten days of antigen stimulation. Expression of **(A)** PD-1, **(B)** CTLA4, and **(C)** TIM-3 on antigen stimulated and unstimulated SMX-NO-responsive T-cell clones. T-cell clones ($5x10^4$/well; total volume, 200 µl, 96-well U-bottomed plate) were stimulated with or without SMX-NO (40 µM) using autologous irradiated EBV-transformed B-cells ($1x10^4$/well). Cultures were incubated in an atmosphere of 95% air / 5% CO_2 / 37°C for 240 hrs. A sample of each clone ($5x10^4$ cells) was removed at various time points and stained using PD-1-PE, CTLA4-APC*, or TIM-3-PE. All flow cytometry was performed using a FACS Canto II machine and FACS DIVA software. Co-inhibitory receptor expression is presented as a measure of MFI.

4.4 Discussion.

T-cell responses are known to be controlled by complex expression patterns of a diverse range of co-inhibitory receptors. Indeed, up to 7 different co-inhibitory receptors are expressed on exhausted T-cells during chronic infection (Blackburn *et al.*, 2009). It is the

cumulative effect of a number of inhibitory pathways, pitted against a similarly diverse number of stimulatory pathways that determines whether an antigenic signal results in peripheral tolerance or an immune reaction. It is therefore important to understand the role and function of each pathway in relation to a specific immune response. Therefore, to assess their role in drug-derived antigen-specific T-cell responses, we have focussed on two distinct T-cell co-inhibitory receptors; CTLA4 and TIM-3. This work aims to expand upon that of chapter 3 by analysing the independent roles and strengths of both co-inhibitory receptor pathways in comparison to that of the PD-L1/PD-1 pathway.

We first assessed the ability for CTLA4 and TIM-3 blocking antibodies to enhance the drug-derived antigen-specific priming of naïve T-cells. Previous CTLA4 blocking studies have shown an enhancement of T-cell responses in relation to autoimmune diseases such as EAE (Karandikar *et al.*, 1996; Perrin *et al.*, 1996), but primarily cancer where treatment leads to tumour regression (Leach *et al.*, 1996). In comparison, fewer studies have reported the individual role of TIM-3 blockade on T-cell responses, however those that have report that blockade results in enhanced T-cell function and subsequent worsening of autoimmune disease in murine models of EAE and type I diabetes (Monney *et al.*, 2002; Sánchez-Fueyo *et al.*, 2003). For this we utilised the *in vitro* T-cell priming assay and stimulated cells with the model drug antigen SMX-NO. Proliferative analysis identified an enhanced proliferative response in the presence of CTLA4 blocking antibodies for 6/9 donors after naïve T-cell priming, but no significant increase was detected in a similar assay with the inclusion of TIM-3 block (3/3 donors). On comparison of blocking CTLA4 to that of PD-L1 (i.e. fold increase in T-cell proliferation), it is clear that PD-L1 block has a much greater regulatory role during primary T-cell responses to SMX-NO, with an average 4.4-fold increase with PD-L1 block, compared to a 2.5-fold increase for CTLA4 block. This data concurs with the generally wider reported use of PD-1 pathways within the immune system than CTLA4, which infers a greater role for PD-1 than CTLA4 in the control of these T-cell responses. This is also supported by

the work of Parry *et al* who studied T-cell activation-induced gene transcripts, and found the generation of just 67% of transcripts was hindered by CTLA4 ligation, in comparison to 90% upon PD-1 activation (Parry *et al.*, 2005). These data clearly indicate a much greater inhibitory role for PD-1 than CTLA4, and the authors conclude that the enhanced inhibitory effect of PD-1 is due to it blocking more membrane-proximal signalling events than CTLA4. In stark contrast to the data discussed above, several murine studies have shown a more aggressive and severe autoimmune phenotype is induced by CTLA4 deficiency (Tivol *et al.*, 1995; Waterhouse *et al.*, 1995) than that of PD-1 deficiency (Nishimura *et al.*, 1999; Nishimura *et al.*, 2001), suggesting that CTLA4 more heavily regulates these responses. Unsurprisingly, as there is a high degree of homology between mouse and human CTLA4 (Harper *et al.*, 1991), anti-CTLA4 biologicals are also associated with more frequent and serious immune-mediated adverse events than anti-PD-L1 therapies in human cancer trials (Brahmer *et al.*, 2012; Hodi *et al.*, 2010). Although the reasoning behind the increased severity of anti-CTLA4 treatment remains unclear, our data indicate that it is unlikely connected to naive T-cell responses.

While the role of TIM-3 is well-characterised in mice, the corresponding picture in human cells is less well defined. Previous studies have inferred that there may be functional discrepancies between human and mouse TIM-3. For instance Hastings *et al* found that upon TIM-3 activation, murine cells undergo cell death, whereas when TIM-3 was blocked on human cells, no reduction in cell death was observed. Moreover, an increase in T-cell proliferative capacity was not seen. Thus, the authors proposed that human T-cells are regulated through a different mechanism to murine cells (Hastings *et al.*, 2009). Our data supports these observations in human cells, where drug-derived antigen-specific proliferative responses were not enhanced by TIM-3 blockade. However, we were able to detect an enhanced secretion of cytokines after the inclusion of TIM-3 blocking antibodies, particularly for the Th1-associated cytokine IFN-γ. Therefore, while TIM-3 appears to be

ineffective at regulating the proliferative response of human T-cells, its role may be limited to specific functions including the management of cytokine secretion.

We also wanted to determine the influence of these regulatory pathways on secondary T-cell reactions by looking at memory T-cell responses to SMX-NO using cells from healthy donors. In a similar fashion to naïve T-cell priming, blocking TIM-3 did not increase the memory T-cell response in any donor (3/3). While blocking CTLA4 was still able to enhance memory drug-derived antigen-specific responses, it did so in a smaller proportion of donors (3/6) than for primary T-cell responses (6/9). These data are in contrast to PD-L1 blocking assays which did not enhance memory T-cell responses. These data therefore support a greater role for co-inhibitory regulation during naïve T-cell priming (primary T-cell responses) than memory (secondary) T-cell responses. It is thought that these pathways act by modulating the activation thresholds of T-cells where co-stimulatory pathways lower thresholds and make it easier to stimulate T-cells, while co-inhibitory pathways increase thresholds and make t-cell activation more difficult. Indeed, this is the proposed mechanism of action for PD-1 in the negative selection of T-cells in the thymus (Nishimura *et al.*, 1999). This may be due to more stringent requirements for the activation of naïve T-cells than memory T-cells. The physiological advantage of this is to prevent the activation of naïve T-cells unless a true antigen is encountered, which simultaneously produces danger signals, and so provides ample co-stimulation and overcomes the high activation threshold set by multiple layers of co-inhibitory signalling. The requirement for additional signalling minimises the risk of a naïve T-cell response to non-harmful or self-antigen, a requirement which is unnecessary for memory T-cells due to their pre-defined antigen-specificity. A number of studies support the need for increased antigen concentration alongside multiple co-stimulatory signals for a naïve T-cell response to peptide-antigen, in contrast to memory T-cells which can be stimulated by immobilised anti-CD3 alone (Croft *et al.*, 1994; Dubey *et al.*, 1996; Sagerström *et al.*, 1993). It is however important to clarify that while naïve T-cell

responses may be more highly regulated by co-signalling, these pathways still regulate memory T-cell responses as highlighted by the small number of enhanced memory responses we detected with the addition of CTLA4 block. Zimmermann *et al* detailed this when they demonstrated that activation thresholds for both naïve T-cells and T-cell clones were similar, each requiring the triggering of approximately 8000 TCRs, but concluded that both naïve and memory T-cell activation thresholds fell significantly in response to co-stimulatory signalling (Zimmermann *et al.*, 1999). Thus, the previously mentioned enhanced adverse effects with anti-CTLA4 therapy opposed to anti-PD-1 biologicals may relate to effects on secondary T-cell responses.

We must stress at this point that these conclusions are based solely on our data which presently details comparatively low number of donors for certain conditions i.e. during the activation of naïve or memory T-cells ± TIM-3 block, or the priming of naïve T-cells ± PD-L1 block. This occurred as we were unable to perform the simultaneous analysis of individual blocks as we were restricted by the quantity of cells recovered after *in vitro* priming. Therefore due to time restrictions, the role of specific pathways was not characterised in as many donors as were others. However, our data displays a clear initial indication for the comparative role of these three co-inhibitory pathways and future work will build upon the current data set to verify these results using more donors.

These co-inhibitory pathways appear to play an important role in the control of primary drug antigen-specific T-cell responses. It may therefore be presumed that as the requirements for a response change, from initial activation to prevention of an overwhelming response, so does the expression profile of these receptors. While the expression of CTLA4 on naïve T-cells is thought to be low and inducible on both $CD4^+$ and $CD8^+$ T-cells upon activation (Takahashi *et al.*, 2000), the independent TIM-3 profiles of $CD4^+$ and $CD8^+$ T-cells are less well-defined. Hastings *et al* found that TIM-3 expression was generally restricted to $CD4^+$ T-

cells, which were only found in human lymph nodes, with peripheral $CD4^+$ T-cells showing little expression until induced post-stimulation (Hastings *et al.*, 2009). The authors additionally report the detection of TIM-3 expression on up to 2% of $CD8^+$ T-cells suggesting that the activity of these cells may be minimally manipulated by this receptor. Indeed, Sakhdari *et al* observed that TIM-3 blockade can increase the release of perforin and granzyme B and thus enhance $CD8^+$ T-cell cytotoxicity (Sakhdari *et al.*, 2012). Therefore, we set out to analyse the expression of both CTLA4 and TIM-3 during the activation of both naïve and memory T-cells, looking at $CD4^+$ and $CD8^+$ T-cell subsets individually.

We were able to identify the expression of both receptors on $CD4^+$, but also $CD8^+$ naïve and memory T-cells isolated from peripheral blood. In contrast to the studies mentioned previously, exposure of naïve T-cells to SMX-NO did not initially enhance CTLA4 expression, but in line with previous reports citing an increase after 2-3 days (Walunas *et al.*, 1994), we observed a slight increase in the percentage of naïve T-cells expressing CTLA4 between 48-72 hrs after re-stimulation with SMX-NO. Likewise, memory T-cell expression of CTLA4 was enhanced after restimulation with SMX-NO. Although $CD4^+$ T-cells were found to have higher basal expression of CTLA4, in both naïve and memory cultures, the maximum increase in $CTLA4^+$ cells upon stimulation with antigen was greater for $CD8^+$ cells than $CD4^+$ T-cells. Indeed, the fold increase on memory $CD8^+$ T-cells was double that on $CD4^+$ T-cells, representing a 6% and 2% increase, respectively. This more prominent increase in CTLA4 expression on the $CD8^+$ T-cell fraction indicates that these cells are more highly regulated by CTLA4 during drug-derived antigen-specific T-cell responses. Additionally, the inclusion of CTLA4-blocking antibodies minimally enhanced CTLA4 expression. This slight elevation indicates that cells may be able to enhance CTLA4 expression in a compensatory manner to account for increased regulatory pressures.

In comparison, the addition of TIM-3-blocking antibodies did not generally affect the expression of TIM-3 on T-cells. We observed that the proportion of naïve and memory TIM-3^+ cells reduced after both initial antigen exposure and re-stimulation, but increased dramatically towards the end of both culture periods. Specifically, we identified both an upregulation the day prior to restimulation, and an increased upregulation 2-3 days after restimulation with SMX-NO. The physiological basis for this expression pattern is to allow for a strong initial response by reducing expression before preventing a long-lasting, potentially damaging, response by increasing expression at later time-points. The proportion of TIM-3 expressing cells increased after antigen stimulation for both $CD4^+$ and $CD8^+$ T-cells subsets. In both naïve and memory cultures, although basal expression on unstimulated $CD8^+$ T-cells was higher than on $CD4^+$ T-cells, the percentage of TIM-3^+ cells increased by a similar amount upon antigen stimulation for both $CD4^+$ and $CD8^+$ T-cells (naïve $CD4^+$, 42.4%; naïve $CD8^+$, 47.9%; memory $CD4^+$, 48.3%; memory $CD8^+$, 49.5%). The similar upregulation of TIM-3 on $CD4^+$ and $CD8^+$ T-cell, as well as between naïve and memory T-cells, indicates that TIM-3 equally regulates all responsive T-cells during a drug-derived antigen-specific response. The expression profiles of both CTLA4 and TIM-3 during *in vitro* naïve T-cell priming differ from that of PD-1 expression. Indeed the number of PD-1^+ T-cells was increased up until re-stimulation, before falling gradually thereafter; indicating PD-1 has a more prominent role in regulation during the initial encounter of naïve T-cells with antigen. In contrast, TIM-3 and CTLA4 expression was enhanced most significantly 48-72 hrs after restimulation, implying that these pathways have a more prominent role in late stage T-cell response opposed to during the initial activation.

In chapter 3, we reported that PD-1 expression was not associated with T-cell exhaustion; a quiescent state characterised by an inability to proliferate and produce cytokines (Wherry and Ahmed, 2004). Previous assessment of the functionality of PD-1^+ TIM-3^+ tumour-infiltrating T-cells found that these cells represented an exhausted phenotype, with other

studies in concurrence that TIM-3 expression can identify exhausted T-cells in patients with HIV or HCV infection (Golden-Mason *et al.*, 2009; Jones *et al.*, 2008). However, upon analysis of both our $CD4^+$ and $CD8^+$ T-cells, the proportion of actively proliferating cells expressing either CTLA4 or TIM-3 was several fold higher than for non-dividing cells as quantified by CFSE-incorporation (CTLA4: CD4, 3.5-fold; CD8, 3.5-fold; TIM-3: CD4, 6-fold; CD8, 4-fold). Simply, this data identifies that the expression of both these co-inhibitory receptors is increased on actively proliferating T-cells, and therefore the expression of these receptors individually is not associated with an inactive, exhausted state.

More detailed analysis of CTLA4 and TIM-3 expression on SMX-NO-specific T-cell clones confirmed that, similar to PD-1, the level of receptor expression can vary widely among clones from the same donor. Importantly, the level of expression did not strongly correlate with the strength of the SMX-NO-specific T-cell responses, as measured by their ability to secrete cytokines or proliferate. This supports our earlier finding using memory T-cells direct from healthy donor peripheral blood, that these co-inhibitory pathways do not highly regulate secondary T-cell responses. However, measurement of these responses relate to a drug-derived antigen-stimulation period of just 48-72 hrs. When we measured the expression of CTLA4 and TIM-3 over numerous time-points up until 240 hrs post SMX-NO-stimulation on T-cell clones, expression of both receptors increased significantly after 48-120 hrs. This implies that while these pathways do not perhaps play a major role in dampening the onset of a secondary T-cell response, they may well play a role in dampening an elongated reaction to ensure protection against a runaway T-cell response.

In conclusion, we have been able to show that CTLA4 is able to regulate drug-derived antigen-specific naïve T-cell priming, but that this regulation is less effective than that of the PD-1/PD-L1 pathway. In contrast, both of these pathways were less influential on secondary, memory T-cell responses, with CTLA4 displaying an enhanced regulatory role during memory

T-cell responses compared to PD-1. Analysis of CTLA4 expression during drug-derived antigen-specific T-cell priming highlighted that this receptor is only upregulated after re-stimulation. At a similar time-point, PD-1 expression remains high but is rapidly down-regulated following a significant increase in expression after initial drug antigen exposure. Additionally, the maximal increase in CTLA4 expression on $CD8^+$ memory T-cells was far greater than that on $CD4^+$ naïve T-cells. Therefore, the identified expression profiles support the proliferation data in that while PD-1 has a more dominant role in early T-cell responses and continues to exert a considerable effect throughout, CTLA4 begins to have a much larger effect during the latter stages of T-cell activation inclusive of memory T-cell responses. Meanwhile, we found that TIM-3 had little to no influence of the regulation of proliferative responses in relation to either naïve T-cell priming or secondary memory T-cell responses, despite rapid upregulation towards the end of antigen-specific responses. We were however able to detect an increase in Th1-specific cytokine secretion with the addition of TIM-3 blocking antibodies, indicating that the role of TIM-3 may be limited to the regulation of particular T-cell functions in humans. Additionally, PD-1, CTLA4, and TIM-3 expression is increased on both activated $CD4^+$ and $CD8^+$ T-cells and thus their expression cannot be used as individual markers of T-cell exhaustion. These data build upon that of chapter 4 to provide a foundation to explore CTLA4 and TIM-3 expression and activity in prospective studies of drug immunogenicity.

Chapter 5: *In vitro* characterisation of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses from drug-naïve donors and allergic patients

Contents

5.1 Introduction.

Drug hypersensitivity can be detrimental to multiple organs, with the most commonly affected being the skin. These reactions are potentially life-threatening and thought to be mediated by T-cells, which have been shown to be activated in hypersensitive patients by a number of suspect drugs including SMX (Mauri-Hellweg *et al.*, 1995). Drug hypersensitivity remains a clinical problem as reactions are almost impossible to predict and difficult to diagnose. This is partly because mechanisms of T-cell activation have not been fully defined. Herein, we focus on SMX, a sulphonamide antibiotic used for the treatment of opportunistic infections, to investigate the origin and functionality of drug and drug metabolite-specific T-cells.

SMX is often marketed alongside trimethoprim as an antibiotic combination therapy known as co-trimoxazole. This formulation is commonly administered to treat the opportunistic infections that arise in patients with underlying immune compromising conditions such as HIV infection or cystic fibrosis (Lavergne *et al.*, 2010). However, SMX exposure is associated with the development of cutaneous hypersensitivity reactions in up to 8% of the general population (Alfirevic *et al.*, 2009). Furthermore, in patients with HIV, 30% receiving co-trimoxazole prophylactically experience SMX hypersensitivity, rising to 50% in those receiving treatment for infection (Pirmohamed and Park, 2001).

T-cells isolated from the blister fluid of patients with co-trimoxazole-induced TEN are cytotoxic and responsive to both SMX and the downstream metabolite SMX-NO (Naisbitt *et al.*, 2001; Nassif *et al.*, 2002). Unfortunately, animal models poorly reflect the human response. This is epitomised by the administration of SMX-NO to rats, which results in the formation of anti-SMX-hapten IgG antibodies and SMX-NO-specific T-cells, whilst no such effect is identified for SMX itself. This animal model therefore infers that oxidative metabolism of SMX to SMX-NO is a pre-requisite for immunogenicity (Gill *et al.*, 1997).

However, it is clear from human studies that both SMX and SMX-NO are indeed able to stimulate human T-cells and that this occurs via differing mechanisms. Chemically stable SMX stimulates T-cells via a direct processing-independent MHC-TCR interaction. Meanwhile, SMX-NO binds to the cysteine residues of proteins, both serum and cellular, to generate a number of potentially immune-activating neo-antigens and thus activate T-cells via a hapten mechanism (Figure 5.1). Although many T-cells from hypersensitive patients respond only to either SMX or SMX-NO, cross reactive T-cells can also be detected (Callan *et al.*, 2009; Castrejon *et al.*, 2010; Elsheikh *et al.*, 2011; Mauri-Hellweg *et al.*, 1995; Naisbitt *et al.*, 2001; Schnyder *et al.*, 2000; Schnyder *et al.*, 1998; Schnyder *et al.*, 1997).

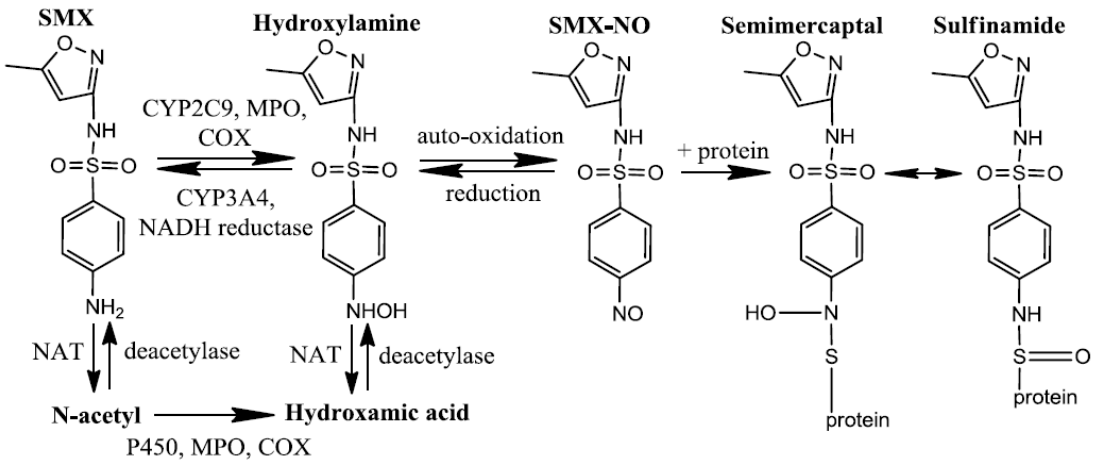


Figure 5.1. Schematic representation of sulfamethoxazole (SMX) metabolism, highlighting the formation of the protein-reactive metabolite sulfamethoxazole-nitroso (SMX-NO).

It has been reported that both drug and metabolite are able to promote the maturation of healthy donor-derived DCs, a critical step in the efficient presentation of drug-antigens on APCs to passing T-cells (Sanderson *et al.*, 2007). However, in contrast to hypersensitive patients, previous studies from healthy human donors have shown that although SMX-NO activates T-cells from all drug-naïve donors, SMX stimulates T-cells in just 30% of these

individuals (Engler *et al.*, 2004). These studies focused on the use of PBMCs or whole T-cell populations, and thus despite extensive research into the mechanisms of SMX-induced T-cell activation, the origin of SMX- and SMX-NO-responsive T-cells has not been defined. Specifically, it is not known whether previously identified SMX-NO responsive T-cells in healthy donors originate from the naïve or memory T-cell compartment. The presence of T-cells with reactivity towards SMX in drug-naïve donors may indicate that responses occur due to cross-reactivity with T-cells specific for as yet undetermined peptides.

5.2 Chapter aims.

(1) To assess the propensity for naïve T-cells from drug-naïve healthy donors to be primed to SMX or SMX-NO.

(2) To determine whether SMX- and/or SMX-NO-specific memory T-cells are present in the peripheral circulation of drug-naïve healthy donors.

(3) To characterise the phenotype and antigen cross-reactivity of SMX- and/or SMX-NO-specific T-cell clones generated from healthy donors.

(4) Elucidate the functional similarities between drug antigen-specific T-cells generated from healthy drug-naïve donors and hypersensitive patients.

5.3 Results.

5.3.1 Memory and naïve-primed T-cells from healthy donors proliferate in the presence of SMX-NO, but not SMX.

Using the *in vitro* human T-cell assay, previously utilised in chapter 3, naïve T-cells from healthy drug-naïve donors were primed to SMX-NO (40 μM). Upon restimulation, antigen-

specific activation was clearly detectable (SMX-NO, 20-40 μM; $p \leq 0.05$) using cells from all donors (Figure 5.2a; four representative donors shown). Similarly, memory T-cells isolated from all drug-naïve donors proliferated in response to SMX-NO (20-40 μM, $p \leq 0.05$) after an 8-day culture period with antigen (Figure 5.2a, representative donors shown).

In stark contrast, for all donors (9/9 individuals), the priming of naïve T-cells to SMX (1 mM) was unsuccessful as assessed by T-cell proliferative responses after SMX re-stimulation comparable to non-drug treated controls (SMX 1-2 mM). Likewise, a SMX-induced proliferative T-cell response (SMX 1-2 mM) was not detected using memory T-cells from these donors (Figure 5.2b, representative donors shown). To show that antigen-specific responses did not fail due to the lack of functional T-cells, PHA was successfully used to non-specifically stimulate T-cells (figure 5.3; PHA, 20 μg/ml; $p \leq 0.05$). Both SMX-NO- (Figure 5.3a) and SMX-(Figure 5.3b) primed naïve and memory T-cell cultures displayed a lack of cross reactivity in response to SMX (1 mM) and SMX-NO (40 μM), respectively.

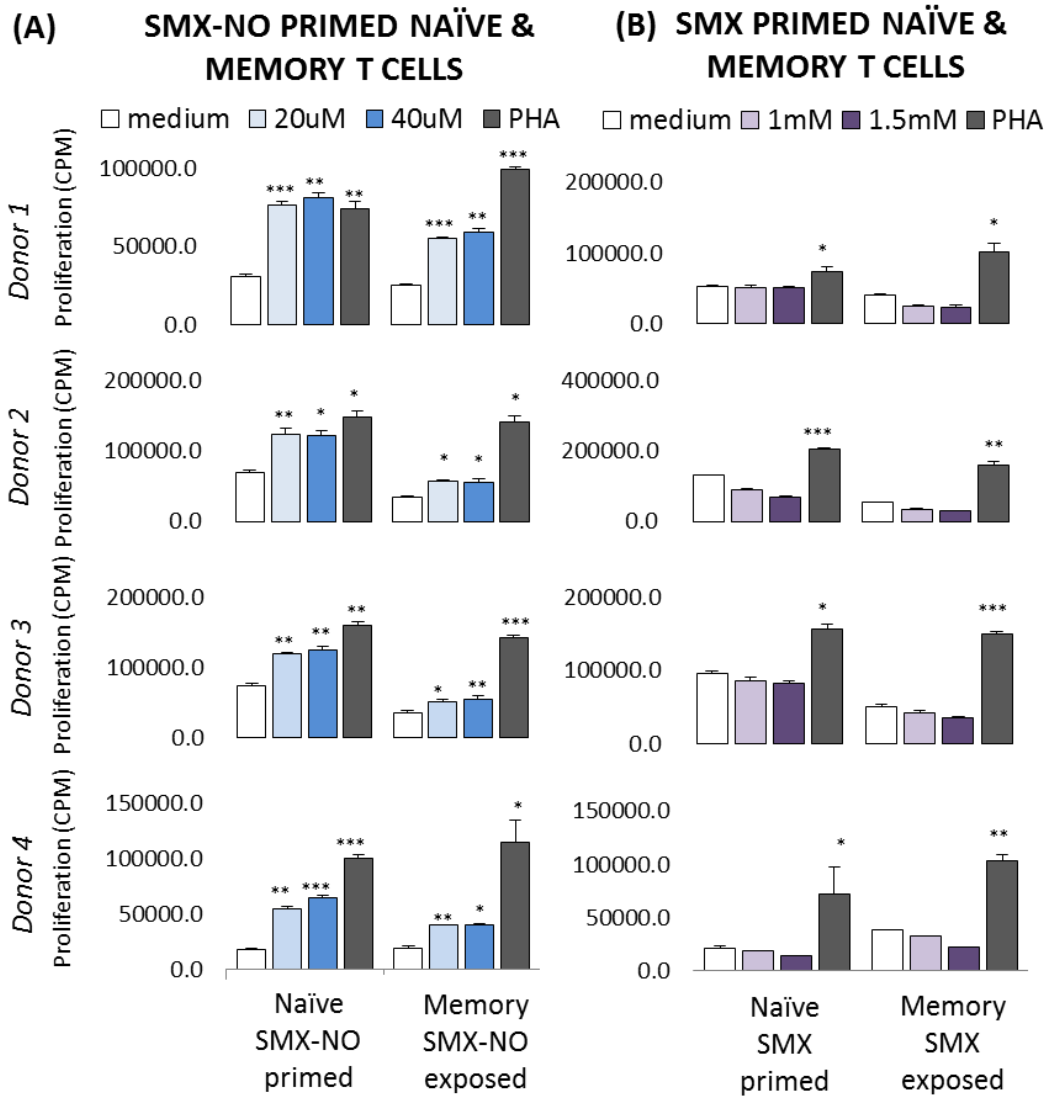


Figure 5.2. **Antigen-specific proliferation of SMX-NO-, but not SMX-, primed naïve and memory T-cells from healthy donors. (A)** Naïve or **(B)** memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) or SMX (1 mM) for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. SMXNO- and SMX cultured T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (20-40 µM) or SMX (1.0-1.5 mM), respectively. After culture for 48 hrs in similar conditions as before, proliferation was measured by adding [^{3}H]-thymidine (0.5 µCi/well) for a final 16 hr incubation. Data from four representative donors shows the mean ± SD of triplicate cultures. Statistical significance denotes a significant increase in proliferative response compared to 'medium only' treated wells (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

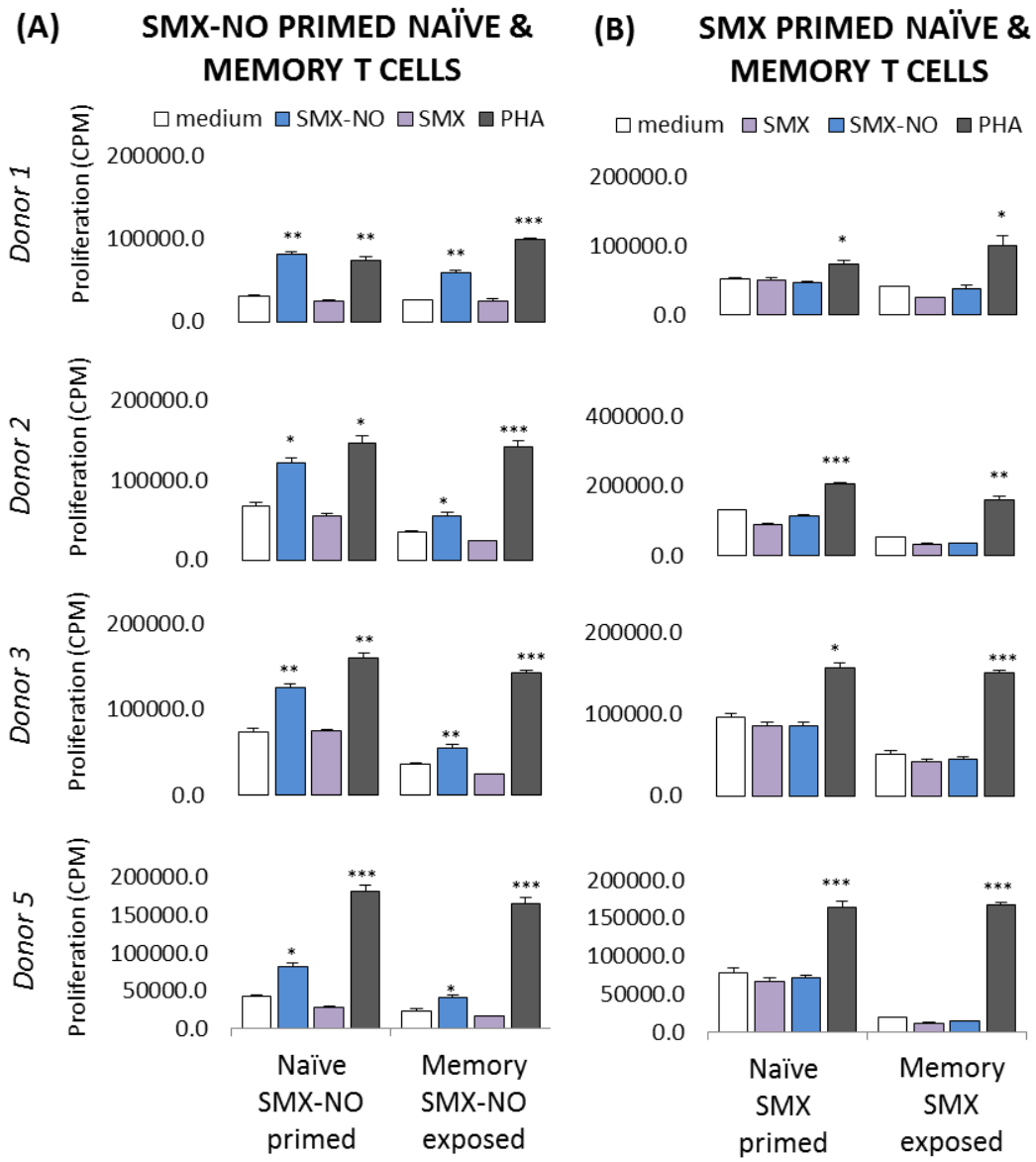


Figure 5.3. **Cross-reactivity of SMX- and SMX-NO-primed naïve and memory T-cells from healthy donors.** Proliferative response of **(A)** SMX-NO and **(B)** SMX-primed naïve and memory T-cells after exposure to both SMX and SMX-NO. Naïve or memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of SMX-NO (50 µM) or SMX (1 mM) for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. Cultured T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate; n=3) with fresh mature DCs ($4x10^3$/well) and SMX-NO (40 µM) or SMX (1 mM). After culture for 48 hrs in similar conditions as before, proliferation was measured by adding [^{3}H]-thymidine (0.5 µCi/well) for a final 16 hr incubation. Data from four representative donors shows the mean ± SD of triplicate cultures. Statistical significance denotes a significant increase in proliferative response compared to 'medium only' treated wells (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

5.3.2 Drug-derived antigen-specific cytokine secretion from both memory and antigen-primed naïve T-cells from healthy donors.

Cytokine secretion was used as an alternative assessment of T-cell activation after naïve T-cell priming or exposure of memory T-cells to drug or metabolite. IFN-γ and IL-13 were used interchangeably upon the activation of drug-derived antigen-specific responses, as both cytokines were normally detected. In one donor (Figure 5.4), enhanced IFN-γ secretion was detected after both the priming of naïve T-cells and exposure of memory T-cells to SMX-NO (Figure 5.4a; mean ± SD donor spot counts: naïve SMX-NO-primed; medium, 58.0 ± 15.6 spot forming units (sfu); 20 µM SMX-NO, 108.5 ± 6.4 sfu; 40 µM SMX-NO, 149.5 ± 4.9 sfu / memory SMX-NO; medium, 85.5 ± 4.9 sfu; 20 µM SMX-NO, 184.5 ± 20.5 sfu; 40 µM SMX-NO, 170.5 ± 13.4 sfu). Similar to the proliferation data, SMX-induced responses were not detected (Figure 5.4b; representative donor spot counts: naïve SMX-primed; medium, 33.0 ± 17.0 sfu; 1 mM SMX, 29.0 ± 7.1 sfu; 2 mM SMX, 16.5 ± 4.9 sfu / memory SMX; medium, 119.0 ± 0.0 sfu; 1 mM SMX, 82.5 ± 20.5 sfu; 2 mM SMX, 70.5 ± 17.7 sfu).

However, T-cells from 2 drug-naïve donors (donor 1 and donor 6) were identified as responsive to both SMX and SMX-NO through an enhanced IL-13 secretion in the presence of antigen. Of note, PBMCs from both of these SMX-responsive donors failed to proliferate after stimulation with SMX or SMX-NO in an LTT (Figure 5.5) and SMX-induced responses were not detected by measurement of proliferative capacity for these donors in the priming assay. Initial SMX reactivity in Donor 1 (Figure 5.6a) was identifiable by a clear increase in IL-13 secretion from naïve T-cells primed to SMX in the presence of drug (average IL-13 spot counts: medium, 63.5 ± 71.4 sfu; 1 mM SMX, 83.0 ± 49.5 sfu; 1.5 mM SMX, 121.5 ± 6.4 sfu). For memory T-cells exposed to SMX, IL-13 secretion was also detected in response to drug, despite high background cytokine secretion (IL-13 spot counts: medium, 103.5 ± 9.2 sfu; 1 mM SMX, 130.5 ± 0.7 sfu; 1.5 mM SMX 129.5 ± 57.3 sfu). SMX-NO-specific IL-13 secretion

was also identified from SMX-NO primed naïve T-cells (average IL-13 spot counts: medium, 58.5 ± 20.5 sfu; 20 µM, 130.5 ± 10.6 sfu; 40 µM SMX-NO, 146.5 ± 26.2 sfu) and SMX-NO-exposed memory T-cells (average IL-13 spot counts: medium, 40.5 ± 10.6 sfu; 20 µM, 157.5 ± 0.7 sfu; 40 µM SMX-NO, 131.5 ± 14.8 sfu).

For Donor 6 (Figure 5.6b), an increase in IL-13 secretion could be observed for naïve T-cells primed to SMX in the presence of drug (average IL-13 spot counts: medium, 187.5 ± 23.3 sfu; 1.5 mM SMX, 231.5 ± 2.1 sfu). However, for memory T-cells exposed to SMX, no increase in IL-13 secretion was detected with the addition of drug following a quantitative comparison of spot forming units between drug and non-drug treated wells (IL-13 spot counts: medium, 60.5 ± 36.1 sfu; 1.5 mM SMX 26.5 ± 10.6 sfu). SMX-NO-specific IL-13 secretion was also identified from SMX-NO primed naïve T-cells (average IL-13 spot counts: medium, 526.5 ± 23.3 sfu; 20 µM, 608.0 ± 60.8 sfu), and SMX-NO-exposed memory T-cells (average IL-13 spot counts: medium, 44.5 ± 17.7 sfu; 20 µM, 126.5 ± 38.9 sfu; 40 µM SMX-NO, 132.5 ± 62.9 sfu), albeit with high background cytokine secretion.

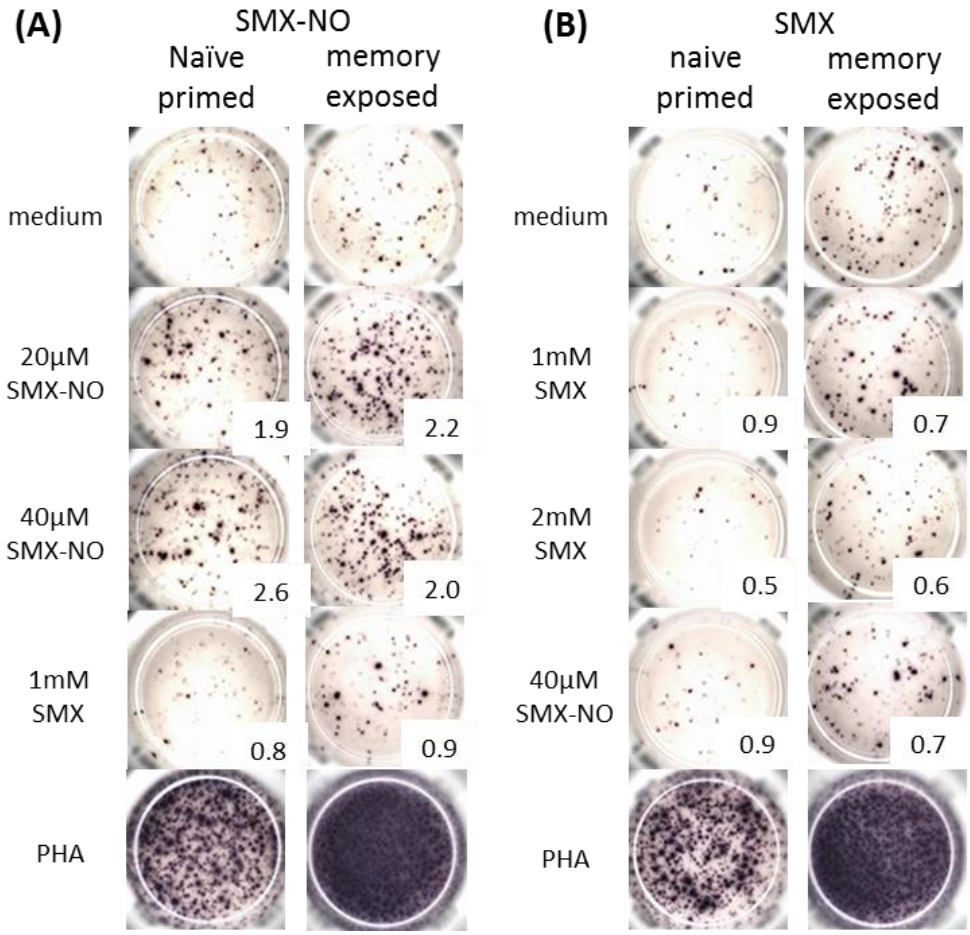


Figure 5.4. Antigen-specific cytokine secretion from SMX-NO-, but not SMX-, primed naïve and memory T-cells from a healthy donor. Naïve or memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from donor 7 were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of **(A)** SMX-NO (50 µM) or **(B)** SMX (1 mM) for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. In accordance with manufacturer's instructions, ELISpot plates were coated with IFN-γ capture antibody and incubated at 4°C overnight. T-cells cultured with SMX-NO and SMX were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well ELISpot plate) with fresh monocyte-derived DCs ($4x10^3$/well) and SMX-NO (20-40 µM) or SMX (1-2 mM). The cells were incubated for 48 hrs in a similar atmosphere as before. The ELISpot plates were then washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader. The stimulation index (SFU of drug-treated wells / SFU of 'medium-only' treated wells) is shown.

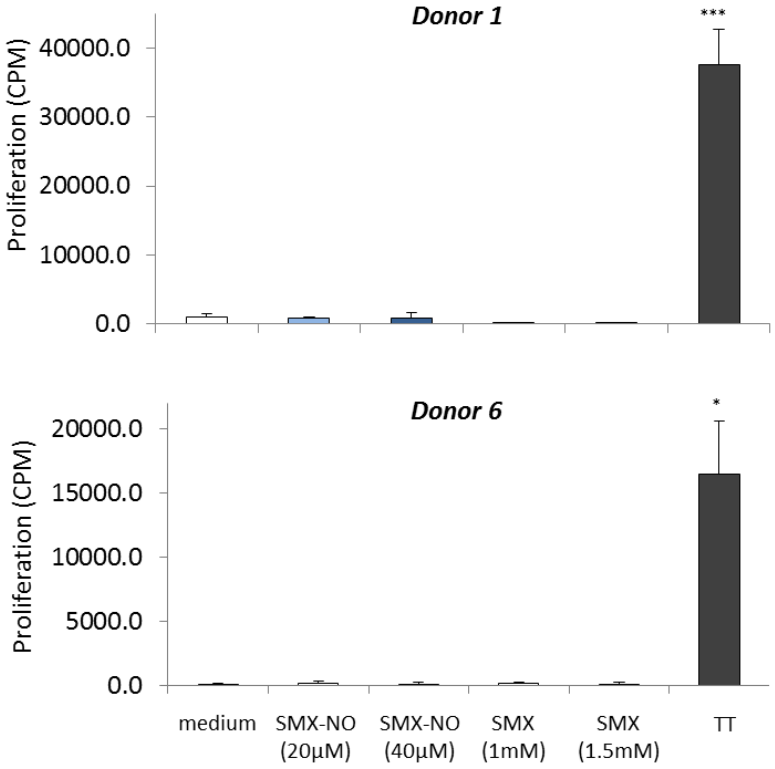


Figure 5.5. **Proliferation of PBMCs (LTT) from healthy donors 1 and 6 in response to SMX and SMX-NO.** Healthy donor PBMCs were plated ($1.5x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate) with SMX-NO (20-40 µM), SMX (1-1.5 mM) or Tetanus Toxin (TT; positive control; 10 µg/ml). Cultures were incubated for 5 days in an atmosphere of 95% air / 5% CO_2. Proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a further 16 hr incubation. Data are presented as the mean ± SD; experiments were conducted in triplicate. Statistical significance denotes a significant increase in proliferative response compared to 'medium only' treated wells (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

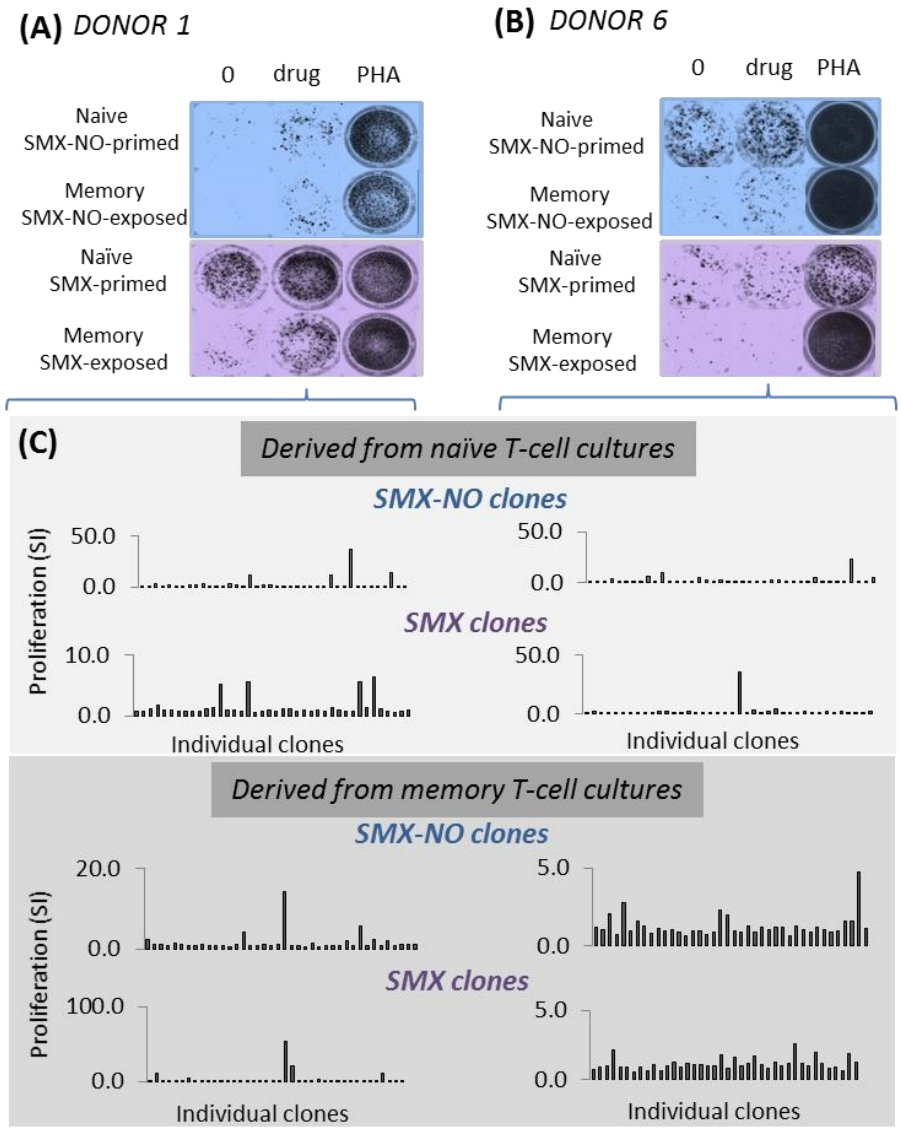


Figure 5.6. SMX- and SMX-NO-induced activation of naïve and memory T-cells from two healthy donors. (A, B) Naïve or memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) from two healthy donors were cultured with autologous mature autologous monocyte-derived DCs ($8x10^4$/well) and SMX (1 mM) or SMX-NO (40 µM) for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. In accordance with manufacturer's instructions, ELISpot plates were coated with IL-13 capture antibody and incubated at 4°C overnight. T-cells cultured with SMX and SMX-NO were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well ELISpot plate) with fresh monocyte-derived DCs ($4x10^3$/well) alongside SMX (1 mM) or SMX-NO (40 µM), respectively. The cells were incubated for 48 hrs in a similar atmosphere as before. The ELISpot plates were then washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader. **(C)** T-cell clones generated from naïve and memory SMX- and SMX-NO-responsive cultures from healthy donors 1 and 6. Individual clones ($5x10^4$/well; total volume, 200 µl; 96-well U-bottomed plate) were cultured with SMX (1 mM) or SMX-NO (40 µM) and autologous irradiated EBV-transformed B-cells ($1x10^4$/well) for 48 hrs in an atmosphere of 95% air / 5% CO_2 / 37°C. Proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a further 16 hr incubation. Experiments were conducted in duplicate. Data presented as clone stimulation index (drug-treated well average / non-drug treated well average).

5.3.3 Generation of drug- and drug metabolite-responsive T-cell clones from SMX-responsive drug-naïve donors.

We next generated T-cell clones from the two SMX-responsive individuals as to confirm the presence of SMX-responsive T-cells, and assess the functionality of these cells in more detail. From donor 1, SMX-responsive T-cell clones were generated from both naïve-primed (n=5) and memory-exposed (n=7) cultures (Table 5.1a). In addition, SMX-NO-responsive clones from both naïve T-cell priming (n=10) and memory cultures (n=7) were generated. Both SMX- and SMX-NO-responsive T-cell clones from the naïve compartment were found to be a mixture of $CD4^+$ and $CD8^+$ T-cell clones, while those from the memory compartment were solely $CD4^+$ T-cells.

From donor 6, 11 SMX-responsive T-cell clones were generated from naïve T-cell priming cultures, as were 6 SMX-responsive T-cell clones from the memory T-cell compartment, with both groups consisting of both $CD4^+$ and $CD8^+$ responsive T-cells (Table 5.1b). SMX-NO-responsive T-cell clones were also generated from naïve T-cell cultures primed to SMX-NO (n=11), all of which were found to be $CD4^+$ T-cells, while SMX-NO-responsive T-cell clones from memory T-cell cultures (n=6) were a mixture of $CD4^+$ and $CD8^+$ T-cells. The initial testing of T-cell clones from SMX- and SMX-NO-treated cultures is summarised in figure 5.6c.

Table 5.1. Characteristics of naïve- and memory T-cell-derived SMX- and SMX-NO-responsive T-cell clones from two healthy donors. T-cell clones from healthy donors **(A)** 1 and **(B)** 6. Individual clones ($5x10^4$/well; total volume, 200 µl; 96-well U-bottomed plate) were cultured with SMX (1 mM) or SMX-NO (40 µM) and autologous irradiated EBV-transformed B-cells ($1x10^4$/well) for 48 hrs in an atmosphere of 95% air / 5% CO_2 / 37°C. Proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a further 16 hr incubation. Experiments were conducted in duplicate. Data presented as clone stimulation index (drug-treated well average / non-drug treated well average). For analysis of CD4/CD8 co-receptor expression, each clone was pipetted into two tubes ($5x10^4$ cells/tube) and resuspended in 3 ul CD4-APC* or 3 µl CD8-PE. Cells were incubated in the dark and on ice for 20 mins. Cells were washed, resuspended in FACS buffer, and analysed on a FACS Canto II machine using cyflogic software.

(A) DONOR 1

	Naïve SMX-NO	Memory SMX-NO	Naïve SMX	Memory SMX
no. of clones tested	144	144	120	168
no. of specific clones	10	7	5	7
Proliferative SI range of antigen-specific clones	1.7-36.8	2.0-14.3	1.7-6.3	1.7-54.2
T cell phenotype	CD4 & CD8	CD4 only	CD4 & CD8	CD4 only

(B) DONOR 6

	Naïve SMX-NO	Memory SMX-NO	Naïve SMX	Memory SMX
no. of clones tested	144	144	114	144
no. of specific clones	11	6	11	6
Proliferative SI range of antigen-specific clones	1.7-22.5	1.6-4.8	1.7-35.2	1.7-2.6
T cell phenotype	CD4 only	CD4 & CD8	CD4 & CD8	CD4 & CD8

5.3.4 Cross-reactivity of SMX and SMX-NO-specific T-cell clones generated from drug-naïve donors.

It has been previously shown that SMX-responsive T-cell clones from hypersensitive patients are highly specific (Castrejon *et al.*, 2010). Thus, we wanted to assess the cross-reactivity of drug- and drug metabolite-specific T-cell clones generated from drug-naïve donors. Both

SMX- and SMX-NO-responsive clones generated from naïve T-cell priming were found to be highly specific and were not cross-reactive with SMX-NO and SMX, respectively (Figure 5.7a and b). In contrast, 3/5 SMX-responsive T-cell clones from the memory compartment proliferated in the presence of SMX-NO (Figure 5.7a) although these responses were much weaker than for SMX. Despite the confirmation of cross reactivity by statistical significance for all three of these clones, only SMX 'clone 3' was clearly cross-reactive at both SMX-NO concentrations used (20 μM SMX-NO, $p = 0.012$; 40 μM SMX-NO, $p = 0.008$; 1 mM SMX, $p < 0.001$; 1.5 mM SMX, $p = 0.009$). The two other cross reactive SMX-responsive clones were only weakly cross reactive to just one SMX-NO concentration (SMX clone 5: 20 μM SMX-NO, $p = 0.067$; 40 μM SMX-NO, $p = 0.013$; SMX clone 7: 20 μM SMX-NO, $p = 0.032$; 40 μM SMX-NO, $p = 0.076$). Only two SMX-NO-responsive memory T-cell clones were assessed for cross reactivity (Figure 5.7b). One was highly specific and showed no cross reactivity. The other meanwhile responded strongly to all concentrations for both SMX and SMX-NO, with the strength of response similar for both parent drug and metabolite (SMX-NO clone 6: 20 μM SMX-NO, $p < 0.001$; 40 μM SMX-NO, $p = 0.017$; 1 mM SMX, $p < 0.001$; 1.5 mM SMX, $p < 0.001$).

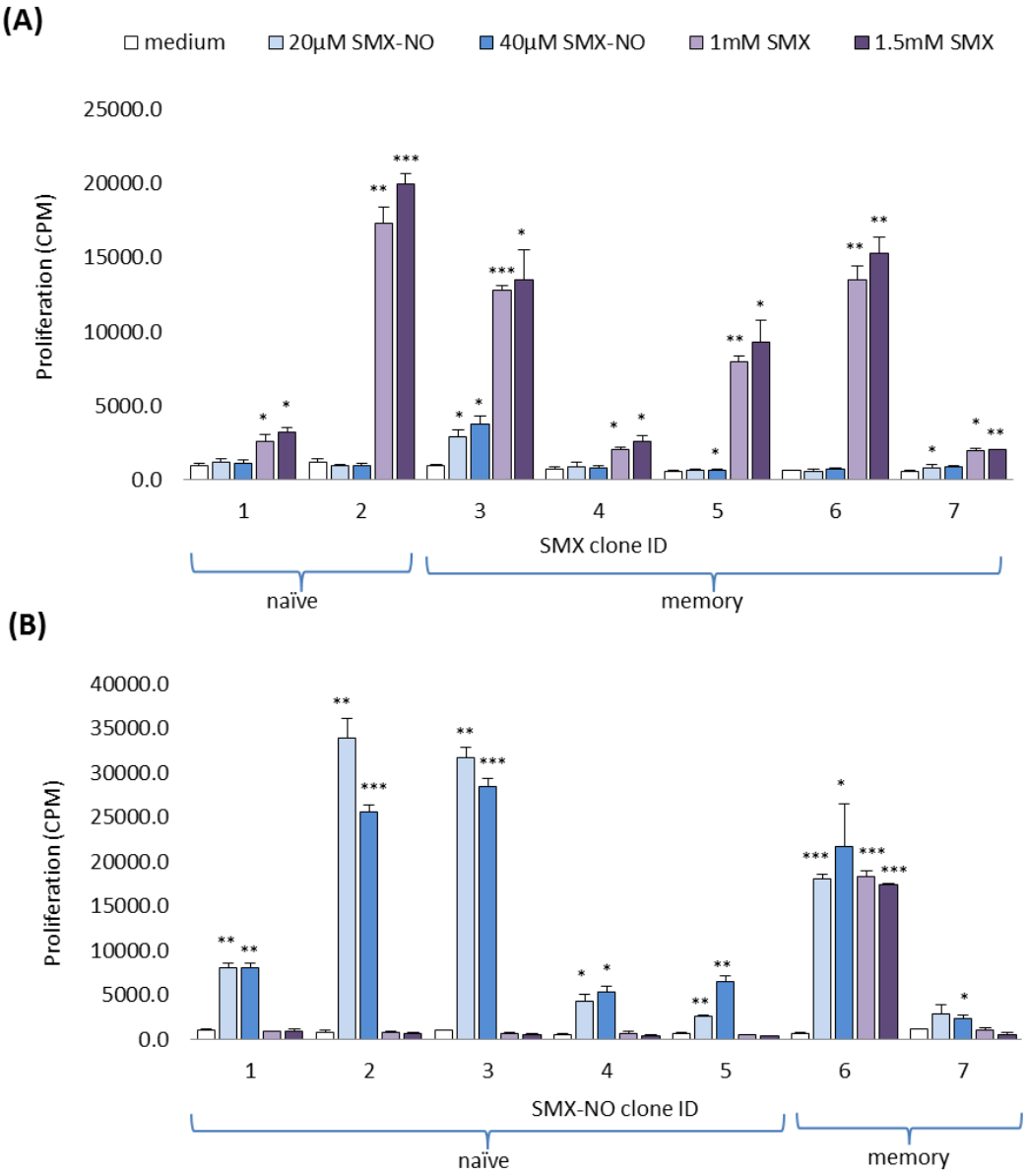


Figure 5.7. Cross-reactivity profile of naïve and memory T-cell-derived SMX- and SMX-NO-responsive T-cell clones from healthy donors. (A) SMX- and **(B)** SMX-NO-responsive T-cell clones ($5x10^4$/well; total volume, 200 µl, 96-well U-bottomed plate) were stimulated with SMX (1-1.5 mM) and SMX-NO (20-40 µM) using autologous irradiated EBV-transformed B-cells ($1x10^4$/well). After incubation in an atmosphere of 95% air / 5% CO_2 / 37°C for 48 hrs, proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a further 16 hrs. Data are presented as the mean ± SD; experiments were conducted in triplicate. Statistical significance denotes a significant increase in proliferative response compared to 'medium only' treated wells (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

5.3.5 SMX-responsive T-cell clones from drug-naïve donors secrete a mixed panel of cytokines/cytolytic molecules.

The activity of SMX-responsive T-cell clones generated from SMX-responsive drug-naïve donors was assessed by measuring cytokine secretion in the presence of SMX. As a comparison, two SMX-NO-responsive clones were also assessed (Figure 5.8a). Two SMX-responsive T-cell clones generated from the naïve compartment (Figure 5.8b), and two from the memory compartment (Figure 5.8c) were analysed for the secretion of the cytokines IFN-γ, IL-13, IL-5, and IL-17, and the cytolytic molecules granzyme B, and perforin. All clones were found to secrete all of the assessed cytokines and cytolytic molecules apart from IL-17, showing a similar secretion panel to SMX-NO-specific T-cell clones.

5.3.6 SMX-responsive $CD8^+$ and $CD4^+$ T-cell clones were MHC class I- and II-restricted, respectively.

SMX-responsive T-cell clones generated from both the naïve and memory compartments from drug-naïve donors were assessed for MHC class I- and II-restriction through the addition of MHC class I and II blocking antibodies. Only seven clones were tested from the two SMX-responsive donors due to limitations in the number of cells. Two $CD8^+$ T-cell clones from donor 6 were assessed for MHC class restriction (Figure 5.9b); one from the memory compartment and one from naïve T-cell priming. Both $CD8^+$ T-cell clones displayed a significant reduction in SMX-specific proliferation in the presence of MHC class I blocking antibodies, but were not MHC class II-restricted (naïve SMX 42: MHC class I block, $p = 0.023$; MHC class II block, $p = 0.501$) (memory SMX 31: MHC class I block, $p = 0.008$; MHC class II block, $p = 0.131$).

For donor 1, a total of 5 $CD4^+$ SMX-specific clones were assessed (Figure 5.9a); 3 from the memory compartment and 2 from naïve T-cell priming. Despite one SMX-responsive clone from the memory compartment appearing unaffected by either MHC class I or class II block, the two other memory SMX clones were MHC class II-restricted (Memory SMX 84: MHC class I block, p = 0.332; MHC class II block, p = 0.015) (Memory SMX 4: MHC class I block, p = 0.281; MHC class II block, p = 0.006). The proliferative response to drug from both SMX-responsive clones generated from naïve T-cell priming was also restricted by blocking MHC class II, despite the response in one clone also being simultaneously class I restricted (Naive SMX 42: MHC class I block, p = 0.302; MHC class II block, p = 0.004) (Naive SMX 22: MHC class I block, p = 0.014; MHC class II block, p = 0.030). The reason for one clone being both MHC class I- and II-restricted, as mentioned previously in this thesis, may potentially indicate that it is not a 'true clone' but a T-cell line derived from more than one cell perhaps relating to errors during the serial dilution phase of T-cell cloning. This well may therefore contain both $CD4^+$ and $CD8^+$ T-cells. Alternatively, this dual MHC blockade could be a consequence of non-specific binding of antibody resulting in the false reduction of proliferation in the presence of an MHC class I-blocking antibody.

MHC class II restricted clones from donor 1 were subject to a more in depth HLA-analysis through the addition of antibodies designed to block HLA-DR, HLA-DP, and HLA-DQ alleles individually (Figure 5.10). For this analysis, a total of four SMX-responsive clones were analysed; two from the memory compartments and two from naïve T-cell priming. The proliferative response from one SMX clone from the naïve compartment was not significantly reduced by the addition of any HLA-blocking antibodies (naïve SMX 42). All three other clones did however respond to antibody blockade, with significant reductions in proliferative capacity in response to SMX seen with the addition of HLA-DR or HLA-DP blocking antibodies. Blocking HLA-DQ failed to alter proliferation in comparison to SMX alone (Naïve SMX 102: HLA-DR block, p = 0.002; HLA-DP block, p = 0.004; HLA-DQ block, p = 0.332) (Memory SMX

84: HLA-DR block, p < 0.001; HLA-DP block, p < 0.001; HLA-DQ block, ≤= 0.274) (Memory SMX 4: HLA-DR block, p = 0.032; HLA-DP block, p = 0.031; HLA-DQ block, p = 0.726).

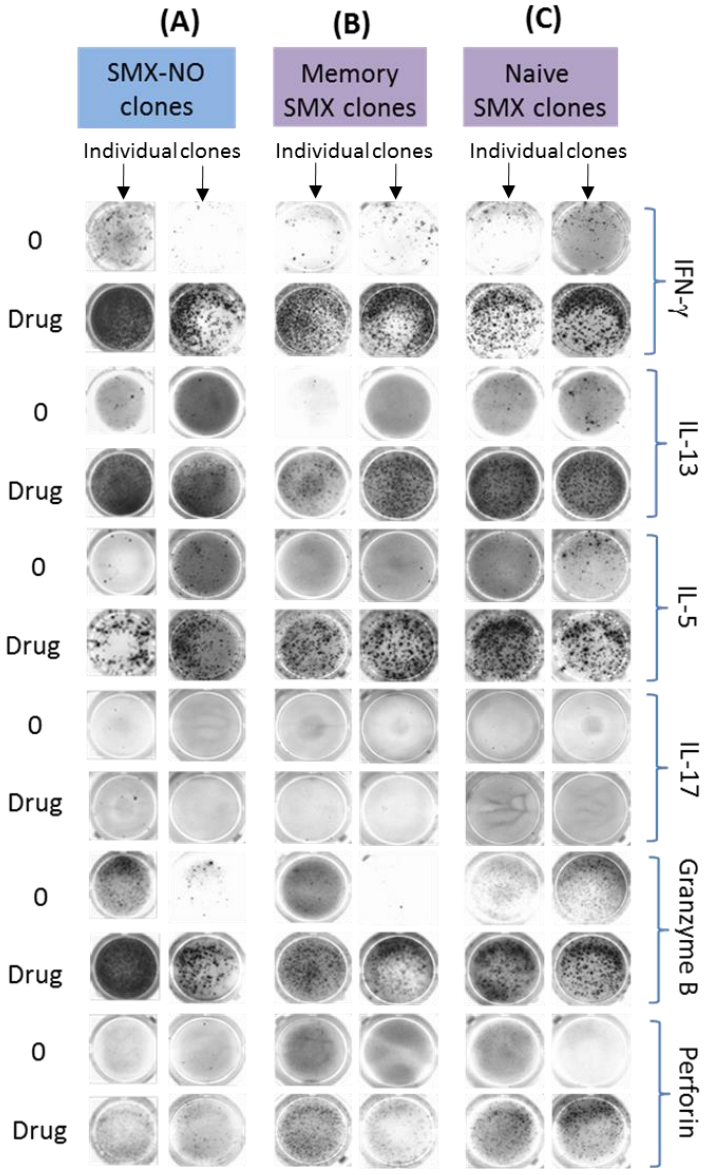


Figure 5.8. Similar cytokine and cytolytic molecule secretion by SMX and SMX-NO-responsive T-cell clones derived from healthy donors. In accordance with manufacturer's instructions, ELISpot plates were coated with IFN-γ, IL-13, IL-5, IL-17, IL-granzyme B, or perforin capture antibody and incubated at 4°C overnight. **(A)** SMX-NO-responsive T-cell clones ($5x10^5$/well; total volume, 200 µl; 96-well ELISpot plate) were cultured with SMX-NO (40 µM) and autologous irradiated EBV-transformed B-cells ($1x10^4$/well) in an atmosphere of 95% air / 5% CO_2 / 37°C for 48 hrs. **(B)** Memory-derived SMX-responsive and **(C)** naive-derived SMX-responsive T-cell clones were cultured under similar conditions but in the presence of SMX (1mM). The ELISpot plates were washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader.

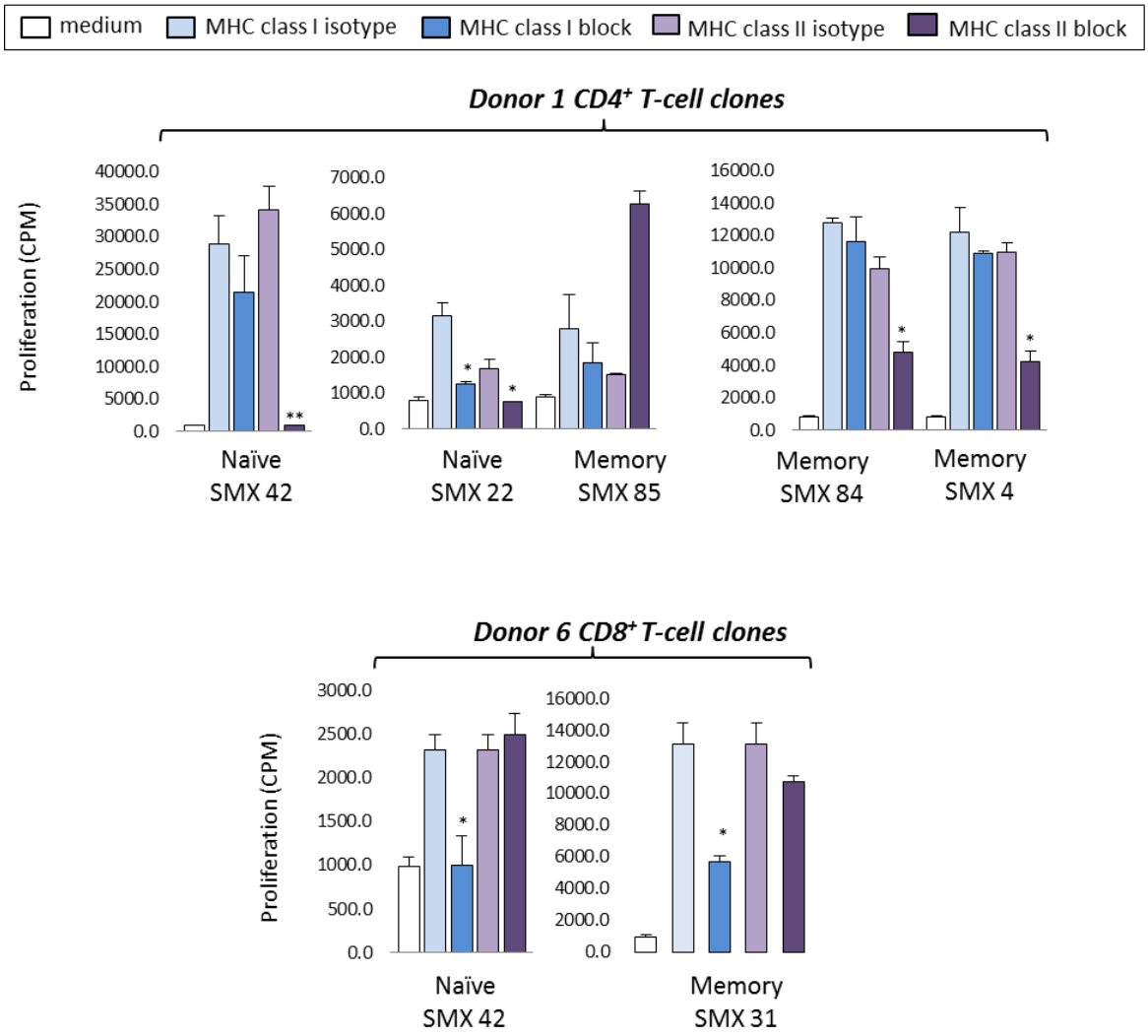


Figure 5.9. MHC-restriction of $CD4^+$ and $CD8^+$ SMX-specific T-cell clones derived from healthy donors. Naïve and memory-derived $CD4^+$ and $CD8^+$ SMX-specific T-cell clones ($5x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate) from healthy donors were stimulated with SMX (1 mM) using autologous irradiated EBV-transformed B-cells ($1x10^4$/well) ± MHC class I- and class II-blocking antibodies or their respective isotype controls (10 µg/ml). After culture for 48 hrs in an atmosphere of 95% air / 5% CO_2 / 37°C, proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a further 16 hrs. Data are presented as the mean ± SD; experiments were conducted in triplicate. Statistical significance denotes a significant reduction in proliferative response compared to respective isotype control treated wells (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

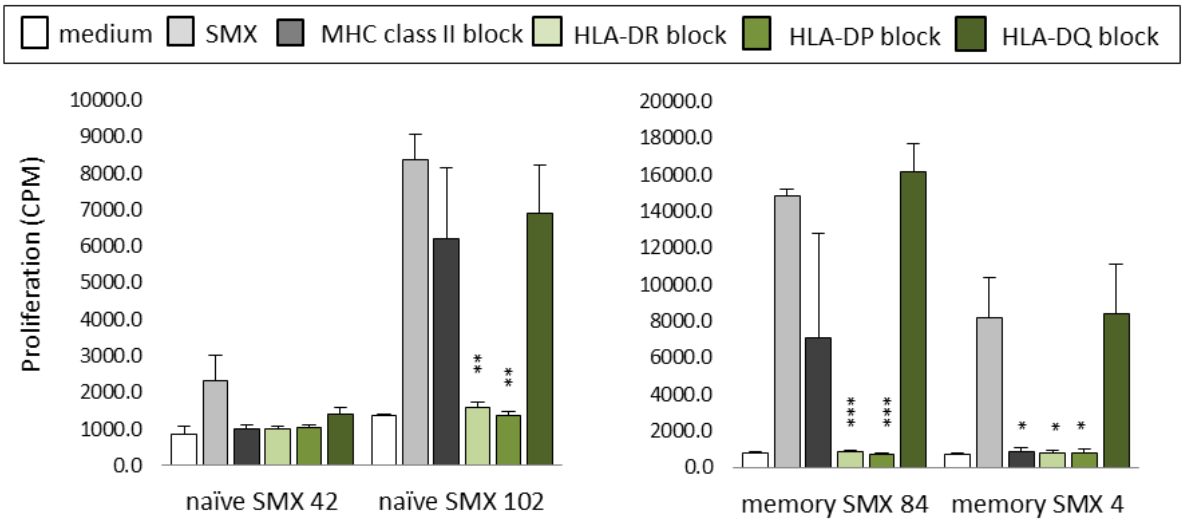


Figure 5.10. Individual blockade of HLA-DR/DP/DQ of 'Donor 1' MHC class II-restricted SMX-responsive T-cell clones. Naïve and memory-derived $CD4^+$ SMX-specific T-cell clones from donor 1 ($5x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate) were stimulated with SMX (1 mM) using autologous irradiated EBV-transformed B-cells ($1x10^4$/well) in the presence and absence of MHC class II-blocking antibodies, individual HLA-DR/DP/DQ-blocking antibodies, or their respective isotype controls (10 µg/ml). After culture for 48 hrs in an atmosphere of 95% air / 5% CO_2 / 37°C, proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for the final 16 hr of the experiment. Data are presented as the mean ± SD; experiments were conducted in triplicate. Statistical significance denotes a significant reduction in proliferative response compared to 'drug only' treated wells (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

5.3.7 Full HLA-typing of both SMX-responsive donors.

As previous HLA-association studies looking at SMX-hypersensitivity have not ruled out a minor role for HLA alleles in the predisposition to hypersensitivity, we characterised the full HLA type of each SMX-responsive donor. While both donors were found to have the same HLA-DQA11 phenotype, for all other HLA subsets the two donors were genetically dissimilar (Table 5.2).

Table 5.2. Full HLA-type of SMX-responsive healthy donors. Healthy donor DNA was isolated from blood samples using the magnetic separation module-1 (Chemagen). DNA samples were then shipped to, and HLA-typed by Histogenics (Ossining, NY, USA).

	MHC class I						MHC class II					
	HLA-A		HLA-B		HLA-C		HLA-DRB		HLA-DQB		HLA-DQA	
	A1	A2	B1	B2	C1	C2	DRB11	DRB12	DQB11	DQB12	DQA11	DQA12
Donor 1	02:01:01G	02:01:01G	39:01:01G	44:02:01G	05:01:01G	07:02:01G	01:01:01	03:01:01G	02:01:01G	05:01:01G	01:01:01G	05:01:01G
Donor 6	02:03:01	26:01:01	15:19	51:01:01	04:03:01	14:02:01	09:01:02G	15:02:01	03:03:02G	05:02:01G	01:01:01G	03:01:01G

5.4 Discussion.

Hypersensitivity to the antibiotic SMX is well-studied; some T-cells from hypersensitive patients are directly stimulated by the parent drug, some by the oxidative metabolite SMX-NO, and a minority are cross-reactive (Castrejon *et al.*, 2010; Mauri-Hellweg *et al.*, 1995). In

contrast, it was shown by Engler and colleagues that while the majority of healthy donor PBMCs can be stimulated to respond to SMX-NO through repetitive exposure to the drug metabolite, only 3/10 individuals were responsive to SMX (Engler *et al.*, 2004). However, these responses were identified using whole blood lymphocytes and no-one has since characterised the individual role of naïve and memory T-cells from drug-naïve donors to identify the origins of these responses. As there is lack of suitable animal models that reflect the hypersensitive human response, investigation of these responses warranted the use of an *in vitro* human T-cell assay.

We were able to successfully prime naïve T-cells to SMX-NO from all donors in concurrence with previous studies that identified SMX-NO-specific T-cell responses in 9/10 drug-naïve donors (Engler *et al.*, 2004). Additionally, we were able to identify memory SMX-NO-specific T-cell responses from all of these donors. In contrast, SMX-responsive T-cell responses were generally not detected from either the naïve or memory subsets. However, upon analysis of cytokine secretion from these cultures, it was found that the secretion patterns from 2 donors indicated SMX-specific IL-13 secretion. Interestingly, LTTs from these donors were negative for both SMX and SMX-NO. Thus the initial *in vitro* culture with antigen was able to expand the responsive memory population to detectable levels. We were subsequently able to generate a number of SMX and SMX-NO-responsive T-cell clones from the naïve and memory cultures from both donors. These data indicate that both the priming of naïve T-cells, but also the activation of pre-existing memory T-cells by SMX-derived antigens, may be involved in the aetiology of SMX-hypersensitivity. Furthermore, as these individuals have not previously been exposed to SMX, the detection of SMX/SMX-NO-specific memory responses raises the possibility that certain individuals in the general population have peptide-specific $CD4^+$ and $CD8^+$ T-cells which are reactive against SMX/SMX-NO-modified peptides through some form of molecular mimicry within the MHC antigen-binding cleft. This idea, termed heterologous immunity, has been identified numerous times previously regarding viral cross-

reactivity (Chen *et al.*, 2001; Selin *et al.*, 1998; Yang *et al.*, 1989). However, more recently this theory has been applied to the development of drug-hypersensitivity in relation to T-cell responses to the drug ABC. ABC-responsive T-cells were isolated from all drug-naïve donors who were HLA-B*57:01 positive, from both the naïve and memory compartments. The authors of this study were able to further prove that specific HLA-B*57:01-restricted epitopes originating from a viral antigen could mount cross-reactive T-cell responses to ABC-dependent synthetic epitopes, thus proving that viral-specific T-cells have the propensity to cross-react with MHC-restricted drug-antigen (Lucas *et al.*, 2015). The exact nature of the antigen(s) responsible for the initial priming of naïve T-cells is unknown. However, if the antigen is pathogen-derived then it could be that a large proportion of the population has circulating T-cells able to cross-react with drugs and/or their metabolites. Indeed, our data are the first to show that both hapten and parent drug can stimulate pre-existing memory T-cells from drug-naïve donors.

Previous immunohistological studies characterising the phenotype of T-cells infiltrating the inflamed skin of patients with maculopapular skin rashes describe the presence of large numbers of $CD4^+$ T-cells and lower numbers of $CD8^+$ T-cells (Pichler, 2003; Pichler *et al.*, 2002). In concordance with this, we were able to generate both $CD4^+$ and $CD8^+$ SMX- and SMX-NO-responsive T-cell clones from both the naïve and memory compartments from drug-naïve donors. Furthermore, in most cultures consisting of both $CD4^+$ and $CD8^+$ T-cells, the majority of antigen-responsive clones were $CD4^+$, apart from for SMX-specific clones generated by naïve T-cell priming from donor 1 where 3/5 clones were $CD8^+$. It was also found that all drug- and metabolite-responsive T-cell clones generated from naïve precursors displayed a total lack of cross-reactivity between SMX and SMX-NO. This reflects the scene in hypersensitive patients both in terms of responsive-cell phenotype, and that T-cell clones are generally highly antigen-specific (Schnyder *et al.*, 2000). In contrast, we identified a

number of cross-reactive SMX- and SMX-NO-responsive T-cell clones derived from memory precursors. Thus, due to the distinct lack of cross reactivity with antigen-responsive clones from naïve T-cell priming, our data indicate that the minority of cross-reactive T-cells observed in hypersensitive patients are likely derived from pre-existing memory T-cells.

Both naïve and memory SMX-specific T-cell clones in our study secreted a mixed panel of cytokines including the Th1 cytokine IFN-γ, the Th2 cytokines IL-13 and IL-5, and also the cytolytic molecules granzyme B and perforin; a panel similar to that secreted by SMX-NO-specific clones (Figure 5.8). This details a similar expression profile to that previously observed using SMX (metabolite)-specific T-cells isolated from hypersensitive patients which secreted IFN-γ, IL-5, and IL-13 (Castrejon *et al.*, 2010; Elsheikh *et al.*, 2011; Schnyder *et al.*, 2000). The release of granzyme B and perforin concurs with findings that SMX-responsive $CD4^+$ and $CD8^+$ T-cells from hypersensitive patients can be activated by the drug to secrete cytolytic molecules. Despite this, it is important to bear in mind that keratinocytes are killed by drug-responsive $CD4^+$ T-cells (Schnyder *et al.*, 1998).

Although previous investigations have only found significant HLA associations with SMX hypersensitivity in non-HIV-positive hypersensitive individuals (Özkaya-Bayazit and Akar, 2001; Roujeau *et al.*, 1986), defining the role of the MHC in the presentation of SMX-derived antigens to T-cells is important for understanding the mechanism of these reactions. The activation of our SMX-specific clones was MHC-restricted, with as expected, $CD4^+$ and $CD8^+$ clones showing a dependence on MHC class II and I molecules, respectively. Furthermore, the class II-restricted responses found in donor 1 were generally found to be HLA-DR and HLA-DP-restricted. While a previous study screened for class II gene associations for co-trimoxazole hypersensitivity in HIV positive patients and found no significant HLA associations, the authors conceded that they could not rule out a minor HLA-specific

influence (Alfirevic *et al.*, 2009). Thus while predisposition to SMX-hypersensitivity may be minimally affected by specific HLA-alleles, these reactions are likely regulated by a number of other immunological factors such as those that manipulate naïve T-cell activation thresholds.

In conclusion, we have shown the activation of both naïve and memory T-cells from drug-naïve donors by SMX and SMX-NO. This indicates that both the priming of naïve T-cells, but also the activation of pre-existing memory T-cells by SMX-derived antigens, may play a role in the onset of SMX-hypersensitivity. As these memory T-cell responses were detected in drug-naïve donors, this indicates that SMX-derived antigens can activate T-cells previously primed to as yet undetermined peptides. In contrast to T-cell clones generated from naïve T-cell priming, we identified that both hapten- and parent drug-responsive T-cell clones derived from memory precursors were cross-reactive and thus likely represent the minority cross-reactive T-cell fraction previously observed using cells from hypersensitive patients. Furthermore, these responses are reliant on the development of antigen-responsive, MHC-restricted $CD4^+$ and $CD8^+$ T-cells which overall secrete a range of cytokines including IFN-γ, Il-13, and IL-5. This lies in agreement with previous studies detailing the function of SMX responsive T-cells from hypersensitive patients. These important functional similarities between our *in vitro* data and previous patient data validates that these *in vitro* assays are useful tools for the detailed investigation of drug-induced T-cell responses and for defining response mechanisms.

Chapter 6: Comparison of allergic patient and *in vitro* activated healthy donor T-cell responses to *p*-Phenylenediamine (PPD) and Bandrowki's Base (BB).

Contents

6.1 Introduction.

Drugs are a frequent source of hypersensitivity reactions. However, hypersensitivity is also associated with exposure to cosmetic products (de Groot, 1987). These reactions can be severely debilitating, with those on the face and neck potentially life threatening as the associated swelling can lead to respiratory difficulties (Kind *et al.*, 2012; Pot *et al.*, 2013). These more severe reactions are commonly associated with the hair dye product, PPD. PPD was first patented for hair colouring in 1883 (McFadden *et al.*, 2011) and is now a common component of hair dye and henna tattoos, however its use is associated with hypersensitivity reactions that present as ACD (Aeby *et al.*, 2009; Jenkinson *et al.*, 2009a; White *et al.*, 2006b). Three quarters of women in North America have used hair dye products (Zhang *et al.*, 2009), 70% of which contain PPD. This figure, combined with the fact that in the EU alone during 2007 spending on hair care related products was roughly 16 billion euros (Bonefeld *et al.*, 2010), indicates that a large percentage of the population is exposed to PPD. Furthermore, this problem is only likely to increase as in emerging countries, cosmetic products do not legally have to label PPD containing products (Thyssen *et al.*, 2008). This raises an important question: why do only a small number of individuals who dye their hair, often for many years, develop ACD?

Extensive research has outlined the potential for PPD to form a range of antigenic intermediates by processes of oxidation, including PPD-benzoquinone and PPD-benzoquinonediimine, both of which can form protein adducts (Figure 6.1). Furthermore, PPD-oxidation products have an enhanced ability to activate DCs, when compared with non-oxidised PPD (Aeby *et al.*, 2008). While both of these oxidation products are inherently unstable and so unable to be investigated individually, spontaneous oxidation ensures their presence during *in vitro* culture with PPD. Further oxidation and self-conjugation results in

the formation of a rearrangement product of the trimer known as BB (Coulter *et al.*, 2009; Coulter *et al.*, 2007b; Jenkinson *et al.*, 2009a). Due to their ability to instigate mouse lymphocyte proliferation, PPD and BB are classified as strong and extreme sensitizers, respectively (White *et al.*, 2006b).

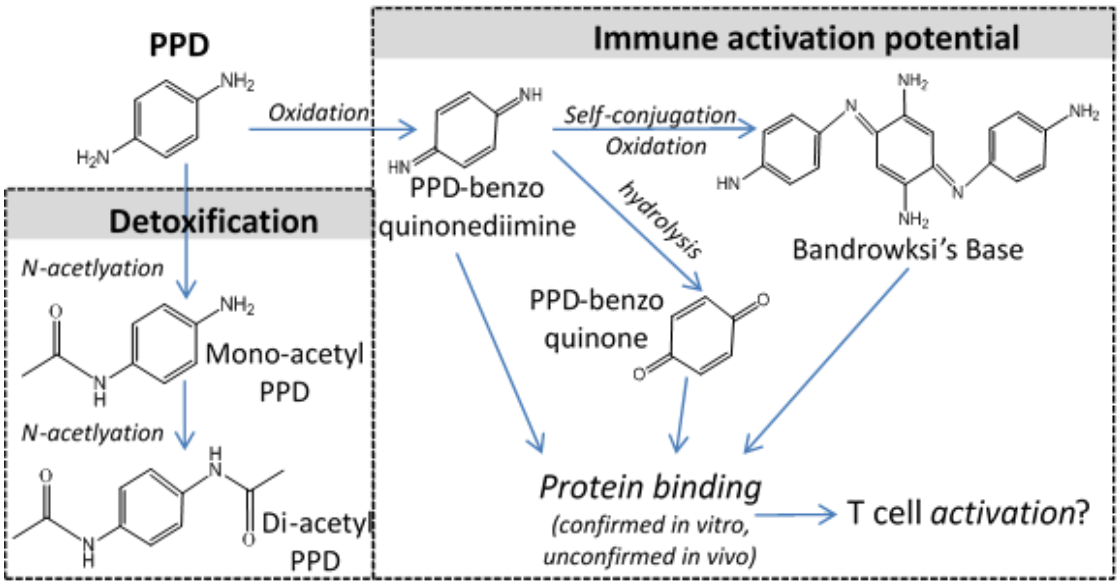


Figure 6.1. Schematic representation of *p*-phenylenediamine (PPD) metabolism and spontaneous oxidation pathways. Adapted from pot *et al* (Pot *et al.*, 2013).

PBMCs from individuals with PPD-induced ACD are activated *ex vivo* with PPD and BB; however, there is often little or no cross-reactivity with both PPD- and BB-responsive clones detected in patient blood. These data suggest that PPD and BB selectively prime naïve T-cells in patients with ACD. Somewhat surprisingly, and similar to SMX hypersensitivity detailed in chapter 5, the response profile is vastly different between patients and healthy donors. While PPD has previously been shown to mature DCs (Coulter *et al.*, 2007a), PBMCs from healthy donors are activated with BB, but not PPD, and these BB responses are of similar strength to those in allergic patients (Coulter *et al.*, 2009; Coulter *et al.*, 2008; Farrell *et al.*, 2009). Collectively, these data indicate that (1) BB-specific T-cell responses develop in most,

if not all, individuals, but this does not result in ACD, and (2) the priming of T-cells to PPD is only observed in a small portion of the population and this is an important early step in the development of ACD. Importantly, previous studies focus on PBMCs or whole T-cell populations and thus the origin of the T-cell mediated response in allergic and tolerant individuals remains unknown.

As PPD fails to instigate T-cell responses in mice after systemic administration, it is classified as a selective human T-cell allergen (Farrell *et al.*, 2009). Thus it is important that the investigation of the immunogenic potential of PPD-derived antigens should be performed using human T-cell models. We have therefore utilised the *in vitro* human T-cell assay, first used in chapter 3, to characterise the propensity of PPD and BB to activate naïve and memory T-cells from healthy donors. Moreover, we continue to address the role of the newly discovered Th17 and Th22 T-cell subsets, and identify how healthy donor responses relate to those from allergic patients *ex vivo*.

6.2 Chapter aims.

(1) To assess the propensity for PPD or BB to activate naïve and memory T-cells from healthy donors.

(2) To characterise the phenotype and cross-reactivity of PPD- and/or BB-specific T-cell clones generated from healthy donors and allergic patients.

(3) To address the role of Th17 and Th22 T-cell subsets in PPD-induced ACD by utilising responsive cells from allergic patients.

(4) Elucidate the functional similarities between PPD- and/or BB-specific T-cells generated from healthy donors and allergic patients.

(5) To assess the role of MHC molecules in the presentation of PPD to T-cells.

6.3 Results.

6.3.1 T-cells from PPD-allergic patients proliferate in the presence of PPD and BB with little cross-reactivity.

Both PPD and BB were shown to stimulate allergic patient PMBC proliferation as measured by [^{3}H] thymidine incorporation. The strength of the proliferative response to PPD was weaker than that observed with BB (Figure 6.2a). Patient PBMCs were then cultured with either PPD or BB for 2 weeks and T-cell clones generated. From five patients, a total of 105 PPD-specific and 122 BB-specific T-cell clones were generated out of a potential 912 and 740 cell-seeded wells from PPD- and BB-exposed PBMCs, respectively (Figure 6.2b). Unfortunately, due to a lack of clinical data regarding the severity of each patient's response, we were unable to correlate the *in vitro* response, in terms of the strength and frequency of T-cell clones, with the patch test grading. Both PPD and BB-responsive clones were isolated from each allergic patient. Thirty five PPD and BB-responsive clones were assessed for cross-reactivity. Approximately 25% of PPD-responsive clones demonstrated low levels of reactivity to BB (Figure 6.3a). In contrast, a weak PPD-specific proliferative response was detected with only 1 BB-responsive clone (Figure 6.3b; cross-reactive clone not shown). Greater than 90% of BB-specific clones and most PPD-specific clones were found to be $CD4^+$ T-cells. However, 15 PPD-specific clones (37.5%) expressed the $CD8^+$ co-receptor.

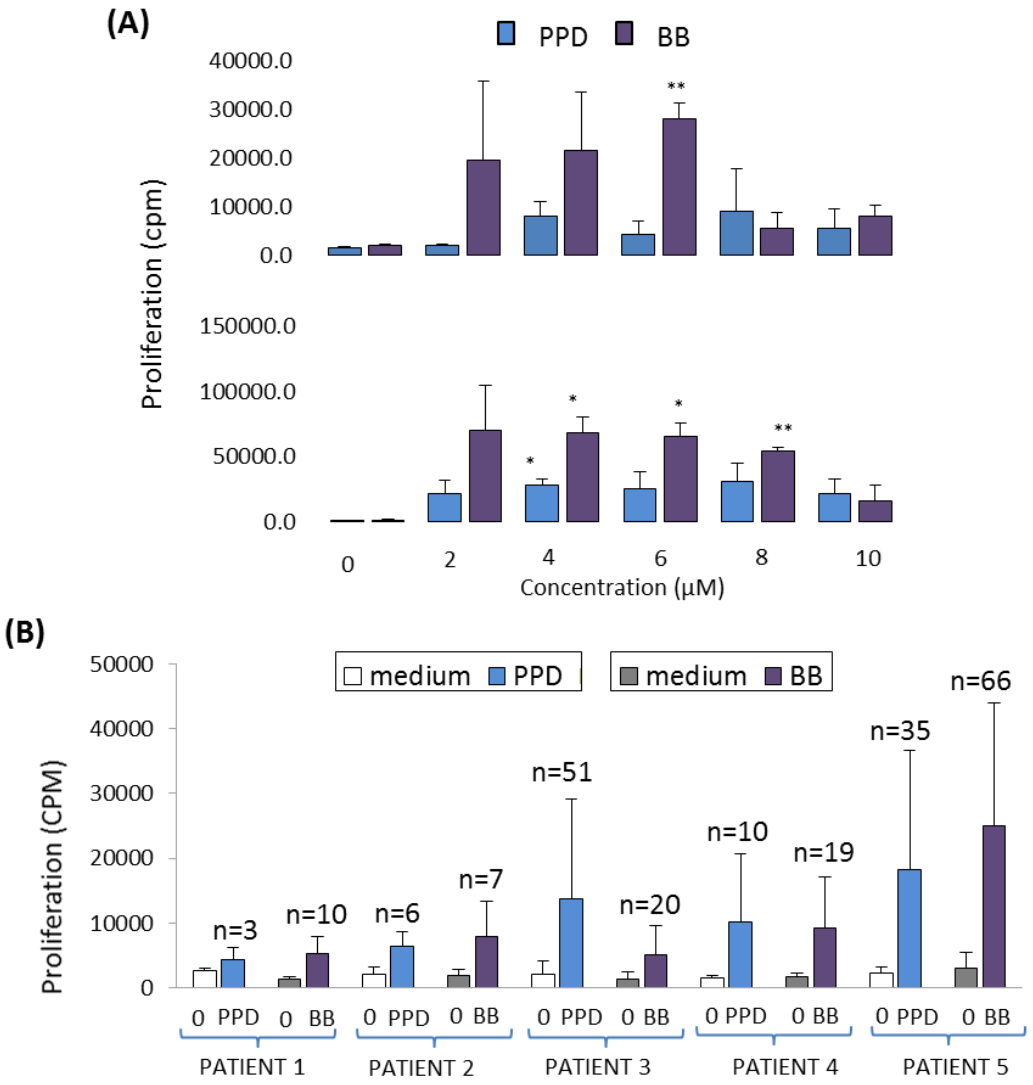


Figure 6.2. Activation of T-cells from PPD-allergic patients with PPD and BB. (A) Allergic patient PBMCs ($1.5x10^5$/well; total volume, 200 µl) were plated with PPD and BB (2−10 µM). Cultures were incubated for 5 days in an atmosphere of 95% air / 5% CO_2 / 37°C. Proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a final 16 hr incubation. Data from 2 representative patients are shown. Experiments were conducted in triplicate. Statistical significance denotes a significant increase in proliferative response compared to 'medium only' treated wells (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$). **(B)** Combined proliferative response of PPD- and BB-responsive clones from each of five patients with ACD (n = number of clones generated). Individual clones ($5x10^4$/well; total volume, 200 µl; 96-well U-bottomed plate) were cultured with PPD or BB (5 µM) and autologous irradiated EBV-transformed B-cells ($1x10^4$/well) for 48 hrs in an atmosphere of 95% air / 5% CO_2 / 37°C. Proliferative responses were then measured as above. Experiments were conducted in duplicate. The average proliferative counts from all clones were combined. All data are presented as the mean ± SD.

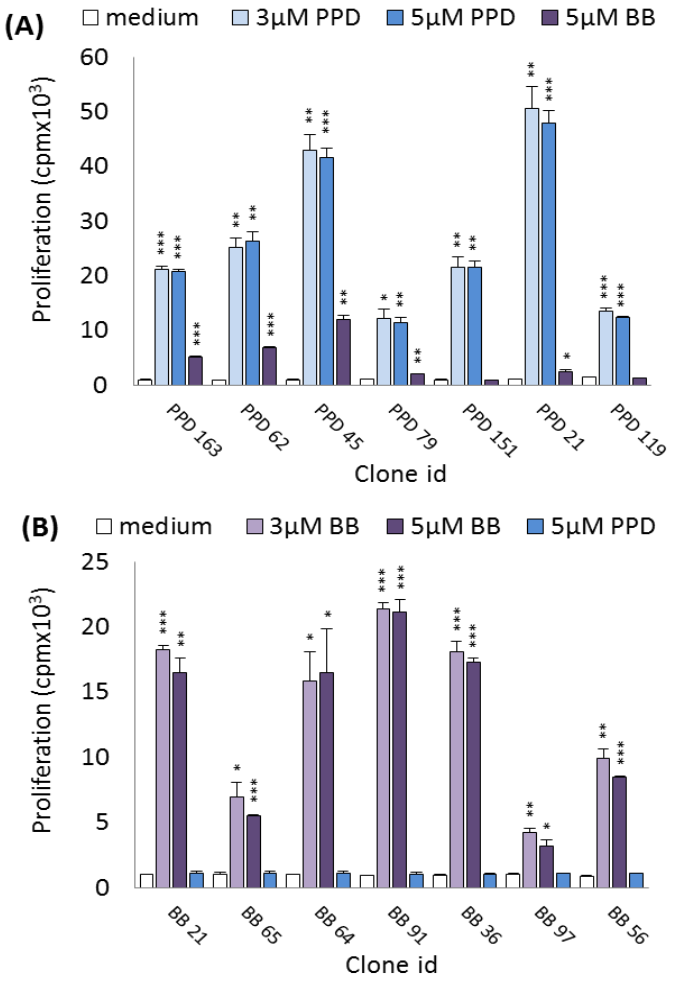


Figure 6.3. **Cross-reactivity of PPD and BB-responsive T-cell clones from PPD-allergic patients.** Activation of 7 representative **(A)** PPD- and **(B)** BB-responsive T-cell clones ($5x10^4$/well; total volume, 200 µl, 96-well U-bottomed plate) with PPD and BB (3−5 µM) using autologous irradiated EBV-transformed B-cells ($1x10^4$/well). After incubation in an atmosphere of 95% air / 5% CO_2 / 37°C for 48 hrs, proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a further 16 hrs. Data are presented as the mean ± SD; experiments were conducted in triplicate. Statistical significance denotes a significant increase in proliferative response compared to 'medium only' treated wells (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

6.3.2 PPD- and BB-responsive T-cell clones from PPD-allergic patients secrete a mixed profile of cytokines, including IL-22.

The profile of cytokines and cytolytic molecules secreted from PPD- (Figure 6.4a) and BB-responsive (Figure 6.4b) T-cell clones was analysed using granzyme B, IFN-γ, IL-13, IL-17 and IL-22 ELISpot. All patient T-cell clones, whether BB or PPD-responsive, were stimulated to

proliferate at the time of the cytokine analysis. $CD4^+$ and $CD8^+$ T-cell clones secreted a similar panel of cytokines, including IFN-γ, IL-13, and IL-22. Secretion of IL-17 was not detected. Antigen-driven secretion of the cytolytic molecule granzyme B was observed with certain PPD- and BB-responsive clones.

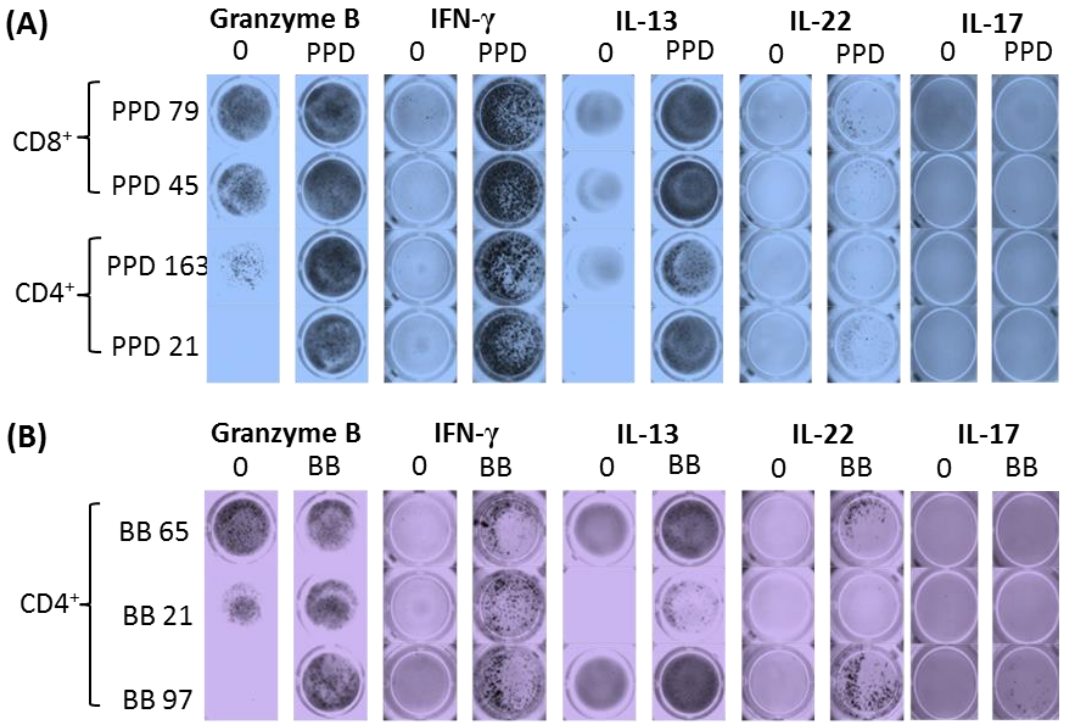


Figure 6.4. **Cytokine and cytolytic molecule secretion by PPD and BB-responsive T-cell clones from PPD-allergic patients.** In accordance with manufacturer's instructions, ELISpot plates were coated with granzyme B, IFN-γ, IL-13, IL-22, or IL-17 capture antibody and incubated at 4°C overnight. **(A)** PPD-responsive $CD4^+$ and $CD8^+$ T-cell clones or **(B)** BB-responsive $CD4^+$ T-cell clones ($5x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate) were cultured in antibody-exposed wells with PPD (5 µM) or BB (5 µM) and autologous irradiated EBV-transformed B-cells ($1x10^4$/well) in an atmosphere of 95% air / 5% CO_2 / 37°C for 48 hrs. The ELISpot plates were washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader.

6.3.3. MHC-blocking confirmed the presence of functional $CD4^+$ and $CD8^+$ PPD-responsive patient T-cell clones.

A panel of $CD4^+$ and $CD8^+$ PPD-specific T-cell clones were cultured with MHC class I or class II blocking antibodies alongside their respective isotype control antibodies. [^{3}H] thymidine

incorporation was then used to assess the proliferative response of these cells in response to antigen stimulation. As expected, PPD-specific $CD8^+$ T-cell responses were MHC class I restricted. Moreover, the response of $CD4^+$ PPD-specific T-cell clones were blocked in the presence of the anti-MHC class II antibody. One $CD4^+$ T-cell clone (PPD clone 163) displayed a significant reduction in proliferation in the presence of MHC class I and class II blocking antibodies (Figure 6.5).

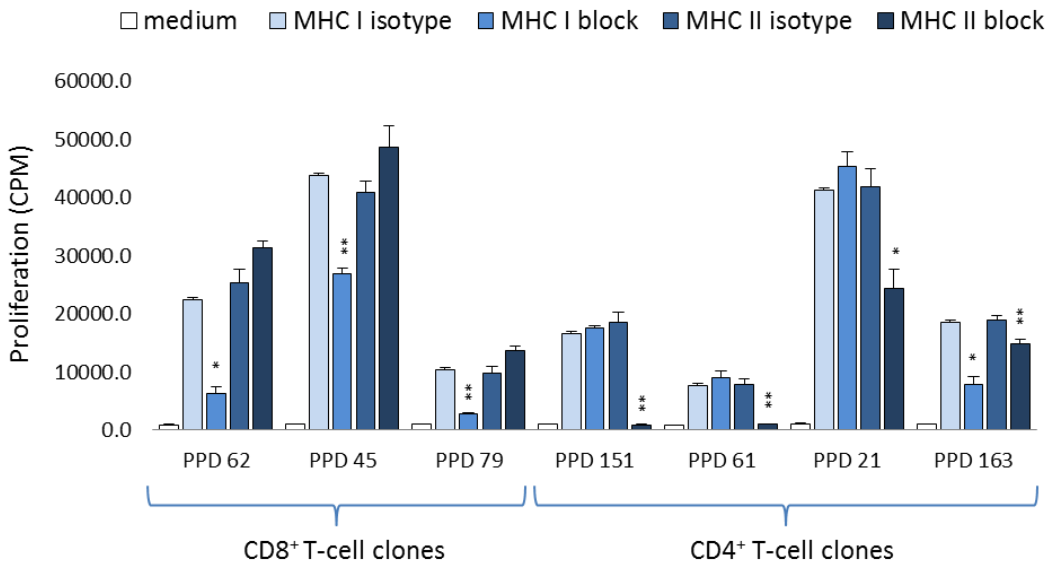


Figure 6.5. MHC-restriction of $CD4^+$ and $CD8^+$ PPD-specific T-cell clones from PPD-allergic patients. PPD-specific T-cell clones ($5x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate) were stimulated with PPD (5 µM) using autologous irradiated EBV-transformed B-cells ($1x10^4$/well) ± MHC class I and class II-blocking antibodies or their respective isotype controls (10 µg/ml). After culture for 48 hrs in an atmosphere of 95% air / 5% CO_2 / 37°C, proliferative responses were measured through the addition of [^{3}H]-thymidine (0.5 µCi/well) for a further 16 hr incubation. Data are presented as the mean ± SD; experiments were conducted in triplicate. Statistical significance denotes a significant reduction in proliferative response compared to respective isotype control treated wells (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

6.3.4. Memory and naïve-primed T-cells from healthy donors proliferate in the presence of BB, but not PPD.

Autologous mature DCs were combined with naïve or memory T-cells and PPD or BB for 1 week. [^{3}H] thymidine incorporation was initially used to assess the ability of these cells to

respond to PPD or BB. Upon restimulation of BB-treated memory T-cells, a clear proliferative response was seen in the presence of BB with cells from 7/7 donors (2.5 μM, $p \leq 0.05$) (Figure 6.6a, four representative donors shown). Proliferative responses of memory T-cells to PPD (2.5-5.0 μM) were not detected in any donor. Priming of naïve T-cells against BB was also observed using cells from all seven healthy donors studied. Following BB stimulation of the BB-primed T-cells, proliferative responses were readily detectable (2.5 μM, $p \leq 0.05$) (Figure 6.6b, four representative donors shown).

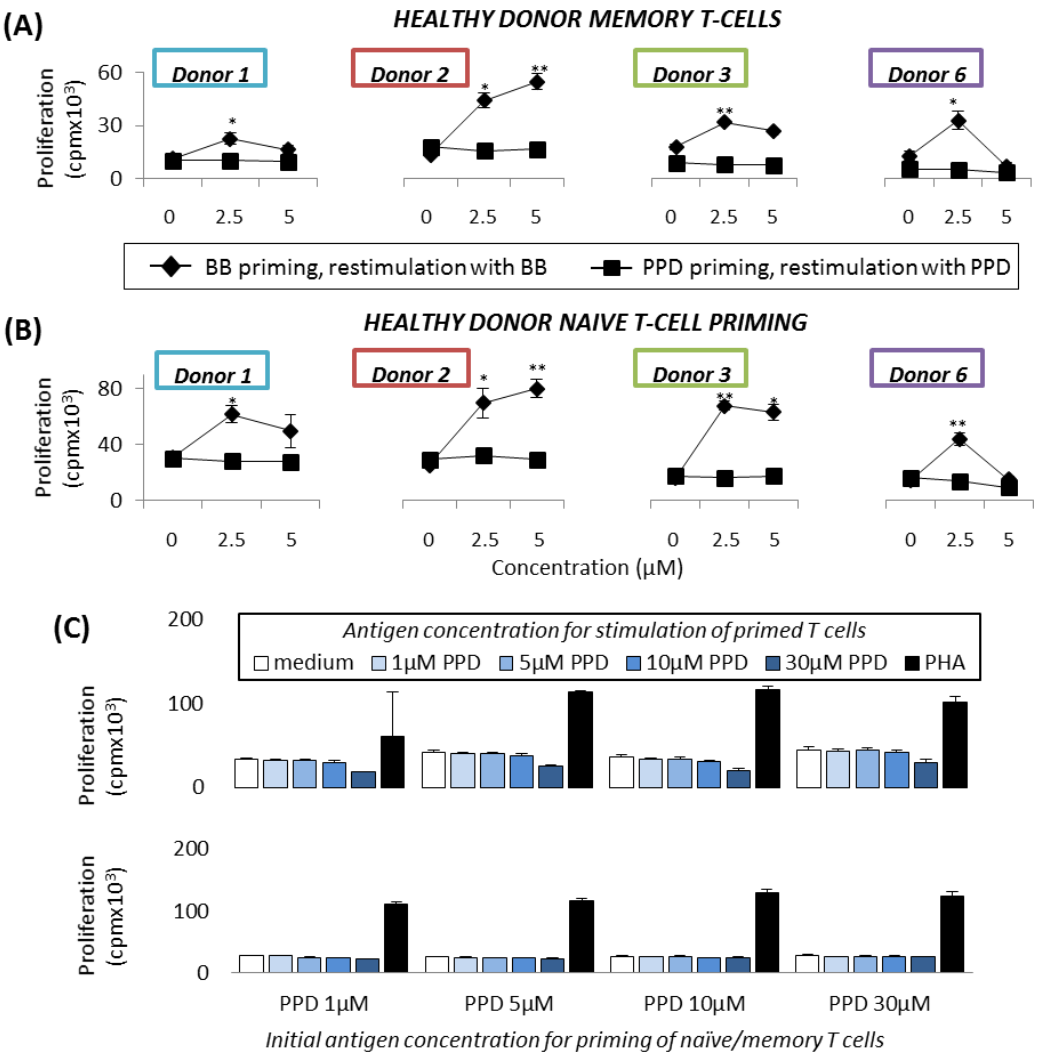


Figure 6.6. Antigen-specific activation of BB-, but not PPD-, primed naive and memory T-cells from healthy donors. (A) Memory or **(B)** naive T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of PPD or BB (5 µM) for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. T-cells cultured with PPD and BB were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate) with fresh monocyte-derived DCs ($4x10^3$/well) and PPD or BB (5 µM), respectively. Cultures were kept in similar conditions as previously for 48 hrs, prior to the measurement of proliferative responses through the addition of [^{3}H]-thymidine (0.5 µCi/well) for the final 16 hr of the experiment. **(C)** Naive T-cells were primed as before with PPD at 1−30µM for 7 days. Cells were then restimulated using 1-30 µM PPD or PHA (20 µg/ml) as a positive control. Cultures were incubated for 48 hrs and proliferation measured as above. All data show the mean ± SD of triplicate cultures. Statistical significance denotes a significant increase in proliferative response compared to 'medium only' treated wells (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$).

Concentrations of PPD that activated PBMCs and T-cells from patients with ACD did not prime naïve or memory T-cells from healthy donors (n=7) (Figures 6.6a and 6.6b). To investigate whether the failure to activate naïve T-cells with PPD related to differences in

sensitivity of cells from allergic patients and healthy donors, the priming experiments were repeated using an expanded range of PPD concentrations. Lower (1 μM) and higher concentrations (10-30 μM) of PPD failed to prime healthy donor naïve T-cells (Figure 6.6c). To show that antigen-specific responses did not fail due to the lack of functional T-cells, PHA was used to non-specifically stimulate T-cells (PHA, 20 μg/ml; $p \leq 0.05$).

6.3.5. Memory and naïve-primed healthy donor T-cells secrete a mixed panel of cytokines in the presence of BB, but not PPD.

Cytokine secretion was used as an alternative assessment of T-cell activation after naïve T-cell priming or exposure of memory T-cells to PPD or BB. This provided a similar response profile to that observed when using proliferation as a measure of T-cell activation (Figure 6.7, four representative donors), with no detectable PPD-specific response in any donor, but a BB response from all donors (naïve BB primed: unstimulated 69.7 ± 65.7 sfu, BB stimulated 153.1 ± 70.8 sfu, $p < 0.005$; naïve PPD primed: unstimulated 87.7 ± 94.3 sfu, PPD stimulated 89.9 ± 94.7 sfu, not significantly different; memory BB primed: unstimulated 78.4 ± 62.7 sfu, BB stimulated 156.8 ± 72.3 sfu, $p < 0.05$; memory PPD primed: unstimulated 138.4 ± 102.1 sfu, PPD stimulated 135.1 ± 109.0 sfu, not significantly different). The range of cytokines able to be analysed fluctuated from donor to donor due to the availability of cells. For two donors we were able to look at a mixed panel of cytokines using ELISpot (donor 6, IFN-γ & IL-13; donor 7, IFN-γ, IL-13 & IL-22). BB-primed T-cells from naïve and memory compartments secreted all three cytokines in response to BB, but not PPD (Figure 6.8).

6.3.6. BB-responsive T-cell clones from healthy donors show no reactivity to PPD.

In initial experiments, naïve and memory T-cells from healthy donors were cultured with autologous mature DCs and BB (5 μM) for 1 week. The primed T-cells were then tested for reactivity against PPD and BB. The BB-responsive T-cells deriving from naïve and memory compartments were not activated with PPD from 7/7 donors (Figures 6.9a and 6.9b, respectively). To further analyse the cross-reactivity of PPD and BB, BB-responsive T-cell clones were generated subsequent to priming and tested for reactivity with PPD. Following expansion, clones from naïve and memory compartments were seen to proliferate, using [^{3}H] thymidine incorporation, in the presence of BB, but not PPD (Figure 6.9c). These data emulate the lack of cross reactivity observed with BB-responsive clones isolated from patients with ACD (Figure 6.9d).

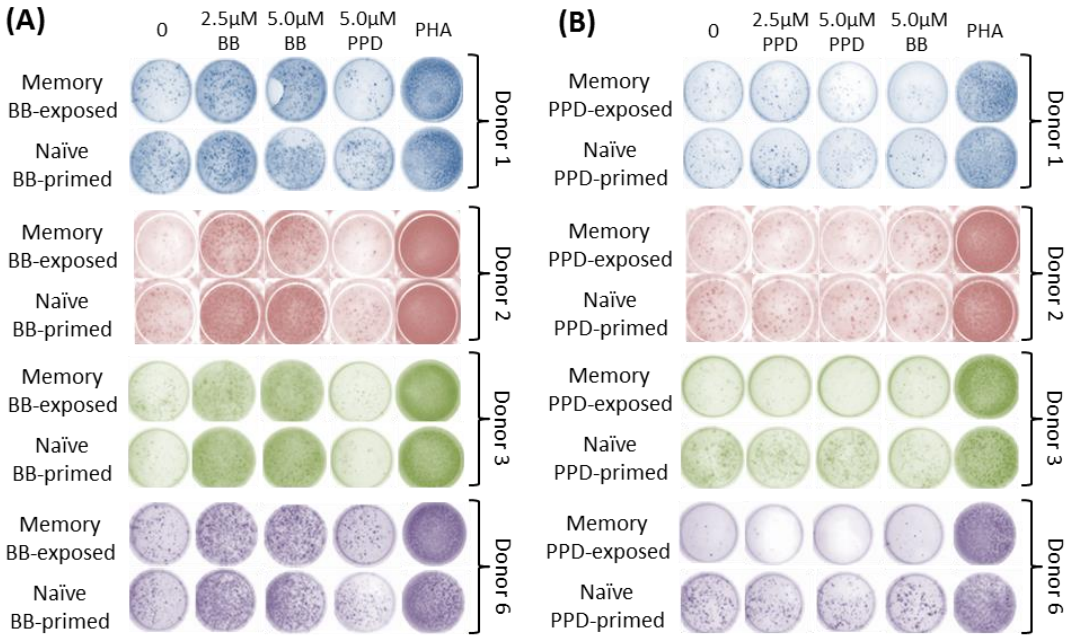


Figure 6.7. Antigen-specific IL-13 secretion from healthy donor BB-, but not PPD-, primed naive and memory T-cells. Naïve or memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of **(A)** BB or **(B)** PPD (5 µM) for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. In accordance with manufacturer's instructions, ELISpot plates were coated with IL-13 capture antibody and incubated at 4°C overnight. T-cells cultured with PPD and BB were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate) with fresh monocyte-derived DCs ($4x10^3$/well) and PPD or BB (5 µM) in antibody-exposed wells. The cells were incubated for 48 hrs in a similar atmosphere as before. The ELISpot plates were then washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader.

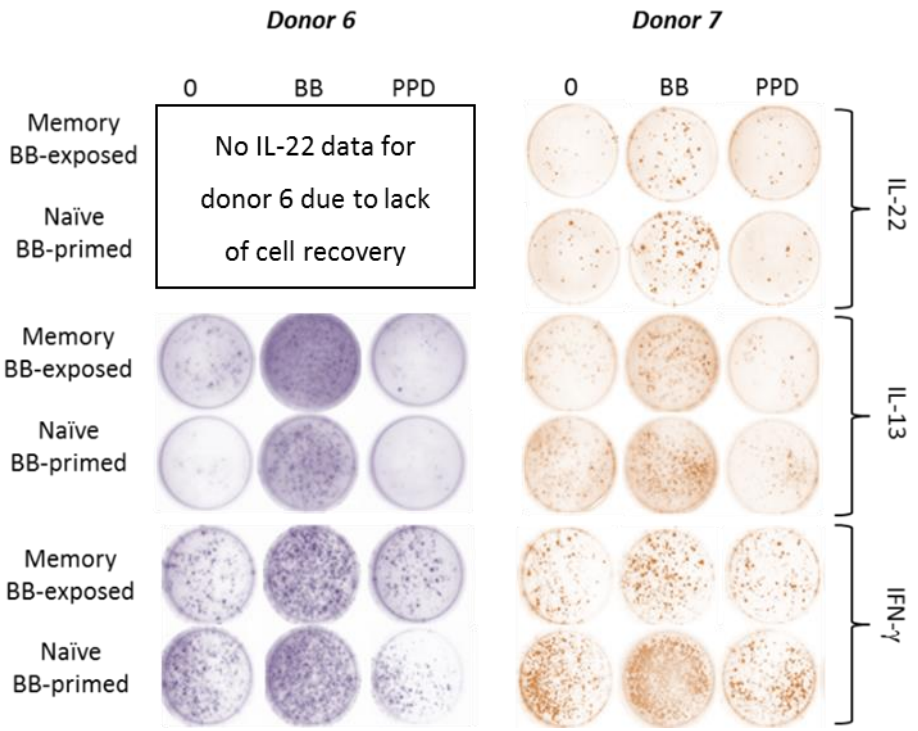


Figure 6.8. BB-induced secretion of IL-22, IL-13, and IFN-γ secretion of naïve and memory T-cells from 2 healthy donors. Naïve or memory T-cells ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) were cultured with mature autologous monocyte-derived DCs ($8x10^4$/well) in the presence of BB (5 µM) for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. In accordance with manufacturer's instructions, ELISpot plates were coated with IFN-γ, IL-13, or IL-22 capture antibody and incubated at 4°C overnight. T-cells cultured with PPD and BB were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate) with fresh monocyte-derived DCs ($4x10^3$/well) and PPD or BB (5 µM) in antibody-exposed wells. The cells were incubated for 48 hrs in a similar atmosphere as before. The ELISpot plates were then washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELISpot reader.

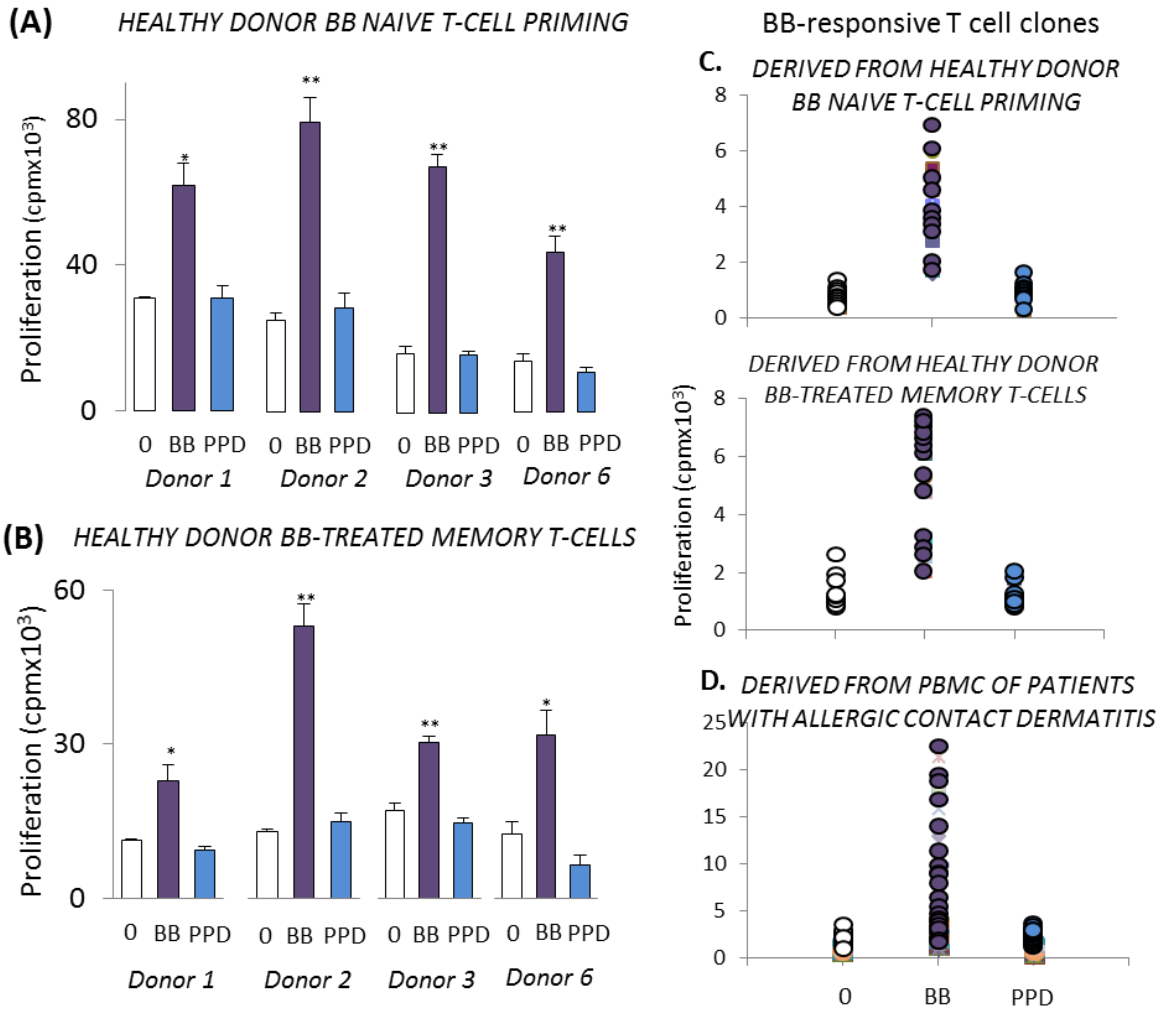


Figure 6.9. Cross-reactivity of healthy donor-derived BB-responsive T-cell lines and clones. (A) Naive or **(B)** memory T-cells from 4 donors ($2.5x10^6$/well; total volume, 2 ml; flat-bottomed 24-well plates) were cultured with autologous mature monocyte-derived DCs ($8x10^4$/well) in the presence of BB (5 µM) for 7 days in an atmosphere of 95% air / 5% CO_2 / 37°C. T-cells were harvested and re-plated ($1x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate) with fresh monocyte-derived DCs ($4x10^3$/well) and PPD or BB (5 µM). Cultures were kept in similar conditions as previously for 48 hrs, prior to the measurement of proliferative responses through the addition of [^{3}H]-thymidine (0.5 µCi/well) for the final 16 hr of the experiment. Data show the mean ± SD of triplicate cultures (*$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$). **(C)** Cross reactivity of BB-responsive T-cell clones derived from healthy donor naive and memory T-cells cultures with BB, as described above. T-cell clones ($5x10^5$/well; total volume, 200 µl; 96-well U-bottomed plate) were stimulated with PPD or BB (5 µM) using autologous irradiated EBV-transformed B-cells ($1x10^4$/well). After culture in an atmosphere of 95% air / 5% CO_2 / 37°C for 48 hrs, proliferative responses were measured as above. **(D)** Cross-reactivity of BB-responsive T-cell clones derived from allergic patients, similar methods used to those above.

6.3.7. HLA-typing of both patient and healthy donor cells failed to identify any potential HLA-risk allele associable to PPD-induced ACD.

Predisposition to certain drug hypersensitivity reactions is now associated with specific HLA alleles, however, a similar association for PPD-induced ACD has not been assessed. To ascertain whether patient PPD-specific responses were HLA allele associated, cells from each patient were HLA-typed for HLA-A, HLA-B, HLA-C, HLA-DR, and HLA-DQ (Table 6.1). Cells from healthy donors that responded to BB, but not PPD, were also HLA-typed for comparison to patient genotypes in the event of identifying a potential allele association (Table 6.2). Although a handful of similar alleles appear in 2-3 patients, no allele was found to be present in all patients.

Table 6.1. Full HLA-type of healthy donors. Healthy donor DNA was isolated from blood samples using the magnetic separation module-1 (Chemagen). DNA samples were then shipped to, and HLA-typed by Histogenics (Ossining, NY, USA). All donors displayed both naïve and memory T-cell responses to BB, but not PPD

	MHC class I						MHC class II					
	HLA-A		HLA-B		HLA-C		HLA-DRB		HLA-DQB		HLA-DQA	
	A1	A2	B1	B2	C1	C2	DRB11	DRB12	DQB11	DQB12	DQA11	DQA12
Donor 1	26:01:01G	31:01:02G	40:06:01G	51:02:01	03:04:01G	15:02:01G	09:01:02G	11:01:01G	03:01:01G	03:03:02G	03:01:01G	05:01:01G
Donor 2	02:01:01G	68:02:01G	15:10:01	18:01:01G	03:04:02	05:01:01G	01:01:01	03:01:01G	02:01:01G	05:01:01G	01:01:01G	05:01:01G
Donor 3	01:01:01G	02:01:01G	08:01:01G	57:01:01G	06:02:01G	07:01:01G	03:01:01G	03:01:01G	02:01:01G	02:01:01G	05:01:01G	05:01:01G
Donor 4	02:01:01G	02:01:01G	44:02:01G	44:02:01G	05:01:01G	05:01:01G	04:04:01	07:01:01G	02:01:01G	03:02:01G	02:01	03:01:01G
Donor 5	26:01:01G	31:01:02G	14:01:01	44:02:01G	05:01:01G	08:02:01G	13:06	07:01:01G	02:01:01G	06:03:01G	01:03:01G	02:01
Donor 6	02:01:01G	23:01:01G	44:02:01G	50:01:01	05:01:01G	06:02:01G	03:01:01G	04:01:01	02:01:01G	03:01:01G	03:01:01G	05:01:01G
Donor 7	02:11:01	11:01:01	07:05:01G	40:06:01G	07:02:01G	15:02:01G	11:01:01	15:01:01G	03:01:01G	06:01:01G	01:03:01G	05:01:01G
Donor 8	03:01:01G	03:01:01G	07:02:01G	52:01:01G	07:02:01G	12:02:02	03:01:01G	15:01:01G	02:01:01G	06:02:01G	01:02:01G	05:01:01G

Table 6.2. Full HLA-type of PPD-allergic patients. PPD-allergic patient DNA was isolated from blood samples using the magnetic separation module-1 (Chemagen). DNA samples were then shipped to, and HLA-typed by Histogenics (Ossining, NY, USA).

	MHC class I						MHC class II					
	HLA-A		HLA-B		HLA-C		HLA-DRB		HLA-DQB		HLA-DQA	
	A1	A2	B1	B2	C1	C2	DRB11	DRB12	DQB11	DQB12	DQA11	DQA12
Patient 1	02:01:01G	11:01:01	27:04:01	35:01:01G	03:03:01G	12:02:02	11:01:01	15:01:01G	03:01:01G	06:02:01G	01:02:01G	05:01:01G
Patient 2	03:01:01G	03:01:01G	35:01:01G	44:02:01G	04:01:01G	05:01:01G	01:01:01G	07:01:01G	02:01:01G	05:01:01G	01:01:01G	02:01
Patient 3	02:01:01G	02:01:01G	08:01:01G	39:01:01G	07:01:01G	12:03:01G	03:01:01G	11:01:01	02:01:01G	03:01:01G	05:01:01G	05:01:01G
Patient 4	02:01:01G	02:01:01G	07:02:01G	37:01:01	06:02:01G	07:02:01G	04:04:01	10:01:01	03:02:01G	05:01:01G	01:01:01G	03:01:01G
Patient 5	11:01:01G	32:01:01G	13:02:01G	51:01:01G	06:02:01G	14:02:01	11:04:01	13:01:01	03:01:01G	06:03:01G	01:03:01G	05:01:01G

6.4 Discussion.

Since the late 19th century, PPD-induced ACD has been reported in those using hair colouring products (Jacob and Goldenberg, 2014). PPD and the oxidation product BB are known to stimulate T-cells from patients with ACD (Coulter *et al.*, 2007a), whist T-cells from healthy

donors are activated selectively with BB. These data, combined with the observation that less than one fifth of PPD patch test positive patients respond to BB (White *et al.*, 2006c), imply that PPD-derived antigens prime T-cells in susceptible patients and that these T-cells drive ACD. Investigations to determine the underlying mechanisms in animal models have been unable to detect a PPD response. This is epitomised by a study where mice were vaccinated sub-cutaneously with BB, and subsequent BB-exposure was found to stimulate T-cells. However, PPD was unable to induce a similar response in PPD-vaccinated animals, nor did these mice respond to PPD-protein adducts (Farrell *et al.*, 2009). This highlights fundamentally different responses to PPD between humans and other animals. Therefore, we have utilised an *in vitro* human T-cell assay to assess the origins and functionality of PPD- and/or BB-responsive T-cells in healthy donors, and validate our findings by way of comparison to PPD-derived antigen-specific T-cells from allergic patients.

To confirm the patch test diagnosis of PPD-mediated ACD, PBMC responses in the presence of PPD and BB were assessed using the LTT. Both PPD and BB were able to stimulate patient PBMCs to proliferate, with BB proving to elicit substantially higher responses than PPD. This reflects previous findings where PPD and BB have been shown to activate T-cells from allergic patients, and that BB is considered to be a stronger sensitizer than PPD (Coulter *et al.*, 2007a; Sieben *et al.*, 2002; White *et al.*, 2006c). To analyse these responding T-cells in more depth, T-cell clones were generated from PBMCs cultured with PPD and BB. PPD- and BB-responsive T-cell clones were generated from all allergic patients. Upon phenotyping these clones, it was found that the vast majority (22/24) of BB-responsive clones were $CD4^+$, as were most PPD-specific clones. Antigen-specific activation of these $CD4^+$ T-cell clones was subsequently found to be MHC class-II restricted. However, 15/40 PPD-specific clones expressed the CD8 co-receptor. As expected in these cells, PPD-induced activation was significantly reduced by the inclusion of an MHC class I-blocking antibody. Together these data suggest that PPD may be the central driving force behind the production of effector T-cells at the site of the allergic

reaction. Although predisposition to a number of drug-induced hypersensitivity reactions is now associated with specific HLA alleles, a similar association for PPD-induced ACD has not been assessed. Therefore we characterised the full genotype of each PPD-allergic patient. The presence of a specific allele in all patients would indicate a strong genetic association and warrant further investigation. No HLA allele was present in all patients, and although a number of alleles were identified in multiple patients, these alleles could also be identified in healthy PPD-tolerant donors. Whilst we concede that this data is limited by a small number of patients (n=5), it appears unlikely that the expression of a single HLA-allele represents a major predisposing factor for PPD-induced ACD.

Whilst BB-responsive T-cell clones from patients displayed a general lack of cross reactivity with PPD, a number of PPD-responsive clones demonstrated weakly positive responses to BB. Despite these differences, both PPD- and BB-responsive T-cell clones were found to secrete a similar panel of cytokines including IFN-γ, IL-13, and the cytolytic molecule granzyme B, with no clear distinction observable between $CD4^+$ and $CD8^+$ clones. Apart from one BB-responsive clone, none of the clones secreted IL-17. Interestingly, clones were also found to secrete IL-22 which is widely associated with enhanced skin inflammation (Cho *et al.*, 2012). Although the secretion of IL-22 is associated with both Th17 and Th22 cells, the release of IL-17 is only found from Th17 cells. Thus these data indicate the presence of a Th22-mediated response rather than that of Th17 during allergic responses to PPD-containing hair dyes. This links in with current knowledge that IL-22 is an important tissue-specific cytokine that is thought to be important in the generation of skin related immunological reactions. Indeed, increased circulating IL-22 has been identified in patients with psoriasis, and in the lesional skin of patients with ACD where it is enriched in comparison to the peripheral blood (Akdis *et al.*, 2012; Cavani *et al.*, 2012; Eyerich *et al.*, 2010; Fujita, 2013).

The second part of this investigation focussed on the priming of healthy donor naïve and memory T-cells to PPD and BB, and aimed to establish the optimal *in vitro* exposure conditions for the antigen-MHC-TCR interaction. Two batches of autologous DCs were used to mimic the sensitisation and elicitation phases of ACD, and proliferative responses and cytokine secretion were measured following antigen stimulation to provide a robust readout system. IFN-γ, IL-13, and IL-22 were used as the primary cytokine readouts as they are each secreted by antigen-specific T-cells from patients with ACD (see above).

Enhanced proliferative responses and cytokine secretion were detected following secondary stimulation of BB-exposed memory T-cells with BB. In contrast, PPD-responsive memory T-cells were not detected. These data are in agreement with the work of Coulter *et al* (Coulter *et al.*, 2007b) outlined previously. Unlike our work in chapter 3 where the identification of memory antigen-specific T-cells indicated heterologous immunity, these responses were not detected in those who had not previously encountered PPD-containing products, but in PPD-tolerant healthy donors. Therefore the presence of BB-responsive memory T-cells from all donors was somewhat expected due to likelihood of widespread sensitisation to PPD amongst the general population (Coulter *et al.*, 2007b). We also studied the activation of T-cells from the naive compartment. Similar to the memory response, PPD-induced naïve T-cell activation was not detected. For all donors, BB primed naive T-cells proliferated and secreted cytokines following BB re-stimulation. In fact, the BB-specific proliferative response following re-stimulation was greater in naive-primed cells, when experiments using naive and memory cells were compared. BB-responsive T-cell clones were generated from both the naive and memory compartments to explore cross reactivity. As expected, T-cell clones were found to proliferate in the presence of BB, but not PPD, in concurrence with the lack of cross reactivity of BB-specific T-cell clones from patients with ACD. Collectively, these data show that (1) BB-responsive memory T-cells circulate in most healthy donors, and (2) that BB is a potent activator of naive T-cells.

In summary, the observation that memory T-cells are not activated in the presence of PPD confirms previous negative LTT data and indicates that it is possible to fundamentally distinguish between allergic and tolerant individuals based on the response of memory T-cells to PPD. Meanwhile, PPD-specific activation of naive T-cells was not detected. Experiments were conducted on 7 healthy donors using 5 µM PPD under conditions that allow for PPD oxidation and the irreversible modification of protein with PPD haptens (Cho *et al.*, 2012; Coulter *et al.*, 2007b; Jenkinson *et al.*, 2009a; Jenkinson *et al.*, 2009b); thus, it is likely that priming of naive T-cells to PPD is detected in fewer than 20% of the human population. It is interesting to speculate why responses to PPD were not detected. First, it is possible that T-cells are activated in patients with ACD in the presence of specific environmental factors that are not present in the *in vitro* experiments. This seems unlikely however since the assay has been developed to provide relevant co-stimulatory signalling; regulatory T-cells are removed and DCs are matured to up regulate cell surface receptors and bypass the requirement for PPD-specific DC signalling. Second, alternative (higher or lower) PPD concentrations might be required for the optimal priming of naive T-cells. To address this possibility, experiments were conducted using a range of PPD concentrations up to the maximum tolerated in the cell assay without causing overt toxicity. Activation of naive T-cells was not observed with high or low concentrations of PPD. Third, it is possible that PPD-derived antigenic determinants are preferentially presented to T-cells in the context of specific HLA alleles as has been demonstrated in a range of drug hypersensitivity reactions (Pavlos *et al.*, 2012). This seems feasible as early experiments exploring the quantitative relationship between applied doses of PPD to skin and the induction of sensitisation indicate a range of sensitivities (see *(Goebel et al., 2012)* for a review of the data). To assess the potential role of HLA-alleles in the predisposition of individuals to PPD-induced ACD, we investigated the HLA-type of cells from both the allergic patients and healthy donors used in our study. We were unable to identify a dominant HLA-allele based on the lack of one allele

shared by all five patients. Our small study number means that we were unable to assess weaker HLA associations despite the presence of certain alleles in 2-3 out of the five patients. Therefore, while it appears unlikely that the presence of a single HLA-allele is ultimately responsible, without confirmation by a larger-scale study, it remains a distinct possibility that a small number of HLA-alleles play a less dominant role in the onset of these reactions.

Despite extensive research, a great deal of controversy and confusion still surrounds the mechanistic basis of hair dye allergy. It remains unclear whether (1) the initial priming to PPD or BB is the basis for ACD, and (2) what factors predispose certain individuals to become sensitised. This is partly because of a distinct lack of sensitive *in vitro* assays to investigate the mechanistic basis of ACD. Herein, we have utilised an *in vitro* DC priming assay to generate T-cells that mirror the *in vivo* phenotype. We were able to prime naive T-cells from human donors to BB but not PPD, which supports previous findings that BB, but not PPD, responses are detectable in tolerant individuals, while responses to PPD are only observed in patients with ACD. PPD-responsive T-cells from patients were $CD8^+$ and $CD4^+$, which were MHC class I- and II-restricted, respectively, and secreted a range of Th1/Th2 cytokines. Moreover, we identified the secretion of IL-22, but not IL-17, from PPD-activated patient T-cell clones indicating a role for Th22 T-cells in PPD-induced ACD. It is now critical that future research is directed towards characterising the factors which promote PPD-specific T-cell responses in patients with ACD.

Chapter 7: General Discussion.

The term adverse drug reactions (ADRs) refers to a wide array of unfavourable effects, ranging from minor coughs or rashes, to those with life-threatening consequences. Adverse reactions are classified into two separate classifications; type A and type B. While type A are predictable, related to the known pharmacological actions of the drug, and account for 8/10 of ADR-related hospital admissions, type B reactions are much rarer and are responsible for the majority of ADR-induced deaths due to their traditionally idiosyncratic nature (Routledge *et al.*, 2004). Combined with the detrimental health effects on patients, these reactions also place a huge financial burden on healthcare services, with ADR-related in-patients accounting for 1.6 million bed days per year in England alone (Davies *et al.*, 2009). Additionally, the financial implications for pharmaceutical companies are staggering on consideration that ADRs continue to be the leading reason for lead-compound attrition during the drug-development process. As the development of a new compound is estimated to cost $868 million, but may be as high as $2 billion, and take the best part of 10 years, the withdrawal of a drug from market represents a real threat to the survival of any company (Adams and Brantner, 2006).

Hypersensitivity reactions are classified as type B ADRs, and are known to be immune-mediated as antigen-specific T-cells can be identified from not only the peripheral blood of patients, but also from inflamed skin at the site of reaction and blister fluid (Beeler *et al.*, 2006; Naisbitt *et al.*, 2001; Nassif *et al.*, 2002). While extensive research has enhanced our understanding of the immunological basis of these reactions, including the mechanisms by which a T-cell can be stimulated by a drug or chemical (hapten, prohapten, PI concept, altered peptide concept (Illing *et al.*, 2012; Landsteiner and Jacobs, 1935; Norcross *et al.*, 2012; Pichler *et al.*, 2011)), much remains undefined. Indeed the field still requires a better

understanding of (a) the factors responsible for predisposing certain individuals to hypersensitivity, (b) the antigenic origins of the subsequent primary T-cell response, and (c) the role of the newly identified Th-cell subsets in disease aetiology; all of which have been subject to investigation within this thesis.

Due to a lack of suitably representative animal models, recent experimental emphasis has been placed on the use of *in vitro* human-based assays. Traditionally, *in vitro* human cell studies have relied on the culture of antigen-specific T-cells isolated from hypersensitive patients. Whilst this can be helpful in establishing the exact antigen responsible for immune stimulation (Porebski *et al.*, 2011), the use of patient samples is limited to the analysis of memory T-cell responses, and we gain no insight into the development of a primary T-cell response to these antigens. Therefore in the last half-decade, much effort has focussed on the development of *in vitro* assays to examine the priming of naïve T-cells.

Early investigations in the 1970's were adapted from work detailing the *in vitro* sensitisation of primary T-cells from Guinea pigs (Thomas and Shevach, 1976) and transferred to human T-cells to investigate a similar response to trinitrophenol (TNP), where antigen presentation was performed by PBMCs (Newman *et al.*, 1977; Seldin and Rich, 1978). Since then, a number of adaptations have been employed to optimise this assay. Firstly, improved separation techniques have allowed for the isolation of distinct cell subsets from peripheral blood based on the differential expression of cell surface markers, including CD45RA and CD45RO for naïve and memory T-cells, respectively. Secondly, protocols have been developed for the generation of functionally efficient DCs, capable of processing and presenting antigens, from $CD14^{+}$ monocytes through a short culture with GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). Utilising these techniques, the activation of primary, naive T-cell responses was studied with regards to chemical sensitisation using hapten-modified DCs. Indeed, a number of contact allergens including TNCB were among the first chemicals shown to prime and

activate naïve T-cells *in vitro* (Dietz *et al.*, 2010). However, these responses were poorly reproducible and the strength of T-cell activation was low. Thus thirdly, upon removal of the $CD25^{+}$ cell population, which contains naturally occurring T-regulatory cells, Vocanson and colleagues were able to report an enhanced sensitisation potential where they could detect responses to weakly allergenic compounds which were not previously capable of producing a detectable response. Importantly, similar results were not detected for compounds not associated with sensitisation, including DMSO (Vocanson *et al.*, 2008). Finally, and most recently, the conditions for *in vitro* priming have been further optimised by our group, including the determination of the optimal T-cell:DC ratio, T-cell density, and length of culture (Faulkner *et al.*, 2012). The current assay also manages to evade the necessity of danger signalling *in vitro*, which is required *in vivo* for the full activation of DCs and the consequent up-regulation of co-stimulatory molecules needed to overcome the threshold for T-cell activation (Gallucci and Matzinger, 2001). Using LPS and TNF-α we are able to mimic such signals *in vitro* and are thus able to produce fully matured DCs. Therefore, we are now at a stage where we can explore primary and secondary T-cell responses alongside one another to gain a more comprehensive view of hypersensitivity reactions.

Previously, genetic diversity in signal 1, specifically among HLA alleles, has been associated with drug hypersensitivity. Despite this, and aside from the associations of ABC with HLA-B*57:01, and CBZ with HLA-B*15:02 (Chung *et al.*, 2004; Mallal *et al.*, 2002; Martin *et al.*, 2012), the majority of individuals who carry known HLA risk alleles do not develop clinically relevant immunological reactions when exposed to a culprit drug. Similarly, genetic association studies have widely discredited a major role for genetic variations in drug metabolising enzymes for predisposition to hypersensitivity (O'Neil *et al.*, 2002; Pirmohamed *et al.*, 2000). However, while the formation of signal 1 is required for T-cell signalling, it is signal 2 that determines whether this translates into an inflammatory or toleragenic response. Therefore this represents a distinct, but equally critical component of T-cell

activation which functions through a wide array of both co-stimulatory and co-inhibitory receptors that act to decrease and increase T-cell activation thresholds, respectively. Polymorphisms in several co-inhibitory pathways are known to be associated with the dysregulation of T-cell activation and the subsequent onset of T-cell-induced autoimmune diseases (Grattan *et al.*, 2002; Koguchi *et al.*, 2006; Nishimura *et al.*, 1999; Niwa *et al.*, 2009). Thus, it is distinctly possible that varied expression and/or function of these pathways between individuals could predispose an individual to drug-induced hypersensitivity by tipping the balance in favour of immune activation. However, how these receptors affect drug-antigen specific T-cell activation remains unexplored. To address this, we utilised an *in vitro* human T-cell assay to (a) inhibit receptor pathways and thus effectively 'peel away' and reveal the influence of individual layers of regulation, and (b) use flow cytometry to assess the kinetics of co-inhibitory receptor expression during the drug antigen-specific activation of T-cells.

Two of the most defined co-inhibitory pathways are PD-1 and CTLA4. They are considered to be critical checkpoints in T-cell regulation as their individual knockdown leads to overwhelming lymphoproliferation in mice, ultimately resulting in death (Nishimura *et al.*, 2001; Tivol *et al.*, 1995; Waterhouse *et al.*, 1995). CTLA4 has two ligands, CD80 and CD86, which it shares with the co-stimulatory receptor CD28 and thus these opposing pathways act to competitively inhibit one another (Teft *et al.*, 2006). PD-1 also has two well-defined ligands, PD-L1 and PD-L2, which upon interaction with PD-1 interfere with T-cell responses upstream to that of CTLA4 (Yokosuka *et al.*, 2012). This more membrane proximal interference is the proposed reason why the activation of PD-1 regulates a far greater number of CD3-induced transcripts than CTLA4 (Parry *et al.*, 2005). While there is a wide range of other co-inhibitory pathways, we were also particularly interested to assess the role of the lesser known receptor TIM-3, which mediates its function through binding to galectin-9 leading to the death of predominantly Th1-specific T-cells. Our interest in TIM-3 lies with

its apparent close expression pattern with PD-1. This was highlighted in recent cancer-model studies where all TIM-3$^+$ T-cells which were infiltrating the tumour co-expressed PD-1, and these PD-1$^+$ TIM-3$^+$ T-cells represented the majority of infiltrating T-cells thus inferring that together these pathways represent a formidable immunological barrier to T-cell activation (Sakuishi *et al.*, 2010). Importantly, we found the expression of all ligands associated with PD-1, CTLA4, and TIM-3 on the monocyte-derived DCs used in our investigations.

We first assessed the individual roles of PD-L1 and PD-L2 on naïve T-cell activation (chapter 3). To do this we used the model immunogen SMX-NO to stimulate T- cells partly due to its stability and defined chemistry, but also because our group have previously reported the successful priming of naïve T-cells from healthy donors to SMX-NO (Faulkner *et al.*, 2012). Blockade of PD-L1 significantly enhanced both the proliferative capacity and cytokine secretion of SMX-NO-primed naïve T-cells in response to restimulation with drug-antigen. Of note, an antigen-specific response was also detected upon blocking PD-L1 in a donor who did not initially respond to SMX-NO. Furthermore, we were also able to detect a drug-derived antigen-specific response after priming naïve T-cells from HLA-B*57:01 positive healthy donors to flucloxacillin, a response which was otherwise undetectable. These data highlight a substantial enhancement in assay sensitivity with the inclusion of PD-L1 blocking antibodies. In stark comparison, the inclusion of PD-L2 blocking antibodies did not alter the activation of T-cells, and when used in combination with PD-L1 block, effectively subdued the enhanced response seen by blocking PD-L1 alone. Our data indicate that PD-1 activation by PD-L1 is highly immune-inhibitory, whereas PD-L2 has the propensity to be co-stimulatory and may even act to regulate the inhibitory effect of PD-L1. In comparison, we found that PD-L1 blockade was not associated with a similar increased response using SMX-NO-exposed memory T-cells. The strong co-inhibitory role of PD-L1, but not PD-L2, lies in concurrence with that of antigen-specific responses in mice identified by Chikuma *et al* (Chikuma *et al.*, 2009). It may be that the enhanced co-inhibitory role of PD-L1 compared to PD-L2 may relate

to the observation that this ligand can also bind to CD80, thus preventing the co-stimulatory interaction between CD80 and CD28 (Butte *et al.*, 2007). Interestingly, our data opposes that of brown and colleagues where blocking PD-L1 did not alter T-cell responses, while blocking PD-L2 enhanced the proliferative response 3-fold (Brown *et al.*, 2003). However, this contrasting data likely relates to the use of immature DCs as conceded by the authors. Therefore while our study relates to the effect of PD-1 ligands during strong TCR-engagement, it is likely that during sub-optimal TCR signalling, the same rules may not apply.

As both CTLA4 ligands share costimulatory function, we blocked the CTLA4 receptor itself to focus on the effects of co-inhibitory signalling during the drug-derived antigen-induced activation of both naïve and memory T-cells (chapter 4). CTLA4 blockade during the priming of healthy donor naïve T-cells to SMX-NO enhanced subsequent T-cell responses, but less so than PD-L1 blockade indicating a prominent, yet inferior role for CTLA4 during drug antigen-specific naïve T-cell priming. This finding correlates with the higher level of T-cell transcriptional control exerted by PD-1 than CTLA4 by Parry *et al* (Parry *et al.*, 2005). However, the onset of more serious immune-mediated adverse effects after knockdown of CTLA4 in mice indicates that CTLA4 has a larger role in the control of immune-responses than PD-1. Similarly, more frequent and serious adverse effects have been reported with the use of anti-CTLA4 than anti-PD-L1 biologicals in human-based clinical trials. Specifically, the anti-CTLA4 therapy ipilimumab is associated with grade 3 or 4 immune-mediated adverse effects in 10-15% of patients, in comparison to 9% who received a similar biological blocking PD-L1 (Brahmer *et al.*, 2012; Hodi *et al.*, 2010). Interestingly, we identified that in contrast to blocking PD-L1, using CTLA4 blocking antibodies significantly enhanced the memory T-cell response to SMX-NO in a number of donors. Thus our data imply that the regulatory dominance of PD-1-PD-L1 over CTLA4 during naïve T-cell activation may switch when dealing with secondary T-cell responses. Moreover, this suggests that the enhanced severity of

adverse effects with anti-CTLA4 therapy may relate to the dysregulation of secondary T-cell responses.

It is important to bear in mind that overall, our data indicate a greater regulatory role for T-cell co-inhibitory pathways during primary, rather than secondary T-cell responses. Indeed, the enhanced memory T-cell response after blocking CTLA4 was detected in fewer donors than for primed naïve T-cell responses. This enhanced stringency on naïve T-cell activation is enforced to prevent the activation of T-cells unless a harmful antigen is encountered, in which case danger signals will be provided leading to ample co-stimulation able to overcome the high activation threshold set by multiple layers of co-inhibitory signalling. The requirement for additional signalling minimises the risk of a naïve T-cell response to non-harmful or self-antigen, a requirement which is unnecessary for memory T-cells due to their pre-defined antigen-specificity (Dubey *et al.*, 1996).

TIM-3 is relatively well-characterised in mice, however its comparative role in humans is less defined. While it has previously been shown that TIM-3 activation on murine cells leads to cell death, no reduction in cell death and no increase in T-cell proliferative capacity was observed in humans. This infers that TIM-3 has differing roles in murine and human physiology (Hastings *et al.*, 2009). Our data supports these previous observations from human cells, as we were unable to detect an increase in proliferation for either naïve or memory T-cells activated by SMX-NO in the presence of TIM-3-blocking antibodies. Additionally, it has previously been reported that anti-TIM-3 antibodies can enhance the secretion of Th1/Th17 cytokines from human cells (Hastings *et al.*, 2009). We observed an enhanced secretion of cytokines after the inclusion of TIM-3 blocking antibodies, particularly for the Th1-associated cytokine IFN-γ. Thus, while TIM-3 is ineffective at regulating the proliferative response of human T-cells, its role may be limited to specific functions including the management of cytokine secretion.

The function of these pathways is ultimately dependent on receptor expression. Thus, to gain a more detailed understanding on the role of these T-cell receptors during drug-derived antigen-specific T-cell responses, we explored the kinetics of PD-1, CTLA4, and TIM-3 expression. We found that PD-1 expression increased both after initial antigen exposure, and more so after re-exposure to antigen, peaking at around 24-48 hrs post restimulation for both naïve and memory T-cells. In contrast, CTLA4 expression was not initially increased by stimulation with antigen, but in line with previous reports citing an increase after 2-3 days (Walunas *et al.*, 1994), we observed a slight increase in the percentage of T-cells expressing CTLA4 between 48-72 hours after re-stimulation with drug. In comparison, the expression pattern of TIM-3 was vastly different to that of both PD-1 and CTLA4 as the proportion of TIM-3^+ cells was seen to fall after both initial antigen exposure and re-stimulation, but increased towards the end of both cultures. As the number of PD-1^+ T-cells dramatically increased until shortly after re-stimulation, before falling gradually thereafter, our data indicate that PD-1 has a more prominent role in regulation during the initial encounter of naïve T-cells with antigen. In contrast, the expression profile of CTLA4, but particularly TIM-3, increased approximately 3 days post restimulation with antigen implying that these pathways have a more prominent role during the latter stages of a T-cell response to drug-derived antigens. Additionally, in cultures with the addition of PD-L1- or CTLA4-blocking antibodies, we report a slight increase in PD-1 and CTLA4 expression after re-stimulation with antigen, respectively. In contrast, no increase in TIM-3 expression was observed after the inclusion of TIM-3 blocking antibodies. This indicates that T-cells are somewhat able to enhance the expression of a specific co-inhibitory receptor to compensate for increased regulatory pressures upon that receptor. However, as we have previously demonstrated the ability to enhance the antigen-specific T-cell response by blocking an individual receptor, the extent to which one co-inhibitory receptor (i.e. PD-1) can be upregulated to compensate for

another (i.e. CTLA4) is unknown. Indeed, the simultaneous assessment of co-inhibitory pathways is currently being investigated within our group.

To look at the individual expression profiles in more detail, we analysed the expression of each inhibitory receptor on $CD4^+$ and $CD8^+$ T-cell cells individually. In both naïve and memory cultures, the basal expression of all three receptors on unstimulated T-cells was much higher on $CD8^+$ T-cells. Despite this, a similar increase in the percentage of PD-1^+ or TIM-3^+ cells upon antigen stimulation was identified for both $CD4^+$ and $CD8^+$ T-cells in both naïve and memory cultures (increased percentage of PD-1^+ cells after antigen stimulation: naïve $CD4^+$, 26.9%; naïve $CD8^+$, 27.2%; memory $CD4^+$, 19.3%; memory $CD8^+$, 16.1%)(increased percentage of TIM-3^+ cells after antigen stimulation: naïve $CD4^+$, 42.4%; naïve $CD8^+$, 47.9%; memory $CD4^+$, 48.3%; memory $CD8^+$, 49.5%). Thus our data suggests that both PD-1 and TIM-3 regulate both $CD4^+$ and $CD8^+$ T-cell responses to a similar extent *in vitro*. In contrast, while CTLA4 also increased on both naïve and memory T-cells after antigen-stimulation, the fold increase on $CD8^+$ T-cells was almost double that on $CD4^+$ T-cells (naïve T-cells: $CD4^+$, > 1.3-fold; $CD8^+$, > 2.1-fold; memory T-cells: $CD4^+$, > 2.0-fold; $CD8^+$, > 4.0-fold). Overall, the expression of CTLA4 was more prominent on activated memory rather than naive T-cells, but also on the $CD8^+$ fraction indicating that these cells are more highly regulated by CTLA4 during drug-specific T-cell responses.

While our assessment of co-inhibitory receptor expression and function on T-cell lines enhances our understanding of how these pathways regulate drug-specific T-cell responses, it was vital that we combined the two to show how expression affects function. To do this we first had to up-date the classification of the response in question. Hypersensitivity responses are traditionally characterised as Th1/Th2 based upon cytokine secretion profiles. However, recently this repertoire has been expanded with the discovery of Th17 and Th22 subsets. Both Th17 and Th22 have dual capabilities; they can induce an anti-inflammatory

innate immune response in keratinocytes to protect skin, but they also have a pro-inflammatory function. While both Th17 and Th22 can secrete IL-22, only Th17 are capable of secreting IL-17 and thus these subsets can identified by the secretion profile of these two cytokines. (Cavani *et al.*, 2012). As both Th17 and Th22 subsets appear to have important consequences in the skin, it is vital to assess their respective roles in drug-induced cutaneous reactions. Upon the priming of naïve T-cells from healthy donors to SMX-NO, we identified the secretion of IL-22, but not IL-17, alongside that of IFN-γ and IL-13. On further analysis using SMX-NO-responsive T-cell clones we looked at an expanded secretory profile and identified the secretion of IL-22 alongside IFN-γ, IL-5, IL-13, granzyme B, and FasL. Once again, secretion of IL-17 was not detected. This consistent secretion profile indicated that Th22, but not Th17, plays a role in SMX-specific T-cell reactions. Upon fine examination of these data, we subsequently discovered the presence of two distinct subsets of T-cell clones that either secrete Fas-L/IL-22 or granzyme B, which was confirmed utilising cells from hypersensitive patients. To our knowledge, this is the first data to show the production of IL-22 alongside IFN-γ by antigen-specific T-cells from drug-hypersensitive patients (Gibson *et al.*, 2014).

With the SMX-NO-response fully characterised we could ascertain whether co-receptor signalling pathways affect the functionality of drug-derived antigen-specific T-cell responses. This represents an important question as high PD-1 expression has previously been used to identify exhausted T-cells. Indeed, the increased expression of a range of immune-inhibitory receptors is associated with T-cell exhaustion (Radziewicz *et al.*, 2007; Zhou *et al.*, 2011). In contrast, others report that PD-1^{hi} cells are fully functional and can control viral replication, and maintain effector T-cell responses during chronic infection (Duraiswamy *et al.*, 2011; Reiley *et al.*, 2010; Zelinskyy *et al.*, 2011). As exhaustion relates to T-cells which are unable to produce cytokines or proliferate (Wherry and Ahmed, 2004), we generated T-cell clones from these T-cell lines to assess the association between the individual cell expression of a

receptor and function. We found that individual T-cells express a wide range of PD-1, CTLA4, and TIM-3. Specifically, we detailed the expression of each receptor on 40 clones and found a 4-fold variation in PD-1, a 10-fold variation in TIM-3, and a 27-fold variation in CTLA4. Importantly, the level of expression of all three receptors individually was not associated with the proliferative capacity of these T-cell clones. Thus, our data disproves that individual high expression of a co-inhibitory pathway can be used to determine T-cell exhaustion. The association of a particular pathway with T-cell exhaustion is tricky as those cells with higher expression of co-inhibitory pathways are often older and thus have a lengthier replication history. It is therefore likely that these cells express a broad range of inhibitory receptors (Wei *et al.*, 2013). Indeed, recent studies highlight that T-cell exhaustion is likely a consequence of a complex signalling pattern between a variety of immune-inhibitory pathways (Blackburn *et al.*, 2009).

For the second section of this project we wanted to investigate the origin of drug antigen-responsive T-cells in hypersensitive patients (chapter 5, summarised in figure 7.1). For this we focussed on those responses deriving from the sulfonamide antibiotic SMX, which is associated with the development of severe hypersensitivity reactions in up to 8% of the general population (Alfirevic *et al.*, 2009). T-cells responsive to SMX and the metabolite SMX-NO can be detected in all SMX-hypersensitive patients. In comparison, while previous studies report that lymphocytes from 9/10 drug-naïve donors respond to SMX-NO, lymphocytes from just 30% of drug-naïve donors respond to SMX (Engler *et al.*, 2004). However, as these drug-naïve donor responses were assessed from whole lymphocyte populations, the individual roles of naïve and memory T-cells remain undefined. We therefore utilised the optimised *in vitro* human T-cell assay to unearth the origins of SMX-derived antigen-responsive T-cells from healthy donors.

We identified that SMX-NO was able to prime naïve T-cells, but also activate memory T-cells in all healthy donors. In contrast, neither naïve nor memory T-cell proliferation was detected in response to SMX. Alternative analysis of T-cell activation by cytokine secretion revealed similar results using cells from one donor. Interestingly however, SMX-specific IL-13 secretion was detected from 2 drug-naïve donors. Upon the generation of T-cell clones from these two donors, we found that SMX-specific T-cell clones had been generated from both the naïve and memory cultures. Indeed, our data are the first to show that both hapten and parent drug can stimulate pre-existing memory T-cells from drug-naïve donors. Whether these data infer that these donors would develop SMX-hypersensitivity upon clinical treatment is not elucidated. However, as these donors have no history of SMX exposure, the identification of both SMX and SMX-NO-responsive memory T-cells strongly indicates that these individuals contain peptide-specific $CD4^+$ and $CD8^+$ T-cells which are reactive against SMX/SMX-NO-modified peptides through some form of molecular mimicry. Heterologous immunity has been linked to drug hypersensitivity previously. In the case of ABC, Lucas *et al* outlined the potential for cross-reactivity between drug and pathogen-derived antigens in an MHC-restricted fashion (Lucas *et al.*, 2015). However, further investigations beyond the scope of our work here will be required to assess the nature of potentially cross-reactive peptides with regards to SMX-hypersensitivity.

As this was the first time that individually SMX-responsive naïve and memory T-cells had been isolated, we generated T-cell clones from the two SMX-responsive donors to characterise these responses in more detail. We successfully generated SMX- and SMX-NO-responsive T-cell clones, with the majority of clones from all but one culture being $CD4^+$ T-cells. This correlates with the previously reported dominant $CD4^+$ phenotype from SMX-hypersensitive patients (Pichler, 2003; Pichler *et al.*, 2002). Moreover we found that the SMX- and SMX-NO-responsive T-cells, functionally mirror those previously identified from

hypersensitive patients by generally lacking cross-reactivity (Castrejon *et al.*, 2010). We observed that all T-cell clones generated from naïve T-cell priming displayed a complete lack of cross-reactivity, while a subset of both SMX- and SMX-NO-responsive T-cell clones derived from memory T-cell cultures were cross-reactive, and thus likely represent the cross-reactive fraction identified in hypersensitive patients.

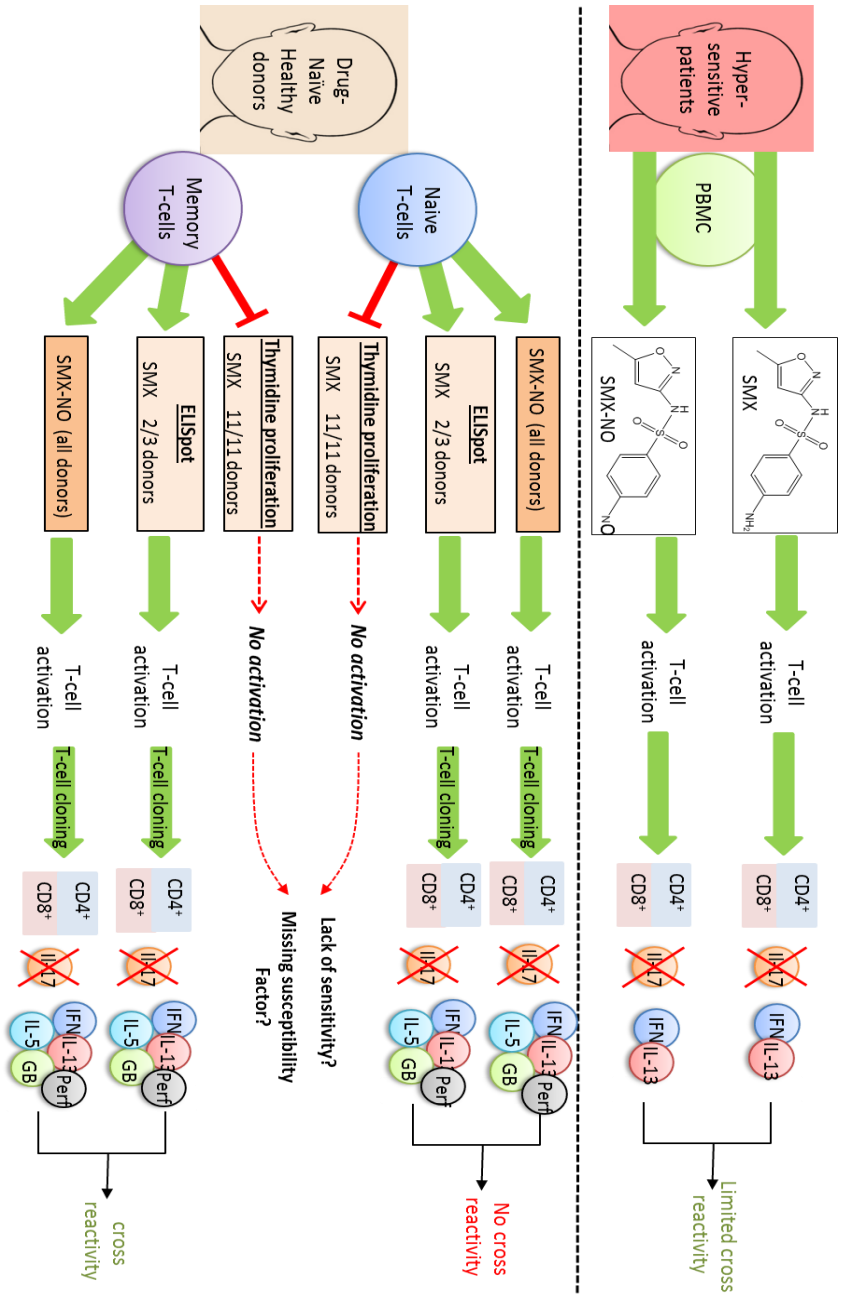


Figure 7.1 Final overview of the T-cell responses which have been detected from SMX-allergic patients and healthy donor naïve and memory T-cell subsets. Patient response data sourced from (Castrejon *et al.*, 2010; Lochmatter *et al.*, 2009).

While the investigation of drug-induced T-cell responses has been the main focus of this work, chemical antigens are also a frequent source of hypersensitivity reactions. Indeed there is a wide range of chemicals known to induce contact hypersensitivity reactions including metals such as nickel, rubber accelerators, preservatives, fragrancies, and certain components of 'tear-free' shampoos (Militello *et al.*, 2006). Severe reactions are associated with a common component of hair dyes, PPD, which causes ACD with a prevalence of 1% in the general population (Kind *et al.*, 2012). PPD forms a range of intermediates through processes of oxidation including PPD-benzoquinone and PPD-benzoquinonediimine. While these oxidation products not only have an enhanced ability to activate DCs in comparison to non-oxidised PPD, they can also form protein adducts (Aeby *et al.*, 2008). Unfortunately, they are inherently unstable, and so it is difficult to investigate the different oxidation products individually. Despite this, spontaneous oxidation ensures their presence during *in vitro* culture with PPD. Further oxidation and self-conjugation results in the formation a rearrangement product of the trimer, known as Bandrowki's Base (BB), which along with PPD is able to stimulate patient T-cells (Coulter *et al.*, 2007a). In comparison, only BB is capable of stimulating similar responses in healthy donors. This healthy donor response to BB requires previous sensitisation, indicating that much of the human population is likely sensitised, potentially due to the use of PPD to dye everyday items such as clothes (Coulter *et al.*, 2007b). The reason why only a small number of individuals who dye their hair respond to PPD and develop ACD remains undefined.

To investigate the mechanisms of PPD-induced ACD (chapter 6, summarised in figure 7.2) we first characterised the T-cell response of allergic patients to both PPD and BB. The stimulation of PBMCs using an LTT confirmed each patient's allergic diagnosis as both PPD and BB produced proliferative responses. BB generally produced much greater stimulation than PPD in conjunction with the local lymph node assay where PPD is ten times less potent than BB.

However, we must reiterate that PPD is still a strong sensitizer and a PPD-concentration of just 0.01% can invoke a response in some allergic patients (McFadden *et al.*, 2011; White *et al.*, 2006a). To further characterise the allergic response, we generated 105 PPD- and 122 BB-responsive clones from a total of 5 allergic patients. More than 90% of BB-responsive clones were characterised as $CD4^+$ T-cells. Most PPD-responsive clones expressed $CD4^+$; however 37.5% of PPD-activated T-cell clones expressed the $CD8^+$ coreceptor implicating PPD as the central driving force behind the production of effector T-cells during an allergic reaction. After the generation of $CD8^+$ T-cell clones, our group previously reported surprise that they did not observe the secretion of cytotoxic molecules as would be expected (Coulter *et al.*, 2009). Indeed, secretion of such molecules is associated with the development of murine ACD (Kehren *et al.*, 1999). In this investigation we were able to detect the secretion of the cytotoxic molecule granzyme B from $CD8^+$ PPD-responsive T-cell clones, alongside IFN-γ and IL-13. In concordance with previous investigations, both PPD and BB-responsive T-cell clones from patients were mostly highly-specific and generally lacked cross-reactivity. Thus, these data indicate that both antigens may selectively prime naïve T-cells in allergic patients.

To assess the role of primary and secondary T-cell responses in healthy donors, we initially cultured T-cells with 5 μM of PPD or BB as this concentration has been shown to induce maximal proliferative responses in patient cells (Coulter *et al.*, 2007b). In our investigation, both naïve and memory T-cells were observed to proliferate and secrete cytokines in response to BB. Neither the activation of pre-existing memory T-cells nor the priming of naïve T-cells was seen with PPD (5 μM). Moreover, both higher and lower alternative concentrations of PPD which fall within the stimulation range of T-cells from allergic patients failed to induce a response. Despite a lack of PPD-response, cytokine profiling of the BB-response from both naïve and memory T-cells identified the secretion of IL-22 alongside IFN-γ and IL-13, and a lack of cross reactivity with PPD. These data represent similar findings to

those from patient T-cell clones highlighting the suitability of this assay to assess chemical-induced hypersensitivity reactions (Gibson *et al.*, 2015).

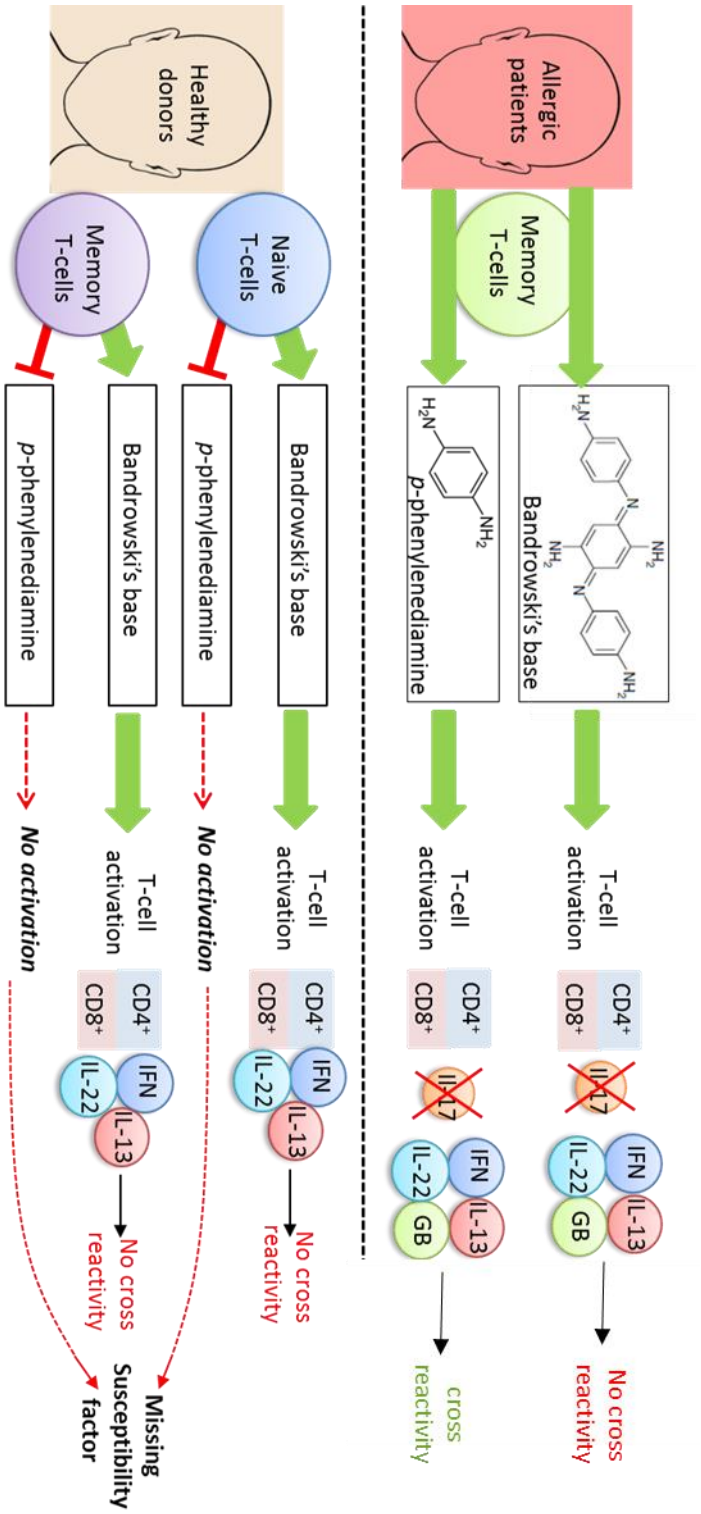


Figure 7.2 Final overview of the T-cell responses which have been detected from PPD-allergic patients and healthy donor naïve and memory T-cell subsets.

Overall, our investigations have highlighted the high degree of complexity involved in the elicitation of a T-cell response to a drug or chemical-derived antigen. Firstly, the development of tolerance versus hypersensitivity is likely a fine balance of multiple factors including HLA-alleles, variants in drug metabolising enzymes, but also co-signalling pathways. Indeed, by investigating the function and expression kinetics of particular co-inhibitory receptors within the complex signal 2 framework of interacting pathways, we have shown that a number of receptors can regulate both the drug-derived antigen-induced activation of primary and secondary T-cell responses; some of which have a predominant effect either during early (PD-1) or later-stage (CTLA4, TIM-3) T-cell activation, on $CD4^+$ or $CD8^+$ T-cells (CTLA4), or may regulate specific effector functions of T-cells such as the seemingly distinct role of TIM-3 on human T-cell cytokine secretion. Validating the influence of co-signalling genetic variants to predispose an individual to hypersensitivity requires a gene-association study, which is costly, time-consuming, and requires a large cohort number. However, polymorphisms in co-inhibitory pathways have previously been linked with autoimmune diseases and thus the cumulative effect of several pathways may represent a significant risk in the development of hypersensitivity reactions. Further complexity lies in the antigenic origins of a response to drug-antigen. We identified that both naïve T-cells and pre-existing memory T-cells from drug-naïve donors can be activated with both parent drug (SMX) and hapten-metabolite (SMX-NO). While we ascertained that T-cell clones derived from memory T-cells are cross-reactive and thus likely represent the small number of cross-reactive T-cells detected from hypersensitive patients *ex vivo*, the lack of previous exposure to SMX indicates that these T-cells were initially primed to an unrelated and undetermined peptide antigen. Similar heterologous immunity has been proposed for T-cell responses to ABC. The definition of the exact nature of the peptide antigens capable of inducing a T-cell response is critical to understand these reactions.

In conclusion, our data highlights a number of distinct functional similarities between our *in vitro* data and data gathered from hypersensitive patients *ex vivo* thus validating the use of these *in vitro* assays as useful tools to investigate both drug- and chemical-induced hypersensitivity reactions. As such, we are currently in the process of adapting this assay by way of miniaturisation to maximise the number of donors and/or antigens that can be assessed simultaneously, to form a suitable pharmaceutical platform for the safety testing of drugs and chemicals. However, the current absence of appropriate susceptibility factors are likely responsible for the lack of response to PPD. Indeed, the future incorporation of (a) genetically-validated risk factors through the use of blocking antibodies and/or cells of a particular genotype, alongside (b) defined peptide-antigens, will provide an advanced platform to study and predict the potential of a new chemical entity to cause a high incidence of hypersensitivity reactions.

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