The Role of T-helper 17 and T-helper 22 Lymphocytes in Beta-Lactam Hypersensitivity

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

Andrew Sullivan

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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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Andrew Sullivan (B.Sc, M.Res)

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Finally finished this goddamn thesis, knew I would get round to it eventually!

CONTENTS

ABBREVIATIONS

aa – amino acids

ABC – abacavir

ACD – allergic contact dermatitis

ACN - acetonitrile

ADR – adverse drug reaction

AGEP – acute generalised exanthematous pustulosis

AhR – aryl hydrocarbon receptor

AHS – abacavir hypersensitivity syndrome

ALP – alkaline phosphatase

ALT – alanine aminotransferase

ANOVA – analysis of variance

APC – antigen presenting cell

APC^2 – allophycocyanin

Bak – Bcl-2 homologous antagonist killer

Bax – Bcl-2 associated x protein

Bcl-6 – B-cell lymphoma 6

Bcl-10 – B-cell lymphoma 10

BLIMP – PR domain zinc finger protein 1

BSA – bovine serum albumin

CBZ – carbamazepine

CCL – chemokine ligand

CCR – chemokine receptor

CD – cluster of differentiation

CFSE – carboxyfluorescein diacetate succinimidyl ester

CLA – cutaneous leukocyte antigen

COX – cyclooxygenase

CPM – counts per minute

CRM – chemically reactive metabolite

CSA – cyclosporin A

CTL – cytotoxic T-lymphocyte

CTLA4 – cytotoxic T-lymphocyte associated protein-4

CXCL – CXC chemokine ligand

CXCR – CXC chemokine receptor

CYP – cytochrome enzyme

DAMP – damage associated molecular pattern

DC – dendritic cell

DHS – drug hypersensitivity syndrome

DIHS – drug induced hypersensitivity syndrome

DILI – drug induced liver injury

DMSO – dimethyl sulfoxide

DNA – deoxyribose nucleic acid

DNCB – dinitrochlorobenzene

DRESS – drug reaction with eosinophilia and systemic symptoms

EBV – Epstein-Barr virus

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme linked immunosorbent assay

ELISpot – enzyme linked immunosorbent spot 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

GABA – gamma-aminobutyric acid

GATA – anti-GABA transporter

GM-CSF – granulocyte macrophage colony stimulating factor

HBSS – Hanks' balanced salt solution

HEPES – hydroxyethyl piperazineethanesufonic acid

HIV – human immunodeficiency virus

HLA – human leukocyte antigen

HRP – horseradish peroxidise

h – hours

HSA – human serum albumin

ICAM – intracellular adhesion molecule

ICOS – inducible co-stimulator

ICOSL – inducible co-stimulator ligand

IFN – interferon

Ig – immunoglobulin

IL – interleukin

IP$_3$ – inositol triphosphate

ITAM – immunoreceptor tyrosine based activation motif

IV – intravenous

Jak – janus kinase

LAT – linker for activation of T-cells protein

LC – langerhans cell

Lck – lymphocyte specific protein tyrosine kinase

LPS – lipopolysaccharide

LTT – lymphocyte transformation test

MAPK – mitogen activated protein kinase

MFI – mean fluorescence intensity

MHC – major histocompatability complex

mins – minutes

MPE – maculopapular exanthema

NADH – nicotinamide adenine dinucleotide

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

PAX – paired box transcription factor

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffered saline

PD – programmed death

PE – phycoerythrin

PHA – phytohemaglutinin

PI – pharmacological interaction

Pip - piperacillin

PKC – protein kinase C

PLC – peptide loading complex

RANTES – regulated upon activation, normal T-cell expressed and secreted

RORγt – retinoic acid receptor related orphan nuclear receptor gamma

ROS – reactive oxygen species

RPMI – Roswell Park Memorial Institute

SD – standard deviation

SEM – standard error of mean

SFU – spot forming units

SI – stimulation index

SJS – Stevens Johnson syndrome

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

TGFβ – transforming growth factor beta

T_h – T-helper lymphocyte

TEN – toxic epidermal necrolysis

TIM – T-cell immunoglobulin and mucin domain protein

TLR – toll like receptor

TNF – tumour necrosis factor

T_{reg} – T-regulatory cell

TT – tetanus toxoid

v/v – volume / volume

WHO – world health organisation

w/v – weight / volume

zap-70 – zeta chain associated protein kinase 70

β2m – beta 2 microglobulin

PUBLICATIONS

Papers

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Abstracts

Poster - _Investigating the role of B-cells in the progression of delayed type drug hypersensitivity in patients with cystic fibrosis_. EAACI DHM5, Munich 2012.

Poster – _Detection of interleukin-22 secreting T-cells in piperacillin hypersensitive patients with cystic fibrosis_. EAACI DHM6, Bern 2014

Talk – _Drug-specific CD4$^+$ and CD8$^+$ T-cells isolated from skin and blood of hypersensitive patients secrete interleukin-22, but not interleukin-17_. EAACI Winter Conference, Les Arcs 2015 – Awarded travel scholarship.

Talk – _CD4$^+$ and CD8$^+$ T-cells isolated from blood and skin of patients with beta-lactam hypersensitivity secrete interleukin-22, but not interleukin-17_. EAACI World Congress, Barcelona 2015 – Awarded travel scholarship.

Poster – _Interleukin-22 and its role in piperacillin hypersensitivity reactions_. British Toxicological Society Meeting, Edinburgh 2015.

Poster – _The role of interleukin-22 in beta-lactam hypersensitivity reactions_. EAACI DHM7, Barcelona 2016 – Awarded travel scholarship.

ABSTRACT

Beta-lactam antibiotics are used to treat recurrent opportunistic infections that occur in patients with cystic fibrosis. However, the incidence of reported hypersensitivity in these patients is greatly higher than the healthy population. Due to the delayed nature of beta-lactam hypersensitivity, a T-cell mediated immune response is implicated. Type IV drug hypersensitivity is a major clinical concern, with a cutaneous aetiology driven through antigen specific T cells. Pro- and anti-inflammatory cytokines can be detected in reactions to β-lactam drugs in responsive T cells from hypersensitive patients. Classical T_h1/T_h2 phenotypes are currently used to classify the reactions, though these do not accurately characterise the function of the immune cells. In addition, this classification does not reference the newer T_h subsets such as T_h9, T_h17 and T_h22.

To characterise the nature of the T-cell response observed in piperacillin hypersensitive patients with cystic fibrosis, T-cell clones from both blood and inflamed skin of hypersensitive patients were generated, then characterised in terms of phenotype and function. Additional investigations into PBMC responses were conducted. Naïve T cells from healthy donors were also primed to piperacillin, as well as attempted regulation of the cytokine response through modulation of a selected nuclear receptor involved in the progression of a T_h22 mediated response.

Drug-specific clones were generated from both blood (n=570, 84% CD4) and skin (n=96, 83% CD4) samples obtained from patients hypersensitive to piperacillin. All clones secreted high levels of IFN-γ and IL-13. Interleukin-22, perforin and granzyme B were secreted in over 50% of clones, with none from either blood or skin showing any detectable level of IL-17A. Naïve T cells primed to piperacillin via autologous dendritic cells showed proliferative responses ($p=0.001$, SI>2). Clones generated from primed T-cells showed similar patterns of cytokine secretion when compared alongside clones generated from hypersensitive patients. Significant differences in chemokine receptor expressions were observed between blood-derived piperacillin-specific clones, skin-derived piperacillin-specific clones and skin-derived non piperacillin-specific clones. CLA, CXCR6 and CCR1 expression was higher on piperacillin-specific skin derived clones when compared to non-piperacillin specific skin derived clones ($p=0.01$). CCR2, CCR4, CXCR1 and E-cadherin were higher on skin specific clones when compared to blood specific clones ($p=0.01$). Piperacillin specific clones isolated from blood and skin of hypersensitive patients, as well as healthy donor PBMC migrated in the presence of chemokines specific to their respective cell surface receptors, with migration to CCR4 and CCR10 most prevalent. In addition, modulation of the aryl hydrocarbon receptor showed that an abrogation of the cytokine response was observed in cells treated with an AhR inhibitor. This abrogation was only observed in the secretion of interleukin-22, a key cytokine in a T_h22 response.

Currently, T-cell mediated hypersensitivity involving drug binding to the HLA molecule has only been shown for HLA class I molecules; no data has provided evidence for HLA class II interactions with drugs being able to activate CD4+ T-cells. To investigate whether HLA class II molecules binding to drugs could activate T-cells, an investigation into the COX-2 specific NSAID lumiracoxib was performed. Lumiracoxib was withdrawn from use after incidences of liver toxicity were reported in 2008.

Utilising a naïve T-cell dendritic cell co-culture assay, attempts were made to generate drug-specific responses from lymphocytes to either lumiracoxib or its major / minor metabolites. No positive responses were generated, with all assays performed showing no activation of drug-specific T-cells following re-challenge with the drugs.

In conclusion, the data generated over the course of this thesis has shown that there is a subset of piperacillin-specific T-cells in hypersensitive patients with cystic fibrosis that secrete IL-22, IFN-γ, perforin and granzyme B in response to antigen challenge. No cells secreted IL17, suggesting a strong T_h22 phenotype rather than T_h17. In addition, The AhR signalling pathway is also heavily implicated in the development of hypersensitivity, giving further evidence for the role of both IL-22 and T_h22 lymphocytes in beta-lactam hypersensitivity.

CHAPTER 1 – GENERAL INTRODUCTION

1.1　THE IMMUNE SYSTEM

The primary role of the immune system is to recognise, protect and mount a response to antigens that are present throughout the body (Akira, Uematsu et al. 2006). The response that is generated can vary greatly, due to the numerous components involved in the immune response. Antigens can be both micro and macro in size, with different cells and molecules responsible for the immune response in each instance.

Physiologically, the immune system functions to protect the body from harmful foreign molecules, including bacteria and viruses. Early stage responses are mediated through the innate immune system, with the adaptive immune system complementing and eventually being the primary driving force behind the response (Medzhitov and Janeway Jr 1997).

In order to be able to mount a response to a wide array of antigens, both T- and B-lymphocytes undergo genetic recombination, developing a diverse range of receptors able to recognise many different molecules. All cells involved in the immune response in blood, as well as tissues, are generated in either the bone marrow or the thymus, with cells maturing and later having the ability to migrate to their site of residency or action (Cooper 2015).

The innate immune response is mediated through physiological barriers such as the skin, alongside inflammatory cells such as mast cells and phagocytes, but does not confer long term immunity. The adaptive immune system is triggered after the innate immune system, conferring immunity to specific pathogens. It

is mediated through the T- and B-lymphocytic responses that are long lasting, conferring a memory immunity for multiple years after initial exposure.

Due to the nature of the response times, the innate immune system responds to a broad array of different antigens, with little specificity for individual molecules. In contrast, the delayed adaptive immune response is highly specific and able to recognise individual antigens. The physiological role of both the innate and adaptive immune systems are described in greater detail in the following sections.

1.1.1 INNATE IMMUNE SYSTEM

Innate immunity involves both physiochemical (skin) and inflammatory mediators such as defensin, as well as cellular components of the innate immune system that can work independently (i.e. cells such as phagocytes and natural killer cells). Working in concert with each other, components of the innate immune system provides a highly effective defence to most micro-organisms and pathogens. The complex interplay of cytokine secretion, as well as interactions with the complement system comprises the humoral barrier, providing an additional layer of immediate immune defence to multiple pathogens (Fearon and Locksley 1996; Abbas 2010).

The innate immune response is triggered through receptor recognition of pathogen-associated molecular patterns (PAMPs). These pathogen recognition receptors (PRRs) can be soluble or associated with cell surfaces, with expression highest on innate immune cells such as macrophages and dendritic cells (Medzhitov and Janeway Jr 1997). Soluble PRR activation by PAMPs leads to multiple effects, including up-regulated phagocytosis, lysosome interactions triggering microsomal lysis and direct action of the soluble PRR molecules on the microorganisms themselves (Medzhitov and Janeway 2002). Cell-associated PRR activation bring about cytokine secretion cascades, resulting in the release of inflammatory mediators, as well as increased phagocytosis similar to the soluble PRR response. Also involved in the innate immune system, are the danger associated molecular patterns (DAMPs) (Kumar, Kawai et al. 2011). These molecules are released during non-senescent necrotic

death as well as traumatic tissue damage, are also able to trigger an innate immune response (Pétrilli, Dostert et al. 2007).

1.1.1.1 CELLULAR COMPONENTS – MONOCYTES

Monocytes are a type of white blood cell comprising up to 10% of the peripheral leukocytes found in human blood. Furthermore, a large reservoir, approximately half of the total monocyte population are found in the spleen (Swirski, Nahrendorf et al. 2009). They are progenitor cells able to differentiate into three distinct cell types; macrophages, dendritic cells and foam cells (Huh, Pearce et al. 1996). They have a diverse array of functions throughout the body, including differentiation to the aforementioned cells, phagocytosis and cytokine production (Turrini, Ginsburg et al. 1992; Friebe, Siegling et al. 2004). Phagocytosis occurs primarily through the opsonisation of pathogens via proteins such as antibodies, in addition to the activation of specific complement receptors (Newman and Tucci 1990).

Monocyte's ability to differentiate *in vivo* has been reported for many decades, with initial work by Ebert and Florey showing terminal differentiation into macrophages as far back as 1939 (Ebert and Florey 1939). Migration into peripheral tissues occurs through the action of chemokines such as CCL2 and CCL7 – allowing for differentiation into the previously mentioned cell types (Shi and Pamer 2011).

It has been reported that there are two distinct subsets of monocytes, based on their expression of CD14 and CD16; a $CD14^+CD16^-$ and a $CD14^+CD16^+$ variant.

Specific phenotypic differences can be noted between these two monocytes, with the CD14$^+$CD16$^-$ expressing higher levels of CCR2 compared to the CD14$^+$CD16$^+$ subtype. However, The CD16$^+$ cells have been shown to express higher amounts of the MHC class II molecule, suggesting that these are more likely to be involved in antigen presentation. Both classes of monocyte are able to differentiate into macrophages in the presence of cytokines such as GM-CSF and IL-4 (Ziegler-Heitbrock, Fingerle et al. 1993; Sallusto and Lanzavecchia 1994; Weber, Belge et al. 2000).

More recently, a third subtype of monocytes has been reported, a CD14$^+$CD16$^+$CD64$^+$ phenotype. This type of monocyte secretes large amounts of TNFα and IL-6 following phagocytosis. The authors suggest that these cells have the propensity to differentiate into either macrophages or DCs, depending on local environmental stimuli (Grage-Griebenow, Flad et al. 2001; Grage-Griebenow, Zawatzky et al. 2001).

1.1.1.2 CELLULAR COMPONENTS – MACROPHAGES

Derived from monocytes present in the blood, macrophages are CD14$^+$ expressing cells (Khazen, M'Bika et al. 2005). When fully mature, they express additional markers specific to macrophages including CD11b, CD40, CD64 and CD68 (Joshi, Fong et al. 2009). They are involved in both the innate and adaptive immune response, with their primary roles throughout the body to act as antigen presenting cells for the immune system, as well as having a functional role in the phagocytosis of cell fragments. Phagocytosis of

microorganisms triggered by IFN-γ proceeds through the actions of endosomal enzymes. Activation of macrophages leads to the presentation of antigens in the context of MHC class I and II molecules to specific helper T-lymphocytes (Mosser 2003; Mantovani, Sica et al. 2004).

1.1.1.3 CELLULAR COMPONENTS – DENDRITIC CELLS

Dendritic cells are the primary antigen presenting cells of the immune system. They develop from monocytes in the bone marrow, and are able to migrate and reside in different tissues monitoring damage and infection (Banchereau and Steinman 1998). They are present in most tissues, and usually have specific names depending on their location; for example, in skin, they are called Langerhans cells, while microglia are in the brain and spinal cord (Ginhoux, Tacke et al. 2006).

Presentation through dendritic cells occurs via the following process: first, the engulfment of extracellular protein antigens which are then processed into smaller peptide fragments. Secondly, these fragments are presented by MHC on the surface of the dendritic cells to allow for recognition by T-lymphocytes (Ingulli, Mondino et al. 1997). Antigen presentation occurs typically on fully matured dendritic cells. The immature cells express lower levels of the co-stimulatory molecules CD80 and CD86. These cells are implicated in phagocytosis rather than antigen presentation (Zheng, Manzotti et al. 2004; Spaggiari, Abdelrazik et al. 2009). Maturation from immature dendritic cells is triggered by PAMP / DAMP signals from bacterial components such as LPS.

High levels of immature dendritic cells are found in the extremities of the body, such as the epidermis, due to the higher rate of contact with antigens. Encountering an antigen drives immature dendritic cells to phagocytose pathogens and migrate to lymph nodes, where they prime naïve T-lymphocytes (Kapsenberg 2003). This process leads to the formation of a pool of residual memory T-lymphocytes specific to the presented antigen. Presentation via immature dendritic cells often leads to immune tolerance through regulatory T-cell (T_{reg}) expansion, as opposed to the immune response triggered through mature dendritic cell presentation. T-lymphocyte activation mediated through dendritic cells occur through two distinct pathways; co-stimulatory receptor ligand interactions (non-specific to the antigen), and direct antigenic stimulation (Steinman 1991). Dendritic cell function is up-regulated via IL-4 and GM-CSF, and in certain in vitro situations these molecules are used to drive a monocyte culture to mature into a terminal dendritic cell phenotype, along with the addition of LPS and TNFα (Dieu, Vanbervliet et al. 1998).

1.1.1.4 CELLULAR COMPONENTS – GRANULOCYTES

These cells are a subclass of leukocytes that digest microorganisms through phagocytosis. They are a broad subset of cells, encompassing basophils, eosinophils and neutrophils (Bochner, Luscinskas et al. 1991). Basophils are the least abundant type of granulocyte; they contain many granules inside the cell filled with pro-inflammatory mediators. Like all granulocytes, basophils are able to be recruited to tissues from the circulating blood. When activated, they act to release histamine and leukotrienes and are involved in the IgE

response, similar to mast cells (Ochensberger, Daepp et al. 1996). Neutrophil migration is mediated via IL-8. Microorganisms are attacked by neutrophils, leading to phagocytosis and pathogen digestion (Zeilhofer and Schorr 2000; Nathan 2006). Eosinophils are important cellular mediators in the final phase of mast cell mediated immune reactions. They have the ability to secrete multiple cytokines such as IFN-γ, IL-5 and IL-10. They migrate in response to eotaxin and IL-5, allowing for a positive feedback loop to be established upon contact with foreign parasites (Yamaguchi, Suda et al. 1988). Eosinophils exert their effect through the cationic granules that are present inside the cells (Dyer, Percopo et al. 2010).

1.1.1.5 CELLULAR COMPONENTS – NATURAL KILLER CELLS

Natural Killer (NK) cells are lymphocytes that display the CD56 cell surface marker (Chan, Sin et al. 1997). They do not express antigen-specific receptors on the cell surface, though they are involved in pathogen immunity through the production of cytokines (Cooper, Fehniger et al. 2001). They are a commonly found cell type, accounting for approximately 30% of all cells generated from lymphoid progenitors. They can be found in tissues such as the spleen and liver, after maturation in the bone marrow and lymph nodes (Morris and Ley 2004). Cell death triggered by the NK cell activation pathway is generally mediated through the production of the cytotoxic molecules including perforin and granzyme B. However, activated NK cells can also produce high levels of IFN-γ (Trapani 1995). IFN-γ increases the effector function of macrophages and promotes cell mediated immune responses.

Major histocompatability complex receptors (MHC) are cell surface proteins involved in the presentation of peptide fragments to T-cells. MHC I inhibitory receptors are known to be involved in the immune response, with the activation of these receptors triggering secretion of cytokines such as GM-CSF and TNF-a, which can lead to the killing of infected cells (Doucey, Scarpellino et al. 2004). In addition, activation of NK cells leads to an increase in IL-12 production, which is involved in the polarisation of the T_h1 lymphocytic response (Lawson 2012).

1.1.2   ADAPTIVE IMMUNE SYSTEM

The adaptive, or acquired, immune system's primary goal is to target and destroy invading pathogens that are noxious to the body, as well as being able to destroy the harmful products of these pathogens. The innate and adaptive immune systems do not work in isolation; rather, they function alongside one another with multiple interactions found between them on a cellular and molecular level. The innate immune system is also known to be able to trigger an adaptive immune response. Immunological memory allows for a rapid response of the immune system upon re-exposure to a previously encountered antigen (Kurtz 2004). A multitude of cells are responsible for the immune response that is triggered upon exposure to a pathogen, with a summary of the main cell types to follow. Critical to the adaptive immune response are lymphocytes, generated from stem cells present in bone marrow. These cells can regulate both humoral and cellular immunity (Morgan, Ruscetti et al.

1976). Broadly, lymphocytes can be split into two distinct categories: B-lymphocytes and T-lymphocytes. These are classified through their mechanisms of antigen recognition, as well as effector function. B-lymphocytes differentiate into either long lived memory cells, or effector plasma cells which secrete antibodies, while T-lymphocytes can differentiate into a multitude of different effector cells, whose primary response to pathogens is mediated through the large scale production of both pro- and anti-inflammatory cytokines.

T-lymphocytes all express multiple T-cell receptors (TCR) on their surfaces, which in turn are responsible for the recognition of different peptide fragments presented in the context of MHC molecules (*figure 1.1*) on antigen presenting cells (Hogquist, Jameson et al. 1994). The resulting TCR–MHC interaction leads to clonal expansion and triggering of a highly specific and targeted immune response that is able to combat infection.
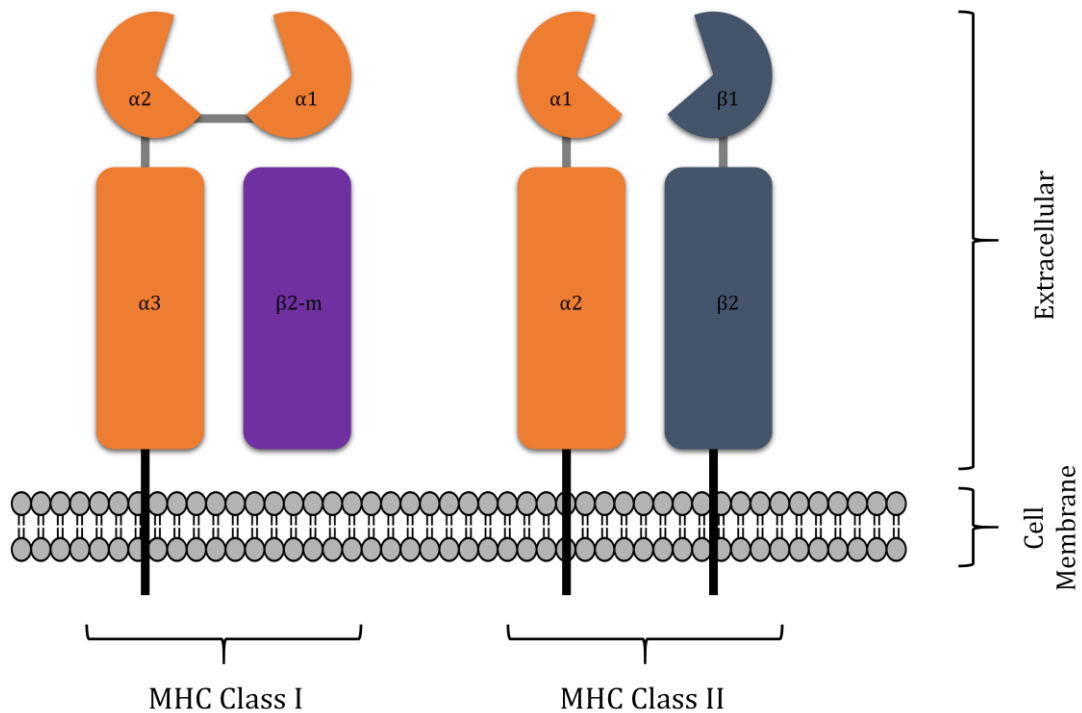
Figure 1.1. _Structure of the MHC displayed on the cell surface._ _MHC class I molecules present antigens to CD8+ T-lymphocytes. They are composed of an alpha chain that is able to signal intracellularly, as well as a beta-microglobulin to complete the structure._ _MHC class II molecules present antigens to CD4+ T-lymphocytes, which are made of an alpha and beta subunit._

1.1.2.1 CELLULAR COMPONENTS – CYTOTOXIC T-LYMPHOCYTES

Cytotoxic T-lymphocytes are primarily CD8+ and are involved in the targeting of infected or otherwise damaged cells. They are activated through a MHC class I restricted molecular interaction, which involves the presentation of specific antigenic fragments to the T-cell through TCR interactions via MHC class I presentation from antigen presenting cells (Norment, Salter et al. 1988). They are responsible for the targeting and killing of specific cells through a cell death pathway involving cytotoxic molecules including perforin, granzyme B

and fas ligand, as well as granulysin. These molecules are released from T-lymphocytes following antigen stimulation (Kaech, Tan et al. 2003). Perforin elicits its action through binding with granzymes, which form a co-complex on the surface of the target cell membrane. This complex allows increased influx of granzyme A and B into the target cell, leading to toxicity (Trapani 1995). Granzymes induce programmed cell death following release from cytotoxic T-cells or NK cells. They exert their action through caspase activation, in addition to increasing the membrane permeability of mitochondria through recruitment of signalling molecules *Bak* and *Bax* (Wei, Zong et al. 2001). Fas ligand binds to the CD95 fas receptor on the cell membrane. Binding triggers a signalling pathway which results in the activation of the caspase apoptotic cell death pathway through caspase-8 activation and signalling to caspase 3 (Andersen, Schrama et al. 2006). The effect of granulysin is brought about through the up-regulation of lipid-degrading enzymes, which result in the loss of structure of cell membranes and the triggering of cell death (Stenger, Hanson et al. 1998).

1.1.2.2 CELLULAR COMPONENTS – HELPER T-LYMPHOCYTES

This subset of T-lymphocyte is involved in the activation of immune cells such as macrophages and phagocytes. Once matured, helper T-cells (T_h) cells express CD4 on their surface which interacts with MHC class II molecules and stabilises the APC T-cell interaction during antigen presentation (Snijdewint, Kaliński et al. 1993). Initially present as naïve helper T-cells, these progenitors can be activated though antigen exposure to the TCR, with resulting progeny

able to become effector, memory or regulatory T-cells, depending on conditions. The differentiation of helper T-lymphocytes occurs through both interleukin-2 (proliferation) acting in an autocrine fashion, alongside interleukin-4 (polarisation) on the STAT4/STAT6 pathway (Kaplan and Grusby 1998). The classic definition of helper T-lymphocytes involves two subsets; T_h1 and T_h2, though newer classifications have been proposed in recent years (***figure 1.2***).
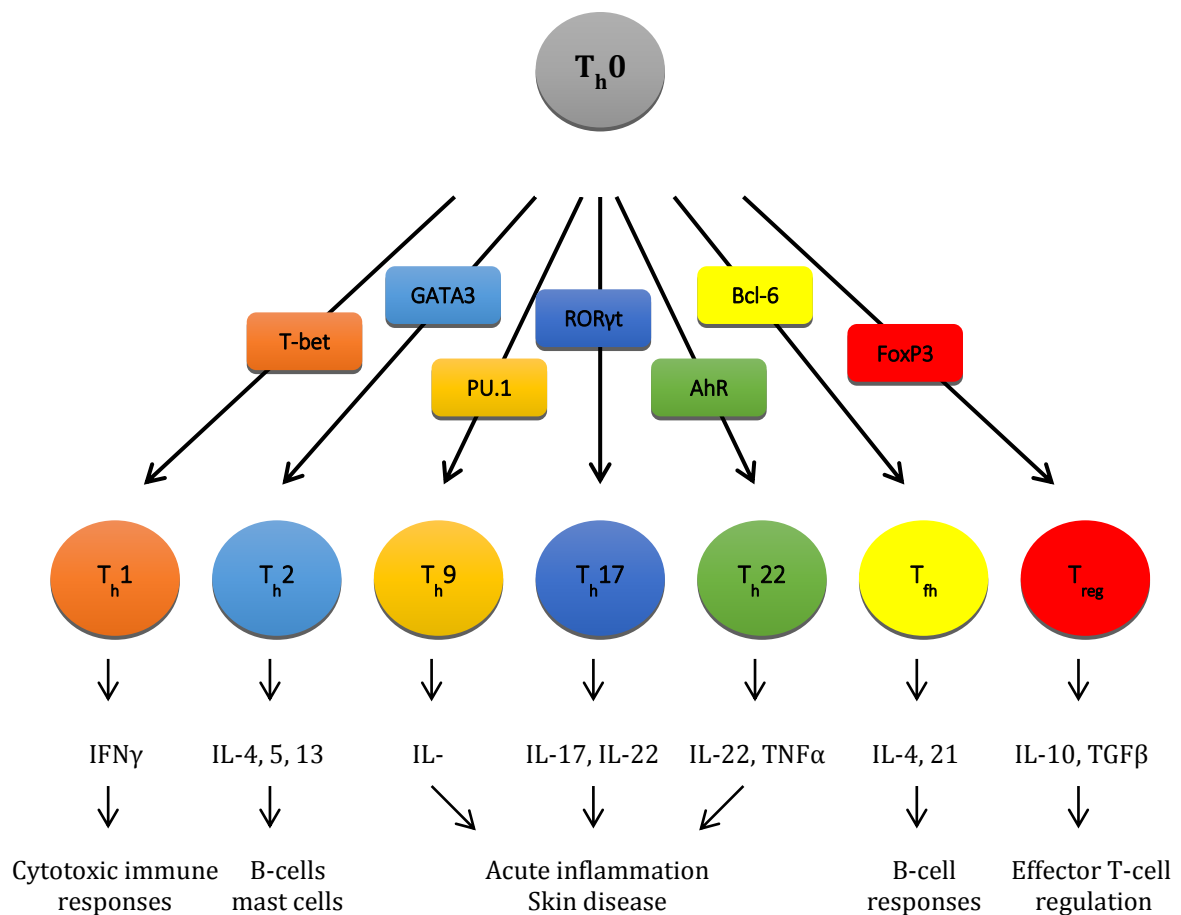
Figure 1.2. *Maturation of Naïve T-lymphocytes. T-cell plasticity can result in a wide range of differentiation pathways. Shown is the differentiation into multiple helper T-cell subsets, with the master transcription factor for each pathway highlighted. Primary secreted cytokines, as well as effector function are also shown below each helper T-cell*

pathway. T-bet (T-box transcription factor), GATA3 (Anti-GABA transporter-3), PU.1 (Macrophage transcription factor), RORγt (Retinoic acid receptor-related orphan nuclear receptor gamma), AhR (Aryl hydrocarbon receptor), Bcl-6 (B-cell lymphoma 6), FoxP3 (forkhead box P3).

1.1.2.2.1   Helper T-lymphocytes – T_h1 / T_h2

Helper T-cells are critical to the continued function of the adaptive immune system. The cytokines they secrete are integral to the production of cytotoxic T-cells, as well as humoral responses. Moreover, T_h cells are involved in class switching and the production of IgE antibodies from B-cells.

After initial activation, proliferative T-lymphocytes have been shown to polarise into either a T_h1 or T_h2 subset (Abbas, Murphy et al. 1996). T_h1 are traditionally associated with a pro-inflammatory phenotype, with high levels of IFN-γ and TNFα secretion. Differentiation of T-lymphocytes into a T_h1 subtype is driven by interleukin-12, alongside the transcription factors STAT4 and T-bet (Szabo, Kim et al. 2000). Further downstream, the activation of STAT1 suppresses interleukin-4 production, needed for T_h2 differentiation. It has been shown that the immune response to infections, chiefly bacteria and protozoa activate a T_h2 response. These bacteria can be killed through the action of iNOS – and the resulting free radicals -produced as a result of IFN-γ mediated macrophage activation (Mills, Kincaid et al. 2000). In addition, T_h1 cells promote NK cell and cytotoxic CD8$^+$ T-cell activation linking to the pro-inflammatory T_h1 model (Constant and Bottomly 1997). Conversely, T_h2 cells are involved in the immune response that targets threats from extracellular pathogens, such as parasitic helminths (Berger 2000). They are generally

considered to be anti-inflammatory, with the cytokines interleukin-5, interleukin-13 and TGFβ secreted. The presence of interleukin-4 is needed for T_h2 polarisation, driven through the activation of transcription factors STAT6 and GATA3 (Zheng and Flavell 1997). In contrast to T_h1 cells, T_h2 lymphocytes are known to recruit eosinophils, mast cells and basophils, as well as acting upon B-cells to trigger the secretion of antibodies. Additionally, T_h2 cells provide a positive feedback loop for rapid recruitment of these cells following antigen stimulation (Bonecchi, Bianchi et al. 1998). Interleukin-10, another cytokine produced from T_h2 cells, has been shown to suppress the polarisation of naïve T_h0 cells into T_h1 cells, as well as inhibiting dendritic cell function (Steinbrink, Jonuleit et al. 1999).

This dual classification has been shown to have its limits however. Interleukin-10 is also known to suppress both T_h1 and T_h2 cells, while continuing to stimulate antibody production. The role of regulatory T-lymphocytes are also not considered in the suppression and activation of $T_h1/2$ cells, with external factors also known to be involved in this regulatory process (Tosolini, Kirilovsky et al. 2011).

Recently, newer subclasses of helper T-lymphocytes have been identified, including T_{fh}, T_h9, T_h17 and T_h22. These do not fit into the old T_h1/T_h2 paradigm, showing that though the T_h1/T_h2 duality is useful as a base, there is a need for greater detail and definition of the roles of these newly discovered helper T-lymphocyte subsets.

Follicular helper T-lymphocytes (T_{fh}) cells are one of the classes that are not able to fit into the existing T_h1/T_h2 paradigm. They are CD4^{+} cells expressing high levels of CXCR5; a receptor for B cell homing. They are found in lymphoid tissues such as the spleen and lymph nodes (Crotty 2014) and are responsible for the production of antibodies through their actions in germinal centres, allowing for somatic hypermutation, cell expansion and class switching to occur (Fazilleau, Mark et al. 2009). Effector B cells are produced as a result of the action of T_{fh} cells in the germinal centres. CD278, encoded by the ICOS gene, has been shown to be integral to the development of T_{fh} cells through studies on knockout mice (Akiba, Takeda et al. 2005). Paracrine activation of the CD40 receptor expressed on B cells by T_{fh} through CD40 ligand binding is also critical for the differentiation that occurs in germinal centres (McHeyzer-Williams, Okitsu et al. 2011). Their role in the maturation and expansion of antibody responses make them integral to the immune responses mounted to antigenic challenge. Additionally, the interplay between interleukin-21 and its activation of CD4^{+} T-cells are dependent on this. A transcription factor that has been identified in the progression of T_{fh} development is Bcl-6. Bcl-6 is known to be modulated through interleukin-4 dependent STAT activation (Harris, Chang et al. 1999; Ichii, Sakamoto et al. 2002).

T_h9 cells were first described in mice. They secrete interleukin-9 as their primary cytokine (Jäger, Dardalhon et al. 2009; Soroosh and Doherty 2009). In contrast to other helper T-lymphocyte subtypes however, these cells do not secrete interleukin-10. Furthermore, regulatory transcription factors that are

essential for differentiation into other T$_h$ subsets (e.g. T-bet, RORγt and Foxp3) are not expressed by T$_h$9 cells (Kaplan 2013). Interestingly, GATA3 and STAT6 – responsible for T$_h$2 differentiation – are expressed at low levels, suggesting that they are involved in T$_h$9 differentiation (Goswami, Jabeen et al. 2012), alongside other cytokines such as interleukin-9 and interferon-α (Schmitt, Klein et al. 2014). Though relatively little is known about their role in humans, recent studies have implicated T$_h$9 cells in multiple conditions, such as asthma and melanoma (Robinson 2010). Their primary roles are thought to be the recruitment of effector cells such as eosinophils and NK cells, as well as promoting the survivability of dendritic cells. T$_h$9 cells are implicated in ulcerative colitis, with a positive feedback loop of interleukin-9 thought to be important for increased intestinal permeability mediated by gut bacteria (Kaplan, Hufford et al. 2015). In addition, recent research has outlined a potential role for T$_h$9 cells in the progression of skin disease, with pro-inflammatory and paracrine functions. Secretion of both TNFα and granzyme B were detected from activated T$_h$9 cells, suggesting that they can directly induce tissue injury (Schlapbach, Gehad et al. 2014).

1.1.2.2.3 Helper T-lymphocytes – Th17

T$_h$17 cells are responsive to signals from both the IL-1R and IL-23R (Chung, Chang et al. 2009). They are considered highly pro-inflammatory due to their large scale production of interleukin-17, TNFα, interferon-γ and MIP-3α (Zambrano-Zaragoza, Romo-Martinez et al. 2014). Primarily involved in microbial defence to pathogens, several studies have shown that they are able

to trigger neutrophil migration (Pelletier, Maggi et al. 2010). Also, T_h17 cells are considered critical in the progression of autoimmune disorders such as psoriasis and asthma, as well as certain cutaneous reactions (Singh, Yamamoto et al. 2014). They are found in high concentrations in barrier environments such as the mucosa and the skin. T_h17 differentiation from T_h0 cells can occur through a cocktail of cytokine signalling, comprising of interleukin-1β, interleukin-6, interleukin-23 and TGFβ (Harrington, Hatton et al. 2005). Interleukin-23, another cytokine secreted from T_h17 cells, has been shown to be involved in chronic inflammation, as well as tissue damage. Another cytokine produced from T_h17 cells, interleukin-21, has been shown to up-regulate T_h17 differentiation in an autocrine and paracrine fashion (Korn, Bettelli et al. 2007). Multiple transcription factors are responsible for the differentiation of naïve T-cells into T_h17 cells, the two key factors being RORγt and STAT3 (Chaudhry, Rudra et al. 2009). An orphan nuclear receptor; RORγt is found in tissues, with promising treatments for the autoimmune disorder rheumatoid arthritis focused on inhibiting this transcription factor, halting T_h17 differentiation (Huang, Xie et al. 2007). STAT3 activation occurs following interleukin-6 exposure. Nuclear translocation is critical for the differentiation of naïve T-cells into T_h17 lymphocytes (Stockinger and Veldhoen 2007). Both interleukin-6 and TGFβ are essential for the differentiation to occur, with both of these cytokines also shown to be inhibited by the presence of T_h1 and T_h2 cytokines. Activation of STAT3 triggers the up-regulation of RORγt which ultimately leads to lineage differentiation into T_h17 cells.

Nuclear translocation is involved in the regulation of gene expression. This is key in helper T-cell differentiation through the ability to activate or suppress certain transcription factors that mediate differentiation, such as both RORγt and STAT3 in the T_h17 pathway. In the case of STAT regulation, tyrosine phosphorylation of the proteins allows for direct binding of the molecule to the promoter regions of target genes. This cytokine-mediated regulation is dependent on the transfer of STAT molecules between the nucleus and cytoplasm; aberration of the cytokine signalling pathway halts STAT translocation and gene expression, leading to a termination of the differentiation pathway (Meyer and Vinkemeier 2007).

1.1.2.2.4   Helper T-lymphocytes – Th22

In 2009, both Trifari *et al*. and Duhen *et al*. independently described a novel subset of helper T-lymphocytes that were distinct from the existing groups. They were characterised by their secretion of interleukin-22 in the absence of interleukin-17, and were so termed T_h22 cells (Duhen, Geiger et al. 2009; Trifari, Kaplan et al. 2009). This important distinction showed that T_h17 and T_h22 cells were separate from each other, rather than variants of the same sub type (Eyerich, Eyerich et al. 2009). T_h22 cells are relatively poorly understood, with little research being conducted exploring their effector function in disease conditions.

Naïve T_h differentiation into T_h22 cells occurs through the presence of the polarising cytokines interleukin-6, as well as TNFα, with further polarisation

observed following addition of interleukin-1β (Fujita, Nograles et al. 2009). In contrast, the presence of TGFβ will inhibit T$_h$22 cell differentiation, instead driving towards a T$_h$17 phenotype (Bettelli, Carrier et al. 2006). An integral part of T$_h$22 differentiation is the presence of the aryl hydrocarbon receptor, with no detectable levels of either RORγt (T$_h$17) or T-bet (T$_h$1) – further underlining the evidence for T$_h$22 cells being a distinct subset rather than a subset of either T$_h$17 or T$_h$1 cells (Esser, Rannug et al. 2009; Jia and Wu 2014). T$_h$22 cells ability to secrete interleukin-22 in the absence of interleukin-17 has been previously characterised, though recent studies have shown a propensity for these T$_h$22 cells to secrete both interferon-γ alongside interleukin-22 (Fujita, Nograles et al. 2009). This could explain the paradoxical nature of the responses that are observed following T$_h$22 activation. For example, T$_h$22 cells have been shown to exert both a pro- and anti-inflammatory effect following activation and cytokine release.

The main function of T$_h$22 cells is to protect the host from invasive pathogens. In addition to this, epithelial barrier control and airway remodelling have also been shown to involve IL-22 (Souwer, Szegedi et al. 2010; Basu, O'Quinn et al. 2012). Several pieces of evidence suggest that IL-22 is also involved in cutaneous pathological conditions. First, the interleukin-22 receptor is located exclusively on epithelial cells such as keratinocytes (Eyerich, Eyerich et al. 2009). Second, T$_h$22 cells have been shown to express high levels of the skin homing chemokine receptors CCR4 and CCR10, allowing for migration to sites of tissue inflammation (Fujita 2013). Thirdly, activated T$_h$22 cells secrete CCL17, which is a ligand for the CCR10 receptor, suggesting a positive feedback mechanism for recruitment and activation (Trifari, Kaplan et al. 2009; Zhang,

Li et al. 2011).   Finally, several studies have shown that expression of T_h22 cells is upregulated in disorders such as psoriasis, atopic dermatitis and inflammatory bowel disease (Eyerich, Eyerich et al. 2009; Nograles, Zaba et al. 2009; Kagami, Rizzo et al. 2010; Kagami, Rizzo et al. 2010; Dige, Stoy et al. 2013).

1.1.2.3 CELLULAR COMPONENTS – REGULATORY T-LYMPHOCYTES

Regulatory T-lymphocytes are characterised as possessing the $Foxp3^+CD25^+CD4^+$ phenotype (Allan, Passerini et al. 2005; Sakaguchi, Ono et al. 2006).   Their functional role in the body is to modulate and suppress the immune system preventing uncontrolled tissue injury.   Suppression of the lymphocytic response is driven via cytokine modulation, through the action of cytokines such as TGF-β or IL-10 which are known to have a subduing effect on the immune system, or through an immunomodulatory receptor pathway via CTLA4 signalling (Rubtsov, Rasmussen et al. 2008; Wing, Onishi et al. 2008). Regulatory T-cells are an important self-check point for autoimmune disorders, as well as tolerance to self-antigens.   TGFβ has been shown to be integral in the development of naïve $CD4^+$ T-cells into regulatory T-cells (Huber and Schramm 2006; Afzali, Lombardi et al. 2007).   They have been shown to express a wide range of TCR with a diverse array of αβTCR present on their cell surface.   This is due to their need for recognition of a wide array of antigens that are encountered throughout the body.

1.1.2.4 CELLULAR COMPONENTS – B-LYMPHOCYTES

CD19^{+} B-lymphocytes are responsible for both antibody production and secretion. They express immunoglobulins across their cell surfaces, which are essential for antigen recognition (Rickert, Rajewsky et al. 1995). Cross-linking of receptors on the cell surface triggers activation and eventual maturation into either plasma cells or memory cells as shown in ***figure 1.3*** (LeBien and Tedder 2008). Plasma cells are responsible for antibody secretion and are essential for complement fixation and the removal of viral fragments (Pelanda and Torres 2012; Nutt, Hodgkin et al. 2015). They are functionally distinct from the other cell type produced following maturation, expressing a CD27^{+}CD138^{+} phenotype (Caraux, Klein et al. 2010). Memory B cells are long lasting, conferring recognition to a previously exposed antigen for years without re-challenge. Upon re-challenge, the memory cells are able to trigger a rapid response through polyclonal activation and mediate their response through highly precise IgG and IgA immunoglobulins (Kurosaki, Kometani et al. 2015).
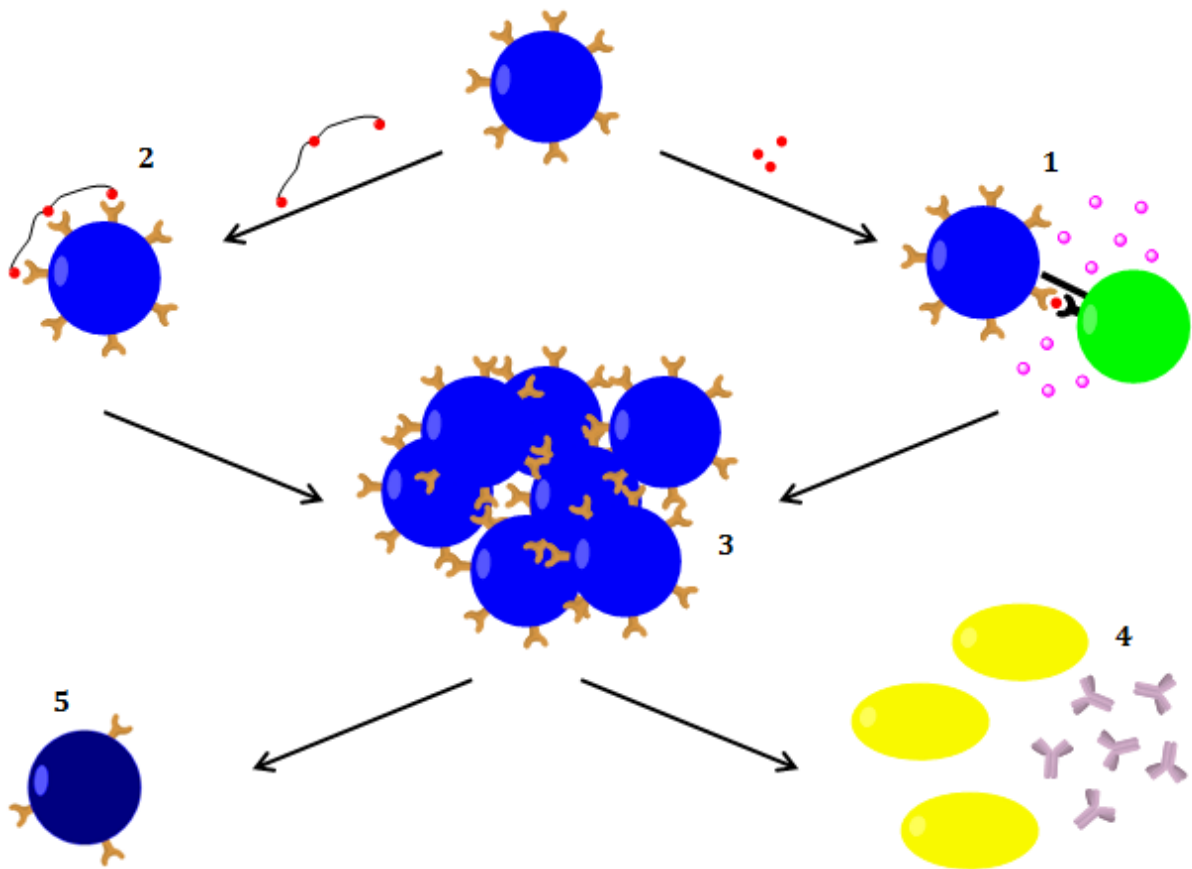
Figure 1.3*. The developmental pathway of B cells.* *Activation of B cells occurs in two ways:*

Thymus-dependant activation (1) involves the recognition of peptide fragments bound to MHCII molecules on APC's by T_h cells, leading to the release of cytokines including IL-4, activating B cells. Independent activation (2) can occur through the cross-linking of multiple IgM antigen receptors on the cell surface by the antigen itself. This can occur with antigens such as bacterial polysaccharides. Following B cell activation, clonal proliferation occurs, (3) resulting in the differentiation into either plasma cell or memory B cells. This proliferation can occur locally, or in the light zones of germinal centres located within the lymph nodes. Activated B cells can undergo somatic hypermutation leading to class switching, involving the rearrangement of heavy chain C regions of the Ig molecule. Class switching favours IgG, IgA and IgE over IgM and IgD. Antigen specificity is retained, allowing for different immunoglobulins to be produced. Plasma cells (4) display high Ig secretion, though they no longer respond to specific T_h signalling. Memory B cells (5) are the progeny of the cells produced in the germinal

centres and are very long lived. Specificity gained during the differentiation in the germinal centres allows the immune response to be triggered quicker and at lower antigenic concentrations when compared to the primary response.

These immunoglobulins are targeted to a single antigen through hypervariable sections located on the antigen binding site (Litman, Rast et al. 1993). Antigen-immunoglobulin binding serves either to inactivate the antigen through steric hindrance, or allowing the recognition by effector cells to target the antigen and mount a suitable immune response. This is accomplished through activation of the complement system, allowing for recognition by target cells such as phagocytes through a process known as opsonisation (Ravetch and Bolland 2001; Rus, Cudrici et al. 2005). ***Figure 1.4*** outlines the maturation of B-lymphocytes in the germinal centers.

These antibodies are either soluble in the plasma of the blood, or bound to the B-cell membrane in the form of a B-cell receptor (Borghesi and Milcarek 2006). These immunoglobulins are found in five classes; IgA (dimer), IgD (trimer), IgE (trimer), IgG (trimer) and IgM (pentamer) (Woof and Burton 2004). IgM and IgD are considered part of the primary immune response, as they are found expressed as receptors on the surface of naïve B-lymphocytes (Goding 1978).
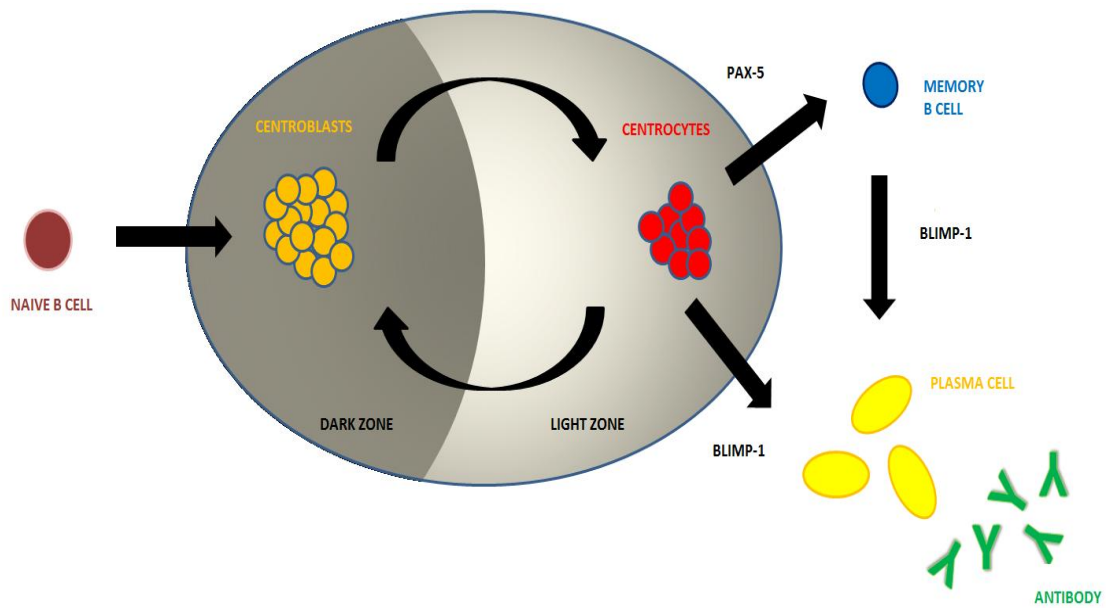
Figure 1.4.   *Germinal Center Activation.* *Clonal proliferation is typically observed in germinal centers (GC) located in the lymph nodes. These GC are locations of very high B cell proliferation. Within these centres, two distinct regions can be observed; light and dark zones, which are located at opposite poles. These poles contain two different populations of the cells: centrocytes aggregate in the light zone, while centroblasts are present in the dark zone. The maturing B cells in the centroblasts undergo somatic recombination responsible for increasing the specificity of the cells to the antigen, along with proliferation. Centrocytes begin to display surface antibodies and are the sub-populations that eventually mature into the progeny cells, either memory B cells or plasma cells. These are formed through multiple signalling pathways, two of the most critical being BLIMP-1 / PAX-5. BLIMP-1 is known to trigger a halt in cellular proliferation.   Co-signalling through the up-regulation of CXCR4 causes cellular migration from the germinal centres to the surrounding tissues.*

1.1.3 CUTANEOUS IMMUNE SYSTEM

The cutaneous immune system is able to be considered a distinct arm in the host immunity in its own right. It provides twofold protection; first, the skin itself is a physical barrier preventing pathogens from entering the body. Second, it houses several cell lineages that are able to counter challenges from invading pathogens (Boguniewicz and Leung 2011). There are large numbers of T-cells present in the skin in the absence of pathogenic challenge, showing that this is a major site of action for the lymphocyte-driven immune response. Studies have estimated the number of T-cells present in the skin to be between 4 billion and 20 billion. T-cells primarily have a memory T-lymphocyte phenotype illustrating previous encounter with an antigen (Bos, Zonneveld et al. 1987; Bos, Hagenaars et al. 1989; Clark, Chong et al. 2006). Studies have shown the upregulation of T_h1, T_h2 and T_h17 lymphocytes in numerous inflammatory skin conditions, though little work has been conducted in regards to the newer T_h9 and T_h22 phenotypes (Di Cesare, Di Meglio et al. 2008; Di Cesare, Di Meglio et al. 2009). However, there is evidence that keratinocytes can produce IL-23, one of the signals required for differentiation into a T_h17/T_h22 phenotype, potentially implicating these subsets in cutaneous reactions (Piskin, Sylva-Steenland et al. 2006).

In the skin, the primary antigen presenting cells are Langerhans cells, which are a subset of dendritic cells. They fulfil the role of antigen presentation in the skin, alongside cutaneous dendritic cells. They are found in the epidermis and are characterised by co-expression of CD207 (langerin) and CD1a (Cella, Sallusto et al. 1997; Bursch, Wang et al. 2007). Langerhans cells migrate to the

lymph nodes following antigen exposure, allowing for antigen presentation to naïve T-lymphocytes. They express surface molecules involved in T-lymphocyte recruitment, activation and inhibition, suggesting an ability to also regulate unwanted immune responses (von Bubnoff, Bausinger et al. 2004). For example, in models of contact hypersensitivity, Langerhans cells have been shown to be involved in the suppression of the immune response (Grabbe, Steinbrink et al. 1995).

Key to the cutaneous immune response is the role of resident memory T-lymphocytes. These are lymphocytes that are located within tissue and do not migrate around the body, as seen with other memory T-lymphocyte subsets such as effector memory (T_{em}) or circulating memory (T_{cm}) cells (Heath and Carbone 2013; Sathaliyawala, Kubota et al. 2013).

These tissue resident memory T-lymphocytes (T_{rm}) are located in the skin and do not migrate to other areas of the body. They are generated following initial antigenic exposure and remain in the affected site for many years following the original challenge, conferring immunity at the site (Masopust, Vezys et al. 2001; Gebhardt, Wakim et al. 2009). They are characterised through the expression of CD103, CD69 and CD8, though CD4 expressing resident memory cells have also been reported (Mackay, Rahimpour et al. 2013; Schenkel, Fraser et al. 2014). Studies have also shown that TGFβ is a critical component of maturation and residence in the skin, due to its upregulation of the expression of CD103 (Casey, Fraser et al. 2012). These memory lymphocytes allow for rapid response to pathogenic exposure without the need for priming and activation by antigen presenting cells in the lymph nodes, allowing for direct

action at the site of pathogen exposure. They have also been shown to be able to differentiate in an antigen-independent fashion, suggesting that location rather than antigenic signalling is a main driving force behind differentiation into a T_{rm} phenotype (Casey, Fraser et al. 2012).

An integral part of cutaneous lymphocytes is the expression of CLA, with over 85% of cutaneous inflammatory lymphocytes shown to express this compared to approximately 5% of circulating lymphocytes (Picker, Michie et al. 1990; Picker, Terstappen et al. 1990). Elegant work by Clark *et al.* showed that over 98% of CLA-expressing T-lymphocytes reside in the skin (Clark, Chong et al. 2006). This, in addition to other surface molecules such as CCR4 and CCR10 are indicators of lymphocyte residence or migration to the skin (Campbell, Haraldsen et al. 1999). CCR10 is of particular interest in cutaneous immunity, due to the high expression of its ligand, CCL27, by keratinocytes (Morales, Homey et al. 1999).

Keratinocytes are one of the major cell types present in the skin. They reside in the outermost layer of the epidermis, where they respond to PAMP signalling. Keratinocytes express several PRR that help to initiate an immune response upon pathogen exposure (Lebre, van der Aar et al. 2007). They are able to secrete multiple cytokines in response to signalling pathways, including TNFα, fibroblast growth factor (FGF) and interleukin-6 (Kupper 1990; Teunissen, Bos et al. 1998). The majority of the cytokines secreted by keratinocytes are not constitutively expressed and are pro-inflammatory in nature. However, there is also evidence for the secretion of β-defensins by keratinocytes that are involved in anti-microbial defence, as well as insulin-like

growth factor (IGF-1), responsible for epidermal maintenance (Seo, Ahn et al. 2001; Edmondson, Thumiger et al. 2003). These cytokines also have the ability to recruit effector cells, such as neutrophils (Reich, Papp et al. 2015). Activation of the keratinocytes via surface expressed triggered TLR often promotes a strong T_h1 response, providing further evidence for their pro-inflammatory role in cutaneous immunity (Miller and Modlin 2007). NK cells have also been shown to migrate to inflamed skin via chemokine signalling from molecules such as CCL5 and CXCL10, both of which are secreted by keratinocytes (Ottaviani, Nasorri et al. 2006).

1.1.4   CYTOKINES: PRODUCTION, RECEPTORS AND FUNCTION

Cytokines are small molecular weight proteins that are integral in cell signalling, proliferation and maturation of other cells. They can also act on themselves (autocrine signalling) or on cells adjacent to them (paracrine signalling), in addition to other more distant locations (Sriuranpong, Park et al. 2003). They are produced from a milieu of cell types including both T- and B-lymphocytes, as well as others such as macrophages and NK cells. Cytokines elicit their function through interaction with receptors located on immune and tissue cells (Stamenkovic, Clark et al. 1989; Fiorentino, Zlotnik et al. 1991; Street and Mosmann 1991; Fehniger, Shah et al. 1999).

T_h1 cytokines are perceived to be pro-inflammatory in nature and exert their effects via the triggering of cellular immune responses. These cytokines include both TNFα and interferon-γ. T_h2 cytokines are more modulatory, with

both pro-and anti-inflammatory cytokines found in this class. They are involved in the induction of an antibody-mediated responses and include cytokines such as interleukin-4, interleukin-13 and TGFβ (Lucey, Clerici et al. 1996; Marzi, Vigano et al. 1996; Hoffmann, Cheever et al. 2000).

There are well over 100 different cytokines that have been characterised – though for the remainder of this section two will be focused on for; the pro-inflammatory cytokine interferon-γ, and the more recently identified interleukin-22. Interferon-γ is classified as a type 2 interferon, sharing little structural homology with other interferons, as well as being coded by a different chromosomal locus. It is coded for by the IFNG gene located on chromosome 12 (Naylor, Sakaguchi et al. 1983). Interleukin-22 is part of the IL-20 subfamily of cytokines, involved in both pro- and anti-inflammatory responses (Sa, Valdez et al. 2007). These cytokines have been chosen due to IFN-γ being the most widely studies pro-inflammatory cytokine produced in response to a wide range of stimulation, as well as IL-22 being indicated in cutaneous reactions.

1.1.4.1 PRODUCTION

These cytokines are produced from multiple cell types, chiefly in response to signals from other cytokines acting upon specific receptors. Interferon-γ is secreted from NK T-lymphocytes, B-lymphocytes and dendritic cells, as well as $CD4^+$ and $CD8^+$ T-lymphocytes. It is produced as a result of cytokines that originate from professional antigen presenting cells – chiefly as a result of

signalling by interleukin-12 and interleukin-18. Interleukin-12 is known to polarise naïve T-lymphocytes towards the inflammatory T_h1 phenotype. In addition, pathogen recognition by macrophages serves to induce a strong IL-12 secretory response, leading to the recruitment of NK T-lymphocytes and interferon-γ secretion. The active molecule itself is a dimer formed from two monomeric structures of the IFN molecule (Ying, Durham et al. 1995; DeMarco, Fink et al. 2005; Chan, Crafton et al. 2006).

Interleukin-22 is secreted from T_h17 and T_h22 lymphocytes, NK cells and fibroblasts (Wolk and Sabat 2006). Its production is induced by secretion of interleukin-23 and interleukin-1β from activated dendritic cells (Satpathy, Briseno et al. 2013), as well as through presentation of lipid antigens in the skin by Langerhans cells via a CD1a restricted pathway (Colonna 2010). The aryl hydrocarbon receptor, a transcription factor, is integral to the generation of interleukin-22 (Esser, Rannug et al. 2009).

1.1.4.2 RECEPTORS

As previously stated, cytokines exert their functions through interactions with specific receptors located on target cells. Cytokine binding induces a downstream signalling pathway that triggers altered gene regulation in the nucleus of target cells, leading to altered protein synthesis. Signalling through the cytokine receptors occurs through a JAK/STAT mediated pathway, which can lead to either genetic regulation directly, or the activation of the caspase pathway. The latter pathway results in activation of the caspase-dependant

apoptosis pathway through upregulation of caspase 9 (Darnell, Kerr et al. 1994; Cardone, Roy et al. 1998; Rawlings, Rosler et al. 2004).

The interferon-γ receptor is heterodimeric, comprising of two chains involved in ligand binding (IFNGR1) and signal transducing (IFNGR2). The IFNGR2 chain is constitutively expressed throughout cell phenotypes, with regulation of this signalling chain responsible for the modulation of interferon-γ through differential expression (Park-Min, Serbina et al. 2007). T_h1 cells have low levels of this chain, while T_h2 cells express it in large amounts. However, the presence of IFNγ leads to T_h1 activation, while conversely inhibiting T_h2 cells. This explains the pro-inflammatory phenotype shift that is observed in the presence of interferon-γ, in addition to providing a positive feedback loop to upregulate cytotoxic cell proliferation in response to infection. Signalling occurs through phosphorylation of the JAK1-STAT1 pathway, resulting in the binding of STAT1 to promoter genes, regulating IFNγ gene expression (Gough, Levy et al. 2008).

Interleukin-22's receptor comprised of a heterodimer consisting of an IL22RA1 and an IL10RB subunit. Though the IL10RB monomer is found throughout the body on a wide range of cell types, the IL22RA1 subunit is only expressed on tissues such as the endothelium and skin. This provides a basis for the hypothesis for interleukin-22 to be involved in cutaneous immune responses (Kotenko, Izotova et al. 2001). Interestingly, a soluble inhibitor of interleukin-22's function, the IL-22 binding protein (IL-22BP/IL22RA2) has also been identified. It shows homology with the IL22RA1 monomer found in the receptor of the cytokine (Kotenko, Izotova et al. 2001). This homology and

opposing effects could explain the paradoxical physiological / toxicological actions of interleukin-22. Similar to interferon-γ, signalling via the IL-22 receptor occurs through the JAK/STAT pathway, through phosphorylation cascades (Lejeune, Dumoutier et al. 2002). In addition, interleukin-22 also triggers ERK and MAPK signalling in endothelial cells (Ouyang, Rutz et al. 2011). In contrast to the IFNγ pathway, is the fact that activation of the IL-22 receptor causes a negative feedback loop to be instigated (Wolk, Witte et al. 2010). One of the downstream effects following IL-22 signalling is the degradation of the IL22RA1 monomer, preventing a recursive loop of activation through reduction of active receptors (Weathington, Snavely et al. 2014).

1.1.4.3 FUNCTION

Cytokine functions vary from activation of both the innate and adaptive immune response, to gene regulation through nuclear receptors. IFNγ has been shown to have both immunomodulatory and stimulatory effects, as well as being able to directly act to inhibit viral replication (Flynn, Chan et al. 1993). It triggers the upregulation of MHC class I molecules on the surface of antigen presenting cells allowing for greater recognition of peptide fragments in response to intracellular pathogens (Yang, Xiang et al. 1995). In addition, IFNγ acts to induce the TAP transporter, integral to peptide transport and loading in the lumen of the endoplasmic reticulum (York and Rock 1996). Expression of multiple genes are altered by IFNγ, for example CXCL9 (upregulation, T-lymphocyte chemoattractant), IRF1 (upregulation, apoptosis mediator), PKR

(upregulation, anti-viral enzyme) and c-myc (downregulation, cell cycle) (Takizawa, Ohashi et al. 1996; Taniguchi, Lamphier et al. 1997; Obaya, Mateyak et al. 1999; Gil, Bohn et al. 2001). The synergistic effect of IL-18 and IL-12 in the induction of a T_h1 mediated response is integral to its role in the response to an intracellular pathogen, and the resulting pro-inflammatory phenotype that is observed (Dinarello, Novick et al. 1998).

The overall function of IL-22 is not fully elucidated. It is produced primarily from T-lymphocytes such as NK cells, as well as both CD4^{+} and CD8^{+} T-cells (Zheng, Danilenko et al. 2007; Spits, Artis et al. 2013). Interleukin-22 is thought to be involved in the defence against extracellular pathogens, as well as maintenance of homeostasis in tissues (Sonnenberg, Fouser et al. 2011; Rutz, Eidenschenk et al. 2013; Zenewicz, Yin et al. 2013). These protective functions have been outlined in both the intestine and lungs, though expression of the IL-22 receptor suggests this protective function could extend to other tissues. In addition, fibroblasts increase protease production following IL-22 stimulation, leading to wound repair (McGee, Schmidt et al. 2013). However, studies have also looked at the pathogenic role of interleukin-22 in multiple diseases. Over expression of IL-22 has been implicated in conditions such as psoriasis, rheumatoid arthritis and enthesitis (Wolk, Haugen et al. 2009; Sherlock, Joyce-Shaikh et al. 2012; Zhang, Li et al. 2012). Though there is evidence for the pro-inflammatory nature of interleukin-22 signalling, these are predominately tied to pathogenic conditions. The majority of the characterised functions of IL-22 seem to be protective in nature, such as the association between IL-22 expression and

liver function protection through STAT3 signalling (Hanash, Dudakov et al. 2012).

The wide effects of cytokines produced by immune cells are part of complex signalling pathways that modulate a variety of downstream effects to promote immune responses against invading pathogens. However, uncontrolled cytokine production may promote autoimmune diseases in susceptible individuals.

1.2   DRUG METABOLISM

Drug metabolism has been a term used since the 1950's to describe the biomedical modification of xenobiotics by specialised enzyme systems. Drug metabolites are eventually removed from the body through either biliary or renal excretion. Most drugs are highly lipophilic and as such poorly excreted. To counteract this, metabolism occurs which transforms the drugs into more water soluble compounds allowing for simpler excretion. The primary site of drug metabolism is the liver, evidence for localised metabolism being involved in the progression of hypersensitivity reactions has been hypothesised (Ju and Uetrecht 1999; Oesch, Fabian et al. 2007). Metabolism of drugs can lead to the formation reactive metabolites that bind covalently to protein to generate the antigen which can be recognised by the immune system (Lavergne, Park et al. 2008).

Sulfamethoxazole is used to treat infections in patients with cystic fibrosis and HIV. Pichler and Tilch cloned T-cells from SMX hypersensitive patients, with the parent drug found to activate both CD4^{+} and CD8^{+} proliferative responses and cytokine secretion (Pichler and Tilch 2004). Importantly, PBMC from hypersensitive patients are also activated by a metabolite, SMX-NO. It can mimic the action of the parent drug (or vice versa) by binding directly to MHC binding peptides to activate CD4^{+} and CD8^{+} T-cells (Mauri-Hellweg, Bettens et al. 1995; Schnyder, Mauri-Hellweg et al. 1997).

As a result of the potential involvement of reactive metabolites in drug hypersensitivity a number of drugs are withdrawn from use after initial greenlighting by the medical community. Although this may prevent

unforeseen harmful side effects and save the pharmaceutical industry hundreds of millions of pounds, it prevents a more detailed understanding of the role of metabolism in hypersensitivity reactions.

1.2.1 DRUG METABOLISING ENZYMES

The majority of drug metabolism (upwards of 95%) occurs in the liver (Uetrecht and Naisbitt 2013). However, other locations are involved in metabolism of drugs, including immune cells, which have been shown to express low levels of cytochrome P450 enzymes. Furthermore, skin expresses its own compliment of drug metabolising enzymes which can selectively metabolise certain drugs (Swanson 2004).

Enzymes responsible for drug metabolism are classified into two categories: microsomal and non-microsomal. Microsomal enzymes are located in the smooth endoplasmic reticulum of tissues, and can be induced by certain drugs, such as rifampicin and alcohol (Madan, Graham et al. 2003). This class of enzymes contains the commonly expressed P450 enzymes, as well as others such as epoxide hydrolases and uridine diphospho-glucuronosyltransferase. Non-microsomal enzymes are located in the cytoplasm. These are non-inducible and are generally involved in conjugation reactions, such as in the case of esterases. Metabolism generally occurs in two steps, phase I, followed by phase II metabolism. This sequential metabolism was first put forward in 1983, and is explained in greater detail below (Neuberger and Smith 1983).

1.2.2 PHASE I METABOLISM

The two most important families of phase I metabolising enzymes are the cytochrome P450 enzymes and the flavin-containing monooxygenases (FMO). Typically, the end result of phase I metabolism is to attach small functional

groups such as sulfhydryls (-SH), hydroxyls (-OH) or carboxyls (-COOH) to the parent compound. These functional groups serve to make the overall structure more polar, allowing for conjugation via phase II enzymes (Park, Pirmohamed et al. 1992; Pirmohamed, Madden et al. 1996; Stachulski and Lennard 2000).

Cytochrome P450 enzymes are a large superfamily of enzymes located in the endoplasmic reticulum of tissues, predominantly the liver. This superfamily is responsible for three quarters of all metabolism that occurs throughout the human body (Berka, Hendrychová et al. 2011). The primary transformation that is catalysed by the P450 family is oxidation (Werck-Reichhart and Feyereisen 2000). Over 50 distinct P450 enzymes have been identified, though only a fifth are involved in drug metabolism to any significant degree. The CYP superfamily are polymorphically expressed, with different isoforms of the enzymes directly responsible for altered metabolic activity of specific compounds. Examples of this include the CYP2D6 family and their role in antipsychotic drug metabolism, as well as CYP3A5 polymorphisms (Gillam, Guo et al. 1995; Hustert, Haberl et al. 2001) Some treatments and drugs are contra-indicated in patients known to express certain gene polymorphisms.

The major CYP superfamily consists of 3 sections, with a total of 8 subclasses (CYP1A, CYP1B, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A). These enzyme families account for 95% of all drug metabolism through the CYP pathway, with CYP3A4 and CYP2D6 alone responsible for over half of the drug metabolism (Wilkinson 2005).

FMO's are the other major enzyme involved in phase I metabolism, involved in both NADPH-dependent oxidation and reactive intermediate generation. There are 5 subclasses of FMO's, labelled FMO1-5. They are known to play a

role in the metabolism of drugs; FMO1 is involved in sulfamethoxazole metabolism and FMO3 is involved in tamoxifen metabolism (Krueger, VanDyke et al. 2006; Vyas, Roychowdhury et al. 2006). Like CYP enzymes, the FMO's are located in the endoplasmic reticulum, and often work in concert with the CYP enzymes for drug detoxification.

1.2.3 PHASE II METABOLISM

Phase II metabolism acts upon the newly acquired functional groups that were added to drugs during the functionalisation process that occurred through phase I metabolism. The goal of phase II conjugation reactions is to increase the molecular weight and assist excretion of the product. Though the majority of compounds undergo both phase I and II metabolism, there are cases where conjugation can occur directly without the need for functionalisation (Kassahun, Mattiuz et al. 1997). Typical conjugates include glutathione (conjugating with the reactive intermediate NAPQI) or glycine. A number of transferases are involved in the conjugation to less toxic compounds, such as the glutathione-s-transferases (Hayes, Flanagan et al. 2005; Perricone, De Carolis et al. 2009).

1.2.4 GENETIC POLYMORPHISMS

Allelic variance and single nucleotide polymorphisms lead to inter-individual variations in drug metabolism. Identification of polymorphic enzymes involved in the metabolism of drugs associated with hypersensitivity could

explain the idiosyncratic nature of the reactions. Metabolism is rarely either 'on' or 'off'; rather, a sliding scale of fast and slow metabolisers (Holstein, Plaschke et al. 2005; Kirchmair, Williamson et al. 2013). Despite this, it is still unclear how variable metabolic rates impact on the development of hypersensitivity.

As individuals are exposed to different amount of potentially toxic metabolites through the metabolic variability, there is the potential for increased danger signals to be presented to the immune system. If an individual was a poor metaboliser of an allergenic drug, this would lead to increased T-lymphocyte activation due to a more favourable environment for T-cell priming.

One of the most widely studied compounds in relation to metabolic variability is the sulphonamide sulfamethoxazole. Both the parent compound and the metabolite sulfamethoxazole-nitroso are detoxified through the NAT1 and NAT2 enzymes. Poor metabolism of these compounds has been linked with an increase in hypersensitivity reactions (Pirmohamed, Alfirevic et al. 2000). This is shown to be directly related to the metabolic activity of the NAT2 enzyme. However, no significant associations were found between phenotype and hypersensitivity prevalence of the NAT or CYP2C9 (the enzyme involved in the formation of SMX-NO) following analysis in a HIV-positive cohort (Rieder, Shear et al. 1991; Pirmohamed, Alfirevic et al. 2000). These data suggest that there are multiple determining factors in the development of hypersensitivity.

1.3   MECHANISMS OF LYMPHOCYTE ACTIVATION

To understand mechanisms of immune cell activation, a number of terms must be defined. First, an immunogen is any substance that 'triggers an immune response'; secondly, a hapten is 'a low molecular weight compound capable of irreversibly modifying macromolecules'; and finally an antigen is something that 'binds with high affinity to immunological receptors' (Uetrecht and Naisbitt 2013). These terms are used consistently throughout this thesis.

1.3.1 ANTIGENIC STIMULATION OF T-LYMPHOCYTES: TCR ACTIVATION, SIGNAL 1, 2, + 3

Activation of T-lymphocytes requires three signals from an antigen presenting cell, with the different signals termed 1, 2 and 3. Firstly, signal 1 is provided through the interaction between a peptide-MHC complex and the appropriate TCR. Triggering the TCR is critical for T-cell activation. However, in the absence of other signals the T-cell will be tolerised and become anergic. Full activation only occurs when a co-stimulatory signal, also known as signal 2, is present. Co-stimulatory signals are antigen non-specific, yet are essential for full activation. This involves a complex interplay of co-stimulatory and co-inhibitory signals, where activation only occurs when the co-stimulatory signals predominate. Signal 3 describes the cytokines released from antigen presenting cells following antigen uptake. Though not essential for T-lymphocyte activation, signal 3 is critical to fully design the nature of the cellular immune response (Curtsinger, Schmidt et al. 1999).

Displayed on the surface of cells, MHC I and MHC II exist to display peptide fragments on the cell surface and allow recognition by T-lymphocytes. Both MHC I and MHC II are similar in structure. However, they differ markedly in the pathway of antigen presentation, and the peptides that interact with the peptide binding groove. MHC I molecules are expressed on all nucleated cells and serve to present peptides of an intracellular origin to CD8$^+$ T-lymphocytes. These MHC class I molecules are assembled through interactions between the MHC class I heavy chain (45 kDa) and a chaperone protein calnexin in the endoplasmic reticulum. Further incorporation of β_2 microglobulin (12 kDa)

leads to the formation of the peptide loading complex (Heemels and Ploegh 1995). This can then associate with the proteins TAP1 and TAP2 (<u>T</u>ransporters associated with <u>A</u>ntigen <u>P</u>resentation), allowing for incorporation of processed peptide fragments (between 8-10 amino acids in length) located in the cytosol into the fully formed MHC class I molecule. These TAP proteins are located exclusively in the membrane of the ER and in the cis-golgi complex, with their main function to transport intracellular peptide fragments for antigen presentation. As members of the ABC transporter family, they are activated through ATP-dependent binding (Ortmann, Androlewicz et al. 1994). Once a bound peptide is incorporated into the MHC class I molecule located in the membrane of the endoplasmic reticulum (ER), it then dissociates from the ER and is transported via the golgi apparatus to the surface of the cell for CD8^{+} T-cell recognition. Peptide presentation occurs on the extracellular portion of the MHC, with the α1 and α2 subunits forming the peptide binding site. This binding site is involved in direct interactions with the T-cell receptor, while the α3 subunit interacts with the CD8 molecule that is co-expressed on the T-cell. Multiple pockets are located in the peptide binding groove, termed A-F, which can incorporate bulky side chains of certain amino acids in the presented peptide fragments. These pockets also act to anchor the peptide in the binding site, through hydrophobic interactions between the amino acids on the peptide and the binding groove (Madden, Gorga et al. 1992). Both the A and F pocket are known to show conserved structures – 95% of amino acids present in the F pocket are one of 7 different AA (Elvin, Potter et al. 1993).
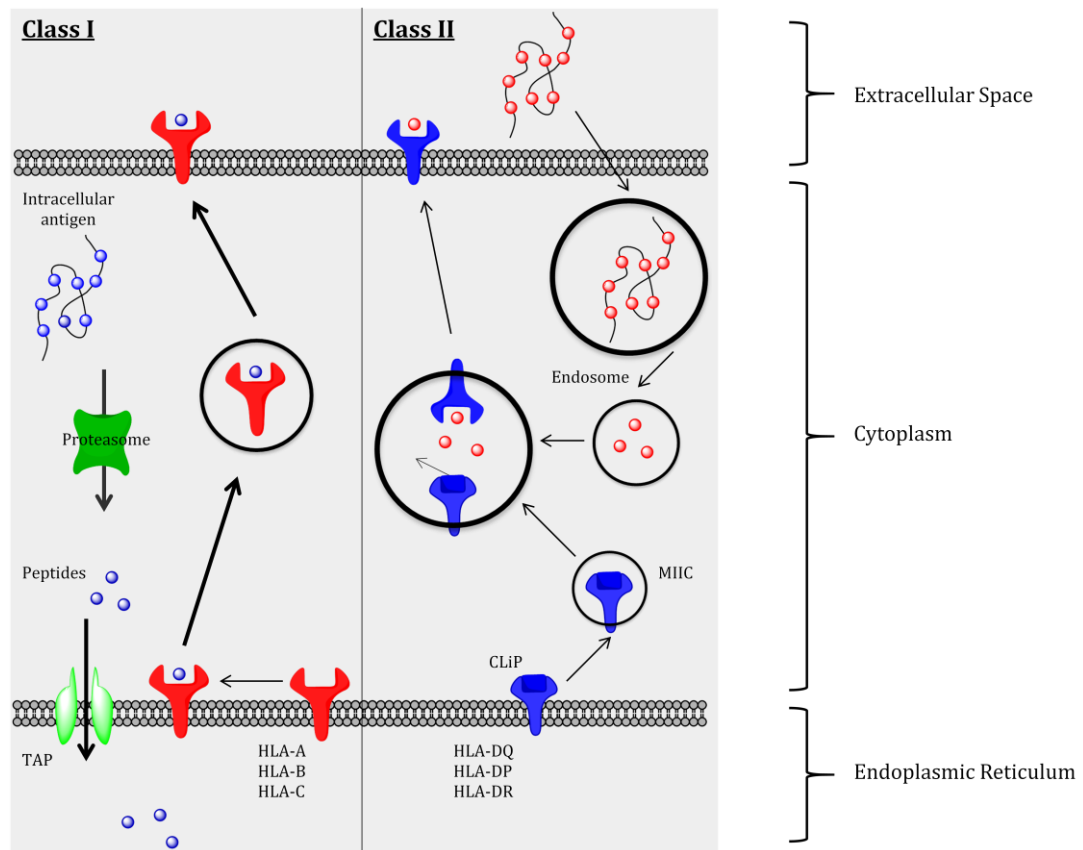
Figure 1.5. *Mechanisms of antigen processing and presentation.*

Class I *– Intracellular antigens are processed into peptide fragments by the proteasome. These fragments are then transported inside the endoplasmic reticulum via the TAP transporter, then loaded onto the MHC I molecules present there. These MHC I molecules then is transported via the golgi complex to the cell surface via vesicles, where it is expressed and able to interact extracellularly with CD8+ T-lymphocytes.*

Class II *– Extracellular antigens are phagocytosed into the cell, then processed in the phagosome to form peptide fragments. These fragments are then carried in the endosome to a lysosome. MHC class II molecules bound to a stabilising Li chain dissociate from the endoplasmic reticulum and transport to the lysosomes via the golgi. Once in the lysosome, the Li chain is degraded by proteases. Additionally, the CLiP dissociates from the MHC II molecule, allowing for peptide loading and presentation. These lysosomes then transport the MHC II molecules to the cell surface for presentation to CD4+ T-lymphocytes.*

MHC II binds to peptides originating from the extracellular environment. These peptides are formed through the phagocytosis of extracellular proteins through the interaction of lysosomes. Taken up from the extracellular space, antigens are degraded as a result of the vesicle pH. These lysosomes contain enzymes which are involved in the antigen processing pathway. Vesicles containing the processed peptides then fuse with other vesicles containing the unbound MHC class II molecules, allowing for antigen binding and migration to the surface of the cell. Antigen binding to the MHC class II molecule is mediated through the actions of the Class II associated invariant chain (CLiP) peptide. This peptide is situated in the peptide binding groove, halting other peptide presentation. Removal of the CLiP peptide is regulated through HLA-DM binding, allowing for exogenous peptide loading and presentation (Santambrogio, Sato et al. 1999; Thayer, Ignatowicz et al. 1999).

Peptides derived from processed proteins eventually activate CD4$^+$ T-lymphocytes. MHC II molecules are found on the surface of antigen presenting cells and contain two glycoprotein subunits – the α and β chains. Expression can be up-regulated following inflammation and exposure to other co-stimulatory ligands. MHC classes are further subdivided, with MHC I containing HLA-A, HLA-B and HLA-C structures, while MHC II contains HLA-DP, HLA-DQ and HLA-DR (Denzin and Cresswell 1995). In most cases professional antigen presenting cells such as dendritic cells express MHC class I and II and are involved in antigen presentation. Other cells such as B-lymphocytes and macrophages also express MHC class I and II. In contrast, most tissue cells express MHC class I – but not MHC class II (Ting and Trowsdale 2002). MHC presentation is summarised in *figure 1.5*.

Antigen presentation can also occur via an alternate mechanism. Cross-presentation is integral to the recruitment of CD8^{+} T-lymphocytes in the response to extracellular antigens and involves presentation on MHC I molecules via a subclass of dendritic cells (Joffre, Segura et al. 2012).

While MHC class I are known to present small peptide fragments between 8-10 amino acids, MHC class II are able to present larger fragments of 12-25. These peptides interact with grooves inside the MHC structure prior to translocation of the MHC molecule to the plasma membrane for antigen presentation. MHC binding involves a complex array of Van der Waals interactions as well as specific hydrogen bonds. In the MHC class I, peptide 5 is integral to successful binding in the TCR/peptide complex (Rudolph and Wilson 2002). MHC class II binding is less dependent on peptide interactions, with the antigenic binding groove more open when compared to MHC class I. It is because of this that the MHC class II are able to present more complex, longer peptide fragments (Neefjes and Ovaa 2013).

Following successful MHC-TCR interactions, downstream signalling is initiated. This leads to proliferation of the T-lymphocytes and regulation of cytokine secretion. CD4 / CD8 co-receptors are expressed on the T-cell surface, leading to TCR clustering and eventual activation. TCR phosphorylation by *Lck* leads to the recruitment of *Zap-70*, the main signalling pathway in T-cell activation. This in turn phosphorylates other molecules including *LAT*, leading to a signalling cascade through the *LAT* signalosome (Smith-Garvin, Koretzky et al. 2009). Release of inositol triphosphate (IP3) and diacylglycerol (DAG) follow, with resulting *MEK/ERK* signalling from DAG through the *Ras* protein leading

to CD69 upregulation on the cell surface, a hallmark of cell activation (D'Ambrosio, Cantrell et al. 1994). IP3 signals lead to the release of stored $Ca2^+$ from the endoplasmic reticulum. This influx is critical in the initiation of cellular production of interleukin-2, needed for continued lymphocyte survival (Liu, Bunnell et al. 1998).

Co-signalling pathways are also essential for the modulation of T-cell activation. If Signal 1 is present along co-stimulatory signal 2, then activation will occur. However, if a co-inhibitory signal 2 dominates then it will lead to cellular anergy and tolerance (Sharpe and Abbas 2006). In addition, it has been shown that co-signalling pathways are able to modulate and even prevent the differentiation of T-lymphocytes into subsets, such as T_h1 and T_h17 (Mizui, Shikina et al. 2008).

A well characterised signal 2 pathway involves the activation CD28, expressed on T-lymphocytes, and its ligands CD80 and CD86 expressed on the surface of antigen presenting cells. Early modulation of the signal 2 pathways looked at this interaction, with inhibition of CD28 shown to induce T-cell anergy (Harding, McArthur et al. 1992). CD80/CD86 are expressed solely on activated antigen presenting cells, providing a checkpoint for autoimmunity (Chen and Flies 2013). This CD28 – CD80/CD86 interaction was shown to be a co-stimulatory signal, through its activation of phosphokinase C (PKC), triggering the production of interleukin-2 through signalling via NFAT (Acuto and Michel 2003).

Multiple co-inhibitory signalling pathways have recently been identified. Programmed death-1 (PD-1) interacts with its ligand PD-L1 and is a key

regulator of T-cell inhibition. It is widely expressed on a variety of cell types and has been shown to regulate autoimmunity (Nishimura, Nose et al. 1999). The receptor is only expressed on active cells, with the ligands induced by pro-inflammatory mediators such as IFNγ and GM-CSF (Keir, Freeman et al. 2007). Activation of the receptor leads to the recruitment of SHP-1 and SHP-2 proteins, which in turn act to dephosphorylate *Zap-70*, causing inactivation of the lymphocyte through a cessation of the signalling pathway (Sheppard, Fitz et al. 2004).

Other co-inhibitory pathways are also beginning to be elucidated, all as a component of a complex signalling cascade as outlined in the comprehensive review by Chen and Flies (Chen and Flies 2013). It is important to note that the signalling pathways here, such as CTLA4 and TIM-3, work in concert with each other to provide an overall response in T-lymphocyte activation or inhibition.

Signal 3 is a much less defined pathway, mediated through the action of cytokines released by dendritic cells. Naïve lymphocytes that are activated in the absence of signal 3 cytokines show poor viability and effector function (Mescher, Curtsinger et al. 2006). As the naïve lymphocytes are only responding to peptide fragments, rather than the whole antigen itself, it is interesting to note that specific antigen targeting responses are mounted by the differentiated lymphocytes. These lymphocytes have never encountered the original antigen, so it is reasonable to assume that the final activation signal comes from the dendritic cells themselves (Curtsinger and Mescher 2010). These polarising signals come in the form of cytokines secreted from

the dendritic cells located at the site of antigenic encounter (Kalinski, Hilkens et al. 1999). The full complement of polarising cytokines needed to drive the differentiation into effector and helper T-lymphocyte subsets have already been explained in section 1.1.2.2, but are also summarised below in ***figure 1.6***.

Naïve T-lymphocyte Polarisation		
Terminal Differentiation	Polarising Conditions	Transcription Factor
T_h1	IL-2, IL-12	T-bet / STAT4
T_h2	IL-2, IL-4	GATA3 / STAT5
T_h9	TGFβ, IL-4, IL-9	PU.1
T_h17	TGFβ, IL-1β, IL-6, IL-21,	RORγt
T_h22	IL-6, TNFα	AhR
T_{fh}	IL-21, CD278	Bcl-6

Figure 1.6 *Polarising conditions for naïve T-lymphocyte differentiation. Different helper T-lymphocyte subsets can be polarised based on the cytokine milieu that is present signalling to the naïve lymphocytes. The polarising cytokines are listed for each T-helper subset, with the master transcription factor also noted for each case.*

1.3.2 HLA POLYMORPHISMS

Human leukocyte antigen (HLA) alleles that code for MHC proteins are a key component of variable presentation of peptides to circulating T-lymphocytes. These alleles are contained in the HLA gene complex, located on chromosome 6, with approximately $3x10^6$ base pairs in this region. It is the most polymorphic gene region in the entirety of the human genome (Parham and Ohta 1996).

These polymorphisms are one of the key driving factors behind the development of hypersensitivity reactions to drugs. This genetic linkage was first hypothesised in 1999, with carbamazepine hypersensitivity identified in a pair of monozygotic twins (Edwards, Hubbard et al. 1999). Following on from this, other HLA polymorphisms have been associated with drug induced hypersensitivity, the most extensively studied being abacavir – HLA-B*5701 linkage, which now precludes individuals with this HLA phenotype from being treated with the compound (Mallal, Nolan et al. 2002; Mallal, Phillips et al. 2008). Only in cases where a patient's genotype is known to be HLA-B*5701 negative will abacavir treatment be implemented (Martin, Klein et al. 2012).

Carbamazepine is the only other drug aside from abacavir to require HLA genotyping prior to treatment, due to its strong association with HLA-B*1502 in a Han Chinese population. Similar to abacavir, it has a strong specificity and sensitivity for individuals with this genotype (Chung, Hung et al. 2004). Both carbamazepine and abacavir CD8$^+$ T-cell responses are restricted to the risk allele, with no cross-reactivity with other alleles observed.

Multiple other HLA associations have been reported with different drugs (*figure 1.7*), however their low predictive values make genetic screening for these polymorphisms unrealistically expensive to prevent hypersensitivity. For example, HLA-B*5701 is also associated with development of flucloxacillin hypersensitivity (Daly, Donaldson et al. 2009). However, only around 1 in 1000 individuals with this HLA allele will go on to develop flucloxacillin hypersensitivity (Andrews, Armstrong et al. 2010). Additionally, flucloxacillin is known to share sequence homology with other HLA alleles, including HLA-B*5801. This homology manifests in the ability of flucloxacillin-specific T-

lymphocytes being restricted primarily to HLA-B*5701, but also HLA-B*5801 to a lesser degree (Monshi, Faulkner et al. 2013). Other polymorphisms that have been implicated in drug hypersensitivity include lumiracoxib – HLA-DRB1*1501, allopurinol – HLA-B*5801 and nevirapine – HLA-B*3505 (Chung, Hung et al. 2007; Chantarangsu, Mushiroda et al. 2009; Tassaneeyakul, Jantararoungtong et al. 2009; Singer, Lewitzky et al. 2010). Multiple mechanisms have been proposed to explain how drugs are able to activate T-lymphocytes. These are discussed in greater detail below.

HLA Class	Drug	Association	Adverse Event
Class I Association	Abacavir	B*57:01	AHS
	Allopurinol	B*58:01	HSS / SJS / TEN
	Carbamazepine	A*31:01 B*15:02	SJS / TEN
	Co-Amoxiclav	A*02:01 B*07:02	DILI
	Flucloxacillin	B*57:01	DILI
	Nevirapine	B*35:05	Cutaneous Reactions
Class II Association	Aspirin	DPB1*03:01	Asthma
	Co-Amoxiclav	DRB1*15:01 DQB1*06:02	DILI
	Lumiracoxib	DRB1*15:01 DQB1*06:02 DRB5*01:01 DQA1*01:02	DILI Hepatic Failure
	Nevirapine	Cw4	Cutaneous Reactions
	NSAIDs	DR11	Cutaneous Reactions
	Ximelagatran	DRB1*07 DQA1*02	DILI

Figure 1.7. *Genetic associations with drugs known to cause adverse reactions in humans. Class I and class II associations have been observed in multiple drugs with varying odds ratios. Certain compounds have been shown to have more than one association, such as the multiple HLA-D alleles with lumiracoxib, or both class I and II associations, such as nevirapine. Multiple adverse reactions have been reported as a result of individuals with these alleles being administered the compound.*

1.3.3 HAPTEN HYPOTHESIS

The hapten hypothesis originating from the 1930s is the oldest recognised model for explaining the chemical basis of drug hypersensitivity. Landsteiner and Jacobs' seminal work in 1935 utilised the model hapten dinitrochlorobenzene (DNCB) to explore the effect of chemical exposure on the sensitisation of guinea pigs (Landsteiner and Jacobs 1935). Their data suggested that the chemical reactivity of DNCB and more specifically the modification of proteins, via attacking specific nucleophilic amino acid residues led to an antigen specific immune response. Studies conducted in subsequent years support the original hapten concept and show that the potential of a chemical to activate lymphocytes relates directly to the reactivity of low molecular weight compounds (Kitteringham, Maggs et al. 1985; Pickard, Smith et al. 2007). Critical to the hapten hypothesis is the concept that the administered chemical itself is initially a non-immunogenic compound – the lymphocyte response is only observed once the chemical is bound covalently to a protein – this protein conjugate may then be immunogenic, with the ultimate antigen being peptides derived from the processed conjugate. Many drugs are administered as inert compounds, which are only able to form protein conjugates following metabolism; these compounds are termed pro-haptens (Naisbitt, Williams et al. 2001; Posadas and Pichler 2007).

Investigations into specific locations of protein binding have shown distinct areas of interactions, with electrophilic locations on the compounds and nucleophilic residues on proteins integral to the lymphocyte activation,

confirming Landsteiner's hypothesis put forward in 1936 (Chipinda, Ajibola et al. 2010).

Work conducted by Weltzien's group showed that penicilloyl peptide epitopes designed to bind to specific MHC class II molecules were able to be recognised and trigger an antigen specific response in a classical hapten mechanism from antigen-specific T-cells (Padovan, Bauer et al. 1997). This, as well as the ability of metal ions to interact with autologous proteins, was eloquently reviewed by Martin, showing that hapten mediated lymphocyte responses were critical to allergic diseases caused by low molecular weight compounds (Martin 2004).

Recent studies using cloned T-lymphocytes reveal that lymphocyte responses develop in one of two ways; either through direct modification of peptides embedded in surface MHC molecules by the low molecular weight compound itself (processing independent) or through the formation of a protein conjugate which is encountered, phagocytosed and processed via antigen presenting cells, which in turn present the MHC bound peptide antigen to the T-lymphocytes (Whitaker, Meng et al. 2011; El-Ghaiesh, Monshi et al. 2012; Jenkins, Yaseen et al. 2013; Yaseen, Saide et al. 2015).

Drug metabolites have been linked to several forms of idiosyncratic drug reaction, with an increased level of reactive metabolite thought to correlate with increased risk of an adverse drug reaction (ADR) (Nakayama, Atsumi et al. 2009). However, other studies have shown that certain drugs are associated with a high incidence of ADRs yet no reactive drug metabolites are formed during administration, such as in the case of ximelagatran, so the role of

reactive drug metabolites in idiosyncratic drug reactions is still not fully elucidated (Uetrecht 2008).

1.3.4 ALTERED SELF PEPTIDE REPERTOIRE

Recent research has highlighted the fact that a section of the MHC gene plays a major role in the development of drug hypersensitivity. These human leukocyte antigen alleles are highly heterogeneous coding regions involved in the presentation to TCR of peptides via the MHC molecules (Madden, Garboczi et al. 1993). A strong association between abacavir and HLA-B*5701 was elucidated by Mallal in 2002. Subsequently, abacavir has been used to study drug-specific T-cell responses and the results obtained led to the development of the altered self-peptide hypothesis (Mallal, Nolan et al. 2002). This theory works on the basis that abacavir is able to interact at specific locations within the antigen binding groove on the MHC molecule. When present, these drugs then alter the self-peptide repertoire that usually interacts at that location. The presence of the drug alters the tertiary structure of the MHC molecule, leading to antigenic recognition of a normally harmless peptides (Martin, Nolan et al. 2004; Chessman, Kostenko et al. 2008; Bharadwaj, Illing et al. 2012). Specifically, abacavir interacts non-covalently with the F pocket of the antigen binding cleft, but only in cases where the HLA-B*5701 allele is present (Illing, Vivian et al. 2012; Norcross, Luo et al. 2012). This leads to the binding of almost 900 novel self-peptides. It is believed that one or more of these peptides trigger an immune response, specifically cytotoxic CD8^{+} lymphocytes.

As a result of this, patients are now screened for the HLA-B*5701 allele before the commencement of abacavir therapy. This is the first case of a genetic test being implemented to avoid adverse effects based on known outcomes, suggesting that many other drug reactions have the potential to be acting in a similar manner. However, to date abacavir is the only drug that has been shown to activate T-cells via this mechanism (Yun, Adam et al. 2012).

1.3.5 PHARMACOLOGICAL INTERACTION OF DRUGS WITH IMMUNE RECEPTORS

Chemically inert drugs have been shown to activate lymphocytes through mechanisms independent of either processing by antigen presenting cells or metabolism. Multiple drugs have been shown to elicit activation of T-cells through this mechanism, including sulfamethoxazole, carbamazepine and lamotrigine (Elsheikh, Castrejon et al. 2011). This direct interaction occurs in a reversible interaction between drug and the MHC molecule. In contrast to the hapten hypothesis, this method of activation occurs rapidly, in a matter of seconds. This timeframe suggests that the activation must be occurring independently of antigen processing (Schnyder, Burkhart et al. 2000; Pichler, Beeler et al. 2006), which requires more time to take place.

This hypothesis is supported further by the following study results; firstly, pulsing experiments that incorporate the drug in question point towards non-covalent MHC-peptide binding, due to the fact that washed antigen presenting cells do not activate T-lymphocyte responses (Schnyder, Mauri-Hellweg et al. 1997). This implies that the antigen presenting cells processing machinery is

not involved in the presentation of the drug to the TCR. Secondly, drug activation leads to downstream receptor signalling, including Ca^{2+} release. This release is delayed with protein antigens due to the time required for processing. In contrast, drug-specific responses are rapid in nature, which precludes the involvement of antigenic processing (Zanni, von Greyerz et al. 1998). Finally, antigen presenting cells that are incapable of protein processing, such as in the case of fixation via glutaraldehyde, are shown to stimulate T-lymphocyte activation in *in vitro* assays with drugs such as lamotrigine, sulfamethoxazole and carbamazepine (Schnyder, Mauri-Hellweg et al. 1997; Naisbitt, Farrell et al. 2003; Wu, Sanderson et al. 2006).

The PI concept is a distinct method of activation from the classical hapten hypothesis, though it does not preclude the possibility that a combination of both hapten and PI activation might be active in certain drug hypersensitive patients. For example, both sulfamethoxazole and sulfamethoxazole-nitroso are capable of activating T-lymphocytes isolated from the skin and blood of patients suffering with sulfamethoxazole hypersensitivity (Nassif, Bensussan et al. 2002; Farrell, Naisbitt et al. 2003). The metabolite response is dependent on adduct formation and antigen processing. In contrast, responses to the parent drug occur rapidly and are best explained by the PI concept. The key differences between these proposed mechanisms of T-cell activation by drugs are highlighted in ***figure 1.8 and 1.9***.

	Hapten Hypothesis	Direct Metabolite Binding	PI Concept	Altered Peptide Concept
A - Pulsing APC with drug antigen	✓	✓	✗	✓
B - Processing Inhibition	✓	✗	✗	✓
C - Glutathione	✓	✓	✗	✗
D - Enzyme Inhibition	✓	✓	✗	✗
E - T-cell activation Kinetics	Delayed	Rapid	Rapid	Delayed

Figure 1.8. *Experimental evidence to differentiate between the different pathways of drug (metabolite)-specific T-cell activation.*

(A) - Pulsing experiments involve the culture of APC for (1 – 16h) followed by repeated washing steps to remove non-covalently bound drug. The pulsed APC are then used in T-cell assays as the source of antigen. (B) - Antigen processing can be blocked via the addition of several chemicals to APC. (C) - Glutathione blocks the protein-reactivity of cysteine-reactive drug metabolites. It can be added to T-cell assays with no known non-specific effects. (D) - Enzyme inhibitors can be added to T-cell assays to block drug metabolism. Experiments to date have been limited as it is not known which drugs are metabolised in the assay. (E) - It is possible to monitor the kinetics of T-cell activation by measuring internalisation of T-cell receptor expression or T-cell calcium release. Antigen processing takes several hours, hence the response is delayed.

1.3.6 DANGER HYPOTHESIS

First proposed in 1994, the danger hypothesis was outlined by Matzinger. It states that the immune response can be triggered upon the recognition of certain 'danger signals' – usually as a result of cellular stress or damage – though the lack of these signals can lead to immune tolerance (Matzinger 1994; Anderson and Matzinger 2000; Pirmohamed, Naisbitt et al. 2002). As discussed earlier, a full response by the immune system is only triggered with the presence of three signals, while activation of the immune cells occurs following the recognition of two signals (Curtsinger, Schmidt et al. 1999).

According to Matzinger, danger signalling through exposure to PAMPs or DAMPs will upregulate signal 2 (dendritic cell co-stimulatory interactions) and promote a pathogenic response. Danger signalling was initially discussed in terms of drug hypersensitivity in a review by Pirmohamed (Pirmohamed, Naisbitt et al. 2002). This is an attractive proposition as many drug metabolites are intrinsically toxic. Thus, products released by the necrotic cells might act as DAMPs and activate dendritic cells. In support of this concept, chemical sensitisers which are directly reactive and damage cells at low concentrations upregulate dendritic cell co-stimulatory receptor expression and cytokine release. Sanderson *et al* showed that metabolism of SMX to a nitroso metabolite activates dendritic cells (Sanderson, Naisbitt et al. 2007). However, studies with other drugs have not been forthcoming, therefore the importance of drug specific dendritic cell signalling remains open to debate.
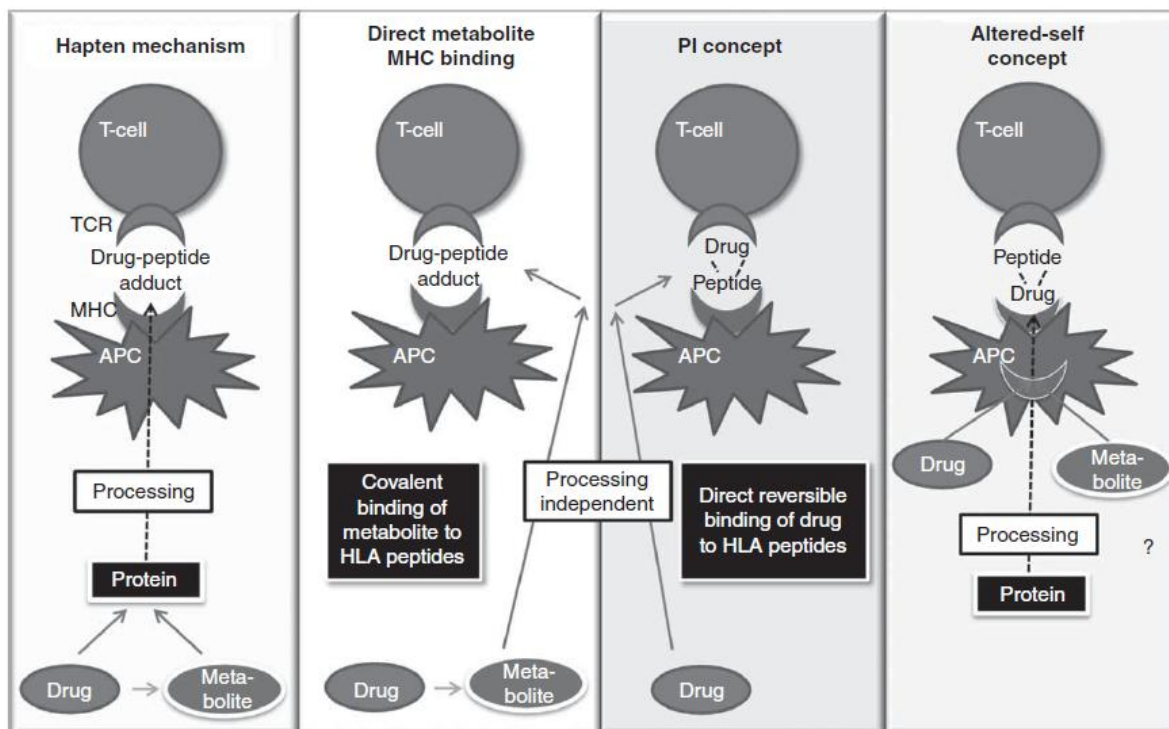
Figure 1.9. *Pathways of drug (metabolite)-specific T-cell activation. Chemically reactive metabolites (and directly reactive drugs) activate T-cells via a hapten mechanism and through direct modification of MHC or MHC binding peptides. It remains to be determined whether drug metabolite MHC binding can alter the repertoire of self-peptides displayed by MHC molecules. Drugs and stable metabolites activate T-cells through direct MHC binding.*

1.4   ADVERSE DRUG REACTIONS

In recent years, there has been a proliferation of drugs used for the treatment of a multitude of illnesses and disease. As a result of this, adverse reactions to drugs are becoming more common (Pirmohamed, James et al. 2004). Due to the cost, time and negative treatment opinions, the need for understanding the molecular mechanisms of adverse drug reactions is more important than ever. The cost of withdrawals of drugs associated with adverse reactions, when factoring in research costs can run into hundreds of millions of pounds, so the economic reasons for elucidating mechanisms are also clearly relevant. Summarised in *figure 1.10* are the compounds that have been withdrawn from use since 1961. Genetic polymorphisms, drug composition and structure, gender, age, and underlying clinical conditions all contribute towards susceptibility to ADRs (Pirmohamed, Breckenridge et al. 1998). Clinical manifestations include the relatively mild skin rashes, urticaria and fixed drug eruptions. A lower number of patients develop serious skin reactions such as Stevens-Johnson syndrome and toxic epidermal necrolysis (Lazarou, Pomeranz et al. 1998). Other organs including the liver and kidneys can also be involved either in isolation or as a component of a generalised hypersensitivity syndrome (Edwards and Aronson 2000; Larrey 2001).

1.4.1 ADR - DEFINITION

As defined by the World Health Organization, an adverse drug reaction is 'a response to a drug which is both noxious and unintended, and which occurs at doses normally used in man for the prophylaxis, diagnosis, or therapy of disease, or for the modifications of physiological function.' (WHO, 1972). While a broad, encompassing statement, there has been controversy regarding the wording, with some scientists arguing that it is not accurate enough. Edwards and Aronson took issue with the 'noxious' part of the definition in 2000, arguing that it precludes ADR that are not harmful, but merely inconvenient (Edwards and Aronson 2000). In response to this, they proposed an alternative definition; 'an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product.' This updated definition is widely accepted, and allows for a broader range of symptoms to be included and ultimately defined in the context of ADR.

Drug Name	Year Identified	Clinical Manifestation
Benfluorex	2009	Pulmonary Hypertension
Benoxaprofen	1982	DILI
Clioquinol	1975	Myelo-optic Neuropathy
Indoprofen	1984	GI Bleeding
Lumiracoxib	2007	DILI
Noscapine	1991	Gene Toxicity
Roziglitazone	2010	Cardiovascular Events
Suprofen	1987	Renal Dysfunction
Temafloxacin	1992	Haemolytic Anaemia
Thalidomide	1961	Teratogenesis
Ximelagatran	2006	DILI
Zimeldine	1983	Hypersensitivity

Figure 1.10. _Approved drugs subsequently withdrawn from marker since 1961. Selected drugs withdrawn from use since 1961 due to resulting adverse effects. Adapted from Stephen's detection and evaluation of adverse drug reactions,_ Talbot et al _2012._

1.4.2 ADR - CLASSIFICATION

Adverse drug reactions are normally classified into two distinct types; Type A (dose-dependent) and Type B (independent of dose, idiosyncratic).

Type A reactions are known, predictable responses that develop in response to the pharmacological action of the drug in question. Approximately 85% of reported drug reactions are Type A, with the observed responses increasing in severity in a dose-dependent manner (Bates, Cullen et al. 1995). The observed symptoms in Type A reactions are usually resolved in response to cessation of treatment. These reactions can be triggered through multiple factors, such as intentional overdose, impaired metabolism and drug retention.

Type B reactions are both unpredictable and not directly dose-dependent, with multiple factors influencing their progression (Pirmohamed, Madden et al.

1996; Zaccara, Franciotta et al. 2007). Often referred to as 'bizarre', due to their idiosyncratic nature, meaning that they are not able to be predictable from the known pharmacology of the drug in question. Typically, Type B responses involve the triggering of the hosts immune system, leading to reactions commonly being referred to as hypersensitivity reactions. Both the adaptive and innate immune system are implicated in the progression of these reactions.

These classifications are somewhat broad, and by their nature do not take into consideration a milieu of different potential influencing factors. Recent discoveries into the linking of certain drug hypersensitivity cases to HLA associations, such as in the case of abacavir, allopurinol and carbamazepine, mean that these classifications are becoming outdated (Hung, Chung et al. 2005; Mallal, Phillips et al. 2008; McCormack, Alfirevic et al. 2011). An ever expanding list of HLA-drug associations means that these interactions are playing a key role in certain reactions, and as such need to be considered in future classifications.

Edwards and Aronson again attempted to expand the classification with their own recommendations, using dose-related (Augmented), non-dose related (Bizarre), dose- and time-related (Chronic), time-related (Delayed), withdrawal (End of use) and therapy failure (Failure), though these are less common than the traditional Type A/B definitions (Edwards and Aronson 2000).

1.4.3 ADR - EPIDEMIOLOGY

Many studies have investigated the relative frequency of drug reactions as a cause for hospital admission. Of these, it is estimated that 6.5% of hospital admissions were due to adverse drug reactions, with upwards of 100,000 annual hospital admissions related in some way to drug exposure (Lazarou, Pomeranz et al. 1998; Pirmohamed, James et al. 2004). It is well known that adverse drug reactions can play a major role in overall patient mortality, with ADRs listed as the 7th most frequent death contributing factor (Lavergne, Park et al. 2008). However, other studies have investigated this in detail, and have found additional contributing factors to the relative abundance of ADR mortality. These factors include race, socio-economic status and age (Dormann, Neubert et al. 2004; Shepherd, Mohorn et al. 2012). In cases where a specific predetermining risk factor has been investigated, there have been reports suggesting that increased incidences of ADR occur in patients with viral infections (Levy 1997; Shiohara, Inaoka et al. 2006), specifically HIV (Lucas, Chaisson et al. 1999). Polypharmacy (van der Ven, Koopmans et al. 1991; Pirmohamed and Park 2001) might also be a contributing factor in chronic conditions such as cystic fibrosis (Whitaker, Naisbitt et al. 2012).

Recently, investigations into genetic polymorphisms have assisted in our understanding of potential mechanisms of ADR. Mutations in coding genes for enzymes involved in drug metabolism can lead to an altering of the function of the enzyme. This alteration can take the form of increased activity (fast-metaboliser), decreased activity (slow-metaboliser) or even an inactivation of the enzyme itself (no metabolism) (Meyer 2000; Pirmohamed and Park 2001).

These alterations can lead to cases where a normally therapeutic dose of drug can build up and cause ADR when they would not be expected. This differential toxicity has been reported with several drugs (Shenfield 2004; Empey 2010). The cytochrome P450 (CYP) enzymes are well known to be highly polymorphic, and as such can show different metabolic activity, therefore drug metabolism can differ between otherwise similar individuals (Zhou, Liu et al. 2009).

1.4.4 DHR - DEFINITION

As a subset of ADR, drug hypersensitivity reactions (DHR) are defined in relation to ADR. They are defined as 'an adverse drug reaction with an immunological aetiology to an otherwise safe and effective therapeutic agent, when administered at recommended doses'. Drug hypersensitivity reactions are complex, immune-mediated reactions with a wide array of symptoms ranging from mild to very severe, along with a host of risk factors (Pohl, Satoh et al. 1988).

1.4.5 DHR - CLASSIFICATION

The classical definition of drug hypersensitivity reactions was provided in 1963 by Gell and Coombs, with immune-mediated reactions classified into 4 distinct categories (Gell and Coombs 1963). These categories were defined primarily through the time of onset of symptoms, with consideration also of

the underlying mechanistic aspects of the response being observed. These categories were termed I-IV, as discussed below and in ***figure 1.11***.

Type I: An immediate response following sensitisation, with IgE the primary mediator. These are caused through antigen cross-linking of receptors on the surfaces of immune cells such as mast cells, triggering histamine, cytokine and eicosanoid release. These mediators cause a clinical response, the most severe of which is anaphylaxis.

Type II: These are cytotoxic reactions, considered semi delayed. They are mediated through natural killer cells along with complement fixation, and are triggered through either IgG or IgM antibodies that are produced by B-cells. Examples of this type of reaction include haemolytic anaemia and thrombocytopenia.

Type III: These are also semi-delayed reactions, again triggered through either IgG or IgM antibodies. This time binding to soluble antigens occurs, as opposed to cell derived antigens in Type II reactions. Excess antigen in the system causes the development of immune complexes. These types of reactions include vasculitis and lupus.

Type IV: These reactions are typically delayed onset reactions, with observable symptoms not noticeable until days after initial exposure to the antigen. They are primarily T-lymphocyte mediated. Both cytokines and cytolytic molecules released from the T-lymphocytes are responsible for initiating the observed clinical response, with multiple diseases known to be triggered in this way, such as drug hypersensitivity syndromes, leishmaniasis, urticaria and toxic epidermal necrolysis.

Due to the wide range of mediators, as well as the differing effector molecules produced, the classic model of Type IV was deemed insufficient, with further stratification required to allow for greater understanding of the mechanisms involved. In 2003, Pichler set out to better define the Type IV reactions, with further sub-division into Type IVa-d, utilising the cells involved in different conditions (i.e. monocytes, neutrophils, eosinophils and cytotoxic T-lymphocytes) to establish the classification (Pichler 2003). The expanded classification is summarised in detail in *figure 1.12*.

	Type I	Type II	Type III	Type IV
General Phenotype	Anaphylaxis	Cytotoxicity	Immune Complex	Delayed
Initiation Time	<1h	<2h	4-8h	>48h
Antibodies	IgE	IgG, IgM	IgG, IgM	N/A
Effector Cells	Basophils, Eosinophils	Antibodies, Complement Cascade	Neutrophils, Complement Cascade	Lymphocytes
Drugs	Cephalosporin	Penicillin	Penicillin	Piperacillin
Examples	Hay Fever	Thrombocytopenia	Rheumatoid Arthritis	Contact Dermatitis

__Figure 1.11.__ Hypersensitivity classification. Type I – IV hypersensitivity can be distinguished through multiple different parameters, shown on the left of the table. Key to the classification is the time of onset, as well as the effector cells that primarily mediate the resulting hypersensitivity.

1.4.6 DHR - EPIDEMIOLOGY

Drug hypersensitivity reactions, although relatively rare are of major clinical concern, and are responsible for a significant percentage of hospital admissions each year (Einarson 1993; Gomes and Demoly 2005). They belong to the Type B styled reactions, where known pharmacology cannot be relied on to predict a patient response. A multi-hospital study conducted in Boston reported a hypersensitivity incidence of 6.1% in 4031 patients during a 6-month period (Bates, Cullen et al. 1995), Koch et al. described a 4.5% incidence rate in a cystic fibrosis patient cohort (Koch, Hjelt et al. 1991), with a meta-analysis performed by Lazarou *et al*. drawing similar results (Lazarou, Pomeranz et al. 1998). However, recent reviews have stated that the overall prevalence of DHR is not fully understood, due to the relative lack of data sets available (Gomes and Demoly 2005; Demoly, Viola et al. 2007). A common cause of DHR is the beta-lactam class of antibiotics (Idsoe, Guthe et al. 1968). Several studies suggest a DHR incidence rate of between 1-8% to the commonly prescribed benzyl-penicillin (Macy and Contreras 2014). Solensky *et al*. stated that when a patient is shown to be sensitive to one beta-lactam, the likelihood of responding to another is increased, making the identification of DHR more complicated (Solensky 2003). Multiple drug hypersensitivity is becoming a major clinical concern, especially in patients routinely exposed to multiple drug regimens (Whitaker, Naisbitt et al. 2012).

	Effector Cells	T-Lymphocyte Secretions
Type IV a	Monocytes	IFNγ
Type IV b	Eosinophils	IL-4, IL-5
Type IV c	Keratinocytes	Perforin, Granzyme B, Fas Ligand
Type IV d	Neutrophils	IL-8

Figure 1.12. _Hypersensitivity Type IV classification._ _Type IV hypersensitivity reactions have been further classified based on the recommendations set forward by Pichler in 2000. These take into consideration the effector cells that mediate the immune response, as well as the major secretions from the activated T-lymphocytes._

1.4.7 DHR - DURATION

As previously mentioned, the Gell and Coombs classification system took into consideration the relative time of onset of the hypersensitivity in its division of DHRs (Gell and Coombs 1963). These can range from immediate, up to months after initial administration. The majority of T-cell mediated reactions take between 1-30 days for symptoms to occur. Proliferation of T-lymphocytes occurs a couple of days after exposure to the antigen, and can continue for several weeks.

1.4.8 DHR - DIAGNOSIS

Currently, the diagnosis of DHRs is only able to be confirmed through the use of _in vivo_ skin tests. Well established protocols have been established to carry out skin prick, patch and intradermal tests (Brockow, Romano et al. 2002). However, in all of these tests there are disadvantages, such as the patient compliance due to the invasive nature of the tests, along with the relatively low

sensitivity levels, which can sometimes lead to false-negatives being obtained. In cases of uncertain response, drug challenge tests can be implemented. Challenge tests can lead to the triggering of latent severe symptoms, resulting in patient discomfort or even death, and as such are rarely conducted unless under strict medical guidelines and supervision (Messaad, Sahla et al. 2004).

Drug-specific T-lymphocytes can be isolated from peripheral blood, along with both blister fluid and inflamed skin samples of patients with suspected DHRs. These drug-specific lymphocytes are known to be long lasting, with the lymphocytes being able to be detected in patients years after the last administration of drug (Bøyum 1976; Dunbar, Ogg et al. 1998).

Because of this availability of drug-specific lymphocytes from patients, *in vitro* tests provide a potentially valuable way to investigate the pathogenesis and mechanisms underlying drug hypersensitivity reactions, with minimal danger or discomfort to the patient. Though better than many clinically used skin tests, sensitivity of potential *in vitro* tests are still not completely 100%, so accurate diagnosis is still reliant on patient histories. The following assays rely on isolated PBMC from patients, so their usage is dependent on access to these PBMC samples, either fresh or from a cryopreserved sample, in addition to having a researcher that is trained in the specific techniques.

1.4.8.1 T-LYMPHOCYTE CLONING

Drug-specific T-lymphocytes often represent a small subset of the total lymphocyte population. Because of this, T-cell cloning allows for the expansion of the drug-specific population, eliminating the problems of low cell numbers.

T-cell cloning is performed using a standardised procedure that has been established for many decades (Lamb, Eckels et al. 1982; Altman, Moss et al. 1996; El-Ghaiesh, Monshi et al. 2012). Using the isolated T-lymphocyte clones, cellular phenotypes, effector function, proliferative responses and cell surface markers can be characterised. As previously stated, drug-specific T-lymphocytes are a small proportion of the total lymphocyte population. For this reason the use of T-cell cloning is highly prevalent and important in the understanding of the pathomechanisms of drug hypersensitivity reactions.

1.4.8.2 LYMPHOCYTE TRANSFORMATION TEST

The lymphocyte transformation test (LTT) (also known as a lymphocyte proliferation assay) is the most widely used *in vitro* method to diagnose potential drug hypersensitivity reactions (Pichler and Tilch 2004). The test works through the measurement of proliferation of T-lymphocytes in response to drug exposure over a set time frame, when compared to control samples. This is done through the incorporation of radioactive thymidine, which is used in DNA synthesis. Thymidine does not allow additional replication to occur, acting as a terminal base, so the assay itself is time sensitive. LTT's have a broad application domain, with the assays dependent on only T-lymphocytes and the drug in question. Both immediate and delayed-type hypersensitivity can be investigated through an LTT, further enhancing its effectiveness (Nyfeler and Pichler 1997). Both sensitivity and specificity in the assay are variable, with much depending on the drugs therapeutic dose and potential for cross reactivity (for example, piperacillin, a beta-lactam antibiotic does not

cross react with other beta-lactams, while flucloxacillin and dicloxacillin have been shown to cross react) (Monshi, Faulkner et al. 2013). Generally, a small peripheral blood sample is sufficient to conduct the assay, with peripheral blood mononuclear cells isolated and used for analysis. Cultures with the drug are incubated for 96h before thymidine addition, with readouts taken 16h later. A positive result is interpreted with a stimulation index, where control proliferation is measured versus antigenic proliferation, with a stimulation index (SI) of greater than 2 considered a positive result.

1.4.8.3 FLOW CYTOMETRY

Flow cytometry is a technique that can identify cell surface markers, along with the ability to sort cell populations into functionally distinct subsets. Diagnosis of a T-lymphocyte response can occur through the investigation of distinct cell surface markers, either from peripheral blood or tissue-specific cells. These markers can also be used to identify antigen-specific T-lymphocyte subsets.

T-lymphocyte activation can be characterised through the identification of activation markers, such as CD69. Other markers of activation include CD40L and CD71 (Simms and Ellis 1996; De Rosa, Herzenberg et al. 2001).

By utilizing fluorescent antibodies, multiple markers can be investigated concurrently, with highly specific sub-populations of cells being able to be identified through the relative expressions of each marker.

A proliferative response can also be studied using the fluorescent biomarker carboxyfluorescein diacetate succinimidyl ester. This biomarker halves its intensity upon each subsequent cell division following drug stimulation of

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lymphocytes. The biomarker itself is incorporated into the cells via interactions with intracellular proteins (Lyons and Parish 1994; Lyons 2000).

1.4.8.4 INTRACELLULAR STAINING

A subdivision of flow cytometry, intracellular cytokine staining allows the investigation of the cytokine profile of antigen stimulated cells. Different subsets of T-lymphocytes triggered by an antigen are phenotypically similar; there are no cell surface markers that can definitively distinguish between a T_h1 and T_h2 lymphocyte response. However, these cells can be characterised through their ability to secrete different cytokine profiles, such as the pro-inflammatory IFN-γ for T_h1 and classically anti-inflammatory IL-4 and IL-13 for T_h2. Using fluorescent antibodies specific to different cytokines, intracellular staining allows the investigation of multiple lymphocyte subsets through their cytokine secretion profiles (Scheibenbogen, Letsch et al. 2005; Horton, Thomas et al. 2007).

1.4.8.5 ENZYME LINKED IMMUNOSORBENT SPOT ASSAY

An enzyme linked immunosorbent spot (ELISpot) assay is designed to characterise cytokine secretion from single cells, both when resting and following antigen stimulation. It is a very quick and easy assay to perform and can give highly sensitive and specific readouts. T-lymphocytes are the primary cell type used for this type of investigation, with a large panel of cytokines able to be looked at using relatively low cell numbers. Activated T-lymphocytes will

secrete a milieu of both pro- (IFN-γ, TNF, etc.), anti-inflammatory (IL-4, IL-13) and regulatory (TGFβ, IL-10) cytokines. ELISpots allow for detection of low frequency cytokine secreting cells to be investigated in a short time frame (McCutcheon, Wehner et al. 1997; Crotty, Aubert et al. 2004; Farrell, Lichtenfels et al. 2013; Gibson, Ogese et al. 2014).

In addition, effector molecules such as perforin and granzyme B are also able to be detected via ELISpot (Engler, Strasser et al. 2004). High levels of sensitivity are reported with this assay. Antigen specific IFNγ secreting T-cells have recently been detected in greater than 90% of penicillin hypersensitive patients using ELISpot (Rozieres, Hennino et al. 2009). Compared with a similarly designed ELISA assay, positive results are able to be detected at levels lower than ELISA, though ELISA assays allow for storage of supernatants for multiple analysis in the future, whereas ELISpots rely on the availability of viable lymphocytes.

It is important to note that while the ELISpot displays very high sensitivity, it does possess a relatively low specificity range. For instance, limits are present in the number of 'spots' that are able to be detected following successful assay development. This is dependent on the ability of the image display to discern between different unique spots. For this reason, even the most precise images will not be able to detect more than approximately 500 spots per well of an ELISpot, despite the presence of many thousands of cells capable of secreting cytokines. The term 'spot forming unit' is employed to address this issue, where a spot forming unit can potentially be many cells all secreting cytokines in the same general location on the ELISpot well, while not being sufficiently dispersed to be individually identified by the ELISpot imaging software. This

solves the dichotomy of there being tens of thousands of cells per well, while having fewer than 500 'spots'.

T-cell activation requires an interaction with APCs and antigen that provides a sufficiently strong signal to activate the T-cell. The level of antigen presented by individual APCs may not be optimal, some of the T-cells may not interact with APC (T-cell : APC ratio is 5:1) and the T-cell may not be able to respond; all of which can account for the lack of cytokine secretion relative to cell numbers. The ability of the T-cell to respond is also dependent on the cell being in the resting stage, or G_0, of the cell cycle (Lea, Orr et al. 2003). The T-cell clones are not synchronised with respect to the cell cycle during these assays, as well as potentially continuing to proliferate in culture due to the presence of IL-2. Thus, the number of cells in G_0, and therefore cells that are responsive, may be low.

1.4.8.6 MULTIPLEX

A multiplex (Luminex) assay is similar to an ELISpot in many ways. The primary difference is the ability to investigate multiple cytokines in a single assay. The fluorescent beads linked to antibodies are used to allow for different spectral characteristics to be incorporated into the assay design. This allows for multiple cytokine readouts from a single cell population. The assay, while functionally superior to the traditional ELISpot, is less widely used due to the relative high cost of the assay (Siawaya, Roberts et al. 2008).

1.4.9 CUTANEOUS DHR

Hypersensitivity reactions that occur following drug exposure are able to target many organs simultaneously.   Common targets of these reactions include the liver, kidneys, heart and skin, with the skin reactions being the most commonly described (Roujeau 2005). The skin is the largest organ in the human body, so its prevalence in drug hypersensitivity reaction symptoms is not surprising. Approximately 3% of cases of hospital admittance due to drug reactions report skin involvement. The high prevalence of blood vessels in the skin means T-cell reactions are more likely to occur here, with relatively easy supply to the organ when compared with other organs.   Skin reactions themselves can vary greatly, both in appearance and relative severity. Mild cutaneous reactions in the form of maculopapular exanthema and urticaria are much less dangerous, as well as much more common. Though rare, reactions such as toxic epidermal necrolysis and Stevens-Johnson syndrome are very dangerous, with a high mortality rate observed. Other reactions can include photosensitivity, fixed drug eruptions and urticaria, of differing severity (Juhlin, Michaëlsson et al. 1972; Pichler 2003). Cutaneous reactions can be broadly classified into different groups through the cytokines secreted from activated T-lymphocytes. $CD4^+$ lymphocytes are known to be involved in the progression of conditions such as maculopapular exanthema; $CD8^+$ lymphocytes can be responsible for more serious conditions, due to their cytotoxic nature.

1.4.9.1 MACULOPAPULAR EXANTHEMA

The most common drug hypersensitivity reaction is the development of maculopapular exanthema. These reactions are seen with beta-lactam antibiotics, quinolones and allopurinol, amongst others (Romano, Fonso et al. 1995). Following drug administration, reactions are observed after approximately a week, with outward symptoms continuing for several days following drug cessation, with MPE reported to be responsible for half of all cutaneous drug reactions (Bigby 2001; Valeyrie-Allanore, Sassolas et al. 2007). MPE is characterised by an increased temperature and itching, as well as the hallmarks of the condition, the development of pink macules on the surface of the skin, which can develop further into a maculopapular rash. This response is caused through the actions of activated drug-specific CD4$^+$ cells that secrete IFNγ and cytolytic molecules (Blanca, Torres et al. 2000; Pichler 2002). Additional studies support a role for chemokines such as RANTES, CCL27 and eotaxin (Posadas, Padial et al. 2002; Tapia, Padial et al. 2004). Due to the mild severity of the reaction, treatments are fairly standard. These include the cessation of drug, as well as brief courses of antihistamines to counteract more severe outbreaks.

1.4.9.2 DRUG REACTION WITH EOSINOPHILIA AND SYSTEMIC SYMPTOMS

Also called drug hypersensitivity syndrome, drug reaction with eosinophilia and systemic symptoms (DRESS) is known to primarily target the liver (though kidneys and lungs can also be damaged), and presents with numerous

symptoms including a fever, skin rash and eosinophilia (Cacoub, Musette et al. 2011). Disease progression is mediated through interleukin-5 secretions from activated T-lymphocytes, leading to the recruitment of eosinophils. Studies have shown that a critical checkpoint in the pathogenesis of DRESS involves the reactivation of latent human herpes virus 6 + 7 (Descamps, Valance et al. 2001). Viral reactivation is also thought to be critical for the clinical manifestation of DRESS, with 76% of patients showing viral reactivation in a recent study (Picard, Janela et al. 2010). DRESS is primarily CD8^{+} T-lymphocyte mediated, with their role shown to be the targeting of reactivated viruses. Half of investigated CD8^{+} T-lymphocytes showed a response to EBV epitopes, further consolidating their role in the disease. Though the T-lymphocyte response is characterised by a classic delayed type IV reaction, the actual mechanism is yet to be fully defined. Multiple drugs have been implicated in the development of DRESS. These include allopurinol, carbamazepine and sulfamethoxazole, amongst others. Withdrawal of the offending drug, alongside systemic steroids are the primary treatment method of DRESS, which has a reported mortality of 8% (Peyriere, Dereure et al. 2006). Interestingly, in contrast to other forms of DHR, reactions continue to develop for several weeks after drug cessation. This is thought to be due to the virus specific CD8^{+} T-cells.

1.4.9.3 STEVENS-JOHNSON SYNDROME

Stevens - Johnson syndrome (SJS) is observed in around 6 cases per million people per year, and is a major cause of mortality in cutaneous drug

hypersensitivity (Ward, Archambault et al. 2010). SJS presents with keratinocyte cell death, alongside skin detachment. Diagnostically, between 10%-30% of total body skin detachment is used to determine a case of SJS. Many drugs have been implicated in the pathogenesis of SJS, including antibiotics, anti-inflammatories and anti-convulsants (Roujeau, Kelly et al. 1995; Harr and French 2012). SJS pathogenesis is poorly understood, with the underlying mechanisms not fully elucidated. Both NK T-lymphocytes and cytotoxic T-lymphocytes have been implicated in the condition, leading to keratinocyte death. Two main pathways are responsible for the keratinocyte death and resulting clinical symptoms, both mediated through effector molecules secreted by activated cytotoxic T-lymphocytes: the fas ligand mediated pathway and the perforin/granzyme B mediated pathway (Abe, Shimizu et al. 2003; Chung, Hung et al. 2008). Granulysin has been shown to be present in greater concentrations than other cytotoxic molecules in the blister fluid of patients, with blister fluid containing high levels of granulysin used to generate SJS-TEN like responses in mice. In addition, several HLA associations have been identified, which can increase the likelihood of the hypersensitivity occurring, with the majority of these associations being drug-specific. There are few treatments for both SJS and TEN, with primary methods of treatment including intravenous Ig, alongside high strength corticosteroids, though the efficacy of these are controversial (Faye and Roujeau 2005; Teo, Tay et al. 2009).

1.4.9.4 TOXIC EPIDERMAL NECROLYSIS

Toxic epidermal necrolysis is reported in 2 cases per million people per year, making it a rare form of SJS. It is also considered a more severe form of SJS, with diagnosis occurring when over 30% of total body skin detachment is seen (Schwartz, McDonough et al. 2013). Again, this is accompanied by large scale keratinocyte cell death. In both SJS and TEN, additional symptoms are observed, typically multi-organ failure and high fevers. Similarly, TEN is observed with a wide spectrum of drug classes, such as the antibiotics, anti-convulsants, antifungals and allopurinol which are all known to cause TEN (Schwartz, McDonough et al. 2013). The pathomechanism is not fully understood, though recent studies have suggested that the keratinocytes themselves play a critical role, with T-lymphocyte activation occurring through presentation of the drug via MHC I present on the keratinocyte cell surface (Wei, Chung et al. 2012). Blister fluid analysis from patients with TEN show high levels of both NK T-lymphocytes and cytotoxic T-lymphocytes, in addition to increased levels of granulysin. Again, a lack of effective treatments impacts on patient prognosis – with mortality of patients reported between 10-50% of SJS / TEN cases (Gomes and Demoly 2005; Schneck, Fagot et al. 2008). Recently, peptide therapy designed to impact on granulysin using siRNA decreased immune responses when targeting CD8^{+} receptors, suggesting a potential novel mechanism to treat cases that do not respond to established treatment methods (Wang, Chung et al. 2013).

1.5   CYSTIC FIBROSIS

Cystic fibrosis (CF) is a lethal autosomal recessive disease that is typified by progressive destruction of the lung tissue leading to respiratory insufficiency of the airways. Impaired mucocilliary clearance leads to chronic infections with gram-negative bacteria such as *pseudomonas aeruginosa*. Cystic fibrosis has an effect on all exocrine glands, including the liver, pancreas and sweat glands. This is a major cause of mortality in patients with cystic fibrosis; liver failure as a result of cirrhosis is the second leading cause of death in these patients. It is the most common recessive disease in people with northern European descent, and upwards of 4% of that population is thought to be a carrier of at least one copy of the mutated CFTR gene (Bobadilla, Macek et al. 2002). Normal healthy CFTR genes code for an ion transporter involved in the movement of chloride ions across epithelial cells.

1.5.1   GENETICS

The condition is caused by mutations in both copies of the cystic fibrosis transmembrane conductance regulator gene, which is located on chromosome 7, specifically in the q31.2 region (Rommens, Iannuzzi et al. 1989). As CF is a recessive disorder, both copies of the gene are required to be mutated, with a person with only one mutated copy of the gene showing no symptoms. There is a population split across the globe of relative frequencies of cystic fibrosis, with it most commonly seen in northern Caucasian populations, while much rarer in sub-Saharan African populations (O'Sullivan and Freedman 2009).

Over 1500 different mutations have been characterised, though the most common is the ΔF508 deletion, which is observed in up to 70% of all cases worldwide (Bobadilla, Macek et al. 2002). This mutation causes a misfolding of the protein due to a deletion of the amino acid at position 508 (phenylalanine). The misfolded protein is unable to be trafficked to the membrane of the cell surface, triggering degradation prior to leaving the endoplasmic reticulum. This results in a lack of a CFTR protein at the cell surface, causing multiple problems related to chloride ion transport (Dalemans, Barbryt et al. 1991).

As it is not a sex chromosome-linked disease, CF affects both men and women equally.   The high prevalence of the CF mutation suggests some sort of heterozygous advantage as a way to explain how a lethal condition could be so widespread. Some studies have shown a link between cholera resistance and CF carriage, suggesting a role in water reabsorption as a potential protective mechanism in that disease. Salmonella also requires a healthy working CFTR

transporter for successful entry into cells (Pier, Grout et al. 1998; Ma, Thiagarajah et al. 2002).

1.5.2 PATHOGENESIS

Though the main site affected in cystic fibrosis is the lungs, additional effects can be observed in other locations such as the pancreas and intestines. Mutations that occur in the CFTR gene lead to reduced amount of ion transport across epithelial membranes. Alterations in chloride ion transport leads to many effects, such as a build up of salt (sodium chloride) outside the cell. This is due to the reduced transport of both chloride and sodium ions to the intracellular space, though only in the sweat glands. In all other cells this process is reversed, necessitating the treatment of patients with hypertonic saline solutions. In cases of cystic fibrosis, the faulty CFTR channel prevents re-absorption of chloride ions from happening, resulting in the excess amounts of salt excreted in sweat. The salty sweat that is produced is one of the main diagnostic tests for CF, excluding a genetic screen (Stern 1997; Mishra, Greaves et al. 2005).

The main adverse effect that is observed in patients with CF is the production of thick mucus. This mucus is formed through abnormally high sodium influx in lung epithelial cells from the airway surface liquid due to the defective CFTR transporter. Sodium is able to re-enter the cells through a different transporter, meaning its influx is much higher when compared to chloride (Welsh 1990; Kibble, Neal et al. 2000). In turn, this triggers the influx of water molecules from the liquid, causing a reduction in the volume of this liquid,

making it thicker. This thick mucus proves an excellent breeding ground for opportunistic infections such as *pseudomonas*., due to the reduction in effective ciliary function. These recurrent infections are often the cause of death in patients with CF, due to their recurrent nature and reduced effect of the immune system to fight off continual attack. Colonies of immovable bacteria, termed biofilms, exist in the lung and act as the sites of origin for new infections. These biofilms are present due to the idea breeding grounds located in the lungs and are typically resistant to multiple antimicrobial agents. Biofilms form through structural changes in the bacterium such as *pseudomonas*, with the loss of motility via flagella removal a key component in the generation of antimicrobial resistance. Initially, in early life *staphylococcus* predominates, with *pseudomonas* present in over 80% of patients once reaching adulthood (Oliver, Cantón et al. 2000). Nasal polyps are also more common in patients with CF, resulting in laboured breathing, in addition to the respiratory problems caused via mucus build up.

A common side effect observed in the patients with cystic fibrosis is that of pancreatic insufficiency. This causes a deficiency in digestive enzymes due to loss of pancreatic cells leading to malabsorption. The weight loss that is caused by this condition is also a major indicator for a CF diagnosis. Islets of langerhans present in the pancreas are responsible for the production of insulin. Damage to these cells can cause resulting cystic fibrosis-related diabetes. Other conditions that are observed in patients with cystic fibrosis include 'clubbing' of the fingertips and infertility, with 97% of males infertile. This infertility is due to a condition termed 'congenital absence of the vas deferens' (Borgo, Mastella et al. 1990; McCallum, Milunsky et al. 2000).

1.5.3   TREATMENTS

Currently, there is no known cure for cystic fibrosis, so all treatment methods come under palliative care, or the treatment of secondary issues that are caused as a result of the CF condition. Patients are often involved in lifelong physiotherapy sessions to improve airway function, as the mucus build up leads to extreme difficulty breathing if left unchecked. Lung transplants are often a last resort for patients with extensive airway scarring. A dual lung transplant is required to avoid cross-contamination of any bacteria present in the host lungs. Due to the pancreatic damage that is often seen in CF, diabetes is common among patients. As a result, insulin injections are a common prescription to allow for stable levels of blood glucose to be maintained (Belkin, Henig et al. 2006; Alves Cde, Aguiar et al. 2007; Davies, Alton et al. 2007).

A key therapeutic method used in the treatment of patients with cystic fibrosis is the inhalation of a hypertonic saline solution (6%). This inhalation is done to improve mucus clearance from the airways of people with cystic fibrosis, countering the thickened mucus found as a result of the defective CFTR transporters triggering excessive water reabsorption from the airway surface liquid. Application of a hypertonic saline solution increases the salt content of the airway surface liquid, leading to an increase in osmosis to rehydrate the liquid. This decreases mucus viscosity as well as improving ciliary function for an increase in mucus removal.

Aerobic fitness is also considered to be an important factor in patient outlook. It is measured through VO_2 peak values, with a markedly increased survival rate in patients with cystic fibrosis where their aerobic fitness was maintained. The recurrent infections that are present throughout a patient's life mean that people with CF are usually on at least one course of antibiotics. This is done to hopefully not allow the bacterial colony to get a foothold in the airways, though the presence of biofilm colonies of infections such as *pseudomonas* in cystic fibrosis means that patients are never considered to be fully free from infections (Lyczak, Cannon et al. 2002; Flume, O'Sullivan et al. 2007). Treatment methods utilising aerosolised antibiotics such as Aztreonam, levofloxacin and tobramycin are employed to improve lung function in cases of chronic infections. This treatment does have side effects however. In patients with CF, there is a much higher reported incidence of allergy to beta-lactam antibiotics. One in 3 patients are known to be allergic to at least one drug, with 20% showing multiple beta-lactam hypersensitivity. This compares to an incidence rate of approximately 2% in the general population (Whitaker, Naisbitt et al. 2012).

Doring *et al*. stated that based on current study evidence, '*prophylactic treatment with anti-pseudomonal antibiotics are not recommended to prevent* P. aeruginosa *infections in patients with cystic fibrosis*', though they do go on to state that the use of early antibiotic eradication therapy in the treatment of *pseudomonas* shows beneficial effects (Doring, Flume et al. 2012). In a study investigating the incidence of piperacillin hypersensitivity, between 26-50% of CF patients were shown to be hypersensitive to the drug. Careful management of the antibiotic courses is often required, as certain commonly prescribed

antibiotics can have long term side effects, such as kidney damage with tobramycin (Hoffmann, Rubin et al. 2002). In addition to this, prophylactic treatment with multiple courses of antibiotics greatly increases the chance of antibiotic resistance. Increasingly, combination therapies are being used to counter this threat of resistance. One such example of combination therapy is the co-administration of piperacillin and tazobactam in targeting *p. aeruginosa*. Piperacillin is a powerful broad-spectrum antibiotic used to treat gram-negative infections which exerts its mechanism of action through the inhibition of bacterial cell wall synthesis. Tazobactam is prescribed alongside this compound due to its synergistic mechanism of action. It is a β-lactamase inhibitor, acting to prevent the breakdown of piperacillin by enzymes produced by gram-positive bacteria. This confers additional activity to the piperacillin compound. This resistance is of paramount concern for clinicians to consider, as antibiotic resistance is increasingly becoming the major barrier for the treatment of illnesses, due to the relative scarcity of new drug classes currently in development.

Research has been implemented to develop new compounds that are able to treat cystic fibrosis in novel ways. One such compound is the new lumacaftor / ivacaftor combination therapy. The two different drugs work in synergy with each other via different mechanisms of action. Lumacaftor acts as a chaperone during protein folding, increasing the transport of CFTR proteins to the membrane surface (Kuk and Taylor-Cousar 2015). This counters the defect observed in the Δ508 deletion, where proteins are not transported to the surface. Additionally, ivacaftor acts upon CFTR transporters present on the membrane surface by binding to the ion channel itself, increasing chloride

transport by increasing the probability of the ion channel being open (McPhail and Clancy 2013). Though this combination therapy has shown to be potentially life changing in its effects, its prohibitive cost (>£250,000 / year) means that the treatment is not widely available in the UK, depending on the health care systems.

1.6   BETA LACTAM ANTIBIOTICS

Antibiotics are among the most widely prescribed medications around the world. Antibiotics fall into several classes, with one of the most popular being the beta-lactam class (Elander 2003). These drugs are characterised by their common beta lactam ring, alongside different side chains. They exert their action through the inhibition of cell wall synthesis in bacteria, via the interaction with the penicillin binding proteins. These proteins catalyse the cross linking of the bacteria cell wall, meaning that the bacteria are unable to generate a solid continual cellular outer layer once exposed to the beta-lactam. They are bactericidal, and irreversibly bind with the serine residue 403 at the active site of the penicillin binding protein (Waxman and Strominger 1983; Fisher, Meroueh et al. 2005).

Due to the widespread use of beta-lactam antibiotics, resistance has begun to become to represent an important clinical problem. Bacterial methods of resistance occur either through utilising altered penicillin binding proteins to synthesise their cell walls, such as in the case of MRSA, or through the production of the beta-lactamase enzyme. This enzyme hydrolyses the active beta-lactam ring of the antibiotic and renders it inactive (Drawz and Bonomo 2010). Newer treatment methods counteract this through co-administration with a beta-lactamase inhibitor such as clavulanic acid, but resistance is still of major concern to treatment regimens.

One such antibiotic that is used in treatment of bacterial infections is piperacillin.   Usually co-administered with the beta-lactamase inhibitor tazobactam, piperacillin is a broad spectrum beta-lactam antibiotic used in the

treatment of a range of infections, most notably in pseudomonas related conditions. An intravenous antibiotic, approximately 30% of piperacillin is bound to serum proteins. It undergoes renal excretion, with a short half-life in plasma of around 1h (Sorgel and Kinzig 1994). It is excreted largely unmetabolised, though a minor desethyl metabolite is formed through N-dealkylation in the liver (Ghibellini, Bridges et al. 2007).

Drug-proteins adducts have been implicated in the development of beta-lactam hypersensitivity reactions (Park and Kitteringham 1990; Evans, Watt et al. 2004; Pichler, Naisbitt et al. 2011). Piperacillin binds selectively to lysine residues on proteins. In *in vitro* assays, piperacillin binds to human serum albumin, with the resulting adducts able to activate patient T-cells (El-Ghaiesh, Monshi et al. 2012). Furthermore, piperacillin albumin adducts have been identified in the blood of patients following a 14 day treatment course. Typically, piperacillin hypersensitivity is reported in patients with underlying medical conditions such as cystic fibrosis, or those who show hypersensitivity to other drugs in the beta-lactam class (Weiss and Adkinson 1988; Koch, Hjelt et al. 1991; El-Ghaiesh, Monshi et al. 2012). Symptoms of piperacillin hypersensitivity include maculopapular exanthemas, fever and other mild cutaneous reactions. Reactions have a delayed onset, with the mean time to the development of clinical symptoms reported to be 9.1days, though alternative studies have reported high frequency of immediate reactions, suggesting alternative mechanisms of action (Whitaker, Naisbitt et al. 2012; Roehmel, Schwarz et al. 2014). Although comparatively rare, immediate hypersensitivity is also observed in multiple beta-lactams. These are a Type I reaction mediated through the IgE molecule, and can range in severity from

mild urticarial rashes to life-threatening anaphylaxis. The hypersensitivity reaction subsides upon cessation of drug. Studies have shown that piperacillin-specific T$_h$2 lymphocytes can be isolated from the blood of patients with piperacillin hypersensitivity (El-Ghaiesh, Monshi et al. 2012).

Investigations have been conducted into the cross-reactivity that is observed with the beta-lactam class of antibiotics. The beta-lactams are a broad class, consisting of penicillins, cephalosporins, carbapenems and monobactams. Cross reactivity is reported between different compounds in the same class, as well as between classes.

The principle site of immunogenicity in these compounds are the beta-lactam ring, the thiazolidine or dihydrothiazolidine ring and the side chains. Studies highlight that cross-reactivity is dependent on similar side chains between compounds. This theory is further enhanced by the fact that penicillins that share a common group in their side chain are often seen to cross react with one another.

In studies comparing responses between penicillins and cephalosporins, low cross-reactivity is observed. Independent groups reported the incidence rate of cross-reactivity at around 10%, though this is reduced when comparing responses to the newer generation of cephalosporins. For example, Buonomo *et al.* showed an 18.5% incidence of cross-reactivity with cephalosporins when investigating patients with confirmed ampicillin hypersensitivity (Romano, Guéant-Rodriguez et al. 2004; Antunez, Blanca-Lopez et al. 2006; Buonomo, Nucera et al. 2014).

In contrast to this, a comparison between penicillins and carbapenems showed little to no cross-reactivity, with work performed by Romano's group showing

an incidence rate of approximately 1% (Romano, Gaeta et al. 2010). The cephalosporins are split into two sub classes; the methoxyimino R1 side chains and the aminocephalosporins. Structural similarity at the R1 side chain position is critical for cross-reactivity in these compounds, with no cross-reactivity reported between the two sub-classes. However, it is important to note that the incidence of cross-reactivity is dependent on multiple factors which are still not fully elucidated. For example, amoxicillin and cefadroxil are different compounds which share the same side chain, yet only have a reported cross-reactivity of 40% (Miranda, Blanca et al. 1996).

1.7   THESIS OUTLINE

The aims of this thesis were to investigate the role of both pro-and anti-inflammatory cytokines in the progression of piperacillin hypersensitivity in patients with cystic fibrosis. To fully understand the pathomechanisms that were involved, a number of analytical, biological and functional experiments were designed, using blood samples from both healthy volunteers and cystic fibrosis patients.   Also, inflamed skin samples were obtained from the hypersensitive patients with cystic fibrosis.

Specific aims of this thesis include:

- Investigation of the feasibility of priming naïve T-cells to piperacillin.

- Exploration of the phenotype and cytokine secretion from piperacillin primed T-cells.

- Characterisation of the phenotype and function of drug-specific T-cells isolated from the blood and inflamed skin of hypersensitive patients.

- Delineate if a typical T_h1 of T_h2 response is observed.

- Investigation of the COX-2 selective NSAID lumiracoxib and its role in drug hypersensitivity.

CHAPTER 2 – MATERIALS AND METHODS

2.1 REAGENTS AND MATERIALS

2.1.1 REAGENTS

Lymphoprep was purchased through Axis-Shield (Dundee, United Kingdom). [^{3}H]-methyl tritiated thymidine was from Moravek (California, United States). Recombinant interleukin-2 was purchased from Peprotech (London, UK). Both protein standards as well as readymade Bradford reagent was purchased from Bio-Rad Laboratories (Hemel-Hempstead, UK). Propan-2-ol was purchased from Fischer Scientific (Loughborough, UK). Dimethyl sulfoxide was purchased from Sigma Aldrich (Dorset, UK). Western blot developing and fixation solutions were purchased from Kodak (Watford, UK). Meropenem, sulfamethoxazole-nitroso and piperacillin were obtained from AstraZeneca (Luton, UK), Dalton Pharma Solutions (Toronto, Canada) and Wockhardt Limited (Wrexham, UK). Lumiracoxib and its metabolites were a gift from Novartis (Surrey, UK). Cyclosporin-A was purchased from Fluka Analytical (Dorset, UK). Pooled human serum was bought from Innovative Research (Michigan, USA), while foetal bovine serum was bought from Invitrogen (Paisley, UK). Tetanus toxoid (pure) was purchased from Statens Serum Institut (Copenhagen, Denmark). All fluorescent antibodies purchased for flow cytometry analysis were from either BD Biosciences (Oxford, UK) or R&D Systems (Minnesota, USA). All chemokine antibodies were purchased from eBioscience (Ireland, UK).

Any other solvents, reagents and supplies were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise mentioned.

2.1.2 MATERIALS

All ELISpot kits and developing substrates were purchased from Mabtech (Stockholm, Sweden), with the exception of fas ligand, which was from AbCam (Cambridge, UK). The trial kits of interleukin-22 ELISpots were a gift from Niklas Ahlborg of Mabtech. Multi-screen ELISpot assay plates were bought from Millipore (Watford, UK). Interleukin-22 ELISA kits were purchased from eBioscience (Ireland, UK). Melt-on wax scintillator sheets, glass fibre filter mats and plastic sample wallets were purchased from Perkin-Elmer Life Sciences (Massachusetts, USA). Magnetic bead separation kits were bought from Miltenyi Biotech (Surrey, UK). Cell culture plates, flasks and dishes were purchased from Thermo Scientific (Hemel-Hempstead, UK). Western blot nitrocellulose membranes and Hyperfilm ECL were purchased from GE Healthcare Life (Buckinghamshire, UK).

Any other solvents, reagents and supplies were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise mentioned.

2.2 CELL CULTURE MEDIUM

T-lymphocyte culture medium contained RPMI-1640, supplemented with 10% human antibody serum, penicillin (100U/ml), streptomycin (0.1mg/ml), transferrin (25µg/ml), L-glutamine (2mM) and HEPES buffer (25mM). All assays were cultured in media absent of both penicillin and streptomycin, though media that contains this combination is also suitable for use. Normally, work with beta-lactams is conducted in the absence of both penicillin and streptomycin.

Antigen presenting cells in the form of Epstein-Barr transformed B-lymphocyte lines were cultured in media containing RMPI-1640, supplemented with 10% foetal bovine serum, penicillin (100U/ml), streptomycin (0.1mg/ml), L-glutamine (2mM) and HEPES buffer (25mM).

Chemotaxis buffer contained RPMI-1640 supplemented with 0.5% bovine serum albumin.

Cells were frozen for long term storage using a freezing medium containing 80% foetal bovine serum and 20% DMSO. This freezing medium was then combined in a 1:1 ratio with standard lymphocyte culture medium to a volume of 1ml prior to freezing and storage.

Flow cytometry medium (FACS Buffer) contained Hanks balanced salt solution, supplemented with 10% foetal bovine serum and 0.03% sodium azide (NaN3).

Magnetic cell separation medium (MACS buffer) contained 2.5g BSA, 2mL EDTA (500mM), 48mL HBSS for a 10x strength stock solution. Dilution to 1x strength was done via 1:10 dilution with HBSS.

2.3   INSTRUMENTATION

Assays incorporating radioactive [^{3}H]-thymidine were harvested using a cell harvester (TomTec, USA) and analysed via the Micro Beta Trilux Counter (Perkin Elmer, Cambridge, UK).   ELISpot assays were read using an AID ELISpot Reader (Cadama Medical, Stourbridge, UK).   ELISA plates were read using an MRX plate reader (Dynex, Lincoln, UK).   Flow cytometry readings were taken using a BD FACS Canto II flow cytometer (BD Biosciences, Oxford, UK) and analysed using a combination of Cyflogic (CyFlo Ltd.) and Flowing 2 (Turku University, Finland).   Bradford assays were read using an MRX plate reader (Dynex, Lincoln, UK).   All columns and magnets used for the magnetic cell separations were purchased from Miltenyi Biotech (Surrey, UK).   All statistical analysis was performed using SigmaPlot 13 software (SyStat Software Inc.).

2.4 SAMPLE RECRUITMENT

All patient samples were screened prior to being taken. Cystic fibrosis samples were taken from patients of known allergic status to drugs, through the Regional Adult Cystic Fibrosis Unit in St James' Hospital in Leeds. Healthy volunteer samples were used for naïve controls. These people have previously not been exposed to the drug in question, to the best of their knowledge. All patients agreed with the written informed consent provided. The research study was approved through the local research ethics committees. Patient samples were collected after study approval from the East Leeds Ethics Committee. Healthy volunteer samples were approved by the Liverpool Local Research Ethics Committee.

2.5 METHODS

2.5.1 PERIPHERAL BLOOD MONONUCLEAR CELL ISOLATION

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples obtained from either volunteers or patients. Peripheral blood was obtained via a trained phlebotomist in heparinised vacutainer vessels. In a 50ml tube, 25ml of lymphoprep was added, with blood carefully layered on top using a syringe and quill. Erythrocytes were separated through density centrifugation (400g, 25min, 17°C, no brake). A buffy coat layer was generated which contained the required PBMCs. This layer was carefully extracted into a fresh tube using a pasteur pipette. Two further washes using 50ml of Hank's balanced salt solution were performed to ensure all remaining lymphoprep was removed. PBMCs were then counted via trypan blue exclusion, with 10μl of cell suspension added to an equal volume of trypan blue (0.2% w/v) and counter via a haemocytometer, using a Lecia DME light microscope (Lecia Microsystems, Milton Keynes, UK). The total cell number of viable cells was recorded as follows: observable viable cells * magnification factor * dilution factor. Percentage viability from initial blood separations was regularly >98%.

Isolated PBMC were frozen and stored for further analysis. The PBMC were frozen in a freezing mixture (80% FBS, 20% DMSO) and equal parts cell culture medium at a density of 1×10^7 cells/ml. These were stored in cryovials and placed into a Mr Frosty container for 24h at -80°C. Once frozen, the cells were then transferred into either a -150°C freezer or a liquid nitrogen tank for long term storage. In cases when cells were used immediately, cells were

transferred into the appropriate media for experimental conditions, stated as appropriate.

2.5.2 MAGNETIC SEPARATION OF CELL SUBSETS

Following on from PBMC isolation, cell subsets are able to be magnetically separated in order to be used in multiple assays. First, the PBMCs were re-suspended in MACS buffer (800μl / $1x10^8$ cells). CD14 microbeads were then added (200μl per $1x10^8$ cells), mixed and incubated in the dark for 15min, 4°C. Additional MACS buffer was then added (15ml per $1x10^8$ cells) to wash cells which were then re-suspended in MACS buffer (500μl per $1x10^8$ cells) and passed through a magnet holding an LS column. The column was washed using additional MACS buffer for a total flow through of 15ml. Cells that flow through are CD14$^-$ and were used for the next stage, while a plunger was used on the column to elute the CD14$^+$ cells. Columns were pre-wet with 3ml of MACS buffer prior to sample addition.

Next, the CD14$^-$ cells were re-suspended in MACS buffer (400μl per $1x10^8$ cells) and Pan-T biotinylated antibody cocktail was added (100μl per $1x10^8$ cells). These cells were mixed and incubated in the dark for 15min, 4°C. Next, MACS buffer (300μl per $1x10^8$ cells) and anti-biotin microbeads (200μl per $1x10^8$ cells) were added and incubated for 15 min, 4°C. Cells were then washed with MACS buffer (15mL per $1x10^8$ cells) and passed through another LS magnetic column. The flow through are CD3 positive cells, while a plunger was used to elute the remaining CD3 negative cells in the column.

Regulatory T-lymphocytes and memory T-lymphocytes were removed from the CD3$^+$ cells using positive selection with CD25 and CD45RO microbeads. The isolated CD25$^+$ (regulatory T-lymphocytes), CD45RO$^+$ (memory T-lymphocytes) and CD45RO$^-$ (naïve T-lymphocytes) were then able to be used in future assays. All cells were able to be incorporated into assays directly following separation, or could be frozen down (1x10^7 per ml) for storage and future analysis.

2.5.3   PROLIFERATION ASSAY

Immune responses are able to be easily measured through the use of a proliferation assay. T-lymphocyte clones (5x10^4, 50µl) were cultured in a 96-well U-bottomed cell culture plate, with antigen presenting cells in the form of irradiated autologous cells (1x10^4, 50µl). The remainder of the well volume was made up of either drug at differing concentrations, cell media alone (negative control) or PHA (5µg/ml, positive control) (all 100µl). Cells were then incubated at 37°C, 5% CO_2 for 3 days, with tritiated [^{3}H]-thymidine (0.5µCi/well) added for the last 16h of culture. Cells were then harvested onto a printed filter mat, sealed with wax and contained in a scintillation sheet. The readouts were measured on a MicroBeta Trilux counter through scintillation counts, with readouts displayed in the form of CPM. An SI of greater than 2 was considered a positive result.

2.5.4 LYMPHOCYTE TRANSFORMATION TEST

Lymphocyte Transformation Tests (LTTs) were performed on PBMC isolated from patient's blood samples. The LTT was used to investigate the presence of memory T-lymphocytes that showed drug-specific reactivity, as described elsewhere (Pichler and Tilch 2004). Briefly, PBMCs (1.5×10^5/well, 100µl) were cultured with drug (differing concentrations, 100µl) in a 96-well U-bottomed cell culture plate, in triplicate. A range of drug concentrations were used to establish an optimal response range. Tetanus toxoid (0.5µg/ ml, 100µl) was used as a positive control, with culture media alone as a negative control. Once all conditions were added, plates were incubated for 6 days at 37°C, 5% CO_2. Sixteen hours prior to the end of the incubation, tritiated [^{3}H]-thymidine was incorporated into the assay (0.5µl/well). Upon the end of the incubation plates were harvested using a MicroBeta Trilux counter, as described previously.

2.5.5 ELISA

ELISA stands for enzyme-linked immunosorbent assay, and is used in the quantification of a substance – typically an antigen or cytokine – present within a solution. In our work the solutions that were tested were supernatants taken from the wells of conducted assays, aiming to measure the total amount of interleukin-22 that had been produced. Briefly, a capture antibody (100µl/well, 10µg/ml) was coated onto the wells of a 96-well flat bottomed plate, diluted in coating buffer (HBSS containing 0.5% FBS), and then left overnight at 4°C. The wells were then washed (250µl/well, 5x, PBS), and assay

diluent added to each well for 1h. The wells were then washed (250μl/well, 5x, PBS) and samples are added (100μl/well). The plate was then incubated for 2h at 17°C. Plates were then washed again, and detection antibody (100μl/well, 1μg/ml) added to each well diluted in assay diluent for 1h. Wells were then washed and streptavidin-HRP (100μl/well, 1μg/ml) added to each well for 30min. Wells were then washed and substrate solution (TMB, 100μl/well) added to each well for 15min. Finally, the stop solution (1N NaOH, 50μl/well) was added to halt the reaction and the resultant colour changes were read at 450nm.

2.5.6   BRADFORD ASSAY

The Bradford Assay is a simple, quick assay used to measure protein concentrations. This is generally performed prior to an ELISA or Western blot, in order to establish a concentration curve through known quantities of a protein - typically BSA - allowing the identification of an unknown sample. Unknown protein concentration samples were then compared against a known protein concentration curve to obtain total protein values, through the use of a spectrophotometer. Once all standards (5μl/well, 1.25μg/ml – 10μg/ml) and unknowns (5μl/well) were set up in triplicate, coomassie stain (150μl) was added to each well, with the resultant colour change read off at 595nm. Values obtained were then analysed with the known protein concentrations used to generate a dilution dependant concentration curve. This curve was then used

to read off the unknown spectrophotometric values, giving a reading for protein concentration.

2.5.7 ELISpot

The enzyme-linked immunosorbent spot assay (ELISpot) is similar in design to the ELISA, however it allows for the characterisation of low levels of cytokine secretions from T-lymphocytes themselves, without the need for supernatants (Engler, Strasser et al. 2004; Zhang and Lehmann 2012). ELISpot assays were used to quantify the number of IFN-gamma and interleukin-22 secreting cells, in addition to other cytokines. Briefly, Multi-screen HTS filter plates were pre-wet with 100μl of activation mix (70% EtOH, 30% d.H$_2$0), then washed with d.H$_2$0 (5x, 200μl), coated with a capture antibody (15-60μg/ml, 100μl) and stored overnight at 4°C. Next, the wells were washed of capture antibody using sterile PBS (250μl, 5x) and then blocked for 2h with standard cell culture medium (250μl). This media was then removed, and T-lymphocytes (5x10^4, 50μl), irradiated autologous antigen-presenting cells (1x10^4, 50μl) and the appropriate culture condition (positive control, negative control, drug, 100μl) were added to the well. Cell culture medium alone was used for a negative control, with PHA (5μg/ml) used for a positive control. The plates were then incubated at 37°C, 5% CO$_2$ for 48h. After incubation, the cells were discarded and the wells washed with PBS (250μl, 5x). Biotinylated antibodies to specific cytokines were then added to each well (1μg/ml, 100μl) in a solution of PBS containing 0.5% FBS. This was then incubated at room temperature for 2h,

and then the wells were washed with PBS (250μl, 5x). Streptavidin-ALP was then diluted in the PBS containing 0.5% FBS and added to each well for 1h (1μg/ml, 100μl). The liquid was then discarded and the wells washed with PBS (250μl, 5x). A solution of BCIP/NBT substrate (100μl) was added to each well for 15min for spot formation to occur. The reaction was then halted using slow running tap water, with the wells left overnight to dry prior to being read on an AID plate reader.

2.5.8   FLOW CYTOMETRY

Flow cytometry was used to investigate the cell surface phenotype and function of T-lymphocytes. Cells ($1x10^5$, 200μl) were incubated with antibodies conjugates to different fluorochromes (5μl) in FACS buffer for 25min, 4°C in the dark. For example: CD3-APC, CD4-PE, CCR7-FITC, CXCR1-APC. These cells were then washed and re-suspended in either fresh FACS buffer (200μl) or para-formaldehyde (4% v/v, 200μl). Fresh FACS buffer was used to re-suspend cells for same day analysis, with para-formaldehyde used in cases where analysis was planned on future days. Cell data was analysed using a FACS Canto II machine. A minimum of $1x10^4$ events were used for analysis acquisition.

2.5.9 T-LYMPHOCYTE CLONING

T-lymphocyte cloning uses small initial amounts of lymphocytes to generate a large population of clones for analysis and comparison assays. This is done through a serial dilution method, with each individual clone that is generated comprising of a population of identical cells generated from an initial single cell precursor.

Briefly, PBMCs from either patient or volunteers, in addition to isolated skin lymphocytes ($1x10^6$, 500µl) were cultured with drug (500µl) at optimal concentration in a 48-well plate for 2 weeks. On days 6 and 9 the wells were supplemented with interleukin-2 (200U/ml) to induced T-lymphocyte expansion.

On day 14, the cells were then harvested and used for a serial dilution. Cells were plated out into 96-well U-bottomed cell culture plates at concentrations of 0.3, 1, 3 and 10 cells per well. They were then re-stimulated with allogeneic irradiated PBMC ($5x10^4$/well), PHA (2µg/ml) and interleukin-2 (75U/ml). These cells were fed every 2 days using cell culture media supplemented with interleukin-2 (75U/ml). Wells that contained clones that were growing well were placed into a fresh 96-well cell culture plate and allowed to expand, through splitting the well as required. This was determined through a large confluent pellet being present in the bottom in the well, in addition to the medium beginning to turn yellow in between normal feeding schedules. Specificity to the cultured antigen was assessed using a standard proliferation assay performed in duplicate wells.

Once antigen-specific T-lymphocytes were identified, they were re-stimulated every 3 weeks to maintain a sufficient level of proliferation with allogeneic irradiated PBMC ($5x10^4$/well), PHA ($2\mu g$/ml) and interleukin-2 (75U/ml).

2.5.10 EBV GENERATION

Epstein-Barr virus transformed B-cell lines were used throughout the work conducted as autologous antigen-presenting cells in multiple assays. These were generated through the following method. First, PBMCs ($2-4x10^6$) from a donor were incubated in supernatant taken from the B9.58 cell line. The supernatant (10ml) was filtered through a $0.45\mu m$ filter attached to a syringe prior to addition to the PBMC, in order to remove any of the cell line from the suspension. Cyclosporin A ($1\mu g$/ml) was added to the supernatant to inhibit T-lymphocyte proliferation. The PBMC were then incubated overnight at 37°C, 5%$C0_2$. Next, the cells were washed and re-suspended in antigen-presenting cell medium containing cyclosporin A ($1\mu g$/ml) and plated into a 24 well plate at a final volume of 2ml. These plates were then incubated for 2 weeks, with fresh antigen-presenting cell media containing CSA added every 3 days. After 2 weeks, antigen-presenting cell media without CSA was used to feed the cells, in order to allow for expansion. When sufficient expansion had occurred, cells were transferred into a free-standing cell flask, and fresh media was used to maintain healthy cells and continued to be grown as necessary, normally being fed twice a week.

2.5.11 ANTIGEN PULSING ASSAYS

Antigen-pulsing assays are used to look at drug-protein binding and to assess the requirement for antigen processing that may be used in the lymphocytes. Epstein-Barr virus transformed B-cells were cultured with drug for 16h, then washed with HBSS in order to remove any free drug. These antigen-presenting cells were then included in assays in the place of non-antigen pulsed cells, in order to investigate if presentation occurs. Generally, T-lymphocytes ($5x10^4$/well, 50μl) and irradiated drug-pulsed antigen-presenting cells ($1x10^4$/well, 50μl) were cultured with T-lymphocyte medium (100μl). Soluble drug at optimal concentration is included as a positive control in place of T-lymphocyte medium alone (Yaseen, Saide et al. 2015).

2.5.12 WESTERN BLOT ANALYSIS

Protein samples isolated from previous cell culture assays were harvested from the supernatants were aliquoted out (10μl) were loaded into the appropriate wells on a 10% SDS-polyacrylamide gel for electrophoresis (1h, 300V, 60mA). The separated proteins were transferred from the gel to a nitrocellulose membrane (1h, 300V, 250mA). Blocking for non-specific binding was performed through the use of 2% non-fat powdered milk in TST buffer (Tris-HCl 10mM, NaCl 150mM, Tween-20 0.05%; pH 8.0) for 2h at 17°C. The presence of drug-protein adducts was detected through the addition of primary antibody (mouse anti-penicillin, 1:20,000) diluted in 2% milk / TST overnight at 4°C. The following day, unbound antibody was washed from the

sample using PBS-Tween-20 (5x wash). A secondary antibody (goat anti-mouse HRP, 1:1,000) was then diluted in milk / TST and added to the membrane for 2h at 17°C. Unbound antibody was then again washed off using PBS-Tween-20 (5x wash). Membrane bound signals were then detected through ECL and developed using standard radiography film in a dark room for differing times (up to 30s) before images were taken.

2.5.13 MHC RESTRICTION ASSAY

Anti-human HLA-A, B and C antibodies (MHC class I) and anti-human HLA-DP, DQ and DR antibodies (MHC class II) were utilised in some reactions to block MHC class I and II interactions (5µg/ml, 100µl/well). Briefly, autologous EBVs were incubated with either the class I or II blocking antibodies ($1x10^4$/well, 50µl) for 30min, then cultured with generated T-lymphocyte clones. Proliferation readouts as well as ELISpot analysis was performed following a 48h incubation at 37°C, 5% CO_2.

2.5.14 T-LYMPHOCYTE PRIMING ASSAY

Using a recently established method (Faulkner, Martinsson et al. 2012) it is possible to prime naïve t-lymphocytes to respond to certain compounds, using autologous dendritic cells as the primary source of antigen presentation. By using this assay we were able to investigate potential lymphocytic responses without the need for patient samples.

CD14$^+$ cells are cultured in the presence of GM-CSF (800U/ml) and IL-4 (50µg/ml) for 6 days. On day 6, TNF-α (50µg/ml, 3µl/ml) and LPS (1mg/ml, 6µl/ml) were added to induce maturation into mature dendritic cells. These cells (2x10^4/well) are then incubated with autologous naïve T-lymphocytes (2.5x10^6 per well) in a 48-well cell culture plate containing the drug aiming to be primed to at optimal concentration. The drugs used in this assay were piperacillin, sulfamethoxazole-nitroso, lumiracoxib and its minor metabolites (concentrations given in chapters). Sulfamethoxazole-nitroso was included in all assays as a positive control, as 9 out of 10 samples are known to be successfully primed to that compound (personal communication).   These plates were then incubated for 7 days at 37°C, 5%CO$_2$. After the 7 days, freshly matured dendritic cells (1x10^4 per well) are added to the now primed T-lymphocytes (1x10^5 per well) after all cells are washed. Positive controls are obtained using PHA (5µg/ml), negative controls with both media alone and wells lacking fresh dendritic cells.

2.5.15 MEMORY T-LYMPHOCYTE ASSAY

Memory T-lymphocytes allow the investigation of patient responses to previously exposed compounds. Simply, a 48-well cell culture plate was coated with a CD-3 antibody (10µg/ml) overnight at 4°C. The following day, the plates were washed with HBSS (200µl, 5x) and memory T-lymphocytes from healthy volunteers (1x10^6) were added to the wells, in addition to polarising cytokines (T$_h$1, T$_h$2, T$_h$17 T$_h$22) as shown in *figure 2.1* and antigen presenting cells in the

form of EBV (4×10^4). CD28 antibody and medium alone were also run for comparisons. Following incubation of the cells for 6 days at 37°C 5%CO_2, the cytokine secretion of cells were then used in an ELISpot readout (1×10^5 cells/well, 100µl) alongside medium alone (100µl).

Figure 2.1. Culture conditions for lymphocyte polarisation

T_h1	IL-12 (25ng/ml), Anti IL-4 (5µg/ml)
T_h2	IL-4 (25ng/ml), Anti IL-12 (5µg/ml), Anti IFN-γ (200ng/ml)
T_h17	IL-1β (20ng/ml), IL-6 (20ng/ml), IL-23 (20ng/ml), TGFβ (5ng/ml)
T_h22	TNF-α (50ng/ml), IL-6 (20ng/ml)

2.5.16 CHEMOTAXIS ASSAY

The chemotaxis assay is designed to measure the ability of cells to migrate across a semi-permeable membrane in response to certain chemokines, depending on whether the cells display the appropriate chemokine receptors. First, chemotaxis buffer containing the desired chemokine(s) (5-25ng/ml) was placed into the bottom half of a trans-well plate (500µl). The clones (generated from patient blood and skin, as well as priming assay generated clones) were then re-suspended in chemotaxis buffer (1×10^5/ml, 100µl) and placed into the bottom half of the plate. Time points were chosen for cell count

measurement (0h, 1h, 4h, 24h) and cell counts were taken from the bottom half of the trans-well plate (10µl), measuring the total amount of migration across the semi-permeable membrane. Results were recorded as percentage total migration.

2.5.17 SKIN LYMPHOCYTE ISOLATIONS

All skin biopsies were obtained following a positive skin patch test to piperacillin. The samples were taken by a trained clinician under sterile conditions at St James' Hospital, Leeds. The skin biopsy (2mm) was placed into standard lymphocyte culture medium and supplemented with interleukin-2 (75U/ml) for transportation to the laboratory. The sample was then placed into a sterile petri dish and finely minced with a scalpel, then re-suspended in cell culture medium with interleukin-2 (75U/ml, 9ml). This volume was then split into 3 wells of a 12-well plate, and incubated for 5 days at 37°C, 5% CO_2. After the allotted time, the suspension was then passed through a cell strainer (0.45µm) and rinsed into a 50ml tube. The cell suspension was then placed into a 48-well plate, where it was supplemented with irradiated PBMC ($1.5x10^4$/well), PHA (5µg/ml) and interleukin-2 (75U/ml) for 14 days. Following day 14, normal T-lymphocyte cloning was undertaken, as explained earlier.

CHAPTER 3 – DETERMINATION OF THE NAÏVE T-CELL RESPONSE TO PIPERACILLIN USING A DENDRITIC CELL CO-CULTURE T-CELL ASSAY

3.1   INTRODUCTION

Beta-lactam antibiotics are one of the most widely prescribed drugs across the world, with approximately half of all antibiotics prescribed estimated to be from the beta-lactam family (Elander 2003). They all share a common structure, with side chains conferring differential activity centred on the beta-lactam ring which gives them their name. The beta-lactam family of drugs are used in the treatment of bacterial infections. They exert their pharmacological action through the inhibition of cell wall synthesis in the invading bacteria (Waxman and Strominger 1983). This class of antibiotics are extensively prescribed for the treatment of patients with recurrent infections, such as is seen in patients with cystic fibrosis. The fibrosis of the lungs, as well as the increased production of thick mucus that is hard to remove provides an ideal breeding ground for multiple opportunistic bacteria, such as *pseudomonas aeruginosa* (Isles, Maclusky et al. 1984; Oliver, Cantón et al. 2000).

Unfortunately, a much higher incidence of beta-lactam hypersensitivity is observed in patients with cystic fibrosis when compared to the general population. It is not understood why this is, though genetics, overall drug burden and the prophylactic nature of the treatment in these cases are all suggested to play a role. As a result of this, many patients with cystic fibrosis are unable to be treated with conventional beta-lactam antibiotics, resulting in a worsening prognosis. This represents a major clinical concern since the primary cause of mortality in cystic fibrosis is as a result of complications due to infections present in the lung (O'Sullivan and Freedman 2009).

Piperacillin, a powerful broad-spectrum antibiotic, is often contra-indicated in patients with cystic fibrosis, with studies putting the prevalence of hypersensitivity between 26-50% (Koch, Hjelt et al. 1991; Pleasants, Walker et al. 1994; Burrows, Nissen et al. 2007). Patients typically present with maculopapular exanthema, with a cutaneous aetiology the primary manifestation of the delayed type reaction, with the mean time of onset of symptoms after initial administration put at 9.1 days. Because of the delayed onset of clinical symptoms, T-lymphocytes are thought to be the primary drivers of tissue injury. Investigating the role of lymphocytes in the progression of piperacillin hypersensitivity would greatly enhance the understanding of the disease pathology.

In the general population, piperacillin is well tolerated, with incidences of adverse reactions reported in the region of 2%. Classical research defining the hapten hypothesis suggests that the T-lymphocytes need to be presented with a drug modified peptide antigen before a response can occur (Landsteiner and Jacobs 1935; Uetrecht 1997; Park, Pirmohamed et al. 1998; Naisbitt, Gordon et al. 2000). In the case of piperacillin, it is thought that the hapten hypothesis is the primary mechanism for lymphocyte activation. A previously established protocol outlined a way to prime naïve T-lymphocytes to drug derived antigens (Faulkner, Martinsson et al. 2012). This assay utilises naïve T-lymphocytes taken from the peripheral blood. These cells are unique in that they have not been exposed to any form of antigen. In this assay, 'priming' of these naïve T-lymphocytes allows us to study cells that would otherwise be tolerant to the antigen. Thus, it is possible to study the origin of the drug-specific T-cell response and factors associated with T-cell activation and

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polarisation. Dendritic cells are the key antigen presenting cells used for T-lymphocyte priming. They are generated from CD14$^+$ monocytes and matured into dendritic cells through a culture cocktail of both GM-CSF and IL-4, as well as LPS and TNF-α as a final maturation step (Dauer, Obermaier et al. 2003). This maturation step is essential for the development of dendritic cells, with the machinery to successfully present antigenic determinants. In contrast, immature dendritic cells are less suited to lymphocyte activation; their main role involves pathogen recognition via TLR signalling and antigen uptake (Mahnke, Schmitt et al. 2002). The structural differences of immature and mature dendritic cells are highlighted in *figure 3.1*.

Multiple studies have highlighted the role of interleukin-17, as well as a newly identified T$_h$17 class of helper T-lymphocytes in hypersensitivity reactions (van Beelen, Teunissen et al. 2007; Joshi, Fong et al. 2009; Peiser 2013). However, previous work in patients with drug hypersensitivity has not studied the T$_h$17 response in any detail. Moreover, one of the cytokines secreted from T$_h$17 cells, interleukin-22, represents a promising candidate for further study. Its receptor is a heterodimer comprised of one unit of both the interleukin-10 receptor 2 and the interleukin-22 receptor 1 (Jones, Logsdon et al. 2008). This interleukin-22 receptor is present in all tissues, such as the skin in high quantity, though is absent on immune cells (Wolk and Sabat 2006). Interleukin-22 is known to be involved in the response to bacterial pathogens, and is produced by both activated T-lymphocytes and to a lesser extent dendritic cells (Zheng, Valdez et al. 2008).
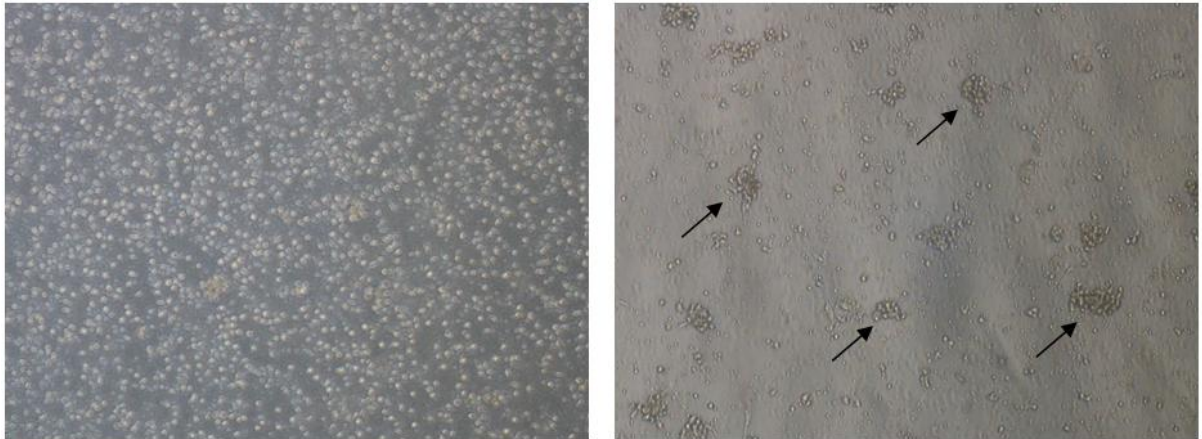
Figure 3.1. *CD14+ cells cultured in a 24-well plate, both pre- and post- maturation via the addition of LPS and TNF-α. The un-matured CD14+ monocytes (left) are shown after 6 days of dendritic cell polarisation using GM-CSF and interleukin-4. These cells are immature and lack confluence. In the CD14+ cells (right) there is clear clumping of the now matured dendritic cells, as highlighted by several arrows on the image. These dendritic cells are not uniform in shape and have a tendency to stick to plates after culture and maturation, requiring scraping from the plate surface to acquire them.*

Drug-specific T-lymphocytes are able to secrete both pro- and anti-inflammatory cytokines in response to stimulation with the appropriate drug-derived antigen (Monshi, Faulkner et al. 2013). In fact, Pichler recently devised a revised classification of delayed type drug hypersensitivity based on the T_h1/T_h2 cytokines secreted from drug specific T-lymphocytes (Pichler 2003). However, relatively little work has been done to characterise the complete profile of cytokines secreted by T-lymphocytes and their overall role in disease progression.

3.2 AIMS AND HYPOTHESIS

Investigation into the mechanisms of T-lymphocyte activation in patients with drug hypersensitivity is accomplished through characterisation of the phenotype and function of antigen-specific T-cells isolated from PBMC. Beta-lactam antibiotics are known to have a high incidence of hypersensitivity reactions and as such the T-cell response has been studied extensively. The generation of T-cell clones responsive to the drugs has enabled researchers to define the way in which the drug activates T-cell and the nature of the induced response. However, these patient studies tell us little about the processes involved in the activation of naïve T-cells as this occurs in patients prior to the appearance of hypersensitivity.

Thus, this chapter focuses on healthy volunteer samples, and the responses that can be generated in response to piperacillin challenge. A dendritic cell co-culture T-cell assay was employed to prime naïve T-cells from donors that had never previously been exposed to the drug. Successful priming allowed us to generate T-lymphocyte clones for further phenotypic analysis and functional characterisation.

The working hypothesis was that:

'Drug-specific T-lymphocytes could be generated in naïve lymphocyte samples following priming using autologous dendritic cells, with the resulting primed lymphocytes able to give an insight into the T-lymphocyte response observed in hypersensitive patients'.

3.3   METHODS

All experimental methods used in this chapter are explained in greater detail in
Materials and Methods Chapter 2. They include:

- Peripheral blood mononuclear cell isolation (*2.5.1*)

- Magnetic separation of cell subsets (*2.5.2*)

- Proliferation assay (*2.5.3*)

- ELISpot (*2.5.7*)

- Flow cytometry (*2.5.8*)

- T-lymphocyte cloning (*2.5.9*)

- EBV generation (*2.5.10*)

- T-lymphocyte priming assay (*2.5.14*)

- Memory T-lymphocyte priming assay (*2.5.15*)

- Chemotaxis assay (*2.5.16*)

3.4 RESULTS

3.4.1 *Healthy volunteer PBMC do not respond to piperacillin exposure* in vitro

In order to fully understand the underlying mechanisms in piperacillin hypersensitivity, our first experiments focused on looking at healthy volunteer samples and if the interaction of T-cells with piperacillin resulted in a detectable proliferative response. Four volunteers were selected, and PBMC were isolated from each individual using a standard protocol. PBMC were used in a lymphocyte transformation test, with differing concentrations of piperacillin up to a maximal dose of 2mM. PHA at a concentration of 5μg/ml was used as a positive control. Proliferative responses to piperacillin were not observed with any concentration of the drug (SI<2). In contrast, lymphocyte proliferative responses above control values were detected in PHA treated wells (SI>2). These results ensured that the samples being used for further analysis did not harbour piperacillin responsive T-cells (***figure 3.2***). Potential T-cell responses to other antibiotics (such as flucloxacillin or meropenem) were not investigated. This was not considered to be relevant, as previous work has shown that piperacillin does not exhibit cross-reactivity to other beta-lactam antibiotics (El-Ghaiesh, Monshi et al. 2012; Yaseen, Saide et al. 2015).
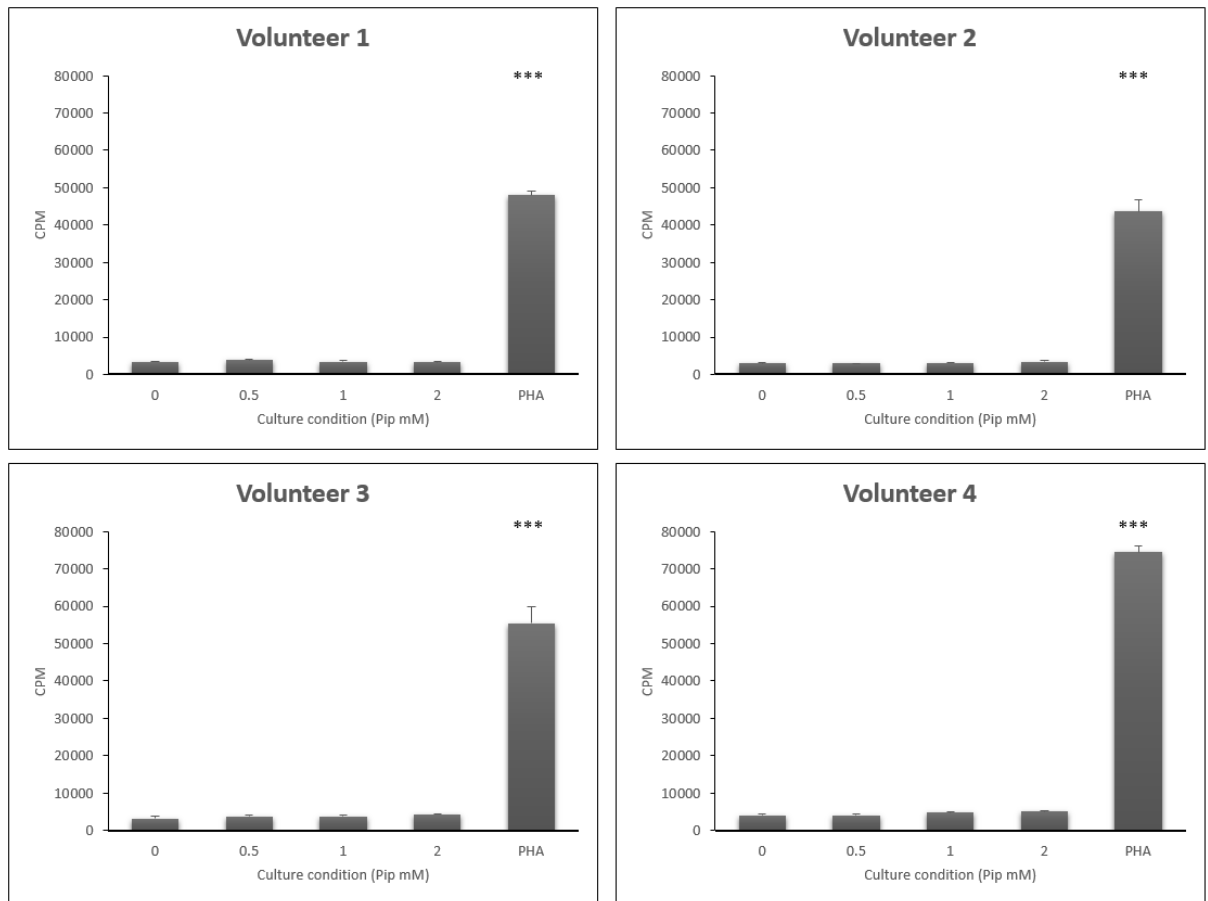
Figure 3.2. _Proliferative responses from 4 healthy volunteers assumed to be naïve to the beta-lactam antibiotic piperacillin._ _PBMCs (1x10⁴, 100μl) were cultured for 5 days in either T-lymphocyte culture media alone, differing concentrations of piperacillin up to a maximal 2mM, or PHA (5μg/ml) as a positive control. Readouts were measured as CPM via [³H]-thymidine incorporation for the final 16h of the experiments. Statistical analysis was performed via a one way ANOVA, compared to the control media well. (P=0.05 *, P=0.005 **, P= <0.001 ***)._

3.4.2 T-lymphocyte cytokine secretion is dependent on the local micro-environment

In order to gain a greater understanding of T-lymphocyte polarisation and the role of the microenvironment, memory T-lymphocytes isolated from healthy volunteers were studies using a simple ELISpot assay. Plates were pre-coated with CD3 antibody. Cells were then cultured in the presence of polarising cytokines for 6 days (T_h1 – IL-12 25ng/ml, Anti IL-4 100ng/ml; T_h2 – IL-4 25ng/ml, Anti IL-12 100ng/ml; T_h17 – IL-1β 20ng/ml, IL-6 20ng/ml, IL-23 20ng/ml, TGFβ 5ng/ml; T_h22 – TNFα 50ng/ml, IL-6 20ng/ml). After development of the assay, lymphocytes exposed to different polarising cocktails were shown to secrete different profiles of cytokines. T_h1 polarised cells secreted high levels of interferon-gamma, T_h2 polarised cells secreted large amounts of the interleukin-13, T_h17 polarised cells secreted interferon-gamma, interleukin-17A, and interleukin-22 and the T_h22 polarised cells secreted high levels of interleukin-22 in the absence of interleukin-17A (**figure 3.3**). This assay was repeated on several occasions with similar results. The plasticity that is shown by T-lymphocytes is shown clearly in this assay, which demonstrates that polyclonal T-cell populations can initiate divergent T-cell responses when required. These responses are at least partly determined by the microenvironment at the time of antigen exposure. In addition, the cells require external stimuli in order to both survive and proliferate. Shown in **figure 3.4** are two well images taken from the same healthy donor. In the well where medium alone is present, the lymphocytes are shown to be sparse and poorly viable. In cells treated with anti CD3 and anti CD28 antibodies, large aggregation and expansion can be observed.

	Interferon-gamma	Interleukin-13	Interleukin-17	Interleukin-22
Control				
CD28 Ab				
T$_h$1 Culture				
T$_h$2 Culture				
T$_h$17 Culture				
T$_h$22 Culture				
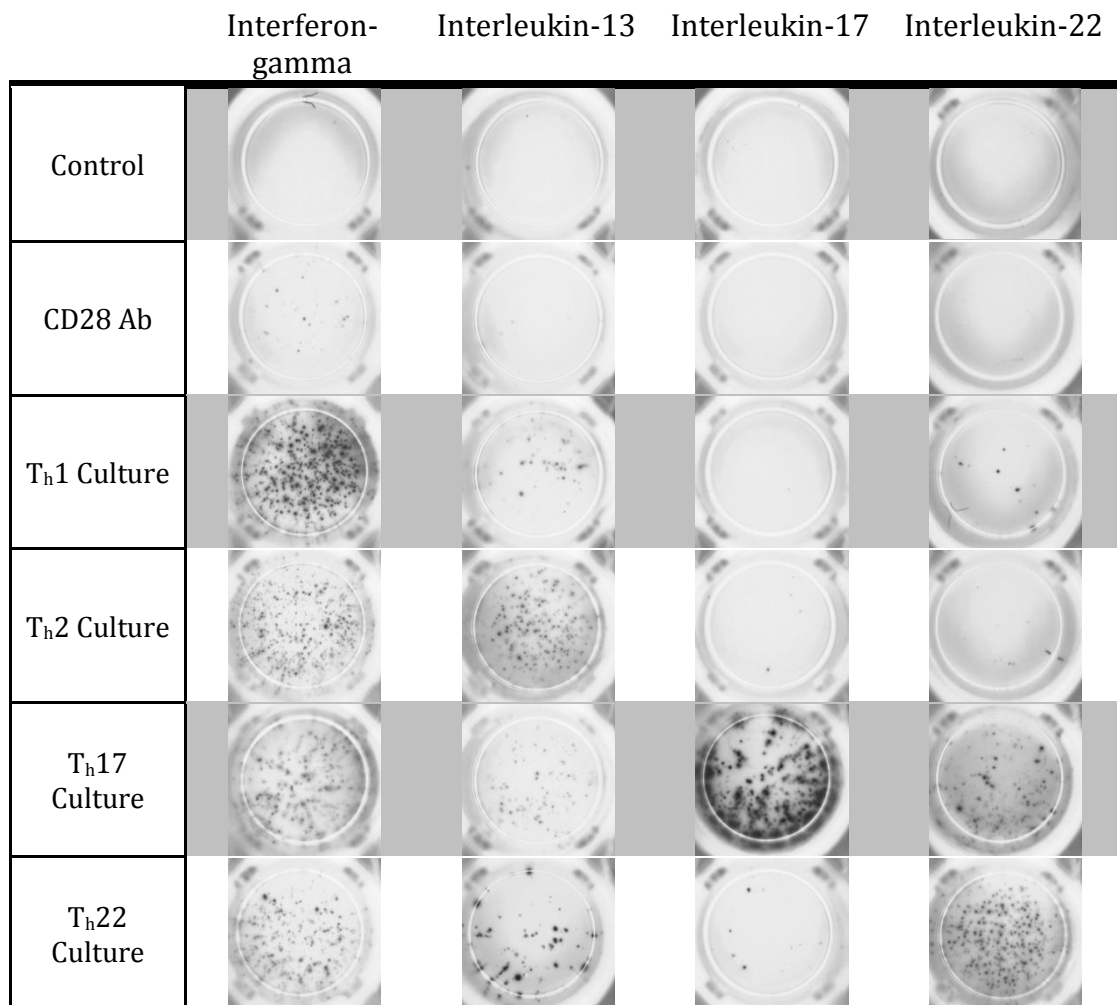
Figure 3.3. *ELISpot well images showing the differing cytokine secretions from a population of memory T-lymphocytes following culture in media containing polarising cytokines.* *High levels of interferon-gamma are seen in T$_h$1 cultures while high interleukin-13 is seen in T$_h$2. T$_h$17 cultures display large secretions of interleukin-17A, with additional interferon-gamma and interleukin-22. T$_h$22 cultures display interleukin-22 secretions in the absence of interleukin-17A, with low levels of both interferon-gamma and interleukin-13 also detected.*
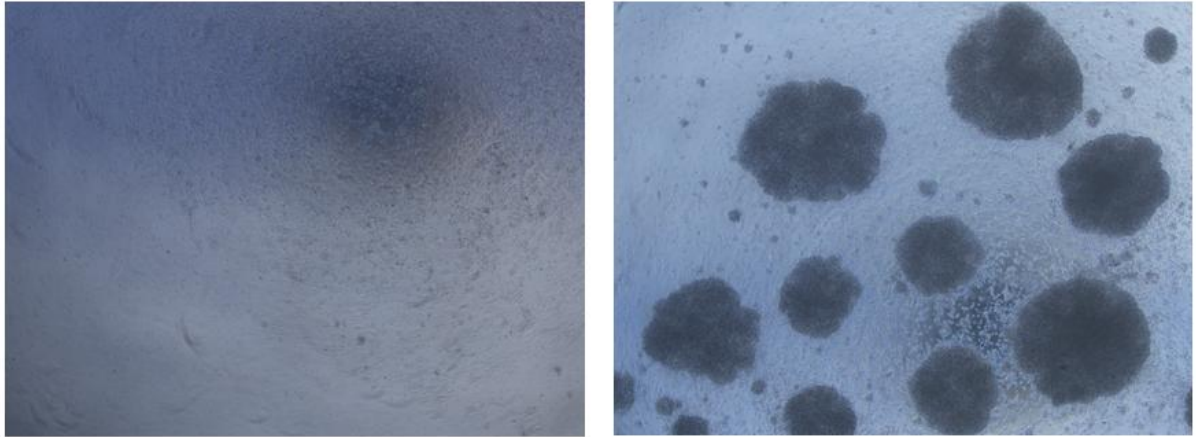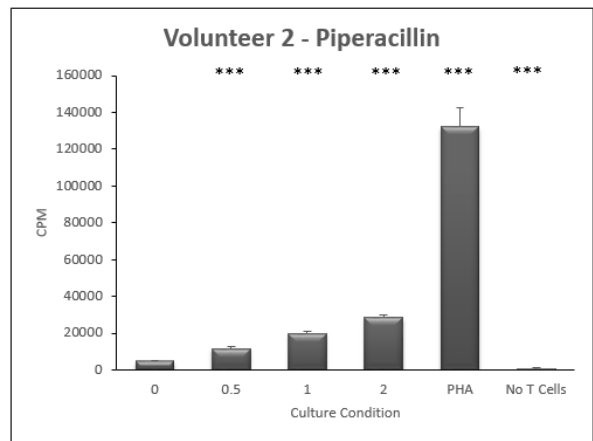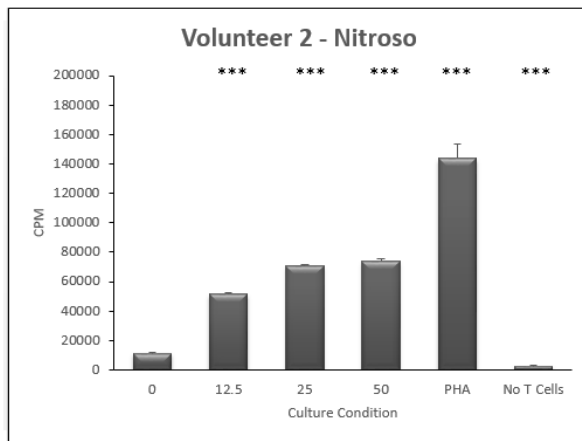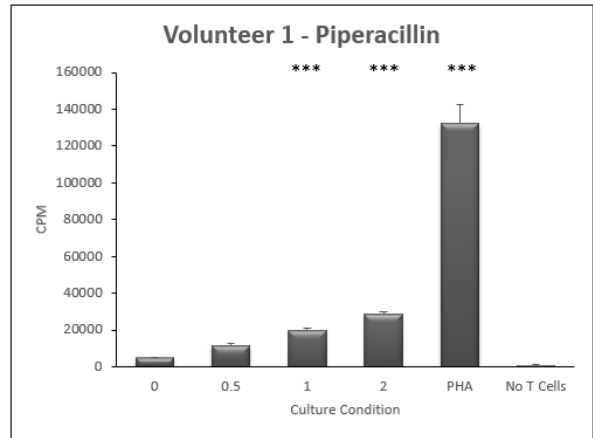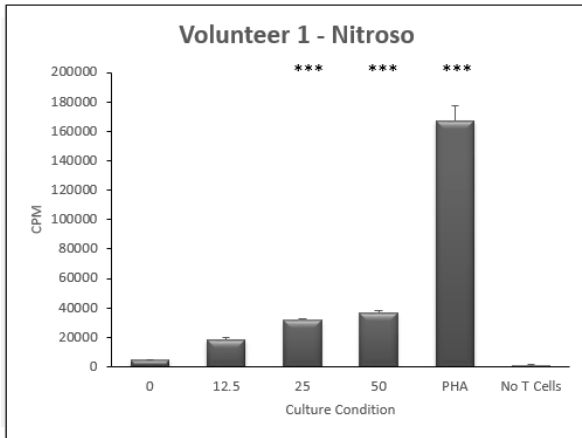
Figure 3.4 _Two representative wells containing memory T-lymphocytes in the absence (left) and presence (right) of external stimuli._ _In cases where external stimuli are not present, lymphocytes are poorly viable and show little to no proliferative activity. However, when external stimuli such as the presence of anti CD3 and anti CD28 antibodies are present, lymphocytes become highly proliferative and aggregate together in response to the stimuli._

3.4.3 Priming of healthy volunteer naïve T-lymphocytes to piperacillin using an autologous dendritic cell co-culture T-cell assay

Following the results showing that the PBMC from volunteers are not responsive to piperacillin, a priming assay was designed utilising autologous dendritic cells and naïve T-cells. First, cells were magnetically separated, then naïve T-lymphocytes were co-cultured with either piperacillin or sulfamethoxazole-nitroso (for a positive control comparison) for two weeks in order to generate a colony of primed T-lymphocytes. Secondly, the primed T-cells were re-stimulated with an additional batch of dendritic cells and drug for 72h, then proliferative responses and cytokine release was measured. In all samples, successful priming was observed to both piperacillin and sulfamethoxazole-nitroso (*figure 3.5*). Proliferative responses were analysed, with an SI greater than 2 considered a positive result. Responses to piperacillin and sulfamethoxazole nitroso were shown to be drug concentration dependant. In some cases, the lowest concentration of tested drug (12.5µm SMX-NO, 0.5mM pip) did not show significant responses, but maximal drug concentrations all expressed highly significant priming for both tested drugs. Moreover, the primed T-cells were not activated with the alternative drug, which confirms antigen specificity (results not shown). Combined results from the four donors are shown in *figure 3.6*. In addition, cytokine secretion from these primed cells was analysed via ELISpot, with secretions of multiple cytokines observed in response to drug challenge (*figure 3.7, figure 3.8*). Maximal cytokine secretion was observed at optimal drug concentration (50µM SMX-NO, 2mM pip). PHA was included as a positive

control to measure total potential secretory ability. Dose dependant secretion of IFNγ, IL-13 and IL-22 was observed in both sulfamethoxazole-nitroso and piperacillin primed conditions. Interleukin-17A was not observed in any condition. An additional condition was tested investigating the response in the absence of T-cells. In these wells, cytokine secretion was abrogated, showing that the cytokines were being produced from the T-lymphocytes. Finally, when compared together, both the sulfamethoxazole-nitroso and the piperacillin primed lymphocytes were shown to have significant dose-dependent responses to differing concentrations of drug challenge (*figure 3.6*).
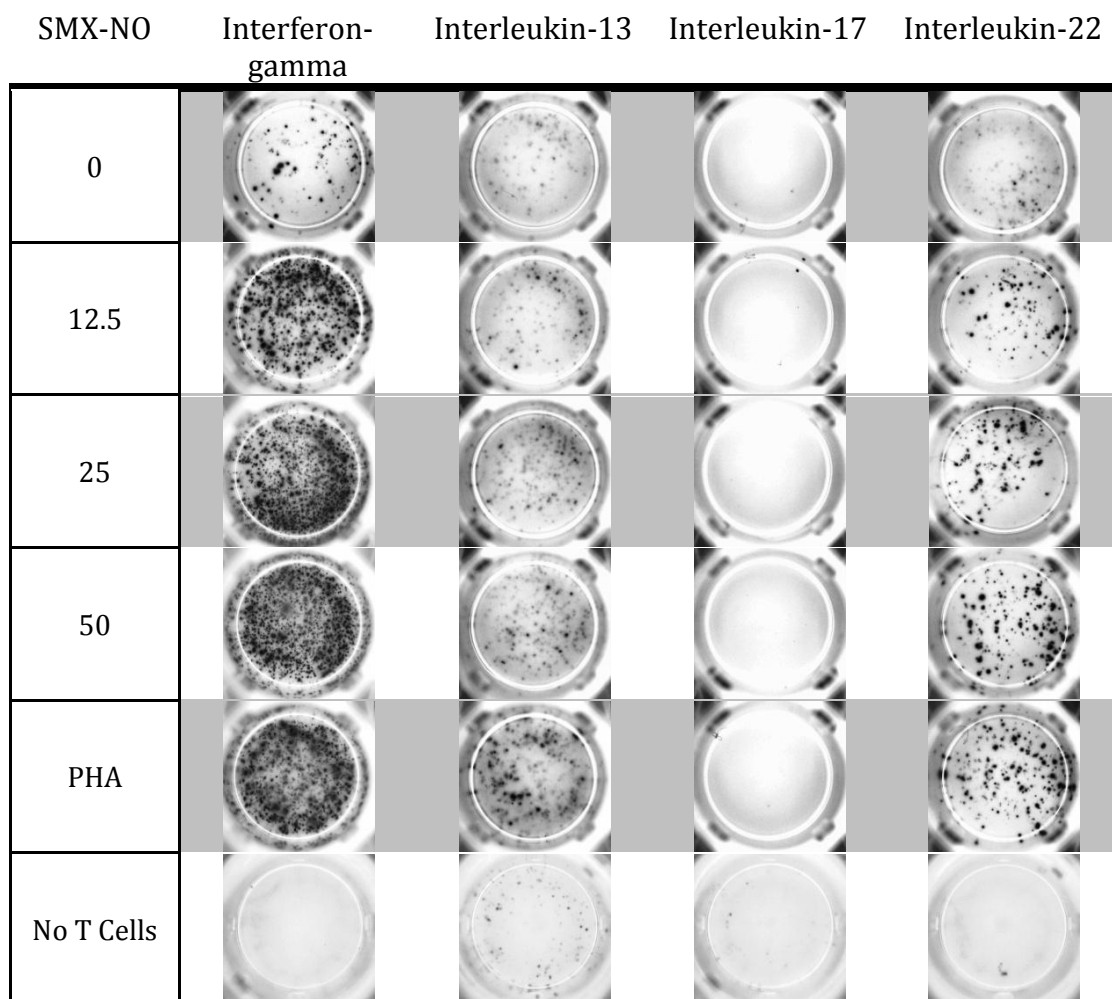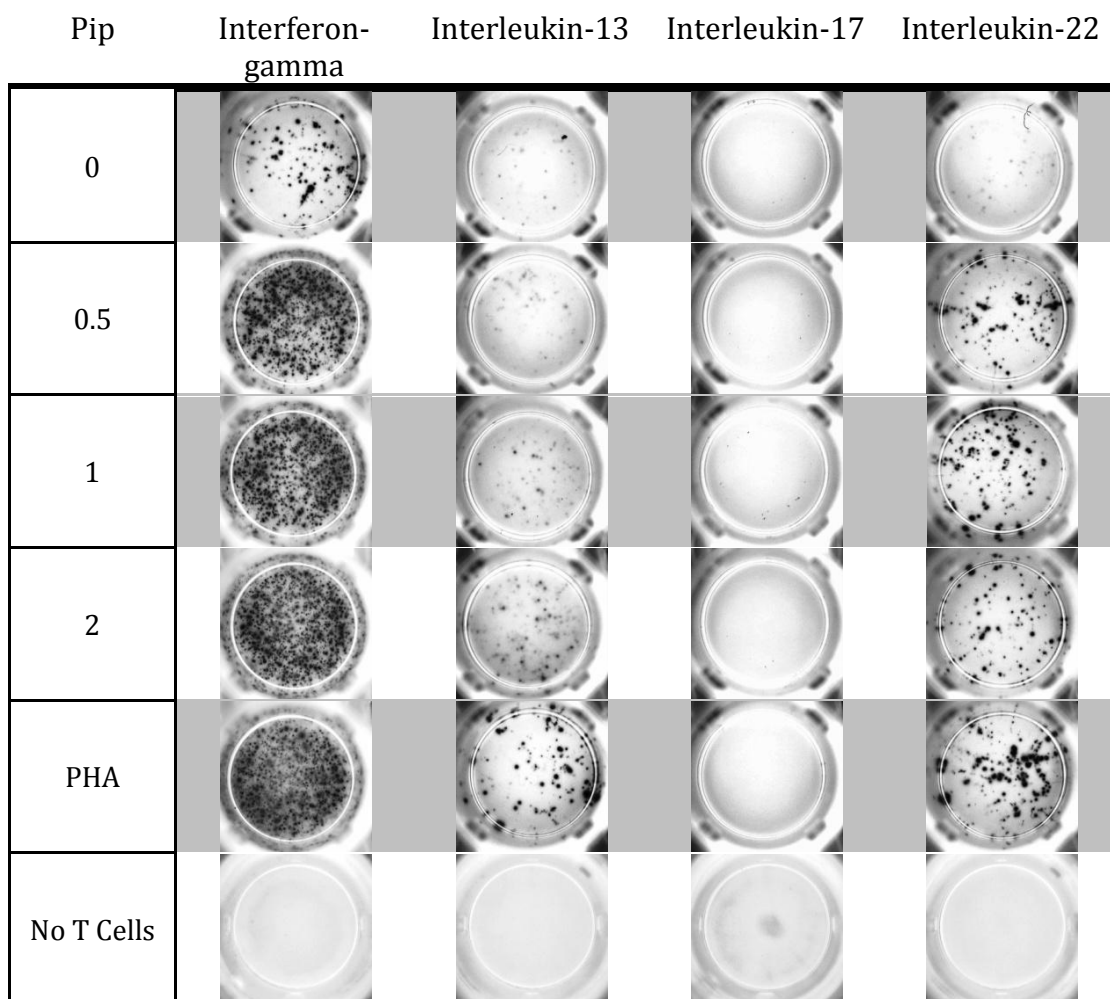
Volunteer 1 - Nitroso

Volunteer 1 - Piperacillin

Volunteer 2 - Nitroso

Volunteer 2 - Piperacillin

Volunteer 3 - Nitroso

Volunteer 3 - Piperacillin

Volunteer 4 - Nitroso

Volunteer 4 - Piperacillin
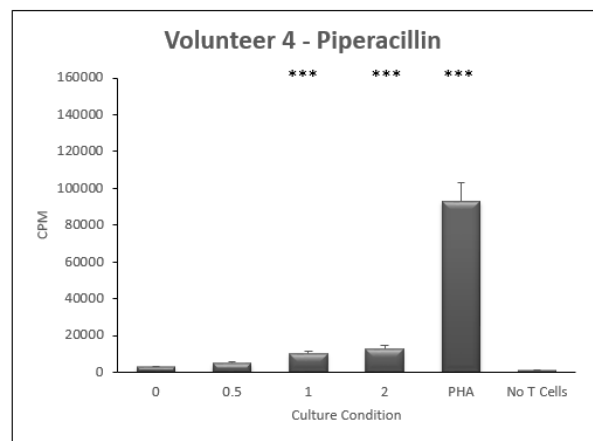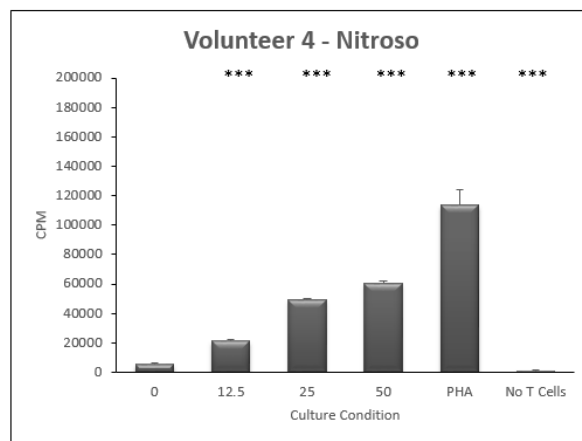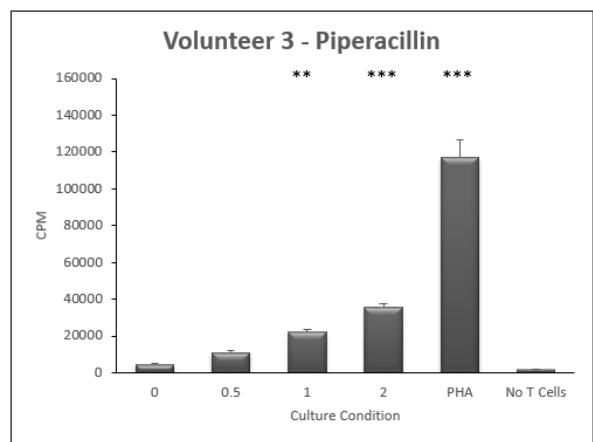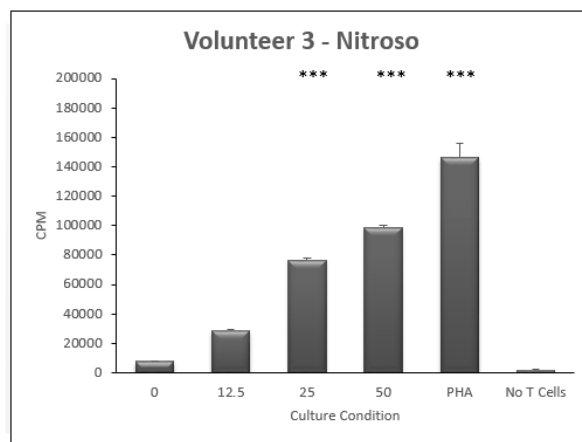
Figure 3.5. _Recall proliferative responses of naïve T-lymphocytes after priming against either sulfamethoxazole-nitroso or piperacillin._ _In each case, proliferation was analysed through the culture of T-lymphocytes (1x10⁵/well, 100µl), antigen presenting cells (2x10⁴/well, 50µl) and the appropriate culture condition (50µl) to a final well volume of 200µl._ _Statistical analysis was performed using a one way ANOVA to compare to the control and test wells._ _All drug treated samples showed a significant increase in proliferation when compared to control wells._ _(P=0.05 *, P=0.005 **, P= <0.001 ***)._
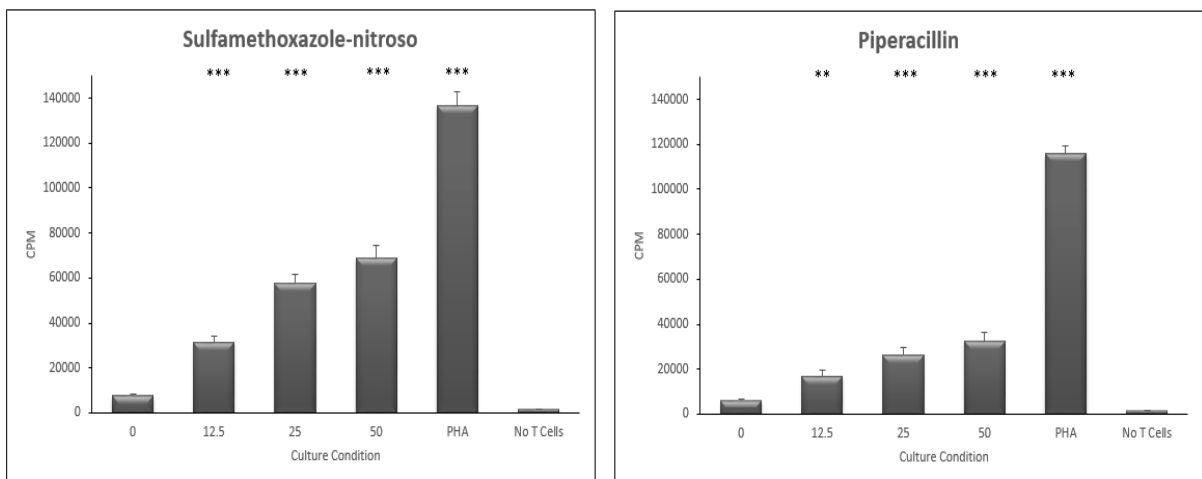
Figure 3.6. _Combined results displaying the proliferative response of primed T-cells from 4 donors following re-stimulation with either piperacillin or sulfamethoxazole-nitroso._ _With both drugs, T-cells from all individuals were combined and the appropriate statistical analysis was performed (one way ANOVA compared to control well)._ _These results mirror the individual proliferation results from the volunteers, with maximal proliferation observed to PHA._ _In both cases, a dose-dependent response is observed to increasing drug concentrations, with the highest response observed at maximal concentrations of the drug._

SMX-NO	Interferon-gamma	Interleukin-13	Interleukin-17	Interleukin-22
0				
12.5				
25				
50				
PHA				
No T Cells				

Figure 3.7. *ELISpot analysis of newly primed T-lymphocytes to sulfamethoxazole-nitroso.* *ELISpot wells containing T-lymphocytes ($1x10^5$, 100µl), antigen presenting cells ($2x10^4$, 50µl) and the stated culture condition (µM) to a final volume of 200µl per well. Analysis was performed using an AID ELISpot reader. Displayed are the results obtained from volunteer 2. Increasing concentrations of drug correlate with a clear dose-dependent response observed in newly primed T-lymphocytes.*

Pip	Interferon-gamma	Interleukin-13	Interleukin-17	Interleukin-22
0				
0.5				
1				
2				
PHA				
No T Cells				

Figure 3.8. *ELISpot analysis of newly primed T-lymphocytes to piperacillin. ELISpot wells containing T-lymphocytes (1x10⁵, 100µl), antigen presenting cells (2x10⁴, 50µl) and the stated culture condition (µM) to a final volume of 200µl per well. Analysis was performed using an AID ELISpot reader. Displayed are the results obtained from volunteer 2. Increasing concentrations of drug correlate with a clear dose-dependent response observed in newly primed T-lymphocytes.*
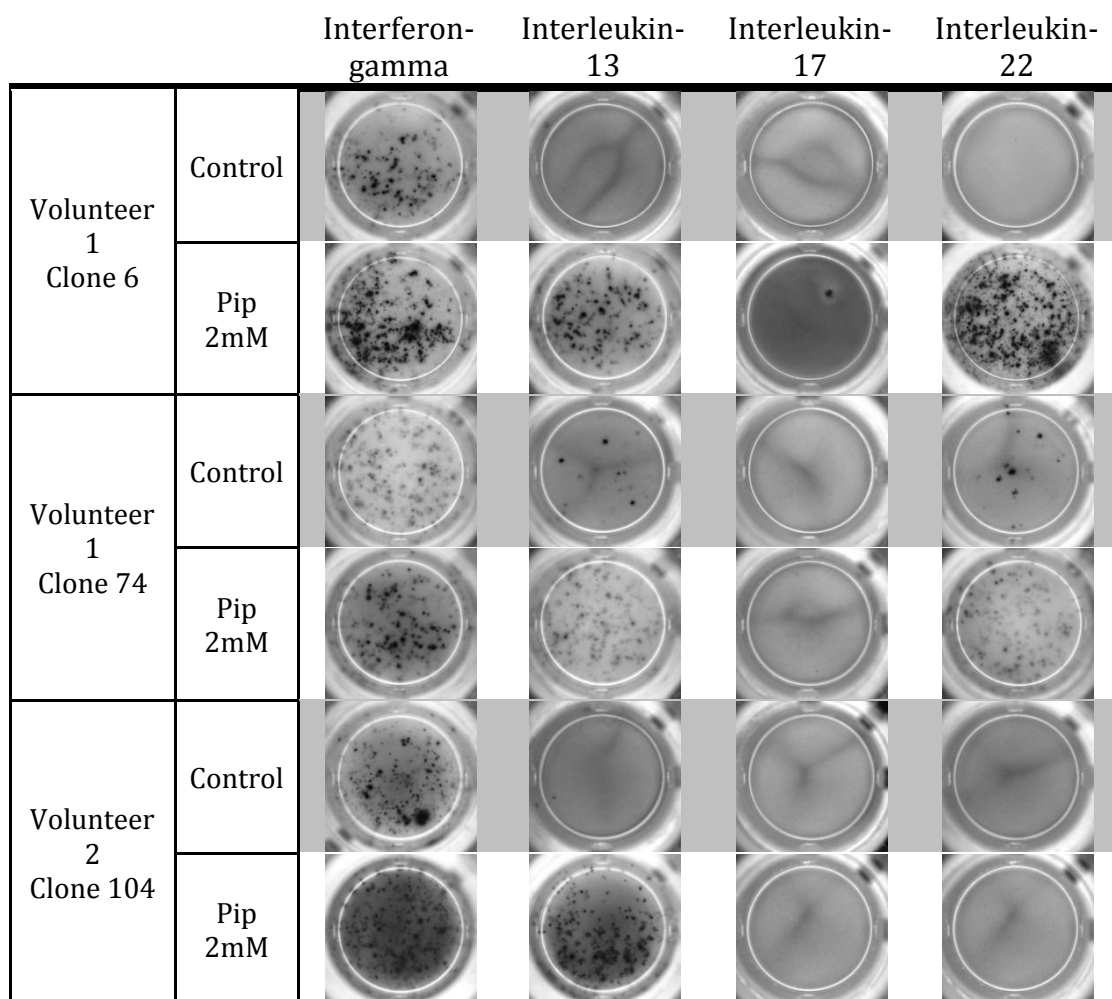
3.4.4 T-lymphocyte clones generated from priming assays secrete IFNγ and IL-13 alongside IL-22

Following the successful T-lymphocyte priming observed in 3.4.3., the newly primed lymphocytes were used to generate T-lymphocyte clones specific to piperacillin. Alongside this, EBV-transformed B-cell lines were generated from all volunteers and used as a ready supply of autologous antigen presenting cells. Five hundred and twenty six clones were generated from the 4 donors. Of these, 64 were activated to proliferate to piperacillin (*figure 3.9*). Clones were initially tested using duplicate control and piperacillin-treated (2mM) wells. A stimulation index value of greater than 2 was taken as a positive result. A predominantly CD8^{+} phenotype was observed in the drug-specific clones, which is the opposite of what is seen in hypersensitive patients (see other chapter results). Across all 4 volunteer samples, similar numbers of both total tested clones and resulting drug-specific clones were observed, with between 117-150 total tested clones and 14-19 drug-specific clones generated. The average percentage generation of drug-specific clones was 12.1% (9.3% - 15.7%). From the drug-specific clones generated (n=64), 49 were shown to be CD8^{+}, giving a CD8^{+} phenotype percentage of 76.6%.
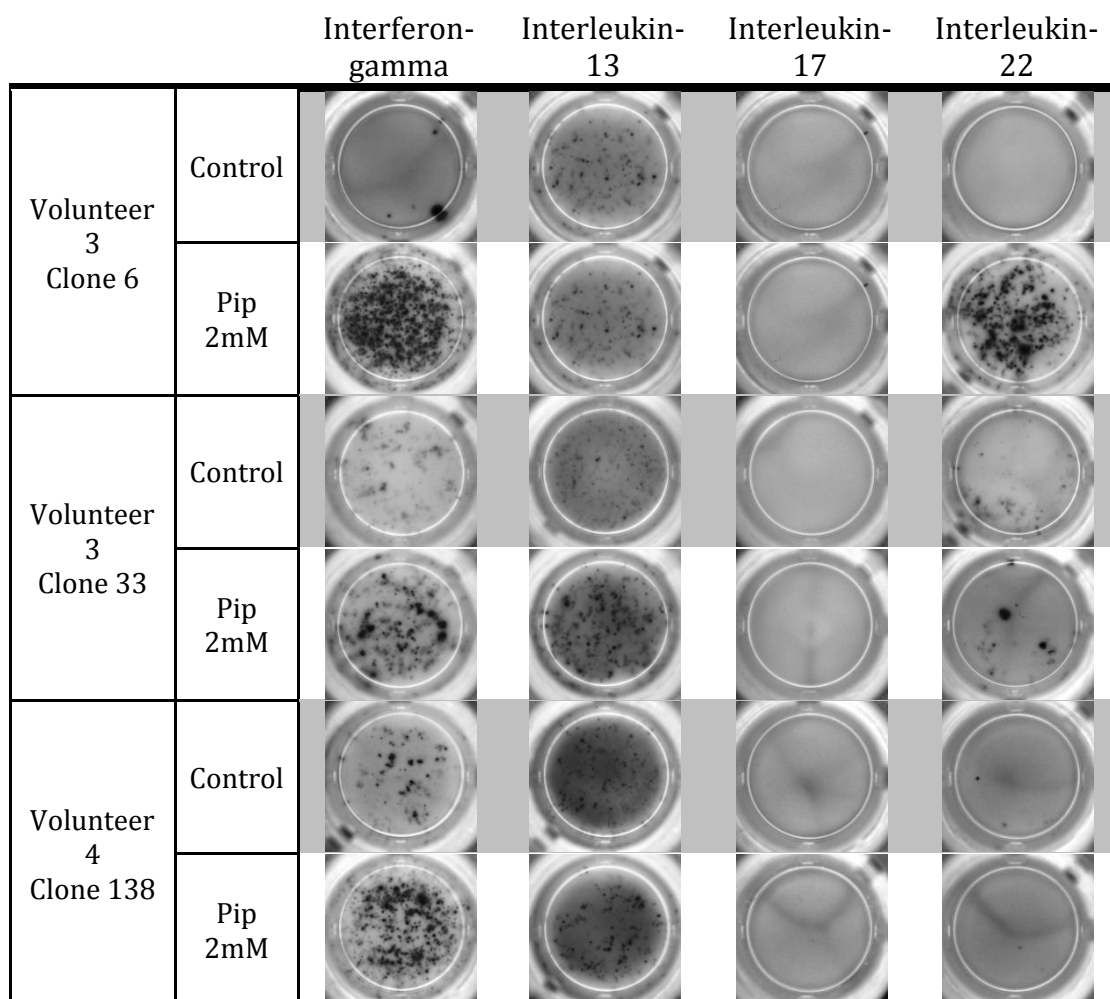
ID	Type	# Tested	# Specific	% CD4+	% CD8+
Volunteer 1	Primed T	150	14	36	64
Volunteer 2	Primed T	121	19	37	63
Volunteer 3	Primed T	138	16	6	94
Volunteer 4	Primed T	117	15	13	87

Figure 3.9. *A table showing the generation of clones from 4 volunteers that had previously been primed to piperacillin. A total of 64 drug-specific clones were generated from 526 tested individual clones.*

From the drug-specific T-lymphocyte clones generated, 10 clones per volunteer were chosen and expanded by repetitive mitogen stimulation for further investigation. The clones were chosen though a combination of initial proliferative values, in addition to overall cell numbers available for analysis. Moreover, CD4+ and CD8+ clones from each volunteer was selected. Clones were found to proliferate in the presence of piperacillin in a dose-dependent manner, with a drug concentration of 1-2mM stimulating maximal responses. Therefore, 2mM was used in ELISpot experiments to profile the cytokines secreted from the piperacillin-specific clones. Multiple cytokines were measured for each drug specific clones; namely interferon-gamma, interleukin-13, interleukin-17A and interleukin-22. Experiments were conducted according to the methods stated in Materials and Methods Chapter 2, as well as following manufacturer's instructions. For all clones tested, secretion of interferon-gamma and interleukin-13 was observed when compared to control wells. Approximately half of all tested clones secreted interleukin-22 at differing levels. In contrast, no interleukin-17A secretion was observed (***figure 3.10, figure 3.11***).

		Interferon-gamma	Interleukin-13	Interleukin-17	Interleukin-22
Volunteer 1 Clone 6	Control				
	Pip 2mM				
Volunteer 1 Clone 74	Control				
	Pip 2mM				
Volunteer 2 Clone 104	Control				
	Pip 2mM				

Figure 3.10. *Cytokine secretion from piperacillin-specific T-lymphocyte clones that have been generated from previously primed naïve T-lymphocytes. T-cells (1x10^{5}, 100μl) were stimulated with autologous dendritic cells (2x10^{4}, 50μl) and culture condition (either culture media alone or piperacillin at 2mM, 50μl) and cytokine secretions then measured by ELISpot. This analysis was undertaken on all clones. Clones selected show secretion of interferon-gamma and interleukin-13, as well as differing levels of interleukin-22. No clones secreted interleukin-17A.*

		Interferon-gamma	Interleukin-13	Interleukin-17	Interleukin-22
Volunteer 3 Clone 6	Control				
	Pip 2mM				
Volunteer 3 Clone 33	Control				
	Pip 2mM				
Volunteer 4 Clone 138	Control				
	Pip 2mM				

Figure 3.11. *Cytokine secretion from piperacillin-specific T-lymphocyte clones that have been generated from previously primed naïve T-lymphocytes. T-cells (1x10^5, 100μl) were stimulated with autologous dendritic cells (2x10^4, 50μl) and culture condition (either culture media alone or piperacillin at 2mM, 50μl) and cytokine secretions then measured by ELISpot. This analysis was undertaken on all clones. Clones selected show secretion of interferon-gamma and interleukin-13, as well as differing levels of interleukin-22. No clones secreted interleukin-17A.*

3.4.5 Chemotaxis to ligands occurs in response to chemokine receptors differentially expressed on clones

Further investigations were undertaken to characterise the phenotype of cell surface chemokine receptors that were expressed on the T-lymphocyte clones. A panel of chemokine receptors, listed in **figure 3.12** were investigated. Analysis of the data was conducted through a combination of Cyflogic and Flowing software. First, the clones were gated using forward and side scatter (FSC / SSC) as shown in **figure 3.13**.

Tube Number	PE	APC	FITC
1	Unstained	Unstained	Unstained
2	CCR1	CCR2	CLA
3	CCR4	CCR6	CD69
4	CCR8	CCR9	CCR5
5	CCR10	CXCR3	CCR3
6	E-Cadherin	CXCR6	Blank

Figure 3.12. *Table displaying the layout for chemokine receptor staining that was used for all analyses. In each tube, $1x10^5$ cells were re-suspended in MACS buffer (200μl) and ran through a FACS Canto II machine to acquire readouts for receptor expression. 4μl of fluorochrome antibodies were added per condition to each well.*
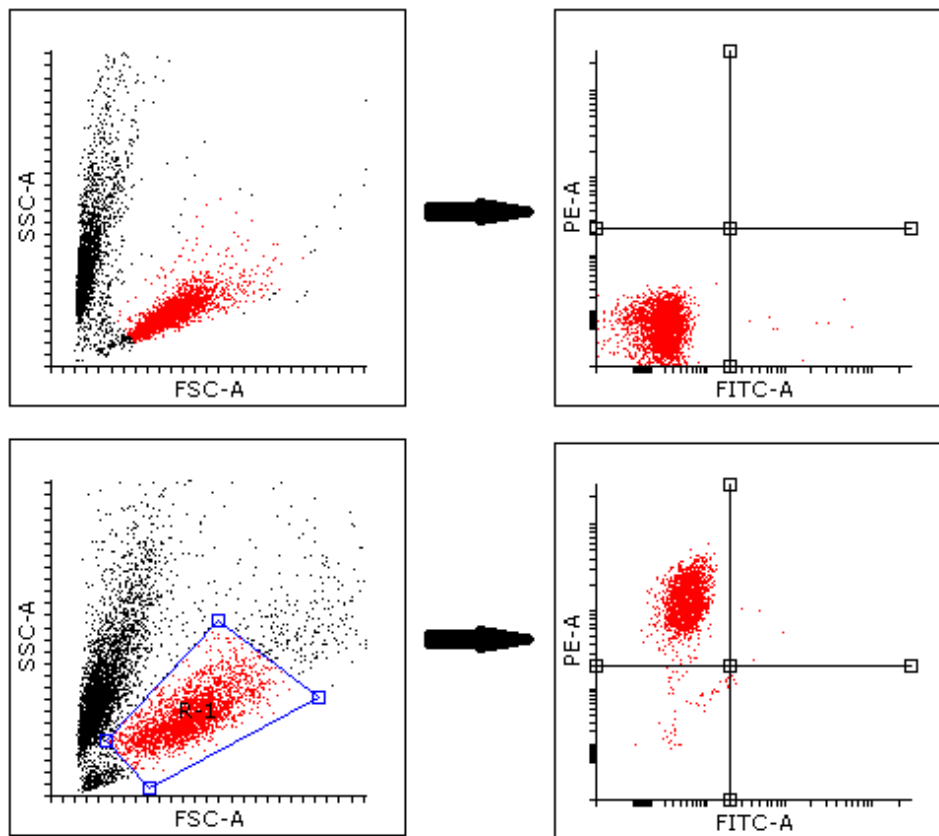
Figure 3.13. *Screenshots of the flow cytometry analysis conducted using Flowing Software. In this image, initial whole event captures are displayed on the left image. The highlighted red area shows where the viable lymphocyte population is located. Gating on this area allows for removal of the background undesirable cells and cell fragments (shown on right). This allows for investigations into only the desired populations through relative fluorochrome expressions on the X and Y axis (example shown is a control unstained sample **top**, with CD4-PE expressing clones shown on the **bottom**).*

All chemokine receptor analysis was performed through comparing the MFI (mean fluorescent intensity) of expressed fluorochromes on clones when compared to control samples stained with a relevant isotype control (***figure 3.14***).

Receptor	Volunteer 1	Volunteer 2	Volunteer 3	Volunteer 4
CCR1	✓✓	✓✓	✓✓	✓✓✓
CCR2	✓✓	✓✓	✓✓	✓✓
CCR3	X	X	X	X
CCR4	✓✓✓	✓✓✓	✓✓✓	✓✓✓
CCR5	✓✓	✓✓	✓✓	✓✓
CCR6	X	X	X	X
CCR8	✓✓	✓✓	✓✓	✓✓
CCR9	✓✓✓	✓✓✓	✓✓✓	✓✓✓
CCR10	✓✓✓	✓✓✓	✓✓✓	✓✓✓
CXCR3	✓✓✓	✓✓✓	✓✓✓	✓✓✓
CXCR6	✓	✓	✓	✓
CD69	✓✓✓	✓✓✓	✓✓✓	✓✓✓
CLA	X	X	X	X
E-Cadherin	✓	✓	✓	✓

Figure 3.14. _Table showing the relative expression of different chemokine receptors in relation to control samples as a comparison of MFI. (✓= MFI difference 1.5-2, ✓✓= MFI difference 2-2.5, ✓✓✓ = MFI difference 2.5+)._

Although clones across volunteers expressed the same chemokine receptors (**_figure 3.14_**), individual clones were shown to express differing levels of each receptor.

The functional consequence of receptor expression was investigated using a chemotaxis assay containing chemokine ligands to respective receptors. Lymphocyte migration was expressed as percentage migration, with receptor expression-dependent migration observed across clones. A total of 12 clones were investigated (3 per volunteer) and analysis performed in the presence of RANTES, CCL2, CCL4, CCL17, CCL25, CCL27, CXCL9 and CXCL16, ligands to CCR1, CCR2, CCR5, CCR4, CCR9, CCR10, CXCR3 and CXCR6 respectively at a number of time points (0h, 1h, 2h, 24h) to measure percentage migration (**_figure 3.15_**). Migration by clones was observed in both a time and receptor

expression dependant manner, with highly expressed receptors correlating with maximal percentage migration.

RANTES - CCR1

CCL2 - CCR2

CCL17 - CCR4

CCL4 - CCR5

CCL25 - CCR9

CCL27 - CCR10

CXCL9 - CXCR3

CXCL16 - CXCR6

__Figure 3.15.__ *Time dependant chemotaxis of T-cell clones towards individual chemokines. A total of 12 clones were investigated (3 per volunteer) over multiple time points (0h, 1h, 2h, 24h). Percentage migration was obtained through cell counts performed at the allotted time points, with total cell number minus cells counted used to work out percentages.*

3.5 DISCUSSION

The method of antigenic recognition in patients with beta-lactam hypersensitivity is hypothesised to involve the binding of the beta-lactam ring to exposed lysine residues on host proteins. In agreement with this hypothesis, sophisticated mass spectromic methods have been used to characterise the site selective binding of various beta-lactam antibiotics to the protein human serum albumin (Jenkins, Meng et al. 2009; Whitaker, Meng et al. 2011). The derived protein adducts are thought to be taken up by antigen presenting cells and processed to liberate a range of peptides, some of which contain the drug bound to lysine. Although the precise nature of antigenic peptides is yet to be defined, research conducted prior to commencement of these studies revealed that albumin adduct active T-cells from hypersensitive patients and fixation of APC which blocks protein processing inhibits the T-cell response (El-Ghaiesh, Monshi et al. 2012). At the St. James' Hospital regional adult cystic fibrosis centre, the majority of patients that are treated there develop hypersensitivity to at least one antibiotic, with multiple drug hypersensitivity a common occurrence. While hypersensitivity reactions in the healthy population are relatively uncommon, it is much higher in patients who are suffering with cystic fibrosis, and as such are exposed to greater and more frequent antibiotic dosing regimens (Parmar and Nasser 2005).

Previous studies have shown that PBMC's from both drug tolerant patients and naïve donors do not respond in a lymphocyte transformation test when stimulated with allergenic drugs at optimal concentrations. *Figure 3.2* shows similar findings with piperacillin. Proliferation of lymphocytes cultured with

piperacillin was not observed. These data show that the volunteer samples used in subsequent investigations throughout the chapter would not respond to the beta-lactam antibiotic piperacillin under normal culture conditions using methods to characterise T-cell responses in drug hypersensitive patients.

The polarisation of helper T-lymphocytes has been extensively reported in multiple studies (Romagnani 1997; Sallusto, Lanzavecchia et al. 1998; Kidd 2003). While the classical T_h1 / T_h2 distinction has been the mainstream profiling conducted for multiple years, recent advances into the lymphocytic phenotypes have led to the discovery of newer subtypes, such as T_h9, T_h17 and T_h22 (Eyerich, Eyerich et al. 2009; Korn, Bettelli et al. 2009; Schlapbach, Gehad et al. 2014). T_h1 cells are considered to be pro-inflammatory, with interferon-gamma the main cytokine secreted from these cells. Other pro-inflammatory cytokines are known to be secreted from these cell types, alongside cytolytic molecules such as fas ligand and granzyme B. In contrast, the T_h2 class of lymphocytes display a more anti-inflammatory phenotype, with low levels of interferon-gamma and higher levels of cytokines such as interleukin-5 and interleukin-13 (Berger 2000). The pro- and anti-inflammatory nature of these two classes are typified though the perception that T_h1 cells are involved in and promote MHC class I driven cytotoxic T-cell responses, with T_h2 secreting cells linked to the promotion of B-cell responses and antibody production. It should however be noted that that separation of T-cells into a T_h1/T_h2 phenotype is not universal. Many antigens stimulate human T-cells to secrete both T_h1 and T_h2 cytokines.

The perception that T_h1/T_h2 polarised signalling describes all forms of immune response is now outdated. It fails to take into account the newer cytokines identified that have integral roles in lymphocyte responses, such as interleukin-17A and interleukin-22. For example, the highly pro-inflammatory interleukin-17A is secreted from T_h17 cells (Miossec, Korn et al. 2009), alongside high levels of interferon-gamma and interleukin-22. T_h22 cells also secrete interleukin-22, but in the absence of interleukin-17A. Interleukin-22 can be considered both pro- and anti-inflammatory depending on the other cytokines being secreted from T-cells, as well as the location of cytokine secretion (Duhen, Geiger et al. 2009). This plasticity of helper T-lymphocytes is demonstrated further in *figure 3.3*. Completely different cytokine secretion profiles are displayed with memory T-lymphocytes when the cells are cultured for 6 days in different cytokine micro-environments. In cases of chronic pathology where tissue microenvironments could contain multiple cytokines, the plasticity of these cells and their potential involvement in hypersensitivity cannot be understated.

An initial investigation into priming naïve T-lymphocytes with autologous dendritic cells and sulfamethoxazole-nitroso was conducted to establish the assay conditions and to serve as a positive control. Sulfamethoxazole-nitroso is considered a 'model hapten' due to its propensity to stimulate T-cells from hypersensitive patients (Castrejon, Berry et al. 2010), as well as in animal models of immunogenicity (Naisbitt, Williams et al. 2001). Moreover, SMX-NO has previously been shown to activate T-cells from healthy drug naïve donors using a range of culture conditions (Pichler 2003; Faulkner, Martinsson et al. 2012). In cases where the underlying mechanisms of sensitisation and

resulting hypersensitivity is unclear, this novel method of using cells from healthy donors could help to elucidate pathways of drug-specific T-cell activation. The work undertaken in this chapter was to explore the activation of naïve T-cells to the beta-lactam piperacillin, which is known to form penicilloyl protein conjugates as a result of the opening of the beta-lactam ring (Weltzien and Padovan 1998; Whitaker, Meng et al. 2011). Successful priming of purified naïve T-cells was observed in 4 volunteers to both sulfamethoxazole-nitroso and piperacillin as shown in *figure 3.5* and *figure 3.6*. Statistically significant priming (P= <0.001) was seen at optimal concentrations of both the nitroso metabolite (at 50µM) and piperacillin (at 2mM). The strength of the induced response to SMX-NO and piperacillin was higher than that observed with 7 other drugs studied in Liverpool (Monshi, Faulkner et al. 2013; Gibson, Ogese et al. 2014; Yaseen, Saide et al. 2015)(unpublished data). This could be due to multiple factors, chief of which would be the simple fact that priming to non-reactive drugs is more difficult, with lower proliferation values observed across the board for compounds activating T-cells via a PI mechanism involving the direct binding of the drug to MHC molecules.

Previous studies have shown that both T_h1 and T_h2 cytokines are secreted from T-cells from beta-lactam hypersensitive patients (Whitaker, Meng et al. 2011). In the original dendritic priming investigation, both pro- and anti-inflammatory cytokines were also found to be secreted from SMX-NO specific T-cells following successful priming (Faulkner, Martinsson et al. 2012). In agreement with this, *figure 3.6* shows that both IFNγ and IL-13 are secreted following re-stimulation of piperacillin naïve T-cells. Furthermore, T-cells

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secreted interleukin-22 following piperacillin and SMX-NO treatment. In contrast, IL-17A secretion was not detected. These data prompted us to investigate the functionality of the drug specific T-cells in greater detail.

Many studies have shown the value of the lymphocyte serial dilution assays for the generation of drug-specific T-lymphocyte clones (Naisbitt, Britschgi et al. 2003; Farrell, Lichtenfels et al. 2013). Previous work in our department have shown that beta-lactam drug-specific clones are able to be generated, with piperacillin clones displaying a predominantly CD4$^+$ phenotype. ***Figure 3.9*** shows that successful generation of drug-specific T-lymphocyte clones is possible after naïve lymphocyte priming. Of the total 526 clones tested, 64 were shown to have a SI greater than 2 indicating drug specificity for a percentage generation of 12.2%. In contrast to previously reported data, the majority of clones generated were of a CD8$^+$ phenotype, although CD4$^+$ clones were also present. This dichotomy of results is difficult to reconcile, though the *in vitro* assay and the 'forced' priming of naïve lymphocytes to a specific compound might explain the disparity.

Clones were shown to secrete both pro- and anti-inflammatory cytokines, though again no interleukin-17A was detected from the clones. While extensive studies have characterised T_h1, T_h2 and T_h17 lymphocytes, no sub-class of cells fully marry up with the cytokine secretion profiles observed in piperacillin specific clones (Zhu and Paul 2008). The presence of interleukin-22 in the absence of interleukin-17A suggests a different sub-class of helper T-lymphocytes, such as the T_h22 class (Akdis, Palomares et al. 2012). This suggestion can be further backed up due to the cutaneous nature of

interleukin-22's physiological action, and its dual pro- and anti-inflammatorial nature (Boniface, Guignouard et al. 2007).

In order to further understand the profile of drug-specific lymphocytes present in piperacillin hypersensitivity reactions, an investigation into chemokine receptor expression was undertaken. Previous studies have highlighted the ability to select lymphocytes from a whole population of cells and cell fragments (Pechhold, Pohl et al. 1994). Viable lymphocytes could be identified through a CD3$^+$ phenotype, with the resulting cells stained with multiple markers of cell surface receptors to a host of chemokines. Of particular interest are the receptors CCR1, CCR4, CXCR3 and CCR10. CCR1 is involved in both lymphocyte recruitment and activation, as well as having a link to both innate and adaptive immunity (Weber, Weber et al. 2001). CCR4 is linked to cutaneous trafficking of lymphocytes, suggesting a key role in cutaneous hypersensitivity syndromes. CXCR3 is found in numerous inflamed tissues, as well as integrin activation (Qin, Rottman et al. 1998). CCR10 is expressed on multiple lymphocytes and its interaction with CCL27 is heavily implicated in T-lymphocyte mediated cutaneous inflammation, again suggesting an important role for the hypersensitivity commonly observed to beta-lactam antibiotics (Soler, Humphreys et al. 2003).

A selection of 10 clones per volunteer were chosen for chemokine analysis, with the resultant expressions recorded as MFI increase above a baseline. Of the receptors that were investigated as shown in *figure 3.14*, some were expressed constitutively across all clones, such as CD69. This is not unexpected, due to the fact that it is a marker of activation which would be

present on dividing lymphocytes such as the tested clones(Cambiaggi, Scupoli et al. 1992).

Significant increases in expression were observed in chemokine receptors, including CCR1, CCR4, CCR10 and CXCR3. These were elevated compared to controls, suggesting a high level of expression across all drug-specific clones. These receptors are all implicated in either lymphocyte trafficking or cutaneous migration which, when taken in tandem with the clones ability to secrete the tissue specific cytokine interleukin-22, suggests that these cells are important mediators of piperacillin hypersensitivity.

Though these clones have been shown to have the potential to migrate in response to chemokines, due to their high receptor expressions proof of migratory ability is required. To accomplish this, chemotaxis assays were employed. The assay used assesses the ability of clones to migrate across a semi-permeable barrier in the presence of differing concentrations chemokines (Gibson, Ogese et al. 2014). Migration in response to ligands was matched to receptors expressed on the cell surfaces. *Figure 3.15* shows that time-dependent migration occurred in response to chemokine ligand presence.

Taken in totality, the naïve T-lymphocytes were successfully primed to piperacillin and elicited responses typical of a hypersensitivity reaction. These primed drug responsive lymphocytes were cloned and expanded. The cloned cells displayed a interleukin-22^{+} cytotoxic CD8^{+} phenotype. In addition, the drug-specific lymphocytes expressed receptors, and were able to migrate in response to chemokines that are implicated in cellular trafficking to inflamed sites, as well as the skin. These data suggest that piperacillin-specific T-

lymphocytes play an integral role in the progression of hypersensitivity. Though this work has only been conducted on volunteer samples, the value of this data cannot be understated and the impetus to investigate the role of drug-specific T-lymphocytes in patients with piperacillin hypersensitivity is now clear. The role of interleukin-22 also seems to be important and this is studied in much more detail in other chapters.

CHAPTER 4 – CHARACTERISATION OF THE PHENOTYPE AND FUNCTION OF PIPERACILLIN-SPECIFIC T-LYMPHOCYTES ISOLATED FROM THE BLOOD OF HYPERSENSITIVE PATIENTS

4.1   INTRODUCTION

Cystic fibrosis is a lethal autosomal recessive disorder which is one of the most common conditions across the globe, especially in western Caucasian populations (Cutting, Curristin et al. 1992). It is estimated that 4% of the population of the United Kingdom are recessive carriers of the gene responsible, with approximately 10,000 people thought to have the condition in the country as of 2014 (www.cysticfibrosis.org.uk). Cystic fibrosis is caused through a mutation of the cystic fibrosis transmembrane conductance regulator protein, which is coded for through the CFTR gene located at position q31.2 on the long arm of chromosome 7 (Rommens, Iannuzzi et al. 1989). This is a highly polymorphic region of coding DNA, which is responsible for the mutations resulting in the condition. Over 1500 individual mutations have been observed, though by far the most common is the ΔF508 deletion (Kerem, Rommens et al. 1989; Bobadilla, Macek et al. 2002). In the case of ΔF508, the deletion of the phenylalanine causes a premature stop codon to exist, resulting in an incorrect tertiary structure of the resultant protein. This misshapen protein is not able to translocate to the cell membrane, and as a result ion transport is halted (Welsh and Smith 1993). The fully functional protein is involved in the transport of chloride ions across cell membranes in the epithelium, through a cAMP- activated ATP anion gated channel (Dalemans, Barbryt et al. 1991). Mutations in the CFTR gene can result in numerous problems, such as insufficient ion transport, complete inactivity of the ion gate or inability of the freshly synthesised protein to translocate to the cell membrane. In cystic fibrosis, the secretion of chloride is reduced, with a

resultant increase in sodium absorption (Sheppard and Welsh 1999). This leads to reduced liquid in the airways, as well as defective ciliary function. A hallmark of cystic fibrosis and a long used diagnostic test involves the investigation of patient sweat, as the excessively salty sweat caused through this ion imbalance is a characteristic of the condition (Mishra, Greaves et al. 2005).

Due to decreased liquid in the airways, patients with cystic fibrosis are known for exhibiting excessively viscous mucus. This is a major impediment to patient quality of life, with repeated physiotherapeutic treatments required to allow for improvement of patient prognosis through mucus removal (Govan and Deretic 1996). This thick mucus is a location where opportunistic infections and bacteria are known to frequent, with the conditions optimal for their survival and growth (Lyczak, Cannon et al. 2002). The effect of these opportunistic pathogens in the airways results in the eventual mortality of over 80% of patients with cystic fibrosis (Turcios 2005; O'Sullivan and Freedman 2009).

Treatment of these infections usually consists of a combination treatment of beta-lactam antibiotic and aminoglycoside (as well as a beta-lactamase inhibitor in some cases). However, a barrier to this treatment regimen exists in the fact that patients with cystic fibrosis exhibit a markedly higher incidence of beta-lactam hypersensitivity when compared with the healthy population (Parmar and Nasser 2005; Burrows, Nissen et al. 2007). As a result of this, treatment regimens are often unsuitable for the patients, resulting in the development of resistant bacteria and the quick exhaustion of traditional

treatment options. Studies have shown that in cases where patients with cystic fibrosis exhibit hypersensitivity to at least one antibiotic, there is a 1 in 5 chance that they will exhibit multiple drug hypersensitivity (Whitaker, Naisbitt et al. 2012). Though reactions to beta-lactam antibiotics are typically delayed-type in nature and therefore mediated through T-lymphocytes and their secretions, immediate hypersensitivity is observed in a small number of cases. In cases of delayed-type piperacillin hypersensitivity, the mean time of onset of symptoms following drug administration is 9.1 days (Whitaker, Meng et al. 2011). Interestingly, severe forms of skin eruption (e.g. Stevens-Johnson syndrome), as well as internal organ damage are rarely seen in the hypersensitive patients.

Multiple drug hypersensitivity is a major clinical concern, as treatment options soon become exhausted. Multiple hypersensitivity is where reactions are observed in at least 2 drugs of a different chemical class (Gex-Collet, Helbling et al. 2005; Pichler, Adam et al. 2010). Due to the high frequency of this condition in patients with cystic fibrosis, more radical treatment methods, such as dual lung transplants, are considered as a last line of treatment. Single lung transplants are often the only available treatment in patients, meaning that lung conditions are often critical to cystic fibrosis patient's prognosis. However, even single lung transplants are considered highly dangerous, due to the risk of a bacterial colony that is present in the un-transplanted lung infecting the newly acquired lung and being unable to be treated (Belkin, Henig et al. 2006).

Cross-reactivity between beta-lactam antibiotics is reportedly low, with few compounds implicated in truly allergic cross reactivity. Aztreonam and penicillins were safely tolerated in 95% of patients in a study (Moss 1991), with others reporting no cross-reactivity observed in piperacillin treated patients (Romano, Guéant-Rodriguez et al. 2004). However, though cross-reactivity is a minor concern, patients often are shown to be sensitised to another compound upon withdrawal of the initial antibiotic, further clouding the treatment options (Atanaskovic-Markovic, Gaeta et al. 2008).

Previous studies in our lab have shown that piperacillin-specific T-lymphocyte clones generated from patients with cystic fibrosis secreted pro-inflammatory cytokines in response to drug challenge, as well as highly significant proliferative responses (Monshi, Faulkner et al. 2013; Yaseen, Saide et al. 2015). The ability to use these clones for further mechanistic studies allows for a greater potential understanding of the underlying mechanism of the hypersensitive response. The observed pro-inflammatory cytokines that were secreted suggested a T_h1 response, though no studies in cystic fibrosis patients have taken into consideration the potential role of newer classes of helper T-lymphocytes, such as T_h17 and its hallmark, interleukin-17A as well as the poorly understood T_h22 class of cells, with its high levels of interleukin-22 secretion. Interleukin-17, in contrast to interleukin-22, is known to be a highly pro-inflammatory cytokine that has been shown to exert its effect through the mediation of certain chemokines implicated in inflammatory conditions (Onishi and Gaffen 2010). It has been shown to be strongly involved in autoimmune disorders, as well as skin conditions such as psoriasis (van Beelen, Teunissen et al. 2007). Integral to its involvement in the inflammatory

response is the triggering of neutrophil and macrophage recruitment, which is a key part of the immune cascade observed in the presence of interleukin-17 (Linden, Hoshino et al. 2000; Sergejeva, Ivanov et al. 2005).

As the receptor for interleukin-22 is located exclusively on tissues such as the skin (Wolk, Kunz et al. 2004), the potential role for interleukin-22 in the progression of cutaneous reactions cannot be overlooked. It can be considered both a pro- and anti-inflammatory cytokine, depending on the poorly defined conditions in which it is generated. In addition, studies have reported that interleukin-22 is critical in bacterial pathogen defence, alongside epithelial remodelling (Sonnenberg, Fouser et al. 2010). Due to these conditions, a potential role for interleukin-17 and interleukin-22 in the progression of the cutaneous symptoms observed in beta-lactam hypersensitivity in cystic fibrosis is a realistic possibility.

4.2   AIMS AND HYPOTHESIS

The high incidence of drug hypersensitivity observed in patients with cystic fibrosis treated with beta-lactam antibiotics is an important clinical concern, yet the underlying pathomechanism that drives this reaction is not yet understood. Through our collaboration with the cystic fibrosis centre at St. James' hospital we had access to patients with cystic fibrosis who were willing to donate samples for research into this iatrogenic disease.

Through the generation of piperacillin-responsive T-lymphocyte clones it was possible to enhance our understanding of the immune pathophysiology that was associated with drug treatment. Since interleukin-17 and interleukin-22 are implicated in other forms of cutaneous disease, alongside the fact that receptors for both cytokines are located in tissues such as the skin, our research focused on the potential role that interleukin-17 and interleukin-22 could play in driving the hypersensitivity reaction. We proposed the following hypothesis:

'Drug-specific T-lymphocytes isolated from patient PBMC secrete tissue-specific cytokines that are responsible for driving observed hypersensitivity reactions.'

4.3   METHODS

All experiments described in this chapter were performed using methods explained in greater detail in Materials and Methods Chapter 2. These include:

- Peripheral blood mononuclear cell isolation (*2.5.1*)

- Proliferation assay (*2.5.3*)

- Lymphocyte transformation test (*2.5.4*)

- ELISA (*2.5.5*)

- Bradford assay (*2.5.6*)

- ELISpot (*2.5.7*)

- Flow cytometry (*2.5.8*)

- T-lymphocyte cloning (*2.5.9*)

- EBV generation (*2.5.10*)

- MHC restriction assay (*2.5.13*)

- Chemotaxis assay (*2.5.16*)

4.4   RESULTS

4.4.1   PBMC isolated from hypersensitive patients proliferate in vitro in the presence of piperacillin

Lymphocyte transformation tests (LTTs) were performed on PBMC isolated from four piperacillin hypersensitive patients with cystic fibrosis. Hypersensitivity to piperacillin had previously been diagnosed, according to clinical criteria. Patients had not been exposed to the drug for several years. T-lymphocytes ($1.5x10^5$ / well, 100µl) were cultured in the presence of different concentrations of piperacillin, up to a maximal 2mM concentration (100µl). After the addition of [^{3}H]-thymidine for the final 16h of the assay (as outlined in 2.5.4), readings were taken as CPM, with a positive result considered to be a SI greater than 2. All four patients showed significant dose-dependent proliferative responses in the presence of piperacillin. In certain circumstances the response observed with piperacillin was above the positive control (tetanus toxoid, 10µg/ml). ***Figure 4.1*** shows the dose-response graphs for four donors whose samples were used throughout this work. An optimal concentration of 2mM of piperacillin was identified, due to the resulting drop in proliferative response observed at a higher 4mM concentration. This drop off was assumed to be due to toxicity, with the drug causing cell death rather than proliferation. PBMC from four tolerant controls, along with healthy donors, were not activated following culture with piperacillin. Proliferative responses were seen in tetanus toxoid treated cells (data not shown).
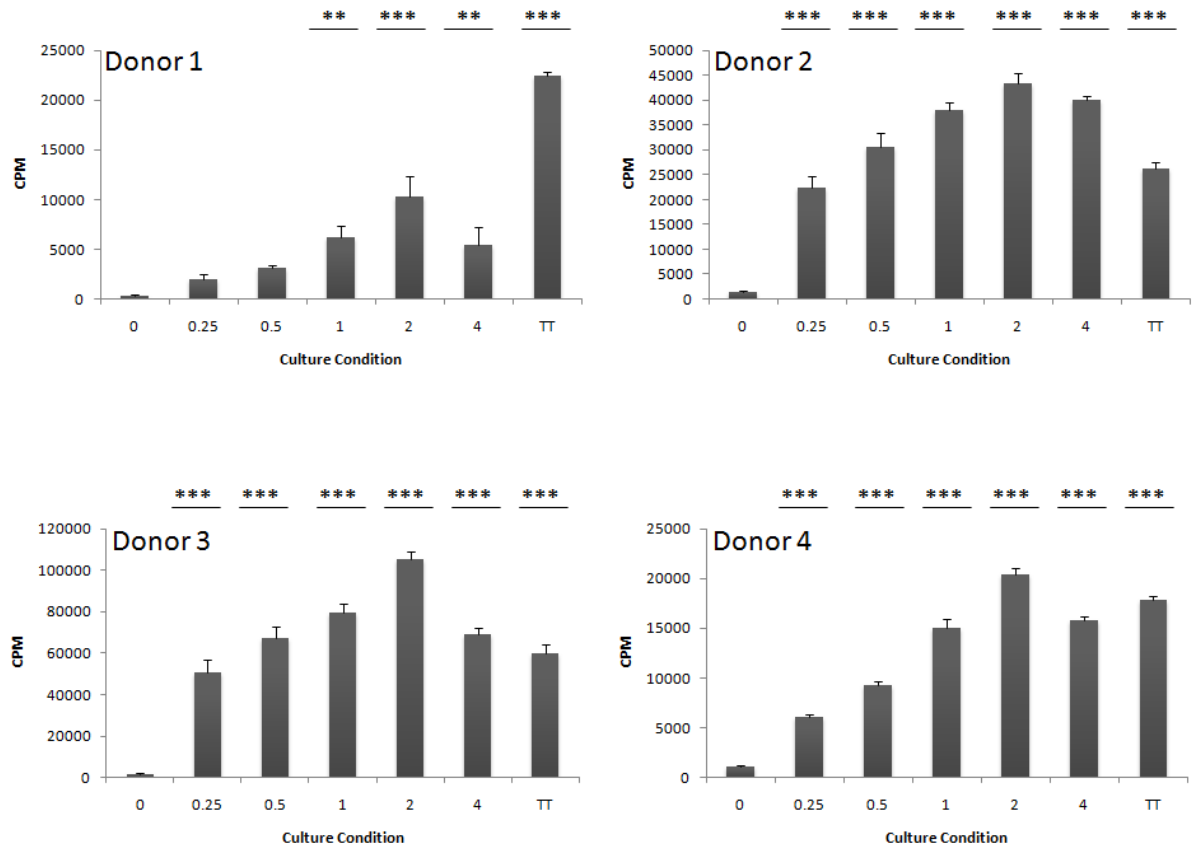
Figure 4.1. *Lymphocytes from hypersensitive patients proliferate in vitro in the presence of piperacillin.* *PBMC were isolated and cultured with increasing concentrations of piperacillin (0.25mM – 4mM), or tetanus toxoid (positive control) for 5 days. [3H]-thymidine incorporation was used to detect antigen-specific T-lymphocyte responses. Readings were taken in triplicate, with statistical analysis performed via one-way ANOVA compared to the negative control.* ** - P=0.005, *** - P=<0.001.*

4.4.2 PBMC isolated from hypersensitive patients secrete interferon-gamma, interleukin-13 and interleukin-22 in response to piperacillin

After positive lymphocyte proliferative responses to piperacillin were detected, further investigations were performed to assess the profile of cytokines secreted. Lymphocytes ($1x10^5$ / well) were cultured with media alone, piperacillin (at an optimal concentration of 2mM), or PHA (5µg/ml) in order to elicit cytokine release. A range of cytokines were investigated, to obtain an overview of the potential for PBMC to secrete T_h1, T_h2, T_h17 and/or T_h22 cytokines. All four hypersensitive patients were investigated, with the results from donors 1 and 4 displayed in ***figure 4.2***. In a resting state when PBMC were cultured with media alone, all donors displayed a low level of interferon-gamma secretion, with no other cytokines detected. Challenge with piperacillin elicited strong cytokine responses, with interferon-gamma, interleukin-13 and interleukin-22 detected from all donors. Interleukin-17A secretion was not detected from piperacillin treated PBMC. Interestingly, interleukin-17A was detected when the PBMC were exposed to the positive control PHA, showing that the cells had the potential to secrete interleukin-17A. Secretion of both interferon-gamma and interleukin-22 in drug and PHA stimulated wells were compared and no significant difference was observed (P>0.05), which suggests drug challenge elicited a near maximal interferon-gamma and interleukin-22 response.
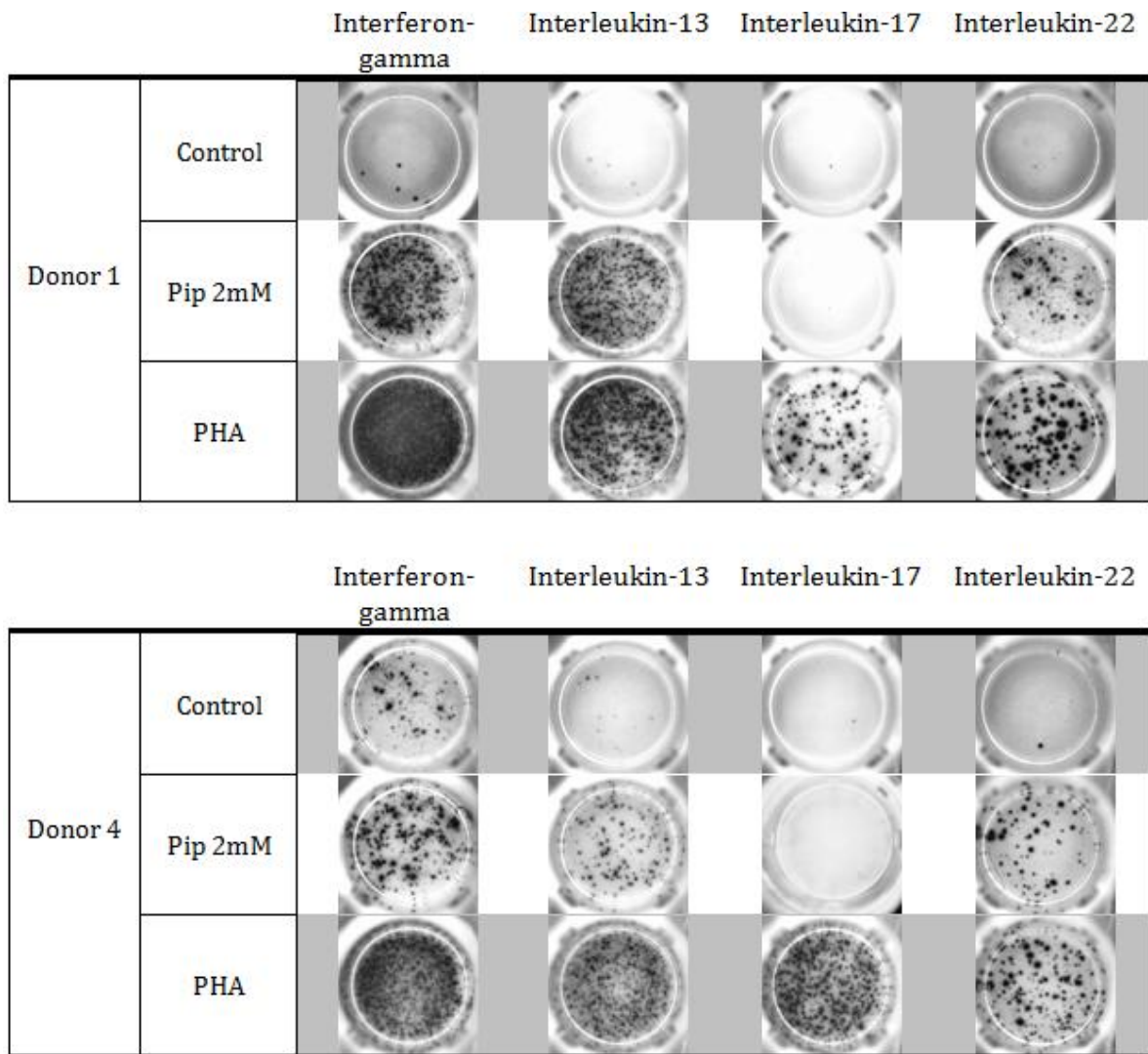
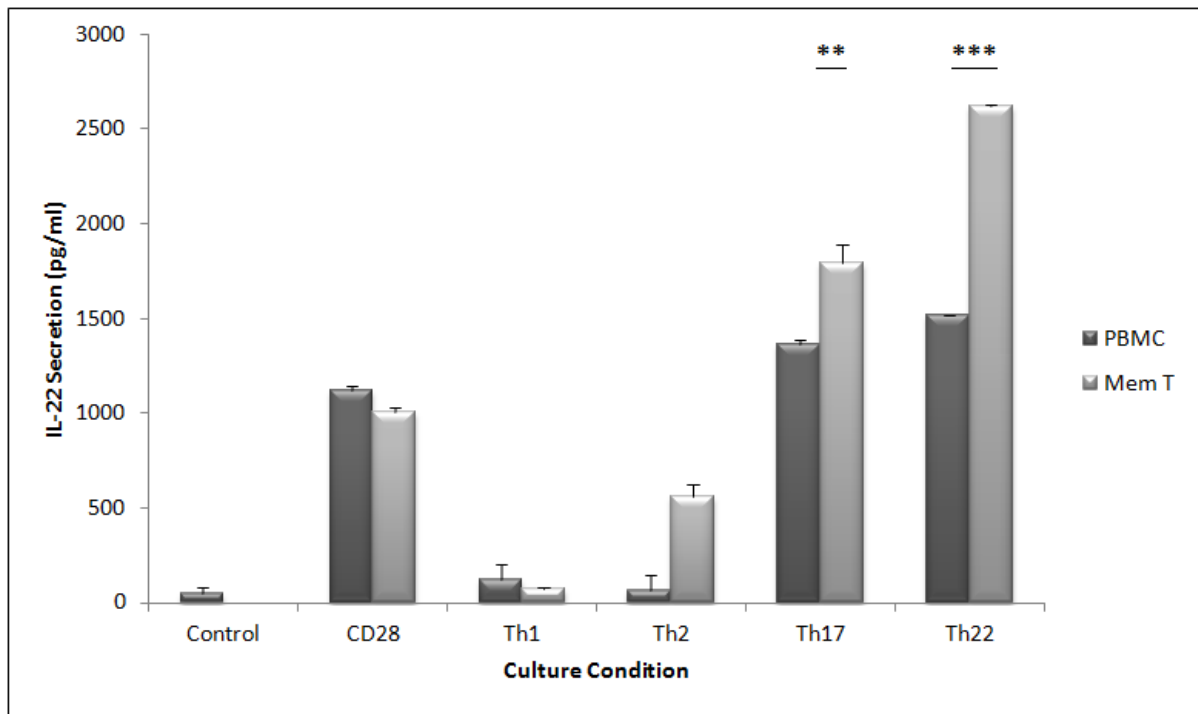***Figure 4.2.*** *Cytokine secretion from hypersensitive patient PBMC in response to piperacillin. PBMC (1x10⁶, 200μl) were cultured with piperacillin or PHA as a positive control for 48h at 37°C, 5% CO₂. The secretion of cytokines were visualised via ELISpot using commercially available kits. Exposure of PBMC to piperacillin resulted in the secretion of interferon-gamma, interleukin-13 and interleukin-22. Interleukin-17A was not observed from drug-stimulated wells. Figure shows representative images from two patients. Other patient responses were comparable.*

4.4.3 Quantification of interleukin-22 secretion from patient PBMC and memory T-lymphocyte cultures

Though ELISpot analysis is useful for low level quantification of cytokine secretions, spot-forming units do not allow for accurate quantification of total cytokine levels. In order to address this, supernatants were harvested from T-lymphocyte cultures following drug exposure for 2 days and assessed for the presence of interleukin-22 via ELISA. PBMC and magnetically separated memory T-lymphocytes from 4 hypersensitive patients were used for this analysis, under different cytokine polarising conditions (see **figure 2.1 – Chapter 2**). Similar levels of interleukin-22 were detected following piperacillin exposure to PBMC and memory cell lines exposed to the control CD28 in the absence of polarising conditions. Exposure to T_h1/T_h2 polarising conditions significantly reduced the levels of interleukin-22 from piperacillin treated PBMC and memory T-cells (**figure 4.3**). In contrast, a significant increase in interleukin-22 was detected with memory T-lymphocytes exposed to piperacillin under T_h17 and T_h22 polarising conditions. Negligible levels were observed in the medium control for both cell types. High levels (1500pg+) of interleukin-22 were detectable in both the T_h17 and T_h22 conditions. Though secretion levels were similar, higher interleukin-22 levels where seen from the memory T-lymphocytes.

Figure 4.3. *Quantification of interleukin-22 secretion from hypersensitive patient PBMC and memory T-lymphocytes following exposure to piperacillin under polarising conditions. Interleukin-22 was measured after 2 days in culture supernatants via ELISA. Interleukin-22 in media control wells in the absence of piperacillin (not shown) was subtracted from the drug-treated wells. CD28 stimulation was used as a positive control. Statistical analysis was performed via Students' T-test comparing CD28 positive control values to differing polarising conditions (** = P> 0.005, *** = P>0.001).*

4.4.4 Drug-specific T-lymphocyte clones generated from patient PBMC mainly express a CD4+ phenotype

PBMC isolated from patients hypersensitive to piperacillin were cultured for 14 days in the presence of piperacillin (2mM), with the resulting T-lymphocyte lines serially diluted to generate individual clones. Clones that expanded to approximately $1x10^6$ cells were tested for piperacillin specificity, with a SI greater than 2 considered a positive response when tested with 2mM piperacillin (cpm drug-treated / cpm control) (*figure 4.4*).

In total, 1440 clones were generated from the four hypersensitive patients. Following specificity testing, 570 clones produced a stimulation index of 2 or greater; hence, an antigen-specific generation rate of 39.6%. It should be noted that the majority (361) of these piperacillin specific clones were from a single donor (*figure 4.5*).

All piperacillin specific clones were phenotyped by flow cytometry. Four hundred and ninety four were shown to be CD4+, with 77 recorded as CD8+. Of these clones, the majority were frozen down and stored in liquid nitrogen for potential future analysis, with 40 well growing clones selected for expansion and further analysis.

Figure 4.4. *Piperacillin specific proliferative responses of clones generated from* *hypersensitive patients.* A stimulation index of greater than 2 was considered to be a positive result (shown on the graph as the horizontal line). All piperacillin responsive
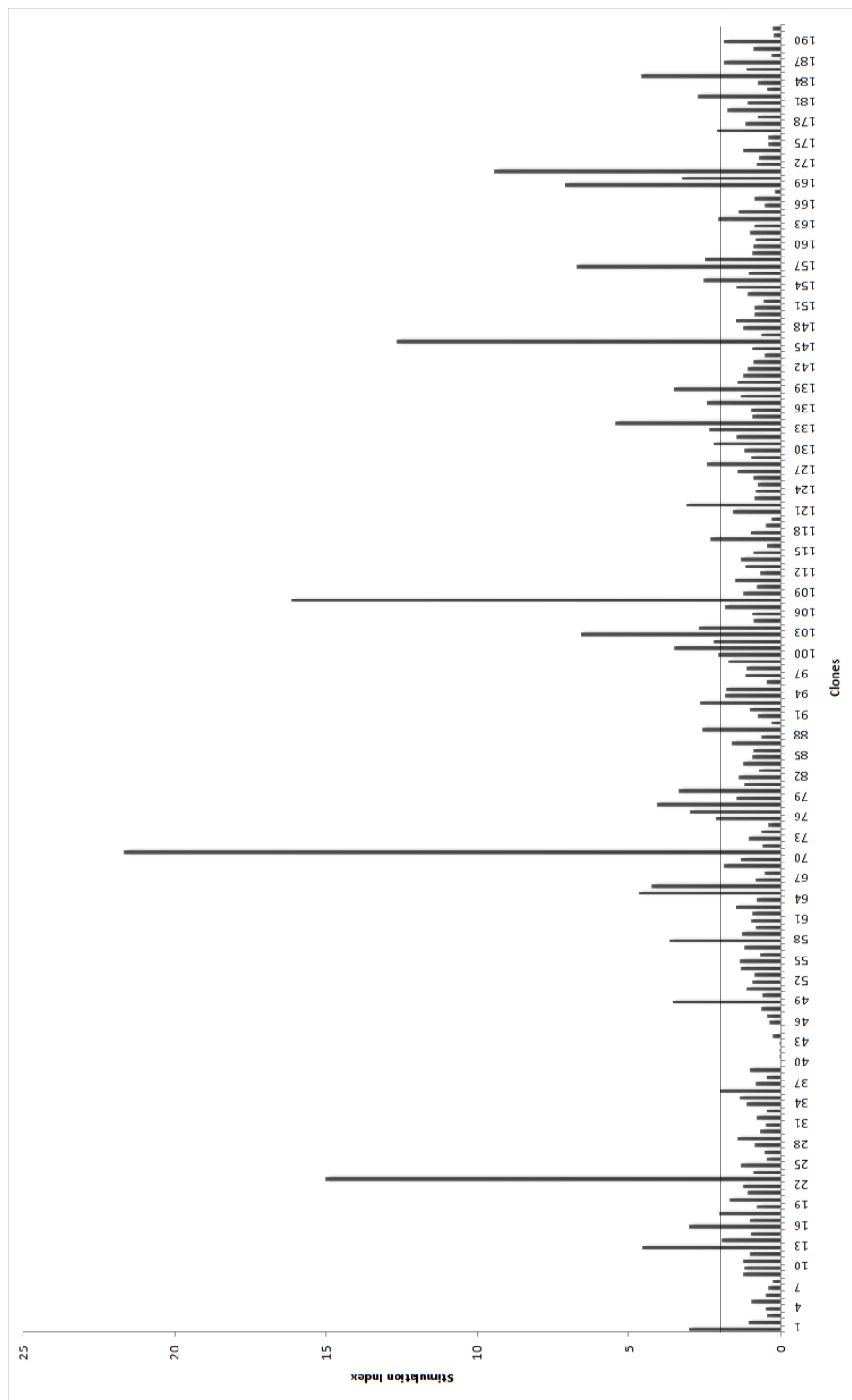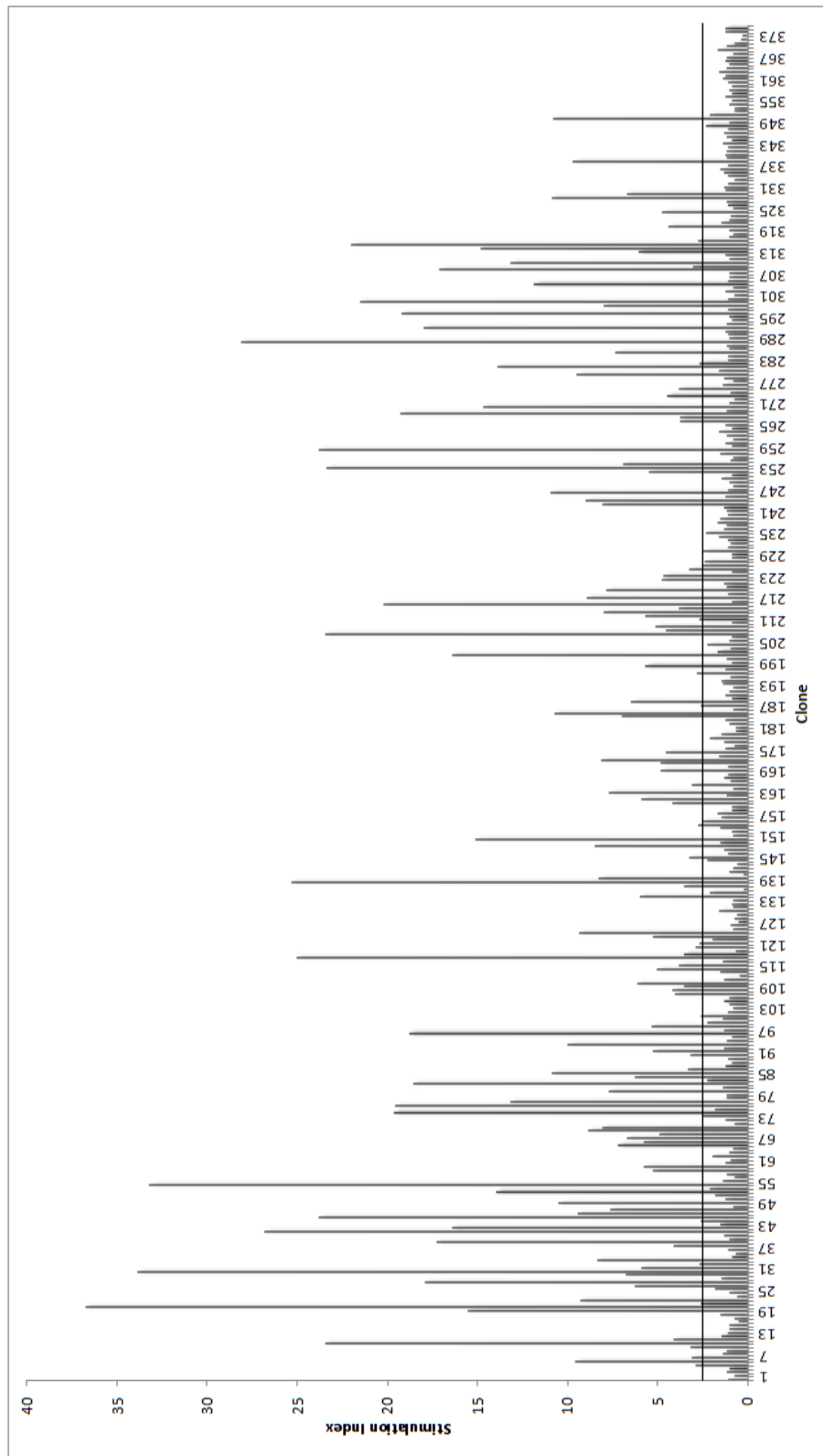
clones were further expanded for analysis.

Figure 4.4. *Piperacillin specific proliferative responses of clones generated from hypersensitive patients. A stimulation index of greater than 2 was considered to be a positive result (shown on the graph as the horizontal line). All piperacillin responsive clones were further expanded for analysis.*

ID	Type	# Tested	# Specific	% CD4+	% CD8+
Donor 1	Blood	768	361	87	13
Donor 2	Blood	104	34	84	16
Donor 3	Blood	376	134	89	11
Donor 4	Blood	192	41	77	23
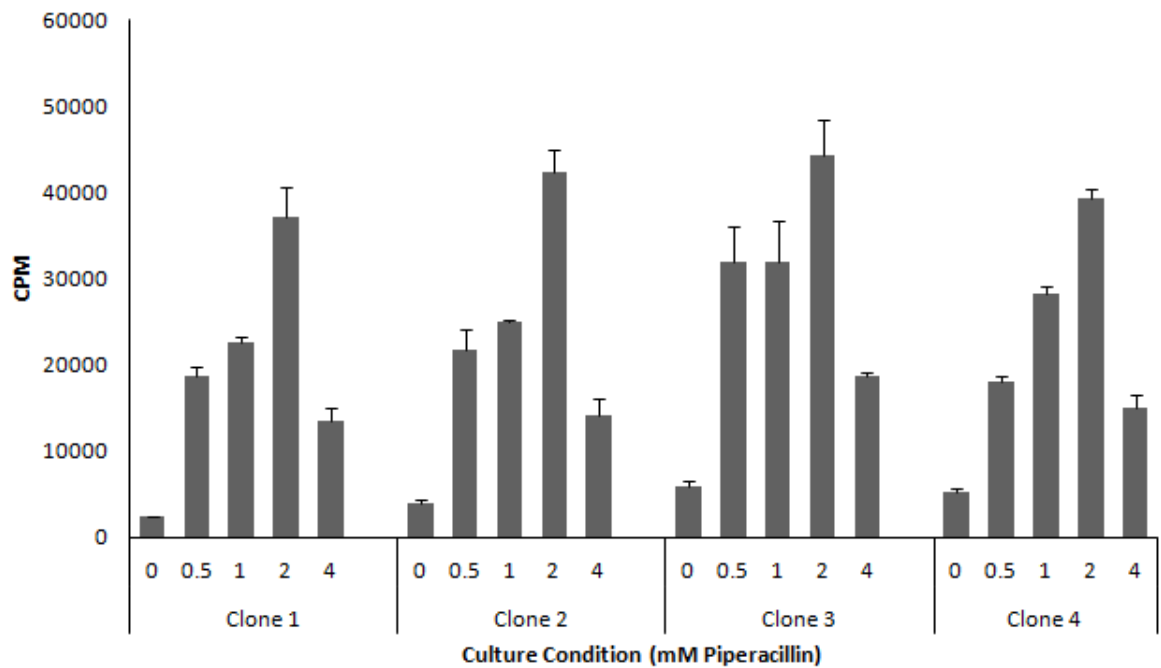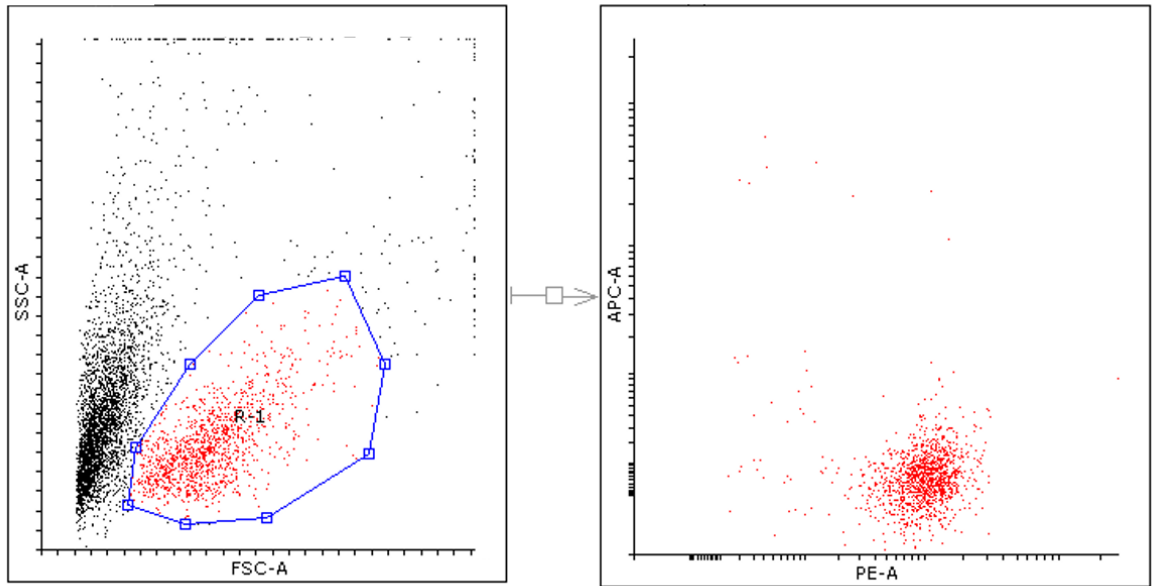
Figure 4.5. ***(Top)*** *Table showing the generation of piperacillin-specific T-lymphocyte* *clones from hypersensitive patient PBMCs.* *A total of 1440 clones were generated from four patients, with 570 piperacillin-specific clones detected. Clones (5x10⁴ cells/well) were cultured with autologous EBV (5x10³/well) in the presence of piperacillin for 48h, with [³H]-thymine (0.5µCi/well) added 16h prior to finishing. Specific clones were determined via a SI of greater than 2. CD phenotyping was conducted via flow cytometry to all piperacillin specific clones.* ***(Middle)*** *Flow cytometry trace showing phenotyping* *of tested clones.* *A minimum of 5x10⁵ events were used for CD phenotyping, with an either/or gating mechanism employed. PE-CD4 and APC-CD8 antibodies were used to phenotype clones.* ***(Bottom)*** *Dose-dependent proliferative responses of 4 representative* *piperacillin-specific clones following exposure to piperacillin.* *Clones (5x10⁴ cells/well) were cultured with autologous EBV (5x10³/well) in the presence of differing piperacillin concentrations (0.5-4mM) for 48h, with [³H]-thymine (0.5µCi/well) added 16h prior to finishing. Maximal responses were observed at 2mM, with toxicity observed at 4mM.*

4.4.5 Drug-specific T-lymphocyte clones show a pro-inflammatory cytokine secretion profile, including high levels of interleukin-22

Following the successful generation of antigen-specific clones, 10 clones from each patient were expanded in order to investigate the cytokines secreted. A panel of both CD4$^+$ and CD8$^+$ clones were selected for analysis. Representative ELISpot images are shown in ***figure 4.6***. High levels of interferon-gamma and interleukin-13 were secreted from all piperacillin treated clones. Interleukin-22 was detected in over 60% of tested piperacillin-specific CD4$^+$ and CD8$^+$ clones. Piperacillin specific activation of the clones did not result in the secretion of interleukin-17A. Further investigations were conducted to assess the secretion of cytotoxic molecules perforin, granzyme B and fas ligand. These were performed via ELISpot using the same clones discussed above, with representative results shown in ***figure 4.7***. Again, drug specific responses were detected in all tested clones, though the levels of cytolytic molecules secreted varied with different clones. Both CD4$^+$ and CD8$^+$ clones secreted high levels of granzyme B. Perforin and fas ligand were secreted in lower levels than granzyme B in response to drug challenge (***figure 4.8***).

		Interferon-gamma	Interleukin-13	Interleukin-17	Interleukin-22
Donor 1 Clone 59	Control				
	Pip 2mM				
Donor 1 Clone 295	Control				
	Pip 2mM				
Donor 1 Clone 300	Control				
	Pip 2mM				

		Interferon-gamma	Interleukin-13	Interleukin-17	Interleukin-22
Donor 3 Clone 223	Control				
	Pip 2mM				
Donor 3 Clone 275	Control				
	Pip 2mM				
Donor 3 Clone 352	Control				
	Pip 2mM				

Figure 4.6. *Piperacillin specific cytokine secretion from hypersensitive patient clones.* *Six representative clones are displayed, 10 clones per donor were investigated. T-lymphocyte clones ($1x10^5$/well, 100µl), autologous EBVs ($2x10^4$/well, 50µl) and culture condition (culture media alone / piperacillin 2mM, 50µl) were incubated for 48h at 37°C, 5% CO_2. Spot forming units were measured via ELISpot. Visual comparison was conducted through an AID plate reader. High levels of interferon-gamma, interleukin-13 and interleukin-22 were observed in both $CD4^+$ and $CD8^+$ clones following piperacillin exposure.*

		Perforin	Granzyme B	Fas Ligand
Donor 1 Clone 59	Control			
	Pip 2mM			
Donor 1 Clone 295	Control			
	Pip 2mM			
Donor 1 Clone 300	Control			
	Pip 2mM			

		Perforin	Granzyme B	Fas Ligand
Donor 3 Clone 223	Control			
	Pip 2mM			
Donor 3 Clone 275	Control			
	Pip 2mM			
Donor 3 Clone 352	Control			
	Pip 2mM			

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Figure 4.7. *Piperacillin specific secretion of cytolytic molecules from hypersensitive patient clones. Six representative clones are displayed, 10 clones per donor were investigated. T-lymphocyte clones ($1x10^5$/well, 100µl), autologous EBVs ($2x10^4$/well, 50µl) and culture condition (culture media alone / piperacillin 2mM, 50µl) were incubated for 48h at 37°C, 5% CO_2. Spot forming units were measured via ELISpot. Visual comparison was conducted through an AID plate reader. Detectable amounts of perforin, granzyme B and fas ligand were observed in both CD4$^+$ and CD8$^+$ clones following piperacillin exposure.*
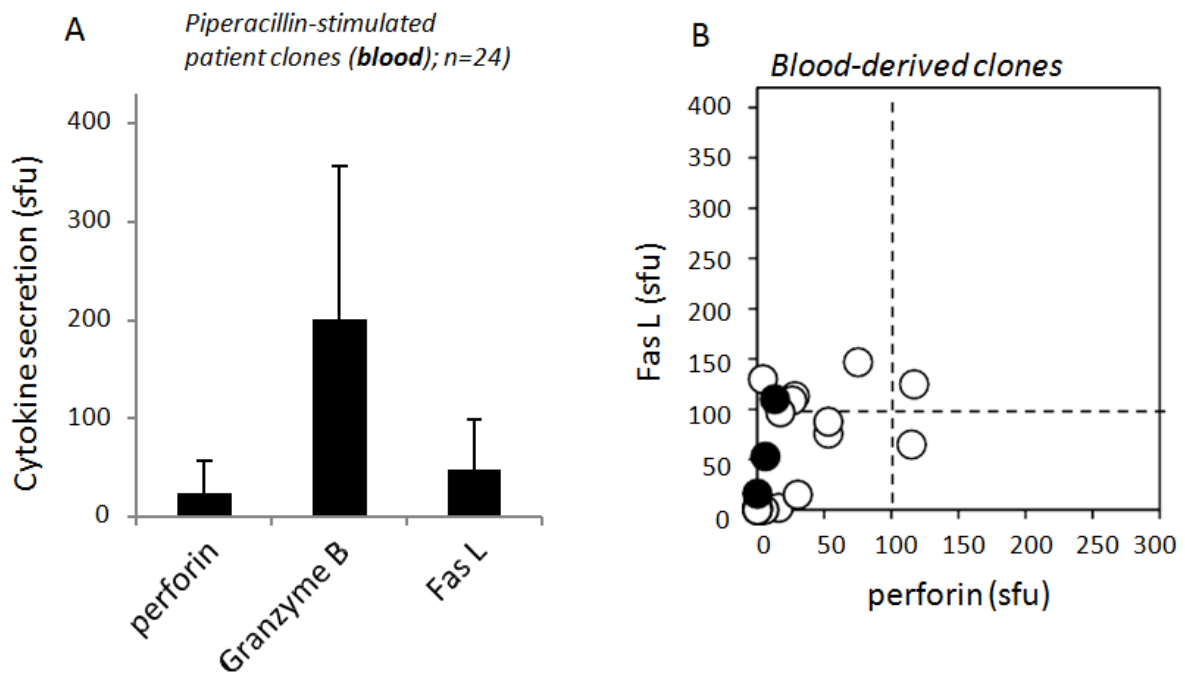
Figure 4.8*.   Cytotoxic molecules secreted from piperacillin-specific clones from hypersensitive patients. Results displayed from 24 clones across 3 donors. T-lymphocyte clones (1x10^5/well, 100μl), autologous EBVs (2x10^4/well, 50μl) and culture condition (culture media alone / piperacillin 2mM, 50μl) were incubated for 48h at 37°C, 5% CO₂. Visual comparison was conducted through an AID plate reader, with SFU recorded. CD4^+ and CD8^+ clones are shown in white and black circles respectively.*

The total spot forming units (SFU) in drug treated and control wells were then counted across all clones and compared. These comparisons are shown in ***figure 4.9***. Statistical analysis was performed using a Student's T-test. A significant increase in cytokine secretion was observed between control and drug-treated wells for interferon-gamma, interleukin-13, interleukin-22, granzyme B and fas ligand (p=<0.001), as well as perforin (p=0.005).
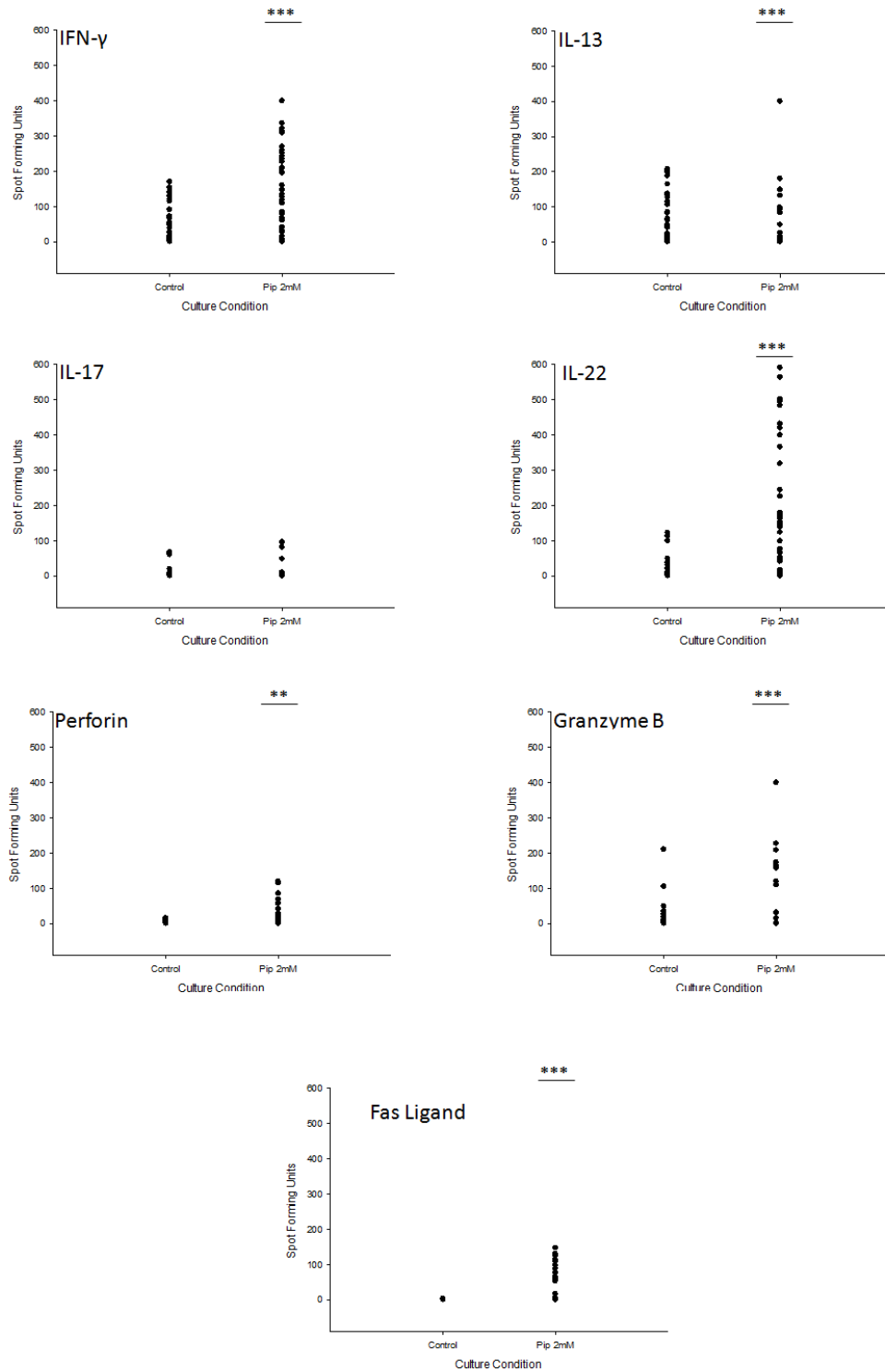
***Figure 4.9.*** *Cytokine and cytolytic molecule secretion from piperacillin-specific clones. Values were compared group-wise across both culture conditions, with significant secretion differences noted in all tested secretory molecules, with the exception of interleukin-17A. (n=30, Students' T-test, * - p=0.05, ** - p=0.005, *** - p=<0.001). Visible spots were identified using an ELISpot reader and expressed as SFU.*

### 4.4.6 Drug-specific T-lymphocyte clones express multiple chemokine receptors on their surface, and migrate in response to specific ligands in a dose- and time-dependent manner

Further investigations were conducted on piperacillin specific and non-drug specific clones isolated from the same patients in order to characterise chemokine receptor expression on the cell surface. Due to limited cell numbers, receptor expression was only measured on clones from 3 of the patients. The results (***figure 4.10***) show significant differences between piperacillin specific and non-specific clones. CCR1, CCR9, CCR10 and CD69 were expressed at significantly higher levels on drug-specific clones, while CCR2, CCR3, CXCR1 and E-cadherin were expressed at higher levels on the non-drug specific clones.
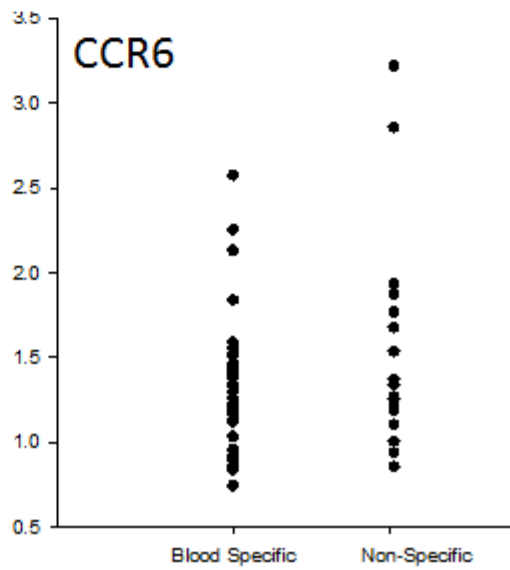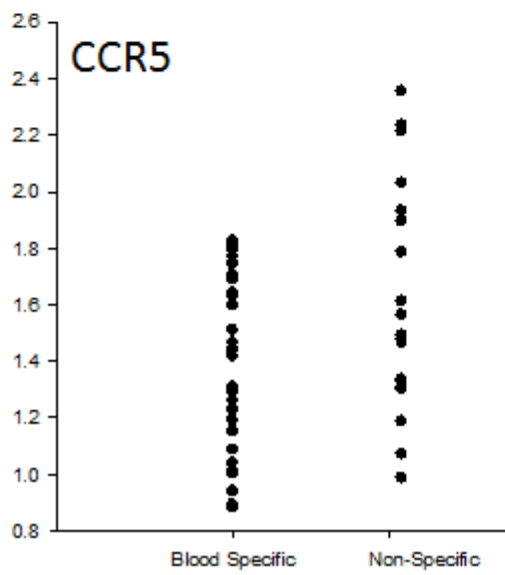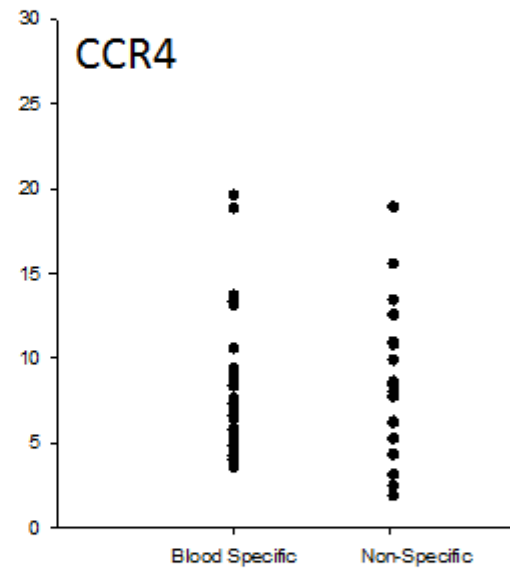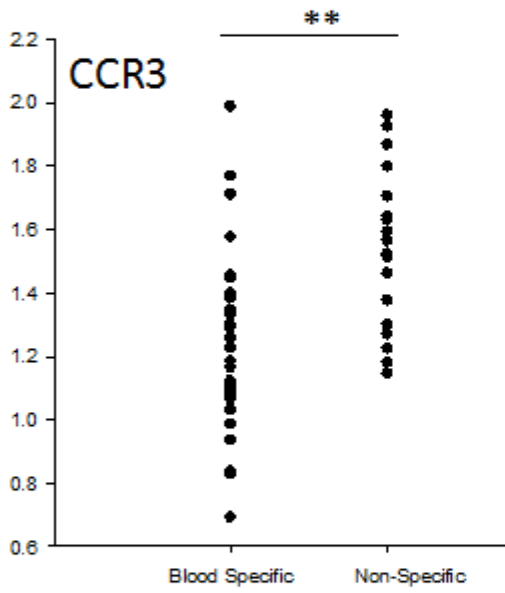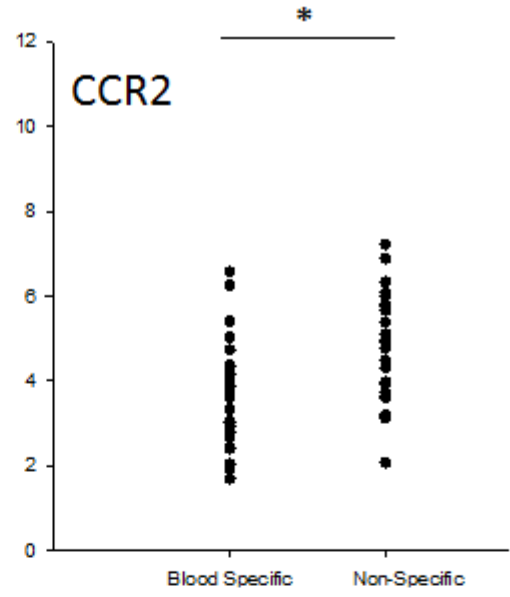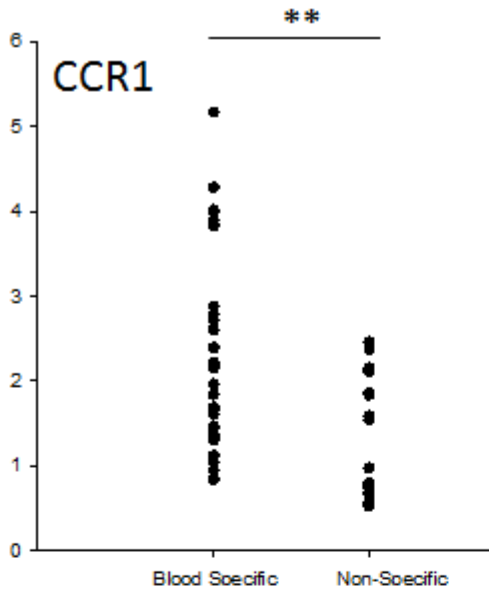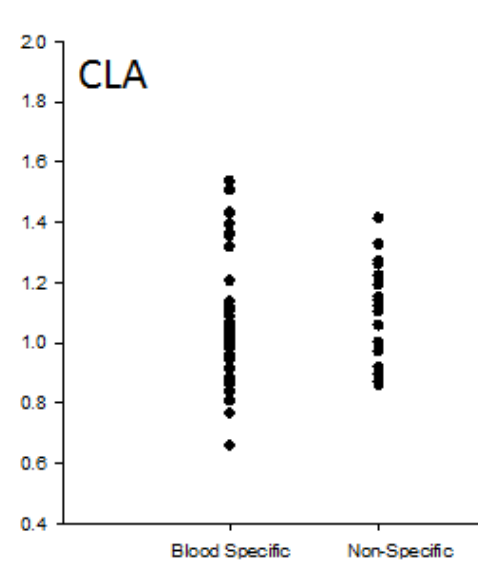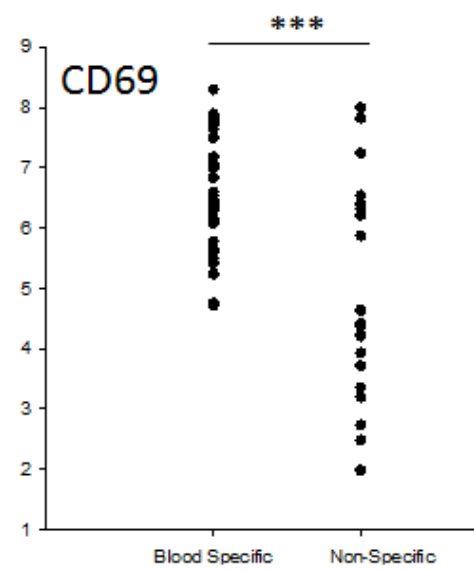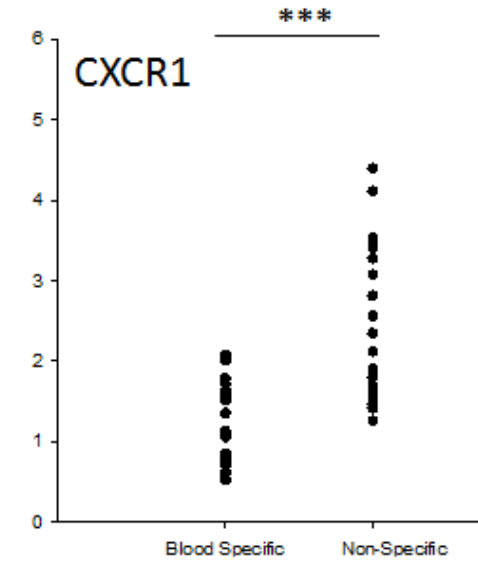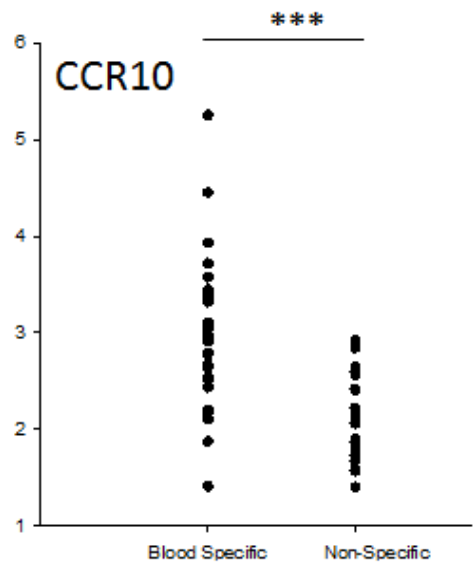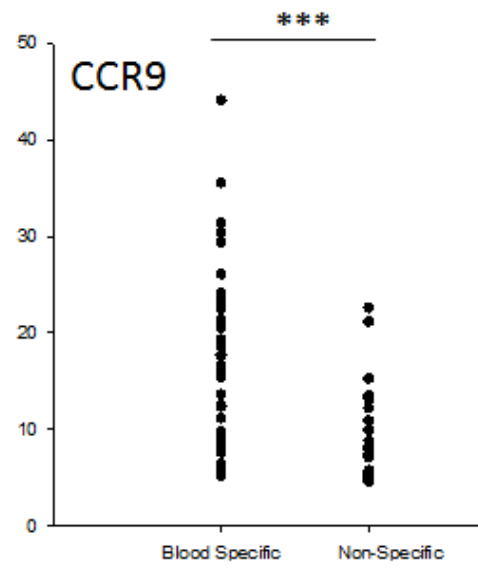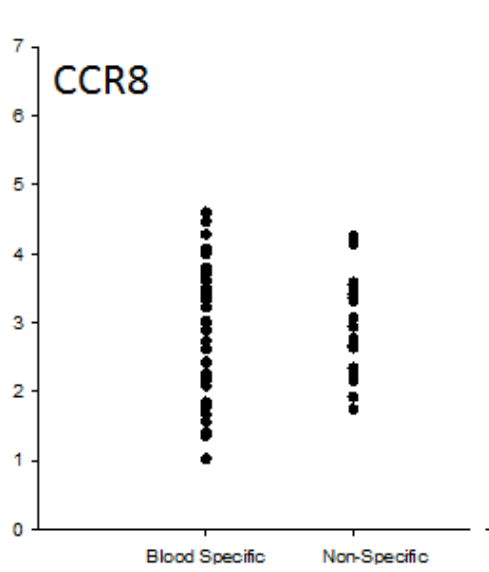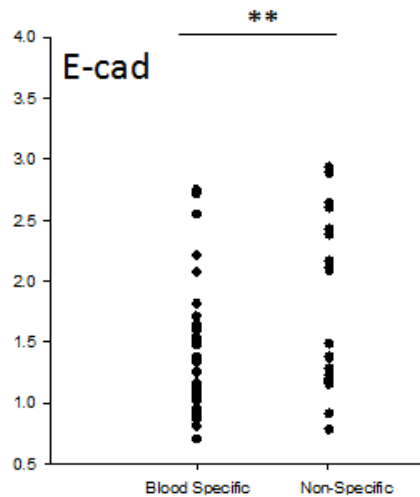
Figure 4.10. *Chemokine receptor expression on piperacillin-specific T-cell clones derived from blood of hypersensitive patients. Piperacillin-specific and piperacillin non-specific T-cell clones (SI< 2) derived from the blood of hypersensitive patients (n=36 per group) were analysed for chemokine receptor expression using flow cytometry. Expression is recorded as fold expression above isotype control. Statistical analysis performed via Student's T-test (* - p=0.05, ** - p=0.005, *** - p=<0.001).*

A chemotaxis assay was designed and used to investigate the ability of clones to migrate in response to exposure of specific ligands that are known to bind to different receptors. The experimental conditions described in chapter 3 were used to measure percentage migration across a semi-permeable membrane. Migration of greater than 20% of each clone was observed with ligands for CCR1, CCR4, CCR9 and CCR10. The movement of cells was time dependant, with the greatest effect noticed after 24h (***figure 4.11***). These data align with the relatively high expression of receptors on the cell surface.

RANTES - CCR1
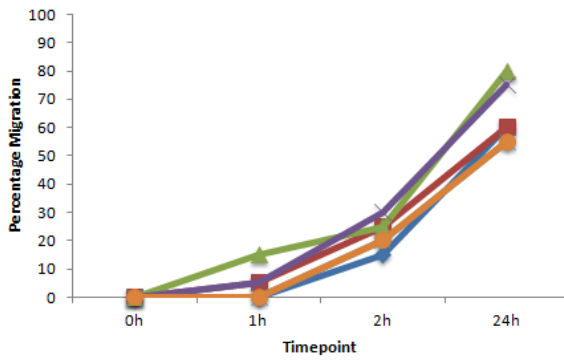
CCL2 - CCR2

CCL17 - CCR4

CCL4 - CCR5

CCL25 - CCR9

CCL27 - CCR10

CXCL9 - CXCR3

CXCL16 - CXCR6

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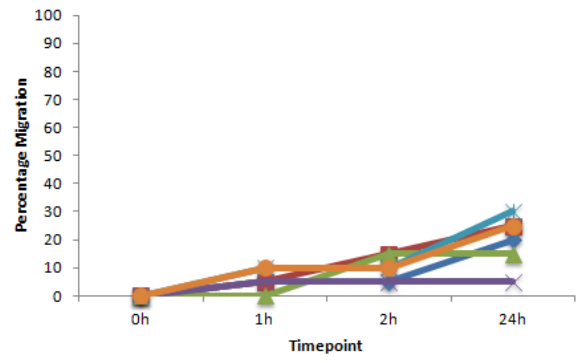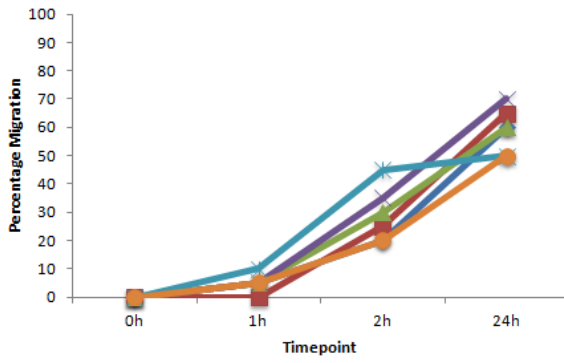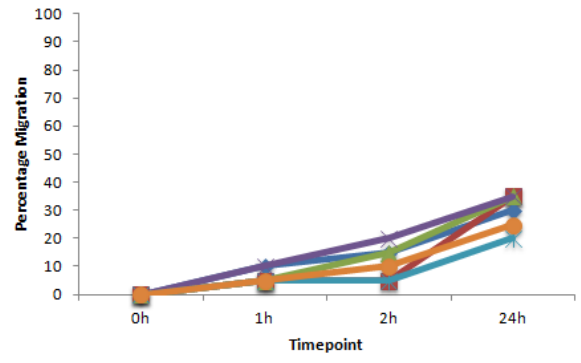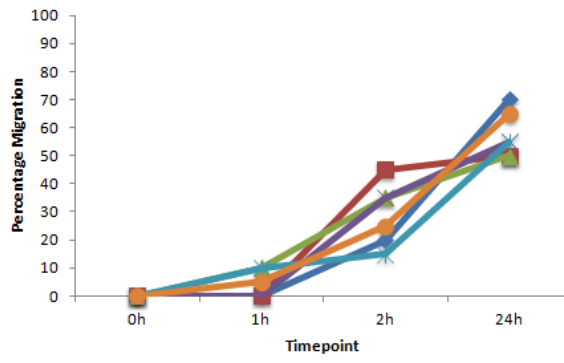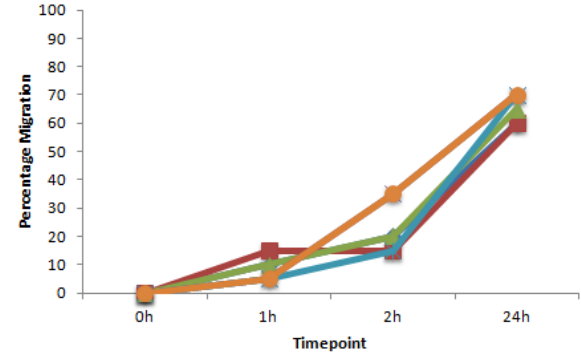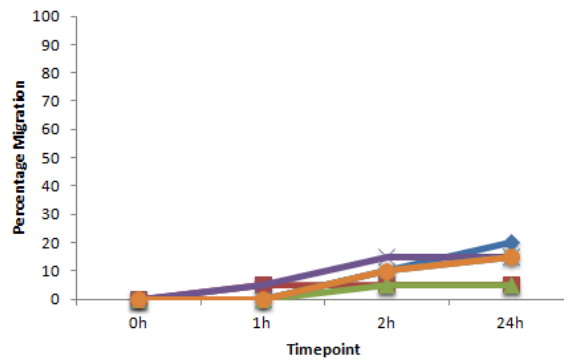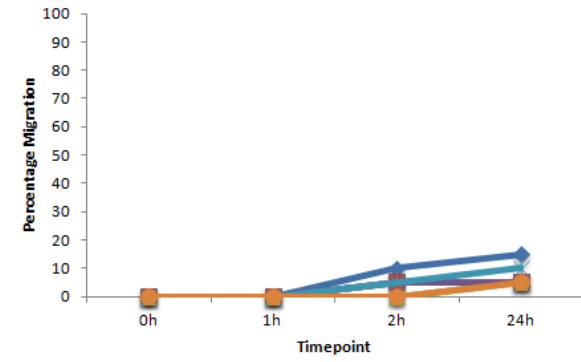
Figure 4.11. *Migration of clones in response to specific chemokines.* *1x10^5 cells were placed in the top of a trans-well plate, with chemokines in the lower half. Migration was measured as percentage total migration across the membrane at specific time points, with percentages worked out through visual analysis through counting using a haemocytometer. Above each graph the respective ligand-receptor combination is detailed.*

4.5   DISCUSSION

Cystic fibrosis is the most common lethal autosomal recessive disorder, which is responsible for a high incidence of mortality across the western world. The opportunistic infections that are the by-product of the condition contribute to the patient mortality, with over 80% of cases reported to end with infection-related mortality (O'Sullivan and Freedman 2009). To combat these recurrent infections, there is widespread prescription of prophylactic antibiotic treatments, foremost amongst which are the beta-lactam and aminoglycoside classes which are sometimes inhaled to reach the site of infection (Hansen, Pressler et al. 2005; McCoy, Quittner et al. 2008). In the general population, these compounds are well tolerated, with low incidences of adverse events or hypersensitivity reported. However, in patients with cystic fibrosis, the incidences of adverse effects are vastly increased (Allan, Moss et al. 1975; Wills, Henry et al. 1998; Ogese, Saide et al. 2015). This increase may be due to the widespread use, with patients treated with multiple antibiotics to combat the infections (Chmiel, Aksamit et al. 2014). The development of adverse reactions often precludes treatment options, thereby greatly complicating patient management. In one study, the incidence of hypersensitivity to at least one beta-lactam antibiotic was put at one in three, compared to approximately 2% in the general population (Whitaker, Naisbitt et al. 2012). In order to obtain a greater understanding of the underlying mechanisms involved in the hypersensitivity reactions, *in vitro* assays have been developed to characterise the phenotype and function of drug specific immune cells from hypersensitive patients.

It has been reported that PBMC isolated from the peripheral blood of patients with cystic fibrosis show a positive proliferative response to a range of antibacterial agents in a lymphocyte transformation test (Lavergne, Whitaker et al. 2010; El-Ghaiesh, Monshi et al. 2012; Jenkins, Yaseen et al. 2013; Naisbitt, Nattrass et al. 2014). This assay involves culturing patient PBMC with the suspect drug for 6 days and then analysis of proliferation through the incorporation of [^{3}H]-thymidine. The positive lymphocyte transformation test results have been replicated with the current patient cohort (results shown in *figure 4.1*). PBMC from all hypersensitive patients demonstrated a significant proliferative response to the drug piperacillin above control values, with a maximal response observed at 2mM piperacillin. Interestingly, the response of piperacillin was higher than that of the positive control tetanus toxoid response in several patients. Stimulation index values were not shown, but ranged from 19 – 62 at 2mM piperacillin. Piperacillin tolerant and healthy volunteer PBMC from 4 donors were also tested using the lymphocyte transformation test (LTT), with no significant lymphocyte proliferation to piperacillin detected. In contrast, a significant response to tetanus toxoid was observed. These data confirm that the LTT is both a sensitive and specific *in vitro* assay for the diagnosis of piperacillin hypersensitivity in patients with cystic fibrosis.

Cytokine secretion from PBMC was investigated following piperacillin stimulation, with the mitogen PHA used as a positive control. Multiple studies have shown that both pro- and anti-inflammatory cytokines can be secreted from PBMC of beta-lactam hypersensitive patients (Brander, Mauri-Hellweg et al. 1995; Rodriguez-Pena, Lopez et al. 2006; Lochmatter, Beeler et al. 2009;

Torres, Mayorga et al. 2014). This is mirrored in the results that were generated in this chapter, with high levels of interferon-gamma and interleukin-13 observed following drug stimulation of hypersensitive patient PBMC. The primary objective of this chapter was to establish whether interleukin-17 and interleukin-22 are also secreted by drug-responsive T-lymphocytes. PBMC from all patients secreted interleukin-22 following piperacillin stimulation (*figure 4.2)*. However, interleukin-17A secretion was not detected. PBMC from the same patients were shown to secrete interleukin-17A (alongside other cytokines) in PHA-treated wells; thus confirming that the lack if IL-17 in piperacillin wells was not due to a technical deficiency. Interleukin-17A is an integral cytokine secreted by T_h17 lymphocytes (Annunziato, Cosmi et al. 2007; Korn, Bettelli et al. 2009; Waite and Skokos 2012). The absence of this cytokine in drug-treated PBMC wells suggests that this subclass of lymphocytes is not involved in piperacillin hypersensitivity. T_h22 secreting lymphocytes on the other hand show a strong interferon-gamma$^+$ interleukin-22$^+$ phenotype (Duhen, Geiger et al. 2009), which is observed in the ELISpot results, suggesting that these lymphocytes may be driving the hypersensitivity response.

Interleukin-22 has been implicated in the progression of cutaneous disorders, primarily due to the fact that the heterodimeric interleukin-22 receptor is expressed at high levels exclusively in tissues such as the skin (Logsdon, Jones et al. 2002; Wolk, Kunz et al. 2004). In order to investigate IL-22 secretion in greater detail, supernatants isolated from piperacillin treated cells were assessed using an interleukin-22 ELISA. Since the positive LTT data are observed several years after the hypersensitivity reaction, it is likely that the

drug-specific IL-22 is being secreted from memory T-lymphocytes. To investigate this, whole PBMC, as well as magnetically separated memory T-lymphocytes, were cultured with piperacillin. In both cases, high levels of interleukin-22 were quantified via ELISA (*figure 4.3*). When the experiments were repeated under T_h17 and T_h22 polarising conditions, higher levels of interleukin-22 secretion was detected in the drug treated culture supernatants. These data confirm that piperacillin specific interleukin-22+ memory T-lymphocytes circulate in the peripheral blood of hypersensitive patients several years after clinical symptoms subside. Somewhat surprisingly, both T_h1 and T_h2 culture conditions reduced the level of interleukin-22 in the supernatant of piperacillin stimulated cells. Collectively, these data suggest that a T_h22 polarising condition likely dominates in hypersensitive patients at the time of drug treatment.

Clonal expansion following serial dilution is an important technique used for the investigation of typically low abundance antigen specific lymphocytes (Engler, Strasser et al. 2004; Wu, Farrell et al. 2007; Ko and Chen 2012). For example, functional analysis of drug-specific PBMC by flow cytometry is almost impossible, due to the low frequency of antigen specific lymphocytes – reportedly less than 1:10,000. The availability of clones allows a detailed analysis of cellular phenotype and function of individual cells. PBMC from the four hypersensitive patients described in the LTT experiments were used in the cloning procedure. In total, 1440 clones were expanded from the serial dilution plates across the 4 donors, with 570 clones shown to be responsive to piperacillin (i.e. with a stimulation index of greater than 2 (*figure 4.5*)). These clones were shown to be primarily CD4+, with 494 clones displaying this

phenotype. However, a small percentage of clones were CD8^{+}. These cells were used alongside the CD4^{+} cells for a more detailed functional analysis. The detection of a dominant CD4 phenotype on drug responsive clones is in agreement with previous studies (Hertl, Bohlen et al. 1993; Pichler, Zanni et al. 1997; Wu, Farrell et al. 2007; Monshi, Faulkner et al. 2013). These data contrast with the CD8^{+} clones generated in chapter 3 from healthy volunteers following priming. This disconnect between observed results shows that the priming assay is not completely mimicking the observed patient data. Despite these differences, assessment of the phenotype and function of T-lymphocytes specific to piperacillin from patients and healthy volunteers represents a useful comparative tool. The difference between patients and volunteers likely relates to the different routes of drug antigen exposure. *In vitro* piperacillin binds almost exclusively to HSA (Jenkins, Yaseen et al. 2013), forming an adduct and possibly a hapteneic antigen. However, it should be noted at high concentrations, the drug also likely binds directly to the surface MHC molecules. In contrast, in patients, cells are exposed to much lower drug concentrations where the piperacillin hapten might modify specific proteins to generate an antigen for T-lymphocytes.

Subsets of clones from each patient (i.e. 10 per patient) were chosen based on replication rate and CD phenotype for further analysis. These clones were then placed into an ELISpot assay to assess drug specific cytokine secretion phenotypes. T_h1, T_h2, T_h17 and T_h22 cytokines were investigated in order to define the T_h phenotype that was displayed by individual drug-specific clones. Other investigations have shown that high levels of interferon-gamma are secreted by beta-lactam specific T-lymphocyte clones (Lochmatter, Beeler et al.

2009; Torres, Mayorga et al. 2009), which is in agreement with the data that was generated in these experiments (*figure 4.6*). In addition, high levels of interleukin-13 were secreted from all clones. Several clones secreted IL-13 in the absence of drug treatment, suggesting that this cytokine can be constitutively expressed under standard cell culture conditions. Clones from hypersensitive patients also secreted high levels of interleukin-22. A similar profile of cytokines was secreted from clones isolated from healthy volunteers. The lack of interleukin-17A secretion from T-cell clones from hypersensitive patients and volunteers agrees with the PBMC ELISpot data (*figure 4.2*), which showed that although the PBMC have the ability to secrete interleukin-17A (as shown by the PBMC wells stimulated with PHA), no drug response was detected. The absence of interleukin-17 from drug-treated PBMC and clones suggests that piperacillin specific IL-17 secreting T-lymphocytes do not circulate in the blood of hypersensitive patients. Collectively, the data shows that drug treatment of CD4+ T-cells in the presence of autologous antigen presenting cells is associated with the secretion of T_h1 (IFN-γ^{high}), T_h2 (IL-13^{high}) or $T_h1/2$ (IFN-γ^{high}, IL-13^{high}) cytokines. IL-22 secretion was seen with piperacillin-stimulated T_h1, T_h2 and $T_h1/2$ clones, whereas IL-17 was not detected.

In addition to the assessment of cytokines, specific cytotoxic molecules were investigated, in order to determine whether drug-specific clones have the potential to damage target cells. It has been shown previously that perforin and fas ligand are both secreted by cytotoxic CD8+ T-lymphocytes recruited to the skin of patients with a variety of inflammatory conditions (Kehren, Desvignes et al. 1999; Nassif, Bensussan et al. 2002; Kish, Gorbachev et al.

2012).   Therefore, if drug-specific clones secrete these markers, it would provide a potential mechanism for the observed tissue injury.   Perforin (Voskoboinik, Smyth et al. 2006), granzyme B (Trapani and Smyth 2002) and fas ligand (Waring and Mullbacher 1999) are all known to mediate targeted cell killing, through various apoptotic pathways.

Perforin, granzyme B and fas ligand were all secreted from drug stimulated clones.   In cases where perforin and granzyme B was secreted, detectable levels were also seen in control wells, while fas ligand was only detected in wells treated with the drug (*figure 4.7*).   Two distinct phenotypes were observed, a perforin⁺granzyme B⁻fas ligand⁺ and a perforin⁻granzyme B⁺fas ligand⁻ phenotype.   These phenotypes were detected in IL-22 secreting and IL-22 negative clones.   The differing phenotypes were also observed when CD4⁺ and CD8⁺ clones were analysed (*figure 4.8*).

Previous studies have shown that multiple chemokine receptors are expressed on the cell surface of drug-specific lymphocytes (Schaerli, Britschgi et al. 2004; Gibson, Ogese et al. 2014), with differing phenotypes expressing a wide variety of potential chemokine receptors.   Peripheral circulating lymphocytes have the ability to migrate in response to chemotactic signalling (Sallusto, Lenig et al. 1999; Zlotnik and Yoshie 2000).   Specific chemokine receptors are implicated in homing, such as CCR9 and gut homing (Pabst, Ohl et al. 2004), as well as the role of CCR4 and CCR10 in skin homing (Soler, Humphreys et al. 2003; Fujimoto, Uratsuji et al. 2008).   Based on these findings, a number of chemokine receptors expressed on drug specific and non-specific clones were profiled and compared.

A number of significant differences were observed between the drug specific and non-specific clones (***figure 4.10***). Of particular interest was the low expression of CCR3 on the drug specific clones, a receptor involved in eosinophil recruitment (Forssmann, Uguccioni et al. 1997). Eosinophils are rarely found in inflamed skin of beta-lactam hypersensitive patients. In addition, high expression of CCR10 and CD69 expression suggest that the drug-specific clones are both active and have the capacity to migrate towards skin (Moretta, Poggi et al. 1991; Homey, Alenius et al. 2002). Overall the expression pattern suggests that drug-specific blood derived lymphocytes have the ability to migrate to the skin of hypersensitive patients.

A chemotaxis assay was performed (***figure 4.11***) to investigate the migratory potential of the drug specific clones. Similar responses to previous chapter work were observed - with migration occurring in a time- and expression-dependent manner. In cases where no significant increase in receptor expression was observed, such as in the case of CCR2 (a monocyte chemotaxis receptor) (Han, Tangirala et al. 1998) and CXCR3 (involved in integrin activation) (Rottman, Smith et al. 2001), little migration was observed. CCL17 and CCL27, ligands involved in the homing of leukocytes towards skin both promoted migration of the clones (Campbell, O'Connell et al. 2007).

These data taken together show that a T-lymphocyte response is generated following drug exposure in cases of piperacillin hypersensitivity. These T-lymphocytes show a T_h22 phenotype, with high levels of interferon-gamma and interleukin-22 secreted in the absence of interleukin-17A. In addition, multiple cytotoxic markers are secreted from the drug-specific lymphocytes

upon challenge *in vitro*. These drug-specific lymphocytes thus have both the potential to migrate to the primary site of beta-lactam hypersensitivity (i.e. the skin), as well as the ability to recruit other cell types to the location and cause tissue injury (Aberer, Bircher et al. 2003; Torres, Blanca et al. 2003; Romano, Blanca et al. 2004; Blanca, Romano et al. 2009). It has been recently shown that for every blood derived antigen specific T-lymphocyte there is an identical cell that resides in the tissue at the site of inflammation. Moreover, authors of the paper propose that the tissue resident cells respond rapidly following repeated antigen exposure whereas the circulating cells are recruited to the tissue to enhance the duration of the response (Gaide, Emerson et al. 2015; Watanabe, Gehad et al. 2015). Thus, the final step of the thesis was to investigate the cells present at the site of the reaction; i.e. lymphocytes that are derived from inflamed skin, and to compare the phenotype, function and migratory capacity of drug specific blood and skin derived cells.

CHAPTER 5 – CHARACTERISATION OF THE DRUG-SPECIFIC LYMPHOCYTIC RESPONSE THAT IS OBSERVED IN INFLAMED SKIN OF HYPERSENSITIVE PATIENTS WITH CYSTIC FIBROSIS

5.1   INTRODUCTION

Previous work characterising the cellular pathophysiology of drug hypersensitivity has focused on investigating lymphocytes extracted from peripheral blood; these PBMC are not the best way to investigate these reactions. Increasingly, tissue samples and lymphocytes extracted from the site of the reaction are becoming the gold standard for mechanistic investigations. In cases of beta-lactam hypersensitivity, where cutaneous symptoms are highly common, this means being able to sample from the site of rash or pathology present on the skin of the patient as a result of the hypersensitive response.

In tissues such as the skin, different cells exist in their own microenvironments. Studies have reported that tissue-resident cells are located in these areas, and therefore are highly likely to be involved in immune responses mounted against invasive pathogens. These tissue resident cells are not present in the circulation, and as such do not normally respond to circulating antigens that are solely located in the periphery (Clark, Chong et al. 2006; Clark 2010; Heath and Carbone 2013; Mueller, Gebhardt et al. 2013).

In general, three types of memory T-lymphocytes are involved in immune regulation throughout the body; central memory T-lymphocytes, effector memory T-lymphocytes and resident memory T-lymphocytes (Sallusto, Geginat et al. 2004; Sathaliyawala, Kubota et al. 2013). While the first two populations are present throughout the circulation, the resident memory T-

lymphocytes are solely located in their specific tissues, and do not show migratory ability.

In addition to these tissue specific lymphocyte sub-populations, there are also resident antigen presenting cells located in specific tissues (Constant, Brogdon et al. 2002; Winau, Hegasy et al. 2007). Normally, dendritic cells present antigens via MHC to the T-lymphocytes (Lanzavecchia and Sallusto 2001). However, in places such as the skin, cells like langerhans cells take up this role and become the primary route through which antigens are presented (Romani, Koide et al. 1989; Merad, Ginhoux et al. 2008). As such, the complex interaction between these specialised antigen presenting cells and the resident T-lymphocytes could provide an insight into the progression of hypersensitivity in cases where a cutaneous aetiology is present.

Recent work has demonstrated that T-lymphocyte mediated cell death of keratinocytes can be brought about in a model of allergic contact dermatitis (Pennino, Eyerich et al. 2010). These skin resident T-cells were responsible for the antigen non-specific killing of keratinocytes through secretion of pro-inflammatory cytokines, directly contributing to the observable disease phenotype.

It has been documented that lymphocytes express multiple cell surface receptors (Valiante, Uhrberg et al. 1997; Baecher-Allan, Brown et al. 2001; Brigl and Brenner 2004). These range from the receptors that are present on all lymphocytes, such as the CD3 molecule, to the highly specialised markers like CD64 on monocytes (and, by extension, macrophages) and specific keratins in keratinocytes (such as keratin-1) (Krüger, Büning et al. 2001;

Murphy and Reiner 2002; Roth, Kumar et al. 2012). Chemokines that can be secreted in response to antigenic challenge have been shown to induce chemotaxis of lymphocytes in simple chemotaxis assays (Ogese, Saide et al. 2015). Based on this, there is the potential of circulating systemic lymphocytes being recruited to areas normally reserved for localised lymphocytes, such as the tissue resident memory T-lymphocytes (Cinamon, Grabovsky et al. 2001; Stein and Nombela-Arrieta 2005).

Specific chemokines have been shown to be involved in this ability for chemotactic migration to occur. In the case of lymphocytes, these chemokines include RANTES, CCL2 and CCL25 (Utsunomiya, Tani et al. 1997; Uehara, Song et al. 2002). Of these the most interesting for further study is CCL27, due to the specific implication of its ability to trigger migration to cutaneous locations (Homey, Alenius et al. 2002; Soler, Humphreys et al. 2003). This potential ability for migration, in addition to the ability of T-cells to secrete interleukin-22 would provide strong support for the role of these mediators in cutaneous hypersensitivity reactions to drugs.

5.2   AIMS AND HYPOTHESIS

Due to the fact that the primary response observed in patients exhibiting hypersensitivity to piperacillin is cutaneous in nature, it is critical to investigate the cytokine, lymphocytic and pathophysiological micro-environment at the site of reaction, namely in the skin. To do this, skin biopsies were obtained from patients following a positive skin test to allow for characterisation of the lymphocytes in inflamed skin.

The cytokines interleukin-17A and interleukin-22 are both implicated in pro-inflammatory conditions related to the skin. However, our previous work suggested that interleukin-22 is secreted from drug specific T-cells in the absence of interleukin-17A. Thus, a T_h22 rather than a T_h17 response may be dominant in hypersensitive patients.

Therefore, the aim of this chapter was to characterise the T-lymphocyte response in inflamed skin of piperacillin hypersensitive patients.   Our hypothesis was that;

'Drug-specific T-lymphocytes present in the inflamed skin of piperacillin-hypersensitive cystic fibrosis patients would show a T_h22 phenotype, and respond to drug challenge'.

5.3   METHODS

All experimental methods mentioned in this chapter are explained in greater detail in Materials and Methods Chapter 2. These include:

- Peripheral blood mononuclear cell isolation (*2.5.1*)

- Magnetic separation of cell subsets (*2.5.2*)

- Proliferation assay (*2.5.3*)

- Lymphocyte transformation test (*2.5.4*)

- Bradford assay (*2.5.6*)

- ELISpot (*2.5.7*)

- Flow cytometry (*2.5.8*)

- T-lymphocyte cloning (*2.5.9*)

- EBV generation (*2.5.10*)

- Western blot analysis (*2.5.12*)

- Chemotaxis assay (*2.5.16*)

- Skin lymphocyte isolations (*2.5.17*)

5.4   RESULTS

5.4.1   *Isolation of T-lymphocytes from inflamed skin of hypersensitive patients with cystic fibrosis*

Critical to the understanding of the lymphocytic response occurring in skin is the ability to isolate and culture antigen-specific lymphocytes from the tissue. Previous studies have outlined a method for this extraction, utilising both expansion and magnetic bead separations of a small skin biopsy obtained following a skin test. Briefly, skin was mechanically dissected and incubated in medium containing interleukin-2 to allow the T-cells to migrate out of the tissue. After 5 days T-cells were expanded with the mitogen PHA. On day 14, an aliquot of the cells were cultured with titrated concentration of piperacillin and irradiated autologous EBV transformed B-cells as antigen presenting cells. After a further 14 days, [3H]-thymidine was added and proliferative responses measured 16h later. The cells proliferated in response to drug exposure in a dose-dependent manner, as show in ***figure 5.1***. The observed responses from these assays mirror responses shown in chapter 4 with PBMC from the same hypersensitive patients.
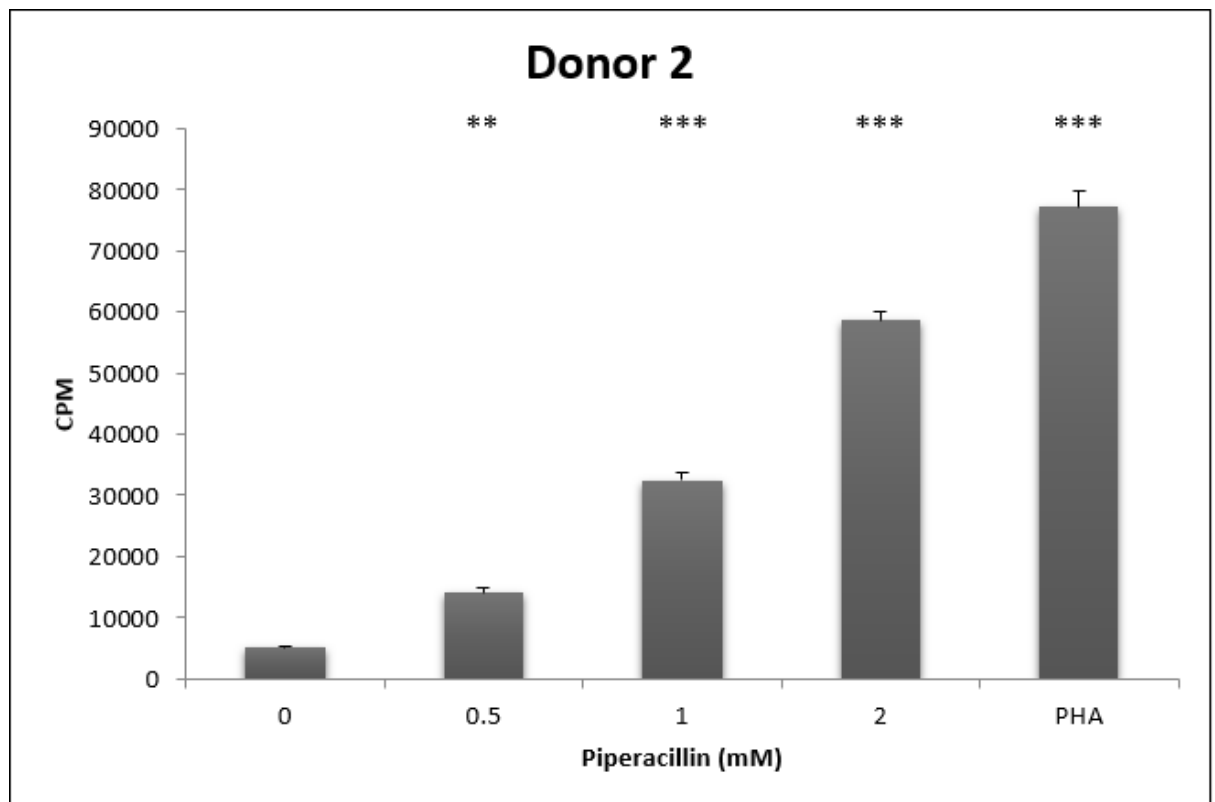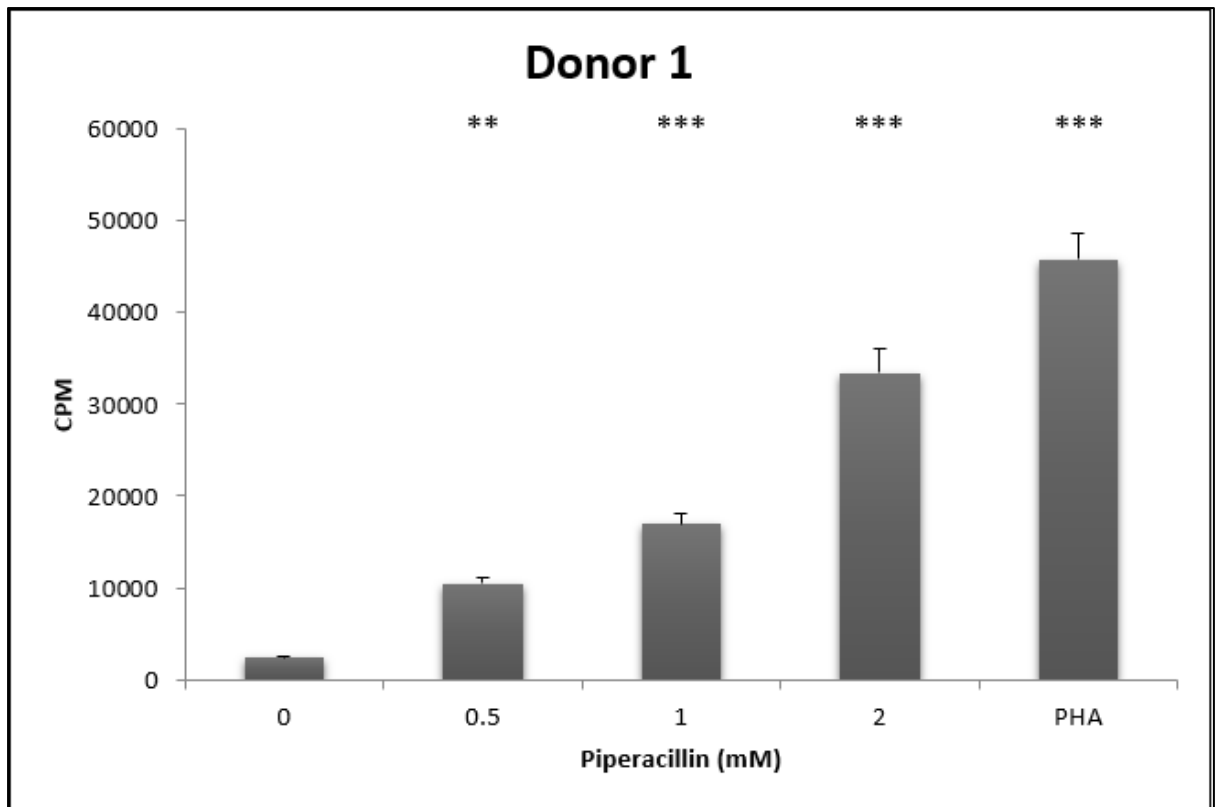
Figure 5.1. *Proliferation of T-cell lines from skin of hypersensitive patients following challenge with different concentrations of piperacillin, with medium alone as a negative*

control, as well as PHA (5µg/ml) as a maximal stimulation positive control. Lymphocytes were isolated from 2 skin samples from 2 patients. $1.5x10^5$ cells were cultured per well in a 96-well plate with piperacillin and antigen presenting cells. $^3[H]$-thymidine was added after 5 days with the assay harvested 16h later. Results were obtained via beta-counter analysis, with CPM as the readout value. Significant stimulation is shown in all drug-treated wells above control. Significance was obtained through one-way ANOVA analysis versus the control samples. ** - P = 0.005, *** - P = <0.001.

5.4.2 Keratinocytes isolated from inflamed skin samples do not present modified piperacillin to resident lymphocytes

Keratinocytes could present piperacillin protein adducts to T-cells to trigger an immune response. To investigate this, a simple western blot assay was conducted with differing concentrations of both piperacillin and primary human keratinocytes isolated from the skin biopsy taken from hypersensitive patients, to see if protein adducts with piperacillin were observed.

Keratinocytes were cultured for 5 days in the presence of piperacillin. After 5 days, culture supernatants were collected and analysed. Upon analysis, no modifications of the serum proteins were observed (*figure 5.2*), with a single band observed following development. This band correlated with the expected results from the unmodified protein, with no other bands being visible under multiple exposure times. This suggests that the only compound available for presentation to the keratinocytes was an unmodified piperacillin molecule, as no other bands were detected.
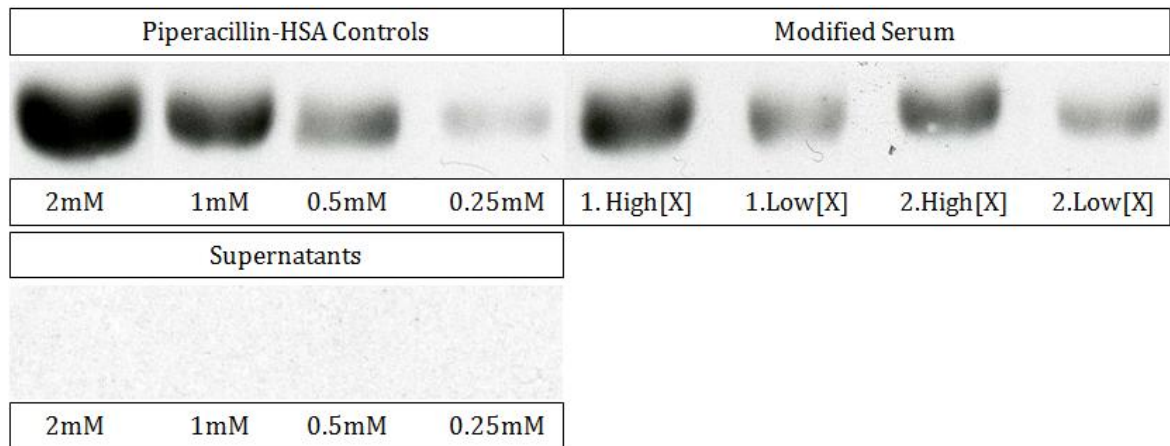
Figure 5.2. *Western blot analysis of supernatants collected from a keratinocyte assay performed on purified patient samples.* *Piperacillin-HSA controls (top left) corresponded with the same band location as the patient supernatants of low and high concentrations of drug (top right). No modification of serum was observed (bottom left). Only a single band was observed at 66.5kDa. Shown above are results from a western blot using anti benzyl-penicillin antibody, with a 10 second development time. The lack of multiple bands suggests no processing is occurring via keratinocyte action.*

5.4.3 Generation of drug-specific T-lymphocyte clones from skin-derived lymphocytes

Clones were generated using the established protocols discussed in previous chapters. Successful generation of T-lymphocyte clones was observed, with a total of 86 piperacillin-responsive specific clones generated across the 2 patient samples. 690 clones were tested in total, giving a specificity rate of 12.5%. In addition, these clones expressed a strong CD4$^+$ phenotype, with 83% of clones shown to express this marker. CD8$^+$ clones were also observed (17%), though at much lower quantities. A clone was considered to respond when a reading greater than 2 on the stimulation index was observed (cpm drug treated / cpm control) as shown by the horizontal line in *figure 5.3*.

Donor 1

Clone #

Stimulation Index

Donor 2
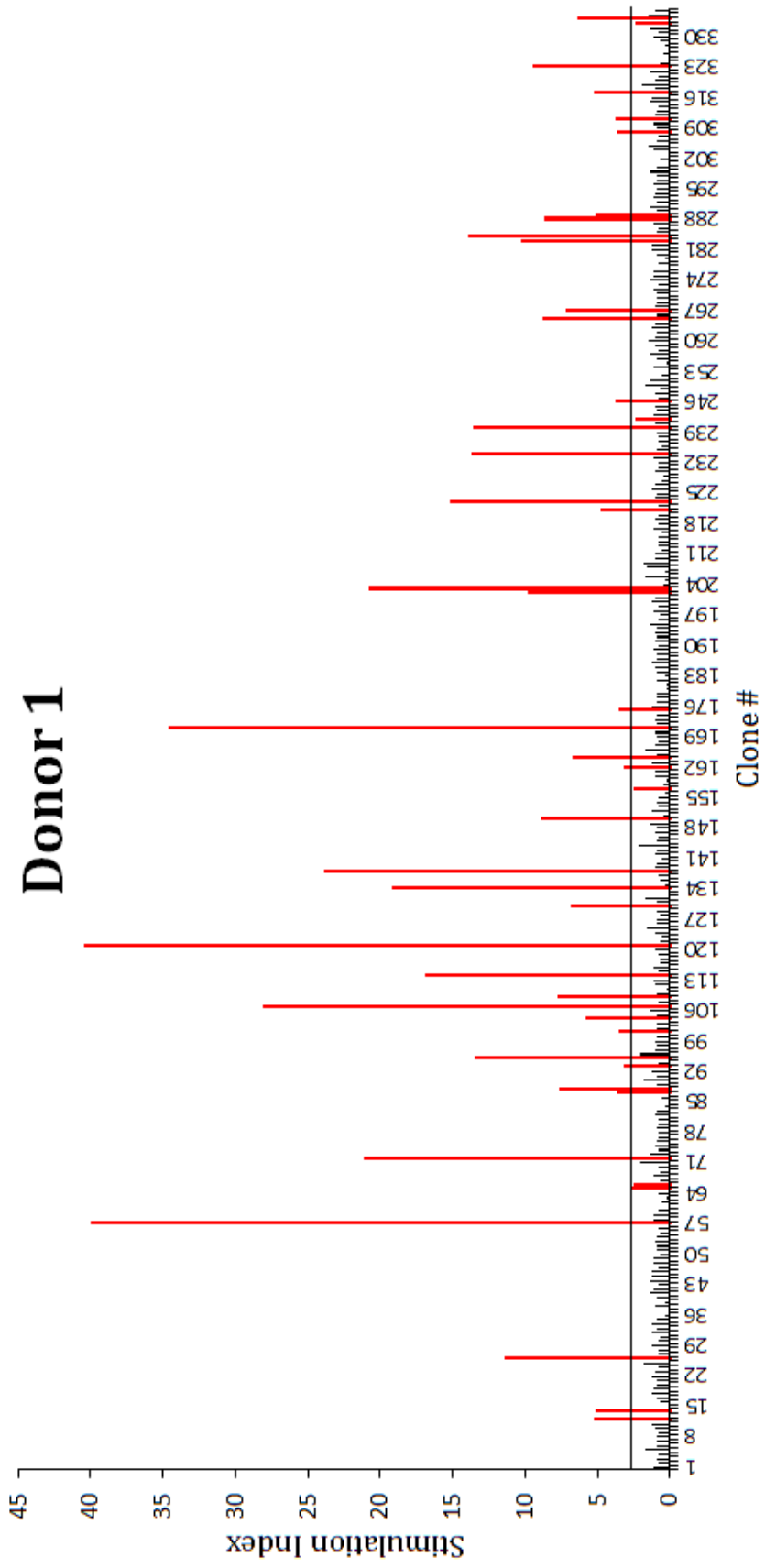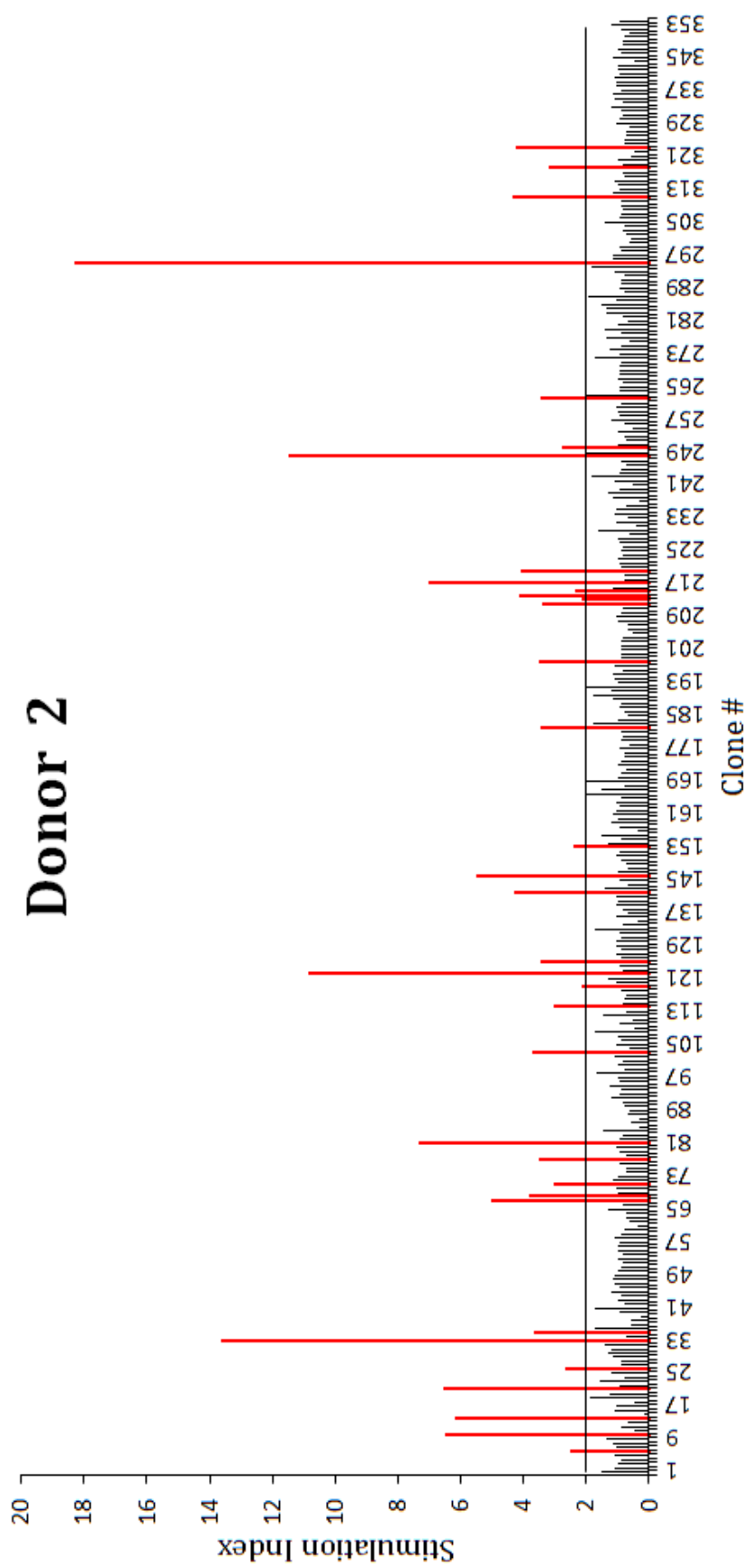
__Figure 5.3.__ Proliferation of piperacillin specific T-cell clone generation from skin of two hypersensitive patients. The clones were generated though a serial dilution technique (0.3cells/well – 10cells/well) and cultured for 2 weeks, with clones showing expansion picked and used for specificity analysis. Proliferative responses were analysed by culturing T-cells either in the presence or absence of piperacillin (2mM) for 48h in triplicate. Radioactive ^{3}[H]-thymidine was incorporated into the assay for the final 16h, with the plates then harvested and proliferation measured via a beta counter as CPM. Clones with a stimulation index of SI>2, where the SI is calculated as (CPM drug treated / CPM control) were selected for further analysis.

5.4.4 Drug-specific T-lymphocyte skin clones express a pro-inflammatory T_h22 cytokine profile, in the absence of interleukin-17A

A total of 12 clones from each patient were chosen for analysis of cytokine secretion via ELISpot. In total, 7 cytokines and cytolytic molecules were investigated; interferon-gamma, interleukin-13, interleukin-17A, interleukin-22, perforin, granzyme B and fas ligand (**figure 5.4**). Interferon-gamma and interleukin-13 secretion was detected from all of the piperacillin-treated clones. The predominant phenotype that was observed was that of a pro-inflammatory nature, with high levels of interferon-γ. Interleukin-22 was detected from over 50% of clones. These clones secreted differing levels of the cytotoxic molecules fas ligand, granzyme B and perforin following exposure to piperacillin. Interleukin-17A was not found to be secreted from any tested clones.

Additionally, the cytokines secreted from these clones were compared to piperacillin non-responsive clones isolated from the same biopsy. The non drug-specific clones did not secrete cytokines in the presence of piperacillin (**figure 5.5**). However, interferon-γ, interleukin-13, interleukin-22 (from certain clones), perforin, granzyme B and fas ligand were detected when the clones were activated with PHA.

		Interferon-gamma	Interleukin-13	Interleukin-17A	Interleukin-22	Perforin	Granzyme B	Fas Ligand
Donor 1 Clone 72	Control							
	Pip 2mM							
Donor 1 Clone 121	Control							
	Pip 2mM							
Donor 1 Clone 157	Control							
	Pip 2mM							
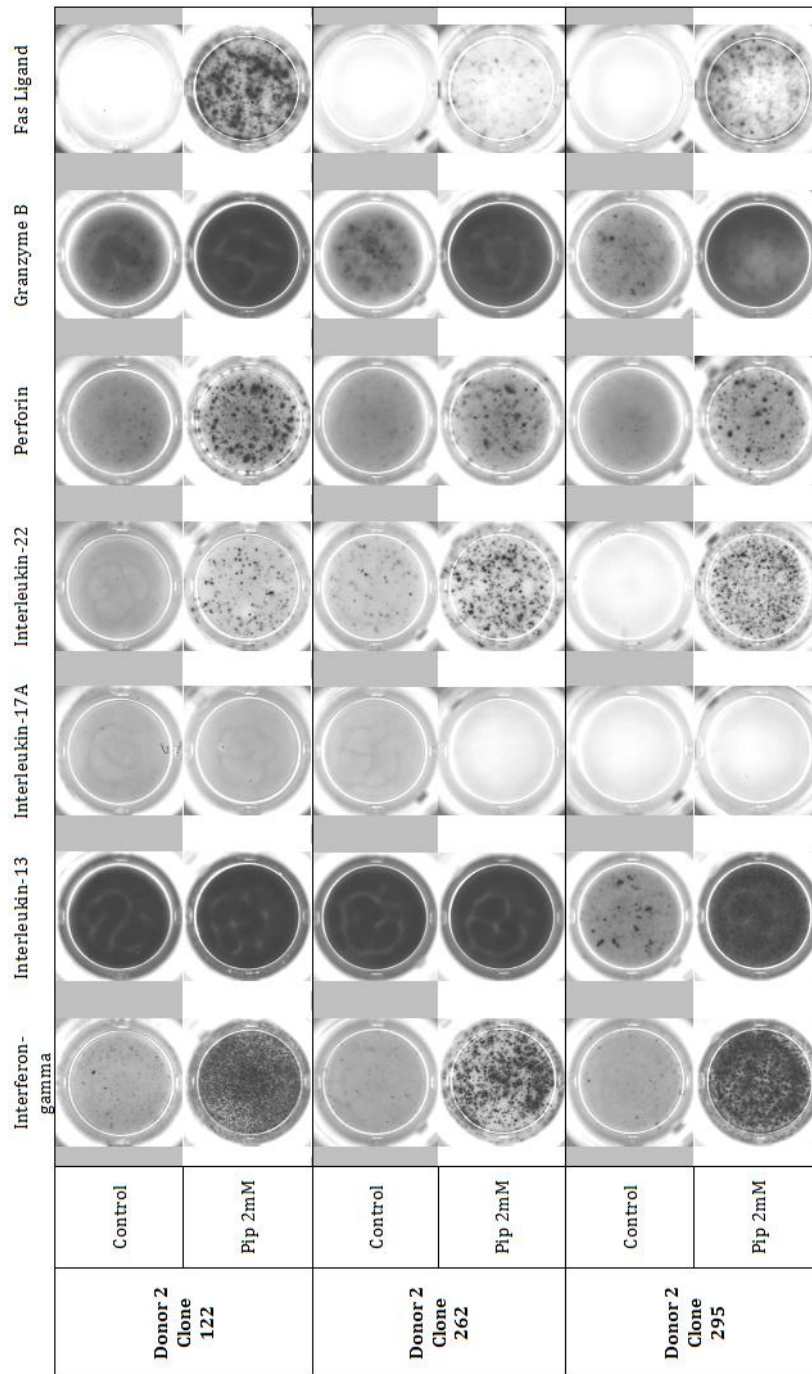
Figure 5.4. *Cytokine secretion from a selection of piperacillin-responsive clones generated from the skin of hypersensitive patients. ELISpot analysis was performed following drug-treated (2mM piperacillin) and control (medium alone) treatment. Testing was conducted to interferon-gamma, interleukin-13, interleukin-17A, interleukin-22, Perforin, Granzyme B and fas ligand.*

Figure 5.5. _Cytokine secretion from a selection of clones that were non-responsive to piperacillin, generated from the skin of hypersensitive patients._ _ELISpot analysis was performed following drug-treated (2mM piperacillin) and control (medium alone) treatment. Testing was conducted to interferon-gamma, interleukin-13, interleukin-17A, interleukin-22, Perforin, Granzyme B and fas ligand._

5.4.5 Expression of chemokine receptors on T-cells from blood and skin of hypersensitive patients

Following on from previous thesis chapters' investigations with blood derived clones, the expression of different chemokine receptors was examined to determine if any functional differences could be observed between blood and skin derived cells (*figure 5.6)*. Expression of 12 chemokine receptors, CLA and E-CAD were compared on piperacillin-specific blood and skin-derived clones (n=24-36 per group). Skin-derived clones that were not activated with piperacillin were used as an additional comparator. Blood and skin-derived piperacillin-specific clones expressed significantly higher levels of CCR1, when compared with non-specific clones. Blood-derived piperacillin-specific clones expressed higher levels of CCR9 and CCR10 when compared with the skin-derived piperacillin-specific and non-specific clones. In contrast, CXCR1, CLA and E-CAD were expressed at higher levels on the piperacillin-specific skin-derived clones. Finally, CCR2, 3 and 4 were expressed at slightly higher levels on skin-derived clones, when the two populations of piperacillin-specific clone were compared.

The movement of piperacillin-specific blood- and skin-derived clones towards 6 chemokines (CCL2, 4, 17, 25, 27 and CXCL16) was measured using trans-well plates to elucidate whether receptor expression was associated with different migratory characteristics as shown in *figure 5.7*. All clones were found to migrate across the trans-well membrane, in a time-dependent manner, in the presence of the CCR4 and CCR10 ligands CCL17 and CCL27, respectively.

Furthermore, the blood-derived clones, which expressed significantly higher levels of CCR9, migrated in the presence of CCL25, a ligand for CCR9.
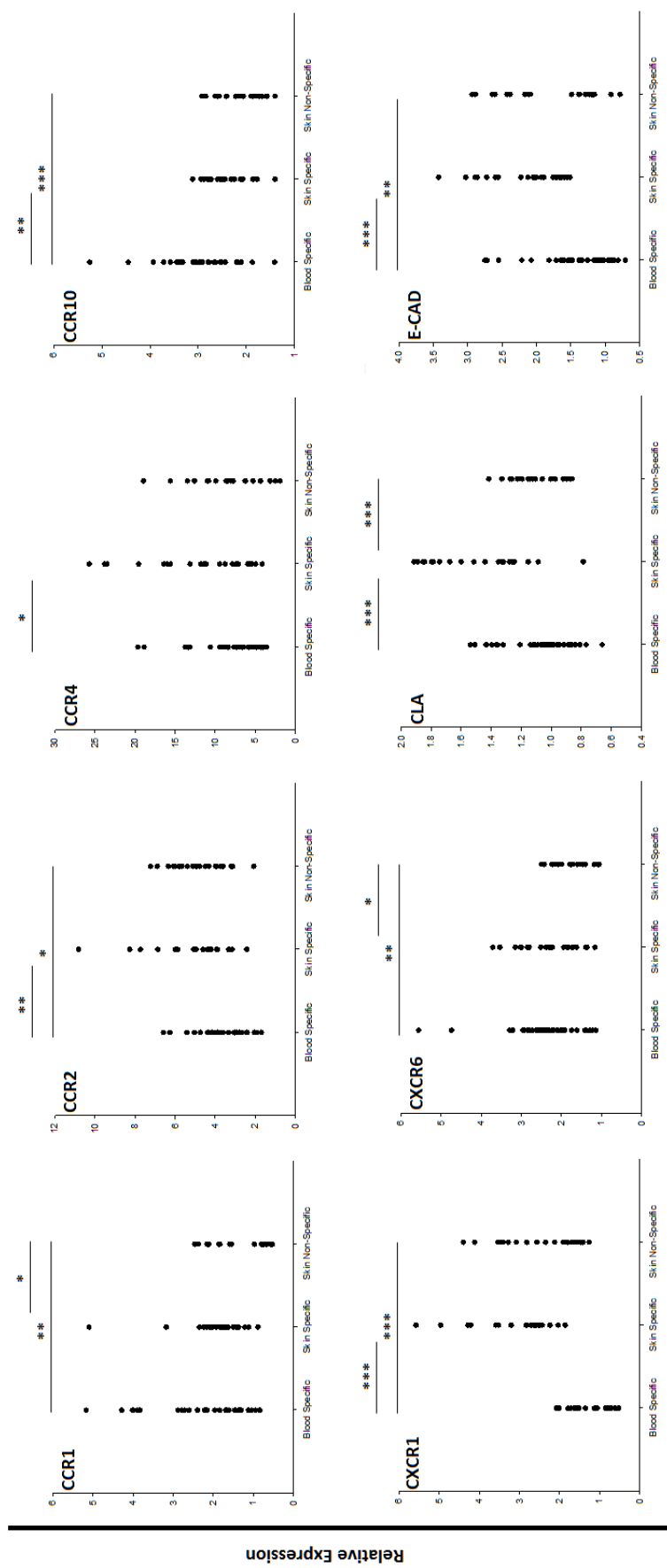
__Figure 5.6.__ *Chemokine receptor expression on the cell surface of piperacillin-specific blood and skin derived clones.* *Non responsive clones isolated from skin of the hypersensitive patients were used as an additional comparison. Clone specificity in all cases was determined through stimulation index values (SI <2 = non-specific, SI >2 = specific). Relative chemokine receptor expression was determined through a fold-change in expression of stained cells compared to isotype controls via flow cytometry. CCR1, CCR2, CCR4, CCR10, CXCR1, CXCR6, CLA and E-Cad were investigated, covering multiple T_h subsets and homing abilities. * - P=0.05, ** - P=0.005, *** - P=<0.001.*
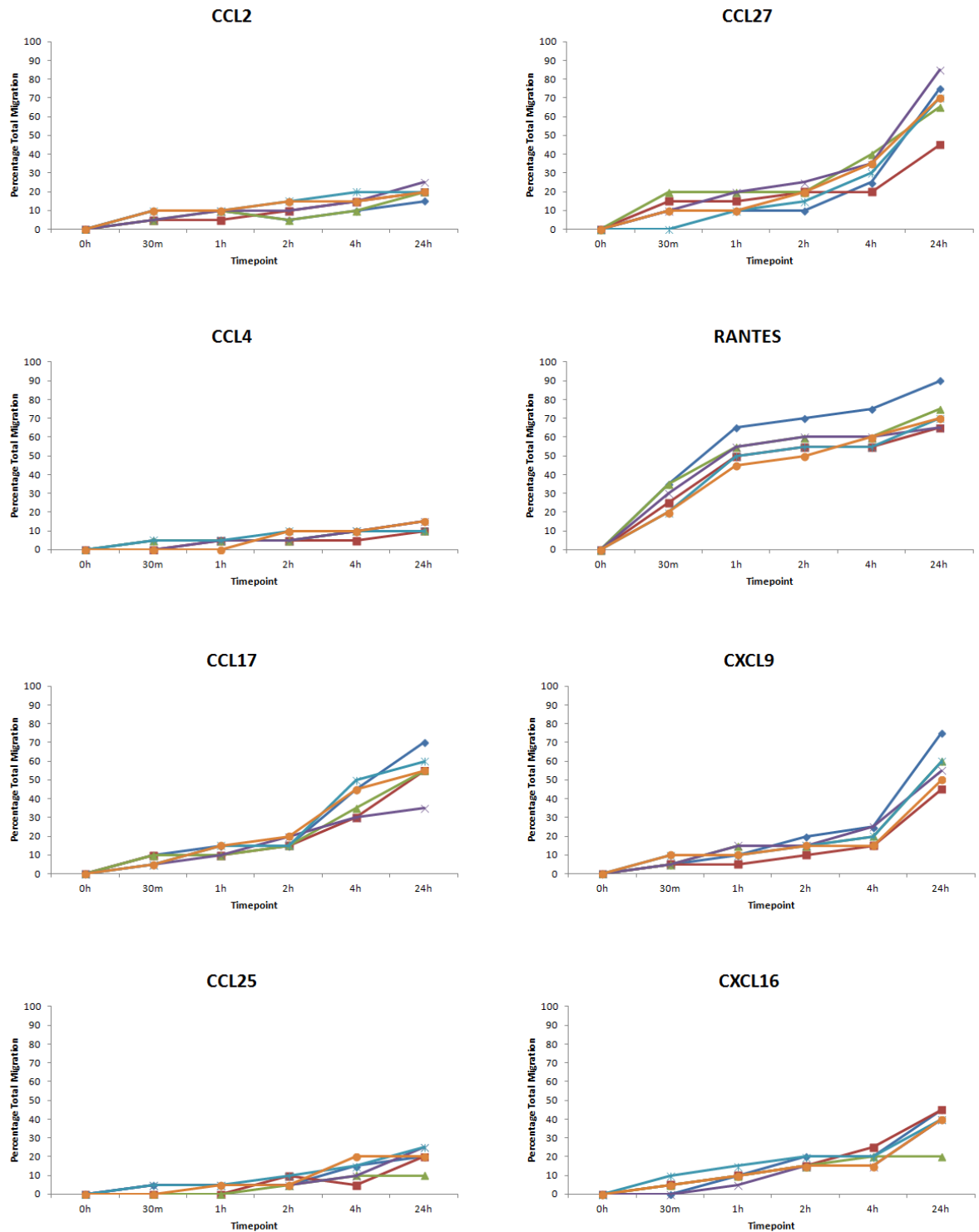
Figure 5.7. _Migration of skin derived piperacillin specific clones in response to specific chemokines._ _1x10^5 cells were placed in the top of a trans-well plate, with chemokines in the lower half. Migration was measured as percentage total migration across the membrane at specific time points, with percentages worked out through visual analysis_

through counting using a haemocytometer. Above each graph the respective ligand-receptor combination is detailed.

Data generated so far in this thesis suggests that piperacillin specific T-cells display a T_h22 phenotype. In order to investigate the origins of the drug specific T-cells, the aryl hydrocarbon receptor was identified as a target for modulation. Previous studies have identified a link between the development of a T_h22 phenotype and aryl hydrocarbon receptor signalling, so modulation of this pathway could lead to further proof that T_h22 secreting T-cells are important in the development of piperacillin hypersensitivity.

To test this hypothesis, naïve T-lymphocytes were cultured with piperacillin in a dendritic cell priming assay as described in **chapter 3**, with the addition of either an aryl hydrocarbon receptor agonist or antagonist in the priming co-cultures. These modifications gave the potential for a polarised T_h22 response to be either driven (in the case of the agonist) or abrogated (for the antagonist). The proliferative responses of these modulated priming assays are shown in **figure 5.8**. Both modulated conditions showed successful priming with piperacillin comparable to earlier results, as indicated through the PHA proliferative readings. However no significant proliferative response was observed in antagonist-treated cells, in opposition to the significant responses seen in agonist conditions. Priming of the naïve T-lymphocytes was also conducted to nitroso-sulfamethoxazole as in previous experiments as a model hapten.

To investigate whether the generation of piperacillin-specific IL-22 secreting T-cells is regulated through aryl hydrocarbon receptor (AhR), naïve T-cell

priming was studied in the presence of an AhR agonist (VAF347) or antagonist (CH-223191). Re-stimulation of T-cells primed in the presence of the AhR agonist resulted in the secretion of interferon-γ, interleukin-13, interleukin-22, perforin, granzyme B and FasL. T-cells primed in the presence of the AhR antagonist secreted IFN-γ, IL-13, perforin, granzyme B and FasL following re-stimulation with piperacillin; however, IL-22 secretion was no longer detected (***figure 5.9, figure 5.10***).
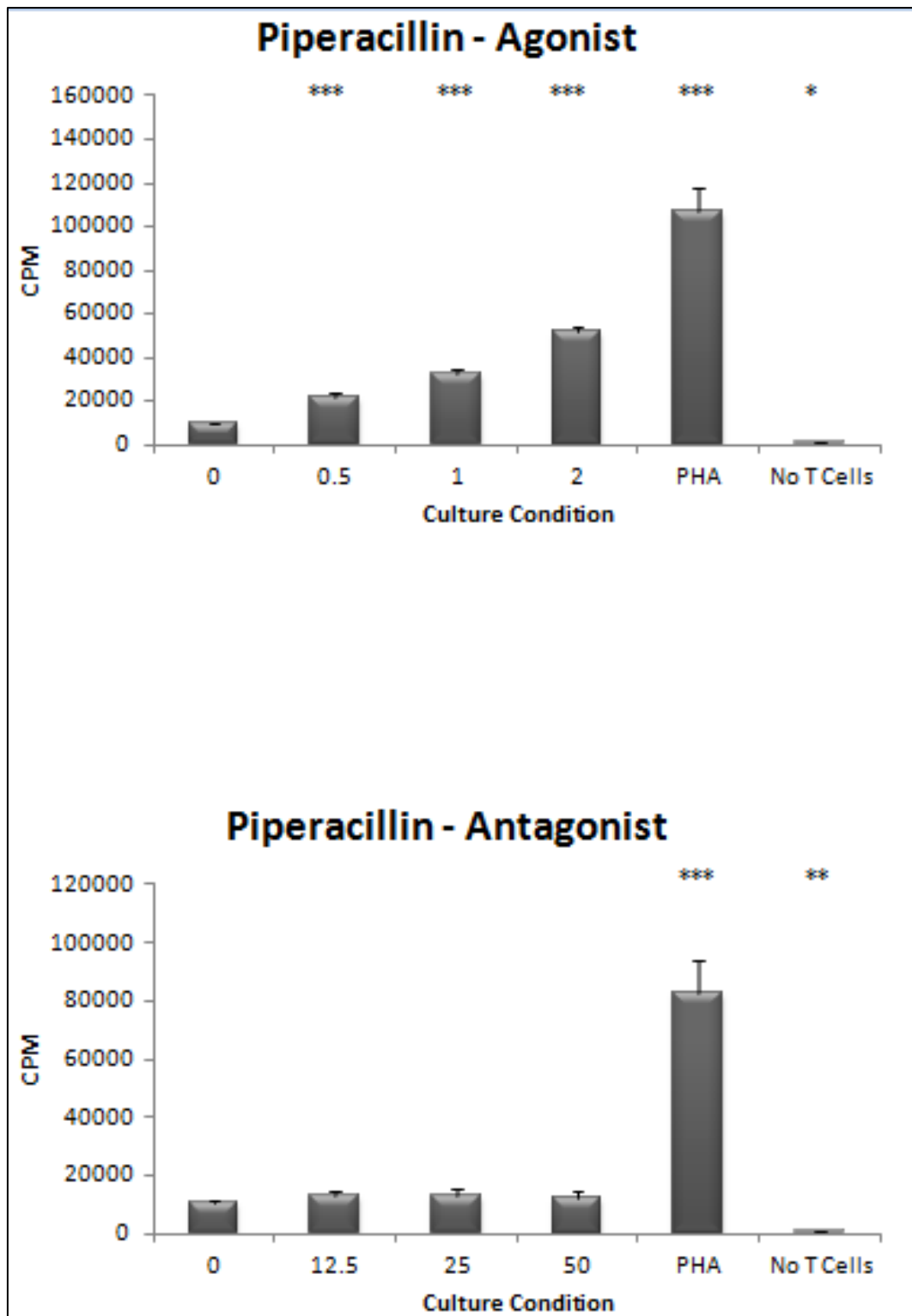
Figure 5.8. *Proliferative responses following naïve T-cell priming with aryl hydrocarbon receptor modulators.* *The agonist used was VAF347 (50nM), with the antagonist used being CH-223191 (100nM).* *Proliferation was measured via incorporation of radioactive ${}^3[H]$-thymidine, then reading via a beta-counter. PHA (5µg/ml) was included as a positive control, with wells containing only mature dendritic*

cells but no primed lymphocytes included as a negative control. Statistical significance was determined through one-way ANOVA analysis compared to the 0 control well. * - P=0.05, ** - P=0.005, *** - P=<0.001.

Donor A + Agonist	Inteferon-gamma	Interleukin-13	Interleukin-17A	Interleukin-22	Perforin	Granzyme B	Fas Ligand
0							
0.5							
1							
2							
PHA							
No T Cells							

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Donor C + Agonist	Inteferon-gamma	Interleukin-13	Interleukin-17A	Interleukin-22	Perforin	Granzyme B	Fas Ligand
0							
0.5							
1							
2							
PHA							
No T Cells							

Donor A + Antagonist	Inteferon-gamma	Interleukin-13	Interleukin-17A	Interleukin-22	Perforin	Granzyme B	Fas Ligand
0							
0.5							
1							
2							
PHA							
No T Cells							

Figure 5.9. *Cytokine secretions from naïve T-cells primed to piperacillin in the presence of an AhR modulator. The agonist used was VAF347 (50nM), with the antagonist used being CH-223191 (100nM). ELISpots to titrated piperacillin (mM), as well as PHA (5µg/ml) as a positive control were performed. 0 corresponds to medium alone (no pip). The 'No T Cells' condition is a negative control with dendritic cells only (+2mM pip). Interferon-gamma, interleukin-13, interleukin-17A, interleukin-22, perforin, granzyme B and fas ligand were all investigated.*
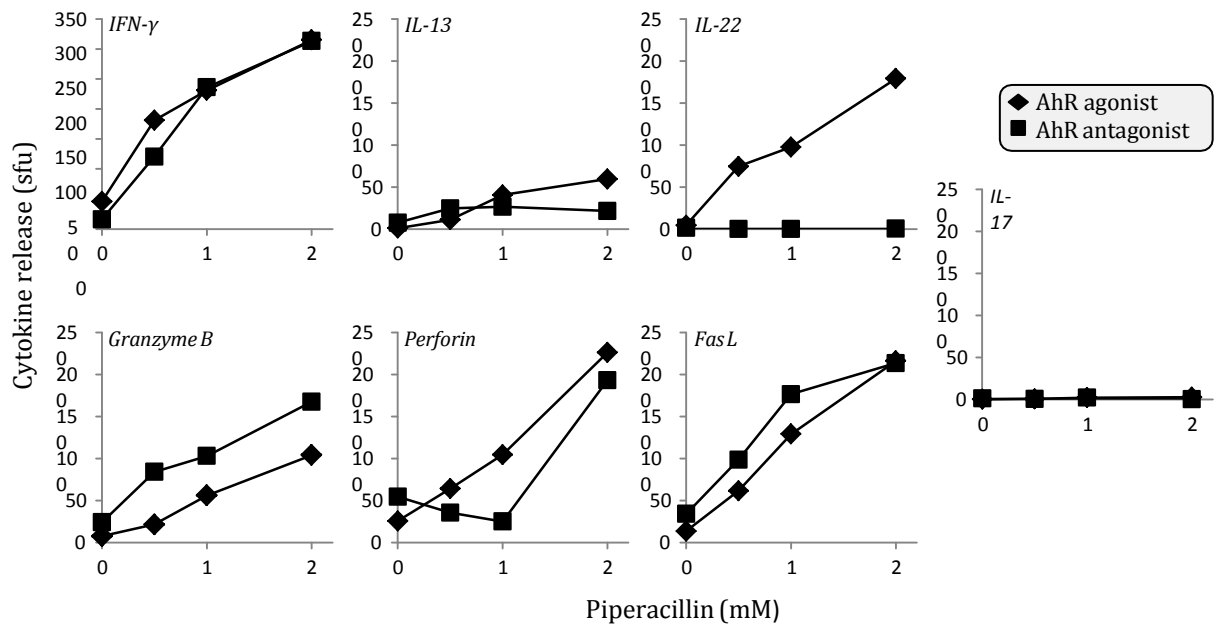
Figure 5.10. *Cytokine secretions from naïve T-cells primed to piperacillin in the presence of an AhR modulator. The agonist used was VAF347 (50nM), with the antagonist used being CH-223191 (100nM). ELISpots to titrated piperacillin (mM), as well as PHA (5µg/ml) as a positive control were performed. Interferon-gamma, interleukin-13, interleukin-17A, interleukin-22, perforin, granzyme B and fas ligand were all investigated. Results were recorded using an AID ELISpot reader, with SFU taken as a measure of cytokine secretion.*

5.5   DISCUSSION

Recent advances in the understanding of beta-lactam hypersensitivity have focused on the mechanistic causes of the condition. The current most valuable way of investigating these conditions is to look at the site of the reaction itself, rather than from peripheral blood PBMC samples as have been conducted in the past. Piperacillin hypersensitivity, the focus of the study, is a delayed reaction with the mean time of symptom onset estimated to be 9.1 days after initial drug administration (Whitaker, Naisbitt et al. 2012). The skin is the major site of tissue injury; however, a small number of patients present with drug-induced fever and arthralgia with no apparent skin injury.

In order to investigate cutaneous reactions associated with piperacillin exposure, lymphocytes were isolated from the reaction site. Following skin testing, drug specific T-cells were clones and phenotyped to explore their function.

Previous studies have formulated a method to isolate resident lymphocytes from skin biopsies (Clark, Chong et al. 2006; Lorenz and Stebut 2014). Three distinct sub-types of memory T-lymphocytes exist throughout the body that could be responsible for the reactions that are observed: T_{cm} central memory T-lymphocytes, T_{em} effector memory T-lymphocytes and T_{rm} resident memory T-lymphocytes (Shin and Iwasaki 2013). Of these, the resident memory T-lymphocytes might be the cells involved in the development of hypersensitivity reactions, due to the tissue selective nature of the observed response.

The ability of hypersensitive patient PBMC to respond to drug *in vitro* when placed into a simple proliferation assays has been reported by several independent research groups (Disis, Schiffman et al. 2000; Naisbitt, Farrell et al. 2003; Merk 2005). Less is known about the ability for cells isolated from specific reaction sites to respond to drug challenge. Little work has been done to investigate the role of skin derived T-cells in drug allergy. Only Nassif and Britschgi have looked into skin-derived T-cells in disease states, so much more work is needed to elucidate pathomechanisms of tissue injury (Britschgi, Steiner et al. 2001; Nassif, Bensussan et al. 2002). In ***figure 5.1***, the responsiveness of skin derived T-cells to piperacillin is tested through using a proliferation assay and piperacillin. The positive responses observed in both patients show that the isolated lymphocytes respond vigorously in response to drug challenge. These data indicate that; first, the protocol was successful at isolating lymphocytes from skin, and second, that the isolated lymphocytes are able to respond to the drug challenge. Thus, the cells obtained through this protocol are viable, responsive to the drug in question and suitable for further mechanistic investigations.

Piperacillin is a powerful broad spectrum beta-lactam antibiotic used in the treatment of multiple recurrent infections, typically seen in patients suffering with cystic fibrosis. It has a molecular weight of 517 and can form a reactive penicilloyl intermediate through breaking of the beta-lactam ring. The derived intermediate will bind irreversibly to protein lysine residues, most notably human serum albumin (Paterson and Bonomo 2005; Ghibellini, Bridges et al. 2007). Tissue injury in the skin is mediated primarily through the actions of lymphocytes against keratinocytes (Barbaud, Béné et al. 1997; Nestle, Kaplan

et al. 2009). To investigate whether piperacillin modifies skin proteins, keratinocytes cultured with piperacillin in serum containing medium was conducted (*figure 5.2*). Piperacillin was found to bind covalently to serum albumin; however, modification of keratinocyte protein from both hypersensitive patients was not detected. These data are in agreement with our previous work using immune cells in serum containing medium (Whitaker, Meng et al. 2011; El-Ghaiesh, Monshi et al. 2012). Piperacillin was found to bind to selective lysine residues on albumin in a dose and time-dependent manner. Moreover, at non-toxic concentrations (similar to those used throughout this work), albumin binding effectively prevented the generation of piperacillin adducts with cells. How these *in vitro* data relate to piperacillin protein binding is not known. Experiments are underway using biopsy specimens to determine whether selective piperacillin protein adducts are formed in patient skin, as well as whether quantitative differences in the levels of binding are seen in tolerant and hypersensitive patients.

Antigen-specific lymphocytes typically make up a small percentage of the total circulating lymphocyte population. Due to the small numbers of piperacillin-specific lymphocytes that would be obtained from a relatively small punch biopsy, the easiest (and most suitable) way to assess the potential reactivity of these lymphocytes is through clonal expansion and analysis of individual cells. The method utilised for this cloning procedure is the same as described in earlier chapters, with the purified cutaneous T-lymphocytes used for the cloning procedure. Piperacillin responsive clones were isolated from the skin of both hypersensitive patients (*figure 5.3*). In total, 690 clones were tested across both patients, with 96 shown to be drug responsive under these

conditions – giving a relative specificity rate of 13.9% for all the tested clones. This value is comparable with the number of piperacillin specific clones identified in patient blood. As shown below in *figure 5.11*, all the clones were tested for expression of phenotypic CD4 or CD8 markers. The clones showed a predominately CD4+ phenotype, though clones expressing CD8+ were also detected in lower frequencies in both patients.

ID	Type	# Tested	# Specific	% CD4+	% CD8+
Donor 1	Skin	354	48	89	11
Donor 2	Skin	336	48	82	18

__Figure 5.11.__ Generation of piperacillin specific T-cell clones from two patients hypersensitive to piperacillin. Clones were isolated from a 2mm punch biopsy. Isolated cells were then magnetically separated, with the purified T-lymphocytes cultured in the presence of PHA for 2 weeks to expand any specific cells. Serial dilutions were then performed on both patient samples, with clones that were growing picked and used for specificity testing. Specificity to piperacillin was determined through a proliferation assay where a stimulation index greater than 2 was positive (cpm drug treated wells / cpm control wells). Phenotypic CD4/CD8 analysis was performed through flow cytometry, with expression of either CD4 or CD8 as acquired by the cytometer.

Once the phenotype was recorded, clones were then selected for further in depth analysis, primarily the investigation of cytokine secretion and migratory characteristics.

Alongside the investigations of interferon-gamma, interleukin-13, interleukin-17A and interleukin-22, the cytotoxic markers perforin, granzyme B and fas ligand were also tested. These markers, if present, could explain why the drug-

specific T-cells cause tissue injury. Other studies have shown that cytolytic markers are secreted by drug specific T-cells from patients with hypersensitivity reactions (Eyerich, Eyerich et al. 2009). Thus, their involvement in piperacillin hypersensitivity was investigated (Yawalkar, Hunger et al. 2001; Posadas, Padial et al. 2002; Pichler 2003). Ten clones from each patient shown to be proliferative in the presence of piperacillin were tested (*figure 5.4*).

Given that human skin is protected by skin-resident and re-circulating T-cells with distinct functional activity, it was important to investigate T-cells isolated from inflamed skin (Watanabe, Gehad et al. 2015). These studies gained further credence when Gaide *et al.* demonstrated, using a mouse model of contact dermatitis, that skin resident and central memory T-cells derive from a common origin (Gaide, Emerson et al. 2015). The authors reported that skin resident cells responded rapidly following antigen challenge, whereas central memory cells were recruited to the tissue more slowly to mediate a delayed response. Skin biopsies were taken from 2 of the 4 hypersensitive patients following positive skin testing. Piperacillin-specific clones were generated by serial dilution and mitogen stimulation then subjected to detailed phenotypic and functional analysis. Similar to the blood-derived clones, approximately 85% of piperacillin-responsive clones were CD4^{+} and drug treatment resulted in the secretion of IFN-γ, IL-13 and IL-22, but not IL-17. However, eleven out of twelve skin-derived CD4^{+} and CD8^{+} IL-22 secreting clones were found to secrete IFN-γ. IL-22 binds to receptors expressed on cells such as keratinocytes to mediate innate responses in skin. Although IL-22 has been described as a protective cytokine, it also promotes pathogenic responses

when secreted, as we have found, in the presence of other cytokines (Mirshafiey, Simhag et al. 2015). For example, CD4$^+$ and CD8$^+$ T-cells that secrete IFN-γ and IL-22 are implicated in the pathogenesis of chronic skin conditions such as psoriasis (Kagami, Rizzo et al. 2010).

Fas L, perforin and granzyme B are T-cell secretory molecules that act in unison to induce apoptosis in tissue cells. Early studies by Posadas *et al.* reported up-regulated levels of perforin and granzyme B in PBMC isolated from patients with acute mild and severe forms of drug-induced skin injury (Posadas, Padial et al. 2002). Moreover, a strong correlation was observed between the level of cytolytic molecules and severity of the disease. In contrast, fas ligand was only observed in patients with Stevens-Johnson syndrome and toxic epidermal necrolysis. More recently, Zawodiniak *et al.* utilised a granzyme B ELISpot to detect cytotoxic T-cells in blood of patients with various forms of drug-induced skin injury (Zawodniak, Lochmatter et al. 2010). Herein, we utilized drug-specific clones isolated from blood and skin of the same hypersensitive patients to measure and compare fas ligand, perforin and granzyme B secretion. Piperacillin specific skin-derived clones secreted high levels of granzyme B when activated with drug, which to some extent explains the findings of Zawodiniak *et al*. In contrast, perforin and fas ligand secretion was largely restricted to the skin-derived clones. These data show that (1) drug-specific skin resident T-cells are the most likely mediators of tissue injury and (2) analysis of blood-derived T-cells alone underestimates the importance of cytolytic molecules in the disease pathogenesis.

The same analysis was performed on clones generated from skin, which were not stimulated to proliferate with piperacillin (***figure 5.5***). PHA was also used

as a positive control to assess the maximal secretory ability of the clones. As expected, these clones did not secrete cytokines or cytolytic molecules in response to piperacillin. Interestingly, the clones secreted multiple cytokines including interleukin-17A and interleukin-22 following PHA stimulation. Furthermore, interleukin-17A was secreted from several clones following PHA treatment, showing that the cells present in the skin have the potential to secrete interleukin-17A, but do not do so following drug challenge. The collective data found from ELISpot analysis of specific and non-specific clones isolated from the skin of patients indicates that drug specific T-cells display a T_h22 phenotype. If IL-17A is involved in the disease pathogenesis, it must be secreted from non drug-specific T-cells via bystander activation as has been noted recently in patients with contact dermatitis (Büdinger, Neuser et al. 2001).

The next step was to investigate the different markers that are expressed on both specific and non-specific clones isolated from patient skin. In the previous chapter, chemokine receptors expressed on the surface of blood specific piperacillin clones were measured, with differing levels of all tested receptors noted (see **Chapter 4**). The same chemokine receptors were tested on skin-derived clones, with the aim being to compare and contrast any differences between the three different classes that were studied; piperacillin blood-specific clones, piperacillin skin-specific clones and piperacillin skin non-specific clones. Chemokine receptors have different roles, with each of them involved in chemotaxis to specific ligands. Chemokine receptors CCR1, CCR4 and CCR10 are highly expressed on T-cells that migrate to skin. The data shown in the previous chapter demonstrated that piperacillin-specific blood

derived clones migrate in the presence of ligands for CCR4 and CCR10. CCR1 has been determined to be involved in the recruitment of both activated T-lymphocytes and monocytes in response to ligand activation, such as through the RANTES molecule. CCR4 has previously been shown to play a role as a chemoattractant on circulating memory lymphocytes, while CCR10 is heavily implicated in skin homing of T-lymphocytes (Hudak, Hagen et al. 2002; Campbell, O'Connell et al. 2007; Xia, Hu et al. 2014).

Significant differences were noted between blood and skin derived clones, alongside non drug specific clones. CCR2, CCR4, CXCR1, CLA and E-cadherin were all shown to be up-regulated in skin-specific clones when compared to blood specific clones. Both CLA and E-cadherin are strongly implicated in skin homing (Mendes-Aguiar, Gomes-Silva et al. 2009). CCR4 and CXCR1 are involved in lymphocyte recruitment and activation, as well as neutrophil activation (Cummings, Martin et al. 1999). These differences noted between the blood and skin derived clones show that there are subsets of lymphocytes in hypersensitive patients that can migrate to the skin and activate an immune response. Comparisons between specific skin derived and drug non-specific clones showed significant differences in CCR1, CXCR6 and CLA expression. All were up-regulated on drug-specific clones. CXCR6 interacts with the macrophage recruitment molecule CXCL16, while CCR1 interacts with RANTES – involved in recruitment to sites of inflammation – as well as MIP-1a, another molecule involved in the recruitment and activation of macrophages (Cook 1996; Galkina, Harry et al. 2007). These differences show that there is a recruitment pathway for specific lymphocytes to activate and migrate to the skin in response to drug exposure in patients with hypersensitivity.

Additionally, skin derived drug specific clones exhibit multiple macrophage recruitment pathways (Soler, Humphreys et al. 2003).

After confirming that the piperacillin-specific circulating and resident T-lymphocytes express skin homing characteristics, the next step was to confirm if they had the ability to migrate. To do this, a chemotaxis assay was performed similar to the previous chapter, this time utilising the skin-specific clones. The same ligands were incorporated into the assay, with CCL2, CCL4, CCL17, CCL25, CCL27, RANTES, CXCL9 and CXCL16 taken as ligands for the chemokine receptors CCR2, CCR5, CCR4, CCR9, CCR10, CCR1, CXCR3 and CXCR6 respectively.

Migration of the clones occurred in a time-dependent manner. In addition, the total percentage migration that was seen from the respective clones was associated with the relative expression of the receptors on the clones, as shown in *figure 5.7*.

A subset of T-cells expressing skin homing chemokine receptors CCR4 and CCR10 have been shown to secrete IL-22 and hence are thought to be important mediators of skin pathophysiology (Duhen, Geiger et al. 2009). Furthermore, ligands for CCR4 (CCL17) and CCR10 (CCL27), which contribute towards the recruitment of IL-22 secreting cells, have been found at high levels in lesional skin of patients with atopic dermatitis (Homey, Alenius et al. 2002; Kakinuma, Wakugawa et al. 2003). Skin-derived CD4$^+$ and CD8$^+$ clones expressed CCR4 and CLA in higher levels than the blood-derived clones. Alternatively the blood-derived clones expressed higher levels of CCR10. Most importantly, both skin and blood-derived clones migrated towards CCL17 and

CCL27 (i.e., ligand for the 2 chemokine receptors). Interestingly, the blood derived clones expressed high levels of CCR9; a receptor more traditionally associated with homing towards the gastrointestinal tract (Zabel, Agace et al. 1999) and migrated in the presence of the CCR9 ligand CCL25, which suggests that drug-specific T-cells in blood have the capacity to migrate to different locations around the body. This data, when taken together with earlier results, gives strong evidence that skin resident and skin migrating T-cells with a T_h22 phenotype are involved in piperacillin hypersensitivity. Additional studies are required to explore the role of IL-22 secreting T-cells in other forms of drug hypersensitivity.

In order to explore the origins of piperacillin specific T_h22 cells, the aryl hydrocarbon receptor, a transcription factor, was identified as a potential regulatory factor. The AhR is involved in the T_h22 signalling pathway, as well as the induction of several metabolising enzymes including members of the P450 superfamily (Ramirez, Brembilla et al. 2010; Basu, O'Quinn et al. 2012). To test whether AhR signalling regulates the production of T_h22 secreting cells, AhR modulators were incorporated into the dendritic cells naïve lymphocyte priming assay described in **Chapter 3**. Both an agonist (VAF347) and an antagonist (CH-223191) were incorporated into the priming stage of the assay, in order to assess the resulting phenotype of the cells present post priming. The initial aim was to assess if successful priming would occur under these conditions containing the AhR modulators. As seen in ***figure 5.8***, the priming assay was able to be completed in both cases of agonist and antagonist presence. The data shown is from three separate assays using different volunteer samples, with successful priming to piperacillin shown in the agonist

treated cells. The values obtained using agonist-contained priming conditions were comparable to the untreated priming values as shown in **Chapter 3**, suggesting that the presence of a T_h22 polarising AhR agonist had no effect on the ability for piperacillin priming to occur. However, in the case of the AhR antagonist-treated primed cells, no significant priming was noted at all concentrations of the drug. PHA treated cells were still responsive, showing the cells were still viable. This antagonist-treatment halted the ability of the priming assay to respond to piperacillin, suggesting that the addition of an AhR antagonist halted the ability for the cells to respond to piperacillin as was normally seen through other priming assays which contained no modulators of the receptor. This would confirm the theory that a T_h22 response is involved in the piperacillin response.

The T-cells primed in the presence of AhR agonist and antagonist were investigated via ELISpot using a panel of cytokines: interferon-gamma, interleukin-13, interleukin-17A, interleukin-22, perforin, granzyme B and fas ligand. Shown in ***figure 5.9*** are the complete cytokine secretion profiles secreted by piperacillin specific T-cells from two of the three healthy volunteers.

The key difference between the agonist-primed and antagonist-primed T-cells was the level of interleukin-22 secreted from piperacillin specific T-cells. In the agonist-treated cells, interleukin-22 secretion was observed, which is consistent with results obtained earlier chapters. However, in antagonist-treated cells, interleukin-22 secretion was completely abrogated, with no detectable interleukin-22 observed upon optimal drug challenge.

The transcription factor AhR regulates the differentiation of naïve T-cells into IL-22 secreting cells. Selective AhR antagonists have been shown to perturb production of IL-22 secreting cells from naïve pre-cursors, whereas memory T-cells are refractory to AhR regulation (Veldhoen, Hirota et al. 2008; Baba, Rubio et al. 2012). T-cells primed to piperacillin in the presence of VAF347 secreted IFN-γ, IL-13 and IL-22 alongside cytolytic molecules perforin, granzyme B and Fas L following re-stimulation with the drug. In parallel experiments with CH-223191, the differentiation of naïve T-cells into piperacillin-specific IL-22 secreting cells was blocked, while all other secretory molecules were detected at essentially the same level. Thus, AhR signalling is critical for the generation of drug-specific IL-22 secreting T-cells.

Some slight reduction in perforin secretions were noted across all donor samples in the antagonist-treated cells, potentially suggesting that the antagonist to the AhR might reduce the level of cytotoxicity induced by antigen specific T-cells; however, this requires further investigation.

Taken as a whole, the data collected from investigations into patient skin samples strongly suggest that a T_h22 phenotype predominates amongst lymphocytes that are responsive to piperacillin. Also, the presence of an aryl hydrocarbon receptor antagonist in the priming of naïve lymphocytes gave evidence to suggest a role for both interleukin-22 and the T_h22 lymphocyte subset in the development and progression of piperacillin hypersensitivity.

CHAPTER 6 – INVESTIGATION INTO THE IMMUNOGENICITY OF THE COX-2 SELECTIVE NON-STEROIDAL ANTI-INFLAMMATORY LUMIRACOXIB

6.1 INTRODUCTION

Non-steroidal anti-inflammatory drugs are one of the most widely prescribed drugs across the globe (Conaghan 2012). They do not contain an active opioid group, and as such are widely used in the treatment of pain, especially in cases where addiction could become, or is already a problem (Holdgate and Pollock 2004). They exert their mechanism of action through the inhibition of the cyclooxygenase enzyme, with the majority of NSAIDs inhibiting both the COX-1 and COX-2 isoforms non-selectively (Cryer and Feldman 1998; Knights, Mangoni et al. 2010). Selective inhibition of the COX enzymes comes about through the structural difference that is conferred through an amino acid substitution at position 523 between the two isoforms; COX-1 contains an isoleucine while COX-2 contains a valine (Gierse, McDonald et al. 1996; Kurumbail, Stevens et al. 1996; Walker, Kurumbail et al. 2001). Cyclooxygenase is involved in the production of prostaglandins from the precursor arachidonic acid. These prostaglandins are the signalling molecules in many inflammatory pathways, and as such are involved in the production of pain (Vane 1971; Marnett, Rowlinson et al. 1999).

The NSAID class has numerous properties; however, they are chiefly involved in the regulation of fever through their antipyretic activity. Fever is caused through the action of prostaglandin E2. Inhibition of the COX enzyme leads to reduction of levels of this prostaglandin, therefore reducing the body temperature (Aronoff and Neilson ; Rainsford 2007).

NSAIDs are generally well tolerated, with side effects of a mild nature being the most prevalent. Of these side effects, dyspepsia and mild bleeding via gastric ulcers are the most commonly reported (Green 2001; Rostom, Dube et al. 2002). However, there have been incidences where potentially life threatening adverse drug reactions have been observed, such as myocardial infarction and kidney failure (De Broe and Elseviers 1998; Trelle, Reichenbach et al. 2011).

Of the two isoforms, both COX-1 and COX-2 have different expressions and mechanisms of action. COX-1 is constitutively expressed throughout the body and is involved as a 'housekeeping' enzyme in a multitude of physiological processes. COX-2 however is involved exclusively in inflammatory processes, and as such is considered a highly promising target for potential future drugs (Dubois, Abramson et al. 1998; Fitzpatrick 2004). Development of these COX-2 selective inhibitors was implemented for this reason, alongside the reduced risk of the generation of ulcers (Traversa, Walker et al. 1995).

These COX-2 selective inhibitors became collectively known as 'coxibs'. Targeting the COX-2 enzyme selectively is possible through the isoleucine > valine substitution mentioned earlier. This substitution allows a small hydrophobic side pocket on the COX-2 enzyme to be interacted with by selective compounds, resulting in binding to the open site that is not present on the COX-1 enzyme due to the steric hindrance of the larger isoleucine amino acid structure (Dubois, Abramson et al. 1998). Unlike the non-specific NSAID-enzyme reactions, the coxibs exhibit irreversible binding to their receptors. These coxibs initially appeared to be successful in targeting the desired receptor and in the treatment of pain. However, beginning in 2004 with

rofecoxib, multiple serious adverse effects were reported resulting in the withdrawal of several drugs in this class. A number of the coxibs were withdrawn due to reported serious cardiovascular events, though other toxicities and adverse conditions were reported (Bresalier, Sandler et al. 2005; Nussmeier, Whelton et al. 2005; Solomon, McMurray et al. 2005).

One of these drugs, lumiracoxib, is an analogue of the widely prescribed painkiller diclofenac as shown in **figure 6.1**. It was originally approved in 2006 for the treatment of rheumatoid arthritis and acute pain, yet was withdrawn a year later due to serious liver damage resulting in the need for liver transplantation (Zhang, Ding et al. 2006; Geusens and Lems 2008). The TARGET study, involving 18,000 subjects, investigated the safety and efficacy of this drug alongside naproxen and ibuprofen yet no serious adverse effects were reported (Schnitzer, Burmester et al. 2004).
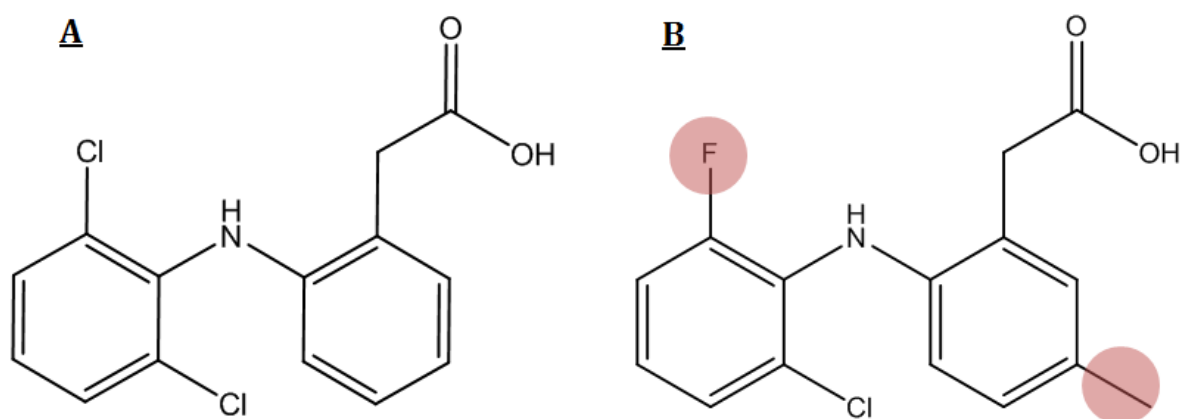
Figure 6.1. *Chemical structures of diclofenac (A, left) and lumiracoxib (B, right). Lumiracoxib is an analogue of the NSAID diclofenac, with a similar structure. Highlighted on the structure of lumiracoxib (B) are the differences in structures between the drugs, with a fluorine replacing a chlorine on a benzene ring and an additional methyl group on the other benzene ring. Diclofenac is often found in salt form, with*

either sodium or potassium being commonly used – turning the carboxylic acid –COOH
group into a –COO⁻ Na⁺/K⁺.

Numerous major and minor metabolites are formed following hepatic metabolism of lumiracoxib. The mechanism of liver injury is yet to be fully defined - though a recent genetic study has implicated a potential involvement of the adaptive immune system, with a HLA-DRB1*15:01⁺ haplotype representing an important risk factor (Singer, Lewitzky et al. 2010). It should be noted that drug-specific T-cells have not been characterised in patients with liver injury as it is no longer possible to access such patients due to drug withdrawal.

Strong genetic associations have been reported for a wide range of drugs over recent years. The most widely studied of these is the association of abacavir hypersensitivity with expression of the HLA-B*57:01 gene, as reported by Mallal in 2002 (Mallal, Nolan et al. 2002; Mallal, Phillips et al. 2008).

T-lymphocyte mediated responses can be classified into two broad categories: Class I and Class II. Lumiracoxib is associated with a HLA class II allele; thus, one would expect that the majority of T-cells activated with the drug would be CD4⁺. It is important to emphasise that the drug HLA class II interactions are much less well defined that the class I associations. In fact to date, nobody in the field has shown that drug binding to an HLA class II molecule leads to the activation of CD4⁺ T-cells.

6.2   AIMS AND HYPOTHESIS

The withdrawal of multiple 'coxib' class drugs has removed a valuable tool in the treatment of both pain and fever. The serious adverse effects that are observed, such as the liver toxicity in the case of lumiracoxib, are so rare that even large scale FDA approved studies failed to identify the potential of the drug to cause liver injury. The withdrawal of drugs at this late stage is a major clinical and financial problem for drug companies, as the development of compounds up to the point of approval can regularly exceed $1 billion. If anything could be done to provide a clearer understanding of these rare, idiosyncratic reactions in a way that either clarifies the benefit / risk ratio early in development, or provides a risk mitigation plan, then it is clearly of great financial and treatment benefit.

As such, work in this chapter sought to investigate whether T-lymphocytes could be successfully primed to either lumiracoxib or its metabolites. The investigation of lumiracoxib-specific clones would allow for the greater understanding of the mechanism involved in the pathogenesis of the liver toxicity.

Therefore, the hypothesis for this chapter of work was that:

'The response observed in lumiracoxib-induced liver injury is due to activation of CD4$^+$ T-lymphocytes only in individuals expressing the risk allele HLA-DRB1*15:01.'

6.3 METHODS

All experiments that were performed in this chapter were explained in greater detail in Materials and Methods Chapter 2. These include:

- Peripheral blood mononuclear cell isolation (2.5.1)
- Magnetic separation of cell subsets (2.5.2)
- Proliferation Assay (2.5.3)
- ELISpot (2.5.7)
- Flow cytometry (2.5.8)
- T-lymphocyte cloning (2.5.9)
- EBV generation (2.5.10)
- T-lymphocyte priming assay (2.5.14)

6.4 RESULTS

6.4.1 Lumiracoxib and its metabolites show clear dose-dependent toxicity when cultured with PBMC from healthy volunteers

In order to investigate potential lymphocytic involvement in the progression of lumiracoxib-induced liver injury, the ability to incorporate the drug into our *in vitro* assays had to be established. Through collaboration with Novartis, samples of the parent compound lumiracoxib and its major / minor metabolites NVP-LBH019, NVP-LBK286, CGS036501 and NVP-LBG677 were obtained (shown in ***figure 6.3***). All of these compounds were not soluble in normal culture media, meaning that dimethyl sulfoxide was used as a solvent to dissolve any drug prior to dilution of stock in medium.

Compound Name	Observed Toxicity (µM) (>LD50)	Optimal Value Chosen (µM)
Lumiracoxib	300	100
NVP-LBH019	100	50
NVP-LBK286	100	50
GCS036501	100	30
NVP-LBG677	30	10

Figure 6.2. *Inhibition of lymphocyte proliferation with lumiracoxib and its metabolites. $1x10^5$ donor PBMC were cultured per well in triplicate on a 96well U-bottomed plate. Concentrations of the compounds were co-cultured with the PBMC for 5 days in a semi-log manner (0.1µM-1mM), with PHA added to each well (2µg/ml) on day 3. Proliferative responses were recorded through the incorporation of ^{3}[H]-thymidine (0.5µl/well) for 16h and analysis through a beta-counter.*

These compounds were tested in a standard proliferation assay to explore the maximum concentrations that do not inhibit the T-cell response. As shown in ***figure 6.4,*** a range of concentrations were tested in order to determine optimal values for use. Inhibition of lymphocyte proliferation occurred at different concentrations for each of the compounds. In each case, maximal toxicity was observed at the highest two doses (1000μM and 300μM), with differing toxicity observed at lower concentrations. Based on the results obtained, the optimal concentrations of each compound for incorporation into the *in vitro* T-cell priming studies was determined and shown in ***figure 6.2***.

NVP-LBH019   NVP-LBK286   CGS036501   NVP-LBG677
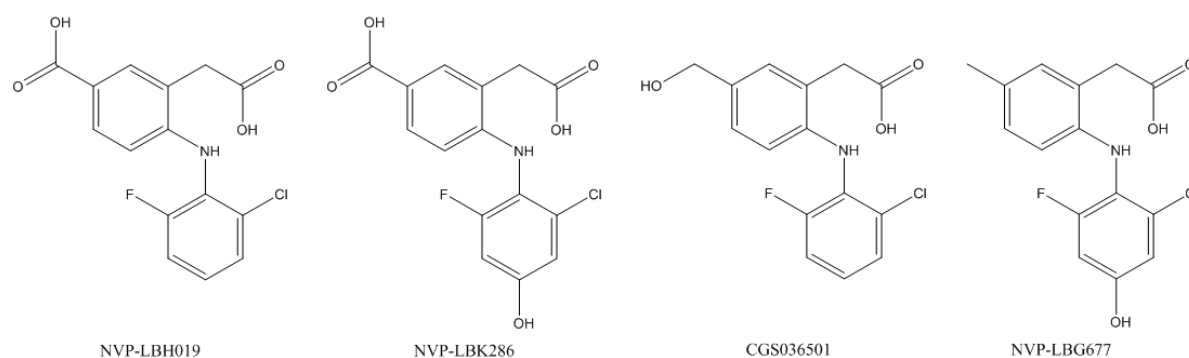
Figure 6.3. *<u>Chemical structures of the four investigated lumiracoxib metabolites.</u> All metabolites share a similar structure with the parent compound lumiracoxib; with the same double benzene rings, -COOH carboxylic acid and –NH amine groups present on all of the compounds. Changes in chemical structure occur on the lower benzene, with the presence or absence of an –OH hydroxyl group, as well as on the top benzene structure with the carboxylic acid group changing into either an –OH hydroxyl or –CH$_3$ methyl group.*
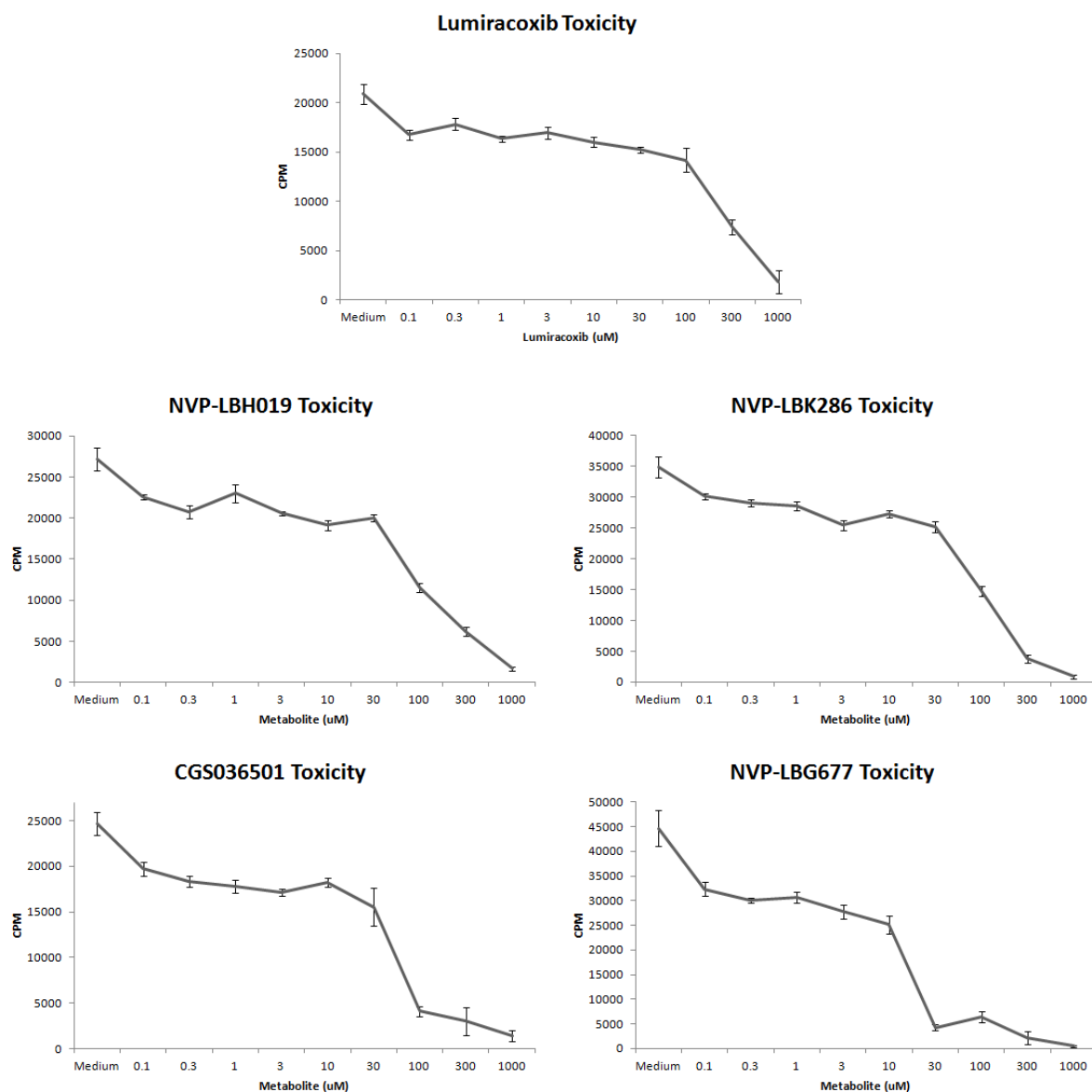
Figure 6.4. _Inhibition of lymphocyte proliferation following culture of PBMC with lumiracoxib and its metabolites. Lumiracoxib and the metabolites were tested in a standard lymphocyte proliferation assay in order to determine optimal concentrations for future assay incorporation. $1x10^5$ donor PBMC per well were cultured in triplicate in 96well U-bottomed plates. Concentrations of the drug compounds were co-cultured with the PBMC for 5 days in a semi-log manner (0.1μM-1mM), with PHA added to each well (2μg/ml) on day 3. Proliferative responses were recorded through the incorporation of ^{3}[H]-thymidine (0.5μl/well) for 16h and analysis through a beta-counter. Differing LD50 were observed for each compound._

6.4.2 Priming of naïve T-lymphocytes from HLA-DRB1*15:01⁺ individuals to lumiracoxib was unsuccessful

Utilising the method described in previous chapters, the next stage of the investigation was to explore whether the naïve T-lymphocytes that were isolated through magnetic separation from PBMC of HLA-DRB1*15:01⁺ individuals were primed to lumiracoxib. Priming experiments were conducted using the optimal values as described in ***figure 6.2***. In total, three donors containing the HLA-DRB1*15:01⁺ allele were primed to the parent drug lumiracoxib. No significant proliferative responses were observed when the primed naïve T-cells were re-challenged with lumiracoxib or SMX-NO, an irrelevant drug control (***figure 6.5***). Significant responses were observed from PHA-stimulated cells, confirming that the cells were viable, but not responsive to the drug (SI of PHA-stimulated conditions 6.7-12.3, omitted on graphs for clarity). Additionally, priming was conducted to the metabolites of lumiracoxib as discussed in ***6.4.1***. Similarly, no significant responses were observed when the primed cells were re-stimulated with the test compounds (***figure 6.6)***. Proliferative responses were measured using CFSE staining and analysis, looking at the percentage of dividing cells in a population. This assay was used to explore whether it was more sensitive than [³H]-thymidine incorporation for the detection of drug-specific T-cell responses. No increase in a dividing cell population was observed with the lumiracoxib metabolites indicating that lymphocytes were not activated. Priming of naïve T-cells and CFSE staining was conducted with sulfamethoxazole-nitroso as a positive control, which has been previously shown to prime naïve T-lymphocytes. As expected, T-cell

proliferation was observed when the primed cells were co-stimulated with SMX-NO.
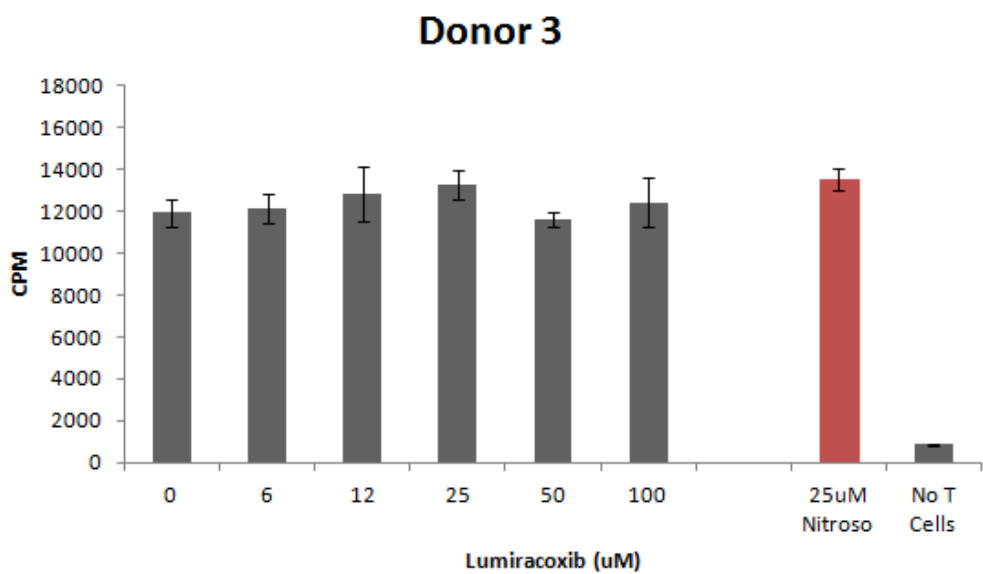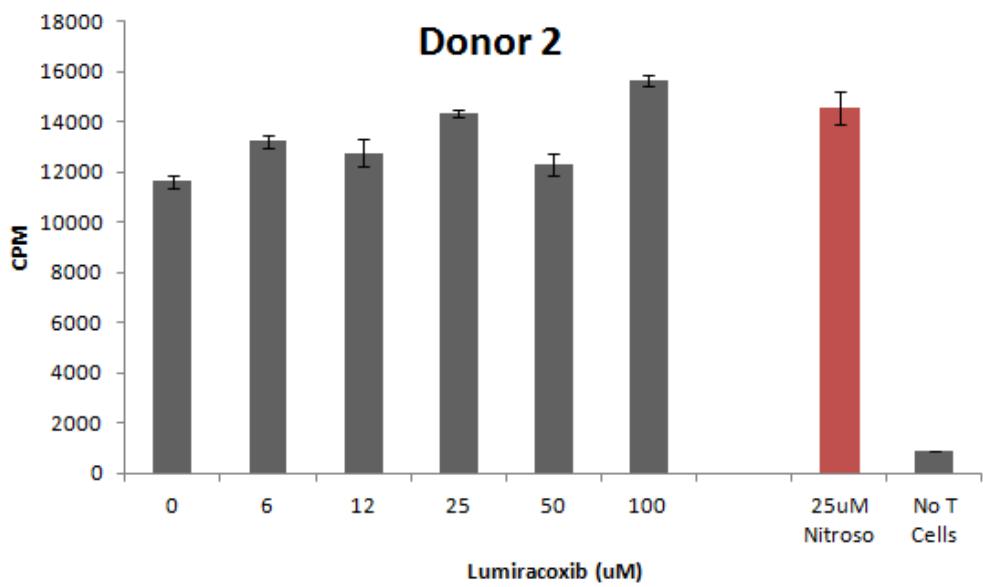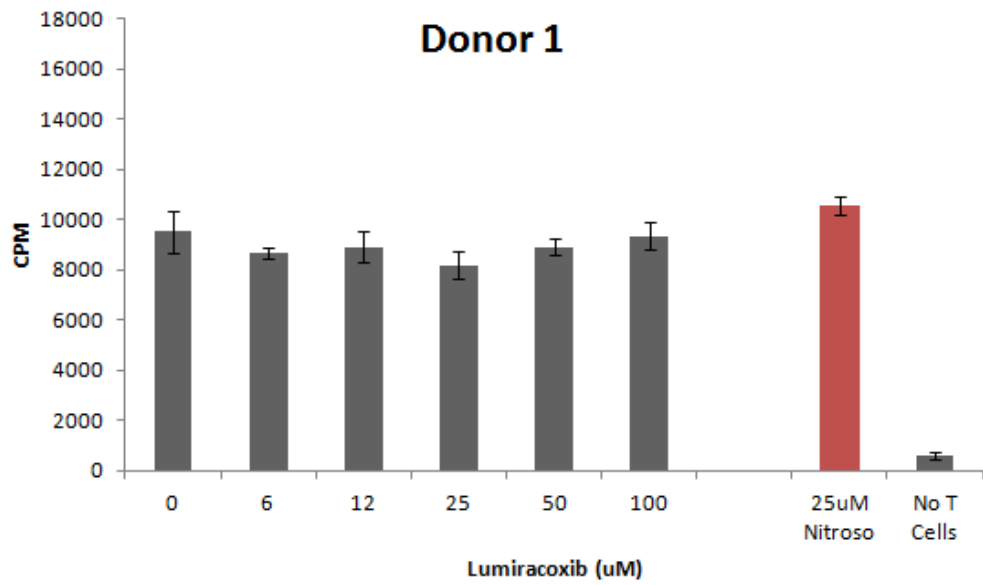
*__Figure 6.5.__ Proliferative responses of naïve T-lymphocytes following priming to lumiracoxib. Naïve T-lymphocytes were isolated from a HLA-DRB1*15:01⁺ donor and placed into a dendritic cell priming assay as described in detail in **Chapter 2**. Priming was conducted to lumiracoxib (well concentration 100µM) based on results obtained in previous assays shown in **figure 6.2**. Following 2 week priming culture, proliferative responses were recorded using differing concentrations of lumiracoxib, with 25µM sulfamethoxazole-nitroso included as a non-specific activation control and no T-lymphocytes included as a negative control – the dendritic cells present being stimulated with lumiracoxib (100µM). Proliferation was recorded via ³[H]-thymidine incorporation (0.5µl/well) for 16h and analysis through a beta-counter. No statistically significant responses were recorded for any of the conditions (NS = P>0.05, one-way ANOVA, comparison to non-specific stimulation via 25µM sulfamethoxazole-nitroso).*
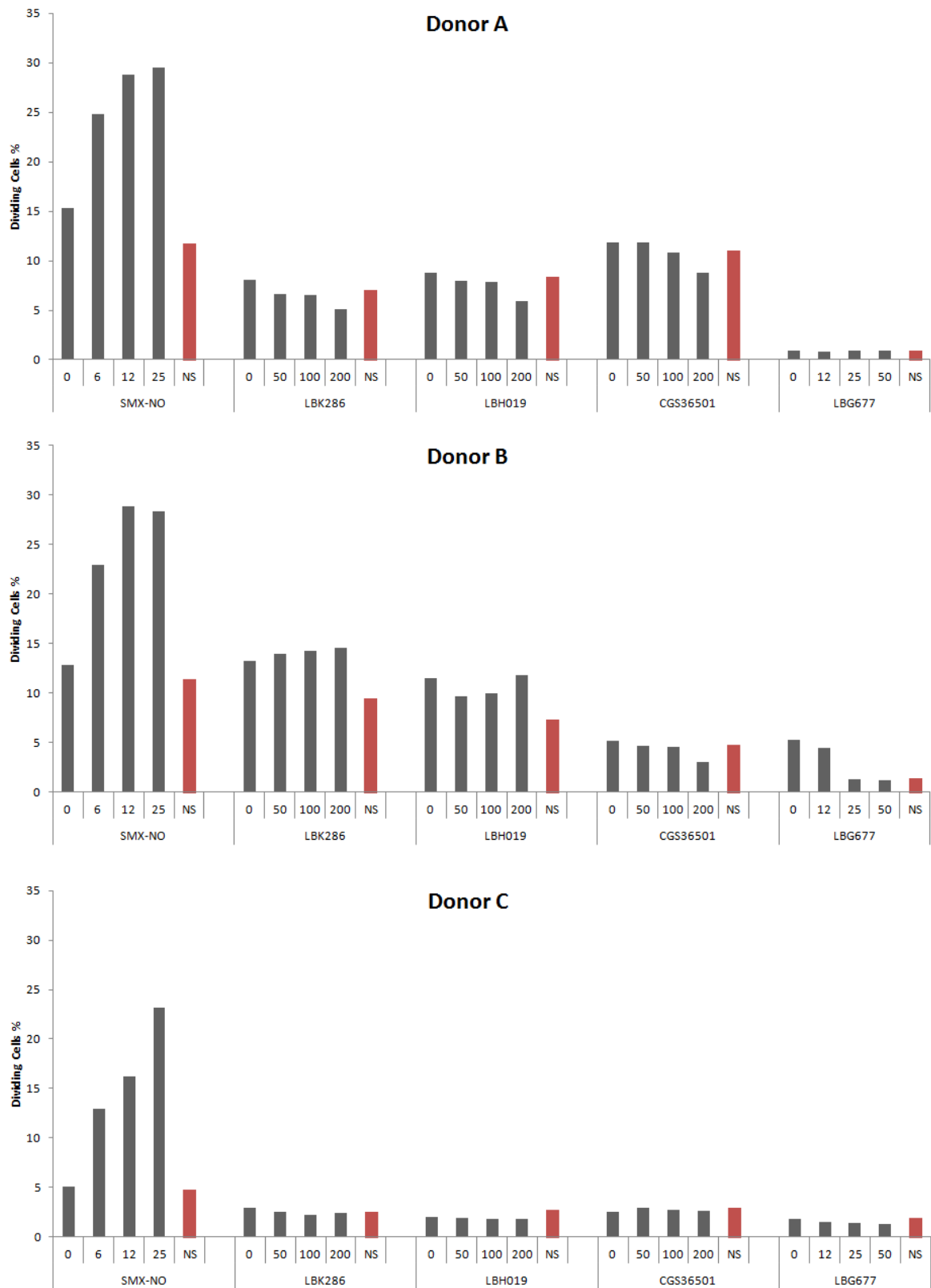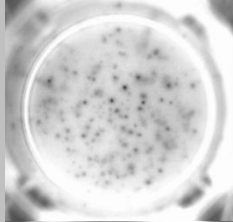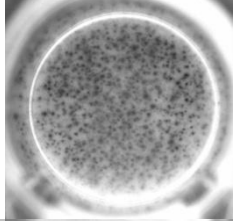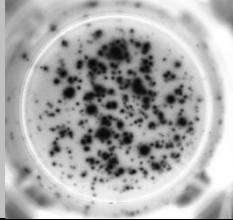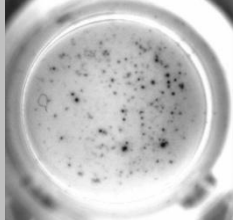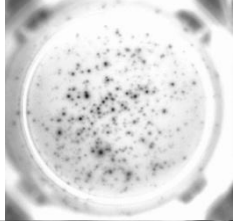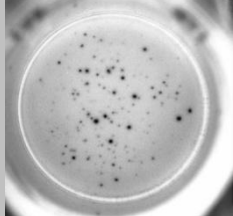
Figure 6.6. _Percentage of dividing cells following priming to different lumiracoxib metabolites and sulfamethoxazole-nitroso using CFSE staining. For each HLA-_

DRB1*1501⁺ donor, CFSE staining was performed to investigate the population of dividing cells as a measure of proliferation and response to drug re-challenge following priming. For each donor, priming was conducted to either sulfamethoxazole-nitroso (as a positive control) or the lumiracoxib metabolites as stated in **figure 6.2**. *For CFSE analysis via flow cytometry, 0.5µl of 5mM CFSE antibody was cultured with the primed lymphocytes prior to analysis, with 1x10⁴ cells used for each culture condition. Values were calculated as percentage expression of CFSE stain compared to unstained control. In sulfamethoxazole-nitroso primed conditions, non-specific stimulation was tested with lumiracoxib (100µM). In lumiracoxib-metabolite primed conditions, non-specific stimulation was tested using sulfamethoxazole-nitroso (25µM).*

6.4.3 Lumiracoxib (metabolite) -specific lymphocytes from HLA-DRB1*15:01⁺ individuals are not detected following bulk culture of PBMC

An alternative approach to detect drug responsive T-cells in PBMC of healthy drug naïve donors is to simply culture PBMC with drug in what is commonly referred to as a bulk culture. The PBMC can be re-stimulated with drug and autologous APC every 2-4 weeks. To investigate whether newly primed lymphocytes harvested from the dendritic cell priming assay secrete cytokines following drug (metabolite) treatment, T-cells were incorporated into an ELISpot assay. Due to the low numbers of cells available for the assay, only interferon-gamma was investigated. **Figure 6.7** shows that interferon-gamma was not secreted from the drug (metabolite) treated conditions, with both of the conditions showing clear wells. PHA stimulated wells showed high levels of interferon-gamma secretion, confirming cell viability.

	Control	Lumiracoxib	PHA
Donor 1			
Donor 2			
Donor 3			

	Control	Metabolite	PHA
Donor A			
Donor B			
Donor C			

__Figure 6.7.__ Interferon-gamma ELISpot analysing the secretion of cytokines from lumiracoxib primed T-lymphocytes. *The primed T-cells were re-stimulated with APC and lumiracoxib (metabolite) alongside positive and negative controls. Negative control conditions were achieved through medium + cells alone. PHA (2μg/ml) was used as a positive control. Representative metabolite results shown (CGS036501). All results were comparable. $5x10^4$ cells/well for each donor and condition were tested. No significant differences were seen through visual or statistical analysis of the control and drug-treated wells for either the lumiracoxib or metabolite-treated conditions (NS P=>0.05, t-test).*

6.4.4 Drug-specific T-lymphocyte clones are not detected from HLA-DRB1*15:01⁺ naïve lymphocyte cultures after priming to lumiracoxib

In order to investigate whether drug-specific lymphocytes were present following the priming of naïve T-cells with lumiracoxib, cloning was performed using cells harvested from the priming assay. Due to time limitations, only 4 of the samples were used for cloning – two each from the lumiracoxib and metabolite (-501 and -677) conditions. **Figure 6.8** shows the results of the clone generation. Over 900 clones were generated. However, no clones displayed specificity to either lumiracoxib or its metabolites. Upon initial testing, several clones' stimulation index values were shown to be above 2 – indicative of a positive response – but these were shown to be false positives upon retesting at a later date.

Donor	Drug	# Clones Tested	# Clones Specific
Donor 2	Lumiracoxib	213	0
Donor 3	Lumiracoxib	194	0
Donor B	NVP-LBH019	153	0
	NVP-LBK286	35	0
	CGS036501	66	0
	NVP-LBG677	88	0
Donor C	CGS036501	124	0
	NVP-LBG677	72	0

Figure 6.8. *Table showing the clone generation of T-cell clones from lumiracoxib primed cells. Clones were generated through the serial dilution using 0.3, 1, 3, 10 cells/well. Clones were re-stimulated on day 14 and expanding clones were picked for analysis. In total, 945 clones were tested, with 0 shown to be specific to the tested compound (specificity rate 0.0%). Specificity was determined through proliferation analysis where a stimulation index of greater than 2 was considered to be specific for the drug in question (SI = cpm drug-treated / cpm control).*

6.4.5   Long term bulk priming of naïve lymphocytes to lumiracoxib and its metabolites shows intermittently specific responses

All of the previous T-cell priming experiments yielded negative results. Thus, one final investigation was conducted prior to concluding that it was not possible to isolate lumiracoxib-responsive T-cells from healthy donors expressing the risk allele. In this case, an extended priming assay was conducted, with PBMC cultured with the compounds for 10 weeks prior to use in proliferation assays and T-cell cloning. These cells were fed with fresh medium containing the drugs over the course of the incubation period, with allogeneic irradiated PBMC used as antigen presenting cells added every 2 weeks.

In total, PBMC from 6 donors expressing HLA-DRB1*15:01 were investigated, with cultures performed to the parent compound lumiracoxib along with the metabolites CGS036501 and NVP-LBG677, giving a total of 18 different culture conditions. As shown in *figure 6.9*, the vast majority of the tested drug-treated PBMC primed bulks did not show a significant response upon re-challenge with the compounds after 10 weeks (SI value <2). However, one lumiracoxib primed sample, as well as 2 GCS036501 samples did display an SI value of greater than 2, meaning potentially that drug-responsive T-cells were present. These 'successfully' primed samples were used for the generation of T-cell clones. Of the clones generated (n=75), none were activated with the test compounds (SI<2).

Lumiracoxib Long Term Bulk

CGS036501 Long Term Bulk

NVP-LBG677 Long Term Bulk
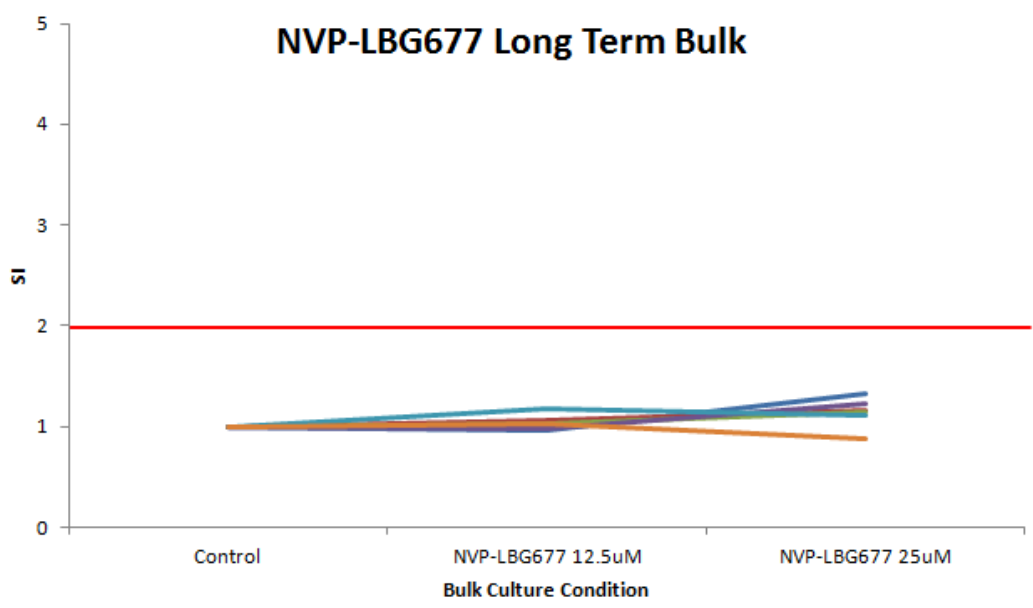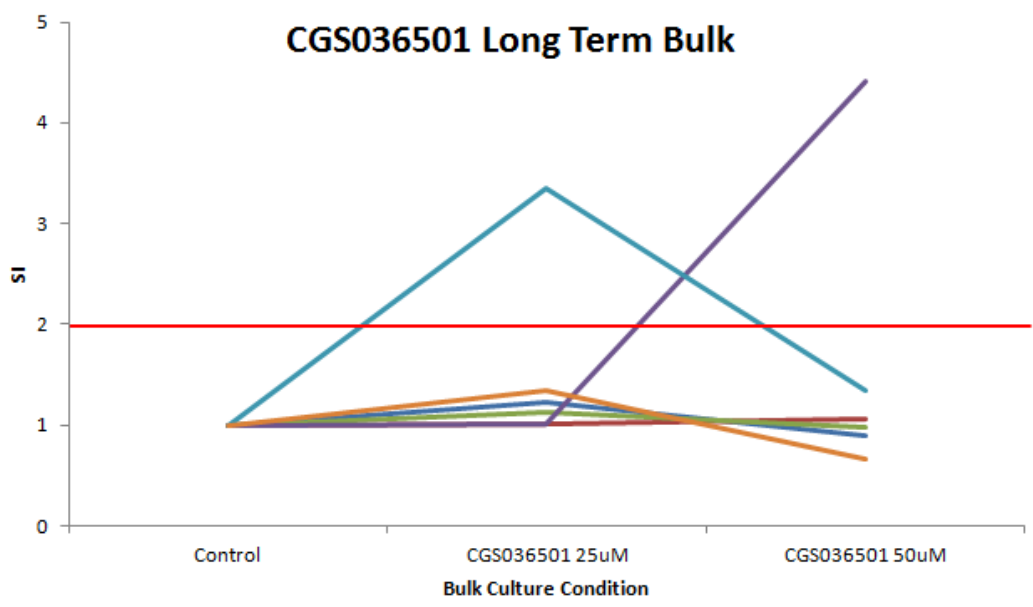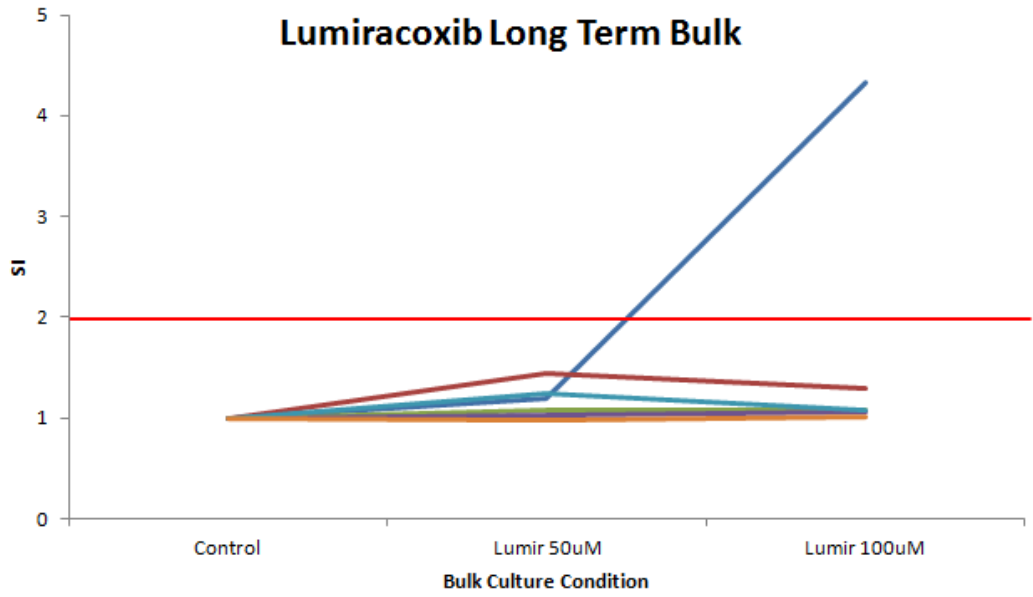
***Figure 6.9.** Proliferative responses observed following long term bulk priming to lumiracoxib and its metabolites. Six donors expressing the HLA-DRB1*15:01+ allele were investigated for long term (10-week) bulk priming, followed by clone generation as described in **Chapter 2**. Analysis was performed after 10 weeks via a proliferation assay, where a stimulation index of greater than 2 was considered to be specific to the tested compound (SI = cpm drug-treated / cpm control). The SI value of 2.0 is shown by a horizontal red line on the graphs. The same six donor samples were used for all three bulk priming conditions.*

6.5 DISCUSSION

Drug hypersensitivity reactions are major clinical concerns that are still poorly understood. Though strides have been taken in recent years to uncover the mechanisms involved in drug hypersensitivity, there is still a lot more to be learned about this condition, both from a mechanistic and genetic standpoint. Of the research that has already been reported, there are two distinct classes of drug hypersensitivity reaction; those restricted to HLA class I and class II alleles. Of the available data, Class I reactions predominate, with these reactions being observed in multiple drugs. Flucloxacillin (Monshi, Faulkner et al. 2013; Nattrass, Faulkner et al. 2015), abacavir (Chessman, Kostenko et al. 2008), carbamazepine (Mauri-Hellweg, Bettens et al. 1995; Wu, Farrell et al. 2007) and allopurinol (Pichler 2004; Roujeau 2006) have all been shown to elicit a class I restricted reaction. Moreover, the expression of a risk allele is associated with the drug-specific activation of CD8$^+$ T-cells. Of the previously mentioned compounds, abacavir is the best studied. Expression of the HLA allele HLA-B*57:01 correlates with the development of abacavir hypersensitivity (Martin, Nolan et al. 2004; Chessman, Kostenko et al. 2008; Mallal, Phillips et al. 2008). The correlations between flucloxacillin and HLA-B*57:01, carbamazepine and both HLA-A*31:01 as well as HLA-B*15:02 and allopurinol and HLA-B*58:01 have also been described (Hung, Chung et al. 2005; Man, Kwan et al. 2007; Daly, Donaldson et al. 2009; McCormack, Alfirevic et al. 2011). Each of these associations lead to a drug specific CD8$^+$ T-lymphocyte mediated reaction.

Lumiracoxib was withdrawn after reports emerged of acute liver toxicity after widespread usage of the compound (Singer, Lewitzky et al. 2010). Though the compound itself is no longer used, the availability of metabolites make lumiracoxib an ideal candidate to explore whether drug binding to the HLA risk allele is associated with the specific activation of CD4$^+$ T-cells.

First, toxicity assays were conducted in order to optimise the final drug concentrations for use in the immunological investigations. In total, 5 compounds were tested in order to determine optimal assay drug concentrations. Different values were obtained for each of the compounds (i.e. different LD$_{50}$ were observed). Based on these data, concentrations were selected for the analysis of T-cell responses (***figure 6.2, figure 6.3***).

Lumiracoxib was initially used to investigate the priming of naïve T-lymphocytes using a dendritic cell priming assay. In total, 3 HLA-DRB1*15:01$^+$ volunteer samples were studied, with results shown in ***figure 6.5.*** No significant priming to lumiracoxib was observed. This was assessed by studying proliferative responses and cytokine release following re-stimulation of the primed T-cells. Sulfamethoxazole-nitroso was used as a positive control to confirm the functionality of the assay.

Following from these negative results with lumiracoxib, further assays were conducted, this time using lumiracoxib metabolites (structures shown in ***figure 6.3***). Again, three volunteer samples were used for priming, with all four metabolites used in separate experiments with cells from each volunteer. In addition, priming was again conducted to sulfamethoxazole-nitroso, as this compound has previously been shown to successfully prime naïve T-

lymphocytes (Faulkner, Martinsson et al. 2012). As seen in ***figure 6.6***, similar results were obtained with the lumiracoxib metabolites. No significant priming of naïve T-cells was observed with the metabolites. In contrast, the nitroso primed cells showed significant dose-response proliferation following re-stimulation. Thus, it is highly likely that priming to either lumiracoxib or its metabolites had not occurred.

The data obtained in ***figure 6.6*** shows CFSE analysis of cell populations post priming. CFSE staining has been reported as an accurate and sensitive way to measure proliferation of a population as total percentage of dividing cells (Lyons, Blake et al. 2013). This was performed via flow cytometry analysis, with CFSE incorporation used to measure dividing cells to assess whether it is a more sensitive readout to detect drug antigen-specific T-cell responses.

Though no observable proliferation was observed following priming to either lumiracoxib or its metabolites, there is a chance that the readouts were not sufficiently sensitive to detect low numbers of antigen specific T-cells. ELISpots have been shown to provide highly sensitive results that otherwise would not be able to be detected through traditional proliferative analysis (McCutcheon, Wehner et al. 1997).

To investigate whether this is the case, ELISpot assays were performed using the primed cells looking for interferon-gamma secretions when the lymphocytes were cultured with medium alone, drug (either lumiracoxib or its metabolites) or PHA. Neither the lumiracoxib nor the metabolite primed conditions showed interferon-gamma secretions following drug re-challenge. The drug treated wells were comparable with the control wells, once again

suggesting that the lymphocytes cultured in the priming assay do not have the ability to respond to lumiracoxib challenge. Interestingly, the cells do show a strong secretory response when challenged with PHA, confirming cell viability and cytokine secretion capacity.

Though negative results were obtained from both proliferation assays and ELISpot analysis, the rarity of the reported liver injury suggests that lymphocytes would only be detected in a small number of patients (Schnitzer, Burmester et al. 2004). To investigate whether T-cells were present in low numbers, serial dilution was utilised to generate clones that may be specific to either lumiracoxib or its metabolites. These were performed using the primed lymphocytes from the earlier assays. In total, 945 potential clones were generated across 4 donor samples, both to lumiracoxib and the four tested metabolites, with no specific clones generated. Initially, several potential clones exhibited specific responses with stimulation index values of greater than 2. However, these were shown to be false positives, as none of the clones were specific upon multiple retests. Only certain primed bulks were chosen to be used in the clone generation stage due to poor cell numbers from other donors as well as time limitations.

A final attempt was made to generate a T-cell response *in vitro* to these compounds, through the use of a modification to the original priming assay (Faulkner, Martinsson et al. 2012). This was done through the culture of HLA-DRB1*15:01+ donor PBMC *in vitro* as a bulk for 10 weeks, with regular additions of fresh medium, the compounds and APC at their established optimal concentrations. This extended time span would potentially give cells

more time to expand; therefore drug-specific T-cell responses might be detectable. As shown in **figure 6.9**, long term bulk priming was conducted to lumiracoxib, as well as the metabolites CGS036501 and NVP-LBG677. These bulks were set up to six donors in each case, with the results of drug challenge shown in the figure. Interestingly, there were a total of 3 bulks that showed a significant response to the compounds upon challenge (two from the same donor across different compounds). These results were obtained using proliferation as a readout. These responses suggest that there is the potential for lymphocytes from HLA-DRB1*15:01$^+$ individuals to respond to lumiracoxib and its metabolites *in vitro*, though the success rate is still low. It is worth noting however, that clone generation from these long term bulks was conducted in cases where initial specificity was seen, with no drug-specific clones being detected. The bulks themselves were of poor viability at the end of the long term bulk priming.

All of this data taken as a whole would suggest that the *in vitro* study of lumiracoxib-induced T-cell responses is unable to be conducted using established methods. It is still possible that reactions are mediated by T-cells; however, new methods must be developed to study this and other forms of HLA class II restricted drug hypersensitivity. Furthermore, HLA-DRB1*15:01 was utilised as a risk allele for this work. There are several other haplotypes that have been shown to be involved in lumiracoxib-induced liver injury which were not looked at (Singer, Lewitzky et al. 2010; Alfirevic and Pirmohamed 2011; Pavlos, Mallal et al. 2012). It is therefore reasonable to hypothesise that one of these other alleles could be involved in the observed hepatotoxicity.

CHAPTER 7 – FINAL DISCUSSION

Before commenting on the results that this thesis has generated as a whole, first certain limitations of the study need to be addressed. While every effort was made to generate robust data, some problems emerged that were unable to be avoided. Firstly, the availability of patient samples was somewhat limited. The samples from patients with cystic fibrosis were kindly donated to us from the St James' Regional Cystic Fibrosis Center in Leeds. These samples were provided based on availability of patients, in addition to given consent and reactions that were occurring during the study. Critically, skin samples were only obtained from two patients due to these factors, predominantly consent. While understandable, the lack of a readily available patient cohort to obtain multiple clinical samples limited the scope of the investigations that were able to be undertaken throughout this thesis. Though efforts were made to keep a supply of working samples (via freezing of excess cells, supernatants etc.), the small number of patient samples available for research was a major limitation. Replication of this data would be made easier through the availability of a replicate cohort of patients with cystic fibrosis, though the feasibility of this is questionable.

In addition; time, cost and cell number constraints were present throughout the work in multiple experiments. While attempts were made to replicate data using sets of samples, limitations in available cell numbers meant that replicates were low. However, statistical analysis was still able to be performed on many of the experiments. Importantly, the skin sample work is worthy of further study. The ethics obtained for these experiments allowed for

a single small punch biopsy to be obtained following an intradermal test. This resulted in <4 million T-cells being isolated from the sample, greatly limiting the work that could be performed. In addition, no repeat samples were able to be obtained, along with the lack of relevant ethics to obtain healthy volunteer skin samples for comparison. Further work would be enhanced through the investigation of these responses in both patients and volunteers.

Adverse reactions to drugs are a major concern for healthcare across the globe. They are one of the most common causes of hospital admissions, with studies putting the relative frequency of hospital admissions due to ADRs as high as 7% (Pirmohamed, James et al. 2004). The problems caused by these reactions to drugs can be classified in two broad terms: on target (type A) and off target (type B). The type A reactions are the most widely reported type of adverse reaction, and can usually be rectified through alteration of the drug dosage, as the resultant side effects are due to the known pharmacology of the compound. Type B reactions are much less prevalent, but can cause life-threatening complications that cannot easily be predicted through known pharmacological mechanisms (Guengerich 2006; Bender, Scheiber et al. 2007; Zaccara, Franciotta et al. 2007). These are a major concern both for healthcare professionals, due to their serious nature, and drug manufacturers, as the complications from type B reactions can often lead to the withdrawal of a drug. This is important for two reasons: first, bringing a compound to market can regularly exceed $1billion; second, these withdrawals can often affect distribution of life-saving drugs, orphan drugs or drugs which are the only currently available treatment for a specific condition (Kaplowitz 2005; Ulrich 2007).

Understanding of the mechanistic basis of hypersensitivity reactions was compiled in 1963, with the classification proposed by Phillip Gell and Robert Coombs (Gell and Coombs 1963; Descotes and Choquet-Kastylevsky 2001). This was the first time that the different clinical manifestations of hypersensitivity had been considered with the goal of elucidating the mechanisms that were involved. It wasn't until the 5th edition of the book, published in 1993, that a separate focus was placed on mechanisms of allergy and IgE-mediated diseases. The Type I-IV classification that was outlined by Gell and Coombs was based on the time to onset of reaction and the functional mediators that bring about the allergic disease. In subsequent decades most mechanistic studies focused on Type I immediate reactions involving IgE antibodies and the activation of mast cells. In contrast, the other forms of hypersensitivity, especially the delayed T-lymphocyte mediated Type IV reactions, remained relatively poorly understood.

The Gell and Coombs classifications are still useful today. For example, β-lactam antibiotics cause reactions in patients that fall under each of Gell and Coombs classification (Torres and Blanca 2010). However, the classification does have important limitations. First, antigen-specific antibodies and T-cells are detectable in individual patients presenting with a hypersensitivity reaction and this cannot be rationalized according to Gell and Coombs classification. Secondly, it does not account for the different populations of T-cell with different effector functions that have been described in the immunological literature. For this reason, expansion of the classification was required. The delayed type hypersensitivity (Type IV) was expanded upon by

Pichler, subdividing them into Type IVa-d (Pichler 2003). This expansion was designed to take into consideration the T-cells involved in different reactions.

This classification was well received, allowing the greater understanding of role of T-lymphocytes in hypersensitivity reactions with a delayed onset, but different clinical presentations. However, once again it is obvious that the classification currently in use is not completely fit for purpose. Recent research has elucidated newer reactivity conditions, such as DIHS / DRESS, as well as newer helper T-lymphocyte subclasses such as the T_h9 / T_h17 / T_h22 phenotypes (Bettelli, Carrier et al. 2006; Ben M'Rad, Leclerc-Mercier et al. 2009; Kagami, Rizzo et al. 2010; Schlapbach, Gehad et al. 2014). Furthermore, in the last decade our knowledge of the mechanistic basis of drug hypersensitivity has increased exponentially. For example, we now know that (1) herpes virus reactivation is implicated in certain reactions (Picard, Janela et al. 2010), (2) HLA allele expression is an important determinant for susceptibility (Pirmohamed, Ostrov et al. 2015) and (3) specific forms of drug-induced kidney and liver injury should fall under the definition of drug hypersensitivity (Wuillemin, Terracciano et al. 2014; Kim, Saide et al. 2015). Despite this, our knowledge of the T-cells that instigate and/or regulate drug hypersensitivity reactions has not progressed significantly beyond the classification proposed by Pichler.

Work conducted over the recent years has shown that both $CD4^+$ and $CD8^+$ memory T-cells are able to secrete unique cytokine profiles upon stimulation, with the characteristic secretions being; T_h1 - IFN^{high} $IL-4^{low}$, T_h2 - IFN^{low} $IL-4^{high}$ $IL-5^{high}$ $IL-13^{high}$, T_h17 - $IL-17A^{high}$ $IL-22^{high}$ and T_h22 - $IL-17A^{low}$ $IL-22^{high}$.

This picture is not as clear as it could be however, due to the ability of both IL-17A and IL-22 to be secreted by T_h1/T_h2 cells. The potential role in skin-mediated conditions of both IL-17A and IL-22 is highlighted through the presence of the receptors for these cytokines being expressed on keratinocytes, along with other epithelial cells. In addition, T_h17 and T_h22 cells are known to express skin-homing chemokine receptors, further enhancing their potential involvement (Boniface, Bernard et al. 2005; Nograles, Zaba et al. 2008; Cho, Suh et al. 2012).

Due to the fact that human skin is known to be patrolled by populations of both circulating and resident memory T-lymphocytes, the work conducted throughout this thesis focused on the investigation of drug-specific T-lymphocytes from both blood and skin of patients that were hypersensitive to piperacillin (Watanabe, Gehad et al. 2015). The role of interleukin-22 especially was of interest, and such investigations into the aryl hydrocarbon receptor were undertaken to assess its involvement in the generation of drug-specific IL-22 secreting T-cells. Piperacillin was chosen for two reasons. First, it is known to form drug antigens both in vitro and directly in patients post administration through lysine modification of proteins (Whitaker, Meng et al. 2011). Second, it is commonly prescribed and its use is associated with a high incidence of hypersensitivity.

Investigations fell into three distinct sections; (1) assessment of the phenotype and function of both blood-derived T-lymphocytes from hypersensitive patients, (2) comparison of the drug-specific lymphocytes from blood and inflamed skin and (3) analysis of healthy volunteer samples and priming of

naïve T-cells. Because of the functional protection given to human skin by resident and recirculating T-lymphocytes, it was of critical importance to compare the T-cell populations in blood and skin. These comparisons were given further importance after a mouse model of contact dermatitis showed that skin resident memory T-lymphocytes and central memory T-lymphocytes both come from a common origin (Gaide, Emerson et al. 2015).

The generation of piperacillin specific T-lymphocyte clones from both the blood and skin of hypersensitive patients showed dominant $CD4^+$ phenotype, with approximately 85% of drug-specific T-cells isolated from blood and the tissue shown to be $CD4^+$. The skin-derived specific clones were shown to secrete interferon-gamma, along with interleukin-22 and cytolytic molecules. Though interleukin-22 has been reported as a protective cytokine, there is also evidence that it can be involved in the pathogenesis of certain diseases such as psoriasis (Kagami, Rizzo et al. 2010). Collectively, the data generated shows that interleukin-22 signalling is involved in piperacillin-mediated maculopapular eruptions. The most interesting outcome of the project was detected when the profile of cytokines secreted by blood and skin-derived clones were compared, as shown in ***figure 7.1***. Piperacillin-specific blood derived clones showed a mixed T_h1, T_h2 and T_h1/T_h2 profile when analysed, with the $CD8^+$ clones shown to display predominantly a T_h2 section profile. In contrast, the skin-derived piperacillin-specific clones showed a much more highly polarised profile, with strong T_h1 / T_h2 responses being observed. In this case, the $CD8^+$ clones secreted a T_h1 profile following drug stimulation. Analysis of the interleukin-22 secreting clones from the skin showed a strong T_h1 profile, suggesting that the hypersensitivity observed in patients following

piperacillin exposure is likely mediated through the action of a subset of T_h1^+ interleukin-22 secreting T-lymphocytes.
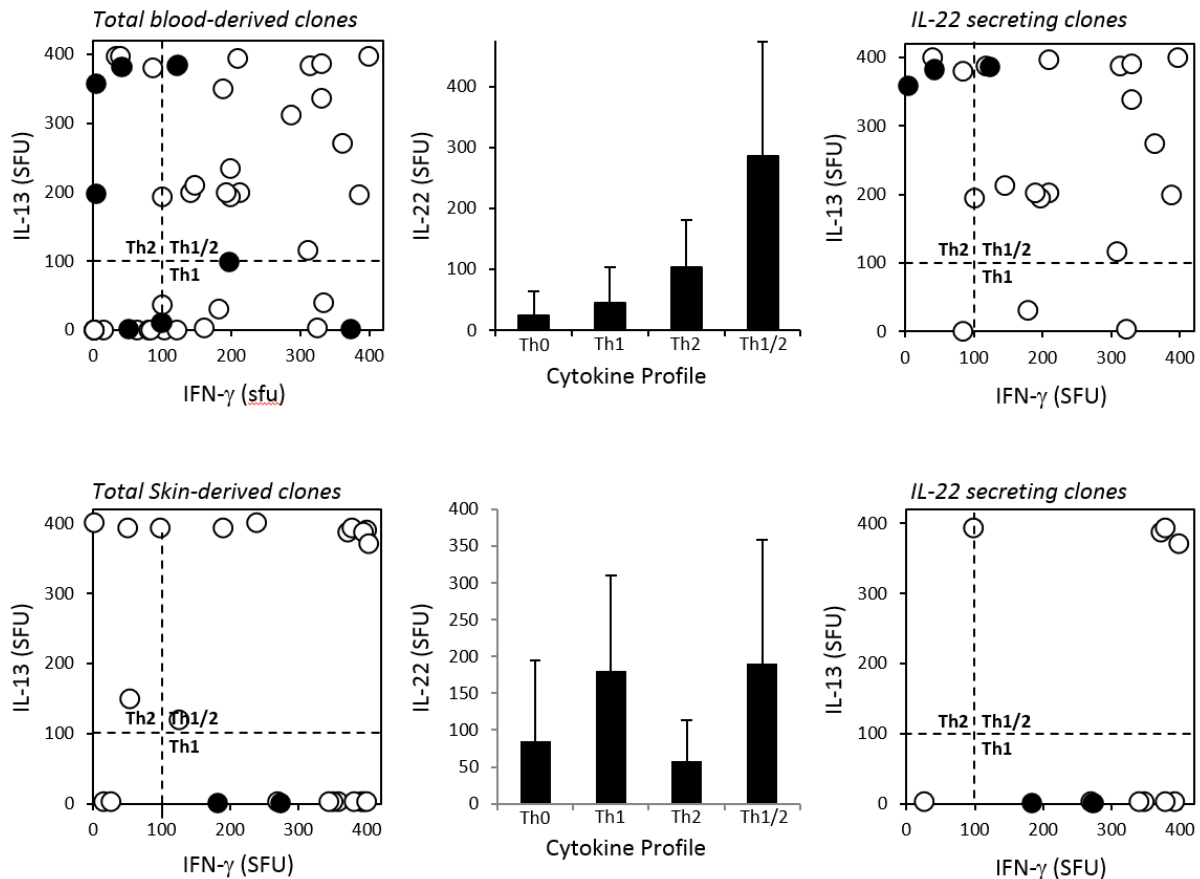
Figure 7.1. *Profile of cytokines secreted from piperacillin-specific T-cell clones derived from blood and inflamed skin of hypersensitive patients. T-cell clones (n=43, blood; n=24, skin) were cultured with irradiated antigen presenting cells and piperacillin (2mM) and cytokine secretion was quantified by ELISpot. (Left) Comparison of IFN-γ and IL-13 secreted by individual blood- and skin-derived T-cell clones. Dashed lines at 100 spot forming units (SFU) were used to classify clones according to classical T_h1, T_h2, T_h1/T_h2 fingerprint. Shaded circles depict CD8+ clones. SFU in drug-treated wells with SFU in control wells subtracted are shown in all images. (Center) Mean ± SD IL-22 secretion from T_h1, T_h2 and T_h1/T_h2 clones. (Right) Comparison of the level of IFN-γ and IL-13 secreted by individual IL-22high (IL-22 SFU of 100 or above in piperacillin-treated wells with control values subtracted) blood- and skin-derived T-cell clones. Each circle represents an individual clone; shaded circles depict CD8+ clones.*

Analysis of the migration of the clones confirmed that they were able to migrate towards skin through the expression of skin-homing chemokine receptors (e.g., CDR4 and CDR10). A subset of T-lymphocytes that are known to express high levels of CCR4 and CCR10 have been shown to secrete IL-22. For this reason, they can be considered to have a role in skin-targeting reactions such as those seen in piperacillin hypersensitivity (Duhen, Geiger et al. 2009). In addition, ligands for CCR4 and CCR10, which contribute towards the recruitment of IL-22 secreting cells, have been found in the skin of patients with atopic dermatitis (Homey, Alenius et al. 2002; Kakinuma, Wakugawa et al. 2003). Importantly, piperacillin-specific skin-derived CD4+ and CD8+ clones expressed CCR4 and CLA in higher levels than the blood-derived clones. In contrast, the blood-derived clones expressed higher levels of the skin-homing CCR10. Critically, both skin and blood-derived clones migrated towards CCL17 and CCL27, ligands for the skin-homing chemokine receptors. Blood derived clones also expressed high levels of CCR9, a receptor closely associated with homing towards the gastrointestinal tract (Zabel, Agace et al. 1999), and migrated in the presence of the CCR9 ligand CCL25, which suggests that drug-specific T-lymphocytes that are circulating in the blood have the capacity to migrate to different locations around the body.

Investigations were then performed using naïve T-lymphocytes from healthy donors to explore whether it was possible to prime T-cells to piperacillin *in vitro*. Following re-stimulation of the primed cells with piperacillin, significant proliferation was observed. Importantly, the piperacillin-specific T-cells secreted the same cytokines as the patient cells (i.e., IFN-γ, IL-22 and IL-13). Moreover, piperacillin-specific IL-17 secretion was not detected. Importantly,

IL-22 was seen in the absence of T_h22 polarizing cytokines such as IL-6 and TNF-α (Duhen, Geiger et al. 2009). In fact, priming of naïve T-cells to piperacillin under T_h17 or T_h22 polarizing conditions did not increase the levels of IL-22 secreted from primed cells. T-cells cloned from the piperacillin-primed naïve T-cells were skewed towards a $CD8^+$ phenotype, in contrast to the highly $CD4^+$ patient clones, and drug stimulation resulted in the secretion of high levels of IFN-γ and moderate levels of both IL-13 and IL-22. Analysis of individual clones revealed that the three clones secreting high levels of IL-22 were $CD8^+$ and they did not secrete T_h1 or T_h2 cytokines.

The aryl hydrocarbon receptor regulates the differentiation of naïve T-lymphocytes into IL-22 secreting cells. Selective AhR antagonists have been shown to inhibit production of IL-22 secreting cells from naïve pre-cursors, whereas memory T-cells are refractory to AhR regulation (Veldhoen, Hirota et al. 2008; Baba, Rubio et al. 2012). In the final component of the work, naïve T-lymphocytes were primed against piperacillin in the presence of an AhR agonist (VAF347) and antagonist (CH-223191). T-lymphocytes primed to piperacillin in the presence of VAF347 secreted IFN-γ, IL-13 and IL-22 alongside cytolytic molecules perforin, granzyme B and fas ligand following re-stimulation with the drug. Concurrent experiments with CH-223191 showed that the differentiation of naïve T-cells into piperacillin-specific IL-22 secreting cells was blocked, while all other secretory molecules were observed at similar levels. Therefore, it has been shown that AhR signalling is critical for the generation of drug-specific IL-22 secreting T-lymphocytes.

The majority of studies exploring the relationship between expression of an HLA risk allele and development of a hypersensitivity reaction have focused on HLA class I linked reactions. For example, carbamazepine's linkage with HLA-B*31:01, flucloxacillin and HLA-B*57:01, allopurinol with HLA-B*58:01 and abacavir's strong association with HLA-B*57:01 have all been shown to relate to the activation of drug-specific T-lymphocytes. The HLA class I associations lead to the selective activation of CD8$^+$ T-cells (Mallal, Phillips et al. 2008; Tassaneeyakul, Jantararoungtong et al. 2009; Monshi, Faulkner et al. 2013). This work is very useful for studying the underlying genetic components of certain drug hypersensitivity reactions, though does not take into account the MHC class II response that can also be present. For this reason, we chose to investigate lumiracoxib – a drug associated with HLA-class II-restricted liver injury. The programme of work utilized established cell culture methods to determine whether it was possible to generate drug-specific T-cells in donors expressing the risk allele and, if so, characterise the phenotype of the responding cells and HLA restriction. Sulfamethoxazole nitroso was used as a positive control, which has previously been shown to stimulate CD4$^+$ and CD8$^+$ T-cells from healthy volunteers to proliferate and secrete cytokines. In contrast to sulfamethoxazole nitroso, T-cells responsive to lumiracoxib were not detected. In subsequent experiments lumiracoxib metabolites kindly donated by Novartis were tested using the same experimental design. However, similar negative results were obtained with the metabolites. It is possible that drug (metabolite)-responsive T-cells are being generated in low numbers in the *in vitro* culture conditions. For this reason, T-cells were cloned from the lumiracoxib and lumiracoxib metabolite cultures. Almost 1000 clones

were tested, but none displayed reactivity against lumiracoxib or its metabolites. These results could be due to a number of reasons, of which two merit the most consideration. Firstly, the knowledge of genetic associations have greatly enhanced our ability to target medications for different people, however there is a chance that our knowledge of the HLA associations with each compound is incomplete. Lumiracoxib hepatotoxicity is known to be linked with a $-DQA1*01:02^+$-$DQB1*06:02^+$-$DRB1*15:01^+$-$DRB5*01:01^+$ haplotype, yet the undertaken work only focused on the HLA-$DRB1*15:01^+$ allele (Singer, Lewitzky et al. 2010). It is therefore possible that susceptibility relates to an alternative (or multiple) HLA allele. Also, though *in vitro* work aims to replicate the conditions found in an organ, specific conditions are not easily mimicked. The hepatotoxicity observed in lumiracoxib reactions could be due to reactive metabolites and protein adducts formed directly in hepatocytes themselves, meaning that the correct antigen would not be generated in the *in vitro* conditions (Li, Slatter et al. 2008). Work with sulfamethoxazole and its metabolite sulfamethoxazole-nitroso has shown that cross-reactivity does not always exist between related compounds; thus, the metabolites potentially responsible for lumiracoxib's liver injury might not cross react with the parent compound (Wulf and Matuszewski 2013).

In conclusion, this work shows that both circulating and skin-resident $CD4^+$ and $CD8^+$ T-lymphocytes that secrete IL-22, but not IL-17, alongside cytolytic molecules are important mediators of maculopapular drug eruptions in humans – with the differentiation of naïve T-cells into drug-specific T_h/T_c22 cells being dependent on AhR receptor signalling.

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