Characterisation of the chemical basis of T-cell mediated β-lactam hypersensitivity reactions

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

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October 2015

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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Fiazia S. Yaseen

For my grandparents

Hajan Sharifan Bibi and Haji Mohammed Shafi
Hajan Naziran Bibi and Haji Mohammed Amanat Ali

Acknowledgements

786 92

Al-ḥamdu lillāh, I am grateful to Allah for giving me the strength, endurance and knowledge to complete this PhD.

I would first like to thank my supervisors Dr Dean Naisbitt and Professor BK Park for their guidance. Dean, your passion is infectious; I never thought I'd get excited reading results off a screen! Without your help and encouragement I'm not sure I would have made it and I hope you know how much I appreciate the opportunity, support and time you have given me. I must also thank ITTP for the funding and Dr Andy Smith.

I am completely indebted to the queens of mass spec; Dr Roz Jenkins and Dr Xiaoli Meng. I am so grateful for your infinite knowledge, help and patience. Through all the ups and downs you always found time for me and never once rolled your eyes as I asked the most random and silly questions! I couldn't have done this without you.

Thank you to all the patients, volunteers, research nurses and clinical staff who made this work possible, especially Dr Paul Whitaker and the team at Leeds Cystic Fibrosis Unit. Thank you to those in the lab for all your help and expertise; John Farrell, Lee, Han (my journey started with you and Junnat!), Sammy and Jane. And the fabulous liver team; Row, James, Lorna, Chris and Fang, who welcomed me to the fold and made the late nights so much easier.

I have to say a massive thank you to all my friends in the cell lab. Monday, Andy, Mohammed, Maike, Katy, John, Arun, Andrew, Will, Zaid, Ryan and Toru you made coming to work such a pleasure. The DJN group has given me the kindest, smartest friends in Catherine, Sally and Manal I will be forever grateful your love, encouragement and positivity. Who knew the hood is an ideal place to talk it out!

Massive thank you to my PhD buddies, you have been there through all the ups and downs, science and otherwise. Ali, Áine, Ágnes, Alix, Holly, Harriet, Jo, Lewis, Liz, Pika, Rach and Andrew Swale you made this journey so much easier.

Penny and Claire I am so grateful for your love and support. Seeing your faces always cheered me up and I am proud to call you my friends. Even though you'll always be the doctors everyone else means! And thank you to Emma, Robyn and Laura for reminding me of the world outside of science, bringing me back to earth and leaving a smile on my face every time. You guys are amazing.

I could not have done this without my amazing family. My parents Yasmin and Yasin, and Thia Jee and Kanie, I will forever be grateful for your love, support and dua's. I am nothing without you. My wonderful siblings Kashif, Aftab, Ashia, Bilal and Aisha thank you for everything. Aisha you're about to start this yourself and I hope to give support and not as many distractions as you did me! Bilal, always knowing how to put a smile on my face, thank you. Insha'Allah in a few short years I will be congratulating you Dr! Mamu Sajid, Baji Shafia, Qasim and Ishmael I am so grateful Allah brought you over here, I don't know what I'd do without you. And thanks to my extended family for their support from afar, from Scotland to Pakistan to New Zealand.

Finally thank you to my wonderful new family for all your love, smiles and encouragement. Naila, Tanveer and Iqra you have welcomed me with open arms, I am blessed to have you with me. And Zuhaib, my amazing husband always knowing the right thing to say. Thank you for your love, support and kindness. I look forward to starting our next chapter together, side by side.

Contents

Abbreviations

ADR	Adverse drug reaction
ACN	Acetonitrile
AGEP	Acute generalised exanthematous pustulosis
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
amu	Atomic mass unit
APC	Antigen presenting cell
BSA	Bovine serum albumin
CCR	Chemokine receptor (C-C) motif
CD	Cluster of differentiation
cDNA	Complimentary DNA
CF	Cystic fibrosis
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CFTR	Cystic fibrosis transmembrane conductance regulator
cpm	Counts per minute
CSA	Cyclosporin
CTL	Cytotoxic T-lymphocyte
Da	Daltons
DAG	Diacylglycerol
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DILI	Drug-induced liver injury
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNCB	Dinitrochlorobenzene
DNP	Dinitrophenol
DRESS	Drug reaction with eosinophilia and systemic symptoms
EBV	Epstein-Barr virus
ECL	Electrochemicaluminescence
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FasL	Fas ligand
FBS	Foetal bovine serum
Fig	Figure
FITC	Fluorescein isothiocyanate
Flu	Flucloxacillin
FoxP3	Forkhead box P3
FSC	Forward side-scatter
GM-CSF	Granulocyte macrophage colony-stimulating factor
h	Hours
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HMGB1	High-mobility group box 1

HSA	Human serum albumin
I.P.	Intra-peritoneal
I.V.	Intra-venous
ICAM	Intercellular adhesion molecules
IFNγ	Interferon-gamma
Ig	Immunoglobulin
IL-*	Interleukin factor *
ITAM	Immunoreceptor tyrosine-based activation motifs
Kb	kilo base
LAT	Transmembrane adapter protein linker for the activation of T-cells
Lck	Lymphocyte-specific protein tyrosine kinase
LCMS/MS	Liquid chromatography tandem mass spectrometry
LFA-1	Lymphocyte function-associated antigen
LN	Lymph node
LPS	Lipopolysaccharide
LTT	Lymphocyte transformation test
MAPK/ERK	Mitogen-activated protein kinases/ Extracellular signal-regulated kinases
Mb	Megabase
MHC	Major histocompatibility complex
MPE	Maculopapular exanthema
MRM-MS	Multiple reaction monitoring-Mass spectrometry
N/ A	Not applicable
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer
NRTI	Nucleoside reverse transcriptase inhibitor
OR	Odds ratio
PAMPS	pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pH	Power of hydrogen
pi	Pharmacological interaction
Pip	Piperacillin
RA	Retinoic acid (vitamin A)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SFC	Spot forming cell
SFU	Spot forming unit
SI	Stimulation index
SJS	Stevens-Johnson syndrome
SMX	Sulfamethoxazole
STAT	Signal transducer and activator of transcription
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TEN	Toxic epidermal necrolysis

TFA	Triflouroacetic acid
Th1	T-helper-1
Th2	T-helper-2
TLR	Toll-like receptor
TNF - α	Tumour necrosis factor alpha
TNTC	Too numerous to count
T_{regs}	Regulatory T-cells
U/L	Units per litre
UK	United Kingdom
ULN	Upper limit of normal
USA	United States of America
v/v	volume/volume
WHO	World health organisation
w/v	weight/volume
ZAP 70	ζ-chain-associated protein kinase 70
β2m	β 2 microglobulin

Publications

Papers

Yaseen, F. S., Saide, K., Kim, S. H., Monshi, M., Tailor, A., Wood, S., Meng, X., Jenkins, R., Faulkner, L., Daly, A. K., Pirmohamed, M., Park, B. K., and Naisbitt, D. J. (2015) Promiscuous T-cell responses to drugs and drug-haptens, *J Allergy Clin Immunol*.

R. E. Jenkins, F. S. Yaseen, M. M. Monshi, P. Whitaker, X. Meng, J. Farrell, J. Hamlett, J. P. Sanderson, S. El-Ghaiesh, D. Peckham, M. Pirmohamed, B. K. Park and D. J. Naisbitt
β-lactam antibiotics form distinct haptenic structures on albumin and activate drug-specific T-lymphocyte responses in multiallergic patients with cystic fibrosis. *Chem Res Toxicol*, 26(6): 963-975. (2013)

Xiaoli Meng, Fiazia S Yaseen, Jenkins R, Earnshaw C, Whitaker P, French N, Pirmohamed M, Naisbitt DJ and Park BK.
Quantitative analysis of the absolute thresholds of piperacillin antigen exposure required for T cell activation
(Submitted to Journal of Allergy and Clinical Immunology)

Abstracts

Fiazia Yaseen, Manal Monshi, John Farrell, Paul Whitaker, Daniel Peckham, B. Kevin Park and Dean J. Naisbitt

Multiple drug hypersensitivity in patients with cystic fibrosis: characterization of highly drug-specific T-cell clones

Poster presentation, Drug Hypersensitivity Meeting, Munich (2012)

Fiazia Yaseen, Manal Monshi, John Farrell, Paul Whitaker, Daniel Peckham, B. Kevin Park and Dean J. Naisbitt

B-lactam hypersensitivity in patients with cystic fibrosis: characterization of highly drug-specific T-cell clones

EAACI Immunology Winter School, Pichl (2013)

Xiaoli Meng, Fiazia Yaseen, Caroline Earnshaw, Rosalind Jenkins, Paul Whitaker, Neil French, Pirmohamed M, Naisbitt DJ and Park BK

Quantitative Analysis of The Absolute Thresholds of Piperacillin Antigen Exposure Required for T cell Activation

Poster presentation, Drug Hypersensitivity Meeting, Bern (2014)

Abstract

The idiosyncratic nature of drug hypersensitivity reactions poses a major clinical problem as they are difficult to predict and can be severe in nature. β-lactam antibiotics provide the cornerstone of treatment for pulmonary exacerbations in patients with cystic fibrosis (CF). Unfortunately, their use is complicated due to a high rate of delayed-type hypersensitivity reactions. This body of work was undertaken to investigate the chemical and cellular mechanisms of β-lactam-induced T-cell mediated hypersensitivity.

Approximately 20% of patients with CF develop multiple drug hypersensitivity (MDH). Peripheral mononuclear blood cells (PBMCs) isolated from 4 patients, but not controls, were activated with three drugs (piperacillin, meropenem & aztreonam). T-cell clones were isolated from the MDH patients and characterised for phenotype, function and cross reactivity. Piperacillin-, meropenem- and aztreonam-responsive T-cell clones (mainly CD4+) proliferated and released IFN-γ following drug stimulation. Clones were drug-specific; cross reactivity was not observed. All 3 drugs formed structurally distinct haptenic structures with specific lysine residues on HSA in a time- and concentration-dependent manner.

β-lactam antibiotics are widely thought to activate immune cells through the covalent modification of proteins, but the link between conjugate formation and an immune response is still to be defined. T-cell clones from piperacillin hypersensitive patients were stimulated by piperacillin-pulsed antigen presenting cells (APCs) in a time dependent manner and T-cell responses were inhibited following inhibition of protein processing. Synthetic conjugates generated via two methods were investigated. Conjugates were similar in terms of sites of modification and relative level of binding at each site. Conjugate 1 did not stimulate T-cells while responses were observed with conjugate 2. The higher concentration of unbound piperacillin detected in conjugate 2 led to the hypothesis that HSA conjugates may facilitate the transfer of piperacillin to appropriate binding sites on MHC molecules to initiate a T-cell response.

T-cell clones were subsequently generated from PBMC cultured with piperacillin conjugate 1. Several clones were stimulated with conjugate 1, but not the parent drug highlighting the existence of T-cells with specificity against different drug-derived antigens.

Using a synthetic piperacillin-modified HSA peptide (PIP-K541 peptide) as a standard, a novel mass spectrometric method was developed to quantify the level of piperacillin protein binding needed to activate T-cells. Levels of PIP-K541 in patient plasma ranged from 2.5-6.5%. *In vitro* modification ranged from low (1%) coinciding with weak proliferative responses piperacillin-specific T-cell clones to high at around 4%. From these experiments it was possible to estimate that 2.8% modification of albumin is sufficient for effective T-cell activation.

The β-lactam antibiotic flucloxacillin is associated with a high incidence of liver reactions. Genome-wide association studies identified HLA-B*57:01 as an important susceptibility factor. CD8+ T-cell clones from patients with liver injury were activated by flucloxacillin to proliferate and secrete cytokines in an HLA-B*57:01 restricted and dose-dependent manner. T-cell activation was detected with both soluble drug and drug-pulsed APC. In contrast, clones from volunteers were only activated with soluble drug and the response was not HLA-B*57:01 restricted.

This work quantifies β-lactam-protein binding in patients and relates this to T-cell activation. Furthermore, the immunological studies show that T-cells from hypersensitive patients are activated via multiple pathways.

Chapter 1: General Introduction

1.1 Introduction

β-lactam antibiotics comprise one of the most common groups of therapies used to treat bacterial infections (Cho *et al.*, 2014) yet the high rate of hypersensitivity reactions, particularly in patients with cystic fibrosis, is a major health concern. Immune-mediated reactions are often difficult to predict and can be severe or even fatal and thus are a significant problem for clinicians and in the drug development pathway. Furthermore, recent studies have reported that for a number of adverse drug reactions susceptibility is increased with possession of particular human leukocyte antigen, or HLA, alleles.

A number of β-lactam antibiotics were investigated throughout this thesis both with and without genetic associations. The molecular basis, and mechanisms involved in these immune-mediated adverse reactions are not fully understood. This work has been developed to elucidate the mechanisms and involvement of drug-specific T-cells in the pathogenesis of β-lactam hypersensitivity and drug-induced liver injury.

The immune system is one of the most complex systems within the human body comprising a wide range of cells and molecules with explicit roles in order to defend the host. This chapter introduces the main components that make up the immune system providing a general base knowledge of the pathways involved in keeping the human body healthy. As with any drug, before administration to patients the relative risk-benefit must always be considered. As a "foreign substance" there is a significant risk of the body treating drugs as a harmful entity and so, in some cases, the immune system will respond as such. Thus drug hypersensitivity reactions are seen. The clinical manifestations of drug hypersensitivity and the molecular pathways for T-cell activation by drugs are

detailed in this chapter. The chapter then progresses to factors that can influence the development of drug hypersensitivity with a particular focus on HLA associations. Finally, a brief introduction into the methods and β-lactam antibiotics used in hypersensitivity research, and specifically in this thesis, are detailed.

1.2 The immune system

The human body's self defence mechanism against various pathogens, viruses, bacterial infections and some chemical toxins is an intricate mix of cells and molecules that make up the immune system. Its job is to recognise and protect the host from harmful invading pathogens. Furthermore, discrimination between an individual's own cells and those that pose a potential threat is essential for the prevention of autoimmune disorders.

The nature of the immune response is usually divided into two categories – innate or adaptive. Innate immunity is characterised as an immediate yet non-specific response whereas adaptive immunity is a targeted, pathogen specific response.

1.2.1 Innate immunity

The innate immune system provides the first line of defence against invading microbes and pathogens that are likely to cause infection. As the early defence mechanism, innate immunity provides an initial and rapid generalised response.

The first barrier between the host and harmful microbes from the external environment is a physical one. The skin and epithelial linings of the

gastrointestinal and respiratory tracts are parts of the innate system, which are constantly active. The other components are ready and waiting to provide a quick response to an invading microbe.

The innate immune system is limited in the number of microbial products or pathogen associated molecular patterns (PAMPs) that it can recognise and therefore react to. Typically, it recognises structures that are either not present on mammalian cells or characteristic of microbial pathogens. Structures can include microbe specific nucleic acids or complex carbohydrates which are unique to microbes' for example LPS in gram negative bacteria. Pattern recognition receptors like the toll-like receptors bind such PAMPs (Abbas *et al.*, 2010). Essentially one of the defining features of the innate immune system is its ability to distinguish between self (mammalian) and non self (microbial) molecules (Janeway Jr *et al.*, 2002).

Innate immunity not only recognises microbes, it also helps eliminate injured host cells which may have become infected due to stress or trauma. Trauma which leads to tissue injury augments T-cell activity as injured host cells often express molecules and up regulate co-receptors that are not common in healthy cells. The innate immune system will therefore recognise the injured cell (Stoecklein *et al.*, 2012).

While providing the initial defence mechanism the innate immune system is also pivotal in stimulating the adaptive immune system. Molecules produced during the innate response can determine the nature of the adaptive response and thus both systems are interlinked.

1.2.2 Adaptive immunity

In contrast to the innate immune system, the adaptive immune responses are triggered by the specific recognition of antigens by lymphocytes. The adaptive immune system plays a major role in delayed drug hypersensitivity, and is discussed in further detail later in this chapter.

T- and B-lymphocytes are the major effector cells of the adaptive immune system which recognise invading pathogens and in turn mount an immune response. Receptors on these cells form antigen binding sites and their great diversity allow such a vast range of antigens to be determined. Naive T- and B-cells reside in secondary lymphoid tissues such as the spleen and lymph nodes. Dendritic cells migrate to these tissues and present captured antigens to the adaptive immune cells which are recognised via T- and B-cell receptors. This leads to clonal expansion and thus a large pool of antigen-specific T- or B-cells. Some of these cells can remain dormant and circulate for a lifetime giving rise to immunological memory permitting a rapid response following a second exposure to the same antigen (Parkin *et al.*, 2001). This is the basis of adaptive immunity and indeed of vaccinations.

Innate and adaptive immunity involve both cellular components and humoral immunity. Humoral immunity is distinct due to the involvement of antigen-specific antibodies formed through the activation of B-cells and their subsequent maturation and expansion of antibody-secreting plasma cells and memory cells (LeBien *et al.*, 2008). A cellular response, as the name indicates, involves a range of cell-mediated responses with phagocytes, antigen-specific T-cells and the release of specific cytokines in addition to a range of other components all involved. Antibodies are not involved in a cell-mediated

response however certain components do overlap between humoral and cellular responses. For example, the secretion of specific cytokines from T-cells which induce Ig isotype switching (Parkin *et al.*, 2001).

I have briefly mentioned just a few of the cells involved in the immune system. However, these are only a subset of the vast array of cells and tissues involved and the next sections gives greater detail of such components.

1.3 Cells and tissues of the immune system

Leukocytes (immune cells) are derived from hematopoietic stem cells in the bone marrow. Here, depending on a variety of stimuli, the appropriate progenitor cells will give rise to all the different cell types of the immune system.

1.3.1 Phagocytic cells

A major component of the innate immune system, the phagocytes main function is to identify and remove microbes. This is achieved by engulfing the pathogen, i.e. bacteria, and destroying the ingested pathogen. Phagocytes can be split into two major lineages: mononuclear phagocytes or polymorphonuclear granulocytes.

Monocytes, a mononuclear phagocyte, are derived from common myeloid progenitor cells and circulate in the peripheral blood (Radtke *et al.*, 2013). These can then migrate through blood vessel walls into various tissues and organs throughout the body. Here, monocytes mature into macrophages, with the location and thus function of the macrophage determining its nomenclature. For example, macrophages that reside in the central nervous system are called

microglial cells and those located in the liver are known as Kupffer cells. Monocytes/macrophages possess many lysosomes that contain enzymes, which can digest and break down invading bacteria. They can live for months and are widespread throughout the body (Male *et al.*, 2007).

Neutrophils, polymorphonuclear granulocytes, are short lived, however they comprise the majority of circulating leukocytes and 95% of circulating granulocytes. They possess acidic lysosomes and intracellular granules which contain a variety of specific, often toxic, materials to destroy microbes including acidic hydrolases, myeloperoxidase and lactoferrin(Male *et al.*, 2007). Vacuoles which contain the ingested microbe (phagosome) fuse together with the lysosomes to become phaglysosomes whereupon the killing takes place. Neutrophils can also release their granules and cytotoxic molecules extracellularly and thus recruit certain cell types to the site of tissue damage. Chemokines such as interleukin-8 (IL-8) promote neutrophil migration to specific sites (Mitsuyama *et al.*, 1994). Phagocytes also kill ingested microbes through a process called an oxidative burst where we see the formation of reactive oxygen species (ROS) and nitric oxide (NO) which can damage biomolecules and enzymes (Slauch, 2011).

Mast cells, basophils and eosinophils comprise the remaining 5% of circulating granulocytes. Both basophils and mast cells carry histamine, an inflammatory mediator. Degranulation, where such a mediator is released, is often stimulated by the presence of an allergen or parasite. Both cell types also have numerous high affinity Fc receptors for IgE and cross linking of these molecules can cause activation and degranulation. Eosinophils hold granules containing major basic protein, which can phagocytose and kill microbes directly. They also produce

inflammatory mediators such as IL-2, IFN-γ, IL-4, IL-5, IL-10 and can synthesise leukotrienes and prostaglandins (Rothenberg *et al.*, 2006).

Dendritic cells (DCs) are another mononuclear phagocyte with a distinct shape. They have long protruding dendrites, which assist in engulfing microbes. DCs are a crucial link between the innate and adaptive immune responses due to their role as professional antigen presenting cells (APCs). They shall be discussed in this context later in section 1.4.

1.3.2 Lymphocytes

Lymphocytes are one of the major cell types involved in, and responsible for adaptive immune responses. T and B lymphocytes (T and B cells) comprise the majority of lymphocytes. An antigen is any substance that can be targeted by receptors and which elicit an immune response. The epitope, or antigenic determinant is the part of that antigen which is recognised by those receptors by either T- or B-cells or even antibodies. They are capable of recognising antigenic determinants, and it is this specificity and memory which makes the system adaptive. All lymphocytes are derived from haematopoietic stem cells in the bone marrow (Male *et al.*, 2007). From there, a number of distinct subsets of lymphocytes are generated with differing functions. T-cells mature and develop completely in the thymus before entering the circulation whereas B-cells partially mature in the bone marrow.

1.3.3 B-lymphocytes

B-cells play a critical role in humoral immunity due to their main function: the production and secretion of antibodies. Membrane bound antibodies on the surface of B-cells are known as immunoglobulins (Ig) and act as antigen

receptors. Following antigen exposure, combined with a second signal usually from an activated T-cell, B-cells can also be induced to proliferate and differentiate into antibody secreting plasma cells. Secreted antibodies reside in the circulation and populate the interstitial fluid part of tissues and also mucosal secretions. These antibodies halt the harmful effect of pathogens in numerous ways. Through binding directly to pathogens, a term called neutralization; they can halt toxic effects, inhibit viral components and thus halt infections (Abbas *et al.*, 2010). Furthermore, antibodies can activate the complement system which consists of distinct plasma proteins that react with each other and coat the pathogen. This system can lead easier identification for phagocytes, also known as opsonisation, enhancing the immune response. This can directly kill bacteria thus aiding phagocytosis.

Cytokines from helper T-cells can induce Ig isotype switching. Naive and plasma B-cells express lower affinity IgD and IgM whereas mature, memory B-cells express the secondary isotypes IgA, IgE and IgG. These long lasting memory B-cells circulate in a resting state, ready to respond rapidly when they encounter antigen (Parkin *et al.*, 2001).

1.3.4 T-lymphocytes

T-cells, while involved in the humoral immune response, are also major players in the cellular immune response. They are distinguished through the variety of T-cell antigen receptors (TCRs) of which there are two well defined types: either α and β, or γ and δ. Both types of receptors are associated with the CD3 complex and thus expression of CD3 can identify T-cells *in vitro*. The vast majority of blood T-cells, up to 95%, are αβ T-cells with the remainder

consisting of γδ T-cells and natural killer cells (or NKT cells) (Abbas *et al.*, 2010).

Each T-cell will only express one subset of TCR, which in turn recognises a small proportion of an antigen, or peptide, which is presented by the major histocompatability complex (MHC). It is therefore the TCR that determines the antigen specificity. Following recognition of antigenic peptide, the specific T-cells bearing the appropriate TCR will proliferate and differentiate giving rise to a large population of effector T-cells (Teff); thus, resulting in the specific immune response. T-cell selection begins in the thymic cortex where cells co-express CD4 and CD8 giving rise to a double positive CD4+ CD8+ thymocyte population which will carry specific TCRs (Bonilla *et al.*, 2010). Thymocytes that do not show affinity for the MHC-peptide complex will ultimately die of neglect via apoptotic mechanisms (Starr *et al.*, 2003). Those thymocytes that recognise the MHC-peptide complex are positively selected, migrate to the thymic medulla and as a consequence lose the co-expression of CD4 and CD8. Depending on the complex recognised (via MHC class I or II) the double positive T-cells are predetermined to become either CD4+ or CD8+ single positive T-cells (Romagnani, 2006) (Germain, 2002).

The effector function of T-cell subsets are often generalised with CD4+ T-cells considered as helper cells and CD8+ T-cells cytotoxic however the reality is not quite so clear cut with some CD4+ T-cells also possessing cytotoxic function (Marshall *et al.*, 2011).

CD4+ T-cells have a central role in the adaptive immune response chiefly in cell mediated immunity and are activated by antigen presenting cells (APCs), which

present peptides to the TCR via MHC class II. There are two particularly well defined subsets of CD4+ T-cell, T-helper cells Th1 and Th2. Understanding of the two distinct populations first came about through identification and analysis of CD4+ T-cells in mice by Mosmann and Coffman and they termed them Th1 and Th2 cells (Mosmann *et al.*, 1986; Zhu *et al.*, 2010). Naive, or Th0 cells, will differentiate into either Th1 or Th2 cells following activation through antigen exposure. The subsequent presence of particular stimuli will go on to stimulate Th1/Th2 differentiation. Interleukin-12 (IL-12) and interferon-γ (IFN-γ) are critical cytokines for the development of Th1 cells and the presence of IL-4 and IL-2 is important in the downstream cascade for the production of Th2 cells (Luckheeram *et al.*, 2012). It is also important to note that often Th1 stimulating cytokines will be inhibitory for Th2 cell differentiation and vice-versa. For example IL-4 will inhibit Th1 cell development and IFN-γ is inhibitory for Th2 cells and thus halts a humoral immune response. Distinct immune responses will therefore arise depending on the responsible pathogen.

Not only does the cytokine environment influence CD4+ T-cell differentiation, the subtypes also have specific cytokine secretion profiles, which distinguishes their function and can aid in their classification. Th1 cells mainly secrete the pro-inflammatory cytokine IFN-γ and IL-2, which promotes CD8+ T-cell proliferation (Zhu *et al.*, 2010). Th1 cells and their cytokines promote cell mediated immunity through the recognition of microbial antigens. IFN-γ activates macrophages for enhanced microbial killing and stimulates IgG production by B-cells thus inducing opsonisation (Abbas *et al.*, 2010; Luckheeram *et al.*, 2012). Th2 cells mediate the adaptive immune response to infection through a humoral immune response. IL-4, IL-5 and IL-13 are the

primary Th2 cytokines. IL-4 and IL-13 stimulate the production of specific IgE antibodies by B-cells. IgE can thereby form cross links with Fcε receptors on mast cells and basophils which induces the secretion and production of active mediators like histamine and further cytokines including tumour necrosis factor (TNF-α) (Murphy, 2008) (Abbas *et al.*, 2010). IL-5 activates eosinophils. They release granular contents that are able to aid in the destruction of the invading extracellular parasite(Zhu *et al.*, 2008).

Naive CD8+ T-cells are activated by APCs which present peptides to the TCR via MHC class I. This antigen-dependant stimulation leads to the clonal expansion and migration of differentiated cytotoxic T lymphocytes (CTLs) into tissues. These are the effector CD8+ T-cells and are particularly important in the defence against viruses. CTLs will specifically kill target cells displaying peptides from cytosolic pathogens that are presented via the MHC class I molecule. Cytotoxic molecules including granzyme-B, perforin and granulysin are characteristically produced via CTL as well as IFN-γ (Belz *et al.*, 2010).

CTLs, and effector CD4+ T-cells, are generally short lived but a small portion give rise to memory T-cells, which can reside for a lifetime. Following re-exposure to the offending antigen, memory T-cells (derived from both CD4+ and CD8+ T-cells) can respond rapidly, inducing the quick and strong proliferation of effector T-cells. Memory T-cells can be further subdivided into central or effector memory T-cells; Tcm or Tem. Generally CD4 give rise to Tcm CD8 to Tem. Tcm cells home to lymph nodes and express L-selectin and CCR7. They can secrete IL-2 but following proliferation will differentiate to effector T-cells for

an enhanced immune response. Tem in contrast have an enhanced intrinsic effector function producing IFN-γ, IL-4 and IL-5 in a matter of hours following antigen recognition (Sallusto *et al.*, 2004).

Regulatory T-cells, Tregs, suppress lymphocyte activity to help control immune responses. In vitro they can be identified through their expression of CD4 and CD25 and also the transcription factor FoxP3, the classical marker for Tregs (Vignali *et al.*, 2008).

Recently a new Th cell lineage has been discovered, Th17, which does not produce the classical Th1/Th2 cytokines. They characteristically produce the pro-inflammatory cytokines IL-17, IL-21 and IL-22 and are thought to be involved in a variety of immune mediated diseases such as rheumatoid arthritis and multiple sclerosis (Waite *et al.*, 2012).

NKT cells comprise a small portion (15%) of lymphocytes and have a large, granular morphology. They respond quickly to eliminate infected cells, particularly from viruses, and also tumour cells via the secretion of perforin and granzyme B. NK cells do not require activation to kill target cells and instead rely upon the expression of a variety of inhibitory and activating receptors. Inhibitory receptors such as the killer cell Ig-like receptor (KIR) recognise different HLA-A, -B and –C alleles on MHC class I molecules which are expressed on healthy cells. Activating receptors such as Dap-12 and NKG2D recognise ligands on cells that are stressed or infected. The combination of receptors will therefore help determine the activation state of the NK cell. In addition,

activators such as type I IFN, IL-12, IL-18, IL-15 and IL-2 contribute to cell effector function, proliferation and cytotoxicity (Vivier *et al.*, 2008).

1.3.5 Antigen Presenting Cells (APCs)

Antigen presenting cells (APCs) are the link between the innate and adaptive immune system. These cells capture, process and present antigens to lymphocytes to initiate the adaptive T-cell response. Dendritic cells (DCs) are the major professional APCs though macrophages and B-cells also possess APC functions in cell mediated and humoral responses respectively. Langerhans cells, a specialised subset of DCs are resident in the skin.

DCs are derived from precursor cells in the bone marrow whereby they migrate to the periphery and reside as immature DCs (iDCs) in the epithelium awaiting potential pathogens. The spherical morphology of iDCs aid in efficient antigen capture and phagocytosis. Following activation by pathogens, a mature phenotype will develop, which is essential for the initiation of T-cell responses. In contrast to iDCs, mature DCs (mDCs) main function is to presents antigens to the TCR. During maturation large cytoplasmic protrusions or dendrites are formed. Furthermore, there is a reduced endocytic capacity, an increased expression of the costimulatory molecule CCR7 (aids migration to secondary lymphoid organs), increased expression of MHC class II molecules for antigen presentation and also IL-12 secretion, which aids Th1 development (Kapsenberg, 2003). DC maturation is further triggered through contact with PAMPs via toll-like receptors and through the release of damage-associated molecular patterns (DAMPs) i.e. heat shock proteins, HMGB1 and uric acid (Shi *et al.*, 2003). Table 2.1 details the toll-like receptors, the PAMPs that bind them

and the DAMPs associated. Drugs can affect DC maturation and antigen presentation. For example, amoxicillin increases the expression of the maturation markers HLA-DR, CD80 and CD86 on DCs from hypersensitive patients (Rodriguez-Pena *et al.*, 2006).

TLR	PAMP	DAMP
TLR1	Bacterial triacylated lipopeptides	Human β-defensins
TLR2	Bacterial lipoproteins	Heat shock proteins (HSP)
	Peptidoglycan	HMGB1 (high mobility group box 1)
	Lipoteichoic acid	
TLR3	Viral double stranded RNA	Self double stranded RNA
	polyinosinic-polycytidylic acid (poly IC)	MRNA, HSP, HMGB1, hyaluronan fragments
TLR4	Lipopolysaccharide (LPS), bacterial (Gram negative bacteria)	HMGB1
	Mannans fungi	Fibrinonectine
	Glycoinositolphospholipides parasites	A surfactant
	Envelope proteins of the virus	Lipoproteins
	HSP	HSP
TLR5	Flagellin flagellated bacteria	
TLR6	Lipoproteins mycoplasma	Heat shock proteins (HSP)
	Lipoteichoic acid	HMGB1
	Zymozan of *Saccharomyces cerevisiae*	
TLR7&8	Viral single stranded RNA	Self single-stranded RNA
TLR9	Bacterial and viral unmethylated CpG DNA	Self DNA
TLR10	Unknown	Unknown
TLR11	Profilin	

Table 1.1 *Toll-like receptors and their associated PAMPs and DAMPs*
Table is adapted from Essakalli *et al* and Abbas *et al.* (Abbas *et al.*, 2010; Essakalli *et al.*, 2009)
TLR = toll-like receptor, PAMPs = pathogen associated molecular patterns, DAMPs = damage-associated molecular patterns

1.4 Immune Activation

1.4.1 Antigen presentation

The presentation of antigens by APCs to T-cells is the bridge between innate and adaptive immunity. As mentioned earlier, antigens are recognised by T-cells via specific TCRs through the context of an MHC molecule. These are cell surface molecules encoded by HLA (human leukocyte antigen) genes on chromosome 6. There are two distinct pathways in which APCs process and present antigens to the T-cell. The route followed is dependent on whether the antigen is of an intracellular or extracellular origin.

Intracellular antigens are often formed due to a viral infection. The virus attaches to the host membrane and injects its DNA or RNA into the cells. The virus then produces and envelopes itself in a similar membrane to the host cell, allowing it to fuse and enter the cell through endocytosis. Once entered, the virus replicates and protein products are released into the cell cytosol. These proteins are degraded by a nuclear or cytosolic proteasome whereby short chains of amino acids, or peptides, are produced. The peptides (8-10 amino acids in length) are transported in to the lumen of the endoplasmic reticulum (ER) via transporter associated with antigen processing (TAP) and loaded onto the MHC I molecule. The peptide-MHC complex can then be released and is transported in vesicles to the plasma membrane via the golgi (Pamer *et al.*, 1998). Now present on the cell surface, intracellular derived peptide-MHC class I complex can be recognised by TCRs on CD8+ T-cells (Figure 1.1).

Extracellular antigens can only be processed by specialised APCs such as DCs, B-cells or macrophages due to the need for phagocytosis. Once internalised and contained within an endosome, the pathogen is digested by enzymes into peptide fragments (Neefjes *et al.*, 2013). In parallel, MHC II molecules are transported from the ER in membrane enclosed vesicles. The two are then able to fuse, which allows the peptide to be loaded onto the MHC binding groove. The peptide-MHC complex then translocates to the cell surface allowing recognition by TCRs on CD4+ T-cells and thus initiating immune response (Figure 1.1).

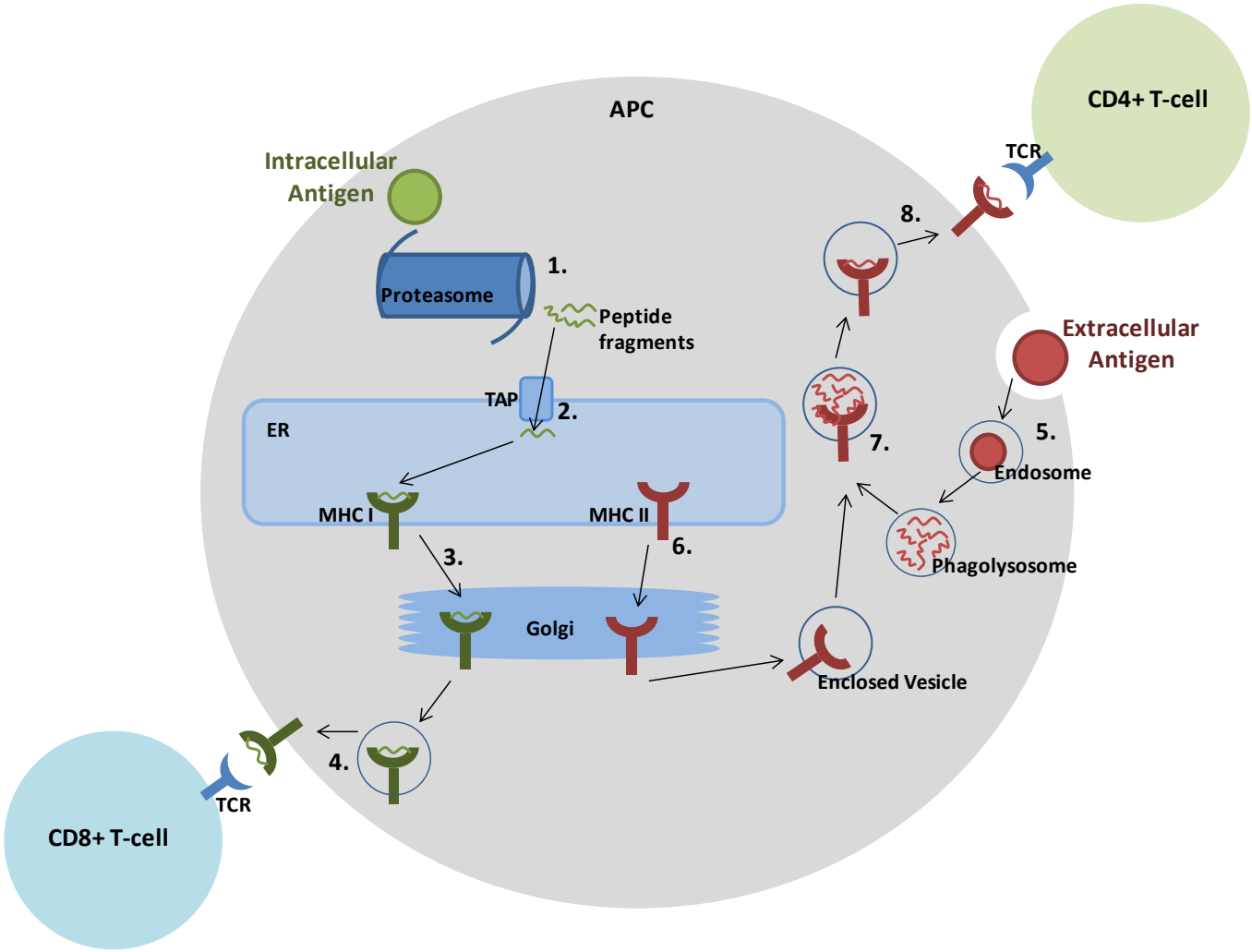


Figure 1.1 *Antigen processing and presentation*.
Intracellular antigens are degraded by proteasomes (1) into peptide fragments and transported via TAP to the ER (2) where they are loaded on to MHC I. The peptide-MHC complex is trafficked to the golgi (3), loaded onto vesicles and displayed on the cell surface (4) allowing recognition by the TCR.
Extracellular antigens are internalised within an endosome and digested into peptide fragments (5). MHC II molecules meanwhile are transported (6) allowing formation of the peptide-MHC complex (7). This complex then translocates to the cell surface (8) displaying the antigen for TCR recognition.

1.4.2 TCR activation

Following recognition of the peptide-MHC complex, a signalling cascade is initiated which ultimately results in the proliferation and differentiation of T cells and the release of specific cytokines. Binding of the peptide-MHC complex to the TCR is the first signal (signal 1) but in itself is not sufficient to initiate an

immune response. Co-stimulatory molecules are involved in the second, signal 2, which promotes the survival and differentiation of the T-cells. The best characterised pathway involves the B7 molecules, B7-1 and B7-2 also known as CD80 and CD86 respectively, expressed on APCs and the T-cell receptors CD28 and CTLA-4 (Murphy, 2008). Engagement between the co-stimulatory receptors can either be stimulatory (CD28) or inhibitory (CTLA-4) ensuring a balanced and appropriate immune response; positive co-stimulatory signals will promote activation while T-cell tolerance will be induced through a negative second signal or the absence of co-stimulation.

Constitutively expressed on the cell surface, CD28 will supplement and sustain T-cell responses and survival allowing cytokines to initiate the clonal expansion or growth and differentiation of T-cells. The co-stimulatory receptor signal transduction enhances T-cell survival through the up regulation of the anti-apoptotic protein Bcl-xl, aids IL-2 secretion and lowers the T-cell activation threshold, which prevents antigen-engaged cells from entering a state of anergy (Acuto *et al.*, 2003). Following T-cell activation CTLA-4 expression is up regulated and engagement with CD80/CD86 will result in negative regulation of the T-cell immune response through inhibition of CD-28 mediated signalling (Sharpe *et al.*, 2002).

Further co-stimulatory molecules include CD40 and its accessory molecule, CD40 ligand, CD27 and CD70 and the integrin LFA-1, which play a role maintaining T-cell proliferation and TCR interaction (Bachmann *et al.*, 1997; Murphy, 2008). Once activated, the secretion of specific cytokines is critical in the differentiation from naive CD4/CD8 to mature effector T-cells, which will no longer require, or need as much, co-stimulation to exert their immune effects.

T-cell activation in turn will trigger further signalling cascades involved in the development of the immune response. This includes the phosphorylation of immunoreceptor tyrosine based activation motifs (ITAMs) by the Src kinases Lck and Fyn (Brownlie *et al.*, 2013). Further downstream this can go on to activate MAPK pathways thus activating enzymes (e.g. ERK, JNK), enhancing production of transcription factors (e.g. NFAT, STAT3) and facilitating the release of signalling molecules (Ca^{2+} and DAG) (Smith-Garvin *et al.*, 2009).

The above summary of the immune system, its functions and the components involved provides a basic insight into this complex network of events that help to protect against infection. The immune system is the human bodies' in built safety system to help combat potential threats. Unfortunately it can sometimes mistake drugs, which are given to help a patient, as harmful. Indeed, the medicine given to patients as treatment can, on occasion, contribute additional problems rather than providing relief.

1.5 Adverse drug reactions

An adverse drug reaction, or ADR, is defined as "an unwanted or harmful reaction following the administration of a drug or combination of drugs under normal conditions of use, which is suspected to be related to the drug" (MHRA, 2014a).

ADRs are a major clinical problem. A prospective study here in the UK, carried out in 2004, looked at over 18,000 patients and found approximately 6.5% of hospital admissions were due to an ADR (Pirmohamed *et al.*, 2004). These data are consistent with another meta-analysis of prospective studies that attributed

the incidence of ADRs as 6.7% (Lazarou *et al.*, 1998) as is the data regarding the distribution of age/sex (Martin *et al.*, 1998). Most patients recovered from their ADRs; however 2.3% of the reactions were classified as being directly responsible for fatality (Pirmohamed *et al.*, 2004). Therefore, when applying the results to the cause of death amongst all hospital patients, ADRs are responsible for up to 0.2% (Lazarou *et al.*, 1998; Pirmohamed *et al.*, 2004). Lazarou *et al.* therefore propose that ADRs are between the fourth and sixth leading cause of death in the USA.

Not only are ADRs a significant health issue in terms of patient safety they can have severe economic consequences. It is estimated that ADRs cost the NHS in the UK £466 million each year (Pirmohamed *et al.*, 2004).

Furthermore ADRs represent a serious obstacle in the development of new therapies and medicines. 10% of all new drugs between 1975 and 1999 acquired black box warnings or were withdrawn, with 2.9% of new chemical entities ultimately being removed from the market. Half of the withdrawals transpired within two years drug approval (Lasser *et al.*, 2002). Although stringent pre-clinical/clinical testing strategies are in place to limit the chance of safety concerns arising after drugs enter the market, as the figures show, unanticipated ADRs still arise. In these cases, pharmaceutical companies lose significant amounts of money following withdrawal of a compound. The cost of bringing a new drug to market is estimated to be between $500 million and $2000 million, a significant investment (Adams *et al.*, 2006).

1.5.1 Classification of ADRs

Due to the wide array of drugs that have been reported to cause adverse reactions, and the form of reactions that occur a classification system has been developed to describe the effects seen. Generally, ADRs are grouped into two major categories type A (on-target, augmented) and type B (off-target, bizarre) reactions (Park *et al.*, 1998).

Type A reactions, the most common ADR, are dose dependent and a result of the exaggeration of a drugs' pharmacological effect. Drug metabolism, transporters and the effects of co-administered treatments can all contribute to the development of Type A reactions. This type of reaction can be controlled by an adjustment of the dose and careful monitoring. For example, hypotension that arises from the use of anti-hypertensive drugs can be resolved through dose reduction.

Type B reactions are less frequent, affecting a small subset of individuals. However, due to their unpredictable and often severe nature, they are a leading cause of drug withdrawal. Type B drug reactions are thus often termed as idiosyncratic. Effects are not related to the drugs' pharmacological action and so it can be difficult to determine the underlying mechanisms involved. Therefore, complete removal of the offending drug is often required. Examples include the development of Stevens-Johnson syndrome, a severe skin reaction that develops when patients are exposed to several oral drug classes including the aromatic anticonvulsants.

Less common ADRs may also occur in a small number of individuals whereby the above classifications are not appropriate (Edwards *et al.*, 2000):

Type C, chronic, reactions arise with the use of a drug for a prolonged period of time e.g. iatrogenic Cushing's syndrome due to high glucocorticoid levels following prednisolone treatment.

Type D, delayed, reactions can occur many years after drug treatment had ceased e.g. the development of tumours following treatment with alkylating agents. Type D reactions can also be used to describe the adverse effects of teratogenic drugs on the children of mothers taking these drugs while pregnant. Thalidomide is one such drug associated with birth defects.

Type E, end of use, reactions occur after drug withdrawal e.g. the sudden cessation of β-blockers can result in angina and following termination of phenytoin (anticonvulsant) withdrawal seizures can occur. Re-introducing the drug and gradually withdrawing it can avoid this type of reaction.

A greater understanding of the mechanisms involved the development of ADRs is essential. By noticing potential risk areas early on and with improved drug screening we can hope to reduce the number of unsafe drugs that reach the market.

1.5.2 Drug-induced liver injury (DILI)

With incidences between 1 in 10,000 and 1 in 100,000 new drug exposures, drug-induced liver injury (DILI) is a rare but serious health condition, associated with high morbidity and mortality (Holt *et al.*, 2006). Due to its low frequency this idiosyncratic adverse drug reaction is often missed in development and trials of new drug treatments. Adverse effects only become apparent after its distribution to the wider population, meaning DILI is the most

common cause of drug attrition, withdrawal and post-marketing regulatory action (Watkins, 2005). The lack of pre-clinical predictive and diagnostic tools or suitable animal models makes it difficult to identify hepatotoxic drugs during the developmental phases of new chemical entities (Kaplowitz, 2005). Measurements of serum liver enzymes such as alanine aminotransferases (ALTs), which are released from dying hepatocytes, are taken during clinical trials. Mild elevations, 2-5 x the upper limit of normal (ULN) requires more frequent monitoring with severe elevations of 10 x ULN necessitating the immediate withdrawal of the drug and termination of the trial (Abboud *et al.*, 2007). High bilirubin levels indicate the metabolism of haem has been compromised and so levels of bilirubin are also measured alongside ALTs which together help diagnose hepatocellular injury (Antoine *et al.*, 2008). The combination of jaundice (bilirubin at over 2 x ULN) and hepatocellular injury (ALT levels at more than 3 x ULN) is the foundation of Hy's law. Hy's law cases have a 10% - 50% mortality rate (Björnsson, 2006).

Though a panel of liver tests are conducted, diagnosis of DILI is complicated. It can be difficult to determine if the drug in question is actually responsible, a term known as "guilt by association" and so other aetiologies such as underlying disease states must be correctly excluded. The use of certain hallmarks of DILI can help in diagnosis including the time for onset, a diminished reaction following removal of drug or indeed a severe and rapid reaction following drug rechallenge (Lee *et al.*, 2005). Confounding factors including polypharmacy, hepatitis infection, congestive heart failure and patients' medical history can all lead to incorrect diagnosis.

DILI diagnosis and research becomes even more complicated when you consider the involvement of the immune system. In recent years more and more evidence has come to light regarding the role of the immune system in the pathogenesis of idiosyncratic DILI (Uetrecht, 2013). Key components of the innate immune system including natural killer cells (NK cells), NK cells with T-cell receptors (NK T-cells) and kupffer cell (liver resident macrophages) are all enriched in the liver allowing immune mediation (Yano *et al.*, 2014). The adaptive immune system is also implicated as a promoter of DILI as many drugs including halothane, diclofenac and carbamazepine which are known to cause DILI, also result in hypersensitivity and immune reactions (Ju *et al.*, 2012). Furthermore, certain drugs associated with liver injury, including flucloxacillin and lumiracoxib have known HLA associations (Daly *et al.*, 2009a; Singer *et al.*, 2010). Several immune mediators have been implicated in the induction of hepatotoxicity via an excessive inflammatory response. TNF-α, IL-1β, high-mobility group box 1 (HMGB1) and IL-17 have all been suggested as mediators which induce hepatocyte cell death (Yano *et al.*, 2014; Zhu *et al.*, 2013). Though more and more information comes to light, mechanisms of DILI remain poorly understood and innovative research methods to study and understand DILI are required.

1.6 Drug hypersensitivity

ADRs that have an immunological aetiology are defined as drug hypersensitivity reactions. They account for up to a third of all ADRs. Also referred to as drug allergies, they affect between 10 and 20% of hospital patients and are also common within the general population affecting up to 7% (Gomes *et al.*, 2005).

Immune-mediated reactions to otherwise safe and effective drugs occur at therapeutic doses and are therefore sometimes referred to as Type B reactions. Since the immune system is involved in the pathogenesis and as they are only observed in a small percentage of the population, reactions are almost impossible to predict. However, scientific advances continue to enhance our understanding of these complex cases, and it is this area on which my thesis hopes to contribute knowledge.

Antibiotics, namely penicillins, are the most common causative drug in drug hypersensitivity accounting for almost 56% of cutaneous drug eruptions (Fiszenson-Albala *et al.*, 2003). However, a wide range of agents are implicated in the development of drug hypersensitivity (Table 1.2). Moreover, the range of clinical manifestation observed differ not only in severity but also with regards to the cytokine profiles and the lymphocytes involves. CD4+ T-cells are implicated in MPE and AGEP while CD8+ T-cells are known to play an important role in SJS and TEN (Nassif *et al.*, 2004; Pichler *et al.*, 2002).

DRESS	AGEP	SJS/TEN
Abacavir Allopurinol Carbamazepine Dapsone Lamotrigine Minocyclin Phenytoin Sulfamethoxazole Sulfasalazine Vancomycin	Aminopenicillins Cephalosporins Celexoxib Dilitiazem Macrolides Quinolones	Allopurinol B-lactams Carbamazepine Lamotrigine Nevirapine Oxicam NSAIDs Phenytoin Sulfonamides Tramadol

Table 1.2 *Drugs commonly associated with hypersensitivity reactions*

1.6.1 Clinical manifestations

Though drug hypersensitivity reactions can target a number of organs in the human body including the liver, kidney, heart and lungs the most commonly affected organ is the skin. This is unsurprising owing to its large surface area, the network of blood vessels involved and therefore the potential for the recruitment of vast numbers of immune cells.

1.6.2 MPE

The majority of cutaneous drug reactions present as maculopapular exanthema (MPE) accounting for up to 95% of drug-induced skin reactions (Bigby, 2001; Hunziker *et al.*, 1997). Reactions consist of diffuse cutaneous erythema and can range from mild rashes to more severe manifestations where pustules develop, with itching and fever also adding to the list of symptoms. CD4+ T-cells are the major cell type involved in the induction of MPE, alongside a variety of secreted molecules including granzyme B, perforin, IL-5 and eotaxin which are also known to mediate the development of the disease (Blanca *et al.*, 2009; Fernandez *et al.*, 2009). Furthermore, the cocktail of cytokines released promotes the recruitment of further cells, such as eosinophils, to the affected site (Yawalkar, 2005). Though reactions can be seen 1-2 days after the start of drug treatment, more commonly MPE is seen between 4 and 14 days following drug administration.

1.6.3 DRESS

DRESS, also known as drug hypersensitivity syndrome, affects skin and multiple internal organs including the lungs, kidney and most commonly the liver. The recruitment of eosinophils mediated by activated T-cells and subsequent IL-5

secretion is a major part of the pathophysiology of DRESS (Choquet-Kastylevsky *et al.*, 1998). Further symptoms include high fever and skin rash. The mortality rate is around 10%, however administration of corticosteroids when organ involvement is apparent can dramatically improve prognosis (Bocquet *et al.*, 1996). Reactivation of herpes virus 6 and 7 (HHV-6 and HHV-7) is often observed in DRESS, and measurements of increased specific IgG and viral DNA reactivation can be used as a tool for diagnosis (Kano *et al.*, 2004; Suzuki *et al.*, 1998). However, these are often not detected until 3 – 5 weeks after symptoms develop and so viral reactivation may occur as a consequence of T-cell activation (Shiohara *et al.*, 2006). Reactivation of further virus has also been implicated including the rise of immunoglobulins against Epstein-Barr virus (EBV) and cytomegalovirus (CMV) (Kano *et al.*, 2004; Picard *et al.*, 2010). Picard *et al*. found activated CD8+ T-cells in all DRESS patients and viral reactivation in around 76%. Furthermore, approximately half of the CD8+ T-cells involved responded to at least one EBV epitope. However, the role of drug and viral specific T-cells in the disease pathogenesis is still to be fully determined.

Anticonvulsants are more often than not the culprit drug involved in DRESS, in so much that anticonvulsant hypersensitivity syndrome (AHS) was originally used to describe DRESS symptoms.

Other drug classes including sulphonamides, allopurinol and anti-retroviral drug such as abacavir are also known to cause DRESS (Walsh *et al.*, 2013).

1.6.4 Acute generalised exanthematous pustulosis (AGEP)

An uncommon cutaneous reaction mediated by IL-8 secreting T-lymphocytes, AGEP is characterised by the vast number of very small, non follicular sterile

pustules that form mainly in the folds of the skin (Britschgi *et al.*, 2001; Sidoroff *et al.*, 2001). Fever and neutrophilia with/without eosinophilia are also seen. (Roujeau *et al.*, 1991). Antibiotics are the leading causative drug involved in AGEP, with aminopenicillins, macrolides, quinolones and sulphonamides all implicated (Sidoroff *et al.*, 2007). Without clinical intervention, mortality from AGEP is around 5% (Roujeau, 2005).

1.6.5 Fixed drug eruptions

Fixed drug eruptions (FDE) are a distinct type of cutaneous reaction where eruptions recur at the same site often following sensitization to a particular drug. Characteristically, after healing a residual hyperpigmentation can remain on the skin (Lee, 2000). An influx of intraepidermal CD8+ T-cells which reside within the lesions of FDE are thought to contribute to local tissue damage (Shiohara, 2009).

1.6.6 Stevens-Johnson syndrome/ toxic epidermal necrolysis (SJS/TEN)

SJS/TEN is the most severe cutaneous hypersensitivity reaction. Both are diseases (SJS and TEN) which affect the skin and mucous membrane causing blistering skin lesions with the separation of the epidermis from the dermis, accompanied with the death of keratinocytes (the predominant cell type in the epidermis). SJS and TEN are now commonly referred to as two parts or spectrums of the same disease with the extent of epidermal separation used as the major clinical diagnostic feature (Mockenhaupt, 2011). Skin detachment from the body's surface area at a rate of 10% or less is classed as SJS,

detachment of 10-30% is the overlap between SJS and TEN and so classed as SJS/TEN and over 30% is the most severe – TEN (Bastujigarin *et al.*, 1993).

The apoptotic death of keratinocytes in the epidermis is thought to eventually lead to necrosis and skin detachment, which is characteristically seen in patients (Paul *et al.*, 1996). NK T-cells and cytotoxic T-cells are implicated as the effector cells particularly as skin lesions contain high numbers of these cells (Chung *et al.*, 2008). Blister fluid is also found to contain up to 4 times the expression of granulysin compared to perforin, Fas ligand and granzyme-B. These recent findings suggesting granulysin is a key mediator for the death of keratinocytes and development of SJS and TEN (Chung *et al.*, 2008). Furthermore, granulysin may even be an important diagnostic tool as levels correlate with clinical severity (Gerull *et al.*, 2011).

SJS/TEN has high rates of mortality, between 20 and 25%, which may be due to the idiosyncratic nature of the disease, limited information regarding its pathogenesis and lack of treatment options (Mockenhaupt *et al.*, 2008). Fortunately cases of SJS/TEN are rare – up to 6 cases per million people per year for SJS and an incidence of up to 1.2 cases per million people per year for TEN (Roujeau *et al.*, 1994).

1.6.7 Time course

The time between initial drug exposure and development of clinical features of a reaction are important for the treatment and diagnosis. Many drug hypersensitivity reactions take between one and 11 days for clinical manifestations to appear and these reactions are known as delayed-type (Pichler *et al.*, 2010).

The activation of T-cells and the molecular changes that subsequently occur are a key feature of delayed type hypersensitivity and helps explain the lag in presentation of clinical manifestations. Moreover, the production of functional cytotoxic molecules including perforin and granzyme B can take up to 7 days following drug exposure and antigen stimulation (Ortiz *et al.*, 1997).

At the other end of the scale immediate hypersensitivity reactions, as the name suggests, occur immediately with symptoms generally manifesting within minutes (Gould *et al.*, 2003). In these reactions antigen cross links IgE antibodies with their high affinity Fc receptors on mast cells leading to the rapid release of cytotoxic molecules (Corry *et al.*, 1999).

With the vast range of clinical manifestations that can arise with hypersensitivity reactions, classification is essential to help separate the different clinical features seen and ultimately aid in the treatment and research of these adverse drug reactions.

1.6.8 Classification of immune reactions

In 1963 Gell and Coombs first categorised hypersensitivity reactions into four major groups, determined by the time to clinical manifestation and according to the type of immune response and the mechanisms involved. Type I, Type II and Type III reactions are antibody-mediated whilst Type IV reactions are T-cell mediated. Though this classification system is still relevant and widely used, more recently further sub divisions of Type IV reactions have been introduced (Pichler, 2003). Table 1.3 highlights the key features of each type of reaction.

Type I reactions are mostly immediate, IgE-mediated which result from the cross-linking of receptors on mast cells and basophils leading to the quick

release of histamine and leukotrienes. Broncho-constriction and anaphylaxis are common clinical manifestions.

Type II, or cytotoxic reactions are mediated by IgG and IgM antibodies which bind to cells. This leads on to binding of complement proteins and the rupture of cells.

Type III reactions are IgG or IgM mediated reactions which can occur due to the presence of high antigen concentrations in the circulation. Serum sickness and vasculitis are type III reactions.

Type IV reactions are mediated by T-cells and are mainly delayed-type hypersensitivity reactions. The wide range of delayed type IV reactions can be divided further into four classes depending on cell involvement; monocytes, eosinophils, cytotoxic T-cells and neutrophils are characteristically involved in Type IVa, IVb, IVc and IVd respectively. Many of the clinical symptoms of drug hypersensitivity detailed earlier including MPE, DRESS and SJS/TEN, are a result of a type IV reaction and it is these type IV delayed hypersensitivity reactions in which this thesis focuses on.

Although these immune reactions have been grouped it is important to note that, as is to be expected with such a complicated system involved, there is often crossovers between the different categories. This is especially true with regard to specific mediators that are released.

1.6.9 Sensitisation and Elicitation

The first exposure to an antigen is known as sensitisation. Here the antigen binds to proteins and is taken up by antigen presenting cells (APCs) and proceed to the draining lymph node. Antigen derived peptides are presented to

CD4+ and CD8+ T-cells which then enter the lymphatic system and general circulation. This in effect is the priming of naive T-cells. When the body is re-introduced to the same antigen, again protein binding occurs yet now antigen specific CD4+ and CD8+ T-cells from the draining lymph node travel to the site where the antigen is bound (Weber *et al.*, 2015). This is the elicitation phase where responses, including T-cell mediated tissue damage, are seen quickly. While priming of cells is seen across the board in hypersensitivity, this mechanism of sensitization and elicitation has been studied extensively and is characteristic of contact hypersensitivity (an inflammatory skin disease triggered by repeated exposure to allergens).

Gell and Coombs classification	Type of immune response	Pathological characteristics	Clinical symptoms	Drug binding	Cell type
Type I (anaphylactic)	IgE	Mast-cell degranulation	Uticaria and anaphylaxis	Covalent	B-cells/Ig
Type II (cytotoxic)	IgG and FcR	FcR dependent cell destruction	Blood cell dyscrasia	Covalent	B-cells/Ig
Type III (immune complex)	IgG and complement or FcR	Immunocomplex deposition	Vasculitis	Covalent	B-cells/Ig
Type IVa (delayed type)	Th1 (IFN-γ)	Monocyte activation	Eczema	Covalent/non-covalent	T-cells
Type IVb (delayed type)	Th2 (IL-5, IL-4)	Eosinophilic inflammation	MPE, bullous exanthema	Covalent/non-covalent	T-cells
Type IVc (delayed type)	CTL (perforin, granzyme B)	CD4+/CD8+ cytotoxicity	MPE, eczema, bullous exanthema, pustular exanthema	Covalent/non-covalent	T-cells
Type IVd (delayed type)	T-cells (IL-8)	Neutrophil recruitment and activation	Pustular exanthema	Covalent/non-covalent	T-cells

Ig – immunoglobulin, FcR – Fc receptor, CTL – cytotoxic T-lymphocytes
Adapted from Pichler, 2003

Table 1.3 *Classification of immune mediated drug reactions.*

1.7 T-cell activation by drugs

Traditionally, peptides are main activators of a cellular immune response, combating potential threats to the host. Unfortunately, small molecules i.e. drugs, can also activate the immune system to a detrimental effect as seen in drug hypersensitivity. In this thesis I shall be focussing on delayed, Type IV hypersensitivity reactions in the hope of furthering the understanding of this adverse reaction.

In order to describe the molecular mechanisms and immunological basis of drug-specific activation of T-cells, a number of specific terms are used (Uetrecht, 2013);

Hapten – these are any low molecular weight compounds that are able to bind and modify macromolecules such as proteins in an irreversible manner. Binding almost always involves the formation of a covalent bond between the compound and protein.

Antigen – a molecule that is able to bind with immunological receptors.

Immunogen – these are molecules that are able to trigger an immune response.

Co-stimulatory molecule – these substances interact with DCs to stimulate or polarise an immune response.

1.7.1 Drug protein binding

Owing to their low molecular weight and small size, drugs are not likely to act as traditional antigens. Instead it is suggested they bind to host proteins forming a hapten which becomes immunogenic. The work of Landsteiner and Jacobs who first described drug-protein binding and the formation of haptens,

has since been expanded significantly with detailed information now available for the binding sites of a number of drugs.

It is now well known that the penicillin class of β-lactam antibiotics typically become immunogenic following covalent, non reversible, binding to a carrier protein. As the most abundant serum protein, human serum albumin (HSA) is commonly used to characterise drug-protein binding. The work of Levine et al established that the formation of a stable, covalent bond between penicillin and lysine residues on proteins is an essential step to sensitisation, furthermore they found that the benzylpenicilloyl group is the major haptenic determinant (Levine *et al.*, 1961b; Levine *et al.*, 1969). In recent years advanced mass spectrometers coupled with an improvement in methods, techniques and expertise, particularly here at the University of Liverpool, has allowed the relationship between the chemistry of drug-protein binding not only to be characterised but for this to be related back to stimulation of drug-specific T-cells. Indeed HSA binding has been characterised for a number of β-lactam antibiotics. Benzylpenicillin binds to 14 lysine residues whilst 18 penicilloylated lysine residues were dectected with the rearrangement product benzylpenicillic acid with selectivity at positions 199 and 525 respectively (Meng *et al.*, 2011a). Flucloxacillin-HSA binding has also been characterised with modified lysine residues detected, furthermore modification has been shown *in vivo* (Jenkins *et al.*, 2009a). The sulphonamide, sulfamethoxazole (SMX) forms a nitroso metabolite SMX-NO following oxidative metabolism which is able to bind and modify intracellular proteins. Callan et al were able to chemically define SMX-NO modification with HSA, glutathione *S*-transferase π and a synthetic peptide (DS3) using mass spectrometry. Selective binding occurred at cysteine residue

47 with glutathione S-transferase π and with HSA at Cys34 which formed only the *N*-hydroxysulfinamide adduct (Callan *et al.*, 2009). A stable sulfenic acid form of Cys34 in HSA exists *in vivo*, however the biological consequences of this or the modifications described above have not yet been defined. Abacavir research has finely defined the great selectivity of abacavir to HLA-B*57:01, with the interaction of abacavir with MHC mapped to the antigen-binding F pocket and the importance of serine at position 116 and aspartate at position 114 highlighted for drug antigen presentation (Chessman *et al.*, 2008). A change in these specific residues was able to alter structure of the F pocket in such a way as to change the repertoire of peptides bound to HLA-B*57:01. The formation of haemoglobin-abacavir aldehyde (ABCA) adducts in patients however suggest that parallel hapten mechanism may be involved in the activation of T-cells. Using mass spectrometric methods ABCA modifications of HSA have beed defined in patients taking abacavir. Modifications were not exclusive to one amino acid; adducts were detected on Lys159, Lys190, His146 and Cys34.

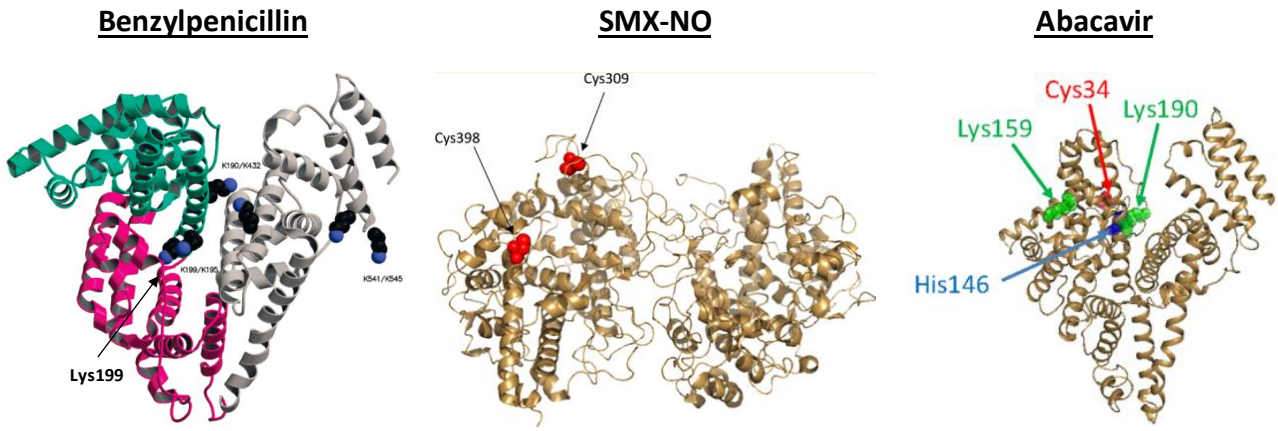


Figure 1.2 *HSA ribbon structures with sites of modification*
Model HSA structures highlighting amino acid modification sites for benzylpenicillin, sulfamethoxazole and abacavir *in vitro*. Structures taken from (Diaz *et al.*, 2001), (Callan *et al.*, 2009) and (Meng *et al.*, 2014).

Antigenicity and in turn immunogenicity is integral for the initiation of an immune response

A number of mechanisms have been proposed by which T-cells are activated following interactions with small molecules/drugs. These are outlined below.

1.7.2 Hapten hypothesis

Early research in the 1930s provided the basis for the hapten hypothesis. Landsteiner and Jacobs were looking to understand the mechanisms by which small molecules activate T-cells. They were able to sensitise guinea pigs to the low molecular weight, chemically reactive compound, dinitrochlorobenzene (DNCB). They went on to report protein modification and formation of conjugated antigens in the animal leading to the hypothesis that protein haptenation by DNCB may play an important role in the immune response observed (Landsteiner *et al.*, 1935; Landsteiner *et al.*, 1936).

Owing to the small size of low molecular weight compounds such as drugs it is unlikely they are able to act as antigens directly. These compounds or their metabolites might therefore act as haptens, inducing immune responses through the covalent binding of proteins in the host. The hapten hypothesis is outlined in a schematic in Figure 1.3.

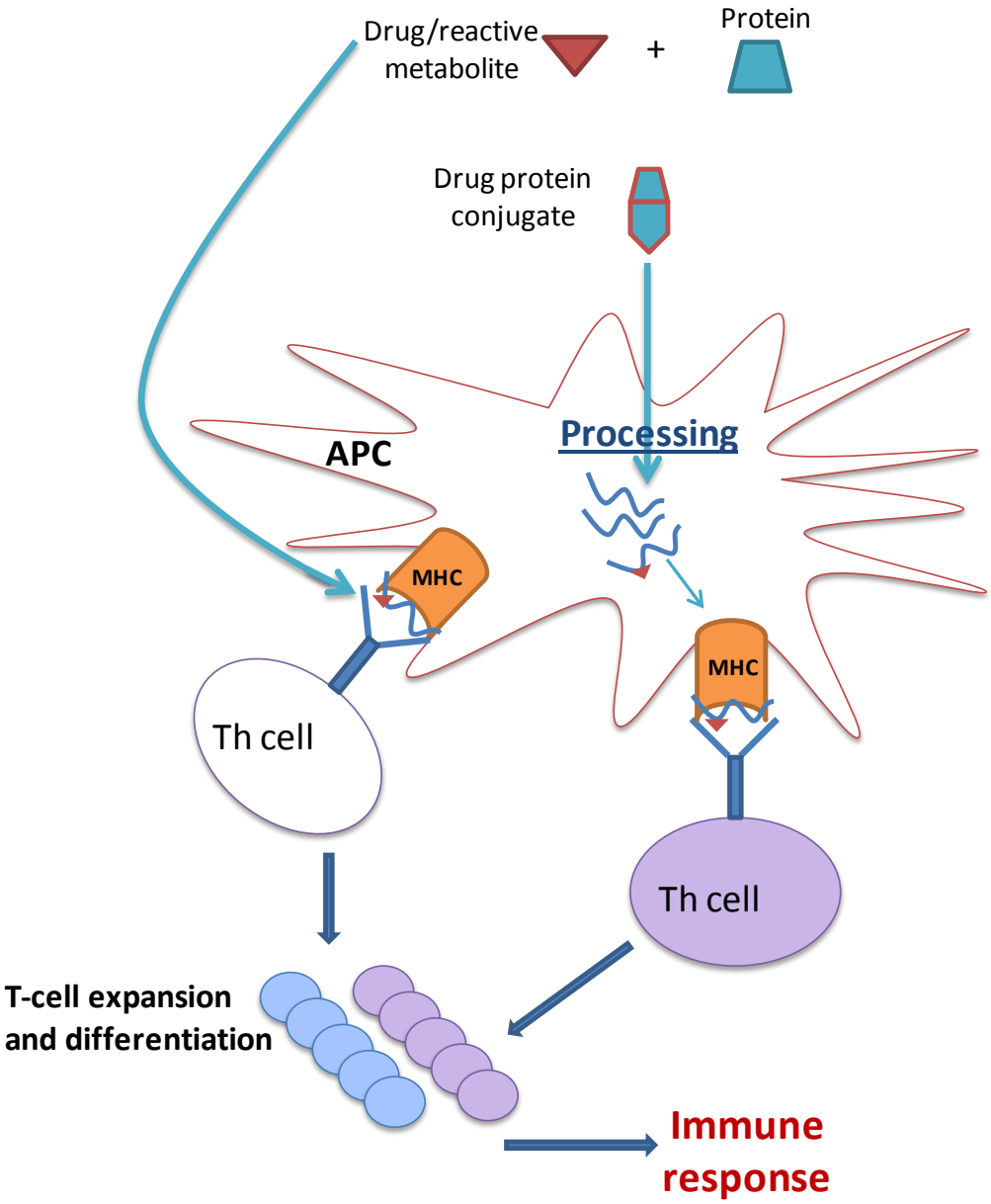


Figure 1.3 *The hapten hypothesis.*
Drug/reactive metabolites bind and covalently modify host proteins which are processed (see Figure 1.2) and the subsequent peptide fragments displayed via MHC molecule for recognition by the TCR on T-cells. Presentation via MHC I will elicit a CD8+ T-cell response and via MHC II the immune response will be mediated by CD4+ T-cells. The drug or reactive metabolite my also modify the MHC-peptide complex directly for presentation to T-cells.

Interactions between the nucleophilic sites on proteins and the electrophilic sites of low molecular weight compounds form the basis for the hapten hypothesis. Binding to specific lysine or cysteine amino acid residues has been seen with the β-lactam antibiotics piperacillin and flucloxacillin and the sulphonamide sulfamethoxazole (SMX) (Callan *et al.*, 2009; El-Ghaiesh *et al.*, 2012; Jenkins *et al.*, 2009a). Indeed, penicillin is one drug where the chemical and molecular basis of hypersensitivity seems to be well defined by the hapten hypothesis.

Many drugs however are not able to react with proteins directly. For example, SMX requires bioactivation through drug metabolising enzymes to form SMX hydroxylamine, and the reactive nitroso metabolites (Castrejon *et al.*, 2010a; Elsheikh *et al.*, 2011). Using T-cells isolated from sensitised mice and T-cell clones form allergic patients Elsheikh et al were able to show the need for processing for T-cell stimulation to occur. APCs were also shown to metabolise SMX in this study, subsequently forming a T-cell antigen from the resultant protein binding (Elsheikh *et al.*, 2011). In further experiments, nitroso derivatives of SMX including SMX-NO (the reactive metabolite which irreversibly binds to protein) were synthesised and administered to mice for 2 weeks. The splenocytes of sensitised mice proliferated and secreted a number of Th2 cytokines following stimulation with the nitroso metabolite and not SMX, the parent compound illustrating importance of metabolism (Castrejon *et al.*, 2010b). Similar results were also observed in T-cell clones generated from allergic patients (Castrejon *et al.*, 2010a)

Continuing with the hapten hypothesis, once protein modification has been achieved, these proteins are processed and cleaved into peptide fragments that

associate with MHC molecules. From there the peptide is presented to, and recognised by specific TCRs. Peptides presented via MHC class I molecules initiate a CD8+ T-cell response, whilst CD4+ responses are a result of MHC class II presentation.

It is also possible for the formation of novel or neo-antigens whereby following modification of the protein by the offending drug, newly synthesized determinants i.e. peptides are able to bind and stimulate an immune response in the absence of the drug. Indeed it is difficult to determine exactly which peptide fragment will elicit the hypersensitivity reaction as a number of drug-modified peptides whilst antigenic are not immunogenic.

The importance of peptides in the development of an immune reaction has been investigated. Using synthetic TNP-peptides, the work of Weltzien and Martin have described specific differences in the MHC binding sites for peptides with the haptens bound in different positions. Two functionally different TNP epitopes were defined. The major epitope for class I MHC-restricted T-cells is peptides with TNP-Lys substitutions at positions 4 and anchor residues at positions 5 and 8. A highly unique set of peptides with lysine modification by TNP at position 7 were able to induce specific, position 7 T-cells, but not other TNP-specific T-cell clones highlighting the importance of the hapten binding site (Martin *et al.*, 1992; Martin *et al.*, 1993; Von Bonin *et al.*, 1992).

1.7.3 Pharmacological Interaction of drugs with immune receptors (PI concept)

Some drug-specific T-cells have been found to become activated via an MHC restricted but processing-independent manner. The hapten theory fails to explain the ability of otherwise non-reactive compounds to stimulate an immune response and so another mechanism has been proposed – the p-i concept (Figure 1.4). The p-i hypothesis suggests that direct, non-covalent interactions occur between the drug, TCR and MHC molecule, which are labile and reversible, but sufficient to activate T-cells (Pichler, 2002). A number of drugs have been found to activate T-cells via the p-i mechanism including carbamazepine (Wu *et al.*, 2006), lamotrigine (Naisbitt *et al.*, 2003b) and SMX (Schnyder *et al.*, 1997). T-cells activated in this manner respond rapidly to drug with Ca2+ influx seen almost immediately (Zanni *et al.*, 1998). Following interaction between the T-cell and APC there is a rapid redistribution of certain organelles and following TCR engagement a rapid intracellular Ca^{2+} endoplasmic reticulum store is released. Indeed the intracellular concentration of Ca^{2+} can increase from 50-100nM to around 1μM following engagement of the TCR (Joseph *et al.*, 2014). This pattern of activation is incompatible with the hapten hypothesis as the time involved is not sufficient for processing, suggesting these pathways are bypassed. Furthermore, it has been observed that certain drugs activated T-cells via MHC molecules expressed on gluteraldehyde fixed APCs, thus preventing antigen processing but not presentation (Schnyder *et al.*, 1997). The p-i theory states that there is no *de novo* antigen formation and T-cell priming; drugs may be able to stimulate

memory and effector cells directly via TCR as these have a much lower threshold for activation (Adam *et al.*, 2011).

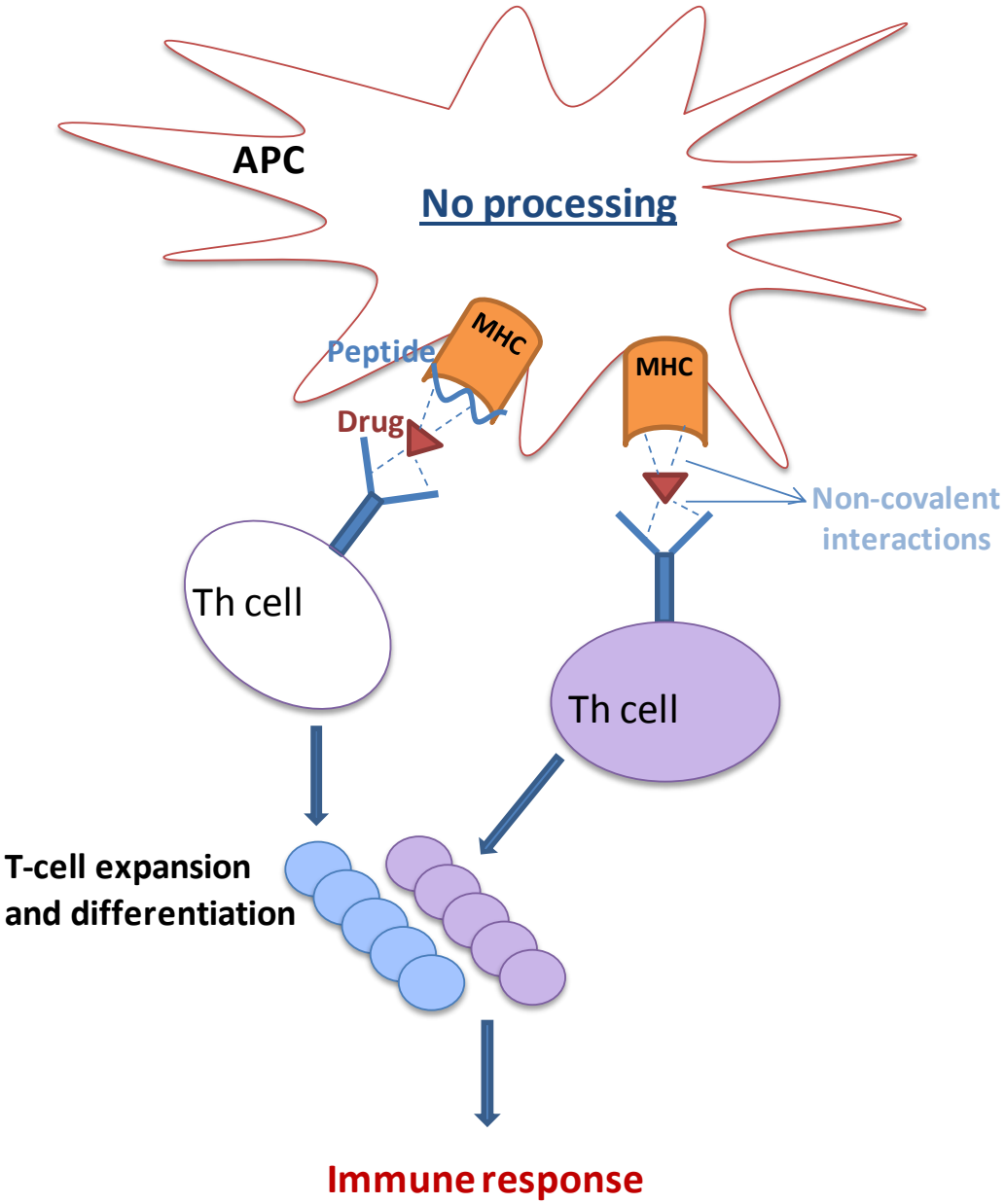


Figure 1.4 *The p-i concept.*
Drug/reactive metabolites bind directly to the TCR and/or MHC molecule in a non-covalent manner, thus eliciting an immune response. Hydrogen bonds, Van der Waals forces and hydrophobic interactions may be involved. A peptide may or may not be present.

1.7.4 Altered self-peptide repertoire hypothesis

MHC genes encode for an array of cell surface glycoproteins, which in humans are known as HLAs. These are either class I or II genes (MHC I, MHC II) and initiate specific immune responses through the presentation of peptides to the TCR as previously detailed. In certain cases of drug hypersensitivity strong links have been found between the offending drug and specific HLA alleles. I will discuss these associations in further detail in later sections.

The nucleoside reverse transcriptase inhibitor (NRTI) abacavir in particular, has a strong association with the HLA-B*57:01 allele (Chessman *et al.*, 2008; Mallal *et al.*, 2002) in so much that genetic testing for the risk allele (HLA-B*57:01) is highly recommended before commencing therapy (Hughes *et al.*, 2004; Rauch *et al.*, 2006). It is possible that abacavir hypersensitivity reactions occur via the hapten hypothesis; HSA adducts with the aldehyde metabolite have been characterised and could possibly play a role in abacavir toxicity (Charneira *et al.*, 2011). Conversely the PI concept has also been suggested as a possible pathway (Adam *et al.*, 2011). More recently however the altered peptide hypothesis has been introduced. The altered self-peptide repertoire hypothesis seems to most accurately describe the chemical basis of abacavir hypersensitivity. This model, illustrated in Figure 1.5, proposes that the drug settles in a particular site within the antigen binding cleft which leads to a change in the repertoire of self-peptides that can interact with the MHC molecule i.e. specific HLA (Bharadwaj *et al.*, 2012; Ostrov *et al.*, 2012). These peptides are subsequently presented to the TCR and thus immune response is initiated. Abacavir interacts specifically with the F pocket of HLA-B*57:01 in a non-covalent manner and so there are changes in the configuration of the

antigen binding cleft (Illing *et al.*, 2012; Norcross *et al.*, 2012). The repertoire of self-peptides that are able to be presented is thus changed and this is the basis for the hypersensitivity reaction.

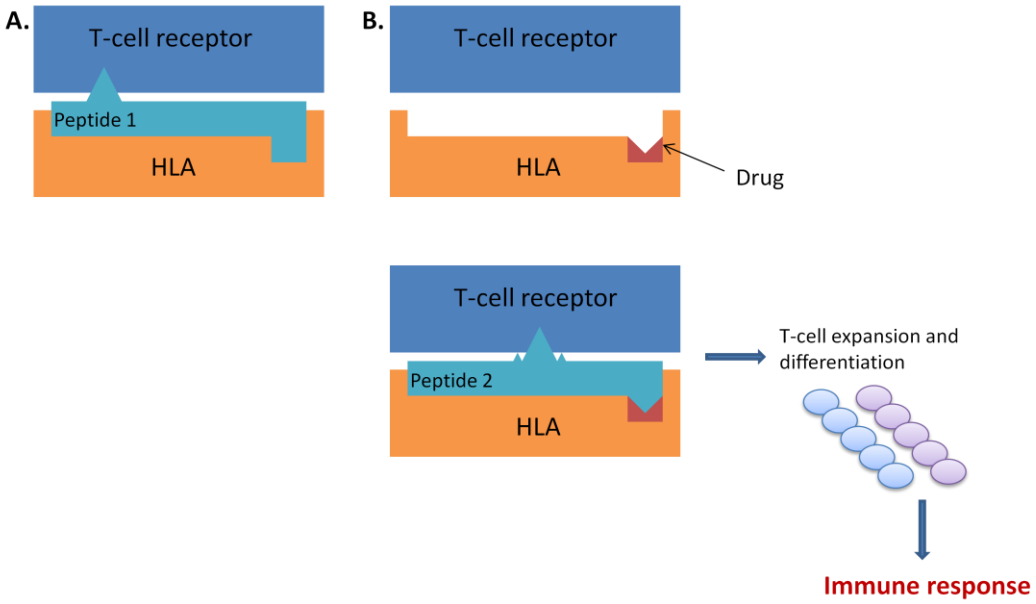


Figure 1.5 *The altered peptide repertoire hypothesis.*
A. Normal interaction of peptide 1 with HLA molecule and TCR. **B.** Presence of drug within the peptide binding groove of the HLA molecule alters the repertoire of peptides (peptide 2) presented by the HLA to the TCR which goes on to initiate the immune response/hypersensitivity.

Whilst this hypothesis and previous work has allowed abacavir binding to be elucidated we still have not determined how this translates to T-cell activation. Furthermore, we are yet to determine the time frame in which this hypothesis acts highlighting the need for further research in drug hypersensitivity reactions to fill the gaps in knowledge.

Recently Bell *et al* were able to describe two pathways in which T-cells were activated by abacavir. In one set of abacavir specific CD8+ T-cell clones abacavir-pulsed APCs were able to stimulate T-cells, in addition responses were

blocked when the processing capacity of APCs was blocked. Other T-cell clones however were activated by abacavir in the absence of APCs, forgoing the need for processing suggesting that T-cells can be activated by abacavir through direct interaction between T-cell and MHC (Bell *et al.*, 2013).

It is important to note that the mechanisms proposed for drug hypersensitivity reactions are not mutually exclusive and indeed parts of each may be involved in the development of an immune response to a drug.

1.7.5 Danger hypothesis

Matzinger proposed the danger model in 1994 stating the body does distinguish between self and non-self and instead immune responses are determined according to the context in which an antigen is presented (Anderson *et al.*, 2000; Matzinger, 1994). Danger signals initiate and activate immunity whereas in the absence of such signals, tolerance is induced.

Three signals are thought to be required to fully complete an immune response with two needed for activation of the immune system (Curtsinger *et al.*, 1999). Specificity of the immune response is provided by the first signal – interaction between the TCR and the MHC-peptide complex (Danese *et al.*, 2004). Activation occurs following a second signal whereby co-stimulatory molecules such as CD40 and CD80 present on APCs, interact with those on T-cells (CD40L, CD28). This signal 2 is essential for the proliferation and clonal expansion of T-cells, for the release of cytokines and for T-cell effector function. Without a second signal however, tolerance to the antigen in question will be observed. Pro-inflammatory cytokines, such as IFN-γ can up regulate co-stimulatory molecules and receptors on APCs further inducing/driving immune responses.

A wide array of co-stimulatory and inhibitory receptors have been reviewed in detail quite recently (Chen *et al.*, 2013).

Modulation of signal 2 can occur through exogenous PAMPS (LPS, peptidoglycan) and endogenous DAMPs released from dead/damaged cells (heat shock proteins, HMGB1). These can contribute to danger signals which are the basis for the danger hypothesis.

Numerous drugs have been found to induce danger signalling and thus immune activation. SMX-NO, the metabolite of SMX, increases the expression of CD40 on DCs (Sanderson *et al.*, 2007), abacavir induces redistribution of heat shock protein in APCs (Martin *et al.*, 2007)and amoxicillin has been found to drive DCs to a mature phenotypes (Rodriguez-Pena *et al.*, 2006).

Viruses including herpes virus 6 (HHV6), human immunodeficiency virus (HIV) and Epstein Barr virus (EBV), have all been implicated in DRESS yet their role in providing danger signals is not fully understood and can complicate diagnosis (Descamps *et al.*, 1997; Descamps *et al.*, 2003; Phillips *et al.*, 2007).

Tissues have the potential to act as a source of further danger signals which may help explain the localised release of such signals and why some reactions are confined to a particular organ.

The third signal is derived from polarising cytokines which act on T-cells and lead to an either Th1 or Th2 immune response (Pirmohamed *et al.*, 2002).

Chemically reactive drug metabolites can act as antigens as part of signal 1 and may also induce cell damage, promoting signal 2 and 3 and thus initiating a full immune response as seen in drug hypersensitivity (Li *et al.*, 2010; Park *et al.*, 2001).

1.8 Factors that predispose drug hypersensitivity

As previously mentioned, ADRs are a major health concern, and a vast range of reactions can develop (Davies *et al.*, 2009). A number of risk factors are known to predispose certain populations to the development of adverse reactions. Large numbers of associations have been found linking particular HLA alleles to reactions caused by certain drugs as detailed in Table 1.3. In addition to genetic variation in MHC, polymorphisms in drug metabolism enzymes can also play a role in the development of ADRs. Mutations in the genes that encode these enzymes can lead to alterations in their activity; an increase, decrease of even complete lack of activity may be observed resulting in a wide variation of toxicities seen in individuals (Meyer *et al.*, 1997). N-acetyltransferases are involved in acetylation and ultimately in the detoxification of a number of drugs including isoniazid, sulphonamides, dapsone and even caffeine (Vatsis, 1995). Polymorphisms in the NAT1 and NAT2 genes, which code for N-acetyltransferase can result in an either fast or slow acetylator phenotype. Fast acetylators often have low concentrations of the parent drug remaining whereas the opposite is true for slow acetylators and so the risk of a reaction may differ between these 2 groups (Shenfield, 2004). Indeed, slow acetylators have a higher risk of developing certain ADRs (Kosseifi *et al.*, 2006) however, it is not always so clear cut. For example, fast acetylators of isoniazid are not necessarily more likely to avoid hepatotoxicity (Gardiner *et al.*, 2006) whilst a slow acetylator phenotype or genotype is not like to be a risk factor for the development of SMX hypersensitivity in HIV-positive patients (Alfirevic *et al.*, 2003). Highly polymorphic cytochrome P450 enzymes such as CYP2C9, CYP2C19 and CYP2D6 are also implicated in toxic adverse drug reactions

(Johansson *et al.*, 2011). Though many drug metabolising enzymes and their genetic variations are involved in the development of an array of adverse reactions, there is currently no strong link to the development of immune reactions (Gardiner *et al.*, 2006).

Other risk factors include gender, with females reported to have a higher predisposition for ADRs, viral infections particularly HIV, and polypharmacy (Fattinger *et al.*, 2000; Saiag *et al.*, 1992).

1.8.1 Major Histocompatability Complex (MHC) molecule

Recently, genes within the MHC region have been linked to the development of an ADR. The MHC region, on the short arm of chromosome 6, is the most polymorphic region within the human genome and this high rate of heterogeneity results in wide inter-individual variation within the population (Parham *et al.*, 1988). Evolutionarily, maintenance of such high rates of polymorphisms in this region implies it is advantageous to possess a heterozygote genotype (Doherty *et al.*, 1975). In humans, MHC class I and MHC class II genes are known as human leukocyte antigen, HLA, I and II.

MHC Class I

Nearly all nucleated cells express MHC I molecules. The MHC molecule consists of the polymorphic transmembrane α heavy chain, encoded by the HLA-A, HLA-B and HLA-C loci, and a light chain called β2 microglobulin (β2m) (Johnson, 2000) as detailed in Figure 1.5. The β_2m is not membrane bound and is thought to play a key role in the transportation of new MHC molecules. The folded α1 and α2 domains form the peptide binding groove whereby the peptide (9 – 11

amino acids in length) can settle and bind through a combination of hydrogen bonds and Van der Waals forces (York *et al.*, 1996).

MHC Class II

MHC II molecules are only expressed on professional antigen presenting cells such as DCs, B-cells and macrophages. The HLA-DR, HLA-DQ and HLA-DP loci encode for MHC II (Lienert *et al.*, 1996). The structure of MHC II differs from MHC II as the β chain is anchored (Figure 1.6). Furthermore, longer peptides (14 – 20 amino acids) are able to accommodate the peptide binding groove due to its more open and flexible structure (Nojima *et al.*, 2003).

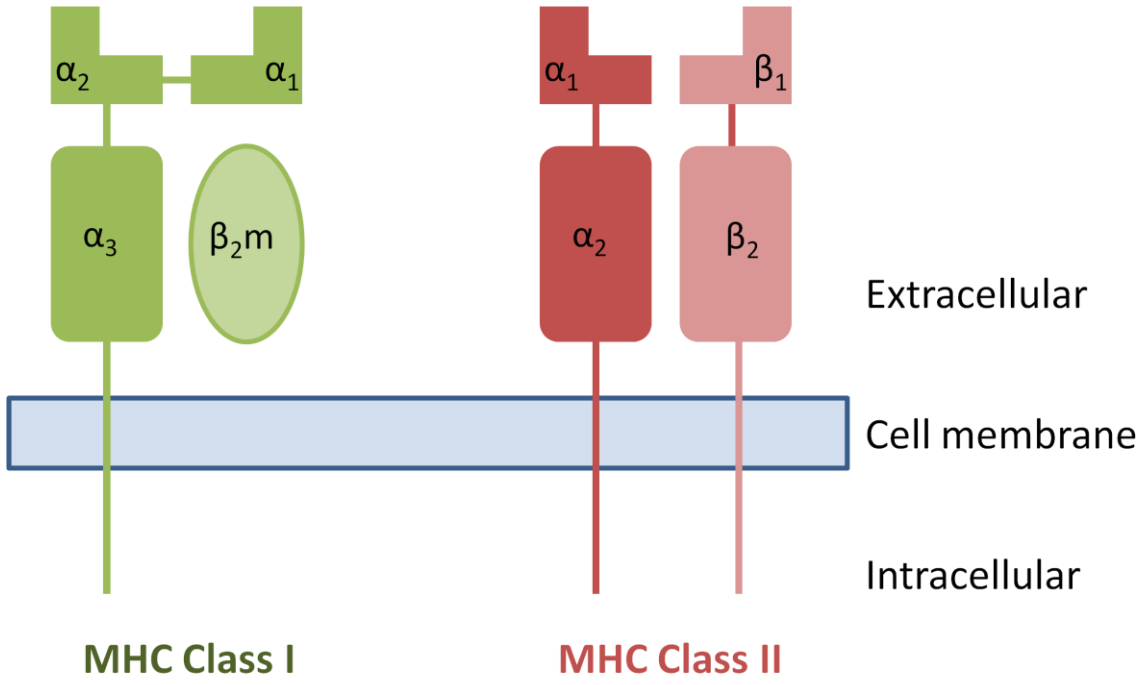


Figure 1.6 Structure *of major histocompatability complex molecule, Class I and Class II.*

Not only do these genes encode for the MHC molecules, they also include genes relating to the complement cascade, heat shock proteins, cytokines and other inflammatory mediators (Horton *et al.*, 2004).

1.8.2 HLA nomenclature

MHCs were first discovered in mice after tissue transplant experiments and soon after, work in the 1950's by Dausset and colleagues, revealed the existence of HLAs (Kelley *et al.*, 2005). When first discovered, the huge diversity of HLA alleles was unlikely to have been expected and so over the years it has been necessary for the naming system to evolve (Albert *et al.*, 1978; Marsh *et al.*, 2010). Indeed regular meetings take place to discuss and revise the ever expanding HLA allele classification. The current method for the nomenclature of HLA alleles is detailed in Figure 1.7.

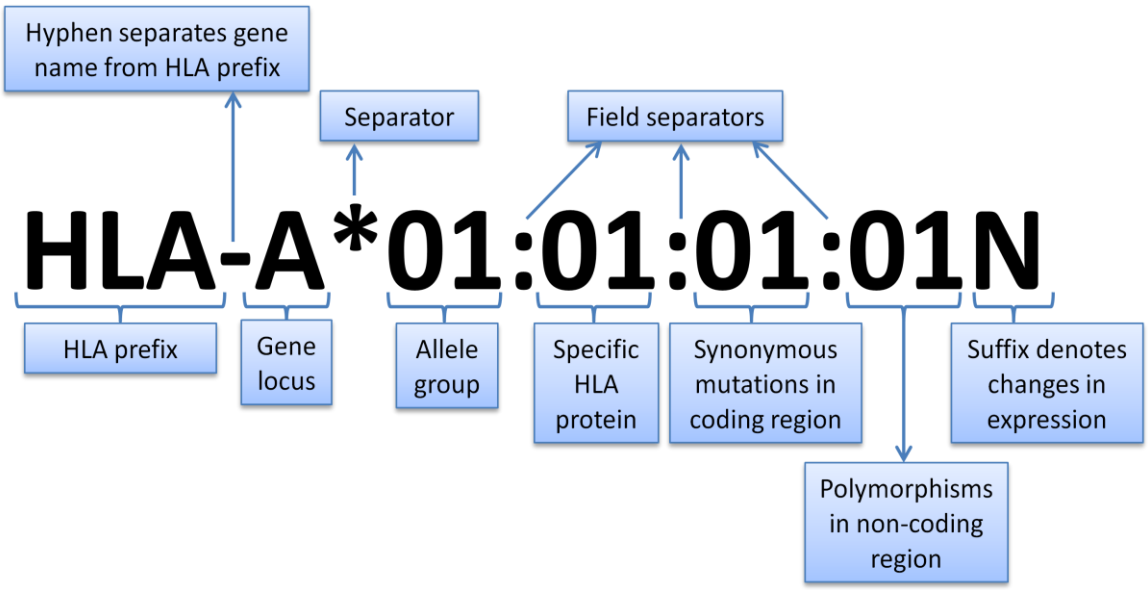


Figure 1.7 *Nomenclature of HLA alleles.*

The letter after HLA identifies the particular gene locus i.e. A, B, C etc. and the asterix indicates molecular typing techniques have been utilised to assign this allele. Each HLA allele then has up to four sets of unique, specific numbers separated by colons. The first two numbers correspond to the allele type

usually assigned by serology. The next two digits represent the subtype and specific HLA allele and signify differences in amino acid sequences. Numbers are assigned sequentially as new DNA sequences/alleles are discovered. The third set of digits represents alleles that differ through synonymous nucleotide substitutions i.e. polymorphisms that do not alter the peptide sequence. The fourth set of numbers can also identify polymorphisms in the non-coding region. Letters at the end of the series of numbers provide information on allele expression:

N – Null allele (not expressed on cell surface)

L – Low expression

S – Expressed as a soluble product

C – Expressed in the cytosol (not on cell membrane)

A – Aberrant expression

Q – Questionable expression (expression effected by mutation) (Tait, 2011)

Over recent years, genome wide association studies have highlighted links between HLA genetics and the development of a hypersensitivity reaction. Table 1.4 lists a number of drugs with known HLA associations which predispose certain populations for the development of a reaction.

	Drug	Adverse reaction	HLA association	Reference
MHC I association	Abacavir	Hypersensitivty	B*57:01	Mallal et al., 2002
	Allopurinol	SJS/TEN and DIHS	B*58:01 in Han Chinese	Hung et al., 2005
	Carbamazepine	SJS/TEN	B*15:02 in Han Chinese A*31:01 in Caucasians	Chung et al., 2004 McCormack et al., 2011
	Flucloxacillin	DILI	B*57:01 in Caucasians	Daly et al., 2009
	Lamotrigine	SJS/TEN	B*38 in Caucasians	Lonjou et al., 2008
MHC II association	Anti-tuberculosis drugs (isoniazid, rifampicin)	DILI	DQB1*02:01 in Indians	Sharma et al., 2002
	Aspirin	Asthma	DPB1*03:01 in Koreans	Choi et al., 2004
	Hydralazine	Systemic Lupus	DR*4	Batchelor et al., 1980
	Nevirapine	Hypersensitivity	DRB1*01:01 in Caucasians	Martin et al., 2005
	Ximelagatran	DILI	DRB1*07:01 and DQA1*02:01 in Caucasians	Kindmark et al., 2008
Mixed	Aminopenicillins	DIHS	A2 and DRw52	Romano et al., 1998
	Co-amoxiclav	DILI	DRB1*15:01 A*02:01	Lucena et al., 2011
	D-penicillamine	Proteinuria	B8, DR3, DR1	Wooley et al., 1980

Table 1.4 *Drug reactions with HLA associations.*

For a number of drugs, upon discovering a HLA association, research has led to a link between the drug and the immune response that is observed. Abacavir is a key example whereby binding sites to HLA-B*57:01 has been characterized in the development of hypersensitivity. HLA allele associations for flucloxacillin and carbamazepine have also been investigated (Lichtenfels *et al.*, 2014; Monshi *et al.*, 2013). For other drugs however it has been difficult to relate the HLA association and its involvement to the reaction observed in clinic. Despite extensive investigations with the use of *in vitro* models, a mechanism for the development of ximelagatran induced liver injury is yet to be defined (Keisu *et al.*, 2010). With such a complex system it can be difficult for researches to elucidate mechanisms involved, develop appropriate models and to relate *in vitro* findings to the clinic.

1.9 Drug hypersensitivity reactions – diagnosis and experimental approaches

In the clinic, skin tests are the only diagnostic tool routinely used for hypersensitivity reactions with numerous publications providing guidelines for carrying out these skin tests (Barbaud *et al.*, 2001; Barbaud *et al.*, 1998; Brockow *et al.*, 2002). There are three classic methods of skin tests; skin prick, intradermal and patch tests. Skin prick tests, whereby a needle with the suspected allergen pricks the skin, is followed up with intradermal testing (injection of up to 50µl of drug solution) if results are negative (Torres *et al.*, 2003). Skin prick and intradermal test are normally carried out where the reaction is thought to be IgE mediated/immediate hypersensitivity, whereas patch tests are normally performed in cases of delayed hypersensitivity (Demoly *et al.*, 2005). Suspect chemical entities are applied to the upper back with the use of adhesive tape with reactions noted (Barbaud *et al.*, 1998). However these traditional skin test often give false-negative results, have low sensitivity and in the case of intradermal tests, are invasive (Barbaud *et al.*, 2001; Strauss *et al.*, 2001). Re-challenging patients in provocation tests are useful for the confirmation of culprit drug following inconclusive results after skin testing. This must be carried out under careful medical observation due to the risk of re-activation of symptoms and so in severe reaction i.e. in SJS, these test are discouraged (Romano *et al.*, 2004).

The diagnosis of delayed hypersensitivity, particularly T-cell mediated reactions, can be complemented by the use of *in vitro* techniques which help

characterise clinical symptoms and highlight the drug responsible for such a reaction. These tests are not widely used in the clinic due to various reasons including the availability of freshly isolated peripheral blood mononuclear cells, the expertise required to conduct such assays/training researchers to carry out the tests and the lack of high throughput methods. The use of patient cells *ex vivo* is however at the centre of drug hypersensitivity research. The *in vitro* techniques described below can offer a safe alternative to often invasive skin tests and importantly in research, allow a better understanding of the molecular mechanisms of the reactions seen in the clinic.

1.9.1 Lymphocyte transformation test (LTT)

The lymphocyte transformation test (LTT) is used to detect drug-specific T-cell responses and the most commonly utilised *in vitro* assay for patients with suspected drug allergies (Nyfeler *et al.*, 1997). Peripheral blood mononuclear cells (PBMCs) are isolated from hypersensitive patients, exposed to the culprit drug and the proliferation of memory T-cells previously sensitised are measured (Luque *et al.*, 2001; Nyfeler *et al.*, 1997; Pichler *et al.*, 2004). Sensitized lymphocytes generate cytokines including IL-2, undertake blastogenesis and the subsequent proliferative response is measured and quantified through the incorporation of ^{3}H-thydmidine during the synthesis of DNA (Merk, 2005). The stimulation index (SI), the ratio of antigenic proliferation in comparison to proliferation in the absence of antigen i.e. cell culture medium, is used to express lymphocyte proliferation. An SI of 2 or more is considered a positive response.

The LTT can be used in delayed and also immediate hypersensitivity reactions though particular drugs have higher specificity including the β-lactam antibiotics, sulphonamides and anticonvulsants (Luque *et al.*, 2001; Merk, 2005). Tolerant or naive individuals would not display positive responses. Studies have shown the usefulness of LTTs in diagnosing penicillin allergy in delayed hypersensitivity, with sensitivities between 60% and 75% (Rozieres *et al.*, 2009). Although non-invasive, the length of the assay (six days) and the requirement of radioactive isotopes do not translate well clinically.

1.9.2 Enzyme-linked immunospot (ELISpot)

The enzyme-linked immunospot (ELISpot) assay has a shorter duration than the LTT (four days), is sensitive and allows the detection and characterization of drug-specific cytokine secreting T-cells (Czerkinsky *et al.*, 1988). T-cells secrete an array of cytokines following activation. Pro-inflammatory (generally Th1) cytokines such as IFN-γ, TNF-α, IL-2, IL-6 and IL-12, Th2 anti-inflammatory cytokines such as IL-4, IL-5, IL-10, IL-13and TGF-β and other secreted molecules such as granzyme B, perforin and Fas ligand can be investigated (Lehmann *et al.*, 2012; Lochmatter *et al.*, 2009; Romagnani, 1992; Romagnani, 2006). This assay provides more detailed information on the biological function of the T-cells that mediate the hypersensitivity reaction.

IFN-γ is one of the major cytokines implicated in numerous delayed hypersensitivity reactions and drug-induced exanthemas. Drug-specific IFN-γ secreting T-cells have been detected and quantified (Naisbitt *et al.*, 2005; Teraki *et al.*, 2003; Yoshimura *et al.*, 2004). While playing an important role in Th1 responses and serving as a cytotoxicity marker, IFN-γ can also contribute to the

Th2 response through its ability to upregulate MHC II molecules on the surface of CD4+ T-cells (Beeler *et al.*, 2006). Sensitivity from PBMC cultures in the IFN-γ ELISpot can reach 90% and up to 100% specificity can be seen in penicillin-induced MPE (Porebski *et al.*, 2011; Rozieres *et al.*, 2009).

Multiplex, or Luminex, assays also quantify drug-specific cytokine secretion by T-cells (Beeler *et al.*, 2006; Chen *et al.*, 2009). Capture antibodies directed at each target molecule/cytokine are bound to colour-coded beads and are detected and quantified by a fluorescently labelled reporter. As each antibody is coupled with a specific bead, multiple beads can be added and therefore multiple cytokines can be examined in a single sample.

1.9.3 Flow cytometry

Flow cytometry allows for the identification of multiple cell surface markers and subsequently the phenotypic analysis and characterisation of cells involved in drug hypersensitivity. Cell surface markers on activated T-cells can be used to detect drug-specific T-cell populations and thus can be utilised in the diagnosis of hypersensitivity (Hari *et al.*, 2001). The surface molecule CD69 is one such marker that has been used as a marker of delayed drug hypersensitivity and is comparable to LTT in terms of sensitivity and specificity (Beeler *et al.*, 2008). Other cell surface markers of drug-specific T-cell activation include CD25, CD40L CD71 and HLA-DR which are upregulated in sensitized individuals (Beeler *et al.*, 2006; Porebski *et al.*, 2011).

As with the LTT, flow cytometry can also be used to measure the proliferative response of previously sensitised memory T-cells. Drug-specific T-cells are stained with the fluorescent dye carboxyfluoescein succinimidyl ester (CFSE),

which binds to amino groups of intracellular proteins and becomes integrated within the lipid membrane. During a drug-specific proliferative response, the intensity of CFSE fluorescence will decrease by half with each cell division and so the the number of divisions can be determined and evaluated (Beeler *et al.*, 2006).

Following stimulation by a drug or antigen Th1, IFN-γ secreting cells and Th2, IL-4 secreting cells play an important role in the development and nature of the immune response (Del Prete *et al.*, 1993; Mosmann *et al.*, 1996). Intracellular cytokine staining, ICS, uses flow cytometry to evaluate such cytokine secretion as these two subsets of cells do not possess cell surface markers. Fluorescent antibodies for cytokines, including IFN-γ and IL-4, form the basis of ICS and with flow cytometry we can determine their involvement in drug induced immune response.

1.9.4 T-cell cloning

In some individuals it can be difficult to detect drug-specific T-cells as part of a PBMC culture using the above described methods. The use of T-cell clones in assays adapted from those previously described provides an alternative allowing research to focus specifically on antigen-specific cells. Using the well-established method of serial or limiting dilution, long lived drug-specific T-cells can be isolated from allergic individuals and expanded to generate drug-specific T-cell clones (Mauri-Hellweg *et al.*, 1995; Staszewski, 1984). Figure 1.8 provides further details of the processes involved in the generation of drug-specific T-cell clones. T-cell clones can then be fully characterised in terms of phenotype, for the expression of specific markers, proliferative capacity and their cytokine

secretion prolife determined. This allows the pathomechanism of drug hypersensitivity to be investigated.

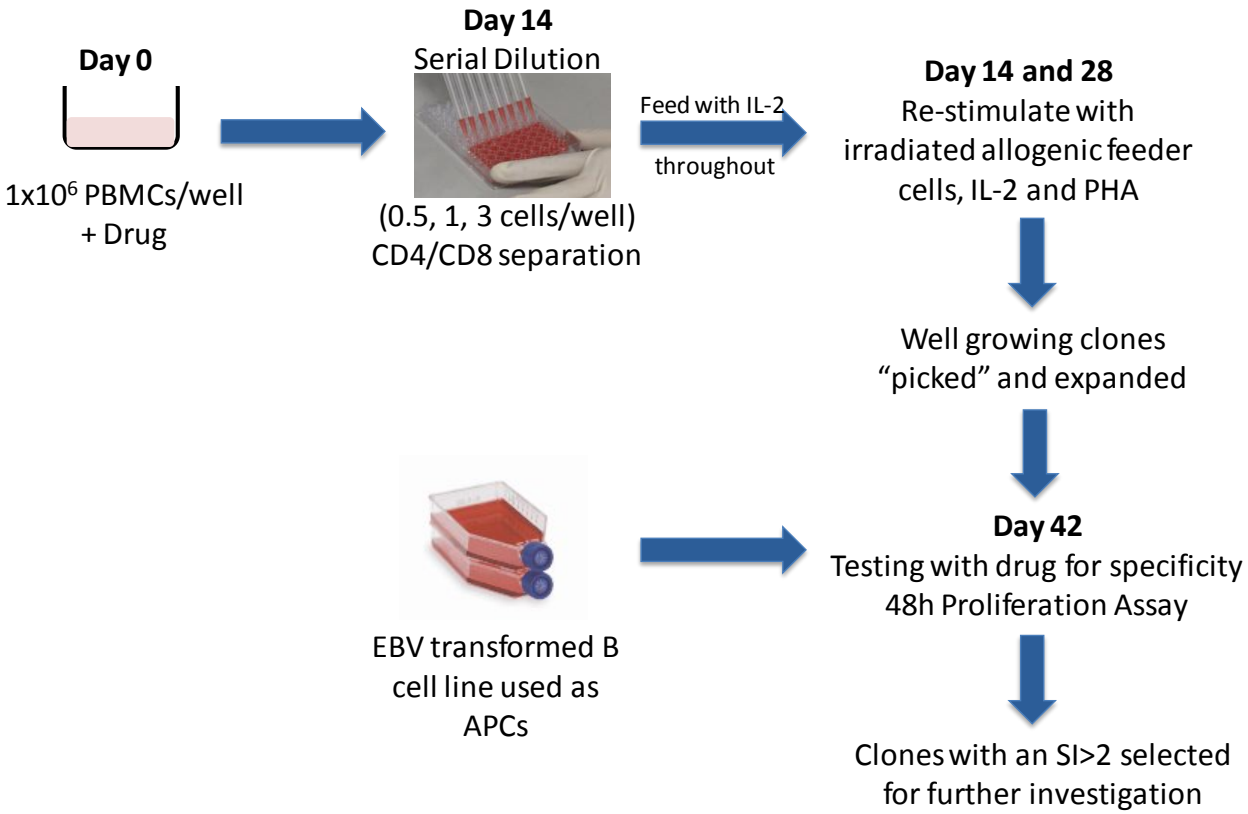


Figure 1.8 *T-cell cloning*
Timeframe and process involved in the generation of antigen-specific T-cell clones

1.10 Cystic fibrosis and drug hypersensitivity

Cystic fibrosis (CF) is one of the most common lethal recessive genetic diseases in the Caucasian population with over 8,500 sufferers and 2 million carriers in the UK (cftrust.org.uk, 2012). Faulty or absent chloride channels in the airways and digestive tract are caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene. Cells are therefore unable to release chloride and sodium reabsorption increases, leading to the accumulation of thick, mucous secretions in the airways. This environment is exploited be bacteria meaning CF patients frequently develop infective pulmonary

exacerbations, destruction of airways and bronchiectasis (Turcios, 2005). Though great improvements have been made in treatment and quality of life, as patients age pseudomonal infections reach up to 80% prevalence with a strong correlation between lung disease and patient morbidity and mortality (Kirkby *et al.*, 2009; Parmar *et al.*, 2005). Antibiotics are therefore prescribed both for treatment and prophylactically.

Unfortunately, allergy to β-lactam antibiotics is a major health concern in patients with CF. Sufferers are three times more likely to develop hypersensitivity reactions compared to the general population with a prevalence of up to 50% (Burrows *et al.*, 2007; Parmar *et al.*, 2005). Delayed type hypersensitivity is most commonly observed in CF patients with a mean time of onset around 9 days after β-lactam administration (Whitaker *et al.*, 2011b; Wills *et al.*, 1998). MPE is the most common manifestation with reactions also consisting of fever, nausea and flu-like symptoms. Less frequently seen, immediate hypersensitivity reaction accounted for 6% of β-lactam reactions with no severe skin reactions and systemic involvement reported (Whitaker *et al.*, 2012).

A number of factors may be responsible for the higher rates of hypersensitivity reactions observed in patients with CF namely the high doses of intravenous antibiotics delivered on a frequent basis increasing risk for sensitisation. With multiple exposures, there is likely to be increased sensitisation and ultimately elicitation of an immune response. Where a bacterial illness occurs, the inflammatory response in lung of CF patient consists of a chronic acute response. High concentrations of neutrophils are observed and as a result, high levels of circulating IL-8, IL-6, IL-17 and in particular the cytokines IL-1β and

TNF-α provide danger signals which could also increase risk of sensitisation (Whitaker *et al.*, 2012). Although the CFTR mutation is still to be fully investigated for its influence on inflammatory state, CFTR knockout mice have been observed mounting an exaggerated cytokine response to bacteria (Heeckeren *et al.*, 1997). An inflated Th2 response in CFTR deficient lymphocytes compared to controls has been described suggesting that the CFTR mutation may contribute to an increased risk of hypersensitivity (Mueller *et al.*, 2011).

1.11 Piperacillin

Patients with CF are prescribed a range of drugs throughout treatment including the anti-pseudomonal antibiotic piperacillin. Part of the penicillin class of antibiotics, the broad-spectrum piperacillin contains a β-lactam ring and is often given in combination with the β-lactamase inhibitor, tazobactam improving its activity against β-lactamase producing resistant bacteria (Speich *et al.*, 1998). Biliary excretion of piperacillin is around 15% in rats and approximately 37% in rabbits whereas in humans, it is negligible (Brogard *et al.*, 1994; Calhoun *et al.*, 1987; Ghibellini *et al.*, 2006). Studies using human liver microsomes and *in vivo* duodenal samples showed metabolism of piperacillin into desethyl-piperacillin through *N*-dealkylation. Furthermore a glucuronide conjugate of the desethyl metabolite has been detected (Ghibellini *et al.*, 2007; Komuro *et al.*, 1997; Minami *et al.*, 1991). As with all patients with CF, piperacillin is given intravenously for rapid absorption, often at relatively high doses and alongside an aminoglycoside. Piperacillin and its desethyl metabolite is able to bind to proteins in plasma (between 20% and 30%) and the formation

of these drug-protein adducts may represent functional antigens which are able to cause hypersensitivity reactions (Sorgel *et al.*, 1993; Whitaker *et al.*, 2011b). Hypersensitivity to piperacillin is most commonly delayed cutaneous reactions consisting of MPE and whilst piperacillin metabolites have been identified liver reactions have not been reported. Research on piperacillin hypersensitivity has allowed T-cell responses to piperacillin to be characterised and piperacillin adducts to be identified. Indeed piperacillin-specific lymphocytes have been identified and isolated in allergic patients and with the generation of piperacillin-specific T-cell clones a predominantly Th2 cytokine secretion profile has been determined (El-Ghaiesh *et al.*, 2012; Whitaker *et al.*, 2011b).

1.12 Flucloxacillin

The β-lactam antibiotic flucloxacillin is used to treat staphylococcal infection. It inhibits peptidoglycan synthesis and so inhibits bacterial cell-wall synthesis (Van Langevelde *et al.*, 1998). Commonly prescribed to the general population, flucloxacillin is generally well tolerated although it is allied with the manifestation of cholestatic liver disease, which affects bile flow. This is seen at a frequency of between 7 and 8.5 in every 100,000 first-time users (Russmann *et al.*, 2005). Metabolism of flucloxacillin via the CYP3A4 enzyme forms a 5-hydroxymethyl metabolite, which has been implicated in biliary epithelial cell cytotoxicity though hepatocyte toxicity was not observed (Lakehal *et al.*, 2001). It has been also been shown that flucloxacillin is able to bind to liver proteins in rat hepatocytes with drug modified albumin detected (Carey *et al.*, 2005).

Susceptibility to flucloxacillin-induced liver injury is strongly associated with the expression of HLA-B*57:01. A genome-wide association study (GWAS)

showed 84% of patients with flucloxacillin-induced liver injury carried the risk allele compared to 5% in the control population (Daly *et al.*, 2009b). Hypersensitivity reactions to the NRTI abacavir, used as part of combination therapy for HIV, is also associated with HLA-B*57:01 indicating possible T-cell involvement, though it is unclear why the liver is targeted in the case of flucloxacillin. Metabolism may play a role as both flucloxacillin and the 5-hydroxymethyl metabolite have been found to bind to human serum albumin (HSA) and at similar residues both *in vitro* and *in vivo* with modified peptides detected in patient plasma (Jenkins *et al.*, 2009a). Hapten formation may therefore play a role in adverse reaction to flucloxacillin.

Recently Monshi *et al.* highlighted the immune basis for flucloxacillin-induced liver injury through the characterisation of flucloxacillin-specific T-cell clones isolated from patients with liver injury. T-cell activation was processing dependent with the level of flucloxacillin binding to albumin correlating with activation of T-cells. Interestingly, other β-lactam antibiotics stimulated flucloxacillin-responsive T-cell clones whereas abacavir, which is also associated with HLA-B*57:01 restricted hypersensitivity, did not induce stimulation in flucloxacillin-responsive clones. Using a panel of APCs expressing a range of HLA-B alleles, HLA specificity was investigated. Flucloxacillin activation of clones was restricted by the risk allele HLA-B*57:01 and the structurally similar HLA-B*57:01 (Monshi *et al.*, 2013).

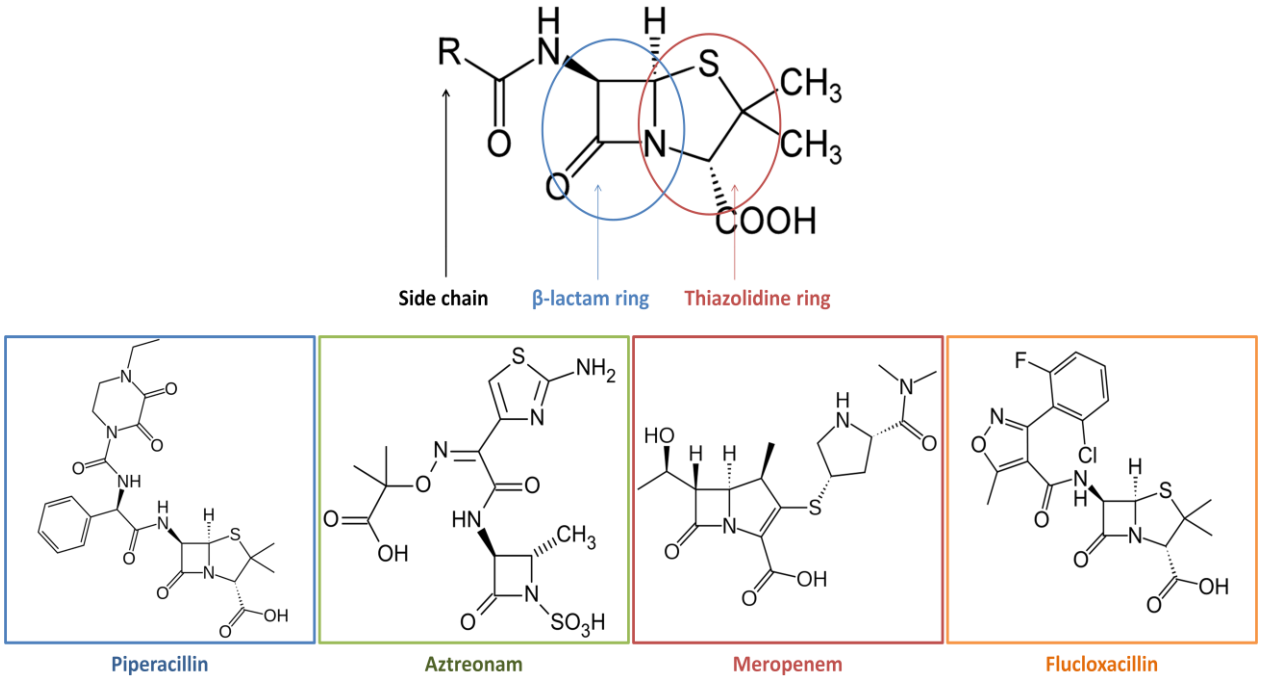


Figure 1.9 *Drug structures*
Core structure of the penicillin class of antibiotics is shown. The chemical structures of piperacillin (a penicillin), aztreonam (a monobactam), meropenem (a carbapenem) and flucloxacillin (a penicillin) are also shown.

1.13 Aims and objectives of thesis

The aim of this thesis was to investigate T-cells and their role in delayed hypersensitivity particularly in patients with skin and liver reactions to β-lactam antibiotics. Through an array of *in vitro* techniques I aimed to characterise drug antigen formation and further our understanding of the relationship between drug-protein binding and the initiation of an immune response that results ultimately in tissue injury in susceptible patients.

Specific aims of this thesis were:

- To investigate multiple drug hypersensitivity and T-cell cross reactivity between different classes of β-lactam antibiotic (piperacillin, aztreonam and meropenem).

- To explore T-cell responses to drugs and drug haptens in human patients expressing HLA risk alleles.
- Develop methods to quantify drug-protein adducts *in vitro* and in patient plasma.
- To explore the relationship between adduct formation and the activation of patient T-cells.

Chapter 2: Material and Methods

2.1 Materials and reagents

Cell culture flasks and multi-well plates were purchased from Thermo Scientific (UK). Lymphoprep™ was purchased from Axis-Shield (Dundee, UK). Human AB serum was purchased from Innovative Research (Michigan, USA). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, UK). Cyclosporin-A was bought from Fluka Analytical (Gillingham, Dorset, UK). Multisort bead separation kits were purchased from Miltenyi Biotec (Surrey, UK). Purified tetanus toxoid was bought from Statens seruminstitut (Copenhagen, Denmark). Recombinant human interleukin-2 (rhIL-2) was purchased from Peprotech (London, UK). Printed glass fibre filter mats, meltilex melt on scintillator sheets and sample bags for microbeta trilux were from Perkin Elmer Life Sciences (Waltham MA, USA). Tritiated [^{3}H]-methyl thymidine was purchased from Moravek (California, USA).

Multiscreen$_{HTS}$ filter plates for ELISpot were purchased from Millipore (Watford, UK). Interferon-γ, interleukin-13, interleukin-5, granzyme-B and perforin ELISpot kits including antibodies and substrate solution were purchased from Mabtech (Stockholm, Sweden). Fas ligand ELISpot kit was purchased from Abcam (Cambridge, UK).

HPLC grade methanol, analytical grade acetonitrile and all LC/MS grade solvents were purchased from Fisher Scientific (Loughborough, UK).

Reducing Laemmli sample buffer. Precision plus protein standards and Bradford reagent was purchased from Bio-Rad (Hertfordshire, UK). Chemiluminescent substrate was purchased from Thermo Scientific (UK).

Nitrocellulose membrane and Hyperfilm ECL was obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). Developer and fixer solution were from Kodak.

CD4-APC, CD8-PE and purified anti-human HLA-ABC-PE and FITC mouse anti-human HLA-DR, DP and DQ monoclonal antibodies were purchased from BD Biosciences (Oxford, UK). Mouse anti-penicillin antibody was bought from AbD Serotec (Oxford, UK). Polyclonal goat anti-mouse immunoglobulins/HRP was purchased from Dako cytomation (Glostrup, Denmark). Anti-flucloxacillin antibody was a gift from Frank Van Pelt (University College Cork, Ireland). Anti-rabbit IgG peroxidise secondary antibody produced in mouse was from -Aldrich (Dorset, UK)

Collagenase A or IV was purchased from Roche (Basel, Switzerland) or Sigma Aldrich (St. Louis, MO). Collagen-I coated plates were from BD Beckinson (San Jose, CA).

Meropenem, aztreonam and flucloxacillin was purchased from AstraZeneca (Luton, UK), Bristol-Myers Squibb Pharmaceuticals (Dublin, Ireland) and Wockhardt UK Ltd (Wrexham, UK) respectively. Insulin-transferrin-selenium was from Life Technologies (Carlsbad, CA).

All other solvents, chemicals and reagents were of analytical grade purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

2.2 Devices, lab-ware and instrumentation

MS and LS columns were purchased from Miltenyi Biotec (Surrey, UK). The liquid scintillation counter used in proliferation assays was the MicroBeta Trilux counter (PerkinElmer, Cambridge, UK). AID ELISpot reader was from

Cadama Madical (Stourbridge, UK). FACs analysis was acquired on BD FACSCanto II flow cytometer (BD Biosciences, Oxford, UK) and post acquisition analysis of flow cytometry data (FCS) files were done using Cyflogic software (CyFlo Ltd). Protein concentration was determined using MRX plate reader from Dynex (Lincoln, UK). C18 ZipTips were purchased from Millipore UK Ltd. Amicon Ultra-4ml centrifugal filter units were bought from Merk Millipore Ltd (Carrigtwohill, Ireland). Mean values and standard deviations were calculated and statistical analysis (paired T tests, Mann-Whitney, one-way ANOVA) was performed using GraphPad Prism (GraphPad Software).

2.3 Patient and volunteer recruitment

CF patients involved in piperacillin, meropenem and aztreonam work attended the Regional Adult Cystic Fibrosis Unit, St James's University Hospital, Leeds, UK. A tolerant CF group consisted of CF patients who had not previously had an adverse reaction to repeated antibiotic therapy. Naive controls were healthy volunteers who had never received the antibiotic in question. Written informed consent was obtained from all patients and the study was approved by the Leeds East Ethics Committee.

For DILI patients and healthy volunteers of the HLA-typed cohort, approval for the study was obtained from the Liverpool Local Research Ethics Committee. Informed written consent was obtained from all patients and participants involved.

2.4 Cell culture medium

Culture medium for T-lymphocytes comprised of RPMI 1640 supplemented with 10% human AB serum, HEPES (25mM), penicillin (1000 U/ml), streptomycin (0.1 mg/ml), L-glutamine (2mM) and transferrin (25µg/ml). Flucloxacillin-specific T-cells were generated and cultured in medium free from penicillin and streptomycin. Assays were conducted using penicillin-free media.

Antigen presenting cells (APC, EBV-transformed B-cells) were maintained in medium comprised of RPMI 1640 supplemented with 10% foetal bovine serum, HEPES (25mM), penicillin (1000 U/ml), streptomycin (0.1 mg/ml) and L-glutamine (2mM)

Media for use with hepatocytes comprised of Williams E supplemented with 1% insulin-transferrin-selenium, 2mM L-glutamine, 10-7M dexamethasone and streptomycin (100µg/ml).

2.5 Methods

2.5.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of patients and volunteers which were collected into heparinised vacutainer tubes. Blood (25ml) was delicately layered on top of lymphoprep (25ml) and the erethrocytes sedimented by density centrifugation (400g, 25mins at room temperature). The buffy coat layer containing the PBMCs was then carefully aspirated using a Pasteur pipette. PBMCs were washed twice in Hanks balanced salt solution (HBSS) to remove any remaining lymphoprep and resuspended in

10ml HBSS. A 10µl aliquot was added to an equal volume of trypan blue (0.2% w/v) and a 10µl aliquot of the stained cells counted using a Neubauer haemacytometer under a Lecia DME microscope (Leica Microsystems, Milton Keynes, UK). Cells for subsequent assays were also counted using Neubauer haemacytometer. Cell viability was assessed by trypan blue exclusion and calculated using the following equation: *percentage viability = viable cells ÷ total cells x 100*. Percentage viability was typically >95%.

PBMCs were again spun down and resuspended in FBS containing 10% DMSO at a density of 1 x 10^7 cells/ml for 24 hours in a Mr Frosty at -80°C. Cells were then frozen in liquid nitrogen or at -150°C for long term storage until required. Alternatively PBMCs were transferred to appropriate media for immediate use in experiments.

2.5.2 T-cell cloning

2.5.2.1 Generation of autologous antigen-presenting cell lines

Epstein-Barr virus (EBV) transformed B-cell lines were used as APCs in all T-cell clone assays. These were created from PBMCs by transformation with the supernatant from the virus-producing cell line B9.58. 5ml of supernatant was filtered through a 0.45µm syringe filter onto a PBMC pellet containing 5 x 10^6 cells. Cyclosporin A (CSA) was added to inhibit the proliferation of T-lymphocytes. After incubation overnight at 37°C under an atmosphere of 95% O_2/5% CO_2, cells were washed and resuspended in APC medium supplemented with CSA (1µg/ml) and transferred to a 24-well plate for cell culture. Fresh APC cell culture medium was added twice a week to maintain cells. CSA was omitted from the culture medium after two weeks to enhance B-cell proliferation. Cells

were transferred to a tissue culture flask when confluent and were maintained with fresh APC culture media twice a week.

2.5.2.2 Generation of antigen-specific T-cell clones

Through T-cell cloning we aim to generate antigen-specific cultures derived from a single cell. PBMCs from patients and volunteers (1x 10^6; 500µl) were cultured with 500µl of the drug in T-cell media in three wells of a 48-well cell culture plate. For piperacillin a concentration of 2mM was used, and for meropenem, aztreonam and flucloxacillin 0.5-1mM drug was used. On days 6 and 9 cell cultures were supplemented with IL-2 (200IU/ml) to expand the number of antigen-specific T-cells.

For the generation of flucloxacillin-specific T-cell clones, CD4+ and CD8+ cells were first separated on day 14 using a CD8 Multisort kit. Cells were harvested and resuspended in PBS containing 0.5% BSA and 2mM EDTA. Magnetic CD8 microbeads were added to the cells and incubated for 15 minutes at 4°C. Cells were then washed twice and added to an MS column held in a magnet. Negative unlabelled cells are able to pass through the column and collected as the CD4+ fraction. The column is then removed from the magnet and CD8+ cells flushed out.

The separated cells and, for piperacillin, meropenem and aztreonam, the un-separated bulk cells were then cloned (on day 14) using an established serial dilution method (Wu *et al.*, 2006). Cells were seeded in 96-well (u-bottom) plates at densities of 0.3, 1 and 3 cells/well and re-stimulated with allogenic irradiated PBMCs (feeder cells, 5 x 10^4 cells/well, PHA (1µg/ml) and IL-2 (60 U/ml). Well growing clones were transferred to new plates and split as required. Antigen specificity was assessed by proliferation assay (see 2.5.4). T-

cell clones were re-stimulated every 14 days as described above in order to maintain T-cell proliferation.

2.5.3 Lymphocyte transformation test (LTT)

LTT assays were performed on PBMCs isolated from the peripheral blood of hypersensitive patients to detect the presence of drug-specific memory T-cells using an established protocol (Nyfeler *et al.*, 1997) provided an overall view of the nature of the immune response. Briefly PBMCs (0.15 x 10^6, 100µl) were cultured with 100 µl drug (0.125 – 8 mM) in 96-well u-bottomed cell culture plates in triplicate. Tetanus toxoid (0.5µg/ml) was used as the positive control and T-cell culture medium for the negative. Plates were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 for 6 days with tritiated [^{3}H]-thymidine (0.5µl per well) added in the last 16 hours. Plates were harvested on day 6 and radioactive transmission from each well was read using a MicroBeta Trilux counter. Dividing cells incorporate the radiolabelled thymidine into newly synthesised DNA. Data is expressed as counts per minute for each well (cpm) with lymphocyte proliferation quantified using the stimulation index (SI), (cpm in drug treated culture/cpm in non treated control culture). Those with an SI of 2 or more are considered positive (Pichler *et al.*, 2004).

2.5.4 Proliferation assay

Similar in principle to the LTT assay proliferation assays allow us to determine proliferative activity and in doing so gives us information on the extent of the extent of the immune reaction. Using this assay we are able to get a measure of the T-cell response and can confirm antigen specificity. T-cell clones are primarily used in proliferation experiments.

Penicillin and streptomycin-free T-cell media is used throughout. T-cell clones (5 x 10^4, 50µl) are cultured in 96-well u-bottomed plate alongside irradiated autologous APCs (1 x 10^4, 50µl) and 100µl of the drug which is being investigated. Drug concentrations are made up at twice that required to account for 1:2 dilution in cell culture plate. Cells are cultured for 72 hours at 37°C under an atmosphere of 95% O_2/5% CO_2. Tritiated [^{3}H]-thymidine (0.5µl per well) is added in the last 16 hours and proliferation measured by scintillation counting (MicroBeta Trilux counter). An SI ≥ 2 is considered a positive result.

2.5.5 Enzyme-linked immunospot (ELISpot) assay

The ELISpot assay is a quick and effective assay that allows for the detection and characterisation of low frequency drug-specific cytokine secreting T-cells (Czerkinsky *et al.*, 1988). ELISpot kits with the appropriate antibodies are widely available for a vast range of cytokines. The majority of assays conducted as part of this thesis were for the detection of IFN-γ. MultiscreenHTS ELISpot filter plates are coated overnight at 4°C with 100µl capture antibody (15ug/ml for IFN-γ, 10µg/ml for IL-13). The following day wells are washed five times with sterile PBS and blocked with 200µl T-cell culture media (penicillin and streptomycin-free media used throughout) at room temperature for 30 minutes. Drug-specific T-cell clones (5 x 10^4, 50µl) are cultured in the ELISpot plate alongside irradiated autologous APCs (1 x 10^4, 50µl) and 100µl of the drug which is being investigated. Drug concentrations are made up at twice that required to account for 1:2 dilution within the plate. Plates are incubated for 48 hours at 37°C under an atmosphere of 95% O_2/5% CO_2. After 48 hours cells are discarded and the wells washed five times with 200µl PBS. Biotin labelled

detection antibody (specific for each cytokine) is diluted to 1µg/ml in PBS containing 0.5% FBS and 100µl added to the wells. The plate was incubated at room temperature for 2 hours before wells were washed five times with PBS (200µl). Streptavadin-ALP was diluted 1:1000 in PBS containing 0.5% FBS and 100µl added to wells and left to incubate at room temperature for 1 hour. Wells were then washed five times with PBS (200µl) and BCIP/NBT substrate added (100µl, 15mins) for spot visualisation. Wells were the washed under slow running tap water. A schematic of the ELISpot assay is illustrated in Figure 2.1. Wells were left to dry completely and spots were visualised and counted on an AID ELISpot reader.

When PBMCs are used 5 x 10^5 cells (in 100µl) are added to each well and APCs omitted from the assay.

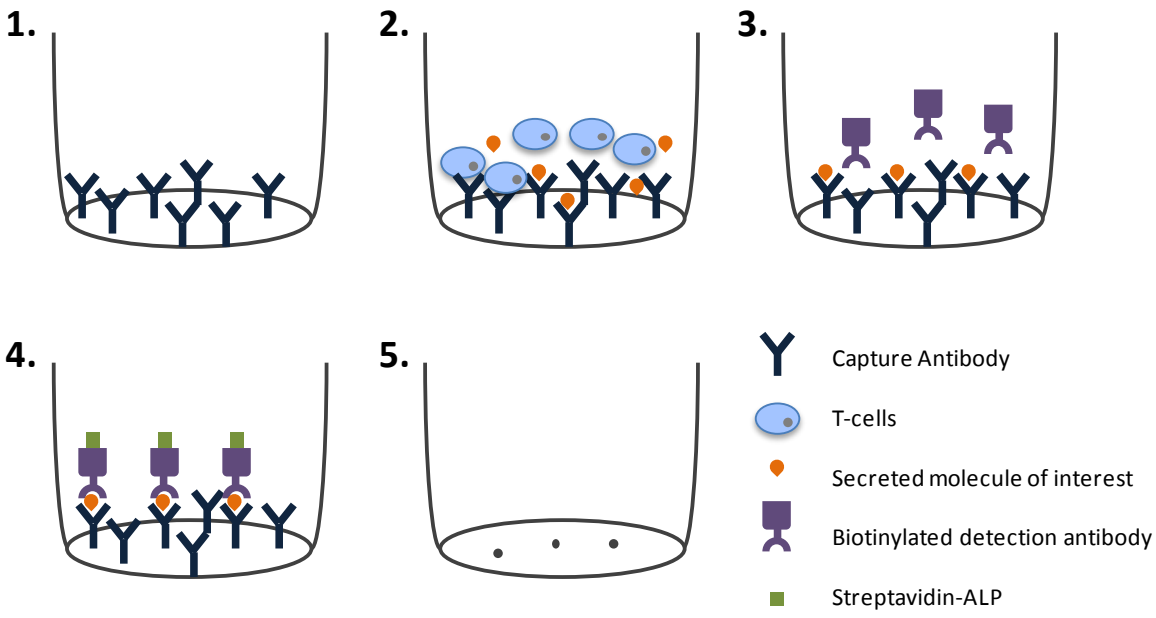


Figure 2.1 *Schematic representation of the ELISpot assay to detect cytokine secretion*
Wells are coated with a capture antibody for the cytokine of interest. Cells are co-cultured with drug for 48hours at 37°C. The molecule of interest, if secreted by cells, is captured. Cells are removed and a biotin labelled detection antibody (specific for each cytokine) is added. Strepdavidin-conjugated alkaline phosphatase is added which binds to the secondary biotinylated antibody. Upon addition of BCIP/NBT substrate solution alkaline phosphatase catalyses a reaction which results in a colour change and the formation of distinct dark spots.

2.5.6 Liquid chromatography/Tandem mass spectrometry

Over the past two decades advances in mass spectrometry methods has allowed the field of proteomics to emerge and improved protein characterisation. This sensitive technique is used to detect, identify and quantify molecules based on their weight (mass, m) and charge (z). The samples, or analytes, in our case will consist of small peptides/fragments. All mass spectrometers have three main components; an ion source, a mass analyser and an ion detector. The first step is ionisation; at least one electron is removed to provide a positively charged ion. The ions are accelerated through the machine and deflected with lighter and more positively charged ions deflected more. The beam of ions is detected and recorded.

With tandem mass spectrometry (MS/MS) following a first round of MS, distinct ions of interest are selected according to their mass to charge ratio (m/z) and fragmented further. In the second round of MS the fragments are further separated based on individual m/z.

Liquid chromatography (LC-MS/MS) is used to pre-fractionate peptides by in-line reversed phase chromatography meaning the MS instrument analyses a fewer species in a chromatographic timescale. Each multiply-charged peptide ion is bombarded with nitrogen which provides a known fragmentation profile at the peptide bond between amino acid residues. The mass analyser detects the series of ions as a mass ladder, with the mass difference between each step equivalent to the mass of one of twenty common amino acids. The peptide sequence can therefore be derived.

Multiple Reaction Monitoring MS (MRM-MS) provides further sensitivity and specificity for the detection and quantification of peptides in complex mixtures.

A triple quadripole instrument (a QTRAP 5500 hybrid quadripole-linear ion trap mass spectrometer was used) is able to select ions of specific a mass to charge ratio (m/z) in the first and third quadrupoles using different voltage settings across the four rods which make up the quads. The precursor ion of interest is preselected in Quadrupole 1 (Q1) while Q2 acts as a collision chamber wherein the peptides are fragmented into partial sequences. A small number of sequence-specific fragment ions are therefore analysed in Q3 rather than a full MS/MS scan. Thus the parent ion is selected in Q1 and fragmented in Q2 whilst a characteristic fragment in may be selected in Q3 (Gillette *et al.*, 2013). The transition (combination of parent ion mass and fragment ion mass) is designed to be diagnostic for the peptide of interest, but must also be detected with good sensitivity. The selection of MRM transitions is therefore critical for sensitive and specific peptide quantification.

2.5.7 LC-MS analysis of drugs

Meropenem and aztreonam were dissolved in 50% methanol/0.1% formic acid to concentrations of 0.25 µM and 2.5 µM, respectively, and infused at 5 µl/min directly into a QTRAP 5500 hybrid quadrupole-linear ion trap mass spectrometer (AB Sciex, Foster City, CA, USA) fitted with a turboion source. The ionspray potential was set to 5500 V and the interface heater was turned off. For meropenem, an ion of *m/z* 384 was selected manually for fragmentation to generate a full product ion scan, plus the resulting fragment ions of *m/z* 254 and 340 were further fragmented. For aztreonam, an ion of *m/z* 436 was fragmented to generate a full product ion scan, and the fragment ion of *m/z* 356 was further fragmented. The MS^3 scans were acquired to aid identification and

reveal potential diagnostic fragment ions to aid in the detection of covalently bound drug in studies of modified protein.

2.5.8 Time- and concentration-dependent modification of human serum albumin by meropenem, aztreonam and piperacillin

Human serum albumin (66 mg/ml, 1 mM) was incubated with piperacillin, meropenem and aztreonam (0.1mM, 1 mM, 10 mM, 20 mM or 50 mM) in 200 µL phosphate buffer, pH 7.4 at 37°C for 24h. In separate experiments the drugs were incubated at a molar ratio of drug to protein of 20:1 for 24, 48, 72 and 96 h under the same experimental conditions. Drug was removed by precipitation of the protein with 9 volumes of ice-cold methanol followed by centrifugation at 14000xg and 4°C for 10min. This was repeated once more, and the protein pellet reconstituted in 50 µL phosphate buffer. The protein was reduced by incubation with 10 mM DTT (w/v) at room temperature for 15 min, and alkylated by incubation with 55 mM iodoacetamide (w/v) for a further 15 min at room temperature. The samples were again subjected to methanol precipitation and were reconstituted in 50 mM ammonium bicarbonate buffer and the concentration was determined by Bradford assay (Bradford, 1976). The modified albumin was diluted to 1.6 mg/ml in 50 µl ammonium bicarbonate buffer. Trypsin (2 µg) was added, and the samples were incubated overnight at 37°C. The digestions were desalted using C_{18} Zip-Tips (Millipore) and dried prior to LC-MS/MS analysis.

2.5.9 LC-MS analysis of drug-modified peptides

For multiple reaction monitoring (MRM) detection of modified peptides, samples were reconstituted in 2% ACN/0.1% formic acid (v/v), and aliquots of

2.4-5 pmole were delivered into a QTRAP 5500 (AB Sciex) fitted with a NanoSpray II source by automated in-line liquid chromatography (U3000 HPLC System, 5 mm C_{18} nano-precolumn and 75 µm x 15cm C_{18} PepMap column [Dionex, California, USA]) via a 10 µm inner diameter PicoTip (New Objective, Massachusetts, USA). A gradient from 2% ACN/0.1% FA (v/v) to 50% ACN/0.1% FA (v/v) in 60mins was applied at a flow rate of 280 nL/min. The ionspray potential was set to 2,200-3,500V, the nebuliser gas to 19 and the interface heater to 150^{o}C.

In order to acquire unequivocal MS/MS characterisation of modified peptides, samples were also analysed using a Q-TOF instrument. Samples were delivered into a Triple TOF 5600 mass spectrometer (AB Sciex) by automated in-line reversed phase liquid chromatography, using an Eksigent NanoUltra cHiPLC System (AB Sciex) mounted with microfluidic trap and analytical column (15cm×75µm) packed with ChromXP C_{18}-CL 3µm. A NanoSpray III source was fitted with a 10 µm inner diameter PicoTip emitter (New Objective). Samples were loaded in 0.1% formic acid onto the trap which was then washed with 2% ACN/0.1% FA for 10mins at 2 µL/min before switching in-line with the analytical column. A gradient of 2-50 % (v/v) ACN/0.1 % (v/v) FA over 90 min was applied to the column at a flow rate of 300 nL/min. Spectra were acquired automatically in positive ion mode using information-dependent acquisition powered by Analyst TF 1.5.1. software, using mass ranges of 400-1600 amu in MS and 100-1400 amu in MS/MS. Up to 25 MS/MS spectra were acquired per cycle (approximately 10Hz) using a threshold of 100 counts per sec, with dynamic exclusion for 12secs and rolling collision energy. Sequence coverage was determined using ProteinPilot software v4.0 with the Paragon™ algorithm

(Shilov *et al.*, 2007) and the most recent version of the SwissProt database. Modified peptides were identified by filtering for specific fragment ions in PeakView 1.1.1 (AB Sciex) and manual inspection of the spectra.

To detect drug haptens, raw LC-MS/MS data were perused in order to identify peptides known to be modified with other β-lactam antibiotics (Jenkins *et al.*, 2009b; Meng *et al.*, 2011b; Whitaker *et al.*, 2011b) which were present with unexplained mass additions in samples exposed to meropenem or aztreonam. These data were used to design transitions specific for drug modified peptides. For meropenem, the *m/z* values were calculated for all possible peptides with a missed cleavage at a lysine residue. To these were added the masses of the proposed haptens (339, 384, 397 and 440 amu) and the parent ion masses were then paired with the proposed fragment masses of 175, 205 and/or 232 amu. For aztreonam, the mass of the proposed hapten (435 amu) was added to the peptide masses and paired with the proposed fragment ion mass of 237 amu.
MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles to maximize specificity, they were optimised for collision energy and collision cell exit potential, and dwell time was 20ms. MRM survey scans were used to trigger enhanced product ion MS/MS scans of drug-modified peptides, with Q1 set to unit resolution, dynamic fill selected and dynamic exclusion for 20s. Total ion counts were determined from a second aliquot of each sample analysed on the same instrument by conventional LC-MS/MS. Drug peak areas were determined by MultiQuant 1.2 software (AB Sciex). Epitope profiles were constructed by comparing the relative intensity of drug peaks for each of the modified peptides within a sample and normalisation of those

signals across samples using the total ion counts. In order to confirm the identity of the fragment ions used in the MRM transitions, MS^3 experiments were performed on selected peptides modified with each of the proposed haptens: MRM-triggered enhanced product ion MS/MS scans of drug-modified peptide were in turn used to trigger information-dependent MS^3 scans, with two MS/MS scans and four MS^3 scans per cycle. Piperacillin modified peptides were analysed as described previously (Whitaker *et al.*, 2011b).

2.5.10 Flow cytometry for T-cell phenotyping

T-cell clones were examined for CD4 and CD8 cell surface expression using flow cytometry. T-cell suspensions (100µl) were stained with fluorescent antibodies; CD4-APC (3µl) and CD8-PE (3µl) and incubated in the dark for 20 minutes at 4°C). Cells were then washed and resuspended in 200µl FACs buffer. Cells were acquired using FACS Canto and analysis conducted using Cyflogic. A minimum of 50,000 lymphocytes were acquired using forward and side scatter characteristics.

2.5.11 Antigen pulsing assays

Pulsing assays allows us to investigate drug-protein binding and presentation. APCs are incubated or pulsed with the drug overnight (16-24 hours). Cells are then washed extensively with HBSS to exclude free drug. These cells are then used in place of the regular non-pulsed APCs in our standard proliferation and ELISpot assays with T-cell clones (see 2.5.4.). T-cell clones (5×10^4, 50µl) are cultured with irradiated autologous drug-pulsed APCs (1×10^4, 50µl) and 100µl of the T-cell media. Soluble drug (100µl) is used as positive control.

2.5.12 Fixation of APCs

The fixation assay is used to determine the role of intracellular processing in APCs as part of the T-cell immune response. APCs (2 x 10^6 cell/ml) were washed twice with HBSS to exclude FBS and resuspended in 1ml HBSS. Glutaraldehyde (25%, 1µl) was then added to the cell suspension mixed gently for 30 seconds. Glycine (1M, 1ml) was quickly added and the cells agitated for a further 45 seconds. Cells were washed three times to remove glutaraldehyde and resuspended in T-cell media. T-cell clones were T-cell clones (5 x 10^4, 50µl) are co-cultured with irradiated glutaraldehyde-fixed APCs (1 x 10^4, 50µl) and 100µl of drug. Cells are incubated for 3 days at 37°C under an atmosphere of 95% O_2/5% CO_2 with tritiated [^{3}H]-thymidine (0.5µl per well) added in the last 16 hours. Proliferation is measured through scintillation counting with an SI ≥ 2 is considered positive.

2.5.13 Absolute quantification

2.5.13.1 Reagents

The following products were purchased from Sigma-Aldrich (Gillingham, UK): HSA (97-99%), Fmoc-L-amino acids, trifluoroacetic acid (TFA), N,N'-Dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt) , N-methylmorpholine (NMM), dimethylformamide (DMF) , piperidine, and dichloromethane(DCM). Invitrogen (Paisley, UK) provided fetal bovine serum. Radiolabeled thymidine was obtained from Moravek International Limited (CA, USA). Trypsin was obtained from Promega (Madison, WI, USA). Piperacillin was kindly provided by Dr Whitaker from Regional Adult Cystic Fibrosis Unit, St. James's Hospital, Leeds.

2.5.13.2 Synthesis of penicillin modified peptides

The synthesis of penicillin modified peptides was achieved by Fmoc chemistry in solution phase. Amino acid side chain protection was effected by the following: triphenilmethyl (Trt) for glutamine; tert-butyl (tBu) for aspartic acid, glutamic acid, and threonine; and benzyloxycarbonyl (Z) for C-terminus lysine. The synthesis was initiated with lys(Z)-OBn salt as shown in Scheme 1. The coupling reactions were activated by means of addition of N,N'-Dicyclohexylcarbodiimide (DCC), in the presence of 1-hydroxybenzotriazole (HOBt) and a base such as N-methylmorpholine (NMM). The Fmoc deprotection step was accomplished twice with 20% piperidine in chloroform for 10 min. The efficiency of these reactions was evaluated by the ninhydrin colorimetric reaction. Once the synthesis was complete, the deprotection processes were carried out following a series of sequential steps: firstly to remove benzyl or Z groups by catalytic transfer hydrogenation; secondly to remove Fmoc with 50% diethylamine in acetonitrile; thirdly to precipitate the peptides with cold diethyl ether. The crude peptides were purified by semi-preparative HPLC on a C18 column (details) with a linear gradient of 95-50% solvent A (0.05% TFA in water) in solvent B (0.05% TFA in acetonitrile) over 30 min at a flow rate of 5mL/min. The purity of peptides was determined by UV spectroscopy and the structure of the peptides were characterised by MS/MS analysis. The 1H NMR spectra were recorded in $CDCl_3$ or MeOD at 400 MHz on a Bruker Advance NMR spectrometer. This was conducted by Dr Xiaoli Meng, University of Liverpool.

2.5.13.3 Isolation of HSA from plasma

HSA was isolated by affinity chromatography. In brief, a POROS anti-HSA affinity cartridge (Applied Biosystems, Foster City, CA, USA) attached to a Vision Workstation (Applied Biosystems) was used to affinity capture HSA which was then eluted with 12 mM HCl. Protein was methanol precipitated, processed as described previously, and analysed by reversed phase LC-MS.

2.5.13.4 Mass spectrometric analysis of Pip modified HSA

Analyses were performed on a 5500 QTRAP® hybrid triple-quadrupole/linear ion trap instrument with Nanospray® II source (AB SCIEX, Foster City, CA, USA). MRM transitions specific for drug modified peptides were selected as follows: the m/z values for all possible modified peptides with a missed cleavage at the modified lysine residue were used together with a fragment mass of 160 corresponding to the cleaved thiazolidine ring of the drug. Notwithstanding the disparity in the ionisation efficiency of the peptides, relative MRM peak heights for each of the modified peptides were determined by MultiQuant software version 2.0 (AB SCIEX) to achieve the absolute quantity of the modified peptides. The total ion count for the whole digest for each sample was normalised to an internal synthetic peptide: in this way, the MRM signals were adjusted for differences in sample loading on-column. Further details of MS method are provided in supplementary methods. Calibration Curves for the synthetic standards were constructed by synthetic peptides (30, 75, 150, 225, 375, 750 fmol).

2.5.14 Generation of synthetic piperacillin-HSA conjugates

Human serum albumin (66 mg/ml, 1 mM) was incubated with piperacillin (at ratios of 50:1, 250:1 and 500:1) in phosphate buffer, pH 7.4 at 37°C for 24h. Conjugates were then processed used two methods as described below.

- **Conjugate 1**

Following 24 hours incubation piperacillin-HSA conjugates were transferred to Amicon Ultra 4ml and 15ml Filter spin columns for removal of free drug. Conjugates were washed 5-6 times with phosphate buffer (5000g, 10mins at 4°C). Conjugates generated through this method with spin columns are referred to as S-conjugates.

- **Conjugate 2**

Piperacillin-HSA incubations are transferred to 15 or 50ml tubes for removal of free drug by MeOH precipitation. 9 x volume of ice-cold methanol is added to the culture followed by centrifugation at 4000RPM at 4°C for 15 minutes. Protein pellets are then washed three times with equal an equal volume of ice-cold methanol before being reconstituted in phosphate buffer. Conjugates generated through methanol precipitation are referred to as M-conjugates.

LC-MS/MS analysis was used to confirm drug modification of HSA in both sets of conjugates. The protein was reduced by incubation with 10 mM DTT (w/v) at room temperature for 15 min, and alkylated by incubation with 55 mM iodoacetamide (w/v) for a further 15 min at room temperature. The samples were subjected to methanol precipitation and reconstituted in 50 mM

ammonium bicarbonate buffer. Concentration was determined by Bradford assay (Bradford, 1976). The modified albumin was diluted to 1.6 mg/ml in 50 µl ammonium bicarbonate buffer. Trypsin (2 µg) was added, and the samples were incubated overnight at 37°C. The digestions were desalted using C_{18} Zip-Tips and dried prior to LC-MS/MS analysis.

All conjugates were diluted in RPMI 1640 to appropriate concentrations for use in T-cell assays.

2.5.15 Western blotting

Protein samples (conjugates or cell lysates) were treated with reducing Laemmli buffer for western blot analysis at a ratio of 70 to 30% sample buffer to reducing agent. Samples were boiled for 10 minutes in preparation for western blot analysis. Aliquots of protein samples (5µg, 10µl) were loaded onto 10% SDS-polyacrylamide gel for separation by electrophoresis (300V, 60mA, 1 hour). Separated proteins were transferred from gel onto nitrocellulose membrane (300V, 250mA, 1 hour). Non-specific binding was blocked in 2.5% non-fat dry milk prepared in Tris/saline/Tween buffer (TST; 150mM NaCl, 10mM Tris-HCL, 0.05%Tween 20 [pH 8]) for 2 hours at room temperature. Immunodetection of drug protein adducts was performed by incubating the blot with primary antibody diluted in 2.5% milk/TST at 4°C overnight. Unbound antibody was removed by washing with PBS-Tween. This was followed by incubation with secondary antibody diluted in 2.5% milk/TST for 2 hours at room temperature. Unbound antibody was removed by washing with PBS-Tween. Signals from the membrane were detected by ECL and developed in dark room using autoradiography film before scanning.

	Piperacillin		Flucloxacillin	
Primary	**Antibody**	**Dilution**	**Antibody**	**Dilution**
	Mouse anti-penicillin	1:20,000	Rabbit anti-flucloxacillin	1:2,000
Secondary	**Antibody**	**Dilution**	**Antibody**	**Dilution**
	Goat anti-mouse HRP	1:1,000	Anti-rabbit IgG	1:1,000

Table 2.1 *Antibodies and concentrations used for western blot analysis*

2.5.16 Hepatocyte culture and flucloxacillin modification

Primary human hepatocytes were isolated using a previously described 2-step collagenase method (Berry *et al.*, 1969; LeCluyse *et al.*, 2005; Seglen, 1976). Briefly, liver resections were received as surgical waste (Aintree hospital, Liverpool, United Kingdom) with full patient consent and ethical approval. The resections were perfused with HEPES-buffered saline (HBS; 10mM Hepes, 5mM KCl, 136mM NaCl, 5g/l glucose), followed by digestion with Collagenase A or IV in HBS containing 700µM $CaCl_2$. The capsule was then opened and the digested cells separated using gauze. The suspension was centrifuged twice at 80xg for 5 minutes at 4°C, and resuspended in Williams E medium. Cells were plated onto Collagen-I coated 6-well plates at 1 x 10^5 cells/ml, 2mls per well, in hepatocyte media. After 3 hours incubation at 37°C under an atmosphere of 95% O_2/5% CO_2, non-adherent cells were washed away and the culture medium replaced. Cell media was changed the next day and replaced with either hepatocyte media or RPMI 1640 supplemented with 10% human AB serum, HEPES (25mM), streptomycin (100µg mg/ml), L-glutamine (2mM) and transferrin (25µg/ml). Cells were treated with flucloxacillin (0 – 4mM) for 24 or 48 hours after which cells were harvested. The dry cell pellets and supernatants were recovered and

stored at -80°C for immunoblot and LC/MS analysis. Protein concentrations were determined by Bradford assay.

2.5.17 MHC restriction assay

Anti-human HLA-A, -B, -C (MHC I) and anti-human HLA-DP, -DQ, -DR (MHC II) antibodies were used to determine if drug presentation to T-cell clones were restricted by MHC I or MHC II. APCs (1 x 10^4, 50µl) were pre-incubated with either MHC I or MHC II blocking antibodies (5µg/ml) at 37°C under an atmosphere of 95% O_2/5% CO_2 for 30 minutes. The APCs were then co-cultured with drug-specific T-cell clones (5 x 10^4, 50µl) with or without soluble drug for 72 hours. Tritiated [^{3}H]-thymidine (0.5µCi per well) was added in the last 16 hours and proliferation of T-cells measured through scintillation counting.

2.5.18 APC mismatch assay

Flucloxacillin-specific T-cell clones (5 x 10^4, 50µl) were co-cultured in 96-well u-bottom plates with either autologous or heterologous EBV-transformed B-cells which were used as APCs. Cells were exposed to flucloxacillin, or medium as a negative control, for 72 hours. A selection of heterologous APCs were chosen based on the expression of MHC class II molecules specifically HLA-B expression. EBV-transformed B-cells were generated from HLA-B*57:01 positive volunteers and a vast range of other HLA-B alleles were selected to enhance variety of allelic expressions available for tests. Cells were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 with tritiated [^{3}H]-thymidine (0.5µCi per well) added in the last 16 hours and T-cell proliferation measured through scintillation counting. Cytokine secretion was determined using IFN-γ ELISpot.

Chapter 3: Characterisation of drug specific T-cells to investigate β-lactam cross reactivity in patients with multiple drug hypersensitivity and elucidation of haptenic structures

3.1 Introduction

Cystic fibrosis (CF) is a fatal inherited disorder, of particular prevalence within the Caucasian population, with over 10,000 affected by the disease in the UK and 1 in 25 carriers of the faulty gene responsible for the condition (www.cysticfibrosis.org.uk, 2014). The development of CF is linked withthe long arm of chromosome 7 particularly the gene sequence that encodes cystic fibrosis transmembrane conductance regulator (CFTR) protein (Riordan *et al.*, 1989; Tsui *et al.*, 1985; Wainwright *et al.*, 1985). CF is an autosomal recessive disease arising due to mutations in the highly polymorphic region of DNA which codes for the CFTR protein (Gibson *et al.*, 2003; Knowlton *et al.*, 1985).

A single mutation, whereby a single phenylalanine residue at position 508 (ΔF508) has been deleted in the CFTR sequence, is accountable for the majority (68%) of CF causative mutations (Kerem *et al.*, 1989). However, great mutational heterogeneity exists in the remaining third of CF alleles. Over 1700 mutations have been identified and implicated in CF with varying frequencies and distribution (Bobadilla *et al.*, 2002; Cuthbert, 2011). CFTR is a cAMP-activated ATP-gated anion channel that transports chloride across epithelial cell membranes, and mutations in the region encoding CFTR can lead to an absent, faulty or defective CFTR protein function, particularly the ability to transport chloride across cell membranes (Rowe *et al.*, 2005). Chloride secretion across the apical airway epithelial cell membrane is reduced and sodium absorption is increased basolaterally. As a result, the transport of fluids and electrolytes is disrupted, airway surface liquid is reduced and ciliary function becomes defective (Matsui *et al.*, 1998; Rowe *et al.*, 2005). The environment provided by the thick viscid secretions formed in the airways and

gastrointestinal system of CF patients is prone to bacterial infection and colonization. Pulmonary exacerbations, airway inflammation and bronchiectasis are a frequent occurrence and unfortunately the chronic bacterial infections and concomitant airway destruction experienced by CF patients, will prove fatal in 80 to 95% of patients (Lyczak *et al.*, 2002; Turcios, 2005).

Intravenous drug therapy usually comprising an aminoglycoside and a β-lactam antibiotic is the foundation for the treatment of respiratory infections in patients with CF. Unfortunately, a high frequency of hypersensitivity reactions can restrict the therapeutic choices available. A number of studies have reported the development of an allergic reaction to be around 26% - 50% in CF patients which is in stark contrast to reaction prevalence of 1% - 10% in the general population (Burrows *et al.*, 2007; Parmar *et al.*, 2005; Pleasants *et al.*, 1994; Whitaker *et al.*, 2011c).

At the Leeds Adult Cystic Fibrosis Unit, only 6% of reactions induced by β-lactam antibiotics are classified as immediate reactions, and no severe skin reactions or systemic involvement was encountered (Whitaker *et al.*, 2012). Allergy to β-lactam antibiotics is normally a non-immediate or delayed-type IV hypersensitivity reaction with a mean time of onset around 9 days after drug exposure, with rashes, fever, nausea and flu-like symptoms most commonly observed (Whitaker *et al.*, 2011b).

If an allergic reaction to one β-lactam antibiotic is encountered, another drug often from a different class of antibiotic is given in replacement. It is unfortunate however, that up to 20% of CF patients develop allergic reactions to multiple antibiotics thus limiting treatment options (Whitaker *et al.*, 2011c).

The development of multiple drug hypersensitivity (MDH) syndrome whereby hypersensitive reactions are seen with at least 2 drugs can lead to further difficulties in patient cases where, for example, a lung transplant becomes the next step in their treatment. The condition of the lungs is critically dependent on the sustained use of antibiotics due to the nature of CF. Furthermore sufferers are usually restricted to one lung transplant and so it is vital to ensure functional lungs for as long as possible (Whitaker *et al.*, 2012). Patients with MDH may be excluded from the transplant program and so the appearance of a mild rash can therefore lead to serious consequences in a patient's prognosis.

Clinical data suggest the extent of cross-reactivity between the different classes of β-lactam antibiotics is low. Moss *et al.* reported that 19 of 20 patients with previous penicillin or cephalosporin reactions safely tolerated aztreonam (Moss *et al.*, 1991) and rates of cross-reactivity between penicillin and meropenem in patients with immediate reactions are estimated at less than 1%. However, many patients become sensitized to the replacement drug following repeated exposure and still experience Type IV hypersensitivity at a later date.(Atanasković-Marković *et al.*, 2008; Frumin *et al.*, 2009; Moss, 1991). The factors that lead to multiple delayed reactions in β-lactam allergic patients have not been fully elucidated and there is need for a clearer understanding of the drug-specific-immune response to inform drug selection and use.

Previous work in the department has focused on one particular commonly used β-lactam antibiotic, piperacillin. Using piperacillin-specific T-cell clones isolated from piperacillin-allergic CF patients, mechanistic studies could be conducted to further our understanding of the immune pathogenesis in this subset of patients. These studies revealed high drug specificity, both for proliferative

responses of lymphocytes, and with regards to cytokine secretion. Not only was the secretion of classic Th1 cytokine IFN-γ detected, Th2 cytokines (IL-4, IL-5 and IL-13) were also secreted at high levels (El-Ghaiesh *et al.*, 2012). Furthermore, using pulsing assays, whereby free drug is excluded and instead bound to APCs before culture with T-cells, show evidence of time dependant antigen formation for the stimulation of an immune response. A minimum of four hours incubation time (piperacillin and APC) is required to elicit a positive T-cell proliferative response. In addition, using novel mass spectrometric methods we have been able to identify the lysine residues that piperacillin modifies on the model protein human serum albumin (HSA) and thus characterise the hapten formed both *in vitro* and *in vivo*. Modifications were most commonly seen at positions Lys 190, Lys 432 and Lys 541 and indeed levels of modification coincided with the intensity of lymphocyte proliferative response. As drug incubation time and drug concentration increased so too did modification of HSA and T-cell response. Analysis suggested peptide sequences around these lysine positions could be the principal functional epitopes and are likely to be antigenic (Whitaker *et al.*, 2011b).

3.2 Aims and Hypothesis

In our department, work has previously focussed on piperacillin in order to define the mechanisms of T-cell activation using PBMCs from hypersensitive patients. Aztreonam and meropenem from the monobactam and carbapenem classes of β-lactam antibiotics respectively, are further drugs used in the treatment of lung infections in patients with CF. This chapter is focussed on these antibiotics in order to determine if it possible to detect individual T-cell

responses to the three drugs. Through the generation of drug-specific clones, we hope to address the question of cross reactivity and further our knowledge of the immune response in hypersensitive individuals.

We aim to characterize the haptenic structures that piperacillin, meropenem and aztreonam form with lysine residues on human serum albumin (HSA).

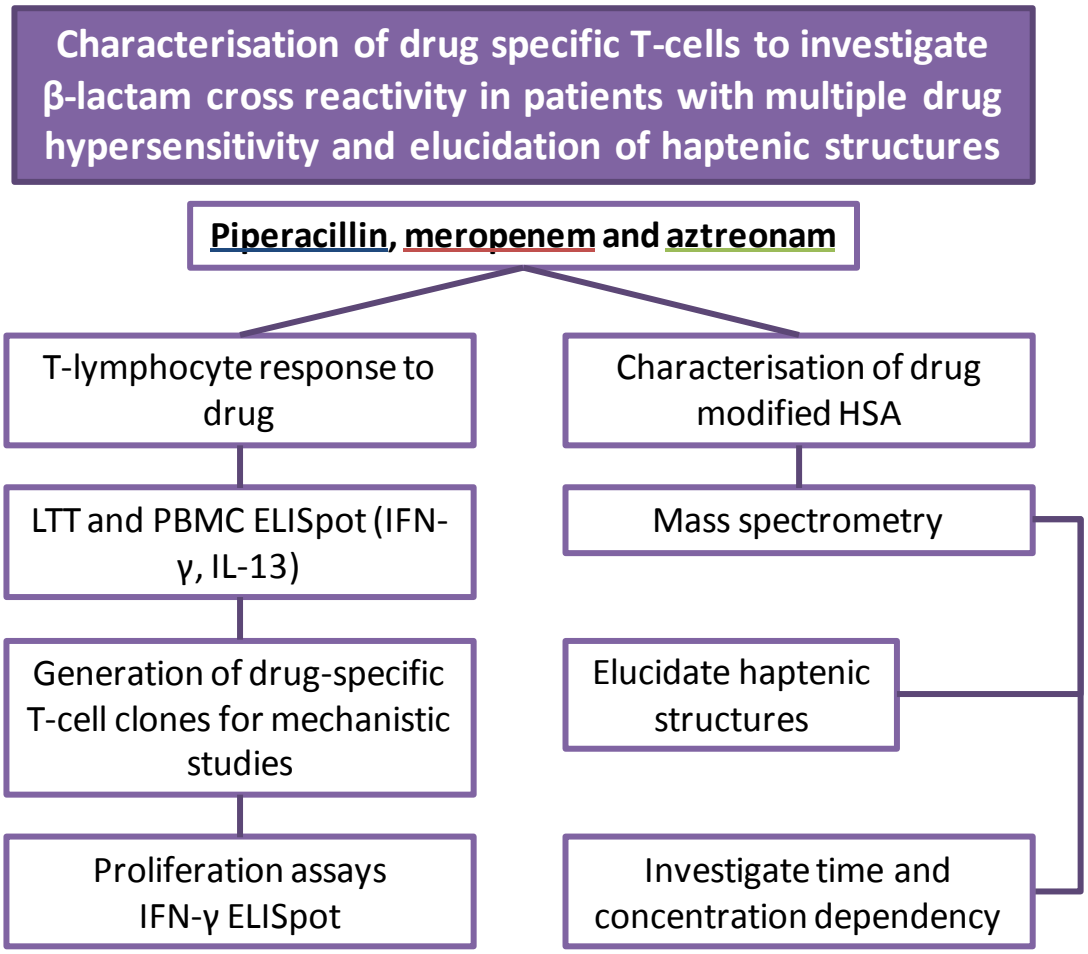
Furthermore, through the isolation of antigen-specific T-cell clones from patients with multiple allergies to β-lactam antibiotics we aim to present a biological read-out to analyse cross-reactivity of these drugs.

From previous literature, with such high rates of hypersensitivity and the structural similarities of the three drugs I propose the following hypothesis:

Drug specific T-cell clones will cross react with each of the three drugs and similar patterns of HSA modification will be observed.

3.3 Methods

The methods used are described fully in Material and Methods, Chapter 2. A brief experimental outline is detailed below.



Scheme 3.1 *Experimental plan and methods used in this chapter*

3.4 Results

A close collaboration with the Leeds Adult Cystic Fibrosis Unit has allowed the recruitment of patients with allergies to multiple β-lactam antibiotics. We were therefore able to utilise PBMCs isolated from patients to examine T-cell responses to piperacillin, meropenem and aztreonam and further understand the immune responses in patients with MDH.

3.4.1 Piperacillin, Meropenem and Aztreonam stimulates PBMCs from allergic patients

PBMCs were isolated from four patients with cystic fibrosis and allergies to the following β-lactam antibiotics; piperacillin, meropenem and aztreonam. Table 3.1 provides a summary of patient information including the clinical features of reactions.

PBMCs from these patients with MDH were stimulated *in vitro* to proliferate with graded concentrations of each drug (0.5 – 2mM). We observed that the proliferative responses were dependent on drug concentrations (Figure 3.1). Furthermore, PBMC cytokine secretion (IFN-γ) and/or IL-13) is observed following drug treatment.

With the exception of patient 3, the strongest responses were seen following piperacillin exposure often differing greatly to the SI's recorded with the other two drugs. This is demonstrated clearly in the case of patient 4 where piperacillin yields an SI of over 60 whilst much weaker responses are seen following exposure to aztreonam and meropenem, <2 and 4 respectively.

Patient ID	Age/sex	Drug reaction	Reaction details	Time to reaction (days)	Time since reaction (years)	Courses prior to reaction	LTT (maximum SI)
1	21/M	Piperacillin Aztreonam Meropenem	MPE, fever MPE MPE	9 4 5	7 5 5	3 2 3	13.3 <2 8
2	26/M	Piperacillin Aztreonam Meropenem	MPE Urticarial rash MPE	5 2 3	2 1 3	3 4 5	31.1 2.4 4.4
3	28/F	Piperacillin Aztreonam Meropenem	Fever, arthralgia Fever, arthralgia Flu-like symptoms, vomiting	9 6 6	6 5 4	7 4 5	6.8 4.1 5.8
4	22/M	Piperacillin Aztreonam Meropenem	MPE/fever Urticarial rash, uticarial Lip swelling	2 1 1	1 1 2	4 2 3	62.5 <2 4

Table 3.1 *Clinical details of allergic patients*
MPE = maculopapular drug eruption, LTT = lymphocyte transformation test, SI = stimulation index.

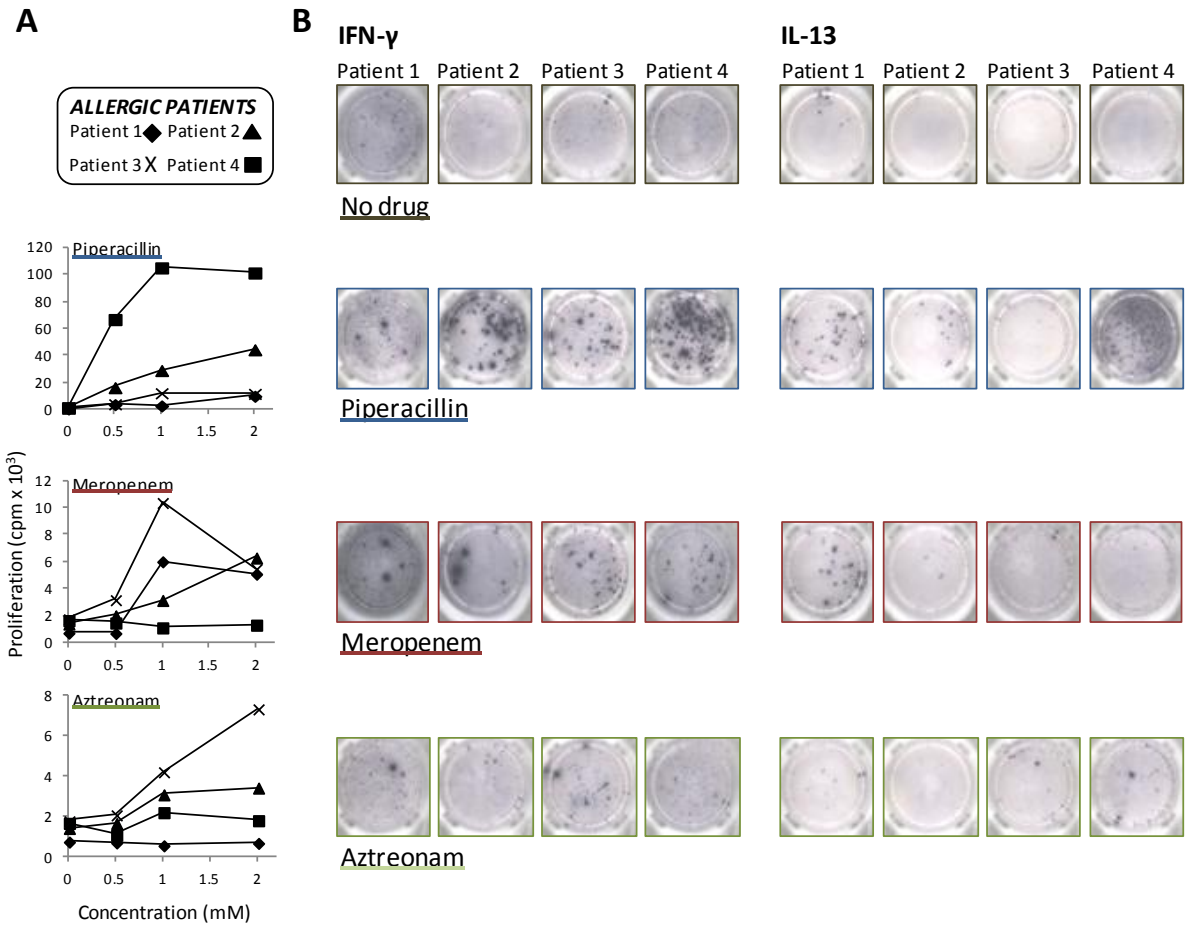


Figure 3.1*Drug-specific stimulation of PBMCs from allergic patients*
(A) PBMCs from 4 patients with MDH were cultured in the presence of piperacillin, meropenem or aztreonam for 6 days. Proliferation of lymphocytes was measured with the addition of [^{3}H]-thymidine in the last 16 hours. Results are shown as mean CPM of triplicate cultures, CPM = counts per minute. Coefficient of variation was consistently less than 20%. **(B)** The drug specific secretion of IFN-γ and IL-13 was determined and visualised by ELISpot assay. Well images from patient PBMCs which were cultured with drug for 48 hours. Plates were developed for spot visualisation according to manufacturer's instructions.

Four drug-exposed tolerant patients were also recruited. These patients had previously been exposed to several courses of the three drugs with no reported allergies. In contrast to MDH patients, PBMCs from tolerant individuals were not stimulated to proliferate or secrete cytokines (Table 3.2). In the LTT, an SI greater than two is considered a positive response. Tolerant patient PBMCs did not yield positive LTTs with any of the three drugs investigated.

Patient ID	Age/sex	Drug reaction	Drug exposure	LTT (maximum SI)
1	28/M	None	Exposed to piperacillin, aztreonam and meropenem in the last three years. One year prior to his death, he required 73 days of antibiotic treatment.	<2
2	24/F	None	Exposed to piperacillin, aztreonam and meropenem in the last three years. In the last 12 months, the patient received 33 days of antibiotic treatment.	<2
3	21/M	None	Exposed to piperacillin, aztreonam and meropenem in the last three years. Averaged 81 days of antibiotic treatment per year.	<2
4	29/M	None	Exposed to piperacillin, aztreonam and meropenem in the last three years. Averaged 14 days antibiotic treatment per year.	<2

Table 3.2 *Clinical details of tolerant patients*
LTT = lymphocyte transformation test, assay conducted with each drug (0.1 – 2mM). SI = stimulation index.

3.4.2 Generation of piperacillin, meropenem and aztreonam-responsive T-cell clones

To further investigate antigen-specificity of T-lymphocytes and the nature of the immune response in patients with MDH we generated drug-specific T-cell clones. One thousand six hundred and sixty-three T-cell clones were generated from the four allergic patients following PBMC stimulation with piperacillin, meropenem or aztreonam. Clones were tested for drug specificity with a total of one hundred and twenty-two found to display reactivity against piperacillin (*n* = 89), meropenem (*n* = 14), or aztreonam (*n* = 22). Figure 3.2 shows the proliferative response of representative clones.

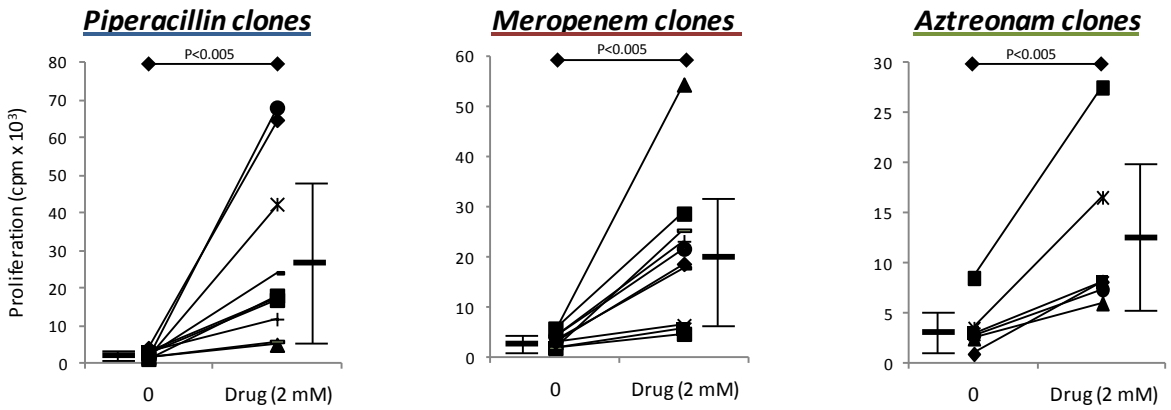


Figure 3.2 *Activation of T-cell clones with piperacillin, meropenem and aztreonam*
Clones were co-cultured with antigen presenting cells and drug (2mM) for 72 hours. Proliferation was measured with the addition of [^{3}H]-thymidine in the last 16 hours. Proliferative responses of representative clones are shown; piperacillin (*n* = 10), meropenem (*n* = 6) and aztreonam (*n* = 10).

The majority of drug-responsive clones were CD4+, however CD8+ and dual positive CD4+/CD8+ clones were also generated from the allergic patients. The dual positive T-cell clones expressed high levels of both CD4 and CD8 proteins. The number of piperacillin, meropenem and aztreonam-responsive clones generated from each patient and their phenotypic qualities are summarised in

Table 3.3. Average CPMs for all specific clones for the drug in question are detailed alongside the standard deviations of all the specific clones. Due to the large range of responses seen (some clones were only just positive with an SI of 2 -3, while others had SI's greater than 20), when grouped together standard deviations were extremely large. If greater number of clones were used it may have been possible to group clones as weak, average and strong responders. However, no previous literature offers advice as to what SI/CPM constitutes a weak or strong response and would therefore be a matter of individual preference. I therefore looked at responses by individual clones rather than solely looking at grouped data.

Fifty-five well growing CD4+ and CD8+ clones were selected from all four allergic patients and utilised in further assays. This pool of T-cell clones were used to characterise concentration-dependent proliferation, cytokine release and cross-reactivity.

Patient ID	Drug	Clones tested (*n*)	Specific clones (*n*)	Proliferation (CPM) Control	Proliferation (CPM) Drug (2mM)	Phenotype (%) CD4+	Phenotype (%) CD8+	Phenotype (%) CD4+/CD8+
1	Piperacillin	58	2	1422 ± 365	10512 ± 7907	100	-	-
	Aztreonam	111	2	2043 ± 788	6683 ± 2042	100	-	-
	Meropenem	111	5	4340 ± 3988	11744 ± 11173	80	20	-
2	Piperacillin	67	23	5477 ± 4137	36004 ± 23732	78	9	13
	Aztreonam	160	6	3529 ± 1761	15711 ± 19623	65	17	17
	Meropenem	100	4	1046 ± 441	2399 ± 938	100	-	-
3	Piperacillin	144	16	8133 ± 4353	53954 ± 18612	50	50	-
	Aztreonam	216	13	8984 ± 5963	23323 ± 12356	84	8	8
	Meropenem	72	1	3504	9550	100	-	-
4	Piperacillin	216	48	2134 ± 2412	24461 ± 18542	88	6	6
	Aztreonam	208	1	6867	33942	100	-	-
	Meropenem	200	5	3795 ± 2827	11493 ± 9648	100	-	-

Table 3.3 *Origin, phenotype and specificity of T-cell clones from allergic patients*
CPM = counts per minute.

3.4.3 Drug-antigen specificity of T-cell clones

Piperacillin, meropenem and aztreonam-responsive CD4+ and CD8+ T-cell clones were co-cultured with drug and EBV-transformed B-cells used as APCs. Clones proliferated in a drug-concentration dependent manner with maximal responses detected between 0.5 and 2mM (Figure 3.3). Fifty-five clones were tested for reactivity against piperacillin, meropenem and aztreonam and responses to each drug were found to be highly drug specific. Clones were stimulated to proliferate (Figure 3.3A) and release IFN-γ (Figure 3.3B) in the presence of the drug to which the PBMC was originally cultured to generate the T-cell clone, but not to the other β-lactam antibiotics. Essentially, a piperacillin-responsive clone, generated via PBMC culture with piperacillin and subsequent serial dilution is piperacillin-specific and will not be stimulated by the other drugs investigated; meropenem and aztreonam. This specificity was also observed in meropenem and aztreonam-responsive T-cell clones.

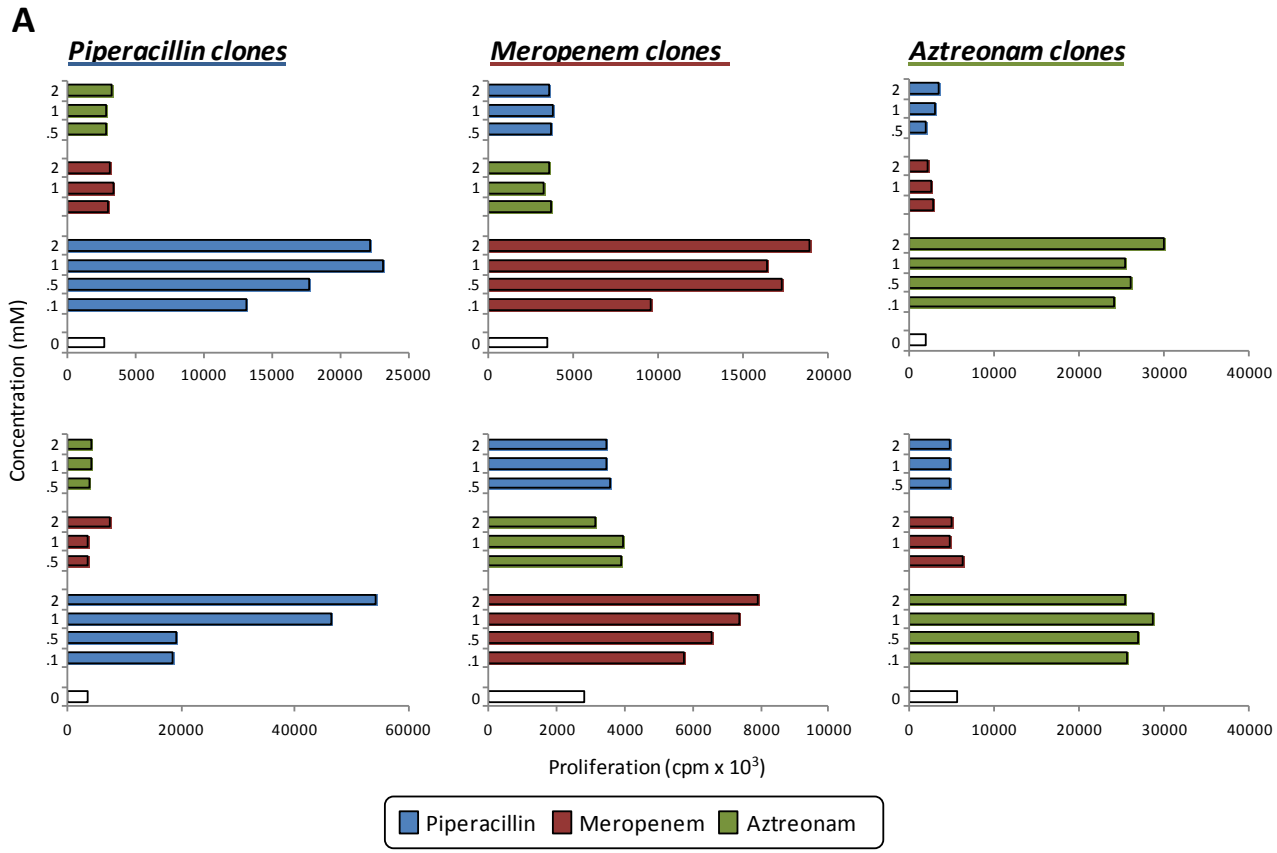


Figure 3.3 *Cross reactivity of piperacillin, meropenem and aztreonam-responsive T-cell clones*
T-cell clones were co-cultured with APCs and drug (0.1 – 2mM) with culture medium serving as negative control. **(A)** Proliferative responses were determined by [^{3}H]-thymidine incorporation which was added in the last 16 hours of the experiment. Results are shown as mean CPM of triplicate cultures. Six representative clones are shown. Coefficient of variation was consistently less than 20%. CPM = counts per minute. **(B)** The drug specific secretion of IFN-γ was determined and visualised by ELISpot assay. Well images from patient PBMCs which were cultured with drug for 48 hours. Plates were developed for spot visualisation according to manufacturer's instructions.

3.4.4 Elucidation of haptenic structures by mass spectrometric analysis of modified peptides

Previous work in our department was performed to fully characterisee the haptens formed from piperacillin binding to HSA and for comparative purposes the MS/MS spectrum of the ^{182}LDELRDEGKASSAK^{195} peptide modified at Lys190 with the cyclised form of piperacillin has been included in Figure 3.4A (Whitaker *et al.*, 2011b). The chemical structure of cyclised piperacillin is shown in Chapter 4, Figure 4.8. We wanted to utilise these techniques to characterise meropenem and aztreonam derived haptens. HSA was exposed to meropenem *in vitro* at a molar ratio of 20:1 (drug to protein) to characterize drug haptens. Careful examination of raw MS/MS data revealed the additions of four different masses (339,383, 396 and 440 amu) to tryptic peptides exhibiting a missed cleavage at lysine residues. Peptide ^{182}LDELRDEGKASSAK^{195} with a mass increase of 383 amu is shown in Figure 3.4B as an example. The retention times of the four different peptide species were identical however suggesting all four had been derived from in-source fragmentation of peptide plus 440 amu.

Mass addition of 339 and 383 amu were associated with drug-specific fragment ions of m/z 175 and 205, whereas mass additions of 396 and 440 were associated with fragment ions of m/z 205 and 232. These data together with the MS^3 spectra for the three fragment ions (Figure 3.5) were interpreted as follows. The drug hapten of 440 amu corresponded to the covalent binding of the parent meropenem iodoacetylated on the nitrogen at the 1' position (Figure 3.6). This gave rise to the immonium ion of Lysine modified with the 3-methyl-3H-pyrrole ring of meropenem (*m/z* 205) and the fragment ion of *m/z* 175 plus acetamide (232 amu; *S*-(5-(dimethylcarbamoyl)-pyrrolidin-2-yl)

carbamothioate). In-source cleavage of the acetamide group from the drug resulted in the drug hapten of 383 amu (440 minus 57 amu). In-source cleavage of the propanol at position 6, the carboxylic acid at position 2, or the trimethylyamine group at position 9' from the iodoacetylated drug hapten gave rise to the observed mass addition of 396 amu (440 minus 44) and the same fragment ions as observed for the hapten of 440 amu. Finally the mass addition of 339 amu was derived from the cleavage of both the acetamide group and the propanol, carboxylic acid, or trimethylamine group from the hapten of 440 amu (440 minus 57 minus 44) and the same fragment ions as observed for the hapten of 383 amu.

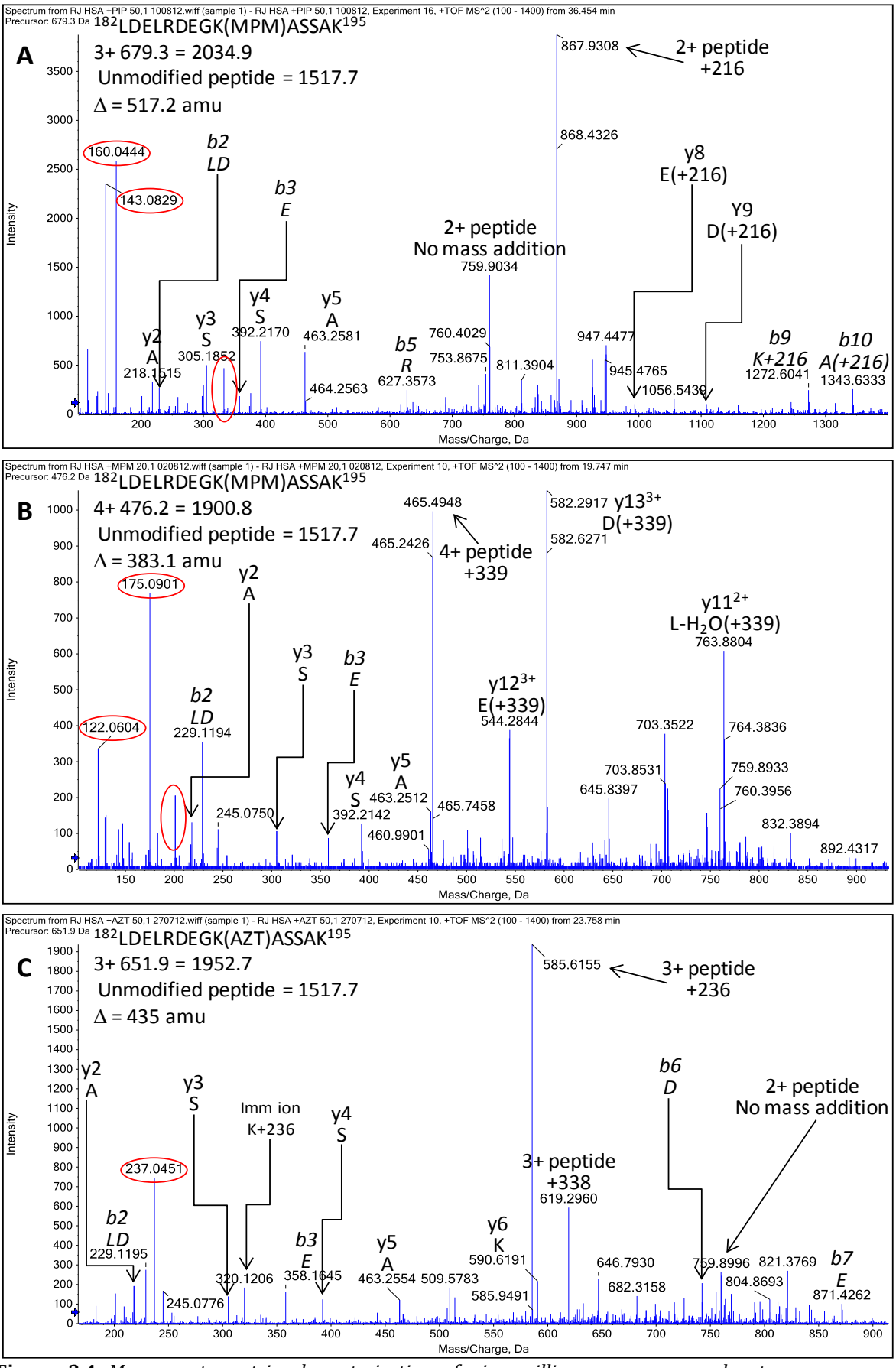


Figure 3.4 *Mass spectrometric characterisation of piperacillin, meropenem and aztreonam haptens formed on HSA*
Representative MS/MS spectra of peptide ^{182}LDELRDEGKASSAK^{195} modified on LYS190 with **(A)** piperacillin **(B)** meropenem and **(C)** aztreonam.

Due to cleaving by trypsin, the same peptides do not appear in the MS analysis of unmodified HSA, so it can be difficult to compare to drug-modified protein. Instead of LDELRDEGKASSAK there will be shorter peptide fragments seen, for example; LDELR, DEG and ASSAK. These are too small to be seen with our standard MS methods. However, the MS/MS spectra of the longer peptide can be seen ^{182}LDELRDEGK^{190} is shown in Figure 3.4.1.

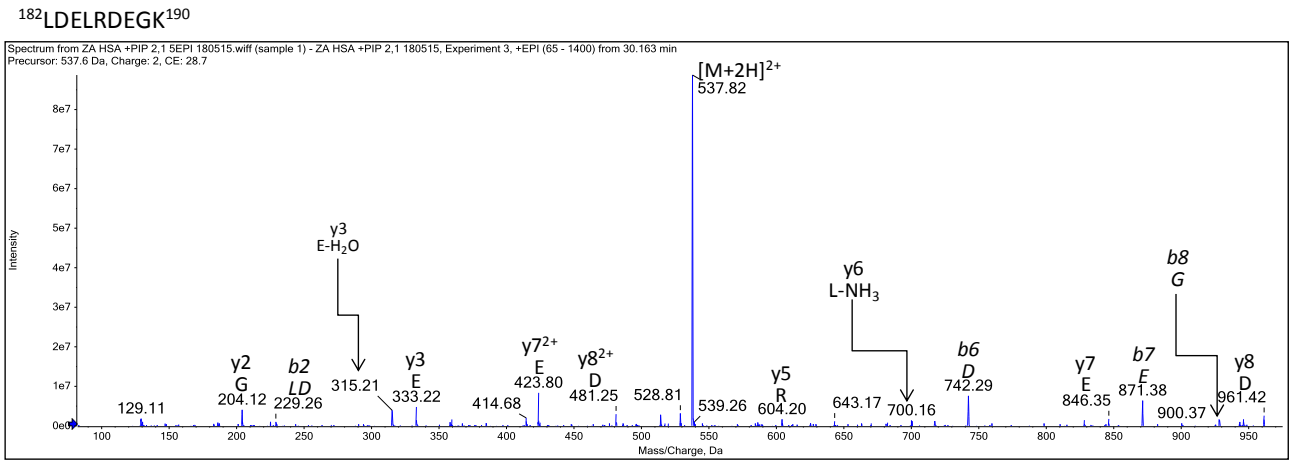


Figure 3.4.1 *MS/MS spectra of peptide ^{182}LDELRDEGK^{190}from unmodified HSA*

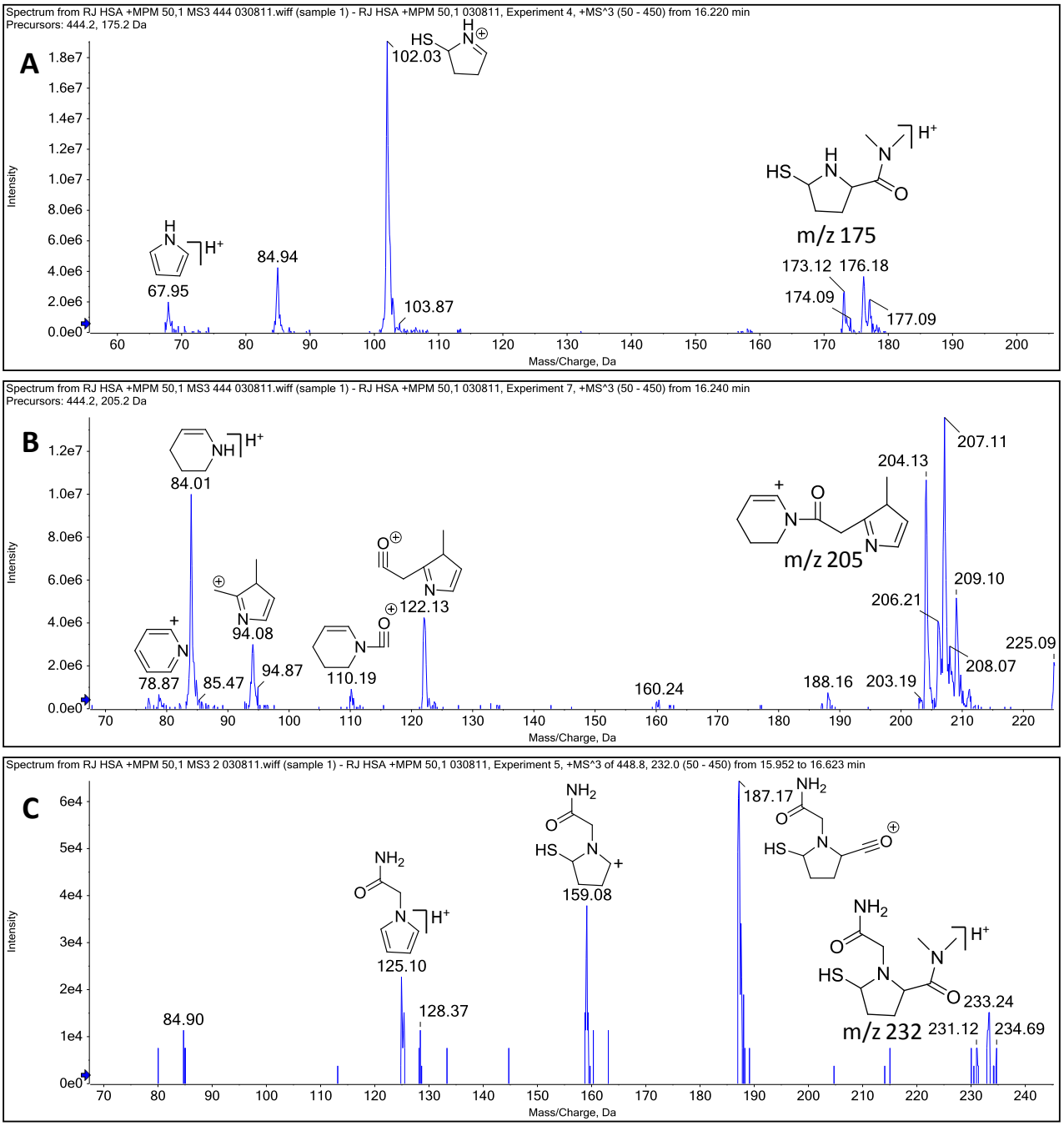


Figure 3.5 *Mass spectrometric characterisation of meropenem haptens formed on HSA*
Representative MS^3 spectra of characteristic fragment ions from meropenem-modified peptides.

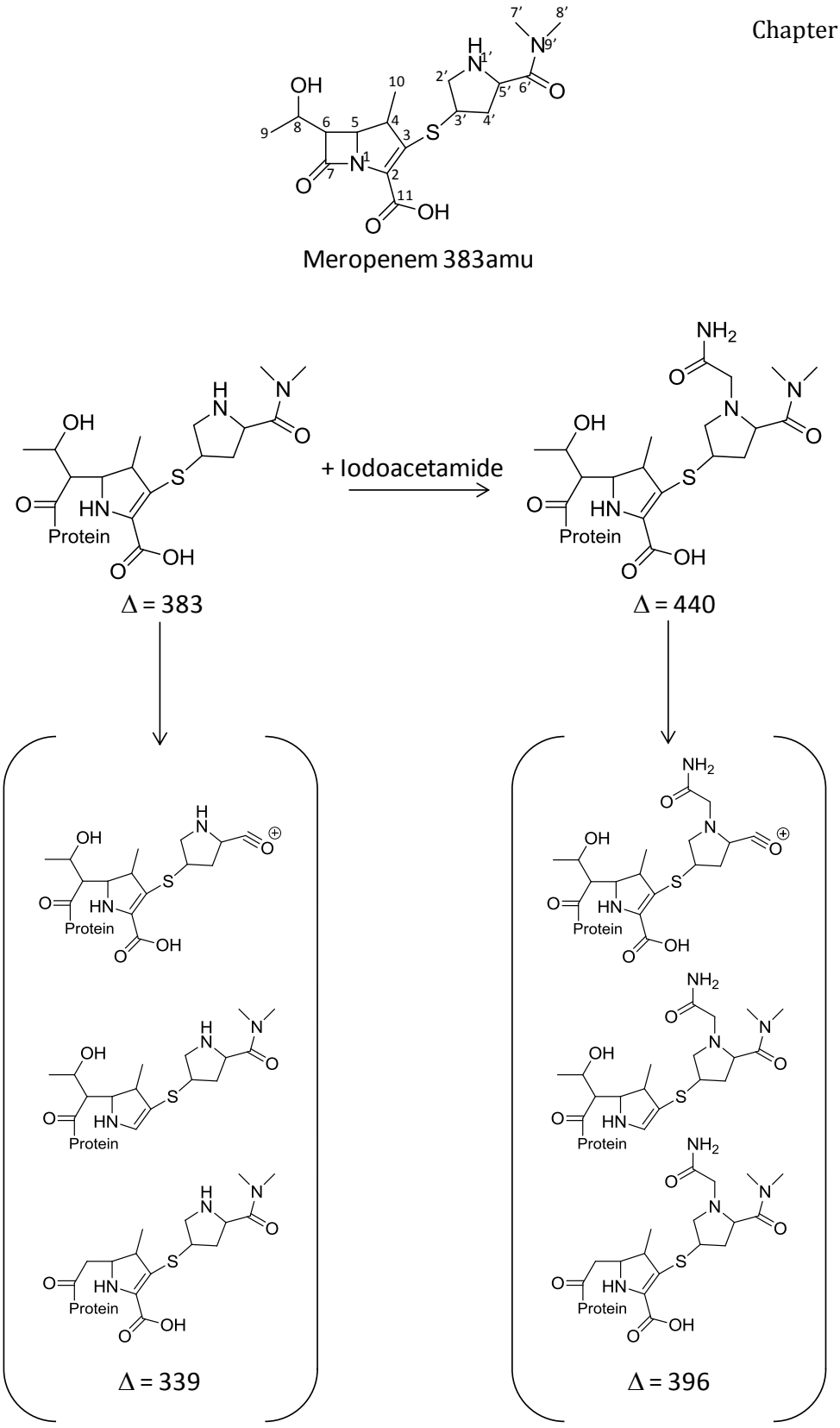


Figure 3.6 *Scheme detailing structure of proposed haptens formed via covalent binding of meropenem to HSA*

Meropenem forms a hapten by the opening of the β-lactam ring and nucleophilic attack by protein lysine residues, resulting in a mass increase of 383 amu for each modified lysine residue. In-source fragmentation of this hapten results in the loss of the propanol at position 6, the carboxylic acid at position 2, or the trimethylamine group at the 9' position (loss of 44 amu; mass increase of 339 amu). The chemistry is complicated by the methods conventionally used to process proteins for mass spectrometry analysis, namely iodoacetylation of cysteine. The drug itself was shown to be iodoacetylated, resulting in two further haptens of 440 and 396 amu.

HSA was modified with aztreonam *in vitro* at a molar ratio of 50:1 (drug to protein) to characterise potential haptenic structures. This higher ratio provided higher levels of HSA modification necessary for our experiments. Nucleophilic attack by the protein lysine residues and opening of the β-lactam ring was the pathway predicted for the covalent binding of aztreonam. This would lead to a mass increase on the peptides of 435 amu (Figure 3.8). The MS/MS spectrum of the peptide ^{182}LDELRDEGKASSAK^{195} modified with aztreonam confirms this (Figure 3.4C), with a mass addition of 435 amu to the peptide plus the presence of partial drug hapten of 338 and 236 amu illustrated in Figure 3.7. We propose the former is formed following the loss of the sulfonic acid and amino groups from position 1''' (Figure 3.8, a → b) and that the latter is formed following the further loss of 2-hydroxy-2methylpropanoic acid (Figure 3.8, c → d). The MS^3 spectrum of characteristic fragment ion from aztreonam-modified peptides is shown in Figure 3.7 below.

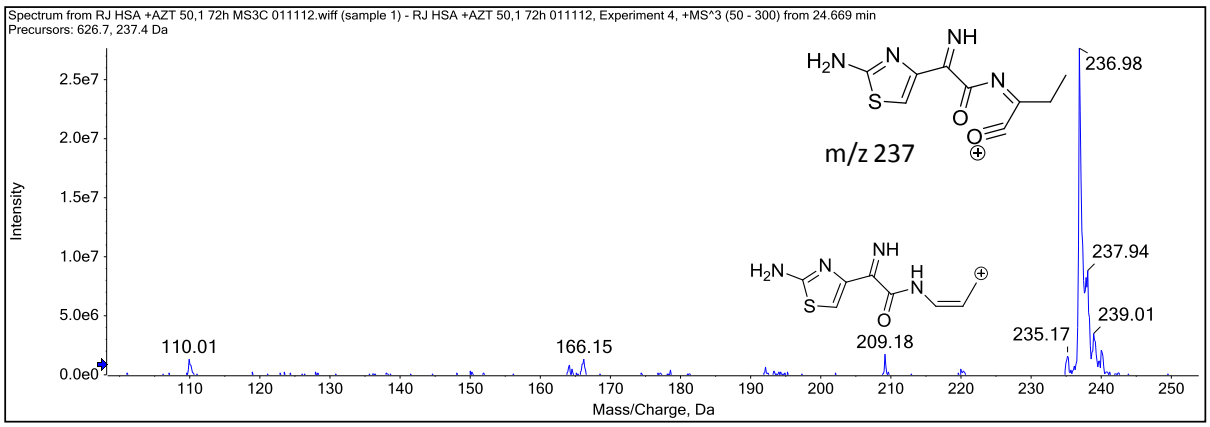


Figure 3.7 *Mass spectrometric characterisation of aztreonam haptens formed on HSA*
Representative MS^3 spectra of characteristic fragment ions from aztreonam-modified peptides.

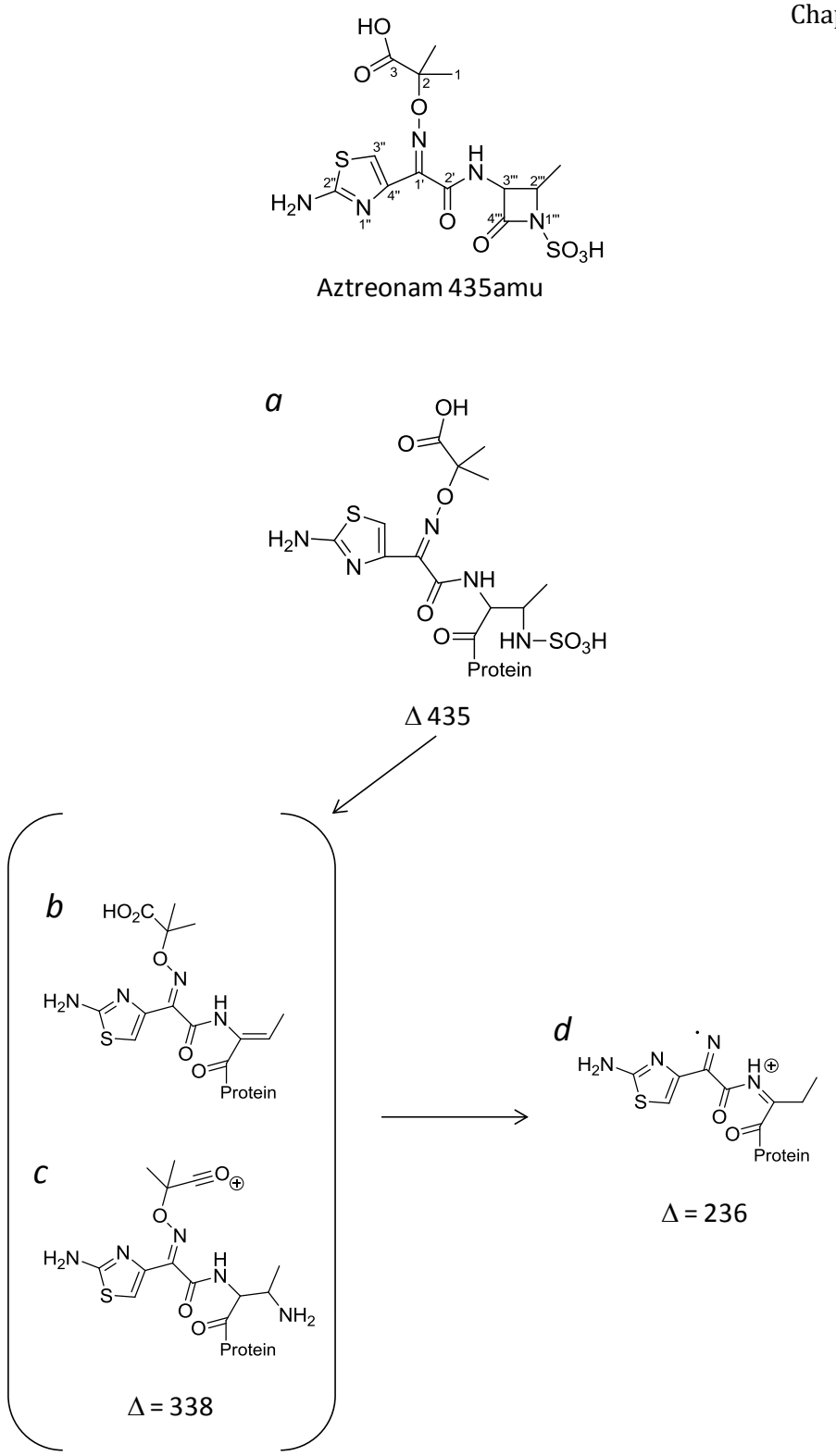


Figure 3.8 *Scheme detailing structure of proposed haptens formed via covalent binding of aztreonam to HSA*
Aztreonam forms a hapten by opening of the β-lactam ring, resulting in a mass increase of 435 amu for each modified Lysine residue. The hapten is labile in the mass spectrometer leading to the detection of partial haptens in the MS/MS spectra of modified peptides of 338 and 236 amu.

3.4.5 Time and concentration dependency of HSA modification with piperacillin, meropenem and aztreonam

Using LC-MRM-MS we have previously characterised the time and concentration dependency of the modification of HSA with piperacillin (Whitaker *et al.*, 2011b). After normalisation for total ion count, the MRM peak areas for the modified peptides were determined in order to assess changes in the relative abundance of those peptides; this is the epitope profile. This revealed that the levels of covalent binding increased with an increased molar ratio of drug to protein and with incubation time up to 72 hours (Figure 3.9C and 3.9D). Piperacillin consistently bound to Lysine at positions 190, 432 and 541 *in vitro* and *in vivo* and drug modifications were detected at an additional 10 out of the 59 Lysine residues in HSA *in vitro* (Figure 3.9B). These are nucleophillic addition reactions, and whilst other nucleophiles exist on HSA (i.e. cysteine and histidine), only lysine forms stable adducts with β-lactams.

A similar approach was adopted to characterise the HSA binding profiles of meropenem and aztreonam. Beginning with meropenem, the MRM peak areas for all four hapten signals were summed for each of the modified peptides. Up to 7 of the 59 Lysine residues in HSA were modified with meropenem after 24 hours (Figure 3.9B). The stability of meropenem in aqueous solution at 37°C is limited so after 8 hours little active drug will be present in the drug HSA incubations (Berthoin *et al.*, 2010; Keel *et al.*, 2011). Similarly, increasing the molar ratio of drug to protein up to a ratio of 20:1 resulted in an increase in signal for each modified peptide. However, no further increase in covalent binding could be detected at molar ratios exceeding 20:1 (Figure 3.9C).

Meropenem is sparingly soluble in water, limiting the maximal drug concentration available for reaction with the protein in aqueous solution.

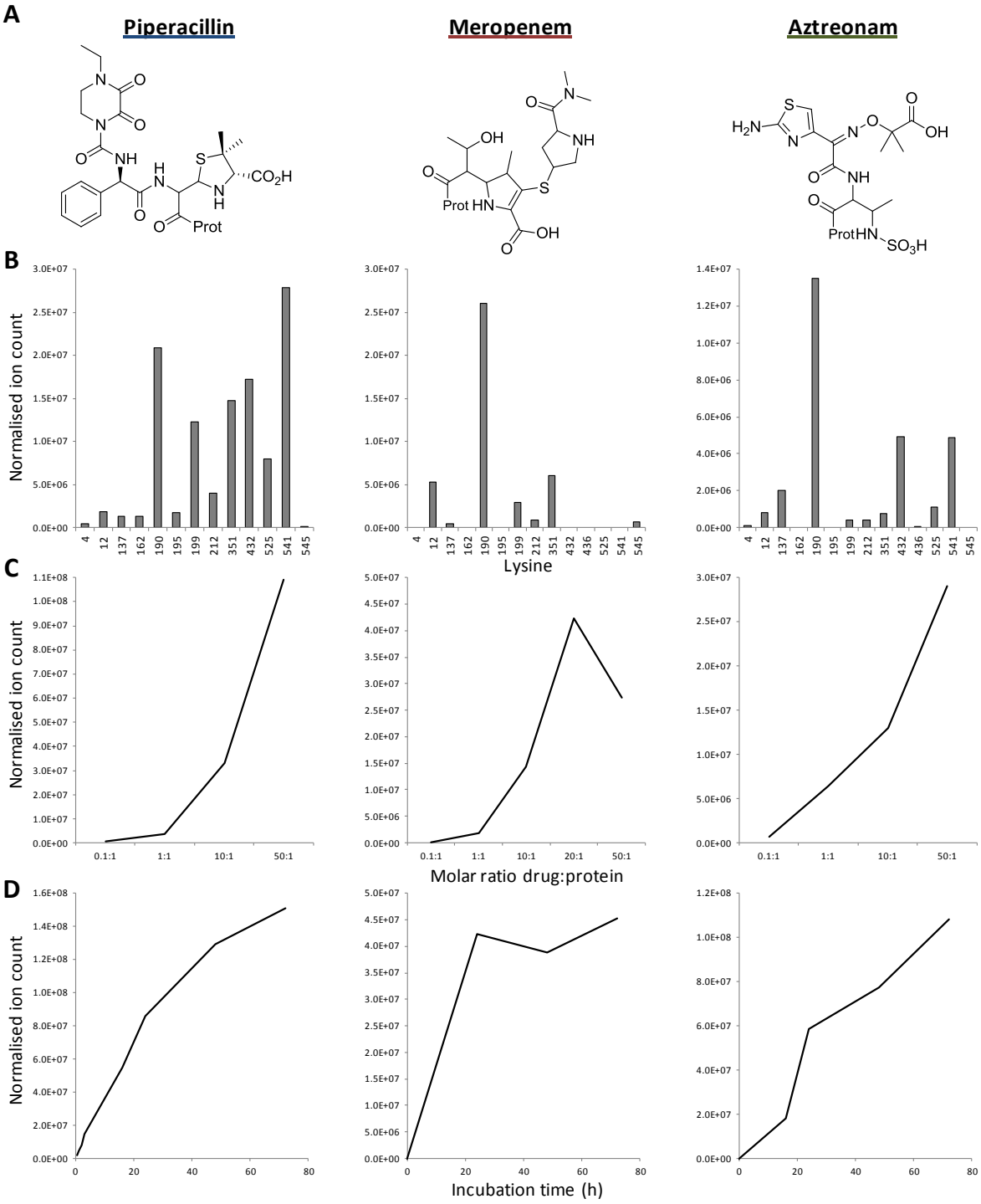


Figure 3.9 *Chemical characterisation of piperacillin, meropenem and aztreonam-HSA covalent binding profiles*
A. Hapten structures of the three drugs as bound to protein (prot). **B.** Epitope profiles showing the lysine residues in HSA which had been covalently modified by the three drugs *in vitro*, as determined by LC-MRM-MS/MS. **C.** Concentration dependency of total drug modification of HSA, as determined by LC-MRM-MS/MS. **D.** Time dependency of total drug modification of HSA, as determined by LC-MRM-MS/MS.

The modification of HSA by aztreonam was detected at 11 Lysine residues after 24 hours at a drug to protein ratio of 20:1. The levels of modification increased progressively with time and increasing drug concentrations (Figure 3.9C and 3.9D).

Whilst the same subset of Lysine residues are targeted by all of the β-lactam drugs investigated here, the relative abundance of modification at each Lysine differs between the three drugs. Lys190 was a primary target for modification with piperacillin, meropenem and aztreonam: the high signal observed was probably not due to the innate greater ionization efficiency of the peptide. We have reached this conclusion as observations from other work in the department has seen the same peptide modified with benzylpenicillin or ampicillin, further β-lactam antibiotics, detected at low relative signal.

3.5 Discussion

It is hypothesised that β-lactam antibiotics form covalent bonds with lysine residues on proteins in drug-exposed patients leading to the generation of antigenic determinants (Batchelor *et al.*, 1965; Jenkins *et al.*, 2009a; Whitaker *et al.*, 2011b). Several studies have shown that processing of β-lactam HSA conjugates liberates peptide sequences that stimulate T-cells, although it must be noted that naturally occurring drug-modified peptides eluted from MHC have yet to be characterised (Brander *et al.*, 1995a; Padovan *et al.*, 1997; Whitaker *et al.*, 2011b). *In vitro*, β-lactam antibiotics can bypass the requirement for protein processing by binding directly to MHC-associated peptides (Brander *et al.*, 1995a; Padovan *et al.*, 1996). Cross-reactivity studies using PBMCs and T-cell clones from penicillin allergic patients have revealed a plethora of drug

response profiles (Mauri-Hellweg *et al.*, 1996; Rozieres *et al.*, 2009; Sachs *et al.*, 2004). In most allergic patients, the peptide-bound penicilloyl ring structure is thought to represent the main structural determinant for T-cells, as β-lactams that contain a different core structure do not stimulate a response. However, individual T-cells can display a range of side-chain reactivity as penicillin, piperacillin and flucloxacillin specific T-cell clones have previously been shown to be cross reactive.

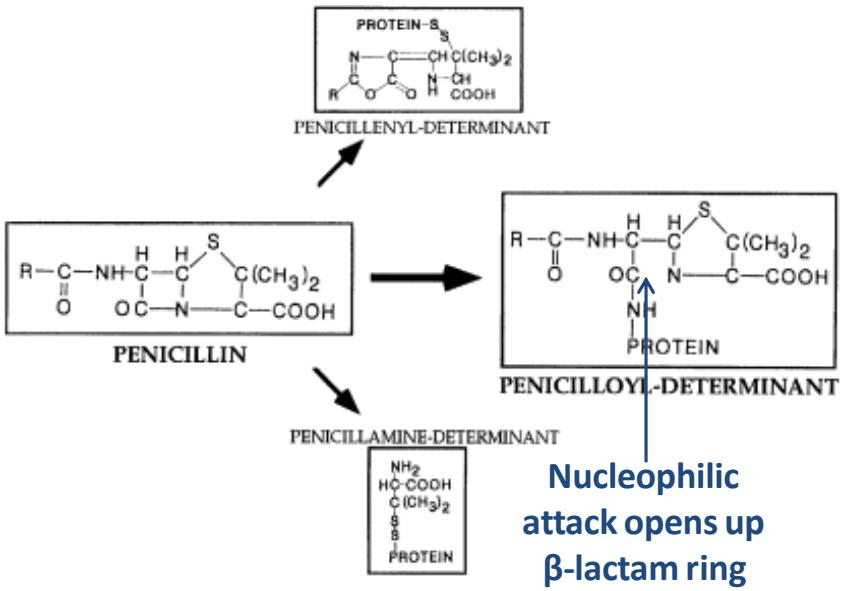


Figure 3.10 *Penicillin derivatives including the penicilloyl ring structure*

Indeed, cross-reactive clones can be stimulated with several penicillins, some of which the allergic patient will not previously have been exposed to. In contrast, other clones are incredibly drug antigen-specific. Our recent studies with mono-allergic patients revealed that piperacillin-responsive PBMCs and clones proliferated and secreted a mixed panel of cytokines including IFN-γ and IL-13 following drug stimulation. Interestingly, piperacillin-responsive clones were not stimulated with other penicillins, whereas several penicillins stimulated flucloxacillin-responsive T-cells (El-Ghaiesh *et al.*, 2012; Monshi *et al.*, 2013;

Whitaker *et al.*, 2011b). Collectively, these data suggest that different antigenic determinants derived from penicillins can be accommodated within the MHC binding cleft without interfering with the T-cell receptor binding interaction. However, the degree of cross-reactivity observed is drug and patient-specific and might be influenced by previous drug exposure. Unfortunately this information was not available to us at the time, although it may be possible to retrieve in the future as part of an ongoing prospective study currently underway at the University of Liverpool. Thus, there is a need to further delineate the structural features that determine T-cell receptor cross-reactivity.

Our investigation focussed on patients with a history of allergic reactions to piperacillin, meropenem and aztreonam. Piperacillin is a member of the penicillin class of antibiotics, which contains β-lactam and thiazolidine ring structures. Meropenem, a carbapenem licensed by the FDA in 1996, contains similar ring structures but the sulphur in the thiazolidine ring has been replaced with a carbon atom, and unsaturation has been introduced. Finally, the monobactam aztreonam is unusual in that the β-lactam ring is not fused to another ring structure. Superficially, the free drugs would appear to show some structural similarities, most notably the presence of the β-lactam ring, and these have been used previously to explain the observed immunological cross-reactivity within this class of antibiotics (Kishiyama *et al.*, 1994; Padovan *et al.*, 1996; Pham *et al.*, 1996; Zhao *et al.*, 2002). However, characterisation of the piperacillin, meropenem and aztreonam haptens formed following ring opening and binding to protein lysine residues reveals markedly different structures (Figure 3.9A) which it would be difficult to argue could drive a cross-reactive response. The chemical differences are further highlighted by the fact that the

drugs favour different covalent binding sites in our model protein, HSA (Figure 3.9B). This suggests that an alternative explanation is required for the multiple drug allergies observed in these patients.

To this end, T-cells from patients with MDH were used as a biological read-out of chemical cross-reactivity. Our objectives were to determine whether it was possible to detect lymphocyte responses to each drug in the allergic patients and to analyse T-cell receptor cross-reactivity through the generation of T-cell clones. Symptoms of the drug-induced immune reactions were classified according to patient's medical records. Patients 1, 2 and 4 developed maculopapular exanthema and/or non-immediate urticarial eruptions against the 3 drugs (Table 3.1). Patient 3 developed less-classical symptoms of drug allergy (arthralgia, nausea and vomiting). Reactions were relatively mild, but in every case treatment had to be discontinued. Each patient received and tolerated several courses of the individual drugs prior to the allergic reaction.

The LTT and IFN-γ ELISpot are the biological assays most commonly applied to diagnose drug allergy *in vitro* (Pichler et al., 2004; Rozieres et al., 2009). Previous studies with piperacillin (mono) allergic patients revealed almost all drug-responsive T-cells proliferate and secrete IFN-γ following drug stimulation (El-Ghaiesh *et al.*, 2012). Utilising both approaches we have shown that piperacillin, meropenem and aztreonam stimulate PBMC from allergic patients, but not drug-exposed tolerant controls, to proliferate and secrete cytokines. These data confirm our previous findings with piperacillin allergic patients and demonstrate that T-cell responses are also readily detectable with meropenem and aztreonam. The drug-specific response was dose-dependent

and in the case of one patient, who was recalled, LTT data was reproducible. PBMCs were stimulated with similar concentrations of the drugs. For three of the patients, piperacillin stimulated the strongest proliferative response and the highest levels of IFN-γ secretion, in comparison to the other drugs (Table 3.1 and Figure 3.1). The number of patients examined was limited due to time, however it is interesting to speculate that this may relate to intrinsic chemical reactivity and differences in the epitope profile of drug protein conjugates formed *in vitro* (Figure 3.2B). Although the absolute level of binding of each of the drugs to protein cannot be defined from the experiments presented here, the data show that piperacillin is more promiscuous in its binding profile suggesting a higher epitope density may be achieved (Figure 3.2B).

Here we have established the range of piperacillin concentrations necessary to elicit a positive T-cell proliferative responses and characterised the hapten formed *in vitro*. However, we are yet to elucidate the threshold of piperacillin haptenation required to trigger an immune response. Quantification of piperacillin modified HSA and subsequent peptides would help relate this to functional T-cell activation. Quantifying levels of piperacillin hapten in patients will aid in determining the minimum quantity of piperacillin antigen required to provoke T-cell activation and immune responses. Using novel mass spectrometric methods we addressed this gap of knowledge in chapter 5.

CD4+, CD8+ and CD4+/CD8+ T-cell clones were isolated from piperacillin, meropenem and aztreonam-stimulated PBMCs to explore T-cell receptor cross-reactivity. CD4+/CD8+ clones expressed high levels of both proteins indicating they somehow evaded thymic deletion. Interestingly, the number of CD4+/CD8+ T-cells is known to increase in certain pathogenic conditions

including certain auto-immune disease and chronic inflammatory disorders such as atopic dermatitis (Bang *et al.*, 2001). Using T-cell clones generated from the PBMCs of patients with MDH we measured antigen-specificity of the T-lymphocytes using proliferation and cytokine release as readouts. No cross-reactivity was observed in over 50 clones that were analysed (Figure 3.3). It may be possible that during the process of generating T-cell clones *in vitro* the clones were driven to become more specific to one particular drug; however this phenomenon had not been noted previously. Indeed, T-cells are more likely to cross-react with other drugs of related structures through epitope spreading. These data clearly demonstrate that non-immediate hypersensitivity reactions to different classes of β-lactam antibiotics in patients with cystic fibrosis are instigated not by cross-reactive T-cells, but through priming of naive T-cells against the different drug antigens. Daubner *et al.* have recently demonstrated that drug-responsive T-cells in certain patients with a history of multiple drug allergy reside in an *in vivo* activated (CD4+CD25dim, with elevated CD38 and PD-1) T-cell fraction (Daubner *et al.*, 2012). It would be interesting to explore whether a similar phenotype of drug-responsive T-cells exist in patients with CF and multiple drug allergies, and whether increased numbers of such T-cells explains why multiple drug allergy is such a common occurrence in CF patients.

Data from this chapter has shown that piperacillin, meropenem and aztreonam-specific T-cell responses are detectable in CF patients with MDH and reactions are likely due to a primary immune response rather that T-cell cross-reactivity. Furthermore this has led to the characterisation of the complex haptenic structures on distinct lysine residues on HSA which provides a chemical basis for the drug specific T-cell response.

Chapter 4: Characterisation of piperacillin-modified human serum albumin and its role in the activation of piperacillin-specific T-cells

4.1 Introduction

Antibiotic hypersensitivity is a major health concern for patients with CF. Hypersensitivity to β-lactam antibiotics occurs in up to 50% of patients with CF compared to an incidence of 0.5-10% in the general population (Burrows *et al.*, 2007; Parmar *et al.*, 2005; Pleasants *et al.*, 1994). Piperacillin, commonly used as part of a treatment regimen for patients with CF, is one such culprit drug implicated in drug hypersensitivity. Symptoms of hypersensitivity include MPE, fixed drug eruptions, fever and flu-like symptoms. Despite the high rates of hypersensitivity, continuation of β-lactam therapy is vital for these patients. The nature of CF means that mucus can quickly accumulate in the airways, an environment which bacteria exploit leading to frequent chronic infections. The condition of the lungs is therefore critically dependent on the sustained use of antibiotics.

Antibiotics are most commonly delivered to patients with CF via an intravenous injection resulting in rapid accumulation of the drug. In the case of piperacillin, patients are typically given 4.5g every 6-8 hours for 2 weeks (CysticFibrosisTrust, 2009). This regimen is in stark contrast to the lower oral dose given to the general population where for acute bronchitis 500mg amoxicillin, three times a day for 5 days is recommended (NICE, 2014). The increased incidence of β-lactam hypersensitivity in patients with CF provides a pool of individuals whereby the mechanisms of drug antigenicity and immunogenicity can be investigated.

The hapten hypothesis is one widely accepted theory for the development of hypersensitivity reactions. It proposes that small molecules form novel antigenic determinants through covalent binding to host proteins and that this

binding interaction determines whether exposure will result in an aberrant immune response (Weltzien *et al.*, 1996b). Drugs, including the β-lactam antibiotics, are thought to activate immune cells through covalent modification of proteins (Padovan *et al.*, 1997; Weltzien *et al.*, 1996b). β-lactam antibiotics form covalent bonds with lysine residues on proteins (Brander *et al.*, 1995a; Padovan *et al.*, 1997). The nature of the binding interaction is well established with binding sites identified in model proteins with numerous drugs including penicillin G, piperacillin, amoxicillin and flucloxacillin (Batchelor *et al.*, 1965; Jenkins *et al.*, 2009a; Levine *et al.*, 1961b; Meng *et al.*, 2011a; Yvon *et al.*, 1990). The β-lactam ring is targeted by nucleophilic lysine residues on proteins. Subsequently, the ring opens allowing the binding of the penicilloyl group and the formation of a drug-protein conjugate with the potential to act as an antigen and activate specific T-cells (Batchelor *et al.*, 1965; Levine *et al.*, 1961b).

Recent advances in mass spectrometry methods have allowed the detailed characterisation of drug modified amino acid residues on proteins, both *in vivo* in plasma samples of hypersensitive patients and *in vitro* (Whitaker *et al.*, 2011b). Penicillin and piperacillin albumin adducts have been shown to stimulate immune cells (Brander *et al.*, 1995a; El-Ghaiesh *et al.*, 2012). Furthermore, El-Ghaiesh *et al* have shown that the activation of T-cells with piperacillin-HSA conjugates is dependent on antigen processing within the antigen presenting cell. The use of solvents including methanol or acetone to precipitate drug-bound proteins, dialysis or chromatography to remove free drug are traditional methods in the generation of drug-protein conjugates. New methods continue to emerge though, specifically the use of centrifugal filters or "spin column" has assisted in the generation of protein adducts that are

"essentially" free of parent drug. With this method there is an absence of solvent in the purification process which has the potential to alter protein structure. In this chapter, in addition to using a previously employed method within the department for the generation of a synthetic piperacillin-HSA conjugate, we used a new method involving centrifugal filters to generate a second synthetic conjugate to investigate further the role of covalent binding in the development of drug hypersensitivity.

The human plasma proteome is complex (over 10,000 proteins - www.plasmaproteomedatabase.org/) so that there is the potential for large numbers of drug-protein adducts and drug modified peptides to be generated. However, HSA is considered as a potential candidate of an endogenous protein carrier for a number of reasons. Primarily, HSA is the most abundant protein in human plasma accounting for 57-71% w/w of total protein (Greenough *et al.*, 2004). 16-22% of an intravenous dose of piperacillin is non-covalently bound to plasma proteins and the majority of covalently bound penicilloyl groups in plasma are bound to HSA (El-Ghaiesh *et al.*, 2012). Furthermore, human serum is also commonly used as a supplement to cell culture media and has been shown to be modified by β-lactams including piperacillin *in situ* (Bertucci *et al.*, 2002; Nerli *et al.*, 1997; Whitaker *et al.*, 2011b).

Though derivatives of the penicillin class of β-lactam antibiotics can form antigenic determinants through binding to cellular proteins, the rate at which this binding occurs is much slower in comparison to serum proteins (Warbrick *et al.*, 1995). Indeed previous work in the department found no evidence for the formation of cellular adducts with piperacillin, albumin was the primary drug target (El-Ghaiesh *et al.*, 2012). Nevertheless, we cannot disregard binding to

cellular proteins as traditional methods for the detection of modified proteins may not be sufficiently sensitive, or indeed inappropriate cells/proteins may have been studied. Recently, using confocal fluorescence microscopy and biotinylated amoxicillin, the higher sensitivity of detection revealed intracellular protein adducts and modified proteins in a range of cell lines (Ariza *et al.*, 2014). However, as the main target protein for penicillins, HSA was used as a model protein to explore the role of piperacillin-protein conjugation in the activation of piperacillin-specific T-cells.

4.2 Aims and Hypothesis

There are limited studies that characterise the chemistry of drug-protein binding and the pathways which link antigen formation to the immune response seen in patients. This chapter aims to begin to bridge this gap of knowledge through:

(1) Generation and characterisation of piperacillin specific T-cell clones from hypersensitive patients to investigate the mechanisms involved in piperacillin protein binding, antigen presentation and T-cell stimulation.

(2) Generation of synthetic piperacillin albumin conjugates using new and established techniques and assessment of their ability to activate T-cells.

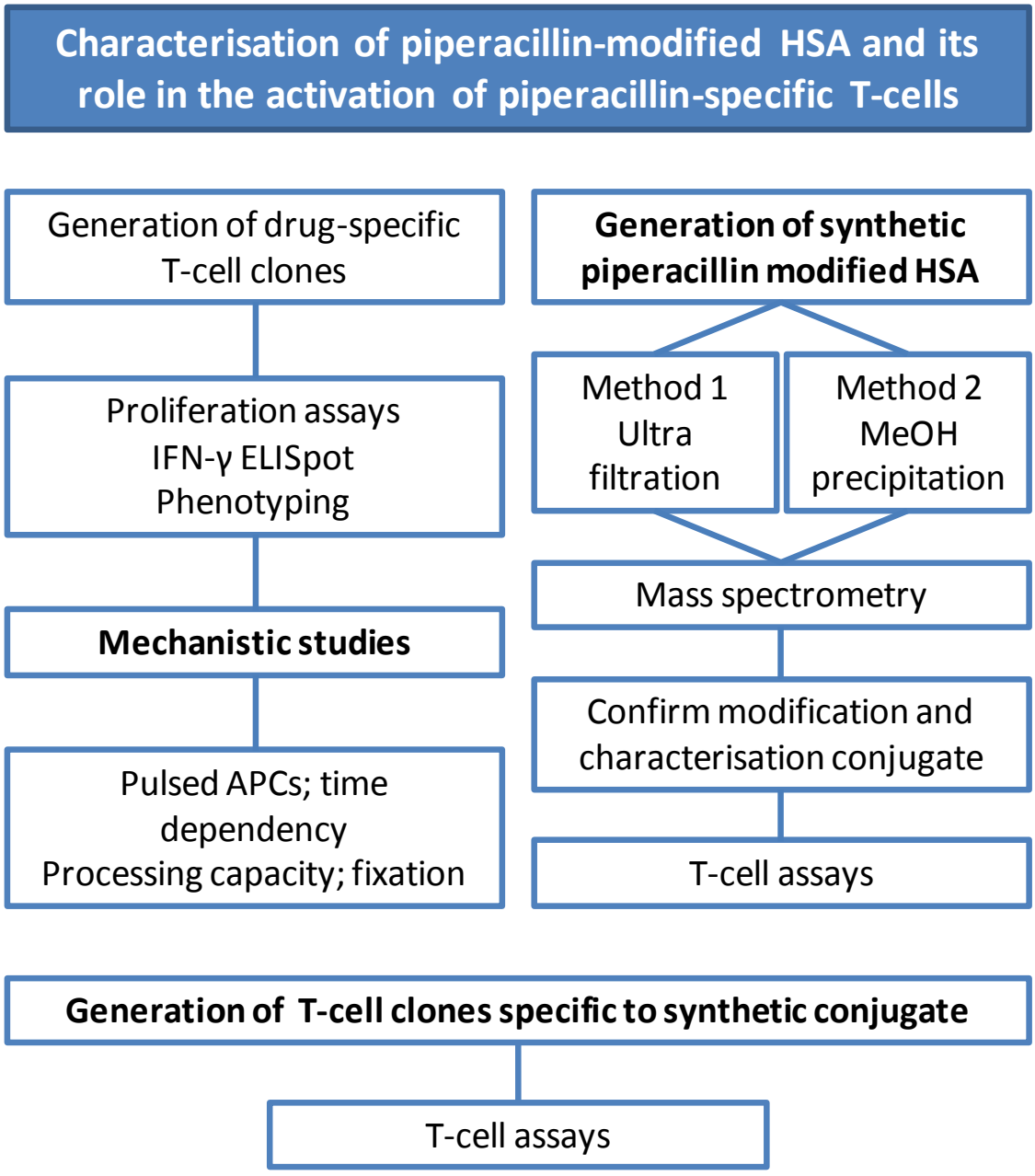
From work previously conducted in this department, and by other groups looking at drug-protein binding I propose that:

(1) Protein binding is an essential part of the mechanism for piperacillin hypersensitivity

(2) Synthetic protein conjugates (made by either method) will stimulate piperacillin specific T-cell clones

4.3 Methods

The methods used are described fully in Material and Methods, Chapter 2. A brief experimental outline is detailed below.



Scheme 4.1 *Experimental plan and methods used in this chapter*

4.4 Results

4.4.1 Piperacillin specific T-cell clones

PBMCs from piperacillin hypersensitive patients (patient details shown in Table 4.1) were used to generate 1,699 T-cell clones of which over 1,000 were specific to piperacillin (Table 4.2). A pool of 123 antigen-specific T-cell clones were expanded for use in assays to assess function and to investigate piperacillin antigenicity and immunogenicity. These experiments took place over the course of a year and clones from each patient used, was dependent on availability at the time. Owing to the large number of clones generated from patient H3 only a small subset were expanded and their phenotype determined. Those not phenotyped were frozen and stored. The majority of the T-cell clones, 91%, were CD4+ with 7% found to be CD8+ and 2% double positive. CD4+ T-cell clones were used in subsequent experiments. Clones proliferated in response to piperacillin in a dose dependent manner and secreted significant amounts of the Th1 cytokine IFN-γ when exposed to the drug. A sample of piperacillin specific clones highlighting proliferative responses, cytokine secretion and characteristic phenotypes are shown in Figures 4.1 and 4.2.

Patient	Age/ Gender	Drug	Reaction	Time from treatment to reaction (days)	Time since reaction (years)	Number of courses prior to reaction
H1	18/M	Tazocin	MPE/Fevers	9	7	3
H2	17/M	Piperacillin	Flu-like illness	2	5	5
H3	19/M	Tazocin	MPE/Fever	2	0.5	4
H4	23/M	Tazocin	MPE	11	4	9
H5	26/M	Tazocin	MPE	5	2	3
H6	28/F	Tazocin	Fever/Arthralgia	9	6	7

Table 4.1 *Patient characteristics*
Clinical details of piperacillin-hypersensitive CF patients. Tazocin = medicine made up of the antibiotic piperacillin and the β-lactamase inhibitor tazobactam, MPE = maculopapular exanthema.

Patient ID	Clones tested (*n*)	Specific clones (*n*)	Phenotype CD4 +	CD8+	CD4+/CD8+
H1	7[a]	2	2	-	-
H2	58[a]	12	12	-	-
H3	1632	1412 (100 expanded, 50 phenotyped)	48	2	-
H4	21[a]	12	12	-	-
H5	67	21	19	1	1
H6	208[a]	26	19	5	2
Total	**1993**	**1485**	**112 (91%)**	**8 (7%)**	**3 (2%)**

Table 4.2 *Number and phenotype of T-cell clones generated*
T-cell clones were generated from 6 piperacillin-hypersensitive patients with CF. [a] specific clones generated by colleagues within the department and kindly donated for experimental work. Flow cytometry was used to determine phenotype of T-cells.

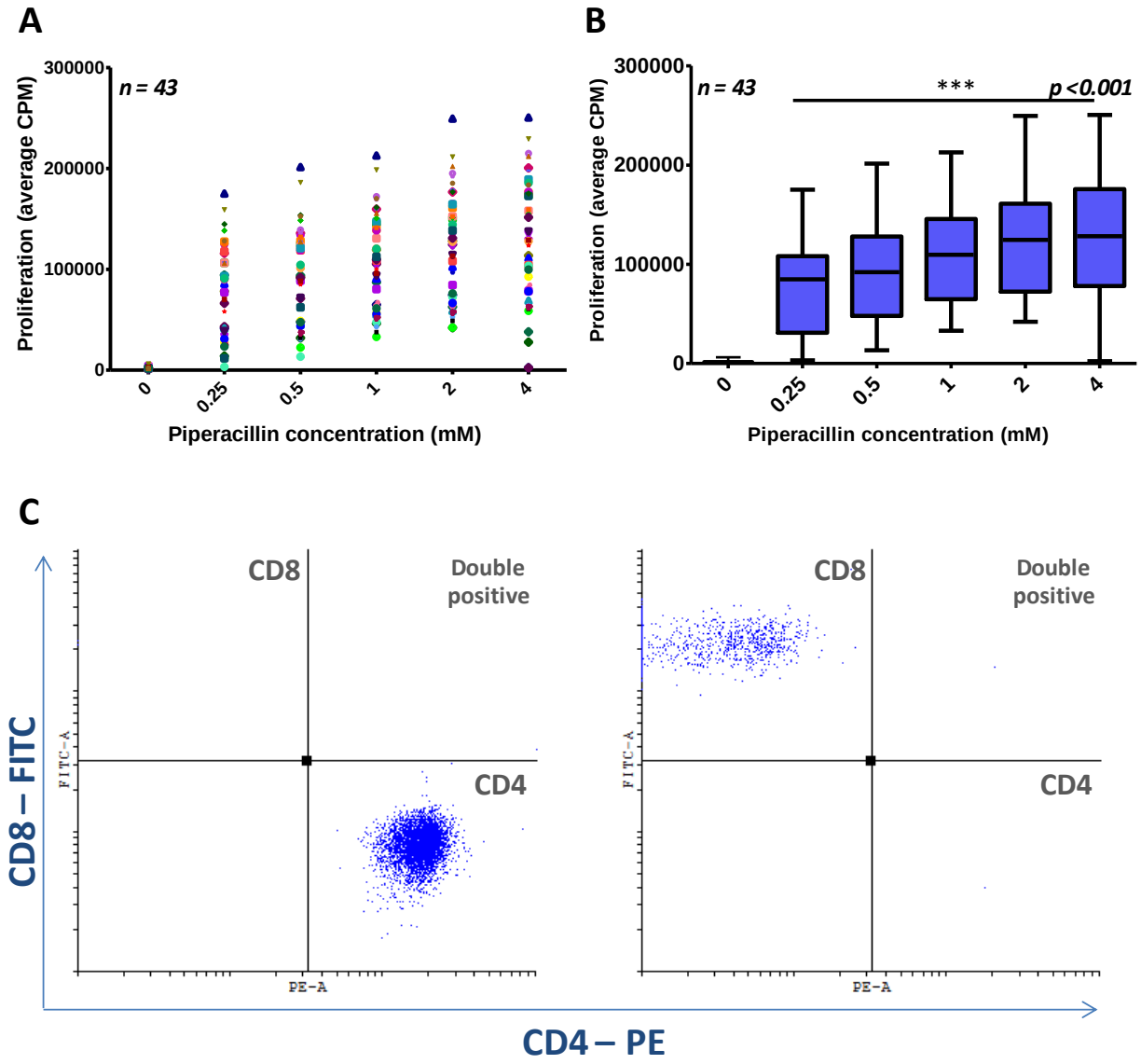


Figure 4.1 *Antigen specific proliferation and phenotype of T-cell clones*
(A) T-cell clones were co-cultured with antigen presenting cells and piperacillin (0 – 4mM) for 96 hours. Proliferation was measured with the addition [3H]-thymidine in the last 16 hours. Each data point represents mean [^{3}H] incorporation from 43 piperacillin specific clones with mean cpm and range shown in **(B)** *** = P value < 0.001
(C) Flow cytometry showing phenotype of T-cells. CD4+ phenotype was observed for majority of T-cell clones. Representative traces for CD4+ and CD8+ T-cell clones are shown here.

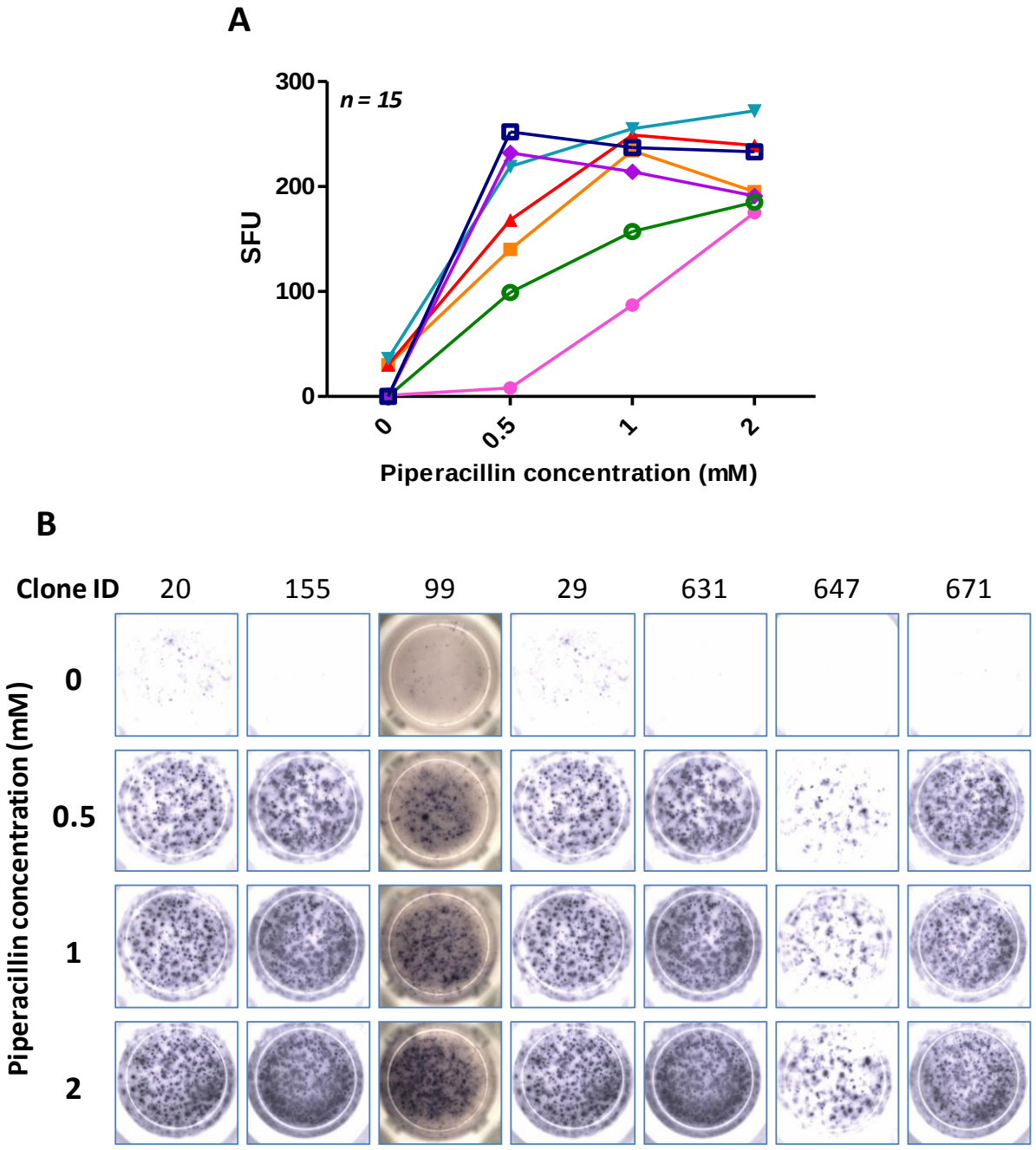


Figure 4.2 *T-cell clones secrete IFN-γ in a dose-dependent manner*
Piperacillin specific secretion of IFN-γ was determined and visualised by ELISpot assay. Plates were pre coated with human IFN-γ antibody overnight. T-cell clones were co-cultured with APCs and piperacillin (0 – 2mM) for 48 hours at 37°C. The plates were then developed according to manufacturer's instructions. Once dry spots were visualised **(B)** and counted **(A)** using AID ELISpot reader. 7 representative clones shown. SFU = spot forming units.

4.4.2 T-cell clones are stimulated by piperacillin pulsed antigen presenting cells

To investigate the relationship between time-dependent piperacillin protein binding and the T-cell response, antigen presenting cells (APCs) were pre-incubated, i.e. pulsed, with piperacillin for 1 – 48hrs to allow antigen formation and processing to occur before use in T-cell proliferation assays. Drug-exposed EBV-transformed B-cells were washed three times to remove unbound piperacillin prior to incubation with piperacillin-specific T-cell clones. T-cell clones were then incubated with pulsed APCs in the absence of soluble drug for 72 hours with [^{3}H] thymidine (0.5µCi) added in the last 16 hours of the incubation to assess lymphocyte proliferation. Clones were activated to proliferate with APCs pulsed with piperacillin for 24 and 48 hours. In contrast, little proliferation was detected at the earlier time points of 1 and 4 hours. The stimulatory response observed after a 24 hour APC pulse was reproducible and comparable to that with soluble drug (Figure 4.3B). This pattern of was observed with multiple clones generated from two piperacillin allergic patients (Figure 4.3A). Representative data from two clones are shown in Figure 4.3B.

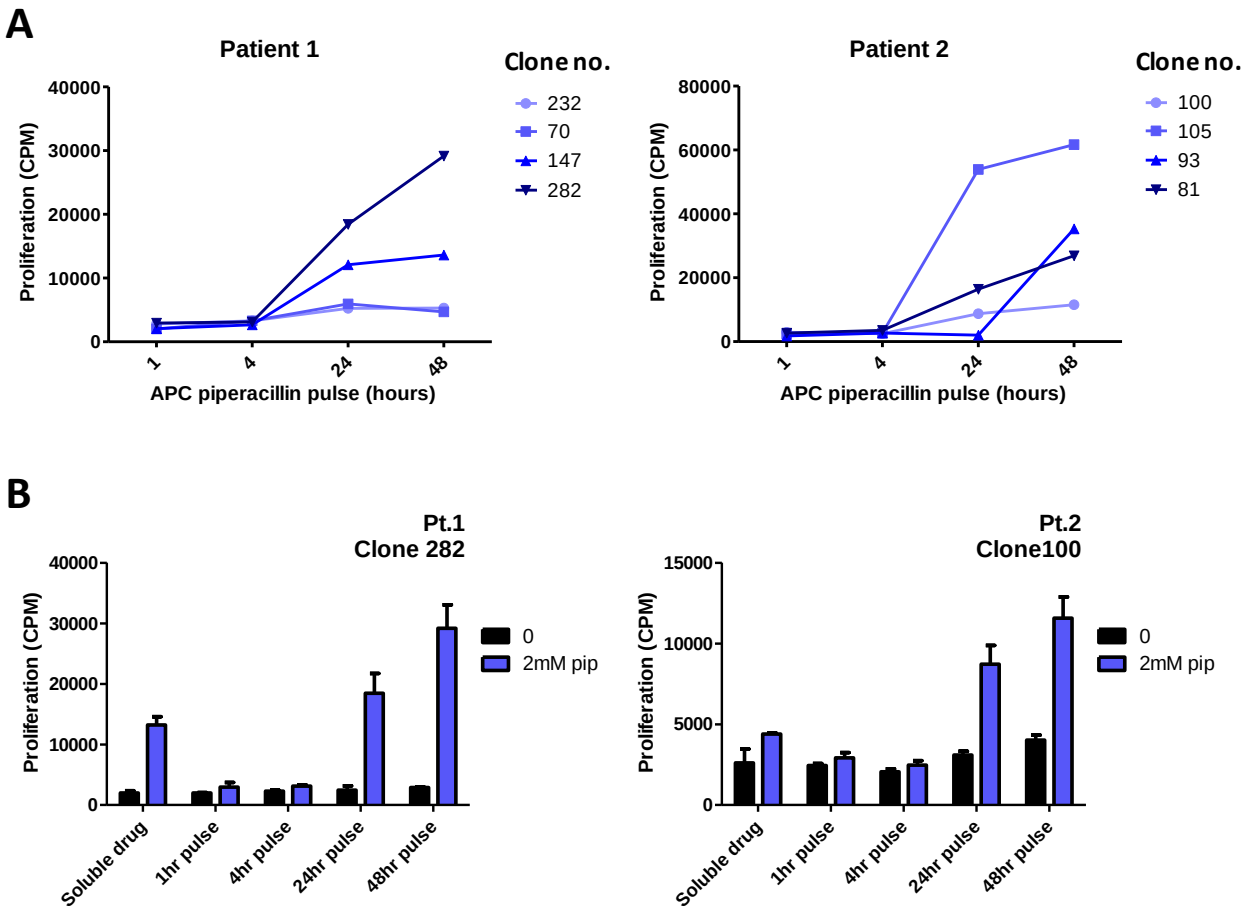


Figure 4.3 *T-cell clones are stimulated by piperacillin pulsed antigen presenting cells*
EBV-transformed B-cells were pulsed with piperacillin (2mM) and culture medium as a control for 1, 4, 24 and 48 hours. Cells were washed three times to remove unbound drug before co-culture with T-cell clones for 72 hours with [^{3}H]-thymidine added in the last 16 hours to assess proliferation. **(A)** 4 clones from two hypersensitive patients were cultured with piperacillin pulsed APCs and proliferation quantified by [^{3}H]-thymidine incorporation. **(B)** Individual data from 2 representative clones with soluble drug included as a comparator are shown (CPM = counts per minute).

4.4.3 Processing is required to activate piperacillin specific T-cell clones

In order to investigate the role of antigen processing in the activation of piperacillin-specific clones, APCs were fixed with 0.025% glutaraldehyde to inactivate protease activity prior to co-incubation with piperacillin-specific T-cell clones and soluble drug. T-cell clones were not stimulated to proliferate when cultured with piperacillin (Figure 4.4).

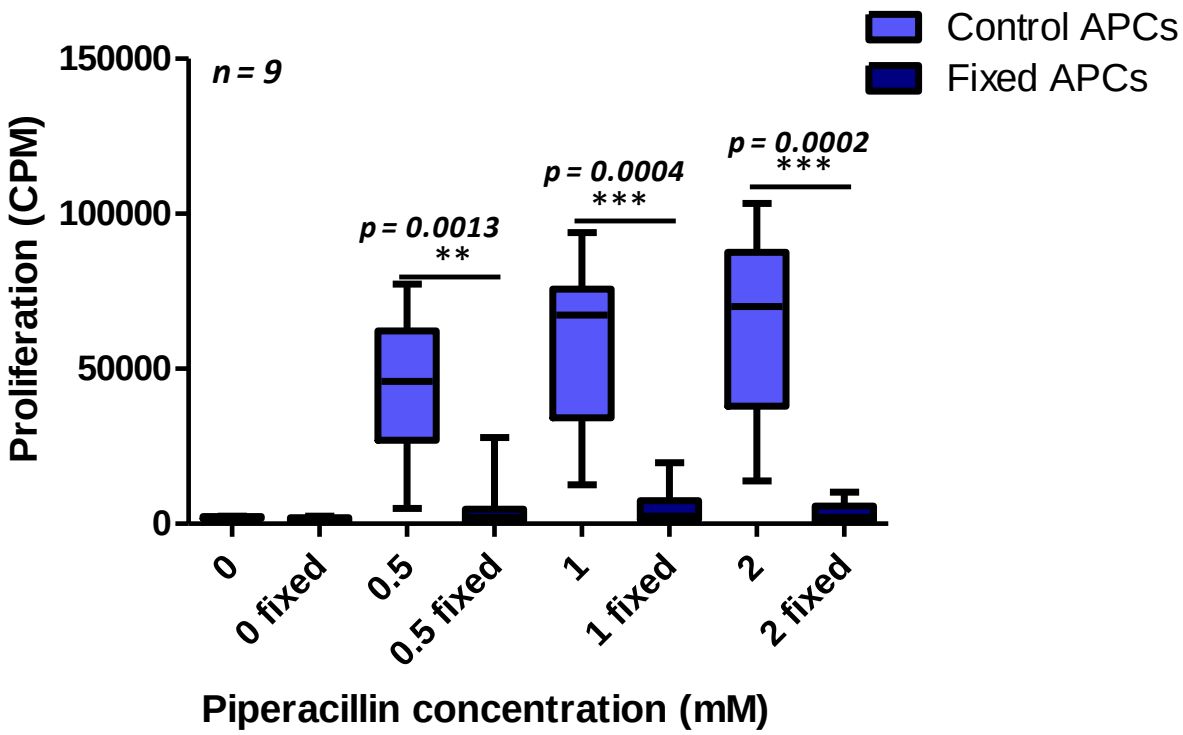


Figure 4.4 *Processing is required for the activation of T-cell clones*
T-cell clones (*n* = 9) were incubated with piperacillin (0 - 2mM) and with either irradiated autologous APCs (control APCs, light blue boxes) or APCs which had undergone glutaraldehyde fixation (fixed APCs, dark blue bars). Proliferation was determined by [^{3}H]-thymidine incorporation, CPM = counts per minute. ** P < 0.01, ***P < 0.001

A minority of clones (approximately 20%) were activated with piperacillin in the absence of APCs (Figure 4.5). These clones were excluded from mechanistic analysis.

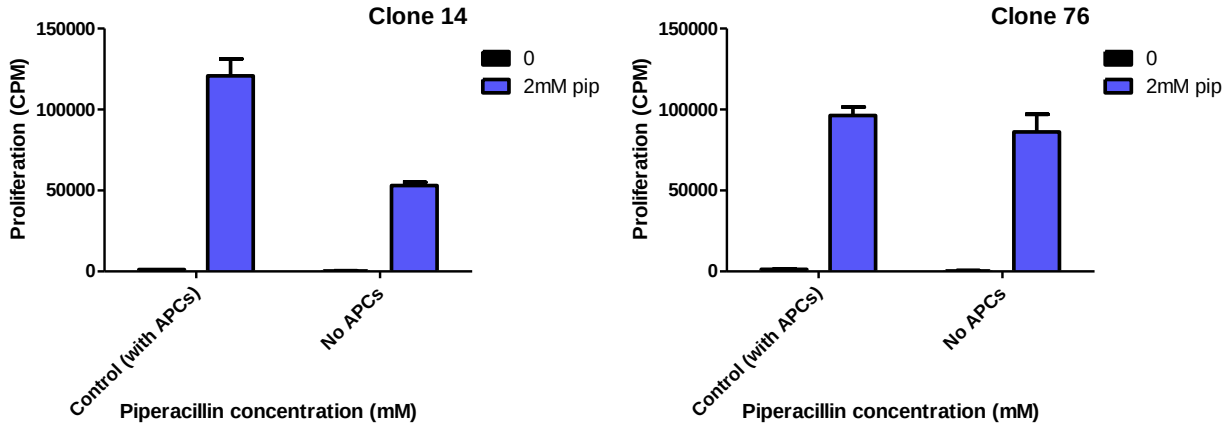


Figure 4.5 *Certain T-cell clones are activated in the absence of APCs.*
A small proportion of clones (~ 20%) were activated with piperacillin alone. Their proliferative capacity in the absence of APCs is shown here alongside control conditions with APCs for comparative purposes. T-cell clones were cultured with 2mM piperacillin and autologous irradiated APCs (standard proliferation assay) or without APCs for 72 hours. [^{3}H]-thymidine was added in the last 16 hours to determine cell proliferation. CPM = counts per minute.

4.4.4 Conjugates

The data presented in Figures 4.3 – 4.5, show T-cell proliferative responses are antigen processing dependent and involve the formation of a drug protein adduct. To explore whether a synthetic model drug protein adduct activates clones, piperacillin-modified HSA was synthesised off-line and added to the T-cell assays as a source of antigen in the absence of free drug. Synthetic albumin conjugates modified with piperacillin have previously been shown to stimulate piperacillin specific T-cell clones (El-Ghaiesh *et al.*, 2012). New methods for the purification of conjugates have since been developed in which the level of free drug is more stringently controlled and measured. With these more defined conjugates, it was possible to investigate mechanisms of T-cell activation in greater detail.

4.4.4.1 Conjugate generation

Two methods for the generation of synthetic piperacillin-HSA conjugates were used and applied for experiments in this chapter. Piperacillin was incubated with HSA at 37°C for 24 hours in phosphate buffer to generate synthetic drug-protein conjugates. Conjugates were then transferred to 15ml spin columns and washed to remove free drug. Hereafter the synthetic piperacillin-HSA conjugates and HSA control conjugates (incubated with phosphate buffer alone) generated via this method shall be referred to as conjugate 1. Conjugate generated and purified via MeOH precipitation is referred to as conjugate 2. Detailed methods are described in Material and Methods Chapter 2 2.5.14. Figure 4.7 briefly summarises methods and profile of conjugates and the 3-D model illustrating sites of modification by piperacillin are shown in Figure 4.6.

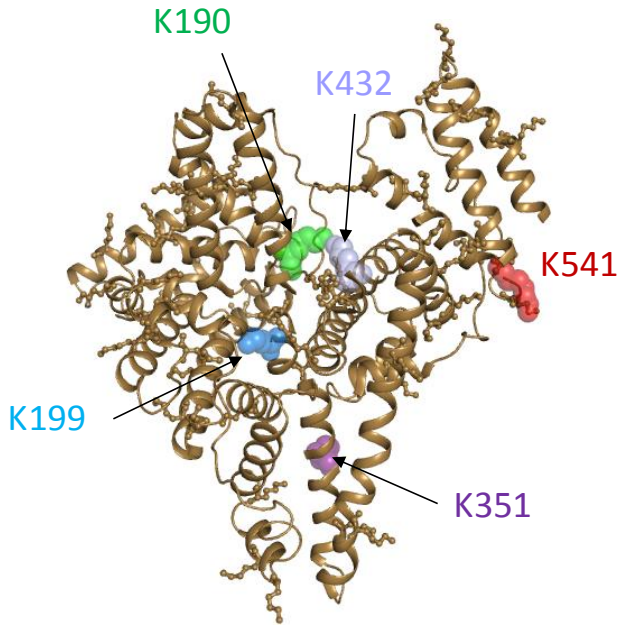


Figure 4.6 *Model of HSA with major piperacillin binding sites at specific lysine residues*

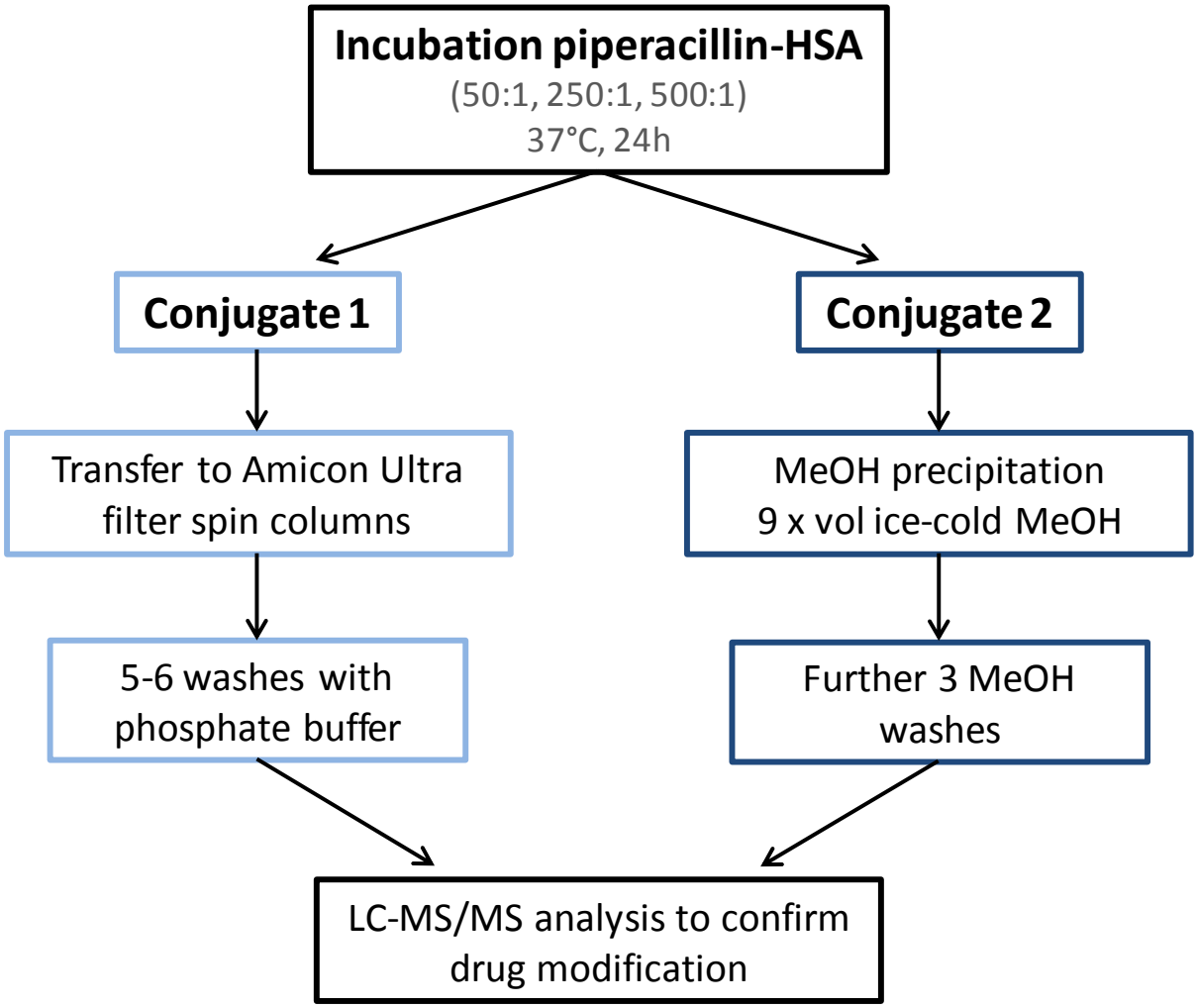


Figure 4.7 *Generation of synthetic piperacillin-HSA conjugates*
Brief outline of conjugate generation is detailed in the scheme above. The epitope profile of each conjugate detailing drug modification sites is also shown.

Analysis by mass spectrometry confirmed modification of HSA by piperacillin in both sets of conjugates generated. Extrapolating the known chemistry of β-lactam antibiotics, protein modification would be predicted to occur through nucleophilic attack of the β-lactam ring, ring opening and covalent binding to protein lysine residues. A hapten with the predicted mass of 517 amu, formed from the direct adduction of piperacillin, was identified (cyclised hapten [1], Figure 4.8). A second hapten of mass 535 amu was also detected. This is thought to have been formed through hydrolysis of the 2,3-dioxopiperazine ring (hydrolysed hapten [2] Figure 4.8)

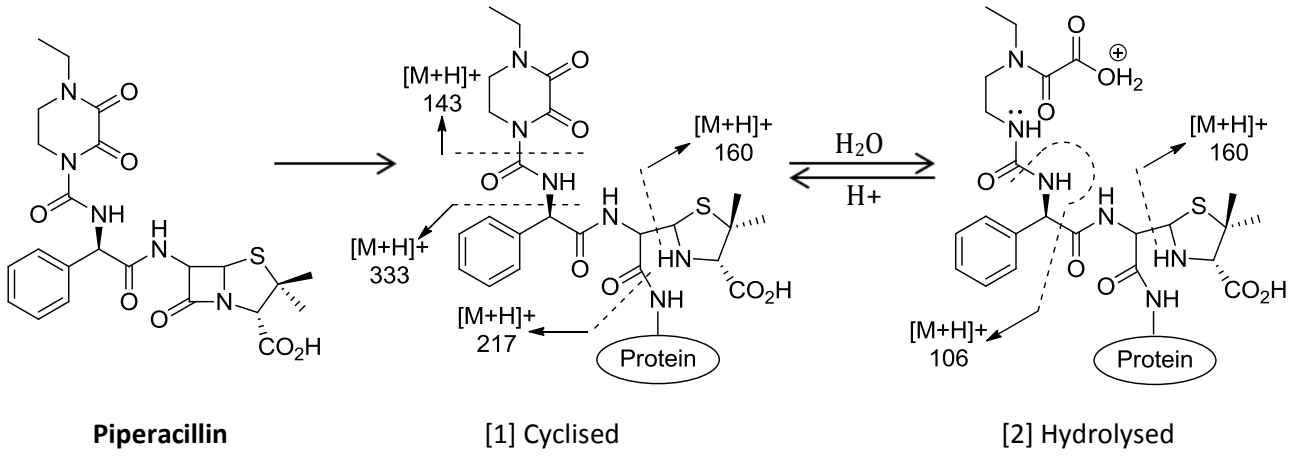


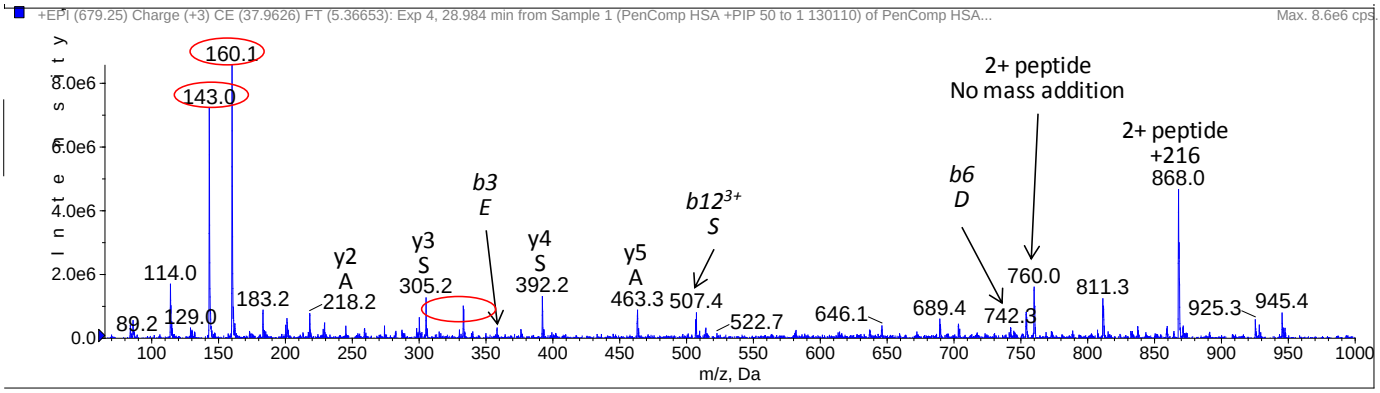
Figure 4.8 *Chemical structures of piperacillin and the resultant cyclised and hydrolysed forms of haptens.* The dominant fragmentation sites induced by MS are also detailed.

The tandem MS spectra of the tryptic peptide ^{182}LDELRDEGK*ASSAK^{195} modified with the cyclised hapten is shown is shown in Figure 4.9A. A triply charged ion at m/z 679.25 was observed, corresponding to the peptide mass with a mass addition of 517 amu; (peptide mass =679.25X3-3=2034.75, Δm=2034.75-1517.77=517). The most characteristic fragmentation ions at m/z 160 and 143 (circled) indicate the incorporation of piperacillin with this peptide. The presence of the abundant ion at m/z 868 which corresponds to the doubly charged peptide mass plus 216 (hapten mass after loss of thiazolidine ring (minus 159) and dioxopiperazine ring (minus 142)) provides further evidence the hapten of mass 517 amu was formed by addition to the β-lactam ring rather than the dioxopiperazine ring.

The mass spectrum of the peptide modified with the hydrolysed hapten which has a mass shift of 535 is shown in Figure 4.9B. It highlights the most characteristic fragment ions were detected at m/z of 106 and 160. In a similar manner to the cyclised hapten, the ion at m/z 867.8 confirmed that nucleophilic addition took place at the β-lactam ring, whilst hydrolysis occurred at the 2,3-dioxopiperazine ring.

A

^{182}LDELRDEGK*ASSAK^{195} + cyclised PIP (3+ 679.25, M*=2034.7, M=1517.8, Δ=516.9)



B

^{182}LDELRDEGK*ASSAK^{195} + hydrolysed PIP (3+ 685.25, M*=2052.7, M=1517.8, Δ=535)

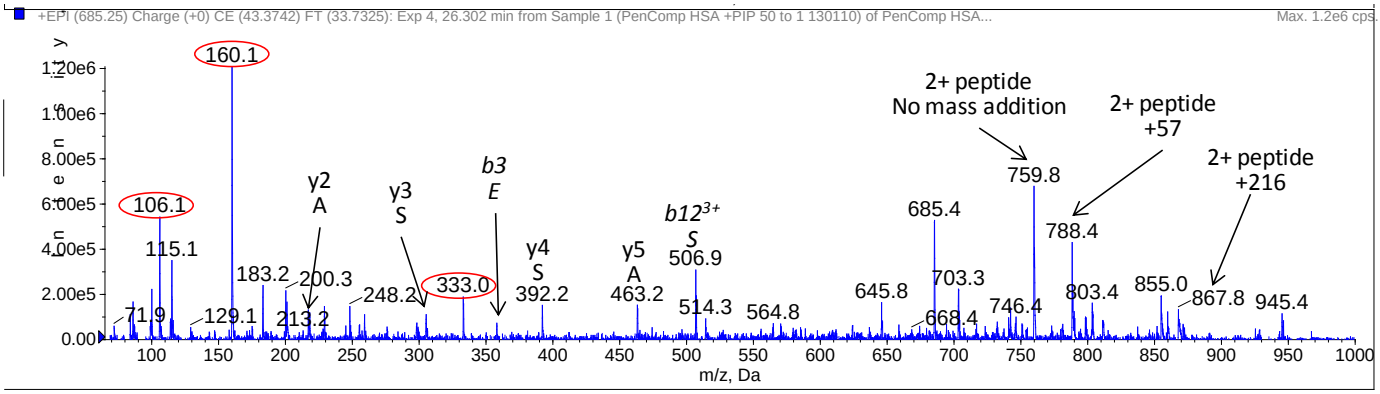


Figure 4.9 Mass *spectrometric characterization of piperacillin haptens formed with HSA in vitro.* Representative MS/MS spectrum of the albumin peptide ^{182}LDELRDEGK*ASSAK^{195} modified at Lys190 by the cyclised and hydrolysed haptens, **(A)** and **(B)** respectively.

Time and concentration dependent piperacillin-modified HSA was also detected by western blot (Figure 4.10A). The anti-penicillin antibody, bound to the protein, is detectable at the band corresponding to a molecular mass of 66 kDA (albumin), This was confirmed by MRM-MS (Figure 4.10B)

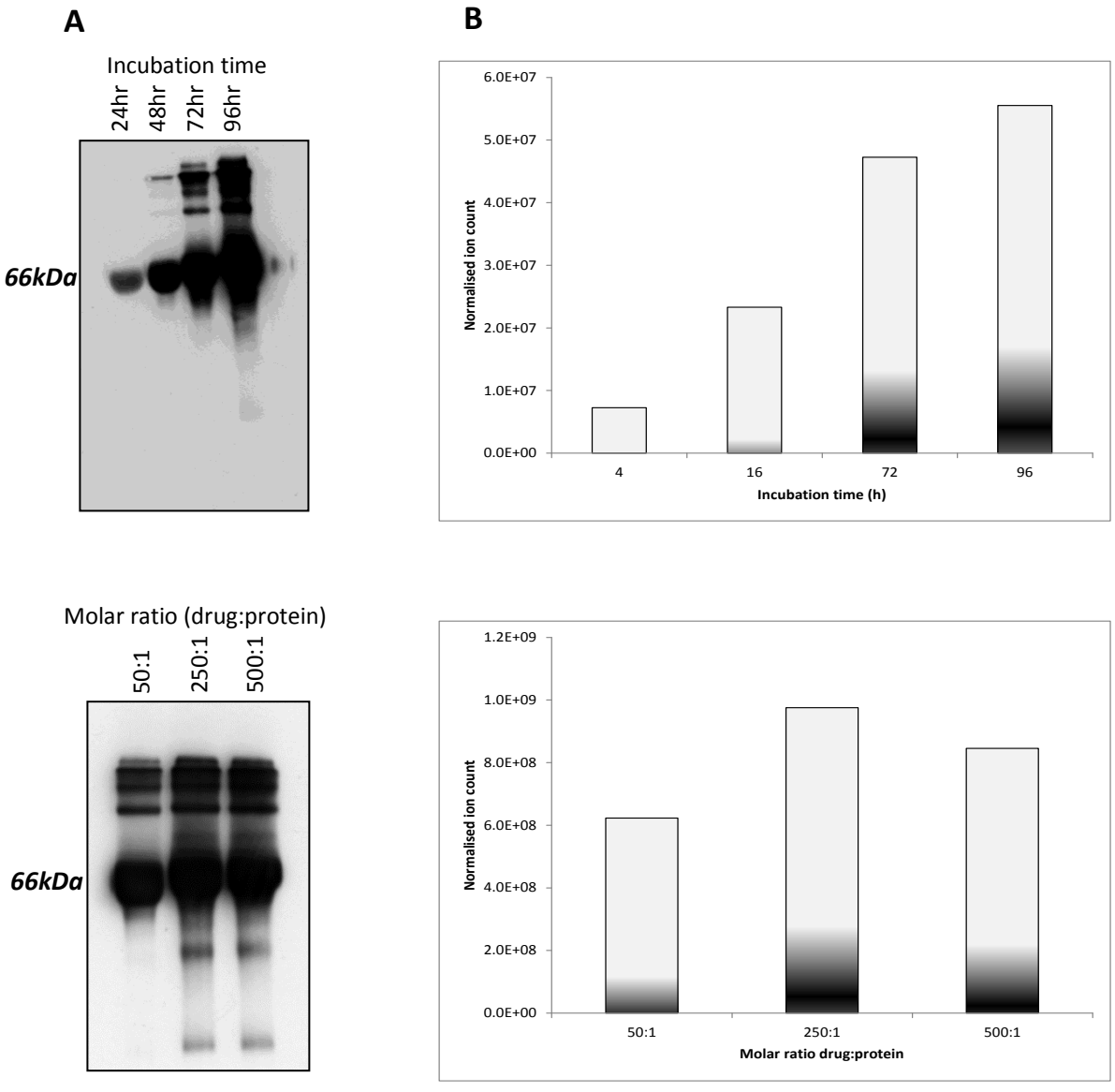


Figure 4.10 *Piperacillin-HSA binding is time and concentration dependent.*
(A) Western blot with anti-drug antibody confirmed piperacillin modification and showed time and concentration dependency. **(B)** MS analysis of time and concentration dependent piperacillin binding. Combined signal for cyclised and hydrolysed adducts are shown. Conjugate at 50:1 ratio was used to investigate time dependency.

The epitope profiles of conjugate 1 and conjugate 2 were strikingly similar (Figure 4.11). Modification of HSA by piperacillin at the Lysine at positions 190, 432 and 542 produced the strongest MRM signals. The hapten was detected at 13 out of 59 lysine residues in HSA for both the hydrolysed and cyclised forms of piperacillin. Table 4.3 details the HSA derived tryptic peptides which contain lysine residues modified by piperacillin.

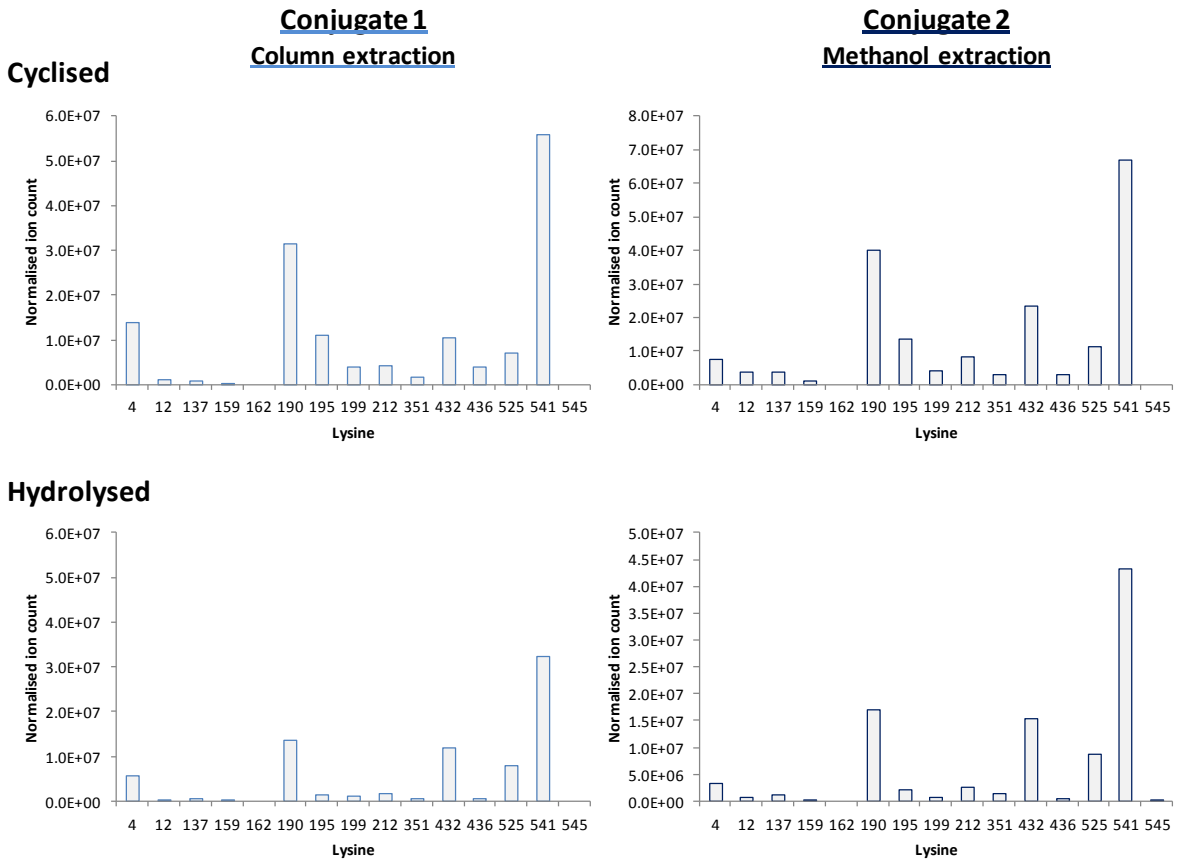


Figure 4.11 *Epitope profile of piperacillin-HSA conjugates.*
Epitope profile of the lysine residues of piperacillin-modified HSA is shown. Normalised AUC of cyclised and hydrolysed haptens.

Lysine residue	Peptide	Cyclised hapten	Hydrolysed hapten
4	DAHK*SEVAHR	✓	✓
12	FK*DLGEENFK	✓	✓
137	K*YLYEIAR	✓	✓
162	YK*AAFTECCQAADK	✓	✓
190	LDELRDEGK*ASSAK	✓	✓
195	ASSAK*QR	✓	✓
199	LK*CASLQK	✓	✓
212	AFK*AWAVAR	✓	✓
351	LAK*TYETTLEK	✓	✓
432	NLGK*VGSK	✓	✓
525	K*QTALVELVK	✓	✓
541	ATK*EQLK	✓	✓
545	EQLK*AVMDDFAAFVEK	✓	✓

Table 4.3 *HSA derived tryptic peptides containing piperacillin modified lysine residues in 2 conjugates.*
Piperacillin:albumin ratio of 50:1, incubation time was 24 hours. The * indicates site of piperacillin modification.

4.4.4.2 Synthetic piperacillin-HSA conjugates purified using centrifugal filters (conjugate 1) do not stimulate piperacillin-specific T-cell clones.

Conjugate 1 was added to co-cultures with piperacillin specific clones and EBV-transformed B-cell lines in a standard proliferation assay. [^{3}H] thymidine was added to the cultures for the last 16 hours of incubation to assess proliferative activity. Piperacillin-specific T-cell clones were not stimulated by conjugate 1 prepared at molar ratios of drug to HSA of 50:1, 250:1 or 500:1 (Figure 4.12).

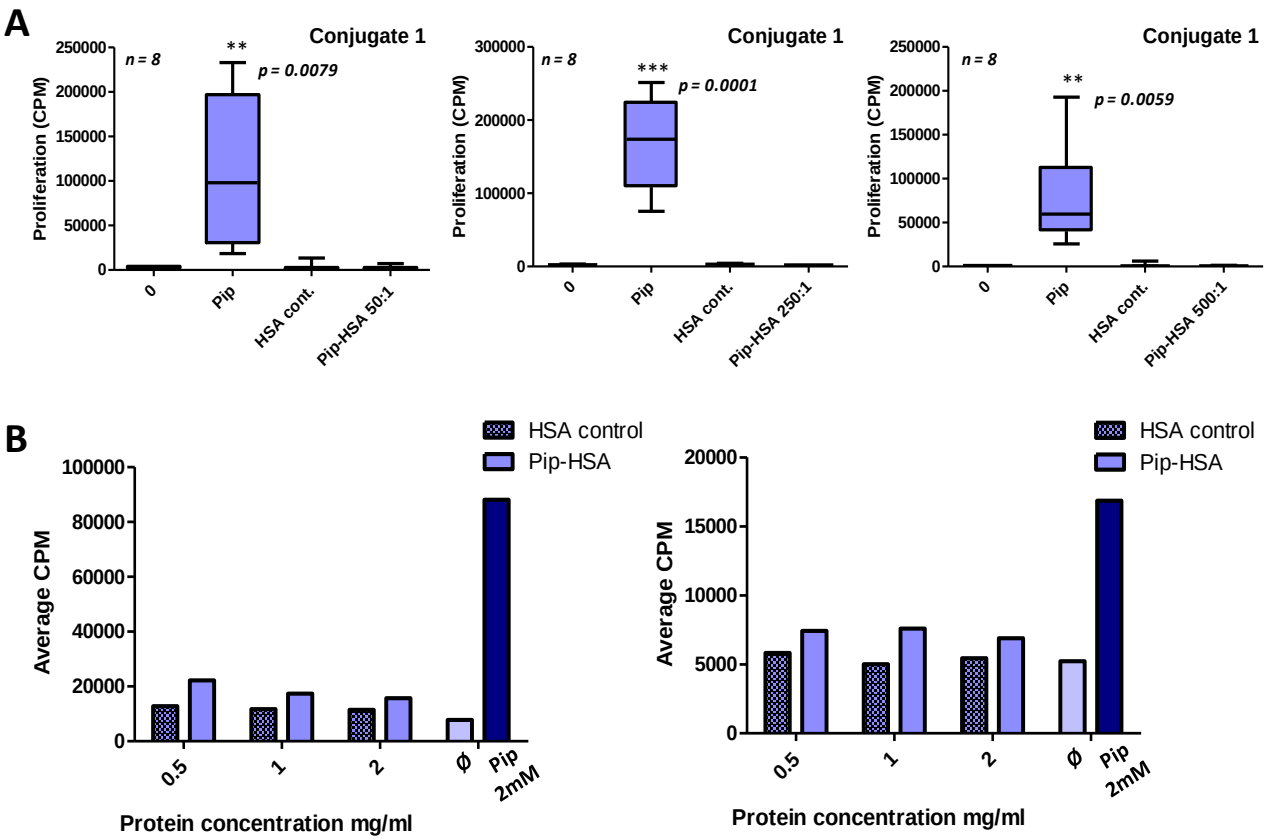


Figure 4.12 C*onjugate 1 does not stimulate piperacillin-specific T-cell clones.*
(A) Piperacillin was incubated with HSA at 50:1, 250:1 and 500:1 molar ratios for 24hrs at 37°C, *n* = 8 for all molar ratios. Conjugates were extracted and purified with ultra centrifugal filters (30K cut off) and protein concentration determined. 4mg/ml S-conjugates, and medium and 2mM soluble piperacillin as negative and positive controls, were co-cultured with piperacillin specific T-cells and autologous APCs for 96hrs. Proliferation was determined by [^{3}H]-thymidine incorporation which was added in the last 16 hours of incubation. **(B)** Dose response data from 2 representative clones. Pip-HSA conjugates at a molar ratio of 250:1 and HSA control were cultured with T-cell clones and APCs at 0.5, 1 and 2mg/ml. Medium alone (Ø) and 2mM piperacillin were included in assay to confirm T-cell activity and specificity. CPM = counts per minute.

4.4.4.3 Synthetic piperacillin-HSA conjugates generated through MeOH precipitation (conjugate 2) stimulate piperacillin-specific T-cell clones.

The data generated with conjugate 1 is in stark contrast to our previous findings in which conjugates prepared at a molar ratio of 50:1 consistently stimulated T-cells to proliferate (El-Ghaiesh *et al.*, 2012). However, the latter were purified by methanol precipitation of the protein which may have resulted in a) sufficient levels of free drug remaining post-precipitation to stimulate a response or b) may have caused the protein to be damaged so that alternative peptides were available for display by MHC class II expressed on APCs. To investigate this further, conjugates were prepared by methanol precipitation to remove free drug, conjugate 2. Conjugate 2 was incubated in co-culture with piperacillin-responsive T-cell clones and autologous EBV-transformed B-cells, and the proliferative responses were recorded. Piperacillin modified conjugate 2 was found to stimulate the proliferation of T-cells (Figure 4.13A). Furthermore, responses to protein concentrations were dose-dependent (Figure 4.13B).

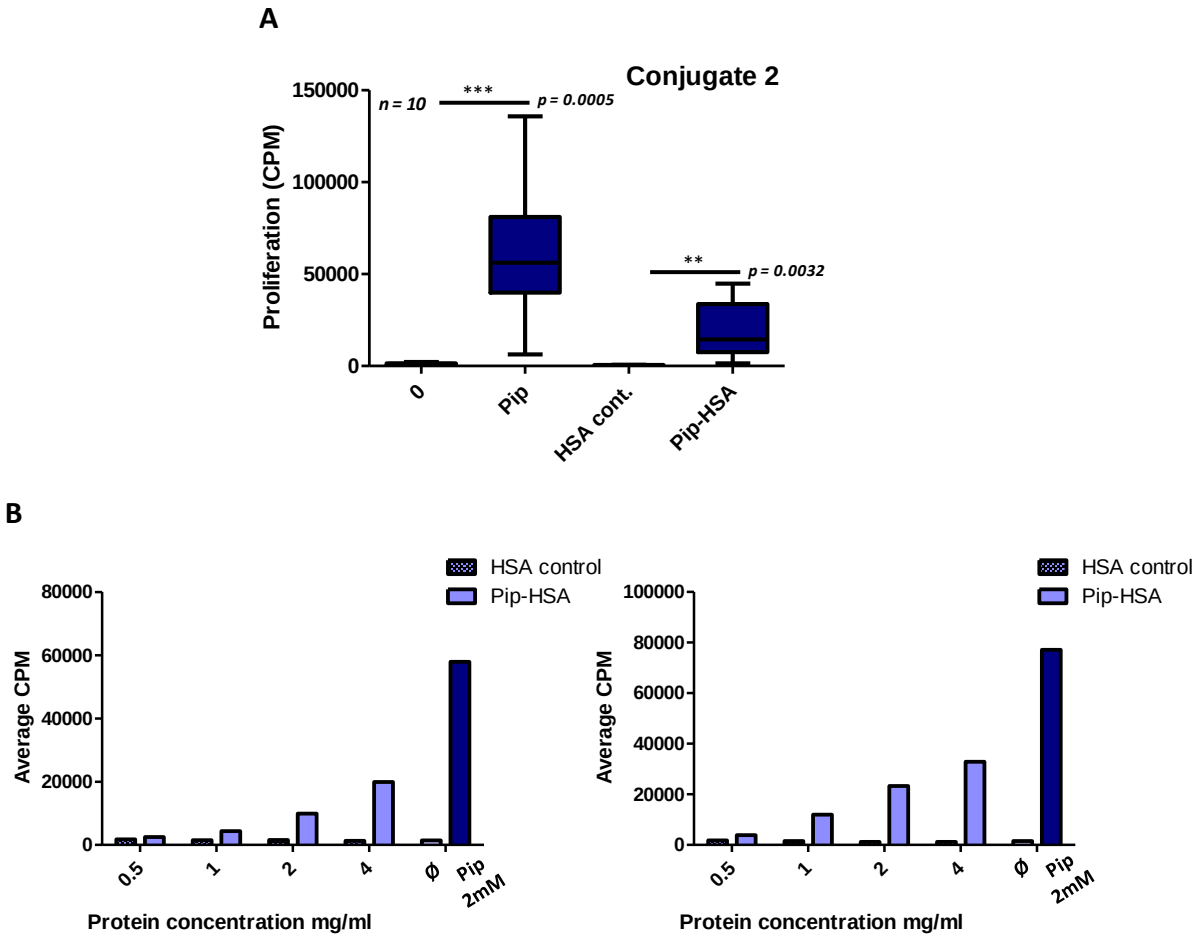


Figure 4.13 *Synthetic piperacillin-HSA conjugates generated through methanol precipitation stimulate piperacillin-specific T-cell clones.*
(A) Piperacillin was incubated with HSA at 250:1 molar ratios for 24hrs at 37°C, *n* = 10. Conjugates underwent MeOH precipitation for purification and protein concentration was determined. 4mg/ml conjugate 2, and medium and 2mM soluble piperacillin as negative and positive controls, were incubated with piperacillin specific T-cells and APCs for 96 hours. Proliferation was determined by [^{3}H]-thymidine incorporation. Data was significantly different. Piperacillin p = 0.0005, Pip-HSA p = 0.0032. **(B)** Dose response data from 2 representative clones. Pip-HSA conjugates at a molar ratio of 250:1 and HSA control were cultured with T-cell clones and APCs at 0.5, 1, 2 and 4mg/ml. Medium alone (Ø) and 2mM piperacillin were included in assay to confirm T-cell activity and specificity. CPM = counts per minute.

To investigate these divergent results in greater detail, T-cell clones were exposed to both synthetic conjugates in the same experiment. Results

confirmed our findings that conjugates generated through MeOH precipitation (C2) stimulated T-cell proliferative responses with representative data from one clone shown in Figure 4.14A. However, when the same T-cell clones were exposed to conjugate 1 (C1, centrifugal filtration method) proliferation was not seen. Furthermore, responses to conjugate 2 were dose dependent (Figure 4.14B).

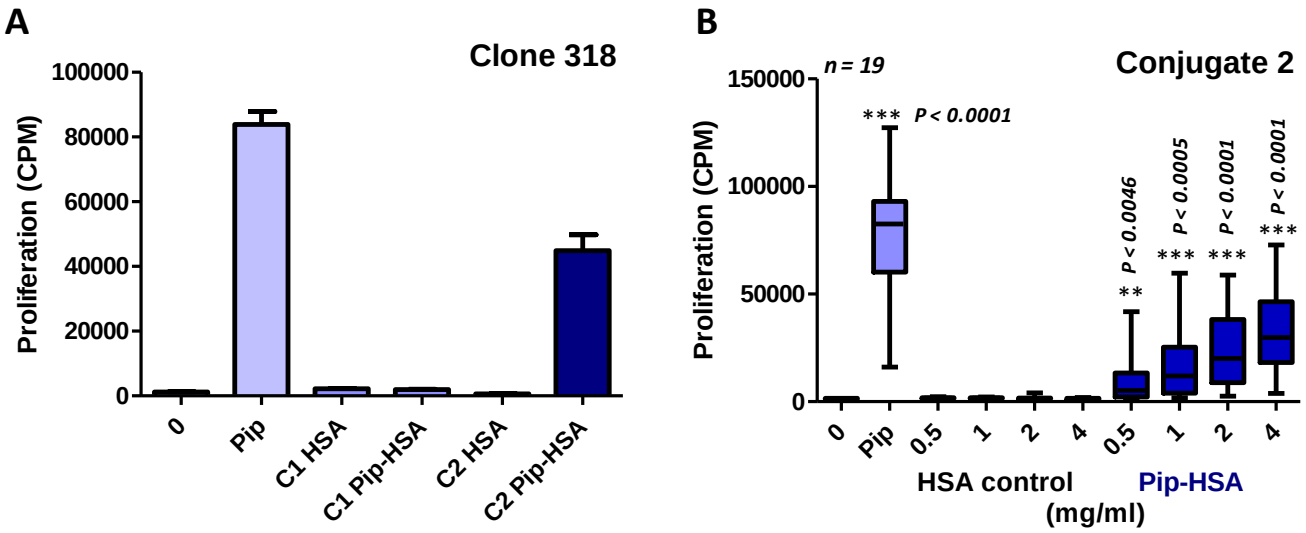


Figure 4.14 *Synthetic piperacillin-HSA conjugates generated through MeOH precipitation stimulate piperacillin-specific T-cell clones in a dose dependent manner.*
(A) Piperacillin was incubated with HSA at a molar ratio of 250:1, *n* = 4. Conjugates were purified either by centrifugal filtration (conjugate 1, C1) or methanol precipitation (conjugate 2, C2). Medium alone and 2mM soluble piperacillin were used as negative and positive controls, and HSA cultured with phosphate buffer alone was used as a negative control for drug modified HSA. T-cell proliferation was determined by [^{3}H]-thymidine incorporation. Representative data from 1 clone of 4 is shown. **(B)** 0.5 – 4mg/ml of HSA conjugate 2 (control and piperacillin modified) were incubated with piperacillin-specific clones and APCs in standard proliferation assay, *n*=19.

4.4.4.4 Piperacillin at µM concentrations is required for a proliferative response in piperacillin specific T-cell clones

Experiments aimed to determine the minimum concentration of piperacillin (soluble drug) required to stimulate piperacillin specific T-cell clones were performed. Clones were co-cultured with EBV-transformed B-cells and exposed to piperacillin at a range of low doses; 10nM, 50nM, 100nM, 100µM, 10µM and 1µM piperacillin. Medium alone and 2mM piperacillin were used as the negative and positive control, respectively. In most clones, piperacillin concentrations of 10µM and over were necessary to stimulate T-cell proliferation to a degree that would be considered positive i.e. an SI of 2 or more (Figure 4.15) (Pichler *et al.*, 2004). A quarter of clones tested (3 out of 12) were stimulated with 10µM piperacillin and 7 clones required at least 100µM piperacillin. However in 2 highly responsive clones, 1µM piperacillin was sufficient in eliciting a positive proliferative response (see clone 411, Figure 4.15B).

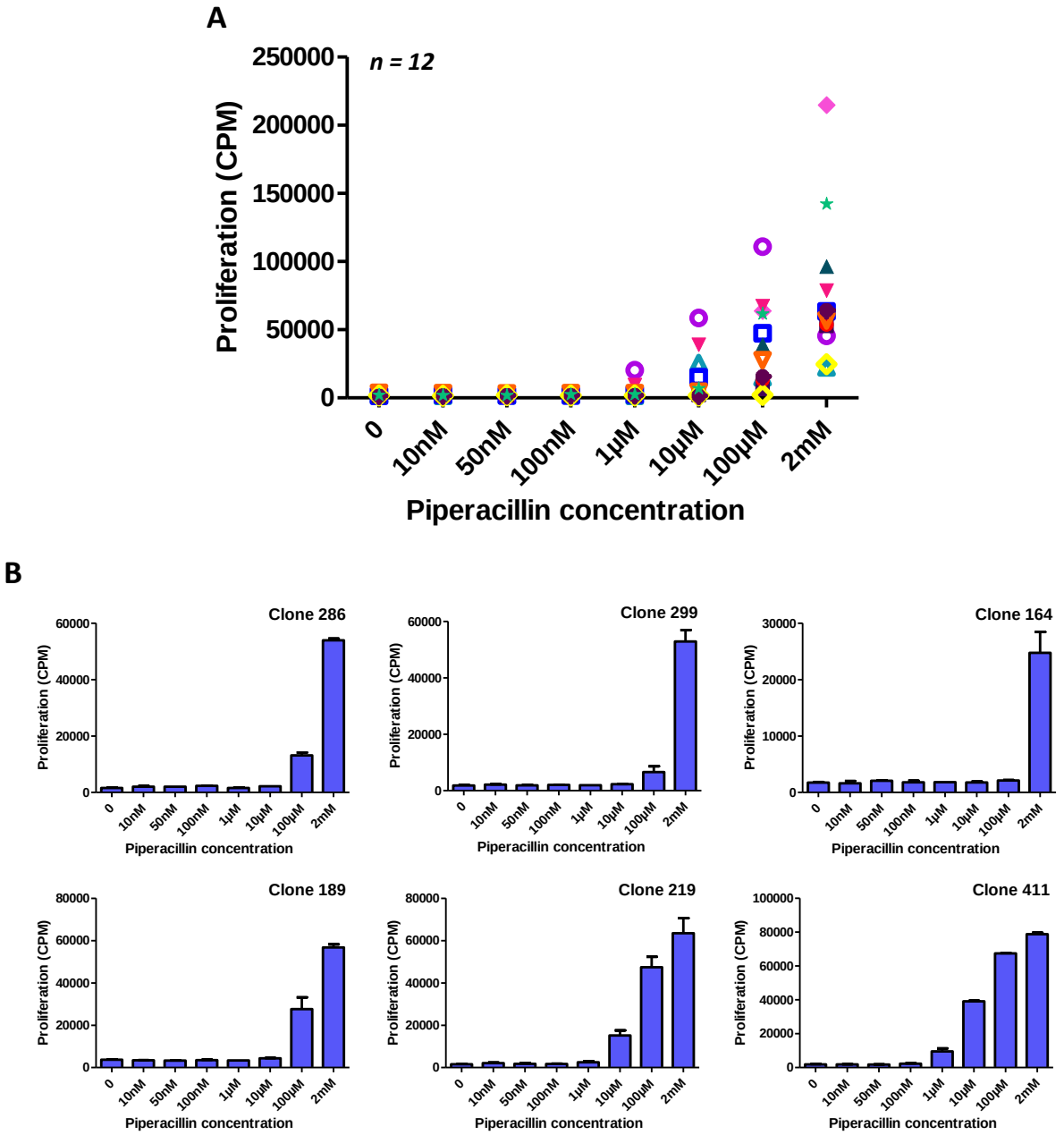


Figure 4.15 *Piperacillin at µM concentrations is required for a proliferative response in piperacillin specific T-cell clones.*
Piperacillin specific T-cell clones were co-cultured with EBV-transformed B-cells and exposed to piperacillin (10nM-100µM). Medium and 2mM piperacillin were used as negative and positive control respectively. T-cell proliferation was determined by [^{3}H]-thymidine incorporation.**(A)** Data from 12 piperacillin specific clones. **(B)** Individual proliferation data from 6 representative clones.

Knowing that at least 1μM piperacillin is required to stimulate T-cell clones, the next step was to determine the amount of free drug remaining in the conjugates used in our assays. This would allow clarification that indeed the drug-protein conjugates were responsible for T-cell proliferation and not free, soluble piperacillin. Samples of conjugates 1 and 2 were processed and analysed by MRM-MS to determine the concentration of unbound piperacillin. The level of free drug as would be present in T-cell culture in our *in vitro* assays in the conjugate 1 was 0.99nM. 150nM piperacillin was present in conjugate 2 (Table 4.4). Quantification of free piperacillin was kindly conducted by Dr Roz Jenkins and Dr Xiaoli Meng (MRC, CDSS).

Conjugate	free pip/mg HSA	free pip in culture @4mg/ml conjugate
1 after spin 5	0.248nM	0.99nM
2 after MeOH wash 3	37.5nM	150nM

Table 4.4 *Level of free piperacillin in conjugates*
The amount of free, soluble piperacillin was quantified for each conjugate at a molar ratio of 50:1, piperacillin:HSA. For conjugate 1 free piperacillin was measure after fifth spin in column, this was used in all T-cell assays. Free iperacillin for conjugate 2 was analysed after third methanol (MeOH) wash which was used in T-cell assays. 0.5 – 4 mg/ml of conjugate were used throughout T-cell assays.

With the level of free piperacillin in both conjugates not sufficient to induce proliferative T-cell responses, we hypothesised the slightly higher concentration of free piperacillin embedded non-covalently in conjugate 2 was somehow being utilised to promote the proliferation of piperacillin specific T-cells. To test this hypothesis, piperacillin specific T-cell clones were cultured in three conditions. The standard proliferation assay (cells and drug cultured in medium) as the control condition and in parallel, cells were cultured with 2mg/ml conjugate 1; either HSA control or drug-modified at a molar ratio of 250:1 of piperacillin to HSA. Free soluble piperacillin (10nM, 100nM, 10µM, 100µM) was spiked into the wells with 2mM piperacillin used as a positive control and T-cell proliferation determined by [^{3}H]-thymidine incorporation. The lower concentrations of piperacillin that were spiked into the conjugate 1 did not result in any T-cell proliferation (Figure 4.16, n=10). However, addition of 2mM piperacillin did induce T-cell proliferation as did conjugate 2, which served as the control.

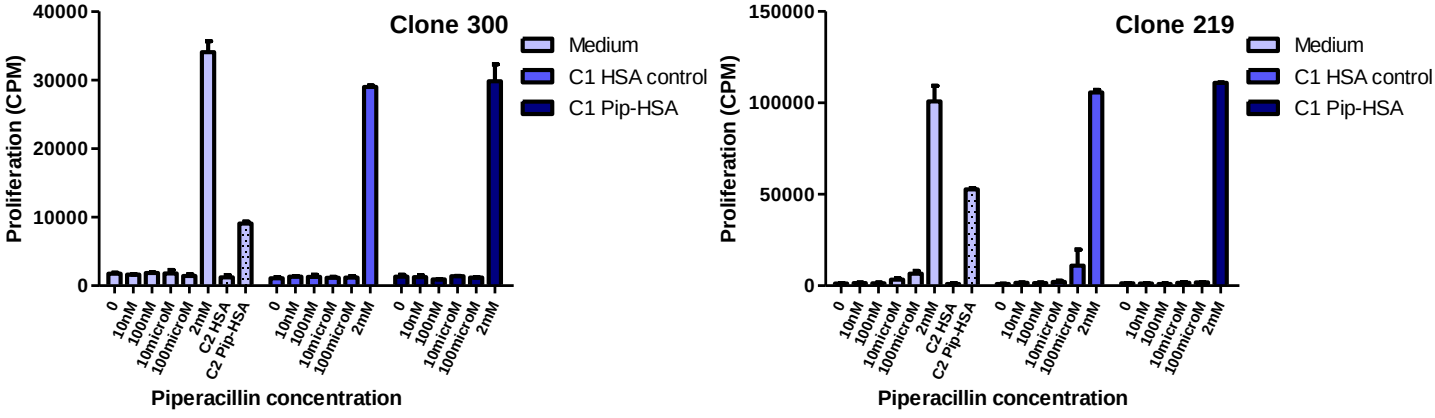


Figure 4.16 *Low dose piperacillin spiked into cell cultures with conjugate 1 do not result in T-cell proliferation.*
Piperacillin specific T-cell clones (*n=10*) were cultured in three conditions; medium as the standard and acting as control, and with 2mg/ml conjugate 1 (C1); either HSA control or piperacillin-modified HSA at a molar ratio of 250:1. Soluble piperacillin (10nM – 2mM) was then spiked into the wells with 2mM piperacillin used as a positive control. Conjugate 2 (C2) were also included in the assay. T-cell proliferation was determined by [^{3}H]-thymidine incorporation. Data from 2 representative clones are shown.

4.4.5 Generation of T-cell clones specific to a piperacillin-HSA conjugate

Furthering investigations of our synthetic piperacillin-HSA conjugate we endeavoured to generate conjugate-specific T-cell clones. PBMCs from hypersensitive patient H3 were isolated and incubated with conjugate 1 for two weeks. T-cell clones were generated through the standard serial dilution protocol. Conjugates at a ratio of 50:1 were used in these experiments.

We were successful in generating 24 T-cell clones specific to the synthetic piperacillin-HSA conjugate with a sample of dose-response data from 14 T-cell clones shown in Figure 4.17A. Proliferation with piperacillin-HSA was dose dependent. Individual data from 6 representative clones are shown in Figure 4.17B. Certain clones were activated to proliferate with as little as 0.25mg/ml of protein conjugate. However, consistent responses were detected with 2mg/ml piperacillin-modified HSA.

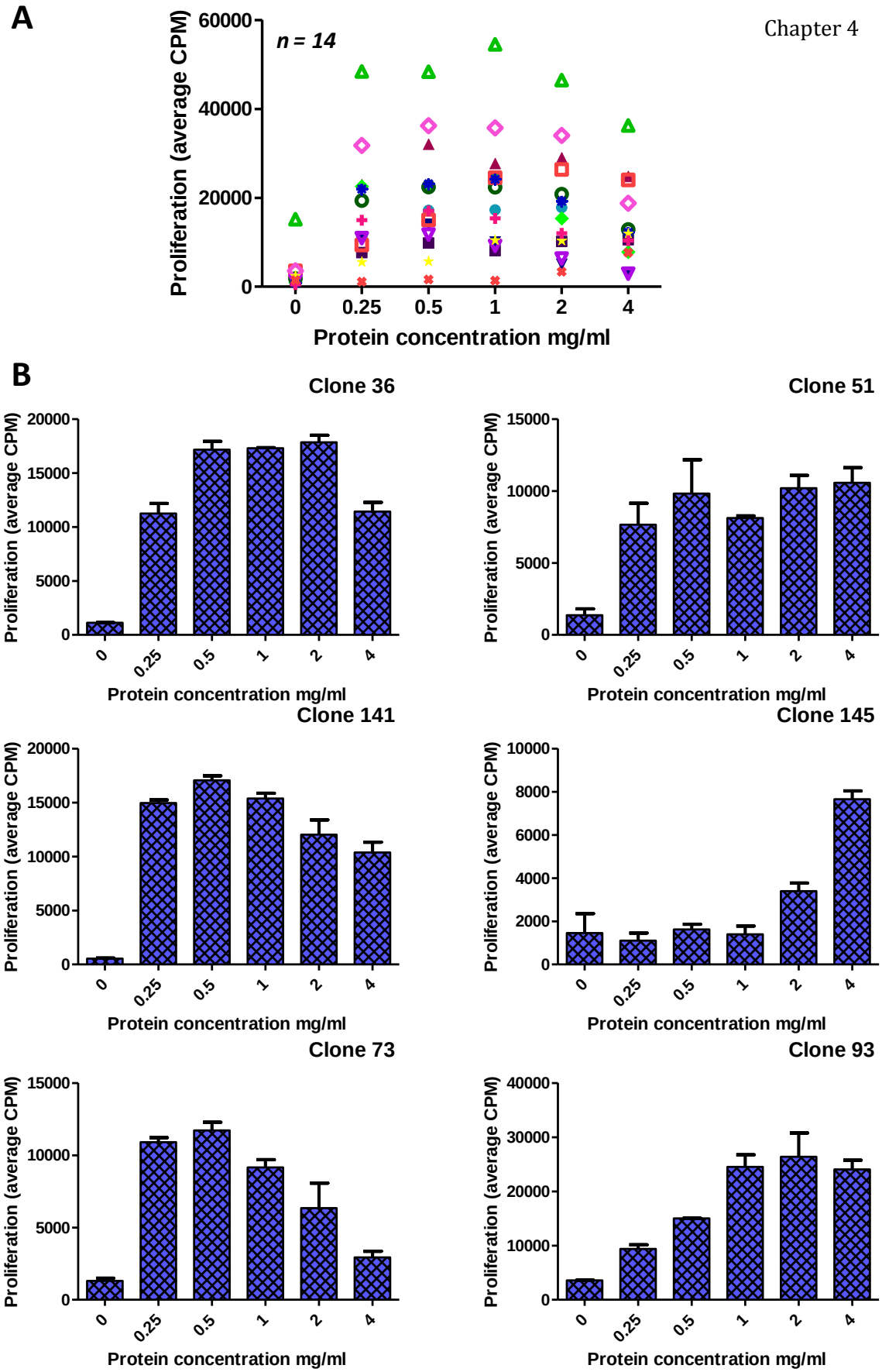


Figure 4.17 *Conjugate primed T-cell clones respond in a dose dependent manner to synthetic piperacillin-HSA conjugate*
(A) T-cell clones were co-cultured with antigen presenting cells and piperacillin-HSA conjugate at 0 – 4mg/ml for 96 hours. Proliferation was measured with the addition [3H]-thymidine in the last 16 hours. Each data point represents mean [^{3}H] incorporation from 14 conjugate specific clones. **(B)** Individual data from 6 representative T-cell clones.

To further explore the specificity of the T-cell clones, cells were exposed to a control HSA which was cultured in phosphate buffer alone (in the absence of piperacillin) and subjected to the normal extraction protocol (HSA control). Moreover, soluble piperacillin (2mM) was included in the assay. Proliferation was not observed following exposure of the T-cell clones (n = 8) to the HSA control (0.25 – 4 mg/ml). However, clones were activated when exposed to the synthetic piperacillin-HSA conjugate. Representative clones are shown in Figure 18. For four of the clones, proliferative responses were seen exclusively with drug modified conjugates (Figure 18A). However, the other clones were also activated with soluble piperacillin (Figure 18B).

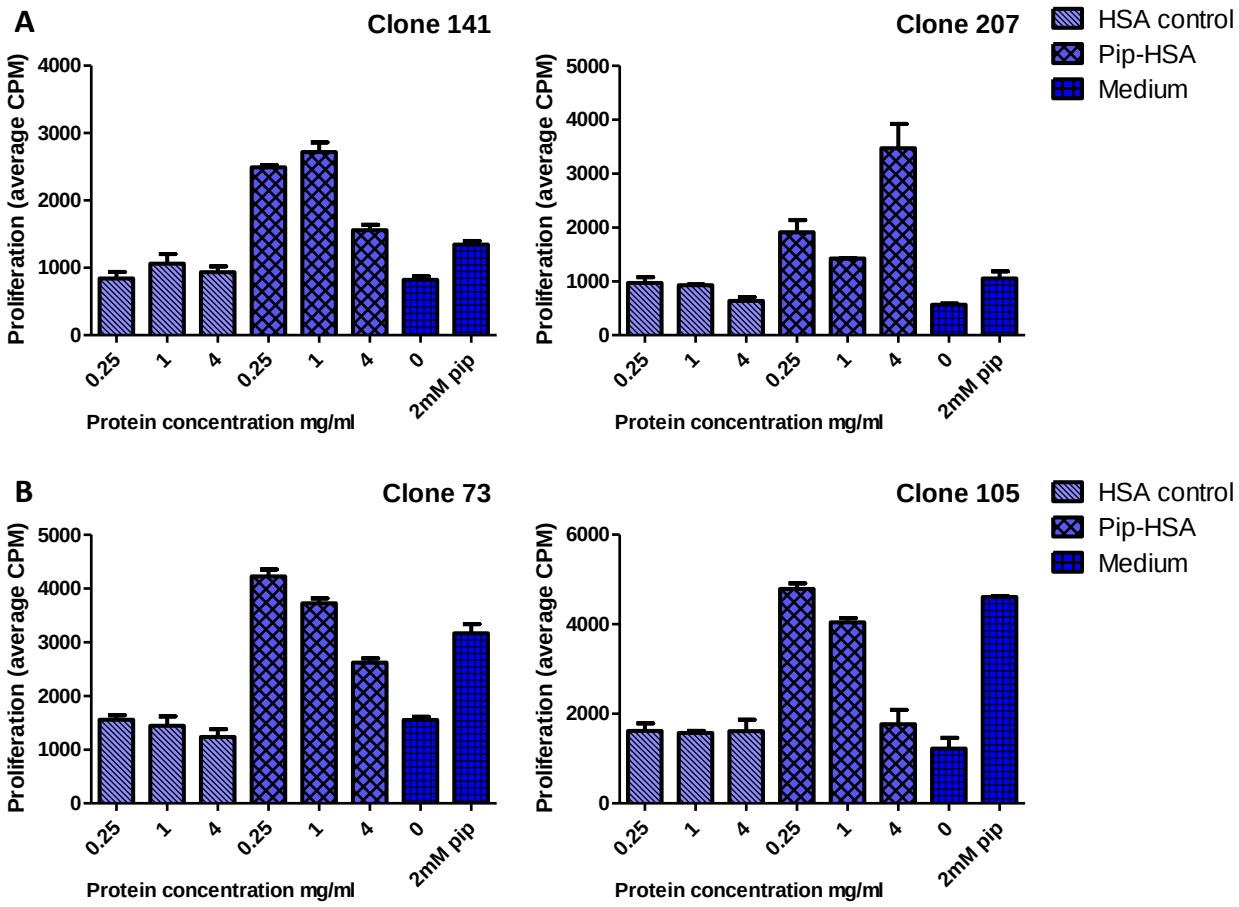


Figure 4.18 *T-cell clones primed to synthetic piperacillin-HSA conjugate do not respond to HSA alone*
(A) T-cell clones (*n* = 8)were co-cultured with antigen presenting cells and conjugates at 0.25, 1 and 4mg/ml of synthetic conjugate, HSA control and piperacillin-HSA (pip-HSA) for 96 hours. Cells were also exposed to soluble piperacillin at 2mM and medium alone as a control. Proliferation was measured with the addition [3H]-thymidine in the last 16 hours. 2 representative clones are shown. **(B)** 2 representative clones which were activated following exposure to soluble piperacillin.

4.5 Discussion

Administration of antibiotics is essential in the treatment regimen for many patients with CF due to the development of regular bacterial infections resulting in pulmonary exacerbations (Turcios, 2005). Unfortunately, between 30% and 50% of patients treated with β-lactam antibiotics develop hypersensitivity reactions (Burrows *et al.*, 2007; Parmar *et al.*, 2005) which is in stark contrast to the rate of hypersensitivity seen in the general population (up to 10%). Hypersensitivity in the CF population is understandably a major health concern for patients and medical health professionals. Little is known about the mechanisms involved in the development of drug hypersensitivity reactions and so the aim of this chapter was to investigate the nature of the piperacillin protein interaction involved in the activation of piperacillin specific T-cell clones.

A number of hypotheses have been proposed to explain drug immunogenicity, one of which is the hapten hypothesis. This theory has been formed around the concept that drugs become immunogenic following covalent binding to larger biological macromolecules such as circulating proteins. Once the haptenic structure has been formed, modification of the protein is able to stimulate an immunological response (Landsteiner *et al.*, 1935). β-lactam antibiotics have been used as a model to study and define this hypothesis (Martin *et al.*, 2014) as opening of the β-lactam ring allows the spontaneous covalent binding of the penicilloyl group to lysine residues on endogenous proteins. Indeed T-cells from penicillin hypersensitive patients have been found to respond to penicillin derived designer HLA-DR binding peptides (Padovan *et al.*, 1997). However, the hapten hypothesis has been challenged by an alternative theory; the PI

concept. Through the use of T-cells isolated from drug allergic patients, Pichler and his co-workers have found that a direct, reversible interaction between the offending drug, MHC molecule and the T-cell receptor itself more accurately describes the nature of the T-cell activation (Pichler, 2002). They have shown that a number of drugs activate T-cells via the T-cell receptor in an MHC allele restricted manner following the blockade of protein processing. Rapid T-cell receptor activation which does not correspond to the length of time required for processing is also observed. Furthermore, inhibition of protein binding does not necessarily inhibit the T-cell response (Gerber *et al.*, 2004; Schnyder *et al.*, 1997).

In light of these findings it is important to further define the chemical basis of drug hypersensitivity.

A panel of over 100 piperacillin specific T-cell clones, generated from PBMCs isolated from piperacillin hypersensitive patients with CF, were used in our assays to investigate (1) the mechanism of piperacillin specific T-cell activation, (2) characterise the nature of drug-protein binding and (3) understand the relationship between the chemistry of antigen formation and drug hypersensitivity. The CD4+ T-cell clones used displayed dose-dependent proliferative responses against the parent drug, which were reproducible with maximum responses seen with 2mM piperacillin. Clones also secreted IFN-γ in a dose dependent manner (Figures 4.1 and 4.2). The Th1 subtype of cells are implicated in the pathogenesis of delayed type hypersensitivity reactions (Kennedy *et al.*, 2008) and IFN-γ secretion has been reported previously with piperacillin specific clones.

With our panel of T-cell clones phenotyped and characterized we began by investigating the mechanisms of antigen presentation. EBV-transformed B cells were used as APCs throughout. Piperacillin-pulsed APCs, with free drug removed by extensive washing, were found to stimulate T-cells in a similar manner to that with soluble drug. The response with pulsed APCs was time-dependent with short 1 and 4 hour piperacillin-pulsed APCs not activating T-cells. In contrast with longer APC piperacillin incubations (48hr), proliferative responses even exceeded that seen with soluble piperacillin (Figure 4.3). 24 – 48 hours is the time required for piperacillin to bind extensively to serum protein (Whitaker *et al.*, 2011b). Considering the hapten hypothesis, we would presume that piperacillin binds to circulating serum albumin in the culture media forming a drug-protein conjugate. APCs would then process the newly formed antigen producing drug modified peptides that are presented via class II MHC molecules to receptors on CD4+ T-cells thus initiating T-cell expansion, differentiation and ultimately resulting in an exaggerated immune response. Glutaraldehyde, which prevents antigen processing but conserves the ability of APC to present antigens, was used to fix APCs before being transferred to a standard proliferation assay with piperacillin and T-cell clones (Shimonkevitz *et al.*, 1983). When these were cultured with fixed APCs and piperacillin, proliferative responses were generally not detected indicating that processing is a requirement for the T-cell proliferative response (Figure 4.4). However, to our surprise, this was not the case for all of the clones tested. Two clones responded with the fixed APCs, with one (clone 76) producing T-cell responses to the same extent as the control, non-fixed APCs. Upon further investigation these T-cell clones were found to self present as the absence of APCs did not

diminish the T-cell response. The presentation of antigens is traditionally thought to occur via cells whose primary function is to present antigens to T-cells, hence the name antigen presenting cell (APC). These include professional APCs such as dendritic cells and B-cells. However, T-cells themselves have the capacity to present antigens made possible due to their ability to synthesize and express MHC molecules (Broeren *et al.*, 1995). Though not fully investigated the ability of T-cells to self present has been reported (Taams *et al.*, 1999). T-cells have been found to possess characteristics associated with professional APCs and when activated T-cells are able to process and display antigens inducing and augmenting T-cell proliferation (Barnaba *et al.*, 1994; Brandes *et al.*, 2005). Indeed, those T-cell clones that were stimulated in spite of fixed APCs appear to have the capacity to self-present the piperacillin antigen without APC involvement.

Previously, synthetic piperacillin albumin conjugates modified at specific lysine residues have been shown to stimulate drug-specific T-cell clones and we wanted to further develop our understanding of the role of drug-protein conjugates in the drug-specific T-cell response (Whitaker *et al.*, 2011b). After 24 hour incubation of piperacillin with HSA (at molar ratios of 50, 250 and 500:1) conjugates were processed through ultra centrifugal filters (30K cut off). Washes within these spin columns removed free drug, purifying the conjugate. However, when T-cells and APCs alongside the synthetic conjugates (conjugate 1), no responses were seen (Figure 4.12). Upon closer inspection we identified differences in the method used to generate said piperacillin-HSA synthetic conjugates compared to synthetic conjugate made previously in the department. Though both involved incubation of piperacillin with HSA,

processing of the conjugates differed. Whilst conjugate 1 utilised spin columns, the other method involved the use of MeOH precipitation to clear free drug. A second conjugate was generated (conjugate 2) using the MeOH precipitation method, similar to previous studies (El-Ghaiesh *et al.*, 2012), this conjugate was able to stimulate piperacillin-specific clones (Figure 4.13) and furthermore responses were dose-dependent.

Western blot and mass spectrometry confirmed piperacillin modified HSA with each conjugate. Importantly, the epitope profiles (lysines modified and relative levels of binding), were similar. Mass spectrometric analysis also revealed that piperacillin formed multiple haptenic structures on lysine residues of both conjugates. As with other β-lactam antibiotics, piperacillin forms adducts with lysine residues through opening of the characteristic β-lactam ring and both hydrolysed and cyclised forms of the piperacillin hapten were present.

Although chemically similar, functionality of the conjugates varied; one stimulated clones to proliferate, the other did not. We postulated that the different methods involved in preparation of the conjugates may have resulted in disparate levels of free drug residing within the conjugate which in turn was resulting in the responses seen with conjugate 2 but not with conjugate 1. Having established that generally μM concentrations (i.e. at least 1μM and normally 10 - 100μM) of piperacillin is required to stimulate piperacillin-specific clones with an SI of 2 or more, we set out to determine the levels of unbound piperacillin in the synthetic conjugates. Mass spectrometric analysis revealed that 0.73nM of free drug was present in conjugate 1. Conjugate 2 contained higher levels of free drug (150nM), however these data clearly do not explain the divergent results observed with the two conjugates. In the final

experiments varied amounts of free piperacillin were spiked in cultures with conjugate 1. We expected to see response at nM concentrations yet to our surprise; proliferation was not detected until μM concentrations of piperacillin were spiked into the assay. Indeed our data seemed to confirm that free piperacillin at levels associated in the conjugates was not stimulating T-cell proliferation and we can say with confidence that free piperacillin alone is not stimulating proliferative responses. Instead, piperacillin bound HSA (either covalently or non-covalently) are clearly playing a pivotal role in the development of the immune response and differences in methods to generate the conjugates are altering the proteins in such a way to allow different functionalities.

Limited data exists regarding the effect of MeOH and/or ultra filtration on the structure of proteins. It is possible that an organic solvent, unlike ultra filtration which does not involve such solvents, could alter the structure of HSA, perhaps through denaturing of folds. Furthermore, whilst numerous MeOH washes would provide a "clean" product a significant proportion of the protein would be lost, whilst the advantage of ultra filtration is that the loss of protein with many washes is restricted. (Bodzon-Kulakowska *et al.*, 2007). In studies with horseradish peroxidise organic solvents were found to affect peroxidise function. Though the enzyme was not completely denatured, changes in the tertiary structure indirectly affected function (Ryu *et al.*, 1992). More recent research has uncovered detailed information regarding structural changes of proteins following solvent extraction. Like ethanol, MeOH has both polar (C-O-H group) and non-polar (C-H bonds) characteristics and is less miscible than water. This enhances protein interactions resulting in reduced protein

solubility and precipitation (Cheng *et al.*, 2011). These properties allow weakening of the hydrophobic bonds within the protein, thus the protein is able to unfold with the extent and reversibility dependent on the solvent used (less polar solvent would render unfolding irreversible) and the length of time to which the protein is exposed to said solvent. When proteins are exposed to more polar solvent like ethanol, and with similar characteristics we can expect this of MeOH too. Following the initial unfolding of the protein, refolding into rod-like structures containing a significant number of α-helices is often seen (Pace *et al.*, 2004). This allows peptide groups to be buried within the structure whilst non-polar side chains are free to interact with the solvent and so the structure, while altered can retain function and is stabilised. Precipitation with MeOH will undoubtedly alter the tertiary structure of HSA during processing of the conjugates and this may well contribute to functionality of conjugate 2. In contrast, processing through ultra filtration and centrifugation spin columns is likely to modify the structure of the conjugates minimally.

It is also possible that HSA may be involved someway in facilitating piperacillin presentation on to MHC molecules thus initiating T-cell activation. Thierse *et al.* investigated the role of HSA in nickel allergy (a common contact allergen), where they described HSA as a "shuttling" molecule. Whilst albumin-nickel complexes (HSA-Ni) activated nickel specific T-cells, serum albumin or the derived peptides were not antigenic (Thierse *et al.*, 2004). Instead HSA-Ni appeared to facilitate the transfer of nickel to high affinity sites between the TCR and MHC molecule. A similar scenario may be present in our T-cell cultures with piperacillin-HSA conjugate 2. The slightly higher levels of free drug may be

directed to appropriate binding sites by piperacillin-HSA thus initiating a T-cell response.

Our last experiments showed the precise and specific nature of the piperacillin hapten-specific T-cell response. We were able to generate clones specific to conjugate 1, where strong proliferative responses were observed in a dose-dependent manner. Interestingly half of the clones tested were exclusively activated by piperacillin-HSA conjugate and not activated by soluble piperacillin highlighting fine specificity of T-cell clones. This trait has previously been observed in penicillin-specific T-cell clones. Clones primed and generated to penicillin G were found to be stimulated with the free drug alone and not to the drug modified conjugate (Brander *et al.*, 1995a). In addition the same specificity was observed in clones generated to penicillin-HSA, whereby penicillin G did not elicit positive proliferative responses. Furthermore, with modifications at Lys190, Lys432 and Lys542 exerting such prominence in our mass spectrometry data we postulate that peptides around these positions could be the principal functional epitopes initiating immune response.

Our data clearly indicate that the development of hypersensitivity is a complicated process. Proteins themselves are complicated in their structure which in turn determines their precise properties and functionality. Though we may have characterised piperacillin binding to HSA which constitutes the majority of circulating protein, the body consists of a vast array of other proteins in various quantities, and so there are an infinite number of possible drug-derived peptides that could stimulate T-cells and promote hypersensitivity.

However, what we have shown is the significant role of drug-protein conjugation in piperacillin hypersensitivity reactions. APCs and antigen processing are pivotal in generating T-cell proliferative responses. Furthermore, drug-modified peptides generated from piperacillin-modified HSA are likely to represent functional T-cell antigens. Indeed they may function as immunogens in patients with CF.

Chapter 5: Quantification of piperacillin-modified peptides *in vitro* and in patients

5.1 Introduction

Previous chapters suggest that covalent binding of piperacillin to proteins form neo-antigens that play a role in the development of an immune response and the overall adverse event (Brander *et al.*, 1995b; Levine *et al.*, 1961a; Park *et al.*, 1998). Piperacillin haptenation has been characterised *in vitro* and *in vivo* in patients exposed to the drug: four types of adducts formed with a core group of lysine residues on human serum albumin (HSA) have been identified. Moreover, as re-iterated in the previous chapter, synthetic piperacillin-HSA conjugates stimulate T cells isolated from patients with piperacillin hypersensitivity in a dose-dependent manner (El-Ghaiesh *et al.*, 2012; Whitaker *et al.*, 2011a). Utilising this knowledge it was possible to investigate further how piperacillin is presented to T-cells and in particular the quantitative relationship between covalent binding and drug immunogenicity. As albumin has been identified as a major protein target for piperacillin *in vivo* and in cell culture, piperacillin-modified HSA was therefore used as a model carrier protein to explore piperacillin antigenicity and immunogenicity.

It has been argued that antigen dose plays a critical role in the incidence and severity of hypersensitive reactions (Aguilar-Pimentel *et al.*, 2010; Long *et al.*, 2011). Furthermore, antigen exposure may have an impact on the characteristics of the responding T-cell repertoire (Kim *et al.*, 2006; Oling *et al.*, 2010). There is a great need to develop sensitive methods to determine the absolute quantity of piperacillin antigen formed *in vitro* and in patients. All of our previous mass spectrometric analyses provide a qualitative assessment of piperacillin-HSA binding and a semi-quantitative analysis of the level of modification at each lysine residue. Quantitative assessment of penicillin hapten

density on protein has been attempted previously by either the penamaldate assay that measures the molar ratio of penicillin groups to protein (Levine, 1962) or a trinitrobenzene sulfonic acid assay that indirectly determines the level of modification by quantification of the free amino groups on protein (Sashidhar *et al.*, 1994).

Neither of these approaches are sensitive enough to monitor the level of piperacillin antigen formed in patients and in cell culture. The use of liquid chromatography coupled with mass spectrometry (LC-MS) together with suitable internal standards is now one of the most widely used techniques for the quantification of conjugates. Over the past two decades advances in mass spectrometry (MS) methods has allowed the field of proteomics to emerge along with improvements in protein characterisation and quantification methods. In particular, MS approaches based on multiple reaction monitoring (MRM), first used for the quantification of low molecular weight analytes, have developed to allow the precise and quantitative analysis of low abundance analytes even in a complex matrix, such as those of protein digests, plasma and urine. The MRM approach targets specific subsets of analytes, and is performed by isolating ions of interest within the mass spectrometer. This analysis is typically operated on tandem quadrupole mass spectrometers (QqQ) that are able to monitor several transitions (precursor/fragment ion pairs) over time, yielding a set of ion traces with the retention time and signal intensity for a specific transition as coordinates. Its targeted nature, high selectivity, and wide dynamic range, has therefore made MRM ideal for absolute quantification purposes (Holman *et al.*, 2012). In this chapter, a novel MRM based MS method

has been developed for quantitative analysis of piperacillin modification *in vitro*, in patients, and *ex vivo* in cell culture.

5.2 Aims and Hypothesis

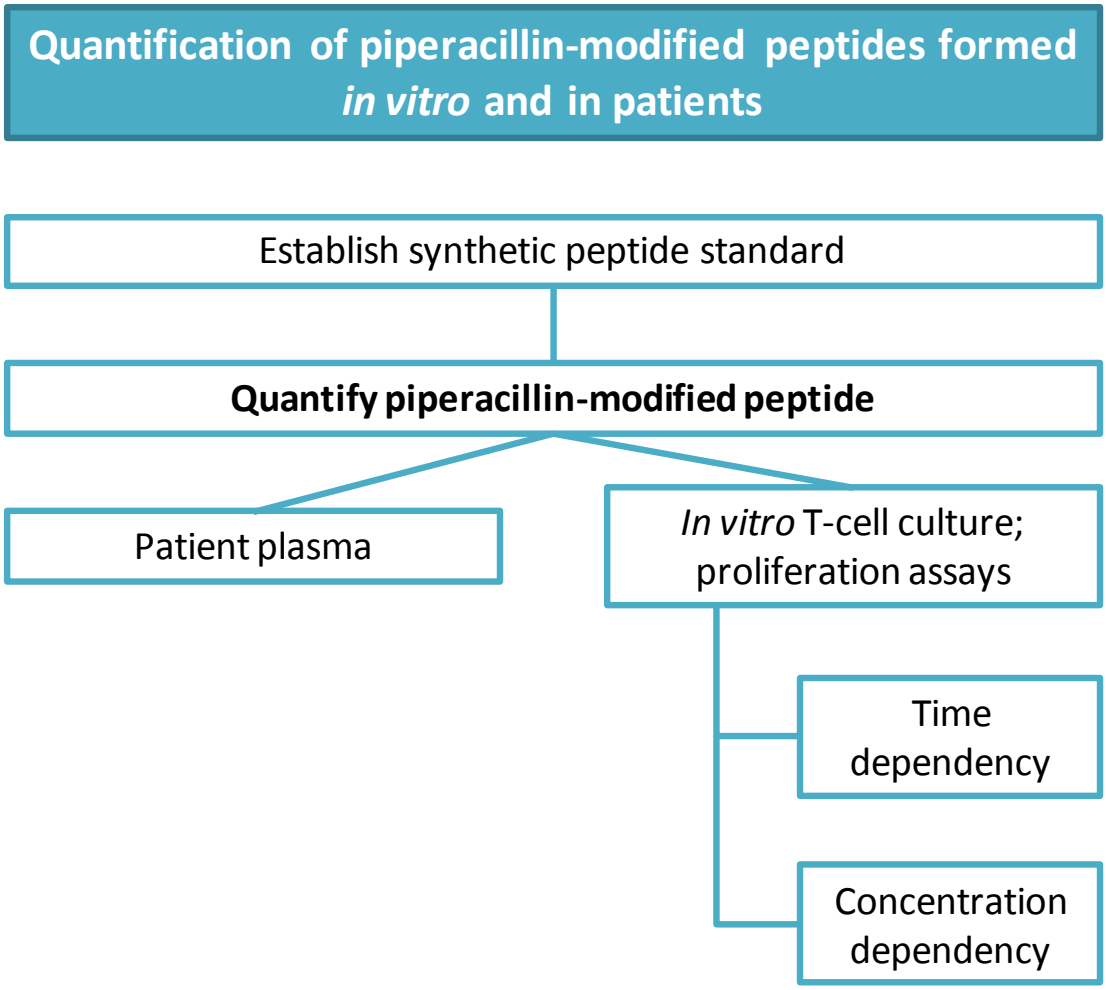
The aim in this chapter was to develop a quantitative method to evaluate the potential association between the antigen dose and piperacillin-specific T cell responses. Novel mass spectrometry methods were developed and used to detect and quantify piperacillin modified peptides in the plasma of patients with CF and from *in vitro* cell culture samples. The results obtained were then related to the immune response seen in *in vitro* T-cell culture. Using the recently established MS techniques in the department I propose the following hypothesis:

There is a clear relationship between the level of piperacillin bound peptides and the in vitro T-cell response.

5.3 Methods

The methods used here are described fully in Material and Methods, Chapter 2.

A brief experimental outline is detailed below.



Scheme 5.1 *Experimental plan and methods used in this chapter*

5.4 Results

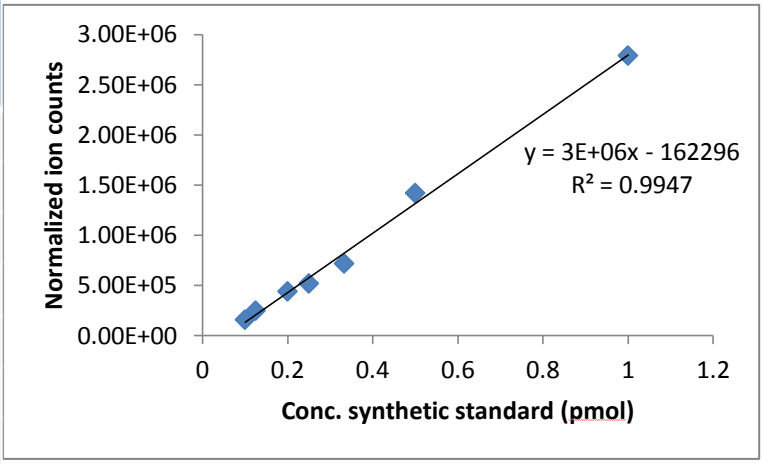
5.4.1 Quantification of drug adduct formation using a synthetic modified peptide

The synthetic piperacillin modified peptide ATK(piperacillin)EQLK (Pip-K541), based on the sequence of HSA incorporating lysine 541, is one of the most consistently detected peptides in patients' plasma. This peptide was therefore used as a standard to quantify the piperacillin modification at this site in HSA. Synthesis of the modified peptide, achieved by Fmoc chemistry in solution phase was conducted by Dr Xiaoli Meng (MRC Centre for Drug Safety Science, University of Liverpool). Protein concentrations of peptide were determined by Bradford assay, and calibration curves for the synthetic standard were constructed using Pip-K541 peptide at 0.1, 0.125, 0.2, 0.25, 0.333, 0.5, 1 pmol on-column. The K541 containing peptide without piperacillin modification, ATKEQLK was used as internal standard in both the modified peptide standard curve and in samples to allow normalization of signals across samples (Figure 5.1). This range of peptide loading was used because it covered the concentration ranges typically seen in both patient samples and *in vitro*.

A

Synthetic standard Pip-K541 Conc. (pmol)	Signal strength (AUC)	Normalised ion count (AUC)
0.1	6.54E+04	1.58E+05
0.125	6.91E+04	2.48E+05
0.2	1.08E+05	4.40E+05
0.25	1.25E+05	5.19E+05
0.333	1.59E+05	7.18E+05
0.5	2.02E+05	1.42E+06
1	2.35E+06	2.79E+06

B



C

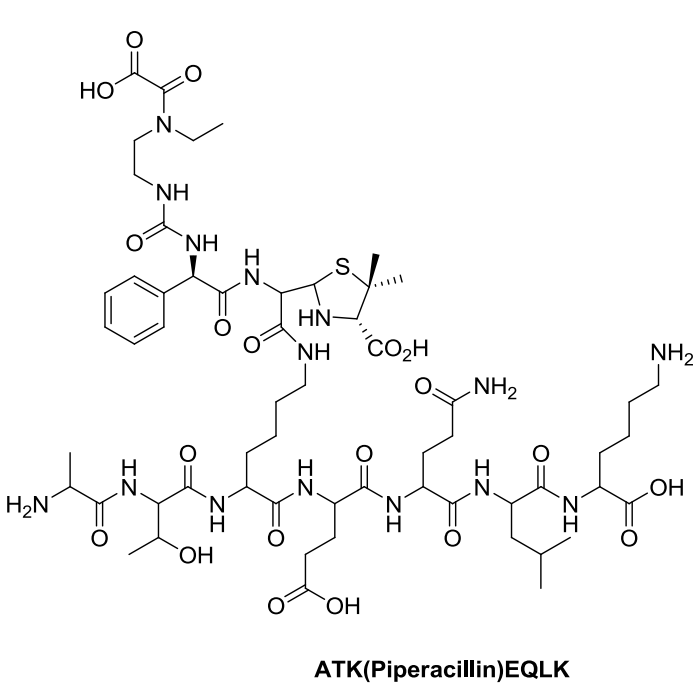


Figure 5.1 *Pip-K541 synthetic peptide standard*
(A) Bradford assay was used to determine protein concentration of synthetic peptide standard before analysis by mass spectrometry. MRM peaks were identified and areas under the curve (AUC) were determined. The ion count was normalised to the UV spectrum (total protein

loading) and the internal standard (IS). **(B)** A standard curve was generated. **(C)** Chemical structure of the Pip-K541 synthetic peptide standard

5.4.2 Piperacillin modified K541 peptide is detectable and quantifiable *in vivo*

Piperacillin modification of HSA in patients was determined at lysine 541 from plasma obtained from ten piperacillin exposed patients from the Regional Adult Cystic Fibrosis Unit at St. James hospital, Leeds. Albumin was isolated from patient plasma on day 14 of treatment by affinity chromatography using a POROS anti-HSA affinity cartridge (Applied Biosystems, Foster City, CA, USA). HSA was processed and analysed by mass spectrometry as described in the methods. The concentration of Pip-K541 peptide was quantified against the synthetic standard. The percentage of modified peptide in relation to the total peptide was also determined (Table 5.1).

Patient	Pip-K541 ion count (AUC)	Normalised ion count (AUC)	Pip-K541 amount (pmol)	Percentage total modification
Pip 1	5.80E+05	6.87E+05	0.282932	5.66%
Pip 2	5.17E+05	5.67E+05	0.243098667	4.86%
Pip 3	2.64E+05	2.83E+05	0.148432	2.97%
Pip 4	5.80E+05	6.57E+05	0.273098667	5.46%
Pip 5	4.74E+05	4.70E+05	0.210598667	4.21%
Pip 6	4.15E+05	4.07E+05	0.189598667	3.79%
Pip 7	7.56E+05	6.32E+05	0.264765333	5.30%
Pip 8	7.45E+05	8.16E+05	0.326098667	6.52%
Pip 9	3.94E+05	2.24E+05	0.128765333	2.58%
Pip 10	1.21E+05	5.50E+05	0.237432	4.75%

Table 5.1 *Quantification of Pip-K541 peptide in vivo*
Plasma from10 piperacillin non hypersensitive patients were collected and prepared for processing. Pip-K541 peptide was identified by MRM/MS, signal strength was normalised and concentration determined against synthetic peptide standard. 5pmol of patient HSA sample was loaded and the percentage of K541 peptide that was modified was determined.

The Pip-K541 peptide was detected in all ten plasma samples which suggests that piperacillin-modification of plasma proteins is universal even in the absence of adverse drug reactions. However, it is important to note that this protein modification does not equal hypersensitivity and we are yet to understand why only a subset of patients become allergic. Using the established standard curve for synthetic piperacillin modified peptide (Figure 5.1), the

percentage of HSA modified by Piperacillin at K541 *in vivo* was found to range from 3% to 6.5% (Figure 5.2).

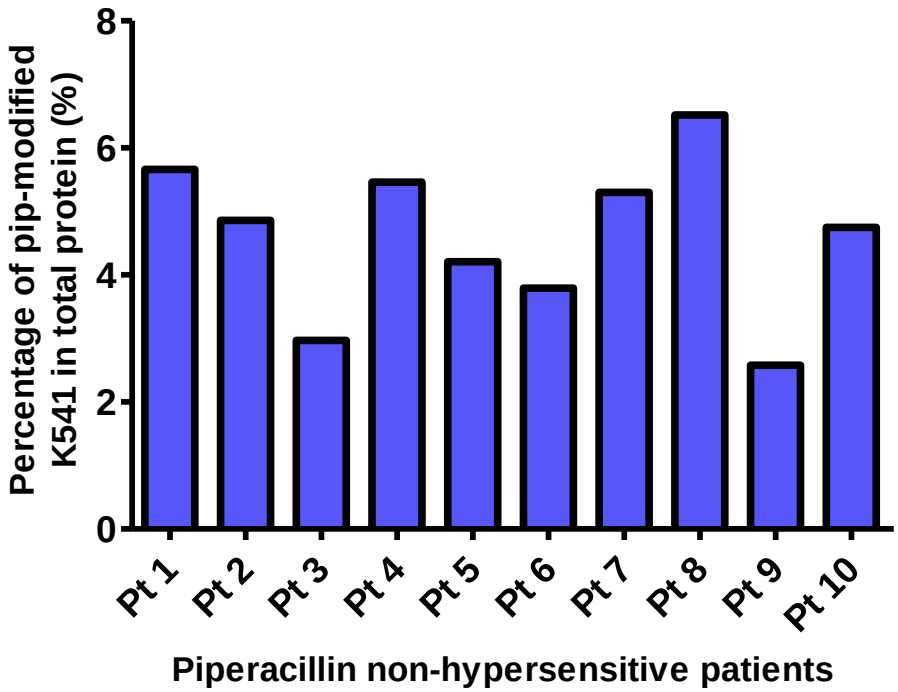


Figure 5.2 *Piperacillin modified K541 peptide in plasma of piperacillin non-hypersensitive patients.*
Pip-K541 peptide present in plasma of piperacillin non-hypersensitive patients. Percentage of total HSA protein that was modified at lysine 541 is shown.

5.4.3 Piperacillin specific T-cell clones

Having generated piperacillin-specific T-cell clones, well growing clones from two patients were selected for use in our assays to relate the level of piperacillin-modified protein to the piperacillin-induced T-cell stimulation seen *in vitro*. Clones responded to soluble piperacillin in a dose-dependent manner. Pulsing assays revealed that T-cell clones were stimulated with APCs that had been pulsed with piperacillin for 24 – 48 hours. Proliferative responses were comparable to that with soluble drug (Chapter 4, Figure 4.3).

5.4.3.1 Piperacillin modified K541 peptide is detectable *in vitro*

The culture supernatants from the T-cell proliferation assays (cultured with APCs and drug) were collected and analyzed for the level of piperacillin-modified K541 peptide. T-cells were treated with 0.1 – 4mM piperacillin to investigate how concentration affects piperacillin modification of HSA and if this correlated with T-cell responses. Four T-cell clones generated from two piperacillin hypersensitive patients were used in a standard proliferation assay with EBV-transformed B-cell lines used as APCs. Supernatants were processed and analysed by MRM-MS as described in methods chapter. A clear dose dependency was observed in the formation of Pip-K541 peptide with the percentage of total peptide that was modified reaching up to 4% (Table 5.2, Figure 5.3). At lower concentrations, 0.1 and 0.5mM piperacillin, levels of modified peptide constituted around 1% of total K541 peptide which coincided with the low levels of proliferation observed with T-cell clones exposed to the lower concentrations of soluble piperacillin.

Piperacillin conc. (mM)	Normalised ion count (AUC)	Pip K541 amount (pmol)	Percentage of total protein
0.1	2078.908	0.021690	0.45%
0.5	57906.205	0.063390	1.25%
1	77504.17	0.075456	1.5%
2	167889.1	0.152488	3.05%
4	256069.3	0.205408	4.1%

Table 5.2 *Modification of Pip-K541 peptide from in vitro cell culture supernatants is concentration dependent*
Piperacillin specific clones from two hypersensitive patients were exposed to 0.1 – 4mM piperacillin in a standard proliferation assay. Combined data is shown. Supernatants from *in vitro* T-cell cultures were collected after 48hrs and processed for mass spectrometric analysis. Pip-K541 peptide was identified by MRM/MS, signal strength was normalised and concentration was determined using the standard curve of the synthetic peptide standard. 5pmol digested protein was loaded on-column and the percentage of peptide modified at HSA K541 was determined.

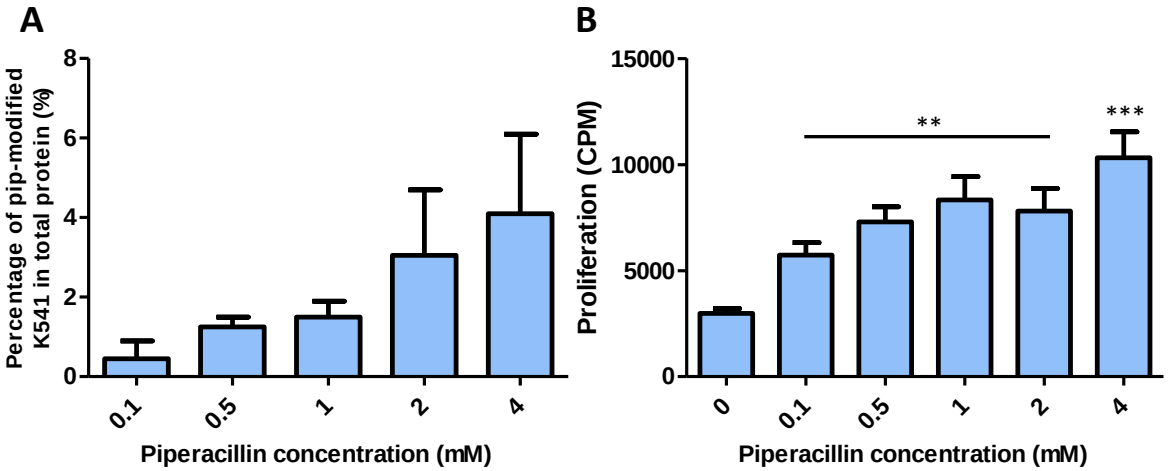


Figure 5.3 *Percentage of Pip-modified K541 peptide formed in vitro is dependent on concentration of piperacillin.*
(A) Pooled supernatants from *in vitro* proliferation cultures from piperacillin specific T-cell clones were analysed by MRM/MS and the concentration of Pip-K5421 peptide quantified. **(B)** Proliferation data from the clones used. T-cell clones were co-cultured with APCs and piperacillin at 0 – 4mM for 96 hours. Proliferation was measured with the addition [^{3}H]-thymidine in the last 16 hours. Supernatants for **(A)** were collected prior to [^{3}H] addition. ** P < 0.01, ***P < 0.001

Having observed an association between the initial concentration of piperacillin exposure and the level of piperacillin modified peptides formed (R^2 = E0.9515/P0.5192), the effect of incubation time was then investigated. Proliferation assays were conducted with 8 piperacillin specific T-cell clones, from 2 patients, co-cultured with APCs and exposed to 2mM piperacillin. Supernatants were collected after 1, 4, 24 and 48 hours incubation and processed for MRM/MS analysis. The formation of modified peptide was shown to be time-dependent (Table 5.3 and Figure 5.4). The percentage of Pip-K541 peptide rose to a maximum of around 4% after a 48 hour time point. Piperacillin modified peptide K541 was also detected at low levels at the shortest time point of 1 hour, with just over 1% of the total protein consisting of this modified peptide. The low levels of haptenation seen at 1 and 4 hours was mirrored in the proliferation data from the same clones, whereby positive proliferative responses were not recorded at the shorter APC piperacillin pulse time points (Figure 5.4B).

Time point	Normalised ion count (AUC)	Pip K541 amount (pmol)	Percentage of total protein
1 hour	17882.92	0.01886	1.26%
4 hours	21090.72	0.022068	1.47%
24 hours	41600.08	0.042577	2.84%
48 hours	60843.99	0.061821	4.13%

Table 5.3 *Quantification of Pip-K541 peptide from in vitro cell culture supernatants-time course.* Piperacillin specific clones from two hypersensitive patients were exposed to 2mM piperacillin in standard proliferation assay. Supernatants from *in vitro* T-cell cultures were collected after 1-48 hrs. Pip-K541 peptide was identified by MRM/MS, signal strength was normalised and concentration determined using standard curve of the synthetic peptide standard. 3 pmol digested protein from supernatant was loaded and the percentage of modified peptide was determined.

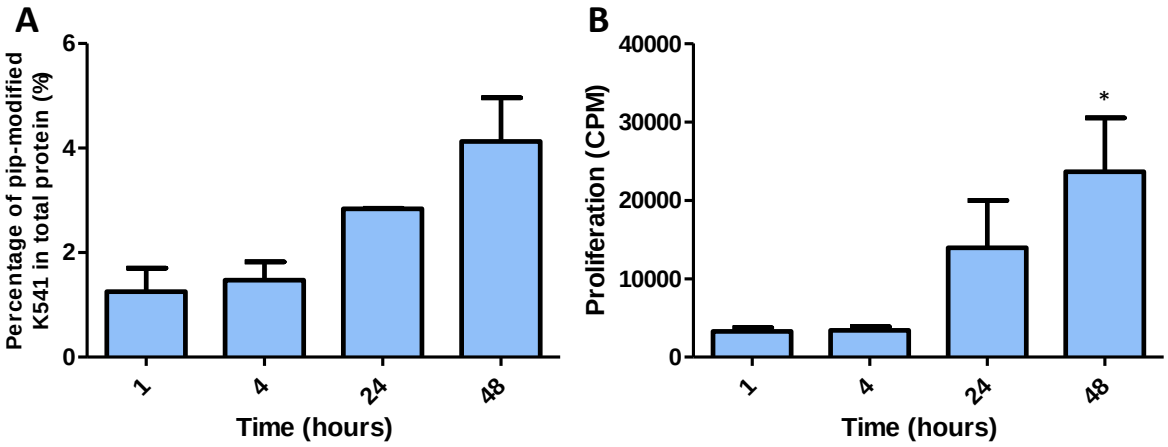


Figure 5.4 *Percentage of Pip-modified K541 peptide formed in vitro is time dependent.*
(A) Pooled supernatants from *in vitro* proliferation cultures from piperacillin specific T-cell clones were analysed by MRM/MS and the concentration of Pip-K5421 peptide determined. Supernatants were taken at 1, 4, 24 and 48 hour time points. Proliferation data from the clones used in assay are shown in **(B)** APCs were pulsed with 2mM piperacillin for 1-48hrs before removal of free drug and culture with T-cell clones. * P=0.0227

Time dependency for the formation of Pip-K541 peptide was also observed at other concentrations of piperacillin exposure. In parallel to this, concentration dependency was again observed in the *in vitro* cell culture supernatants (Figure 5.5).

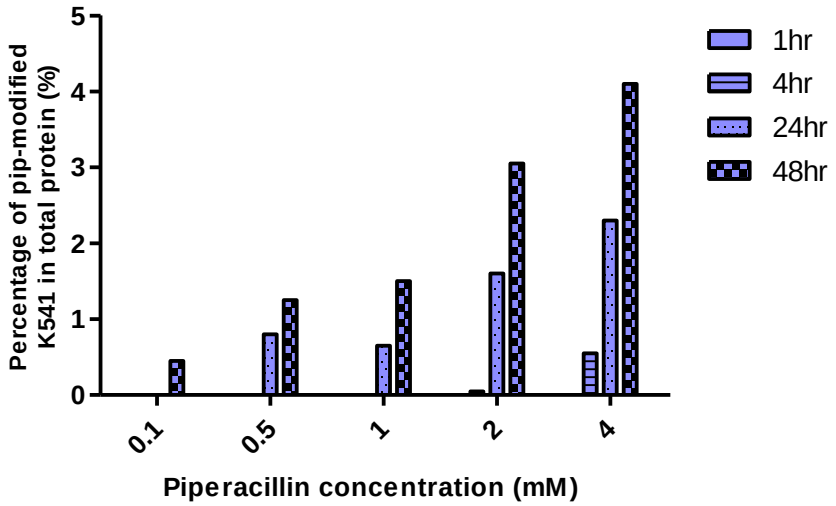


Figure 5.5 Formation *of Pip-modified peptide K541 is time and concentration dependent.* Piperacillin specific clones from two hypersensitive patients were exposed to 0.1 – 4mM piperacillin in standard proliferation assay. Supernatants from *in vitro* T-cell cultures were collected after 1-48 hrs. Pip-K541 peptide was identified by MRM/MS, signal strength was normalised and concentration determined using standard curve of the synthetic peptide standard. 3 pmol digested protein from supernatant was loaded and the percentage of modified peptide was determined.

5.5 Discussion

The penicillin class of β-lactam antibiotics has been widely studied, yet the relationship between levels of circulating drug-protein conjugate and activation of a cellular immune response in patients has not been defined.

Recent studies have shown that piperacillin covalently modifies lysine residues on albumin in plasma from hypersensitive and non-hypersensitive patients and hapten-modified albumin is able to stimulate piperacillin responsive T-cells isolated from hypersensitive patients *ex vivo*. These observations raise the question whether there is a difference in the level of modification in individuals and if there is a threshold of piperacillin antigen exposure required for T-cell activation. We quantified the level of piperacillin modification in patient plasma and in cell culture supernatants using mass spectrometric methods. The level of piperacillin-modified albumin in patient plasma was found to range from 2 to 6%; these figures are similar to the levels of modification needed to activate patient lymphocytes *ex vivo*. Thus, although levels of free drug needed to activate T-cells are significantly higher than plasma concentrations, the level of piperacillin protein adduct are comparable. Our findings also highlight the important roles of antigen dose in the activation of T-cells.

Historically, quantification of drug-modified protein has always been challenging. Emerging mass spectrometric techniques have made it feasible to achieve the relative quantification of protein modification and the absolute quantification when combined with synthetic standards (Gallien *et al.*, 2011; Lange *et al.*, 2008). However, the synthesis of piperacillin-modified peptides is extremely challenging and requires a specific synthetic strategy since direct penicilloylation of peptides occurs mainly on the N-terminal amino group.

Another major obstacle encountered in the synthesis of piperacillin-modified peptides is the acid lability of the piperacillin molecule. Acid treatment of the peptides either during chain elongation or during cleavage of the peptides from the support is a necessary component of peptide synthesis. Consequently, special precautions are required to avoid loss of the piperacillin group during the synthesis of piperacillin bearing peptides. We therefore developed a stepwise method incorporating a piperacillin-lysine conjugate to synthesize piperacillin-modified peptides in which piperacillin is attached to ε-amino group of lysine. The novel synthetic method is based on the use of a specific protecting group for blocking potentially reactive side chain groups of amino acids in the peptide synthesis. Preferred side-chain protecting groups are readily removable under mild conditions at the end of the synthesis without disturbing the piperacillin molecule.

With peptide ATK(Pip)EQLK in hand, we sought to quantify piperacillin HSA adducts in plasma isolated from10 patients with CF. In addition to the quantification of the Pip-K541 peptide we were also able to determine the level of modified peptide with relation to total protein. This was found to range from as little as 2.5% rising up to 6.5% of total protein consisting of modified peptide. The next step was to estimate the minimal quantity of piperacillin haptenation necessary to induce T cell activation. Piperacillin-specific T-cell clones generated from PBMCs isolated from hypersensitive patients with CF were cultured with piperacillin at various concentrations (0.25–4mM), and 25 µl supernatant was removed after 1–48 hours to measure the level of piperacillin haptenation. Pip-modified K541 was detectable at 0.1 mM after a 48 hour incubation; however, the level of modification was low (1%), coinciding with a

weak proliferative response (Figure 5.3). A concentration dependent increase in the level of modification was associated with the proliferation of piperacillin-specific T-cell clones. To quantitatively determine the extent of Pip haptenation required for triggering immune responses, EBV-transformed B-cells, which are used as APCS, were pulsed with piperacillin for 1, 4, 24, and 48 hours prior to washing and exposure to clones. The supernatants were removed to determine the level of piperacillin haptenation in culture. APCs pulsed with piperacillin for 1 and 4 hours did not stimulate T-cells to produce a proliferative response, corresponding with low levels of haptenation (less than 1.5%). In contrast, APCs pulsed with piperacillin for 24 and 48 hours stimulated all clones to proliferate and the strength of the response was stronger to that seen with the soluble drug. It can therefore be estimated that as little as 2.8% of piperacillin haptenation is sufficient for T-cell activation.

It is worth noting that the level of the piperacillin antigen formed in patients (2.58 to 6.52%), was in the same range as that required to activate T-cells *in vitro*, yet not all patients develop an adverse reaction. The inability of piperacillin haptens to induce immune responses in certain patients suggests that hapten modification of protein alone is not sufficient to cause hypersensitivity reactions. This poses the key question: what drives some patients to be allergic and not others? The outcomes of immune responses may be ultimately determined by an orchestral scenario incorporating antigen formation, danger signals, immune regulation and genetic factors.

Accumulative evidence, largely from *in vitro* studies, demonstrate that a high antigen dose favours the development of Th1 cells, whereas a low antigen dose favours the development of Th2 cells(Hosken *et al.*, 1995; O'Garra, 1998;

O'Garra *et al.*, 2011). In addition, a repeated high-dose of antigen has been shown to generate anergy or tolerance of immune responses (Gabrysova *et al.*, 2010; Meiler *et al.*, 2008; O'Garra *et al.*, 2011). Moreover, the IgE response against protein antigens is profoundly influenced by the dose used for sensitization (Barwig *et al.*, 2010). Whether the antigen dose will have impact on allergic phenotypes in CF patients is currently under investigation. A prospective study to monitor the quantitative relationship between antigen formation and immunological responses in hypersensitive patients with CF during the desensitization process will undoubtedly help improve the safety profile of piperacillin in susceptible populations.

Chapter 6: Specificity of T-cell responses in patients with flucloxacillin-induced liver and skin injury and in HLA-B*57:01+ volunteers

6.1 Introduction

Adverse drug reactions pose major problems both clinically and also in the drug development pathway. A number of drug hypersensitivity reactions have been linked with HLA molecules, cell surface glycoproteins expressed on professional APCs. HLAs represent the loci of genes that encode the MHC complex in humans. There are two major classes, MHC I and MHC II, so named due to differences in their molecular structure and function. As heterodimers, both classes of MHC molecule consist of an anchored heavy α chain (extracellular α_1, α_2 and α_3 chain in the case of MHC I and membrane anchored α_1 and α_2 chain in MHC II molecules) and a second light chain β domain. MHC I molecules possess a soluble β domain known as $\beta_2$microglobulin (β_2m) which is not membrane bound, unlike class II molecules whose β domain (β_1 and β_2) is anchored (Madden, 1995). Structural differences allow the two classes of MHC molecule to accommodate different lengths of peptide within their antigen binding grooves. MHC I generally holds peptides between 8 and 11 amino acids in length whilst the peptide binding groove of MHC II molecules can accommodate longer peptides (12-25 amino acids) due to its more open structure (Rammensee, 1995; Speir *et al.*, 2001).

MHC molecules on APCs play a crucial role in the activation of T-cells. They are utilised as a means to display peptide antigens. This process is critical as T-cells are only able to recognise and respond to antigenic peptides that are bound and presented via the MHC complex (Rudolph *et al.*, 2006). In general, peptides derived from endogenous antigens are loaded on to MHC I molecules, which are presented to and recognised by receptors on CD8+ T-cells, whereas CD4+ T-cells express receptors that recognise peptide fragments, which are presented

in the context of MHC II, usually derived from extracellular proteins (Guermonprez *et al.*, 2002; Pamer *et al.*, 1998).

As a highly polymorphic region, a wide range of variant HLA alleles exist, which in turn interact with the wide range of possible peptides. The MHC region is reported to contain around 100 genes that code for molecules involved in antigen presentation and immune regulation (Horton *et al.*, 2004; Mungall *et al.*, 2003). Indeed, due to this high degree of variety, many drug hypersensitivity reactions are directly associated with particular HLA alleles. It is believed that HLA molecules play a direct role in the pathogenesis and development of drug hypersensitivity and also in the induction of self-tolerance (Bharadwaj *et al.*, 2010; Yun *et al.*, 2012). Certain HLA alleles pose as risk factors for the development of hypersensitivity whereas others can provide protection (Mallal *et al.*, 2002; Temajo *et al.*, 2009). The association between HLA-B*57:01 to the antiretroviral drug abacavir and the development of hypersensitivity has been extensively studied (Adam *et al.*, 2012; Chessman *et al.*, 2008; Illing *et al.*, 2012; Ostrov *et al.*, 2012) and results perfectly demonstrate the concept of personalised medicine. Pre-clinical screening of patients to identify the presence of the HLA-B*57:01 allele and exclusion of such patients from abacavir treatment significantly reduces the incidence of hypersensitivity to abacavir and this is now common clinical practice (Hughes *et al.*, 2004; Mallal *et al.*, 2008; Zucman *et al.*, 2007).

Carbamazepine-mediated SJS/TEN in Han Chinese and Thai populations has a strong selectivity as the drug activates CD8+ T-cells in patients carrying the risk allele. Again screening is recommended for possession of HLA B*15:02 (Chen *et al.*, 2011; Chung *et al.*, 2004). More recently HLA-A*31:01 has been implicated

as a risk allele in Caucasian and Japanese populations (McCormack *et al.*, 2011; Ozeki *et al.*, 2011). Drug-specific CD8+ T-cell responses have been detected in donors expressing HLA-A*31:01 and the CD8+ T-cell response was HLA-A*31:01 restricted. However, in the same HLA-A*31:01 positive donors, CD4+ T-cells were detected and the drug was presented to the T-cells by a variety of HLA class II molecules (Lichtenfels *et al.*, 2014).

Thus far, our investigations have focussed on delayed type skin reactions, yet there is an array of ways in which adverse reactions to drugs can present themselves. The β-lactam antibiotic flucloxacillin is widely used for Staphylococcus aureus infections. Unfortunately, in Europeans around 8.5 patients in 100,000 develop cholestatic hepatitis following flucloxacillin treatment (Bharadwaj *et al.*, 2010). In 2009 an association between HLA-B*57:01 and flucloxacillin induced liver injury was identified. Despite the strong association only 1 in every 500 – 1000 individuals with HLA-B*57:01 develop DILI following treatment with flucloxacillin (Daly *et al.*, 2009b). This clearly suggests that a variety of other factors including; age, metabolic capacity and underlying disease states contribute to the development of this iatrogenic disease (Chalasani *et al.*, 2010). Furthermore, possession of the HLA-B*57:01 allele should not be used to exclude patients from flucloxacillin treatment as most will not develop DILI and therefore be prevented from taking an effective drug. This is very different to abacavir where screening is internationally accepted as an essential pre-requisite to therapy. Also unlike abacavir, flucloxacillin does not alter the repertoire of self-peptides binding to HLA-B*57:01 and is instead thought to bind covalently to endogenous proteins before resultant peptides are processed and presented to drug specific T-cells

via HLA-B*57:01 (Monshi *et al.*, 2013; Norcross *et al.*, 2012). It was with the association of abacavir and HLA-B*57:01 that the potential role of HLAs in personalised medicine really came to the forefront. As stronger and clinically relevant HLA associations (unlike flucloxacillin) come to light, personalised medicine becoming routine could be a very real possibility in the future.

Until recently, the role of T-cells in human DILI was not well defined. PBMC proliferative responses to drugs in up to 65% of DILI patients was reported in an early study, suggesting that liver injury might indeed involve an immunopathological mechanism (Maria *et al.*, 1997). However, these T-cells were never characterised in terms of phenotype or function and additional studies have not been forthcoming. Furthermore, liver histology from a patient suffering from DRESS and fulminant liver failure following sulphasalazine treatment revealed an infiltration of granzyme B secreting T-cells in close proximity to apoptotic hepatocytes. These T-cells were thought to be the major cellular effectors for the tissue necrosis observed and *in vitro* proliferation tests confirmed the T-cells were drug specific (Mennicke *et al.*, 2009). However, few studies have taken investigations further in order to fully characterise the role of T-cells in DILI, and information relating HLA risk alleles to T-cell responses is also limited. A recent study by Monshi *et al.* took steps to address this (Monshi *et al.*, 2013). Flucloxacillin-responsive CD4+ and CD8+ T-cells generated from patients with liver injury were characterised in terms of phenotype and function and naive CD45RA+CD8+ T-cells from healthy donors expressing HLA-B*57:01 were shown to be activated with flucloxacillin when the drug antigen was presented by DCs. Furthermore, activation of CD8+ T-cell clones was found

to be processing dependent and restricted by HLA-B*57:01 and the closely related HLA-B*58:01.

In a recent study, flucloxacillin-responsive CD8+ clones from healthy donors with and without the HLA risk allele B*57:01 were generated to evaluate mechanisms of drug antigen presentation (Wuillemin *et al.*, 2013b). Flucloxacillin degrades spontaneously in cell culture medium liberating a hapten that binds covalently to lysine residues on protein (Jenkins *et al.*, 2009b). Hence, it is possible to differentiate between flucloxacillin and flucloxacillin hapten-responsive T-cells through the use of soluble drug and drug-pulsed antigen presenting cells as sources of antigen. T-cells from HLA-B*57:01 negative donors were preferentially activated via a hapten mechanism involving protein processing. In contrast, the parent drug was found to activate most T-cells in HLA-B*57:01+ donors. These data led the authors to conclude that the parent drug drives T-cell responses in HLA-B*57:01+ donors and liver reactions in susceptible patients. In this chapter, we aim to expand on the current information and further investigate the relationship between the genetic association and immune basis for DILI.

6.2 Aims and Hypothesis

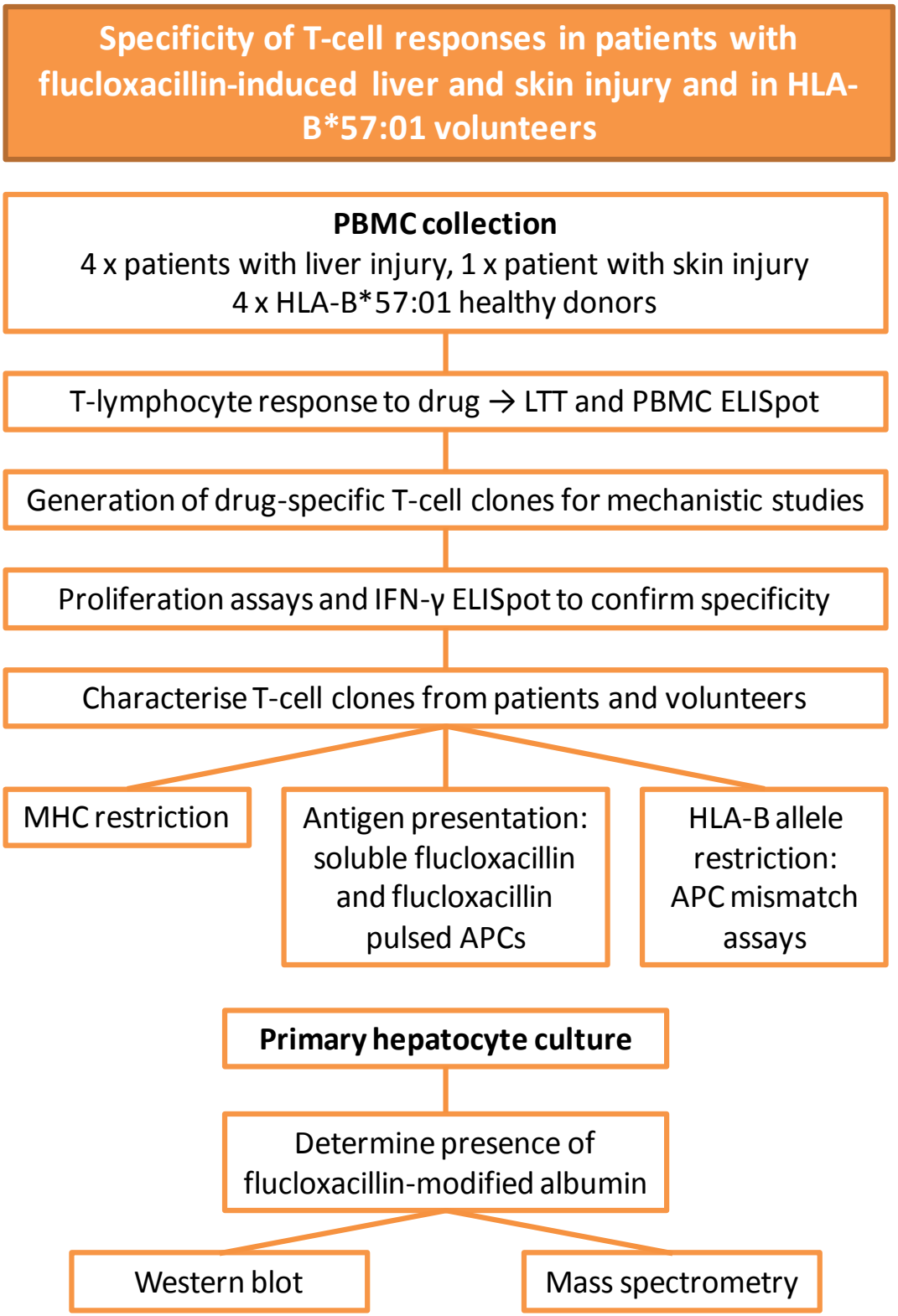
With the discovery of HLA-B*57:01 as a risk factor for flucloxacillin-induced liver injury, this chapter aims to further investigate the flucloxacillin-specific T-cell response in HLA-B*57:01 patients with DILI and a patient expressing the risk allele who developed a serious skin reaction. Specific objectives included; (1) exploration of T-cell responses in HLA-B*57:01 donors and patients to identify differences between patients suffering from different forms of hypersensitivity reactions, (2) investigation of HLA specificity by utilising an array of different APCs expressing different HLA molecules and finally (3) investigation of flucloxacillin binding primary human hepatocytes. With these aims and previous literature in mind I propose the following hypotheses:

(1)Mechanisms for the development of an immune response will differ between patients and volunteers.

(2)Fine specificity to HLA alleles will be observed in flucloxacillin specific T-cells.

6.3 Methods

The methods used in this chapter are described fully in Material and Methods, Chapter 2. A brief experimental outline is detailed below.



Scheme 6.1 *Experimental plan and methods used in this chapter*

6.4 Results

We are fortunate here at the University of Liverpool to have close connections to the Royal Liverpool and Broadgreen University Hospital (RLBUHT) enabling recruitment of patients with flucloxacillin-induced liver and skin injury. Furthermore, the university hosts a unique cohort of 1000 genotyped healthy volunteers. Access to this biobank of lymphocytes from HLA-typed volunteers has also allowed us examine T-cell responses to flucloxacillin lymphocytes from HLA-B*57:01 positive drug naive blood donors.

6.4.1Flucloxacillin specific PBMC response

PBMCs were isolated from patients with flucloxacillin-induced liver injury and from one patient, patient 018, who developed a severe cutaneous hypersensitivity reaction (AGEP) following flucloxacillin exposure. Patient characteristics and HLA type are detailed in Table 6.1. It was interesting to observe that lymphocyte proliferative responses were not detected in any of the DILI patients (Figure 6.1A), though we later used clones generated from these patients to explore mechanisms involved in the T-cell response. Proliferative responses from patient 018 were detected following PBMC isolation and *in vitro* culture with titrated concentrations of flucloxacillin (Figure 6.1C). PBMC responses to flucloxacillin were also detected using an IFN-γ ELISpot, not only in the patient with skin injury but also from the PBMCs of patients with DILI (Figure 6.1B). As shown in Figure 6.1D secretion of IFN-γ was dose dependent and ELISpot assays also showed flucloxacillin induced secretion of IL-13 and Granzyme B (Figure 6.1B).

	ID	Age/ gender	Peak liver function tests at time of liver injury			Time to Onset	RUCAM Score	Since Reaction
			ALT	Bilirubin	ALP	weeks		years
Liver injury	**P1**	61/M	23x ULN	3x ULN	9x ULN	3	7	11
	P2	73/F	13x ULN	12x ULN	2x ULN	4	3	2
	P3	90/F	11x ULN	13x ULN	4.5x ULN	3	6	3
	P4	65/F	4x ULN	36x ULN	2x ULN	3	5	3
Skin injury	**P018***	Reaction 1	2x ULN	0.5x ULN	1.5x ULN	2	-	10
	58/M	Reaction 2	3x ULN	1x ULN	1x ULN	1 day	-	4

Table 6.1 *Clinical details and HLA-B*57:01patients*
Patients 1 – 4 are those with flucloxacillin-induced liver injury
* Patient 018 suffered from a skin reaction following exposure to flucloxacillin. Patient 018 was given flucloxacillin twice over the last 10 years and was diagnosed with AGEP on both occasions. Details of both reactions are shown and despite lower elevated ALT liver function tests were also conducted.
ULN = upper limit of normal (ALT, alanine aminotransferase – 35, bilirubin – 17, ALP, alkaline phosphatase – 125). RUCAM = Roussel Uclaf Causality Assessment Method, cases were evaluated by application of the Council for International Organizations of Medical Science scale. The pattern of liver injury was classified according to the International Consensus Meeting Criteria. 3–5 = possible, 6–8 = probable, >8 = highly Probable. RUCAM score not obtained for patient 018. Diagnosis of DILI was done by expert hepatologists.

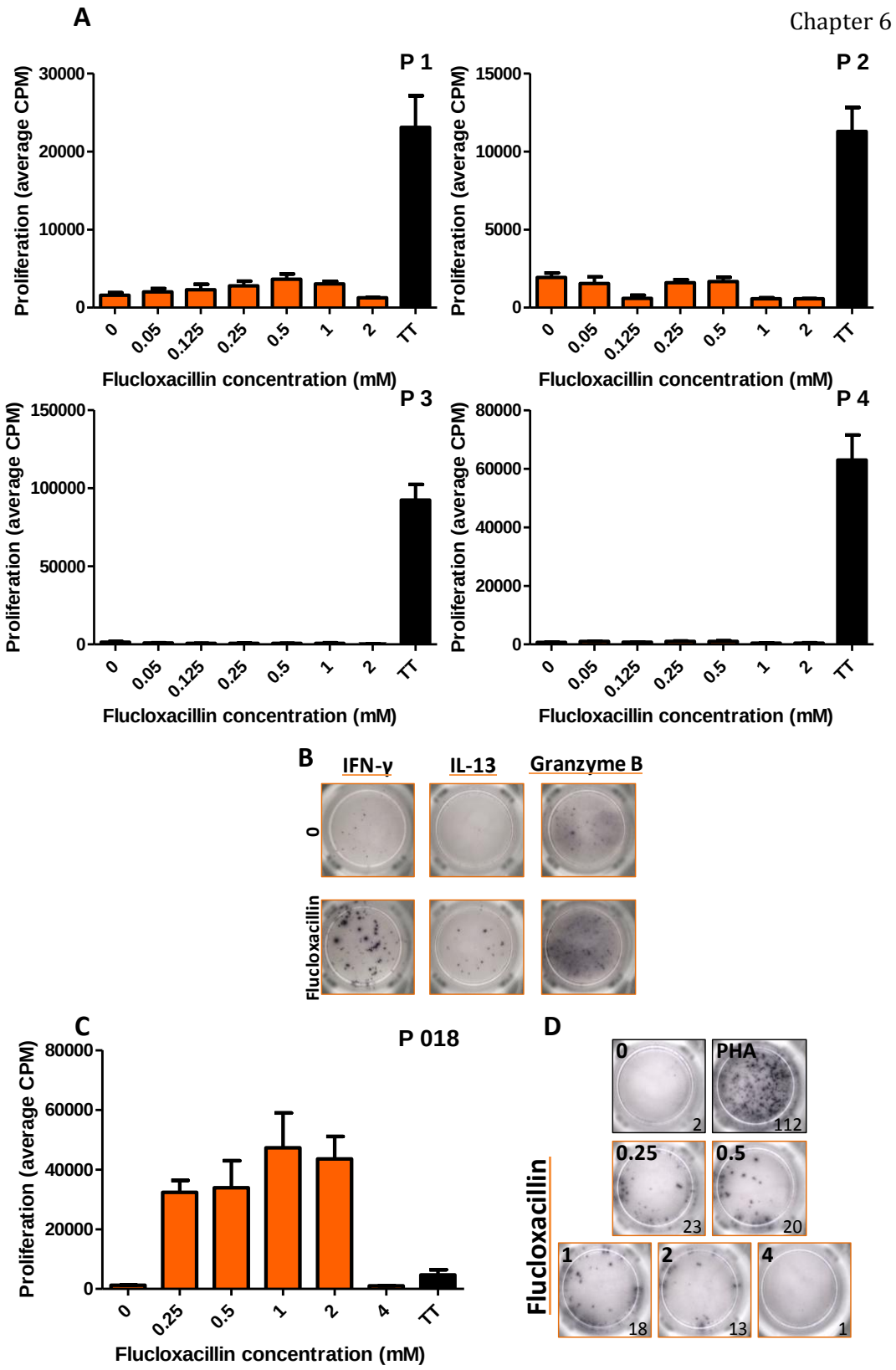


Figure 6.1 *Flucloxacillin specific stimulation of patient PBMCs*
(A and C) PBMCs were incubated with flucloxacillin (0.05 – 4mM) for 6 days with [3H]-thymidine added to cultures in the last 16 hours to detect lymphocyte proliferation, as per LTT assay. CPM = counts per minute. Culture medium (0) and tetanus toxoid (TT, 0.5mg/ml) were used as negative and positive controls respectively. Data from patients with DILI shown in **(A and B)** and LTT of patient 018 with flucloxacillin induced AGEP is shown in **(C)** Flucloxacillin-specific IFN-γ secretion from patient PBMCs was detected by ELISpot assay. Cells were cultured with flucloxacillin (0.25 – 4mM) for 2 days before plates were developed for spot detection. **(B)** Flucloxacillin-specific (1mM) IFN-γ IL-3 and Granzyme B secretion from DILI patient PBMCs using cytokine specific ELISpot assays. **(D)** IFN-γ ELISpot from skin patient 018.

6.4.2 Characterisation of flucloxacillin-specific T-cell clones

PBMCs from patients who had previously suffered from flucloxacillin-induced liver injury and PBMCs from one patient who had experienced a severe skin reaction (AGEP, Pt. 018) following administration of flucloxacillin were used to generate T-cell clones, specific to flucloxacillin (Table 6.2). Finally, flucloxacillin specific clones were also generated from the PBMCS of HLA-B*57:01 positive healthy volunteers from our HLA-typed cohort using our recently developed DC/T-cell co-culture system that effectively primes naive T-cells. The process of T-cell cloning involves the pre-selection of CD8+ T-cells, however magnetic bead isolation is not absolute and though most of the T-cell clones generated were CD8+, 15% of the total flucloxacillin-specific T-cell clones generated were found to be CD4+ T-cells following analysis by flow cytometry. These data agree with our previous study showing CD4+ and CD8+ T-cells in patients with DILI (Monshi *et al.*, 2013). The clones from the patient with flucloxacillin-mediated AGEP were all CD8+.

ID	No. specific clones	CD4+ (%)	CD8 (%)	CD4+/CD8+ (%)
Patient clones				
P 1 - 4	90	8	88	2
P 018	7	0	100	0
HLA*B57:01+ volunteer clones				
V 1	11	100	0	0
V2	28	0	100	0
V3	4	0	100	0
V4	3	0	100	0

Table 6.2 *Number and phenotype of flucloxacillin-specific T-cell clones generated.*
Patient ID's are prefixed by the letter P and healthy volunteers with a V. Majority of clones generated from patients were from those with liver injury (P1-4, see table 1 for clinical details). Patient 018 had previously developed a skin reaction to flucloxacillin.

T-cell clones were assessed for specificity using a standard 72 hour proliferation assay. T-cell clones were co-cultured with EBV-transformed B-cells, which were used as APCs, and flucloxacillin. Cells were incubated at 37°C in an atmosphere of 95%O_2/5% CO_2 for 48 hours with [^{3}H] thymidine (0.5µCi) added in the last 16hrs of the incubation to assess lymphocyte proliferation. CD8+ T-cell clones generated from the PBMCs of patients with liver and skin injury and healthy volunteers were antigen specific and lymphocyte proliferation was dose dependent (Figures 6.2-6.4).

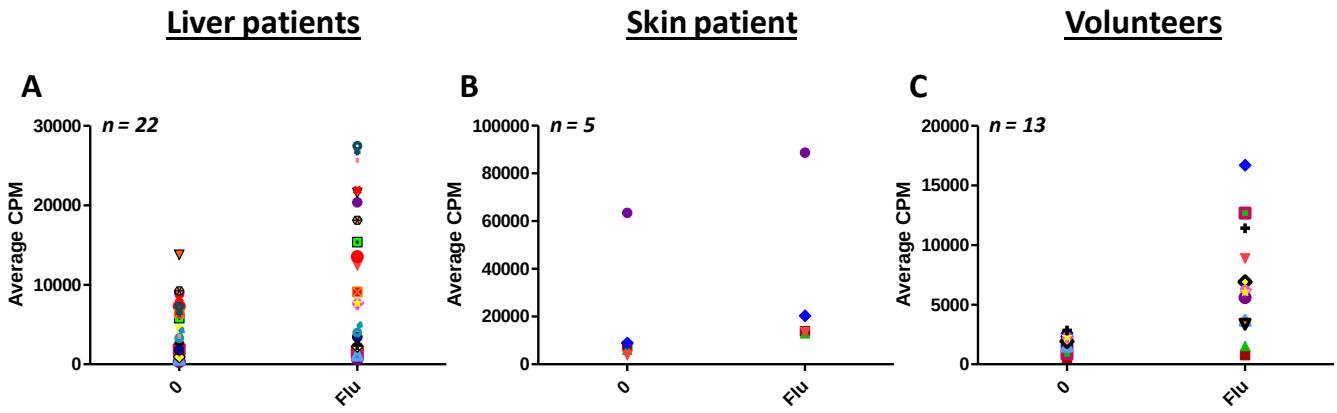


Figure 6.2 *Flucloxacillin specific proliferation of T-cell clones*
Each data point represents mean [^{3}H] incorporation as a measure of proliferation from flucloxacillin specific clones. T-cell clones were generated from patient with **(A),** liver injury, n=22, **(B)** one patient with skin reaction, n=5 and from **(C)** healthy volunteer, n=13. CPM = counts per minute.

Individual proliferation data from nine T-cell clones generated from patients with liver and skin injury are shown in Figure 6.3 and representative cytokine secretion data is presented in Figure 6.4. These data confirm antigen specificity and highlight the range in responses seen. Some T-cell clones react readily following flucloxacillin exposure whereas others respond less vigorously, though still show positive dose dependent proliferative responses and cytokine release. Concentrations of 0.25mM and 0.5mM are often sufficient to elicit a proliferative response or indeed stimulate cytokine secretion, whilst 4mM flucloxacillin appears to be cytotoxic.

We also observed no difference in the dose-dependent response from patients and healthy donors (Figure 6.5).

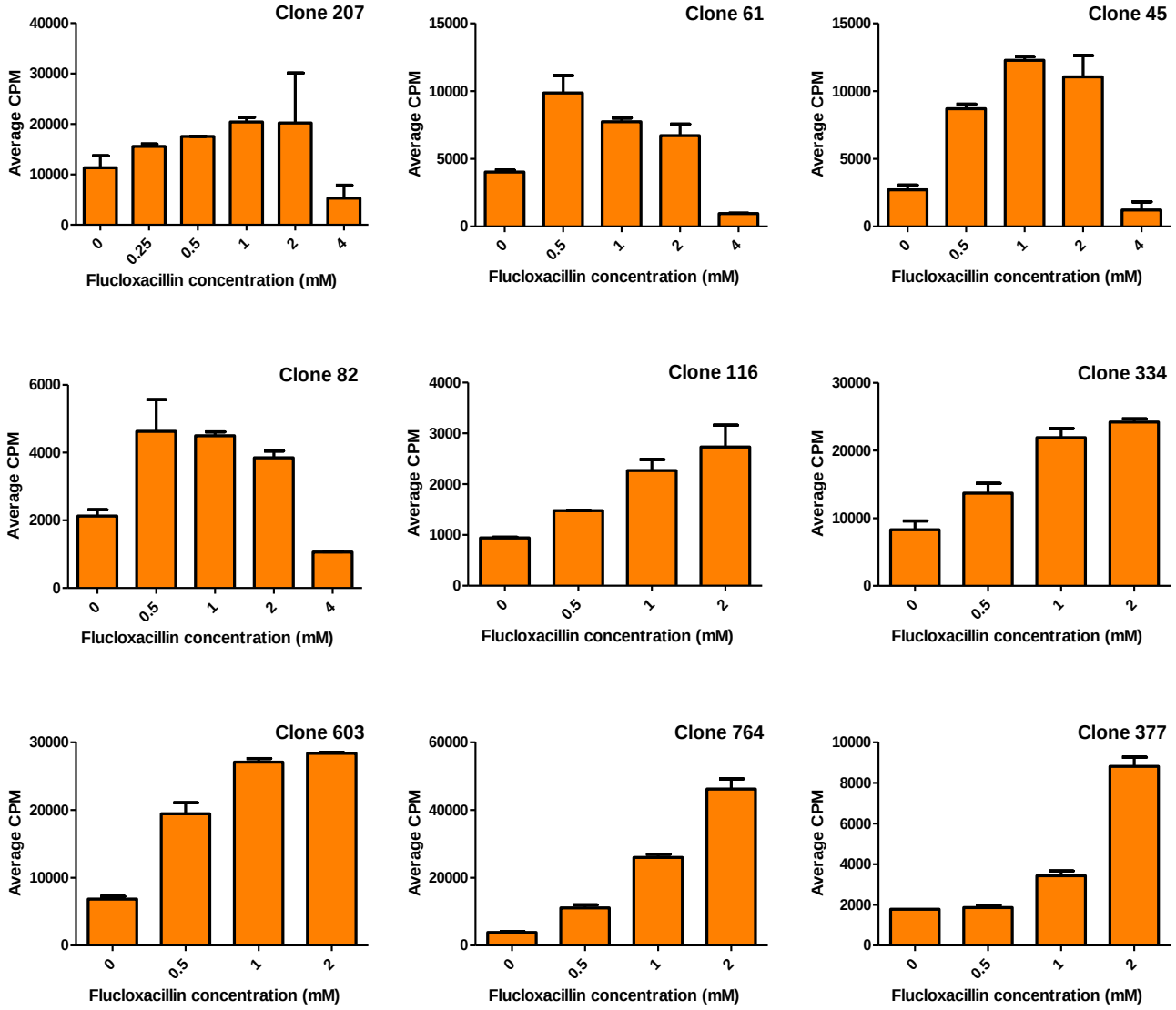


Figure 6.3 *T-cell clones proliferate in a dose-dependent manner*
Proliferation following flucloxacillin exposure (0-4mM) was assessed. T-cell clones were co-cultured with APCs and drug at 37°C in an atmosphere of 95%O_2/5% CO_2 for 72 hours. [^{3}H] thymidine incorporation (0.5µCi) added in the last 16 hours of incubation was used to assess antigen specific proliferation. Data from 9 representative clones shown. CPM = counts per minute.

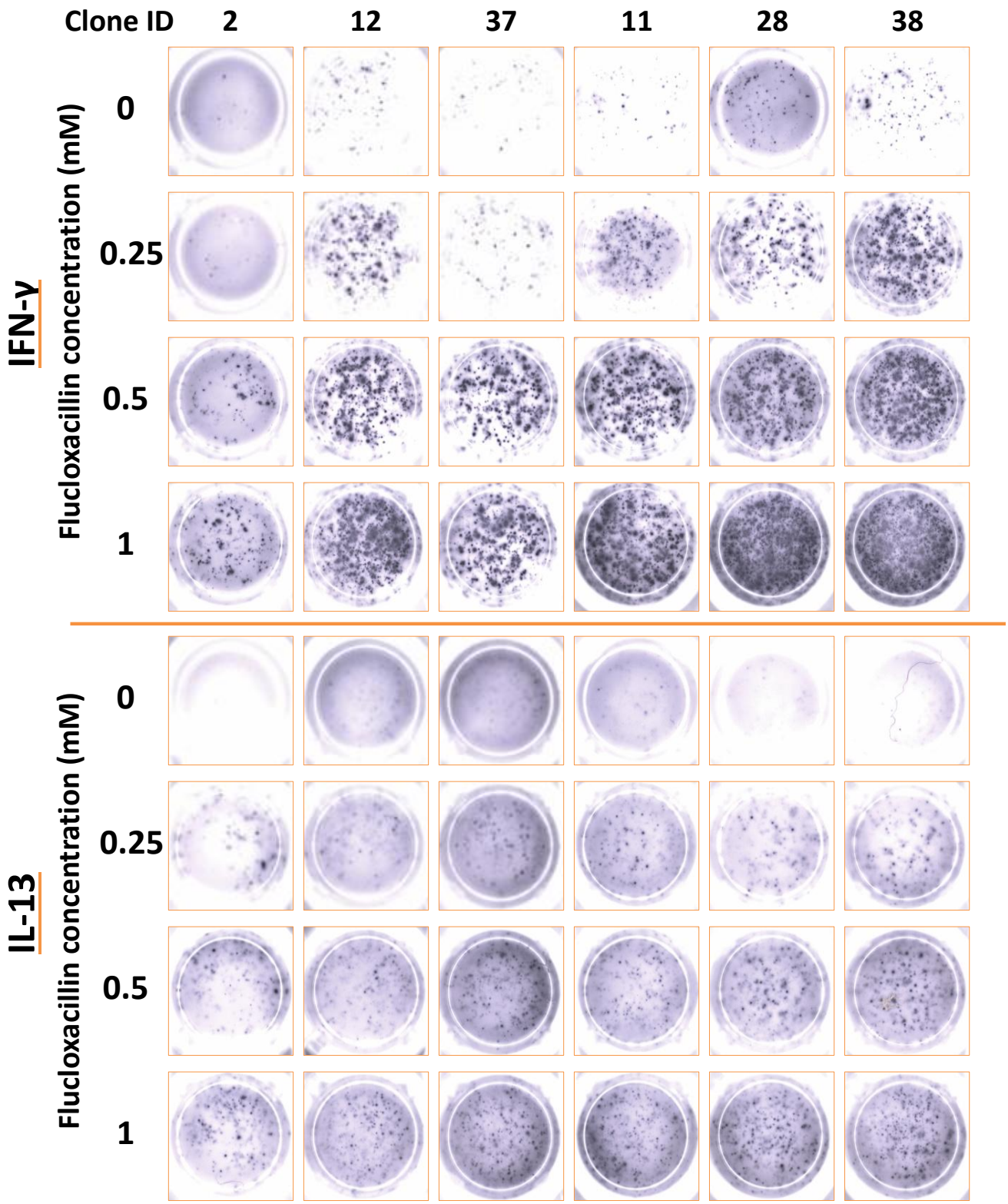


Figure 6.4 *Cytokine secretory response of flucloxacillin specific T-cell clones is dose dependent*
Drug specific secretion of IFN-γ and IL-13 was determined via cytokine specific ELISpot assay. Plates were first coated with specific cytokine antibodies and incubated overnight. T-cell clones were then co-cultured with APCs and drug for 48 hours after which plates were developed for visualisation of spots according to manufacturer's instructions. Six representative clones shown.

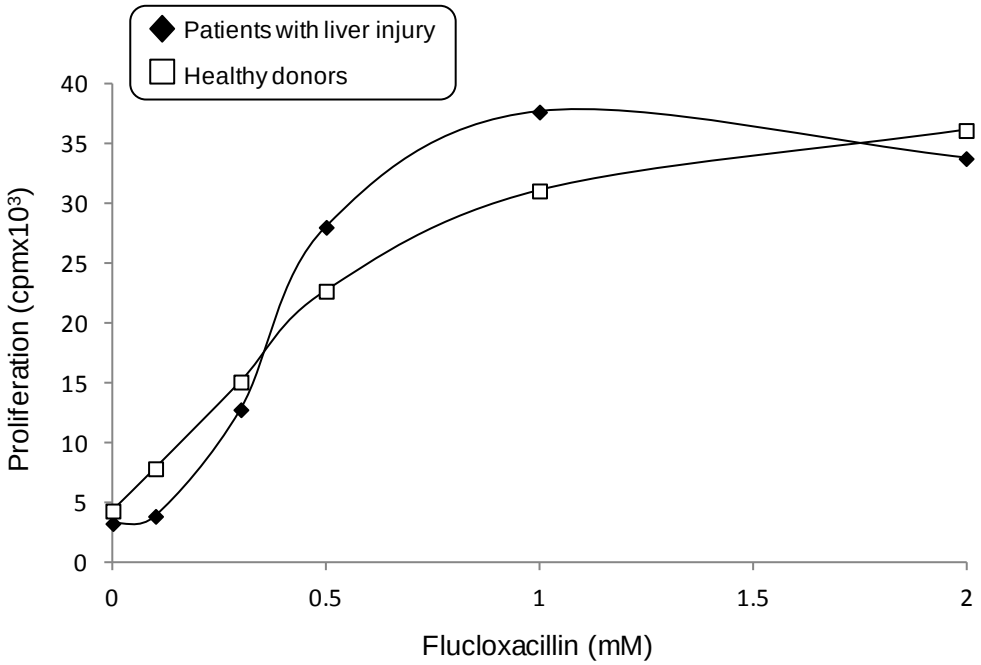


Figure 6.5 *No difference in proliferation is seen between T-cell clones generated from patients and volunteers*. Activation of T-cell clones from healthy donors (n=7) and patients (n=7) with titrated concentrations of flucloxacillin. Proliferation was measured after 72h through the addition of [^{3}H] thymidine. CPM = counts per minute. Mean CPM are shown in the figure. Student T-test was used to compare the response of clones from patient and healthy donors at each drug concentration. Significant differences were not detected.

6.4.3 Activation of flucloxacillin-specific T-cell clones is MHC restricted

Using anti-human MHC I and MHC II blocking antibodies we endeavoured to determine if CD4+ and CD8+ T-cell activation (proliferative activity) was restricted by MHC I or II. A panel of clones generated from both patients and healthy volunteers were tested for MHC-restriction. The proliferation of CD8+ T-cell clones was found to be restricted by MHC I, but not MHC class II (Figure 6.6, clones 764, 122 and 42). The opposite was true of CD4+ flucloxacillin specific T-cell clones; addition of MHC I blocking antibody did not negatively affect proliferative activity whereas blockade of MHC II inhibited activation (Figure 6.6, clones 8 and 3). Finally, MHC I restriction was observed with dual positive T-cell clone 14. No decrease in proliferation was seen following addition of the MHC II blocking antibody. In fact proliferative activity increased suggesting activation of this clone is MHC I restricted. Thus, our results show that the proliferation of T-cell clones from patients and healthy donors are indeed MHC-restricted according to the phenotypic characteristics of the T-cells in question.

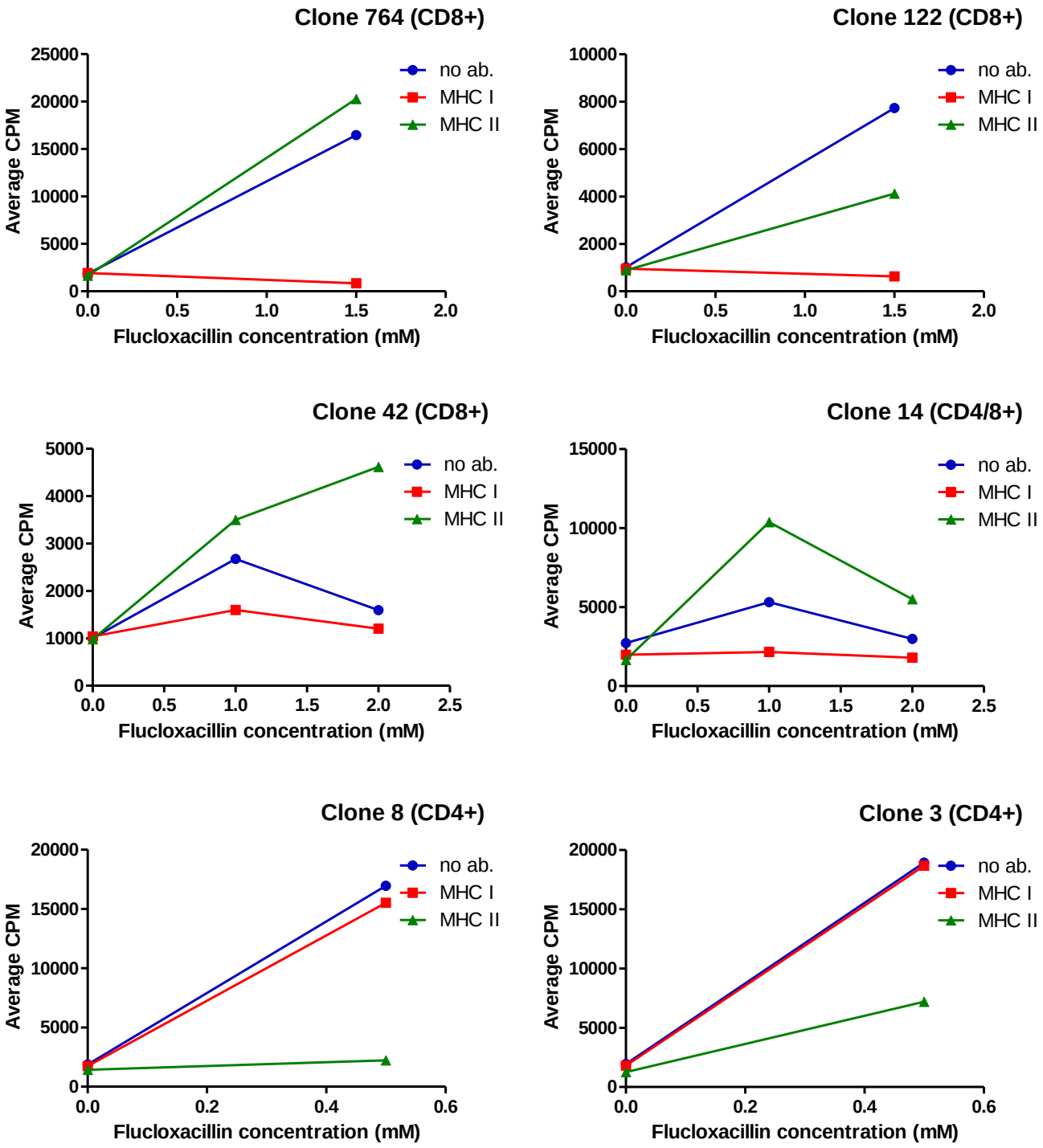


Figure 6.6 *MHC restricted flucloxacillin recognition*
Autologous EBV-transformed B-cells were pre-incubated with or without MHC I or MHC II blocking antibodies (5µl/ml) for 30 minutes at 37°C before co-culture with flucloxacillin-specific T-cell clones in the presence or absence of flucloxacillin. Cells were incubated for 48 hours at 37°C in an atmosphere of 95%O_2/5% CO_2. [^{3}H] thymidine (0.5µCi) was added in the last 16 hours of incubation to determine antigen specific proliferation. Data from 6 representative clones from liver patient and healthy volunteer are shown. CPM = counts per minute.

6.4.4 Activation of clones from healthy donors and DILI patients with soluble flucloxacillin and flucloxacillin-pulsed APCs

The following section focuses on CD8+ T-cell clones from HLA-B*57:01+ DILI patients and healthy donors to explore mechanisms of antigen presentation.

Flucloxacillin degrades spontaneously in cell culture medium liberating a hapten that binds covalently to lysine residues on protein (Jenkins *et al.*, 2009b). It is therefore possible to differentiate between flucloxacillin and flucloxacillin hapten-responsive T-cells through the use of soluble flucloxacillin and flucloxacillin-pulsed antigen presenting cells as sources of antigen.

Flucloxacillin-specific T-cell clones were cultured with autologous EBV-transformed B-cells (APCs) and soluble flucloxacillin (1mM) or APCs that had been pulsed for 16 hours with flucloxacillin (1mM). Proliferative responses were measured using [^{3}H]-thymidine incorporation after 48 hours. CD8+ T-cell clones from healthy donors were activated to proliferate with soluble flucloxacillin, but not flucloxacillin-pulsed antigen presenting cells (Figure 6.7). The results obtained with T-cell clones obtained from DILI patients somewhat contrasted with the results seen from healthy donors; all clones were activated with soluble flucloxacillin and flucloxacillin-pulsed antigen presenting cells.

Binding of flucloxacillin to lysine residues on the model protein human serum albumin was measured to confirm that hapten-protein adducts are formed during the 16h culture period (Figure 6.8A). Importantly, in our next experiment antigen presenting cells were pulsed with flucloxacillin for different durations (4-48h) and responses were not detected in T-cell clones from healthy donors (Figure 6.8B). However, with MHC class I restricted flucloxacillin-specific CD8+ T-cell clones generated from PBMCs of HLA-

B*57:01+ patients with liver injury, different results were obtained confirming the results from Figure 6.7. Clones were activated with soluble flucloxacillin *and* flucloxacillin-pulsed antigen presenting cells and we found that T-cell proliferative responses (n=5 clones) were detected consistently when antigen presenting cells were pulsed with flucloxacillin for 24-48h (and after 4h, to a lesser extent; Figure 6.8B).

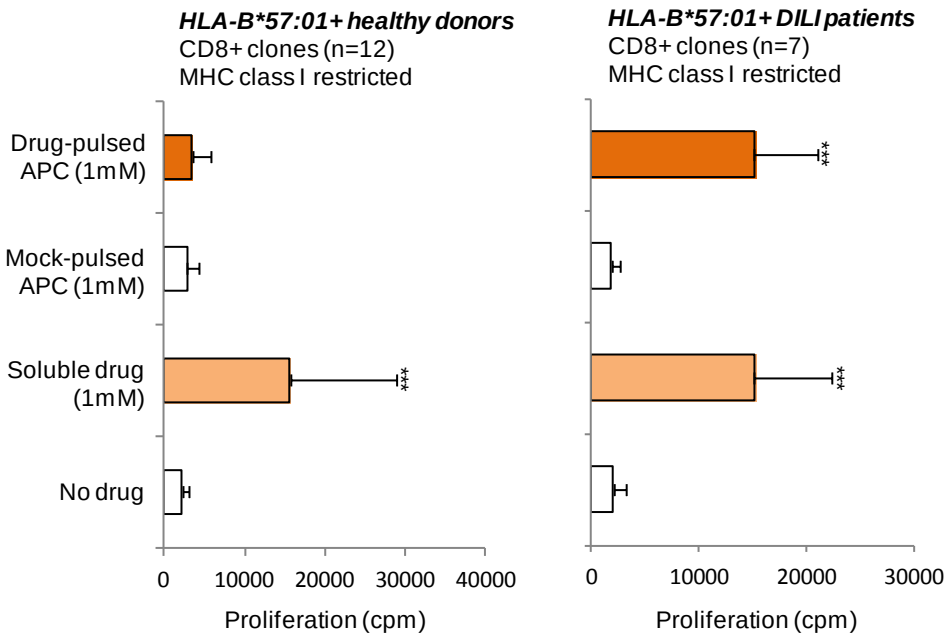


Figure 6.7 *Activation of clones generated from healthy donors and patients with flucloxacillin-induced liver injury with soluble flucloxacillin or flucloxacillin -pulsed APCs.*
Proliferation was measured by the addition of [^{3}H]-thymidine in the last 16 hours of experiment. Data as Mean+/-SD (Student T-test was used to compare datasets [***P<0.001])

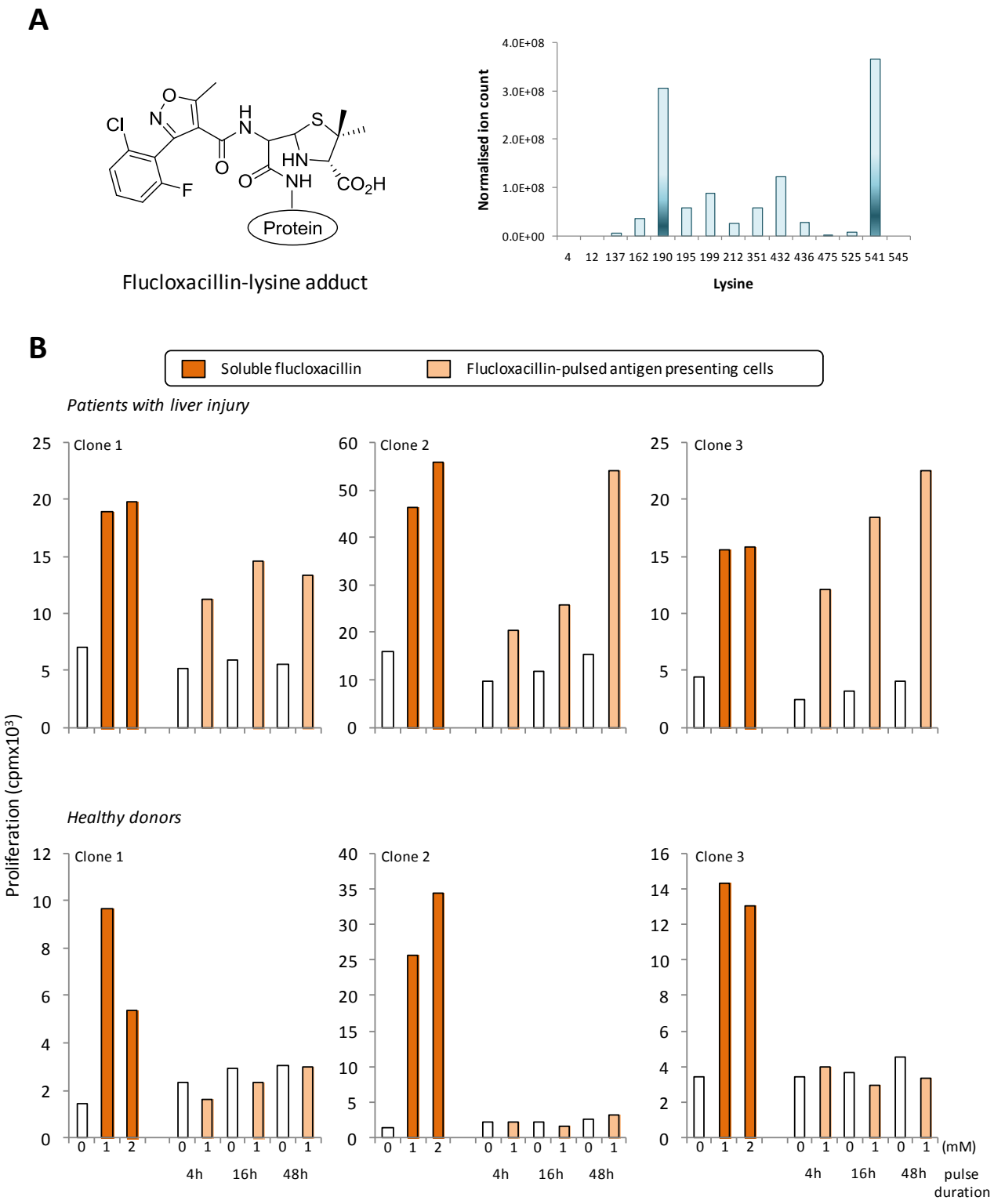


Figure 6.8 *Patient T-cell clones are activated by flucloxacillin-pulsed APCs*
(A) Binding of flucloxacillin to lysine residues on human serum albumin. Mass spectrometry was used to measure the epitope profile of the lysine residues on albumin modified in antigen presenting cell culture after 16h. **(B)** Activation of flucloxacillin-specific CD8+ T-cell clones from patients with liver injury and healthy donors with soluble drug and antigen presenting cells pulsed with drug for 4-48h. Proliferation was measured by the addition of [^{3}H] thymidine in last 16h of culture.

6.4.5 Flucloxacillin derived haptens activate CD8+ T-cell clones from patients with liver injury in a HLA allele-restricted manner

To explore further HLA-specific restriction, in particular whether flucloxacillin haptens are presented to T-cells in a HLA-B*57:01 restricted manner, flucloxacillin-specific T-cell clones from patients with DILI were incubated alongside autologous APCs and a variety of APCs expressing different HLA-B alleles, allowing us to determine the role of certain HLA alleles in the activation of T-cells from DILI patients. All APCs used were transformed B-cells and in initial experiments, clones were exposed to flucloxacillin pulsed APCs, but not the free drug.

Three panels of APCs were generated from the PBMCs of healthy donors selected from our HLA-typed cohort (Alfirevic *et al.*, 2012). Donors were selected to provide APCs that expressed a broad array of HLA-B alleles. Individual clones were cultured with flucloxacillin-pulsed APCs from between 5 and 12 allogeneic donors. T-cell clones were activated in a highly HLA-restricted fashion; proliferative responses were only detected with antigen presenting cells expressing HLA-B*57:01 and in some instances the closely-related HLA-B*58:01 (Figure 6.9B, C, E).

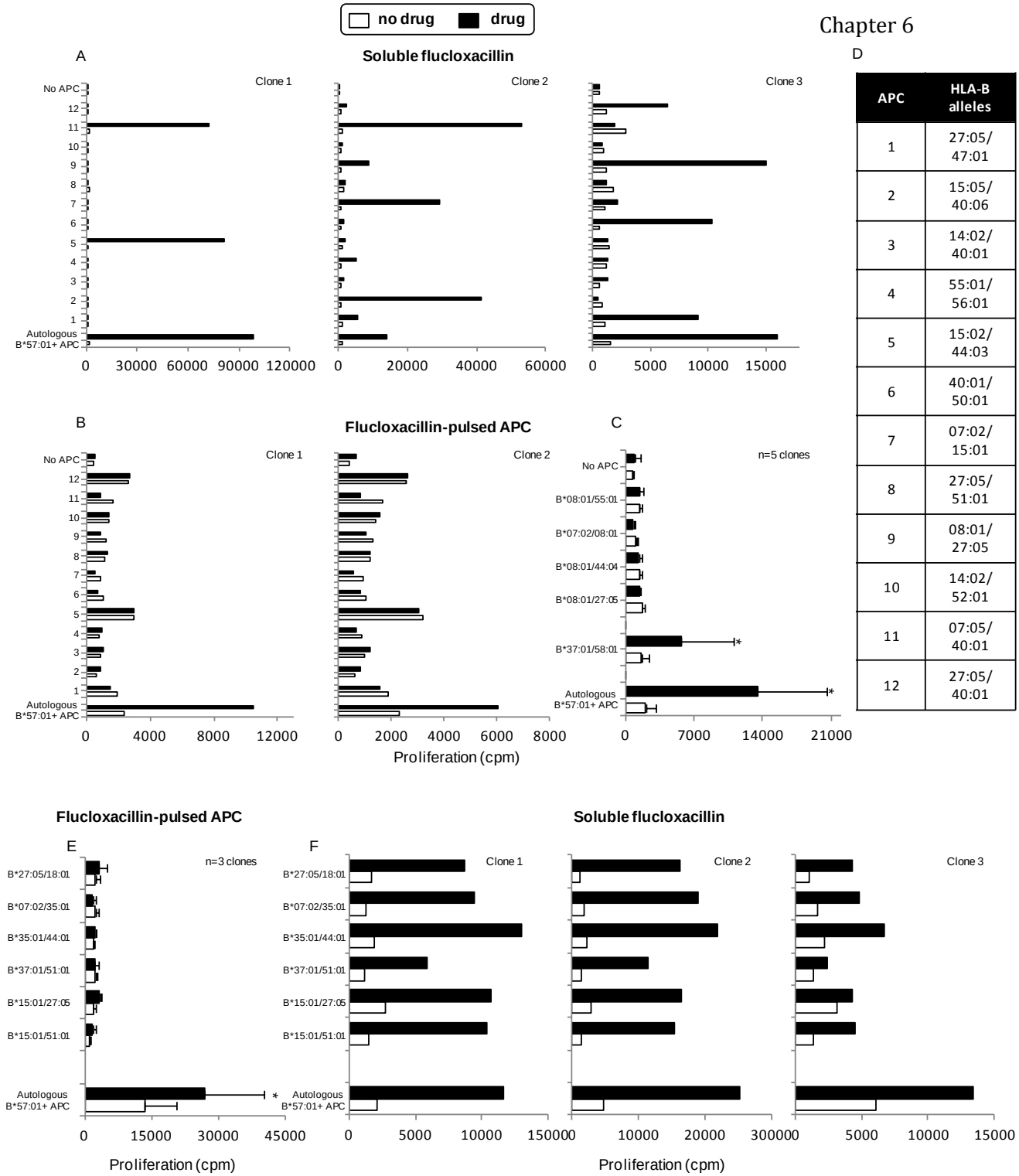
6.4.5 Soluble flucloxacillin activates CD8+ T-cell clones from patients with liver injury in a HLA unrestricted manner

In parallel experiments, additional reactivity was tested using soluble drug as a source of antigen. Certain clones were activated with flucloxacillin in an allele unrestricted fashion (Figure 6.9F; clones 1 and 2); whereas other clones were activated in the presence of 15-50% of the APCs expressing different HLA-B alleles (Figure 6.9A).

6.4.6 Flucloxacillin binding to albumin is readily detectable *in vitro*.

Primary hepatocytes were cultured with flucloxacillin (0 – 4mM) for 48 hours in media containing albumin (R9 media) and serum free media (Williams media) to identify formation of flucloxacillin derived haptens. Western blots with a flucloxacillin-specific antibody (formed in rabbit, kindly donated by Frank Van Pelt, University College Cork, Ireland) confirmed flucloxacillin-modified albumin in the supernatant of cells cultured in albumin-containing media (Figure 6.10A). Formation of the protein adduct was dose dependent. However flucloxacillin-derived haptens were not detected by western blot in viable hepatocytes.

When hepatocytes were cultured with flucloxacillin in serum free Williams media, an array of drug modified proteins were detected. Due to the spread of bands visible we cannot be certain that the band seen at 66Kda (albumin) is indeed flucloxacillin-modified albumin.



D

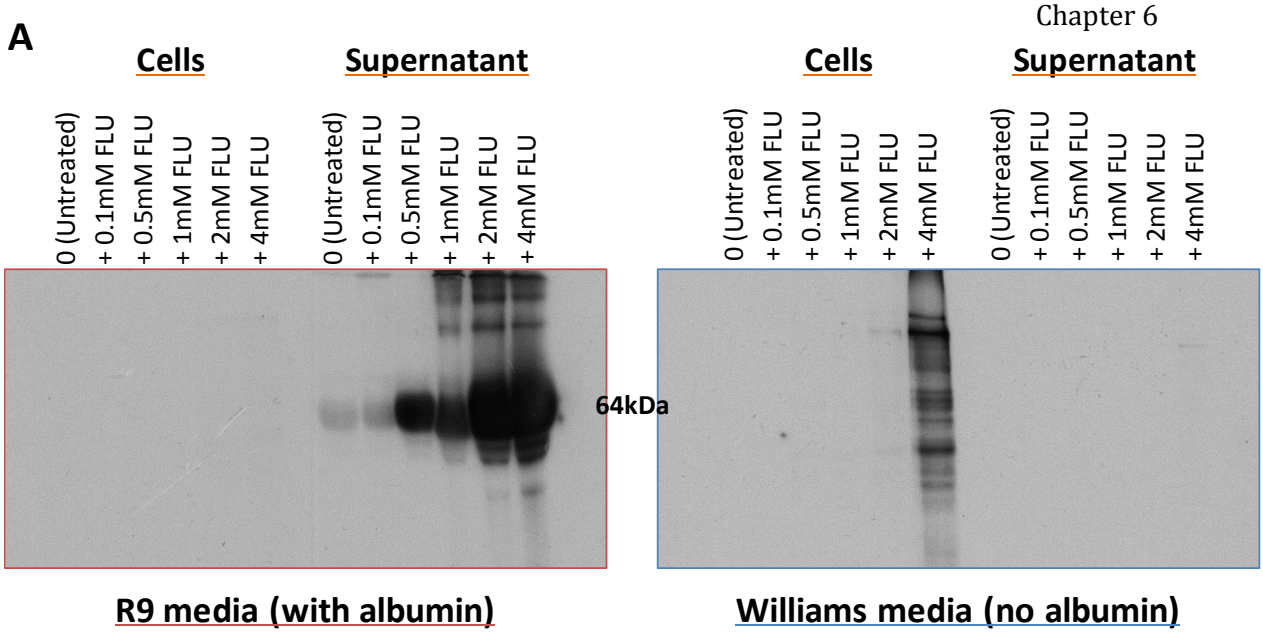
APC	HLA-B alleles
1	27:05/ 47:01
2	15:05/ 40:06
3	14:02/ 40:01
4	55:01/ 56:01
5	15:02/ 44:03
6	40:01/ 50:01
7	07:02/ 15:01
8	27:05/ 51:01
9	08:01/ 27:05
10	14:02/ 52:01
11	07:05/ 40:01
12	27:05/ 40:01

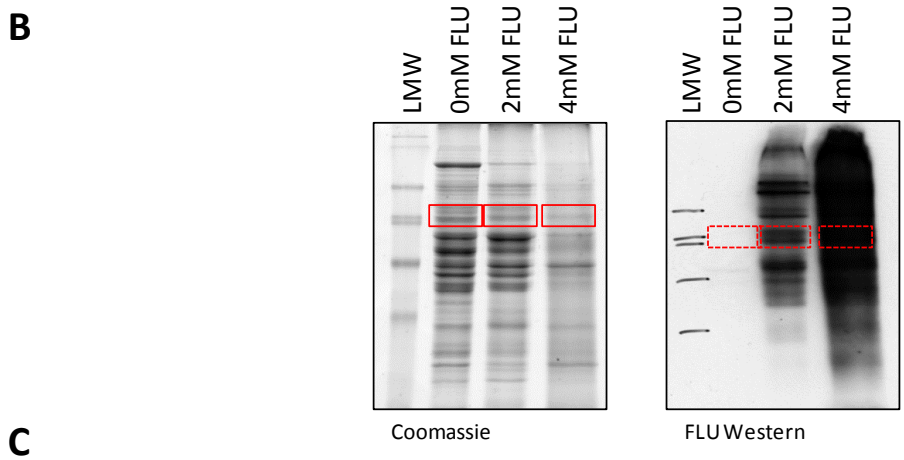
Figure 6.9 *HLA specific recognition of flucloxacillin and activation of antigen-specific patient T-cell clones varies in pulsed and non-pulsed APCs*

Activation of flucloxacillin-specific CD8+ T-cell patient clones with soluble drug **(A, F)** and flucloxacillin-pulsed APCs **(B,C,E)** using 3 panels of allogeneic APCs. **(A and F)** APCs were co-cultured with flucloxacillin-specific T-cell clones and soluble flucloxacillin for 72 hours at 37°C in an atmosphere of 95%O_2/5% CO_2. [^{3}H]-thymidine (0.5µCi) was added in the last 16 hours to determine T-cell proliferation. **(B, C, and E)** APCs were pulsed with flucloxacillin for 24 hours before three washes were applied to remove free drug. APCs were co-cultured with flucloxacillin-specific T-cell clones for 48hours with proliferation measured by the addition of [^{3}H-thymidine. CPM= counts per minute.

D. Table showing HLA-B alleles expressed on antigen presenting cells used in **(A and B)**. **(C and F)** Data as Mean+/-SD (student T-test was used to compare datasets [*P<0.05]).

We therefore continued with a coomassie assay to visualise protein and the appropriate section of the gel was cut out and digested for mass spectrometric analysis. The mass spectrometery work was conducted by Dr Roz Jenkins (MRC Centre for drug safety science). Three flucloxacillin-modified albumin peptides were detected; LAK*TYETTLEK, NLGK*VGSK and K*QTALVELVK. The MS/MS spectrum for peptide LAK*TYETTLEK is shown (Figure 6.10). Although drug-modified peptides were detected at this stage we cannot be certain of the formation of flucloxacillin derived haptens in hepatocytes. In our hepatocyte cultures, we observed significant cell damage when hepatocytes were cultured with 4mM flucloxacillin and in some instances also with 2mM flucloxacillin. Such high concentration may be toxic as cells often did not survive in cultures of 4mM flucloxacillin; these observations mirror the lack of proliferation seen in T-cell cultures at 4mm flucloxacillin. In these preliminary experiments cell damage, breakdown and lysis of cell proteins could provide sites for flucloxacillin binding.





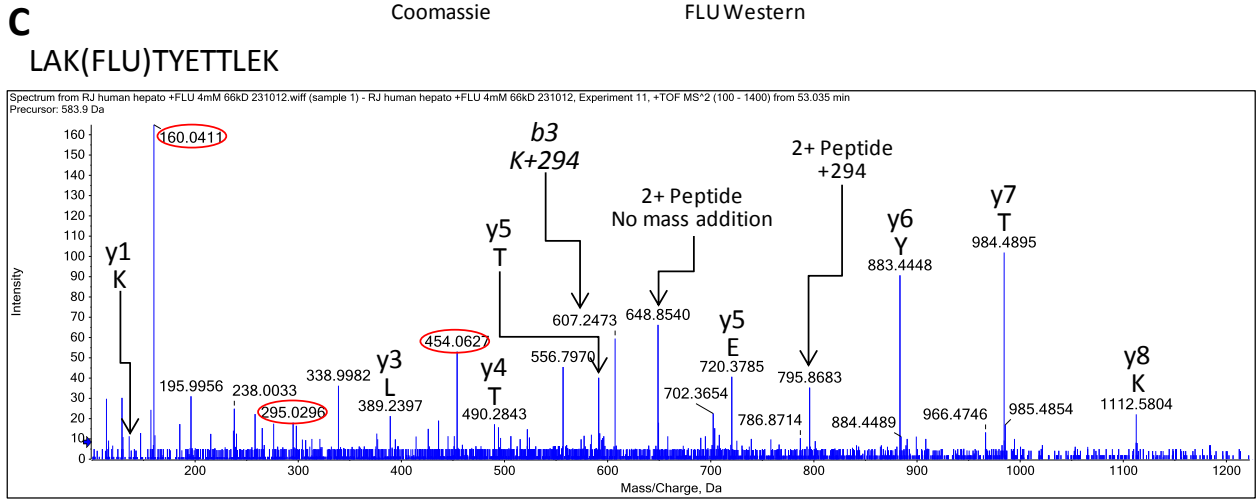


Figure 6.10 *Flucloxacillin-modified albumin can be detected in vitro*
(A) Primary hepatocytes isolated from a liver segment of a consenting patient were seeded onto a collagen I coated 6-well plate (1.5 x 10^6 cells per well) and left to adhere for 3-4 hours. Medium was then replaced to remove unattached hepatocytes and cells were left to culture at 37°C in an atmosphere of 95%O_2/5% CO_2 overnight. Media was removed and cells dosed with flucloxacillin (0 – 4mM) in ether R9 media (supplemented with albumin) or Williams media (serum free). Following 48 hours incubation hepatocytes were harvested and supernatants collected. Protein concentration of the lysed cell pellets and supernatants were determined using Bradford assay and western blot with anti-flucloxacillin antibody conducted. **(B)** Coomassie stain and western of hepatocyte cell lysates. **(C)** MS/MS spectra of flucloxacillin modified LAK*TYETTLEK.

6.5 Discussion

Adverse drug reactions including cutaneous hypersensitivity reactions and DILI, can develop into serious and sometimes fatal problems placing an enormous burden on any health care system (Davies *et al.*, 2009; Lazarou *et al.*, 1998; Pirmohamed *et al.*, 2004). Strong HLA associations for abacavir (Mallal *et al.*, 2002), carbamazepine (Chung *et al.*, 2004; McCormack *et al.*, 2011) and allopurinol (Hung *et al.*, 2005) reactions have been identified, to name a few. Numerous studies have explored the role of HLA alleles in the presentation of drugs to T-cells, particularly with the anti-retroviral drug abacavir allowing the complex structural interactions between the MHC-peptide complex, the drug molecule and the T-cell receptor to be characterised (Illing *et al.*, 2012; Mallal *et al.*, 2008; Yun *et al.*, 2012).

The β-lactam antibiotic flucloxacillin is another drug with a strong HLA-association with an adverse reaction. However this does not translate to the clinical problem. Individuals with the HLA-B*57:01 allele are at an 80-fold increased risk of developing flucloxacillin-induced liver injury but only 1 in 1000 drug-exposed individuals with HLA-B*57:01 would actually develop DILI (Daly *et al.*, 2009b). Thus, pre-clinical screening of patients about to embark on flucloxacillin treatment is not recommended. This highlights that a variety of other risk factors no doubt contribute to the development of most forms of idiosyncratic drug reaction. These could include the chemical properties of the drug in question, viral infection (i.e. HIV and herpes virus), age and gender, T-cell repertoire and other genetic predispositions (Coopman *et al.*, 1993; Guglielmi *et al.*, 2006; Kim *et al.*, 2010; Ko *et al.*, 2011; Schmid *et al.*, 2006; Shiohara *et al.*, 2006; Thong *et al.*, 2011)

The aims of this chapter were to generate CD8+ T-cell clones from DILI patients and healthy donors expressing HLA-B*57:01 after priming to investigate mechanisms of antigen presentation and in particular, how restricted the T-cell response is to HLA-B*57:01. The availability of a flucloxacillin AGEP patient expressing HLA-B*57:01 allowed us also to investigate whether class I restricted CD8+ T-cells circulate in patients with skin reactions. Finally, using primary human hepatocytes, it was possible to characterise binding of the flucloxacillin hapten to cellular and extracellular protein.

Proliferation assays with PBMCs from DILI patients and HLA-B*57:01+ volunteers were largely negative. Indeed no lymphocyte proliferation was seen in any PBMCs aside from those isolated from a patient who developed a severe skin reaction (AGEP) following flucloxacillin treatment. LTT data from this patient showed clear dose-dependent proliferation, with SI's reaching up to 35, much greater than the SI of 3.8 recorded with the positive control (TT).

AGEP, a rare but severe cutaneous adverse reaction, is characterised by its typical morphology of an acute edematous erythema or skin eruption with dozens of small non-follicular neutrophil-rich sterile pustules. These skin symptoms are almost always accompanied by a high fever, leukocytosis (neutrophilia and sometimes eosinophilia) and facial swelling (Halevy, 2009; Speeckaert *et al.*, 2010). Histological studies, positive skin patch tests and positive LTTs have suggested a role for drug-specific T-cells in the pathogenesis of AGEP (Halevy, 2009). Furthermore drug-specific CD4+ and CD8+T-cells have been isolated from the blood and skin of AGEP patients and more recently drug-specific Th17 cells have been identified in the peripheral blood of patients (Britschgi *et al.*, 2001; Filì *et al.*, 2014). Given the immune pathomechanism

involved in the development of AGEP it is perhaps not surprising that PBMCs from the patient with flucloxacillin-induced AGEP proliferated vigorously when stimulated *in vitro* in the presence of the drug.

We should not however rule out immunological involvement in DILI patients, as low levels of PBMC activation with flucloxacillin was detected using an IFN-γ ELISpot assay; antigen-driven cytokine release was observed in all 4 DILI patients with the drug-specific release of IL-13 and granzyme B also detectable in one patient where sufficient cells were available to test (Figure 6.1).

T-cells from flucloxacillin-treated PBMCs of patients with liver and skin reactions were cloned and expanded. Clones were tested for flucloxacillin reactivity and used to characterise HLA restriction and mechanism(s) of drug-specific T-cell activation. In contrast with abacavir, where CD8+ T-cells are exclusively activated with the drug, flucloxacillin-specific CD4+ and CD8+ T-cell clones were generated (Chessman *et al.*, 2008). Activation of the T-cell clones to proliferate and secrete cytokines with flucloxacillin was concentration dependent. T-cell clones were highly sensitive to the dose of flucloxacillin used, with concentration greater than 2mM likely to be toxic in the majority of clones and in some cases even 1mM, whilst doses less than 0.25mM did not often induce positive responses. With a 500mg oral dose of flucloxacillin, peak serum concentrations of 14.5mg/l or 31μM has been reported (MHRA, 2014b). These concentrations of flucloxacillin are not consistent with those required for activation of T-cell clones, yet the antigen formed *in vivo* may not be formed at the same level *in vitro*. In addition, with significant biliary excretion local concentrations of flucloxacillin in the liver are likely to be higher than the recorded plasma concentration. Our first experiments have shown that CD8+ T-

cells from HLA-B*57:01+ patients with liver injury and flucloxacillin-induced skin reaction are activated by the offending drug.

To further explore the functional role of HLA-B*57:01 in the presentation of flucloxacillin to T-cells, T-cell clones generated from patients and from HLA-B*57:01+ volunteers were utilised to define the role of APCs in the T-cell response. APCs were first excluded from the T-cell assay and proliferative responses to the drug were not detected in the absence of APCs.

Flucloxacillin-specific T-cell responses were also dependent on the drug derived antigen interacting with specific MHC molecules as the proliferative response of CD8+ T-cell clones was restricted by MHC I whilst CD4+ T-cells were restricted by MHC class II molecules. Thus to activate T-cells, flucloxacillin or flucloxacillin-derived antigens must interact with HLA molecules expressed on professional APCs.

Many drug hypersensitivity reactions are now associated with expression of specific HLA risk alleles (Daly *et al.*, 2012; Phillips *et al.*, 2011). This has led to a renewed interest in characterizing the drug immunological receptor interaction. Immunology doctrine states that the activation of T-cells is dependent on the binding of MHC bound peptides to specific T-cell receptors. Peptide binding stimulates signal transduction events that ultimately lead to the T-cell response. In the context of drug hypersensitivity, the drug (or product of metabolic or auto oxidation) interacts in some way with this MHC peptide T-cell receptor complex to trigger T-cells that wouldn't be activated in the absence of drug. Drug-responsive T-cells are activated by (1) HLA-restricted peptides derived from hapten protein conjugates (Whitaker *et al.*, 2011b), (2) peptide sequences

displayed by MHC molecules with drugs bound to the antigen binding cleft (Illing *et al.*, 2012) and potentially (3) drugs interacting directly with natural HLA binding peptides (Ostrov *et al.*, 2012; Yun *et al.*, 2014). We wanted to explore these concepts further with respect to (1) the nature of the drug antigen presented in the context of the HLA molecules and (2) whether HLA-B*57:01 is the only HLA molecule that flucloxacillin antigens interact with to activate T-cells.

Wuillemin et al (Wuillemin *et al.*, 2013b) recently generated flucloxacillin-responsive CD8+ clones from healthy donors with and without the HLA risk allele B*57:01 to evaluate mechanisms of flucloxacillin antigen presentation. T-cells from HLA-B*57:01 negative donors were preferentially activated via a hapten mechanism involving protein processing. In contrast, the parent drug was found to activate most T-cells in HLA-B*57:01+ donors. These data led the authors to conclude that the parent drug drives T-cell responses in HLA-B*57:01+ donors and liver reactions in susceptible patients. In initial experiments, we utilized MHC class I-restricted CD8+ clones generated from HLA-B*57:01+ donors after priming naïve T-cells with autologous dendritic cells and soluble flucloxacillin. A detailed description of antigen-specificity, dose-response profile, cellular phenotype and cytokine secretion of flucloxacillin clones generated using this method (and from patients with liver injury) has been reported previously (Monshi *et al.*, 2013). In agreement with Wuillemin *et al*. (Wuillemin *et al.*, 2013b) most clones were activated to proliferate with soluble flucloxacillin, but not flucloxacillin-pulsed antigen presenting cells (Figure 6.7). MHC class I restricted CD8+ T-cells were also generated directly from PBMC of patients with liver injury. Using the

methodological approach outlined above, we obtained somewhat contrasting results with patient clones; all clones were activated with soluble flucloxacillin and flucloxacillin-pulsed antigen presenting cells. Collectively, these data suggest that flucloxacillin haptens drive T-cell responses in patients, whereas the high levels of soluble drug used during *in vitro* priming drives the expansion of clones responsive against the parent drug.

Using CD8+ T-cell clones from patients with liver injury that were activated with soluble flucloxacillin and flucloxacillin-pulsed antigen presenting cells we could probe the specificity of the drug-HLA binding interaction. With three panels of EBV-transformed B-cells, used as APCs, a wide range of HLA-B alleles could be investigated. T-cell clones exposed to pulsed APCs were activated in a highly HLA-restricted fashion; proliferative responses were only detected with antigen presenting cells expressing HLA-B*57:01 and at times the structurally-similar HLA-B*58:01 (Figure 6.9B, C, E). When soluble flucloxacillin was used as the antigen however, additional reactivity was detected with APCs expressing a variety of HLA-B alleles. Certain clones were activated with flucloxacillin in an allele unrestricted fashion (Figure 6.9F; clones 1 and 2); whist others were activated in the presence of a number of APCS expressing different HLA-B alleles (Figure 6.9A). Collectively, these data describe a highly HLA-B*57:01 restricted presentation of flucloxacillin-derived antigens to CD8+ T-cells. Experiments using soluble drug show that at the high drug concentrations required to activate T-cells, binding interactions occur with multiple HLA molecules that are unlikely to occur in patients.

Finally, preliminary work utilising freshly isolated primary hepatocytes was conducted to investigate flucloxacillin binding in these cells. Flucloxacillin-modified albumin was easily detected in the supernatants of hepatocyte cultures with albumin supplemented media via western blot. In serum free media however, binding was only observed in the cell lysates, not the supernatant. Mass spectrometry confirmed the presence of three drug-modified peptides, however the cell damage observed at the high concentrations of flucloxacillin (2-4mM) may have provided binding sites for flucloxacillin that are unlikely to be seen in patients without overt liver injury. Further investigation is therefore required to confirm the formation of flucloxacillin derived haptens in hepatocytes and the relationship between adduct formation and the immune response.

In conclusion, through the study of flucloxacillin-induced liver injury, we have shown that patient T-cells are preferentially activated via a hapten mechanism. Furthermore, the activation of T-cells (presumably by hapten-modified peptides) is highly HLA restricted. These data have important implications for studies focusing solely on drug-specific T-cells derived from healthy human donors.

Chapter 7: Final Discussion

ADRs are a major health concern accounting for around 6.5% of hospital admissions and costing the NHS in the ukover £466 million each year (Pirmohamed *et al.*, 2004). Furthermore, with the time and huge cost involved in the development of a new drug (approx. 10 years, $868 million) withdrawal of a drug due to an ADR can be crippling (Adams *et al.*, 2006). Scientific research, particularly in the early stages of drug development, to predict and therefore avoid such reactions is absolutely necessary.

While Type A reactions are more common, off-target Type B reactions are more serious, frequently leading to drug withdrawals and even mortality due to their unpredictable, idiosyncratic nature (Routledge *et al.*, 2004). Many of these reactions have an immune aetiology and are indeed drug hypersensitivity reactions. It is therefore important for susceptible patients to avoid ADRs if they are preventable.

Major scientific advances in characterising the nature of the drug-specific immune response has led to success stories like the now absent presentation of abacavir hypersensitivity syndrome due to patient pre-screening for HLA-B*57:01 (Mallal *et al.*, 2008). However, there is still some way to go in fully understanding the complexity of drug hypersensitivity and with further research the hope is to manage this form of immunological ADR successfully.

Two major hypotheses exist that help explain the ability of drugs to initiate an immune-mediated response against cells of the host. The first, known as the

hapten hypothesis, details how drugs or their metabolites bind covalently to host proteins. The haptenated proteins are taken up by specialised antigen presenting cells, processed, and cleaved into shorter peptide fragments which are presented to T-cells and their receptors via MHC molecules (Landsteiner *et al.*, 1935; Park *et al.*, 2001). On the other hand, the p-i concept proposes that T-cells can be stimulated without the need for cellular processing. This hypothesis states that non-covalent interactions between the drug itself, the T-cell receptor and the MHC molecule is sufficient to elicit an immune response (Pichler, 2002). Both mechanisms attempt to describe the unique interaction between drug, T-cell receptor, MHC molecule and/or MHC binding peptide. For a number of drugs, years of research have allowed the nature of the interaction to be determined.

DNCB and penicillin are known protein reactive chemicals that bind directly to nucleophilic amino acid residues to generate novel antigenic determinants that activate T-cells via a hapten mechanism (Kitteringham *et al.*, 1985; Landsteiner *et al.*, 1935). The field, however, is complicated by the fact that these chemicals bind to MHC, potentially modifying the structure of the peptide binding groove, and also MHC bound peptides. Thus, drugs that bind covalently to protein have the potential to activate T-cells via 4 pathways. Each of these pathways involves protein processing to liberate antigenic peptides; however, with one pathway, T-cell receptors may be triggered even when protein processing is blocked. Pathway 1 (classical hapten mechanism) involves drug-protein binding, processing of drug-modified protein, liberation of drug modified and unmodified peptides and binding of the modified peptides to MHC molecules

(Adam *et al.*, 2011; Landsteiner *et al.*, 1935; Yun *et al.*, 2012). The second pathway has essentially the same origin as pathway 1; however, here drug-protein binding leads to an alteration of the sites of amino acid cleavage during processing. This subsequently generates a novel peptide sequence (without drug bound), some of which associate with MHC to activate T-cells. Pathway 3 involves the drug binding directly to MHC outside the MHC peptide binding cleft, but in a manner that alters the structure of the cleft. Novel peptides bind to this "altered" MHC activating T-cells (Illing *et al.*, 2012; Norcross *et al.*, 2012). Finally, pathway 4 involves a direct interaction between drug and MHC bound peptide (Gerber *et al.*, 2006; Pichler, 2002; Schnyder *et al.*, 1997). Here, T-cell activation might occur in the absence of protein processing if MHC associated peptides contain drug binding sites. Alternatively, protein processing may liberate "relevant" MHC binding peptides for drug. From this brief discussion one can see the complexity of identifying and defining pathways of drug-specific T-cell activation *in vitro*.

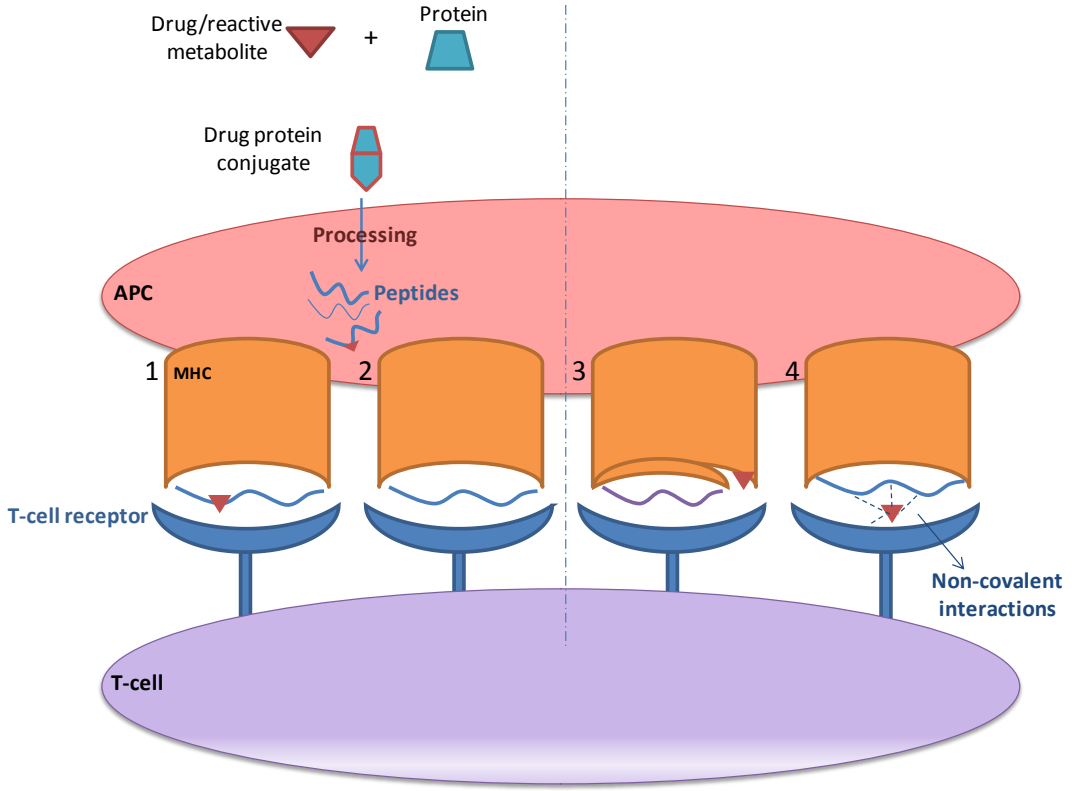


Figure 7.1 *Mechanisms of drug hypersensitivity*
Pathways 1 and 2 involve the formation of haptens and crucially, processing by the antigen presenting cell. Both drug modified or unmodified peptides may be presented to the T-cell in the classical hapten mechanism (1). Drug modification liberates novel peptides which may activate T-cells (2). (3) Altered peptide hypothesis; direct binding of drug to MHC alters structure and the repertoire of peptides able to bind and elicit a response. (4) p-i mechanism whereby drug binds non-covalently and interacts directly with the TCR (with or without peptide present.

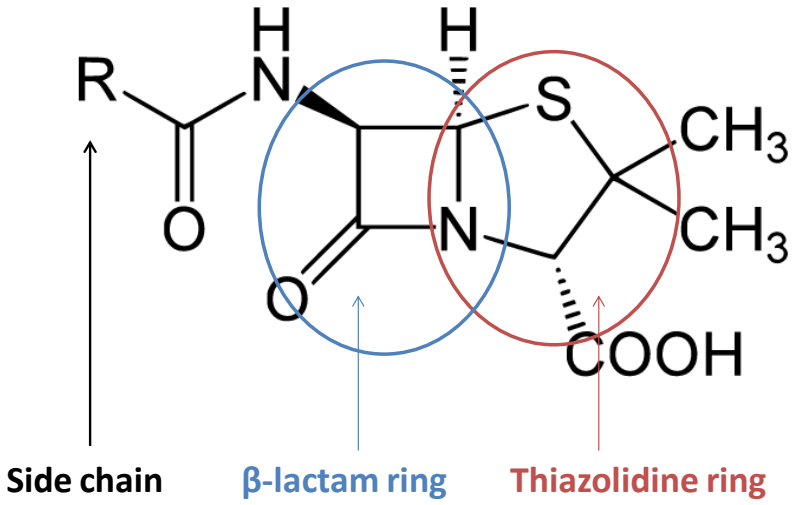
Moreover, as will become clear below, T-cells from the same patient seem to receive drug triggering signals via multiple mechanisms, with the "apparent" dominant pathway determined somewhat by the experimental conditions and the nature of the antigen added to the experiment (Illing *et al.*, 2012). Hence, conclusions relating individual pathways of T-cell activation observed *in vitro* to what occurs in the hypersensitive patient should be made with caution.

Drugs such as carbamazepine, allopurinol and sulfamethoxazole are known to activate T-cells thorough a pathway best described by the p-i concept (Castrejon *et al.*, 2010a; Naisbitt *et al.*, 2003a; Schnyder *et al.*, 1997; Wu *et al.*, 2006; Yun *et al.*, 2012). The parent compound activates T-cells via formation of non-covalent bonds with antigen presenting cells. The response of T-cells is incredibly rapid (T-cell activation is detected in minutes); thus, protein processing is not a requirement for the generation of antigenic determinants. It is MHC-restricted (i.e., specific MHC class I or II blocking antibodies inhibit the response), indicating that the drug interacts with the MHC molecules prior to activating T-cells (Elsheikh *et al.*, 2011; Lichtenfels *et al.*, 2014; Schnyder *et al.*, 1997). *In vitro* assays to diagnose drug hypersensitivity reactions (e.g., the lymphocyte transformation test) rely on the p-i concept, since high levels of protein-reactive metabolite are not formed in cell culture. For carbamazepine and allopurinol, stable metabolites have also been shown to activate T-cells from hypersensitive patients (Braden *et al.*, 1994; Wu *et al.*, 2006). The isolation and characterization of T-cell clones revealed that certain T-cells are in fact activated with the metabolite, but not the parent drug. Thus, one should not conclude that a patient is not sensitized against a drug with a negative response in *in vitro* assay. It might be that the cells are exposed to an irrelevant drug-derived antigen. Sulfamethoxazole is one example where protein-reactive metabolites have been synthesized and methods developed to expose patient cells to the metabolite in a form that does not cause direct toxicity. T-cell responses to metabolites bound covalently to protein and the parent drug bound directly to MHC are detected in all hypersensitive patients (Elsheikh *et al.*, 2011; Schnyder *et al.*, 2000).

Recent studies with abacavir proposed an additional pathway of drug-specific T-cell activation, the so called "altered self-peptide repertoire" hypothesis (Illing *et al.*, 2012; Norcross *et al.*, 2012; Ostrov *et al.*, 2012). Here the authors show that abacavir binds to endogenous MHC molecules (specifically HLA-B*57:01) altering the structure of the peptide binding site and subsequently the peptides that can be incorporated. The authors propose (although are yet to prove) that T-cells involved in abacavir hypersensitivity reactions are activated via these "altered-self", newly loaded peptides. In a way, this concept could be viewed as an expansion of the p-i concept in that the drug binds directly to MHC to activate T-cells. The main difference is that drugs enter antigen presenting cells and bind to MHC prior to peptide loading.

The major objective of my project was to utilize β-lactam antibiotic-responsive T-cells to further our understanding of the interaction of drugs with immune cells. β-lactam hypersensitivity was selected as the paradigm to study since the reactivity and interaction of β-lactam antibiotics with protein has been studied in detail and there was a good foundation on which to build. The drugs in this class of antibiotics all contain the core β-lactam ring structure from which the drugs are named and inhibitory effects on bacterial cell wall synthesis occur. The β-lactam ring structure is often fused to a five-membered thiazolidine ring, as in the case of penicillins, a major class of β-lactam antibiotic, and differing side chains form the wide range of antibiotics available (Figure 7.2). The β-lactam ring is targeted by nucleophilic lysine residues on proteins for antigen

formation, leading to the ring opening and binding of the penicilloyl group



(Batchelor *et al.*, 1965; Levine *et al.*, 1961c).

Figure 7.2 *Core structure of penicillin class of β-lactam antibiotics*

One of the major issues discussed in the field of drug hypersensitivity is whether a drug-peptide adduct is an absolute requirement for the activation of T-cells. Martin *et al.* and Weltzien *et al.* have shown designer "drug-peptide adducts" activate certain T-cells. Thus, these studies provide the framework for my investigations (Martin *et al.*, 1992; Ortmann *et al.*, 1992; Thierse *et al.*, 2005; Weltzien *et al.*, 1996a). Specifically, the authors were able to demonstrate that the location of the hapten on the peptide determines whether or not a T-cell will be activated and the magnitude of the response,

Patients with cystic fibrosis were utilized for many studies, as this population provided easy access to relevant samples. The nature of the disease leads to frequent intake of antibiotics for the maintenance of effective lung function. Unfortunately, a high rate of hypersensitivity (between 26 - 50% compared to 3

- 10% in the general population) is observed in this patient group (Burrows *et al.*, 2007; Parmar *et al.*, 2005; Whitaker *et al.*, 2011c). Hence, the study of β-lactam hypersensitivity is necessary to aid in not only understanding the mechanisms involved in the pathogenesis but also to assist the development of clinical tests for diagnosis. Work completed prior to commencement of my studies revealed the presence of drug-responsive T-cells in peripheral blood of approximately 75% of piperacillin hypersensitive patients. The majority of drug-specific T-cells were CD4+ and were found to secrete an array Th1 and Th2 cytokines following activation (El-Ghaiesh *et al.*, 2012). T-cell responses to other drugs had not been studied.

My first experimental chapter looked closely at multiple hypersensitivity syndrome utilising PBMCs from patients who had developed hypersensitivity to 2 or more of the following β-lactam antibiotics: piperacillin, meropenem and aztreonam. We were able to isolate and expand piperacillin, meropenem and aztreonam specific T-clones from each patient and observed a fine specificity in the T-cell response. No cross reactivity was observed when clones were cultured with the different drugs. Hence, one can conclude that the hypersensitivity reactions that develop in these patients following sequential drug exposure are each dependent on the priming of naïve T-cells and not cross-reactivity. Using mass spectrometric methods we were able to characterise the haptenic structures formed by each of the three drugs on HSA lysine groups. Each drug bound covalently to the same lysine groups (albeit to slightly different extents); thus, the drug-moiety attached to protein and not the

site of modification seems to be the primary driver of the drug-specific T-cell response.

Opening of the β-lactam ring is common to all β-lactam antibiotics and allows for the formation of adducts with proteins including HSA (Batchelor *et al.*, 1965; Levine *et al.*, 1961b). Thus, the next objective of my studies was to focus on one drug, piperacillin, and generate synthetic HSA adducts essentially free of parent drug, and investigate their immunogenicity (i.e., ability to activate T-cell clones from hypersensitive patients). Two synthetic piperacillin-HSA conjugates were generated; methanol-extracted conjugate 2 (see chapter 4) was able to stimulate piperacillin-specific T-cell clones whilst a second conjugate generated via the same method but extracted differently (filtration process to remove free piperacillin; conjugate 1), failed to induce T-cell proliferation. Mass spectrometric analysis of the protein adducts revealed a similar level of modification at the same lysine groups; thus, this did not explain the difference observed in the T-cell assays. Due to these unexpected results, several months of investigation followed in order to define the mechanism of piperacillin-specific T-cell activation and explain the results observed with the different piperacillin HSA conjugates. Despite a strikingly similar mass spectrometric appearance of the two conjugates, conjugate 2 was found to contain a higher level of "free" or at least non-covalently associated piperacillin. Thus, T-cell assays were established utilizing conjugates 1 and 2 and spiked (low) concentrations of free drug. From these experiments it was possible to conclude that T-cells were not activated with free piperacillin via a p-i pathway. This conclusion is further supported by experiments showing that inhibition of

protein processing blocked T-cell responses to piperacillin (chapter 4). Thus, the clones generated following PBMC expansion with parent drug do not seem to be activated with HSA adducts and are not activated with the parent drug. Previously, Thierse *et al* working on mechanisms of contact allergy with nickel proposed that HSA may act as a "shuttling molecule" which in essence brings the metal allergen through the epidermis towards TCR/MHC binding site for T-cell activation (Thierse *et al.*, 2004). This phenomenon was mimicked *in vitro* using nickel bound to HSA through a coordinate linkage. It is possible that a similar situation occurs with piperacillin. If so, the level of "free" piperacillin in conjugate 2 would be shuttled by HSA to relevant protein binding sites, where it facilitates hapten transfer and ultimately the formation of antigenic determinants to stimulate clones. In contrast, the level of free drug in conjugate 1 seems to be insufficient for this process to occur.

In additional experiments the specificity of the piperacillin hapten response was further highlighted. T-cell clones specific to conjugate 1 (which did not stimulate clones specific to soluble piperacillin) were generated. While responses were dose-dependent to conjugate 1, in half of the T-cell clones tested proliferative responses to soluble piperacillin were not observed.

Having demonstrated the ability of drug protein adducts to activate T-cells from piperacillin hypersensitive patients, we went on to relate the absolute concentrations of piperacillin HSA adducts formed *in vitro* and in patients to the activation of T-cells. Utilising mass spectrometric expertise in the department, not only could we detect and identify piperacillin modified peptides, importantly levels were now quantified. Quantification was achieved through

development of a novel method whereby a synthetic piperacillin modified peptide was synthesised and used as a standard. Similar levels of piperacillin-modified peptide were detected *in vitro* and in patient plasma, with 2% - 6% of total protein consisting of modified pip-K541 peptide. With methods in place, time and concentration dependency was explored allowing us to conclude that 2.8% modified peptide was the required threshold to trigger a T-cell response. This is the first time a quantitative analysis of drug modification has been related to the activation of a drug-specific T-cell response. Taking this further, a prospective study would be an ideal next step. Monitoring antigen formation and the immunological/clinical response in tolerant and hypersensitive patients and in hypersensitive patients during desensitization would help improve the safety profile of piperacillin in susceptible populations.

Despite being the first to explore the quantitative relationship between drug protein binding and the T-cell response, we are still a fair distance away from defining the particular hapten-peptide structure(s) required to ligate MHC and activate a primary immune response in patients. Crystallography and computer modelling aligned to mass spectrometric analysis of peptides eluted from MHC molecules would undoubtedly aid in our structural knowledge. However, these studies are complicated because piperacillin-specific T-cell responses are not MHC-allele restricted, i.e., drug-peptide adducts bind to proteins encoded by multiple HLA alleles to activate T-cells. Furthermore, even if it was possible to characterize MHC-binding piperacillin peptide adducts, it is likely that several will need to be synthesized to study T-cell responses and peptide synthesis using drug-modified lysine as starting material is incredibly difficult.

Flucloxacillin, like piperacillin, is from the penicillin class of β-lactam antibiotics. It is commonly used to treat staphylococcal infections. Unlike the other β-lactams investigated in this thesis, flucloxacillin is a cause of acute liver failure, which has a strong association with HLA-B*57:01 (Daly *et al.*, 2009b). Interestingly abacavir, also associated with HLA-B*57:01, is not linked with DILI and currently similarities in mechanisms of reaction have not been determined and so associations with the same allele may be coincidental (Yip *et al.*, 2014). Despite the strong association with HLA-B*57:01, pre-clinical screening is not possible as almost 14,000 patients would need to be screened before DILI is avoided in one patient, thus preventing thousands of patients from receiving effective treatment (Karlin *et al.*, 2014). Investigating the mechanisms involved in the development of flucloxacillin induced adverse reactions would therefore aid our understanding of the disease pathogenesis and in turn findings could be applied to the clinic. Though an HLA association exists for flucloxacillin-induced liver injury, skin reactions to flucloxacillin have not been associated with HLA-B*57:01 or other HLA alleles. In chapter 6 investigations with flucloxacillin focussed on the immune response in patients with skin and liver injury. Experiments with PBMCs showed lymphocyte proliferation was easily detected in a patient with skin injury yet patients with flucloxacillin induced liver injury yielded largely negative results. It was interesting to note that DILI patients and the patient with skin injury were HLA-B*57:01 positive. Clones from both patient groups were largely CD8+ and the drug-specific response was MHC class I restricted indicating that the HLA risk allele may be directly involved in presenting the drug-derived antigen to T-cells. Unfortunately, due to limited availability of T-cell clones from the patient with

flucloxacillin-induced skin injury, additional experiments were not possible. Indeed, a much larger study with an increased number of patients with both DILI and skin injury would be required to reach definitive conclusions.

Utilising a bank of HLA-typed volunteers available at the University of Liverpool we were able to investigate HLA restriction of the flucloxacillin-specific T-cell response in HLA-B*57:01+ donors and compare the findings obtained to patients with DILI. It was interesting to find that CD8+ T-cell clones from HLA-B*57:01+ donors, though activated by soluble flucloxacillin were not stimulated by flucloxacillin-pulsed APCs, an observation paralleling the finding of a recent study with flucloxacillin whereby clones from HLA-B*57:01+ donors were found to be activated preferentially by the parent drug via a p-i pathway (Wuillemin *et al.*, 2013a). CD8+ T-cell clones from HLA-B*57:01+ patients with liver injury however, were activated by both soluble drug and pulsed APCs. Furthermore, the hapten response (i.e., response to drug-pulsed antigen presenting cells) showed fine HLA specificity when clones were cultured with a panel of antigen presenting cells expressing a range of HLA-B alleles; clones exposed to flucloxacillin-pulsed antigen presenting cells only responded in the presence of cells expressing HLA-B*57:01+ and in some cases the structurally similar HLA-B*58:01. This level of restriction was not observed when clones were cultured with the same antigen presenting cells and soluble flucloxacillin, possibly due to the multiple HLA binding interactions that occur when the drug is added at high concentrations.

In final investigations with flucloxacillin we were fortunate to have access to primary hepatocytes. Preliminary studies detected flucloxacillin modified peptides in hepatocyte cell lysates, which suggests that hepatocyte-modified protein may be an important antigen for T-cells and direct the immune response to the liver. In support of this argument, flucloxacillin-specific T-cell clones have been shown to secrete cytolytic molecules including granzyme B, Fas ligand and perforin. Furthermore, CD3+ and CD8+ lymphocytes have recently been shown to infiltrate liver in patients with liver failure and kill hepatocytes *in vitro* (Wuillemin *et al.*, 2014). Finally, the expression of chemokine receptors CCR2, CCR4 and CCR9 on T-cells from HLAB*57:01+ volunteers and patients with DILI suggests that liver injury may result from a mass infiltration of flucloxacillin-reactive cytotoxic T-cells as these receptors are involved in migration and accumulation of immune cells in liver (Mennicke *et al.*, 2009; Monshi *et al.*, 2013). Further work utilising precious liver specimens and T-cell/hepatocyte co-cultures will undoubtedly aid our understanding of the pathogenesis of DILI particularly the contribution of the immune system.

Characterization of drug-modified protein and exploration of the relationship between adduct formation and activation of immune cells has been a common theme throughout my thesis. Drug-modified protein and the peptides generated through protein processing undoubtedly play an important role in the development of drug hypersensitivity and perhaps a pivotal role in DILI. As the most abundant serum protein, much of my research focused on HSA, yet drugs have the potential to bind covalently to a vast number of circulating proteins.

Thus, we must take into consideration the great number of peptide fragments that are generated naturally and those that might participate in the immune response whence modified. Hence, the picture becomes incredibly complicated. Indeed the development of an exaggerated immune response is likely to involve a wide range of peptides which interact with multiple HLA molecules to activate T-cells. Being able to focus directly on the drug-derived peptides which bind to the HLA molecule and trigger the immune response is a natural next step. Abacavir, like flucloxacillin, has an association with HLA-B*57:01 (Hetherington *et al.*, 2002; Mallal *et al.*, 2002). Studies by Chessman *et al* have defined the role of HLA-B*57:01 and the fine specificity of the MHC interaction with abacavir was mapped to the drug-binding F-pocket (Chessman *et al.*, 2008). In recent years Illing *et al* have successfully eluted peptides bound to HLA-B*57:01. Using mass spectrometry they were able to characterise the peptides bound to these HLA molecules. Examination of the peptide repertoire presented by HLA-B*57:01 saw a shift in the nature of peptides bound in abacavir treated cells compared to untreated control cells (Illing *et al.*, 2012). Illing *et al* proposed that abacavir bound specifically to the antigen-binding cleft of HLA-B*57:01 meaning the usual repertoire of peptides able to bond to HLA-B*57:01 was somewhat hindered. From this work the altered self-peptide repertoire hypothesis of drug hypersensitivity was put forward. The methods used here can be transferred and used to build upon the work in this thesis. Indeed, in the final months of the PhD, preliminary work was conducted and methods researched in order to apply elution and mass spectrometry methods to flucloxacillin and so methods are now in place to elute peptides bound to HLA-B alleles from cells cultured with and without flucloxacillin. Figure 3 details the

work flow and methods used to analyze the vast number of MHC binding peptides that are eluted off antigen presenting cells. It will therefore be possible in the very near future, in a similar manner to Illing *et al*, to characterise the repertoire of drug-modified and unmodified peptides which bind to the specific HLA.

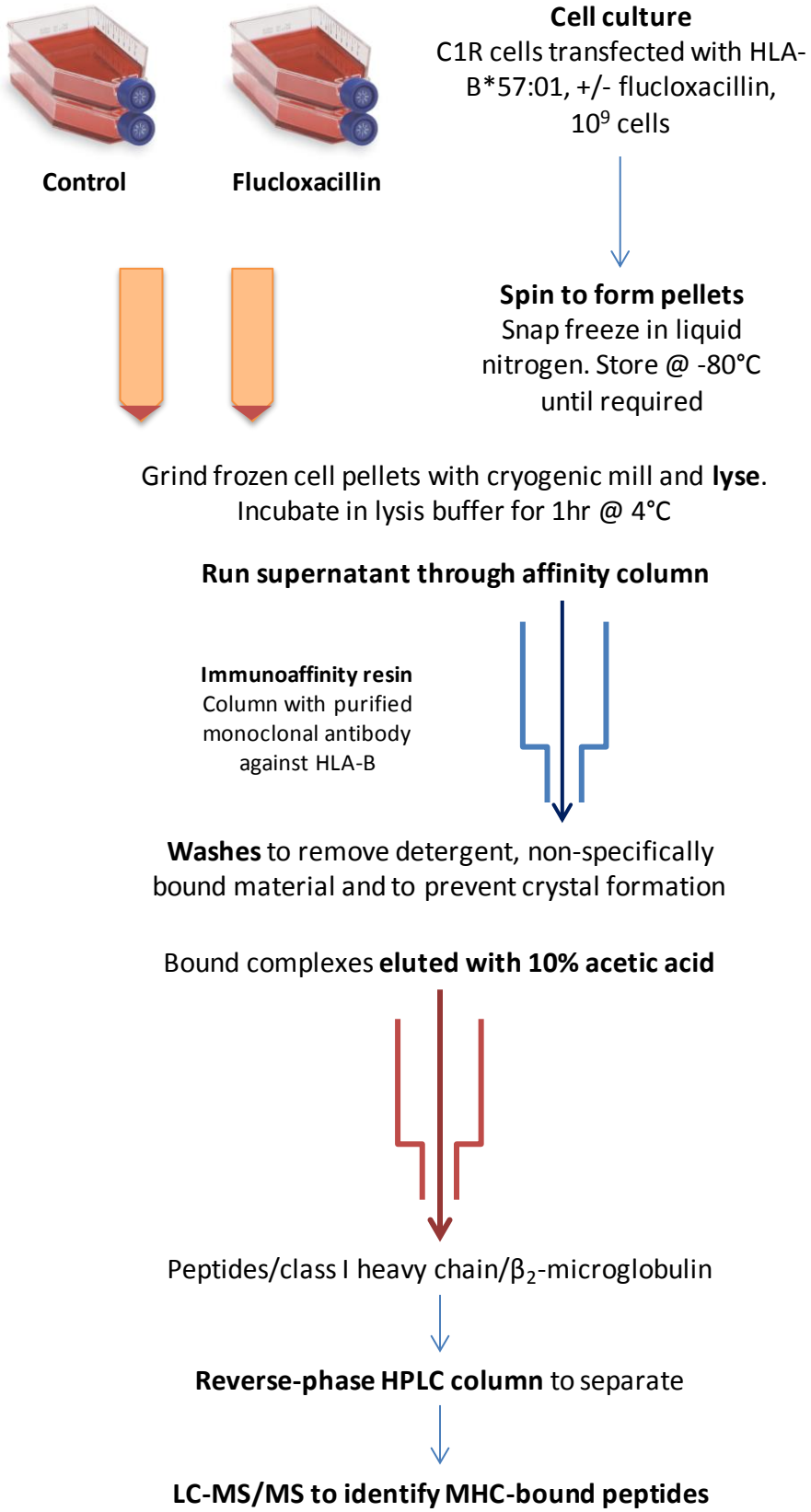


Figure 7.3 *Method flow for peptide elution*

Drug hypersensitivity is an ever evolving field of research. Whilst new findings continue to emerge often further questions arise as a result. Much of the work in this thesis has detailed the *in vitro* situation; thus, findings should be interpreted with some caution as other pathways of drug-specific T-cell activation may occur in patients. Ideally models would provide a situation where the effect of multiple mechanisms can be studied thus providing deeper understanding of the pathogenesis of drug hypersensitivity. Unfortunately the complex nature of the immune system makes the development of *in vitro* models difficult. The situation is further complicated when metabolism is a prerequisite for adduct formation and when investigations with HLA alleles are included. The precise nature of the genetics involved in susceptibility often rules out the development of animal models. Nonetheless, focussing on particular stages in the development of hypersensitivity, as done here, can help piece together and further our understanding of this complex iatrogenic disease. Continuing research will not only lead to improved drug design and development but also translate through to the clinic aiding patient diagnosis in the hope of continually reducing the risk of drug hypersensitivity.

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