Development and use of a mouse model to investigate β-lactam hypersensitivity reactions

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

By

Ryan G Nattrass

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Declaration

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented wholly, or in part, for any other degree or qualification.

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Ryan G Nattrass

This research was undertaken at the Department of Molecular and Clinical Pharmacology, at the centre for Drug Safety Science, University of Liverpool.

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Abbreviations

ADR	Adverse drug reaction
WHO	World health organisation
ACN	Acetonitrile
AGEP	Acute generalised exanthematous pustulosis
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
Amox	Amoxicillin
amu	Atomic mass unit
APC	Antigen presenting cell
BMDDCs	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
CCR	Chemokine receptor (C-C) motif
CD	Cluster of differentiation
cDNA	Complimentary DNA
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CHS	Contact hypersensitivity
cpm	Counts per minute
CSA	Cyclosporin
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
Da	Daltons
DAG	Diacylglycerol
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DILI	Drug-induced liver injury
dLN	Draining lymph node
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNCB	Dinitrochlorobenzene
DNFB	Dinitroflourobenzene
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
Em	Emission
ER	Endoplasmic reticulum
Ex	Excitation
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
GATA	Erythroid transcription factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
h	Hours
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HMGB1	High-mobility group box 1
I.P.	Intra-peritoneal
I.V.	Intra-venous
ICAM	Intercellular adhesion molecules
ICOS	Inducible T-cell co-stimulator
IDILI	Idiosyncratic drug-induced liver injury
IFNγ	Interferon-gamma
IgE	Immunoglobulin E
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
LLNA	Local lymph node assay
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
mir	Micro RNA
MPE	Maculopapular exanthema
N/ A	Not applicable
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer
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
TEN	Toxic epidermal necrolysis
TFA	Triflouroacetic acid
T_H1	T-helper-1
T_H2	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
Veh	Vehicle
w/v	weight/volume
ZAP 70	ζ-chain-associated protein kinase 70
β2m	β 2 microglobulin

Abstract

β-lactam hypersensitivity reactions can be severe and are extremely difficult to predict. Drug-specific T-cells have been identified in blood of patients presenting with cutaneous and hepatic hypersensitivity reactions, indicating that they play a role in the disease pathogenesis. Animal models are highly effective tools that have been used extensively to dissect mechanisms of disease and pathways of disease progression; however, animal models of drug hypersensitivity reactions have proven difficult to develop. The aims of this thesis were to utilize 3 β-lactam antibiotics amoxicillin, piperacillin and flucloxacillin to explore antigen-specific T-cell responses in the mouse and to attempt to develop a model of T-cell-mediated drug-induced liver damage. The project utilized the C57/Bl6 $CD4^+$ T-cell deficient mouse with a mutation in the αβ gene encoding for MHC class II molecules, which has previously been used to investigate skin sensitization to drugs.

In initial experiments, amoxicillin-specific $CD8^+$ T-cell responses were detected both *in vivo* and *ex vivo*. Sensitization was obtained through painting of the drug onto the skin of mice that had been depleted of $CD4^+$ T-cells, which are thought to exert regulatory/suppressor functions. On completion of the sensitization protocol, draining lymph node cells were removed and the drug-specific T-cell response was detected through analysis of proliferation and IFN-γ release. In contrast, proliferative responses and cytokine release were not detected with cells from vehicle control mice.

The study was expanded to include 3 β-lactam antibiotics. Activation of $CD8^+$ T-cells was readily detectable following sensitization with flucloxacillin. In contrast, only weak *ex-vivo* proliferative responses were detected following sensitization with piperacillin, which may relate to the fact that piperacillin preferentially activates $CD4^+$ T-cells in hypertensive human patients. Drug-specific T-cell clones from human patients were generated and tested alongside murine counterparts to provide a detailed assessment of cross-reactivity and variability in the drug-specific T-cell response between species. Amoxicillin and flucloxacillin demonstrated cross-reactivity with both human and murine drug-specific T-cells. Piperacillin cross-reactivity was difficult to assess in mouse. However, human piperacillin-specific T-cells displayed no evident cross-reactivity with amoxicillin or flucloxacillin.

The *ex vivo* activation of flucloxacillin-specific $CD8^+$ T-cells from sensitised mice was discovered to be dependent on the presence of APCs. The concentration of APCs added to cultures of drug-specific draining lymph node cells was directly correlated with the amount of $CD8^+$ T-cell activation. In fact, the removal of APCs ablated the proliferative response and IFNγ secretion when APCs were added to flucloxacillin re-challenged *ex vivo* cultures of flucloxacillin-specific $CD8^+$ T-cells from the draining lymph nodes of sensitised mice.

There are currently no animal models of drug-induced liver injury where the adaptive immune system has been shown to damage hepatocytes. It is therefore difficult to explore the mechanistic basis of the tissue injury. Thus, an aim of the project was to characterize the immunogenicity of flucloxacillin and explore whether flucloxacillin-responsive $CD8^+$ T-cells damage hepatocytes. In initial experiments sensitization was achieved through epicutaneous application. $CD8^+$ T-cells from draining lymph nodes of the flucloxacillin-treated mice proliferated in a concentration-dependent manner following *ex vivo* secondary stimulation. The proliferative response was associated with IFN-γ and granzyme B release. Flucloxacillin-specific hepatocyte toxicity and apoptosis was observed when $CD8^+$ T-cells were cultured with dendritic cells and flucloxacillin for 24h, washed and transferred to the hepatocyte cultures. In contrast, hepatocyte killing was not detected in with T-cells from vehicle control mice. In separate experiments, flucloxacillin-specific T-cells were forced to migrate to the mesenteric lymph nodes using retinoic acid, prior to administration of oral flucloxacillin for 10 days, followed by analysis of liver histology and plasma biomarkers of liver injury. Oral exposure resulted in gall bladder swelling, hepatic mononuclear cell infiltration (especially around the bile ducts) and mild elevations in plasma ALT.

This work has highlighted the usefulness of animal models in studying disease whilst also acting as evidence to the difficulty in developing such models. The experiments show successful sensitization of mice against different β-lactam antibiotics and a promising model to study the role of the adaptive immune system in flucloxacillin-induced cholestatic liver injury.

CHAPTER 1

General Introduction

Contents

1.1. Adverse drug reactions

In 2004 it was reported that 6.5% of hospital admissions were due to adverse drug reactions (ADRs) (Pirmohamed et al. 2004). ADRs can occur to nearly every drug. Reactions have been reported in almost every organ/tissue and range in severity from mild skin reactions to life threatening organ failure. The objective of this first section of the introduction is to define, classify and explain the clinical implications of ADRs.

1.1.1. Definition

The World Health Organisation (WHO) effectively defines ADRs as any "harmful, unintended reaction to medicines that occur at doses normally used for treatment" (www.who.int/mediacare/factsheets/fs193/en/index.htmL).

1.1.2. Classifications of ADRs

ADRs can be classified into 5 groups based on the nature of reaction induced by the drug; Type A, B, C, D, and E reactions (Park et al. 1998). Type A reactions are referred to as "on-target" dose-dependent reactions. Due to the predictable nature of these reactions, they are possible to control through dose alterations. Type A reactions represent the majority of ADRs.

Type B reactions as opposed to type A reactions are "off-target" and rarely dose-dependent. The idiosyncratic nature of the reactions means that they are not related to the primary pharmacology of the drug. Type B reactions require the drug to be removed totally from the system as these reactions have a relatively high mortality rate. An example of a Type B ADR would be a severe skin reaction (e.g. Stevens Johnson syndrome) after administration of anti-convulsants such as carbamazepine.

Other ADRs are known and although they present only in the minority of reactions, are defined as thus (Edwards I. R. and Aronson 2000):

Type C (Chemical)

Type C reactions can be explained by the chemical structure of the drug or metabolite and how it may react in a biological system. An example would be liver toxicity after paracetemol overdose.

Type D (Delayed)

These can occur many years after a treatment; for example, tumours occurring many years after administration of chemotherapeutics.

Type E (end of treatment)

Type E reactions are concerned with withdrawal-like symptoms after an extended drug course is stopped. An example would include seizures after stopping phenytoin.

1.2. Hypersensitivity reactions

Hypersensitivity reactions are off-target type B reactions which account for approximately one sixth of all ADRs (Pirmohamed et al. 2004). They can be allergic or pseudo-allergic reactions with the latter showing symptoms of an allergic reaction, except without a detectable response from the adaptive immune system. Pseudo-allergic reactions mimic the allergic response clinically without any allergy-specific immune mechanism involved (Descotes and Choquet-Kastylevsky 2001). That is to say, pseudo-allergic reactions involve the same mediators as non-pseudo-allergic reactions however pseudo-allergic reactions do not progress due to immunological intolerance but due to pharmaco-toxicological intolerance in relation to a pharmacogenetic predisposition found in select patients

(Descotes and Choquet-Kastylevsky 2001). For example, non-immunological activation of the complement system by contrast media results in the immediate release (<1h, with no requirement of previous sensitisation) of biologically active peptidic by-products, such as the anaphylatoxins, which in turn causes the release of histamine (Bush and Swanson 1991). An allergic drug hypersensitivity reaction can occur to nearly any drug and effect nearly any biological system, but, the most commonly diagnosed reactions are those from antibiotics and antiepileptics and the skin is the organ most commonly affected with an incidence of 2-3% of all hospitalised patients (Bigby et al. 1986) (Hunziker et al. 1997). Allergic reactions are classified as involving immunological memory and highly specific recognition processes where prior sensitisation is a pre-requisite to development of symptoms (Descotes and Choquet-Kastylevsky 2001). If already sensitised, allergic symptoms can vary in time of onset from <1h to over 3 weeks (Pichler 2003).

1.2.1. Classifications of drug hypersensitivity reactions

Gell and Coombs (Gell 1963) classified drug hypersensitivity reactions based on the nature of the induced immune response into Type I, II, III, and IV (Table1:1). Knowledge pertaining to the Type IV delayed hypersensitivity reactions, as well as hypersensitivity reactions in general, has improved greatly since Gell and Coombs first put forward their classification and so delayed-type hypersensitivity reactions were further classified into 4 sub-categories; IVa – IVd. This expanded classification was based around the phenotype and function of T-cells isolated from patients with different forms of hypersensitivity (Pichler 2003).

	Immune Reactant	Antigen	Effector	Example of hypersensitivity reaction
Type I	IgE	Soluble antigen	Mast cell activation	Asthma, systemic anaphylaxis
Type II	IgG	Cell or matrix associated antigen	FcR^+ cells (phagocytes, NK cells)	Haemolytic anaemia
Type III	IgG	Soluble antigen	FcR^+ cells	Serum sickness
Type IVa	IFN-γ, TNF-α (T_H1 cells)	Antigen presented by cells or T-cell stimulation	Macrophage activation	Contact hypersensitivity
Type IVb	IL-5, IL-4/IL-13 (T_H2 cells)	Antigen presented by cells or T-cell stimulation	Eosinophils	Chronic asthma, Maculopapular exanthema
Type IVc	Perforin/ granzyme B (CTL)	Cell associated antigen or T-cell stimulation	T-cells	Contact hypersensitivity, Hepatitis
Type IVd	IL-8, GM-CSF (T-cells)	Soluble antigen presented by cells or direct T-cell stimulation	Neutrophils	AGEP

Table1:1 – Classification of hypersensitivity reactions based on immune reactants, antigens and effector mechanisms. Examples of the clinical hypersensitivity reactions are also listed. Adapted from Pichler (2003).

1.3. The immune system

1.3.1. The innate immune system

The innate immune system is the first line of defence against infectious agents. The mechanisms of the innate immune system are in place even before encounter with microbes and are matured further after contact with them. Innate immunity is, evolutionarily, the oldest mechanism of defence with its co-evolution alongside microbes

making it present in all multicellular organisms including insects and plants. Components of the innate immune system are functional at all times like the skin and epithelial barriers of the gut and respiratory tracts whereas, other parts including phagocytes and the complement system are only activated when in the presence of microbes (Beutler 2004). The cells of the innate immune system include intraepithelial lymphocytes, neutrophils, macrophages, and natural killer (NK) cells. These all contribute to the fight against invading microbes through the presentation pattern recognising receptors to pathogen-associated molecular patterns (PAMPS) (Beutler 2004). PAMPS are an array of microbial products that are present on the surface of microbes. They are essential for the survival of the microbe. However, a variety of pattern recognition receptors on the surface of the cells of the innate immune system interact with PAMPS. The main ones are toll-like receptors (TLRs) of which there are 10 known human variants (Table 1.2). All are responsible for recognising molecules usually expressed on microbial but not mammalian cells. Depending on the TLR activated, the end outcome can be expression of inflammatory cytokines, chemokines, endothelial adhesion molecules, and costimulatory molecules (Tang D. et al. 2012).

Plasma membrane TLRs	PAMPs
1 & 2	Bacterial triacylated lipopeptides
2	Bacterial peptidoglycan, lipoprotein, lipotechoic acid, porins; Viral hemagglutinin
4	Gram negative bacteria LPS, fungal mannans, parasitic phospholipids, viral envelope proteins, host heat shock negative proteins
5	Bacterial flagellin
2 & 6	Bacterial diacylated lipopeptides and lipotechoic acid
10	Partner for TLR2 and shares a variety of agonists with TLR 1(Guan et al. 2010)
Endosome membrane TLRs	
3	Viral double stranded RNA
7	Viral single stranded RNA
8	Viral single stranded RNA
9	Viral and bacterial unmethylated CpG DNA

Table 1.2 – Toll-like receptor molecules with specific PAMPs that bind them. Adapted from Abbas (2010)

The innate immune system also has a role in stimulating an adaptive immune response. For lymphocytes to begin to launch response against a particular antigen they need two signals; 1) is the requirement of a specific antigen to be presented to the cell which ensures that the following response is antigen-specific and 2) is additional stimuli by the innate immune system which can be in the form of either costimulators (T-cells), cytokines (T & B-cells) or complement breakdown products (B-cells). These latter signals ensure that the response is to a dangerous antigen and not just the cell reacting to a non-hazardous antigen. It is

important to note at this point that the innate immune system is incapable of attacking "self" unlike the adaptive immune system which is very capable of auto-immune reactions.

1.3.2. Cells of the innate immune system

1.3.2.1. Mast cells

Mast cells are a granule-containing cells involved with wound repair and defence against pathogens. When activated, mast cells release granules containing chemokines and histamine which dilates blood vessels and recruit neutrophils and macrophages. Mast cells play a central role in allergy and autoimmune diseases such as eczema and asthma (Prussin and Metcalfe 2003) and have a large role in reactions such as immediate hypersensitivity reactions to β-lactam antibiotics where cross linkage of cell surface IgE molecules by drug induces mast cell activation and de-granulation (Gould et al. 2003).

1.3.2.2. Neutrophils

Neutrophils are granule-containing cells capable of phagocytosing pathogens, however, only after these pathogens have been opsonised. They are also one of the first types of cell to migrate toward sites of inflammation and are a marker of acute inflammation (Hickey and Kubes 2009).

1.3.2.3. Eosinophils

Eosinophils are granule-containing cells responsible for combating invading parasites. Upon activation, eosinophils release their granular contents which include reactive oxygen species, enzymes, lipid mediators, growth factors, and a large variety of cytokines. Eosinophils also have a role in eczema and asthma (Hogan et al. 2008).

1.3.2.4. Basophils

Basophils are granular cells similar to mast cells which secrete histamine and heparin when activated under inflammatory conditions. Like eosinophils and mast cells, basophils play a role in parasite infection and allergy. Basophils can be found in usually high numbers around ectoparasite infections (Schroeder 2009).

1.3.2.5. Phagocytes

The main role of the phagocyte is to devour and destroy foreign bodies and pathogens through lysosome injection of digestive hydrolytic enzymes. Mature phagocytes usually reside in tissues but a small population is present in the circulation and are capable of presenting antigens to T-cells. There are a number of phagocytes in different tissues and are named according to the tissue they reside in with phagocytes in the central nervous system being called microglial cells, in the vascular sinusoids of the liver they are called Kupffer cells, in the pulmonary airwaves they are called alveolar macrophages, multinucleate phagocytes in the bone are called osteoclasts (Flannagan et al. 2009).

1.3.2.6. Natural killer cells (NK cells)

Natural killer cells are lymphocyte-like cells which are responsible for recognising stressed, infected or malignant cells; they then kill them by secreting inflammatory cytokines (Biron 1999). When a NK cell encounters a cell the decision whether to kill that cell is finely balanced through a number of activating or inhibitory signals that are generated through detection of the ligands that are present on the surface of that cell. For example class I MHC molecules are interpreted by the NK cell as inhibitory signals and if there are a lack of MHC I molecules on the surface of the cell (which regularly happens when a cell has been infected), the NK cell will receive an imbalance of signals, become activated, and kill the

target cell (Bryceson et al. 2006). NK cells can be identified *ex vivo* through the expression of CD56 and they have been shown to have a role in contact hypersensitivity where NK cells were capable of mediating long-lived, antigen-specific adaptive recall responses independent of B-cells and T-cells to the strong haptens DNCB and oxazolone (O'Leary et al. 2006).

1.3.2.7. Dendritic cells (DCs)

Dendritic cells (DCs) have an important role in immunity through linking the innate immune response to an adaptive immune response. Generally, DCs express PAMPS, and mature from being large round immature cells with low level expression of MHC, to being cells with large membranous projections. This maturation is triggered after coming into contact with molecules such as lipoposaccharides on the surface on gram positive bacteria (Medzhitov et al. 1997) or after release of damage-associated molecular patterns (DAMPs) such as HMGB1, heat shock proteins, and uric acid (Shi et al. 2003) which, apart from morphological changes, induce up-regulation of costimulatory molecules CD80, CD86, and MHC II on the cell surface. This maturation process transforms the DC into a potent T-cell stimulator. Some drugs can influence the maturational state of dendritic cells including amoxicillin which induces the increased expression of CD80 and CD86 on DCs from hypersensitive patients but not tolerant controls (Rodriguez-Pena et al. 2006). DCs develop from stem cells in the bone marrow and there are a number of DC subsets in both mouse and human with varied cell surface molecules and functional responses (Villadangos and Schnorrer 2007). There are two main types of DC, plasmacytoid DCs and conventional DCs. Plasmacytoid DCs require FTL3 ligand to differentiate (Sathe and Wu 2011) and are recognisable from their lack of CD1a, CD11b, and CD11c expression (Satpathy et al. 2012). They are also involved in mounting responses to viruses through their intracellular expression of TLR7/9 and their

ability to secrete large amounts of IFNα/β once activated (Hochrein and O'Keeffe 2008). Mouse and human plasmacytoid DCs can be distinguished through expression of endocytic receptor Siglec-H and CD303, respectively (Satpathy et al. 2012). Conventional DCs are generally thought of as being derived from lymphoid progenitors (Sathe and Wu 2011). Conventional DCs which express high levels of CD11c and MHC II can be divided into two further subsets; 1) lymphoid associated-DCs which reside in the spleen, thymus, and lymph nodes or 2) migratory DCs which reside in the peripheral organs (Kushwah and Hu 2011). Lymphoid associated conventional DCs are also further divided by their CD4/8 cell surface expression where $CD8^+$ DCs are polarised to secrete high amounts of pro-inflammatory IL-12 and presentation of intracellular foreign immunogen whilst $CD4^+$ DCs are more associated with initiating humoral CD4 T_H2 responses (den Haan et al. 2000). $CD4^-$ $CD8^-$ lymphoid associated conventional DCs are also polarised towards initiating humoral T_H2 responses but can, importantly, also secrete TGFβ which results in priming T_{regs} which can lead to the suppression of immune responses (Zhang X. et al. 2005). Migratory conventional DCs encompass cells such as the DCs of the skin like Langerhans cells (LC) and other dermal DCs. These DCs are unique in their ability to migrate from their place of origin to lymphoid organs where they can then present immunogenic antigen to T-cells (Villadangos and Schnorrer 2007).

1.3.3. The adaptive immune system

The cells of the adaptive immune system consist of T and B-lymphocytes and can be found circulating through the lymph as well as scattered across virtually all the other organs and tissues of the body. The role of the adaptive immune system is to be able to respond specifically to a wide variety of microbes which could be introduced at any point in the body. The first step in encountering a foreign microbe is transporting it to the peripheral

lymphoid organ (lymph node) where the antigen is presented by APCs to T and B lymphocytes. Naive lymphocytes migrate through the lymphoid organs and are presented the antigen by the APCs which allow them to develop into either effector cells, which provide immediate action, or memory cells, which "remember" the foreign antigen and provide future action, if required. These memory and effector lymphocytes are effectively transported all around the body to peripheral sites of antigen entry so that an adaptive immune response is able to be launched systemically to the particular antigen.

1.3.3.1. T-lymphocytes

T-cells progenitor cells are generated in the bone marrow and are transported to the thymus where they are required to undergo four selection/differentiation steps in order to become a cell population which express a fully functioning T-cell receptor (TCR) along with co-receptor molecules CD4 and CD8 (Carpenter and Bosselut 2010). These steps generate double positive $CD4^+$ $CD8^+$ thymocyte population which then migrates to the cortex of the thymus for further differentiation into single positive CD4 or CD8 T-cells (Germain 2002). The continued survival of these thymocytes is dependent upon their interaction with self-peptides displayed by residual cortical epithelial cells in regards to MHC I or MHC II presentation. The amount of interaction through MHC I or MHC II molecules will also determine whether the double positive thymocyte will differentiate into a single positive $CD8^+$ or $CD4^+$ T-cell, respectively (Germain 2002). Furthermore, double positive T-cells containing TCRs which do not efficiently interact with self peptide-MHC complexes do not receive survival signals and undergo apoptosis. This mechanism has evolved in an effort to ensure that matured T-cells do not react to self-antigen (Germain 2002). After migration of these single positive T-cells to the thymic medulla and further depletion of cells which recognise self-antigen, the now mature CD4/$CD8^+$ T-cells migrate to periphery secondary

lymphoid organs (Germain 2002). Naturally occurring immunosuppressive $CD4^+CD25^+FoxP3^+$ regulatory T-cells also develop in the thymus with current opinions suggesting that these cells develop from single positive $CD4^+$ T-cells which interact strongly with self-peptides but at a level deemed insufficient enough to warrant deletion (Sakaguchi 2004). This mechanism seems to involve the regulatory T-cell master transcription factor FoxP3 (Sakaguchi 2004) (Picca et al. 2006).

T-cells once mature recognise peptide fragments presented by MHC molecules which have been intracellularly processed and need both this antigenic signal and co-stimulatory signals to become activated. After exposure to an antigen, antigen-specific T-cells with the corresponding T-cell receptor will proliferate and differentiate. T-cells expressing TCRs with no corresponding MHC peptide complex undergo apoptosis through lack of stimulation (Romagnani 2006). T-cells have subsets which are distinguished by their expression markers. T-cells which express CD4 are known to have roles in B-cell differentiation, macrophage activation, and regulation of cell-mediated immunity. They are activated by peptide MHC II complexes. “Regulatory” T-cells which express CD4 and CD25 (most commonly but other phenotypes exist) have the ability to suppress the functions of other T-cells which is an important process in regulation of immune responses and maintenance of self-tolerance. Cytotoxic $CD8^+$ T-cells recognise peptide antigen through MHC I presentation and are responsible for the killing of cells infected with foreign antigens and tumour cells (Romagnani 2006).

1.3.3.2. Memory T-cells

Memory T-cells may be either CD4 or $CD8^+$ T-cells and can be derived from any point of differentiation from naive T-cell precursors. Upon activation, they have the potential to

differentiate into further subsets and/or also retaining their effector and cytokine secretion profiles (Zielinski et al. 2011). Memory T-cells can be further subdivided due to their homing properties with central memory T-cells expressing CCR7 and L-selectin. These cells home to lymph nodes whilst only having limited effector functions but able to undergo brisk proliferative responses to generate many effector cells, upon antigen challenge. In contrast, effector memory T-cells do not express CCR7 or L-selectin and mainly home to peripheral mucosa (Zielinski et al. 2011). They retain effector T-cell function but do not retain significant proliferation potential. Memory T-cells usually develop at the peak of an immune response where a small subset of cells begin to express the IL-17R (Zielinski et al. 2011). Unlike effector and naive T-cells, memory T-cells only require the cytokines IL-15 and IL-17 for their continued survival (Surh and Sprent 2008).

1.3.3.3. $CD4^+$ T-cells

$CD4^+$ T-cells are capable of differentiating into various subsets, each with their own distinct set of cytokines able to perform seperate effector functions. The $CD4^+$ T-helper cells demonstrate a large amount of plasticity in their differentiation ability and are induced to differentiate into subsets of T-helper cells like T_H1, T_H2, T_H17, by the cytokine contents of their surroundings (Zhu and Paul 2010). Two of the best defined subsets of effector $CD4^+$ T-cells are T_H1 and T_H2 cells. The main difference between these two subsets is their secretory cytokines with T_H1 $CD4^+$ T-cells secreting IFNγ, TNFα, and IL-2 and T_H2 $CD4^+$ T-cells secreting IL-4, IL-5, and IL-13 (Hsieh et al. 1993). Differentiation into T_H1 is dependent upon exposure to pro-inflammatory cytokines e.g. IL-12 and IFNγ derived from innate immune cells (Lighvani et al. 2001). Activated $CD8^+$ DCs represent a major source of IL-12 and are believed to be the main drivers of T_H1 deviation (Maldonado-Lopez et al. 1999). Once the naive T-cell has differentiated into a into a T_H1 cell it secretes IFNγ which both upregulates T_H1

differentiation and simultaneously downregulates T_H2 diferentiation. Through a trascription cascade involving transcription factors STAT-1, T-bet, and STAT-4, the T_H1 $CD4^+$ T-cell is able to fully differentiate into an effector cell with the primary objective of removing cells infected with foreign intracellular antigen through macrophage activation (Zhu and Paul 2010). Increased T_H1 differentiation and effector function has been implicated in the pathogenesis of autoimmune diseases including Crohn's disease (Neurath et al. 2002) and lupus nephritis (Ooi and Kitching 2012).

T_H2 $CD4^+$ T-cells differentiate for example under conditions of helminth infection where phagocytosis would be ineffective. The presence of foreign parasites induce the release of IL-4 from mast cells which is detected by naive $CD4^+$ T-cells.IL-2 IL-4 signalling initiates activation of transcription factors including GATA-3, STAT5, and STAT6 (Pai et al. 2004) (Zhu et al. 2003). This leads to the differentiation of T-cells into a T_H2 polarised subset (Zhu and Paul 2008). Differentiated T_H2 $CD4^+$ T-cells produce their effector function through secretion of cytokines IL-4 and IL-13. These cytokines induce production and secretion of specific IgE antibodies by B-cells to opsinise the parasites whist also secreting IL-5. IL-5 may activate eosinophils in the vicinity of the parasite to release their protein-degrading granule contents (Kool et al. 2012). T_H2 cells are also well known for their role in triggering immune responses to innocuous environmental allergens, resulting in chronic inflammation associated with allergic diseases such as eczema, allergic rhinitis, and asthma (Holgate 2012).

Also recently discovered and of note, are T_H9, T_H17 and T_H22. Similar to T_H1 and T_H2 secreting T-cells, naive $CD4^+$ T-cells are promoted to differentiate into the different subsets of T_H cells through exposure to polarising cytokines.

T_H9 $CD4^+$ T-cells differentiate when exposed to IL-4 and TGF-β and fully developed T_H9 cells. T_H9 cells seem to be associated with tissue inflammation and mucus production (Chang et al. 2010). T_H9 $CD4^+$ T-cells are classified according to the production of high amounts of IL-9, a cytokine closely linked to the development of athsma.

T_H17 cells develop when exposed to a cocktail of many cytokines including TGF-β, IL-6, IL1-β, IL-21 and IL-23. When fully differentiated this subset of $CD4^+$ T-cell mainly targets extracellular pathogens and chronic neutrophillic inflammation (Bettelli et al. 2008). T_H17 cells are endowed with the ability to infiltrate tissues are and responsible for autoimmune dieseases such as mutilpe sclerosis (Stockinger and Veldhoen 2007).

T_H22 cells develop in environments containing the cytokines TGF-α and IL-6 and when fully developed are mainly associated with tissue inflammation (Eyerich et al. 2009) as well as being an important component of mucosal antimicrobial host defense (Basu et al. 2012).

Regulatory T-cells or T_{regs} are $CD4^+$ $CD25^+$ T-cells capable of regulating other types of effector T-cells and are present in a variety of tissues with differentiated, distinct roles in each particular tissue. T_{regs} which do not develop in the thymus are deemed "induced" T_{regs} and they differ from T_{regs} which develop in the thymus through being Forkhead box P3 (FoxP3) transcription factor negative (Baron et al. 2007) in contrast to T_{regs} which remain in the lymphoid tissue which are deemed natural T_{regs}. The FoxP3 transcription factor has been linked to fatal autoimune reactions in humans and mice (Hori et al. 2003) with FoxP3 being found to be the master regulator of $CD25^+$ $CD4^+$ T-cells. The implications of this discovery being that FoxP3 is vital for the prevention of certain autoimmune diseases. Natural occurring T_{regs} elicit their suppressive function in response to extremely low doses of antigen in comparison to concentrations required to activate naive $CD4^+$ T-cells (Takahashi et al.

1998). The suppression of effector T-cell functions are achieved through a number of means. T_{regs} are capable of inhibiting IL-2 secretion by responder T-cells, a cytokine required for clonal expansion (Takahashi et al. 1998), they can directly kill effector $CD8^+$ T-cells and NK cells via granzyme and perforin mediated mechanisms (Cao et al. 2007), and they can directly outcompete T-cell interactions with APCs through enhanced expression of LFA-1 (Yamaguchi et al. 2011). $FoxP3^+$ T_{regs} also express the co-inhibitory receptor Cytotoxic T-lymphocyte Antigen 4 (CTLA-4) which can interact with CD80/86 on DCs resulting in the down-regulation of these T-cell co-stimulatory signals for T-cell activation (Wing et al. 2008). The secretion of TGF-β and IL-10 is also an important immunosuppressive mechanism which can induce $CD4^+CD25^+FoxP3^-$ cells to acquire $FoxP3^+$ cell characteristics like increased CD25 and CTLA-4 expression (Chen W. et al. 2003). Natural T_{regs} can also induce peripheral $CD4^+$ T-cells to become "induced" T_{regs} through TGF-β and IL-10 secretion and also through contact-dependent mechanisms (Chen W. et al. 2003) (Zheng et al. 2004).

T_{regs} have been used in a therapeutic manner in mouse models of various diseases. The general protocol is adoptive transfer of T_{regs} from naive mice. This has demonstrated therapeutic effects in animal models of autoimmune encephalomyelitis (Kohm et al. 2002), inflammatory bowel disease coalitis (Kohm et al. 2002), and autoimmune haemolytic anaemia (Mqadmi et al. 2005), to name just three.

1.3.3.4. $CD8^+$ T-cells

$CD8^+$ T-cells are generated in much the same way as $CD4^+$ T-cells where in the lymph organs, antigen is presented to induce antigen-specific clonal expansion of naive $CD8^+$ T-cells. Activated cells differentiate into effector cytotoxic $CD8^+$ T-cells (CTL), and finally migration into other tissues.

Like $CD4^+$ T-cells, $CD8^+$ T-cells can differentiate into different subsets under different cytokine environments. These include the subsets Tc1, Tc2, and Tc17. The Tc1 $CD8^+$ T-cells are comparable to T_H1 type in $CD4^+$ T-cells in regards to the secretion of IFNγ, TNFα, and IL-2, and an effector profile aimed towards combatting bacterial and viral infection (Mosmann et al. 1997) (Kryczek et al. 2007) (Tajima et al. 2011). Unlike $CD4^+$ T-cells however, which readily differentiate into T_H1 and T_H2 cells, $CD8^+$ T-cells appear to prefferentially differentiate into Tc1 $CD8^+$ T-cells with differentiation into Tc2 $CD8^+$ T-cells requiring large concentrations of IL-4 and use of an anti-IFNγ antibody *in vitro* (Sad et al. 1995). If $CD8^+$ T-cells can be induced to differentiate into Tc2 cells, they can be identified through their cytokine secretions with Tc2 cells able to secrete IL-4 and IL-5 and not IFNγ as opposed to Tc1 cells which secrete IFNγ but not IL-4 or IL-5 (Mosmann et al. 1997). It is important to state however, that once differentiated *ex vivo* and adoptively transferred, Tc1 and Tc2 cells are releatively stable and able to retain their cytokine secretion profiles even after 90 days *in vivo* (Cerwenka et al. 1998). As well as Tc1 and Tc2, a further subset of $CD8^+$ T-cells has been discovered which secrete IL-17, these cells have been deemed Tc17 cells (Kryczek et al. 2007) (Tajima et al. 2011). These Tc17 cells differentiate from naive $CD8^+$ T-cells when cultered with TGF-β and IL-6 and furthermore, seem to be affiliated with the tumour microenvironment (Nam et al. 2008) and were capable of exhibiting anti-tumour activity and cytotoxicity as well as Tc1-like cells (Tajima et al. 2011).

To differentiate from being naive to becoming cytotoxic, $CD8^+$ T-cells require co-stimulation from APC as well as peptide presentation via MHC I. The $CD8^+$ T-cells can also recieve differentiation signals from helper $CD4^+$ T-cells; however, this is not a requirement for $CD8^+$ T-cell clonal expansion and CTL development and seems to be more associated with $CD8^+$ T-cell differentiation into memory cells (Kaech and Cui 2012). IL-2 is instrumental in

stimulating $CD8^+$ T-cells to proliferate and become CTL. Upon DC-mediated antigen stimulation in the lymph node, subsequent co-stimulation leading inexorably to T-cell activation, activated antigen-specific $CD8^+$ T-cells begin to secrete IL-2 which works in an autocrine fashion through binding to its high affinity IL-2R on the T-cell surface, thereby driving its clonal expansion and functional differentiation into an effector CTL (Boyman and Sprent 2012).

CTLs target cells infected with intracellular pathogen. This causes the clonal expansion of the CTLs in the first instance and killing is contact dependent, as well as antigen-specific with cytotoxic molecules only being injected into the target cell when the target cells express MHC I complexed to the target peptide (which is the ligand for the T-cell recepter and CD8 corecepter on the CTL). Alongside MHC presentation, binding of adhesion molecule ICAM-1 to the ligand for the CTL ahesion molecule LFA-1 is required. Only when all these connections are made betwixt CTL and target cell, will cytotoxic molecules be released into the intracellular space between CTL and target cell to cause cell death through cytotoxicity by the release of Fas-L, perforin /granzyme B (Nassif et al. 2004), and /or granulysin (Chung et al. 2008). To cause apoptosis, granzyme B must first gain entry into the target cell cytoplasm which is done through the action of secreted membrane disrupting protein perforin (Kagi et al. 1994). Upon entry into the infected target cell, granzyme B causes apoptosis through both caspase-dependent pathways via activation of caspases 8 and 3 (Atkinson et al. 1998) (Medema et al. 1997) and through caspase independent pathways through mitochondrial dysfunction leading to release of cytochrome c and eventual necrotic cell death (Heibein et al. 1999). In addition to perforin and granzyme B killing pathways, cytotoxic $CD8^+$ T-cells can cause cell death through fas-ligand signalling which leads to apoptosis through direct activation of caspase 8 leading to eventual activation of active

caspase 3 and target cell apoptosis (Rouvier et al. 1993). Cytotoxic $CD8^+$ T-cells are not damaged during this process due to a proteolytic enzyme on the surface of the $CD8^+$ T-cell named cathepsin B, which degrades any errant perforin molecules which may have come into its vicinity. Granulysin, as opposed to fas ligand and granzyme B is a secreted apoptotic agent which does not require direct cell-cell contact. Investigations using mice found that granulysin was in high concentration in blister fluid and where upon injection into naive mice, stimulated SJS-like symptoms (Chung et al. 2008). Granulysin is a molecule found only in activated $CD4^+$ and $CD8^+$ T lymphocytes, NK cells and in activated, but not in resting, CTLs (Pena et al. 1997). The mechanism of cell death is achieved through binding to the target cell membrane. Granulysin then activates sphingomyelinase followed by a slow increase in ceramide concentration or induces an increase in intracellular calcium and efflux of intracellular potassium (Saini et al. 2011). Both pathways are linked with fast mitochondrial membrane damage, which leads to cell death via activation of caspase molecules (Li Q. et al. 2005) (Veljkovic Vujaklija et al. 2012).

1.3.3.5. B-lymphocytes

B-cells differentiate in the bone marrow. The main function of this class of lymphocyte is to secrete antibodies. Upon encountering antigen, B-cells will proliferate via clonal expansion to become either plasma or memory B-cells (Parkin and Cohen 2001). Plasma B-cells secrete antibodies of the same antigen affinity as the pre-clonal expanded B-cell-surface antibody. The major difference between B and T-cells is that B-cell receptors interact with antigenic protein, not the derived peptide (Parkin and Cohen 2001). The secreted B-cell antibodies functions include the neutralisation of viral components and opsonisation of antigens in order to aid cell removal through the work of phagocytes or NK cells (Parkin and Cohen 2001). Memory B-cells remain in circulation for the long-term and differentiate to secrete

higher-affinity IgG or IgA antibodies, rather than their plasma and naive counterparts (IgD and IgM) to allow a swift response if the antigen is ever reintroduced (Parkin and Cohen 2001). Immediate hypersensitivity reactions are seen following exposure to foods like peanuts or drugs like β-lactam antibiotics such as amoxicillin. These immediate reactions are caused by B-cells secreting specific IgE molecules (Torres et al. 2003).

1.4. MHC molecules

As previously mentioned, T-cell antigens are presented through either MHC I or MHC II molecules on the surface of APCs to CD8 or CD4 TCRs, respectively.

There are some important differences between mouse and human MHC molecules. For example, once activated, human T-cells express MHC II whilst mice only express MHC II on certain cells e.g. DCs (Mestas and Hughes 2004). All MHC molecules are polymorphic with more than 200 alleles of some human MHC class I and class II genes (Janeway CA Jr 2001.). Furthermore, a human typically expresses around six different MHC I molecules and eight different MHC II molecules on their cells (Janeway CA Jr 2001.). Mouse MHC haplotypes include MHC I - K, D, and L (also called H2-K, H2-D, H2-L), and MHC II - A and E (also called I-A and I-E) (Janeway CA Jr 2001.).

The structure of MHC I molecules consists of one polymorphic light chain (α) and a β-chain (β2m) (Fig 1.1) which is not membrane bound but plays a key role in transporting newly synthesised MHC molecules to the cell surface. Peptides of between 9-11 amino acids in length are presented in the binding groove and are held in place through hydrogen bonds and Van der Waals forces (Janeway CA Jr 2001.).

The MHC II structure differs from MHC I molecules through having the β-chain anchored in the MHC region and the length of presented peptides can be larger than 11 peptides long.

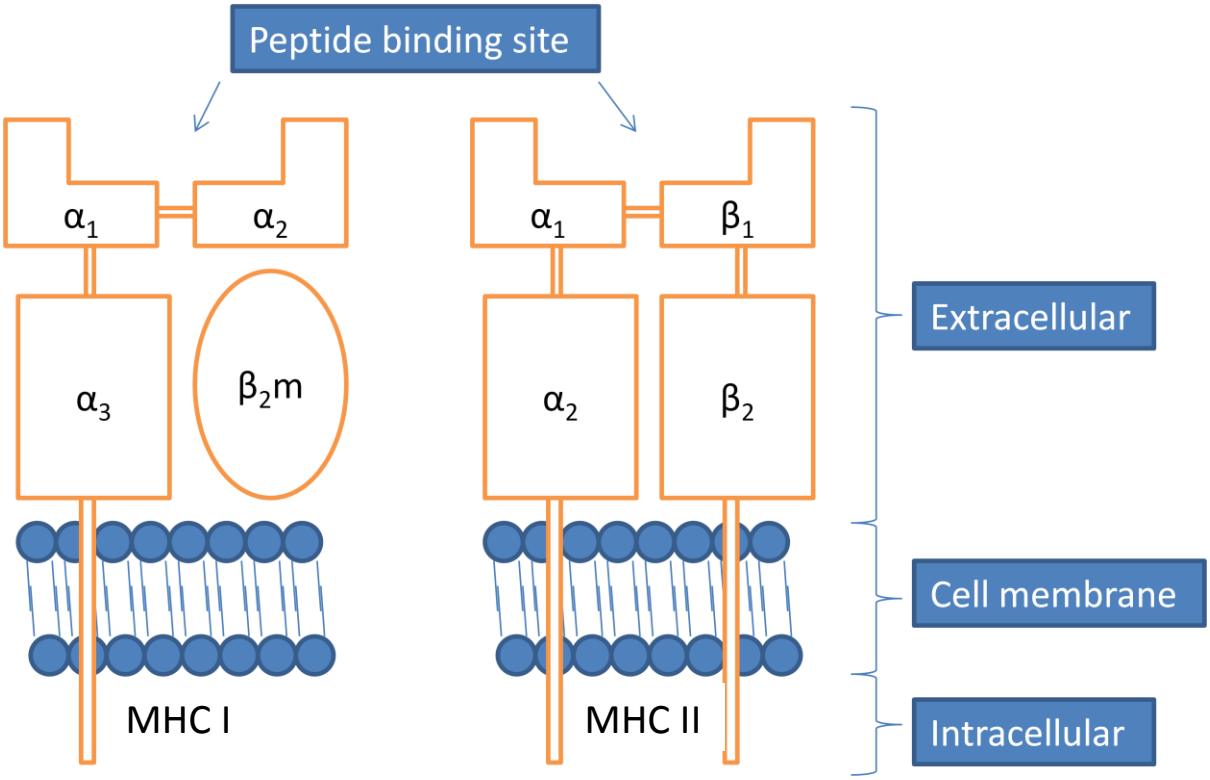


Fig 1.1. The structure of class I and class II major histocompatibility complex molecules

Intracellular foreign peptides are processed and presented via MHC I and extracellular foreign antigens are presented via MHC II (Fig 1.2). Intracellular antigens usually originate from viral origins where a virus enters a cell, replicates and therefore releases viral protein into the host cell cytosol (Neefjes et al. 2011). These proteins are degraded by cytosolic and nuclear proteasomes and the resulting peptides of around 9-11 amino acids in length are translocated to the endoplasmic reticulum (ER) (Neefjes et al. 2011). This translocation is done through transporter associated with antigen presentation (TAP) which allows the peptide to access MHC I molecules (Neefjes et al. 2011). In the ER the MHC I heterodimer is assembled out of the heavy chain, a light chain called β_2 microglobulin (β_2m) and the

peptide itself which sits deeply in the MHC binding groove. After the MHC I molecule is fully assembled it is released from the ER and presented at the cell surface (Neefjes et al. 2011). Uncoupled MHC/peptide molecules are returned to the cytosol for degradation (Hughes et al. 1997) (Fig 1.2). Constraints of the MHC binding are that peptides with a "low-affinity" for the MHC binding site will not be presented at the cell surface and the MHC molecule instead will be further subjected to further rounds of peptide selection and binding (Moremen and Molinari 2006) (Blum et al. 2013).

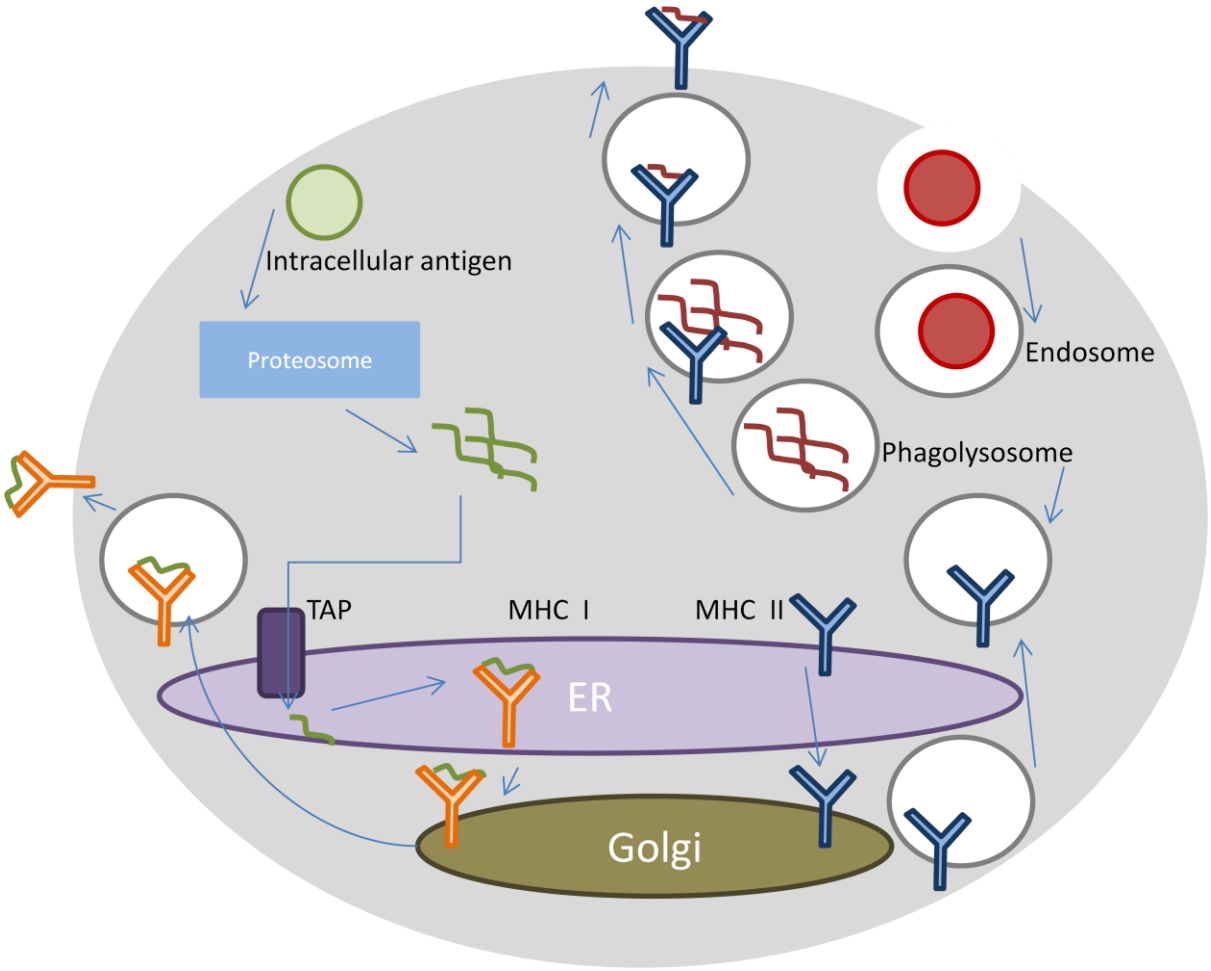


Fig 1.2. The processing of intracellular and extracellular antigen followed by presentation via MHC I, and MHC II molecules, respectively. Intracellular antigens are degraded in the proteasome. Peptides are transported to the ER via TAP where they associate with MHC I molecules. The MHC-bound peptide is then trafficked to the Golgi in vesicles. These then fuse with the phagolysosome where

peptide loading then occurs. The MHC-bound peptide is then transported to the cell surface where the particular antigen is displayed. Figure adapted from Neefjes et al (2011).

Extracellular peptides are presented through MHC II molecules to $CD4^+$ cells and only specific APCs like DCs, macrophages, and B-cells are able to utilise this pathway. Extracellular pathogens are endocytosed and proteins are degraded through enzyme activity to generate peptide fragments. MHC II molecules that have been assembled in the ER are released into the endosome to allow peptide loading to occur in the MHC II binding groove. The MHC II molecules are then fused with the cell surface to present the foreign peptides (Neefjes et al. 2011).

1.4.1. Human HLA types

The MHC region is highly polymorphic and this has been attributed to increases in viral protection offered by heterozygosity (Doherty and Zinkernagel 1975). MHC genes code a large variety of cell surface glycoproteins involved in the presentation of antigens and in humans the genes are called human leukocyte antigen (HLA) class I and II genes.

Human MHC I molecules are encoded by the HLA-A, B, and C loci with MHC II molecules being encoded by HLA-DR, DQ, and DP loci. Certain HLA types have now been associated with particular drug hypersensitivity reactions. For example, carbamazepine hypersensitivity reactions have been linked with HLA type B*15:02 and A*31:01 (Chung et al. 2004) (McCormack et al. 2011), nevirapine hypersensitivity reactions have been linked with DRB1*01:01 (Martin A. M. et al. 2005), and flucloxacillin-induced liver injury has been linked to B*57:01 (Daly et al. 2009).

1.4.2. TCR activation following MHC presentation

Upon MHC presentation to a T-cell via the TCR, the T-cell must first recognise the peptide-MHC molecule presented by the APC which is accomplished through the TCR. The TCR is a multi-protein transmembrane complex consisting of the TCRαβ, CD3εδ, CD3εγ, and CD3ζζ dimers (Kuhns and Davis 2012) and arguably the $TRIM_2$ dimer (Swamy et al. 2010). On the TCR, the TCRαβ dimer has variable antibody domains which bind to MHC-presented peptide as well as the MHC molecule itself (Garcia et al. 1996). Upon successful ligand binding to the TCRαβ, tyrosine residues on the CD3 chains (which are part of the immunoreceptor tyrosine-based activation motif) of the TCR are phosphorylated to instigate further T-cell activation steps. Currently, models propose either a conformational change, aggregation, or segregation of the TCR-CD3 complex however all models result in tyrosine phosphorylation of the cytoplasmic TCR/CD3 ITAMs which is the earliest detectable biochemical step known to be required for TCR triggering (Choudhuri et al. 2005) (Choudhuri and van der Merwe 2007). Co-receptor CD4 and CD8 recognition of sites present on the MHC molecule is crucial for efficient T-cell activation. Co-stimulatory receptor engagement between CD28 on T-cells and CD80/86 on DCs results in the secondary activation signal for T-cell stimulation (Janeway CA Jr 2001.) and is linked to downstream signalling involving P13K leading to the phosphorylation of PIP2 which in turn leads into the cellular pathway described in more detail below (Okkenhaug et al. 2001). The amount of peptide-MHC presentation to the TCR appears directly correlated with the amount of *in vivo* $CD8^+$ memory T-cells generated (Leignadier and Labrecque 2010) and their cytotoxic abilities (Wherry et al. 1999). Contrastingly, a study of *in vivo* progression of a monoclonal $CD8^+$ T-cell responses demonstrated that low-affinity peptide-MHC ligands could induce expansion of functional cytotoxic and memory T-cells (Zehn et al. 2009) (Corse et al. 2011).

Following successful MHC presentation, intracellular signalling is induced to allow for transcription of factors responsible for proliferation, differentiation and/or cytokine/growth factor release. TCR triggering stimulates IL-2 release. IL-2 binds to the IL2-R which leads to clonal expansion and differentiation into either effector T-cells or memory T-cells; immunoreceptor tyrosine-based activation motifs (ITAMs) must be phosphorylated on the TCR/CD3 complex by signalling cascade molecule Lck for this to occur (Zhang W. et al. 1998). Likewise, Zap 70 is recruited to the TCR/CD3 complex where it is activated, inducing phosphorylation of downstream adaptor proteins (Nel 2002) (Smith-Garvin et al. 2009) to ultimately produce DAG. DAG activates the MAPK/ERK pathways to promote activation of transcription factor NF-κB, and IP3 which induces Ca^{2+} release from the ER which, in turn, induces the opening of Ca^{2+} release activated Ca^{2+} channels to increase cytosolic Ca^{2+}. Calcium-bound calmodulin activates the phosphatase calcineurin which promotes IL-2 gene transcription through the transcription factor NFAT. T-cells can then become activated through release and binding of IL-2 to the IL2-R which leads to clonal expansion and differentiation into either effector T-cells or memory T-cells (Kuo and Leiden 1999).

1.5. The liver and immune regulation

Hepatocytes are the main constituents of liver with ~80% of mass consisting of hepatocyte cytoplasm. Functions of hepatocytes include protein synthesis and storage, synthesis of cholesterol, bile acids and phospholipids, detoxification, and modification and excretion of exogenous and endogenous substances. Hepatocytes are the primary producers of serum albumin, the most abundant protein in the body. The liver is somewhat immunosuppressive with more lymphoid cells associated with the innate immune system than the lymphoid cells

found in peripheral blood. In particular, large amount of NK cells are present which comprise 65% of all lymphoid cells found in the liver; however conversely, the amount of adaptive immune $CD3^+$ $CD4^+$ CD8- and $CD3^+$ CD4- $CD8^+$ T-cells is lower in the liver when compared with PBMCs. This balance indicates that the liver immune system is highly polarised to fight pathogens through the actions of the innate immune system (Norris et al. 1998) (Norris et al. 1999).

1.6. The skin and immune regulation

The skin is mainly comprised of keratinocytes which as well as forming a physical barrier against invading pathogens, also help fight them through displaying various pattern recognition receptors such as toll-like receptors. Specialised immune cells are present in the epidermis alongside keratinocytes; these consist of memory T-cells and Langerhans cells (epidermal DCs). Langerhans cells have been discovered to be important in contact hypersensitivity reactions where the immunogen is delivered shallowly into the skin, not penetrating the epidermis (Romani et al. 2012). Langerhans cells have also been shown to have a poor capacity for activating $CD8^+$ T-cells in staphylococcal infected skin leading to resolution of the infection through humoral immune responses. In mice, a subset of $CD103^+$ dendritic cells exist deeper in the skin, in the dermis, as well as in other organs (Ginhoux et al. 2009). This subset seems to have a human equivalent ($CD141^+$ DCs) (Haniffa et al. 2012). In both species this group of DCs has a well-defined role in immunity against viral antigens and preferential presentation to $CD8^+$ T-cells (Bedoui et al. 2009). Another subset of DC found in the skin is a $CD11b^+$DC which seems to preferentially present antigen to $CD4^+$ T-

cells and in particular, regulatory $CD4^+$ T-cells (Haniffa et al. 2012). Collectively, these data indicate that an adaptive response is preferred in the skin.

1.7. T-cell movement and organ infiltration

The ability of antigen-specific T-cells to move around the body to possible sites of infection is a necessary requirement in the adaptive immune system. Leukocytes travel from the blood to peripheral tissues using adhesion molecules. These adhesion molecules include selectins, integrins, and chemoattractant receptors. Selectins promote the rolling movement of leukocytes along endothelial cell surfaces (Luster et al. 2005). Integrins are trans-membrane surface receptors which promote the firm attachment of leukocytes to endothelial cells, and chemoattractants are G-protein coupled receptors which promote the firm adhesion to endothelial cells through integrins and subsequent direct cell migration through tissue gradients (Luster et al. 2005). During inflammation in a tissue, the vascular beds upregulate the ligands for selectins, integrins, and chemoattractants to induce direction cues for inflammatory T-cells, which express the corresponding receptors to enter the tissue of requirement from the vascular system. T-cells can also be "imprinted" with specific allergy-prone organ homing when they are still naive. The three main organs that T-cells are imprinted to are the gut, the skin, and the lung. To imprint T-cells to travel to the gut, the T-cell is stimulated to express the integrin $\alpha_4\beta_7$ and the receptor chemokine 9 (CCR9) (Luster et al. 2005) (Fig 1.3. B). To imprint homing to the skin, T-cells are stimulated to express the lectin cutaneous leukocyte antigen (CLA) along with chemokine receptors CCR4, CCR10, and in some T-cell subsets CCR8 and CCR6 (Januszewicz and Firkin 1988) (Fig 1.3. A). The DCs which originate from specific organs have shown to be able to imprint

homing to naive T-cells through release of environmental cues. DCs originating from the gut secrete retinoic acid metabolites which induces expression of the gut-tropic receptors α4β7 and CCR9 on naive T-cells (Mora et al. 2005); DCs from the skin secrete the active vitamin D3 metabolite which induces CCR10 expression in naive T-cells (Sigmundsdottir et al. 2007). Other chemokine receptors have also been discovered and linked to migration to other organs, like lung (Fig 1.3. C) (Masopust and Schenkel 2013).

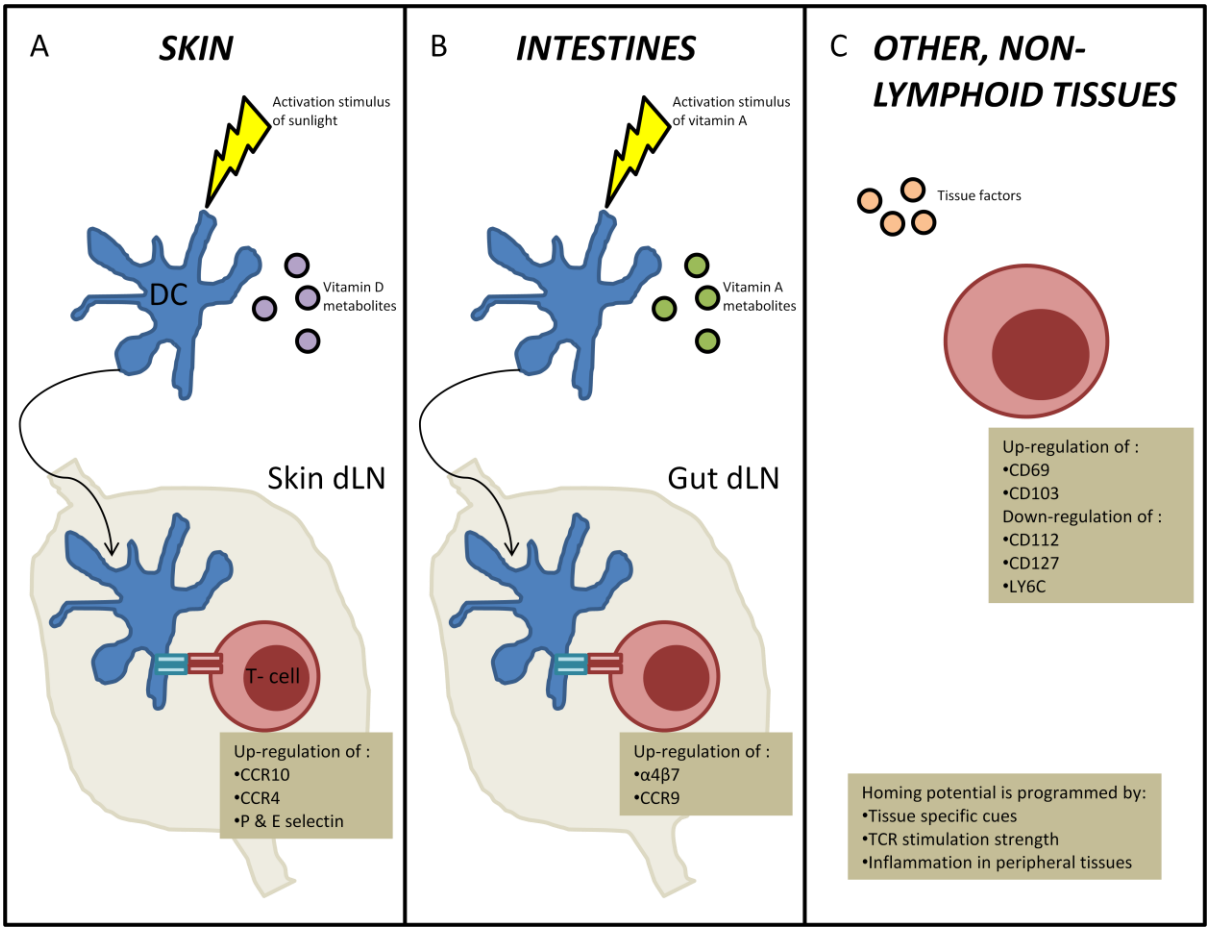


Fig 1.3. T-cell migration in skin (A), gut (B), and other tissues like lung (C). The migration of T-cells is determined by tissue specific vitamins or factors as well as priming location. Adapted from Masopust and Schenkel (2013).

1.8. Contact hypersensitivity (CHS)

Contact hypersensitivity is defined as an immune reaction which usually presents itself after someone has been repeatedly exposed to a particular drug or protein-reactive chemical (Xu et al. 2000). In CHS, when the body is first exposed to the protein-reactive chemical usually via the skin, but not exclusively, the chemical in question first binds to protein. This bound protein is then taken up by APCs in the skin; these cells proceed to travel to the draining lymph nodes where peptides derived from the foreign protein are presented to $CD4^+$ and $CD8^+$ T-cells which then enter the circulation and lymphatic system (Xu et al. 2000). This process is known as sensitisation. The next step in CHS is elicitation; this happens when the body is re-introduced to the protein-reactive chemical after a period of time has passed. The chemical again binds to protein but this time chemical specific $CD4^+$ and $CD8^+$ T-cells from the draining lymph node travel to the site of contact (Nassif et al. 2004). The effector $CD8^+$ T-cells proceed to secrete inflammatory cytokines like IFNγ and cause death of keratinocytes which have been in contact with the altered proteins through cytotoxicity involving Fas-L, perforin/ granzyme B, and granulysin (Chung et al. 2008) (Nassif et al. 2004).

It is known that $CD4^+CD25^+FoxP3^+$ natural T_{regs} are capable of inhibiting the development of CHS through a variety of mechanisms including the production of inhibitory cytokines like IL-10, TGF-β, and IL-35 (Hawrylowicz and O'Garra 2005) (Green et al. 2003) (Collison et al. 2007), direct cytolytic abilities (Zhao et al. 2006), inhibition of proliferation (Thornton and Shevach 1998), and disruption of DC function (Tang Q. et al. 2006) (Kimber et al. 2012). The development of CHS via skin sensitisation has been experimentally demonstrated to be reliant upon TLR2, TLR4 activation, and IL-12 signalling. It is important to note however that only in mice devoid of both TLR signalling and IL-12 signalling was sensitisation inhibited (Martin S. F. et al. 2008) (McFadden et al. 2013).

An example of perhaps the most well known molecule capable of causing CHS is urushiol, the active molecule responsible for the effects of poison ivy. Urushiol requires oxidation via keratinocytes before becoming protein-reactive and able to cause sensitisation but upon repeat exposure, the elicitation response usually leads to painful blisters. Chemicals capable of causing CHS can range in severity from being able to sensitise most individuals at very small concentrations, like nitrohalobenzenes, which have been studied extensively due to their sensitising nature, to extremely weak sensitising chemicals, like drugs such β-lactam antibiotics which only sensitise a very small percentage of the population even at extremely high concentrations (Gielen and Goossens 2001).

1.9. Clinical implications of cutaneous drug hypersensitivity reactions

Drug hypersensitivity reactions most commonly present themselves as a relatively mild skin reaction called maculopapular exanthema (MPE). This condition consists of itching wheals and rashes which can resolve by discontinuing the drug and treatment with either steroid/emollient creams or systemic antihistamines (Rich et al. 2013). Symptoms generally appear 8-11 days after the drug is first given, but in already sensitised patients, symptoms can be seen after only 1-2 days. A myriad of drugs have been reported to cause MPE including β-lactam antibiotics, sulphonamide, and diuretics to name just a few. The more severe form of MPE which also derives from drug exposure is acute generalized exanthematous pustulosis (AGEP) which effects about 1 in every 100,000 drug treatments (Rich et al. 2013). The symptoms for this form of reaction include the appearance of sterile pustules in the skin which usually appear 2-5 days after starting treatment. Healing is evident 5 days after stopping the administration of the drug, but this condition can be fatal with a mortality rate of about 2-4%. The most serious drug induced skin reactions are however Steven's Johnson syndrome (SJS) and toxic epidermal necrosis (TEN) which have a

mortality rate of approx 13% and 39% respectively (Rich et al. 2013). They are rare with SJS affecting ~1 in 100,000 treated patients and TEN affecting ~1 in 1,000,000 treated. They are diagnosed through the amounts of skin detachment (~10%- SJS, 10-20%-SJS/TEN, ~30%-TEN) and the average time for onset of symptoms is around 17 days after the start of treatment (Rich et al. 2013). The turning point from diagnosis of MPE to SJS is often when the exanthema becomes painful and bullae begin to form 12-24 hours later. Removal of the drug is usually enough to prevent the SJS progressing to TEN but common symptoms still include blistering in mucous membranes (e.g. mouth/lips) and possible keratoconjunctivitis (Rich et al. 2013).

1.10. Clinical implications of systemic drug hypersensitivity reactions

Although the skin is usually the organ under duress in drug hypersensitivity reactions, systemic drug hypersensitivity reactions pose a real problem for certain drugs like flucloxacillin, ximelagatran, and carbamazepine.

1.10.1. Drug reaction with eosinophilia and systemic symptoms (DRESS)

Eosinophilia is diagnosed when eosinophils in the blood increase above normal levels. Eosinophilia is only a disorder if it is idiopathic; however, it is often an accompaniment of DRESS (Simon and Simon 2007). DRESS is a term used to define drug hypersensitivity reactions which present with a latency period between onset of symptoms and first exposure to the culprit drug. The term DRESS has been coined to describe a variety of drug hypersensitivity reactions and the main symptoms are cutaneous reactions as described above along with internal organ damage which has clinical overlap with other diseases caused by drugs such as SJS and drug-induced liver injury (Schlienger et al. 1998). The

systemic symptoms which usual present alongside cutaneous symptoms, are predominantly liver orientated but can also include kidney, lung, and heart. The mortality rate of DRESS is around 10% with the majority of these deaths being contributed to liver damage (Peyriere et al. 2006) (Eshki et al. 2009). Other than drug induced, DRESS can also be virus induced. Evidence of this stems from some cases continuing to exacerbate despite discontinuation of the offending drug and evidence of viral infection in laboratory tests (Shiohara and Kano 2007). In patients with sporadic outbreaks of DRESS, tests have shown that the emergence of symptoms coincides with the reactivation of viruses HHV-61, HHV-7, Epstein-Barr virus, and cytomegalovirus (Seishima et al. 2006) (Oskay et al. 2006) (Kano et al. 2006).

1.10.2. Drug-induced liver injury (DILI)

Drugs which induce skin reactions are usually only discovered to do so after the drug has been marketed to the public due to the small percentage of people that are effected, whereas systemic drug hypersensitivity reactions, and DILI, is one of the most common reasons for the prevention of drugs from entering the pharmaceutical market in the first place (Temple and Himmel 2002). DILI is the main reason for acute liver failure having just over 50% of cases attributed to it with 39% being due to hepatotoxicity due to overdose of paracetemol's active metabolite and 13% due to idiosyncratic liver injury caused by other drugs (Maddrey 2000). Relatively recently, an important role has been highlighted for HLA class I and II genes in the development of DILI.

1.10.3. Idiosyncratic drug-induced liver injury (IDILI)

Not all drugs cause one type of liver damage and the effects of a drug can vary in the time of onset of symptoms which can vary from hours after the drug is given to weeks after the drug has been stopped, which is not unusual for DILI caused by antibiotic treatment. When a

delay is observed between drug exposure and onset of liver injury, the reactions is deemed idiosyncratic drug-induced liver injury (IDILI). The mechanism of how this happens is not fully known however, the delayed nature of the reaction strongly indicate the involvement of the adaptive immune system (Adams et al. 2010). Relatively recently, an important role has been highlighted for HLA class I and II genes in the development of IDILI which again implicates the adaptive immune system in the pathogenesis of the disease (Daly and Day 2012).

Interestingly, there seems to be a correlation between drug-HLA associations and the incidence of cholestatic drug-induced liver injury. Cholestasis is known to be a possible route for IDILI by drugs like flucloxacillin. Damage is caused when the transport of bile is halted through disruption in transport proteins in the bile duct. This leads to the build of bile and bile salt in the liver which leads to bile induced liver membrane damage and build up of cholesterol, both disrupt membrane proteins and reductions in the bile salt pool and enterohepatic recirculation, all of which add to liver function impairment and damage (Andrews and Daly 2008). There are a variety of forms of liver damage cause by drugs which can widely vary from drug to drug. Paracetemol had long been a model drug to investigate drug-induced liver injury however the mechanisms are a known pharmacology of the drug; hepatocyte damage occurs due to the protein-reactive metabolite of paracetemol N-acetyl-p-benzoquinoneimine (Dahlin et al. 1984) which at therapeutic doses is detoxified by glutathione however, at overdoses, glutathione is exhausted resulting in the toxic metabolite and subsequent hepatocyte necrosis attributed to mitochondrial death (Masubuchi et al. 2005). Amodiaquine is a malaria drug found to cause liver damage through hepatotoxicity where symptoms appeared weeks to months after drug administration and re-appear after re-administration of the drug strongly indicating the

adaptive immune system (Uetrecht J. 2005). Although, like flucloxacillin the adaptive immune system is culprit, the mechanisms of damage are vastly different between the two drugs highlighting the complexity and variance of IDILI from drug to drug.

1.11. Pathogenesis of drug hypersensitivity reactions

There are currently three mechanisms by which drugs are thought activate T-cells through their TCR; the hapten hypothesis, the Pi mechanism and the altered peptide hypothesis. A hapten is defined as a low molecular weight compound capable of binding to protein to create an immunogenic molecule. An immunogen is any antigen that is capable of inducing a humoral or cell-mediated immune response rather than immunological tolerance. Hence, an antigen is any substance that may be specifically bound by components of the immune system although not every antigen causes an immune response; ones that do are defined as immunogenic.

1.11.1. Hapten hypothesis

The hapten hypothesis states that drugs or low molecular weight compounds under 1000Da in weight must form stable adducts with endogenous protein to be able to induce an immune response (Park et al. 1998). Haptenation of proteins can occur either directly or through metabolic activation. In direct haptenation, like with the classic sensitising agents dinitrochlorobenzene (DNCB) and dinitroflourobenzene (DNFB), the haptenic molecule binds directly to protein in a relatively non-specific manner to modify lysine and cysteine residues generating the immunogenic protein (Knight and Green 1979) (Fig 1.4. A). β-lactam antibiotics are also known to be directly protein-reactive and mass spectrometric techniques have been used in the case of piperacillin (Whitaker et al. 2011) and

flucloxacillin (Jenkins et al. 2009) to determine the exact amino acid residues that are modified by the drugs. However, most drugs only become sufficiently haptenic after bioactivation and this bioactivation is thought to occur after the chemical structure of the molecule has been altered through the action of enzymes such as cytochrome P450 enzymes, which are usually responsible for the detoxification of compounds. However, the oxidation of certain drugs can, ironically, cause them to become protein-reactive. P450 enzymes are found most abundantly in liver but can be also found in skin, gut and kidney. Importantly, immune cells express only low levels of P450 enzyme. However, other enzymes known to cause drugs to become protein-reactive (e.g. NADPH oxidase and myeloperoxidase) are found in phagocytes and other APCs (Sezer et al. 2001).

1.11.2. The Pi concept

The pharmacological interaction or "Pi concept" states that drugs do not need to be bound covalently to protein to become immunogenic and that some drugs can in fact bind directly to the MHC binding grooves to cause T-cell-mediated immune reactions. The Pi concept, as opposed to the hapten hypothesis, states that parent drug is able to "structurally" fit into both the T-cell receptor and the antigen presenting cells-MHC groove to stimulate the T-cells to react (Pichler et al. 2006) (Adam et al. 2011) (Fig 1: 4. B). This concept was devised through experimentation with drug-specific T-cell clones to drugs like sulfamethoxazole where the T-cells were found to react to drugs in an MHC-T-cell receptor dependent way. Furthermore, specific T-cell clones were capable of reacting to drug-treated fixed APCs (Schnyder et al. 1997) (Zanni et al. 1998).

1.11.3. Altered-self peptide repertoire hypothesis

One drug, abacavir, has recently been shown to directly bind to MHC molecules through its association with HLA-B*57:01 allele where up to 50% of patients carrying the allele will develop hypersensitivity reactions whilst patients not carrying the allele are prescribed the drug with absolutely no immunological hypersensitivity reactions predicted (Mallal et al. 2008). This association lead to the screening of patients before prescription of the drug, leading to the first successful example of personalised medicine (Yun et al. 2012). This particular mechanism of the Pi-concept has come to be named the "altered-peptide concept" (Fig 1:4. C). The interaction of abacavir with endogenous HLA-B*57:01 shows the binding interaction changes the confirmation of the peptide binding cleft, altering the repertoire of peptides displayed on the cell surface (Illing et al. 2012) (Ostrov et al. 2012) (Norcross et al. 2012). These data imply that pre-existing peptide-specific T-cells are activated indirectly by abacavir through the display of cross-reacting "altered-self" peptides.

1.11.4. The danger hypothesis

The danger hypothesis simply states that the body does not discriminate between non-self and self but only strives to protect against danger (Matzinger 1994) (Anderson and Matzinger 2000). It is thought that the immune system requires three signals to react fully to an antigen (Curtsinger et al. 1999). Signal 1 involves presentation of an antigen via the MHC to the T-cell receptor and if no other signals are received, then this will result in tolerance to the particular antigen. Signal 2 involves the action of co-stimulatory interaction between APC and T-cells and the release of pro-inflammatory cytokines like IFNγ to enhance presentation. Signal 3 involves the response of specific T_H1 or T_H2 cells through polarising cytokines which specifically target T-cells (Pirmohamed et al. 2002). With respect to drug

hypersensitivity reactions, drugs and/or protein-reactive metabolites have the ability to be presented as part of Signal 1 via a possible drug protein interaction or MHC-T-cell receptor interaction and Signal 2 and 3 can be derived from either surgical trauma, drug derived stress, infection (Uetrecht J. P. 1999), or necrotic or apoptotic cell death. It has been shown that only necrotic cells can activate dendritic cells (Gallucci et al. 1999) but apoptotic cells have been shown to activate cytotoxic T-cells (Shi et al. 2000). It is well documented that patients with viral infections are more susceptible to adverse drug reactions (Sullivan and Shear 2001), however, with respect to danger signals in drug hypersensitivity reactions the specific nature and origin of the danger signals from drug and non-drug signalling is not well defined and more research is needed.

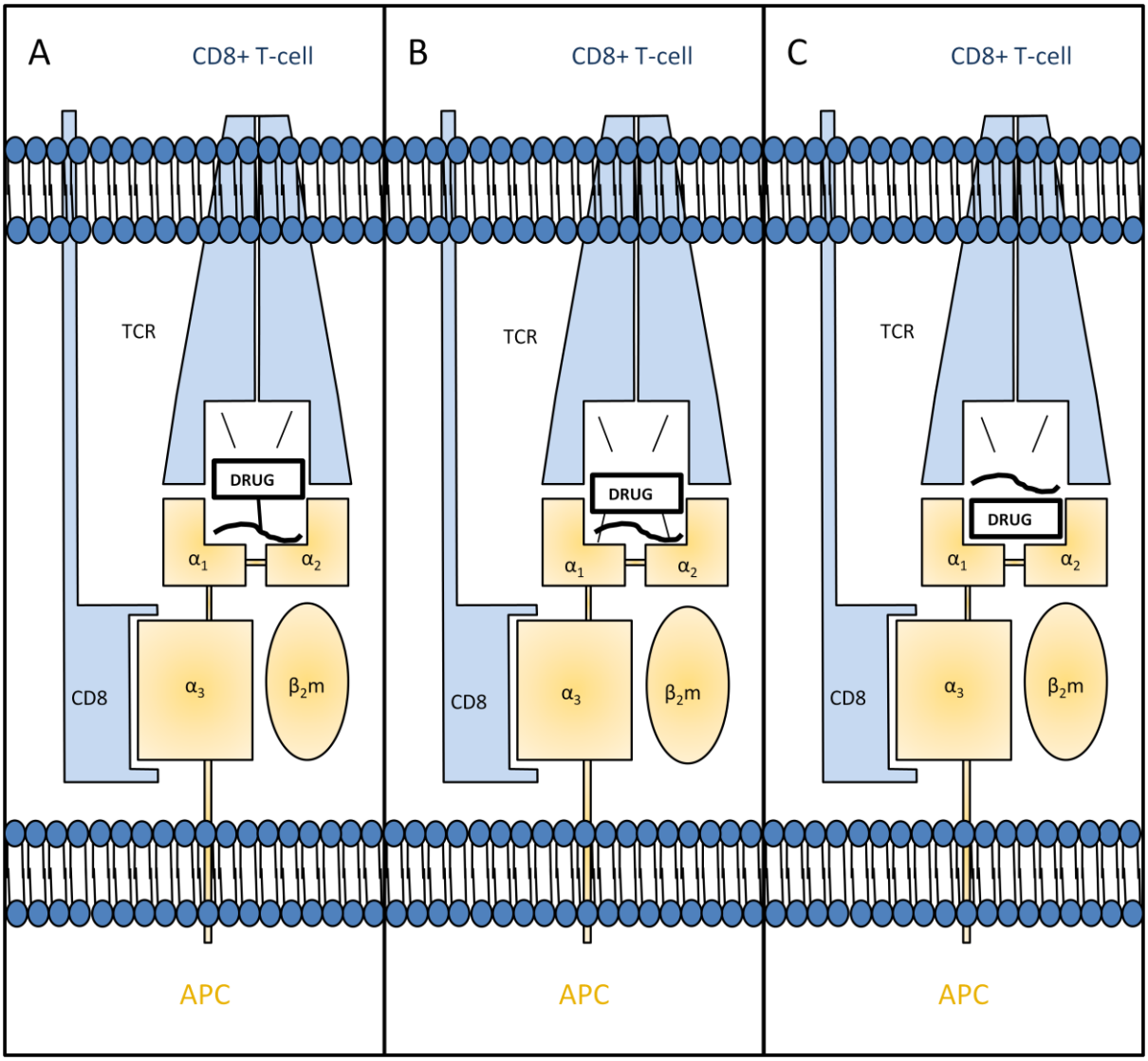


Fig 1.4. The hapten hypothesis, the "Pi-concept", and the altered peptide concept. (A) Hapten hypothesis where the peptide embedded in the MHC I molecule, is covalently bound and altered by protein-reactive drug (e.g. flucloxacillin), creating an immunogenic antigen. (B) The "pi-concept" where the drug is capable of binding directly to the TCR during MHC presentation (e.g. SMX, carbamazepine). (C) Altered peptide concept where the drug is capable of non-covalent binding directly to particular HLA-B associated MHC I molecules, allowing for presentation of "altered-self" peptides to $CD8^+$ T-cells and subsequent sensitisation (e.g. only abacavir to date). Adapted from Adam et al (2011).

1.12. β-lactam antibiotics

Beta-lactam ring-containing drugs are antibiotics that exert their pharmacological effect through binding irreversibly to bacterial penicillin binding proteins involved in the synthesis

of peptidoglycan. β-lactam antibiotics are known to be capable of inducing immediate (IgE mediated) and delayed-type (T-cell-mediated) hypersensitivity reactions.

1.12.1. β-lactam-induced immediate hypersensitivity reactions

Beta-lactam-induced immediate allergic reactions are classified as Type 1 through their quick onset of symptoms (<1h) and release of histamine and other vasoactive inflammatory mediators. The release of these usually generates clinical symptoms of anaphylaxis and rapidly occurring urticaria (Baldo 2014). Before an immediate reaction can instigate in a patient, that patient must first have been sensitised to the β-lactam in question (Stone et al. 2014). Sensitisation occurs when the antibiotic is first introduced to the patient and immunogens created by the presence of the drug are taken up by APCs and presented to T and B-cells in the context of a T_H2 response. This results in drug specific IgE antibodies being produced in response to that particular immunogen if the drug is re-introduced (Stone et al. 2014). The specific mechanisms involved in β-lactams causing the symptoms of immediate reactions are a consequence of three interactions between allergen, antibody, and cell. The produced IgE antibodies mediate this reaction through interacting with their complementary allergens and with mast cells and basophils. The antibodies bind strongly with their FcεRI receptor which is abundantly expressed on mast cells and basophils and form a long-lasting, slowly dissociating complex (Baldo 2014). Interactions of the combining sites of the cell-bound antibodies alongside the actions of the bound allergen allows cross-linkage of adjacent antibodies, aggregation of the FcεRI receptors, and the triggering for rapid release of histamine, platelet activating factor, heparin, chemo-tactic factors, serotonin, and enzymes, all of which leads to the symptoms seen in type I β-lactam hypersensitivity reactions (Kaliner et al. 1972) (Gould et al. 2003) (Baldo 2014).

1.12.2. β-lactam induced delayed hypersensitivity reactions

Beta-lactam containing antibiotics are capable of causing Type IV delayed hypersensitivity reactions which involve drug-specific T-lymphocytes (Pichler 2003). The symptoms of organ damage appear after drug-specific cytotoxic cells are generated through sensitisation via APC presentation of drug-immunogens to naive T-cells which can be polarised towards a T_H1 (Monshi et al 2013) or a T_H2 (El-Ghaiesh et al. 2011) type response (Pichler 2003). This induces clonal expansion of drug-specific T-cells. Upon re-exposure to the particular drug, specific T-cells infiltrate organs, utilise their cytotoxic abilities and secrete inflammatory cytokines in response to the presence of the immunogen. The generation of drug-specific T-cells requires MHC presentation of non-self-protein as previously discussed which in respect to β-lactams, is usually generated through the creation of haptenated protein. There is long-standing evidence of β-lactams covalently binding to protein and specifically, lysine residues (Levine and Ovary 1961) (Jenkins et al. 2009) (Padovan et al. 1997). This occurs through the opening of the reactive β-lactam ring after nucleophilic attack to create stable protein adducts to prime naive T-cells through a widely accepted haptenic mechanism.

Amoxicillin, piperacillin, and flucloxacillin have all been investigated in regards to the hapten hypothesis where human serum albumin has been found to be modified at specific lysine residues (Whitaker et al. 2011) (Jenkins et al. 2009) (Ariza et al. 2012) and synthetic piperacillin albumin conjugates have been demonstrated to stimulate T-cell clones from hypersensitive patients in a concentration-dependent manner (Elsheikh et al. 2010). All these drugs show similar protein-binding patterns and involvement of the adaptive immune system is well defined (Rozieres et al. 2009) (Elsheikh et al. 2010) (Monshi et al. 2013). T-cells affect different organs in susceptible patients with amoxicillin and piperacillin mainly inducing skin rash and flucloxacillin mainly injuring liver. The reason as to this has not been

fully elucidated although a possibility could be that T-cell clones generated from flucloxacillin-sensitised patients mainly express $CD8^+$ (Monshi et al. 2013), while piperacillin-specific T-cell express $CD4^+$ (Elsheikh et al. 2010). Why these two seemingly similar β-lactam antibiotics would show this trend may lie in the mode of sensitisation. Hepatocytes are known to be able to generate albumin which is synthesised in the cytosol. Flucloxacillin is known to be able to bind to liver protein in hepatocytes of rats as Carey and Van Pelt (2005) demonstrated through analysis of liver cytosol of rats dosed systemically with flucloxacillin, where albumin was specifically drug-modified. The mode of sensitisation in flucloxacillin reactions may therefore be directed against intracellular antigen. In contrast, piperacillin and amoxicillin-specific T-cells may be activated by a MHC II-restricted extracellular antigen.

1.13. Experimental approaches to studying delayed-type hypersensitivity

Drug hypersensitivity reactions can be studied through protein modification, antigen presentation, or human T-cell activation *ex vivo*. However, animal models where drug-specific T-cells can be monitored *in* or *ex vivo* represent the only approach where the relationship between drug distribution and the priming of naive T-cells can be studied directly.

1.13.1. Nitrohalobenzenes

Nitrohalobenzenes are a family of haptens which all form the same dinitrophenyl-modified protein adduct. DNCB in particular has been studied extensively in regards to mechanisms of T-cell activation, drug distribution, and protein binding. DNCB is known to be a strong sensitiser capable of inducing cellular immune response in 100% of subjects which is readily detectable after skin challenge with proliferating and IFNγ secreting CD4 and $CD8^+$ T-cells

(Friedmann et al. 1983) (Pickard et al. 2009). Through fixation of APCs with gluteraldehyde it was shown that DNCB requires metabolic activity and the processing of protein antigens to generate a T-cell response (Pickard et al. 2007). With the utilisation of designer peptides with MHC binding motifs, others found that DNCB must bind to specific peptides to become immunogenic with the presence of DNCB in the peptide being an obligatory factor (Martin S. et al. 1993) (Martin S. and Weltzien 1994) (Preckel et al. 1997). Collectively, this research shows that DNCB binds to intracellular and extracellular proteins which generate activated T-cells via protein processing and MHC-restricted antigen presentation.

1.13.2. The local lymph node assay (LLNA)

In 1989 Kimber et al developed the murine local lymph node assay (LLNA) which is an *in vivo* assay capable of determining if a substance was a strong sensitizer through measurement of proliferation of the draining lymph nodes with radioactive [^{3}H] thymidine after painting of the particular hapten onto the skin. The methods for this procedure involve [^{3}H] thymidine being injected I.V., lymph nodes are then isolated and macerated and proliferation counted as scintillation per cell pellet. This was a breakthrough assay to determine strong haptens but however, the assay is not sensitive enough to detect sensitisation to very weak sensitisers including drugs (Kimber et al. 1989).

1.13.3. Effector mechanisms of sensitisation

Work performed by Vocanson et al (2006) demonstrated that $CD8^+$ T-cells were the effector cells in CHS reactions to DNCB. Using mice depleted of $CD4^+$ T-cells, sensitisation and subsequent elicitation responses were observed to be extraordinarily robust. Furthermore, adoptive transfer of DNCB sensitised $CD8^+$ T-cells was able to induce an elicitation response

in naive mice but only after they had been depleted of $CD4^+$ T-cells simultaneously demonstrating the down-regulatory abilities of T_{regs} in the CHS response.

1.13.4. Models of drug hypersensitivity and attempted animal models of idiosyncratic drug-induced liver injury

Animal models capable of showing drug hypersensitivity and involvement of the adaptive immune system are a useful tool in unveiling the mechanisms of specific drug hypersensitivity reactions; examples include the nevirapine skin rash model, halothane, and amodiaquine-induced liver injury. Each of these are briefly discussed below.

1.13.4.1. Nevirapine

The animal model of nevirapine skin rash has been developed in rats as the sensitisation of mice was unsuccessful (Ng et al. 2012). Nevirapine is a drug used to treat patients with HIV infection and soon after market it was discovered to cause varying severities of skin rash and, in rare cases, liver toxicity in females (Pollard et al. 1998). Sensitisation of rats revealed a strong adaptive immune response involving infiltrates in inflamed skin containing large amount of T-cells and macrophages. Depletion of $CD4^+$ T-cells ablated the response and depletion of $CD8^+$ T-cells made the response stronger indicating that in nevirapine hypersensitivity, $CD4^+$ T-cells are the effector cells (Popovic et al. 2006). The mechanism of how the nevirapine activated $CD4^+$ T-cells cause tissue injury has been thoroughly investigated in this model. Oxidation of nevirapine in the liver leads to the generation of 12-OH-nevirapine. This is carried to the skin where metabolism leads to production of 12-OH-nevirapine sulphate in the epidermis. Covalent binding of this metabolite to protein leads to the activation of effector $CD4^+$ T-cells and nevirapine-associated skin rash (Sharma et al. 2013). Mouse models of nevirapine-induced liver injury have been investigated. In C57Bl/6

mice, increased ALT are observed after 3 weeks of treatment. Livers show inflammatory lesions which resolve over time (Ng et al. 2012). This observation indicates that this may in fact be a model of immune tolerance, as opposed to a model of idiosyncratic DILI (IDILI) (Ng et al. 2012).

1.13.4.2. Halothane

The closest animal model of drug-induced liver injury involving the adaptive immune system to date investigates reactions associated with halothane, which is metabolized in liver to ultimately form adducts with liver proteins. Halothane is thought to cause hepatitis through an antibody driven auto-immune reaction with the majority of patients with halothane hepatitis having antibodies in their sera that react with liver microsomal antigens (Canalese et al. 1981) (Bird and Williams 1989). The immunological pathway of mild liver damage in animals is known for halothane. The liver protein adducts are detectable in animals (Neuberger et al. 1987) (Mathieu et al. 1975), and it was recently shown that the injury in this model is associated with an infiltrate of eosinophils which when depleted, reduced injury (Proctor et al. 2013). This type of damage is typical of clinical halothane-induced hepatitis but severe halothane-induced injury seems to progress over time which strongly suggests an adaptive response, however, there has been no direct evidence to date of liver damage in mice caused directly through a drug-driven adaptive immune response.

1.13.4.3. Amodiaquine

Amodiaquine is an anti-malarial drug which was withdrawn from the market due to hepatotoxicity and agranulocytosis. The clinical symptoms of amodiaquine hepatotoxicity resolved quickly after discontinuation but re-introduction of the drug was met with rapid rises in ALT levels, being consistent with an immune-mediated reaction (Uetrecht J. 2005).

Amodiaquine is metabolised to yield a reactive haptenic metabolite (Harrison et al. 1992). After dosing mice and rats for 2-3 weeks with amodiaquine, increases ALT of 2-3 fold control were observed. However, this returned to basal levels after 6 weeks dosing in both species (Shenton et al. 2004). In animals with raised ALT, increases in numbers of $CD4^+$ T-cells and activation of macrophages and B-cells in the spleen was observed along with changes in the liver. Increased expression of immune function-related genes like CD3e and CD4 was observed (Uetrecht J. 2005). Furthermore, the livers of these animals were found to have a mild infiltration of immune cells with raised numbers of $CD4^+$ T-cells, NK cells, and T_H17 cells (Uetrecht J. 2005). The reduction of ALT levels after continued dosing of amodiaquine indicates immune adaptation and tolerance to the drug as opposed to the progression to severe DILI. This adaptation is also seen in humans and is more frequent than the development of severe DILI. In defence of this statement a 3-fold increase in IL-10 liver mRNA was detected during dosing indicating the involvement of regulatory T-cells which are well known for their role in immune tolerance (Langier et al. 2010) (Shevach et al. 2001). It would be of interest to attempt to develop an animal model of DILI in an animal devoid of regulatory T-cells, where $CD8^+$ T-cells are known to be the majority effector cell in the disease.

From this brief discussion, is it clear that animal models of IDILI have been difficult to develop and/or reproduce and most end in failure (Ng et al. 2012) (Uetrecht J. and Naisbitt 2013); because of this there are, currently, no adaptive immunity-based animal models of liver injury from any drug that produces IDILI in humans (Roth and Ganey 2011).

1.14. AIMS

- To establish an animal model of β-lactam skin sensitisation to show drug-specific T-cell responses to amoxicillin, piperacillin, and flucloxacillin.
- Use this model to;
    - Study the phenotype and function of antigen-specific T-cells
    - Study β-lactam T-cell cross-reactivity
    - Investigate whether drug-responsive T-cells kill hepatocytes *in vitro* or *in vivo*

CHAPTER 2

Development and optimisation of the mouse model of contact hypersensitivity to amoxicillin

Contents

2.1. INTRODUCTION

Recently, Vocanson et al (2006) developed a mouse model in which the mice are predisposed to develop CHS responses to weak haptens, haptens which only sensitise a very small percentage of the population even at high doses. The mice used have a mutation in the αβ gene encoding for MHC class II molecules (II°/°) and have reduced numbers of $CD4^+$ T-cells (2.5% of peripheral $CD4^+$ T-cells) (Rozieres et al. 2010). Approximately 50% of the residual $CD4^+$ T-cells in the MHC II KO mice are T_{regs} that can suppress hapten-specific $CD8^+$ T-cell responses (Kish et al. 2007). The IL-10-producing $CD4^+$ FoxP3-T-cells, and natural $CD4^+$ $CD25^+$ $FoxP3^+$ T-cells endowed with down-regulatory abilities circulate in wild type and knockout mice and participate in the resolution of autoimmunity (Bacchetta et al. 2007) (Roncarolo and Battaglia 2007). These cells need to be depleted with an anti-$CD4^+$ antibody before CHS reactions to weak haptens can be observed due to their down-regulatory effect on the effector cells of CHS, $CD8^+$ T-cells (Vocanson et al. 2006).

The anti-$CD4^+$ antibody used throughout this thesis has been proposed to cause depletion of $CD4^+$ T-cells through C3b-receptor-mediated clearance (Alters et al. 1990). This would cause phagocytosis of antibody-bound target cells through interaction between macrophage/monocyte Cb3 with target-cell bound Cb3. This hypothesis has been tested in investigations into systemic lupus erythematosus where patients have been discovered to have defective C3b-mediated clearance (Iida et al. 1982). Investigations using mice pre-disposed to developing this disease have reported that $CD4^+$ T-cell depletion using anti-$CD4^+$ antibody GK 1.5 in these mice is significantly more difficult (Wofsy and Seaman 1987).

The presence of T_{regs} in an MHC II KO mouse raises questions upon the origin/ selection/ differentiation of these cells. In wild-type mice, $CD4^+$ T-cells develop through high-affinity

interaction with self-peptides and MHC II molecules. Interestingly, the interaction of CD4 with the MHC II molecules is not a prerequisite for the development of T_{regs} (Denning et al. 2003) (Kish et al. 2007). The main culprit for T_{regs} development is however the transcription factor Foxp3 (Curiel 2007), which can be induced to be expressed by external cues at low levels in thymocytes in the thymus. This low level expression of Foxp3 thereby induces the cell to express medium levels of CTLA-4 and CD25 along with T_H1, T_H17, and adaptive T_{reg} cytokines which, along with other microenvironment factors, push the cell to develop into a $CD4^+CD25^+Foxp3^+$ T_{reg} and is the likely reason as to why T_{regs} escape deletion in the thymus (Rudensky et al. 2006) (Curiel 2007).

The assays developed by Vocanson et al (2006) demonstrated that $CD8^+$ T-cells were the effector cells in CHS. This was demonstrated through ablation of the elicitation response after administration of an anti-$CD8^+$ antibody and through adoptive transfer of sensitisation to naive mice, via transference of purified $CD8^+$ T-cells from sensitised mice. It is important to point out the limitations of this model in regards to the lack of MHC II molecules and $CD4^+$ T-cells where only $CD8^+$ T-cell-mediated reactions are being measured and possible $CD4^+$ T-cell-mediated reactions will not be able to be investigated or measured. It is also prudent to take the strain choice of C57Bl/6 mice into account when looking at the limitations and bias of the model that has been developed and investigated in this chapter and throughout this thesis. Mice on a C57Bl/6 background have a T_H1 bias towards pathogens whilst other strains like BALB/c bias towards T_H2- predominant responses (Mills et al. 2000).

In this chapter we attempted to establish the model in Liverpool to analyse the drug-specific activation of T-cells *in vitro*. Proliferative responses and IFNγ secretion were measured using [^{3}H] thymidine and IFNγ ELISA, respectively. Furthermore, the activation state of $CD8^+$ T-cells

has been analysed through flow cytometric analysis of $CD8^+$ T-cell activation markers CD28 and "inducible T-cell co-stimulator" (ICOS). $CD8^+$ T-cells are known to up-regulate certain T-cell receptors as a defence against foreign antigens. These include CD28 and ICOS. CD28 and ICOS enhance basic T-cell responses to foreign antigen including proliferation, secretion of cytokines, and up-regulation of molecules that mediate cell-cell interactions (Hutloff et al. 1999). CD25 can also be a marker of $CD8^+$ T-cell activation as CD25 is rapidly up-regulated by antigen-specific $CD8^+$ T-cells after T-cell receptor stimulation (Kalia et al. 2010). DCs also have important roles in activating T-cells through maturation, expression of costimulatory molecules like CD40 and CD86, and antigen presentation via MHC I or MHC II. Interestingly, amoxicillin has been shown to have the ability to drive DCs, from allergic patients, to become semi-mature and activate T-cells (Rodriguez-Pena et al. 2006). Thus, we also analysed the effect of amoxicillin on mouse DC maturation markers *ex vivo*.

2.2. AIMS

- To utilise $CD4^+$ T-cell depleted, MHC II KO mice to investigate the mechanisms of delayed-type hypersensitivity to the β-lactam amoxicillin.

2.3. METHODS

The initial methods described below were performed in Lyon, France where the successful sensitisation of mice to amoxicillin was first described and published (Rozieres et al. 2010) (Vocanson et al. 2006). Subsequent experiments were performed in Liverpool.

2.3.1. Mice

All mice used were between 8-20 weeks of age. Female wild type C57Bl/6 mice were ordered from Charles River (Kent, UK). MHC II KO mice were C57BL/6 mice with a mutation in the αβ gene encoding for MHC class II molecules were provided by C. Benoist and D. Mathis (Harvard Medical School, Boston, MA) for experiments performed in Lyon, France. Liverpool based experiments used C57Bl/6 mice with a mutation in the αβ gene encoding for MHC class II molecules (Charles River - Kent, UK). To deplete $CD4^+$ T-cells from mice, I.P. injections of rat anti-mouse CD4 antibody (GK 1.5 Bioxcell - UK) were administered on day 0 (100µg/mouse) and on day 7 (100µg/mouse) in 100µL PBS. Unless otherwise stated, for *ex vivo* readouts, mouse inguinal dLN cells were pooled from groups of two mice to generate one n-number.

2.3.2. Drugs and chemicals

The strong haptens 2, 4-Dinitro-1-flourobenzene (DNFB), 2, 4-dinitro-1-chlorobenzene (DNCB), 2, 4-dinitro- benzenesulphonic acid (DNBS) and the antibiotic amoxicillin were purchased from Sigma Aldrich (UK). Amoxicillin suitable for I.V. injection was also used for sensitisation experiments in Lyon (Clamoxyl- GlaxoSmithKline, France) and at Liverpool (Amoxil – GlaxoSmithKline, UK). Sulfamethoxazole (SMX) was purchased from Sigma (France).

2.3.3. Sensitisation and challenge

Mice were sensitised over days 3 days through daily painting with either DNFB (50µL of 2.5% solution) or amoxicillin (50µL of 1g/mL) in DMSO (Sigma, UK) on a shaved abdomen of approximately $3cm^2$ surface area. I.V. sensitisation was attempted through injection of 100µL of 10mg/mL amoxicillin via orbital venous injection in Hanks balanced salt solution. In certain experiments amoxicillin was administered orally (10µL of 200mg/mL) in Hanks balanced salt solution. All drug-exposed mice were compared against comparable vehicle exposed controls. Ear challenge was performed on day 8 and 10 with sensitiser/challenger (25µL of 0.5g/mL amoxicillin) (25µL 2.5% DNFB) on each side of one ear as well as SMX (0.25g/mL) to act as a specificity control. The contralateral ear was painted with equal amount of vehicle alone, with increase in swelling being measured through use of a pocket thickness gauge micrometer (Mitutoyo, Kyoto, Japan). Each mouse was treated as one n-number.

2.3.4. Cell isolation

For *ex vivo* readouts; draining lymph node (dLN), spleen, or liver was removed from mice on day 7 or 8. Immune cells were isolated from dLNs and spleens via maceration through 100µm nylon filter (BD Biosciences, UK) and then washed. Splenocytes were exposed to NH_4Cl to lyse blood cells. Livers were perfused with PBS prior to removal and then finely chopped and incubated for 30 minutes at 37°C with DNAse (1mg/mL) (Sigma, France) and collagenase (0.1mg/mL) (Sigma, France). The remaining suspension was passed through 100µm nylon filters and then suspended in 1:1 of 40%:80% Percoll (Sigma, France). After centrifugation, the middle layer of cells (lymphocytes) were collected and washed.

2.3.5. Cell culture medium

Immune cells were incubated in RPMI 1640 (Sigma, UK) supplemented with 10% foetal bovine serum (FBS) (Gibco, UK) L-glutamine (2mM), 2-mercaptoethanol (50µM), and unless otherwise stated, 1000U/mL penicillin and 0.1mg/mL streptomycin.

2.3.6. Generation and isolation of APCs

To generate bone marrow-derived dendritic cells (BMDDCs) the bone marrow was flushed from the femurs of mice with complete culture medium and large pieces of bone were filtered out with a 100µm nylon filter. Cells were incubated with 0.1mg/mL GMCSF (Peprotech, NJ, USA) for 8 days after which immature DCs were harvested. To mature the DCs, LPS (0.5µg/mL) was added to the cultures for 24h before harvesting.

To isolate CD11c positive APCs, the mesenteric LNs were removed from mice and disaggregated into a single cell solution through ~1h incubation with 5mg/mL collagenase D (Sigma, UK). Cells were then washed with Hanks balanced salt solution and incubated with $CD11c^+$ magnetic beads (Miltenyi Biotec – UK) before being run through a magnetic column (Miltenyi Biotec – UK) and then washed, counted, and finally used.

2.3.7. Flow cytometric analysis of bone marrow-derived dendritic cell maturation markers

Bone marrow-derived DCs were generated as previously described and on day 8, were incubated with amoxicillin (0.25mg/mL) or LPS (0.5µg/mL) in petri dishes at $3x10^6$ cells/dish in 10mLs. Medium alone was used as a positive control. After 72h, the surface phenotype of the cells was analyzed using the following antibodies: CD40 (FITC), CD86 (FITC), Class II (PE) (BD Pharminogen, UK), and CD11c (PE -Cy5.5) (Caltag laboratories, UK). Antibodies were diluted with FACS buffer (PBS, 10%FCS, 0.02% sodium azide (Sigma, UK)) and 0.5µL of each

antibody was added to around 500,000 cells. Cells were then incubated on ice for 20 minutes and washed with 1.5mLs buffer prior to analysis on a FACScalibur flow cytometer using CellQuestPro.

2.3.8. Analysis of antigen-specific proliferative responses *ex vivo*

Unless otherwise stated, LNs were pooled from groups of 2 mice to give one n-number. Draining LN (inguinal) and spleen cells were plated out in triplicate ($0.5x10^6$ cells/well) with APCs ($5x10^4$ cells/well) in the presence or absence of antigen and cultured for 5 days at 37°C and 5% CO_2. Proliferation was measured through addition of [^{3}H] thymidine (0.25µCi/well) (Moravek -California, USA) for 16h, and assessment of incorporation into newly synthesised DNA. Plates were harvested and read as cpm per well.

2.3.9. Analysis of antigen-specific IFNγ secretion *ex vivo*

Cell supernatants were removed after dLN cells ($0.5x10^6$ cells/well) and APCs ($2.5x10^4$ APCs ([either $CD11c^+$ cells from non-dLN or BMDDCs]/well) were incubated for 5 days at 37°C and 5% CO_2 with amoxicillin (0, 0.1, 0.25, 0.5, 1mg/mL). Supernatant was frozen at -20°C prior to analysis of cytokine secretion using a mouse IFNγ ELISA kit (R&D systems, UK). ELISA 96 well micro-plates (Mabtech – UK) were firstly coated with 100µL of 4mg/mL capture antibody (rat anti-mouse IFNγ antibody) in PBS, sealed and incubated overnight at 4°C. Plates were then washed with wash buffer (0.05% TWEEN*20 (Sigma- UK)) via wash bottle three times, adding 300µL wash buffer each time. Block buffer (1% BSA (Sigma-UK) in PBS) was then added at 250µL/well and plate incubated at room temperature for 1h, after which plates were washed again. Samples were then added at 50µL neat supernatant and 50µL reagent diluent (0.1% BSA, 0.05% TWEEN*20 in Tris-buffered saline (Sigma- UK)) in duplicate. Standard was added from 2000pg/mL mouse IFNγ down 7 halving concentrations to zero in

reagent diluent. Plates were incubated for 2h at room temperature and then washed. Detection antibody (600ng/mL biotinylated goat anti-mouse IFNγ antibody) was added at 100µL/well in reagent diluent and plates incubated for 2h at room temperature. Plates were then washed and Streptavidin HRP (200x diluted kit stock) was added at 100µL in reagent diluent and plates incubated for 40 minutes at room temperature. Plates were then washed and substrate solution (tetramethylbenzidine) was added at 100µL and plates were incubated for ~20 minutes. After colour change in wells containing standard were clearly visible, 50µL of 1M H_2SO_4 was added, and optical density was determined in a microplate reader set to 450nm. Results were determined using a 4-parameter logistic standard curve plotted on SigmaPlot software.

The number of IFNγ secreting cells per dLN/spleen was determined through IFNγ ELISpot where $0.5x10^6$ cells/well and bone marrow-derived dendritic ($2x10^4$cells/well) were incubated for 48h at 37°C with 5% CO_2. ELISpot plates were processed for IFNγ secreting cells according to the kit instructions (BD Biosciences mouse IFNγ ELISPOT – France). Multiscreen filter plates were coated with IFNγ antibody (15µg/mL) overnight at 4˚C. Wells were washed with PBS (200µl) and blocked for 30 minutes with medium (200µl). Cells from the dLNs of painted mice were added to the plate at $0.25x10^6$cells/well along with matured BMDDCs. Amoxicillin (0.5mg/mL) or SMX (50µg/mL) was added to wells and after 48h, the plate was developed. The wells were washed with PBS and incubated with secondary antibody (diluted 1:1000 in PBS containing 0.5% FBS) for 2h at room temperature. After washing with PBS, Streptavidin-ALP (diluted 1:1000 in PBS containing 0.5% FBS) was added to wells for one hour. Spots were visualised by the addition of BCIP/NBT substrate (100µl, 15 min). The plate was counted on an AID ELISpot reader (Cadama Medical, Stourbridge, UK) when thoroughly dried.

2.3.10. Flow cytometric analysis of splenocytes and lymph node $CD4^+$ and $CD8^+$ T-cells

Splenocytes and mesenteric, auxiliary, and auricular LNs were stained with CD4 (FITC) CD8 (PE) (BD Pharminogen, UK) antibodies (2µL/ sample) for 15 minutes before being washed and analysed on a FACScalibur flow cytometer (BD Bioscience, UK). Cells were initially gated on the FSC to remove cell debris. Samples were analysed using CellQuestPro and analyzed with CyflogicTM software (CyFlo Ltd, Finland).

2.3.11. Flow cytometric analysis of $CD8^+$ T-cell activation markers after sensitisation with amoxicillin

Activation of $CD8^+$ T-cells was analysed in dLN, spleen, and liver of CD4-depleted, amoxicillin-sensitised mice. After lymphocyte isolation, cells were stained with anti-CD8 (PE Cy7), CD4 (APC), CD25 (PE), and ICOS (PE Cy5.5) antibodies (BD Pharminogen, France). Cells were then washed and analysed on a BD LSR II for $CD8^+$ T-cell activation after gating on the FSC to remove cell debris.

2.3.12. Statistics

Unless otherwise stated, statistics were performed on SigmaPlot with significance being measured using the students T-test. Error bars display the standard deviation from the mean.

2.4. RESULTS

2.4.1. Phenotyping of BMDDCs following amoxicillin incubation

To investigate if amoxicillin could induce DC maturation in mice as had been reported using human DCs from allergic patients (Rodriguez-Pena et al. 2006), mouse BMDDCs were generated and cultured with no drug, amoxicillin, or the positive control LPS. Results indicated that culture with amoxicillin for 72h did not alter the phenotype of BMDDCs against cells cultured in medium alone with no increases in the co-stimulatory molecules CD40 or CD86 or mature DC marker CD11c (Fig 2.1.). A small shift in MHC II molecules was detectable in amoxicillin-cultured BMDDCs compared against untreated control however the biological relevance of this small shift is open to question. The positive control LPS induced increased expression of CD40, CD86, and CD11c with a shift in expression of MHC II molecules when compared against untreated control and amoxicillin cultured cells (Fig 2.1.).

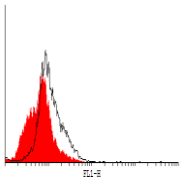
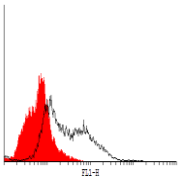
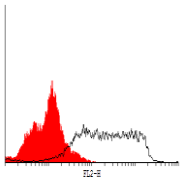
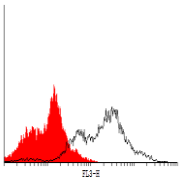
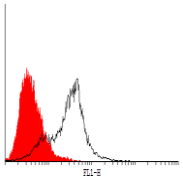
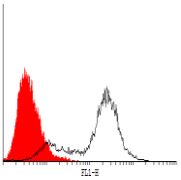
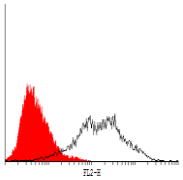
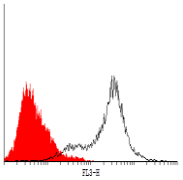
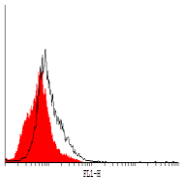
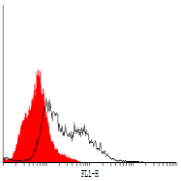
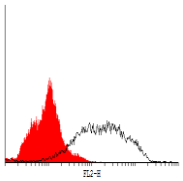
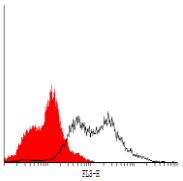
Fluorescent antibody	CD40 (FITC)	CD86 (FITC)	MHC CLASSII (PE)	CD11c (PE-CY 5.5)
Condition	ΠΠ= Un-labelled ΠΠ = Fluorescent antibody labelled			
Untreated				
LPS(0.5µg/mL)				
Amoxicillin (0.25mg/mL)				

Fig 2.1. Amoxicillin does not alter BMDDCs phenotype or induce maturation. BMDDCs were generated through culturing wild-type C57/Bl6 mouse bone marrow with GMCSF (0.1mg/mL) for 8 days. On day 9, cells were further incubated with no drug, amoxicillin (0.25mg/mL) or LPS (0.5µg/mL) for 72 hours before cells were harvested, marked with fluorescent antibodies CD40, CD86, MHC II and CD11c, and analysed for marker expression by flow cytometry. Red histograms indicate un-labelled cells with black line histograms representing labelled cells.

2.4.2. *In vivo* depletion of $CD4^+$ T-cells from MHC II KO mice

To investigate the efficiency of using a CD4 antibody to deplete $CD4^+$ T-cells *in vivo*, I.P. injections of anti-CD4 antibody (100µg injection on day 0 and 7) were administered. Flow cytometric analysis of spleen and lymph node of MHC II KO mice demonstrated effective depletion of residual $CD4^+$ T-cells from the MHC II KO mouse. Similar results were detected

following flow cytometric analysis of lymph node and spleen (Fig 2.2.). Wild type mice showed clear populations of $CD4^+$ and $CD8^+$ T-cells while MHC II KO mice have visibly decreased $CD4^+$ T-cells. MHC II KO mice which received anti-$CD4^+$ T-cell antibody injections expressed no detectable $CD4^+$ T-cells in either lymph node or spleen. Populations of $CD8^+$ T-cells remain constant and apparently un-altered by antibody treatment (Fig 2.2.).

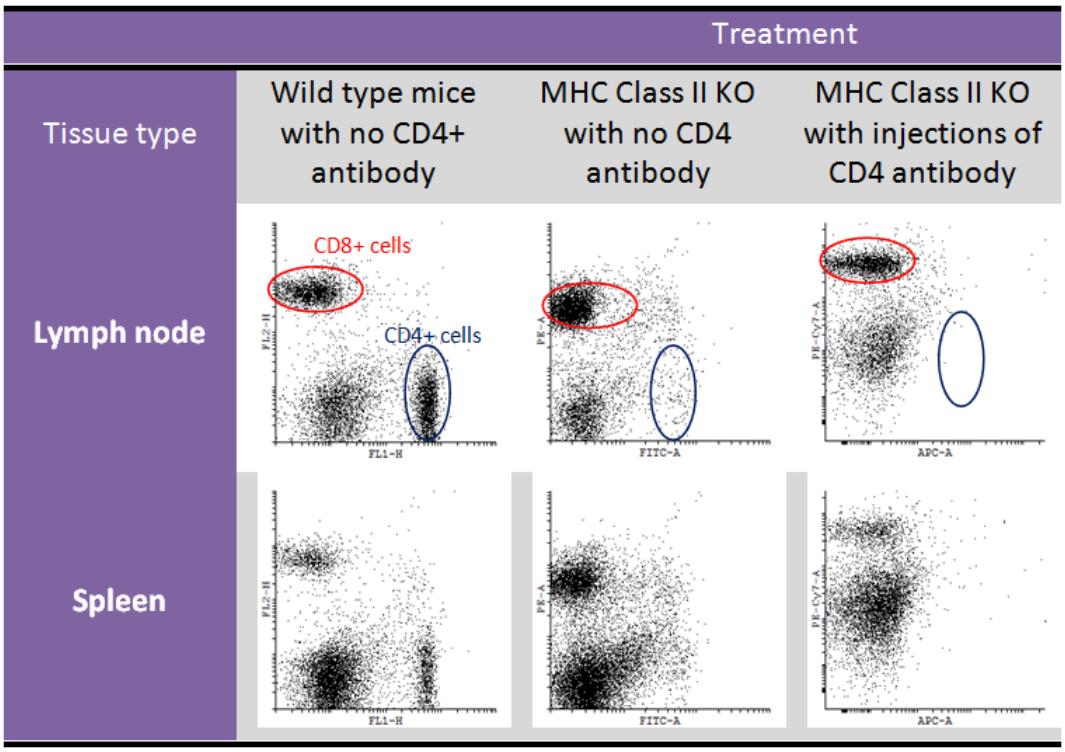


Fig 2.2. Flow cytometry of mouse lymph nodes and spleen showing full depletion of $CD4^+$ T-cells in MHC II KO mice administered I.P. anti-CD4 antibody injections. Wild type and MHC II KO were on a C57/Bl6 background. I.P. injections of CD4 antibody were given to mice on day 0 and 7 with tissues being taken on day 8. Cells were dual stained with CD8 (PE) and CD4 (FITC) fluorescent antibodies.

2.4.3. Assessment of the T-cell response to dinitroflourobenzene

In order to establish *in* and *ex vivo* readouts, MHC II KO mice were painted with DNFB and then analysed for increases in ear thickness after a hapten-specific ear challenge (Fig 2.3.) or via draining lymph node T-cell activation measured via proliferation and IFNγ ELISA (Fig 2.4.). In both instances large, hapten-specific increases were observed. *In vivo* challenge

showed an increase in ear thickness at days 1, 2, and 3 days after the challenge. *Ex vivo* challenge showed hapten-specific increases in proliferation and IFNγ secretion. DNFB was used *in vivo* due to its superior sensitising ability (Friend and Lane 1973). DNBS was used in the *in vitro* assays. This was done because DNFB and DNBS are known to create the same haptenic molecules however DNBS is significantly less toxic to cells in culture compared to DNFB (Akiba et al. 2004).

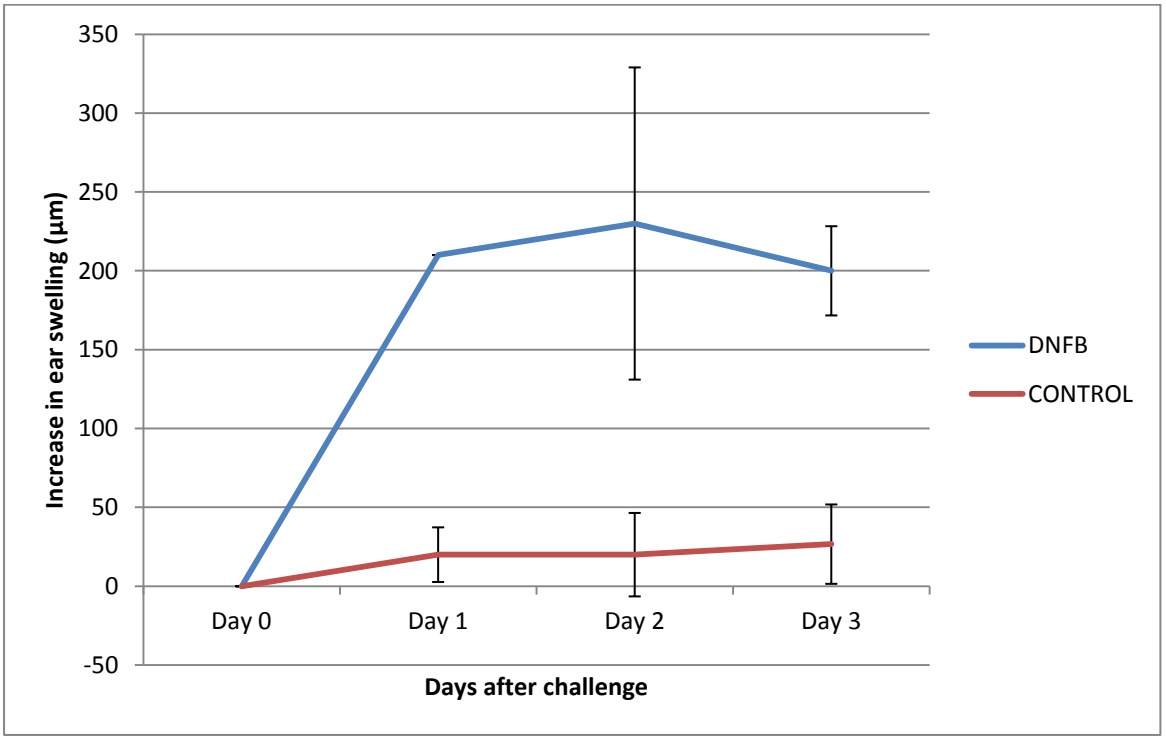


Fig 2.3. Increase in ear thickness after DNFB ear challenge. MHC II KO mice were depleted of $CD4^+$ T-cells and painted with DNFB or vehicle control for three days. Five days later, mice were challenged on one ear with DNFB and with vehicle control on contralateral ear. Results were measured for 3 days after challenge and as increases in hapten challenged ear minus increases in vehicle challenged (Control n=3, DNFB n=2).

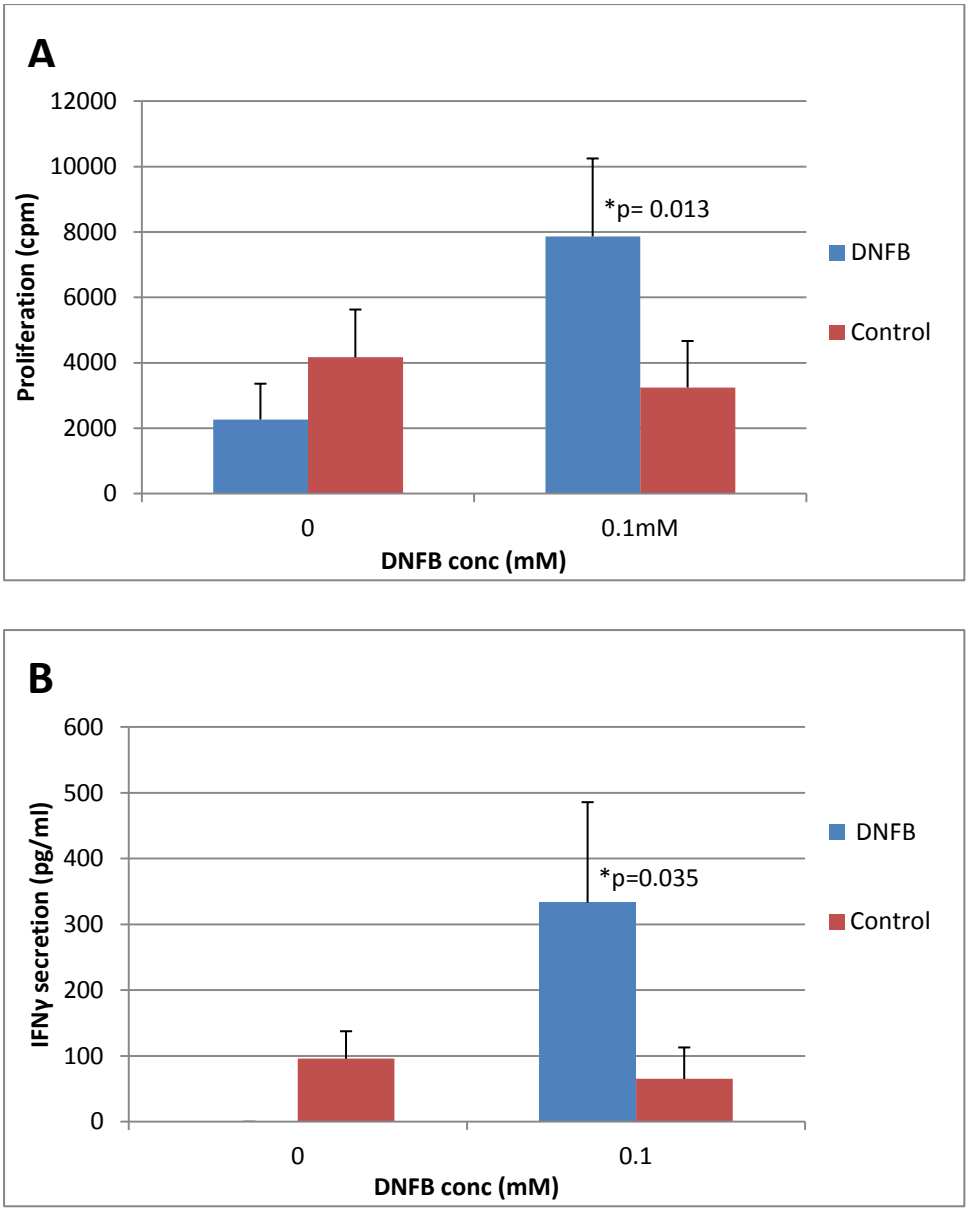


Fig 2.4. Significant antigen-specific increases in proliferation (A) and IFNγ secretion (B) from mouse dLN cells after painting of DNFB onto a shaved abdomen compared to vehicle only painted controls. Mice were painted with DNFB (2.5%) or vehicle only (70% DMSO) on days 1-3. Draining auxiliary LNs were taken on day 8, macerated to a single cell suspension and added at $0.5x10^6$ cells/well with $5x10^4$ BMDDCs in the presence or absence of 0.1mM DNBS. After 5 days incubation, proliferation was measured via [^{3}H] thymidine incorporation and IFNγ content of cell supernatant was measured via ELISA (n=3).

2.4.4. Development of a mouse model of delayed, T-cell-mediated skin sensitisation to amoxicillin

Initial attempts to recreate the mouse model developed by Vocanson et al (2009) using MHC II KO mice depleted of $CD4^+$ T-cells were unsuccessful. Analysis was performed using three readouts; 1. Ear swelling after challenge showed no significant differences in the drug-treated compared to the respective control (Fig 2.5.) 2. Drug-specific T-cell proliferation was not detected (Fig 2.6. A) 3. Furthermore, analysis of IFNγ secretion following amoxicillin treatment *ex vivo* showed no drug-specific increases (Fig 2.6. B).

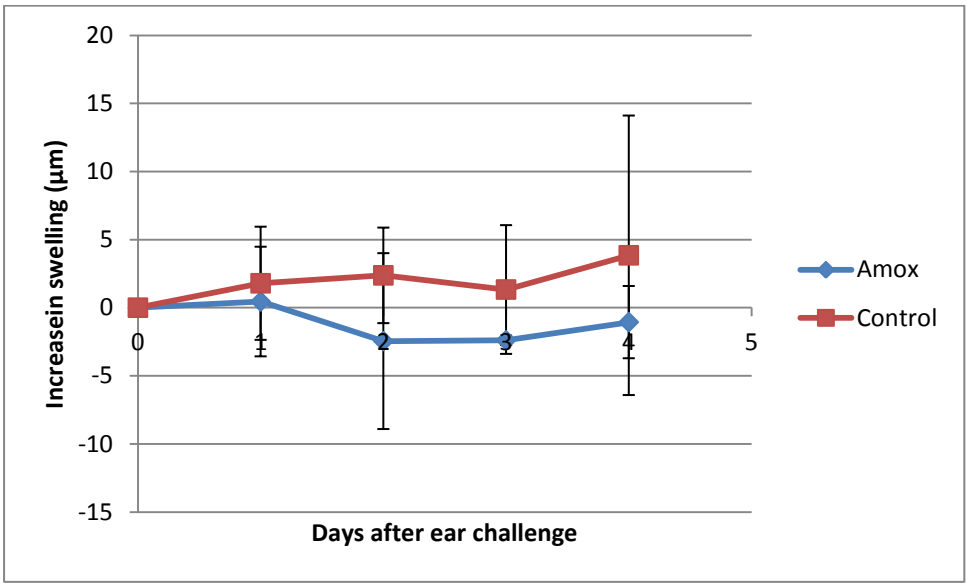


Fig 2.5. Ear swelling challenge of amoxicillin-painted mice showing no increases in swelling compared to vehicle controls. MHC II KO mice were depleted of $CD4^+$ T-cells on day 0 and painted with 50µL of 0.5g/mL amoxicillin or vehicle control for days 1-3 with swelling measured after a challenge on the ear (25ul of 0.5g/mL amoxicillin) on day 8. Ear swelling represents increase in swelling compared to vehicle only challenged ear (n=3). Statistics are shown if $p<0.1$.

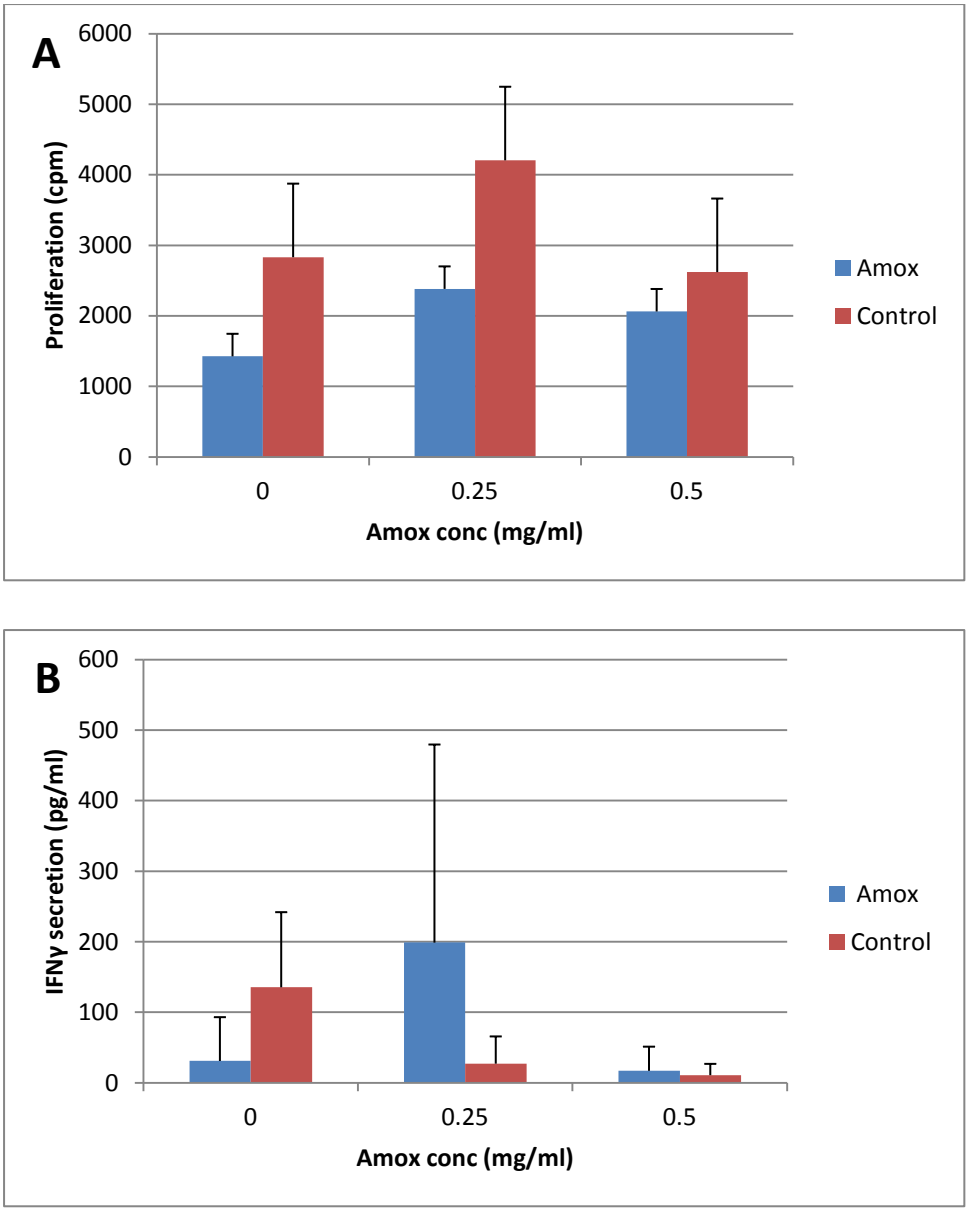


Fig 2.6. Proliferation (A) and supernatant IFNγ content (B) of amoxicillin-painted mouse dLN cells incubated with amoxicillin. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injections of CD4 antibody (100µg) on day 0 and painted with 50µL of 0.5g/mL amoxicillin or vehicle control for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated in triplicate at $0.5x10^6$ cells/well with $5x10^4$ BMDDCs in RPMI medium containing pen/strep for 5 days after which proliferation was measured via [^{3}H] thymidine incorporation and IFNγ content of supernatant was measured via ELISA. (n=3).

2.4.5. Amoxicillin-modified, LPS matured BMDDCs cause non-specific activation in co-cultured mouse draining lymph node cells

As the initial experimental conditions were un-suitable for sensitising mice to amoxicillin, the methods were altered so as to more closely mirror a clinical sensitisation to amoxicillin where human patients are prescribed amoxicillin due to a bacterial infection. The amoxicillin used in the assays was changed to an I.V. grade drug and administered at 50µL of 0.5g/mL. The APCs in the *ex vivo* assay were matured with LPS before incubation with the dLN cells. However, ear swelling analysis after amoxicillin sensitisation and challenge showed no significant increases when compared with vehicle controls (Fig 2.7.). Furthermore, maturation of the BMDDCs with LPS just served to increase the background proliferation and IFNγ release (Fig 2.8.).

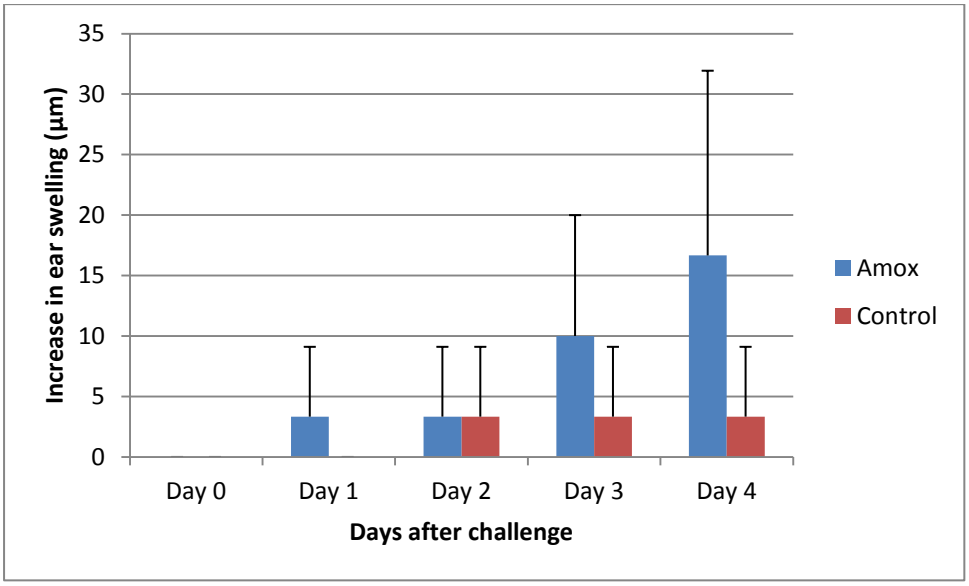
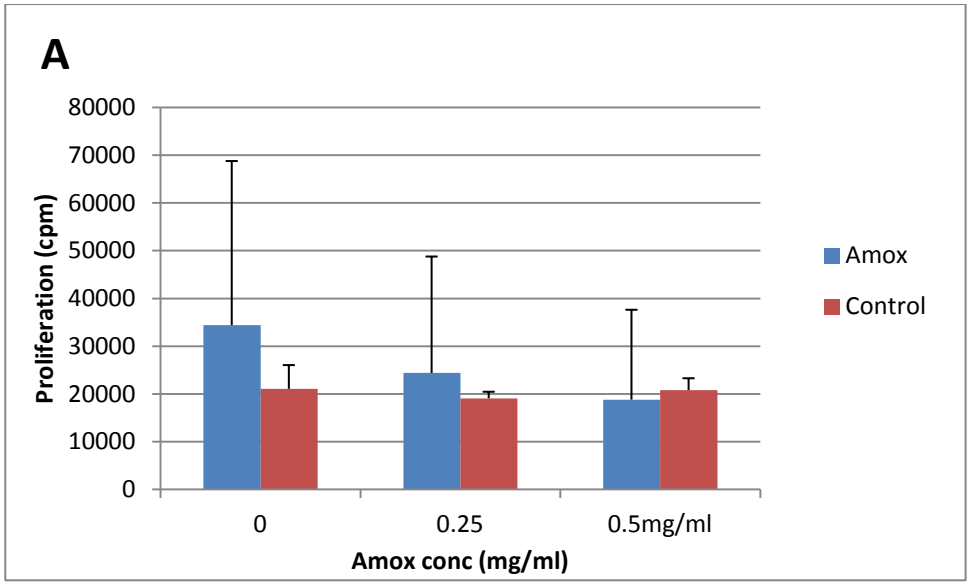


Fig 2.7. Amoxicillin I.V. grade painting and challenge showing no increase in ear swelling. MHC II KO mice were depleted of $CD4^+$ T-cells on day 0 and painted with 50µL of 0.5g/mL I.V. amoxicillin or vehicle control for days 1-3 with swelling measured after a challenge on the ear (25ul of 0.5g/mL amoxicillin) on day 8. Ear swelling represents increase in swelling compared to vehicle only challenged ear plus standard deviation from the mean (n=3). Statistics are shown if $p<0.1$.



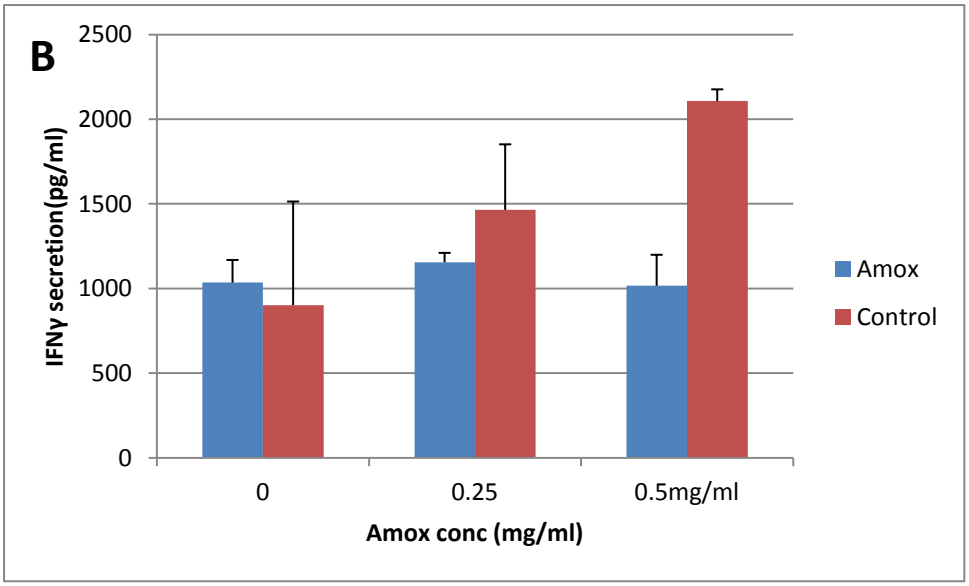


Fig 2.8. Proliferation (A) and IFNγ content of supernatant (B) from I.V. amoxicillin-painted mouse dLN cells incubated with matured BMDDCs. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injections of anti-CD4 antibody (100µg) on day 0 and painted with 50µL of 0.5g/mL I.V. amoxicillin or vehicle control for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated in triplicate at $0.5x10^6$ cells/well with $5x10^4$ matured BMDDCs in RPMI medium containing pen/strep for 5 days after which proliferation was measured via [^{3}H] thymidine incorporation and IFNγ content of supernatant was measured via ELISA. Figures show mean +/- one standard deviation (n=3). Statistics are shown if $p<0.1$.

2.4.6. Optimisation of amoxicillin sensitisation protocol

To rectify the problems encountered in sensitising $CD4^+$ T-cell depleted mice to amoxicillin, a collaboration was established with the creators of the model in Lyon where their

experimental protocols could be observed and documented. These experiments used the same MHC II KO mouse with depletion of $CD4^+$ T-cells using antibodies; however, the Lyon protocol used an increased initial sensitising dose of amoxicillin (50µL 0.5g/mL to 50µL of 1g/mL) and a second ear challenge on day 9. These alterations were sufficient to show an increase in ear thickness in mice painted and challenged with amoxicillin against all controls (Fig 2.9.), thereby sensitising mice to amoxicillin. *Ex vivo* readouts from the dLN and spleens of amoxicillin-painted mice were also increased. IFNγ ELISpot analysing the number of IFNγ secreting cells in the dLNs and spleens of amoxicillin-painted mice were significantly increased against sensitisation-specific controls of amoxicillin (Fig 2.10.). Flow cytometric analysis of the activated $CD8^+$ T-cells in the dLNs of amoxicillin-painted mice revealed an increase in the number of activated T-cells when amoxicillin and control mice were compared (Fig 2.11.).

Other than sensitisation via skin, sensitisation through oral dosing and I.V. injection was investigated. Intra-venous dosing of amoxicillin was performed at 100µL of 10mg/mL amoxicillin and ear challenge yielded significant increases in ear swelling against all respective controls on days 2, 3, and 4 after first ear challenge (Fig 2.9.). *Ex vivo* readouts for I.V. sensitised mice were not as clear cut as for painted mice with no distinct increases in IFNγ secreting cells or amount of activated $CD8^+$ T-cells in the dLNs or spleens. Although no $CD8^+$ T-cells were detectable in the livers of control mice, I.V. administration of amoxicillin caused a consistent increase in the amount of activated $CD8^+$ T-cells in the livers of these mice, when compared against skin painted or orally dosed mice (Fig 2.11.). Mice orally dosed with amoxicillin showed no significant increases in ear swelling after ear challenge (Fig 2.9.) or in *ex vivo* readouts (Fig 2.10.) (Fig 2.11.), showing that oral dosing, is the least efficient route of administration if attempting to induce sensitisation.

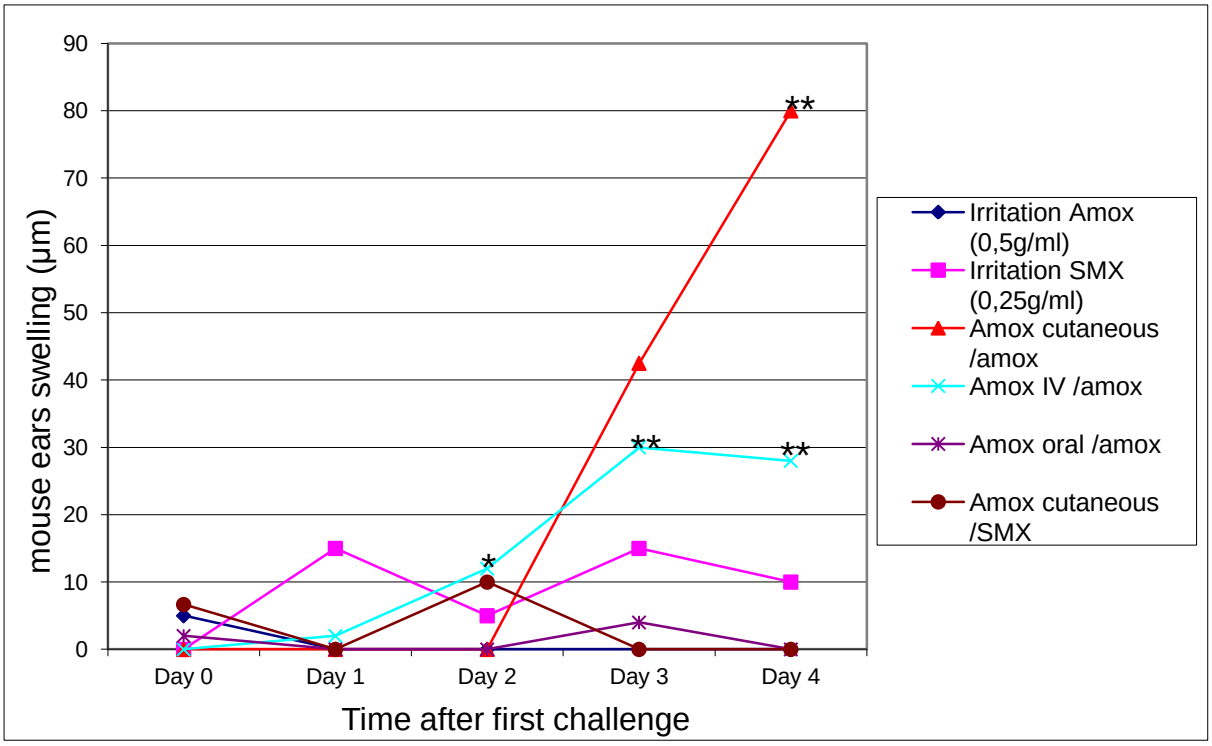


Fig 2.9. Amoxicillin sensitisation and challenge showing significant increases in ear swelling in mice administered amoxicillin I.V. or cutaneously. Mice were depleted of $CD4^+$ T-cells on days 0 and 6 with 100µg and 20µg I.P. injection respectively. Sensitisation was performed on days 1-3 with cutaneous amoxicillin-painted onto a shaved abdomen at 50µL of 1g/mL, oral amoxicillin at 10µL of 200mg/mL and I.V. amoxicillin at 100µL of 10mg/mL via orbital injection. Ears were challenged with 12.5µL on each side of the ear of 0.5g/mL amoxicillin or with 0.25g/mL SMX in 70% DMSO on day 7 and 9. Mice were painted with drug on their left ear and with vehicle only on the contra-lateral ear. Values represent increases in swelling from drug challenged ear minus vehicle challenged ear. Statistical analysis compare amoxicillin-specific swelling against amox cutaneous/SMX (each n-number is indicative of one mouse)(irritation amox and irritation SMX n=2, amox cutaneous/amox and amox cutaneous/SMX n=4, amox IV/amox and amox oral/amox n=5) (*$p<0.01$, **$p<0.001$).

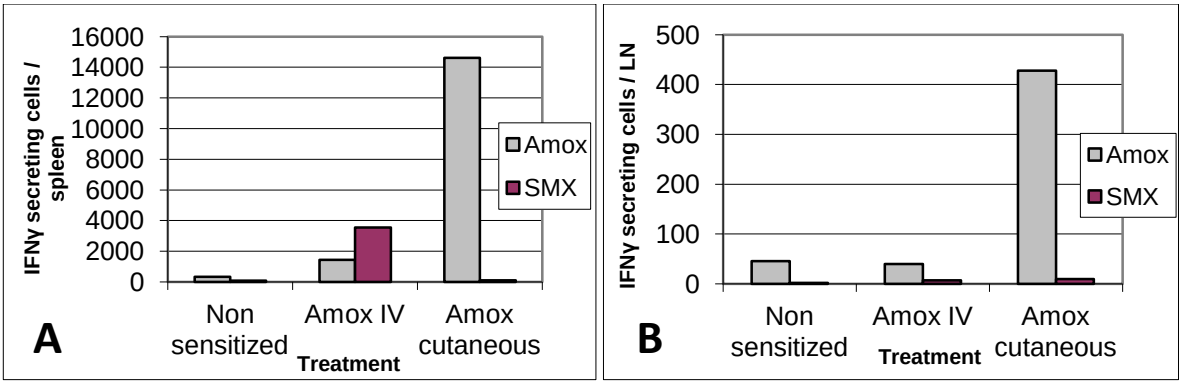
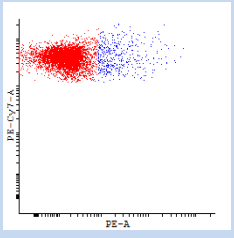
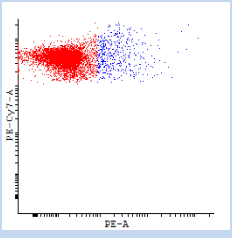
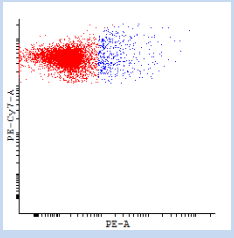
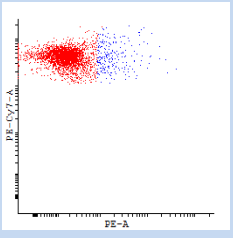
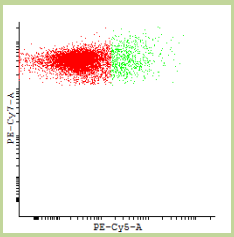
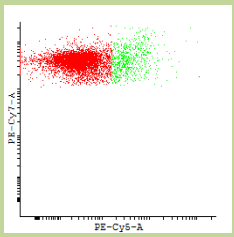
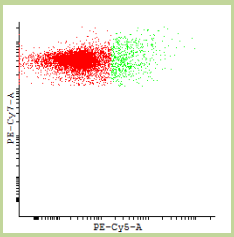
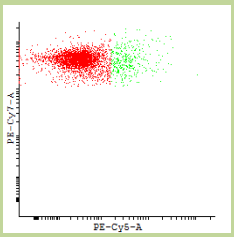
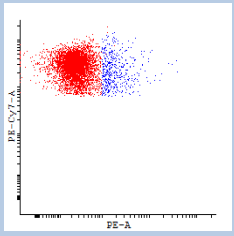
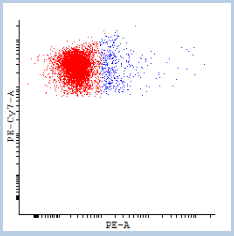
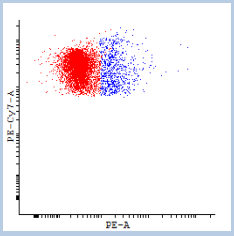
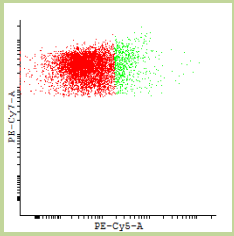
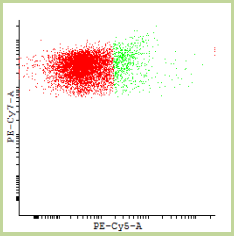
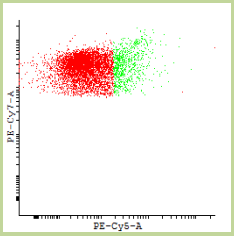
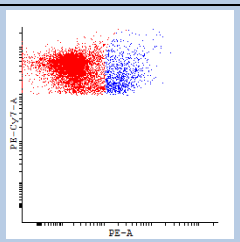
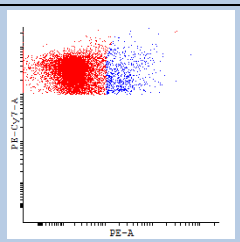
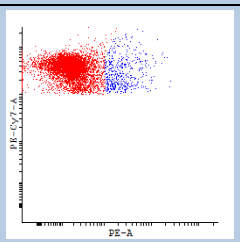
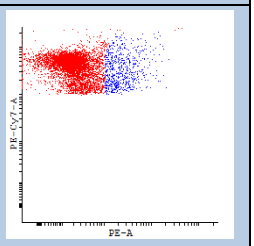


Fig 2.10.IFNγ secreting cells of the spleen (A) and dLN (B) of amoxicillin-sensitised mice. Mice were depleted of $CD4^+$ T-cells on days 0 and 6 with 100µg and 20µg I.P. injection respectively. Sensitisation was performed on days 1-3 with cutaneous amoxicillin-painted onto a shaved abdomen at 50µL of 1g/mL and I.V. amoxicillin at 100µL of 10mg/mL via orbital injection. Spleens and dLNs were collected on day 7, macerated to a single cell suspension through 100µm nylon filter and subjected to a red blood cell lysis. Cells were counted and incubated with no drug, amoxicillin (0.5mg/mL) or SMX (50µg/mL) at $5x10^5$ T-cells/well with $2x10^4$ BMDDCs/well naive for 36 hours at 37°C with 5% CO_2 in ELISpot plates which were analysed for IFNγ secreting cells according to the kit instructions. Cell counts are presented as number of IFNγ secreting cells in whole tissue (cells were pooled from three mice per condition, n=1).

		Amoxicillin route of administration			
	Gating	**None**	**Cutaneous**	**Oral**	**I.V.**
% of $CD25^+$ $CD8^+$ cells	$CD8^+$ / $CD25^+$/ $Icos^+$	6.42%	7.24%	6.57%	6.46%
Lymph Node $CD25^+$, $CD8^+$ cells (blue)	$CD8^+$ / $CD25^+$				
% of $Icos^+$, $CD8^+$ cells		9.58%	10.59%	9.1%	10.33%

Lymph node Icos$^+$, CD8$^+$ cells (green)	CD8$^+$ / Icos$^+$				
% of CD25$^+$ CD8$^+$ cells			7.56 %	6.5%	13.19%
Liver CD25$^+$, CD8$^+$ cells (blue)	CD8$^+$ / CD25$^+$	No CD8$^+$ cells			
% of Icos$^+$, CD8$^+$ cells			10.01%	9.04%	13.27%
Liver Icos$^+$, CD8$^+$ cells (green)	CD8$^+$ / Icos$^+$	No CD8$^+$ cells			
% of CD25$^+$ CD8$^+$ cells		10.95%	7.37%	6.81%	9.68%
Spleen CD25$^+$, CD8$^+$ cells (blue)	CD8$^+$ / CD25$^+$				
% of Icos$^+$, CD8$^+$ cells		12.04%	8.1%	7.17%	10.76%

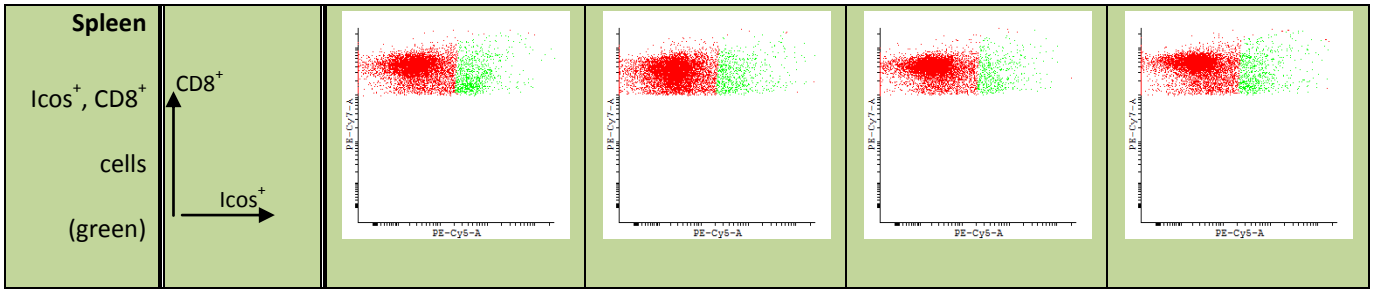


Fig 2.11. Flow cytometric analysis of amount and activation status of, amoxicillin-painted mouse dLN, spleen, and liver $CD8^+$ T-cells. Mice were depleted of $CD4^+$ T-cells on days 0 and 6 with 100µg and 20µg I.P. injection respectively. Sensitisation was performed on days 1-3 with cutaneous amoxicillin-painted onto a shaved abdomen at 50µL of 1g/mL, oral amoxicillin at 10µL of 200mg/mL, and I.V. amoxicillin at 100µL of 10mg/mL via orbital injection. Spleens and dLNs were collected on day 7, macerated to a single cell suspension through 100µm nylon filter and subjected to a red blood cell lysis. Livers were perfused with PBS, cut up finely and incubated for 30 minutes at 37°C with DNAse (1mg/mL) and collagenase (0.1mg/mL). Liver tissue was then macerated into a single cell suspension through 100µm nylon filters, diluted in PBS, washed and resuspended in 1:1 of 40%: 80% Percoll. After centrifugation, the middle layer of cells (lymphocytes) were collected, washed and counted. Cells were labelled with flourochromes CD8 (PE Cy7) CD4 (APC), CD25 (PE) and ICOS (PE Cy5.5) (BD Pharminogen) and were analysed on a BD – LSR II (cells were pooled from three mice per condition n=1)

2.4.7. Successful sensitisation to amoxicillin using knowledge gained from previous failed experiments

The main protocol differences between the Lyon experiments and previous earlier attempts in Liverpool was the increased concentration of painted amoxicillin from 0.5g/mL to 1g/mL and the removal of penicillin from the *in vitro* cell culture medium. The next step therefore was to repeat the experiment in Liverpool with these modifications. Fig 2.12. shows *in vivo* sensitisation of CD4 depleted mice with 1g/mL amoxicillin. A significant increase in ear swelling was observed when amoxicillin treated mice were compared against vehicle controls, after challenge with amoxicillin.

In the previous analysis of T-cell proliferative responses and cytokine release, problems had been encountered when using BMDDCs with large background readings with both readouts. Thus, the *ex vivo* assays were altered to use magnetically separated CD11c positive cells as

APCs from the non-draining lymph nodes of experimental mice. This had the added advantage of reducing the number of mice used in each experiment. Cells from the dLN of amoxicillin-painted mice showing significant increases in proliferation when co-cultured with APCs and amoxicillin at 0.25, 0.5 and 1mg/mL for 5 days (Fig 2.13. A). Significant increases in IFNγ were also detectable by IFNγ ELISA from the dLN cells of amoxicillin-painted mice when cultured with amoxicillin at 0.25 and 0.5mg/mL (Fig 2.13. C). Spleen cells from amoxicillin-painted mice also showed increased drug-specific proliferation however only at the highest drug concentration of 1mg/mL (Fig 2.13. B). Interferon-γ secretion by splenocytes from amoxicillin-sensitised mice was not detectable (results not shown) indicating that spleen cells are not activated to the same extent as dLN cells.

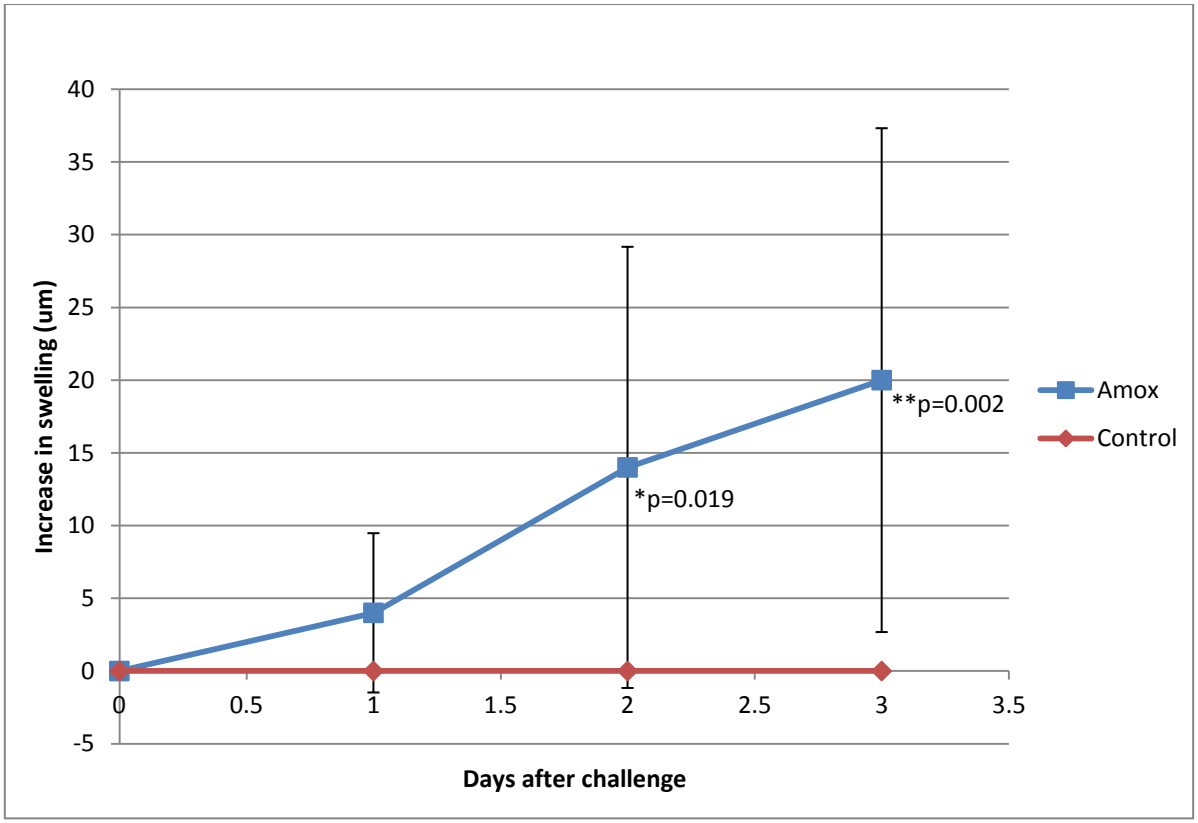


Fig 2.12. Amoxicillin induced ear swelling. MHC II KO mice were depleted of $CD4^+$ T-cells on day 0 and painted with 50µL of 1g/mL I.V. amoxicillin or vehicle control for days 1-3 with swelling measured after a challenge on the ear (25ul of 1g/mL amoxicillin) on day 8. Values represent increase in swelling compared to vehicle only challenged ear (amox n=5, control n=8) (*p<0.0.5, **p<0.005).

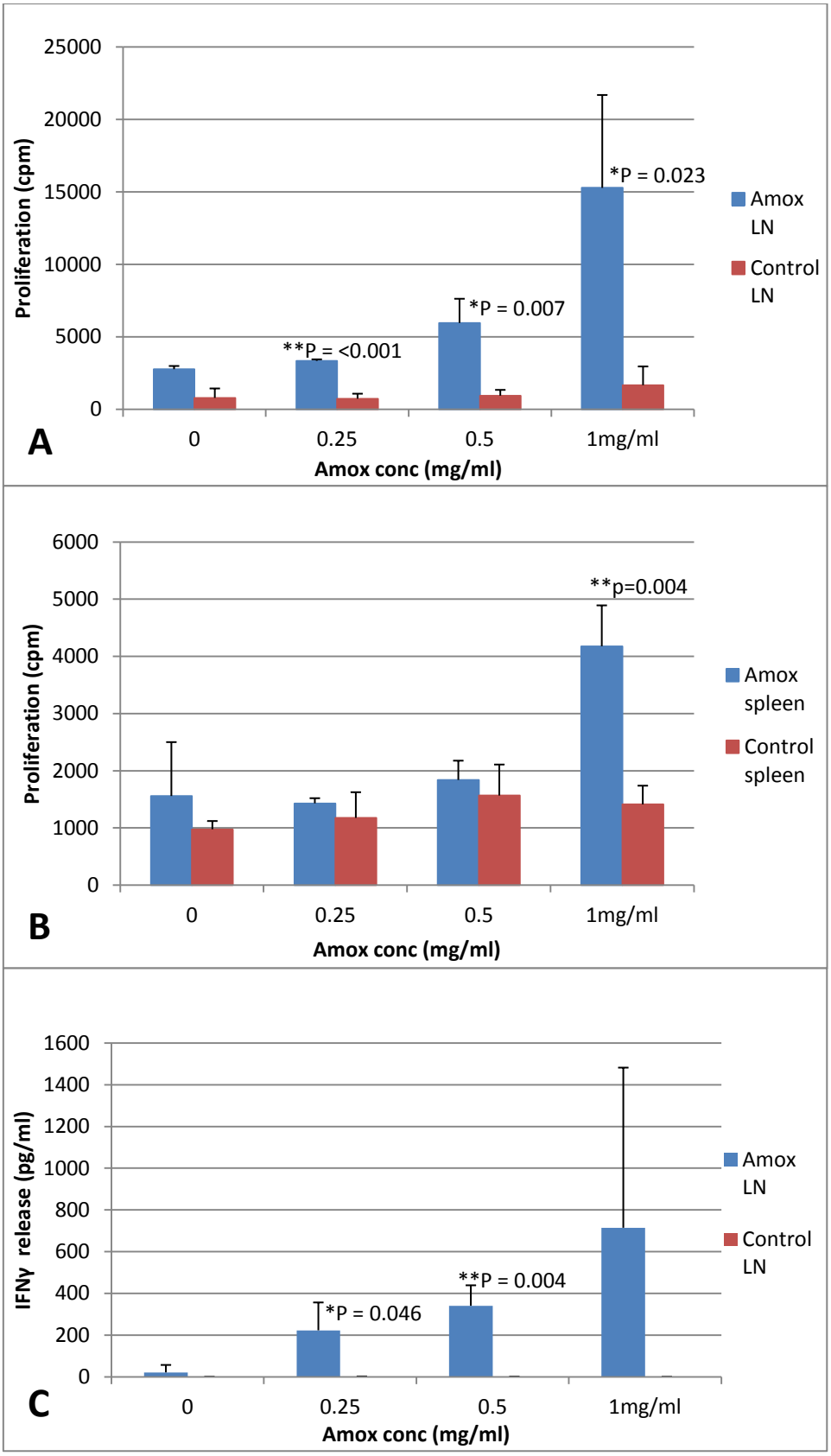


Fig 2.13. Significant, dose-dependent increases in proliferation in dLN (A) and spleen cells (B) with accompanied significant IFNγ release from the dLN of amoxicillin-sensitised mice. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injections of CD4 antibody (100µg) on day 0 and 7. Mice were painted with 50µL of 1g/mL I.V.

amoxicillin or vehicle control for days 1-3. On day 8 the dLNs and spleens were removed, macerated to a single cell suspension and incubated in triplicate at $0.5x10^6$ cells/well with $2.5x10^4$ APCs collected from the mesenteric LN through CD11c positive magnetic isolation. Cells were incubated in RPMI medium containing no pen/strep for 5 days with amoxicillin after which proliferation was measured via [^{3}H] thymidine incorporation after 16h and IFNγ content of supernatant was measured via IFNγ ELISA. (n=3) (*$p<0.05$, **$p<0.005$).

2.5. DISCUSSION

Beta-lactam hypersensitivity is a well-documented clinical problem. Reactions involve the drug-specific activation of patient T-cells, which secrete cytokines and cytolytic molecules that initiate tissue injury. The intrinsic reactivity of the β-lactam ring structure is critical for drug binding to the pharmacological target – membrane and cytoplasmic bacterial penicillin-binding proteins involved in peptidoglycan synthesis. The drug forms an irreversible bond with the protein targets, inhibiting peptidoglycan synthesis. Unfortunately, the β-lactam antibiotics also bind to human proteins generating drug-hapten-protein adducts that at least have the potential to act as antigens and activate an unwanted immune response. Using mass spectrometry, such adducts have been detected in 100% of patients exposed to β-lactam antibiotics including piperacillin (Whitaker et al. 2011), flucloxacillin (Jenkins et al. 2009), and amoxicillin (Jenkins unpublished data). Thus, although the formation of drug-protein adducts is implicated in β-lactam hypersensitivity, it is clearly not the primary factor that determines whether drug exposure will be associated with the development of a hypersensitivity reaction.

The nature of the drug-specific T-cell response in patients with amoxicillin hypersensitivity has been studied in detail with T-cells isolated from the blood of hypersensitive patients showing high T-cell activation upon *ex vivo* drug re-stimulation (Rozieres et al. 2009). $CD4^+$ and $CD8^+$ T-cells from hypersensitive patients have been shown to proliferate and secrete

IFNγ and IL-5 (Rozieres et al. 2009). Most recently, amoxicillin has been shown to regulate dendritic cell toll-like receptor expression and activity selectively in patients with hypersensitivity (Sanchez-Quintero et al. 2013).

Patient studies provide information on the nature of the drug-specific T-cell response, but it is very difficult to study the factors involved in T-cell priming and/or the mechanisms that regulate the primary immune response. Hence, Vocanson et al (2009)(Vocanson et al. 2006)(Vocanson et al. 2006)(Vocanson et al. 2006)(Vocanson et al. 2006) developed a mouse model devoid of $CD4^+$ T-cells, capable of becoming sensitised to weak sensitising molecules including the β-lactam antibiotic amoxicillin. The mechanisms of amoxicillin sensitisation and elicitation of a T-cell response was further dissected by Rozieres et al (2010) using the same mouse model. They demonstrated the accumulation of amoxicillin-specific $CD8^+$ T-cells in the draining lymph nodes of amoxicillin-painted mice and their activation upon *ex vivo* stimulation with amoxicillin. The sensitisation of animals to amoxicillin was strongly inhibited by $CD4^+$ $CD25^+$ regulatory T-cells. Collectively, these data suggest that drug-specific $CD8^+$ T-cells are the culprits in amoxicillin-induced hypersensitivity and are capable of the eliciting effector functions responsible for the severe skin reactions witnessed in delayed hypersensitivity reactions (Rozieres et al. 2010) (Vocanson et al. 2006).

In beginning to prepare for the mouse model, the competencies of the readouts were investigated using the model strong haptens DNCB and DNFB (which both form the same protein-reactive intermediate 2, 4-dinitrophenol). Both *in vitro* and *in vivo* sensitisation were confirmed in animals painted with the haptens, verifying the readouts as suitable to assess sensitisation and elicitation.

Sensitisation of mice to amoxicillin was not as efficient and removal of $CD4^+$ T-cells is required to detect a drug-specific T-cell response (Rozieres et al. 2010). We initially conducted similar experiments in Liverpool with little success. A number of alterations in the methods were required to finally witness a full immunological reaction to amoxicillin.

The experiments performed in Lyon show a clear sensitisation to amoxicillin through drug-specific ear swelling (Fig 2.9.) and increases seen in the number of amoxicillin-specific IFNγ secreting cells in the dLN of amoxicillin-sensitised mice. The difference in the routes of administration show that to achieve maximal sensitisation and elicitation, cutaneous application of drug is required (Fig 2.9) (Fig 2.10.). Fig 2.11. sheds further light on the activation status of $CD8^+$ T-cells in amoxicillin-exposed $CD4^+$ T-cell depleted MHC II KO mice. Cutaneous application of amoxicillin yielded the highest amount activated $CD8^+$ T-cells in the draining lymph nodes, confirming that the best route of administration for generating drug-specific T-cells in the dLN is cutaneous application. These results were however not reciprocated in the percentage of activated $CD8^+$ T-cells in the spleen having negative implications for the further use of splenocytes as a readout of *ex vivo* T-cell activation. From the T-cells isolated from liver, the largest percentage of activated $CD8^+$ T-cells came from the route of I.V. administration and the least from oral administration. These data suggest that a greater number of $CD8^+$ T-cells infiltrate the liver following I.V. administration of drug. Thus it could be interesting to study oral dosing with β-lactams which are known to cause liver damage like amoxicillin/clavulanic acid in rare cases (Kim et al. 2011) and/or flucloxacillin (Monshi et al. 2013).

Although measurement of proliferation through [^{3}H] thymidine incorporation of dLN cells measures whole well proliferation instead of T-cells alone, hapten-specific $CD8^+$ effector cells are known to be responsible for high IFNγ production and have been used by various

research groups as a marker of hapten-specific T-cell activation (Akiba et al. 2002) (Xu et al. 1996) (Kehren et al. 1999). Amoxicillin-specific proliferation and IFNγ secretion was detected in sensitised animals and with both readouts together, this indicates the presence of amoxicillin-specific $CD8^+$ effector cells in the dLN of the amoxicillin-painted mice.

DCs from amoxicillin allergic patients have been reported to become semi-mature upon culture with amoxicillin (Rodriguez-Pena et al. 2006). This data was not able to be replicated in wild type C57/Bl6 mice indicating that the DCs generated in this manner are more like naive or non-allergic patient DCs.

The initial published method of sensitising MHC II KO, $CD4^+$ T-cell depleted mice to weak sensitisers used: amoxicillin at 1g/mL concentration to initially sensitise, *ex vivo* readouts using dLN cells incubated with APCs generated from bone marrow, and medium not containing penicillin (Rozieres et al. 2010). Problems encountered with this method were: (1) getting amoxicillin into solution at 1g/mL which was rectified through obtaining I.V. amoxicillin from the Royal Liverpool Hospital, (2) BMDDCs causing high background T-cell activation in *ex vivo* readouts which was rectified through use of magnetically isolated $CD11c^+$ DCs from non-dLNs, (3) penicillin in medium causing T-cell activation via cross-reactivity which was discovered upon its removal. This optimisation yielded the method which was used throughout the rest of this thesis.

In this chapter we established and refined the Vocanson et al (2006) MHC II KO C57Bl/6 CD4 depleted mouse model in Liverpool. This allowed us to study the priming of naïve $CD8^+$ T-cells to different β-lactam antibiotics, T-cell cross-reactivity with the different drugs (chapters 3 and 4), and attempt to develop an animal model of drug-induced liver injury (chapters 4 and 5).

CHAPTER 3

Cross-reactivity of human and mouse T-cells to the β-lactam antibiotics amoxicillin, flucloxacillin, and piperacillin.

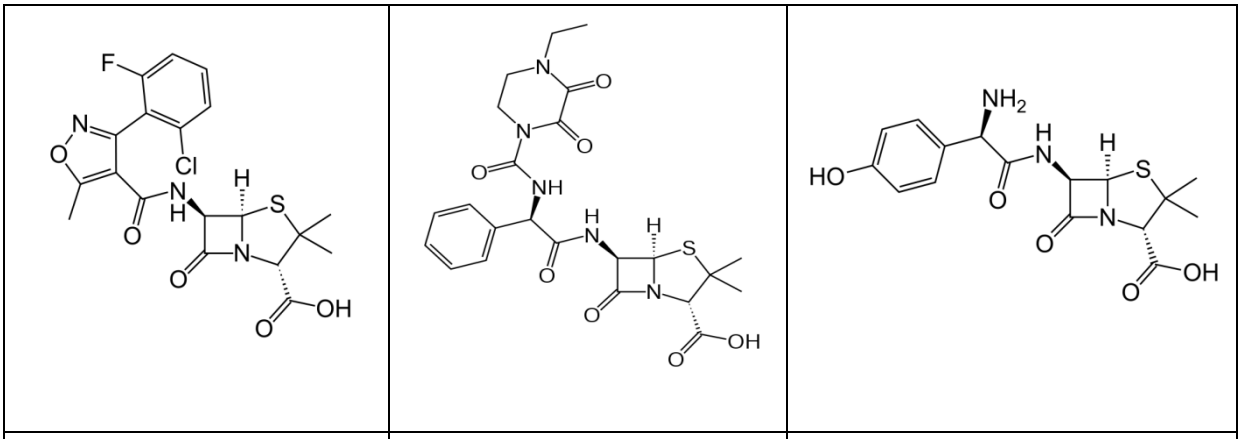
Contents

3.1. INTRODUCTION

T-cells play a central role in the development and elicitation of β-lactam-induced delayed-type hypersensitivity reactions. It is widely accepted that β-lactams cause these reactions through binding to protein to create a haptenic structure which is detected by the cells of the immune system. These T-cells have been isolated and cultured from drug-hypersensitive patients and have been shown to react in an antigen-specific manner (Romano et al. 2004) (Rozieres et al. 2009) (El-Ghaiesh et al. 2011). Due to the similar structure of different β-lactam antibiotics (Fig 3.1.), once a patient is known to be allergic, other β-lactam containing antibiotics are avoided. However, cases of clinical cross-reactivity have been documented with penicillin/cephalosporins (Audicana et al. 1994) (Salvo et al. 2007) (Solensky 2012) and experimentally, the activation of drug-specific T-cells with different drug moieties has not been fully defined (Miranda et al. 1996). To date, investigations have outlined a link between drugs containing very similar side chains. This was discovered in patients allergic to amoxicillin who displayed an immediate reaction to the drug and who also demonstrated clinical cross-reactivity to cefadroxil, which has the same side chain. None of the same patients reacted to cephalosporins to different side chains (Miranda et al. 1996). This theory is however at odds with delayed hypersensitivity reactions with β-lactam containing antibiotics which have different side chains but nevertheless are cross-reactive. (Padovan et al. 1997) demonstrated that highly reactive amoxicillin-specific T-cell clones would react to other penicillins but not to cephalosporins, even cephalosporins with identical side chains. These findings indicate that T-cell clones generally react to the penicilloyl core structure and that some T-cell clones recognise the core structure with accompanying side chains and some do not, reacting to the core structure even when there are large modifications to the side chains (Mauri-Hellweg et al. 1996) (Padovan et al. 1996).

T-cell cross-reactivity has been investigated using human T-cell clones from various different β-lactam hypersensitive patients (e.g. penicillin G, ampicillin, flucloxacillin, amoxicillin and piperacillin hypersensitive patients). Results showed that all β-lactams displayed a degree of cross-reactivity between each other with significant amounts of T-cell proliferation in at least one T-cell clone (Mauri-Hellweg et al. 1996) (Monshi et al. 2013).

The three β-lactam antibiotics used in this study (amoxicillin, flucloxacillin, and piperacillin) are known to bind to lysine residues on protein and in particular, Lys 190 on human serum albumin. Adduct formation is dependent on opening of the β-lactam ring (between N and the double bonded O) following nucleophilic attack to allow the formation of stable protein adducts (Whitaker et al. 2011) (Monshi et al. 2013) (Jenkins et al. 2009) (Ariza et al. 2012).



Flucloxacillin	**Piperacillin**	**Amoxicillin**

Fig 3.1. The molecular structures of the β-lactams flucloxacillin, piperacillin, and amoxicillin.

Patients with a history of delayed-type penicillin allergy are rarely prescribed drugs of similar classes but due to this, case reports of particular cross-reactive drugs are lacking (Yates 2008). Investigations into both mouse and human clone T-cell cross-reactivity has the benefit of discovering potential T-cell cross-reactivity whilst also being able to assess the comparability of the mouse model. It is important to state the limitations of the MHC II KO $CD4^+$ T-cell mouse model in only looking at $CD8^+$ T-cell responses.

3.2. AIMS

- To investigate β-lactam cross-reactivity between amoxicillin, piperacillin and flucloxacillin in a mouse model of delayed-type drug hypersensitivity and compare results against drug-specific human clones.

3.3. METHODS

3.3.1. Drugs and chemicals

Intra-venous grade flucloxacillin and amoxicillin sodium was obtained from Royal University Hospital, Liverpool. Piperacillin sodium, phytohaemagglutinin (PHA), Hanks balanced salt solution, and DMSO were from Sigma, Dorset, UK.

3.3.2. Culture medium

Mouse cells were cultured in RPMI 1640 (Sigma, Dorset, UK) medium supplemented with 10% foetal bovine serum (FBS) (Gibco, UK), 2mM L-glutamine (Sigma, Dorset, UK), and 50µM 2-mercaptoethanol (Sigma, Dorset, UK).

Human cell culture medium was comprised of RPMI 1640 supplemented with human AB serum (10%), HEPES (25mM), L-glutamine (2mM) and transferrin (25µg/mL).

3.3.3. Human Subjects

Drug-specific T-cell clones were generated from patients with an adverse reaction to amoxicillin, piperacillin, or flucloxacillin. Table 3.1. lists the clinical features of the reactions and the number of clones generated. A total of 100mL of blood was collected for PBMC isolation. Approval for the study was acquired from the Liverpool local Research Ethics Committee and informed written consent was obtained from each donor.

3.3.4. Generation of Epstein-Barr transformed-cells

Epstein-Barr virus (EBV) transformed B-cell lines were created from PBMC by transformation with supernatant from the virus-producing cell line B9.58. Lines were maintained in RPMI 1640 supplemented with 10% FBS (Invitrogen, Paisley, UK), 100 mM L-glutamine, 100 µg/mL penicillin, 100 U/mL streptomycin, and used as a source of autologous antigen-presenting cells

3.3.5. Generation of drug-specific T-cell clones from human donors

To isolate peripheral blood mononuclear cells (PBMCs), blood was layered on top of lymphoprep (50:50, blood: lymphoprep) (Axis-shield, Dundee, UK) and the erythrocytes were sedimented via density centrifugation (400g, 25min, room temperature). PBMCs were washed twice in Hanks balanced salt solution to remove any remaining lymphoprep, resuspended, counted via trypan blue exclusion, and plated out in 48 well plates at $1x10^6$ cells/well with drug at 1-2mM. IL-2 (60 U/mL) was added to maintain antigen-specific proliferation. Cultures were supplemented on days 6 and 9 with medium containing IL-2 and on day 14 cells were harvested, washed, and counted. T-cells were then cloned by serial dilution (El-Ghaiesh et al. 2011). Briefly, T-cells were dispensed into 20 96 well plates/drug at 0.3, 1, or 3 cells/well with a stimulation cocktail of $5x10^4$ irradiated PBMCs/well, PHA (5µg/mL), and IL-2 (2.5µL/mL). Cells were cultured for 14 days. Medium was supplemented with IL-2 on day 5 and then every 2 days thereafter (25µL). Growing wells were further expanded into 4 wells prior to assessment of specificity. To test the specificity of the clones, T-cells (0.5×10^5) were incubated with drug (0, 0.5, 1, 2mM) and irradiated autologous EBVs (0.1×10^5). After 48h, [^{3}H] thymidine (0.5µCi) was added, and 16h later proliferation was measured by scintillation counting. Clones with an SI of >2 were chosen for further expansion and cross-reactivity testing.

3.3.6. Analysis of T-cell clone phenotype

T-cell clone phenotyping was performed by flow cytometry on a BD FACSCanto II using anti-CD4, CD8 fluorescent antibodies (BD Biosciences) (see chapter 2).

3.3.7. Activation of human T-cell clones and cross-reactivity

The proliferative response and cytokine release was measured using [^{3}H] thymidine incorporation and IFNγ ELISpot, respectively. Drug-specific T-cell clones ($0.5x10^5$cells/well in duplicate) were incubated for 48h with drugs (0-2mM) and autologous irradiated EBV transformed B-cells ($0.1x10^5$cells/well). For the last 16h [^{3}H] thymidine (0.5µCi) was added to cultures. Interferon-γ secretion of drug-specific T-cell clones was quantified through IFNγ ELISpot where multiscreen HTS filter plates (Millipore, Watford, UK) were coated overnight at 4˚C with IFNγ capture antibody (15µg/mL). The following day, wells were washed five times with PBS and blocked with culture medium (200µl, 30min, room temperature). T-cell clones ($0.5x10^5$) were added to wells with irradiated autologous EBVs (0.1×10^5) and the drug antigen (0-2mM). Plates were incubated at 37˚C in 5% CO_2 and developed after 48h, according to the manufacturer's instructions. At the end of the incubation cells were discarded and wells were washed five times with 200µl PBS. Biotin-labelled detection antibody was diluted to 1µg/mL in PBS containing 0.5% FBS and added to the wells (100µl). The plate was incubated at room temperature for 2h before wells were washed five times with PBS. Streptavidin-ALP was diluted 1:1000 in PBS containing 0.5% FBS and added to wells (100µl, 1h, room temperature). Wells were washed five times with PBS (200µl) and spots were visualised by the addition of BCIP/NBT substrate. The plate was counted on an AID ELISpot reader (Cadama Medical, Stourbridge, UK) when thoroughly dried.

3.3.8. Mice

All mice used were between 8-20 weeks of age and were C57/Bl6 MHC II KOs with a mutation in the αβ gene encoding for MHC class II molecules. To deplete $CD4^+$ T-cells from

mice, I.P. injections of rat anti-mouse CD4 antibody were administered on day 0 (100µg/mouse) and on day 7 (100µg/mouse) in 100µL PBS.

3.3.9. Mouse sensitisation

Mice were sensitised on days 1-3 through painting with flucloxacillin, piperacillin, or amoxicillin (50ul, 1g/mL) in 70% DMSO on a shaved abdomen of approximately $3cm^2$ surface area. Vehicle mice were painted with 70% DMSO only. Tissues were removed on day 8 for *ex vivo* readouts. Unless otherwise stated, for *ex* vivo readouts, mouse inguinal dLN cells were pooled from groups of two mice to generate one n-number.

3.3.10. Mouse cell isolation and analysis of drug-specific proliferative responses

For *ex vivo* readouts dLNs were removed from mice on day 8. Cells were isolated from dLNs via maceration through 100µm nylon filter (BD Biosciences, UK) and then washed and counted. To isolate CD11c positive APCs, the mesenteric LNs were removed from mice and disaggregated into a single cell solution through ~1h incubation with 5mg/mL collagenase D (Sigma, UK). Cells were then washed with Hanks balanced salt solution and incubated with $CD11c^+$ magnetic beads (Miltenyi Biotec – UK) before being run through a magnetic column (Miltenyi Biotec – UK) and then washed, counted, and finally used.

After cell isolation, dLN cells were incubated in U-bottomed 96 well plates for 5 days (5% CO_2 and 37°C) ($1.25x10^5$ cells/well – 200µL) with CD11c positively isolated APCs ($2.5x10^4$cells/well). Amoxicillin, piperacillin, and flucloxacillin were added to cell cultures at concentrations of 0, 0.25 (~0.65mM), 0.5 (~1.1mM), and 1mg/mL (~2.2mM). After 5 days, proliferation was measured through incorporation of [^{3}H] thymidine (0.25µCi/well) (Moravek -California, USA) after 16h into newly synthesised DNA. Plates were harvested and read as stimulation index (SI) per well where the drug-specific increases are determined

through dividing the cpm at drug concentrations by the cpm at zero drug concentrations. Supernatants were collected for IFNγ secretion via IFNγ ELISA (see Chapter 2) (R&D Systems, UK).

3.3.11. Statistics

Unless otherwise stated, statistics were performed on SigmaPlot with significance being devised using the students T-test. Error bars represent one standard deviation from the mean.

3.4. RESULTS

3.4.1. Generation of T-cell clones from hypersensitive patients

The number of T-cell clones generated from hypersensitive patients as well as patient hypersensitivity symptoms and percentage of clone phenotype is described in Table3.1. Table 3.2. describes the phenotypes of clones used in the following experiments.

Medication	Clinical hypersensitivity reaction	Number of clones generated	Number found to be drug-specific	CD phenotype
Amoxicillin	DILI to co-amoxiclavulanic acid	169	67	80.6% $CD8^+$
Piperacillin	Maculopapular skin rash	192	39	87.0% $CD4^+$
Flucloxacillin	DILI	186	23	91.5% CD8

Table 3.1. Allergic patient clinical features and T-cell cloning.

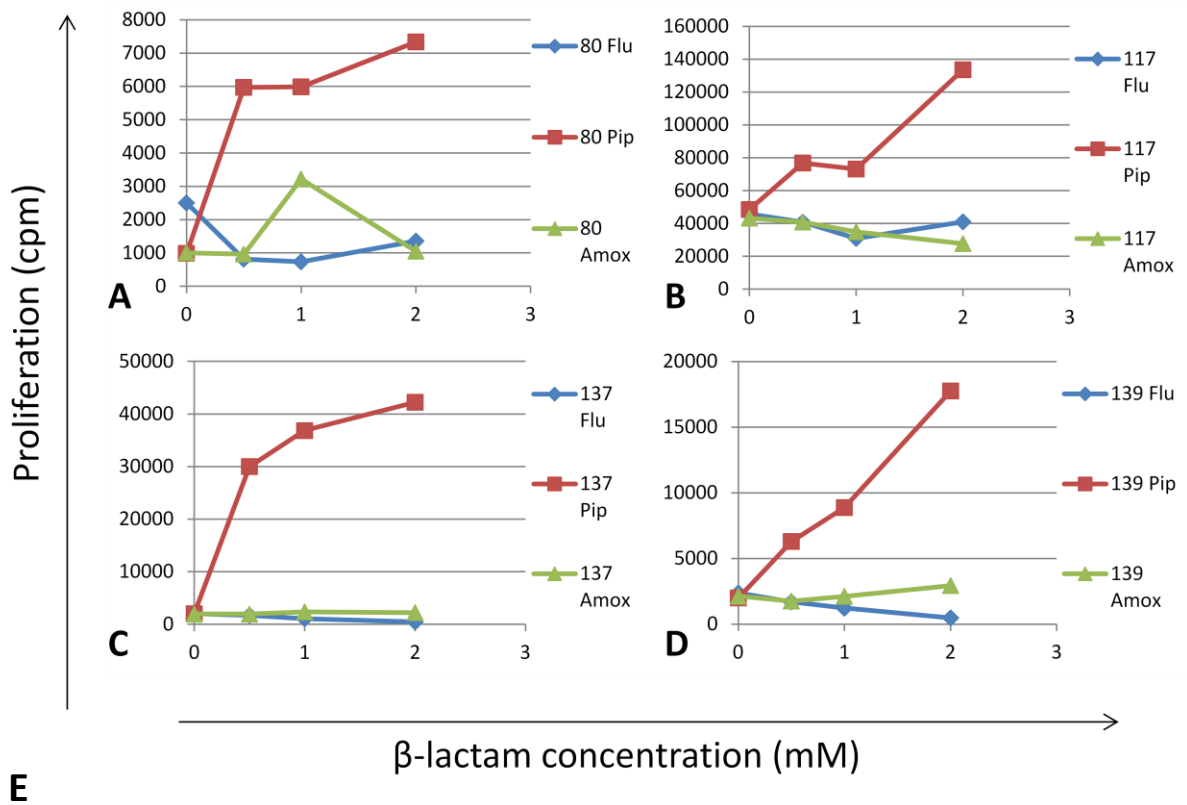
T-cell clone phenotype	$CD4^+$	$CD8^+$	Dual $CD4^+CD8^+$
Amoxicillin clones	75	64, 69, 106	41
Piperacillin clones	36, 80, 117, 137, 139	N/A	N/A
Flucloxacillin clones	N/A	50, 52, 149, 180	N/A

Table 3.2. Phenotype of T-cell clones selected for this study

3.4.2. Activation of human T-cell clones with amoxicillin, piperacillin, and/or flucloxacillin

3.4.2.1. Piperacillin-specific T-cell clones do not cross react with flucloxacillin or amoxicillin

In order to analyse the activation and cross-reactivity of piperacillin-specific T-cell clones, they were incubated separately with amoxicillin, piperacillin, and flucloxacillin *in vitro*. Piperacillin-specific human T-cell clones showed drug concentration-dependent proliferation and increases in the number of IFNγ secreting cells when piperacillin-treated wells were compared against vehicle controls (Fig 3.2.). In contrast, clones did not proliferate or secrete IFNγ when stimulated with flucloxacillin or amoxicillin.



E

Clone no	36			80			137			139		
	flu	*pip*	*amox*	*flu*	*pip*	*amox*	*flu*	*pip*	*amox*	*flu*	*pip*	*amox*
0												
0												
0.5mM												
1mM												
2mM												
PHA-5 µg/mL												

Fig 3.2. Proliferation (A-D) and IFNγ ELISpot (E) showing the activation of T-cell clones from a piperacillin hypersensitive patient with piperacillin but not with amoxicillin or flucloxacillin. Human T-cell clones were generated through serial dilution of PBMCs in the presence of IL2 and piperacillin. Piperacillin-specific T-cell

clones were then incubated at $0.5x10^5$ cells/well with $0.1x10^5$ irradiated EBVs for 48h in the presence of absence of drug. For the proliferation assays (A-D) [^{3}H] thymidine (0.5µCi) was added and 16h later proliferation was measured by scintillation counting. For IFNγ secretion analysis, cells were incubated in ELISpot filter plates for 48h and processed according to the kit protocols (E). All clones were $CD4^+$.

3.4.2.2. Flucloxacillin-specific $CD8^+$ T-cell clones show cross-reactivity to amoxicillin, but not piperacillin

In order to analyse the activation and cross-reactivity of flucloxacillin-specific T-cell clones, they were incubated separately with amoxicillin, piperacillin, and flucloxacillin *in vitro*. Flucloxacillin-specific human T-cell clones proliferated in a drug-concentration-dependent manner when incubated with flucloxacillin (Fig 3.3. A-D). Amoxicillin-specific responses were debatable with 3 of the 4 clones; an SI of 4 or above was recorded with all three clones (Fig3.3. A, B, D). In contrast, piperacillin did not activate the flucloxacillin-specific clones.

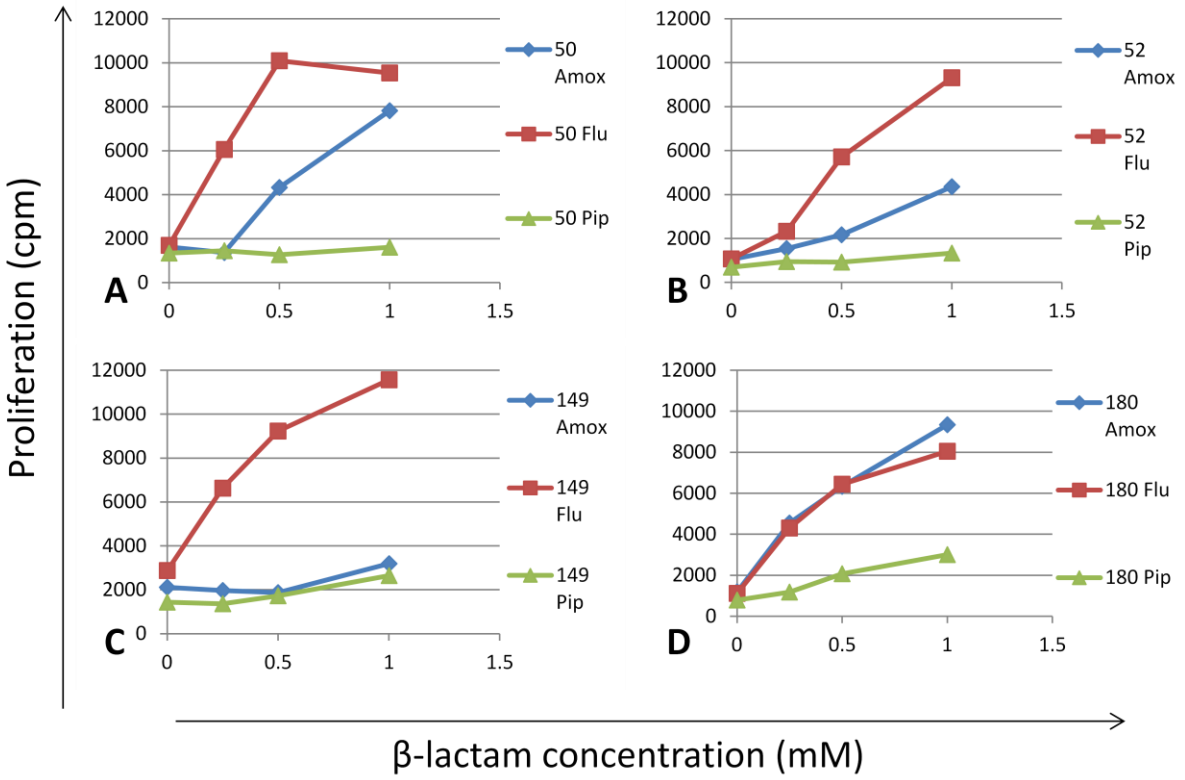
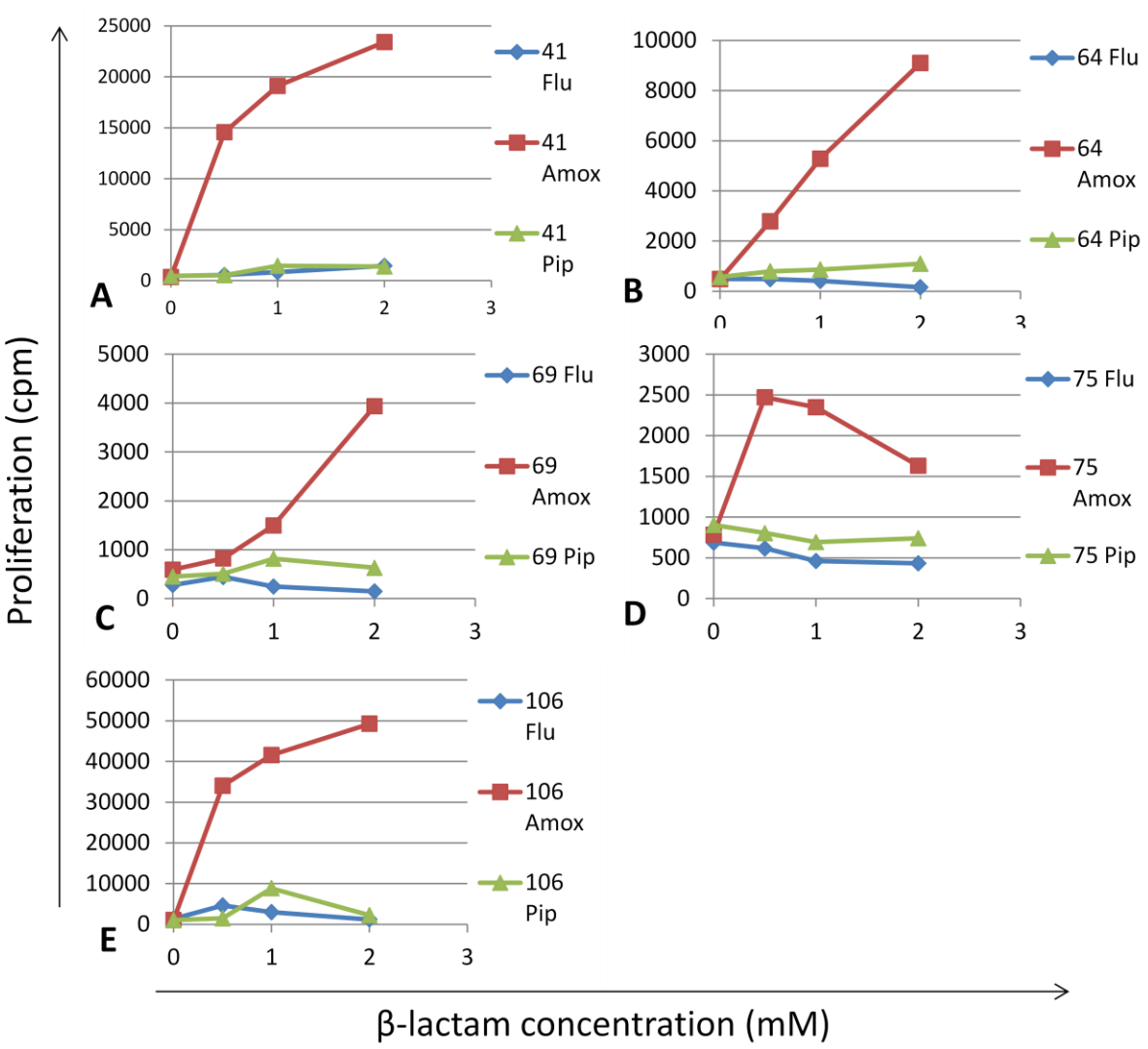


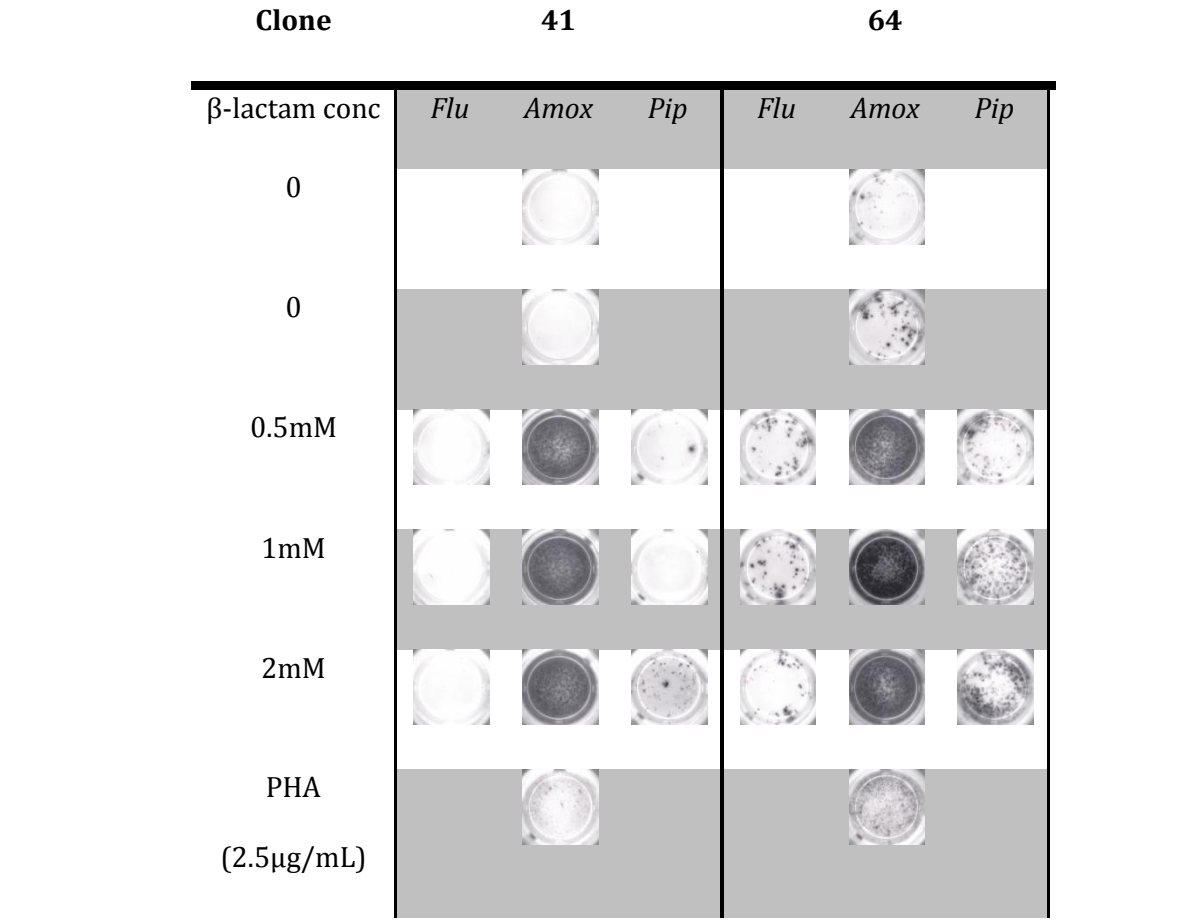
Fig 3.3. Proliferation of flucloxacillin-specific human T-cell clones showing cross-reactivity to amoxicillin but not piperacillin. Human T-cell clones were generated through serial dilution of PBMCs in the presence of IL2 and

flucloxacillin. Flucloxacillin-specific T-cell clones were then incubated at $0.5x10^5$ cells/well with $0.1x10^5$ irradiated EBVs for 48H in the presence or absence of drug (A-D). For the proliferation assay [^{3}H] thymidine (0.5µCi) was added and 16h later proliferation was measured by scintillation counting (A-D). All clones were $CD8^+$.

3.4.2.3. Amoxicillin-specific T-cell clones show limited cross-reactivity to piperacillin

Five amoxicillin-specific T-cell clones were tested for amoxicillin-specificity and cross-reactivity with piperacillin and flucloxacillin. All five clones showed concentration-dependent amoxicillin-specific increases in proliferation of at least 3x the control with accompanied increases in number of IFNγ secreting cells at all three amoxicillin concentrations tested (Fig 3.4.). Consistent increases (SI>3) in proliferation with flucloxacillin incubation were only observed with clones 41 and 106 at 2mM and 0.5mM, respectively (Fig 3.4. A, E). However, the proliferative response was not accompanied with an increase in IFNγ secretion. Weak increases in piperacillin-specific proliferation were detected in two clones with SIs >8 in clone 69 and 106 at 1mM piperacillin incubation (Fig 3.4. C, E). Similar results were obtained when IFNγ secretion was analysed. Piperacillin-specific responses were clearly visible when spot forming units (SFUs) in drug and control wells were compared for clone 106 at 0.5mM piperacillin incubation (Fig 3.4. E).


Proliferation (cpm)
β-lactam concentration (mM)
A
25000
20000
15000
10000
5000
0
0
1
2
3
41 Flu
41 Amox
41 Pip
B
10000
8000
6000
4000
2000
0
64 Flu
64 Amox
64 Pip
C
5000
4000
3000
2000
1000
0
69 Flu
69 Amox
69 Pip
D
3000
2500
2000
1500
1000
500
0
75 Flu
75 Amox
75 Pip
E
60000
50000
40000
30000
20000
10000
0
106 Flu
106 Amox
106 Pip


Clone
41
64
β-lactam conc
Flu
Amox
Pip
Flu
Amox
Pip
0
0
0.5mM
1mM
2mM
PHA
(2.5µg/mL)

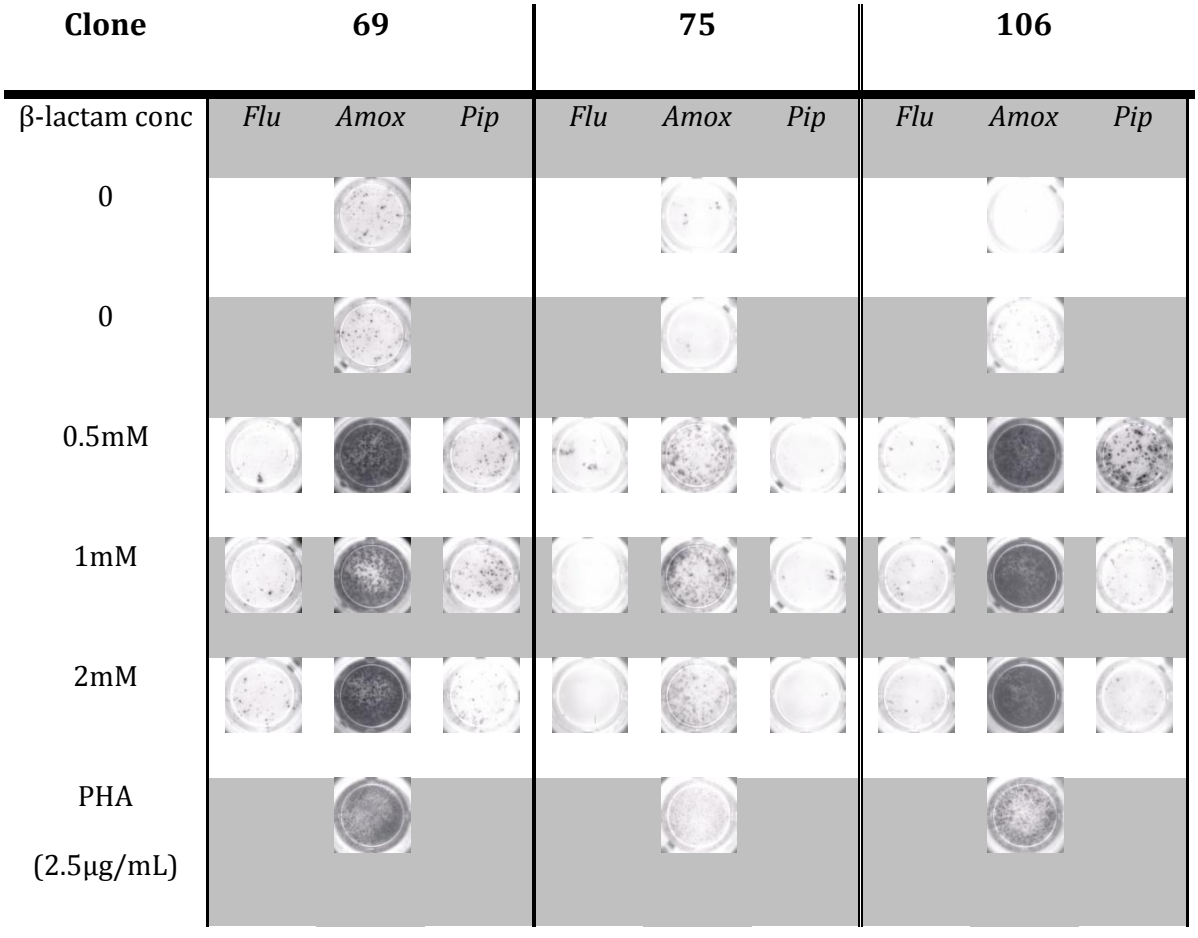

Clone
69
75
106
β-lactam conc
Flu
Amox
Pip
Flu
Amox
Pip
Flu
Amox
Pip
0
0
0.5mM
1mM
2mM
PHA
(2.5µg/mL)

Fig 3.4. Proliferation and IFNγ secretion analysis of the cross-reactivity of amoxicillin patient T-cell clones showing limited cross-reactivity with piperacillin and no significant cross-reactivity with flucloxacillin. Human T-cell clones were generated through serial dilution of one human patient PBMCs in the presence of IL-2 and amoxicillin. Amoxicillin-specific T-cell clones were then incubated at $0.5x10^5$ cells/well with $0.1x10^5$ irradiated EBVs for 48h. For proliferation analysis (A-E) [^{3}H] thymidine (0.5μCi) was added and 16h later proliferation was measured by scintillation counting. For IFNγ secretion quantification, cells were incubated in ELISpot filter plates and processed according to the kit protocols after 48h incubation with figures showing number of SFUs compared to zero control (Table).

3.4.3. $CD8^+$ T-cell reactivity of amoxicillin, piperacillin, and flucloxacillin in a mouse model

In order to analyse the possible cross-reactivity between amoxicillin, piperacillin and flucloxacillin-specific $CD8^+$ T-cells, proliferation and IFNγ secretion of β-lactam painted mouse dLN cells were studied. Proliferation data is presented as stimulation index (SI) where the response is calculated as fold increases compared to control wells. This is to more clearly show drug-specific increase whilst also making the separate experiments easier to compare.

Drug-specific increases in dLN cell proliferation were observed when drug-treated and vehicle control mice were compared (Fig 3.5. A1. B1, C1). Comparable results were obtained when IFNγ secretion was used as a readout. Significant increases in IFNγ secretion were observed when dLN cells from drug and vehicle control mice were compared (Fig 3.5. A2, B2, C2). These data show that mice were successfully sensitised to the β-lactam-containing antibiotics amoxicillin, piperacillin, and flucloxacillin.

The amount of dLN cell proliferation and IFNγ secretion ($CD8^+$ T-cell activation) induced by incubating flucloxacillin-sensitised mouse dLN cells with flucloxacillin (Fig 3.5. C1 & C2) was significantly larger when compared against the proliferation and IFNγ secretion induced in piperacillin-sensitised mouse dLN cells when incubated with piperacillin (Fig 3.5. B1 & B2).

This difference in T-cell activation indicates that in a $CD4^+$ T-cell depleted mouse model, flucloxacillin is more effective at sensitisation than piperacillin.

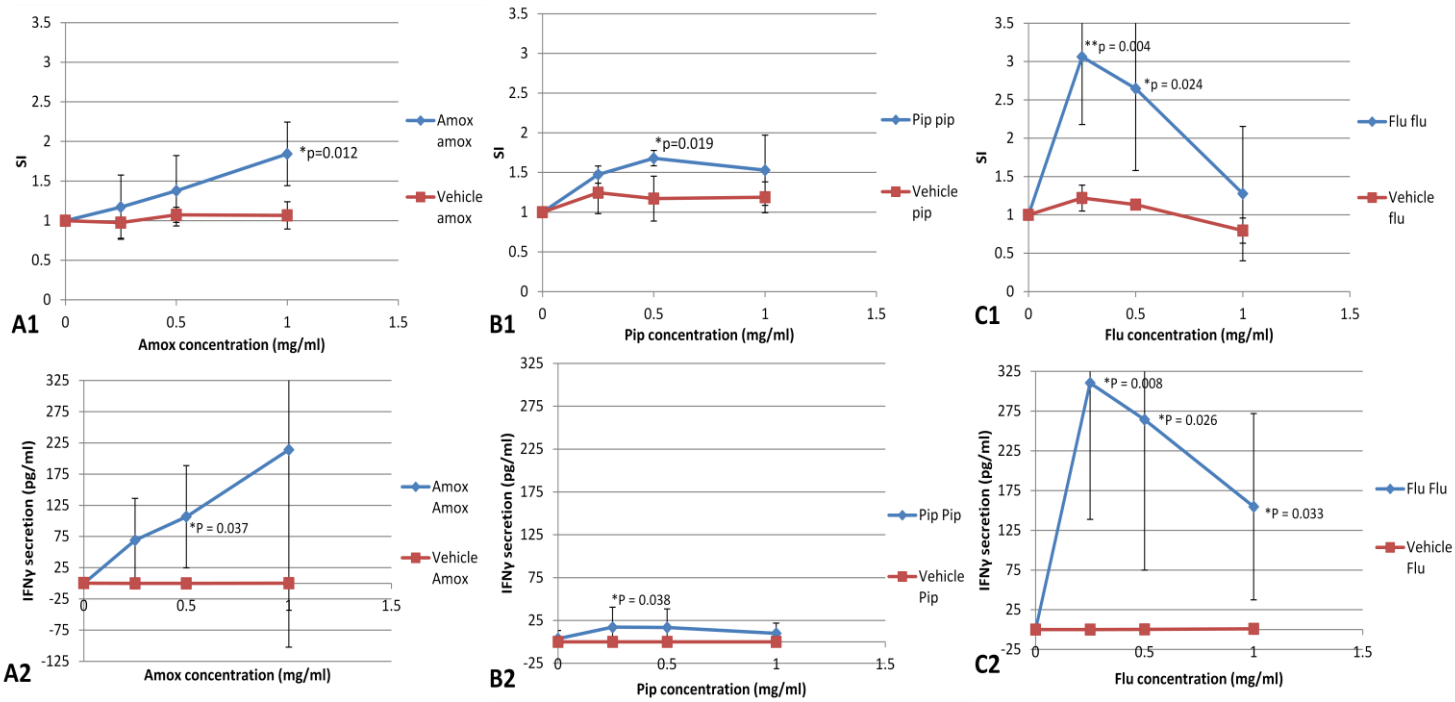


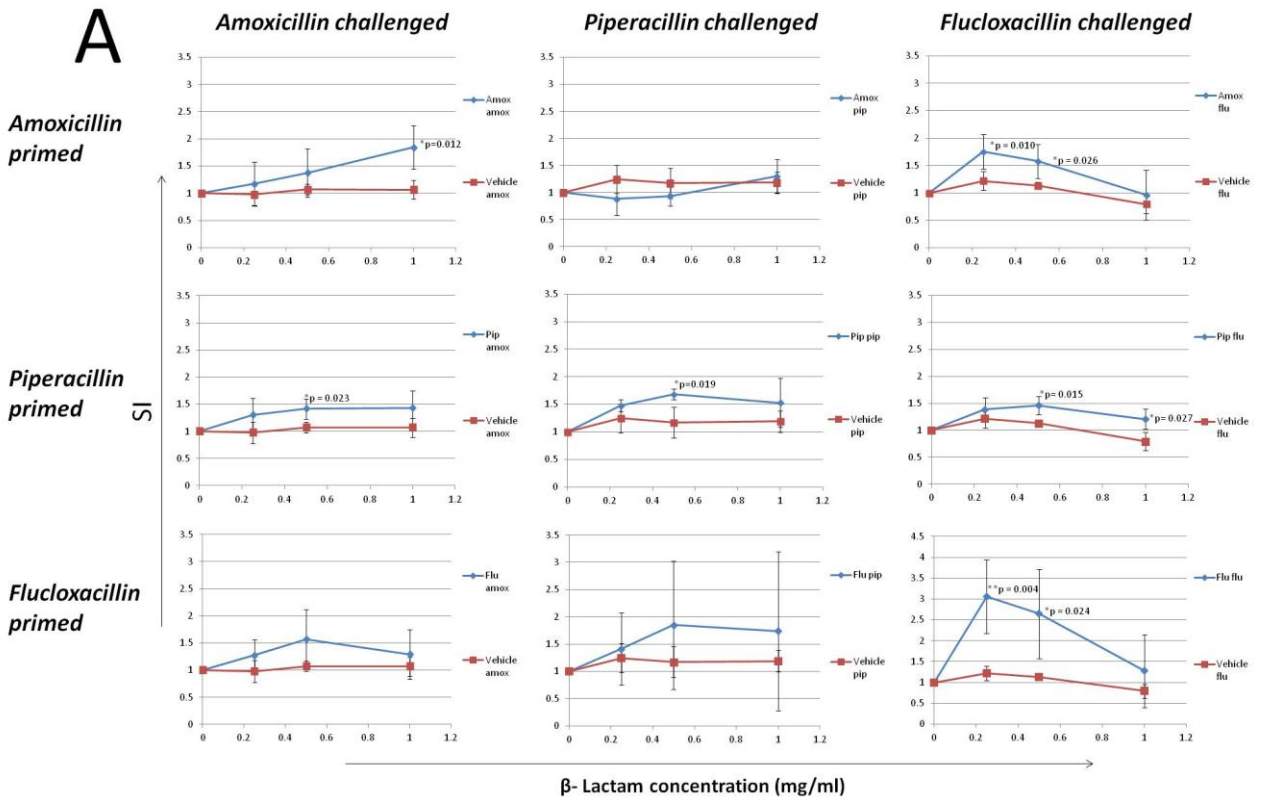
Fig 3.5. Proliferation analysis of the cross-reactivity of amoxicillin, piperacillin, and flucloxacillin-specific mouse dLN cells. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injections of CD4 antibody (100µg) on day 0 and 7. Mice were painted with 50µL of 1g/mL amoxicillin, piperacillin, or flucloxacillin for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated in triplicate at $0.125x10^6$ cells/well with $2.5x10^4$ APCs collected from the non-draining LN through CD11c positive magnetic isolation. Cells were incubated for 5 days after which proliferation was measured via [^{3}H] thymidine incorporation after 16h (n≥3) (A) Results show proliferation data as stimulation index (SI). Immediately prior to thymidine addition, supernatants were removed from wells (100µL) and analysed for IFNγ content through IFNγ ELISA (B). Results show amount of IFNγ secreted to drug, minus IFNγ secreted at no added drug (Amoxicillin n=3, piperacillin n=3, flucloxacillin n=6).

Cross-reactivity between amoxicillin, piperacillin, and flucloxacillin was investigated through *ex vivo* incubation of sensitised dLN cells with all three drugs. Cross-reactivity was assessed via a significant drug-specific proliferation and/or via significant drug-specific increases in IFNγ secretion when compared against vehicle controls.

Amoxicillin-sensitised mouse dLN cells proliferated in a drug-dependent manner when incubated with flucloxacillin (Fig 3.6. A - Amoxicillin-primed). This result was reciprocated in the analysis of IFNγ secretion from amoxicillin-sensitised mice (Fig 3.6. B - Amoxicillin-primed). These data indicate a consistent cross-reactivity between amoxicillin and flucloxacillin in the mouse. Conversely, amoxicillin-sensitised mouse dLN cells did not significantly proliferate or secrete IFNγ when incubated *ex vivo* with piperacillin.

Piperacillin-sensitised mouse dLN cells demonstrated mild proliferative responses following *ex viv*o incubation with amoxicillin, piperacillin, and flucloxacillin (Fig 3.6. A – Piperacillin-primed). However, this cross-reactivity was not evident upon analysis of piperacillin-sensitised mouse dLN cell IFNγ secretion, which showed no drug-specific increases from incubation with amoxicillin or flucloxacillin (Fig 3.6. B – Piperacillin-primed). The contradicting results from piperacillin-sensitised mouse dLN cells indicate a small degree of cross-reactivity between piperacillin with amoxicillin and flucloxacillin.

Flucloxacillin-specific mouse dLN cells upon incubation *ex vivo* with amoxicillin and piperacillin did not significantly proliferate when compared against vehicle controls (Fig 3.6. A – Flucloxacillin-primed). Conversely however, upon *ex vivo* incubation with amoxicillin and piperacillin, flucloxacillin-specific mouse dLN cells secreted significant amounts of IFNγ when compared against vehicle controls. As with piperacillin, these contradicting results indicate a small degree of cross-reactivity between flucloxacillin with amoxicillin and piperacillin.


A
Amoxicillin challenged
Piperacillin challenged
Flucloxacillin challenged
Amoxicillin primed
Piperacillin primed
Flucloxacillin primed
SI
Amox amox
Vehicle amox
*p=0.012
Amox pip
Vehicle pip
Amox flu
Vehicle flu
*p = 0.010
*p = 0.026
Pip amox
*p = 0.023
Pip pip
*p=0.019
Pip flu
*p= 0.015
*p= 0.027
Flu amox
Flu pip
Flu flu
**p = 0.004
*p = 0.024
β- Lactam concentration (mg/ml)

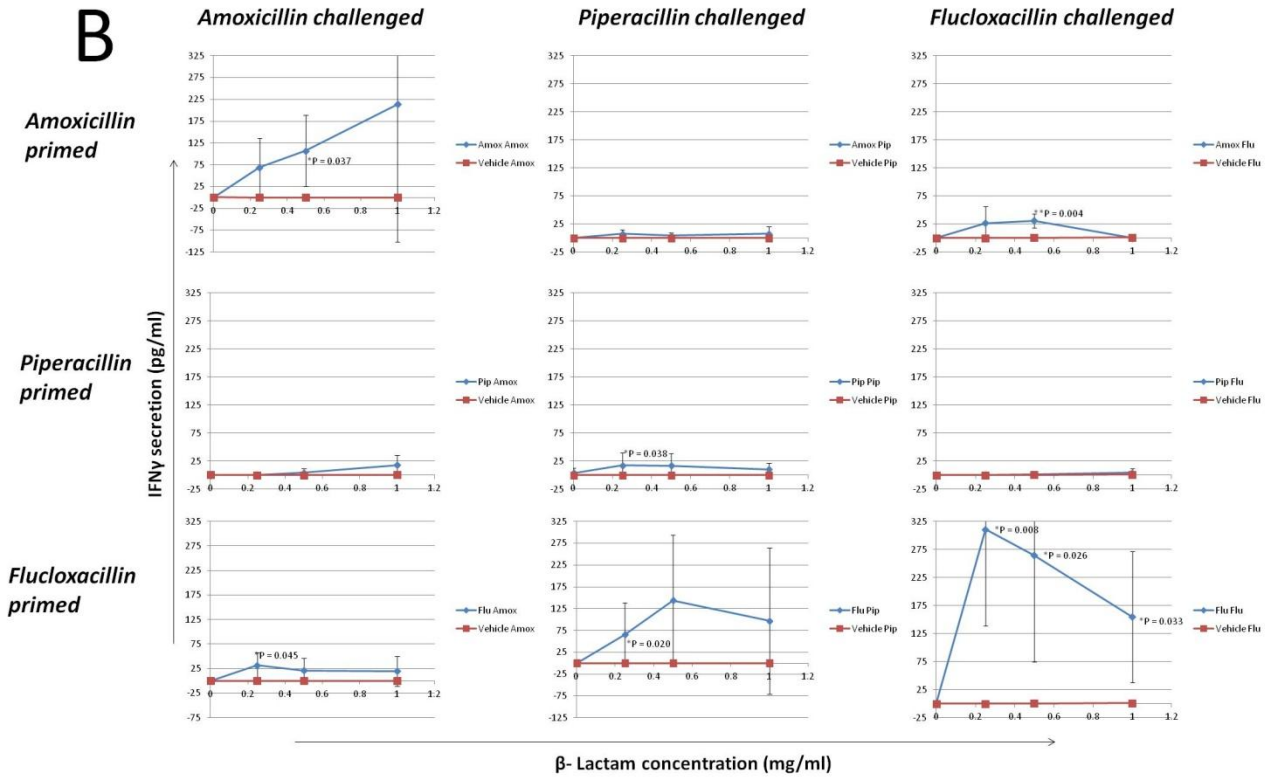

B
Amoxicillin challenged
Piperacillin challenged
Flucloxacillin challenged
Amoxicillin primed
Piperacillin primed
Flucloxacillin primed
IFNγ secretion (pg/ml)
Amox Amox
Vehicle Amox
*P = 0.037
Amox Pip
Vehicle Pip
Amox Flu
Vehicle Flu
*P = 0.004
Pip Amox
Pip Pip
*P = 0.038
Pip Flu
Vehicle Flu
Flu Amox
*P = 0.045
Flu Pip
*P = 0.020
Flu Flu
*P = 0.008
*P = 0.026
*P = 0.033
β- Lactam concentration (mg/ml)

Fig 3.6. Proliferation analysis of the cross-reactivity of amoxicillin, piperacillin, and flucloxacillin-specific mouse dLN cells. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injections of CD4 antibody (100μg) on day 0 and 7. Mice were painted with 50μL of 1g/mL amoxicillin, piperacillin, or flucloxacillin for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated in triplicate at $0.125x10^6$ cells/well with $2.5x10^4$ APCs collected from the non-draining LN through CD11c positive magnetic isolation. Cells were incubated for 5 days after which proliferation was measured via [^{3}H] thymidine incorporation after 16h (n≥3) (A) Results show proliferation data as stimulation index (SI). Immediately prior to thymidine addition, supernatants were removed from wells (100μL) and analysed for IFNγ content through IFNγ ELISA (B). Results show amount of IFNγ secreted to drug, minus IFNγ secreted at no added drug (amoxicillin n=3, piperacillin n=3, flucloxacillin n=6).

3.5. DISCUSSION

In this chapter, T-cell cross-reactivity between amoxicillin, flucloxacillin, and piperacillin was investigated using both a mouse model of β-lactam induced hypersensitivity and human patient T-cell clones *in vitro*. The aim of this was to investigate whether cross-reactivity between the three β-lactams was similar or different in both systems. These particular β-lactam antibiotics were chosen due to their known association with inducing hypersensitivity reactions with different clinical phenotypes in humans. Furthermore, drug-specific T-cells have been isolated from patients with each form of hypersensitivity (Romano et al. 2004) (Rozieres et al. 2009) (El-Ghaiesh et al. 2011). In hypersensitive patients piperacillin and amoxicillin mainly induce skin reactions (Grieco et al. 2005) (Chovel-Sella et al. 2013) with amoxicillin/clavulanic acid also however able to induce drug-induced liver injury (Leise et al. 2014). Flucloxacillin hypersensitivity, conversely, mainly represents itself as drug-induced liver injury (Kaniwa and Saito 2013). Why these structurally similar drugs should target different organs during hypersensitivity reactions is largely unknown, however, an interesting correlation lies in the mechanisms of T-cell activation with piperacillin mainly inducing drug-specific $CD4^+$ T-cells, amoxicillin producing mainly $CD8^+$ T-

cells and flucloxacillin producing nearly entirely drug-specific T-cells that are $CD8^+$ (Table 3.1.).

The phenomenon of cross-reactivity between β-lactam antibiotics occurs through binding of the antibiotic molecule to specific lysine residues on self-protein to create an immunogenic protein structure (Whitaker et al. 2011) (Monshi et al. 2013) (Jenkins et al. 2009) (Ariza et al. 2012); because of the similarities between β-lactam antibiotics, the same immunogenic protein could cause T-cell activation and thus cross-reactivity between two separate β-lactam antibiotics. Both human and mouse T-cells displayed reactivity against flucloxacillin and amoxicillin. This could be because both drugs bind to the same lysine residue to create immunologically similar haptenic molecules which when re-introduced, cause T-cell activation. In respect to the three drugs investigated here, the different phenotypes of clone may play a role in the prevention or allowance of cross-reactivity. For example, piperacillin largely induces drug-specific T-cells that are $CD4^+$ whilst amoxicillin and flucloxacillin mainly induce drug-specific T-cells that are $CD8^+$. This difference in phenotype may induce sensitisation to different peptide epitopes which are not able to be replicated by other β-lactams. This reason implicates a possible mechanism as to why, in human patients, piperacillin-specific T-cells are not cross-reactive with amoxicillin or flucloxacillin (Table 3.3.).

The mouse model of sensitisation to amoxicillin had been successfully established (see chapter 2). Furthermore, as we have isolated and generated amoxicillin, piperacillin, and flucloxacillin human T-cell clones from hypersensitive patients. The next step was to investigate if it was possible to sensitise mice to piperacillin and flucloxacillin. If this was possible, cross-reactivity could be compared in both human and mice. Sensitisation to amoxicillin was repeated alongside attempts to sensitise mice to piperacillin and

flucloxacillin. With flucloxacillin, sensitisation was possible and furthermore, caused even more robust effector T-cell responses upon *ex vivo* drug stimulation than amoxicillin. Piperacillin conversely, displayed very weak effector responses upon *ex vivo* drug stimulation indicating that sensitisation was not as efficient as with amoxicillin or flucloxacillin. A possible reason for the lack of T-cell response may again lie in the mechanism of sensitisation where piperacillin is known to cause sensitisation through mechanisms guided towards producing drug-specific T-cells which are $CD4^+$ (Table 3.1.). In a mouse model devoid of $CD4^+$ T-cells, a lack of drug-specific T-cells is not wholly unsurprising. Another explanation for the lack of substantial piperacillin-specific increases in proliferation and IFNγ could lay in the possibility that piperacillin is capable of generating drug-specific $CD8^+$ T-cells which are of a Tc2 phenotype. This shift in phenotype would explain the lack of IFNγ with Tc2 cells secreting little or no IFNγ and instead secreting IL-4 and IL-5 (Sad et al. 1995). The lack in substantial proliferation could also be partly explained through a shift towards a Tc2 phenotype through Tc2 human T-cell clones being reported to have dampened proliferative responses compared to Tc1, even when incubated with Tc2 related cytokine IL-4 (Vukmanovic-Stejic et al. 2000). The mild increases in IFNγ detected in the piperacillin-specific dLN cell supernatant indicates the very likely presence of Tc1 cells as Tc2 cells do not secrete IFNγ (Sad et al. 1995) (Vukmanovic-Stejic et al. 2000). However, it would be of interest to analyse the cytokine secretions of the piperacillin-sensitised dLN cells to determine whether piperacillin is also capable of generating $CD8^+$ T-cells shifted towards an IL-4 secreting Tc2 phenotype.

Once sensitisation to the three drugs was detectable in the mouse model, cross-reactivity was investigated in the mouse model through *ex vivo* drug incubations and then compared

against human T-cell clone data. All mice and human cross-reactivity data is summarised in Table 3.3.

Human T-cell cross-reactivity	*Amox challenged*	*Pip challenged*	*Flu challenged*
Amox primed	YY	Y	N
Pip primed	N	YY	N
Flu primed	Y*	N*	Y*
Mouse CD8$^+$ T-cell cross-reactivity	*Amox challenged*	*Pip challenged*	*Flu challenged*
Amox primed	YY	N	YY
Pip primed	Y	YY	Y
Flu primed	Y	Y	YY

Table 3.3. Cross-reactivity of mouse and human T-cells summarised. Data is shown as reactivity being evident in proliferation AND IFNγ (n>3) (YY), evident in proliferation OR IFNγ only (n>3) (Y), or not evident in either readout (n>3) (N) (*only one readout)

In the mouse model, it would be feasible to assume that amoxicillin and flucloxacillin were creating similar haptenic proteins in both sensitisation and elicitation leading to cross-reactive activation in both amoxicillin and flucloxacillin-primed dLN cells; as these drugs both bind to the same Lys residues and cause sensitisation via intracellular mechanisms

which yield large amount of $CD8^+$ T-cells (Whitaker et al. 2011) (Monshi et al. 2013) (Jenkins et al. 2009) (Ariza et al. 2012).

In human T-cell clones, amoxicillin-specific T-cells were different from their murine counterparts. No cross-reactivity to flucloxacillin and limited cross-reactivity to piperacillin were observed through analysis of the number of IFNγ secreting cells. Flucloxacillin-specific human T-cell clones showed similarities to the mouse model (i.e. through cross-reactive increases in proliferation to amoxicillin was observed). This was contrasted against the differences of piperacillin cross-reactivity in the mouse which was not detectable in the human data. Further investigation would have been ideal to fully dissect the cross-reactive potential of flucloxacillin-specific human T-cell clones but due to cell numbers this was not possible. Monshi et al (2013) has previously looked at cross-reactivity in flucloxacillin-specific human T-cell clones and found cross-reactivity to both amoxicillin and piperacillin.

The differences in cross-reactivity seen between human and mouse T-cells are not un-surprising due to the differences between the two assays. At the time of sensitisation the protein which the β-lactams would likely bind to whether in human or mouse, would have been human albumin or mouse albumin, respectively, which have approximately 70% amino acid sequence homology (Kosa et al. 1998). This difference in initial protein could lead to differences in the tertiary structure of the haptenic peptides being presented to the T-cells during sensitisation. Protein differences at elicitation could also be a factor with human and mouse T-cells being cultured in medium containing human and bovine albumin, respectively. Bovine albumin is 76% identical to human albumin, in regards to amino acid sequence (Kosa et al. 1998).

The three β-lactam containing antibiotics used here all have structurally different side chains (Fig 3.1.) and displayed a cross-reactivity with at least one other drug in human and/or mice. This indicates that the haptenic peptides being formed are dependent on the core β-lactam structure, and not the side chains. Interestingly, human piperacillin-specific T-cells did not show reactivity to amoxicillin or flucloxacillin in *ex vivo* readouts. Investigations into T-cell cross-reactivity with other β-lactam antibiotics has been performed by Mauri-Hellweg et al (1996) to reveal that non-cross-reactive human T-cells showed an up-regulation of one TCR Vβ-chain whilst broadly cross-reactive human T-cell lines demonstrated a heterogenous TCR usage; which may have been the case with the non-cross-reactive piperacillin-specific human T-cell clones investigated here.

Finally, the human T-cell clones used here are by definition one type of T-cell sensitised to one haptenic peptide and as previously described, β-lactams are capable of binding to multiple lysine residues on protein to create different haptenic peptides, some of which could be generated using other β-lactams. The mouse cells used in these experiments are derived from $CD4^+$ T-cell depleted lymph node and so, are a multitude of $CD8^+$ T-cells, sensitised to a variety of drug bound haptenic peptides; the implications of this being that the murine readouts are a measurement of a multitude of different $CD8^+$ T-cells, and human readouts of one particular haptenic peptide for each clone.

In conclusion, cross-reactivity in human T-cell clones has been analysed to show the possibility of cross-reactivity in all three β-lactams tested. Also, novel data has been generated in mice sensitisation experiments. Data presented show the activation of $CD8^+$ T-cells to the β-lactams flucloxacillin and piperacillin (albeit to a lesser extent). Cross-reactivity was observed between amoxicillin and flucloxacillin. Flucloxacillin sensitisation in mice

seemed efficient with large numbers of drug-specific T-cells showing robust T-cell activation upon *ex vivo* elicitation. This discovery opens up novel experimental possibilities involving flucloxacillin sensitisation, which is known to induce liver injury. There are currently no animal models of flucloxacillin sensitisation at the time of writing, so the development of a mouse model capable of mimicking the human condition could be utilised as a vital tool in dissecting the mechanisms of pathogenesis of flucloxacillin induced liver injury and working to block or counteract these mechanisms.

CHAPTER 4

Investigating flucloxacillin sensitisation and antigen presentation

Contents

4.1. INTRODUCTION

In 1999, carbamazepine-induced mucocutaneous syndrome was observed in monozygotic twins (Edwards S. G. et al. 1999). This opened the field and in 2002 an association between HLA-B*57:01 and abacavir hypersensitivity was discovered (Mallal et al. 2002) (Veronesi et al. 2002). Several HLA types have since been found to contribute to drug hypersensitivity reactions including flucloxacillin-induced liver damage. Expression of HLA-B*57:01 was found to be significantly increased in patients with DILI (Daly et al. 2009). How the presence of the HLA-B*57:01 molecule contributes to the generation of flucloxacillin-induced liver injury is not entirely understood. There has been a proposal that flucloxacillin, when added to *in vitro* PBMC cultures devoid of professional APCs, activated antigen-specific $CD8^+$ T-cells from naive HLA-B*57:01 positive individuals via a direct interaction with MHC and specific TCRs. In HLA-B*57:01 negative individuals, T-cell responses were detectable but the T-cells were activated via a hapten-based mechanism (Wuillemin et al. 2013). Drug-specific $CD8^+$ T-cells from donors carrying the risk allele are HLA-B*57:01-restricted in some individuals (Wuillemin et al. 2013). However, in other donors, other HLA-class molecules present the drug-derived antigen to T-cells (Wuillemin et al. 2013). As mice do not possess HLA-B*57:01 allele or a murine equivalent, flucloxacillin sensitisation using the CD4 depleted mouse model would assume to be proceeding via a hapten-based mechanism, however, as mice have not been sensitised to flucloxacillin previously, this is not certain.

In favour of a hapten-based mechanism of flucloxacillin-induced drug hypersensitivity, flucloxacillin is a β-lactam antibiotic known to be able to bind to lysine residues on albumin to create immunogenic drug-protein antigens capable of stimulating flucloxacillin-specific $CD8^+$ T-cells from allergic human patients. Furthermore, flucloxacillin pulsed antigen presenting cells have been shown to stimulate flucloxacillin-specific human T-cell clones.

This response is blocked when the pulsed antigen presenting cells are fixed to inhibit cellular processing (Monshi et al. 2013). This indicates the binding of flucloxacillin to protein is crucial in the activation and generation of flucloxacillin-specific T-cells in humans. Establishment of a flucloxacillin mouse sensitisation model would allow the determination of whether flucloxacillin-specific T-cell activation was occurring through a hapten or Pi-based mechanism.

4.2. AIMS

To use the mouse model to:

- Analyse mechanisms of flucloxacillin-specific T-cell activation
- Investigate the involvement APCs have on the flucloxacillin-specific T-cell response.

4.3. METHODS

4.3.1. Mice and $CD4^+$ T-cell depletion

All mice used were between 8-20 weeks of age and were C57/Bl6 MHC II KOs with a mutation in the αβ gene encoding for MHC class II molecules (Charles River - Kent, UK). To deplete $CD4^+$ T-cells from mice, I.P. injections of rat anti-mouse CD4 antibody (GK 1.5 Bioxcell - UK) were administered on day 0 (100µg/mouse) and on day 7 (100µg/mouse) in 100µL PBS.

4.3.2. Drugs and chemicals

Intra-venous grade flucloxacillin sodium was obtained from The Royal University Hospital, Liverpool. Phytohaemagglutinin (PHA), Hanks balanced salt solution, and DMSO were from Sigma, UK.

4.3.3. Culture medium

RPMI 1640 (Sigma, UK) was supplemented with 10% foetal bovine serum (FBS) (Gibco, UK), L-glutamine (2mM) (Sigma, UK), and 2-mercaptoethanol (50µM) (Sigma, UK).

4.3.4. Mouse sensitisation

Mice were sensitised on days 1-3 through painting with flucloxacillin (50ul, 1g/mL) in 70% DMSO on a shaved abdomen (approximately $3cm^2$ surface area). Vehicle mice were painted with 70% DMSO only. Tissues were removed on day 8 for *ex vivo* readouts. Unless otherwise stated, for *ex* vivo readouts, mouse inguinal dLN cells were pooled from groups of two mice to generate one n-number.

4.3.5. Cell isolation

For *ex vivo* readouts dLNs were removed from mice on day 8. Immune cells were isolated via maceration through 100µm nylon filter (BD Biosciences, UK) and then washed and counted.

4.3.6. Generation of bone marrow-derived DCs and isolation of CD11c $^{+}$ DCs from lymph nodes

To generate bone marrow-derived dendritic cells (BMDDCs) the bone marrow was flushed from the femurs of mice with complete RPMI medium and large pieces of bone were filtered out with a 100µm nylon filter. Cells were incubated with 5% GMCSF (Peprotech, NJ, USA) for 8 days after which immature DCs were harvested. To mature the DCs, LPS (0.5µg/mL) was added to the cultures for 24h before harvesting.

To isolate CD11c positive APCs, the mesenteric LNs were removed from mice and disaggregated into a single cell solution through ~1h incubation with 5mg/mL collagenase D (Sigma, UK). Cells were then washed with Hanks balanced salt solution and incubated with $CD11c^{+}$ magnetic beads (Miltenyi Biotec – UK) before being run through a magnetic column (Miltenyi Biotec – UK) and then washed, counted, and finally used.

4.3.7. DC pulsing with flucloxacillin

For DC pulsing experiments where DCs were incubated with flucloxacillin before culture with dLN cells, BMDDCs were generated and harvested on day 8 as previously described. Cells were then incubated in 6 well plates ($1.5x10^{6}$cells/well) with flucloxacillin (0.25, 0.5, 1mg/mL) for 24h or at 0.5mg/mL for 1, 4, 24, or 48h (37°C, 5% CO_2). At each time point, cells were washed with medium to remove un-bound drug and added ($2.5x10^{4}$cells/well) to dLN cells ($2.5x10^{5}$cells/well). Readouts were taken after 5 days in culture.

4.3.8. $CD8^+$ T-cell proliferation analysis via CFSE staining

After sensitisation and cell isolation, dLN cells were incubated with CFSE (5mM, 0.5µL) (Ebioscience, UK) for 5 minutes at room temperature. The cells were washed and incubated with flucloxacillin (0.25, 0.5, 1mg/mL) or PHA (10µg/mL) in U-bottomed 96 well plates ($1.25x10^5$ cells/well) (Nunc, UK) with CD11c positively isolated DCs ($2.5x10^4$ cells/well). After 5 days at 37°C and 5% CO_2, cells were then washed, incubated with a fluorescent $CD8^+$ antibody (Allophycocyanin) (2µL/sample) for 15 minutes at 4°C. Cells were then washed and analysed for proliferation on a FACScalibur flow cytometer. FACS traces were gated to exclude dead or dying cells and to focus the analysis on $CD8^+$ T-cells. Data was analysed with Cyflogic (Finland).

4.3.9. Proliferation analysis of dLN cells via [^{3}H] thymidine incorporation

After sensitisation and cell isolation, dLN cells ($1.25x10^5$ cells/well) were incubated in U-bottomed 96 well plates for 5 days at 5% CO_2 and 37°C with either CD11c positively isolated DCs ($2.5x10^4$ cells/well), immature BMDDCS ($2.5x10^4$ cells/well), no addition of DCs, or removal of DCs with CD11c-positive magnetic isolation. After 5 days, proliferation was measured through incorporation of [^{3}H] thymidine (0.25µCi/well) (Moravek -California, USA) into newly synthesised. Plates were harvested and read as scintillation per well.

4.3.10. IFNγ secretion analysis of dLN cells

IFNγ content of supernatant from the proliferation assays described above, was removed on day 5 of culture and analysed through a mouse IFNγ ELISA kit (R&D systems, UK).

Quantification of IFNγ secreting cells was analysed through an IFNγ ELISpot kit (BD Biosciences, UK). Multiscreen filter plates (Millipore, Watford, UK) were coated with IFNγ antibody (15µg/mL) overnight at 4˚C. Wells were washed with PBS (200µl) and blocked for

30 minutes with medium (200µl). Cells from the dLNs of painted mice were added to the plate ($2.5x10^5$cells/well) with and without CD11c positive cells magnetically isolated from the non-dLNs ($2.5x10^4$cells/well). Flucloxacillin (0.25, 0.5 1mg/mL) and PHA (20µg/mL) were added to wells and after 48h, the plate was developed according to the manufacturer's instructions. The wells were washed with PBS and incubated with secondary antibody (diluted 1:1000 in PBS containing 0.5% FBS) for 2h at room temperature. After washing with PBS, Streptavidin-ALP (diluted 1:1000 in PBS containing 0.5% FBS) was added to wells for one hour. Spots were visualised by the addition of BCIP/NBT substrate (100µl, 15 min). The plate was counted on an AID ELISpot reader (Cadama Medical, Stourbridge, UK) when thoroughly dried.

4.3.11. Detection of *in vitro* flucloxacillin covalent modification of human serum albumin by LC/MS

Human serum albumin (1.3 mg, 40µM) was incubated with 400µM flucloxacillin in 500µL phosphate buffer, pH 7.4, at 37°C for 16h. Aliquots of 50µL were removed after 30 min, 1, 2, 3, 16 and 24h and processed for MS analysis. At each time point the drug was removed by precipitation of the protein with ten volumes of ice-cold methanol followed by centrifugation at 14000g at 4°C for 10min. This was repeated three times, and the protein pellet was reconstituted in 25µL phosphate buffer. The protein was reduced by incubation with 10mM DTT w/v at 55°C for 15min, and alkylated by incubation with 166mM iodoacetamide w/v for a further 15min at room temperature. The samples were again subjected to methanol precipitation and were reconstituted in 30µL 50mM ammonium bicarbonate buffer. Trypsin (1µg) was added, and the samples were incubated overnight at 37°C. The digestions were desalted using C18 Zip-Tips (Millipore) and dried prior to LC-MS/MS analysis

For LC/MS analysis, digested and ZipTipped samples were reconstituted in 10µL 5% ACN/0.05% v/v TFA, and aliquots of 0.5–2µL were analysed, whereas cation exchange fractions were reconstituted in 120µL of 5% ACN/0.05% TFA, and aliquots of 60µL were analysed. Samples were delivered into a QSTAR® Pulsar I hybrid MS by automated in-line LC (integrated LCPackings System, 5mm C18 nano-precolumn and 75µm×15 cm C18 PepMap column (Dionex, California, USA)) via a 10µm inner diameter PicoTip (New Objective, MA, USA). For prefractionated samples, the nano-precolumn was washed for 30 min with 5% ACN/0.05% TFA prior to initiation of the solvent gradient in order to reduce the salt content of the sample. A gradient from 5% ACN/0.05% v/v TFA to 48% ACN/0.05% v/v TFA in 60min (unfractionated samples) or 70min (fractionated samples) was applied at a flow rate of 300nL/min. MS and MS/MS spectra were acquired automatically in positive ion mode using information-dependent acquisition (Analyst, Applied Biosystems). Survey scans of 1s were acquired for m/z 400–2000, and the three most intense ions were selected for MS/MS, with accumulation times of 1s and with a dynamic exclusion of 40s. Database searching was performed using ProteinPilot version 2 (Applied Biosystems) against the latest version of the SwissProt database, with biological modifications allowed and with the confidence level set to 90%. Flucloxacillin (mass addition 453amu) or 5'-hydroxymethyl flucloxacillin (mass addition 469amu) were included as high probability user-defined modifications of Lys and carboxamidomethyl as a fixed modification of Cys. The data were also assessed manually for the presence of a dominant fragment ion of 160amu, indicative of cleavage of the thiazolidine ring from the drug adduct and the presence of the remaining adduct fragment of 294amu (flucloxacillin) or 310amu (5'-hydroxymethyl flucloxacillin) present on the lysine residue (Jenkins et al. 2009).

4.3.12. Adoptive transfer of flucloxacillin sensitisation to naive mice via I.V. injection of flucloxacillin-exposed DCs

BMDDCs were isolated as previously described. Retinoic acid (RA) (10nM) (Sigma, UK) was added to the culture medium on day 4. On day 9, cells were washed and incubated with or without flucloxacillin (0.5mg/mL) with all cells being incubated with LPS (0.5µg/mL), RA (10nM) and GMCSF (5%) in medium containing mouse albumin (0.5%) (Sigma, UK) and L-glutamine (2mM). After 16h, cells were then washed and injected at $0.5x10^6$ cells/mouse via I.V. injection. Mice received the BMDDCS exposed to flucloxacillin or the un-exposed BMDDCS. All mice received I.P. injection of CD4 antibody (100µg) and RA (0.04mg) in Hanks balanced salt solution (100µL). After twenty one days, mesenteric lymph nodes were removed and analysed for flucloxacillin-specific proliferation via previously described [^{3}H] thymidine incorporation.

4.3.13. Statistics

Unless otherwise stated, statistics were performed on SigmaPlot with significance being devised using the students T-test. Error bars represent one standard deviation from the mean.

4.4. RESULTS

4.4.1. Flucloxacillin-specific $CD8^+$ T-cell proliferation

To quantitatively analyse the number of $CD8^+$ T-cells that were proliferating in an antigen-specific manner, $CD8^+$ T-cells from the dLNs of flucloxacillin-sensitised mice were analysed through CFSE staining after five days incubation with flucloxacillin. Flucloxacillin-sensitised dLN cells incubated at 0.25-1mg/mL flucloxacillin showed a 5%-11% increase in $CD8^+$ T-cell proliferation (Fig 4.1.). A maximal response was observed with 0.25-0.5mg/mL flucloxacillin indicating that the highest drug-concentration was inhibiting the proliferative response (Fig 4.1.).

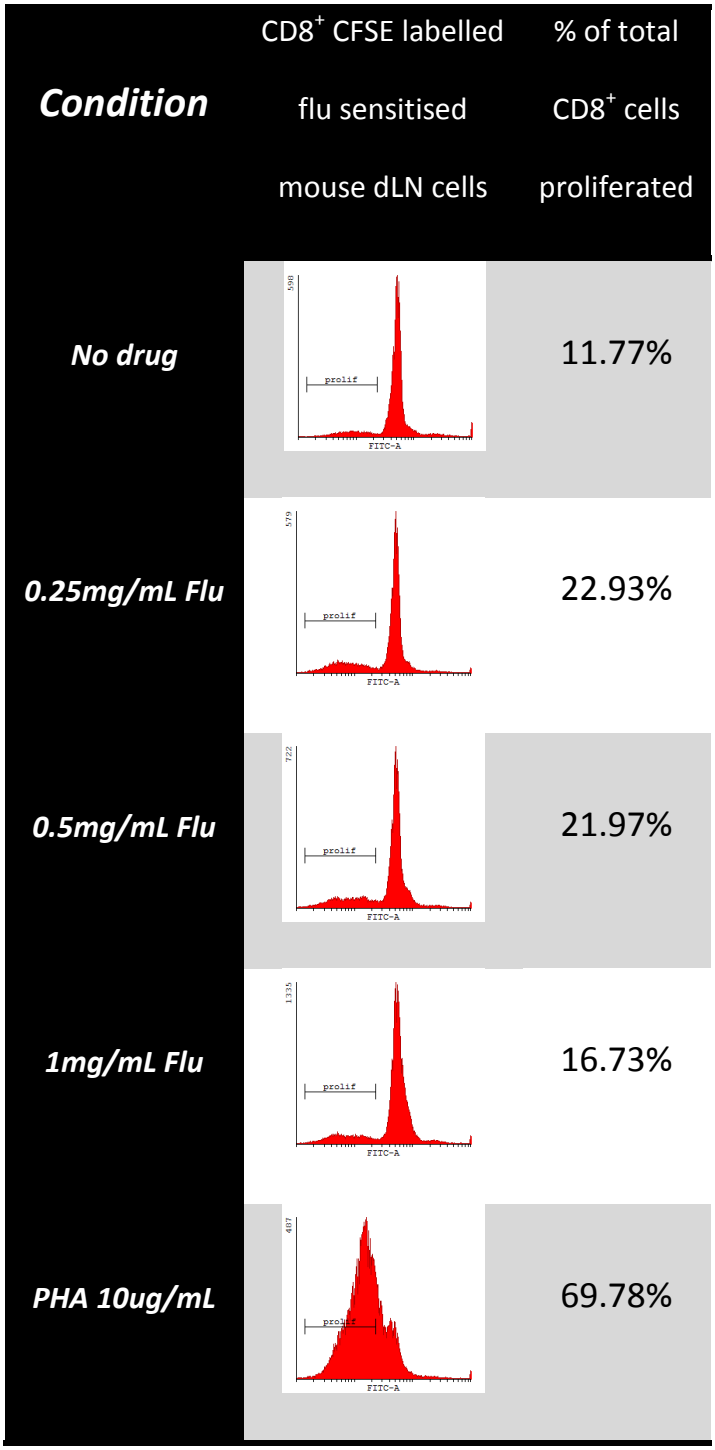


Fig 4.1. $CD8^+$ T-cells from the dLNs of flucloxacillin-painted mice proliferate in an antigen-specific fashion. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injection of an anti-CD4 antibody (100µg) on day 0 and 7. Mice were painted with flucloxacillin (1g/mL, 50µL) for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated in triplicate with APCs collected from the mesenteric LN through CD11c positive magnetic isolation. Cells were incubated for 5 days with CFSE (FITC) and drug after which cells were harvested, washed, incubated with an anti-CD8 antibody (APC) and analysed for proliferation through gating on $CD8^+$ CFSE fluorescence (n=1 with cells pooled from three mice per condition).

4.4.2. APCs are required for flucloxacillin-specific T-cell activation

To investigate the impact of the addition of APCs into the *ex vivo* readouts (proliferation and IFNγ secretion), flucloxacillin-specific T-cells were analysed with varying levels of APCs in the assays. When CD11c positive cells were removed from the assay both flucloxacillin-specific dLN cell proliferation and IFNγ secretion were not detected (Fig 4.2. A1 and A2). When the dLN containing resident DCs were analysed, significant increases in proliferation were observed with flucloxacillin however, a significant increase in IFNγ secretion was not detected in culture supernatant (Fig 4.2. B1 and B2). Finally, when CD11c positive cells were isolated from the non dLN and added to the assay, significant increases in flucloxacillin-specific proliferation and IFNγ secretion were observed (Fig 4.2. C1 and C2). Similar results were obtained in repeat experiments when an IFNγ ELISpot was used to detect $CD8^+$ T-cell activation demonstrating that cytokine secretion was only stimulated upon addition of APCs to the assay (Fig 4.3.).

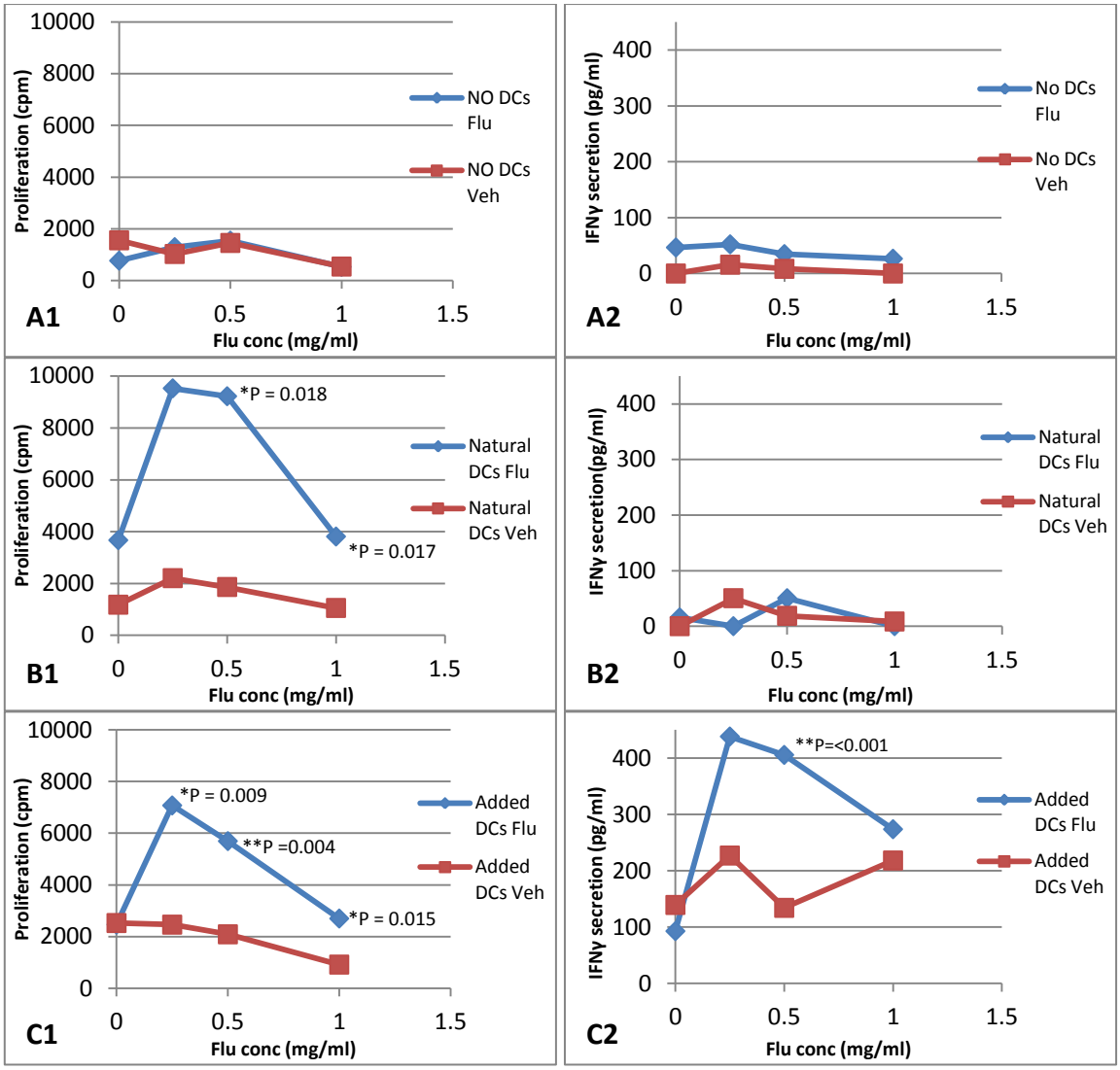
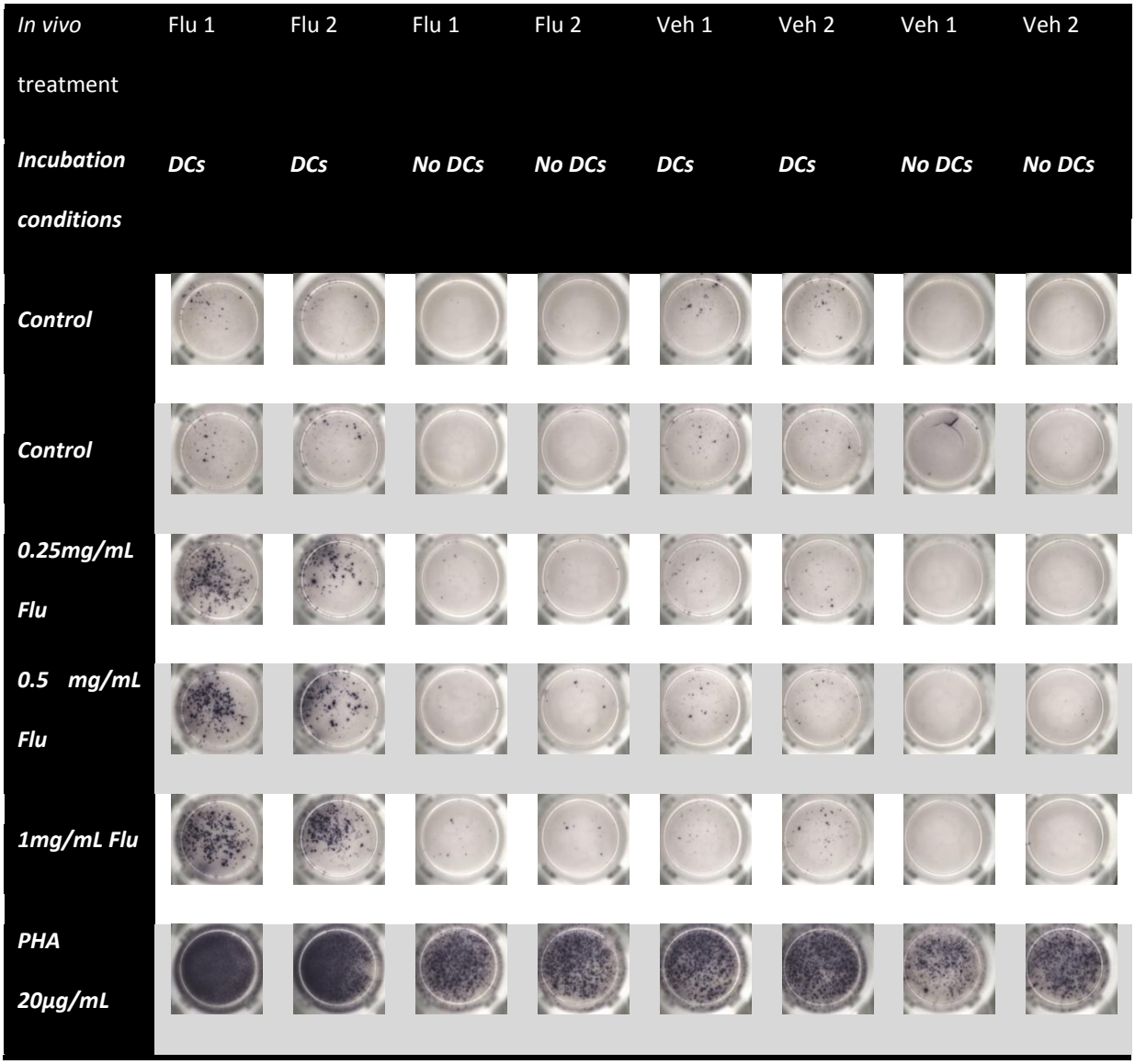


Fig 4.2. *Ex vivo* $CD8^+$ T-cell activation with flucloxacillin requires APCs. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injections of anti-CD4 antibody (100µg) on day 0 and 7. Mice were painted with flucloxacillin (1g/mL, 50µL) or vehicle only for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated in triplicate with; CD11c cells removed (A), no DC addition or removal (natural DCs)(B), or addition of $2.5x10^4$ CD11c positive APCs collected from the mesenteric LN through CD11c positive magnetic isolation (C). Cells were cultured for 5 days at 37°C and 5% CO_2 after which proliferation was measured through [^{3}H] thymidine incorporation after 16h (1) or supernatants were removed and analysed for IFNγ content through IFNγ ELISA (2). P-values were devised using the students T-test or Mann Whitney, where appropriate and are shown if $p<0.05(n=3)$. Standard deviation error bars have been removed to increase data clarity and comparability.



Incubation conditions	Flu 1-DCs	Flu 2-DCs	Flu 1-No DCs	Flu 2-No DCs	Veh 1-DCs	Veh 2-DCs	Veh 1-No DCs	Veh 2-No DCs
0mg/mL Flu	0	0	0	0	0	0	0	0
0.25mg/mL Flu	220.5	77.5	11.5	5.5	0	0	0	8.5
0.5mg/mL Flu	150.5	110.5	12.5	11.5	0	0	0	12.5
1mg/mL Flu	199.5	189.5	18.5	3.5	0	0	0	0
PHA 20ug/mL	TNTC	TNTC	503.5	513.5	400.5	390.5	416	529.5

Fig 4.3. APCs are required to stimulate flucloxacillin-specific $CD8^+$ T-cells to secrete IFNγ. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injections of anti-CD4 antibody (100µg) on day 0 and 7. Mice were painted with flucloxacillin (1g/mL, 50µL) or vehicle only for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated in an ELISpot plate with and without the addition of CD11c positive APCs.

Fig shows the ELISpot wells after development and table shows the number of spot forming cells with control values subtracted (too numerous to count – TNTC) (n=2 shown).

4.4.3. Modification of human albumin by flucloxacillin is time dependent

The covalent modification of human serum albumin was analysed through LC/MS to give a semi-quantitative value of time required for flucloxacillin-induced covalent modification. In short, flucloxacillin was incubated with human albumin for 0.5, 1, 2, 3, and 16h before removal of drug and analysis of covalent binding of flucloxacillin through tryptic digestion and subsequent mass spectrometry. The number of covalently-modified lysine residues is semi-quantified to reveal increasing numbers of covalent-modification over time (Fig 4.4.).

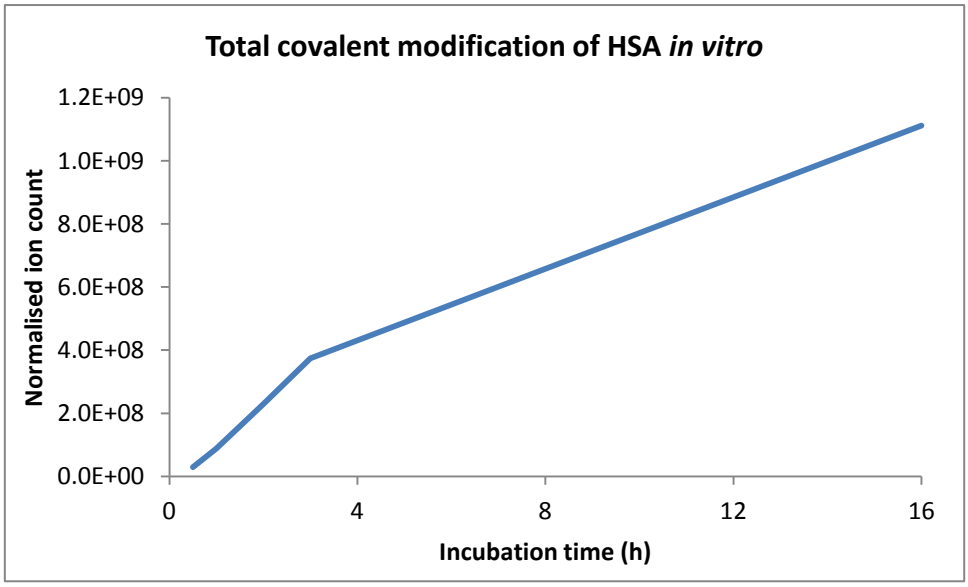


Fig 4.4. Covalent binding of flucloxacillin to human albumin lysine residues increases over time. After in vitro incubation of human albumin (1.3 mg, 40µM) with flucloxacillin (400µM, pH 7.4, at 37°C), tryptic digestion of and MS/MS spectra of tryptic peptides 210–218 and 182–195 revealed flucloxacillin modification of Lys212 and Lys190, respectively. Cleavage of the thiazolidine ring of the adduct yields a characteristic fragment ion of m/z 160 and cleavage of the entire adduct from the peptide yields an ion of m/z 454 in each spectrum. Semi-quantitative analysis of the time dependent increase in the peptides containing modified Lys212 and Lys190 are based on the relevant extracted masses followed by normalisation using the total ion count for the sample.

4.4.4. Flucloxacillin pulsed dendritic cells activate flucloxacillin-specific CD8$^+$ T-cells

As high DC numbers were required for future experiments, CD11c$^+$ DCs isolated from LN were changed to BMDDCs. To explore whether BMDDCs act as suitable APCs and present flucloxacillin-hapten protein complexes, they were incubated with dLN cells to assess the flucloxacillin-specific T-cell activation response (Fig 4.5.). Similar to the dLN cell derived DCs, they induced a significant increase in proliferation upon addition of flucloxacillin at 0.5 and 1mg/mL (Fig 4.5.). Unlike CD11c$^+$ DCs however, the flucloxacillin-sensitised dLN cell proliferative curve started with high backgrounds and remained elevated with increasing flucloxacillin concentrations (Fig 4.5.).

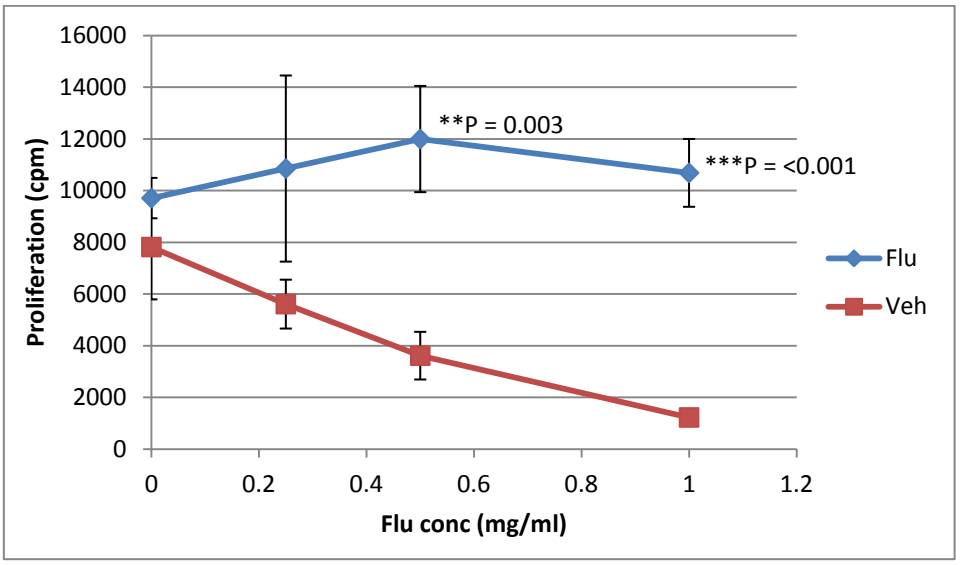


Fig 4.5. Flucloxacillin-specific CD8$^+$ T-cells proliferating with BMDDCs as APCs. MHC II KO mice were depleted of CD4$^+$ T-cells with I.P. injections of anti-CD4 antibody (100µg) on day 0 and 7. Mice were painted with flucloxacillin (1g/mL, 50µL) or vehicle only for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated in triplicate at 1.25x10^5 cells/well with BMDDCs which were added at 2.5x10^4 cells/well. After 5 days incubation, cells were analysed for proliferation through [^{3}H] thymidine incorporation after 16h. P-values are shown if p<0.05 *p<0.05, **p<0.05, ***p<0.005 (n=3).

In subsequent experiments, to determine whether BMDDCs process and present flucloxacillin-hapten protein complexes, BMDDCs were cultured with 0.5mg/mL flucloxacillin for 1, 4, or 24h, then washed and incubated with flucloxacillin-sensitised mouse dLN cells for five days, after which IFNγ secretion was measured (Fig 4.6.). Significant IFNγ release was detected at 24h DC pulsing (Fig 4.6.) when compared to vehicle painted controls. Due to the washing steps in-between the incubation of APCs with flucloxacillin and the incubation of these pulsed APCs with the flucloxacillin specific CD8+ T-cells generated via the mouse model; the drug-specific increases in IFNγ secretion via CD8+ T-cell activation seen in Fig 4.6. are more likely to be through haptenisation of protein and subsequent intracellular processing (hapten hypothesis) than through long term non-covalent binding to the MHC I molecule (pi-concept or altered peptide concept).

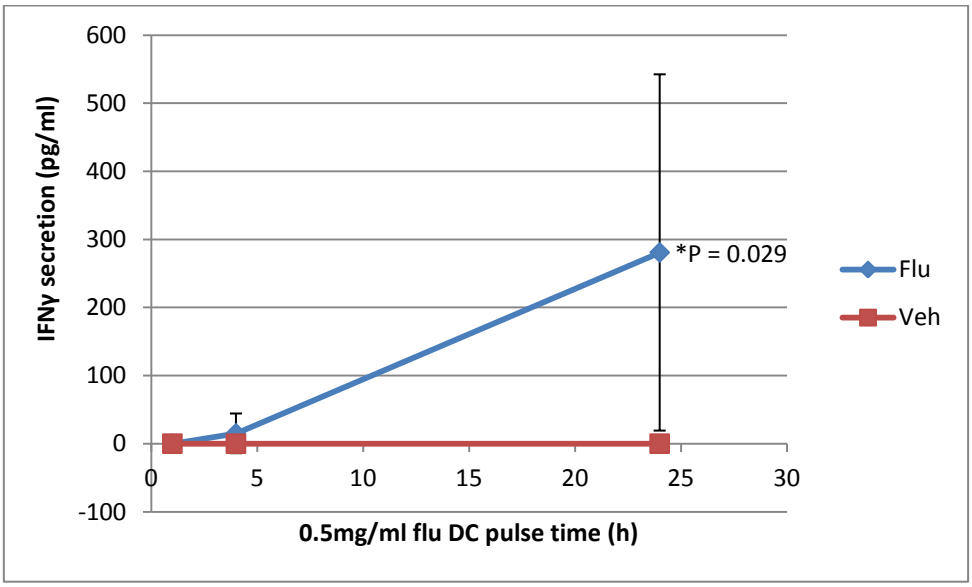
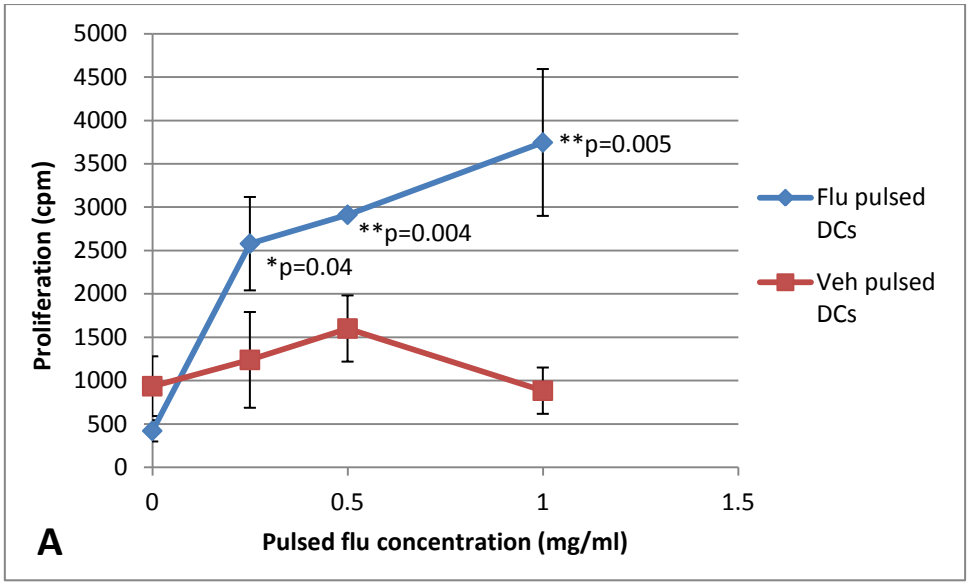


Fig 4.6. Flucloxacillin-specific $CD8^+$ T-cells are activated with BMDDCs pulsed with flucloxacillin for 24h. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injection of anti-CD4 antibody (100µg) on day 0 and 7. Mice were painted with flucloxacillin (1g/mL, 50µL) or vehicle only for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated in triplicate at $1.25x10^5$ cells/well with flucloxacillin treated BMDDCs. BMDDCs were generated and incubated for 1, 4, and 24 hours with flucloxacillin (0.5mg/mL) before being harvested, washed and plated out at $2.5x10^4$ cells/well. There was no soluble drug in assay. After 5 days incubation, cells were analysed for IFNγ secretion through removal of supernatant and analysis by IFNγ

ELISA. P-values were devised using the students T-test or Mann Whitney, where appropriate and are shown if $p<0.05$ (n=4).

To confirm the pulsing experiment, BMDDCs were pulsed for 24h with 0.25, 0.5, and 1mg/mL flucloxacillin, washed to remove un-bound drug and then incubated with mouse dLN cells. Significant proliferative responses were detectable at all flucloxacillin concentrations (Fig 4.7. A). Furthermore, significant IFNγ release from dLN cells was seen at 0.5mg/mL and 1mg/mL flucloxacillin (Fig 4.7. B). Why keeping flucloxacillin in the assay during the five day incubation with BMDDCs causes high background proliferation (Fig 4.5.), whilst pulsing BMDDCs does not (Fig 4.7.), is unknown. One possible explanation could lie in five days in culture with flucloxacillin leading to a maturing of the un-matured BMDDCs.



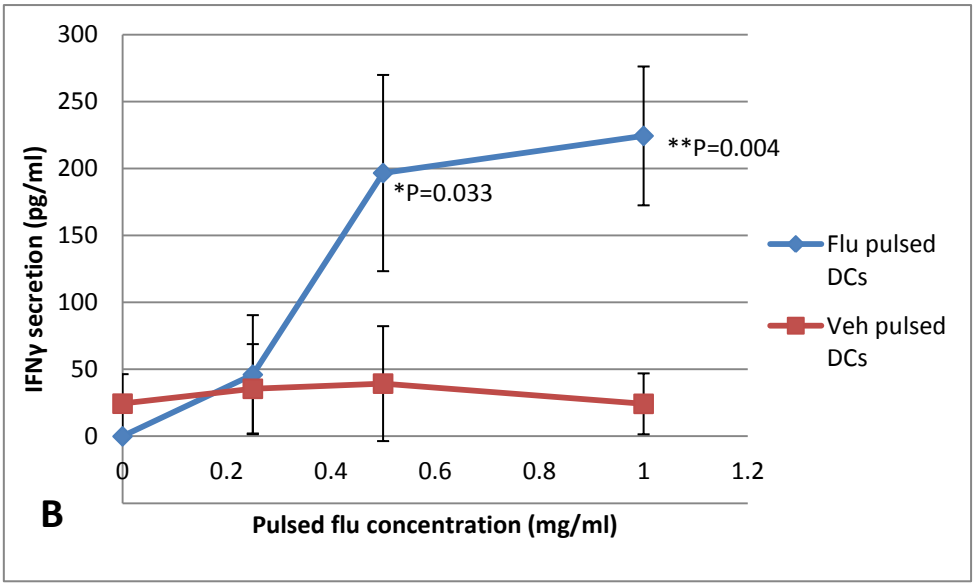


Fig 4.7. 24h flucloxacillin pulsed mouse DCs incubated with dLN cells of flucloxacillin-painted mice showing $CD8^+$ T-cell activation. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injections of anti-CD4 antibody (100µg) on day 0 and 7. Mice were painted flucloxacillin (1g/mL, 50µL) or vehicle only for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated in triplicate with flucloxacillin incubated BMDDCs. BMDDCs were incubated for 24h at 37°C and 5% CO_2 at the stated flucloxacillin concentrations. BMDDCs were thoroughly washed before addition to dLN cells. There was no soluble drug in assay. After 5 days incubation, cells were analysed for proliferation through [^{3}H] thymidine incorporation after 16h (A) or for IFNγ secretion through removal of supernatant and analysis by IFNγ ELISA (B) (n=3).

4.4.5. Adoptive transfer of flucloxacillin pulsed dendritic cells to induce sensitisation in naive mice

Since it was possible to activate flucloxacillin-sensitised $CD8^+$ T-cells with flucloxacillin-pulsed DCs *in vitro*, the next step was to explore whether $CD8^+$ T-cells could be sensitised with flucloxacillin-pulsed DCs *in vivo*. BMDDCs were generated, matured, incubated with RA to imprint gut homing, and then incubated for 24h with flucloxacillin (0.5mg/mL) before being I.V. injected into naive $CD4^+$ T-cell depleted MHC II KO mice at $0.5x10^6$ DCs/mouse. Twenty-one days after immunisation, the mesenteric lymph nodes were removed, macerated to a single cell suspension, and incubated with APC and flucloxacillin for five days after which antigen-specific proliferation was measured via [^{3}H] thymidine incorporation. Mice which were adoptively transferred flucloxacillin-exposed mature DCs demonstrated an antigen-specific increase in proliferation to flucloxacillin when compared to controls (Fig 4.8.).

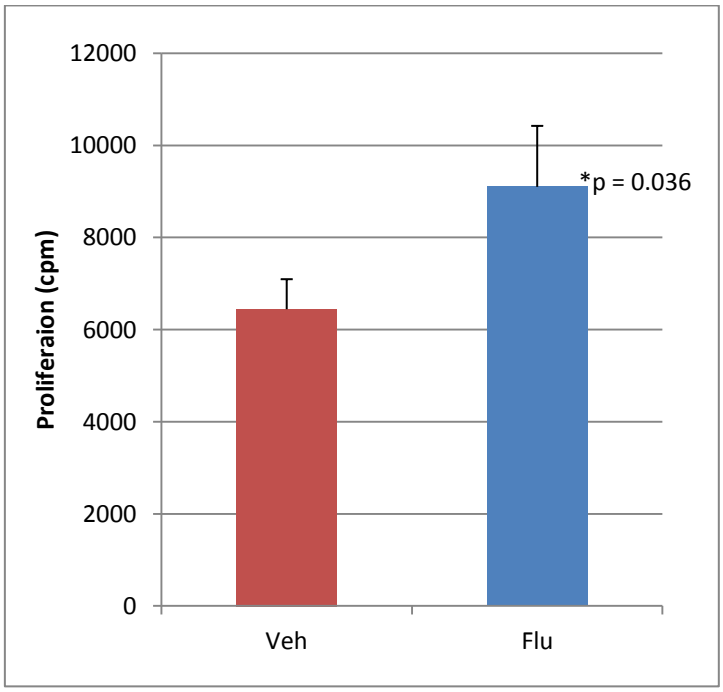


Fig 4.8. Adoptive transfer of flucloxacillin sensitisation to naive CD4 depleted mouse. BMDDCs were generated, matured, incubated with RA (10nM) for 4 days, and with flucloxacillin (0.5mg/mL) or medium only for 16h

before washing and I.V. injecting into a CD4 depleted MHC II KO mice. Mice were left for 21 days before mesenteric LNs were taken, and analysed for flucloxacillin-specific proliferation after 5 days through [^{3}H] thymidine incorporation after 16h (n=3, one mouse per n-number).

4.5. DISCUSSION

The proliferation of drug-specific T-cells in an antigen-specific manner *ex vivo* is commonly used to diagnose drug hypersensitivity reactions in humans. The results shown here indicate that flucloxacillin sensitisation and elicitation in this mouse model is dependent on APCs and that T-cell activation is driven through a hapten-based mechanism dependent on antigen processing by APCs.

Proliferation measured through [^{3}H] thymidine incorporation measures whole well proliferation instead of CD8$^+$ T-cell per se. To confirm that the proliferating dLN cells were in fact CD8$^+$ T-cells, proliferation was also measured through CFSE incorporation and flow cytometry. CFSE is a fluorescent dye, which is split between daughter cells during each cell division. A particular advantage of this technique is that specific cell populations (CD8$^+$ T-cells) can be analysed (Hanafusa et al. 2012). These experiments confirm that flucloxacillin-sensitised CD8$^+$ T-cells were proliferating *ex vivo* in a drug-concentration-dependent manner with maximal proliferation at 0.25mg/mL. Interestingly, a higher drug concentration inhibited the proliferative response indicating that at these concentrations, flucloxacillin inhibits cell division.

To attempt to dissect the mechanism by which flucloxacillin activates T-cells from sensitised mice, the role of APCs was analysed in the *ex vivo* assays. Experimental data showed that the removal of APCs from the assays stopped the activation of the CD8$^+$ T-cells with ablation of proliferation and IFNγ secretion. Furthermore, pulsed and washed APCs were still able to

activate flucloxacillin specific CD8+ T-cells. This CD8+ T-cell activation in the absence of soluble drug *ex vivo* suggests that T-cell activation is progressing via a hapten-based mechanism emulating HLA-B*57:01 negative human patient flucloxacillin hypersensitivity (Daly et al. 2009) (Wuillemin et al. 2013).

In analysis of the APC removal assays, the amount of T-cell activation was directly correlated with the amount of APCs in the assay. Flucloxacillin-specific T-cells incubated with the most APCs (added DCs) significantly proliferated and secreted IFNγ. A reduction in the amount of APC in the assay (natural DCs) induced less flucloxacillin-specific T-cell activation, with significant proliferation but no significant secretion of IFNγ. The same patterns have been reported with the use of human T-cell clones with particular T-cells requiring extra antigen stimulation to secrete IFNγ but not to proliferate (Hecht et al. 1983).

The ability of flucloxacillin-pulsed DCs to induce drug-specific $CD8^+$ T-cell activation has also been investigated using human T-cell clones. These experiments found that human T-cells were activated via a similar hapten mechanism where flucloxacillin-specific $CD8^+$ T-cells were activated by APCs pulsed for 16h with flucloxacillin to yield increases in T-cell activation, measured through up-regulation of activation marker CD107a (Wuillemin et al. 2013). Shorter APC pulses failed to activate T-cells and importantly these time-points correlate with the time required for flucloxacillin to modify protein (Monshi et al. 2013) (Jenkins et al. 2009) (Fig 4.4.).

Adoptive transfer of sensitisation through injection of drug-exposed immune cells has been used historically in immunology research to identify immunogenic compounds or to induce immunity (Elsheikh et al. 2010) (Mitchison 1955). In regards to drug hypersensitivity, adoptive transfer of DCs has been used with drugs like SMX to identify the immunogenic

metabolite SMX-NO (Elsheikh et al. 2010), or with amoxicillin-specific $CD8^+$ T-cells to identify effector mechanisms of CHS (Vocanson et al. 2006). As flucloxacillin-pulsed APCs were found to activate flucloxacillin-specific $CD8^+$ T-cells *ex vivo*, the next step was to investigate whether they could also prime $CD8^+$ T-cells *in vivo*. The benefit if this would be to create a more clinically accurate mouse model of flucloxacillin-induced delayed-type hypersensitivity, where the mesenteric lymph nodes were the site of $CD8^+$ T-cell priming, instead of the inguinal lymph nodes when flucloxacillin is painted onto abdomen skin.

In conclusion, sensitising naive mice to flucloxacillin via adoptive transfer of flucloxacillin-exposed DCs demonstrated that sensitisation, as well as elicitation, to flucloxacillin is most likely mediated through a hapten mechanism.

CHAPTER 5

Use of a mouse model to investigate flucloxacillin-induced liver damage

Contents

5.1. INTRODUCTION

Delayed-type hypersensitivity reactions involving the immune system are a serious complication in both patient treatment and drug development. Although the skin is usually the organ under duress in drug hypersensitivity reactions, systemic drug hypersensitivity reactions pose a real problem for certain drugs like flucloxacillin which primarily causes DILI (Park et al. 2011). There are currently no animal models of DILI to show flucloxacillin damaging liver cells indirectly via activation of adaptive immune responses. However, one group has shown that after high doses of dicloxacillin (600mg/kg, approximately 8x maximum human dose) (a drug structurally similar to flucloxacillin), Th2 expression of the mediated factor IL-4 is increased in blood serum along with ALT and bilirubin levels. Furthermore, the authors stated that neutralisation of IL-4 reduced the hepatotoxicity (Higuchi et al. 2011). This model however, relied on an incredibly high dose of flucloxacillin and showed very few signs of cholestatic liver damage, the mechanism by which dicloxacillin (and flucloxacillin) usually causes liver damage in humans (Park et al. 2011) (Daly et al. 2009).

Flucloxacillin has previously been shown to form liver adducts *in vivo* through western blotting of liver cytosol of rats systemically dosed with flucloxacillin; the adducts formed were found to be at the same molecular weight of mouse albumin (Carey and van Pelt 2005). As previously discussed flucloxacillin and other β-lactams are known to bind to lysine groups on protein to form adducts. Flucloxacillin has been shown to bind specifically *in vitro* to human serum albumin at Lys190 and Lys212. The level of binding of flucloxacillin to specific lysine residues has been shown to correlate with the activation of T-cell clones (Monshi et al. 2013); however the specific binding involved in activating T-cells *in vivo* has not been defined. As human albumin is produced inside hepatocytes, the preferential

activation of $CD8^+$ T-cells in patients with flucloxacillin-induced liver injury (Monshi et al. 2013) could be attributed to flucloxacillin forming an intracellular antigen. If $CD8^+$ T-cells are the primary mediators of flucloxacillin-induced liver injury, immune cells must be prompted to migrate to the liver. The ability of DCs and $CD8^+$ T-cells to migrate to specific lymph nodes is mediated through secreted chemokines able to cause cell migration after binding to G-protein coupled receptors. Dendritic cells from the mesenteric lymph nodes are known to imprint gut homing onto co-cultured T-cells through releasing soluble factors (Mora et al. 2005) and the soluble factor in question was found to be retinoic acid (RA) (Iwata et al. 2004). *In vitro* culture of RA with DCs and T-cells up-regulated gut homing receptor CCR9 expression (Jaensson et al. 2008) and subsequent *in vivo* mesenteric lymph node homing (Svensson et al. 2008). Experiments performed using staining and injection of activated DCs into liver parenchyma showed that three lymph nodes seem to drain the liver, the coeliac and portal nodes with the first mesenteric lymph node performing a secondary role (Barbier et al. 2012). For T-cells to migrate to the mesenteric lymph nodes, expression of integrin α4β7 and the chemokine receptor CCR9 is essential and is usually induced through antigenic stimulation by gut homing DCs (Svensson et al. 2008); however, retinoic acid is known to stimulate the expression of both integrin α4β7 and the chemokine receptor CCR9 (Iwata et al. 2004). Liver cells and their ability to process and present exogenous antigens to activate $CD8^+$ T-cells has previously been investigated: liver sinusoidal epithelial cells and Kuppfer cells both present exogenous antigen to promote $CD8^+$ T-cell proliferation comparable and in instances, increased when compared against the professional APCs (e.g. mature DCs). However, the increased $CD8^+$ T-cell proliferation was not accompanied with increases in IFNγ or T-cell activation markers giving an insight into how antigen presentation could affect liver immune responses. Hepatocytes, although giving reduced amount of $CD8^+$ T-cell

proliferation, were also shown to present exogenous antigen (Ebrahimkhani et al. 2011). This ability of liver epithelial cells to present antigen as efficiently as mature DCs is interesting in regards to flucloxacillin-induced cholestatic liver damage, where the epithelial cells of the bile ducts are the cells under duress. Flucloxacillin-induced cholestasis is defined as being painless jaundice with increased ALT, ALP, and bilirubin levels which are indicative of bile duct obstruction (also known as vanishing bile duct syndrome), where other causes have been eliminated (Russmann et al. 2005) (Miros et al. 1990).

Liver cells contain high amounts of the enzyme ALT which is released when liver cells undergo cell death. ALT levels are detectable in the blood of patients and are used as a marker of liver disease with normal values between approximately 7-50 international units per litre (IU/L) (Panteghini et al. 1983). Although widely used as a liver damage biomarker, ALT is also found, albeit at lower levels, in locations such as the kidney, lung and skeletal muscle which can be problematic as strenuous exercise or unknown factors un-related to liver damage can increase blood serum ALT levels (Giannini et al. 2005). This means ALT levels alone are not a solid readout of liver damage and other liver damage biomarkers are required. Recently, micro-RNAs have been investigated in regards to markers of liver damage detectable in blood serum (Wang H. et al. 2012). MicroRNAs are released from certain tissues cells upon apoptosis or necrosis with most tissues having specific microRNA associations. To investigate possible liver associated microRNAs in DILI, a mouse model of acetaminophen induced liver damage has been reported; a panel of 44 microRNAs were screened and increases in blood serum levels of microRNA mir-122 were observed in mice with liver injury (Wang K. et al. 2009). Mir-122 has since been shown to be increased in the blood serum of human patients with DILI (Wang H. et al. 2012). Blood serum mir-122 levels have been investigated in this chapter alongside let-7d and lin-4 microRNA levels. Let-7d is

believed to become up-regulated during developmental processes (Kloosterman and Plasterk 2006). Lin-4 is a microRNA which acts to dictate the onset of larval stages of developmental events in *C. elegans* (Lee et al. 1993) and has been added to samples manually as an internal normalisation control while let-7d has been used here as an external normalisation control against levels of mir-122.

Alkaline phosphatase (ALP) is a group of enzymes that hydrolyze phosphate esters and which can also be found within a number of human tissues. Clinically, ALP levels can be measured in human serum to give an indication of obstructive and space-occupying lesions of the liver. Cholestatic liver conditions are associated with liver damage through obstructive means and so increased ALP levels can be used as a diagnostic tool for the detection of the early stages of cholestasis (Epstein et al. 1984).

To investigate flucloxacillin-induced liver injury three approaches have been undertaken where flucloxacillin-specific $CD8^+$ T-cells have been directed towards the liver and then an elicitation challenge has been administered in the form of oral dosing at relative therapeutic doses.

1. Mature BMDDCs were cultured with RA and flucloxacillin, then I.V. injected into naive mice. The hypothesis here was that these mature DCs would migrate to the mesenteric lymph nodes and present flucloxacillin altered peptides to $CD8^+$ T-cells which would, in turn, undergo clonal expansion to flucloxacillin-modified peptides.
2. Flucloxacillin-specific $CD8^+$ T-cells from the dLN as well as $CD11c^+$ DCs from the non-dLN were isolated and cultured with RA before injection into naive mice. DCs were also cultured with flucloxacillin prior to injection. The hypothesis here was that all

injected immune cells would migrate to the mesenteric LNs without need for *in vivo* priming of flucloxacillin-specific $CD8^+$ T-cells.

3. To avoid the pitfalls of *ex vivo* cell culture, RA painted directly onto the skin of flucloxacillin-sensitised mice in the hope it would induce mesenteric LN gut homing receptor expression. The hypothesis here was that the skin DCs which migrate to the nearest draining lymph node in the skin to induce clonal expansion to foreign antigen, would be induced to travel to the mesenteric lymph nodes instead.

In this chapter the binding of flucloxacillin to intracellular hepatocyte albumin was also studied alongside the cytotoxic abilities of flucloxacillin-specific $CD8^+$ T-cells after *ex vivo* elicitation and *in vivo* elicitation, in the hope of developing a model of flucloxacillin-induced liver injury, mediated via cytotoxic actions of the adaptive immune system.

5.2. AIMS

- To investigate flucloxacillin binding to protein *in vivo*
- To analyse the cytotoxic ability of flucloxacillin-specific $CD8^+$ T-cells generated in the mouse model against hepatocytes *in* and *ex vivo*.
- To develop a mouse model of flucloxacillin-induced liver injury

5.3. METHODS

5.3.1. Mice

As previously described, all mice used were between 8-20 weeks of age and were C57/Bl6 MHC II KOs with a mutation in the αβ gene encoding for MHC class II molecules. To deplete $CD4^+$ T-cells from mice, I.P. injections of rat anti-mouse CD4 antibody were administered on day 0 (100µg/mouse) and on day 7 (100µg/mouse) in 100µL Hanks balanced salt solution.

5.3.2. Drugs and chemicals

Intra-venous grade flucloxacillin was obtained from Royal University Hospital, Liverpool. Hanks balanced salt solution for hepatocyte isolation (Gibco, UK), Collagenase D (Roche, CA), $NaHCO_3$, HEPES, $CaCl_2$, DNAse and Trypsin inhibitor from soybean were from Sigma, UK.

5.3.3. Dosing to generate flucloxacillin-specific $CD8^+$ T-cells

Mice were sensitised for days 1-3 through painting with flucloxacillin (50ul, 1g/mL) in 70% DMSO on a shaved abdomen of approximately $3cm^2$ surface area. Vehicle mice were painted with 70% DMSO only. Tissues were removed on day 8 for *ex vivo* readouts.

5.3.4. Mouse immune cell isolation

For *ex vivo* readouts dLNs were removed from mice on day 8. Immune cells were isolated from dLNs via maceration through 100µm nylon filter (BD Biosciences, UK) and then washed and counted. To isolate CD11c positive APCs, the mesenteric LNs were removed from mice and incubated with CD11c positive magnetic beads (Miltenyi Biotec – UK) before being run through a magnetic column (Miltenyi Biotec – UK) then washed and counted.

5.3.5. Granzyme B analysis

Flucloxacillin-specific and vehicle only T-cells were plated out ($1.25x10^5$cells/well) with CD11c positive DCs magnetically isolated from the non-dLNs ($2.5x10^4$cells/well) in a pre coated ELISpot plate. Plates were incubated for 48h in a humidified 37 °C CO_2 incubator after which wells were developed according to the mouse granzyme B ELISpot kit instructions (R&D Biosciences, UK). Briefly, cells were removed and wells washed with 250µL of supplied wash buffer four times, completely removing the liquid in-between washes. Detection antibody was added (100µL of kit stock) to wells and plates incubated at 4°C overnight. Washes were repeated and the Streptavidin-AP was added to each well (100µL of kit stock) and plates incubated for 2h at room temperature. Plates were washed again and then BCIP/NBT chromogen was added to each well (100µL) and plates incubated for 1h at room temperature in the dark. Wells were rinsed with de-ionised water and counted on an AID ELISpot reader (Cadama Medical, Stourbridge, UK) when thoroughly dried.

5.3.6. Systemic administration of flucloxacillin for the analysis of drug protein binding

MHC II KO mice were depleted of $CD4^+$ T-cells on day 0 and on days 1-5 were dosed I.P. with flucloxacillin (14mgs, 100µL dH_2O). On day 8 blood was taken from each mouse through cardiac puncture and centrifuged to separate out blood serum to be analysed for flucloxacillin-albumin binding. On day 8 livers were also removed to be fractionated and analysed for flucloxacillin-albumin binding to measure drug antigens.

5.3.7. Liver fractionation to isolate cytosol

Livers were removed after systemic dosing, chopped and homogenised. The liver cytosol was isolated through centrifugation of homogenised liver at 100,000g and removal of the

cytosol containing supernatant as described by Christen et al (Christen et al. 1991). The pellet was resuspended in buffer (0.1 M Tris, 1 mM EDTA, 10% glycerol, pH 7.4) and stored at −70 °C. The protein concentrations of the samples were determined using the method described by Bradford (1976) using the Bio-Rad (Bio-Rad, Hemel Hempstead, U.K.) protein assay reagent.

5.3.8. Western blotting and coomassie staining

Aliquots of 5µg protein were separated by electrophoresis on a 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membrane. Non-specific binding was blocked using Tris/saline/Tween buffer (TST; NaCl, 150 mM; Tris-HCl, 10 mM; Tween 20, 0.05% [pH 8]) containing 10% non-fat dry milk for 16h at 4°C. The blot was incubated with primary anti-flucloxacillin antibody (gift from Prof. Van Pelt, Dublin, Ireland) diluted 1:20,000 in 5% milk/TST for 1h, followed by incubation with HRP-conjugated anti-mouse IgG Ab (Abcam) diluted 1:10,000 in 5% milk/TST for a further 1h. Signal was detected by coomassie blue staining or via ECL (Western Lightning; PerkinElmer, Boston, MA) using autoradiography film and a GS800 calibrated scanning densitometer (Bio-Rad, UK.) (Whitaker et al. 2011).

5.3.9. LC/MS to determine flucloxacillin binding to albumin lysine residues

For LC-MS/MS analysis, digested and ZipTipped snap-frozen liver samples from mice systemically dosed with flucloxacillin were reconstituted in 10µL 5% ACN/0.05% v/v TFA, and aliquots of 0.5–2µL were analysed, whereas cation exchange fractions were reconstituted in 120µL of 5% ACN/0.05% TFA, and aliquots of 60µL were analysed. Samples were delivered into a QSTAR® Pulsar i hybrid MS by automated in-line LC (integrated LCPackings System, 5mm C18 nano-precolumn and 75µm×15cm C18 PepMap column (Dionex, California, USA)) via a 10µm inner diameter PicoTip (New Objective, MA, USA). For

prefractionated samples, the nano-precolumn was washed for 30min with 5% ACN/0.05% TFA prior to initiation of the solvent gradient in order to reduce the salt content of the sample. A gradient from 5% ACN/0.05% v/v TFA to 48% ACN/0.05% v/v TFA in 60min (unfractionated samples) or 70min (fractionated samples) was applied at a flow rate of 300nL/min. MS and MS/MS spectra were acquired automatically in positive ion mode using information-dependent acquisition (Analyst, Applied Biosystems). Survey scans of 1s were acquired for m/z 400–2000, and the three most intense ions were selected for MS/MS, with accumulation times of 1s and with a dynamic exclusion of 40s. Database searching was performed using ProteinPilot version 2 (Applied Biosystems) against the latest version of the SwissProt database, with biological modifications allowed and with the confidence level set to 90%. Flucloxacillin (mass addition 453 amu) or 5'-hydroxymethyl flucloxacillin (mass addition 469 amu) were included as high probability user-defined modifications of Lys and carboxamidomethyl as a fixed modification of Cys. The data were also assessed manually for the presence of a dominant fragment ion of 160amu, indicative of cleavage of the thiazolidine ring from the drug adduct, and the presence of the remaining adduct fragment of 294amu (flucloxacillin) or 310amu (5'-hydroxymethyl flucloxacillin) present on the lysine residue (Jenkins et al. 2009).

5.3.10. Hepatocyte isolation and culture for analysis of immune-mediated killing

MHC II KO mice were anaesthetised with sodium pentobarbital (I.P. ~80µL, 40mg/mL) and once foot reflex had ablated, were surgically opened and the hepatic portal vein exposed and cannulated. The liver was then flushed with wash buffer (Hanks, 900mM $NaHCO_3$, HEPES) for 4 minutes at 12mL/minute and then with digestion buffer (Wash buffer, 5mM $CaCl_2$, 0.5mg/mL collagenase D, 0.07mg/mL trypsin inhibitor from soybean) at 12mL/min until liver began to soften and show striations. The liver was then eviscerated, the

epithelium removed through use of sharp forceps, and the resulting organ placed in a Petri dish. Incubation buffer (Wash buffer, 0.1mg/mL DNAse) was added to just cover the liver and hepatocytes were softly shaken out of tissue. Cell suspension was poured through a 100µm nylon filter to remove large cell clumps then centrifuged (60g, 3 minutes) and washed.

5.3.11. Detection of hepatocyte viability, cytotoxicity and apoptosis

Hepatocytes were isolated, washed, counted via trypan blue exclusion and plated into white flat bottomed 96 well plates at 2500 or 5000 cells/well. $CD8^+$ T-cells were isolated from the inguinal dLNs and plated out with CD11c positive DCs (from the mesenteric LNs) and flucloxacillin as previously described. After 24h T-cells and DCs were washed to remove soluble drug and incubated at $2x10^4$ cells/well with freshly plated out hepatocytes. Plates were incubated for 4h (37°C and 5% CO_2). Cell viability, cytotoxicity, and apoptosis was measured through ApoTox-Glo kit protocols (Promega, UK). Briefly, GF-AFC substrate (100µM) and bis-AAF-R110 substrate (100µM) was added to all wells and incubated for 30 minutes at 37°C after which luminescence was measured at 400Ex/505Em to give a quantitative readout of cell viability and at 485Ex/520Em to give a quantitative readout of cytotoxicity. To measure apoptosis, Caspase-Glo® 3/7 reagent (100µL/well) was added equally to all wells, plates were incubated for 30 minutes at room temperature and caspase activation was then measured through scintillation to give a quantitative readout of apoptosis.

5.3.12. Adoptive transfer of BMDDCs

To generate bone marrow-derived dendritic cells (BMDDCs) the bone marrow was flushed from the femurs of mice with complete RPMI medium and large pieces of bone were

filtered out with a 100µm nylon filter. Cells were incubated with 5% GMCSF (Peprotech, NJ, USA) for 8 days after which immature DCs were harvested. To mature the DCs, LPS (0.5µg/mL) was added to the cultures for 24h before harvesting. RA (10nM) (Sigma, UK) was added for days 4-8. On day 9, cells were washed and incubated with flucloxacillin (0.5mg/mL), LPS (0.5µg/mL), RA (10nM) and GMCSF (5%) in 1640 medium containing 0.5% mouse albumin (Sigma, UK) and L-glut for 16h before injection. Control conditions contained all reagents with the exception of flucloxacillin. Cells were then washed and injected at $0.5x10^6$ cells/mouse I.V. with 2 mice receiving BMDDCS exposed to flucloxacillin and 2 mice receiving un-exposed BMDDCS; all mice simultaneously received I.P. CD4 antibody (100µg) and RA (0.04mgs) in 100ul Hanks balanced salt solution. Twenty-one days later, oral dosing began with all mice receiving 2.3mgs/flucloxacillin/day in 10µL of dH_2O. Readouts of ALT in blood serum were measured on days 0, 2, and 5.

5.3.13. Adoptive transfer of $CD8^+$ T-cells and $CD11c^+$ APCs

Flucloxacillin-specific and control $CD8^+$ T-cells were generated as previously described. $CD8^+$ T-cells ($1.25x10^5$cells/well) were then plated out in U-bottomed 96 well plates with CD11c positive DCs ($2.5x10^4$cells/well) isolated as previously described for 24h in medium containing 0.5% mouse albumin and 10nM RA. T-cells were then harvested, washed, and injected into naive MHC II KO mice at $0.2x10^6$cells/mouse. Alongside T-cell preparation, $CD11c^+$ APCs isolated from mesenteric lymph nodes of MHC II KO mice were incubated in flat bottomed 24 well plates for 24h at $1x10^6$ cells/well in medium containing 0.5% mouse albumin, 10nM RA and with or without 0.5mg/mL flucloxacillin. Cells were then harvested, washed, and I.V. injected alongside non-specific $CD8^+$ T-cells ($0.1x10^6$cells/mouse). Immediately prior to I.V. injections, naive mice were depleted of $CD4^+$ T-cells via I.P. injection of anti-CD4 antibody (100µg) and I.P. injected with RA (14mgs) 100µL Hanks

balanced salt solution. After 3 days, oral dosing began with all mice receiving 2.3mgs/mouse/day of flucloxacillin in 10µL of dH_2O for 10 days. Readouts of mir-122 in blood serum and caspase 3 staining of liver were taken on days 0, 5, and 10.

5.3.14. Painting of retinoic acid and flucloxacillin

Mice were depleted of $CD4^+$ T-cells and painted with flucloxacillin (1g/mL, 50µL) or vehicle alone as previously described. However, in this experiment each mouse received RA (0.5mgs, 50µL) painted onto skin/day. On day 7, oral dosing of flucloxacillin began for 2 days with each mouse dosed with 2.3mgs/flu/day. Readouts consisted of blood serum liver damage biomarkers ALT and mir-122 which were taken prematurely on day 3 of oral dosing due to mouse discomfort caused by skin damage. The experiment was subsequently repeated using reduced concentration of RA (0.1mgs, 50µL) with readouts taken on days 0, 2, 4, and 10 of oral dosing. Readouts included: photography of the gall bladders on day 4 and 10, blood serum analysis of liver damage biomarkers ALT, ALP and mir-122, and histology and immunohistochemistry of the liver.

5.3.15. Histology of liver

After organ dissection, tissues were fixed by immersion in 10% neutral buffered formalin (or 4% PFA). Tissue was removed from fixative and cut to appropriate size/orientation & placed within labelled plastic cassettes. Tissues were then run through a Sakura Tissue Tek VIP E300 Tissue Processor to remove water from tissue & replace it with paraffin wax. Tissues were subsequently placed in metal moulds containing molten paraffin wax and set on cold platform to solidify. Four micron sections were cut on a microtome, floated in water bath and collected onto glass slides. Sections were then dried at 37°C for 30 minutes to allow adherence to slide before staining. Sections were de-waxed in Xylene (Sigma, UK) for 5

minutes and subsequently taken down descending grades of alcohol (100%, 96%, 85%, and 70%) to distilled water. Sections were then stained in either Mayers Haematoxylin (Sigma, UK) or cleaved Caspase-3 monoclonal antibody to clone D175 (5A1E) (Cell Signalling Technology – UK) for 5 minutes. Sections were run under cold distilled water for 6 minutes and then stained sections in eosin for 2 minutes. Sections were taken through 3x 96% alcohol baths, then 3x 100% alcohol baths, then 2x xylene baths, before finally being mounted in DPX mounting medium (Sigma, UK) and allowed to dry. Histological analysis was conducted by Julie Haigh at The University of Liverpool, Leahurst Campus, Neston, United Kingdom.

5.3.16. Measurement of ALT blood serum levels

Mouse blood was left to clot for approximately 3h after which blood serum was separated via centrifugation. Serum samples were diluted in 0.9% saline solution (1;5, 1:10 or 1:20). Sample (30µL) was added to wells in triplicate prior to analysis in the ALT Liquid stable reagent-based kinetic assay (300µL) (Thermo, Waltham, MA), at 37°C, according to the manufacturer's instructions.

5.3.17. Measurement of ALP blood serum levels

As above, mouse blood serum was isolated. Serum sample ALP levels were subsequently processed according to the manufacturer's instructions (Alkaline Phosphatase Assay Kit, Abcam, UK). Briefly, standard curves were set up alongside serum samples where 40µl of the 5mM p-nitrophenyl phosphate (phosphatase substrate which turns yellow when dephosphorylated by ALP) solution was diluted with 160µl assay buffer to generate1mM p-nitrophenyl phosphate standard. 0, 4, 8, 12, 16, 20 nmol/well. Serum samples were diluted 10x with assay buffer and added in triplicate (80µL) to a 96 well plate. P-nitrophenyl

phosphate standards were added to separate wells. p-nitrophenyl phosphate (5mM, 50µL) was then added to each sample well and plates incubated for 60min at 25°C away from light. ALP enzyme solution (10µL) (Abcam, UK) was then added to each well and plates incubated for 60min at 25°C away from light. Stop solution (Abcam, UK) (20µL) was then added to each well and plates read in a microplate reader set to 405nm. ALP activity of samples was calculated through using the formula ALP activity (U/mL) = A / V / T where: A is amount of p-nitrophenyl phosphate generated by samples (in µmol). V is volume of sample added in the assay well (in mL). T is reaction time (in minutes). Standard curves were devised using SigmaPlot.

5.3.18. Measurement of blood serum liver damage biomarker mir-122

miRNA was extracted using an miRNeasy kit (Qiagen, Venlo, Netherlands), following the manufacturer's instructions, with minor modifications. Briefly, 40µL of serum was made up to 200µL with nuclease-free water then combined with 700µL of QIAzol. The sample was mixed and left for 5 minutes before the addition of 140µL of chloroform. Samples were then mixed vigorously for 15 seconds and centrifuged at 12,000g for 15 minutes at 4°C. Equal volumes (350µL) of the upper aqueous phase and 70% ethanol were mixed in a fresh microtube before adding the total volume to an miRNeasy minispin column. The column was centrifuged at 8,000g for 15 seconds at room temperature. The flow-through, containing the small RNA fraction, including the miRNA complement, was mixed with 450µL of 100% ethanol. The elute was then purified using a MinElute kit (Qiagen, Venlo, Netherlands). The small RNA elution mixture was applied to a MinElute column, 700µL at a time. The immobilized RNA was then washed with various buffers before a final 80% ethanol wash. The column was then dried by centrifugation. The small RNA fraction was eluted in 14µL of nuclease-free water and stored at −80°C. MiRNA levels were measured using

Taqman-based quantitative polymerase chain reaction (PCR). The small RNA elute was reverse transcribed using specific stem-loop reverse-transcription RT primers (Applied Biosystems, Foster City, CA) for each target miRNA species, following the manufacturer's instructions. Next, 2µL of RNA was used to produce the complementary DNA (cDNA) template in a total volume of 15µL. Then, 1.33µL of cDNA was used in the PCR mixture with specific stem-loop PCR primers (Applied Biosystems, Foster City, CA) in a total volume of 20µL. Levels of miRNA were measured by the fluorescent signal produced from the Taqman probes on an ABI Prism 7000 (Applied Biosystems). All samples were assayed in duplicate. Levels of miRNA were normalized to levels of ubiquitous let-7d and to externally added lin-4.

5.3.19. Statistics

Unless otherwise stated, statistics were performed on SigmaPlot with significance being devised using the students T-test. Error bars represent one standard deviation from the mean.

5.4. RESULTS

5.4.1. Detection of flucloxacillin-modified albumin peptides *in vivo*

To analyse whether flucloxacillin can covalently modify albumin *in vivo*, the serum of mice dosed systemically with flucloxacillin for 5 days was analysed through western blot with a flucloxacillin-specific antibody. Flucloxacillin-specific binding was strongly detected in serum of all drug-exposed mice (Fig 5.1.). The flucloxacillin-modified band was detected with a molecular weight of approximately 67kDa, the same as mouse albumin. The faint band visible in the serum control is assumed to be non-specific binding, as albumin is known to be especially sticky in *in vitro* culture (Fig 5.1.).

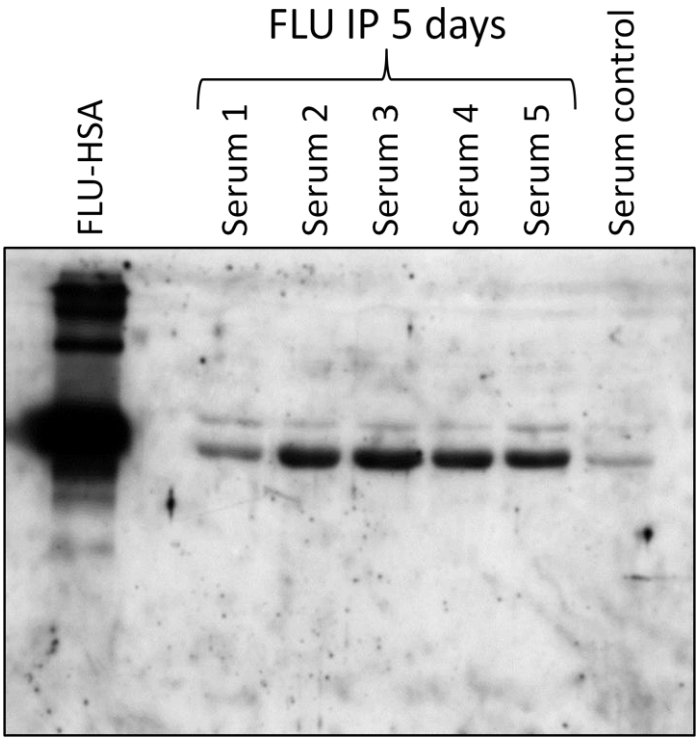


Fig 5.1. Western blot of mouse serum showing a flucloxacillin-modified band at the appropriate molecular weight for mouse albumin. MHC II KO mice were dosed for 5 days with flucloxacillin dosed I.P. Blood serum albumin was then analysed for flucloxacillin binding via western blot using a flucloxacillin-specific antibody. Marker on the left shows human albumin modified with flucloxacillin as reference with serum control being mice dosed with vehicle only. Protein analysis was performed by Roz Jenkins.

Liver cytosol was isolated and proteins present were separated through electrophoresis. The clear bands at the correct molecular weight of mouse albumin were removed (Fig 5.2. A) and analysed via LC/MS to determine if flucloxacillin binding to albumin was detectable. Specific peptides known to have previously been shown to bind to β-lactams were specifically analysed and results showed that for the peptide LDGVK*EK (Fig 5.2. B – underlined), flucloxacillin presence, β-lactam ring cleavage, full length peptide and full length peptide plus the flucloxacillin-molecule was detectable (Fig 5.2. C).

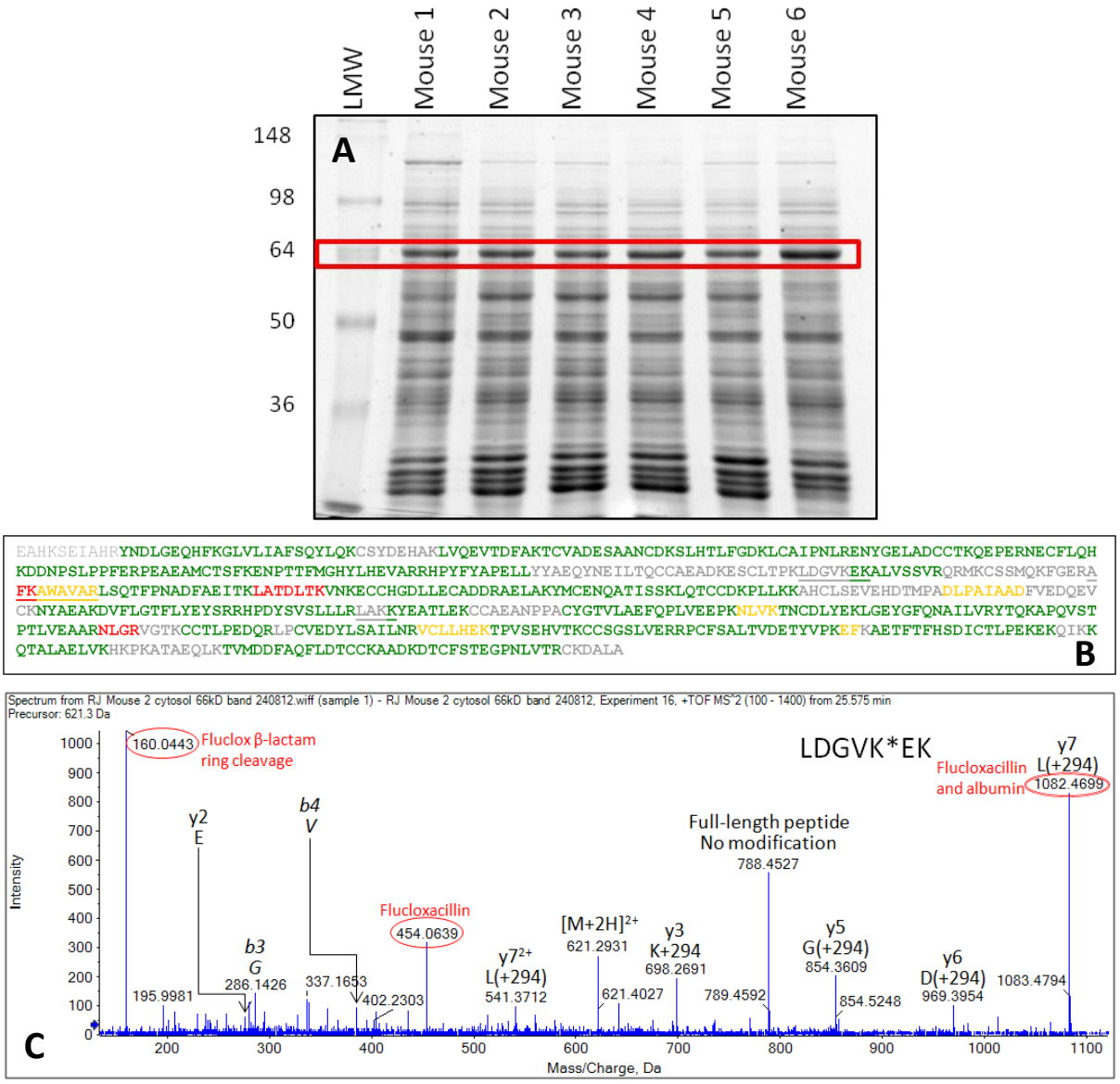


Fig 5.2. *In vivo* binding of flucloxacillin to mouse albumin in liver cytosol. Mice were systemically dosed with flucloxacillin for 5 days via I.P. injection (14mgs/mouse/day) and three days after the final dose, livers were removed, fractionated through centrifugation to isolate cytosol and subsequent cytosol run down a gel and coomassie stained to separate out proteins. Bands at the molecular weight of mouse albumin were cut out (A – outlined), trypsin digested (B - Green = peptide identified at >95% confidence, yellow = peptides identified at >90% confidence, red= low confidence assuming no modification of lysine, grey = not detected in this standard search, underlined peptides are tryptic peptides observed to be modified by flucloxacillin) and analysed through mass spectrometry(C). Peptide fragment LDGV*EK (lys 186) is shown in C from mouse 2's band. Protein binding analysis was performed by Roz Jenkins.

5.4.2. Granzyme B secretion of flucloxacillin-specific $CD8^+$ T-cells

The cytotoxic ability of flucloxacillin-specific $CD8^+$ T-cells was analysed through granzyme B ELISpot. Flucloxacillin-specific dLN cells were found to secrete significantly more granzyme B in response to *ex vivo* stimulation with flucloxacillin, when compared with dLN cells from vehicle control mice (Fig 5.3.).

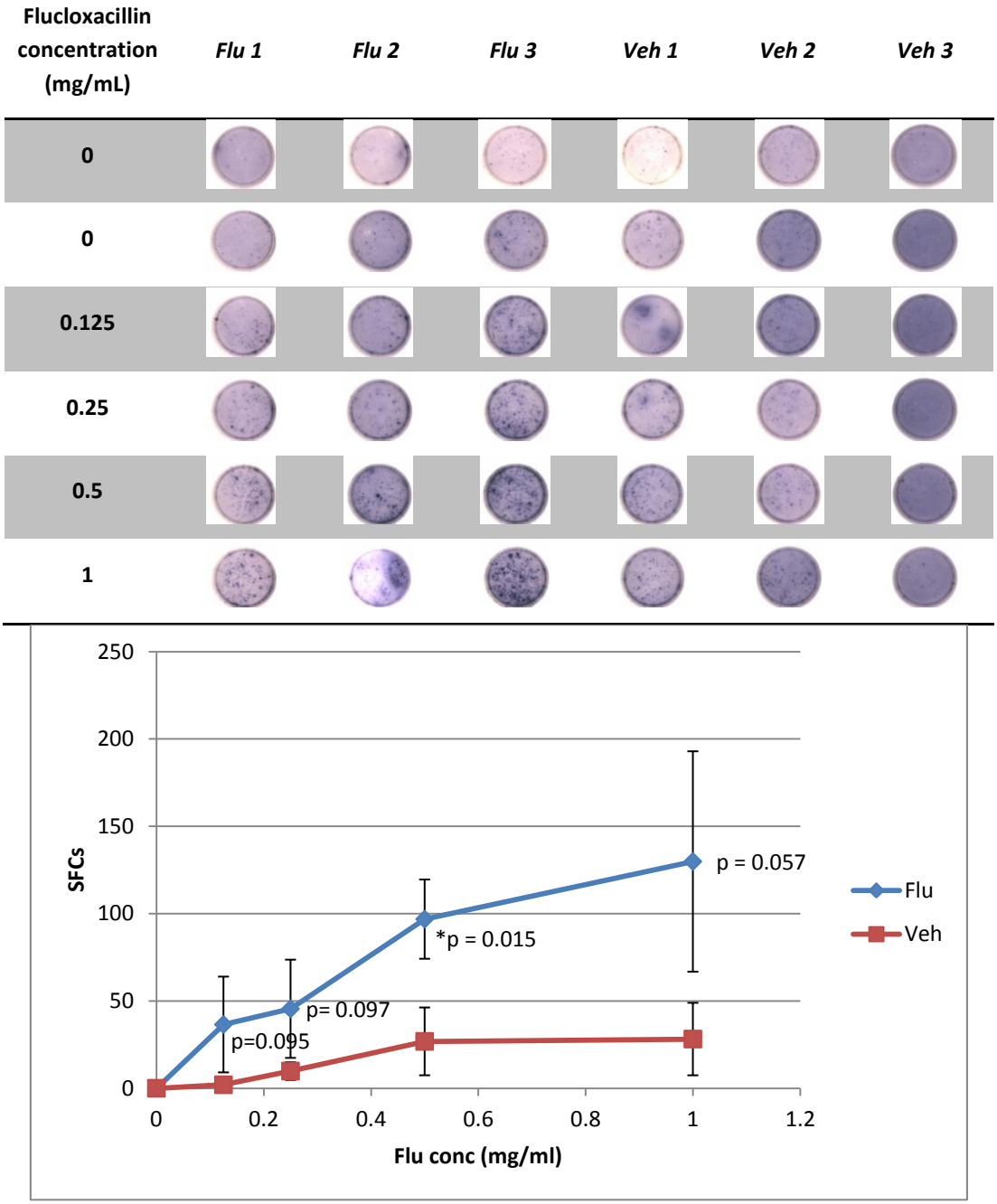
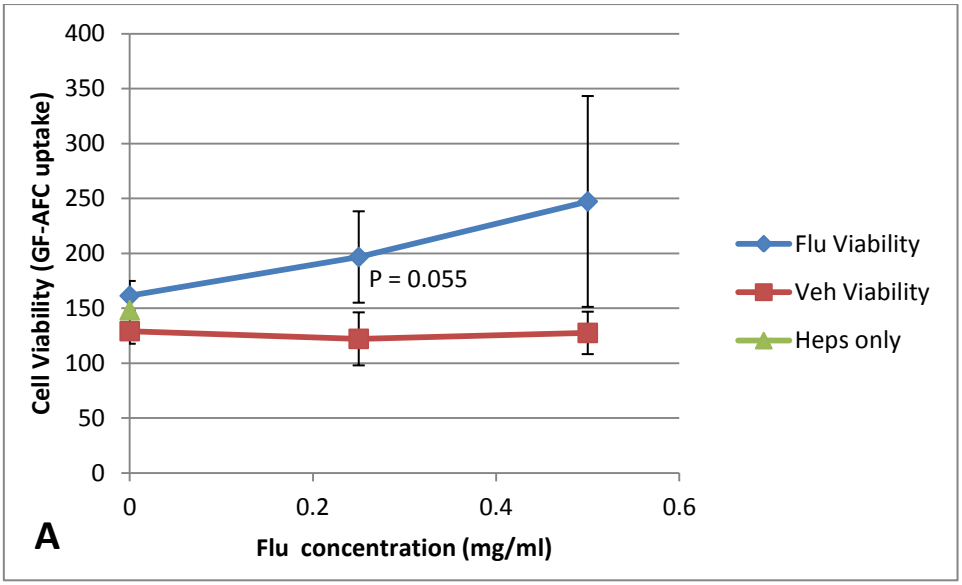
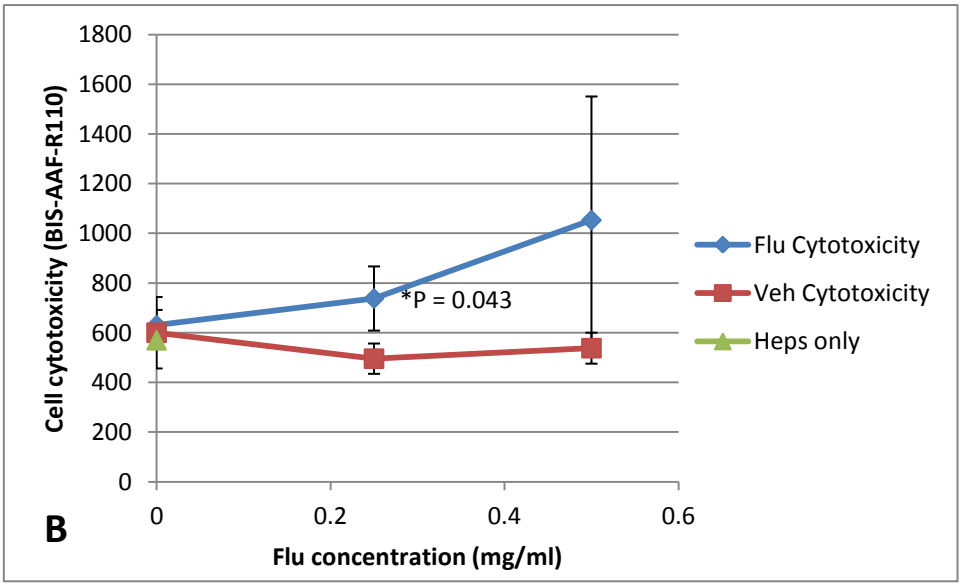


Fig 5.3. Granzyme B ELISpot showing flucloxacillin-specific increases in granzyme B secretion from flucloxacillin-specific $CD8^+$ T-cells. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injections of CD4 antibody (100µg) on day 0 and 7. Mice were painted with 50µL of 1g/mL flucloxacillin or vehicle only for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated at $1.25x10^5$cells/well with $2.5x10^4$cells/well of CD11c positive APCs collected from the mesenteric LN through CD11c positive magnetic isolation. Cells were plated out in an antigen coated plate and after 24h incubation at 37°C and 5%CO_2, were developed according to kit instructions. Table shows single well pictures with Fig showing number of drug-specific SFCs. Each n-number is indicative of one mouse (n=3).

5.4.3. Investigation into the cytotoxic potential of flucloxacillin-specific $CD8^+$ T-cells on hepatocytes *ex vivo*

The ability of flucloxacillin-specific $CD8^+$ T-cells to cause hepatocyte cytotoxicity and apoptosis was measured via luminescence and scintillation respectively. Flucloxacillin-specific $CD8^+$ T-cells were generated through the usual protocol and pulsed with flucloxacillin in the presence of APCs for 24h after which they were washed and added to freshly isolated hepatocytes with readouts being measured 4h later. Significant flucloxacillin-specific T-cell/hepatocyte cytotoxicity and apoptosis was detected at 0.25mg/mL flucloxacillin (Fig 5.4. A, B). Interestingly, un-pulsed $CD8^+$ T-cells caused an increase in apoptosis compared to vehicle T-cells, showing an inherent cytotoxic capability when compared to hepatocytes only or hepatocytes with un-pulsed T-cells. No significant difference was observed between hepatocyte only and vehicle T-cell wells, indicating that the addition of T-cells alone had no effect on the viability, cytotoxicity, and apoptosis of hepatocytes (Fig 5.4. C).


400
350
300
250
200
150
100
50
0
Cell Viability (GF-AFC uptake)
P = 0.055
0
0.2
0.4
0.6
Flu concentration (mg/ml)
Flu Viability
Veh Viability
Heps only
A


1800
1600
1400
1200
1000
800
600
400
200
0
Cell cytotoxicity (BIS-AAF-R110)
*P = 0.043
0
0.2
0.4
0.6
Flu concentration (mg/ml)
Flu Cytotoxicity
Veh Cytotoxicity
Heps only
B

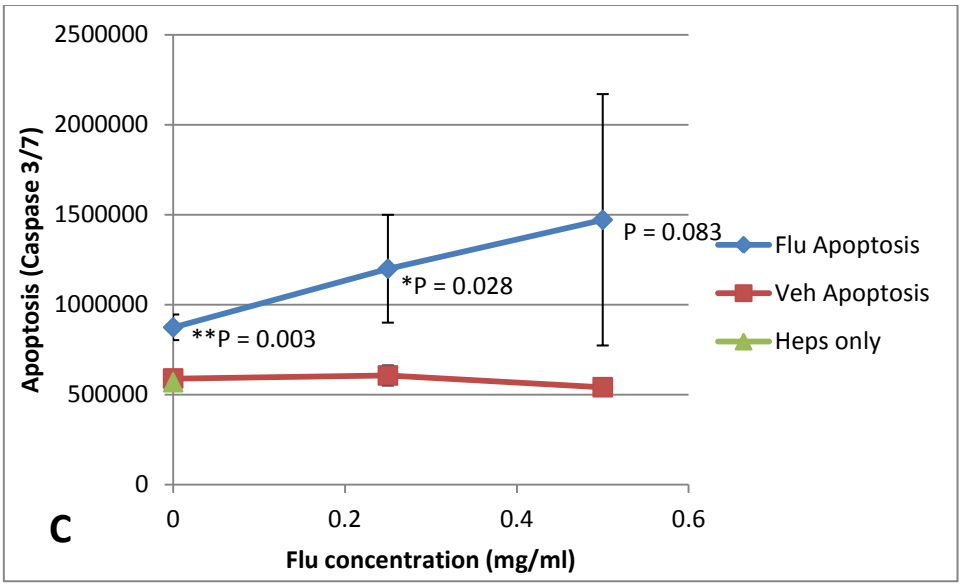

2500000
2000000
1500000
1000000
500000
0
Apoptosis (Caspase 3/7)
P = 0.083
*P = 0.028
**P = 0.003
0
0.2
0.4
0.6
Flu concentration (mg/ml)
Flu Apoptosis
Veh Apoptosis
Heps only
C

Fig 5.4. Flucloxacillin-specific $CD8^+$ T-cells cause drug-specific increases in cytotoxicity and apoptosis in hepatocytes. MHC II KO mice were depleted of $CD4^+$ T-cells with I.P. injections of CD4 antibody (100μg) on day 0 and 7. Mice were painted with 50μL of 1g/mL flucloxacillin or vehicle only for days 1-3. On day 8 the dLNs were removed, macerated to a single cell suspension and incubated at $1.25x10^5$cells/well with $2.5x10^4$cells/well of CD11c positive APCs collected from the mesenteric LN through CD11c positive magnetic isolation. After 24h in culture at stated concentrations of flucloxacillin, cells were washed, counted, and 20000 cells were added to 2500 freshly isolated mouse hepatocytes/well in medium for 4h at 37°C and 5% CO_2 alongside hepatocytes with no added T-cells. Wells were then treated according to Promega APOTOX GLO kit protocols to analyse cell viability (A) and cytotoxicity (B) measured via luminescence and apoptosis (C) measured through scintillation. Statistics were performed using the students T-test. P values of <0.05 were considered significant with $p<0.1$ being shown. Each n-number represents dLN cells from one mouse (n=3).

5.4.4. Attempt to develop a mouse model of flucloxacillin-induced liver injury

Flucloxacillin sensitisation in CD4 depleted MHC II KO mice has been shown to generate drug-specific $CD8^+$ T-cells which become activated upon elicitation with flucloxacillin *ex vivo* to proliferate and generate inflammatory IFNγ (see chapter 4) as well as showing cytotoxic granzyme B secretion and drug-specific increase in hepatocyte cytotoxicity and apoptosis (this chapter). Systemic flucloxacillin dosing has shown that the drug binds to mouse albumin within the liver cytosol. Elsewhere, previous studies have shown that flucloxacillin systemic dosing of rats yielded drug adducts in the liver cytosol, detectable by western blotting (Carey and van Pelt 2005). Finally, I.V. injection of RA treated BMDDCs exposed to flucloxacillin-primed T-cells in the mesenteric lymph nodes of $CD4^+$ T-cell depleted MHC II KO naive mice (see chapter 4). The next step in the development of a mouse model of flucloxacillin-induced liver injury was the elicitation of $CD8^+$ T-cells in the mesenteric lymph nodes of $CD4^+$ T-cell depleted MHC II KO naive mice and subsequent systemic dosing to test the hypothesis that flucloxacillin-specific $CD8^+$ T-cells can cause liver injury.

Three separate experiments were embarked upon in an attempt to develop a mouse model of flucloxacillin-induced liver injury.

(1) $CD4^+$ T-cell depleted MHC II KO mice were adoptively transferred mature BMDDCs exposed to flucloxacillin. Twenty-five days later a 5 day oral dosing regimen of 2.3mgs flucloxacillin/day was initiated with ALT being measured on day 1, 2, and 5 of oral dosing. In this preliminary experiment, a visible increase in ALT levels to over double the respective controls in the serum of mice given flucloxacillin-exposed mature BMDDCs was observed at day 5 of oral dosing (Fig 5.5.) indicating the possibility of mild flucloxacillin-induced liver injury.

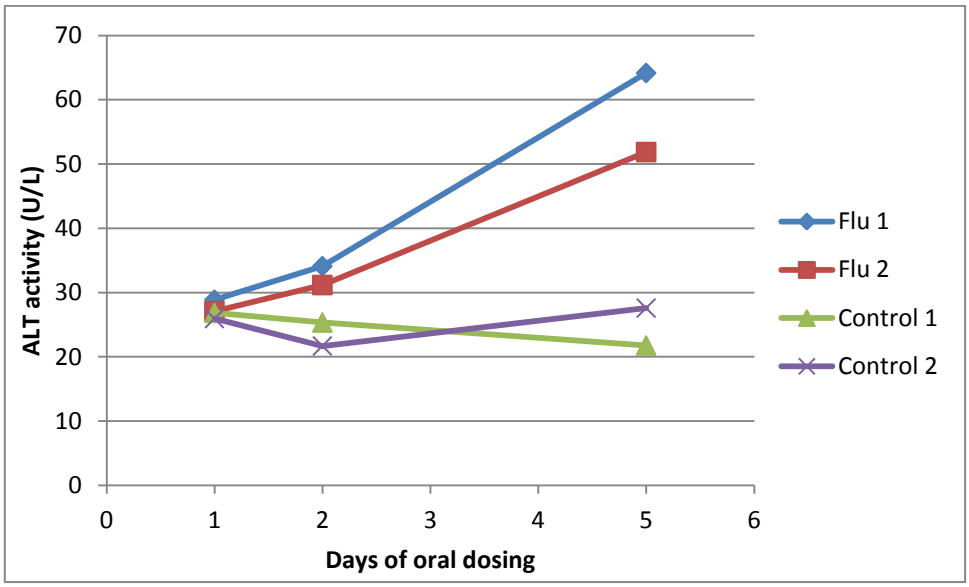
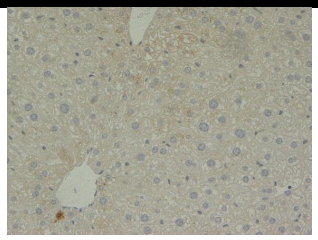
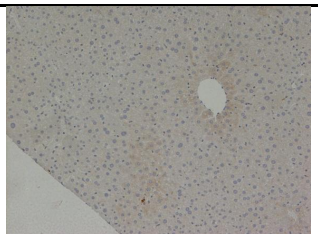
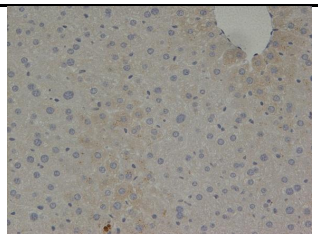
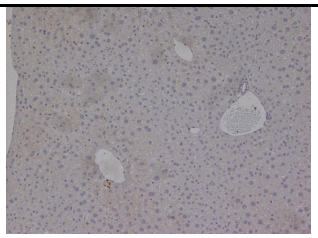
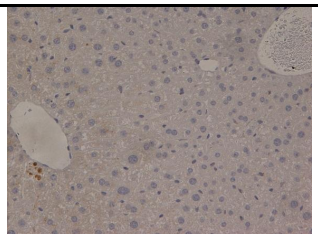
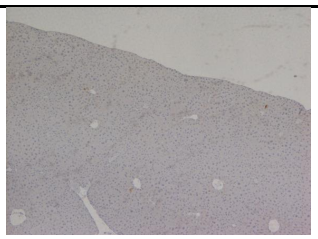
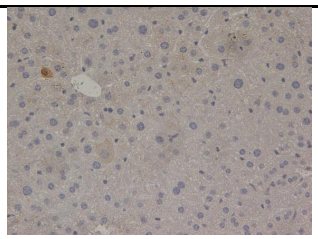
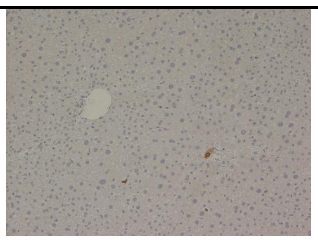
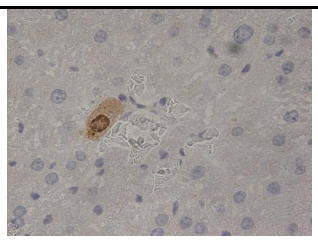


Fig 5.5. Preliminary experiment showing increases in ALT levels in blood serum of orally dosed mice adoptively transferred with flucloxacillin-exposed mature DCs. Bone marrow was removed from the femurs of MHC II KO mice and incubated with GM-CSF for 8 days. RA was added at 10nM on days 4 and 8 with LPS being added on day 8 to mature the DCs. On day 9 BMDDCs were incubated in RPMI containing 0.5% mouse albumin with or without flucloxacillin at 0.5mg/mL and on day 10 the cells were washed and I.V. injected into naive MHC II KO mice at $0.25x10^6$ cells/mouse. Animals also received I.P. injections of 100µg CD4 antibody to deplete $CD4^+$ T-cells and 14mgs RA in 100µL Hanks balanced salt solution to make immune cells migrate to the mesenteric lymph nodes. Twenty-five days after injection of BMDDCs, oral dosing of 2.3mgs/day of flucloxacillin was started in all mice for 5 days. Mice were sacrificed on days 1, 2, and 5, post oral dosing with blood serum being analysed for increases in ALT (n=2 shown).

(2) The ability of flucloxacillin-specific $CD8^+$ T-cells to cause *in vivo* liver damage was further investigated by adoptively transferring cytotoxic $CD8^+$ T-cells as well as flucloxacillin-exposed APCs. CD4 depleted MHC II KO mice were adoptively transferred $CD8^+$ T-cells taken from the dLNs of flucloxacillin or vehicle painted mice, after being incubated for 24h with APCs. Mice also received *in vitro* flucloxacillin-exposed CD11c positive cells from the mesenteric lymph nodes of CD4 depleted MHC II KO mice. After three days, all mice were orally dosed with 2.3mgs/flucloxacillin/day for 10 days with liver damage readouts of mir-122 in blood serum (Fig 5.6. B, C) and histology staining of caspase 3 of the liver (Fig 5.6. A) being investigated on day 0, 5, and 10 of oral dosing. In contrast to the previous experiment, no clear increases in liver damage biomarkers of mir-122 (Fig 5.6. B, C) were observed in animals injected with flucloxacillin-exposed immune cells when compared against relative controls. Additionally, no increases in apoptosing hepatocytes were detected after caspase 3 staining of the liver were witnessed by histological analysis (Fig 5.6. A). These data suggest that the transfer of cytotoxic $CD8^+$ T-cells is not as efficient in transferring sensitisation as transfer of flucloxacillin-exposed mature BMDDCs. ALT levels were not measured due to haemolysis.

Days of oral dosing	Condition of adoptively transferred cells	4x or 10x magnification	20x or 40x magnification
Day 0 of flucloxacillin oral dosing	Flucloxacillin-exposed		
	Vehicle exposed		
Day 5 of flucloxacillin oral dosing	Flucloxacillin-exposed		
	Vehicle exposed		
Day 10 of flucloxacillin oral dosing	Flucloxacillin-exposed		

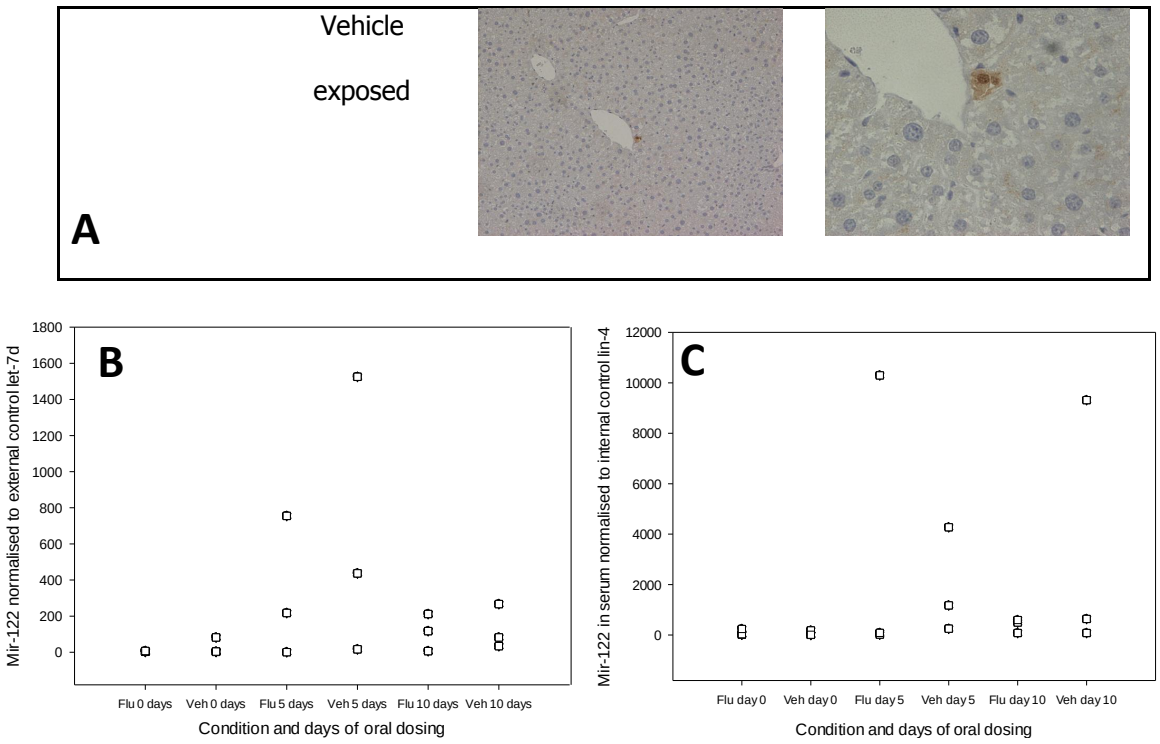


Fig 5.6. I.V. injection of flucloxacillin-exposed $CD8^+$ T-cells and $CD11c^+$ APC from the mesenteric lymph nodes of sensitised mice fail to induce liver damage in naive mice subsequently orally dosed with flucloxacillin. Flucloxacillin-specific $CD8^+$ T-cells were generated through CD4 depletion (day 0) and painting of flucloxacillin onto skin (day 1-3) in MHC II KO mice and maceration of the dLNs 5 days after the last dose (day 8). Flucloxacillin-specific $CD8^+$ T-cells were incubated in media containing mouse albumin and 10nM RA to infer gut homing with CD11cs magnetically separated from the mesenteric lymph nodes. After 24h, cells were washed and I.V. injected at $0.2x10^6$ cells/mouse into naive CD4 depleted MHC II KO mice along with $0.1x10^6$ (24h,10nM RA, 0.5mg/mL flucloxacillin incubated) CD11cs isolated from the mesenteric lymph nodes (day 10). Control mice received $CD8^+$ T-cells from vehicle only painted mice and CD11cs not incubated with flucloxacillin. All mice also received I.P. injections of 14mg/mouse of RA on day 10. On day 13, I.V. injected mice all received oral flucloxacillin of 2.3mgs/mouse/day for 10 days. Readouts of liver histology stained for caspase 3 activation (A)(brown) and mir-122 increase in blood serum (B, C, D) were taken on days 0, 5, and 10 of oral dosing. Histology (A) shows n=1 data representative of full results (n=3) which are fully shown in supplementary figure 1. Complete histological analysis is reported in appendix 1. Mir-122 data is displayed as normalised compared to let-7d (B) and lin-4 (C) (one mouse per n=number. n=3 shown).

(3) Due to the conflicting results from the two initial attempts to develop a model of flucloxacillin-induced liver injury (Fig 5.5.) (Fig 5.6.), a third protocol was designed in an attempt to remove *in vitro* culturing of immune cells and adoptive transfer as this

step had proved to be costly in regards to recoverable cell numbers. Thus, MHC II KO mice were sensitised as before with the added exception of adding RA to the site of drug/vehicle painting to impose mesenteric lymph node homing. In preliminary experiments after 2 days of oral dosing a statistically significant increase in ALT levels (Fig 5.7. A) and visible increases in relative mir-122 levels in the blood serum in flucloxacillin-painted mice was observed (Fig 5.7. B, C). Thus, a large scale experiment was designed incorporating the RA painting to assess the reproducibility of these results.

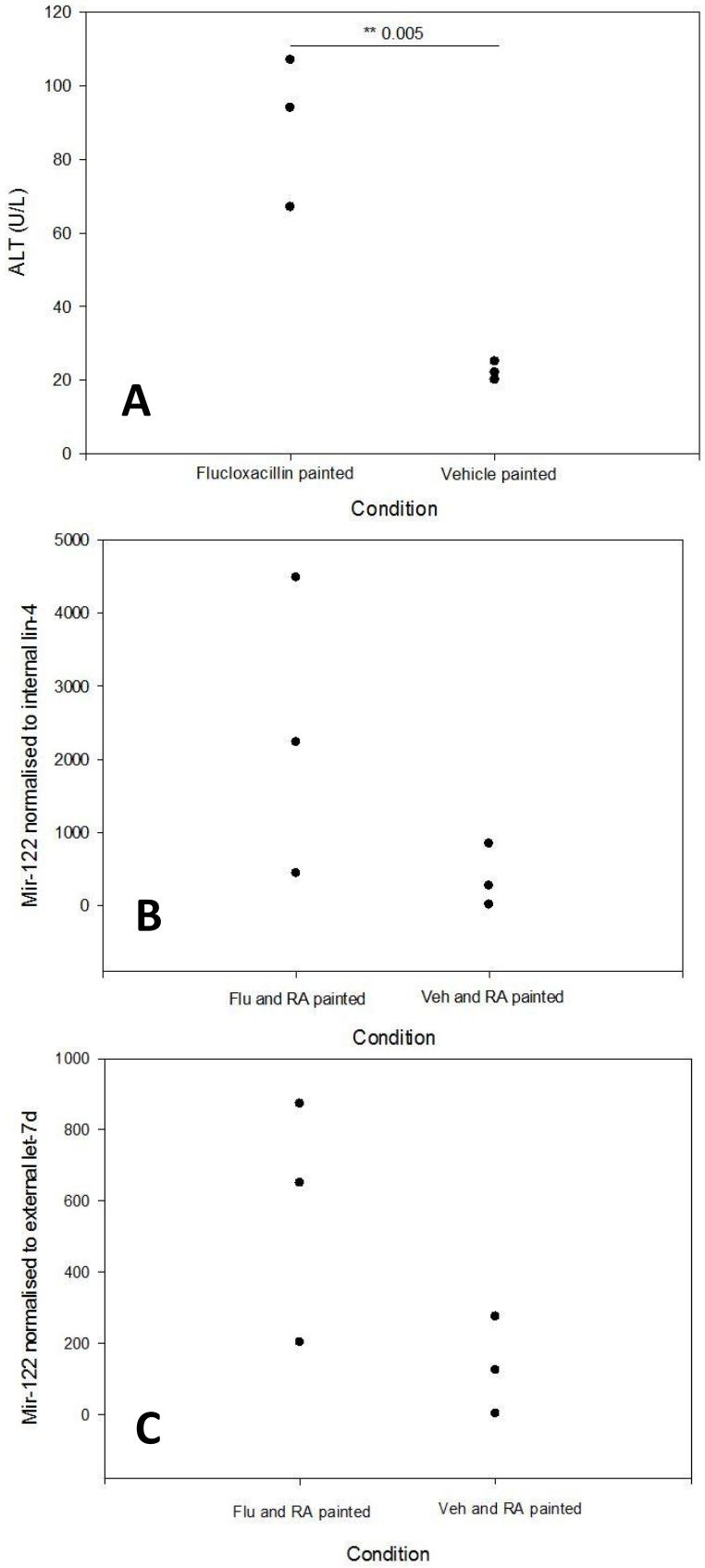
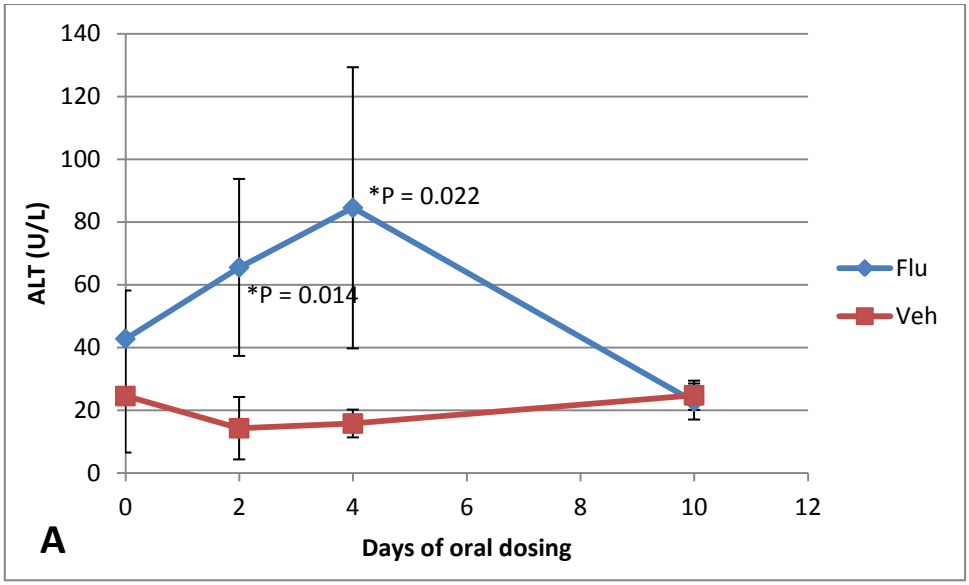


Fig 5.7. Significant increases in liver damage biomarkers in blood serum of CD4 depleted MHC II KO mice after oral dosing of flucloxacillin following painting of flucloxacillin and retinoic acid onto skin. MHC II KO mice were

depleted of $CD4^+$ T-cells through I.P. injections of CD4 antibody on day 0 and mice were painted with 0.5mg/RA/day with or without flucloxacillin on days 1-3. On day 7 mice were orally dosed with flucloxacillin for 2 days with liver damage biomarkers of ALT levels (A) and mir-122 (B, C) being analysed in blood serum on day 8 with mir-122 data being displayed as normalised compared to let-7d (B) and lin-4 (C). P values of <0.05were considered significant and statistics were devised using the students T-test (n=3 shown).

In the repeat of the RA painting experiment (which induced significant increases in ALT levels and increased mir-122 in two out of three animals sensitised to flucloxacillin after 2 days of oral dosing), a consistent increase in ALT levels was detected in the blood serum of mice sensitised and subsequently orally dosed with flucloxacillin for 2 days when compared against relative controls (Fig 5.8. A). Significant increases against controls were also detected in flucloxacillin-sensitised mice at day 4 of flucloxacillin oral dosing. Interestingly, after 10 days of oral dosing ALT levels dropped back to normal levels (Fig 5.8. A). To confirm liver damage, mir-122 levels in blood serum of mice were analysed but no significant increase was observed in orally dosed, flucloxacillin-sensitised mice (Fig 5.8. B). These data indicate that the increases seen in ALT may not have been hepatocyte-related. As flucloxacillin in humans primarily causes DILI through cholestasis, ALP levels in the blood were also measured to reveal no significant increases in flucloxacillin-sensitised mice compared against relative controls (Fig 5.8. C). These data indicate that flucloxacillin sensitisation/ RA painting alongside ten days of flucloxacillin oral dosing in the mouse was not able to demonstrate severe liver damage via cholestatic means.



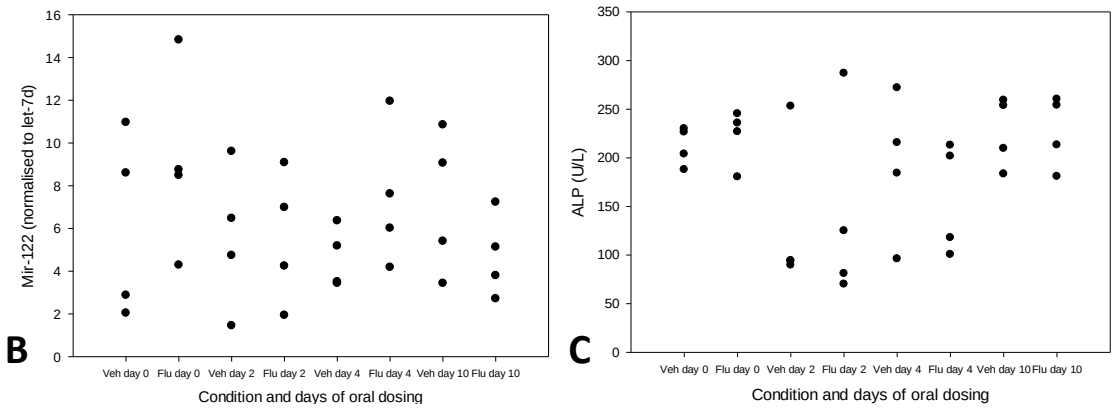


Fig 5.8. Inconclusive results showing significant flucloxacillin-specific increases in ALT in blood serum at days 2 and 5 however accompanied with negative mir-122 and ALP blood serum analysis. Mice were painted with RA (0.1mg/day) and either flucloxacillin (1g/mL, 50µL) or vehicle control for 3 days. 4 days later, all mice were orally dosed flucloxacillin (2.3mgs/day) for 10 days with mice being sacrificed after 0, 2, 4, and 10 days of oral dosing. Blood serum levels of ALT (A), mir-122 (B) (normalised to let-7d), and ALP (C) were analysed. P values of <0.05were considered significant and statistics were devised using the students T-test (n=4 shown).

On removal of the livers on day 4 of oral dosing, an interesting development was encountered where the gall bladders in flucloxacillin-sensitised mice were swollen when compared against vehicle control. Intact gall bladders were photographed and on day 10 of oral dosing to reveal that gall bladders from all mice were swollen with flucloxacillin-sensitised mice gall bladders being particularly engorged (Fig 5.9.). Although blood serum

analysis was negative for biomarkers of DILI, nevertheless the observation of swollen gall bladders upon administration of a drug known to cause cholestasis is intriguing.

	Gall bladder swelling	
Individual mouse data	***Control***	***Flucloxacillin-sensitised***
Day 4 oral dosing		
Day 10 oral dosing		

Fig 5.9. Flucloxacillin-induced swelling of the gall bladders on day 4 of oral dosing in flucloxacillin-sensitised mice and on day 10 in both flucloxacillin-sensitised and control mice. Mice were painted with RA (0.1mg/day) and either flucloxacillin (1g/mL, 50µL) or vehicle control for 3 days. 4 days later, all mice were orally dosed flucloxacillin (2.3mgs/day) for 10 days with mice being sacrificed after 0, 2, 4, and 10 days of oral dosing. Table shows n=1 representative of results (n=1 day 4, n=4 day 10). All results from gall bladder swelling are shown in supplementary figure 2.

As flucloxacillin-specific $CD8^+$ T-cells had been shown to cause increases in hepatocyte apoptosis *in vitro* (this chapter), immunohistochemistry was performed on the livers of the

mice painted with RA/flucloxacillin and subsequently orally dosed to attempt to detect immune-mediated killing of hepatocytes *in vivo*. Livers sections from all mice were stained with caspase-3 and subsequent analysis revealed no significant increases in hepatocyte cell death in flucloxacillin-sensitised animals or after oral dosing (Fig 5.10.). Of the few visible apoptosing cells present, they were deemed not to be hepatocellular but endothelial or stellate cells.

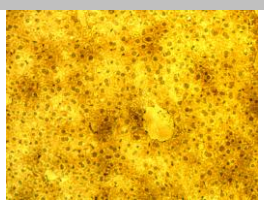
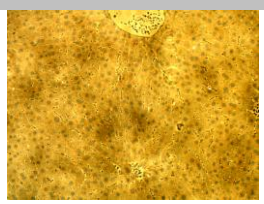
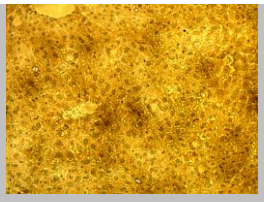
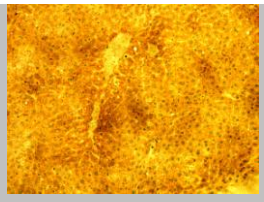
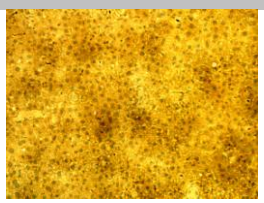
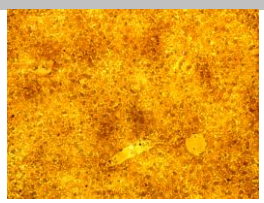
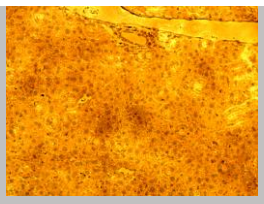
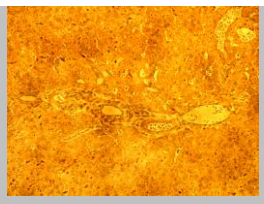
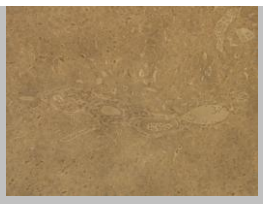
	Vehicle pained		Flucloxacillin-painted	
Day of oral dosing	***Original image***	***False couloured***	***False coloured***	***Original image***
Day 0				
Day 2				
Day 4				
Day 10				

Fig 5.10. Caspase 3 staining of the liver showing no increases in apoptotic hepatocytes. Mice were painted with RA (0.1mg/day) and either flucloxacillin (1g/mL, 50µL) or vehicle control for 3 days. 4 days later, all mice were orally dosed flucloxacillin (2.3mgs/day) for 10 days with mice being sacrificed after 0, 2, 4, and 10 days of oral dosing. Histological analysis for increases in apoptosis was performed through staining liver for caspase 3 (brown). Cell nuclei were stained blue. Table shown n=1 representative of full results (n=4). A complete histological analysis of caspase 3 content is shown in supplementary figure 3.

Liver sections from mice painted with RA/flucloxacillin and subsequently orally dosed for 10 days were H&E stained and imaged to look for any other cellular anomalies which flucloxacillin sensitisation and oral dosing may have induced. Analysis of livers revealed an

increase in infiltrating leukocytes which are especially pronounced at day 10 oral dosing in flucloxacillin-sensitised and vehicle animals (Fig 5.11.).

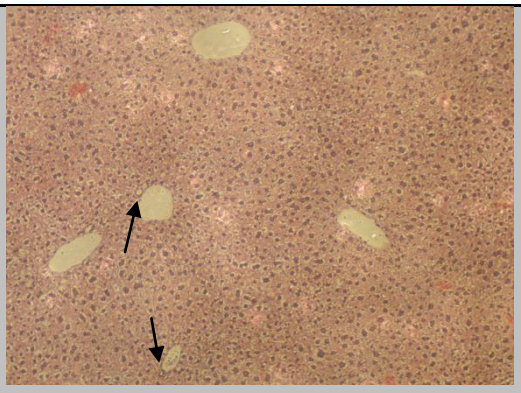
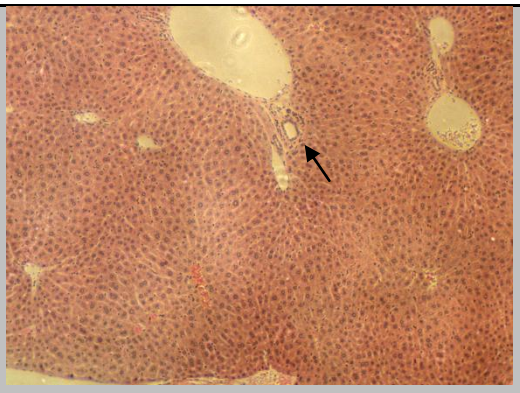
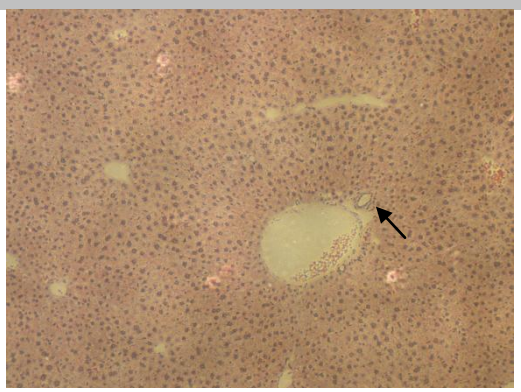
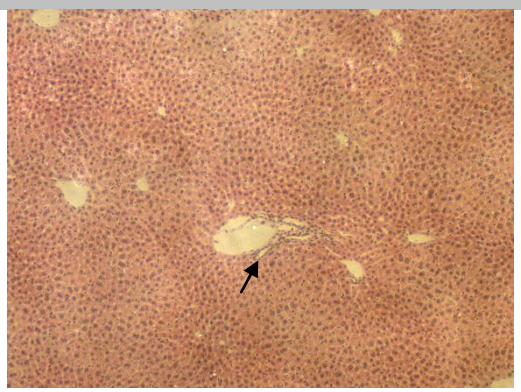
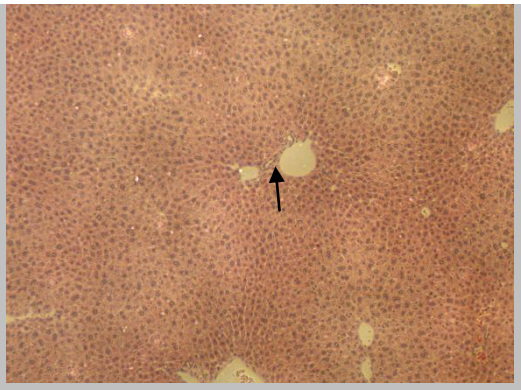
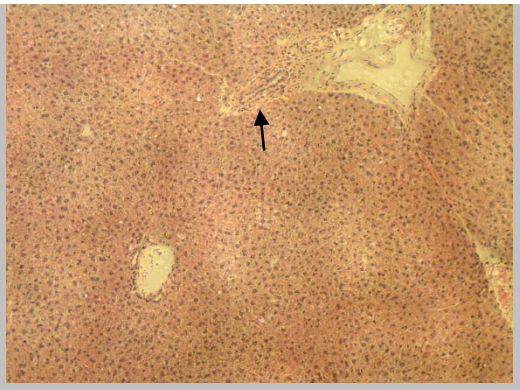
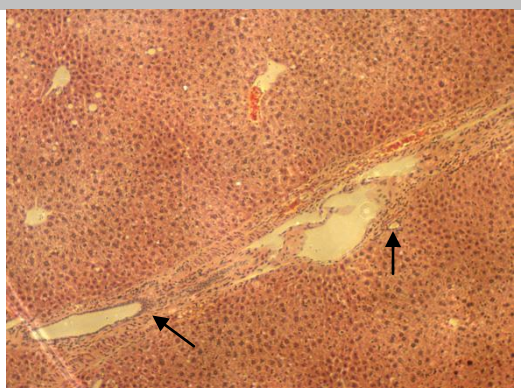
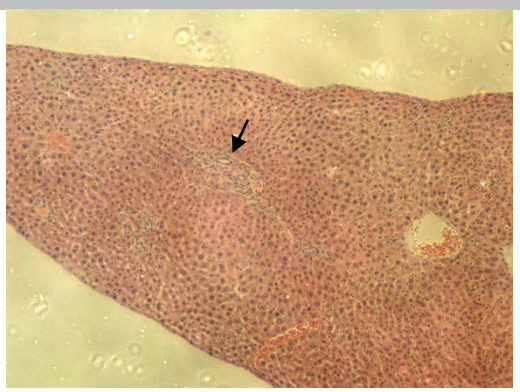
Days of oral dosing	*Vehicle pained*	*Flucloxacillin-painted*
Day 0		
Day 2		
Day 4		
Day 10		

Fig 5.11. H&E stained liver showing increases in infiltrating leukocytes. Mice were painted with RA (0.1mg/day) and either flucloxacillin (1g/mL, 50µL) or vehicle control for 3 days. 4 days later, all mice were orally dosed flucloxacillin (2.3mgs/day) for 10 days with mice being sacrificed on days 0, 2, 4, and 10 of oral dosing. Black arrows highlight bile ducts. Table shows n=1 representative of full results (n=4). A complete histological analysis of H&E stained liver from all mice is shown in supplementary figure 4.

5.5. DISCUSSION

There are a number of mouse models of immune-mediated liver injury which show liver damage; both hepatocellular and cholestatic (Feldman et al. 2013) (Gujral et al. 2003) (Gujral et al. 2004b) (Woolbright et al. 2013). These models were designed with an aim to investigate liver injury induced through viral infection or genetic disease. A few models of particular relevance and interest to the investigations performed here are discussed below.

Biliary atresia is a condition where bile is retained in the gall bladder. This condition is induced through auto-immune reactions following viral infection where disease symptoms are induced through attack of the bile ducts by the immune system (Feldman et al. 2013). If wild-type mice are infected with Rhesus rotavirus at birth they quickly develop bile duct obstruction, biliary atresia, and subsequent liver damage which leads to a survival rate of only 17.5% (Feldman et al. 2013). The investigators of this model put forth a possible mechanism of immune-mediated bile duct damage where B-cells are responsible for presenting foreign antigen to naive T-cells which in turn, stimulate CTL, macrophages and NK cells to induce damage in bile duct cells leading inexorably to biliary atresia (Feldman et al. 2013).

Another technique which has proven insightful is to induce damage and then monitoring the resulting mechanisms. In bile duct ligation (a surgical technique that mimics obstructive cholestasis), analysis of resulting damage in mice has shown a role for infiltrating

neutrophils/leukocytes as animals deficient in CD18 or ICAM-1 were highly protected against bile duct ligation-induced liver injury (Gujral et al. 2003) (Gujral et al. 2004b). Others have used bile duct ligation to analyse the resulting mechanisms of cellular damage. Cell death was found to occur via necrosis, not apoptosis (Woolbright et al. 2013).

Earlier in this thesis I have reported that flucloxacillin-induced liver injury in humans is a phenomenon known to involve drug-specific T-cell activation. The T-cell response is HLA-B*57:01 restricted in the majority of cases (Monshi et al. 2013), which is in agreement with the HLA-B*57:01 and susceptibility to liver injury (Daly et al. 2009). Flucloxacillin-specific T-cells from HLA-B*57:01 positive donors are activated by hapten and Pi-mechanisms (Wuillemin et al. 2013). However, 25% of patients that develop flucloxacillin-induced liver injury do not carry the risk allele. Combined analytical studies monitoring drug-protein binding and immunological studies clearly shows a hapten-based mechanism of $CD8^+$ T-cell activation in these donors (Daly et al. 2009) (Monshi et al. 2013). Although $CD8^+$ T-cells are known to circulate in patients with flucloxacillin-induced liver injury, their role in mediating tissue injury is unknown. The experiments performed here using a mouse model of flucloxacillin sensitisation, shed some light on possible routes of $CD8^+$ T-cell-mediated liver injury.

The objectives of this chapter were to investigate flucloxacillin-induced liver injury via direction of flucloxacillin-specific $CD8^+$ T-cells to the liver. This was to be followed by an elicitation challenge directed at the liver through oral dosing.

The flucloxacillin-specific $CD8^+$ T-cells generated in this mouse model are known to require APCs to respond. These data suggest that the T-cell antigen derives from an intracellular

protein. Flucloxacillin has previously been shown to bind to specific lysine residues on circulating proteins, in particular human serum albumin (Jenkins et al. 2009). In this chapter flucloxacillin-modified lysine residues have been detected in the liver cytosol of mice. These data are in agreement with (Carey and van Pelt 2005) who characterised hepatic adducts in liver of rats systemically dosed with flucloxacillin. The major modified band had a mw of 65-70kDa, the same as rat albumin. So: as flucloxacillin modifies albumin selectively, hepatocytes synthesise albumin, and flucloxacillin-specific $CD8^+$ T-cells become activated upon presentation of flucloxacillin-modified intracellular peptides; the hypothesis in this chapter was that flucloxacillin-specific $CD8^+$ T-cells would cause apoptosis in flucloxacillin-exposed hepatocytes both *in* and *ex vivo*.

In order to test the cytotoxic capabilities of the flucloxacillin-specific $CD8^+$ T-cells, the number of granzyme B secreting cells was quantified through ELISpot. Flucloxacillin-specific T-cells from sensitised mice secreted significantly higher levels of granzyme B following flucloxacillin stimulation (1-2mM), when drug and vehicle treated mice were compared. This granzyme B secretion has also been demonstrated in human $CD8^+$ T-cell clones which responded with a similar dose response (Monshi et al. 2013).

Thus in the presence of APCs, flucloxacillin-specific $CD8^+$ T-cells proliferate, and secrete IFNγ and granzyme B *ex vivo* following drug stimulation. Systemic dosing yields flucloxacillin-albumin adducts in the liver of treated mice. The next step was to culture flucloxacillin-specific $CD8^+$ T-cells with hepatocytes to measure drug-specific T-cell-mediated hepatocyte-killing. Significant increases in hepatocyte cytotoxicity and apoptosis compared against hepatocytes only were observed in wells containing flucloxacillin-specific $CD8^+$ T-cells. These data indicate that drug-specific T-cells are able to kill hepatocytes *ex vivo*. Interestingly drug stimulated and un-stimulated T-cells damaged hepatocytes indicating that they are isolated

from mice in a partially activated state. The ApoTox-Glo assay enables the discrimination between apoptotic and necrotic cell death (Prasad et al. 2012). The majority of dying hepatocytes were killed via an apoptotic pathway. Granzyme B (shown herein) and other molecules such as fas ligand are released from cytotoxic $CD8^+$ T-cells and are known to cause cell death in target cells through activating the apoptotic cascade (Kagi et al. 1994) (Atkinson et al. 1998) (Medema et al. 1997).

The next logical step in the investigation was to determine whether flucloxacillin-specific $CD8^+$ T-cells cause liver injury *in vivo*. In preliminary experiments, mice were adoptively transferred flucloxacillin-exposed mature BMDDCs imprinted with gut homing via RA treatment and then orally dosed in an attempt to induce liver-orientated elicitation *in vivo*. Blood serum ALT levels in flucloxacillin-exposed DC injected mice, were over double the respective vehicle control (vehicle= 21-27U/L, flucloxacillin=51-64U/L).

Animal models of drug-induced liver injury have been able to demonstrate increases in liver damage biomarkers with simultaneous infiltration of immune cells however the models which have come closest have all involved drugs which seem to act via MHC II mediated pathways: these include animal models of amodiaquine (Shenton et al. 2004) and nevirapine hypersensitivity (Shenton et al. 2003). Long-term dosing with these drugs resulted in adaptation with some showing increased numbers of $CD4^+$ regulatory T-cells with eventual reductions in blood serum liver damage biomarkers (Ng et al. 2012). The next step therefore in attempting to develop a complete mouse model of flucloxacillin-induced liver injury would therefore involve long term oral dosing of flucloxacillin/RA painted mice whilst keeping $CD4^+$ regulatory T-cells at depleted levels. This would assess whether the injury would increase or resolve itself which would be of particular interest in this model due to the effector cells being $CD8^+$, not $CD4^+$.

Painting of RA onto skin was more efficient than adoptive transfer showing ALT peaking at over 3x the normal upper limits (vehicle = 20-25U/L, flucloxacillin=67-107U/L), a value deemed to be clinically important in the removal of drugs such as ximelagatran from the market due to hepatocellular and cholestatic damage (Keisu and Andersson 2010). As the mir-122 levels were not as pronounced as the ALT levels with only two out of the three flucloxacillin-painted mice showing increases against vehicle control, it is possible that the increased ALT levels shown in Fig 5.7. A could have been partly attributed to other organs (e.g. kidney, lung or skeletal muscle) which are also known to give increased ALT levels after damage (Giannini et al. 2005). Importantly however, the increases seen in the ALT levels alongside the mir-122 levels and the increases seen in *in vitro* readouts would strongly indicate the involvement of the adaptive immune system in hepatocyte damage in response to flucloxacillin. Upon repeating of this experiment on a larger scale, the increase in ALT levels in blood serum after two days of exposure to oral flucloxacillin was reproducible. Significant amounts of ALT were also detectable after four days of oral dosing with flucloxacillin-sensitised mice having over four times higher ALT levels than their respective controls; however, ALT levels in serum returned to baseline after ten days of oral dosing. The ALT levels from this method of systemic elicitation challenge peaked at around ~120 U/L which is not outstanding in the field of liver toxicity where ALTs can be ten times this level (Imaeda et al. 2009). However, in regards to cholestasis and mouse models of cholestasis where the bile-duct is surgically ligated, ALT levels of ~250U/L and 10 fold increased over respective controls have been published in high impact journals as significant (Gujral et al. 2004a).

Another hypothesis which could explain the retention of bile in the gall bladder and its distension is dysfunction of the sphincter of Oddi (Torsoli et al. 1990). The sphincter of Oddi

is a band of muscle at the end of the biliary tree which controls the flow of bile into the duodenum (Torsoli et al. 1990). Dysfunction in the sphincter of Oddi can lead to gall bladder distension due to the build-up of pressure. The main pathophysiology of sphincter of Oddi dysfunction is through the action of gall stones or damage caused during previous surgeries. Infections of the common bile duct are also capable of narrowing the bile duct however these are also strongly associated with the development of gall stones (Kinney 2007).

Further analyses of blood serum biomarkers of liver damage (mir-122) were also negative, indicating a lack of specific hepatocyte damage. This was further confirmed in the immunohistochemical analyses of caspase-3 in the liver sections, which showed very little hepatocyte apoptosis. The presence of increased ALT with little to no evidence of damage in histological analyses has been described previously by researchers in attempting to develop animal models of DILI (Ng et al. 2012). As a measurement of cholestasis, ALP levels were measured and found to be basal in all mice.

Although not showing large scale liver damage, intriguing morphological-changes were observed on day 4 and 10 of mice exposed to flucloxacillin after painting with RA and flucloxacillin. A large swelling of the gall bladder was observed after 10 days of oral dosing, pointing to a bile duct obstruction and the possible development of flucloxacillin-induced cholestasis in the same manner as humans (vanishing bile duct) (Russmann et al. 2005) (Miros et al. 1990). This hypothesis was further tested through histological examination of the liver and bile ducts. This analysis did not reveal increases in apoptotic hepatocytes but did show significant leukocyte infiltrate. As previously discussed, infiltrating leukocytes/neutrophils have been strongly linked with the development of cholestasis induced through bile duct ligation (Gujral et al. 2003) (Gujral et al. 2004b). Although caspase-3 levels in the liver were negative, the early stages of cholestasis consist of very

limited apoptosis due to the induction of anti-apoptotic proteins (Li Z. et al. 2007). Furthermore, the administration of anti-caspase inhibitors were effective in Fas antibody-induced apoptosis models of liver injury although did very little in a mouse model of cholestatic liver injury via bile-duct ligation (Gujral et al. 2004a). In this model, the cell death in the liver is mainly attributed to necrosis. This is due to infiltrating immune cells, mainly neutrophils, which cause cell death via generating reactive oxygen species (Jaeschke 2011). Taken collectively, the depletion of $CD4^+$ T-cells and sensitisation to flucloxacillin has caused an increase in liver damage biomarkers, a swelling in gall bladder size, and an infiltration of the liver by immune cells which tentatively points to the very early stages of cholestasis through vanishing bile duct syndrome.

Future experiments to test this hypothesis should be conducted to fully dissect the possible potential of this model. For example:

(1) The reproducibility or the positive results and a comprehensive monitoring of the gall bladder swelling.

(2) The ability of RA painted onto skin to induce clonal expansion of drug-specific $CD8^+$ T-cells in the mesenteric LN.

(3) The infiltrating immune cells seen in the livers of mice orally exposed to flucloxacillin should be analysed to: 1. determine their phenotype 2. Explore which cytokines they are secreting (if any), and 3. assess whether flucloxacillin-specific $CD8^+$ T-cells are present.

(4) As ALT levels were increased after two days of exposure to flucloxacillin, readouts at day one of oral dosing should be taken to clarify when ALT levels begin to rise and perhaps more importantly, why, in regards to which particular cell are causing the increase.

(5) A long-term experiment with readouts being taken after three weeks of oral dosing should be organised. In humans, cholestatic liver injury induced by flucloxacillin only presents observable outward symptoms a minimum of three weeks after the initial dose (Andrews and Daly 2008).

In conclusion, a mouse model of flucloxacillin-induced liver injury via cholestasis may have been developed here but further experimentation is required as to whether the model could be developed to reproduce the severe liver damage seen in humans.

CHAPTER 6

General Discussion

Drug hypersensitivity is a serious off-target effect associated with exposure to drugs. A high incidence of hypersensitivity reactions can lead to the drug being withdrawn from the market or restrictions to its use. The majority of hypersensitivity reactions have an immune aetiology.

Drug hypersensitivity reactions have historically been investigated through the use of blood and tissue samples from hypersensitive patients. In early experiments, plasma from hypersensitive patients was screened for expression of anti-drug antibodies. Such antibodies were detected in patients with haematological reactions (Moeschlin and Wagner 1952), which led to the suggestion that they were directly involved in the pathogenesis of drug-induced idiosyncratic disease. Patients suffering from isoniazid hepatotoxicity also displayed antinuclear antibodies (Salazar-Paramo et al. 1992) which have recently been linked directly to isoniazid-induced liver injury (Metushi et al. 2014).

Despite, these data, it is now widely believed that antigen-specific T-cells ultimately control whether a patient will develop a hypersensitivity reaction and the nature/severity of the symptoms following drug exposure. In patients with cutaneous hypersensitivity, T-cells have been isolated from inflamed skin and shown to be drug-specific (Nassif et al. 2004) (Nassif et al. 2002) (Pichler et al. 2011) (Schmid et al. 2002) (Pichler et al. 2002). Furthermore, it is possible to isolate circulating T-cells from blood of the same patients many years after clinical manifestations of the reaction subside. By cloning individual T-cells from patients with different forms of cutaneous hypersensitivity reactions it has been possible to characterize drug hypersensitivity in terms of cellular phenotype and functionality (Pichler et al. 2011) (Pichler 2003). Interestingly, such T-cells express skin-homing chemokine receptors that would allow them to migrate back to skin if the hypersensitive patient was inadvertently exposed to the culprit drug (Wu et al. 2007). Recently, several forms of

cutaneous drug hypersensitivity reaction have been found to be strongly associated with expression of particular HLA class I molecules e.g., abacavir hypersensitivity and HLA-B*57:01 (Mallal et al. 2002), carbamazepine and HLA-B15:02 (Chung et al. 2004), and allopurinol and HLA-B*58:01 (Hung et al. 2005). These data imply that the drug antigens interact in some way with the specific HLA-molecule to active T-cells. Indeed, this has now been shown to be the case. Chessman et al (2008) demonstrated that abacavir hypersensitivity was dependant on cytokine producing cytotoxic $CD8^+$ T-cells and was uniquely restricted to HLA-B*57:01. Direct binding of carbamazepine to HLA-B*57:01 leads to activation of $CD8^+$ T-cells in patients displaying the most severe cutaneous hypersensitive responses (SJS/TEN) (Hsiao et al. 2014). Finally, Yun et al (2003) have recently shown via *in vitro* experimentation with T-cell clones that activation is dependent on the both the presence of HLA-B*58:01 allele and high concentration of drug.

The role of T-cells in patients with liver injury is less well defined. A delayed onset in certain forms of liver injury is indicative of an immunological mechanism. It must be noted however that many people develop liver reactions several months after drug exposure. For example, the median time to onset of hepatic adverse events following exposure to the tyrosine kinase inhibitor lapatinib is 110 days (Spraggs et al. 2012) (Spraggs et al. 2011). This time-course is very different to a classical drug hypersensitivity reaction (e.g., maculopapular eruption, SJS/TEN), which develops 1-4 weeks after initial drug exposure (Romano et al. 2011). A case report describing a patient with DRESS has shown that a hypersensitivity reaction can develop into fulminant liver failure (Mennicke et al. 2009). Histological investigations revealed infiltration of granzyme B secreting $CD3^+$ lymphocytes in close proximity to apoptotic hepatocytes suggesting that T-lymphocytes participate in the liver reaction.

Isolation of drug-specific T-cells from DILI patients, but not drug-exposed controls, provides direct evidence that they participate in the disease pathogenesis. Early studies using the lymphocyte transformation test – a simple *in vitro* assay based on assessment of lymphocyte proliferative responses in drug-treated and vehicle control cultures - detected drug-specific lymphocyte responses in approximately 50% of patients with DILI (Maria and Victorino 1997). Drugs to which T-cell reactivity was detected in more than one patient included co-trimoxazole, allopurinol, fentiazac, captopril, phenytoin, and carbamazepine. More recently, similar data has been reported in Chinese patients with DILI. The lymphocyte transformation test was reported to have a sensitivity (% of DILI patients with a positive lymphocyte transformation test) and specificity (% of control patients with a negative lymphocyte transformation test) of 47.5 and 95.9%, respectively (Chen G. Y. et al. 2012). Importantly, the phenotype and function of the T-cells were not defined in either manuscript and additional studies have not been forthcoming.

Liver dysfunction occurs in approximately 20% of patients exposed to isoniazid. As such, its ability to activate drug-specific T-lymphocytes has been studied in detail. Warrington et al (1982) reported that the lymphocyte transformation test was positive in 95% of DILI cases, whereas lymphocyte responses were not detected in patients receiving isoniazid without evidence of liver damage. Interestingly, the patients' T-cells were activated with the parent compound and/or an isonicotinic acid HSA conjugate. The predictive value of the LTT in isoniazid-induced hepatitis was studied in a cohort of 61 patients receiving isoniazid for chemotherapy. Liver injury was detectable in 58% of the LTT positive group, but also 23% of the LTT negative group (Warrington et al. 1982). From this brief discussion it is clear that there is still much to learn regarding the role of T-cells in DILI. With this in mind, researchers in Liverpool have isolated and fully characterized the function of flucloxacillin-responsive T-

cells from patients with DILI. $CD8^+$ T-cell clones expressing CCR4, CCR9, CCL17, and CCL25, and secreted IFN-gamma, perforin, granzyme B, and FasL following drug stimulation (Monshi et al. 2013). These studies define the immune basis for flucloxacillin-induced liver injury; however additional studies are needed to explore the drug-specific T-lymphocyte response in other forms of DILI.

For an increasing number of hepatotoxic drugs (e.g., flucloxacillin, (Daly et al. 2009) augmentin (Donaldson et al. 2010), lumiracoxib (Singer et al. 2010), lapatinib (Spraggs et al. 2011), ximelagatran (Kindmark et al. 2008), and isoniazid (Daly and Day 2012), genome-wide association studies have identified specific HLA alleles as important susceptibility factors. These data suggest that a highly restricted drug-derived antigen interacts with the protein encoded by HLA risk allele to activate T-cells in susceptible patients. For flucloxacillin, it has been shown that the $CD8^+$ T-lymphocyte response is HLA-B*57:01 restricted, which effectively links the genetic association to the tissue injury. Furthermore, naive $CD45RA^+/CD8^+$ T-cells from volunteers expressing HLA-B*57:01 were found to be activated with flucloxacillin when DCs presented the drug-antigen (Monshi et al. 2013). Similarly, Wuilleman et al (2013) found that it was possible to isolate flucloxacillin-responsive T-cells directly from healthy donor blood lymphocytes using extended 3-8 week culture protocols that involved weekly stimulation with drug and autologous APCs. It should be emphasized that flucloxacillin is the only example to date where it has been possible to define the immunogenetic basis of the liver reaction. It should also be noted that even for flucloxacillin, (1) the majority of individuals who carry the known HLA risk allele do not develop clinically relevant reactions when exposed to the culprit drug and (2) many patients that develop DILI will not carry the risk allele. Thus, HLA associations are not absolute indictors of risk. There is therefore an urgent need to characterize the immunological parameters that are

superimposed on the HLA association and indeed HLA-restricted T-cell activation to determine why particular individuals develop DILI.

The data outlined above indicate that the use of human cells has been extremely important in defining the cellular phenotype and cytokine profile of drug-specific cells; however, this work does not lend itself to interpretation of the *in vivo* situation at the time of a hypersensitivity reaction. In the field of autoimmune disease, animal models have been used to dissect the mechanisms of how the immune reaction develops *in vivo* and strategies to prevent such reactions. As an example, rheumatoid arthritis is a disease involving auto-reactive T-cells. Animal models of the disease have been developed through proteoglycan-induction of rheumatoid arthritis where the adaptive immune system causes joint inflammation (Hanyecz et al. 2004). Investigations using this and other animal models of the disease have lead to numerous human clinical trials (Keystone 2002). From these, a strategy of T-cell inhibition through blockage of signalling through the co-stimulatory molecule CD28 using a fusion protein (known as Abatacept) has demonstrated efficacy in clinical trials leading to the registration of Abatacept for the treatment of rheumatoid arthritis (Genovese et al. 2005).

In the field of drug hypersensitivity, researchers have worked extensively to develop animal models that reproduce the clinical situation in humans. However, the development of an animal model of drug hypersensitivity which fits these criteria has proven to be elusive. Amodiaquine induced liver injury in humans has an immune aetiology and is associated with agranulocytosis and hepatotoxicity which, upon discontinuation of the drug leads to a prompt recovery. Re-challenge with the drug leads to a rapid increase in ALT levels indicating the involvement of the adaptive immune system (Neftel et al. 1986) (Uetrecht J. 2005). Many attempts to create an animal model of amodiaquine have ended in failure.

Dosing the drug at high concentrations for four days in rats revealed an increase in ALT levels but no accompanied histological changes (Gruppi et al. 1995). Administration of the drug alongside a glutathione-synthesis inhibitor revealed centri-lobular necrosis 6h post dose in mice however, this does not resemble the human condition. Administration of a clinically relevant dose of the drug yielded promising results where ALT increased in both rats and mice after 2-3 weeks which was consistent with human data. However, unlike the human condition, continued dosing of the drug lead to a decrease in the ALT levels and instead of exacerbation of injury, adaptation and immune tolerance to the drug was witnessed (Shenton et al. 2004).

Isoniazid is a first line drug for the treatment of *Mycobacterium tuberculosis* however is it associated with liver injury mediated most likely through the actions of the adaptive immune system (Ng et al. 2012). Administration of the drug to both rabbits and rats leads to mild liver damage but neither form of the toxicity is similar to the liver injury witnessed in humans. Rats and rabbits displayed a direct toxicity to an isoniazid metabolite where serum levels of the metabolite correlated directly with increased ALT levels (Mitchell et al. 1976) (Sarich et al. 1999) indicating that these species were not suitable as model of human isoniazid liver injury. In mice, the covalent binding of isoniazid to proteins was detected (Metushi et al. 2011). Mice were immunised with supernatant of liver homogenate in complete Freund's adjuvant in combination with isoniazid in an attempt to develop an immune response. This did lead to auto-immune hepatitis however, when oral administration of isoniazid (which was hypothesised to aggravate the condition) was started, the drug not only did not cause increased DILI, it reduced the hepatitis (Metushi et al. 2011) (unpublished observation) (Ng et al. 2012), suggesting that the drug in this model induces tolerance.

Through the number of failed attempts at developing an animal model of drug hypersensitivity which closely mimics the human condition, there has been one model which did not. Nevirapine is a drug used to treat HIV-1 infection and causes drug mediated skin rash in a minority of patients (Pollard et al. 1998). The incidence of hypersensitivity reaction is higher in females (Wong et al. 2001). When female brown Norway rats are fed nevirapine at 150mk/kg/day they develop red ears after about seven days and skin rash after 14-21 days with an incidence of 100% (Shenton et al. 2003). With this rat model of nevirapine induced rash it was discovered that if the starting dose of nevirapine was lowered before the full dose was administered the rash was prevented, which is indicative of some form of desensitisation (Shenton et al. 2003).

The development of the murine LLNA (Kimber et al. 1989) was an important milestone for the prediction of the sensitisation potential of chemicals and has been used for over 20 years to investigate CHS reactions to haptens. Recently, researchers have used a C57Bl/6 mouse with mutation in the αβ gene encoding for MHC II molecules to sensitise mice to weak sensitisers such as the β-lactam antibiotic amoxicillin (Vocanson et al. 2006) (Rozieres et al. 2010). The mice have vastly reduced numbers of $CD4^+$ T-cells but retain a small population of regulatory T-cells which are $CD4^+$ $CD25^+$. Amoxicillin sensitization in these mice was achieved through direct painting of the drug onto the skin, but only when the residual $CD4^+$ T-cells were depleted. Five days after the last drug exposure, the dLN were removed from the mice to allow isolation and characterization of drug-specific $CD8^+$ T-cells. These data indicate that $CD8^+$ T-cells are the primary mediators of sensitization, while $CD4^+$ T-cells (down) regulate the antigen-specific T-cell response.

The three β-lactam antibiotics used in this study (amoxicillin, flucloxacillin, and piperacillin) are known to bind to lysine residues on protein and in particular, Lys 190 on human serum

albumin. Adduct formation is dependent on opening of the β-lactam ring following nucleophilic attack to allow the formation of stable protein adducts (Whitaker et al. 2011) (Monshi et al. 2013) (Jenkins et al. 2009) (Ariza et al. 2012) (Meng et al. 2011).

The primary aim of this thesis was the use two of these β-lactam antibiotics, amoxicillin and flucloxacillin, which are associated with a high incidence of skin and liver reactions, respectively, to explore antigen-specific immunogenicity in the $CD4^+$ T-cell depleted MHC II KO C57Bl/6 mouse. With knowledge of the flucloxacillin-specific sensitization process we then explored whether it was possible to direct the T-cells to liver and detect evidence of immune-mediated tissue injury.

The development of the mouse model was centred around the work performed by the group of JF Nicolas in Lyon and in particular, around the published papers by Vocanson et al (2006) and Rozieres et al (2010). They were able to show that β-lactam specific $CD8^+$ T-cells generated in this mouse model are drug-specific but demonstrated a degree of cross-reactivity to other β-lactam antibiotics. The constraints of this model were that due to the mouse model being devoid of MHC II molecules and the mice being further depleted of $CD4^+$ T-cells, the mouse model is only capable of analysing effector $CD8^+$ T-cell responses and not $CD4^+$ T-cell responses, whether they be inhibitory or not. Furthermore, as the background of the mice were C57Bl/6 mice, the responses are biased towards a T_H1 phenotype (Mills et al. 2000).

Mice were sensitised to amoxicillin using published protocols; however, drug-specific T-cells were not detected and evidence of sensitisation (i.e. ear selling responses) was not obtained. Thus, a series of experiments were conducted in Lyon in attempt to resolve this issue. After several modifications to the sensitisation protocols it was possible to

characterise amoxicillin-specific $CD8^+$ T-cells that could mediate a contact allergic reaction (chapter 2), successfully repeating the published data by Rozieres et al (2010) and highlighting the effector functions of both T_{regs} and CTL in amoxicillin hypersensitivity. Similar experiments were then conducted with two additional β-lactam antibiotics flucloxacillin and piperacillin. Topical exposure to flucloxacillin was associated with the activation of naive $CD8^+$ T-cells. Following *ex vivo* stimulation a concentration dependant proliferative response and IFNγ secretion were readily detectable. Other than being the first instance of a mouse being successfully sensitised to flucloxacillin, the flucloxacillin-specific T-cells generated displayed the same Tc1 phenotype as flucloxacillin-specific T-cells isolated from human allergic patients (Monshi et al. 2013) (Wuillemin et al. 2013). The comparability between mouse and human continued when attempting to sensitise to piperacillin. In contrast to flucloxacillin, piperacillin stimulated only a weak $CD8^+$ T-cell response which may relate to the fact that piperacillin preferentially activates $CD4^+$ T-cells in hypersensitive patients and induces T_H2 polarised cytokine secretions (El-Ghaiesh et al. 2011). Work performed using human T-cell clones from β-lactam hypersensitive patients also displayed drug-specificity however with accompanied cross-reactivity. The trends of cross-reactivity differed between mouse and human drug-specific T-cells but the overall reactivity of the cells displaying antigen-specificity was similar, indicating core similarities between the drug-specific $CD8^+$ T-cells generated in the mouse model and the human T-cell clones generated from drug hypersensitive patients (Whitaker et al. 2011) (El-Ghaiesh et al. 2011).

The importance of DCs in activating drug-specific $CD8^+$ T-cells has been demonstrated in the work performed in chapter 4 of this thesis. Flucloxacillin-specific $CD8^+$ T-cells required the presence of DCs to launch a full T-cell response (i.e. proliferation and IFNγ secretion) when cultured with flucloxacillin *ex vivo*. If the co-culture concentration of DCs was decreased

when incubating flucloxacillin-specific $CD8^+$ T-cells, a full T-cell response was not induced. Indeed, when DCs were removed from the *ex vivo* culture conditions, the T-cell response to flucloxacillin was ablated indicating the necessity of APCs when presenting the drug-derived antigen to mouse $CD8^+$ T-cell. The activation of $CD8^+$ T-cells by APC with no soluble drug present is indicative of a peptide haptenisation mechanism. However, the discovery of HLA-B*57:01 being associated with flucloxacillin allergic patients (Daly et al. 2009), opened the door for flucloxacillin to be linked to the pi-concept, which recently gained experimental evidence (Wuillemin et al. 2013). This being said, patients can still develop flucloxacillin hypersensitivity via a hapten-mediated mechanism (Wuillemin et al. 2013). Furthermore, priming of naive human T-cells against flucloxacillin hapten has also been demonstrated (Monshi et al. 2013). Genetic screening before administration of a drug is looking to become a large part of future clinical practice. For example, HLA-B*57:01 screening before administration of abacavir is now performed routinely and has decimated the numbers of abacavir induced hypersensitivity reactions (Mallal et al. 2008). Flucloxacillin does not currently require a genetic screen before administration due to a prevalence of only 8.5 in 100,000 patients and HLA-B*57:01 not certifying the development of flucloxacillin DILI (Russmann et al. 2005). The implications of this are that flucloxacillin is going to continue to be prescribed to both HLA-B*57:$01^{+/-}$ patients, highlighting the importance of investigating both the hapten and pi mechanisms regarding onset of liver injury.

If the experiments performed in this thesis were repeated with a $CD8^+$ T-cell deficient mouse where $CD4^+$ T-cells were left un-altered, the continued presence of T_{regs} would assumedly have a negative impact on what effector functions may be elicitated; as has been reported in other animal models of $CD4^+$ T-cell mediated diseases like nevirapine hypersensitivity (Ng et al. 2012).

The mouse model has been developed and utilised in this thesis to demonstrate that upon first contact with flucloxacillin, protein binding occurs on at least one peptide (see chapter 5) to create a haptenic peptide capable of inducing T-cell activation (Monshi et al. 2013) and sensitisation *in vivo* (see chapter 4). The haptenic chemical, if introduced via the skin, is likely detected by $CD103^+$ DCs present in the dermis which preferentially present to $CD8^+$ T-cells (Bedoui et al. 2009). These modified peptides are detectable in the blood serum of systemically dosed animals and more importantly in respect to flucloxacillin, in the liver cytosol (see chapter 5). This detection of modified albumin peptides in the liver cytosol of systemically dosed mice corroborates the work performed by Carey and Van Pelt (2005) who published findings very similar to this, in rat. Demonstration of killing of flucloxacillin-modified hepatocytes by $CD8^+$ T-cells was beyond the scope of this thesis as *in vitro* incubation of flucloxacillin with hepatocytes was not able to yield detectable protein modifications (data not shown). However, this experimental course would provide direct evidence that an intracellular protein conjugate activates the immuno-allergic hepatotoxicity.

Hepatocytes, when infected with foreign antigen (i.e. parasites) are known to be targeted by cytotoxic $CD8^+$ T-cells. For example, upon culture of malaria parasite infected hepatocytes with $CD8^+$ T-cells from a vaccinated mouse, the $CD8^+$ T-cells were discovered to kill the infected hepatocytes via contact-mediated perforin secretion (Trimnell et al. 2009). With regards to DILI, the mechanisms of hepatocyte cell death is somewhat varied from drug to drug with very little direct evidence of an adaptive immune response. Examples include amodiaquine-induced liver injury where in rats dosed for 6 weeks with the drug, a mild increase in $CD4^+$ T-cells, NK cells, and T_H17 cells was documented alongside mild increases in ALT levels (Shenton et al. 2004). For flucloxacillin-induced liver injury, the main evidence of

damage to the liver by the adaptive immune system stems from flucloxacillin-specific $CD8^+$ T-cells being isolatable from hypersensitive patients suffering liver injury (Monshi et al. 2013). In the experiments performed here, a kit designed to look at three readouts of cell viability simultaneously was chosen to analyse the potential-drug-specific killing of hepatocytes by flucloxacillin pulsed $CD8^+$ T-cells from flucloxacillin-sensitised mice. The hepatocyte viability, cytotoxicity, and apoptosis was measured simultaneously through use of Promega's Apotox Glo Triplex assay which has been successfully used as a readout in a variety of cell types (Prasad et al. 2012) (Arnault et al. 2013). The results obtained from the co-culture of hepatocytes with flucloxacillin-pulsed $CD8^+$ T-cells from the dLNs of flucloxacillin-sensitised mice is the first direct evidence for the drug-specific killing of hepatocytes by actions of the adaptive immune system. In support of the cytotoxicity data $CD8^+$ T-cells were found to secrete high levels of granzyme B following T-cell receptor triggering. Granzyme B initiates apoptosis through triggering of the caspase cascade (Chung et al. 2008).

A mouse model of immune-mediated hepatitis has recently been developed where mice were engineered so that exposure to tamoxifen (an oestrogen receptor antagonist) induces partial expression of ovalbumin in hepatocytes (Cebula et al. 2013). Antigen-specific $CD8^+$ T-cells were then adoptively transferred to cause symptoms mimicking immune-mediated hepatitis. These symptoms involved the clearance of antigen expressing hepatocytes through cytotoxic actions of the $CD8^+$ T-cells. This included $CD8^+$ T-cell proliferation and subsequent cytotoxic actions leading to an increase in ALT levels in blood serum. Immunohistochemistry revealed increases in intracellular caspase 3, in ovalbumin expressing hepatocytes (Cebula et al. 2013). Although this animal model is fashioned on immune-mediated hepatitis instead of drug hypersensitivity, the mechanisms of $CD8^+$ T-cell-

mediated hepatocyte killing after presentation of foreign antigen is strikingly similar to the results discovered *in vitro* here with flucloxacillin.

Upon an *in vivo* challenge, flucloxacillin-specific $CD8^+$ T-cells did not demonstrate a clearance of hepatocytes as reported in the mouse model of immune hepatitis (Cebula et al. 2013). However, swelling of the gall bladders and infiltration of leukocytes did tentatively suggest the development of early cholestasis through biliary atresia (see chapter 5). The development of cholestasis by flucloxacillin could be due to the presentational ability of the liver cell subsets (Ebrahimkhani et al. 2011). This "antigen presenting ability" has been gauged through analysing presentation of OVA peptide and subsequent $CD8^+$ T-cell proliferation and activation. Results from this study demonstrated that liver hepatocytes and hepatic stellate cells were not efficient at presenting foreign antigen to $CD8^+$ T-cells. However, Kuppfer cells and perhaps more importantly, hepatic sinusoidal epithelial cells, were capable of presenting foreign antigen which was comparable to the amount of presentation demonstrated by mature spleen DCs (Ebrahimkhani et al. 2011). This is interesting in respect to flucloxacillin-induced liver injury, where injury in human patients is mainly mediated through cholestatic liver damage which involves damage towards the epithelial cells of the bile duct. This may perhaps develop through bile epithelial cells being efficient at presenting flucloxacillin altered peptides to cytotoxic $CD8^+$ T-cells, which in turn destroy the epithelial cells to allow bile build-up and subsequent cholestatic liver damage. A possible mechanism of gall bladder swelling through downstream biliary damage by flucloxacillin could be explained through flucloxacillin creating an immunogenic haptenic protein in the liver. This could lead to bile duct damage through effective antigen presentation by biliary epithelial cells to effector lymphocytes. This ductal damage through cytotoxic $CD8^+$ T-cells could lead to further T-cell-mediated inflammation and through

activation of these T-cells, IFN-stimulation of macrophages could follow. This macrophage activation could lead to the release of nitric oxide, ROS, and TNF with subsequent epithelial cell death via apoptotic and/or necrotic pathways (Sokol and Mack 2001) (Sokol et al. 2003). This inflammatory action could lead to the stenosis of the downstream bile ducts and increase of biliary pressure. This immunological domino effect could explain the apparent biliary atresia and gall bladder swelling seen here.

To fully dissect the potential of the mouse model developed so far in this thesis, several future investigations should be initiated. The possibility of cholestatic liver damage after extended oral dosing (>3 weeks) combined with to assessment of whether the biliary atresia witnessed in the mice would continue and/or cause complications. The killing of specific subsets of liver cells by flucloxacillin-specific $CD8^+$ T-cells should be investigated through *ex vivo* co-culture. Furthermore, measurement of cell death bio-markers should be investigated to analyse whether other liver cells subsets (e.g. bile duct epithelial cells) are preferentially targeted by flucloxacillin-specific $CD8^+$ T-cells. The presence of liver-infiltrating leukocytes also needs to be analysed to determine whether flucloxacillin-specific $CD8^+$ T-cells migrate into hepatic tissue.

If fully elucidated and verified, a mouse model of cholestatic flucloxacillin-induced liver injury which truly mimics the human condition would be an invaluable tool in attempting to dissect the mechanisms of disease pathogenesis. Possibilities of use could also include:

1) Using the model to experiment towards a preventative of flucloxacillin-induced liver injury. As flucloxacillin-induced liver injury is usually only detected after the onset of jaundice (Andrews and Daly 2008), the damage has essentially already been done. Therefore, a preventative measure seems a more feasible goal than a cure. This

could perhaps be achieved through up-regulation of T_{regs}, as the adoptive transfer of T_{regs} in mice has been used to successfully prevent diseases such as autoimmune haemolytic anaemia (Mqadmi et al. 2005), and the development of cholestatic-mediated flucloxacillin-induced liver damage is linked to $CD8^+$ T-cells (Monshi et al. 2013) (Wuillemin et al. 2013) (chapter 5).

2) Antibiotic-resistance is a very real threat to the way humans currently live (Balsalobre et al. 2014). It is imperative that new antibiotics, or new ways of using them, are developed to combat antibiotic-resistant bacteria. If the new antibiotics that are developed are similar to the ones currently used (small protein-reactive molecules), then this model could be used as an early warning system for whether these new molecules could be immunogenic and/or capable of causing cholestatic liver damage.
3) For nevirapine-induced skin rash, it was discovered that in a rat model of the drug-induced disease, a slow increase in concentration of the drug prevented the disease from progressing (Shenton et al. 2003). This discovery was instrumental in changing the way nevirapine was prescribed to humans. The mouse model could be used to investigate whether this particular method of drug introduction could induce flucloxacillin tolerance, leading to the prevention of liver injury.

To summarise, amoxicillin, piperacillin, and flucloxacillin have proven to be of invaluable use when attempting to dissect the mechanisms of drug-specific sensitisation and elicitation, drug-protein binding, and analysis of the processes involved in delayed-type hypersensitivity reactions. Use of a mouse model of CHS altered through $CD4^+$ T-cell depletion to analyse drug hypersensitivity, has shown to be invaluable in investigating the pathways of drug-

specific $CD8^+$ T-cell activation. For the first time, mice have been sensitised to piperacillin and flucloxacillin and furthermore, flucloxacillin-specific $CD8^+$ T-cells have been shown to kill hepatocytes *in vitro*. An *in vivo* oral flucloxacillin challenge did not lead to a mode of flucloxacillin –induced cholestatic liver injury mediated through the actions of $CD8^+$ T-cells. However, un-expectedly, this challenge did demonstrate symptoms consistent with early stages of cholestasis. Further experimentation using the methods developed herein, should be embarked upon in an effort to develop what would be the first animal model of DILI able to demonstrate injury mediated by the adaptive immune system.

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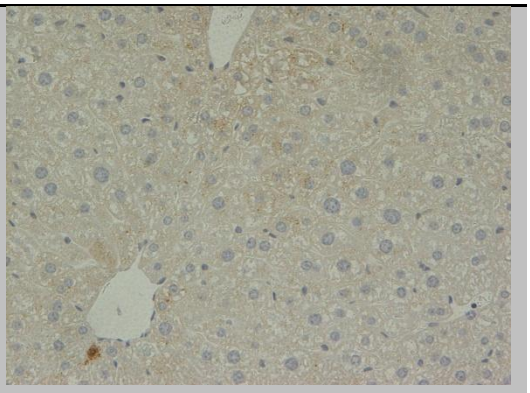
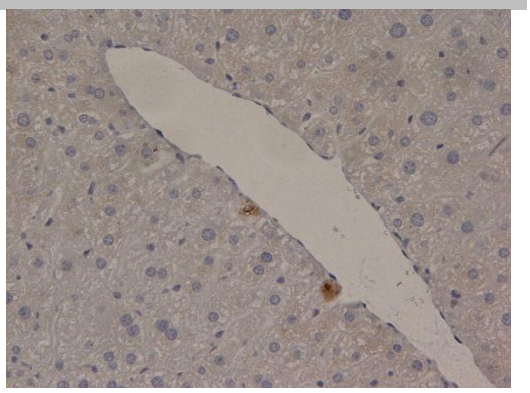
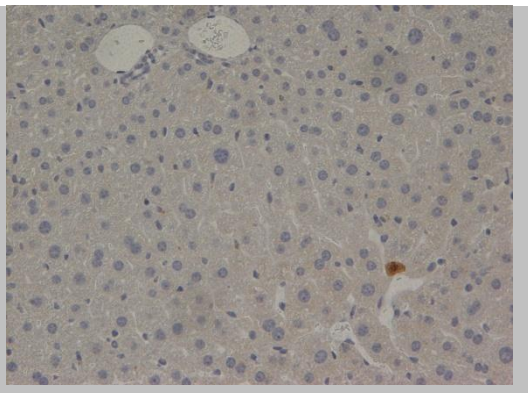
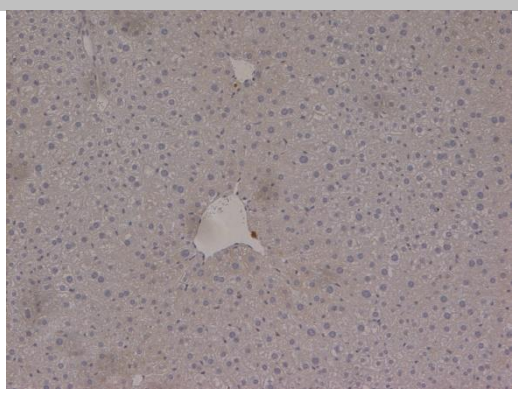
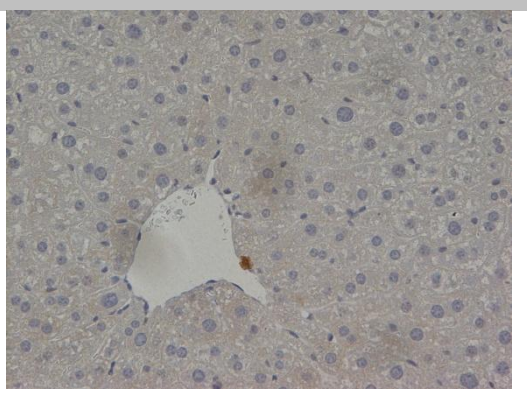
Appendix

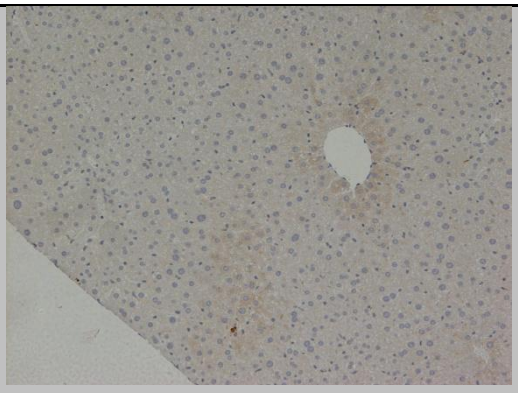
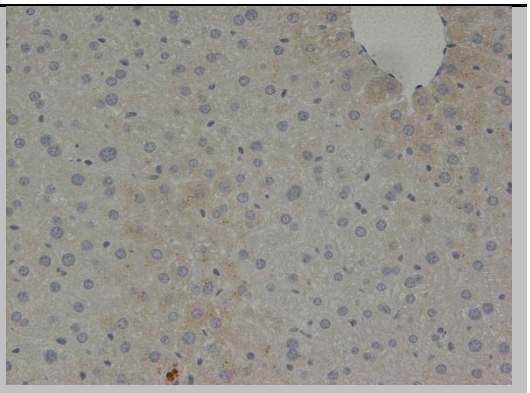
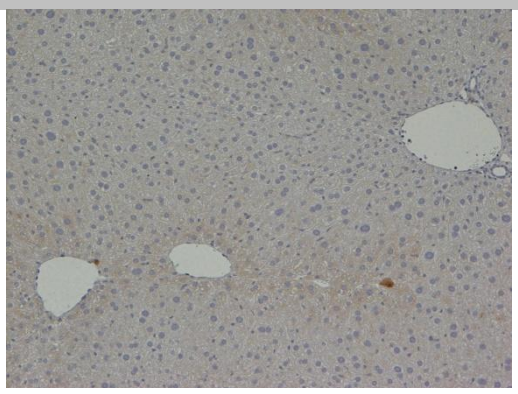
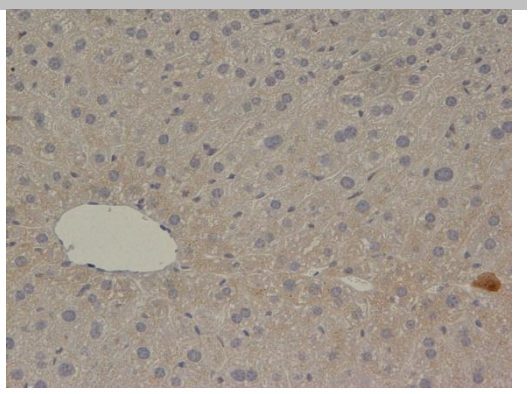
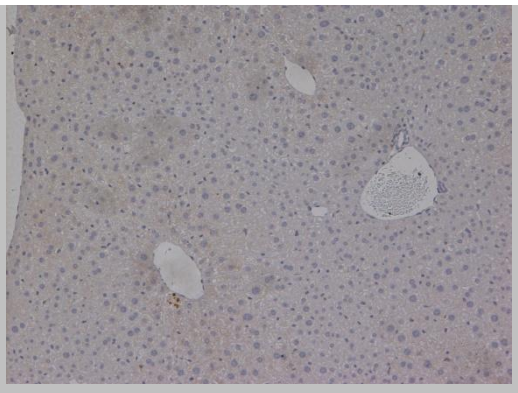
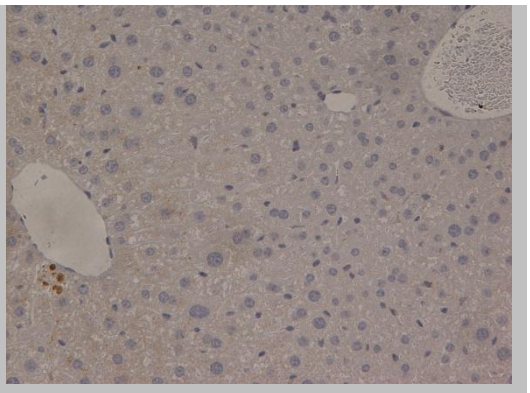
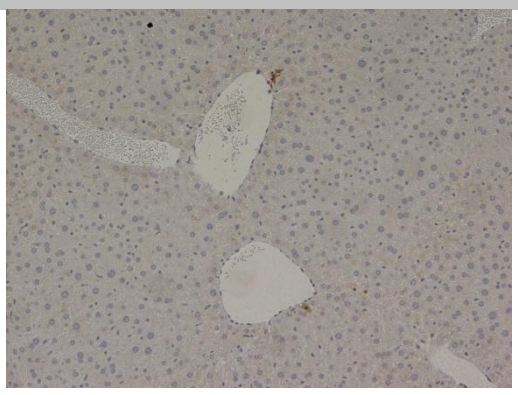
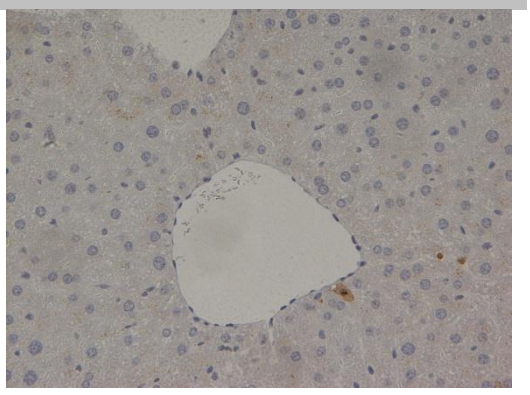
Supplementary figure 1. Flucloxacillin oral dosing of mice adoptively transferred flucloxacillin-specific $CD8^+$ T-cells and flucloxacillin-exposed $CD11c^+$ DCs show no increases in hepatocyte apoptosis.

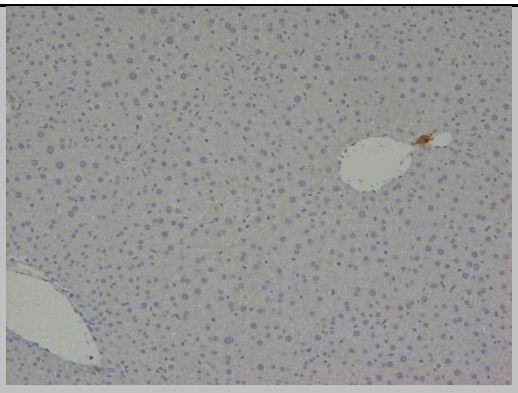
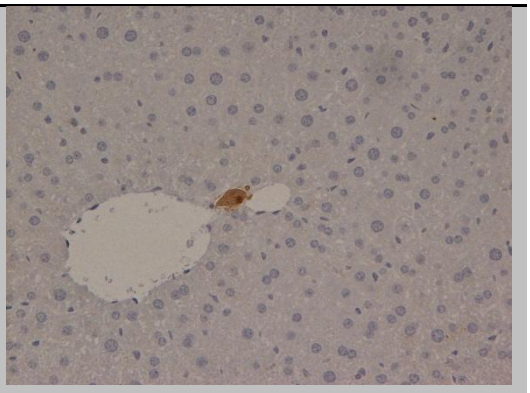
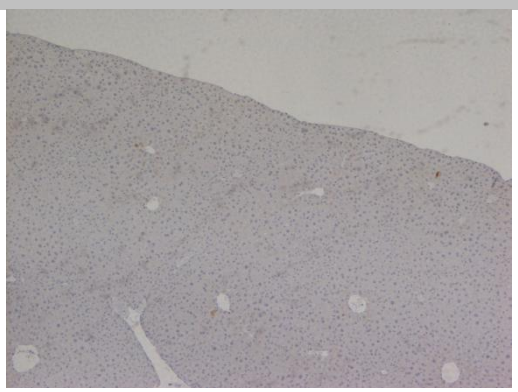
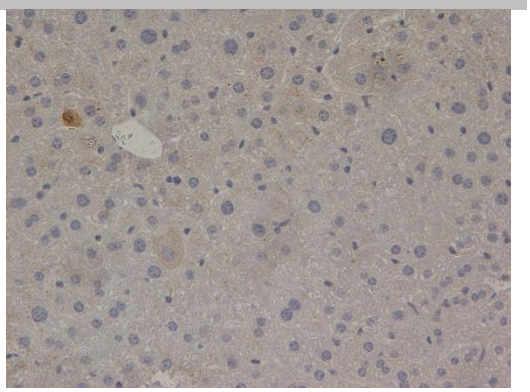
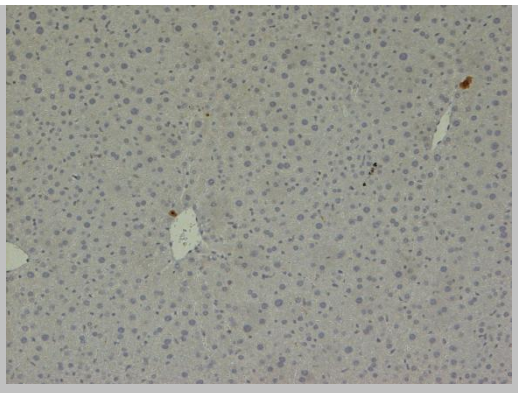
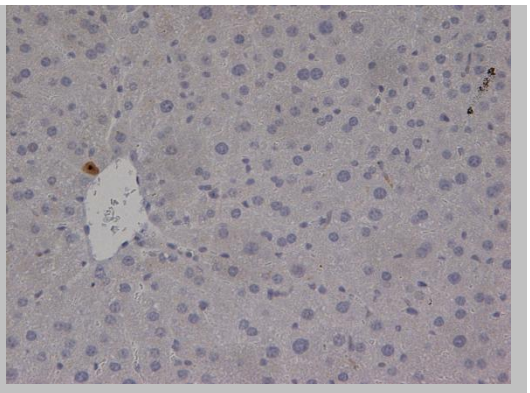
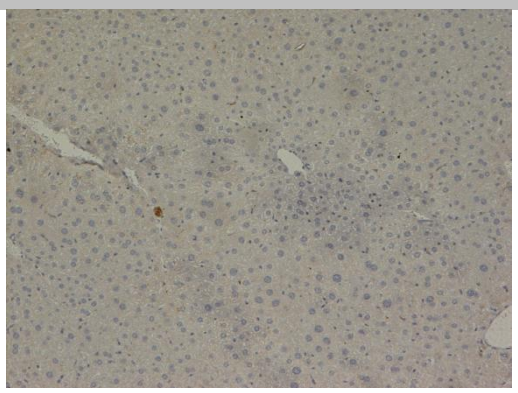
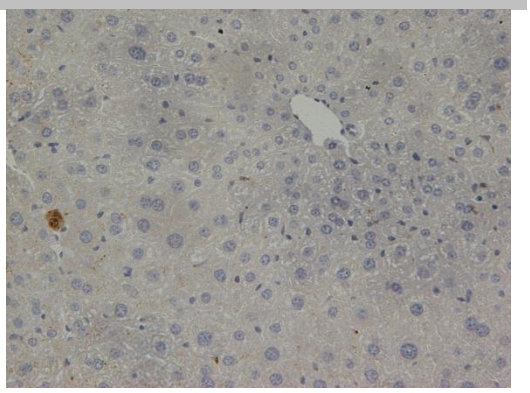
Supplementary figure 2. Flucloxacillin oral dosing of mice painted with RA induces gall bladder swelling in mice sensitised with flucloxacillin after four days of oral dosing and in all mice after ten days of oral dosing.

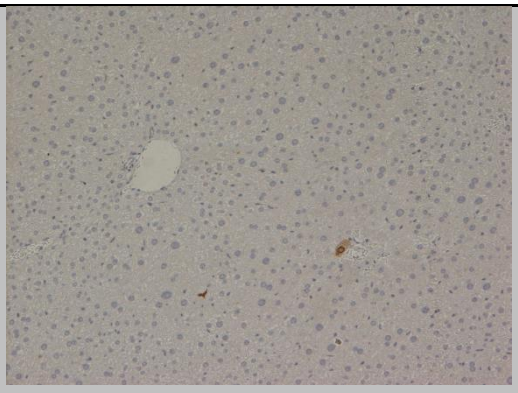
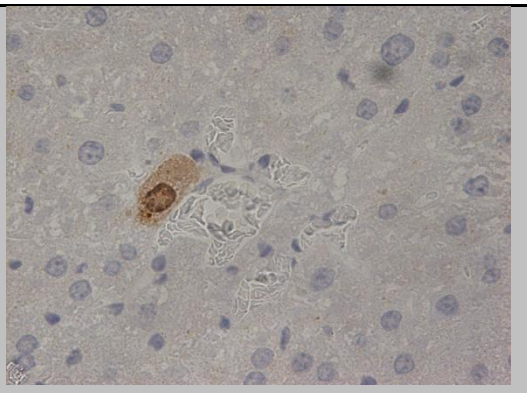
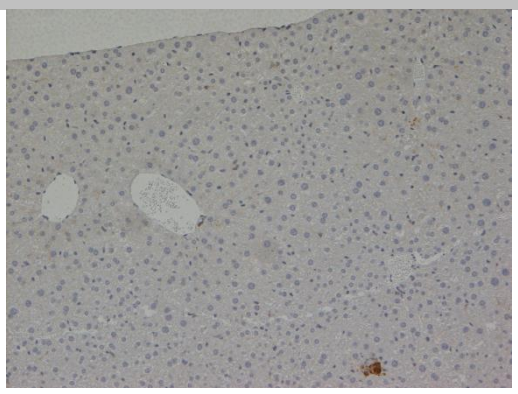
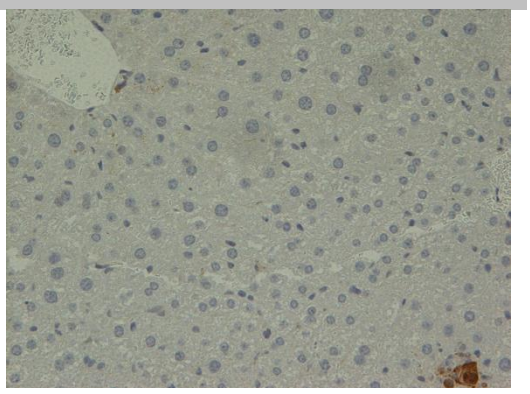
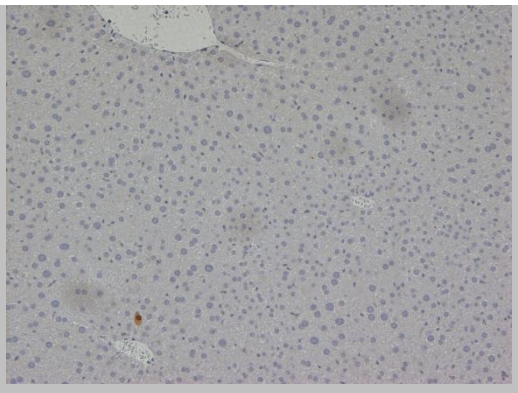
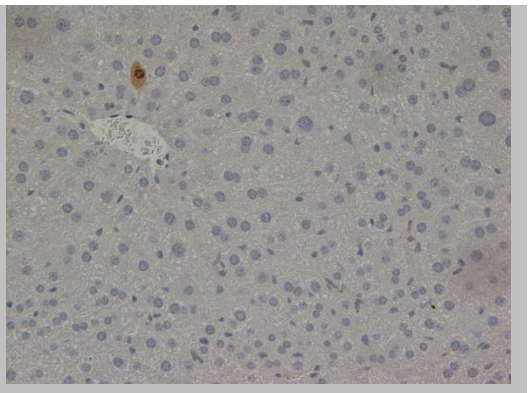
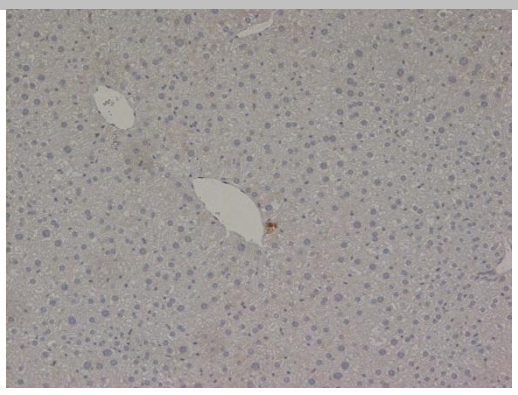
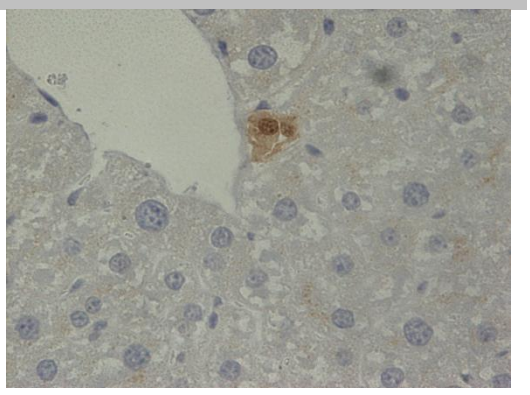
Supplementary figure 3. Flucloxacillin oral dosing of mice painted with RA/flucloxacillin does not induce increases in hepatocyte apoptosis.

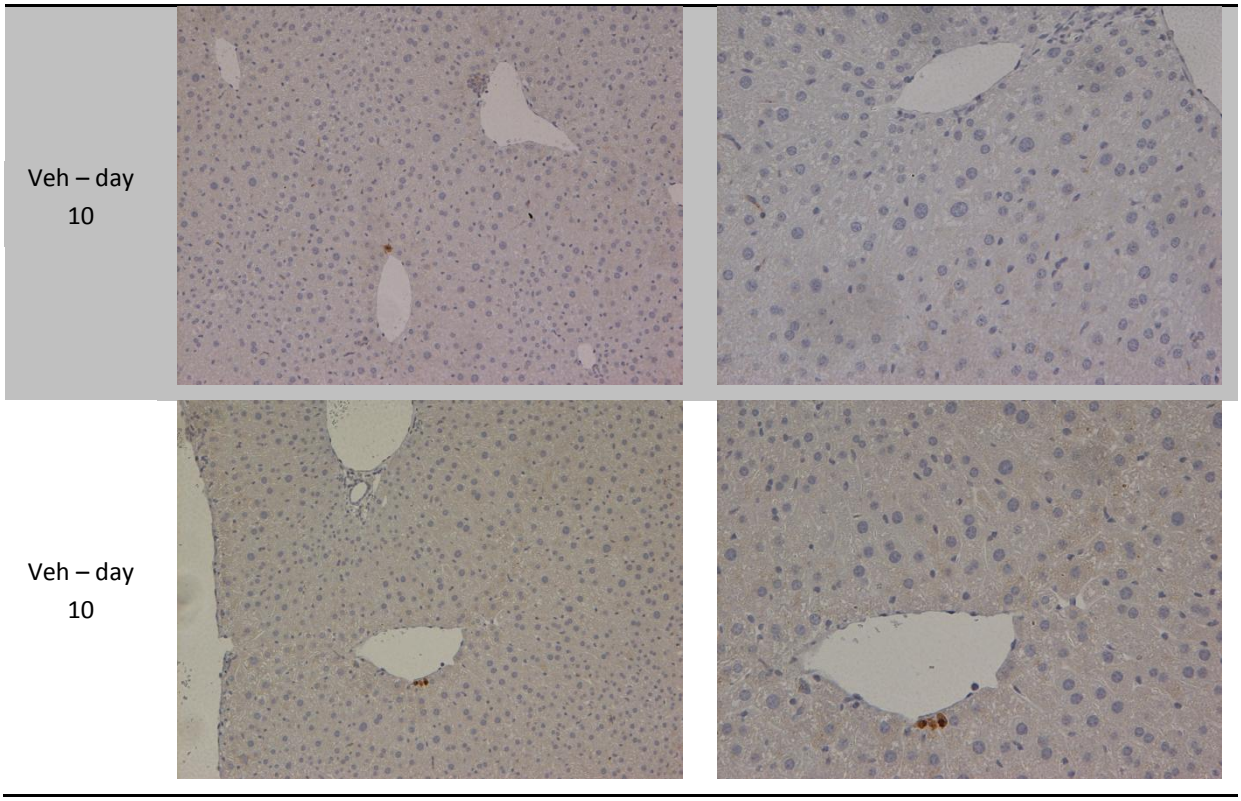
Supplementary figure 4. Flucloxacillin oral dosing of mice painted with RA/flucloxacillin induces infiltration of leukocytes into the liver.

Mouse treatment and days of oral dosing	**10x/4x magnification**	**20x/40x magnification**
Flu – day 0		
Flu – day 0		
Flu – day 0		
Veh – day 0		

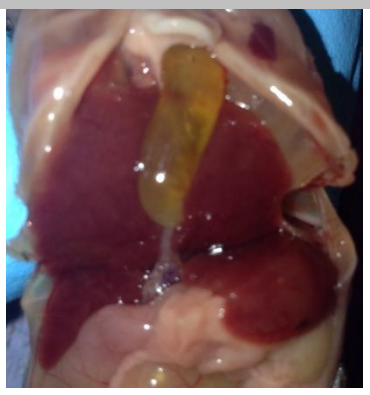
Veh – day 0		
Veh – day 0		
Flu – day 5		
Flu – day 5		

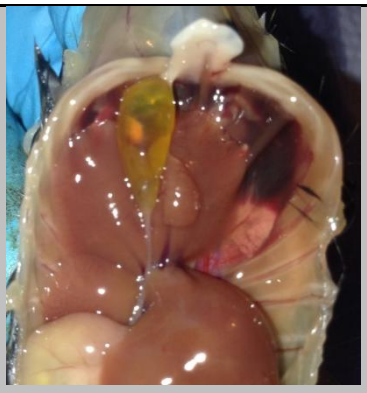
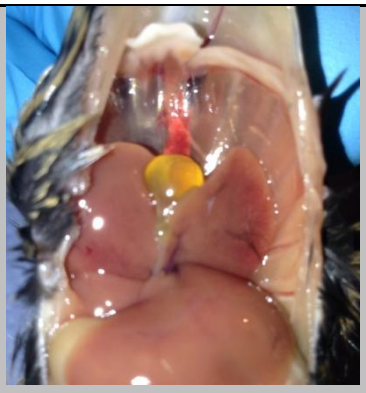
Flu – day 5		
Veh – day 5		
Veh – day 5		
Veh – day 5		

Flu – day 10		
Flu – day 10		
Flu – day 10		
Veh – day 10		

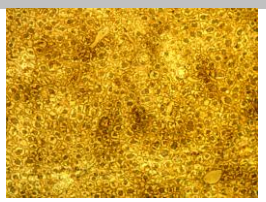
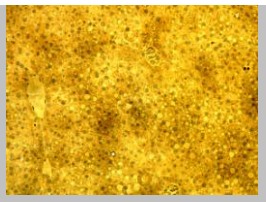
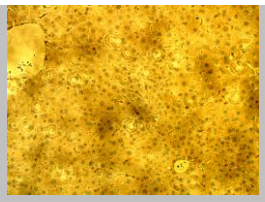
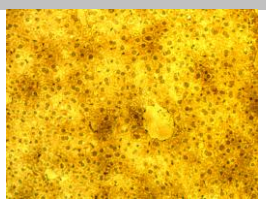
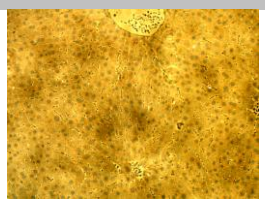
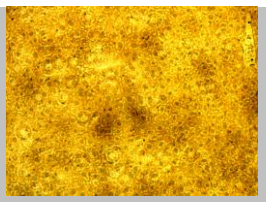
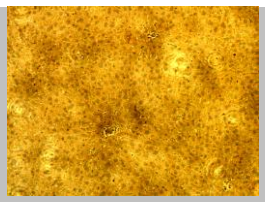
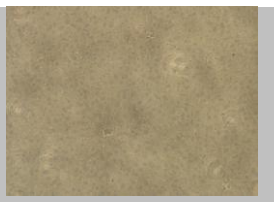
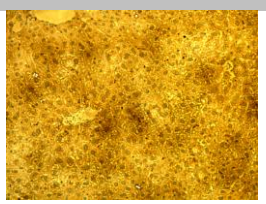
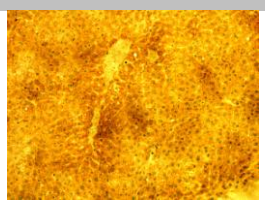
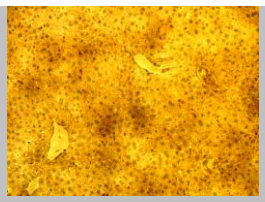
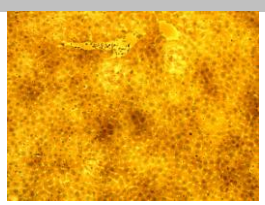


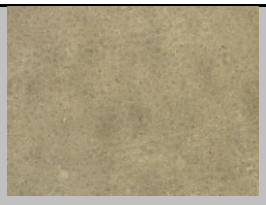
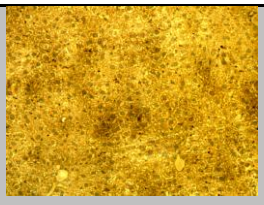
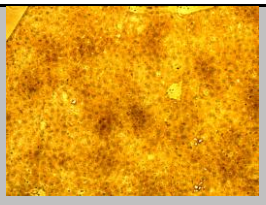
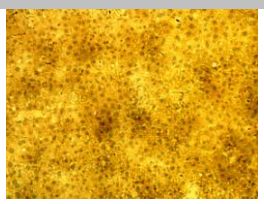
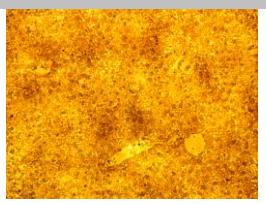
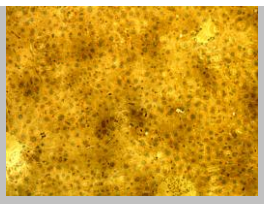
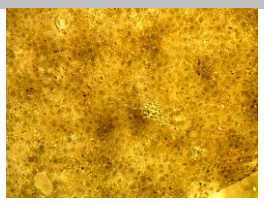
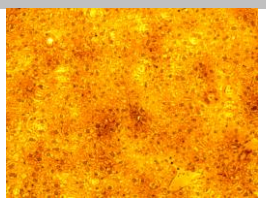
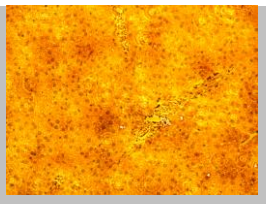
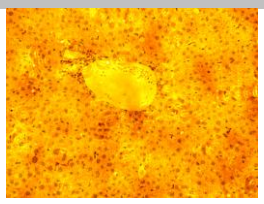
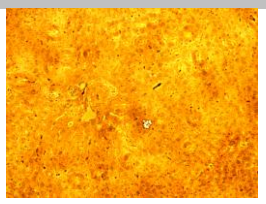
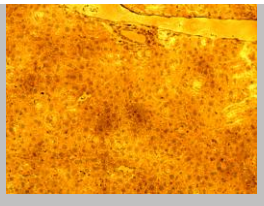
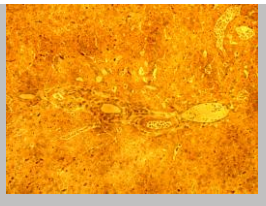
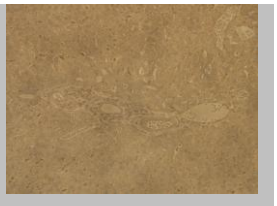
Supplementary figure 1. Flucloxacillin oral dosing of mice adoptively transferred flucloxacillin-specific $CD8^+$ T-cells and flucloxacillin-exposed $CD11c^+$ DCs show no increases in hepatocyte apoptosis.

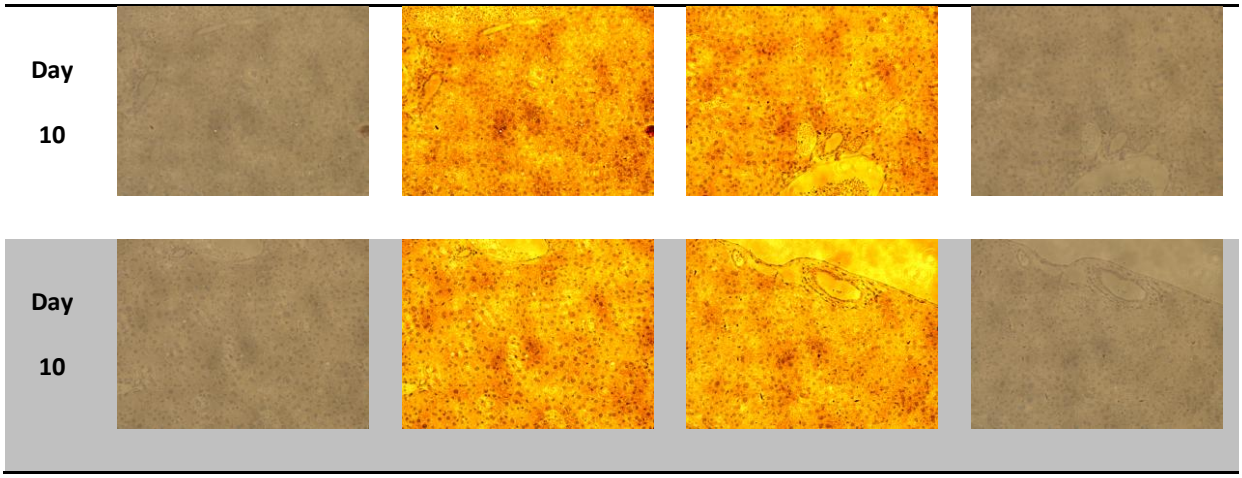
	Gall bladder swelling	
Individual mouse data	***Control***	***Flucloxacillin-sensitised***
Day 4 oral dosing		
Day 10 oral dosing		
Day 10 oral dosing		

Day 10 oral dosing		
Day 10 oral dosing	**N/A**	

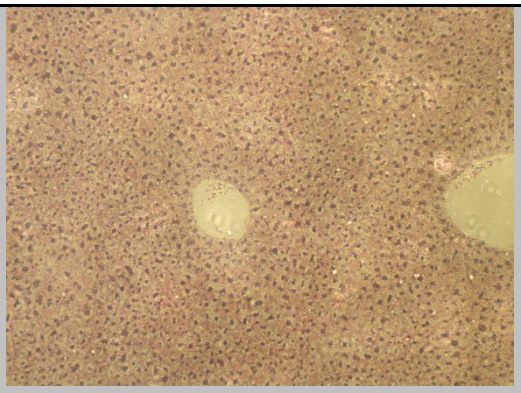
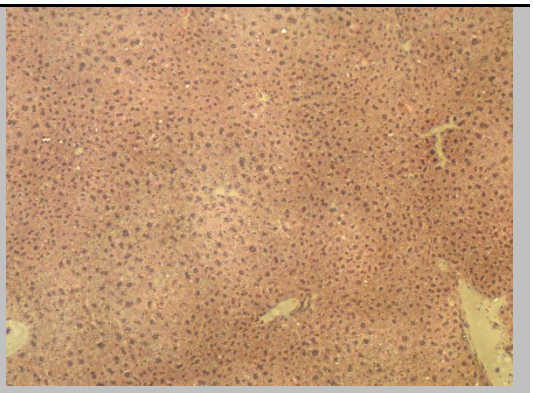
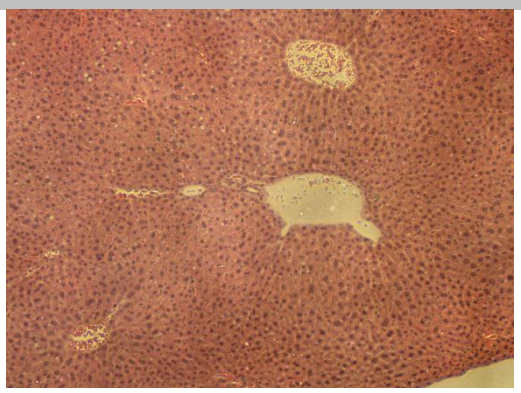
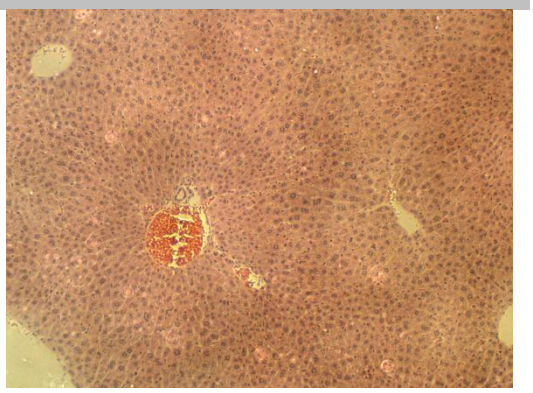
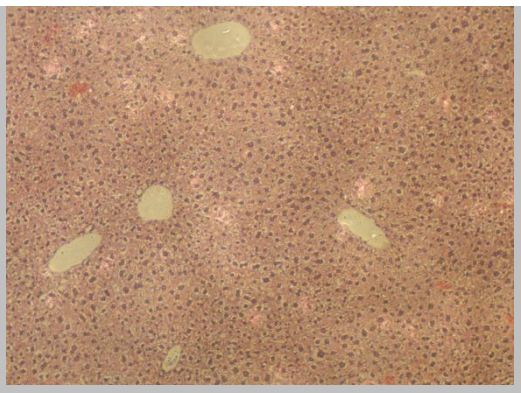
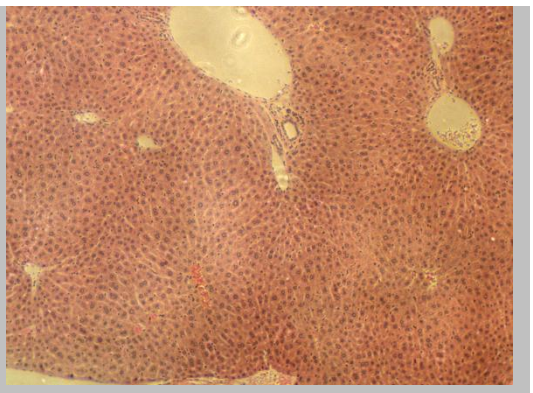
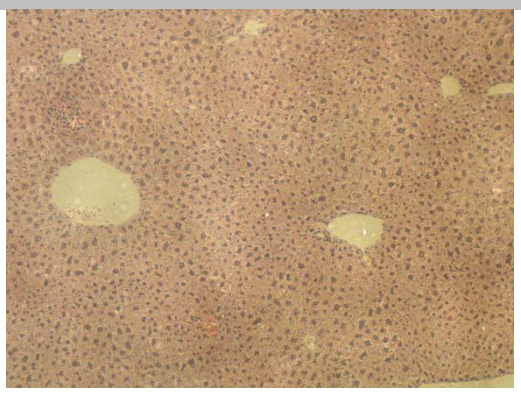
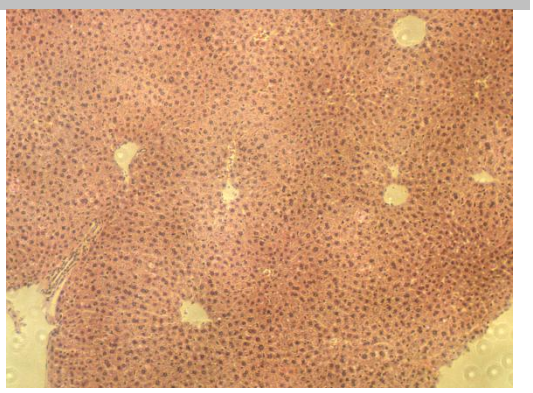
Supplementary figure 2. Flucloxacillin oral dosing of mice painted with RA induces gall bladder swelling in mice sensitised with flucloxacillin after four days of oral dosing and in all mice after ten days of oral dosing.

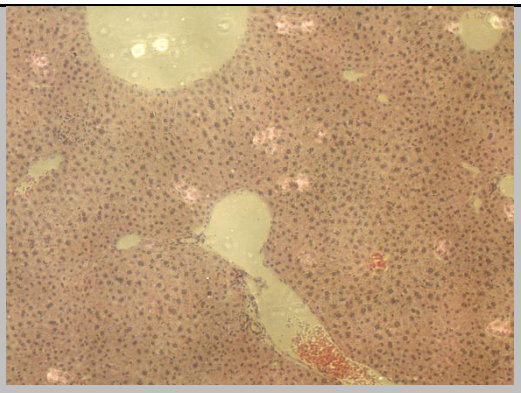
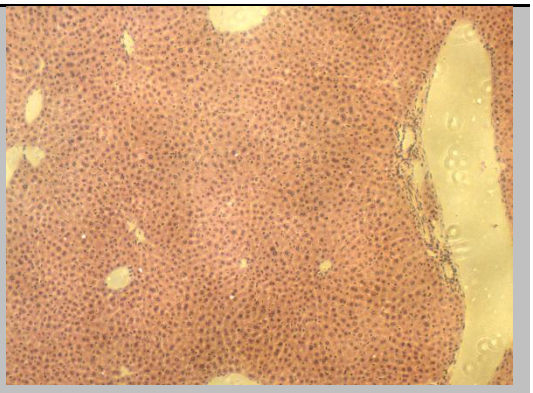
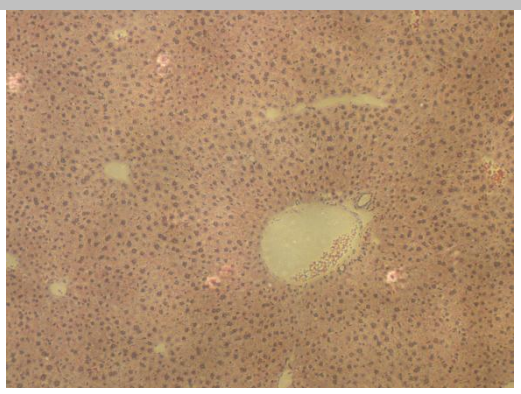
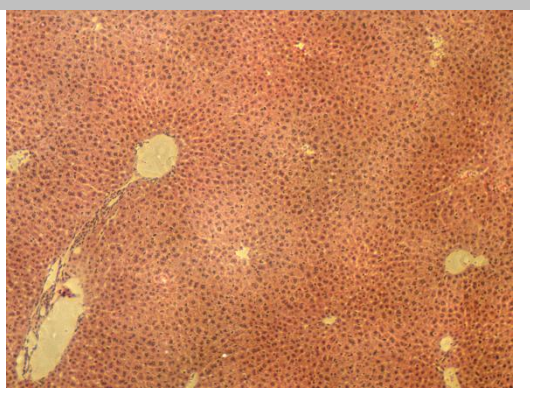
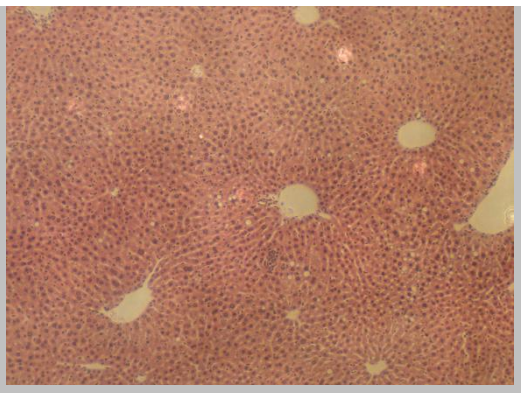
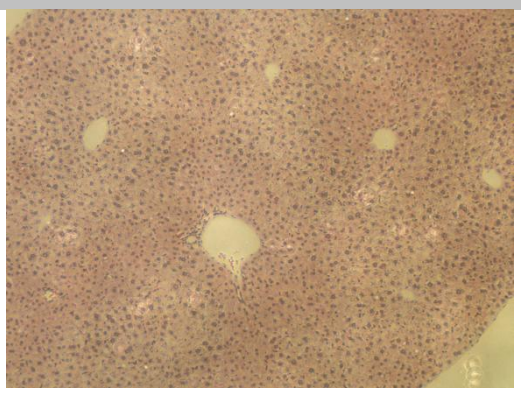
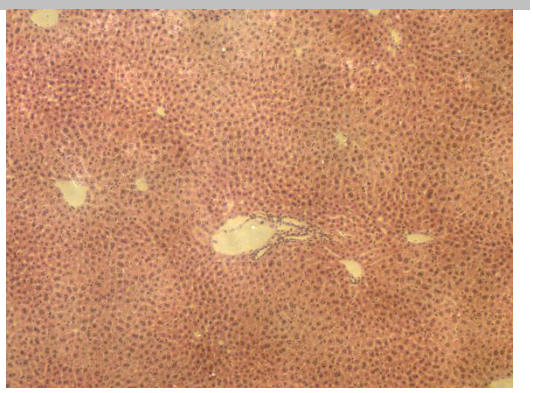
	Control		Flucloxacillin-sensitised	
	Original image	False coloured	False coloured	Original image
Day 0				
Day 0				
Day 0				
Day 0				
Day 2				
Day 2				
Day 2				

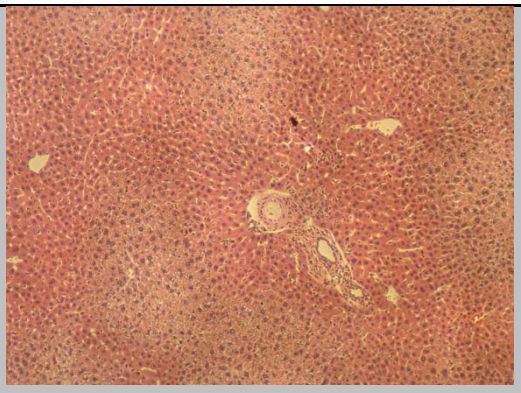
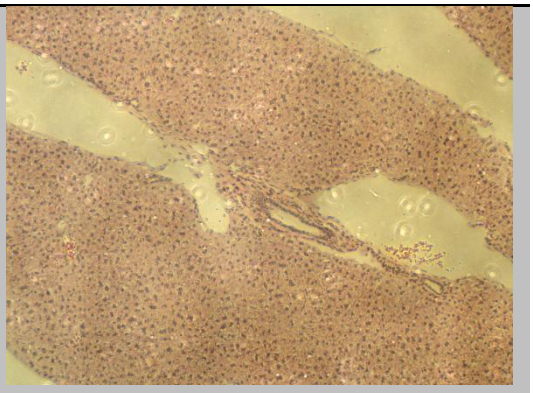
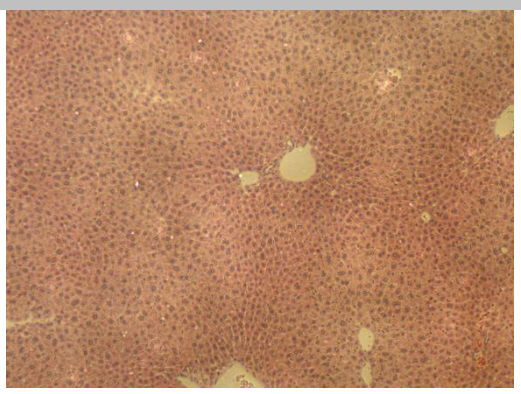
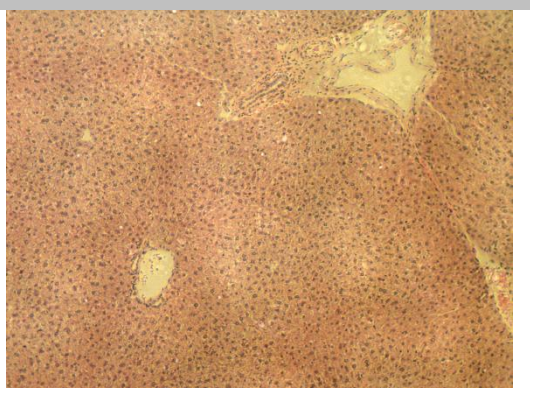
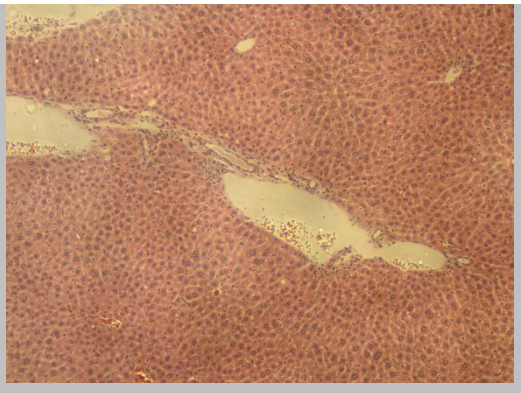
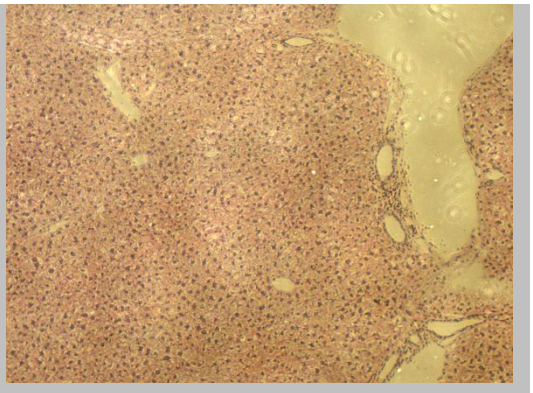
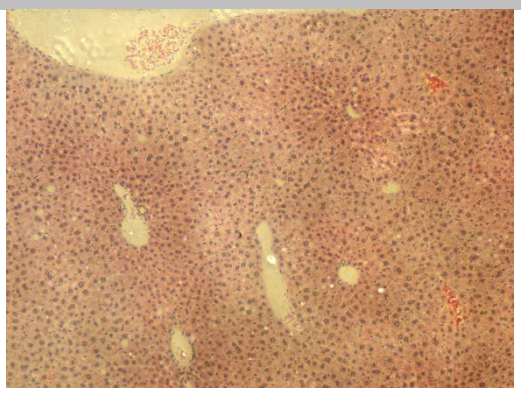
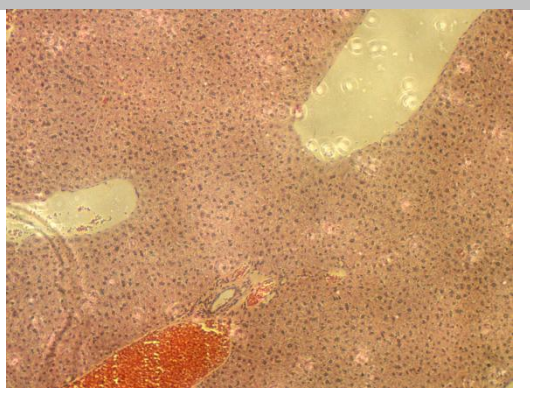
Day 2				
Day 4				
Day 4				
Day 4				
Day 4				
Day 10				
Day 10				

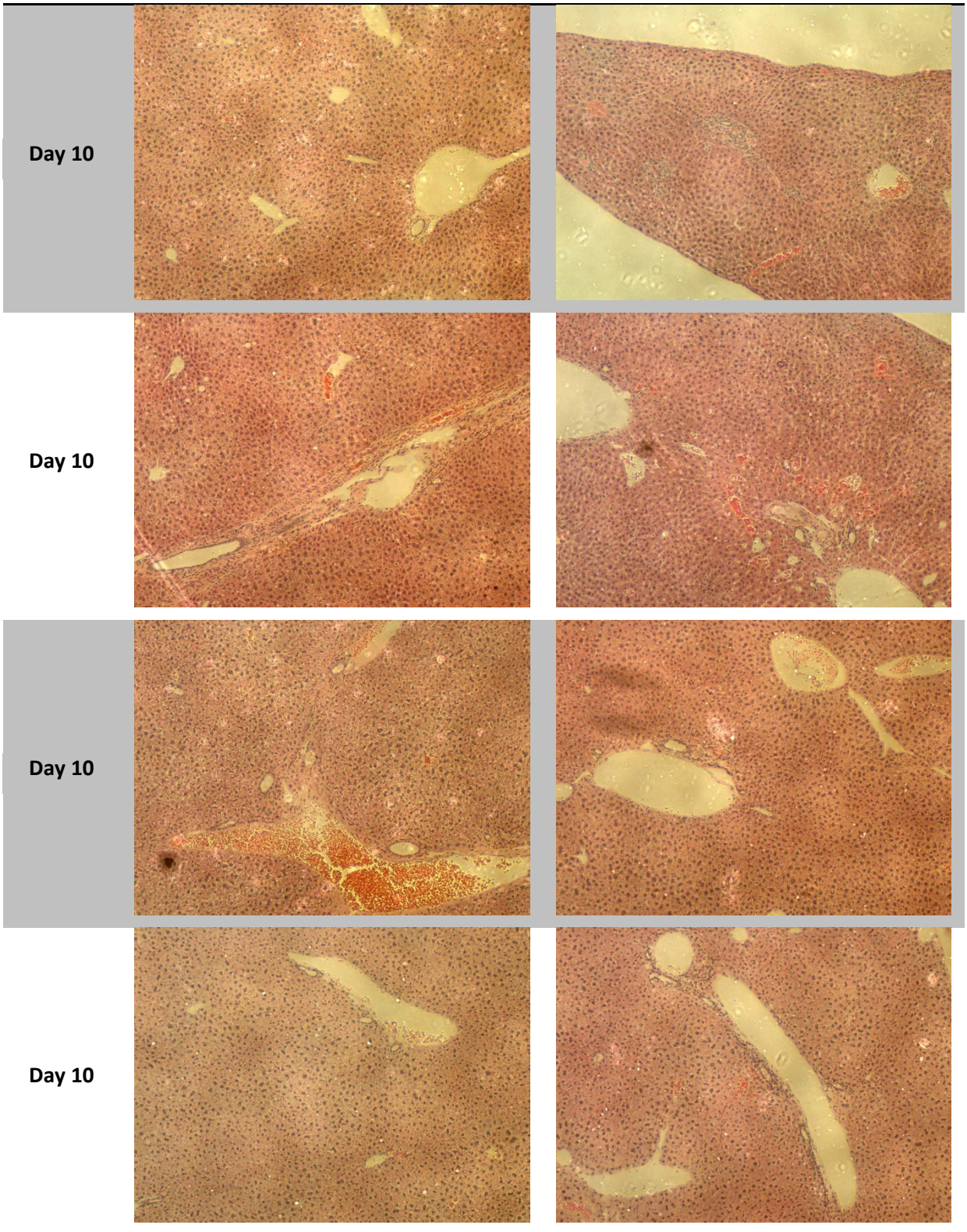


Supplementary figure 3. Flucloxacillin oral dosing of mice painted with RA/flucloxacillin does not induce increases in hepatocyte apoptosis.

Condition / days of oral dosing	*Veh painted*	*Flu Painted*
Day 0		
Day 0		
Day 0		
Day 0		

Day 2		
Day 2		
Day 2		
Day 2		

Day 4		
Day 4		
Day 4		
Day 4		



Supplementary figure 4. Flucloxacillin oral dosing of mice painted with RA/flucloxacillin induces infiltration of leukocytes into the liver.