UNIVERSITY OF LIVERPOOL

# CHARACTERISATION OF THE PHENOTYPE AND FUNCTION OF DRUG-RESPONSIVE T-CELLS IN PATIENTS WITH DRUG-INDUCED LIVER INJURY.

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

**Laila Kafu**

**October 2021**

## DECLARATION

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree.

…….

Laila. M. Kafu (BSc, MSc)

To my beloved late father Emhemed Ben Kafu

## ACKNOWLEDGMENTS

Firstly, I would like to thank my God, the most gracious and merciful for giving me the strength to carry this PhD when things felt out of hand. I am grateful to my financial sponsors Libyan Government for giving me the opportunity to pursue this PhD. I have been fortunate to have had the best supervisor any PhD student could ask for. Dean always had his door open when needed and was always calm when we approached any problems, whether scientific or otherwise. His brilliant scientific mind never failed to guide us throughout our PhD journey. Alongside the experience I have come with from my extensive career, Dean added to this experience by giving me a new perspective on leadership in the scientific and research field. I always remember his famous saying to me, 'Keep going Laila', when I need the extra push or motivation. This journey will forever be cherished in my memory, because it enriched my experiences and made my daily tasks easier to manage. My unreserved thanks to my supervisor, Professor Dean Naisbitt. Thank you to Dean for helping me finish my writing in what felt like an eternal Covid lockdown! Having the right work atmosphere and setup is such an undervalued aspect until Covid showed up. You offered me your office, when I had used up all the time slots available in Harold Cohen library. I would like to thank my colleagues in the lab and throughout the Department of Molecular and Clinical Pharmacology in Translational Medicine at Sherrington building where my research laboratory was located as well as my office; MRC Centre for Drug Safety Science. I would like to thank my colleagues who have made the PhD journey less painful, special thanks to Monday Ogese for his support and guidance in the laboratory; Andy whom I shared orange chocolate, with James, Arun, John who was always helpful in radiating our blood samples for experiments; special thanks to Adam whom I shared with tremendous amount of coffee and pastries to give us the sugar rush we needed to continue working in the laboratory for the whole day; Sean, Kareena, Serat, and Kanoot and our new colleagues. I hope I did not forget anyone of my dear colleagues who have been a great support for me. Thank you to Mubarak and Aziz who were always up for a chat and advice, with you guys I did not miss Arabic and with time we became dear friends. Special thanks to Paul Thomson for all his leniency and support and patience when I embarked on the journey of writing my thesis. Words fail to express my appreciation to my family. My dear husband Mr. Adel Alsadeq, for the support and for taking the load off my shoulders especially that we had to do long distance to balance our responsibilities. I thank my daughters, Alaa and Saja, and my son, Ali, who are the beginning of everything beautiful in my life and my best friends. Alaa, thank you for taking care of your brother and father back home and for Saja who astonishes me with her motivational chat and her understanding for the nature of my work and for Ali who always being in my heart and my mind. I would also like to thank my parents and my siblings for their support and the WhatsApp group chats that were the highlight of my days. Special thanks also to Dr. Ghada Almuntasser, a childhood friend and a dear sister who checks up on me weekly and motivates me to finish my PhD. Thanks to Dr. Afaf Gerushi, who throughout the years have become a friend. I would like to convey special acknowledgement to my friends, Dr. Ruba Bunyan and Dr. Shaymaa Al majmaie who never hesitated to donate blood when I needed.

To my beloved mother, you have been inseparable support with your prayers and kind words. I gift you all the work I have put in this thesis.

Finally, I would like to thank everybody who was important to the fulfilment of this thesis, as well as expressing my apology that I could not mention you personally.

## TABLE OF CONTENTS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| **ACE** | Angiotensin Converting Enzyme |
| **ADE** | Adverse Drug Event |
| **ADRs** | Adverse Drug Reactions |
| **AG nap** | Acyl Glucuronide naproxen |
| **ALF** | Acute Liver Failure |
| **ALP** | Alkaline phosphate |
| **Amox** | Amoxicillin |
| **APAP** | Acetaminophen |
| **APC** | Allophycocyanin |
| **APC** | Antigen Presenting Cell |
| **Bactrim** | Sulfamethoxazole/Trimethoprim |
| **BCIP/NBT** | 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium |
| **BSA** | Bovine Serum Albumin |
| **CCR (#)** | Chemokine receptors |
| **CFSE** | Carboxyfluorescein Diacetate Succinimidyl Ester |
| **CHM** | Commission on Human Medicines |
| **Clav** | Clavulanate |
| **CMV** | Cytomegalovirus |
| **CNS** | Central nervous system |
| **COX** | Cyclooxygenase enzymes |
| **COX- 1** | Cyclooxygenase 1 inhibitor also called constitutive enzyme |
| **COX- 2** | Cyclooxygenase 2 inhibitor also called inducible enzyme. |
| **CPM** | Count Per Minute |
| **CSA** | Cyclosporine |
| **CYP** | Cytochrome P450 |
| **DC** | Dendritic cell |
| **DDs** | Dapsone |
| **DDs-NO** | Dapsone Nitroso |
| **DHR** | Drug Hypersensitivity Reaction |
| **DIAT** | Atabecestat |
| **DILI** | Drug Induced Liver Injury |
| **DILIGEN** | Drug Induced Liver Injury genetic association study |
| **DILIN** | Drug Induced Liver Injury Network |
| **DM nap** | Des methyl naproxen |
| **DMSO** | Di- methyl sulfoxide |
| **DNA** | Deoxyribose Nucleic Acid |
| **DRESS** | Drug Reaction with Eosinophilia and Systemic Symptoms. |
| **EBV-DNA** | Hepatitis B virus DNA quantification |
| **EBVs** | Epstein Barr- Virus |

| | |
|---|---|
| **ECAD** | E-cadherin |
| **ELIspot** | Enzyme-Linked Immunospot Assay |
| **EMA** | European Medicines Agency |
| **ER** | Endoplasmic Reticulum |
| **FACs** | Fluorescence Activated Cell sorting |
| **FasL** | Fas Ligand |
| **FBS** | Fetal Bovine Serum |
| **FcεRI** | High-Affinity IgE Receptor |
| **FDA** | Food and Drug Administration (USA) |
| **FDE** | Fixed Drug Eruption |
| **FITC** | Fluorescein Iso Thiocyanate |
| **GB** | Granzyme B |
| **GM-CSF** | Granulocyte-Macrophage Colony-Stimulating Factor |
| **GSH** | Glutathione |
| **GWAS** | Genome wide association studies |
| **H2O2** | Hydrogen peroxide |
| **HBSS** | Hanks balanced salt solution |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| **HHV-6** | Human Herpes Virus |
| **HHV-7** | Human Herpes Virus |
| **HIV** | Human Immunodeficiency Virus |
| **HLA** | Human Leukocyte Antigen |
| **HSA** | Human Serum Albumin |
| **IAAAS** | Agranulocytosis and A plastic Anemia Study |
| **iDILI** | Idiosyncratic Drug Induced Liver Injury |
| **IFNY** | Interferon - Y |
| **Ig** | Immunoglobulin |
| **IL (#)** | Interleukin (#) |
| **LTT** | Lymphocyte Transformation Test |
| **MFI** | Mean fluorescence Index |
| **MHC** | Major histocompatibility Complex |
| **MHRA** | Medicines and Healthcare Product Regulatory Agency |
| **Mino** | Minocycline |
| **MPE** | Maculopapular Exanthema |
| **MPO** | Myeloperoxidase |
| **mRNA** | Messenger Ribonucleic Acid |
| **NAC** | N-acetyl Cysteine |
| **NAL** | N- Acetyl Lysine |
| **Nap** | Naproxen |
| **NAT** | N- Acetyl Tyrosine |
| **NHS** | National health Service |

| | |
|---|---|
| **NK** | Natural Killer Cell |
| **NSAIDs** | Non-Steroidal Anti-inflammatory Drugs |
| **PAMP** | Pathogen-associated Molecular Pattern |
| **PBMCs** | Peripheral blood mononuclear cells |
| **PBS** | Phosphate Buffer Solution |
| **PE** | R-phycoerythrin |
| **PHA** | Phytohemagglutinin |
| **PRRs** | Pathogen recognition receptors |
| **PV** | Pharmacovigilance |
| **RBMI** | Roswell Park Memorial Institute |
| **RPM** | Revolutions per minute |
| **SCAR** | Severe Cutaneous Adverse Reactions |
| **SCHP** | Sulfachloropyridazine |
| **SFD** | Sulfadiazine |
| **SFM** | Sulfamerazine |
| **SFU** | Spot forming unit |
| **SI** | Stimulation index |
| **SJS** | Stevens-Johnson syndrome |
| **SMX** | Sulfamethoxazole |
| **SMXNHOH** | Sulfamethoxazole hydroxylamine |
| **SMX-NO** | Sulfamethoxazole Nitroso |
| **TAP** | Transporter Associated with antigen Presentation |
| **TCCs** | T-cell clones |
| **TCR** | T cell receptor |
| **TEN** | Toxic Epidermal necrolysis |
| **Th** | T helper cell |
| **TLRs** | Toll like receptors |
| **TNF** | Tumour Necrosis Factor |
| **TT** | Tetanus Toxoid |
| **UNOS** | United Network for Organ Sharing |
| **ULN** | Under limit of normal |
| **WHO** | World Health Organisation |
| **YCS** | Yellow Card Scheme |

## PUBLICATIONS

**Published papers:**

Arun Tailor, † James C. Waddington, † Jane Hamlett, † James Moggs, † **Laila Kafu, †** John Farrell, † Gordon J. Dear, ‡ Paul Whitaker, § Dean J. Naisbitt, † Kevin Park, † and Xiaoli Meng*´ †. **Definition of Haptens Derived from Sulfamethoxazole: In Vitro and in Vivo. Chem Res Toxicol. 2019, 32, 2095–2106.**

Paul J Thomson[1], **Laila Kafu[1]**, Xiaoli Meng[1], Jan Snoeys[2], An De Bondt[3], Dries DMaeyer[3], Hans Wils[3], Laurent Leclercq[2], Petra Vinken[4], Dean J Naisbitt[1]*. **Drug-specific T-cell responses in patients with liver injury following treatment with the BACE inhibitor Atabecestat. J. Allergy Clin.immunol.2020, (11).**

**Manuscripts in preparation are associated with this thesis:**

Paul J Thomson [1†], **Laila M Kafu [1†]**, Nik Fragkas [2†], Thomas Hammond [3], Michael Kammueller [2], Munir Pirmohamed[1], Dean J Naisbitt [1*]. **Characterisation of the drug derivative that stimulates T-cell responses in patients with naproxen-induced liver injury.**

Monday O Ogese[1], Liam Farrell[1], **Laila Kafu[1]**, Serat-E Ali[1], Andrew Gibson[1], Aimee[2] Hillegas, Geoff Williams[2], Melanie Sakatis[2], Dean J Naisbitt[1]*. **Detection of immune-mediated drug-induced liver injury liability during drug development: An in vitro assessment of immunogenicity using blinded compounds.**

**Abstracts:**

Joshua Gardner[1], Sean Hammond[1], Monday Ogese[1], Serat-E-Ali[1], **Laila Kafu[1]**, Samantha Jones[1], Amy Chadwick[1], Catherine Betts[2], Dean J Naisbitt[1]. **Bioenergetic profiling of drug- specific monoclonal T-cell populations.**

**EAACI Drug hypersensitivity meeting 2018, Amsterdam- Poster presentations:**

Arun Tailor[1], James Waddington[1], Jane Hamlett [1], **Laila Kafu [1]**, John Farrell [1], Gordon Dear [2], Paul Whitaker[3], D.J. Naisbitt [1], Xiaoli Meng [1], B. Kevin Park [1]. **Nitroso sulfamethoxazole forms multiple haptenic determinants with cysteine, lysine and tyrosine.**

A. Tailor[1], T. Usui[1], **L. Kafu[1]**, B. Jagota, N[2]. Kitteringham[2], B.K. Park[1], X. Meng[1], D.J. Naisbitt[1]*. **In vitro detection of terbinafine protein adducts and the synthesis of HLA-binding peptides for functional studies.**

## ABSTRACT

Hypersensitivity reactions to drugs are an important cause of patient morbidity and mortality. One of the most difficult problems for the pharmaceutical industry is prediction of drugs with liver liabilities during the early stages of development. There is an incomplete understanding of the pathways of drug-specific immune cell activation and how the induced immune responses result in clinical manifestations in the liver. Thus, the major focus of this thesis was to investigate whether drug-responsive T-cells were detectable in patients with 6 forms of drug-induced liver injury (atabecestat, naproxen, sulfamethoxazole, isoniazid, minocycline and co-amoxiclav) and if so characterise the nature of the induced response, pathways of drug-specific T-cell activation and cross-reactivity with closely related structures.

Atabecestat is an oral β-Secretase enzyme BACE inhibitor that was in development for the treatment of Alzheimer's disease. However, elevations in hepatic enzymes were detected in a number of in trial patients, which resulted in termination of the development programme. CD4+ T-cell clones activated with the DIAT metabolite of atabecestat were detected in patients with liver injury. Lower numbers of clones displayed reactivity against atabecestat. Clones proliferated and secreted IFN-γ, IL-13 and cytolytic molecules following atabecestat or DIAT stimulation. Certain atabecestat and DIAT-responsive clones cross-reacted with N-acetyl DIAT; however, no cross-reactivity was observed between atabecestat and DIAT. $CD4^+$ clones were activated through a direct, reversible compound-HLA class II (HLA-DR) interaction with no requirement for protein processing.

Naproxen is a member of aryl acetic acid class of a non-steroidal anti-inflammatory drugs that induces liver injury in a small number of patients. Naproxen is extensively metabolized in the liver by CYP2C9 to 6-O-desmethylnaproxen. Both the parent drug and the desmethyl metabolite undergo phase-II metabolism to their respective acyl glucuronide conjugated metabolites. CD4+ and CD8+ T-cells from patients with liver injury expressing a range of different Vβ receptors were stimulated to proliferate and secrete cytokines when exposed to desmethyl naproxen, but not with naproxen, or naproxen acyl glucuronide. Activation of the CD4+ clones was HLA-DQ-restricted and dependent on antigen presenting cells. Limited cross-reactivity was observed with naproxen acyl glucuronide. Most clones secreted cytokines with desmethyl naproxen-pulsed antigen presenting cells, while fixation of antigen presenting cells blocked the T cell response.

Previous studies exploring patients with sulfamethoxazole- and co-amoxiclav-induced cutaneous hypersensitivity have shown that nitroso sulfamethoxazole (a metabolite of sulfamethoxazole) and amoxicillin activate T-cells via a hapten mechanism involving antigen processing within antigen presenting cells. Herein, CD4+ T-cell clones were isolated from patients with sulfamethoxazole- and co-amoxiclav-induced liver injury and shown to be activated via the same pathway involving HLA class II restriction and formation of drug protein adducts. Clones displayed differing T-cell receptor and exhibited structural specificity in that they were not activated with structurally-related compounds. Minocycline clones were also generated from patients with liver injury, but could not be expanded sufficiently for detailed functional assessments. Isoniazid-responsive T-cell clones were not detected.

**In conclusion**, these studies demonstrate the selective activation of drug-responsive T-cells in patients with drug-induced liver injury. A variety of *in vitro* assays were used to explore the phenotype and function of the responding cells. Additional research is required to investigate the specific role these T-cells play in the pathogenesis of the adverse event and to define the patient-specific factors that influence susceptibility. A clear understanding of mechanisms will give clear insights to assist pharmaceutical industry in the development of effective and safe drugs.

# CHAPTER 1: GENERAL INTRODUCTION

## CONTENTS

## 1.1 PHARMACOVIGILANCE (DRUG SAFETY) AN OVERVIEW.

In the past, the term pharmacovigilance (PV) was synonymous of post marketing surveillance in terms of adverse drug reactions (ADRs) and it defined the study of the safety of pharmaceutical products (Hughes, 2004). Later, PV encompassed premarketing phases including clinical development of the medicine and registration (Moride et al., 1993, Härmark and Van Grootheest, 2008).

Nowadays, the ways in which diseases are managed and controlled have changed due to advances in medicine and different pharmaceutical preparations. Nevertheless, adverse reaction to the drugs continue to be a hindrance and can cause illness, disability, and even death (Bates et al., 1995). Individual patients may exhibit particular and unpredictable sensitivities to certain medicines. In addition, if more than one medicine is prescribed there is a risk of negative interactions. In the past three decades, many countries have established national PV systems and joined with the World Health Organization (WHO) programme for international drug monitoring at the Uppsala monitoring centre where the global PV network exists. Despite this, less than 27% of developing countries have fully functional systems. The main reasons of reluctance of countries to develop PV systems include a lack of legislation, imbalanced regulatory frameworks due to their infrastructure, lack of resources, and expertise (Pirmohamed et al., 2007). All these factors are significantly important to building integrated PV systems (Figure 1.1).

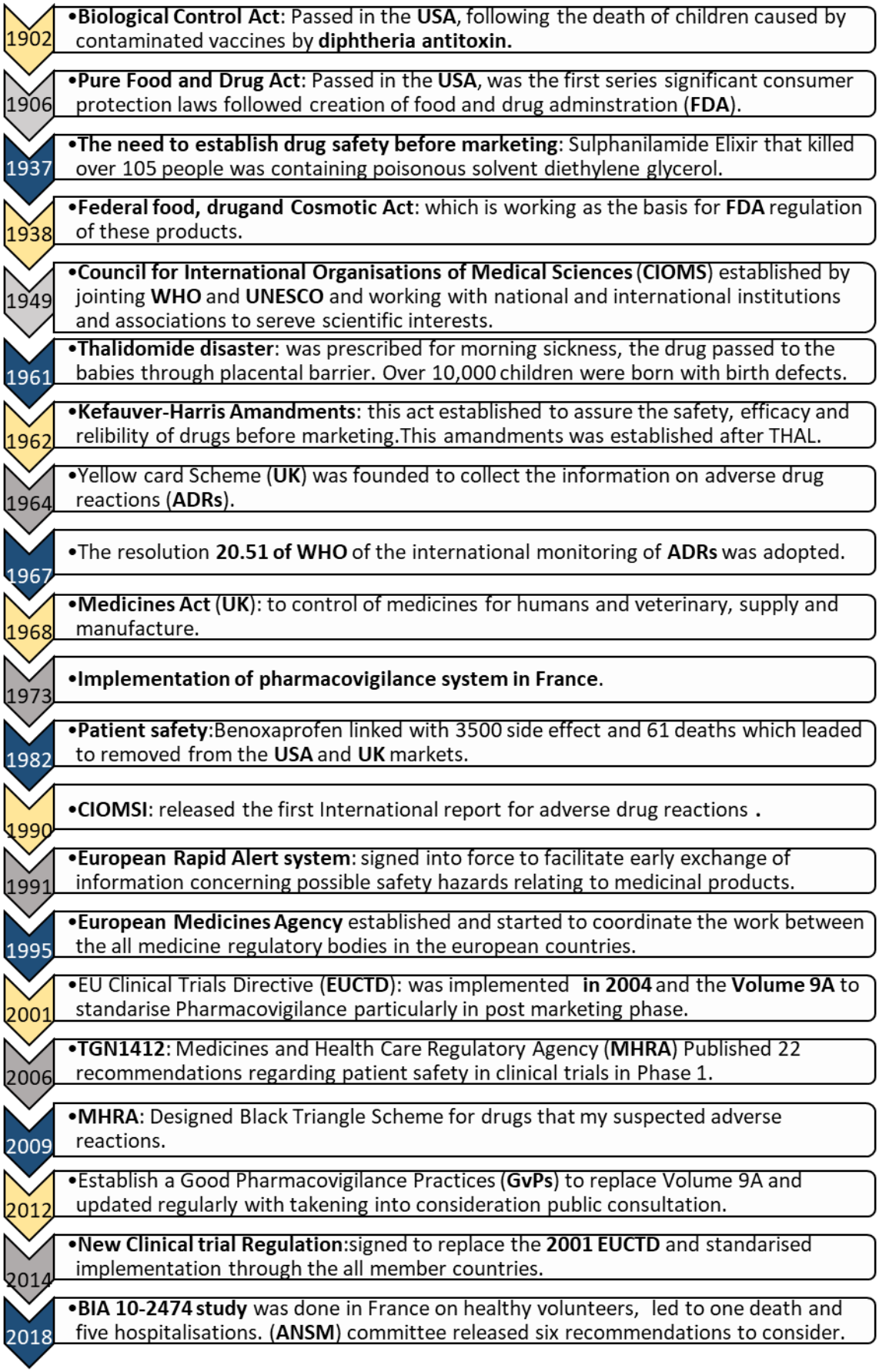



**Figure 1.1. An illustration of the establishment of pharmacovigilance.**

## 1.2 AN OVERVIEW OF ADVERSE DRUG REACTION: LITERATURE REVIEW STRATEGY.

Different online database resources were searched for pooling the all relevant information about ADRs. Databases used were PubMed, Science Direct, Scopus, Web of Science and Cochrane library including systematic reviews and metanalysis. The other general searches were also performed online by using google scholar, the University of Liverpool DISCOVER, and WHO monitoring programme. The search was limited to articles in the English language. The terms used for searching included ADR, drug toxicity, drug hypersensitivity, drug allergy. Eventually, Boolean operators were used to combine or exclude keywords in terms relevant to the search.

## 1.3 DEFINING AN ADVERSE DRUG REACTIONS.

ADRs have been described for as long as medicines have been prescribed. For example, in 1937, 105 people in the USA including approximately 34 children after ingesting elixir sulfanilamide. It was reformulated into a raspberry-flavoured elixir containing a poisonous solvent diethylene glycerol (Ballentine, 1981, Wax, 1995). In 1961, children were born with birth defects (Phocomelia) due to thalidomide (McBride, 1961). Rofecoxib was withdrawn due to an association with increased cardiovascular risk (Sibbald, 2004, Sonawane et al., 2018). These events have caused major concerns in health care sectors and led to changes to regulations by focusing on safety before the post marketing surveillance phase particularly after the thalidomide disaster (Avorn, 2012). However, it is important to know that ADRs are a common problem, which affect patients in primary, secondary and tertiary care. This introduction guides the reader through definitions with regards to ADRs and focuses for the main part on some of the less common, but most serious ADRs that involve inadvertent activation of the hosts adaptive immune system.

Early studies by Cluff et al in 1964 defined ADRs as: "Any adverse response to a medication undesired or unintended by the physician" (Cluff et al., 1964). Other definitions from the same period are unclear and comprise intentional and unintentional overdose as well as some administration errors (Ogilvie and Ruedy, 1967, Hurwitz and Wade, 1969). Therefore, these definitions are less beneficial when analysing adverse reactions when drugs are used at doses intended for therapy.

Forty-eight years ago, WHO defined ADRs as “any noxious and unwitting response to a drug used in humans for prophylaxis, diagnosis or therapy of disease, or for modification of physiological function” (Organization, 1972, Edwards and Aronson, 2000, Lindquist, 2007). However, not all ADRs are harmful such as the irritation that causes dry cough, for example with angiotensin converting enzyme (ACE) inhibitors (Fogari et al., 2011, Barreras and Gurk-Turner, 2003). This definition has remained largely unchanged; however there has been suggestions that other variants are considered. For example, the definition does not indicate any preventable event that may cause patient harm due to medication error in dose concentration or any other reason while the medication is under the control of professionals. Red neck syndrome due to over rapid administration of vancomycin is one example of this. Furthermore, harmful effects caused by contamination in herbal medicines are visible medication errors (Ferner and Aronson, 1999). Edwards and Aronson proposed an all-encompassing definition of ADRs as “An appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product” (Edwards and Aronson, 2000).

## 1.4 DEFINING ADVERSE DRUG EVENTS.

An adverse drug event (ADE) is defined as an unintended injury caused while a patient is taking his treatment or subsequently caused by medical intervention rather than disease processes *per se* (Vincent et al., 2001). According to the Harvard medical practice study II, 3.7% of hospital admissions recorded in the United States and Australia led to adverse events. About 70% of these patients experienced multiple adverse events while 7% of them the disabilities were permanent and 14% contributed to death (Leape et al., 1991, Thomas and Brennan, 2000). This term sometimes is used to express any noxious and unintended response to a drug but this is confusing as the reason or the cause is not known. In this case it remains an unattributed adverse event; if the cause is thought to be drug-related so the adverse event becomes a suspected ADR; if the cause is due to medicinal product, in this case the ADE can be defined as a recognised ADR (Blenkinsopp et al., 2007, Talbot and Aronson, 2012).

## 1.5 DEFINING SIDE EFFECTS OF DRUGS.

The term side effect is defined in 1991 by WHO as any unintended effect related to pharmacological properties of a drug that is normally used in man (Macedo, 2004, Shakir and Wilton, 2000). This definition is ambiguous because the side effect of the drug could be harm or may be beneficial. For instance, treating hypertension with $\beta$ Blockers might relieve patient angina. Also, a beneficial side of tricyclic antidepressants on depressed patients is an anticholinergic action on the irritable bowel.

**1.6 INCIDENCE OF ADRs.**

Several studies have been conducted to assess the importance of ADRs in relation to hospital admissions. These studies indicated about 5% of all hospital admissions related to ADR (Hurwitz and Wade, 1969, Pirmohamed et al., 1998). The length of stay in the hospitals when ADRs are detected is increased, causing a significant economic burden (Classen et al., 1997). In the UK, several prospective studies have been conducted exploring the incidence rate of ADRs in children hospitals, the results showed that the overall of incidence rate was 9.53%. 39.30% of ADRs causing hospital admissions were associated with life-threatening conditions (Choonara and Harris, 1984). A separate study indicated that the incidence rate of paediatric hospital admissions due to ADRs was 2.09%, while the outpatient children incidence rate of ADRs was 1.46% (Impicciatore et al., 2001). A prospective observational study was conducted in Merseyside, UK, to analyse the burden of ADRs for 18,820 admissions in two general hospitals. The result of this study showed that 1225 admissions were due to ADRs. The median bed stay was eight days which is considered to represent 4% of the bed capacity of the hospitals. Furthermore, the National Health Service (NHS) cost of such admissions was £466 M, which causes huge burden on the health care system. The fatality rate was 0.15%. The results of this study necessities measures to be developed to reduce the incidence of ADRs and thus improve the benefit/harm ratio of drugs (Pirmohamed et al., 2004). As indicated above, idiosyncratic toxicities although relatively rare can be life-threatening and as such it is imperative that we understand the disease pathogenesis. In contrast, direct or acute toxicities targeting major organs such as the liver and heart may occur more commonly and these are briefly discussed below. Drug induced liver injury represent

broad spectrum of liver pathologies and can be predictable or unpredictable which is not identified in preclinical tests that currently available (Weaver and Valentin, 2019). It can be acute or chronic. Acute hepatic injury causes necrosis, acute hepatitis and biliary obstruction (Salazar, 1989). Several drugs induce acute hepatitis and fulminant hepatic failure, such as rifampin and isoniazid. Furthermore, methyldopa-induced hepatotoxicity is seen within 4-12 weeks of the treatment with marked elevation of ALT, AST and moderate increase of alkaline phosphatase. Methyldopa has been clearly linked to both acute and chronic liver injury and adverse events are associated with 10% mortality rate (Hayashi and Bjornsson, 2018).

Cardiovascular toxicities can be observed after to several groups of drugs. Drugs can damage to the heart muscle or valves, for instance anthracyclines have been shown to produce cardiomyocyte ultrastructural abnormalities (Ewer and Lippman, 2005, Mamoshina et al., 2021), dilated cardiomyopathy and clinical dysfunction, which leads over time to fibrosis and irreversible dysfunction (Ewer and Lippman, 2005). Also, cardio-toxic drugs can induce QT interval prolongation (Kannankeril et al., 2010, Roden, 2004) and the rise and delay in cardiac repolarisation form ventricular tachycardia which leads to ventricular fibrillation and death (Daly, 2013).

**1.7 RISK FACTORS AND ADRs.**

Several factors are potentially associated with ADRs, particularly those associated with idiosyncratic drug reactions (Pirmohamed et al., 1994, Park, 1986). It is important to study the role of these factors in order to try to gain a deeper understanding of the adverse effects of the drug to try to avoid future ADRs (Hussaini and Farrington, 2014a). Most ADRs are due to the fundamental variability in the

pharmacokinetics, pharmacodynamics seen among patients. Genetic factors are also involved and these are discussed in more detail below (Pirmohamed et al., 1994). The factors most commonly associated with ADRs include;

- Host (age, gender, genetic polymorphism and ethnicity).
- Environment (metabolic syndromes, alcohol intake and chronic viral infection).
- Dosage of therapeutic medication particularly, those medications with a narrow therapeutic range (hepatic metabolism, lipophilicity and biliary excretion).

**1.7.1 PATIENT-RELATED FACTORS (AGE, GENDER AND ETHINICITY).**

Although, the effect of age and gender on the risk of developing ADRs remain controversial, several studies indicated that both factors may be important. Elderly patients who often take multiple medications, or visit many physicians are more susceptible to ADRs when compared with younger age groups (Alomar, 2014). Cholestatic drug-induced liver injury (DILI) is common in elderly people due to the reduction in activity of drug metabolising enzymes, and their capacity to eliminate drugs (Hussaini and Farrington, 2014a, Alomar, 2014, Chalasani et al., 2015). Several studies have shown that the risk of DILI and liver failure is higher in women compered to men. The UNOS liver transplant database registered 76% of 270 transplanted cases presenting with drug-induced acute liver failure as women, in the period from 1990 to 2002. According to drug induced liver injury network (DILIN) reports, the higher incidence rate of DILI in females was 65% (Chalasani et al., 2008). In Chinese populations, the activity of CYP3A4 is higher in women than in men, and is known to alter the metabolism of midazolam in women (Labbé et al., 2000, Waxman and

Holloway, 2009). Potential reasons for female susceptibility to DILI include altered pharmacokinetics and pharmacodynamics of drugs, hormonal effects, interactions with immunomodulating agents and difference in the adverse response of the immune system to some drugs and their metabolites (Amacher, 2014). In contrast, other prospective data from Spain found, no difference in age and gender in the risk of DILI. This study included 603 DILI patients with different age and gender (Lucena et al., 2009, Björnsson and Davidsdottir, 2009, Lammert et al., 2008).

The effect and action of drugs also varies between individuals due to genetic factors which are sometimes related to ethnicity (Torpet et al., 2004). Gene polymorphisms encoding drug metabolising enzymes, drug transporters and receptors are important causes for inter individual differences in drug exposure and influences susceptibility to certain forms of ADR (Sexton et al., 2000, Adkinson Jr, 1984). The genotypes like SLCO1B1 are associated with simvastatin myotoxicity, while human leukocyte antigen (HLA) genotype is an essential predictor of the susceptibility towards multiple forms drug-induced liver and skin toxicity (Daly, 2012). For example HLA-B*57:01 is associated with an increased risk of flucloxacillin-induced liver injury, while the same HLA allele is an absolute determinant of abacavir hypersensitivity reactions (Daly et al., 2009).

### 1.7.2 ENVIRONMENTAL FACTORS.

The development of allergic drug reactions is influenced by associated disease states that alter the metabolic pathways and induce variations in the immunologic responses to drugs. For example, patients with human immunodeficiency virus (HIV) frequently develop idiosyncratic toxicity with anti-infective drugs, especially with

trimethoprim- sulfamethoxazole (van der Ven et al., 1991, Koopmans et al., 1995). Some investigators suggest that a deficiency in glutathione in this patient group may be responsible for the increased incidence of the ADRs as glutathione is involved in the detoxification of reactive drug metabolites (Roederer et al., 1991, Buhl et al., 1989). Moreover, the reactivation of human herpes virus (HHV) 6 and 7 have been linked to an increase in the incidence of DRESS syndromes (drug reaction with eosinophilia and systemic symptoms) (Pichler, 2003, Chung et al.).

Alcohol drug interactions can facilitate the development of ADR by the altering pharmacodynamics and pharmacokinetics of drugs (Bruce et al., 2008). The symptoms of alcohol-drug intake can include nausea, vomiting, headache, loss of coordination and hypotension (Krupski et al., 2009). Certain groups of drugs may cause internal bleeding when taken with alcohol, and the best example of these are NSAIDs, particularly with people who suffer from peptic ulcers or gastritis (Kim et al., 2009).

### 1.7.3 DOSAGE OF THERAPEUTICS.

Development of idiosyncratic ADRs is not related to the known pharmacology of the drug, as such reactions are often stated as being dose independent (Pirmohamed et al., 1998). However, this statement is an over simplification of the clinical picture of idiosyncratic ADRs as most drugs associated with a high incidence of idiosyncratic ADRs are given at a high mass dose. What is clear is that a threshold level of drug exposure is required to activate the hosts effector immune system; however, this threshold differs in patients and may even differ in the same patient throughout their

life as they are exposed to different stress signals and pathogenic species that impact on the bodies regulatory pathways.

Regarding the route of drug administration, intravenous administration is associated with more severe ADRs than oral administration. The best example is vitamin K (anticoagulant reversal) which works (Vermeer and Schurgers, 2000) in the body as a cofactor, in the synthesis of coagulation factors II, VII, IX, and X and proteins C and S (Esmon et al., 1987, IARC, 2000). The intravenous administration of vitamin K causes severe anaphylactoid reactions (Britt and Brown, 2018). The incidence of severe non-immune mediated anaphylactoid reactions is 3 per 10000 administrated doses of vitamin K (Aronson, 2009, Howland, 2006).

**1.8 DETECTING AND MONITORING OF ADRs.**

Monitoring of ADRs describes the prospective supervision, observation, and testing of an ongoing process. Monitoring provides reassurance that the goal has been or will be achieved, or suggests changes that will allow it to be achieved. Drug safety monitoring is an essential element for the effective use of medicines, drug eluting medical devices and for high quality medical care (Coleman et al., 2006). For example, monitoring of drug concentrations can be used to achieve a health benefit, avoid harm, or both. It also has the potential to inspire confidence between patients and health professionals in medicines and participate to growing standards of medical practice by monitoring the progress of the disease and adjusting treatment accordingly (Blenkinsopp et al., 2007). ADRs can be detected in pre-clinical studies where some important specific tests can be assessed such as teratogenicity, mutagenicity and carcinogenicity and a great deal of risk information may be

obtained (Berlin et al., 2008, Van Norman, 2016). Nevertheless, there is a lack of adequate knowledge about genetic predisposition, metabolic idiosyncrasies, and concomitant diseases in pre- clinical phase (Hastings, 2001). Studies at this stage are carried out on animals and in cell culture assays. The disparity in biological processes between humans and animals makes it difficult to predict hypersensitivity reactions for any new drug. This is because experimental animals do not express human MHC molecules, they metabolise drugs differently and are generally maintained under a sterile environment. Thus, they are not exposed to environmental stress signals that alter human immune regulatory pathways, which impact on susceptibility towards the development of an adverse event in humans.(Bala et al., 2005, Jones and Kingery, 2014). Studies are carried out on animals and humans, in the three phases, before authorisation marketing the drug. These studies provide essential information on the efficacy of the drug, however limited knowledge about safety is acquired until drugs are administered to a much wider population (Berlin et al., 2008, Van Norman, 2016). In clinical trials the patient number (up to 1000 patients) exposed to a medication is limited (Seddon, 2006), as is the duration of patient exposure, additionally the adverse effects are only seen in a small percentage of the population (Figure 1.2). Furthermore, restricted patient populations such as pregnant women, children, and elderly people are often excluded from clinical trials. These factors lead to the detection of many ADRs in post marketing surveillance (Figure 1.2), where the prescription can be monitored and case reports collected through spontaneous reporting systems (Steinke, 2019).

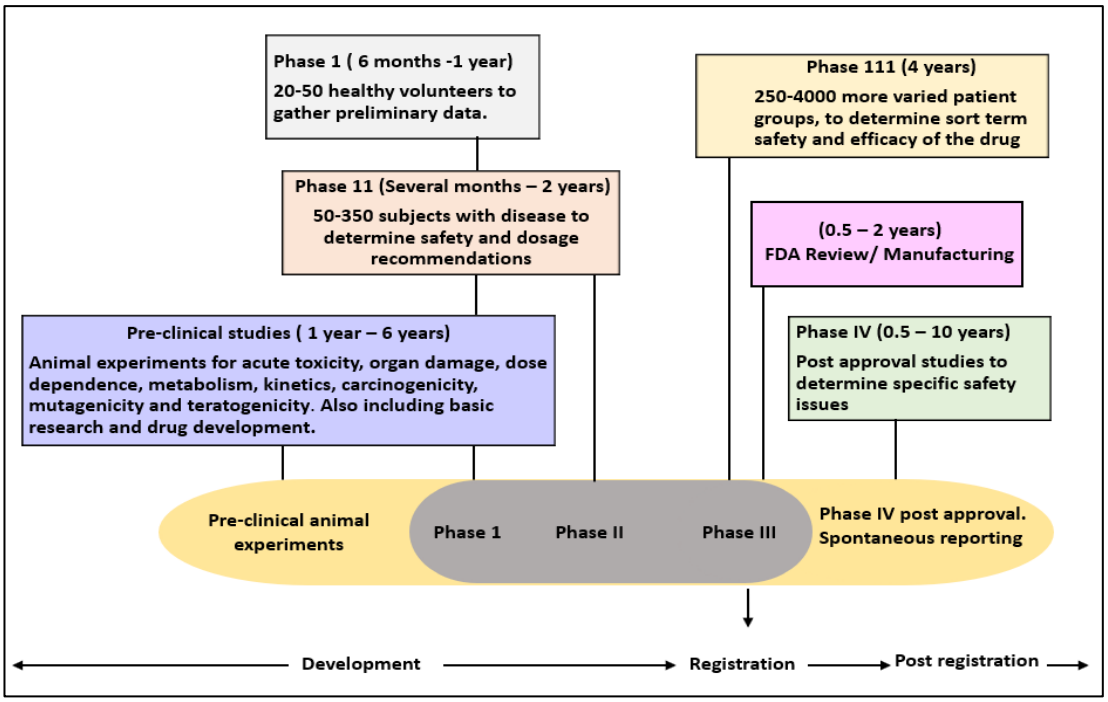



**Figure 1.2. Clinical development of medicines.** The scheme illustrates the three phases of development with the time line before registration and the post-marketing phase IV.

The yellow card scheme is the UKs ADR reporting system operated by the Medicines and Healthcare Products Regulatory Agency (MHRA) and the Commission on Human Medicines (CHM). This system was created in 1964 after the thalidomide tragedy. It allows clinicians to collect, report and monitor any suspected safety concerns from any medicines (Avery et al., 2011).

In 2000 at the first international conference on consumer reports on medicines summarised the potential advantages of patient reports where they have unique perspectives and experiences (Organization, 2000). The MHRA updated the yellow card scheme in 2005 to allow patients to be involved by submitting any suspected reactions including those considered not to be serious (McLernon et al., 2010). Withdrawal of drugs from the market for safety reasons need strong supporting evidence. Case reports and randomised clinical trials are the main source to inform

regulatory bodies about potential withdrawal of drugs from the market as they focus on the quality of the evidence supporting the withdrawal. A total of 22 drugs were withdrawn from the market for safety reasons since the beginning of 1990 to late 1999. 82% of withdrawal were due to serious ADRs including hepatic and cardiac reactions (Arnaiz et al., 2001).

### 1.9 COSTS ASSOCIATED WITH ADRs (ECONOMIC ASPECTS).

ADRs represent a substantial burden on health-care resources in developed and developing countries. Many countries spend huge amount of financial resources, 15% to 20% of hospital budgets to treat the complications of ADRs (Bordet et al., 2001). In 1994, ADR costs to the NHS in England were estimated to be in the region of £380 million (Wiffen, 2002). A more recent study estimated the cost burden to be over £ 450 million in one year (Pirmohamed et al., 2004). In China, the direct costs of ADRs on hospitalized patients were estimated to be ¥603252.81 (Qing-ping et al., 2014). Prolongation of hospital stay due to ADRs in the USA cost up to 30.1 billion dollars, as well as the outpatient care that additionally creates a financial burden (Kalisch et al., 2011, Field et al., 2005).

### 1.10 PHARMACEUTICAL INDUSTRIES AND REGULATORY CONCERNS.

Adverse drug events (ADE) are a major challenge for the pharmaceutical industry. The rigorous regulations imposed on pharmaceutical industries in pharmaceutical formulation during the phases of drug development are established to reduce the number of ADRs, and to ensure safety testing of the compound before market approval (Breckenridge, 2015). However, monitoring of ADRs for investigational and marketed drugs still needs more restrictions and effective regulations to ensure safety and to alleviate any kind of burden on patients. For instance, cancer

chemotherapeutic agent toxicity spectrums vary greatly even in patients with the same type of cancer (Yang et al., 2017). Idiosyncratic ADRs in some cases are not identified until the post marketing surveillance phase. Therefore, the post-authorisation process has been an increasingly vital aspect of pharmacovigilance (Evans, 2012, Medicine, 2012). This issue can be complicated further if the drug is used for the treatment of severely ill patients. Although, significant regulatory efforts have been conducted to reduce the impact of ADRs the considerable cost of taking a standard drug through to market approval only for it to be withdrawn represents a major impediment to Pharma.

**1.11 PREVENTION OF ADRs.**

ADRs are often preventable (Bates et al., 1995). Prospective and retrospective studies have shown that between 20% and 80% of ADEs and ADRs are preventable (Bates et al., 1995, Seeger et al., 1996). Evidently, drug use has a plethora of advantages for ill patients, but their risks should equally be considered before prescribing them to patients. The main characteristics for differentiating preventable from non-preventable ADRs include; dose, ADR reporting, appropriate prescription and effective patient monitoring (Schumock and Thornton, 1992). Another vital characteristic is the use of quality Information technology (IT) to document patient medication history. There are three ways for reducing idiosyncratic ADRs as implemented by (Evans et al., 1994). The use of digital alerts that detect drug allergies enabled physicians either to prevent the progress of ADEs to more severe conditions or to prevent them immediately, where approximately half of the reports were not recognised prior to the digital system. The use of a standardised administration rates for antibiotics, and notifying hospital staff of ADRs to increase

their awareness. In addition, the use of digital alerts enables hospital staff to establish databases and protocols to monitor hospitalised patients drug dosage and modify according to the need. Germany successfully used a system of Automatic Laboratory Signals (ALS) that signposts laboratory values that may indicate ADRs (Azaz-Livshits et al., 1998, Levy et al., 1999, Dormann et al., 2004). The digital system detected ADRs in 377 patients over 6 months, with study staff and chart reviews equally consulted in the process. Although computer systems improve the detection of ADRs, it is important to understand that technical difficulties should be expected and predicted. One way that could improve ALS is by including individual medicines data for more accurate decision support (Dormann et al., 2004). This system is likely to prevent "warning-fatigue" and terminate the use of the decision analysis software.

### 1.12 THE ROLE OF PHARMACOGENETICS/PHARMACOGENOMICS IN ADRs

Pharmacogenomics refers to the convergence between pharmacology and genetics and deals with genetically determined responses to drugs, differences in the metabolism of medications, genders, and people with various medical conditions (Nebert, 1999). Pharmacogenomics is the study of variability in drug response due to heredity and the relation of genes determining drug effect (Evans and Relling, 1999, Phillips et al., 2001, Pirmohamed, 2001).

Certain types of hepatotoxicity associated with drugs such as the β-lactam antibiotics flucloxacillin and co-amoxiclav have been shown to be associated with HLA genotype expressed by the patients (Daly et al., 2009, Lucena et al., 2011). Furthermore, hepatotoxicity due to erythromycin, fenofibrate, methyldopa is associated with HLA-A* 33:01 and HLA-A* 33:03, but it was not observed in all cases (Nicoletti et al., 2017). Also, hepatotoxicity due to the flupirtine is associated with HLA-DRB1*16:01-

DQB1*05:02 (Nicoletti et al., 2016). These and other studies prove that pharmacogenetics plays an important role in determining susceptibility to immune ADRs with the HLA allele potential encoding for the surface receptor that presents the drug antigen to T-cells that mediate the ADR. As mentioned above, abacavir hypersensitivity is the most studied HLA ADR association. A study on 200 Australian HIV+ Western Australian's exposed to abacavir initially revealed the potential association between HLA-B*57:01 and hypersensitivity. The presence of the haplotype HLA-B*57:01, HLA-DR7, and HLA-DQ3 displayed excellent sensitivity, with the positive predictive value at 100% and the negative predictive value was 97%. (Mallal et al., 2002b). Subsequent studies demonstrated that patch test confirmed cases of abacavir hypersensitivity only occur in patients expressing HLA-B*57:01 (Giorgini et al., 2011, Hughes et al., 2008) and that pharmacogenetic screening for HLA-B*57:01 can effectively eradicate abacavir hypersensitivity with significant patient economic benefits (Böhm et al., 2018).

### 1.13 WORLD HEALTH ORGANIZATION (WHO) CRITERIA FOR SEVERITY ASSESSMENT OF ADRS.

WHO has established 3-tired criteria for the definition of seriousness of ADRs to differentiate between the ADR effects**:**

A) **Mild**, drug exposure does not add complications to the original disease and there is no requirement for treatment.

B) **Moderate**, where the vital organs are moderately involved, and the symptoms are clearly marked. However, no circulatory failure or loss of

consciousness is observed. Any observation of biochemical or structural changes will justify classification in this category. Treatments are necessary.

C) **Serious**, this type of ADR, defined as any untoward medical occurrence that at any dose results in persistent or significant disability/incapacity, and/or is life-threatening.

**1.14 CLASSIFICATION OF ADRs.**

ADRs are often classified into two major types; type A (non-patient dependent) is augmented or dose-related (Pirmohamed et al., 1998), and type B is bizarre or non-related dose (Davies, 1977). Both forms of ADR, type A and type B were the original classifications (Rawlins and Thompson, 1991). Later, four further categories were added. Type C reactions relate to dose and time related reactions, type D reactions are delayed reactions, type E reactions are withdrawal or end of use, and the last category type F represents unexpected failure of therapy (Edwards and Aronson, 2000, Hartigan-Go and Wong, 2000, Royer, 1997, Park et al., 2001) . The different types of ADR are described in more detail below.

- **Type A** (pharmacological) which constitute about 80% of ADRs, and are predictable from the drugs known pharmacology (Rawlins, 1977). Type A reactions include:

  **1)** Side effects which are undesirable and unavoidable effects produced by therapeutic drugs doses. All drugs come with side effects, some side effects are minor, and few are serious (JENKINS et al., 1987, Adelman et al., 2002).

  **2)** Toxic effects of drugs that can damage specific organs of the body such as the liver, kidney or systems such as central nervous system (CNS). The toxic

effect of the drug usually develops when the dose of the drug exceeds the therapeutic range. For instance, bleeding with anticoagulants, and serotonin syndrome caused by selective serotonin reuptake inhibitors (Riley and Kohut, 2010, Adelman et al., 2002, Thien, 2006).

**3)** Drug interactions that cause alterations in bioactivation processes, for instance metabolism, which may boost or reduce the effect of simultaneously administrated medications. Multiple drug (polypharmacy) use is one of the most important factors that increases the risk of ADRs. Valproic acid is not an enzyme inducer, but it may cause clinically relevant drug interactions by inhibiting the metabolism of selected substrates, most notably phenobarbital and lamotrigine (Perucca, 2006).

- **Type B** (hypersensitivity or idiosyncratic) account for 10%- 15% of ADR, and affect only a small number of individuals (Uetrecht, 2007). This type of ADR is bizarre, and cannot be predicted during the preclinical or early clinical phase of development (Thien, 2006, Pirmohamed et al., 1998). Reactions are often serious and associated with a high incidence of mortality. The mechanisms associated with this type of ADR are not fully defined, therefore prevention is predominatingly impossible (Uetrecht, 2007, Rubio et al., 2010). Type B reactions are referred to as:

  **1)** Immune mediated (allergic) reactions involve IgE antibodies or effector T cells or more rarely immune complex or cytotoxic reactions (Greenberger, 2006).

  **2)** Non-immune mediated reactions (non-allergic) or drug intolerance which is an inability to tolerate the drug mostly due to inherited genetic variation

specifically in liver enzymes. Drug intolerance can be predictable as a pharmacologic side effects or unpredictable as pseudo allergic (Riedl and Casillas, 2003).

- **Type C** reactions, paracetamol (acetaminophen) hepatotoxicity and Ipomeanol pulmonary toxicity are two examples for this type of ADR, that can either be predicted or clearly justify with regards to the chemical structure of the drug metabolite (Naisbitt et al., 2000). This type of ADR is uncommon, and often occurs when a toxic drug metabolite is formed in a specific organ or tissue.
- **Type D**, known as a delayed reaction, take many months or years to appear. Examples include secondary tumours with immunosuppressant treatment and teratogenicity in children after drugs are taken by mothers during pregnancy (Fetal hydantoin syndrome).
- **Type E**, known as withdrawal reaction after a reduction of the dose suddenly or when the drug treatment is terminated. The probability of a reaction is linked more closely to the duration of treatment rather than the dose (Arulmani et al., 2008, Rohilla and Yadav, 2013). Withdrawal seizures on stopping phenytoin and a withdrawal syndrome on stopping paroxetine both are examples of type E reactions (Perucca and Gilliam, 2012, Rohilla and Yadav, 2013).
- **Type F**, known as unexpected failure of therapy. They are often caused by drug-drug interactions, where one drug increases or decreases the efficacy of the other (Hartigan-Go and Wong, 2000). For example, the interaction of

antibiotics that are used for the treatment of tuberculosis, such as rifampicin, ethambutol, and phenytoin, and anti-convulsant medication (Abajo, 1988).

- **Type G** reactions are known as genetic reactions. This type of reaction is caused by irreversible DNA damage. For instance, thalidomide an antiemetic drug which was given to pregnant women causing limb anomalies, congenital heart disease, and malformations in the eyes (Kim and Scialli, 2011).

**1.15 DRUG INDUCED HYPERSENSITIVITY REACTIONS.**

Many hypersensitivity reactions to drugs are immunologically mediated, and can target different organs. The organs primarily involved are skin, liver, kidney and lungs, and reactions vary in severity from mild conditions to life-threatening conditions such as anaphylaxis, severe cutaneous ADRs including Drug Rash with Eosinophilia and Systemic Symptoms (DRESS), Steven-Johnson syndrome (SJS) and Toxic epidermal necrolysis (TEN) and DILI (Pavlos et al., 2012, Phillips and Mallal, 2010). According to Gell and combs (1963) classification adapted by Pichler and co-workers, immune-mediated ADRs are classified into four main categories illustrated in (Table 1.1) below (Demoly et al., 2014, Posadas and Pichler, 2007).

**Table1.1: Classification of drug allergies adapted from (Pichler, 2003).**

| Reaction type | Type of immune response | Pathophysiology | Clinical symptoms | Typical chronology of the reaction |
|---|---|---|---|---|
| I | IgE | Mast cell and basophil degranulation | Anaphylactic shock, angioedema, urticaria, bronchospasm | Within 1-6 hours after the last intake of the drug |
| II | IgG and complement | IgG and complement-dependent | Cytopenia | 5-15 days after the start of the offending drug |
| III | IgM or IgG and complement or FcR | Cytotoxicity and deposition of immune complexes | Serum sickness, urticaria. Vasculitis | 7-8 days. 7-21 days after the start of the offending drug |
| IVa | Th1 (IFN-c) | Monocytic inflammation | Eczema | 1-21 days after the start of fending drug |
| IVb | Th2 (IL-4 and IL-5) | Eosinophilic inflammation | Maculopapular exanthema. DRESS | 1 to several days after the start of the offending drug. 2-6 weeks after the start of the offending drug |
| IVc | Cytotoxic T cells (perforin, granzyme B, FasL) | Keratinocyte death mediated by T cells CD4 or CD8 | Maculopapular exanthema. SJS/TEN, pustular exanthema | 1-2 days after the start of the offending drug for fixed drug eruption. 4-28 days after the start of the offending drug |
| IVd | T cells (IL-8/CXCL8) | Neutrophilic inflammation | AGEP | Typically, 1-2 days after the start of the offending drug but could be longer. |

Further, another drug allergy classification was adapted by Levine (1966) which relied on the author's clinical experience with penicillin allergy. This classification was based on concealed time between dosage administration and onset of hypersensitivity reaction (Levine, 1966) Figure 1.3.

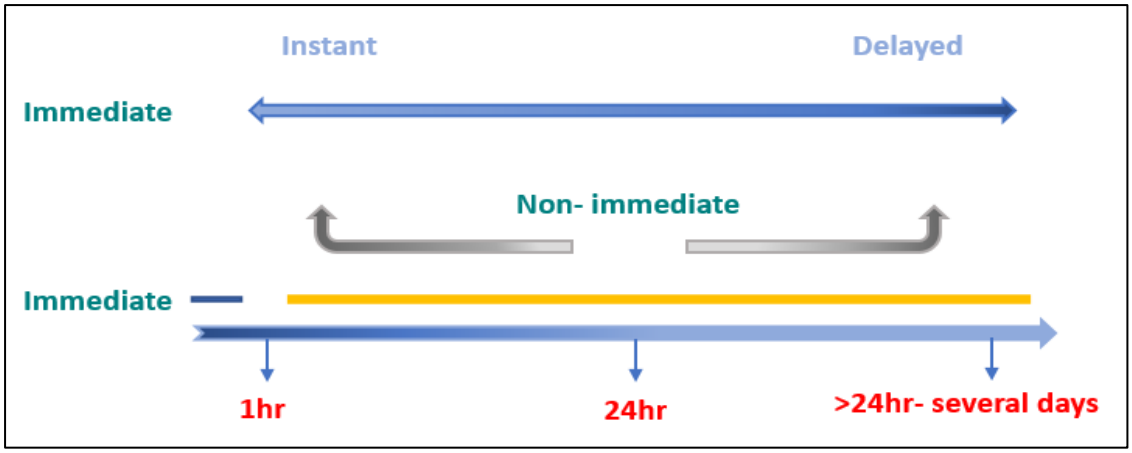



**Figure 1.3. The onset of drug administration and the onset of drug hypersensitivity reaction.** The graph shows the time appearance of delayed (T -cell mediated) drug hypersensitivity reactions. The graph also shows the non-immediate period which is between acceleration and delayed reactions. Thus, drug hypersensitivity can be immediate if <1hour, accelerated from 1-24 hour, and delayed if >24hour.

## 1.16 THE ETIOLOGY AND CLINICAL MANIFESTATIONS OF IMMUNE-MEDIATED DRUG HYPERSENSITIVITY REACTIONS.

### 1.16.1 DRUG INDUCED SKIN DISORDERS.

Skin is an organ commonly targeted by drugs as it is a metabolically and immunologically active site. The skin has a large surface area, contains a vast blood vessel network and as the skin is visible, even mild skin rashes are detectable. Hence, it is difficult to compare the frequency of cutaneous hypersensitivity reactions with reactions that target other organs, where the mild ADR might go unnoticed (Uetrecht and Naisbitt, 2013, Roujeau, 2005).

#### 1.16.1.1 MACULOPAPULAR RASH OR EXANTHEMA (MPE).

MPE accounts for more than 91% of drug-induced skin reactions (Hunziker et al., 1997). The eruption symptoms manifest firstly on the trunk and upper extremities, then become widely distributed with polymorphous appearance (Roujeau, 2005).

This eruption appears from 4-14 days, and continues for 1-2 days after the treatment is terminated (Valeyrie-Allanore et al., 2007). Drug-specific cytotoxic CD4+T cells have a crucial role in skin inflammation via induction and release of a variety of cytokines, cytolytic molecules and chemokines, which all play an important role in the disease pathophysiology (Yawalkar et al., 2000a, Yawalkar et al., 2000c, Tapia et al., 2004, Pichler, 2002). Drugs that are implicated in this type of skin reaction include allopurinol, non-steroidal anti-inflammatory drugs (NSAIDS), β-lactam antibiotics, quinolone and sulphonamide antibiotics. Treatment of MPE starts with discontinuation of the triggering drug, this is at times sufficient with application of emollients to affected area. For more severe reactions systemic antihistamines and topical corticosteroids are administrated (Lee, 2000).

**1.16.1.2 URTICARIA.**

Urticaria is the second most common type of drug-induced skin eruption. It is classified as a form of immediate hypersensitivity and IgE is implicated as an important mediator (Böhm et al., 2018). IgE antibodies bind to the surface of mast cells creating a binding site for the drug antigen. Cross-linking of mast cell associated IgE leads to degranulation and the release of mediators that bring about the adverse event. β-lactam antibiotics are common mediators of this type of ADR (Hunziker et al., 1997). Other drugs that cause urticaria include radiocontrast media, aspirin, anaesthetic muscle relaxants, anti-inflammatory drugs and opiates (Ardern-Jones and Friedmann, 2011). Urticaria lesions are relatively large, raised, pruritic skin lesions. Lesions can appear rapidly and then the skin may return normal a few hours after drug withdrawal (Ardern-Jones and Friedmann, 2011); however, once other urticaria lesions appear, they might also last for days (Uetrecht and Naisbitt, 2013).

#### 1.16.1.3 FIXED DRUG ERUPTION (FDE).

This type of eruption is always caused by drug exposure. The symptoms of this skin rash appear as one or more lesions at the same site every time a same drug is administered. This kind of drug-induced reaction is characterised by the appearance of hyperpigmentation (Shiohara, 2009). The skin eruption is mediated by cytotoxic CD8+ T cells with an effector memory phenotype, and the appearance of these cells is limited to the affected area (Pichler, 2003, Posadas and Pichler, 2007).The most common classes of drugs that are associated with FDE are analgesics (acetaminophen, piroxicam, and mefenamic acid), antibiotics (tetracycline, rifampicin, trimethoprim-sulfamethoxazole, amoxicillin), sedatives and anticonvulsants (e.g., carbamazepine), and certain muscle relaxants such as chlormezanone (Lee, 2000). Generally, the most important step for the treatment of this type of cutaneous adverse reaction is early withdrawal of the culprit drug and short-term systemic steroid therapy (Chung et al., 2016).

#### 1.16.1.4 DRUG REACTION WITH EOSINOPHILIA AND SYSTEMIC SYMPTOMS (DRESS).

The clinical symptoms of DRESS start with severe maculopapular rash, fever, frequent eosinophilia and lymphadenopathy. This is followed by an increase in serum alanine aminotransferase levels and abnormal liver function often resulting in hepatitis (Shiohara, 2007), and haematological abnormalities such as atypical lymphocytes in the circulation, thrombocytopenia, or leukopenia (Roujeau, 2005, Bocquet et al., 1996, Walsh and Creamer, 2011). These symptoms may effect systemic organs such the kidneys or lungs and cause interstitial nephritis, pulmonary infiltrates and arthralgia, which may be life threatening (Guillon et al., 1992). DRESS has an approximate mortality rate of 10% (Uetrecht and Naisbitt, 2013, Proudfoot et

al., 2009). IL-5 secreted from drug-stimulated T cells is thought to play an important role in pathophysiology part of DRESS, by regulating the cellular actions of eosinophils (Choquet-Kastylevsky et al., 1998). Several studies propose that the reactivation of human herpes viruses (HHV-6, HHV-7, EBV and CMV) are associated with the onset of DRESS (Shiohara et al., 2012). For instance, detection of CMV-DNA and EBV-DNA levels have been detected in the blood of patients with fatal cases of DRESS associated with phenytoin and allopurinol (Aihara et al., 2001, Descamps et al., 2003). Virus and drug-specific T-cells are detected in patients with DRESS and currently the role that both T-cell populations play in the disease pathogenesis is not fully defined. The symptoms of DRESS start 2-6 weeks after initiating drug administration (Peyriere et al., 2006). In contrast, re-exposure to the same drug can trigger a second DRESS reaction in 24 hours due to the generation of memory T cells at the time of initial drug exposure (Verma et al., 2013). The most common drugs that are involved in DRESS are anticonvulsants (phenobarbital, phenytoin, carbamazepine, lamotrigine, valproate), antivirals (abacavir, cidofovir, and nevirapine), antimicrobials (dapsone), antibiotics (minocycline, ampicillin, flucloxacillin, sulfonamides), and NSAIDs (diclofenac) (Criado et al., 2012) (Table 1.2). The casual treatment is withdrawal of the trigger drug, and symptomatic treatment. Corticosteroids, and immunosuppressant such as cyclosporine are often used in severe cases (Choudhary et al., 2013).

**1.16.1.5 STEVEN JOHNSON SYNDROME (SJS) AND TOXIC EPIDERMAL NECROLYSIS (TEN)/LYELL SYNDROME.**

This type of drug induced-skin reaction is characterised by the presence of keratinocyte apoptosis (Abe et al., 2003b). These are the most severe types of

cutaneous ADR, but fortunately their appearance is rare (approximately 1-2 cases per million inhabitants per year, and slightly lower at 0.4-1.2 cases per million inhabitants per year for SJS and TEN respectively) (Rzany et al., 1996). The mortality rate due to the severity of TEN is approximately 25%- 30% (Downey et al., 2012, Roujeau, 2005), while fatalities in patients with SJS are less common. The symptoms of SJS/TEN start 1-3 weeks after initiating drug administration (Mockenhaupt, 2009). Clinical symptoms appear initially on the face and trunk. This is followed by mucosal membranes such as mouth, eyes, genitals and intestines (Böhm et al., 2018, Oplatek et al., 2006). Skin involvement with SJS is less than 10%, while the skin involvement with TEN is 30% or more (Uetrecht and Naisbitt, 2013). Patients present with blistering skin, erosions and large areas where the epidermis has separated from the dermis (Bastuji-Garin et al., 1993, Mockenhaupt, 2009). Analysis of blister fluid revealed the infiltration of cytotoxic CD8+T cells and NK cells (Nassif et al., 2004, Le Cleach et al., 2000). Both T cells and NK have the ability to secrete high level of cytolytic granulysin granules which work as a killer that spread keratinocyte apoptosis throughout the skin (Chung et al., 2016, Ernst et al., 2000, Okada et al., 2003). T cell-mediated keratinocyte damage can be increased by the appearance of the proinflammatory cytokine IFN-Y, which increases MHC expression for antigen presentation (Nassif et al., 2004, Yawalkar et al., 2000a). It has also been shown that SJS/TEN syndromes may involve the apoptosis inducing molecule Fas ligand (Abe et al., 2003b). The syndrome is immune-mediated as reoccurrence of symptoms has been detected when patients are inadvertently rechallenged with the suspected medication (Harr and French, 2010, Halevi et al., 2000). Greater than 50% of TEN survivors suffer from long term complications of the disease (Harr and French, 2010).

Triggering TEN/SJS drugs include antibiotics (cephalosporins, aminopenicillins, and sulfonamides), anti-convulsants (carbamazepine, phenytoin, phenobarbital, lamotrigine), allopurinol, antiviral drugs (nevirapine), antifungal drugs (imidazole), and NSAIDS (diclofenac, and Ibuprofen) (Harr and French, 2010, Halevy et al., 2008, Sharma et al., 2008, Ward et al., 2010).

In this type of hypersensitivity reaction there is no specific treatment known to be beneficial. Several treatment programmes have been attempted with limited therapeutic benefit.

**Table 1.2: Drugs that most frequently induce cutaneous hypersensitivity reactions.** Data extracted from open vigil 2.1 Med DRA on 17th of October 2017; US pharmacovigilance data, 2004-2014. The colours indicate to the relative risk of incidence when compared to the other drugs (Bohm, Ruwen et al, 2018). DILI= Drug-induced liver injury; DIA= Drug induced Agranulocytosis; DRESS= Drug reaction with Eosinophilia and systemic symptoms; SCAR= Severe cutaneous adverse reactions. The red cross mark indicates the seriousness of the ADRs, while the orange cross mark indicates moderate severity.

| Drug | DIA | DILI | Hypersensitivity | Anaphylaxis | SCAR | DRESS |
|---|---|---|---|---|---|---|
| Abacavir | | | | | | ✚ |
| Allopurinol | ✚ | | | | ✚ | ✚ |
| Amoxicillin | | ✚ | ✚ | ✚ | ✚ | ✚ |
| Acetaminophen | | | | ✚ | | |
| Carbamazepine | | | | | ✚ | ✚ |
| Carboplatin | ✚ | | | | | |
| Cefazoline | | | | ✚ | | |
| Cefotaxime | | | | | | ✚ |
| Cefuroxime | | | | ✚ | | |
| Ceftriaxone | | | ✚ | ✚ | ✚ | ✚ |
| Cisplatin | ✚ | | | | | |
| Clavulanic acid | | | ✚ | ✚ | ✚ | ✚ |
| Clindamycin | | | ✚ | | ✚ | ✚ |
| Cytarabine | ✚ | | | | | |
| Diclofenac | | | | ✚ | | |
| Doxycycline | | | | | | ✚ |
| Etoposide | ✚ | | | | | |
| Ethambutol | | | | | ✚ | ✚ |
| Fluorouracil | ✚ | | | | | |
| Fluconazole | | ✚ | | | ✚ | ✚ |
| Gemcitabine | ✚ | ✚ | | | | |
| Gadolinium | | | | ✚ | | |
| Isoniazid | | ✚ | | | ✚ | ✚ |
| Ibuprofen | | | | | ✚ | |
| Iopromide | | | ✚ | ✚ | | |
| Lamivudine | | ✚ | | | | ✚ |

| Drug | DIA | DILI | Hypersensitivity | Anaphylaxis | SCAR | DRESS |
|---|---|---|---|---|---|---|
| Lidocaine | | | | + | | |
| Methotrexate | + | | | | | |
| Metronidazole | | | | | + | + |
| Minocycline | | | + | + | | |
| Naproxen | | | | + | | |
| Nevirapine | | + | | | + | |
| Oxaliplatin | + | | | | | |
| Piperacillin | | | | | + | + |
| Phenytoin | | | | | + | + |
| Propofol | | | | + | | |
| Rifampicin | | + | | | + | + |
| Rocuronium | | | | + | | |
| Spironolactone | | + | | | | |
| Sulfasalazine | | | | | + | + |
| Sulfamethoxazole | + | | | | + | + |
| Tazobactam | | | | | + | + |
| Telaprevir | | | + | | + | + |
| Temozolomide | + | | | | | |
| Terbinafine | | | | | + | |
| Trimethoprim | + | + | | | + | + |
| Valproate | | | | | + | + |
| Vancomycin | | | | + | + | + |
| Vincristine | + | + | | | | |
| Zonisamide | | | | | + | + |

### 1.16.2 DRUG INDUCED LIVER INJURY AND FORMATION OF REACTIVE METABOLITES.

Hepatotoxicity remains a major concern following drug exposure, and the most important reason for drug withdrawal during post marketing surveillance. Liver is the major site of drug metabolism which enzymatically transforms chemicals and drugs to larger more polar forms prior to elimination.

The hepatic cytochrome P450 enzymes play an important role in the metabolism process, they convert prodrugs into their active form (Almazroo et al., 2017), convert active drugs into elimination products and in rare cases convert drugs into reactive and potentially toxic species. When enzymes catalyse the conversion of drugs into reactive metabolites the process is referred to as bioactivation. Reactive metabolites may bind covalently to proteins in the liver, and induce ADRs either directly or indirectly through different mechanisms (Amacher, 2012). The difference in metabolic capacity of individuals may influence susceptibility of individuals to DILI. Metabolic capacity is influenced by genetic as well as environmental factors. Alterations in drug metabolism has been proposed as an important factor in susceptibility to drug hypersensitivity reactions targeting liver and other organs; however, to date, there is little direct evidence to date to show that enhanced bioactivation or decreased detoxification leads to a significant shift in risk (Leung et al., 2012). The main two manifestations of DILI are hepatocellular and cholestatic.

#### 1.16.2.1 HEPATOCELLULAR LIVER INJURY.

Hepatocyte liver injury is characterised by elevation of the concentrations of serum aminotransferases: alanine (ALT) and aspartate (AST), and alkaline phosphatase (AP), and manifests as hepatocyte necrosis with or without steatosis (Bleibel et al., 2007). The symptoms usually start 1-3 months after drug exposure, but sometimes

the latency period of DILI can be more than 1 year (Björnsson, 2010). In contrast, in some cases the time to onset is rapid. For example, liver injury caused by telithromycin occurs only a few days after drug exposure (Clay et al., 2006). The differences in reaction timeframe may relate to different mechanisms of tissue injury.

#### 1.16.2.2 CHOLESTATIC LIVER INJURY.

Cholestatic liver injury is characterised by predominant elevation in alkaline phosphatase (ALP), and bilirubin, and portal inflammation with slight hepatocytic injury (Bleibel et al., 2007) . If the ratio of ALP and bilirubin in terms of the number of times is less than 2, it is considered to be cholestatic DILI (Hagley et al., 1993), while if its more than 5, the ADR is considered to be hepatocellular for instance as seen with acetaminophen (Ko et al., 2017). However, if the ratio is between 2 and 5 it is considered to be a mixed type of DILI (Uetrecht and Naisbitt, 2013, Chalasani et al., 2014). There are different risk factors for DILI including; age, sex and genetic polymorphisms of metabolising enzymes P450 (Hussaini and Farrington, 2014b). Drugs classified as causing harm to liver by direct toxic effects include methotrexate-induced liver fibrosis and acetaminophen-associated liver failure. The latter is dependent on dose and duration of exposure to the drug (Lee and Senior, 2005). Other drugs damage the liver indirectly through activation of the hosts innate and/or adaptive immune system. In these reactions, discussed in more detail later, the reaction onset is delayed by weeks or months. Once a hypersensitivity reaction arises, no specific therapy is available except discontinuation of culprit drug to prevent progression of acute liver failure, followed by administration of antihistamine diphenhydramine and hydroxyzine for symptomatic pruritus.

**1.17 THE IMMUNE SYSTEM.**

The immune system is the host defence system. It consists of lymphoid organs, cells and cytokines that protects the human body from a wide variety of microorganisms that cause harm to the human body. It can also distinguish the wide variety of organisms from healthy tissue and respond appropriately. The bone marrow and thymus are the primus of all immune cells found in the blood. The immune system is classified into the innate system, non-specific arm that reacts to a broad array of foreign stimuli, and the adaptive immune system (specific) that responds through the production of antigen-specific antibodies and T cells (Figure 1.4). All immune cells work together in a collaborative and complementary manner to protect the body. The adaptive immune system thus aids and drives the immune response (Medzhitov and Janeway Jr, 1997). Drug hypersensitivity reactions arise when the immune system responds naturally in an appropriate manner to an inappropriate drug stimulus.

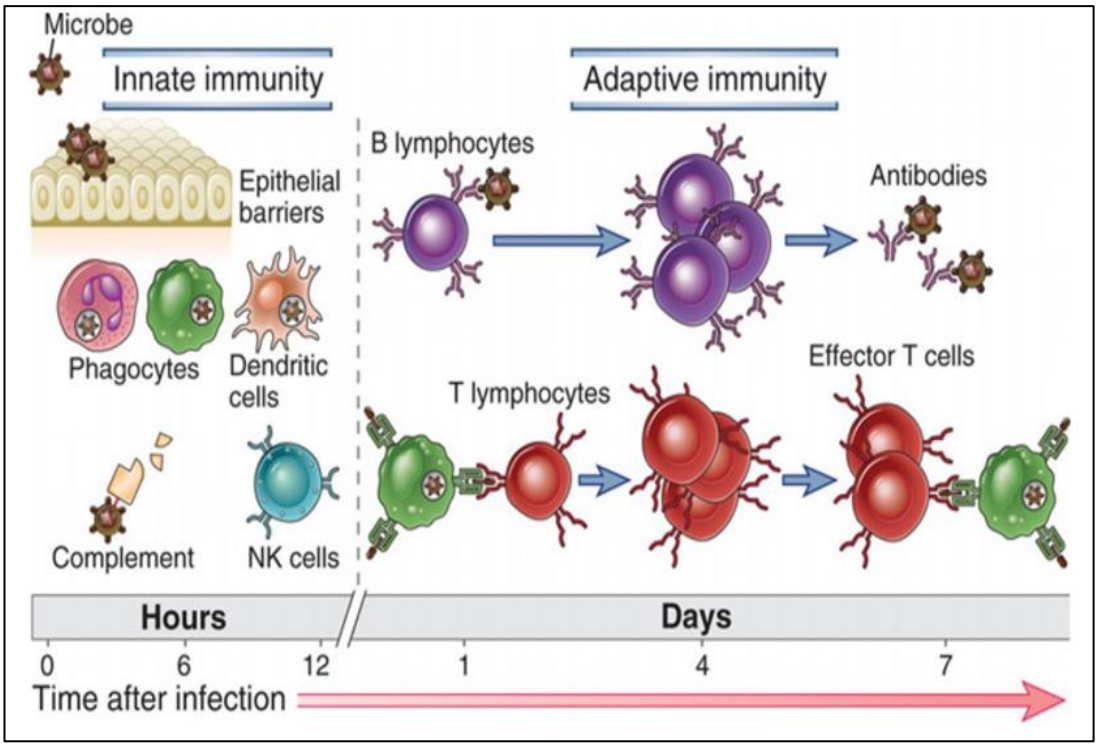



**Figure 1.4. The innate immunity versus the adaptive immunity**. The innate immune system is considered as the first line of defence against non-self-pathogens, and it provides a preconfigured response against any pathogen spread throughout the body. Moreover, the time line of the response is immediate. Whereas the adaptive immune system is considered as the second line of defence and provides a tailored response to each stimulus. The time line of the response after antigen exposure is much longer. The photo was adapted from Abbas et al: cellular and molecular immunology, 7th edition. Copywrite: 2014

### 1.17.1 INNATE IMMUNITY.

The innate immune system consists of all the immune defences that lack immunological memory. It is comprised of physiochemical barrier (epithelial layer) which is considered a first barrier, chemical mediators (defensins) which are antimicrobial peptides (Agerberth and Guðmundsson, 2006), lysosomes and phospholipids. The humoral barrier in the innate system, is made of inflammatory mediators, cytokines as well as coagulation cascades. The innate system limits microorganisms from damaging the tissues they invade, by the activation of innate response rapidly. A primary aspect of the innate immune response is the ability to

recognise pathogen-associated molecular patterns (PAMPS) and prevent or reduce its spreading to prevent any infection (Janeway, 1989). If infectious agents circumvent these barriers, phagocytic cells, i.e. macrophages, monocytes and neutrophils, come into effect. Receptors entrenched in the genome known as pathogen recognition receptors (PRRs) recognise the PAMPs. These PRRs exist in either soluble or a cell-associated form, and can be expressed by dendritic cells, macrophages and neutrophils (Kumar et al., 2009, Kawai and Akira, 2010, Takeuchi and Akira, 2010).

**1.17.1.1 CELULLAR COMPONENTS OF THE INNATE IMMUNE SYSTEM (MEDIATORS).**

It is known that the cells of the innate immune system act as a secondary barrier to microorganisms after the epithelial membranes. These cells are known as phagocytic cells, i.e. macrophages, monocytes and neutrophils and they are acting if the infectious agents circumvent the epithelial membranes. The most important phagocytic cells are macrophages and dendritic cells (DCs). The other cellular components of innate immunity include cells releasing inflammatory mediators, such as mast cells, basophils, eosinophils, and Natural Killer (NK) cells which has ability to recognise malignant cells and the cells who infected by viruses and induce apoptosis to kill them.

**1.17.1.1.1 MACROPHAGES CELLS.**

These cells are antigen presenting cells (APCs) responsible for the phagocytosis of invading pathogens that is critical to both the innate and adaptive immune system (Khazen et al., 2005). The process of phagocytosis is activated by the binding of PAMPs to PRRs present on the macrophages surface, enabling them to engulf and kill

microorganisms. Reactive oxidative species, nitric oxide and lysosomal enzymes are vital components to the process of pathogen engulfment (Aderem and Underhill, 1999). Macrophages then present peptide antigens derived from the engulfed pathogen to T-lymphocytes.

#### 1.17.1.1.2 DENDRITIC CELLS (DCS).

DCs are antigen presenting cells (APC) that guard the immune system. They are the most important phagocytic cells which ingest extracellular antigens (Banchereau and Steinman, 1998). They develop from hematopoietic bone marrow progenitor cells that develop into immature DCs, which are known for their significant endocytic activity and low T cell activation potential. The maturation of DCs is initiated through exposure to danger signals, such as TLR ligands and proinflammatory signals (LPS, TNFα or DAMPs) or heat shock proteins. During the maturation process, the endocytic ability of the cells is downregulated, whereas the lysosomes and antigen processing components of the cells are utilised. Upregulation of peptide-MHC complexes leads to significant expression of costimulatory molecules, such as CCR7, CCR5 and CCR1 as well as IL-2 (Rescigno et al., 1998, Han et al., 2009). DCs initiate antigen specific memory T-cell responses once they mature and are transported to the T cell primary regions of the lymph nodes. DCs also pass antigens captured from external environments to T-cells that are present at low levels in the blood and then migrate to lymph nodes (Banchereau and Steinman, 1998). DCs control the presence of pathogens in the adaptive immune system by stimulating a series of antigen-specific responses (Iwasaki and Medzhitov, 2015). (Mellman, 2013, Hubo et al., 2013). A third signal is also produced that is equally important for naïve T cell activation. Signal III exists through soluble factors like cytokines IL-12, IL-15, IL-6 and TNF-α (Kapsenberg, 2003).

### 1.17.2 ADAPTIVE IMMUNITY.

The adaptive immune system is activated by the innate immune system due to their synchronized complex cellular and molecular interactions (Cooper et al., 2004, Raulet, 2004). The primary function of the adaptive immune system is to kill invading pathogens through release of effector molecules. It is particularly important in the destruction of pathogens that evade the innate immune system (Coico, 2021). The adaptive immune system is also known as acquired or specific immune system.

#### 1.17.2.1 CELULLAR COMPONENTS OF THE ADAPTIVE IMMUNE SYSTEM.

The key components of the adaptive immune system are mononuclear cells monocytes, and lymphocytes that lack granules. They act as arbitrators between cellular and humoral immunity (Miller and Osoba, 1967). Lymphocytes and other cellular elements of blood originate from the same progenitor hematopoietic bone marrow stem cells (Figure 1.4). Lymphocytes are broadly divided into B-lymphocyte and T-lymphocyte categories, the former further differentiate into plasma secreting cells and the latter into cytokine-secreting effector cells (Janeway et al., 2008). Both T-lymphocytes and B-lymphocytes have specific receptors on their surface, and can recognise different antigens according to their structures. The binding of antigen to the antigen specific receptors induce lymphocytes to acquire effector functions and memory phenotypes.

##### 1.17.2.1.1 B- LYMPHOCYTE CELLS.

Humoral immunity helps to protect against various pathogens with the aid of B-cells and their affiliate antibodies. B-cells have the ability to recognise organisms that are not related to the body. B-cells protect against pathogens and secrete relevant

antibodies due to their specific expression of CD19 and their ability to divide into either long-lived memory cells or effector plasma cells. They trigger the humoral immune response due to the binding of antigens to IgM and IgD on naïve B-cells and subsequently differentiate to yield antibody secreting plasma cells and memory B-cells. The activation of B-cells can be tracked by the process of isotype switching that shows the production of antibodies apart from IgM and IgD. The activated B-cells release IgE antibodies that bind to antigens. IgG antibodies are also produced, which are vital for the control of infections in various tissues (Amali et al., 2017).

**1.17.2.1.2 T- LYMPHOCYTES.**

T cells are implicated in cell-mediated immune responses. They are activated by specific antigens they encounter. T cells proliferate and various changes takes place to enable a response by effector cells. There are two major subtypes of T-cells; killer CD8+ T cells and helper CD4+ T cells. Helper T-cells magnify the immune response by cytokine secretion, whereas the cytotoxic T cells kill cells that express the antigen. Helper T cells are often referred to as CD4+ cells because they express a protein called CD4 that associates with T cell receptors (TCR). Pre-cursor thymocytes relocate to the thymus gland from the bone marrow through the bloodstream, after the differentiation stage of hematopoietic stem cells. Thymocytes pass through several distinct intermediate stages (Mombaerts et al., 1992). The first maturational stage is classified as double CD4/8 negative because they do not show expression of these cell surface markers. The double negative cells are subject to rearrangement of TCR-β chains to allow thymocytes to generate CD4+ and CD8+. Following this stage, the rearrangement of TCR-α takes place and mature CD4 or CD8 single positive cells are

generated which then migrate from the thymus gland to the blood (Overgaard et al., 2015).

#### 1.17.2.1.2.1 HELPER T CELLS.

Helper T cells have an important function in the immune system in fighting pathogenic infections. They act as a regulator between innate and adaptive immune responses, and determine the immune response that the body should make for a specific pathogen through the secretion of cytokines (McHeyzer-Williams et al., 2006). These cytokines boost the activity of killer cells and macrophages (Alberts et al., 2002). These cells do not kill the infected cells or pathogens directly as they do not have obvious cytotoxic activity. Helper T cells are classified into different subsets, namely Th1 and Th2 cells. Both Th1 and Th2 cells secrete cytokines in response to antigen stimulation. The output of Th1 cell activation is IFNY, lymphotoxin, IL2 and TNFβ, whereas the output of Th2 cells is IL-4, IL-5, IL-9, IL10 and IL-13 (Zhu et al., 2009). Th2 cells have the ability to activate B cells to produce different antibody classes (Figure 1.5). In general terms, Th1 cells are considered to be proinflammatory which activate macrophages, while Th2 cells are considered to be anti-inflammatory. The production of specific cytokines from stimulated DCs at an area of infection, leads to the activation and polarisation of naïve helper cells into helper subsets (Alberts et al., 2002). This differentiation process also depends on the type of APCs that present the antigen and their phenotype and costimulatory protein molecules expressed (Luckheeram et al., 2012). CD4+ Th1 and Th2 cells express TCR on their surfaces that recognise and interact with the antigenic peptide MHC class II complex.

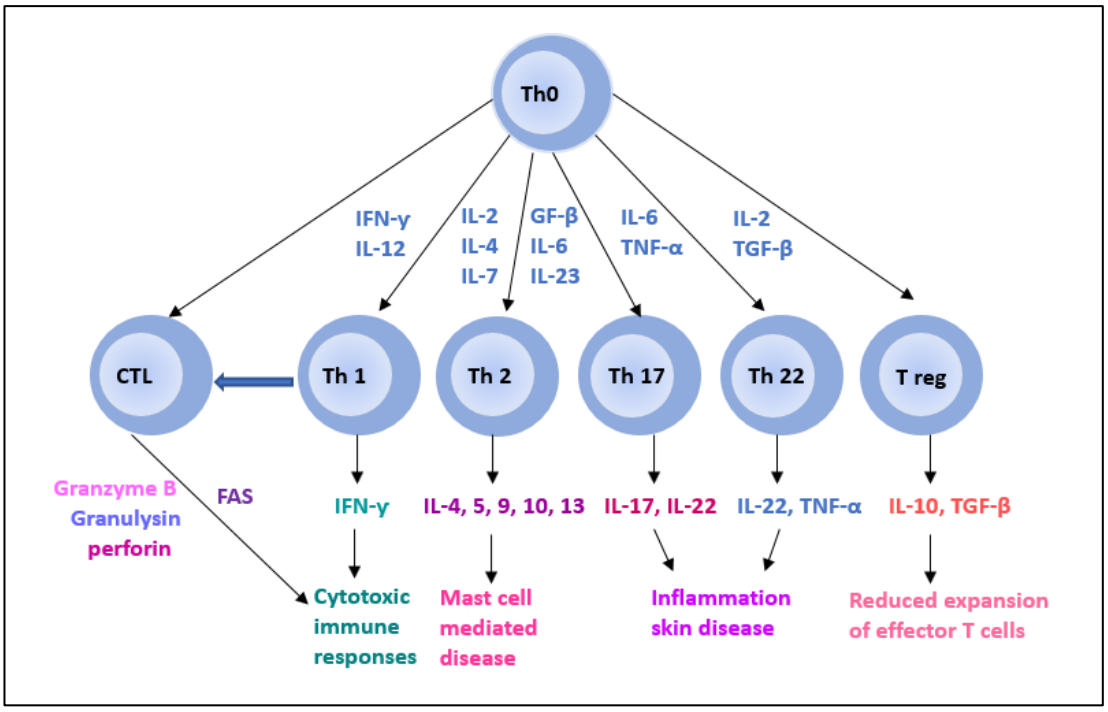



**Figure 1.5. Distinct Th0 subsets producing cytokine control of T cell differentiation.** A naïve T cells (Th0 cells) are a CD4+ T cell subsets in humans and depends on cytokine production and effector functions and are responsible for effects intermediate between those of Th1 and Th2 cells.

**1.17.2.1.2.2 KILLER T CELLS.**

Cytotoxic cells also known have the capability to recognise and interact with the pathogens and kill the infected cells before the harmful microorganism penetrates and infects other cells (Harty et al., 2000). Once the TCR binds to the triggering antigen in a complex with MHC class I, naïve CD8+ T cells become activated and will proliferate. Cytotoxic T cells are produced and when activated they secrete proinflammatory cytokines such as IFNy which has anti-microbial effect. TNF- α that has pro-inflammatory effects, perforin, granzyme and FasL that induce target cell cytotoxicity directly (Lazarevic and Flynn, 2002). The cytolytic perforin protein which form pores allows toxins, ions, and water into infected cells. Other toxins called granulysin and granzyme enter infected cells within the pores and cleave intracellular proteins which leads to cell death through activation of the apoptotic cascade (Radoja

et al., 2006, Demers et al., 2013). The other important CD8+ T cell effector mechanism is through the Fas/FasL pathway. Here, an intracellular death signal is delivered through Fas receptor triggering (Chavez-Galan et al., 2009). FasL on the cell surface of stimulated CD8+ T cells bind to Fas receptors on surface of the target cell (Figure 1.6). This binding causes the Fas molecules to pull together triggering signalling cascades and the induction of apoptosis.

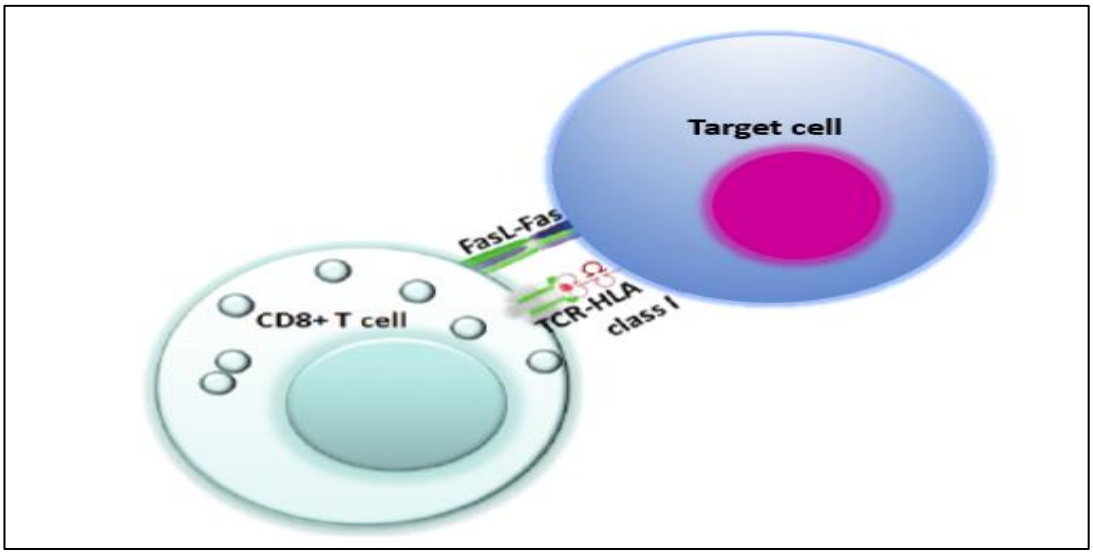



**Figure 1.6. FasL molecule is expressed on cytotoxic cells and recognise the infected cell through Fas molecule receptor on the target cell surface**. The target cell is destroyed by the CD8+ T cell through the recognition of HLA class I restricted peptide antigen. Adapted from Chávez-Galán L et al, 2009.

### 1.18. DIAGNOSIS OF HYPERSENSITIVITY.

A combination of in vivo methods and in vitro laboratory tests are used to assist the diagnosis of hypersensitivity reactions (Hamilton, 2019). However, the strength of the diagnosis of hypersensitivity relies firstly on the clinical history and physical examination to confirm sensitization and to ensure appropriate recommendations and treatments are made (Hamilton, 2010). Mechanistic characterisation of non-

specific mediators such as the level of neutrophils, or eosinophils in the blood, and immunohistochemically stained liver or skin biopsies is important to determine the role of these cells when penetrating specific areas. Three classical skin tests are performed to attempt to diagnose hypersensitivity reactions; firstly epicutaneous patches are applied to identify the potential antigen, or to assess the cross reactivity of the alternative drug (Lopez et al., 2007). Factors that affect the results of the skin test include the presence of T cells in the skin, the ability of skin cells to present the drug after penetrating the skin and the ability of the drug to penetrate the skin (Pichler and Tilch, 2004). Secondly, skin prick tests are commonly used in the diagnosis of hypersensitivity because they are safe, easy, quick and accurate. A small drop of the possible drug antigen is placed on the skin before pricking the skin. Thirdly, the intradermal skin test can be used, but it is considered as less safe. The technique involves injecting small amount of drug antigen followed by the formation of a wheal and redness after 10- 15 minutes. (Brockow et al., 2002). All in vivo tests have the disadvantage that the treatment might harm the patient through the generation of an unwanted T cell response. As such, skin testing is not advised in patients with the most severe forms of cutaneous hypersensitivity reaction or in reactions involving internal organs. To avoid this risk, in vitro tests are also used. These tests described in detail below offer the advantage that they also improve our understanding of immune cell functions and the role of different cell populations in the ADR (Yawalkar et al., 2000b).

### 1.18.1 LYMPHOCYTE TRANSFORMATION TEST.

The aim of the lymphocyte transformation Test (LTT) is to determine whether a patient has developed a T cell response against a certain drug. It is also referred to as

lymphocyte proliferation or stimulation test. It is a commonly used as diagnostic test in the area of drug allergy with limited clinical risk, and it had the advantage that multiple drugs can be assessed in the same experiment (Kano et al., 2007). Several studies indicated that the specificity and sensitivity of LTT, are significantly higher than in skin tests. For instance, the specificity of the LTT is reported as 85%, with a corresponding sensitivity of 74% which is 12% higher than skin tests (Romano et al., 2004, Nyfeler and Pichler, 1997, Pichler and Tilch, 2004). The LTT should be performed 4 to 6 weeks after acute symptoms appeared. Within this time period T cells show high proliferation following in vitro drug stimulation (Hari et al., 2001). Peripheral blood mononuclear cells (PBMCs) separated from blood of patients are cultured with specific drugs for five days. 3[H] - thymidine is then added to measure the proliferation of T cells and the result is expressed as a stimulation index (SI). The raised proliferative response in the presence of provoked drug is interpreted as drug-specific T cell sensitisation. This test depends on the presence of memory T cells that are sensitised to the culprit drug (Luque et al., 2001). The LTT assay can be used for both immediate and delayed hypersensitivity reaction to identify the incriminated drug (Pichler, 2014). In delayed hypersensitivity reactions to β- lactams, the LTT is much more sensitive than skin tests (Luque et al., 2001). The limitation of this assay beside the length test duration, is if the PBMCs contain macrophages >25% as they might produce prostaglandin E2 (PGE2) which inhibits IL2 production that may suppress T cell proliferation (Walker et al., 1983, Rive et al., 2013).

### 1.18.2 ENZYME- LINKED IMMUNOSORBENT ASSAY (ELISPOT).

ELIspot technique is a highly sensitive immunoassay used for diagnosis of drug hypersensitivity. ELIspot is a quantitive assay that measures the secretion of cytokine

at single T cells specific for any antigen. Antigen-specific T cells secrete effector molecules when stimulated and form spots of different sizes on a membrane with different intensities (Sundararaman et al., 2015).The ELIspot assay is mostly used to detect IFN-γ secretion as a primary readout for the stimulation of CD4+ and /or CD8+ T cells; however, a variety of cytokines and cytolytic molecules can be measured in isolation or in tandem to obtain knowledge of the nature of the induced T cell response (Monshi et al., 2013, Naisbitt et al., 2015) . T cells or PBMCs are incubated with the specific drug in ELIspot plates containing variable membranes coated with capture antibodies for a particular cytokine or cytolytic molecule of interest. The T cells secrete cytokines that are captured by the specific antibodies. After an appropriate incubation time, cells are removed by washing and secreted molecules are detected using a detection antibody. The visible spots correspond to an individual cytokine are produced on the surface which can be quantified as a measurement of T cell activity (Lehmann and Zhang, 2012, Cox et al., 2006). The ELIspot technique has a higher sensitivity than the enzyme-linked immunosorbent assay (ELISA) allowing it to detect rare or low frequency human T cells (Tanguay and Killion, 1994).

**1.18.3 FLOW CYTOMETRY.**

Flow cytometry is a powerful effective technique for characterizing T cell phenotype at a single cell level. It is one of the most important tools used in the diagnosis of allergy to drugs and in the management of disease (van Eeden et al., 1999, Liu Yin and Tobal, 1999). Flow cytometry can be used to analyse the expression of drug activated T cell surface markers such as; receptor proteins, costimulatory molecules, chemokine receptors and MHC class II molecules (Shipkova and Wieland, 2012,

Beeler and Pichler, 2006). Flow cytometry utilizes fluorescent-tagged antibodies for each marker, which are measured on different channels of the machine.

Flow cytometry can also be used to evaluate the capacity of T cells to proliferate under different conditions using the fluorescent dye carboxy fluorescein diacetate succinimidyl ester (CFSE). The technique relies on the penetration of CFSE through cellular membranes and its binding with amino groups intracellularly. As a result of stimulation, cells divide and fluorescence halves at each division. This allows the flow cytometer to measure the number of divisions individual T cells go through following antigen stimulation (Gibson et al., 2014b, Quah et al., 2007). The main advantage of CFSE assay is the identification of very low numbers of antigen-specific dividing cells populations (Beeler et al., 2006).

**1.18.4 T CELL CLONING.**

T cell cloning is another in vitro assay which used to characterise drug specific T cells. It can be done when the number of individuals is limited, and when it is difficult to detect drug specific T cells through the use of previously described assays. T cell cloning requires incubation of PBMC for two weeks with the optimal concentrations of specific drug. The expansion of these T cells allows for serial dilution and the generation of monoclonal T cells that can be expanded through mitogen stimulation. Individual cultures are then assessed for antigen-specific proliferative responses before further studies to characterise phenotype and function. T cell clones have been used to define the pathomechanism of different forms of drug hypersensitivity reactions.

**1.19 MAJOR HISTOCOMPATIBILITY COMPLEX.**

The major histocompatibility complex (MHC) called the H-2 complex was discovered in mice and was found to be responsible for the rejection of transplant tissue (Gorer, 1936). In 1950, a similar antigenic system called immune iso antibodies was shown to be expressed on the surface of leukocyte cells (Dausset, 1958). The (MHC) molecules are recognized as a gene complex which are made of glycoproteins. They are located on the surfaces of all nucleated cells, particularly on chromosome 6P21 that have an important role in the regulation of the immune system in humans. Chromosome 6 extends to more than 170 M base pairs that are essential in building of DNA and RNA (Bodmer, 1987). The MHC in humans is defined as a polygenic, where it contains more than 200 different MHC I and MHC II genes with various ranges of peptide binding specificities (Daly et al., 2001). In humans, MHC I and MHC II genes are referred to as human leukocyte antigen (HLA) genes. Genetic variations in HLA has an effect on many diseases, particularly autoimmune diseases as different HLA proteins display different peptide sequences to specific TCR (Trowsdale, 2011, Janeway Jr et al., 2001). These variants are known as alleles and are inherited by an individual from parents (Vandiedonck and Knight, 2009). MHC molecules are classified into MHC class I which is located at the telomere end, MHC class II which is located at the centromere, and MHC class III which is located at the middle region in the short arm of chromosome 6 between MHC class I and MHC class II (Figure 1. 7). Both MHC class I and MHC class II are involved in adaptive immunity, where their biological role is the presentation of peptide antigen to T cells, thus they are clinically important in organ transplantation (Mahdi, 2013). Though, they are similar in their structure, the slight differences are in the sequence of amino acids in the peptide

binding cleft distinguishes the type of antigen presented to CD4+ and CD8+ T cells. MHC class III molecules are implicated in different tasks, and also have genes of unknown immunological function (Xie et al., 2003). MHC class I proteins are found on the surface of almost every somatic cell, they express normal self-antigens and abnormal or non-self-pathogens to the effector cells implicated in cellular immunity. In contrast, MHC class II are found exclusively on DCs, macrophage, and B cells where they present abnormal or non-self-pathogens for the activation of T cells (Udenfriend et al., 1954, Bjorkman and Parham, 1990). MHC class I and MHC class II present antigenic peptides derived from exogenous protein and endogenous protein to CD8+ cytotoxic T cells, and CD4+ helper T cells, respectively (Bharadwaj et al., 2012, Usui and Naisbitt, 2017). MHC class I molecules consist of two polypeptide chains that vary in length, where the longer α-protein chain (spans the cytoplasmic membrane) is encoded on chromosome 6, whereas the shorter β2 microglobulin protein molecule is non-covalently bound to the α chain and is encoded on chromosome 15 (Figure 1.8). MHC class II molecules have two polypeptide chains; α, β chains which both are similar in length and encoded on chromosome 6 (Figure 1.7). Noncovalent bonds link the two chains to one other. MHC class I is encoded by three loci known as HLA-A, HLA-B, and HLA-C, whereas MHC class II is encoded by three loci known as HLA-DR, HLA DQ, and HLA DP heterodimer proteins (Shiina et al., 2004, Williams, 2001).The antigenic peptide binding cleft of HLA class II molecules is open and up to 25 amino acids can be presented (Rammensee, 1995). In contrast the MHC class I peptide binding groove is closed and much shorter peptides are presented to TCR. Both MHC class I and MHC class II expression are regulated by cytokines. The expression of MHC class I and MHC class II molecules can be induced by IFNY. In fact MHC class II

expression can be induced by IFNY on some cell types that do not normally express class II (Zhou, 2009). Thus, a rather complex picture emerges for the rules governing peptide production in the presence or absence of IFN-γ. This situation is thought to be important in autoimmunity (Vardjan et al., 2012).

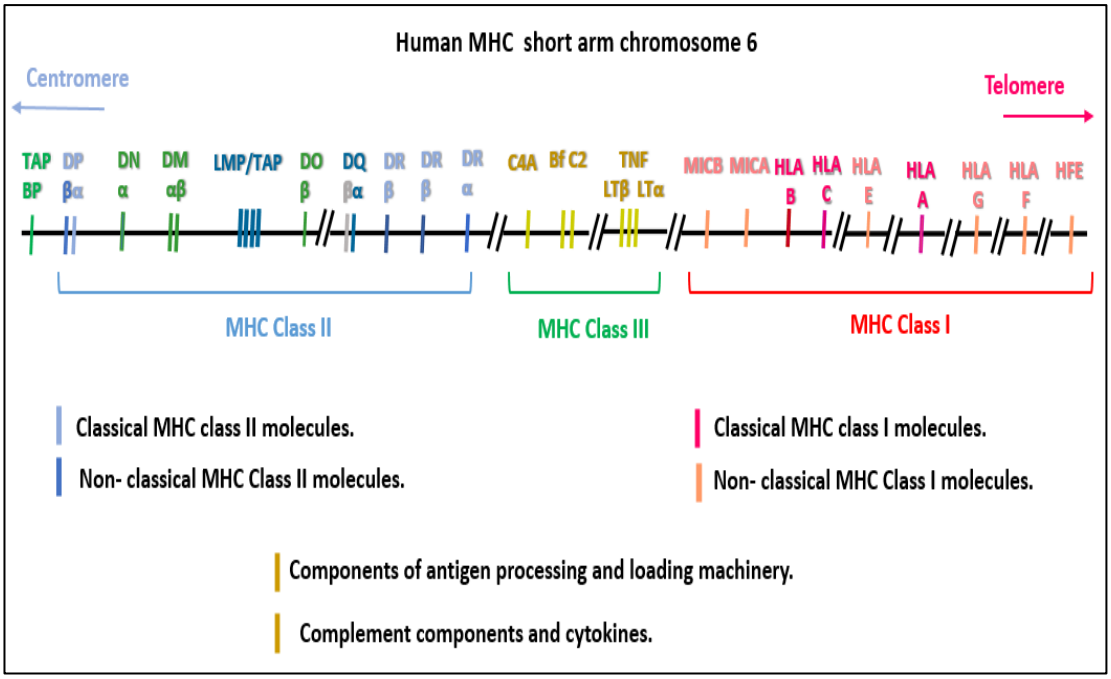



**Figure 1. 7. The organisation of human MHC regions on the short arm of chromosome 6.** HLA molecules span 3.5 million base pairs on the short arm of chromosome 6. They are HLA-DR, HLA- DP, HLA-DQ and A, B, C. Each gene has multiple alleles. Genes of the MHC class III cover proteins of the complement system such as C2, C4, Bf, and TNF.

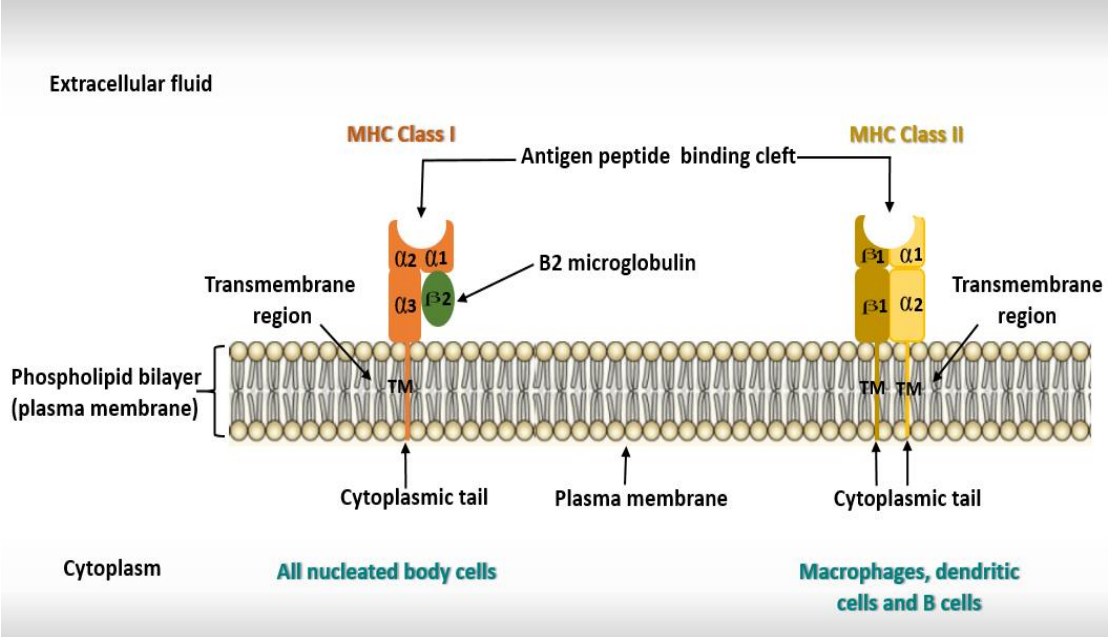



**Figure 1.8. Schematic structure of MHC Class I and MHC Class II molecules**. Both MHC class I and class II have similar structure. In each molecule, a cleft is formed to assimilate antigenic peptides. Humans express an array of different MHC molecules. Each MHC molecule is designed to bind with specific shaped antigenic peptides.

## 1.20 ANTIGEN PROCESSING AND PRESENTATION.

The pathway of antigen processing starts with uptake of antigens followed by the mechanisms that allow formation of MHC class I and MHC class II molecules and their association with peptides for display on the cell surface to T cells. Antigen processing is an important process for an immune response in T cells, where it allows the immune system to survey the intracellular and extracellular environment to identify potential infectious agents, whilst ignoring self-proteins (Figure 1.7). The most important and potent APCs are DCs, but several studies indicate macrophages and B-cells can also perform similar functions with less potency for naive T cell stimulation (Steinman, 1991).

### 1.20.1 ANTIGEN PROCESSING.

Generation of antigenic peptides within APCs starts in the cytosol, with proteolytic digestion of proteins by enzyme proteases generating small peptide (2 to 25 amino acids) fragments. The peptides then associate with MHC class I and II molecules for presentation on the cell surface (Blum et al., 2013).

### 1.20.2 ANTIGEN PRESENTATION.

Antigen processing and presentation allows T cells to recognise peptide antigens and respond in an appropriate manner. There are two sources of antigenic peptide that are derived from endogenous and exogenous antigens (Figure 1.8). MHC class I presentation pathway; antigenic peptides are generated in the cell cytosol. Proteins processed in this manner include invading viruses, intracellular bacteria, or protozoal parasites. These endogenous proteins are modified by ubiquitin which is a 76-amino acid protein, then subjected to digestion by the proteasome and cytosolic proteases (Leone et al., 2013). The antigenic peptides formed then enter into the endoplasmic reticulum (ER) by means of protein transporters associated with antigen presentation TAP 1 and TAP 2 by forming heterodimers in the membrane of the ER to simplify the passage of peptides from inside the ER into the surface of the ER. This active process is an ATP dependent (Blum et al., 2013). The TAP 1 and TAP 2 associates with tapasin protein (TPN), ER p57 and calreticulin (CRT) together via disulphide bonds to form a peptide loading complex. The MHC I heavy chain is initially formed as a linear peptide in the ER, then with the coordination of calnexin and calreticulin, the linear peptide is folded. Binding B2 microglobulin protein and ER (ERp57), TAP 1 and TAP 2 then transfer the peptide loaded MHC-I complex to the antigen cleft. Peptides that enter to the ER through the TAP 1 and TAP 2 together with the peptides that are synthesized

within the ER bind to the MHC via the endoplasmic reticulum resident amino peptidase (ERAP) (Leone et al., 2013). Once the peptide is bound to the MHC class I complex, it starts losing the affinity with tapasin, which allows it to arrive at the cell surface through the Golgi apparatus (Harding and Unanue, 1990). MHC class 1 molecules (Figure 1.9. A) are recognised on the surface of the APC primarily by CD8+ T cells (Harding and Unanue, 1990, Chessman et al., 2008). MHC class II pathway; starts by the APC taking up exogenous protein and internalizing them into endocytic vesicles of the professional APC (Figure 1.9.B) such as DCs, macrophages, and B-cells (Roche and Furuta, 2015). Then the internalized protein is transferred to endosomal / lysosomal vesicles by phagocytosis or endocytosis depending on the type of APC. Digestion of protein into peptide fragments occurs via proteases and cathepsins in acidic media. Meanwhile, the synthesis of MHC class II αβ chain dimers occurs in the ER with an invariant chain (Ii) assisted by calnexin to form new molecules which prevent peptides binding MHC class II inside the ER (Ten Broeke et al., 2013). The newly formed molecules combine with HLA-DM (DM). These molecules (known as Ii loaded MHC class II DM complex), are then transported through the Golgi apparatus into the late endosomes. Once the Ii loaded MHC class II DM complex is located inside the endosome, proteolysis of invariant chain allows freeing of the peptide binding cleft for antigenic peptides (Robinson and Delvig, 2002). However, HLA-DM facilitates peptide entry into the MHC-II cleft and therefore, the MHC molecule loaded with the peptide can be transported to the surface of APCs where it is recognised by CD4+ T cells.

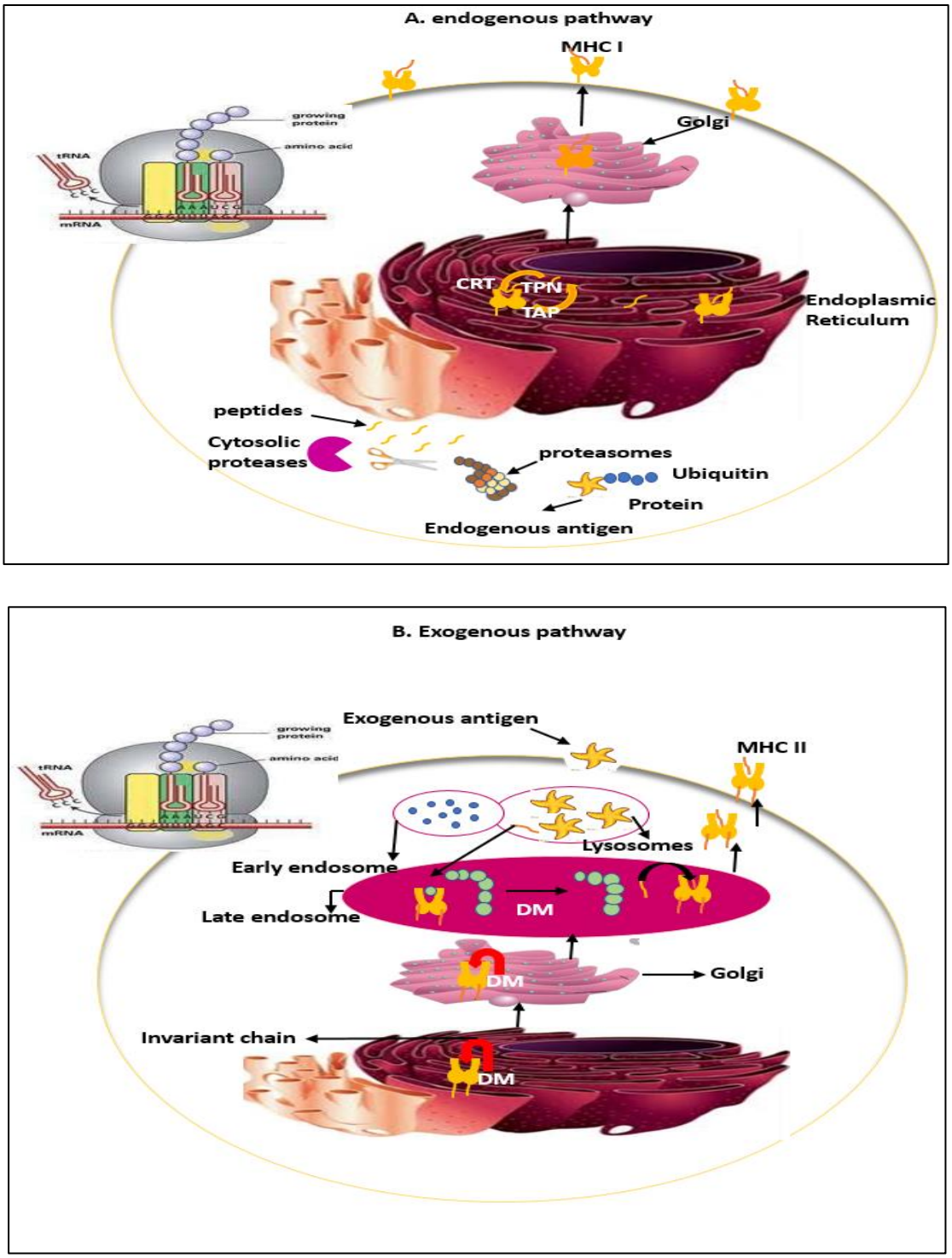



**Figure 1.9. Endogenous and exogenous processing pathways of professional antigen presenting cells.** Endogenous antigens are presented through MHC class I on APC to CD8+ T cells (cytotoxic cell), and exogenous antigens are presented via MHC class II on APC to CD4+ T cells (helper T cells).

**1.21 T- CELL RECEPTOR (TCR).**

TCRs are composed of polypeptide chains, on the surfaces of T cells. Although, each TCR is restricted to identify only a few peptides, with the exception of the peptide recognition site, they are similar in structure. Each TCR chain consists of four domains; variable region (N- terminal), a constant region, a stalk segment consists of short cysteine (the stalk segment forms the inter chain bound between the cytoplasmic tails), and the fourth domain is a transmembrane region. The variable region is composed of three loops, CDR1, CDR2, and CDR3. These are known as the complementarity determining regions (CDRs), where α and β chains form a TCR binding site that is responsible for antigen recognition. The two loops CDR1 and CDR2, contact the side chains of the MHC binding groove, while CDR3 interacts with the peptide ligand and subsequently determine the TCR specificity (Garcia and Adams, 2005). The constant region forms a complex with CD3 co- receptors, CD4 and CD8, all of which transmit signals that generate an antigen recognition site into the T cell, and ultimately leads to the stimulation of downstream signalling cascades (Teng et al., 1998). Once the TCR intracellular signalling cascade is triggered, effector functions are activated within the cell. The polypeptide chains of the TCRs are either αβ or γδ, where the majority of T cells express αβ receptors and restricted by classical HLA class I and class II molecules (Figure 1.10). γδ polypeptide chains are expressed on a small number of TCRs, it has been proposed that these cells may help to combat bacterial and parasitic infections (Rudolph et al., 2006).

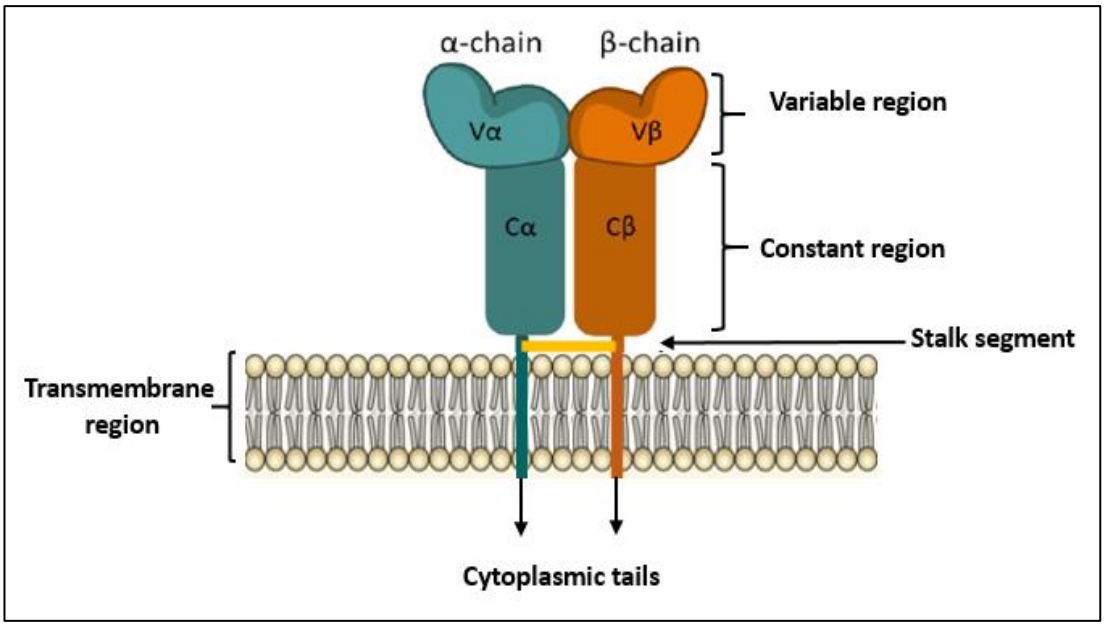



**Figure 1.10. The structure of T- cell receptor adopted from Janeways immunology (Murphy et al., 2008).**

All invading antigens appear to interact with CDR3α and β loops in a similar manner, and both of them are waved around peptide central region (Hennecke et al., 2000).

### 1.22 MECHANISMS OF IMMUNE ACTIVATION.

When examining how a drug activates the immune system, it is important to consider how the drug interferes/alters the MHC peptide TCR binding interaction. The main concepts describing these interactions are referred to as the hapten, pharmacological interaction (p-i), and altered peptide repertoire models and these are discussed below.

#### 1.22.1 HAPTEN CONCEPT.

It was Landsteiner and Jacobs who theorised the hapten model in 1935. The hapten model investigated protein binding interactions with chemical sensitizers, such as dinitrophenols and trinitrophenols. The authors discerned that formation of a covalently-modified drug protein conjugate is critical for initiation of an immune response measured by antibody production in experimental animals. They coined

the term ‘carrier specificity’ describing the importance of the reactive molecule and site of modification within the protein (Cell and Silverstein, 1962). Studies with penicillin further elucidated our understanding of the hapten model and expanded it to drug hypersensitivity. β-lactam antibiotics were initially shown to covalently bind to lysine residues on the surface of proteins (Schneider and De Weck, 1965). It was later revealed that T cells recognize penicillin-modified protein adducts and specific peptide sequences were identified as being important for MHC binding (Weltzien et al., 1996). Furthermore, penicillin-modified peptides have been shown to activate T cells in humans confirming that hapten recognition occurs in human patients that develop an adverse reaction (Padovan et al., 1997). Alteration of the penicillin structure, for example removing the thiazolidine ring and the side chain of the drug prevented T cell activation highlighting the structural specificity of the drug TCR binding interaction (Weltzien and Padovan, 1998). However, to date no crystal structures have been defined showing a drug hapten complexed with a peptide in the binding groove of HLA molecule (Figure 1.11).

Most drugs differ from the β-lactam antibiotics in that they are not directly protein-reactive. Several drugs do however form reactive species through the normal process of drug metabolism. Several groups, including our group, have proposed that these drug metabolites act as haptens, modifying protein and selectively stimulating a T cell response in hypersensitive patients. The sulfonamide antibiotic sulfamethoxazole (SMX) is the most widely studied example as the reactive nitroso metabolite (SMX-NO) can be synthesized for functional studies and patient samples are readily available. SMX-NO binds covalently to serum and cellular proteins including the surface of human neutrophils (Naisbitt et al., 1999). Further, animal studies

illustrated that SMX-NO drives T cell responses in an MHC-dependent manner (Naisbitt et al., 2002, Farrell et al., 2003). The presence of SMX-NO reactive T-cells in hypersensitive patients illustrated that the metabolite was equally likely to be formed in patients and that adduct formation promotes a T cell response (Castrejon et al., 2010). APCs modified covalently with SMX-NO activate T cells. Since these incubations are free of soluble drug it is highly likely that the T cells are stimulated with peptides derived from an SMX-NO-modified protein. Furthermore, fixation of the APCs, which blocks antigen processing, blocked the T cell response. Recently, SMX-NO has been shown to prime naïve T cells from healthy donors once regulatory T cells were removed from the culture conditions (Faulkner et al., 2016).

### 1.22.2 PHARMACOLOGICAL INTERACTIONS (P-I) CONCEPT.

T cells were also shown to respond to drugs such as SMX and lidocaine in a metabolism-independent and processing-independent manner (Schnyder et al., 1997, Zanni et al., 1998). In both instances, T cells reacted in the presence of drug and fixed APCs, while responses were not observed when APCs were pulsed with soluble drug. These data exclude the hapten model. The T cell response was MHC-dependent, but not always limited to a particular HLA allele, suggesting that the drugs might interact with a degree of specificity with specific TCRs (Von Greyerz et al., 2001). This showed some parallels of how drugs could react with other receptors in the body in a pharmacological sense. Accordingly, the model was named the 'pharmacological interactions' model or p-i. model. It has been suggested from in vitro studies that T cells in patients with hypersensitivity can be activated in the absence of innate immune signalling (Pichler, 2005); however this hypothesis is difficult to prove as all patients are exposed to different levels of stress signalling and

immune regulation throughout their lives. Unlike hapten-responsive T cells, certain T cells responsive to SMX showed reactivity with other sulfanilamides (Watkins and Pichler, 2013), indicating the specificity of the drug peptide receptor binding interaction may be less stringent. Recent studies updated the p-i. model to include drug reactions (e.g., carbamazepine and allopurinol) strongly associated with HLA alleles (discussed in detail below). A genome-wide association study (GWAS) portrayed that patients from Taiwanese populations tend to develop hypersensitivities to carbamazepine, if they expressed HLA-B*15:02 (Chen et al., 2011). Upon further analysis, HLA-B*15:02 restricted T cell activation is observed in hypersensitive patients that express the TCR Vβ-11-ISGSY clonotype (Ko et al., 2011). Clearly, in these patients, the interaction of carbamazepine with T cells is dependent on having both, the HLA allele as well as the precise TCR (Wei et al., 2012a). Interestingly, European and Japanese populations equally develop carbamazepine-reactive T cell responses and hypersensitivity reactions when they express HLA-A*31:01 (Lichtenfels et al., 2014). However, this selective drug HLA binding does not describe the complete clinical picture as many individuals that develop carbamazepine hypersensitivity do not express a known HLA risk allele. Similar observations have been described in patients with allopurinol hypersensitivity reactions. Patients that developed severe skin reactions selectively expressed HLA-B*58:01 (Hung et al., 2005). The T cell clones derived from patients were activated when oxypurinol, a stable metabolite of allopurinol, bound directly to HLA-B*58:01. When studies utilised a panel of allogenic HLA-mismatched APCs, the authors found a mixed pattern in which some T cells were restricted to B*58:01 while others showed some cross-reactivity with similar HLA alleles (Yun et al., 2014b). In this instance, the

stable drug metabolite clearly was able to interact with the structurally similar HLA molecules in a direct manner (Figure 1.11).

### 1.22.3 ALTERED PEPTIDE REPERTOIRE CONCEPT.

A strong correlation between abacavir hypersensitivity and HLA-B*57:01 expression has been described (Mallal et al., 2002a). In fact, abacavir is now the archetypal personalized medicine as individuals are pre-screened for HLA-B*57:01 expression before abacavir use and as such cases of abacavir hypersensitivity have been eradicated. Abacavir reacts with T cells in a processing dependent manner and early studies demonstrated that abacavir can form adducts with protein indicative of a hapten pathway (Bell et al., 2013, Meng et al., 2014). However, through a combination of structural HLA analyses, protein mass spectrometry and functional studies, abacavir was shown to activate T cells via an entirely novel pathway and to date this is the only drug that has been shown to interact with immune cells in this way. Abacavir interacts with the F-pocket situated in the binding groove of HLA-B*57:01 and consequently cause an alteration in the presentation of peptides on the cell surface. This led to several studies defining a new TCR-drug-HLA interaction (Illing et al., 2012, Norcross et al., 2012, Ostrov et al., 2012). The Illing et al study observed when comparing the HLA peptide binding repertoire of untreated and abacavir-treated cells that the immunopeptidome differed significantly. In the presence of abacavir altered amino acid residues at the PΩ anchor position were detected. In the absence of the drug the PΩ anchor position prefers amino acids with large aliphatic side chains like phenylalanine and tryptophan. However, upon treating HLA-B*57:01 expressing cells with abacavir, an increase in the amino acids with smaller side chains such as isoleucine and leucine were observed. This alteration in the peptide

repertoire results in the display of novel peptide sequences by HLA-B*57:01 and it is proposed that T cells that trigger the hypersensitivity reaction are activated by some of these peptides. A recent study employing a transgenic mouse model showed that although T cells can be activated by abacavir when isolated ex vivo, these responses could not be replicated in vivo without first dampening immunoregulatory pathways (Cardone et al., 2018). This may partly explain why only approximately 50% of individuals expressing HLA-B*57:01 develop hypersensitivity when exposed to the drug (Figure 1.11).

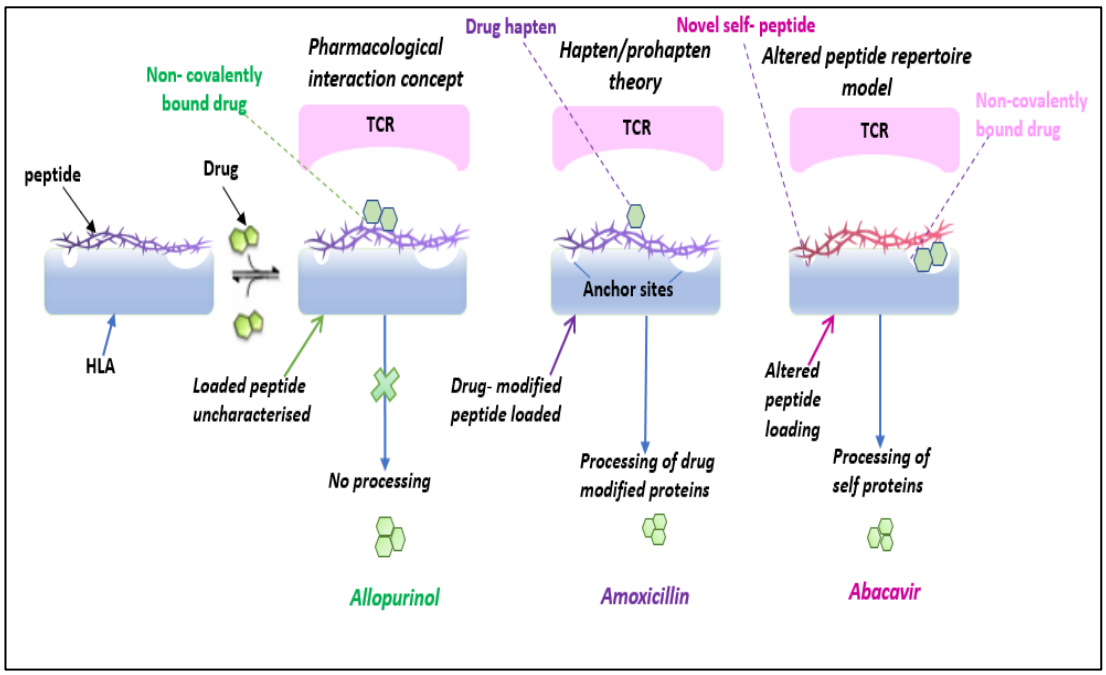



**Figure 1.11. PATHWAYS OF DRUG-SPECIFIC IMMUNE ACTIVATION.** These models show the interaction of drugs with T cells how HLA, peptide and TCRs interact with small molecules. **(A)** P-i (Pharmacological interaction model) concept. Drugs bind directly to the TCR, or HLA peptide molecules where TCR is essential as stabilising of the HLA molecules when the drug is bound. This kind of interaction occurs without the involvement of intracellular processing in the APC or the formation of a drug-protein adduct. **(B)** Hapten / prohapten theory, where the drug or its metabolite binds to protein covalently in the APC. T cells are activated with a drug-modified peptide derived from the original adduct. Amoxicillin is an example of this concept. **(C)** The altered peptide repertoire model, where the drug sits in the pocket of peptide binding cleft, which results in alteration of the peptides displayed. Abacavir is the only example of this model. Adapted from (Bharadwaj et al., 2012).

**1.23 HLA ALLELES NOMENCLATURE.**

In 1968, under sponsoring of WHO, a nomenclature committee for the HLA system was established (Bodmer, 1997). The committee determined if an allele was eligible for a name and then assigned names to specific genes. Accordingly, the committee has overseen the development of a standardized HLA nomenclature system as well as the usage of nomenclature based on serologic specificities, cellular responses and DNA sequences (Hurley, 2020). The identification of a specific HLA facilitates the selection of cells, tissue and organs before transplantation, but is also important in the diagnosis of autoimmune disorders (Matzaraki et al., 2017).Due to the links between expression of specific HLA molecules and development of ADRs, the HLA naming system has also become important to researchers exploring mechanisms of drug hypersensitivity (Illing et al., 2017). Serological techniques were used initially for the detection of HLA polymorphisms of class I and later class II. This was accomplished by the identification of epitopes and agretopes present on HLA molecules that triggered antibody responses (Tait, 2011). Limitations of serological technique appeared when a large number of HLA polymorphisms were studied. Thus, a new nomenclature method was designed to eliminate the limitations and to increase reproducibility (Figure 1.12). These refinements rely on each allele being defined by the name of the locus followed by a separator and up to 4 different sets of numbers, separated by a colon (Marsh et al., 2010). The first 2 fields represents the serological allele family shared by a similar serological behaviour, whereas the second 2 fields are allocated in numerical progression as each new allele is described in terms of synonymous difference inside and outside of the axons (Bodmer et al., 1989, Torres and Moraes, 2011). Eventually, an unrestricted suffix may be added to

the allele number in the form of a letter to specify the allele expression status (Marsh et al., 2010). A list of suffixes is used to indicate the expression status of the allele and these are outlined below:

**N =** null allele (not expressed at the cell surface).

**L =** allele have low levels of cell surface expression.

**S =** soluble allele (secreted protein) but do not located on the cell surface.

**C =** Assigned to allele that produce protein in the cytosol but not on the surface of the cell.

**A =** indicates an aberrant allele but there is a doubt to whether a protein actually is expressed.

**Q =** questionable expression. The mutation seen in the known alleles that affect expression levels in other alleles has been shown to affect normal expression levels in other alleles.

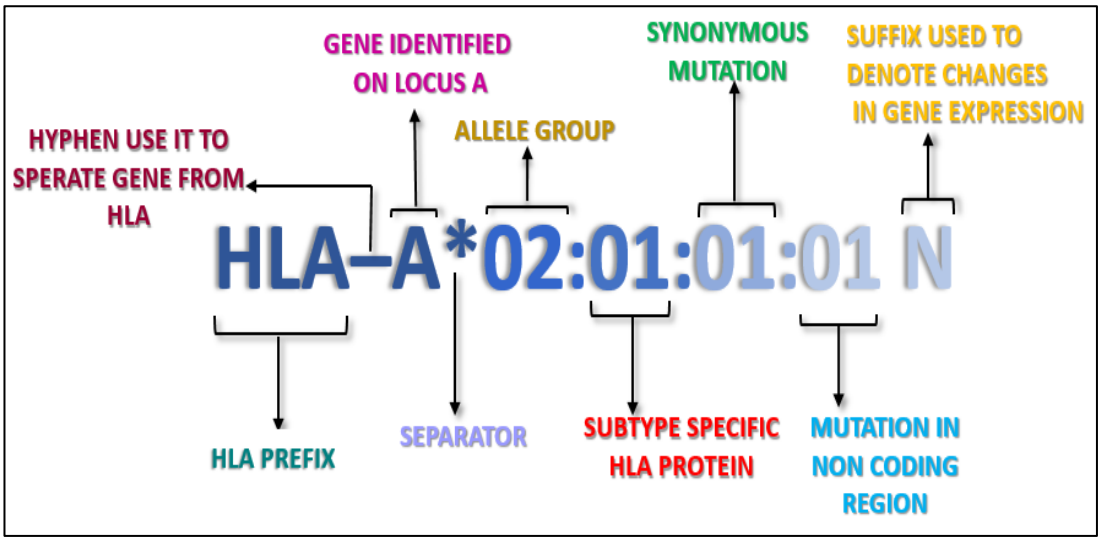



**Figure 1. 12. HLA allele nomenclature**. An illustration of the nomenclature of one allele of the HLA complex.

### 1.24 HLA ASSOCIATIONS WITH ADRs.

In recent years there has been considerable evidence gathered describing strong associations between expression of a single HLA allele and development of a specific form of drug hypersensitivity reaction targeting skin or liver (Table 1.3). For many years it has been known that drugs activate T cells in patients that develop cutaneous hypersensitivity. However, the detection of HLA alleles alongside a delayed onset of liver injury in susceptible patients provided initial evidence that the adaptive immune system may also be involved in liver injury. In recent years our group has focused on 3 forms of liver injury (flucloxacillin-, co-amoxiclav- and tuberculous medication-induced liver injury) and found the presence of drug-responsive T cells only in patients that developed adverse events (Monshi et al., 2013, Kim et al., 2015, Usui et al., 2017b).

**Table1.3: Drugs induced ADRs and HLA associations**. Examples of culprit drugs induced adverse reaction associated with specific HLA alleles. These HLA associations including HLA class I and II alleles. Adopted from (Fan et al., 2017).

| Drug | HLA association | Adverse reaction | population | Reference (s) |
|---|---|---|---|---|
| Amoxicillin-clavulenate | DRB1*15:01 DQB1*06:02 A*02:01-B*18:01 | DILI | Caucasian | Hautekeete ML et al., 1999 |
| Flucloxacillin | B*57:01 | DILI | Caucasian | Daly et al., 2009 |
| Minocycline | B*35:02 | DRESS | Caucasian | UrbanTJ et al., 2017 |
| Lumiracoxib | DRB1*15:01 DQB1*06:02 | DILI | Not available | Singer JB et al., 2010 |
| Isoniazid | DQB1*02:01 DDQA1*02:01 RB1*07:01 | DILI | Indian | Sharma et al., 2002 |
| Ximelagatran | | DILI | Caucasian | Alfirevic et al., 2012 |
| Ticlopidine | A*33:03 | Cholestatic hepatitis | Japanese | Hirata K et al.,2008 |
| Carbamazepine | B*15:02 A*31:01 B* 15:11 | SJS/TEN | Taiwanese, Han Chines, Malaysian, Japanese, Thai Caucasian | Hung S.I et al., 2006 Ozeki T et al., 2011 |
| Allopurinol | B*58:01 | SJS/TEN SCARs | Taiwanese, Han Chinese, Korean, European | Hung, S.I et al., 2005 |
| Nevirapine | DRB1*01:01 DRB1*01:02 B*35 | DRESS | Hispanics, African, Asian, European | Yung J et al., 2011 |
| Phenytoin | B*13:01 B*51:01 | SJS/TEN SCARs | Han Chinese, Thai | Chung WH et al., 2014 |
| Lamotrigine | B*15:02 | SJS/TEN | Han Chinese | Yi-WUshi et al.,2011 |
| Sulfamethoxazole | B*14:01 B*35:01 | DRESS SJS/TEN | Eu, Afro American | Yi-JULi et al., 2020 |
| Abacavir | B* 57:01 | HSS | All | Mallal S et al., 2008 |

DILI= Drug-induced liver injury; HSS= Hypersensitivity syndrome; SJS= Stevens Johnson syndrome; TEN= Toxic epidermal necrolysis; SCARS= Sever cutaneous adverse reactions.

## 1.25 IMMUNE EVIDANCE FOR HYPERSENSITIVITY REACTIONS

### 1.25.1. MINOCYCLINE.

Minocycline use is associated with fulminant hepatic failure, induced lupus, and cholesteric hepatitis with autoimmune features (Gough et al., 1996, Crosson and Stillman, 1997, Masson et al., 1996, Maria and Victorino, 1997a). In a systematic review (Garner et al., 2012) 39 randomized control trials were selected and 6013 participants were studied. The Cochrane Skin Group specialized register was involved in the review process (Lawrenson et al., 2000). Search terms used associated with minocycline were; drug hypersensitivity, adverse drug reaction, liver damage, allergy, chronic hepatitis, and toxicity. These reviews concluded that minocycline therapy resulted in different adverse drug reactions such as severe hepatic dysfunction, autoimmune lupus-like syndrome (Lawrenson et al., 2000, Garner et al., 2012), cholestatic hepatitis with autoimmune features and neutropenia (Bhat et al., 1998) and drug reaction with eosinophilia and systemic symptoms (DRESS) syndrome (Ochsendorf, 2010).

### 1.25.2 PHARMACOLOGY OF MINOCYCLINE.

Minocycline is a semi-synthetic derivative of the tetracycline group of antibiotics (Etebu and Arikekpar, 2016). It is classified as a second-generation tetracycline antibiotic and has a significant level of activity against tetracycline sensitive organisms including tetracycline-resistant staphylococci (Macdonald et al., 1973, Minuth et al., 1974). It has an unrivalled position both chemically and biologically due to the exchange of a dimethyl amino group at C7 and the lack of a functional group at C6 (Jonas and Cunha, 1982). It is an amphoteric substance where the conjugated phenolic enone system extends from C- 10 to C-12, which results in a pKa of 7.5, while

the conjugated trione system extending from C-1 to C-3 is nearly acidic. Minocycline is prepared as dehydrated hydrochloride, thus it is strongly acidic and more soluble in polar solvents and water (Barringer et al., 1974). It chelates with metal ions such as Fe+, Mg+ and Ca+ (Brogden et al., 1975).

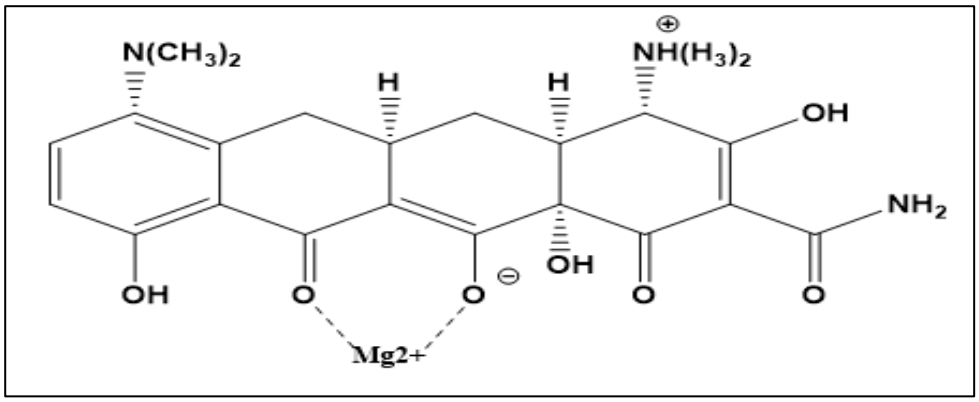



**Figure 1.13. Minocycline (semisynthetic) chelates with divalent and/or trivalent metal ions such as magnesium ($Mg^{2}$+, $Mg^{3}$+), calcium ($Ca^{2}$+, $Ca^{3}$+), and iron ($Fe^{2}$+, $Fe^{3}$+) at the C-11 and C-12 position**. The complex chelates formation are stable and making all tetracyclines including minocycline less effective.

### 1.25.3 ANTI-MICROBIAL ACTION OF MINOCYCLINE.

Minocycline has broad spectrum of antibacterial activity against both aerobic and anaerobic gram-positive and gram-negative bacteria (Nelis and De Leenheer, 1982, Patel et al., 2011). It is similar to other tetracyclines, where it is used in the treatment of respiratory tract infections, skin infections, and infection in the genital and urinary system and used to improve the signs and symptoms of rheumatoid arthritis (Suresh et al., 2004). It is used to treat several bacterial infections such as pneumonia, acne vulgarise (Alarcón, 2000, Rempe et al., 2007, Sweetman, 2009). Minocycline is predominately effective against Propionibacterium more than other tetracyclines (Strauss et al., 2007). For this reason, it is frequently used in the treatment of acne

vulgaris (Abe et al., 2003a). Other microorganisms affected by minocycline broad-spectrum activity are mycoplasma, chlamydia, rickettsia, spirochetes, nocardia, and legionella (Steigbigel et al., 1968).

### 1.25.4 MECHANISM OF ACTION OF MINOCYCLINE

- **INHIBITION OF PROTEIN SYNTHESIS.**

Minocycline, like all other tetracyclines, intercepts organisms through passive diffusion in an energy-dependent transport protein mechanism unique to the bacterial inner cytoplasmic membrane (Sherman et al., 2015). The antimicrobial action of minocycline also targets bacterial ribosomes. Minocycline concentrates intracellularly in susceptible organisms via alteration of the bacterial cytoplasmic membrane and acts by binding reversibly with 30S ribosomal sub-particle. It blocks the binding of aminoacyl-tRNA to the entry point of amino acids in protein synthesis, thus interfering with protein synthesis of bacteria(Buxton and Benet, 2011).

### 1.25.5 PHARMACOKINETICS AND PHARMACODYNAMICS OF MINOCYCLINE.

Minocycline is rapidly and almost completely absorbed in body tissues after oral administration due to high solubility in polar solvents at physiological pH. Minocycline is widely distributed in fluids and tissues of the body (Iwasawa and Kido, 1969, Noble et al., 1967, Kelly and Kanegis, 1967). The absorption of minocycline is about 80% (Brogden et al., 1975). The serum level of the drug within the first hour reaches 95% and the concentration in 2 hours is 2-4 µg/ml (Greig and Scott, 2016). The drug is usually orally administrated in a single dose of 200 mg followed by 100mg twice daily for 7 days. At the end of 24 hours, the level of the drug in serum is 1µg/ml, approximately 76% of minocycline binds with protein thereby the prolonged half-life

of minocycline is 16 - 20 hours. Although minocycline has high protein binding properties, it is highly lipophilic (Macdonald et al., 1973). It rapidly mobilizes from the blood into tissues and reaches target tissues, while also having a superior ability to cross the blood-brain barrier due to its lipophilicity (Plott and Wortzman, 2006, Macdonald et al., 1973).

### 1.25.6 METABOLISM OF MINOCYCLINE.

Hydroxylation and N-demethylation are the major pathways of hepatic minocycline metabolism (Mannargudi et al., 2009, Shu, 1966). However, up to six metabolites of minocycline have been described. The main principle metabolite is 9-hydroxy minocycline (Böcker et al., 1991). 4-epiminocycline is formed as a result of epimerisation of minocycline rather than biotransformation, and the other two metabolites are mono N-demetylated derivatives. Minocycline is also oxidized by myeloperoxidase and HOCL to form protein-reactive metabolites (Udenfriend et al., 1954).

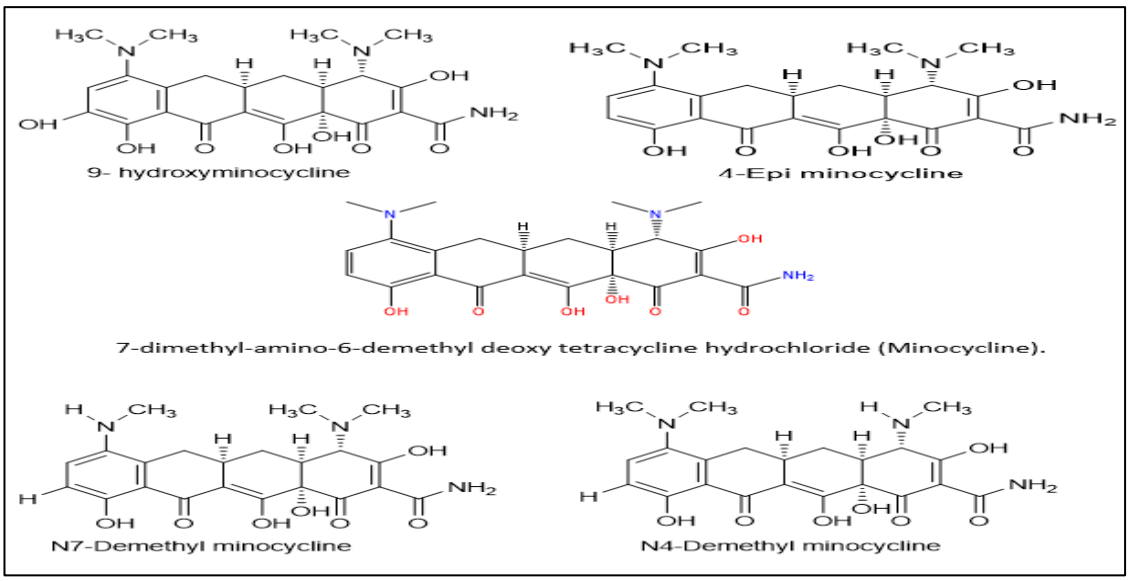



**Figure 1:14. Chemical structures of the major minocycline metabolites.**

### 1.25.7 HUMAN LEUKOCYTE ANTIGEN ASSOCIATIONS WITH MINOCYCLINE-INDUCED DILI.

The HLA-B allele B*35:02 is a rare HLA allele associated with susceptibility to minocycline DILI, which suggests that the drug might interact with this HLA allele to activate patient T-cells. When assessing the HLA types imputed from SNP genotype data in the MHC region, a significant enrichment of the HLA-B*35:02 allele was observed in the cases versus population controls (Urban et al., 2017). The association with the imputed HLA-B*35:02 allele was then confirmed with sequence-based HLA typing (Singer et al., 2010).

### 1.26.1. SULFAMETHOXAZOLE.

Sulfonamide drugs can be classified into two groups; A) antibacterial sulfonamide antibiotics which contain in their chemical structure a five- or six-membered nitrogen-containing ring attached to the N1 nitrogen of the sulfonamide group and an aryl amine group (H2N) at the N4 position of the sulfonamide group (Naisbitt et al., 1999, Brackett et al., 2004, Strom et al., 2003). B) Non- antibacterial sulfonamides do not have an N-containing ring attached to the N1 nitrogen of the sulfonamide group (Knowles et al., 2001, Roth et al., 2018, Verdel et al., 2006). Sulfamethoxazole (SMX) is an antibacterial agent from the sulfonamide group of drugs. It works as a bacteriostatic agent. Sulfamethoxazole is often used with trimethoprim (TMP) as a combination for first-line treatment of urinary tract infections, and it is used in the treatment of pneumocystis carinii pneumonia in patients with HIV infection (Roth et al., 2018, Naisbitt et al., 2001).

### 1.26.2 PHARMACOKINETICS OF SULFAMETHOXAZOLE.

The absorption of sulfamethoxazole is rapid and about 70% of the drug is bound to plasma proteins. The time to reach maximum drug concentration in plasma is 1-4 hours after the drug is taken orally. Sulfamethoxazole is metabolised in the liver by N-acetylation and glucuronidation, and the CYP2C9 enzyme is responsible for the formation of a N4-Hydroxy metabolite (SMX-NHOH) (Cribb and Spielberg, 1990, Cribb and Spielberg, 1992). Autoxidation of the metabolite SMX-NHOH, which circulates in the blood, generates the electrophilic metabolite nitroso sulfamethoxazole (SMX-NO) (Naisbitt et al., 1996, Castrejon et al., 2010).

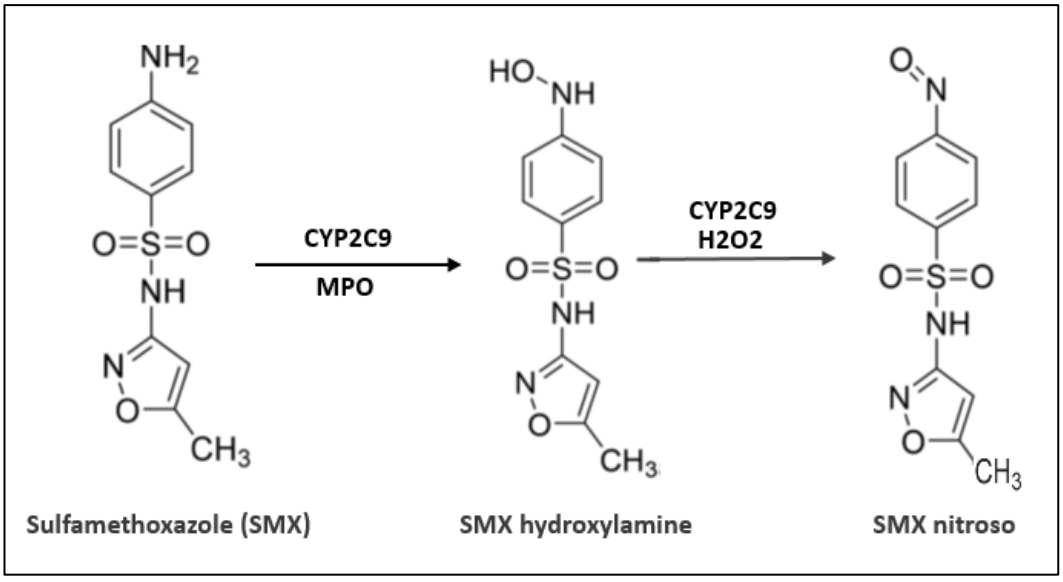



**Figure 1.15. Scheme illustrating the metabolism of sulfamethoxazole involving cytochrome P450- mediated oxidation and formation of the reactive nitroso metabolite.**

### 1.26.3 CLINICAL MANIFESTATIONS OF ADVERSE DRUG REACTIONS TO SULFAMETHOXAZOLE AND ITS METABOLITES.

The most common side effects of SMX are gastrointestinal disturbances such as diarrhea, nausea, stomach upset, and vomiting. The symptoms usually start 7 to 14 days after treatment (Table 1.3). Type 1 hypersensitivity reactions involve immunoglobulin E (IgE) antibodies and clinical symptoms include allergic skin reactions (urticaria and rashes) which occurs within 30 minutes of drug administration, angioedema, and anaphylaxis (Wulf and Matuszewski, 2013). However, more common reactions are type 4 T-cell mediated reactions with a delayed onset. Most studies exploring mechanisms of sulfamethoxazole hypersensitivity have concluded that the formation of chemically reactive metabolites that bind covalently to protein is the initiating event in the development of hypersensitivity reactions (Cribb and Spielberg, 1992, Park et al., 1998, Uetrecht, 1999). These studies largely focus on the direct toxicity of the reactive nitroso metabolite of sulfamethoxazole, which might be involved in stress signalling to the adaptive immune system and help to determine whether drug exposure results in an effector drug-specific T-cell response. In patients with delayed-type cutaneous hypersensitivity, T cells that can be isolated respond to either parent drug or nitroso sulfamethoxazole (Schnyder et al., 2000a, Uetrecht and Naisbitt, 2013). Delayed-type reactions range from mild conditions such as maculopapular exanthema to severe life-threatening conditions such as Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), liver failure and agranulocytosis. Approximately, 30% of patients with the human immunodeficiency virus (HIV) develop allergic reactions due to a treatment combination of sulfamethoxazole and trimethoprim (Naisbitt et al., 2002).

This incidence is approximately 10-fold higher than that seen in the general population.

### 1.26.4 HUMAN LEUKOCYTE ANTIGEN ASSOCIATIONS WITH SULFAMETHOXAZOLE-INDUCED DILI.

Very recently, European and African persons with sulfamethoxazole-induced liver were compared with population controls in HLA sequencing experiments(Li et al., 2020). The analysis found that HLA-B*14:01 was associated with DILI in Europeans, while HLA-B*35:01 may be a risk factor for African people. This data was not available when the studies described below were conducted.

### 1.27.1 ISONICOTINIC ACID HYDRAZIDE.

Isonicotinic acid hydrazide is also known as Isoniazid (INH), is an antibiotic highly effective for the management of tuberculosis (Wang et al., 2016). It is used in combination with rifampicin, pyrazinamide, and either streptomycin or ethambutol (Anti-tuberculosis drugs) when mycobacterium tuberculosis is active (Stuart et al., 2009). Isoniazid is also administrated as prophylactic therapy for; A) human immunodeficiency virus (HIV) patients and purified protein derivative (PPD) reactions. B) People with pulmonary damage on their chest X-ray that is likely to be due to healed tuberculosis. C) People whose PPD reactions convert from negative to positive in two years. Isoniazid has two actions; bacteriostatic and bactericidal. It activity depends on growing mycobacteria (Singh et al., 2008).

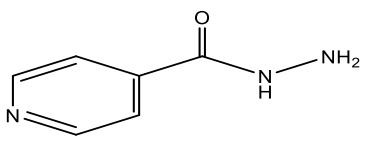



**Figure 1.16. Chemical structure of Isonicotinic acid hydrazide (INH).**

### 1.27.2 ISONIAZID METHABOLISM.

INH is bioactivated directly to a reactive metabolite, this metabolite is thought to contribute to the liver injury detected in some patients. INH-induced liver injury has two phenotypes; mild liver injury, which can resolve during continued therapy, and severe liver injury which is associated with the production of anti-drug/anti-CYP P450 antibodies. Two INH metabolites may be involved in the development of INH-induced liver injury; acetyl hydralazine (AcHz) and hydralazine (Hz) (Metushi et al., 2011).

### 1.27.3 CLINICAL MANIFESTATIONS OF ADVERSE DRUG REACTIONS TO INH.

The side effects of INH-induced liver injury include malaise, fatigue, nausea, fever, vomiting and eosinophilia. ALT and AST elevations are both important markers for hepatocyte injury (Malhame et al., 2016). Jaundice may appear after around 12 weeks of therapy (Chalasani et al., 2015, Black et al., 1975, MADDREY and Boitnott, 1973). INH can also induce lupus-like syndrome (Salazar-Paramo et al., 1992). Deficiency in the production of red blood cells, platelets, and white blood cells by the bone marrow causes aplastic anemia, thrombocytopenia, and agranulocytosis (Young and Maciejewski, 1997). Recently our research group successfully isolated and characterised T-cell clones responsive towards INH from PBMC of patients with liver and skin injury (Usui et al., 2017a).

### 1.28.1 AMOXICILLIN/CLAVULANIC ACID.

Co-amoxiclav (Amox/Clav) is a broad-spectrum antibiotic. It is a combination of amoxicillin and clavulanic acid, which work together to reduce antimicrobial resistance. Amox/Clav as with other β-lactam antibiotics, are frequent causes allergic drug reactions (Chalasani et al., 2008, Horton et al., 1998). The rate of incidence of DILI due to the combination of Amox/Clav is higher (from 3 to 17 per

100,000 prescription) than for amoxicillin alone (Salvo et al., 2007, Rodríguez et al., 1996). Amoxicillin is a semisynthetic penicillin associated with a very low rate of hepatocellular and cholestatic liver injury when used alone, which leads us to indicate that the β- lactamase inhibitor clavulanic acid is responsible for the increased incidence of DILI, with it possibly redirecting the T-cell response towards liver through tissue-specific stress signalling (Chalasani et al., 2008). Recent studies of Amox/Clav DILI performed by the US DILI Network (DILIN), indicated that 11% of all DILI cases out of 1038 were (highly likely) due to Amox/Clav. The Regional Registry of Hepatotoxicity in southern Spain found that Amox/Clav was the drug responsible for the highest number of DILI cases (Delemos et al., 2016, Andrade et al., 2005, Fontana et al., 2005). In Italy, the Italian interregional group of pharmacovigilance (GIF) database for the suspected adverse drug reactions (ADRs), found during the study period (January 1988–June 2005), 1088 reports related to Amox/Clav and 1095 reports related to Amoxicillin alone (Salvo et al., 2007). They concluded that Amox/Clav seems to be associated with a higher risk of (SJS), and hepatitis than Amoxicillin alone. The study also found, there is no significant difference in considered parameters that they considered as a risk factors of DILI such as increasing the age or gender. This conclusion is consistent with the suggestion that there is no correlation between age and gender with susceptibility of benzyl penicillin DILI (Azoury et al., 2018).

### 1.28.2 AMOXICILLIN/ CLAVULANIC ACID MODE OF ACTION.

Clavulanic acid works by inhibiting the beta-lactamase enzyme thereby prolonging the antibacterial effect of amoxicillin (Poirel et al., 2005). Amoxicillin is an effective antibiotic that works by inhibition of cross-linking peptidoglycan (PG) units during

bacteria cell wall synthesis (Josephine et al., 2004). This process leads to either the inhibition of bacterial growth or death (Heesemann, 1993).

### 1.28.3 HUMAN LEUKOCYTE ANTIGEN ASSOCIATIONS WITH CO-AMOXICLAV-INDUCED DILI.

Previous studies reported that Amox/Clav-induced hepatocellular/cholestatic liver injury was associated with expression of several HLA class II alleles. Specifically, the DRB1*1501-DRB5*0101-DQB1*0602 haplotype is found in patients presenting with liver injury. In contrast, other studies found HLA association has a limited impact on the expression of hepatitis. It remains to be proven whether the identified HLA alleles are directly involved in drug binding and the immune response or whether they may link to expression of other HLA alleles through linkage disequilibrium. It is possible that the Amox/Clav antigen binds with a degree of selectivity to a range of HLA class I and class II alleles and expression of these slightly skew susceptibility toward liver injury (Hautekeete et al., 1999, Donaldson et al., 2010, Lucena et al., 2011, Andrade et al., 2004).

## 1.29 AIMS AND OBJECTIVES OF THE THESIS.

The subject of this study was to investigate the immunological aetiology of allergic reactions to drugs from different pharmacological classes, and drugs metabolites by using human lymphocyte in qualitative and quantitive assessments with cellular phenotyping to illustrate the mechanisms of drug antigenicity and immunogenicity. To perform these assessments; we utilised T-cells PBMCs from both healthy donors and clinically diagnosed hypersensitive patients.

The specific aim of my project was to assess whether drug-specific T cell responses are selectively observed in patients presenting with DILI. The project specifically focussed on the following compounds: minocycline, co-amoxiclav, sulfamethoxazole, isoniazid, naproxen and atabecestat. The generation of T cell clones from patients exposed to naproxen and atabecestat allowed me to (1) explore the nature of the drug molecule involved in the adverse event (parent drug or drug metabolite), (2) define pathways of drug presentation to T cells, and (3) characterise the phenotype and function of the responding cells.

# CHAPTER 2: MATERIALS AND METHODS.

## CONTENTS

## 2.1. REAGENTS, CHEMICALS AND DRUGS.

Purified tetanus toxoid (TT) was purchased from Statens serum institute (Copenhagen, Denmark). Phytohaemagglutinin (PHA) was purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Lymphoprep density separation gradient solution was purchased from Axis-Shield (Axis-Sheild, Dundee, UK). Tritiated [3H]-methyl thymidine was purchased from (Moravek California, USA) and from Morovek Bio chemicals (Brea, CA). Dimethyl sulfoxide (DMSO), Hanks balanced salt solution (HBSS) and, bovine serum albumin (BSA), were purchased from Sigma-Aldrich (Dorset, UK). Cyclosporine-A (CSA) was purchased from Fluka Analytical (Dorset, UK). Recombinant human interleukin-2 (IL-2) was purchased from Peprotech (London, UK). Glutaraldehyde and trypan blue solution were purchased from Sigma Aldrich.

## 2.2. TEST KITS.

- ELISpot kits containing coating and detection antibodies along with BCIP/NBT Plus liquid substrate solution were bought from Mabtech (Nacka Strand, Stockholm, Sweden).
- TCR Vβ Repertoire IOTest® beta mark kit was supplied by Beckman Coulter (Indianapolis, USA). Fluorochromes for use in flow cytometry were purchased from BD Biosciences (Oxford, UK).
- Chemokine antibodies were purchased from BD Biosciences (Oxford, UK).
- Anti-human HLA blocking antibodies were bought from Serotec (Kidlington, UK), while the other anti-human HLA-DQ/DR/DP monoclonal blocking antibodies and their respective isotype controls were obtained from BD Biosciences (Oxford, UK). Anti-human HLA-ABC (isotype, IgG1; clone DX17) was purchased them from BD Biosciences (Oxford, UK).

## 2.3. MEDIA AND BUFFERS.

**F1 MEDIUM** for generation of EBV-transformed B-cell lines. The components of this type of media are 500 ml RPMI 1640 supplemented with 10% (V/V) foetal bovine serum (FBS) which was purchased from Invitrogen (Paisley, UK), HEPES (25 mM), L-glutamine (2 mM)**,** streptomycin (0.1 mg/ml) and penicillin (1000 U/ml).

**R9 MEDIUM** for generating bulk T-cell lines and T-cell clones. The components of this type of media are 500 ml RPMI 1640, supplemented with 10% (v/v) human AB serum bought from Innovative Research (Michigan, USA), 25 mM HEPES buffer, 2 mM L-glutamine, 25 µg/ml transferrin, 100 U/ml streptomycin and 100 µg/ml penicillin were purchased from Sigma-Aldrich (Dorset, UK).

**FACS BUFFER FOR CELLULAR PHENOTYPING.** The components of this solution are 500 ml HBSS, 10% (v/v) FBS, 0.2 mg/ml, sodium azide in HBSS.

**PHOSPHATE BUFFER SALINE (PBS).** The components NaCl (80g), $Na_2HPO_4$ (11.6g), $KH_2PO_4$ (2g), KCl (2g) are diluted to 1 litre with distilled water. A further 1:10 dilution was required before use, final pH: 7.0.

**FREEZE MIX.** The component of this preparation is 80% pooled human AB serum/20% DMSO.

## 2.4. PLASTICWARES.

Flasks and Nunc 6-, 24-, 48-, and 96-well plates for cell culture were purchased from Thermo Scientific (Waltham, MA, USA). Printed glass fibre filter mats A for thymidine analysis, sample plastic bags and Meltilex scintillator sheets were bought from Perkin Elmer (Waltham, USA) and Turku (Finland). ELISpot Membrane 96-well multiscreen filter plates were purchased from (Millipore, Watford, UK). Falcon tubes, universal tubes, 15 ml tubes, heparinised vacutainer tubes and safety blood collection set were

purchased from Greiner Bio-One (Kremsmünster, Austria). Cryovials were obtained from Alpha Laboratories (Hampshire, UK) whilst, Nalgene Mr. Sterile and non-sterile FACS Tubes obtained from BD Biosciences (Oxford, UK). Small safety tubes were obtained from Eppendorf (London, UK). Frosties freezer container was purchased from Thermo Scientific (Waltham, MA, USA). Filters 0.22 and 0.45 µm, 50ml syringes and kwills were purchased from Thermo Scientific, (Waltham, MA, USA), Merck Millipore (Burlington, MA, USA), BD Biosciences (Oxford, UK) and VWR (Radnor, PA, USA) respectively. Pasteur pipette were supplied from Elkay (Chicago, IL, USA).

**2.5. DEVICES.**

Laminar air flow system class II (TriMAT2, Contained Air Solutions, Manchester, UK)

Leica DME microscope (Leica Microsystems, Milton Keynes, Germany).

Centrifuge 4000 (Eppendorf).

CO2 Incubator culture from (SANYO).

Micro Beta Trilux 1450 LSC scintillation counting (Perkin Elmer, USA).

Tomtec harvester 96- Mach III M (Receptor technologies, UK).

Fan assisted Oven were filter mats dried (SCIQuip, Shropshire, UK).

Wallac 1495-021 Micro sealer (Perkin Elmer, Waltham, MA, USA).

Dymax 5 pump (Charles Austen Pumps Ltd, Surrey, UK).

Flow cytometry BD FACS CANTO II (BD Bioscience, USA).

Elispot plate reader AID classic (Cadama Medical, Stourbridge, UK).

**2.6. HUMAN SUBJECTS.**

Up to 108 ml of whole blood was collected from HLA typed healthy donors and from patients clinically diagnosed as with liver injury. Blood sample was obtained with full ethical approval and written informed consent was obtained prior to sampling.

## 2.7. T- CELL EXPERIMENTAL PROCEDURES.

### 2.7.1 T-CELL CHARACTRISATION TESTS.

These tests comprise quantitative and qualitative assessments of T-cell responses to drugs. The next two figures represent the methods used throughout this thesis to demonstrate and study of T lymphocytes responses to antigen (Figure2.1), and antigenicity and immunogenicity of drugs (Figure 2.2).

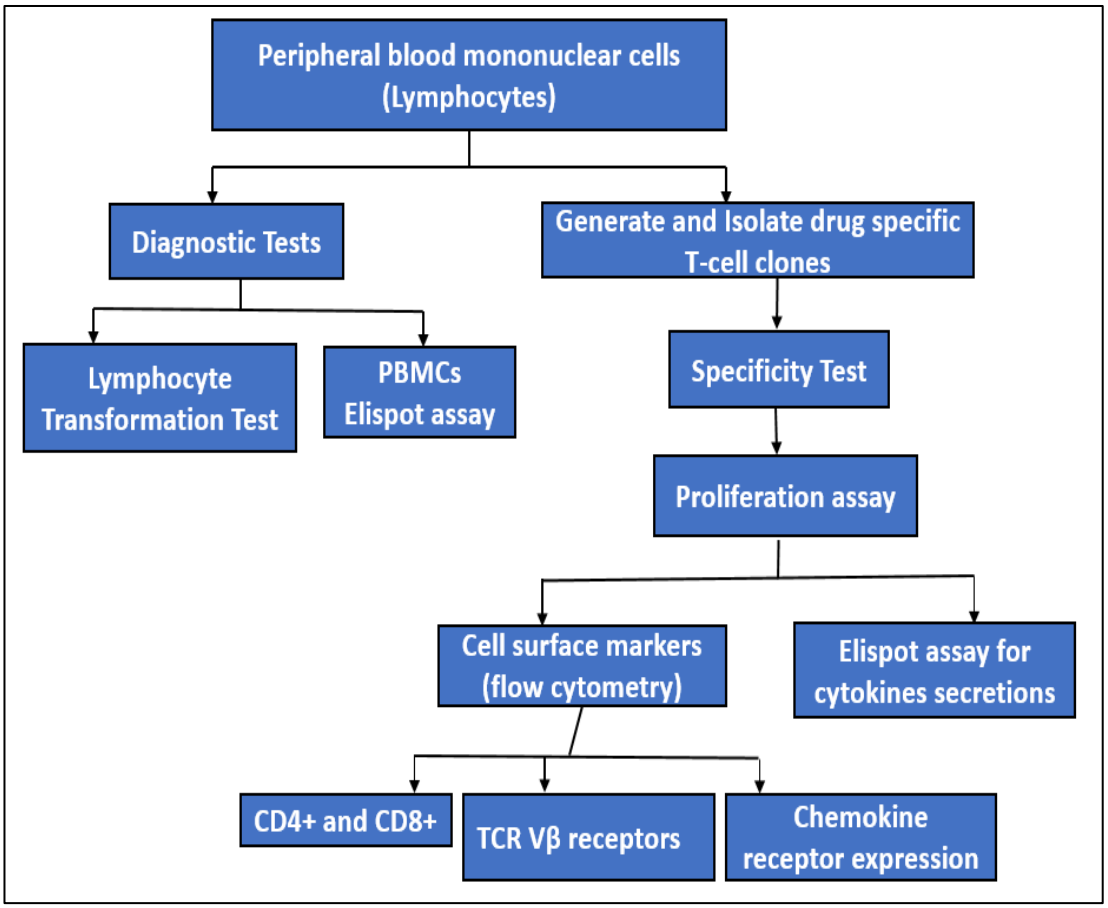



**Figure 2.1. An illustration of the methods used to study T-cell response to drugs/antigen.**

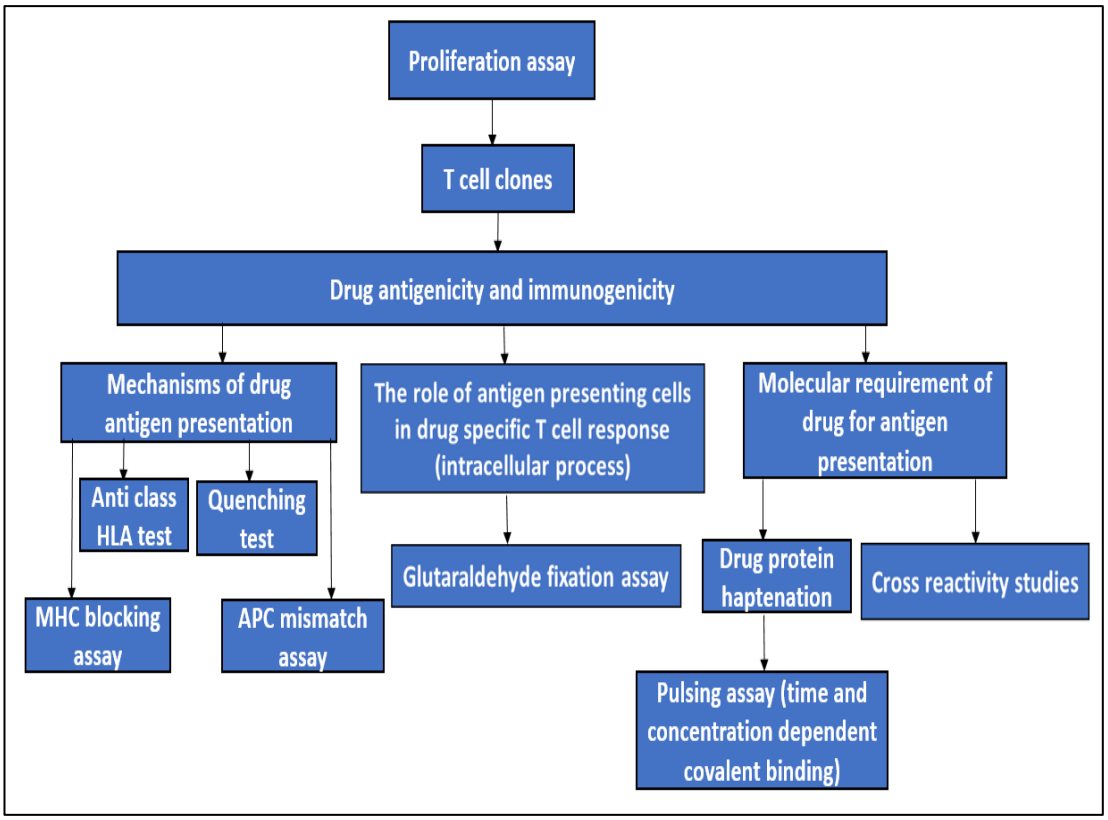



**Figure 2.2. An outline of the methods employed in the study of antigenicity and immunogenicity of the drugs.**

### 2.7.2. ISOLATION OF PBMCS FROM VENOUS BLOOD.

Blood collected in heparinised vacutainer tubes was layered onto Lymphoprep density gradient separation solution (25 ml) (total of 4 tubes per average of 100 ml blood donation) using a 50ml syringe and a kwill. Following the layering tubes were centrifuged at 2000 revolutions per minute (rpm) for 25 minutes with low acceleration and with zero brake to avoid the disruption to density layers formed during the process. The lymphocyte layer (buffy coat) was removed carefully, using a Pasteur pipette to a new 50 ml falcon tube, and washed with Hanks balanced salt solution (HBSS). The pellet (cells) was resuspended in HBSS and spun at 1800 rpm for 15 min with full acceleration and with the brake activated and, the supernatant was discarded. The

cells were transferred into one fresh tube and washed with HBSS to remove any remaining Lymphoprep solution. Cells were centrifuged again at 1500 rpm for 10 minutes; the supernatant was disposed and the cells were resuspended in culture media before counting. To count the cells; a mixture was prepared from a 10 µl cell suspension, 10 µl trypan blue (0.2% w/v) and HBSS to a suitable dilution factor to ensure accurate counting. An aliquot of 10 µl of the mixture was placed on a Neubauer haemocytometer (Sigma-Aldrich) under a Leica DME microscope (Leica Microsystems, Milton Keynes). The viability percentage of the PBMCs was calculated as follows: **percentage viability = viable cells ÷ total cells × 100**. The viability percentage was ≥ 95% for all the PBMC isolations. Cells were used in quantitative and qualitative assessments or frozen for future use.

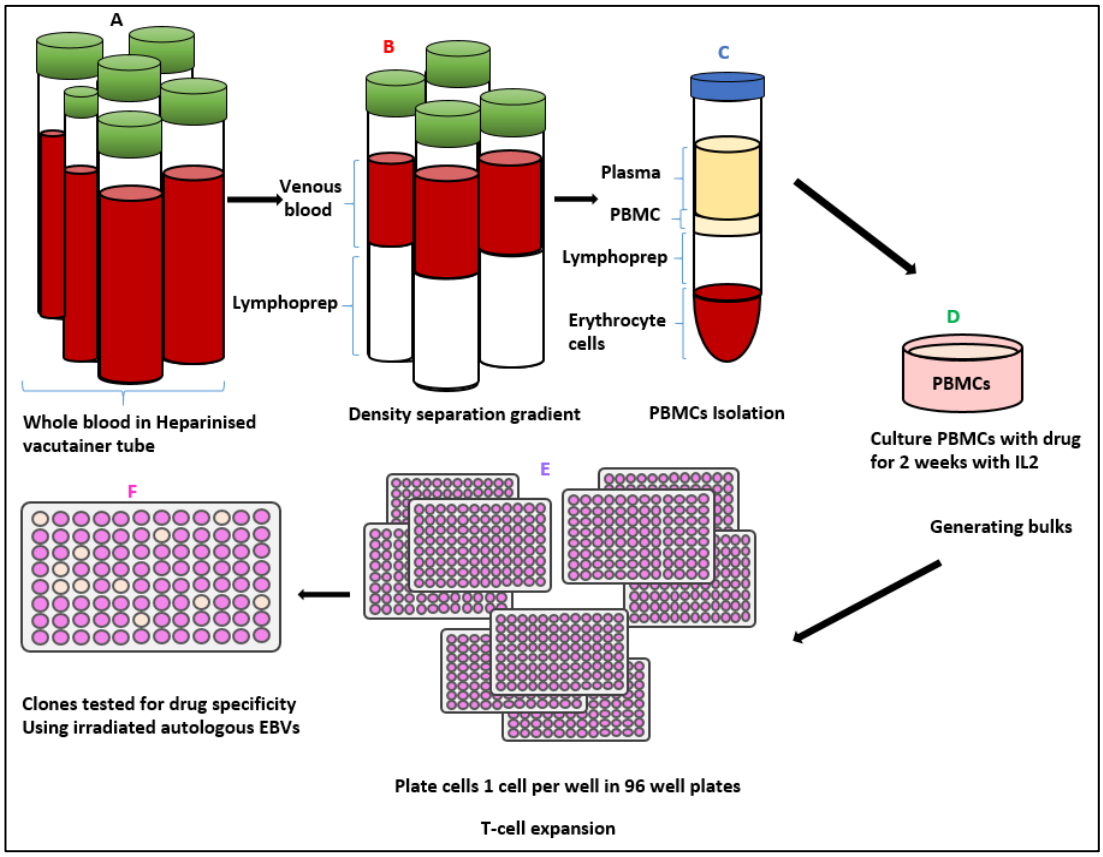



**Figure 2. 3. Isolation of PBMCs.** Workflow steps starting from collecting venous blood to generate drug specific T-cell clones.

### 2.7.3. CRYOPRESERVATION AND THAWING PBMC.

Cryovials were labelled with donor number, cell type, date and the initial of researcher. All cryo preservative steps were carried out in ice. The PBMCs, T-cell clones, were resuspended in R9 at a concentration of 10-20 x $10^6$ cells/ ml and frozen at the concentration of (500 µL; 5 x $10^6$cells / vial). 500 µL of freeze mix (80% human AB serum/ 20 % DMSO) was added slowly drop by drop to 500 µL cells. The cells were mixed gently after each drop of freeze mix to ensure steady mixing of the two solutions and to avoid making bubbles. The contents of 1ml were transferred into 1.8 ml cryovial and frozen in Mr frosty freezing container at -80$^o$C freezer for 24 hours. The following day, frozen PBMCs or T-cell clones were transferred to a -150$^o$C freezer or liquid nitrogen for long term storage.

To thaw cryopreserved PBMCs or T-cell clones cryotubes were transferred from the -150$^o$C freezer to a 37$^o$C water bath and gently agitated until the contents were thawed. The cells were gently mixed with 8 ml R9 after being transferred to a falcon tube. Cells were washed immediately and centrifuged at 1500 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellets were resuspended in 5 ml R9 media.

### 2.7.4. DETECTION OF DRUG SPECIFIC PBMC RESPONSES USING THE LYMPHOCYTE TRANSFORMATION TEST (LTT).

PBMC concentration was adjusted to 1.5x$10^6$cells /ml in R9 media and cells were cultured (100µl; 150,000 cells/well) in triplicate wells per condition in a U-bottom 96-well plate with drug (100µl) at twice the desired concentration due to further dilution in the cell R9 media already added. The first wells were untreated contained R9 media (100µl) used as negative control while the last wells contained tetanus toxoid

resuspended in R9 (0.1µg/ml) or PHA as a positive control. Plates were incubated at 37°C, 5% $CO_2$ for 6 days. The proliferation of the PBMCs was measured using [3H] - thymidine incorporation, which was added for the last 16 hours of the incubation. The cells were harvested onto filter paper and sealed, then read using a Microbeta counter (Scintillation Counter). It is important to note, some drugs and their metabolites such as; SMX and SMX NO were dissolved in DMSO, and their stock solutions were diluted in R9 culture media to the desired concentration. The final concentration of DMSO (< 0.5%) used was non-toxic to the cells and did not *per se* induce proliferation of T-cells.

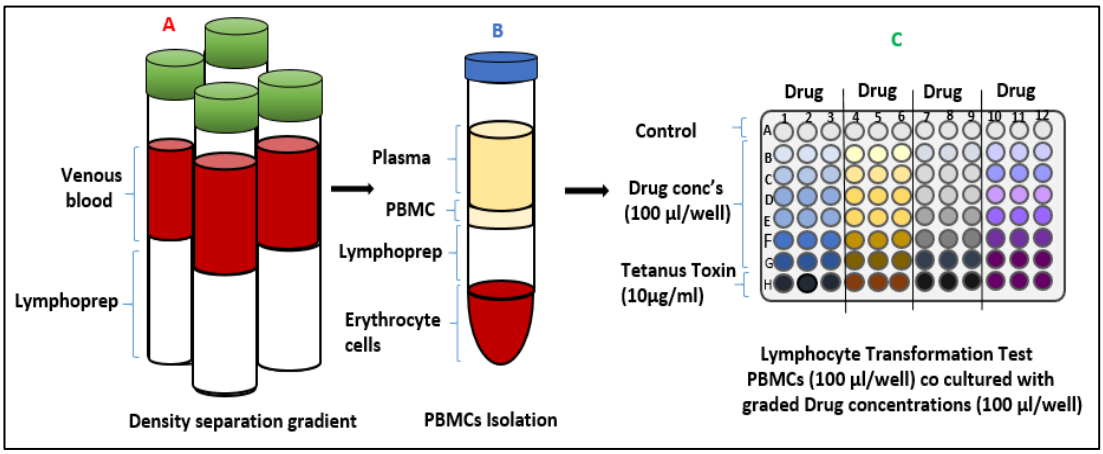



**Figure 2.4. Lymphocyte transformation Test (LTT).** Work flow starting from separation of PBMCs to performing LTT using graded concentrations of the drug. PBMCs with R9 are using as negative control, and Tetanus toxoid with PBMS are using as positive control in this test.

### 2.7.5. GENERATION OF EBV-TRANSFORMED B-CELLS.

The cell line B9-58 acts as a source of Epstein - Barr virus (EBV). Autologous Epstein-Barr virus transformed B-cell lines were generated from (5x $10^6$) PBMCs of hypersensitive patients or healthy donors, and used as antigen presenting cells (APCs) in all assays with T-cell clones. Virus producing B95.8 cells were spun at 1500 rpm for 10 minutes and 8 mL of the supernatant was removed from confluent cultures.

Supernatant was passed through a 0.22µm filter to remove any remaining cellular substance and supplemented with cyclosporine A (CSA; 1µg/mL) to prevent T-lymphocyte proliferation. Cells were incubated at 37°C overnight under an atmosphere of 5% $CO_2$. After the transformation process, cells were centrifuged at 1500 rpm for 5 min, the supernatant was discarded. Cells were resuspended in F1 media and supplemented with (1µg/mL) CSA to prevent the growth of untransformed cells. The cells were then transferred into a 24 well plate at various cell concentrations. Culture media (F1) was replaced twice a week with fresh media supplemented with CSA (1µg/ml) and after 3 weeks cells were fed with F1 without CSA. Once the cells showed growth in clusters without any turbidity in media or any kind of cellular contamination, they were transferred to a cell culture flask with regular feeding twice a week with F1 alone. The cells in this stage were a ready source of immortalised antigen presenting cells.

### 2.7.6. GENERATION OF DRUG SPECIFIC T-CELL LINES AND T-CELL CLONES FROM PBMCS.

#### 2.7.6.1. DRUG SPECIFIC T-CELL ENRICHMENT.

Bulk T-cell lines were generated by incubating PBMCs in medium (2x $10^6$ cells/ml) in the presence of the 2x concentration of the drugs/metabolites in 48 well plates (500µl; 1 x $10^6$ cells/well) for a period of 14 days. On day 6 and 9, cells were fed with culture media suplemented with IL2 (2µl/ml), at 37°C and 5% $CO_2$ to enrich the population of antigen specific T-cells. The optimal dose concentration of the drug was chosen to generate bulks. For drugs that had not been studied in the lab before, the highest non toxic dose concentration was used in addition to half of the

concentrations based on lymphocyte toxicity test results.In separate experiments, the bulk t-cell cultures were generated with multiple concentrations of the drug. T-cells were mixed on day 14 before conducting the serial dilution experiment.

**2.7.6.2. SERIAL DILUTION OF BULKS CULTURES TO GENERATE ANTIGEN SPECIFIC T-CELL CLONES.**

Antigen enriched PBMCs were serially diluted into 3 densities: 0.3 cells /well, 1 cell/well and 3 cells/well after diluting the bulks. A stimulation cocktail was prepared by resuspending irradiated allogenic PBMC as feeder cells ($5x10^4$/well) in 45 ml culture media supplemented with IL2 (2µl/ml) and mitogen phytohaemagglutinin (PHA; 5µg/ml). The cell mixtures were plated into 96 well U- bottom plates (100µl/well) according to the three different concentrations. Plates were incubated at 37°C, 5% $CO_2$, and fed with R9 supplemented with 2µl/ml IL2 at 25µl/well on day 5 and then every 2 days to generate T cell clones. Well growing clones were picked and transferred to a new 96 well plate and expanded into 2 wells and later across 4 wells and fed for another 14 days.

**2.7.7. TESTING CLONES FOR ANTIGEN SPECIFICITY USING [3H]-THYMIDINE INCORPORATION (INITIAL TEST).**

Autologous EBVs transformed B-cells (EBVs) are considered to be ready to use in all characterisation tests, once they are in clumps and in bright yellow media. Autologous EBVs were centrifuged in 1500 rpm for 10 min, the supernatant was removed and the cells suspended in R9 media to a cellular concentration of $0.2x10^6$/ml. Autologous EBVs were irradiated for 20 min to stop the proliferation of the cells but allowing for antigen processing and presentation.

The drug induced proliferation was measured by culturing T-cell clones ($5x10^4$/50 µl/well) with irradiated autologous EBVs ($1x10^4$/50 µl/well) and an optimal concentration of drug (100 µl/well) in two wells, and R9 media (100 µl/well) was added to the other two wells. Plates were incubated for 48 hours in at 37°C, 5% $CO_2$. Tritiated thymidine (0.5 µCi) was added for an additional 16 hours before the tested clones were harvested onto filter mats using a Tomtec harvester 96 – Mach III M connected with a Dymax 5 pump. Filter mats were dried in a fan assisted oven at $80^oC$, and scintillation sheets melted over them. A beta counter (scintillation counting) used to analyse and read the measurements of [3H]-thymidine uptake by T-cell clones. The proliferative responses of the T-cell clones were measured as counts per minute (cpm). Stimulation index (SI) was calculated by dividing the average extent of the proliferation of the drug treated wells by the proliferation of the cells in R9 media. T-cell clones with proliferative SI equal to 1.5 - 2 were considered as antigen specific cell clones.

**2.7.8. RESIMULATION AND EXPANSION OF ANTIGEN SPECIFIC T-CELL CLONES.**

Each drug specific T-cell clone with a proliferative stimulation index of 1.5 - 2 or greater was picked from the remaining 2 untested wells and transferred to sterile FACs tube. Cells were washed in R9 media then centrifuged at 1500 rpm for 5 min. They were then resuspended in 330 µl R9 media in a single well of a 48 well plate. Stimulation cocktail was prepared that contained irradiated allogenic PBMCs (irradiation prevents proliferation while maintaining cell surface marker expression) at 1.5x $10^6$cells / ml, PHA (10µg/mL) and IL-2 (5µL/mL) to expand the cells. Each drug specific T-cell clone was added in 330 µL R9 media to wells containing an equal volume of the restimulation cocktail. The following day, cells were fed with 330 µLR9

supplemented with 2 µL/ml IL2. This process was repeated three times a week after removing 330 µL culture supernatant from the top of each well without disturbing the T-cell clones. When T-cells became confluent cells, the wells were split across multiple wells. The cells were incubated at 37°C, 5% $CO_2$ and maintained in culture for 14 days with regular feeding before using in functional assays.

### 2.7.9. QUANTITITVE ASSESSMENT OF T-CELL CLONE RESPONSES TO DRUGS (SECOND TEST).

Drug-specific T-cell clones ($5x10^4$cells in 50µl/well) were cultured with irradiated autologous EBV ($1x10^4$cells in 50µl/well) and range of drug concentrations including the optimal dose concentration in U bottom 96 well plate in duplicate. 100µl R9 medium was added to untreated wells which was used as a negative control, and 5µg/ml (100 µL/well) PHA was used as positive control. Plates were incubated for 48 hours at 37°C, 5% $CO_2$. [3H]-thymidine (0.5 µCi) was added (25 µl/well) in the last 16 hours of incubation. Cellular proliferation was measured using scintillation counting.

### 2.7.10. ANTIGEN SPECIFICTY AND ANALYSIS OF CYTOKINE SECRETION FROM DRUG SPECIFIC T CELL CLONES VIA ENZYME LINKED IMMUNE ABSORBANT SPOT (ELISPOT) ASSAY.

The qualitative assessment of cytokine and cytolytic molecule secretion upon drug treatment can be achieved by Elispot assay. The membranes at the bottom of the wells of Elispot plates were pre-wetted with 35% ethanol (15 µl/well) for 2 min and washed five times with sterile water (200 µl/well). The stock concentration of IFNY 1mg/ml coating antibody was diluted to 15µg/ml; IL13, IL17 diluted from 0.5mg/ml to 10 µg/ml in HBSS and IL22 is 20 µl/ml capture antibody was used. The Elispot plate

was coated with (100 µl/well) of capture antibodies and incubated overnight at 4$^{o}$C. The following day, the coated filter wells of Elispot plates were subjected to five washes with HBSS (200 µl/well), and blocked with R9 media (200 µl/well) for 30 minutes at room temperature to prohibit non-specific binding. TheR9 media was then removed from the wells. Autologous EBVs were irradiated (1x10$^{4}$/ 50µL/ well) for 20 min, and plated with drug-specific T-cell clones (5x10$^{4}$/50µL/well) to each Elispot plate well in the presence or absence of (100µl/well) the optimal drug concentration. The plate was incubated for 48 hours at 37$^{o}$C; 5% $CO_2$. Following the incubation period, the cells in the wells were discarded and the filter wells were washed five times with PBS. Biotin conjugated detection antibodies specifically adhered to the cytokine bound antibodies, which were diluted according to the manufacturer instructions in PBS containing 0.5% FBS (100µl/well), and incubated for 2 hours at room temperature. The contents of the plate were discarded, and the filter wells were washed five times with (200 µL/well) PBS, followed by addition of Streptavidin-ALP (alkaline phosphatase). Streptavidin-ALP was diluted into 1:10000; 100 µL/well in PBS-0.5% FBS to bind with the biotin part of the antibodies. The plate was incubated for 1 hour at room temperature and was subsequently washed with PBS / 0.5% FBS. Eventually, 100 µL/well of sterile filtered substrate coloured solution (BCIP/NBT) was added and incubated for 15-20 minutes in dark place at room temperature. The plate was extensively washed with cold water to stop colour development. The plate was then left to dry overnight at room temperature. An Elispot (AID classic) reader was used to inspect and count the spots. The presence of spots illustrates that cytokines have been secreted by the cells upon drug treatment.

Analysis of ELISpot results was performed with the positive threshold set as the number of spot-forming units (SFU).

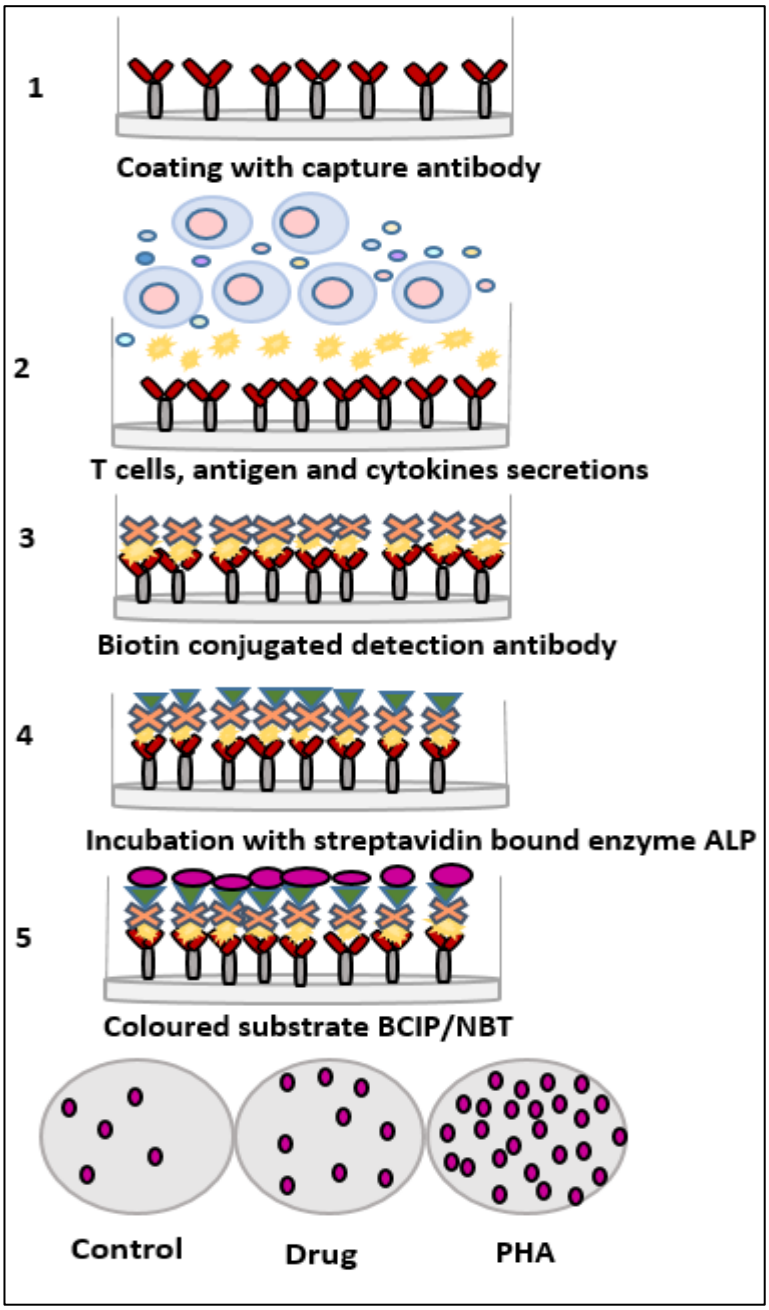



**Figure 2.5. Detection of cytokine secretion using ELISpot assay.**

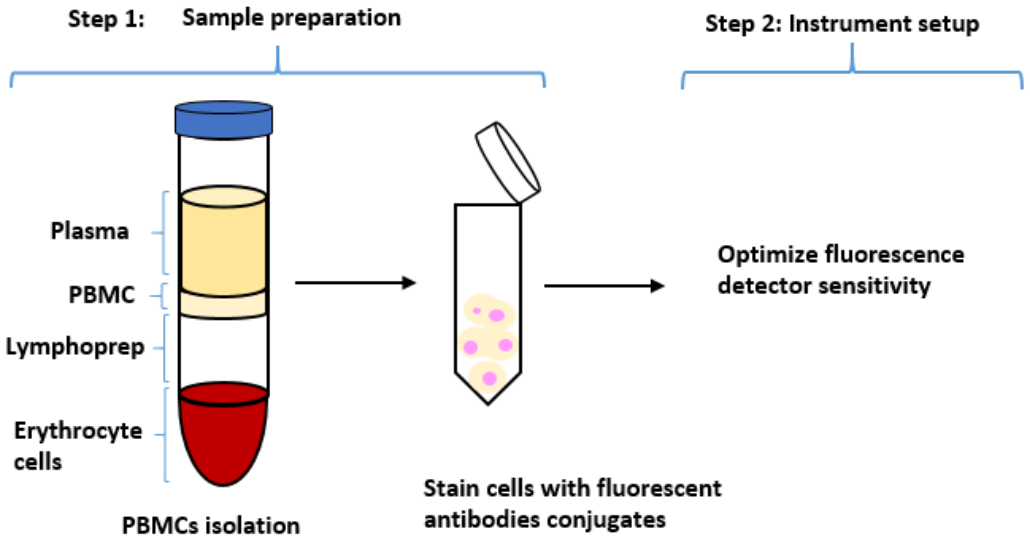
### 2.7.11. FLOW CYTOMETRIC PHENOTYPING OF T-CELLS IN TERMS OF CD4, CD8 CHEMOKINE RECEPTORS EXPRESSION, AND TCR Vβ EXPRESSION.

Flow cytometry was used for detection of specific markers on the surface of the T-cells by staining the cell population with fluorophore-bound antibodies to initially identify CD4 and CD8. T- Cell clones ($5x10^4$ cells; 50µL) were transferred to a FACs tube on ice, stained with 1.5µL of mouse monoclonal FITC conjugated anti-CD4 antibody, and PE conjugated anti-CD8 antibody and incubated at $4^oC$ for 20 minutes. One tube containing unstained or isotype-stained cells was prepared under the same condition and used as a control. Following the incubation, cells were washed with 500 µL of FACs buffer and centrifuged in 1500 rpm for 5 minutes at $4^oC$. Supernatant was then removed and the cells were resuspended in 200 µL FACs buffer for immediate analysis. If the analysis was to be conducted the next day, the cells were resuspended in 0.4% paraformaldehyde instead of FACs buffer and stored at $4^oC$. The expression of cell surface molecules on the T-cell clones was measured by using a FACS CANTOII flow cytometer.

The analysis of chemokine receptor expression was carried out similarly by taking 5 tubes of each T cell clone and using panels of three antibodies with FITC, PE and APC conjugated fluorophores for the assessment of 15 different receptors in a single test. The antibodies from the main subfamilies CXC, CC, CX3C and XC were chosen to analyse the cell surface receptors. T- cell clones ($5x10^4$ cells; 50µL) were transferred to a FACs tubes on ice. Three antibodies from different panels (3 x 3µl) were added to the cells in FACs tubes, and incubated at $4^oC$ for 20 minutes. Following the incubation period, cells were washed with FACS Buffer (500 µL / tube) and the supernatant was discarded. The cells were then resuspended in 200 µL / tube FACs

buffer. Chemokine receptor expression was measured by flow cytometry. The expression was presented as mean fluorescence intensity of the whole population of each clone. The fluorescence intensity indicates how mush photon is emitted and the extent of the emission depends on the concentration of the excited fluorophore. Mean Fluorescence index= Fluorescence intensity of stained cells/ Fluorescence intensity of unstained cells.

Analysis of TCR Vβ expression was performed where, the total of 9 FACs tube were used, one of them was used to gate the T cell population during flow cytometry, and the other 8 contained were required for TCR Vβ analysis. Each TCR Vβ antibody cocktail (FITC, PE, FITC/PE) was designed to investigate three TCRs, twenty-four staining antibodies in total. 50µL T cell suspension was pipetted into each tube and each TCR Vβ antibody (3 µL) cocktail was added individually to the respective eight tubes. Tubes were incubated at room temperature for 20 minutes, washed with FACS buffer (500 µL / tube) to remove any unbound antibodies. The TCR Vβ of the T cell clones was analysed by using flow cytometry.



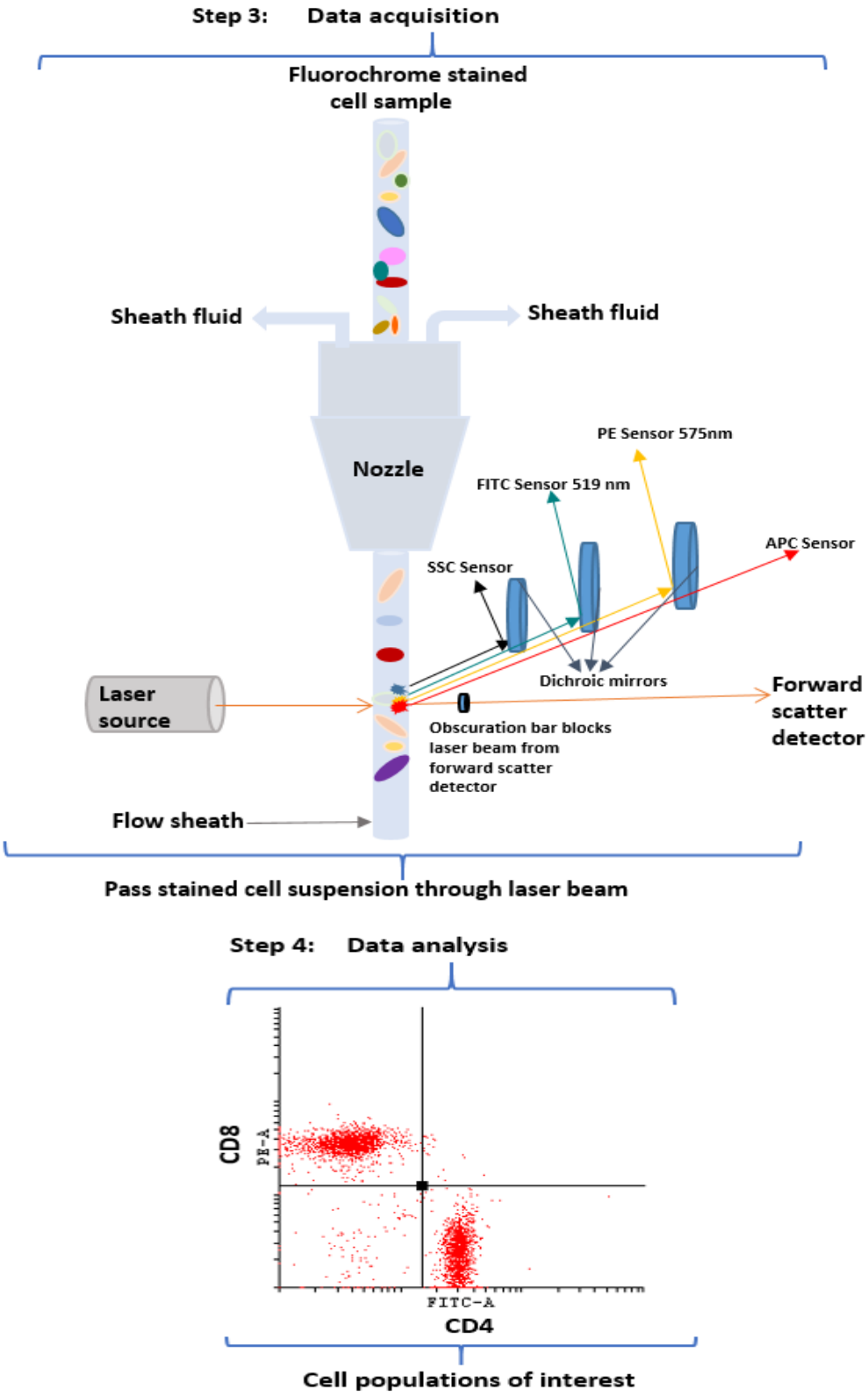



**Figure 2. 6. Four steps to elucidate the cellular phenotype of T-cell clones using flow cytometry. 1)** Isolation of the PBMCs and the cell population is stained with florescent antibodies. Multiple markers are used in a single sample, and the individual fluorochrome is associated with different light wavelengths. **2)** Instrument setup according to the manufacturer instructions. **3)** Cells are passed through the flow cell tube in fast flowing sheath fluid, where each single cell passes through the channel of the laser beam. Fluorochromes bound to markers on the cell surface and emit light at their specific

wavelengths, and this is detected with different sensors (SSC, FITC, PE and APC sensors). The side scatter (SSC) sensor provides information based on the cell size and granularity, while forward scatter (FSC) sensor is responsible for characterisation of the cell population. **4)** The analysis of the T-cell population (lymphocytes) is gated on a dot plot.

### 2.7.12. ACTIVIATION DRUG RESPONSIVE T CELL CLONES IN THE ABSENCE OF APC.

The dependence on antigen presenting cells for activation of drug specific T-cell clones was evaluated by co culture of T-cell clones ($5\times10^4$ cells, 50 µL) and drug in presence and absence of irradiated autologous EBV-transformed B-cells ($1x10^4$cells, 50 µL). Assays were conducted in a 96-well U-bottom microplate using T-lymphocyte culture medium as negative control in a total volume 200 µL. The plates were incubated at (37°C, 5% CO2) for 48 hours. Control wells without APCs were also added to measure possible 'self-presentation' which may occur with T-cells in the presence of the optimal drug concentration. Cellular responses were quantified via either analysis of proliferation via scintillation counting or via analysis of cytokine secretion using the Elispot assay.

### 2.7.13. ANALYSIS OF T CELL CROSS REACTIVITY TEST WITH DIFFERENT CONCENTRATIONS OF DRUGS / METABOLITES.

Qualitative assessment of T cell proliferation and cross reactivity was performed through the general protocol of T-cell proliferation assay by uptake of [3H] – thymidine while cytokine secretion was measured using Elispot. The T-cell clones were co cultured with the different concentrations of drugs, drug metabolites and structurally related drugs from the same or different pharmacological class, in the presence of irradiated autologous EBVs.

### 2.7.14. ASSESSMENT OF PATHWAYS OF ANTIGEN PROCESSING DURING THE ACTIVATION OF THE DRUG T CELL CLONES.

The assessment of antigen presenting cell processing was carried out by stimulation of the specific T cell clones by pulsing APCs with drug and by fixed APCs prior to addition to T-cell assays.

#### 2.7.14.1. PULSING OF ANTIGEN PRESENTING CELL.

The pulsing assay determines the importance of protein binding in the T-cell response. Qualitative assessment of T cell proliferation was performed through the general protocol of T-cell proliferation assay by uptake of [3H] – thymidine while cytokine secretion was measured using Elispot. Autologous EBV- transformed B-cells (2 x$10^6$/ml) were centrifuged to remove F1 medium and R9 was added. The EBV suspension was cultured with the desired concentration of drug antigen for different time periods between 1-16 hours (37°C, 5% $CO_2$), and with R9 media in control wells. EBVs were washed extensively with R9 to remove any free drug. Non-pulsed fresh EBVs were irradiated alongside EBVs pulsed with drug. Drug-specific T cell clones were co-cultured with non-pulsed fresh irradiated EBVs (in the presence and absence of soluble drug as a control) and with drug pulsed irradiated EBVs for 48h (37 °C, 5% CO2). Described-cell proliferative responses and cytokine release were then measured as described above. T-cell activation with antigen pulsed EBVs is indicative of protein adduct formation being required for T-cell activation, while the absence of T cell responses in antigen-pulsed wells indicates that the parent drug may be binding directly to surface HLA molecules.

**2.7.14.2. FIXATION OF ANTIGEN PRESENTING CELLS.**

Autologous EBVs ($2x10^6$/ml) were prepared, washed in HBSS and resuspended in 2 ml HBSS. Glutaraldehyde (0.05%, 1ul/ml) was added to cells and agitated for 30 seconds. Glycine (2ml, 1mM) was quickly added to stop the reaction and the cells were agitated for 45 seconds. Fixed cells were washed and suspended in 5ml R9 media. Glutaraldehyde fixed autologous EBVs ($1x10^4$cells; 50 µl) were then added to T-cell clones ($5x10^4$cells; 50 µl) in the presence or absence of drug. Plates were incubated for 48 hours at (37 °C, 5% $CO_2$). Proliferative responses were measured using [3H]-thymidine incorporation via scintillation counting as described. Alternatively, cytokine secretion was measured using Elispot. The inhibition of T cell responses in the presence of glutaraldehyde fixed EBVs indicates that antigen processing is needed for T-cell activation.

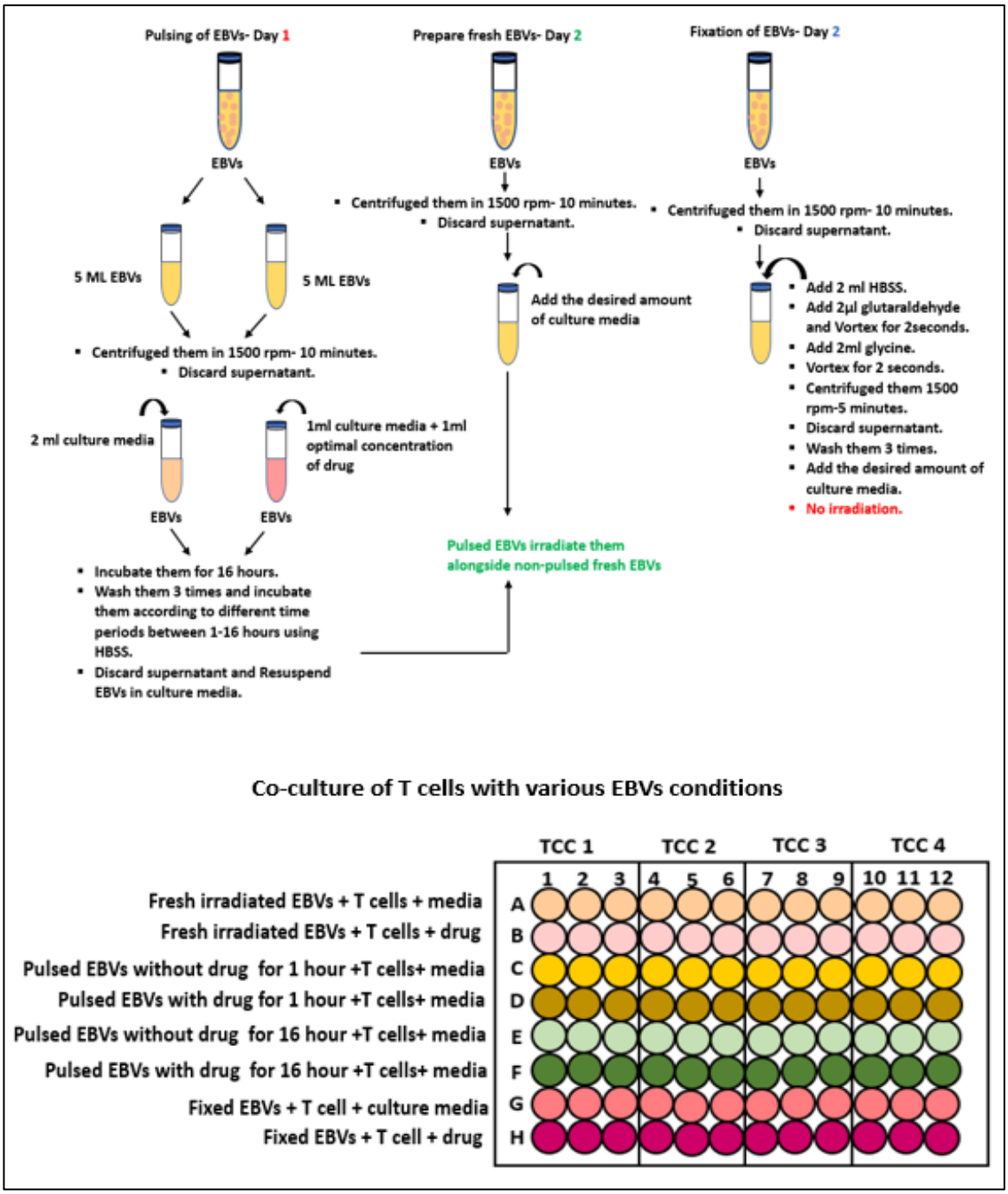



**Figure 2.7. flow diagram to elucidate pulsing and fixation of antigen presenting cell assays.** **In day 1,** pulsing EBVs steps, EBV-transformed B cells were treated with the drug for different periods of time and wash them repeatedly to remove free drug prior to culturing with T cells. **In day 2,** unpulsed EBVs were treated with the desired concentration of the drug. **In day 2,** T-cell clones were incubated with the optimal drug concentration in the presence of glutaraldehyde- fixed autologous EBVs. The positive control used in this assay is the study drug. 3H-Thymidine was added for the final 16 hours of incubation and the activation of T cell clones was measured using scintillation counting. Alternatively, cytokine secretion was measured using ELIspot.

### 2.7.15. MHC RESTRICTION OF T-CELLS.

MHC restriction assays were carried out to determine whether the antigen presentation to drug specific T-cell clones was dependent on MHC class I or class II. Anti-human HLA-ABC and HLA-DR/DQ/DP monoclonal antibodies were used to block MHC antigen interactions by binding with MHCI and MHCII molecules and preventing T cell activation. Autologous EBVs ($1x10^4$, 50 µL) were irradiated and incubated with either MHC I or MHC II blocking antibodies at a concentration 5 µg/mL and their respective isotype controls at $37^oC$, 5% $CO_2$ for 30 minutes. Drug specific T cell clones ($5x10^4$cells; 50 µl) were co cultured with the EBVs ($1x10^4$, 50 µL) with or without drug (optimal concentration) for 48 hrs. The T-cell response were measured through characterization of proliferation or cytokine release. Similar MHC restriction experiments were performed for the subclasses of MHCII (HLA-DQ/ DR/ DP).

## 2.8. STATISTICAL ANALYSIS.

Stimulation index (SI) was used to measure the proliferation of T-cells. If SI is 1.5 or greater, it would be considered as an antigen specific response. The response would then be confirmed in multiple repetitive experiments. Stimulation index was calculated as (average of treated conditions/ average of untreated control). Also, it is important to know SI does not consider standard deviation within mean values and is not a useful measure of variability within data replicates. Standard error of the mean (SEM) was used to measure how far the sample mean of the data is likely to be from the true population mean. The (SEM) was calculated in most statistical analysis of the experiments. The statistical analysis for detection of proliferative responses and cytokine release was performed utilising Mann–Whitney test and or student T-test, where appropriate.

# CHAPTER 3: CHARACTRISATION OF THE DRUG DERIVATIVE THAT STIMULATES T-CELL RESPONSES IN PATIENTS WITH NAPROXEN-INDUCED LIVER INJURY.

## CONTENTS

## 3.1 INTRODUCTION

Naproxen ((+) -(S)-2-(6-methoxy-2-naphthyl) propionic acid)), is a propionic acid derivative which is of the 2- aryl acetic acid class (Kelly et al., 1989, DeArmond et al., 1995). It is among a group of drugs classified as non-steroidal anti-inflammatory drugs (NSAIDs) and is stereo-chemically pure when compared with the other drugs from the same group (Davies and Anderson, 1997). It is also the only NSAID presently marketed as a single enantiomer.

Naproxen (Nap) is one of the most commonly prescribed over-the-counter drugs. It has analgesic and anti-inflammatory properties which have been widely used in the treatment of acute and chronic rheumatoid arthritis, osteoarthritis, acute gout, ankylosing spondylitis, and other inflammatory diseases (Davies and Anderson, 1997).

Hepatic injury is one of the possible side effects associated with Nap therapy (Victorino et al., 1980). Frenger and Morbach reported two rheumatic cases where jaundice appeared with slight and transient changes in serum transaminase levels during the treatment period with naproxen (Frenger and Morbach, 1973). Bass, Law, and Knight reported that Nap therapy caused jaundice in one patient with mild back pain history and spinal osteoarthrosis treated with Nap. Victorino and her colleagues showed that the clinical and biochemical changes of Nap-induced liver injury were compatible with the histological findings after liver biopsy (Bass, 1974, Law and Knight, 1976, Victorino et al., 1980).

The modality of Nap-induced liver injury varies between mild elevations of liver enzymes to severe hepatic failure (Ali et al., 2011) with enzyme elevations ranging from hepatocellular to cholestatic injury (D'Amore et al., 1996, Demirag et al., 2007).

Patients with liver injury report an elevation in liver enzymes over 1-6 weeks after commencing therapy.

Naproxen undergoes extensive hepatic metabolism with the cytochrome P450 isoforms CYP1A2, CYP2C8, and CYP2C9 catalysing the formation of 6-O-desmethylnaproxen (Falany et al., 2005a, Bowalgaha et al., 2005). Both this primary metabolite and the parent drug (Figure 3.1) undergo phase II glucuronidation (Skonberg et al., 2008), with 95% of an ingested dose recovered in a conjugated form in urine (Falany et al., 2005b). Acyl glucuronidation is a common metabolic route for carboxylic acid NSAIDs (e.g., naproxen, ibuprofen, diclofenac, and lumiracoxib) and although a pivotal detoxification pathway, acyl glucuronides are unstable and undergo hydrolysis and acyl migration (Van Vleet et al., 2017, Bowalgaha et al., 2005). As a consequence of this, NSAIDs bind covalently to cellular macromolecules such as albumin in the liver, with examples including diclofenac, zomepirac, benoxaprofen and ibuprofen (Iwaki et al., 1999, Bischer et al., 1995, Presle et al., 1996, Seitz et al., 1998, Bailey and Dickinson, 1999, Qiu et al., 1998). Two discrete adducts are formed; (i) transacylation adducts through direct modification of nucleophilic amino acids and (ii) a Schiff base adduct retaining the glucuronide structure through the reaction of isomeric glucuronides with amine nucleophiles followed by Amadori rearrangement (Hammond et al., 2014). Benet et al (Benet et al., 1993) demonstrated a relationship between the first-order degradation rate of acyl glucuronide metabolites to a plethora of drugs and covalent binding to albumin, suggesting that acyl glucuronide adduction of protein may be responsible for the liver reactions associated with NSAIDs.

Although the involvement of the adaptive immune system in the mediation of naproxen-induced liver injury has not been explored, drug-specific T cells have been implicated in hepatic adverse events associated with a number of therapeutics including flucloxacillin (Monshi et al., 2013), amoxicillin-clavulanate (Lucena et al., 2011) and anti-tuberculous drugs (Usui et al., 2017a). Drug-specific T cells are detected in peripheral blood of patients with tissue injury, but not drug-exposed healthy controls, and the result is T cell proliferation and the secretion of effector molecules such as perforin and granzyme B. In the case of flucloxacillin, the drug-derived antigen interacts with a degree of selectivity with the HLA molecule HLA-B*57:01 identified in genetic studies (Daly et al., 2009, Monshi et al., 2013, Wuillemin et al., 2014). Furthermore, effector T cells have been shown to infiltrate the liver of patients with sulfasalazine- (Mennicke et al., 2009), flucloxacillin- (Wuillemin et al., 2013) and atabecestat-induced liver injury (De Jonghe et al., 2020), indicating that they play a direct role in the tissue damage.

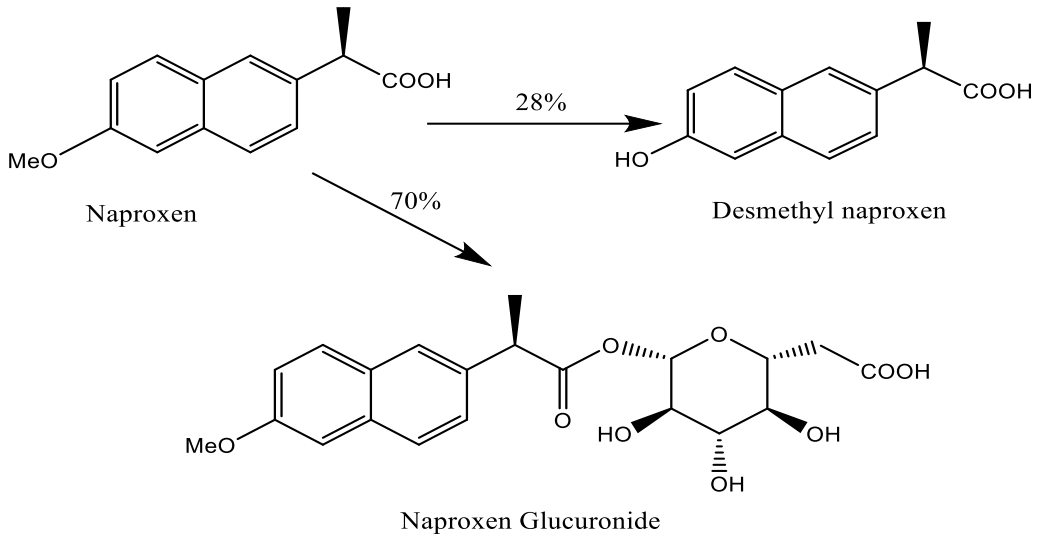



**Figure 3.1. Naproxen metabolism.** The chemical structures of the parent drug naproxen (Nap), and its major metabolites. Adapted from (Bougie and Aster, 2001).

### 3.2 CHAPTER AIMS.

To investigate the role of the adaptive immune system in the liver reactions observed in patients exposed to naproxen through the generation of T cell clones, we characterised (1) the major antigenic determinants, (2) pathways of naproxen metabolite presentation to T-cells and (3) the phenotype of the responding clones.

### 3.3 METHODS

The following methods have been used in this chapter which are prescribed in more detail in Materials and Methods Chapter 2. These include:

- LYMPHOCYTE TRANSFORMATION TEST (2.7.4).
- T-CELL CLONING (2.7.5and 2.7.6).
- QUANTITIVE AND QUALITATIVE ASSESSMENT OF CLONE RESPONSE AND REACTIVITY TO DRUGS (2.7.6.1 and 2.7.6.2).
- ELISPOT ASSAY (2.7.10).
    - CYTOKINE SECRETION PROFILE.
- FLOW CYTOMETRIC PHENOTYPING OF T-CELLS (2.7.11).
    - CD4 AND CD8.
    - CHEMOKINE RECEPTORS.
    - TCR Vβ MULTIPLE CHAINS EXPRESSION.
- ASSESSMENT OF MHC RESTRICTION OF ANTIGEN PRESENTING CELLS.
- INVESTIGATION OF ANTIGEN PROCESSING OF T-CELLS.
    - PULSING ASSAY (2.7.14.1).
    - GLUTARALDEHYDE FIXATION ASSAY (2.7.14.2).

### 3.4 STATISTICAL ANALYSIS.

Mean values and standard deviations were calculated, and statistical analysis was performed using the student's t-test unless stated otherwise.

## 3.5 RESULTS

### 3.5.1 LYMPHOCYTE TRANSFORMATION TEST (LTT) WITH IDILI PATIENT PBMCS.

Three subjects aged 30 to 57 years (confirmed cases of naproxen iDILI) were enrolled in the study. PBMC were isolated from fresh blood samples and stimulated *in vitro* with graduated concentrations of naproxen (50- 400µM), desmethyl naproxen (50-600 µM), and naproxen acyl glucuronide (50- 400µM). The clinical details of patients are described in (Table 3.1).

**Table 3.1: Clinical Details of Patients with Naproxen Induced Liver Injury**

| Patient | Gender | Age | Peak liver function tests | | | Underlying disease | Details of reaction | Subject |
|---|---|---|---|---|---|---|---|---|
| | | | ALT* (IU/L) | ALP* (IU/L) | Bilirubin* (Umol/L) | | | |
| 1 | Female | 57 | 1286 | 912 | 98 | Cholecystitis | Jaundice cholelithiasis | DILI |
| 2 | Female | 31 | 535 | 146 | 0.99 | Polyarthritis | Jaundice cholelithiasis | DILI |
| 3 | Female | 51 | 596 | 268 | 47 | Cholecystitis | Jaundice, cholelithiasis | DILI |

**the limit of normal range: ALT (2-53 IU/L); ALP (40-130 IU/L); bilirubin (3-17Umol/L)*

Dose-dependent proliferative responses with stimulation index (SI) of 2 or above was seen with naproxen with PBMC from donor 1, while with naproxen acyl glucuronide with PBMC from donors 1, 2 and 3 was not detected (Figure 3.2A). Furthermore, the proliferation was observed with graded concentrations of desmethyl naproxen with PBMC from donor 1 and 3 (Figure 3.2A). Elispot was carried out to assay the cytokines (IL22, IFNγ) secreted from patient PBMC after drug (metabolite) treatment. Non-significant secretions for IL22 by PBMCS in response to graded concentrations of Nap, DM nap, and AG nap was observed (data not shown), while the significant secretion for IFNγ by PBMCS in response to different concentrations of desmethyl naproxen (DM nap) was seen (Figure 3.2B).

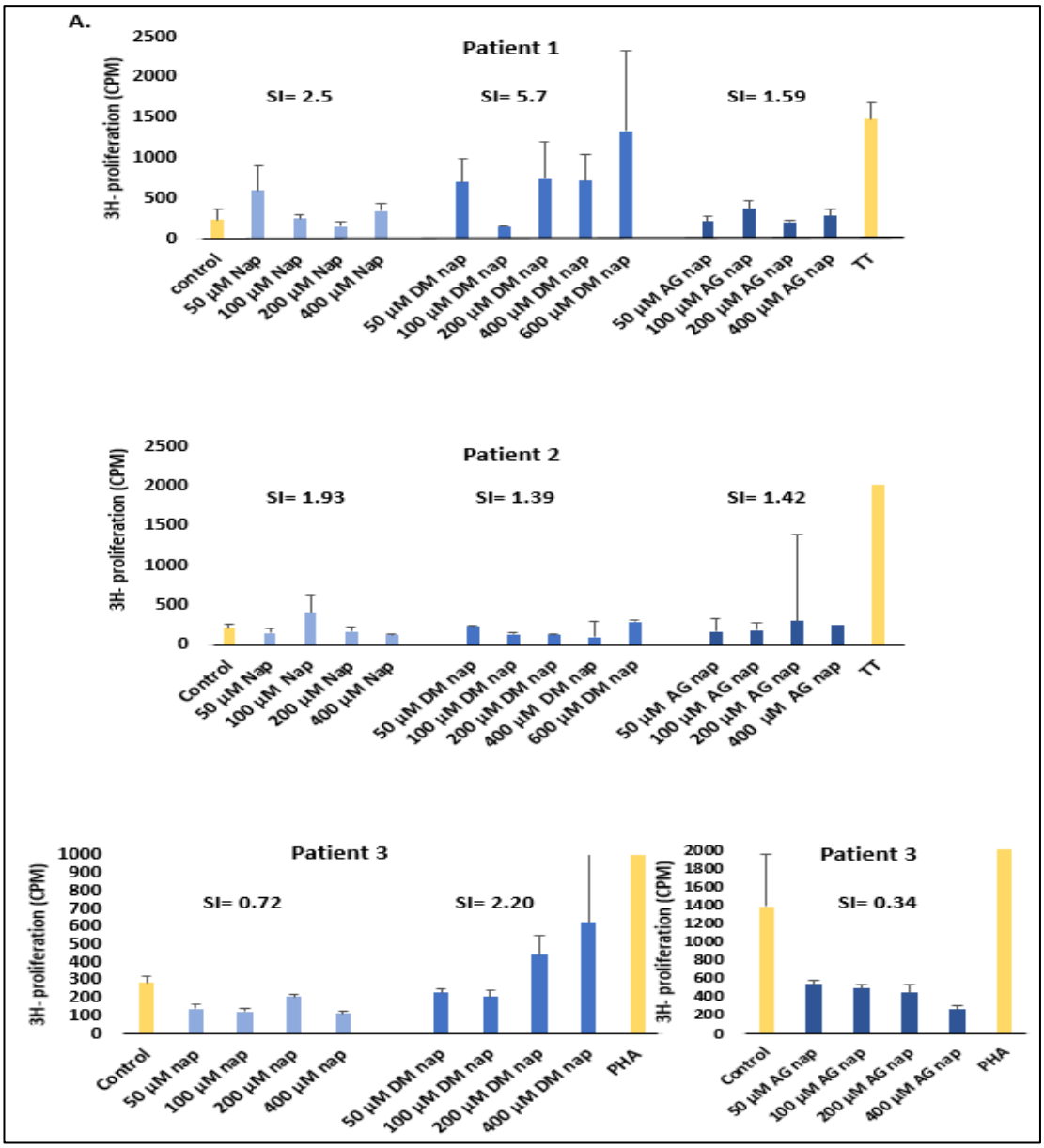



**Figure 3.2. Activation of PBMCs of naproxen iDILI patients with naproxen (Nap), desmethyl naproxen (DM nap) and naproxen acyl glucuronide (AG nap)**. **A)** LTTs were performed on PBMCs of three naproxen hypersensitive patients. PBMCs were cultured in triplicate (1.5x $10^5$ cells per well/100 µl) and incubated with graduated concentrations of Nap (50-400µM), DM nap (50-600µM)and AG nap(50-400µM)in 96 well plate and incubated for 6 days (37°C, 5% $CO_2$). Tetanus toxoid (TT) (5 µg/ml) and culture medium were used as positive

and negative controls, respectively. Thymidine (0.5µCi 3H) was added for the final 16 hours of incubation. T- cells proliferation with 3H-thymidine incorporation evaluated by using scintillation counting. Data are presented as mean ±SD of drug metabolite bound to albumin. T-tests were used for comparisons at the same time points. Lymphocytes from NAP DILI patient 1 were incubated in an ELIspot plate pre-coated for IL22 and IFN-γ with NAP, DM nap, and AG nap a period of 2 days. Phytohemagglutinin (PHA) (10 µg/mL) and medium were used as positive and negative controls, respectively. The ELIspot plates were developed following the manufacturer's instructions and counted using ELIspot AID reader. **B)** ELIspot plate pre-coated for IFN-γ with DM nap treated wells.

### 3.5.2 GENERATION OF DESMETHYL NAPROXEN-RESPONSIVE T-CELL CLONES.

To further investigate DILI patient T cell responses to Nap and its metabolites, T- cell clones were generated from naproxen, desmethyl naproxen, and naproxen acyl glucuronide T cell lines and tested for drug specificity. A total of 34 DM nap responsive T-cell clones were detected on initial testing (DM nap 200 µM, duplicate cultures) from a total of 750 DILI patient 1 T cell clones tested (Figure 3.3A). Eleven of these T cell clones displayed dose-dependent proliferative responses in the presence of DM nap and were expanded for more detailed mechanistic investigations (8 of the clones are shown in Figure 3.3C). Far fewer DILI patient 1 T cell clones displayed proliferative responses to nap, AG nap and none of these were deemed drug-responsive in dose-response studies. From a total of 211 T cell clones generated from DILI patient 2 DM nap T cell lines, 1 was stimulated to proliferate in the presence of DM nap on initial testing and repeat dose-response studies (Figure 3.3B). Nap and AG nap responsive T cell clones were not detected in dose-response studies. Drug-responsive T cell clones were not detected from DILI patient 3.

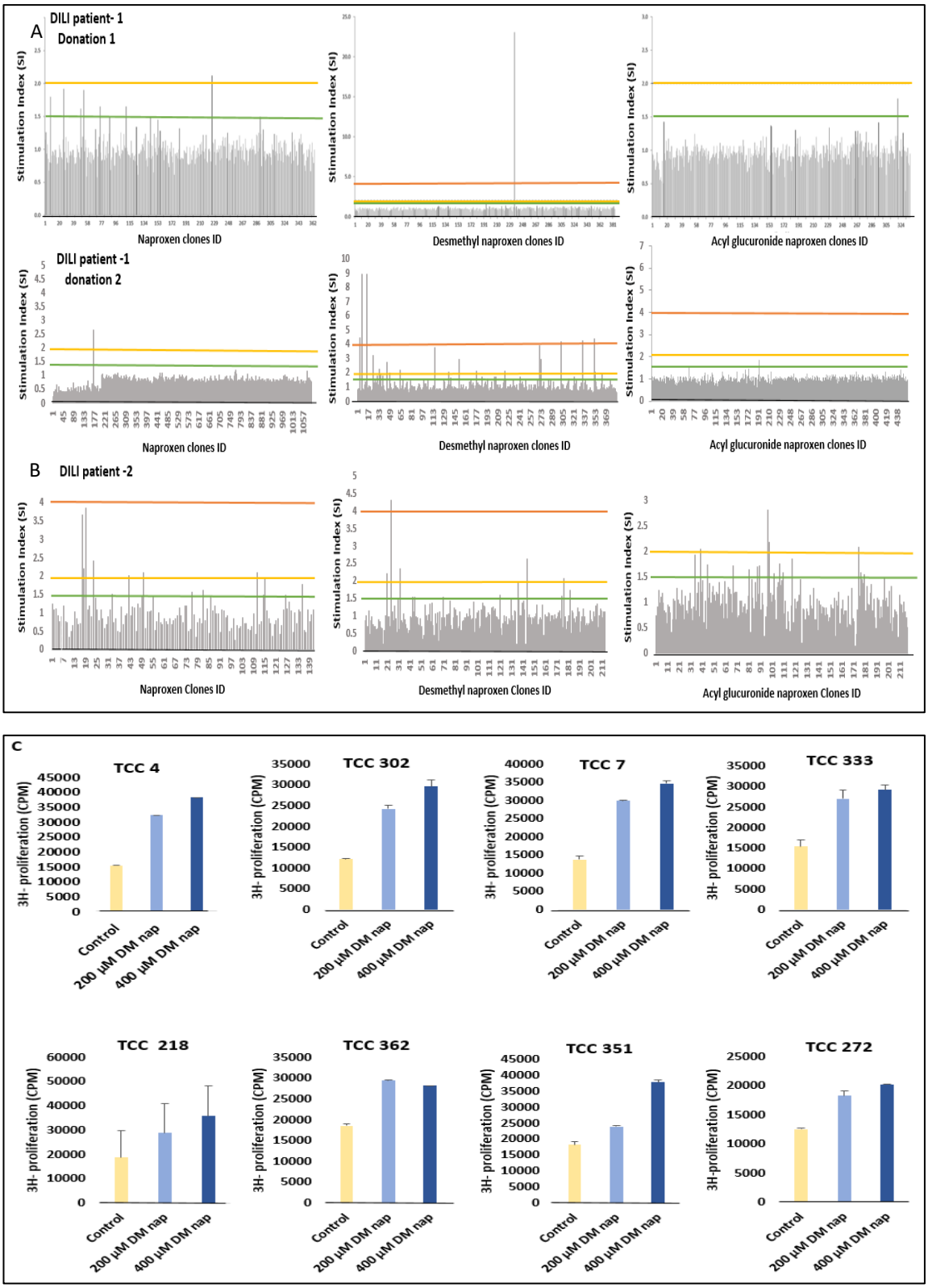
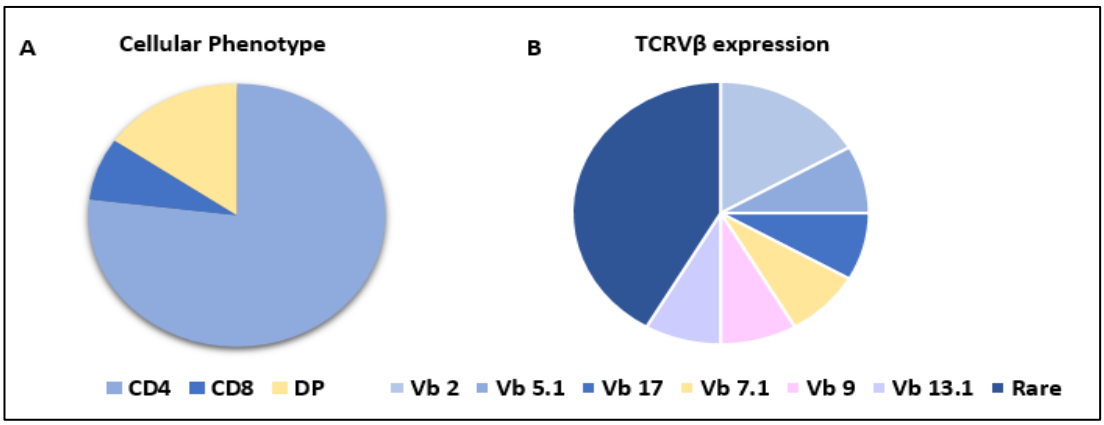



**Figure 3.3. Initial drug specificity testing of Cell clones from patients with naproxen (Nap)-induced liver injury. A)** T cell clones (5 x $10^4$ / 50 µL) were incubated with autologous EBV-transformed B cells (1 x $10^4$ / 50 µL) in the presence and absence of NAP, DM nap, and AG nap in a U-bottomed 96 well microplate. Cells were incubated for 48 hours (37°C; 5% $CO_2$) and [$^3$H] thymidine was added for the last 16 hours. Proliferative responses were assessed using scintillation counting. T cell clones with a stimulation index of 1.5 were selected, expanded and subjected to dose-response studies. **B)** Dose-dependent activation of T cell

clones with DM nap. T cell clones were incubated with autologous EBV-transformed B cells and DM nap (200-400 µM) and proliferative responses were measured as described above. **C)** Responses from 8 representative clones are shown. Statistical analysis was performed using The Mann-Whitney test to compare treated conditions with the untreated control at the same time point.

### 3.5.3 PHENOTYPE AND CYTOKINE SECRETION FROM DESMETHYL NAPROXEN SPECIFIC T CELL CLONES.

Following the successful generation of drug-specific T cell clones, ten of the T cell clones generated to DM nap were phenotyped as CD4+, while 2 expressed a CD8+ phenotype and double positive (DP) clones expressed both CD4 and CD8 (Figure 3.4A). The T cell clones expressed a varied TCR-Vβ repertoire with 60% of the TCR-Vβs detected by the commercial staining kit (Figure 3.4B). DM nap-responsive T cell clones exhibited strong IFN-γ and IL-22 secretion in response to incubation with the drug metabolite (Figure 3.4C). IL-17 secretion was detected from one of the DM nap-responsive T cell clones. Four representative DM nap responsive T cell clones were selected for cell surface receptor analysis. Across these four T cells clones a high expression of CCR4 which is predominantly expressed by Th2 cells, cutaneous lymphocyte antigen-positive skin-homing T cells and Treg cells (Yoshie and Matsushima, 2015) and CD69 was observed, while CCR5, CXCR3, CCR2, CCR1, CCR9 and CCR8 expression was identified on one T cell clones (Figure 3.4D).



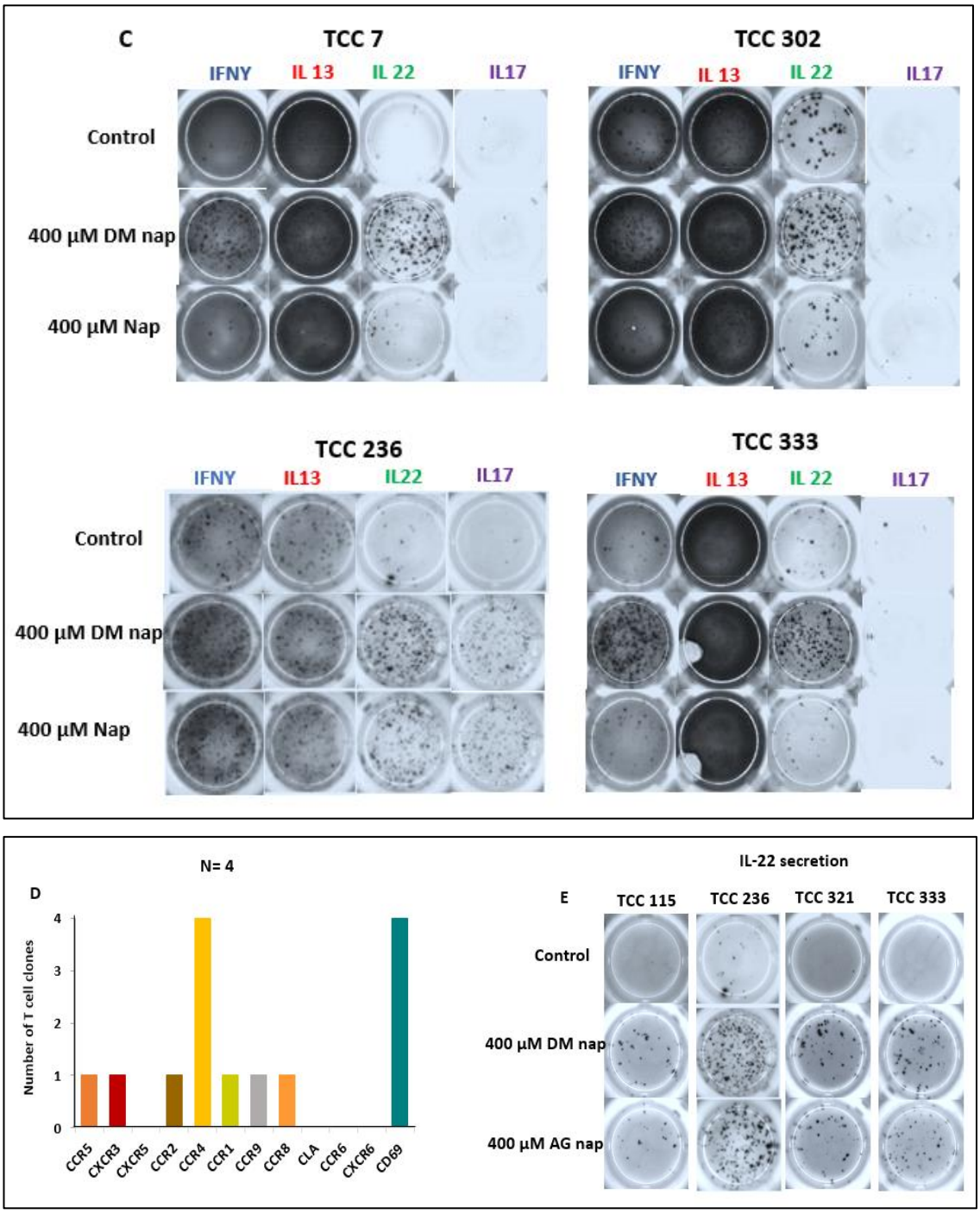



**Figure 3.4. Characterisation of desmethyl naproxen (DM nap)-responsive T-cell clones surface phenotype and cytokine secretion profiles with naproxen (Nap), desmethyl naproxen (DM nap) and naproxen acyl glucuronide (AG nap). A)** CD4/8 phenotype of T cell clones. T-cells (5 x $10^4$; 50 µL) were stained with antibodies CD4-FITC/APC and CD8-PE and incubated for 20 minutes at 4°C. T cell clones were then washed and analysed using flow cytometry (BD FACSCANTO II). DP (double positive) T cell clones expressed high levels of CD4 and CD8. **B)** T cell clones TCRVβ expression. T cells (5x$10^4$/ 50 µL) were stained with T-

cell Vβ receptor antibodies (FITC, PE, FITC-PE) and incubated for 20 minutes. T cell clones were then washed and analysed for TCRVβ expression using flow cytometry. Rare refers to T cell clones where Vβ expression was not detected using a panel of antibodies that covers 80% of known T-cell receptors. **C)** T-cells (5 x $10^4$ / 50 µL) were incubated with autologous EBV-transformed B cells (1 x $10^4$ / 50 µL) in an ELIspot plate, pre-coated for IFN-γ, IL-13, IL-17 and IL-22, in the presence of NAP of DM nap (400µM) for a period of 48 hours (37°C; 5% $CO_2$). The ELIspot plates were developed and counted using an AID ELIspot reader. **D)** T cell clones (50 µL) were stained with antibodies CCR2, CXCR3, CCR1, CCR8, CCR9, CTLA4, CLA, CCR6, CXCR6, CD69, CXCR5, CCR5, E-cadherin and CCR4. T cell clones were then washed and analysed for receptor expression using flow cytometry. Receptor was deemed to be expressed if mean staining intensity exceeded twice the isotype control. **E)** Reactivity of DM nap responsive T cell clones toward AG nap. IL-22 ELIspot was used as a measure of T cell clones activation.

### 3.5.4 DESMETHYL NAPROXEN RESPONSIVE T CELL CLONES DISPLAY STRONG REACTIVITY AGAINST NAPROXEN ACYL GLUCURONIDE, BUT ONLY WEAK AGAINST PARENT DRUG.

Very little cross-reactivity was observed when the DM nap-responsive T cell clones were cultured with NAP; however, one T cell clone (TCC 236) was found to secrete IFN-γ, IL-17 and IL-22 in the presence of both DM nap and the parent drug (Figure 3.4C). Based on the T cell clones cytokine secretion profiles, IL-22 secretion was selected as the readout to assess AG nap cross-reactivity and the pathway of drug presentation to the T cell clones. Interestingly, the DM nap responsive T cell clones (including TCC 236) were stimulated to secrete IL-22 in the presence of AG nap. Figure 3. 4E shows the IL-22 ELIspot data from 4 representative T cell clones. DM nap-responsive T cell clones were not stimulated to secrete IL-22 in the presence of other NSAIDs (ibuprofen, diclofenac, aspirin and acetaminophen) (Figure 3.5 and 3.6).

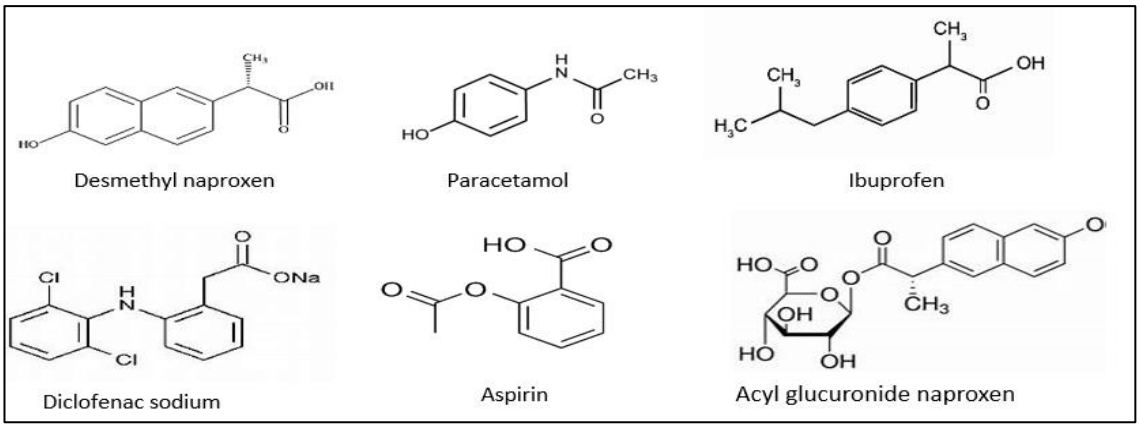



**Figure 3.5. Chemical structures of some non-selective COX inhibitors.** The characteristic feature of nonselective COX inhibitor NSAIDs is the presence of carboxylic acid (COOH) functional group.

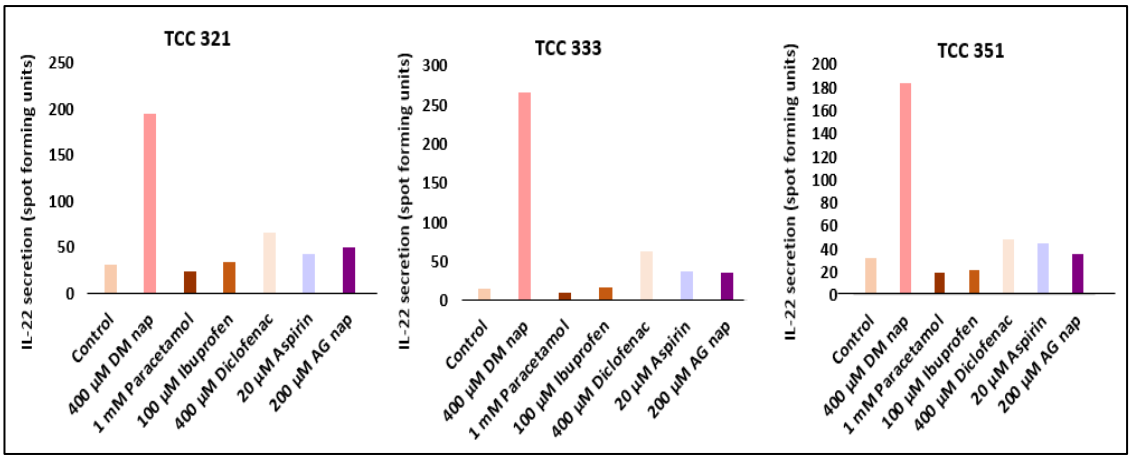



**Figure 3.6. Activation of desmethyl naproxen (DM nap) -responsive T cell clones with other non-steroidal anti-inflammatory drugs and paracetamol.** T-cell clones (5 x $10^4$ / 50 µL) were incubated with autologous irradiated EBV-transformed B cells (1 x $10^4$ / 50 µL) in the presence and absence of DM nap **(**400 µM), paracetamol (1mM), ibuprofen (100 µM), diclofenac (400 µM), aspirin (20 µM) and AG nap (200 µM) in a U-bottomed 96 well microplate. Cells were incubated for 48 hours (37°C; 5% $CO_2$). The ELISpot plate was developed according to the manufacturer's instructions. The plate was left to air-dry overnight, IL-22 secretion was measured using ELIspot reader.

### 3.5.5 HLA-DQ-RESTRICTED ACTIVATION OF DESMETHYL NAPROXEN RESPONSIVE T CELL CLONES.

Incubation of DM nap-responsive T cell clones with the drug metabolite in the presence and absence of EBV-transformed B-cells indicated a complete eradication of IL-22 secretion when antigen presenting cells were removed from the assay (Figure 3.7A). Next, anti-human HLA blocking antibodies were used to explore the dependence of MHC on the activation of CD4+ T cell clones. IL-22 secretion was detected from all T cell clones cultured with DM nap and EBV-transformed B-cells (Figure 3. 7B); however, IL-22 secretion was reduced to basal levels when the antigen presenting cells were pre-treated with an anti-HLA class-II blocking antibody. Such findings were not observed in T-cells co-incubated with EBV transformed B-cells pre-treated with anti-HLA class-I block. In similar antibody blocking experiments with those specific for individual MHC class II alleles, activation of the T cell clones with DM nap was found to be dependent on HLA-DQ (Figure3. 7C).

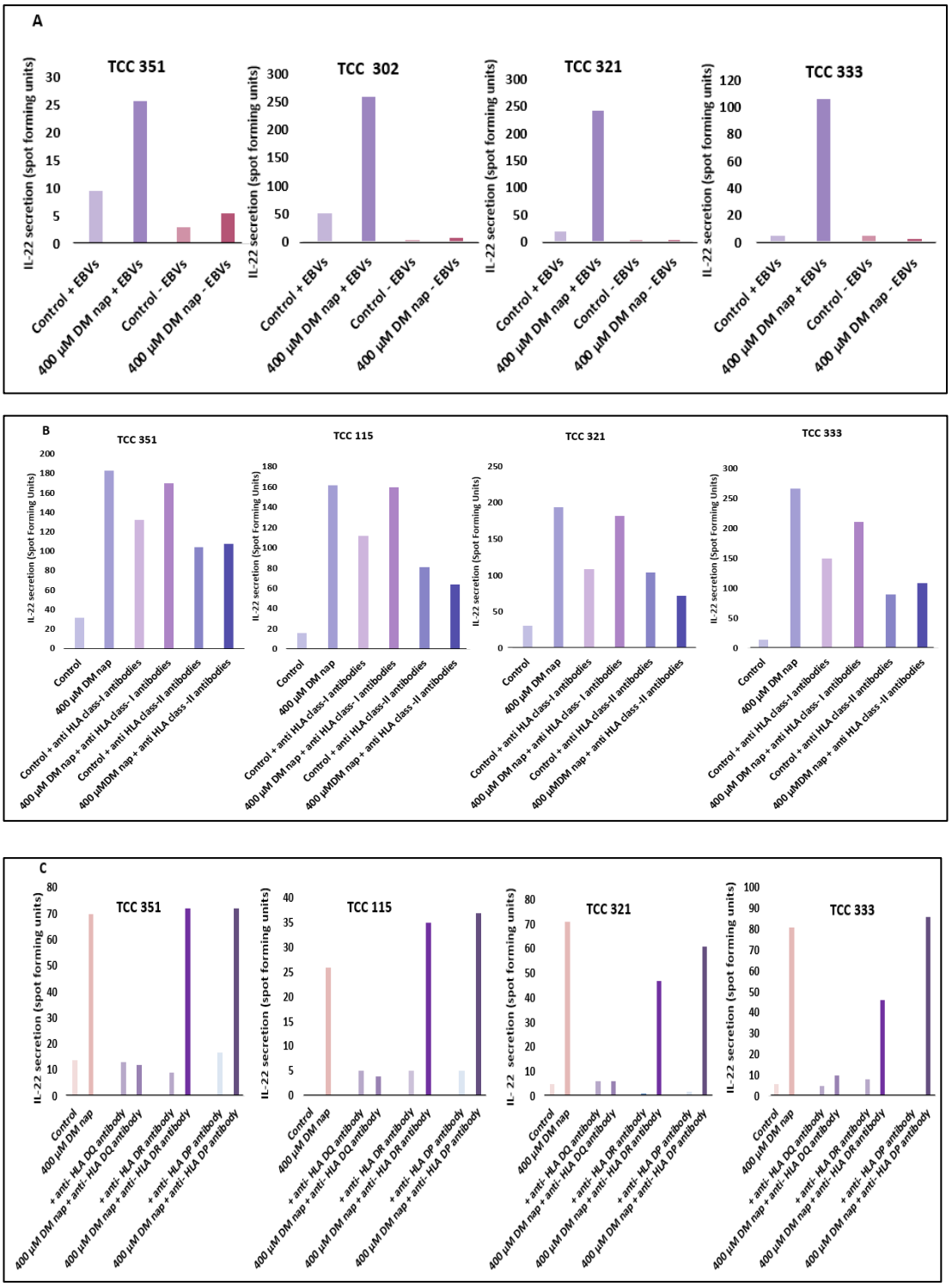



**Figure 3.7. HLA-DQ restricted activation of desmethyl naproxen (DM nap)-responsive T cell clones. (A)** T cell clones (5 x $10^4$ / 50 µL) were incubated with DM nap in the presence or absence of autologous EBV transformed antigen presenting cells (1 x $10^4$ / 50 µL) on an ELIspot plate pre-coated with IL-22 for 48 hours (37°C; 5% $CO_2$). **(B, C)** T cell clones (5 x $10^4$ / 50 µL) were incubated with DM nap and autologous EBV transformed antigen presenting cells (1 x $10^4$ / 50 µL) in the presence and absence of anti-human HLA blocking antibodies; **(B)** HLA class-I and HLA class-II antibodies and **(C)** HLA class-II sub-class (HLA-DP, DQ and DR). EBV-transformed B

cells were pre-treated with antibodies for 20 min. T cell clones activation was quantified via analysis of IL-22 secretion using ELIspot.

### 3.5.6 DESMETHYL NAPROXEN RESPONSIVE T CELL CLONES ARE ACTIVITED VIA A HAPTEN MECHANISM.

EBV-transformed B-cell pulsing experiments were conducted to investigate the nature of the DM nap HLA-DQ binding interaction involved in activation of the T cell clones. In these experiments EBV-transformed B-cells were cultured in the presence and absence of DM nap for 1 and 16 hours. The antigen presenting cells were then washed repeatedly to remove any weakly bound drug before being incubated with the drug specific T cell clones. Figure 3.8 shows that most T cell clones secreted IL-22 in the presence of EBV-transformed B-cells pulsed with DM nap for 1 and 16 hours and that the levels of IL-22 secreted were similar to that observed with the soluble drug metabolite. In contrast, IL-22 secretion was not detected when T cell clones were cultured with DM nap and glutaraldehyde-fixed EBV-transformed B-cells, where fixation blocks antigen processing. One DM nap-responsive T cell clone that proliferated weakly in response to DM nap displayed a contrasting T cell activation pathway. This T cell clone was stimulated to proliferate in the presence of fixed EBV-transformed B-cells, while proliferation was not detected with antigen presenting cells pulsed with DM nap for 16hours (Figure 3.9).

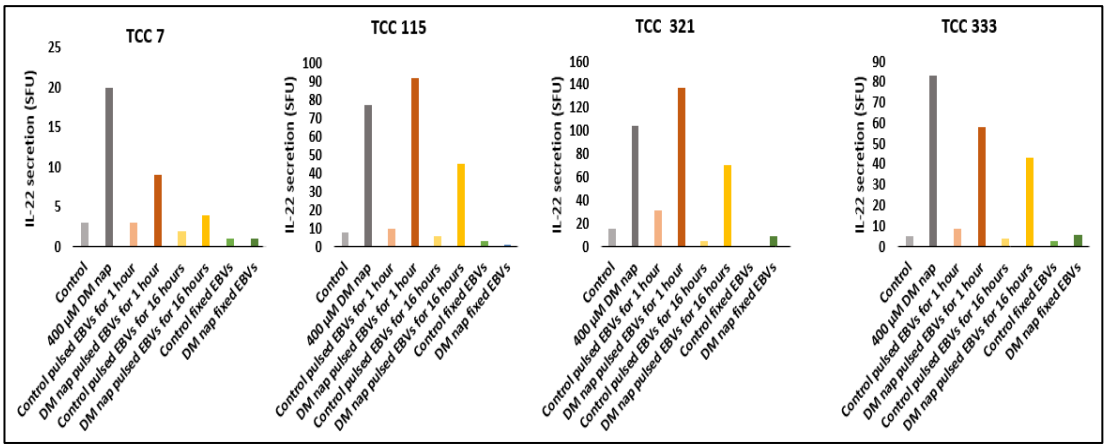



**Figure 3. 8. Activation of T-cell clones with desmethyl naproxen (DM nap)-pulsed antigen presenting cells is dependent on antigen processing. (A)** T cell clones (5 x $10^4$ / 50 µL) were incubated with autologous EBV-transformed B cells (1 x $10^4$ / 50 µL) pulsed with DM nap (400µM) for 1 or 16 hours (brown columns, yellow columns). The DM nap pulsed EBV-transformed B cells were washed repeatedly to remove free compound prior to culturing with T-cells. Furthermore, T cell clones were incubated with DM nap (400µM) in the presence of glutaraldehyde-fixed autologous EBV-transformed B cells (green columns). DM nap was used as a positive control (gray columns). Activation of the T cell clones was measured via IL-22 ELIspot.

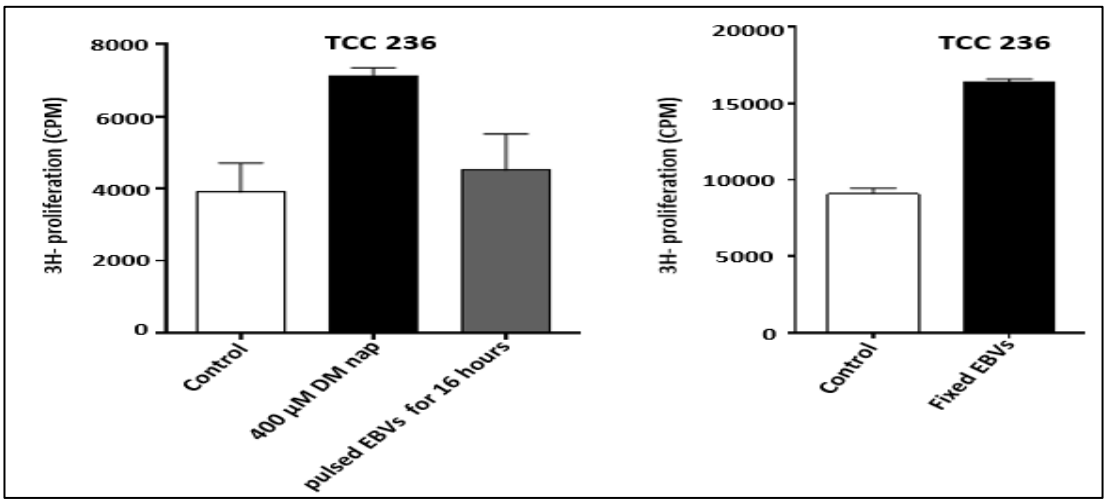



**Figure 3.9. Activation of T cell clone (236) with desmethyl naproxen (DM nap) occurs in the absence of antigen processing.** (A) T cell clone (5 x $10^4$ / 50 µL) was incubated with autologous EBV-transformed B cells (1 x $10^4$ / 50 µL) pulsed with DM nap (400µM) for 16 hours. The DM nap-pulsed EBV-transformed B cells were washed repeatedly to remove free compound prior to culturing with T-cells. Furthermore, TCC was incubated with DM nap (400µM) in the presence of glutaraldehyde-fixed autologous EBV-transformed B cells. Soluble drug was used as a positive control. Activation of the clones was measured via [$^3$H] thymidine.

### 3.6 DISCUSSION

DILI is rare, but often life-threatening. Furthermore, DILI is difficult to detect during preclinical studies of the drug, and if sufficiently serious cases are identified, this can lead to the withdrawal of a drug from the market. (Uetrecht and Naisbitt, 2013). DILI has been diagnosed in patients exposed to a variety of therapeutics and in several cases development of tissue injury is associated with carriage of a particular HLA allele. Examples include HLA-B*57:01 with flucloxacillin (Daly et al., 2009), HLA-B*35:02 with minocycline (Urban et al., 2017), HLA-A*33:01 with ticlopidine (Hirata et al., 2008) and HLA-DRB1*15:01 with co-amoxiclav (Lucena et al., 2011). This in turn suggests that the causative drug (or metabolite derived from the drug) interacts with a degree of selectivity with the HLA protein to activate T cells and that these cells are involved in the disease pathogenesis. The most studied example of an HLA-associated liver reaction is flucloxacillin-induced liver injury, where the drug interacts with HLA-B*57:01 binding peptides to activate CD8+ T cells in patents and similar cells infiltrate liver at the time of the adverse event (Monshi et al., 2013, Wuillemin et al., 2014). It is important to note that expression of an HLA risk allele does not necessarily predispose a patient to a hepatic adverse event. For example, with the flucloxacillin HLA-B*57:01 association, only 1:1000 individuals expressing the HLA allele actually develop liver injury when exposed to the drug (Monshi et al., 2013). Furthermore, many hepatic drug reactions develop in patients expressing no obvious HLA risk allele. This is the case with atabecestat (discussed in chapter 4), a BACE inhibitor whose development for the treatment of Alzheimer's disease was terminated due to liver enzyme elevations in some patients(Henley et al., 2019). Atabecestat-induced liver injury is not associated with expression of an HLA allele; however, (i) T cell

infiltrates have been detected in a liver biopsy sample (De Jonghe et al., 2020) and (ii) T cells responsive towards a primary atabecestat metabolite have been isolated from PBMC of patients with liver injury (see chapter 4).

In this chapter we focussed on the NSAID naproxen, which is used to treat mild to moderate pain via the inhibition of COX-1 and 2 enzymes. Naproxen-induced liver injury is detected with a prevalence of approximately 1-3 cases per 100,000 patients. Naproxen is an interesting exemplar to study, as it is oxidised to 6-O-desmethyl naproxen through phase I metabolism, and both naproxen and desmethyl naproxen are conjugated to form an acyl glucuronide derivative by phase II metabolism. Several previous studies have defined acyl glucuronide conjugates (conjugates of naproxen and its metabolite desmethyl naproxen) as chemically reactive and they bind covalently to proteins both in vitro and in vivo (Vree et al., 1993). Hence, they have the potential to activate patient T-cells through a hapten mechanism; however, to date evidence for drug or drug metabolite-specific T-cell activation in patients with DILI is lacking. Through the use of diagnostic assays with PBMC from patients with naproxen-induced liver injury and drug-(metabolite) specific T-cell cloning we have studied whether drug-responsive T cells are detectable and if so characterised the structures that interact with HLA molecules to stimulate proliferation and cytokine release. T-cells have been isolated and characterised from PBMCs collected from three subjects. The subjects showed proliferative responses and/or cytokine release in the presence of desmethyl naproxen. Previously, Whritenour and colleagues evaluated the sensitivity and specificity of the LTT in patients with drug-induced liver injury. Positive results ranged from 52.9% to 95% for different drugs (Whritenour et al., 2017). However, for certain drugs (e.g., flucloxacillin, atabecestat) diagnostic

PBMC assays yield negative or inconsistent results despite the fact that drug-responsive T-cells are detectable through the generation and characterisation of T-cell clones.

In this study, we were able to expand T-cells from naproxen, desmethyl naproxen and naproxen acyl glucuronide PBMC cultures and conduct serial dilution experiments to generate T-cell clones. Clones were then subjected to proliferation and cytokine release assays as the readout for a drug-specific response. From a total of almost 961T cell clones generated from 2 patients with liver injury, 12 were confirmed as desmethyl naproxen responsive. These T cell clones mainly expressed CD4+ and varied TCR Vβ surface receptor (indicating they derived from different precursors) and were stimulated to proliferate and secrete IFN-γ and IL-22 when exposed to desmethyl naproxen and autologous antigen presenting cells. The *in vivo* C max for naproxen has been measured at 199 µM (Spahn-Langguth and Benet, 1992), a nontoxic concentration for PBMC, and as such initial T cell clone testing and most mechanistic studies were performed using a concentration of 200 µM naproxen and naproxen metabolites. Desmethyl naproxen responsive T cell clones were activated in a dose-dependent manner, with metabolite concentrations as low as 25 µM stimulating proliferative responses and cytokine release. Twenty-five µM is approximately 10-fold higher than the estimated plasma C max of desmethyl naproxen; however, it is possible that desmethyl naproxen may accumulate to significantly higher concentrations in liver due to the variable expression of drug metabolism and transporter enzymes.

Detection of T cells that are predominantly activated by a primary drug metabolite is rare, but not unprecedented. Allopurinol hypersensitivity is mediated by the dose-dependent activation of oxypurinol-specific T cells (Chung et al., 2015, Yun et al., 2014a, Emmerson et al., 1988). Oxypurinol is a metabolite formed through the xanthine oxidase-catalysed metabolism of allopurinol. Similarly, T-cells from patients hypersensitive to carbamazepine are activated with stable metabolites such as carbamazepine 10, 11 epoxide and 10-hydroxy carbamazepine, alongside the parent drug (Wu et al., 2006b, Wei et al., 2012b). These data clearly highlight the importance of studying different forms of a culprit drug in *in vitro* diagnostic assays, especially when the parent compound yields negative results.

The absence of T cells that display activity towards naproxen acyl glucuronide is important because acyl glucuronide metabolites are so commonly cited as culprits in adverse drug reactions with little supportive evidence. Miyashita, Lwamura and their colleagues have observed a correlation between acyl glucuronide metabolites whose parent drugs have been withdrawn from the market due to adverse events and high mRNA expression levels of pro-inflammatory cytokines in PBMC (Miyashita et al., 2014, Iwamura et al., 2015). Hence, it is possible that the acyl glucuronide metabolite participates in the adverse event through the provision of stress signals that are important for priming of naïve T-cells against an alternative structural moiety, in the case of naproxen, the desmethyl metabolite.

Further work aims to characterise the structural features of the desmethyl naproxen-specific activation of CD4+ T cell clones. Firstly, the T cell clones were found to exhibit a degree of intra-structural cross-reactivity in that they were activated with naproxen

acyl glucuronide. However, no cross-reactivity was observed with other carboxylic acid-containing drugs. As the pharmacological target of naproxen and it is metabolites is the enzyme cyclooxygenase and cyclooxygenase binding lead to enzyme inhibition; our data indicates that chemical signalling between the drugs, MHC and the TCR must be significantly more stringent. Secondly, the importance of HLA was considered through omission of antigen presenting cells from the T cell assay and the addition of anti-human HLA blocking antibodies.

These experiments demonstrated (i) the importance of antigen presenting cells for desmethyl naproxen specific T cell activation and (ii) that T cells were triggered when the drug metabolite associated with HLA-DQ molecules. Thirdly, the pathway of desmethyl naproxen presentation to T cells was studied using antigen presenting cell pulsing and antigen presenting cell fixation experiments. Antigen presenting cells pulsed for 1 or 16 hours with desmethyl naproxen activated the majority of T cell clones, while fixation blocked IL-22 secretion. All CD4+ T- cells showed restriction to the HLA-DQ allele antigen by significant decrease of IL 22 secretion after they were triggered when desmethyl naproxen associated with HLA-DQ. Collectively, these data suggest that for T cell activation desmethyl naproxen associates strongly with antigen presenting cells and antigen processing is a prerequisite; however, additional structural studies are needed to define the nature of the drug-HLA-DQ peptide binding interaction. Interestingly, higher levels of IL-22 secretion was observed when T cell clones were incubated with EBV-transformed B-cells pulsed with desmethyl naproxen for 1 hour, as opposed to longer time periods, which suggests that the T-cell stimulatory signal is generated rapidly in the assay. Fixation of antigen presenting cells with glutaraldehyde before addition to T-cell assays containing drug blocked IL-

22 secretion. Thus, T-cell activation is dependent on processing of proteins by the antigen presenting cells. One T cell clone exhibited distinct characteristics from the rest. This T cell clone secreted IL-17 in conjugation with IFN-$\gamma$ and IL-22. Furthermore, the T cell clone was not activated in the presence of desmethyl naproxen pulsed antigen presenting cells, while fixation of the antigen presenting cells did not inhibit the response. These data provide preliminary evidence for a heterologous population of T-cells being detected in the same patient with varying characteristics.

Collectively our findings demonstrate that T cells with specificity towards desmethyl naproxen circulate in patients with naproxen-induced liver injury. It is therefore likely that the hepatic reactions experienced by patients involve the adaptive immune system with CD4+ T cells being the prime mediator. Despite intensive investigations, we found no evidence that naproxen acyl glucuronide activates patient T-cells. The activation of T cells with a phase I drug metabolite differs from previous studies with drugs such as flucloxacillin and ticlopidine, where T cells activated with the parent drug. This has important implications for Pharma trying to determine the chemical entities responsible for DILI as they most consider the entirety of chemical entities formed through drug metabolism (not just the parent drug and haptenic compounds). Furthermore, the data complicates the use of in vitro tests to diagnose DILI and stable metabolites are not available for all compounds.

# CHAPTER 4: CHARACTRISATION OF DRUG- SPECIFIC T-CELL RESPONSES IN PATIENTS WITH LIVER INJURY FOLLOWING TREATMENT WITH THE BACE INHIBITOR ATABECESTAT.

## CONTENTS

**4.1 AN OVERVIEW.**

Alzheimer's disease (AD) is a chronic neurodegenerative disease which is characterised by an impairment in cognitive function beyond the scope of the normal ageing process (Mayne et al., 2020, Vassar, 2005). An imbalance exists between production and clearance of amyloid-β (Aβ) in patients with AD and a common disease characteristic is the presence of neurofibrillary tangles comprised of hyper phosphorylated tau proteins and amyloid plaques (Sanabria-Castro et al., 2017). Amyloid-β is produced from amyloid precursor protein via a cleavage process by BACE-1 in the first and rate limiting step (Sathya et al., 2012). Therefore, it is hypothesised that inhibition of the BACE-1 enzyme can reduce the formation of the toxic amyloid plaques, thereby having a positive impact on the progression of AD (Evin et al., 2010). As such the development of therapeutics which target the underlying cause of AD as opposed to the symptoms only, represents an attractive prospect. Atabecestat (JNJ-54861911) is an orally administered BACE inhibitor (BACE1) developed for the treatment of AD recently withdrawn from clinical trials (Hsiao et al., 2019). The drug functions by inhibiting the BACE processing of amyloid precursor protein (APP), reducing the production of (Aβ) fragments, thereby reducing the synthesis of amyloid plaques (Panza et al., 2018). Through the course of several clinical studies, elevations in hepatic enzymes were observed in patients undertaking atabecestat therapy thereby being considered an adverse drug reaction accredited to the drug (Novak et al., 2020, Panza et al., 2019).

Elevated hepatic enzymes were observed in patients participating in two atabecestat studies with 24% of patients exhibiting >1.5 upper limit of normal (ULN) levels of alanine transaminase (ALT), including 11% with ALT > 3x ULN. These were commonly

associated with increases in aspartate aminotransferase (AST), but only 1 patient had a concomitant increase of bilirubin > 2x ULN in the presence of a normal alkaline phosphatase, indicative of severe hepatocellular injury. Importantly, increases in the level of ALT did not correlate with the concentration of atabecestat (metabolites) in patient plasma. A swift decline in ALT levels was observed in most patients following withdrawal of atabecestat; however, ALT levels continued to increase in the plasma of a limited number of patients. In particular, one patient (patient 6) reported an ALT level of 28.8x ULN one month after cessation of therapy. This, coupled with the immuno-histological detection of T-cell infiltrates in a liver biopsy specimen (De Jonghe et al., 2020) suggests that the adaptive immune system, might be involved in the pathogenesis of the atabecestat-induced adverse event.

Until recently, the involvement of the adaptive immune system in drug-induced liver injury (DILI) was controversial. Maria and Victorino detected drug-responsive T-cells within PBMC isolated from certain patients with DILI (Maria and Victorino, 1997b). However, the drug-responsive T-cells were not characterised in terms of phenotype or function. More recently, the activation of drug-responsive T-cells from the blood of patients to a number of drugs including flucloxacillin-(Monshi et al., 2013) , amoxicillin-clavulanate- (Kim et al., 2015) and tuberculosis medication-induced liver injury (Usui et al., 2017a) has been reported. Furthermore, several HLA allele associations have been identified in patients with DILI for a host of drugs including flucloxacillin (HLA-B*57:01) (Daly et al., 2009), ximelagatran (HLA-DRB1*07:01) (Hirasawa et al., 2017) and lumiracoxib (HLA-DRB1:15:01) (Singer et al., 2010) and in patients with drug rash with eosinophilia and systemic symptoms (DRESS); e.g.,

vancomycin (Konvinse et al., 2019) and dapsone (Zhang et al., 2013) where liver injury develops as a component of a multifaceted disease.

Although not always proven through T- cell studies with patient PBMC, the genetic associations suggest that drugs interact preferentially with the protein (or binding peptide) derived from the HLA of interest to stimulate T- cells that contribute towards the disease pathogenesis. HLA molecules displayed on the cell surface present drugs bound directly via reversible pharmacological interaction (p-i concept) and covalently bound drug peptide adducts generated through protein processing (hapten)(Pichler, 2021).

Atabecestat undergoes metabolism to generate an epoxide metabolite and diaminothiazine (DIAT) each of which are susceptible to cytochrome P450-mediated metabolism liberating products with the potential to bind covalently to protein (Koriyama et al., 2021). DIAT is formed from atabecestat via the carboxylesterase-mediated hydrolysis of the amide bond resulting in loss of the cyano-pyridine ring structure (Ferreira et al., 2021) . In contrast, the epoxide of atabecestat is formed on the amino-methyl-thiazinyl ring through cytochrome P450-3A mediated metabolism.

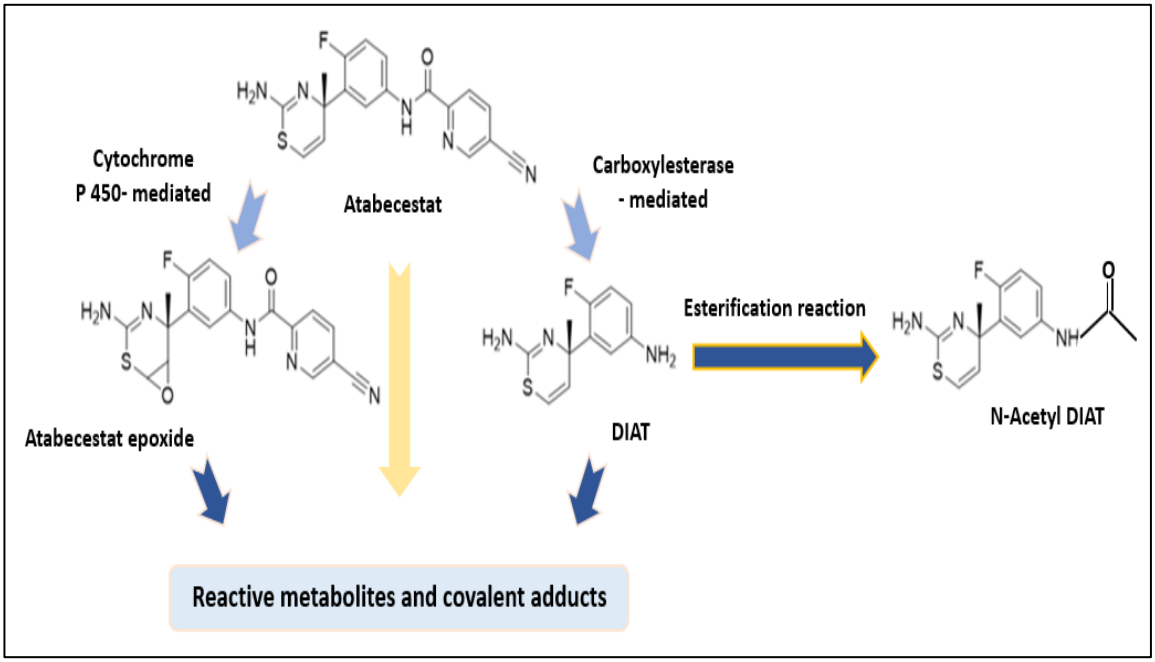



**Figure 4.1. Structure of atabecestat and metabolic conversion to DIAT and atabecestat epoxide.**

As previously described, the LTT assay allows for the diagnosis of circulating drug-(metabolite) responsive T-cells in the peripheral blood of a patient with a suspected drug reaction (Nyfeler and Pichler, 1997). Briefly, the PBMC are co-incubated in the presence of the drug or metabolite(s) in question, then after a period of 6 days, T-cell activity is quantified via a number of methods, with proliferation being the most common. Proliferation amounting to a doubling of proliferation or more between drug treated and control cells is considered a T-cell response to the drug. Indeed, notable success has been garnered with the LTT assay to diagnose both immediate and delayed reactions to beta lactam drugs (Luque et al., 2001). However, it is important to understand that a negative result in the LTT assay does not necessarily rule out the involvement of T-cells in a hypersensitivity reaction to the drug. Additional diagnostic tests, such as PBMC ELIspot, can also be conducted to quantify

memory T-cell responses via secretion of cytokines and cytolytic molecules, again this has the same caveats as the LTT.

T-cell cloning allows for the expansion of a single responsive T-cell which is generated from the lines of patients or healthy donors incubated in the presence of a drug/metabolite. Confirmed responsive T-cells can then be characterised for their phenotype and function by way of cross-reactivity, mechanism of activation and analysis of cellular surface markers. T-cell cloning has successfully identified the involvement of CD4+ and CD8+ T-cells in the hypersensitivity reactions to a number of drugs including abacavir (Bell et al., 2013), carbamazepine (Lichtenfels et al., 2014), allopurinol, flucloxacillin (Yaseen et al., 2015), dapsone-(nitroso) (Alzahrani et al., 2017) and tolvaptan (Gibson et al., 2020).

In the previous chapter we successfully diagnosed naproxen hypersensitivity reactions and characterised drug (metabolite) specific T-cells in hypersensitivity patients, thereby confirming that the adaptive immune system is implicated in the disease pathogenesis. Consequently, in this chapter we adopted a similar approach to explore the role of T-cells atabecestat-induced liver injury.

## 4.2 CHAPTER AIMS.

The aim of this chapter was to elucidate whether atabecestat-responsive T-cells were detectable in atabecestat clinical trial patients that developed elevations in liver enzymes and then generate T-cell clones to characterise phenotype and function of the drug-responsive cells along with pathways of T-cell activation and cross-reactivity with related structures.

**4.3 METHODS.**

The following methods have been used in this chapter which are described in more details in the chapter 2.

- LYMPHOCYTE TRANSFORMATION TEST (2.7.4).
- PBMC ELISPOT ASSAY (2.7.10).
- T-CELL CLONING (2.7.6).
- QUANTITIVE AND QUALITITIVE ASSESSMENT OF T CELL CLONE REACTIVITY TO THE DRUG AND CROSS REACTIVITY WITH STRUCTURALLY RELATED METABOLITES (2.7.6.1 and 2.7.6.2).
- FLOWCYTOMETRIC CD4+ AND CD8+ PHENOTYPING OF T-CELLS (2.7.10).
- FLOWCYTOMETRIC CHARACTERIZATION OF T-CELLS CELLULAR SURFACE RECEPTORS (2.7.10).
- ASSESSMENT OF MHC RESTRICTION OF ANTIGEN PRESENTING CELLS (2.7.15).
- ASSESSMENT OF ANTIGEN PRESENTING CELL PULSING AND FIXATION ON T-CELL ACTIVATION (2.7.14.1 and 2.7.14.2).

## 4.4. RESULTS

### 4.4.1. USE OF LTT AND PBMC ELISPOT FOR DETECTION OF ATABECESTAT AND ITS METABOLITE SPECIFIC T- CELLS IN ATABECESTAT PATIENTS PBMC.

To investigate the role of T-cells in the DILI reactions observed to atabecestat, PBMC were obtained from 14 study patients previously exposed to the drug from 3 clinical studies. Twelve/ fourteen had experienced liver ALT and AST elevations during therapy (Novak et al., 2020). The ALT increase in the selected patients ranged between 3.8 and 29.1x ULN. Table 4.1 summarises the demographics of the patients and details of the adverse event. None of the patients showed skin symptoms or eosinophilia.

**Table 4.1: Patient demographics and details of the adverse events.**

| Patient ID | Gender | Age | Country of origin | First study dayALT>3xULN | Maximum ALT increase (xULN) | Dose at time of reaction | Time to study blood sampling following 1st ALT>3xULN |
|---|---|---|---|---|---|---|---|
| Patient 1 | F | 71 | Belgium | D260 | 4.9 | 10 mg | 758 |
| Patient 2 | F | 66 | Belgium | / | / | 5 mg | / |
| Patient 3 | M | 68 | Belgium | D168 | 4.5 | 10 mg | 1024 |
| Patient 4 | F | 75 | France | D155 | 8.4 | 5 mg | 468 |
| Patient 5 | M | 63 | Sweden | D288 | 7.5 | 50 mg | 691 |
| Patient 6 | F | 76 | UK | D32 | 28.8 | 5 mg | 544 |
| Patient 7 | M | 73 | Spain | D344 | 6.5 | 10 mg | 750 |
| Patient 8 | M | 75 | Belgium | / | / | 50 mg | / |
| Patient 9 | M | 57 | Germany | D84 | 15.7 | 50 mg | 975 |
| Patient 10 | F | 68 | France | D260 | 9.4 | 25 mg | 377 |
| Patient 11 | F | 67 | Australia | D58 | 29.1 | 25 mg | 306 |
| Patient 12 | F | 64 | UK | D115 | 8.6 | 25 mg | 152 |
| Patient 13 | F | 67 | UK | D150 | 4.6 | 5 mg | 141 |
| Patient 14 | F | 76 | USA | D84 | 3.8 | 25 mg | 84 |

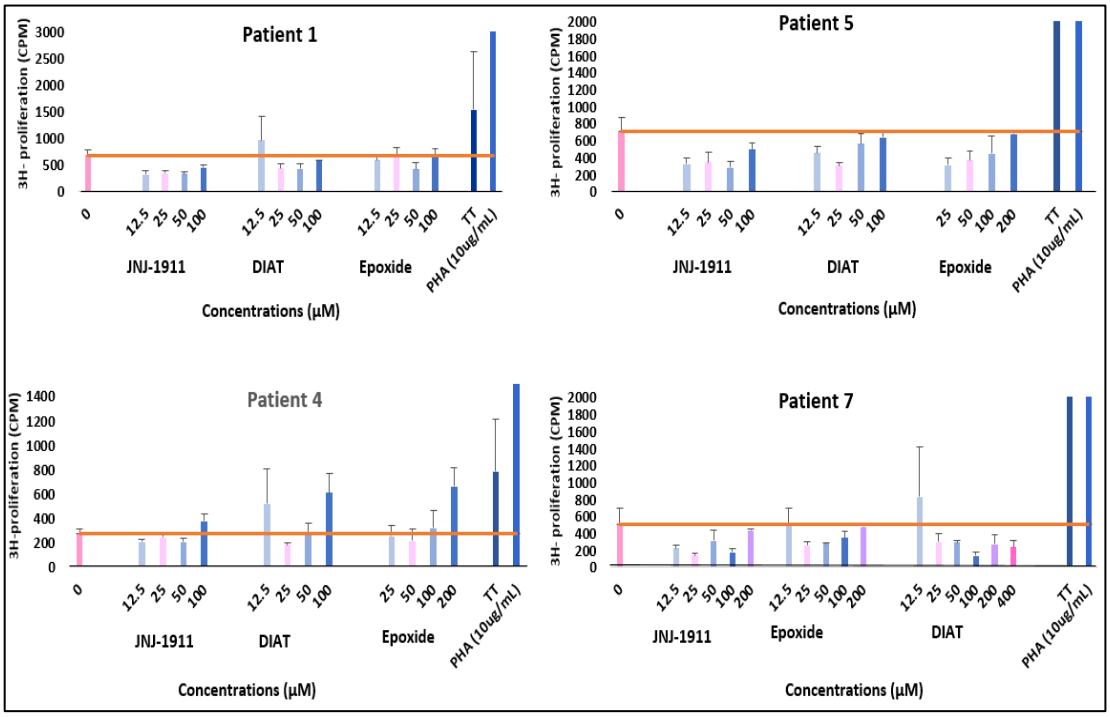
***Abbreviation: / not applicable because there is no elevation on ALT.***

The LTT was conducted on 10 patient PBMCs while ELIspot for IFN-γ and granzyme B was conducted on 9 patients (this included one patient not presenting ALT

elevations). to assess the presence of drug-responsive circulating T-cells in patients with liver injury. PBMCs from 4 subjects were not viable upon defrosting of vials, while cellular yield was low in 1 subject, excluding them from ELIspot assays. Low levels of proliferation (stimulation index [proliferation in compound treated wells/ proliferation in medium wells] 2-2.5) were induced by the DIAT and epoxide metabolites in patients 4, 1 and 7; however, based on the low cpm values of the control condition and the size of the error bars, it is likely this was a false positive (Figure 4.2). All other subjects displayed no proliferation in the presence of any of the study compounds. Strong T-cell proliferation was induced with PBMC from all donors by the positive controls phytohemagglutinin and tetanus toxoid.

Secretion of granzyme B was observed in response to the DIAT metabolite in patient 6, but no other compounds (Figure 4.2). IFN-γ secretion was not detected. Furthermore, no secretion of IFN-γ or granzyme B was observed from PBMC of other patients with any of the study compounds. Strong secretion of IFN-γ and granzyme B was observed in response to PHA. Finally, no proliferation or secretion of IFN-γ or granzyme B was observed in healthy volunteers in response to atabecestat, DIAT and atabecestat epoxide. Results for diagnostic tests for all patients in the study is summarised in Table 4.2.

**A.**




**B.**

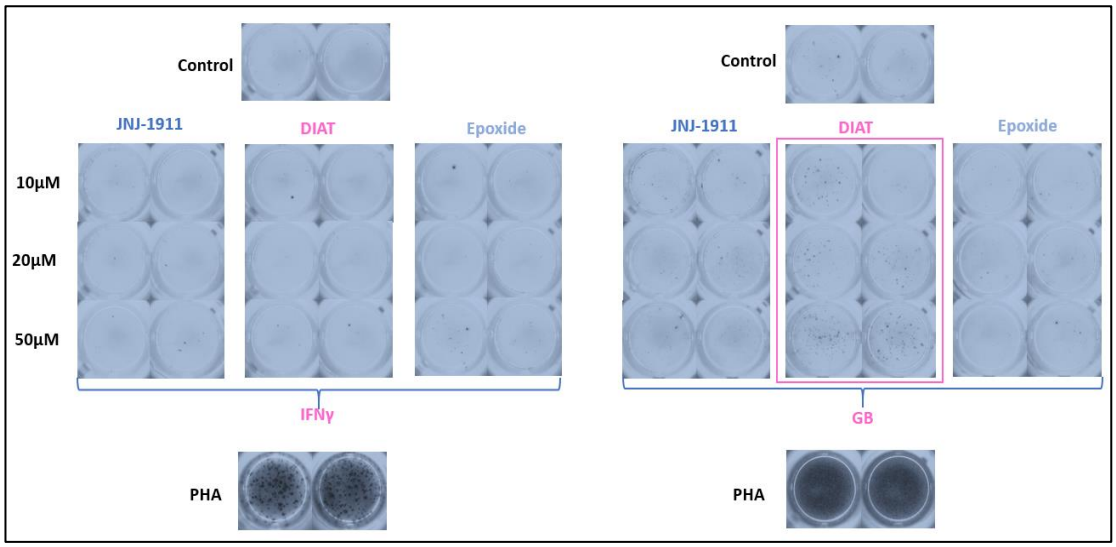



**Figure 4. 2. LTT assay and PBMC ELIspot to atabecestat patients. A)** PBMC co-incubated with the atabecestat, DIAT and epoxide for a period of 5 days in a 96 well U- bottomed plate (37 °C; 5% $CO_2$). Tetanus toxoid (TT) and PHA were used as positive controls and R9 medium as a negative control. Tritiated thymidine was then added for an additional 16 hours and proliferation was assessed by scintillation counting. **B)** PBMCs were incubated in an ELIspot plate, pre-coated for IFNγ and Granzyme B, in the presence of atabecestat, DIAT and epoxide at an array of concentrations (cell numbers permitting), for a period of 48 hours (37°C; 5%$CO_2$). PHA and R9 medium alone were used as positive and negative controls respectively. Following incubation, the ELIspot plate was developed for the respective cytokines following the manufacturer's instructions and counted using ELIspot AID reader.

**Table 4.2: Summary of diagnostic assays in atabecestat patients.**

| Patient ID | PBMC proliferation assay | PBMC ELIspot | |
|---|---|---|---|
| | | IFN-γ | Granzyme B |
| Patient 1 | -ve[1] | -ve | -ve |
| Patient 2 | -ve | -ve | -ve |
| Patient 3 | -ve | n/a[2] | n/a |
| Patient 4 | -ve | -ve | -ve |
| Patient 5 | -ve | -ve | -ve |
| Patient 6 | -ve | -ve | +ve |
| Patient 7 | -ve | -ve | -ve |
| Patient 8 | n/a | n/a | n/a |
| Patient 9 | -ve | -ve | -ve |
| Patient 10 | n/a | n/a | n/a |
| Patient 11 | n/a | n/a | n/a |
| Patient 12 | -ve | -ve | -ve |
| Patient 13 | -ve | -ve | -ve |
| Patient 14 | n/a | n/a | n/a |

[1]Result considered positive when stimulation index (response in drug treated/response in medium control) greater than 2. N/A- assay not conducted due to low cell number/viability.

### 4.4.2. T-CELL CLONING.

T-cell clones were generated from patients with liver injury to assess cellular phenotype, drug cross-reactivity and pathways of drug presentation. Initial proliferation testing of clones was performed with medium and study compounds, atabecestat (10 μM), DIAT (25 μM), acetyl DIAT (25μM) and atabecestat epoxide (10μM), using duplicate cultures. Responsive T-cell clones were generated to atabecestat from patients 4, 5 and 6, while DIAT responsive T-cell clones were generated from patients 1, 5, 6, 9 and 12. Acetyl-DIAT responsive clones were only generated from patient 6, while weakly-atabecestat epoxide-responsive clones were observed in patients 4, 6 and 12. Cloning results from one representative patient (patient 6) is displayed in Figure 4.3, while results to all patients tested are summarised in Table 4.3.

**Patient 6:**

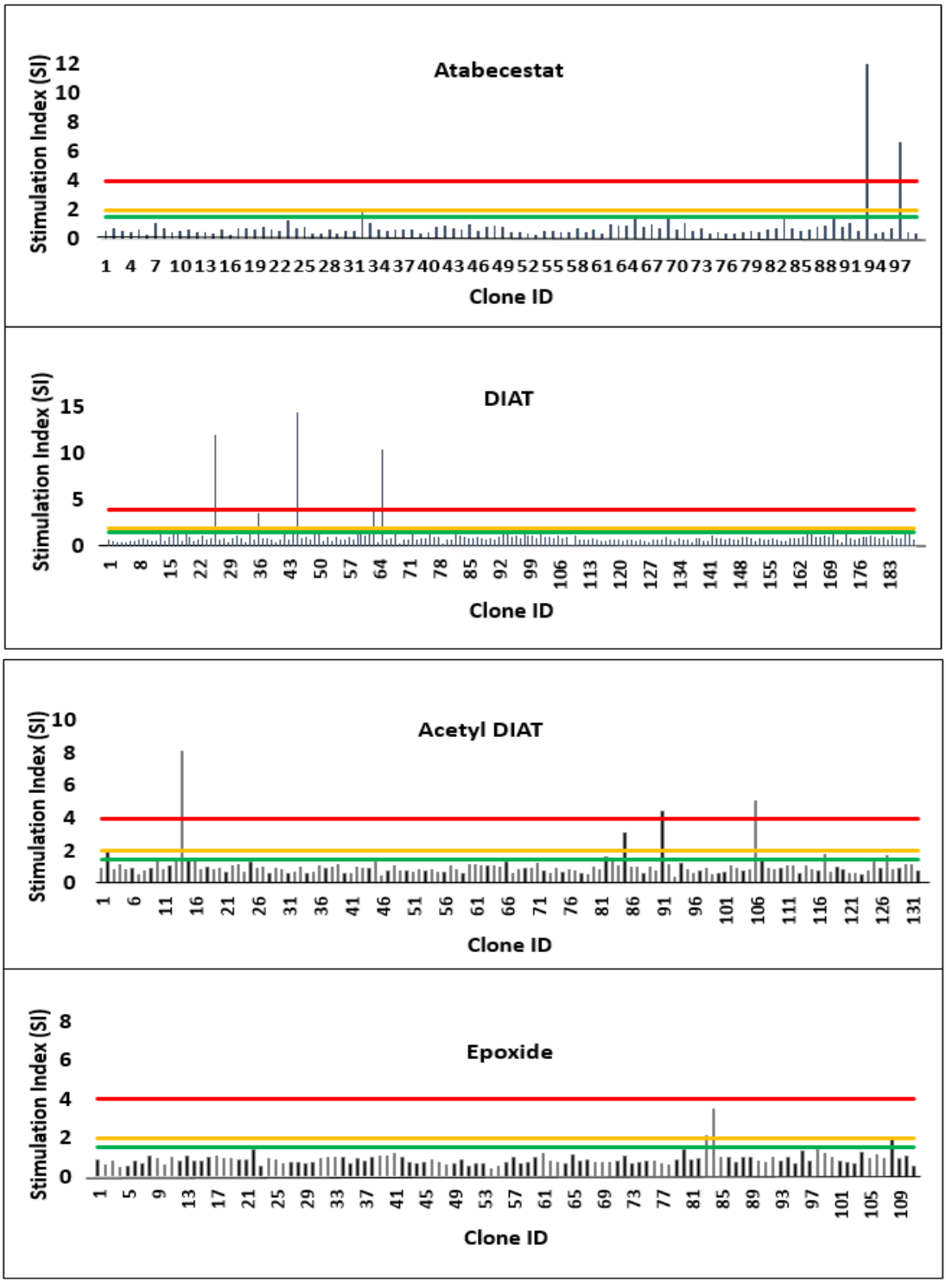
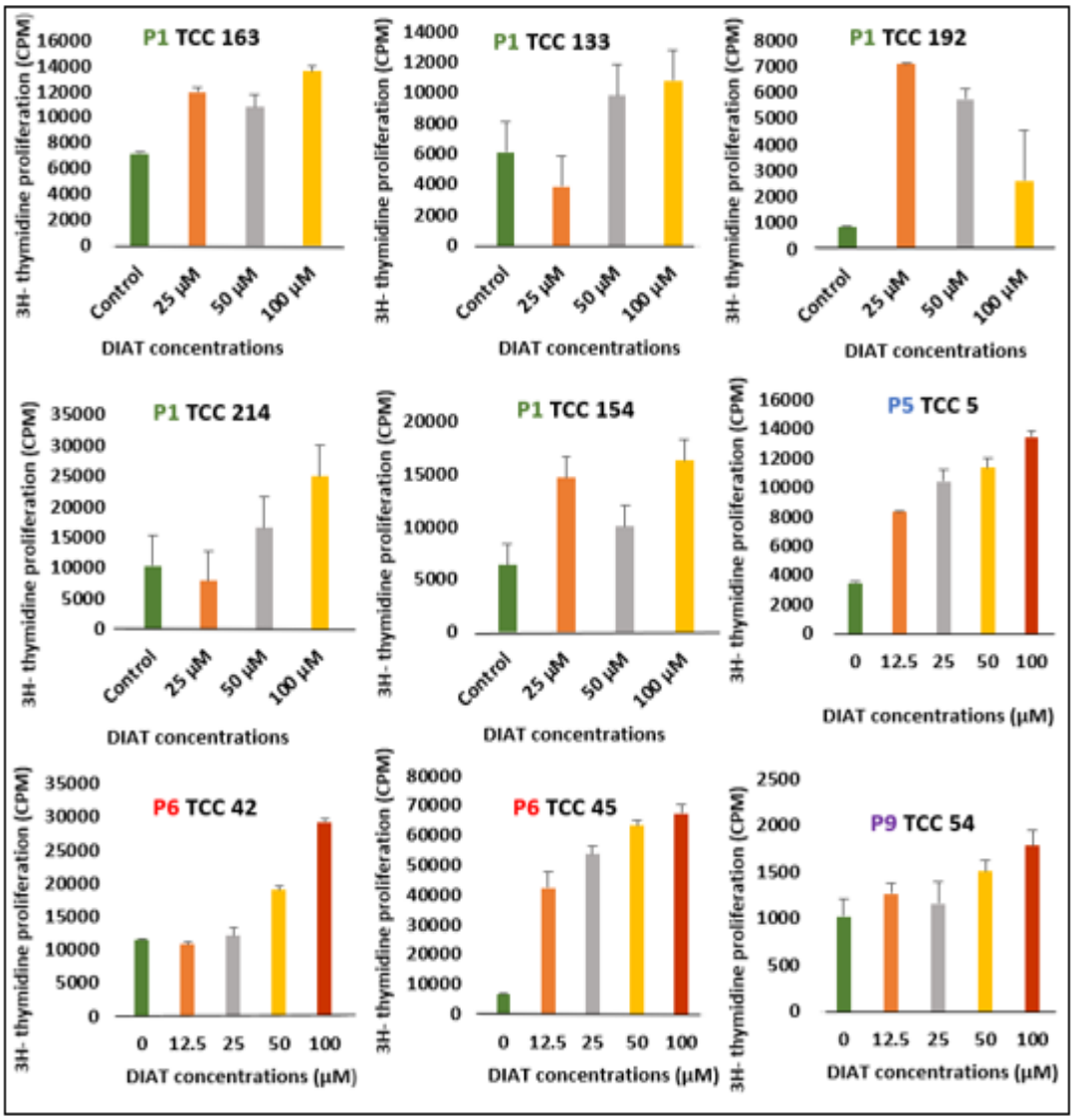


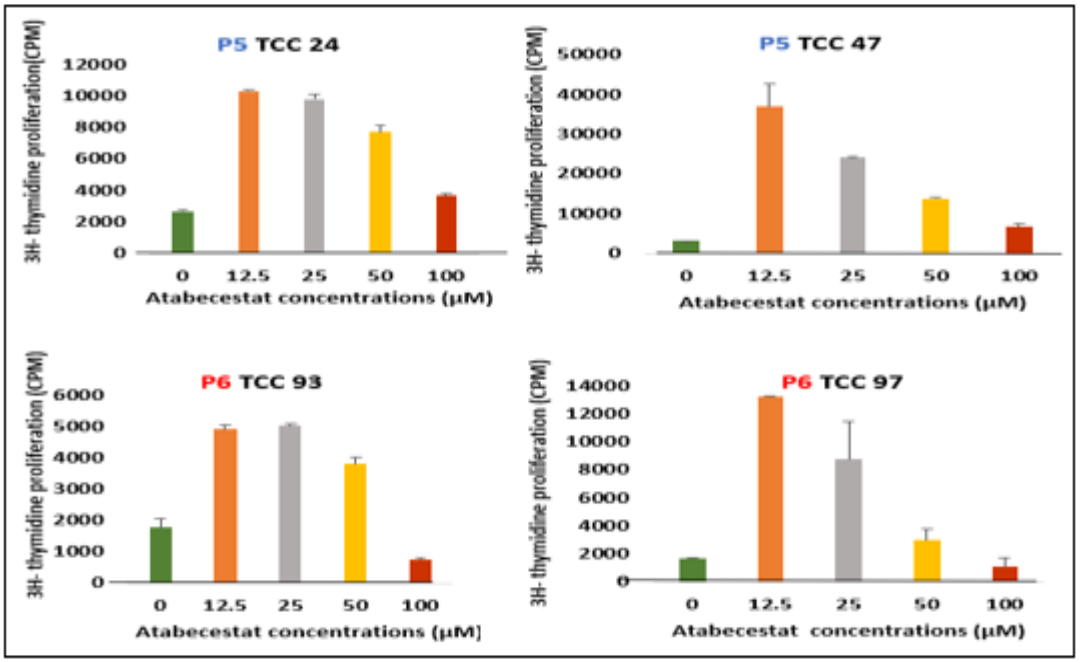

**Figure 4.3. T- Cell cloning to atabecestat-(metabolites) using PBMC from patient 6.** T-cell clones from atabecestat, DIAT, acetyl DIAT, and atabecestat epoxide derived T- cell lines (I.e., each figure panel shows separate clones) were co-incubated with autologous antigen presenting cells in the presence and absence of atabecestat (10µM), DIAT (25µM), acetyl DIAT (25µM), and atabecestat epoxide (10µM). Cells were incubated for 48 hours. [$^{3}$H] thymidine was then added for an additional 16 hours incubation and T-cell proliferation was

assessed using scintillation counting. Clones with a stimulation index of 1.5 (green line) or greater were selected and expanded for further analysis.

### 4.4.3. CONFIRMATION OF T- CELL RESPONSES TO ATABECESTAT-(METABOLITES).

Confirmation of the responsiveness of T-cell clones after the first testing stage was via a dose response study at compound concentrations of 0-100 µM (duplicate conditions per culture condition). Four, nine and three T-cell clones were stimulated to respond in a dose-dependent manner with atabecestat, DIAT and acetyl DIAT, respectively (Figure 4.4). The proliferative response and cytokine secretion from all clones generated from patients 1, 5, 6 and 9 are shown in Figure 4.4. No confirmed responsive T-cell clones were identified to atabecestat epoxide. In some instances, IFN-γ secretion quantified by ELIspot was selected as a readout for confirmation of T-cell activity (DIAT T cell clones 26 and 36, acetyl DIAT T cell clones). The total number of study compound-responsive T-cell clones and percentage of responsive clones generated during initial testing and after dose-responsive assessment from each patient is summarised in Table 4.3.


P1 TCC 163
P1 TCC 133
P1 TCC 192
P1 TCC 214
P1 TCC 154
P5 TCC 5
P6 TCC 42
P6 TCC 45
P9 TCC 54
3H- thymidine proliferation (CPM)
Control
25 µM
50 µM
100 µM
DIAT concentrations
0 12.5 25 50 100
DIAT concentrations (µM)


P5 TCC 24
P5 TCC 47
P6 TCC 93
P6 TCC 97
3H- thymidine proliferation(CPM)
3H- thymidine proliferation (CPM)
0 12.5 25 50 100
Atabecestat concentrations (µM)

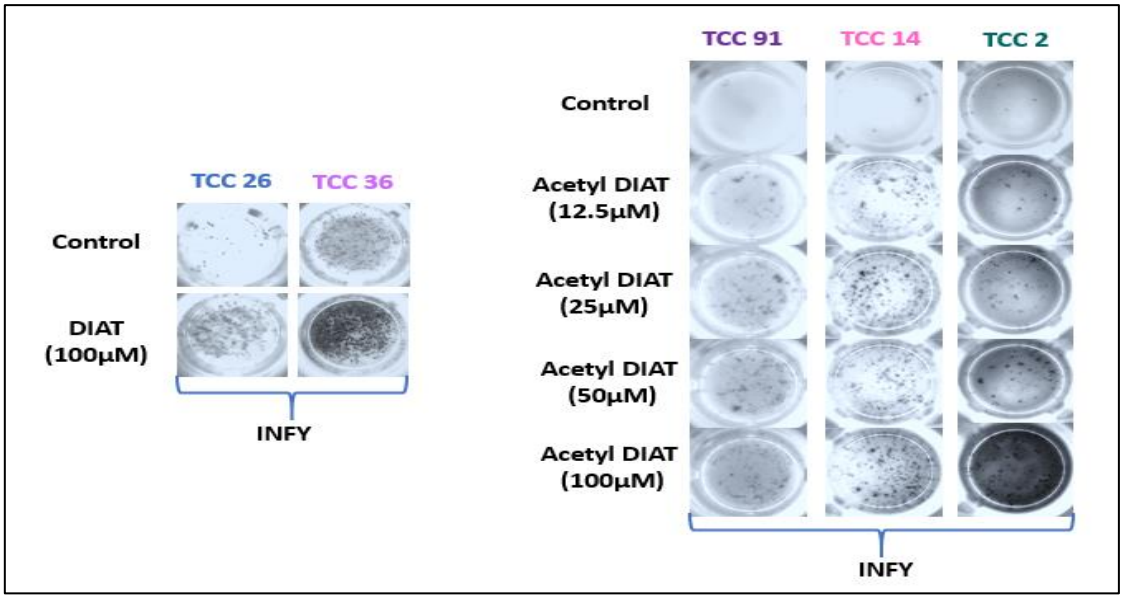



**Figure 4.4. Confirmation of T-cell reactivity towards atabecestat-(metabolites).** T cell clones ($5x10^4$/50 µL) were co-incubated with autologous antigen presenting cells ($1x10^4$/50 µL) in the presence and absence of the study compounds in a 96 well U-bottomed microplate at concentrations of 0-100 µM for 48 hours (37°C; 5% $CO_2$). [$^3$H] thymidine was added for an additional 16 hours and proliferation was measured via scintillation counting. Bars denote mean of duplicate wells. Error bars denote ± SEM. ELIspot images denote IFN-γ secretion from T-cells in the presence of DIAT/Acetyl-DIAT.

**Table 4.3: Summary of drug-responsive T-cell clones generated from PBMC of patient with atabecestat induced DILI.**

| | T- cell cloning to Atabecestat | | | | T- cell cloning to DIAT | | | |
|---|---|---|---|---|---|---|---|---|
| Patient ID | Tested number of clones | Number of drug-responsive clones (test one) | Number of drug-specific clones (repeat testing) | Percentage of responding clones (%) | Tested number of clones | Number of drug-responsive clones (test one) | Number of drug-specific clones (repeat testing) | Percentage of responding clones (%) |
| Patient 1 | 183 | 0 | 0 | 0 | 500 | 16 | 5 | 1 |
| Patient 4 | 37 | 1 | 0 | 0 | 36 | 3 | 0 | 0 |
| Patient 5 | 48 | 2 | 2 | 4.17 | 58 | 4 | 1 | 1.72 |
| Patient 6 | 99 | 5 | 2 | 2.02 | 189 | 19 | 4 | 2.12 |
| Patient 7 | 38 | 0 | 0 | 0 | 19 | 0 | 0 | 0 |
| Patient 9 | 57 | 0 | 0 | 0 | 56 | 2 | 1 | 1.79 |
| Patient 12 | 46 | 0 | 0 | 0 | 22 | 1 | 1 | 4.54 |
| Patient 13 | 16 | 0 | 0 | 0 | 11 | 0 | 0 | 0 |

| | T- cell cloning to acetyl DIAT | | | | T- cell cloning to Atabecestat epoxide | | | |
|---|---|---|---|---|---|---|---|---|
| Patient ID | Tested number of clones | Number of drug-responsive clones (test one) | Number of drug-specific clones (repeat testing) | Percentage of responding clones (%) | Tested number of clones | Number of drug-responsive clones (test one) | Number of drug-specific clones (repeat testing) | Percentage of responding clones (%) |
| Patient 4 | 29 | 0 | 0 | 0 | 20 | 1 | 0 | 0 |
| Patient 6 | 132 | 9 | 3 | 2.27 | 111 | 3 | 0 | 0 |
| Patient 12 | 20 | 0 | 0 | 0 | 23 | 5 | 0 | 0 |

### 4.4.4. PHENOTYPING AND CELLULAR SURFACE RECEPTORS.

With the exception of one CD8+ T-cell clone (patient 6; atabecestat-responsive) and one T-cell clone which was characterised as double positive (patient 5; DIAT-responsive) all responsive T-cell clones to atabecestat, DIAT and acetyl DIAT were phenotyped as CD4+ and three responsive T-cell clones to DIAT were phenotyped as CD8+. Figure 4.5 shows representative flow cytometric dot plots and histograms from 3 T-cell clones across two patients.

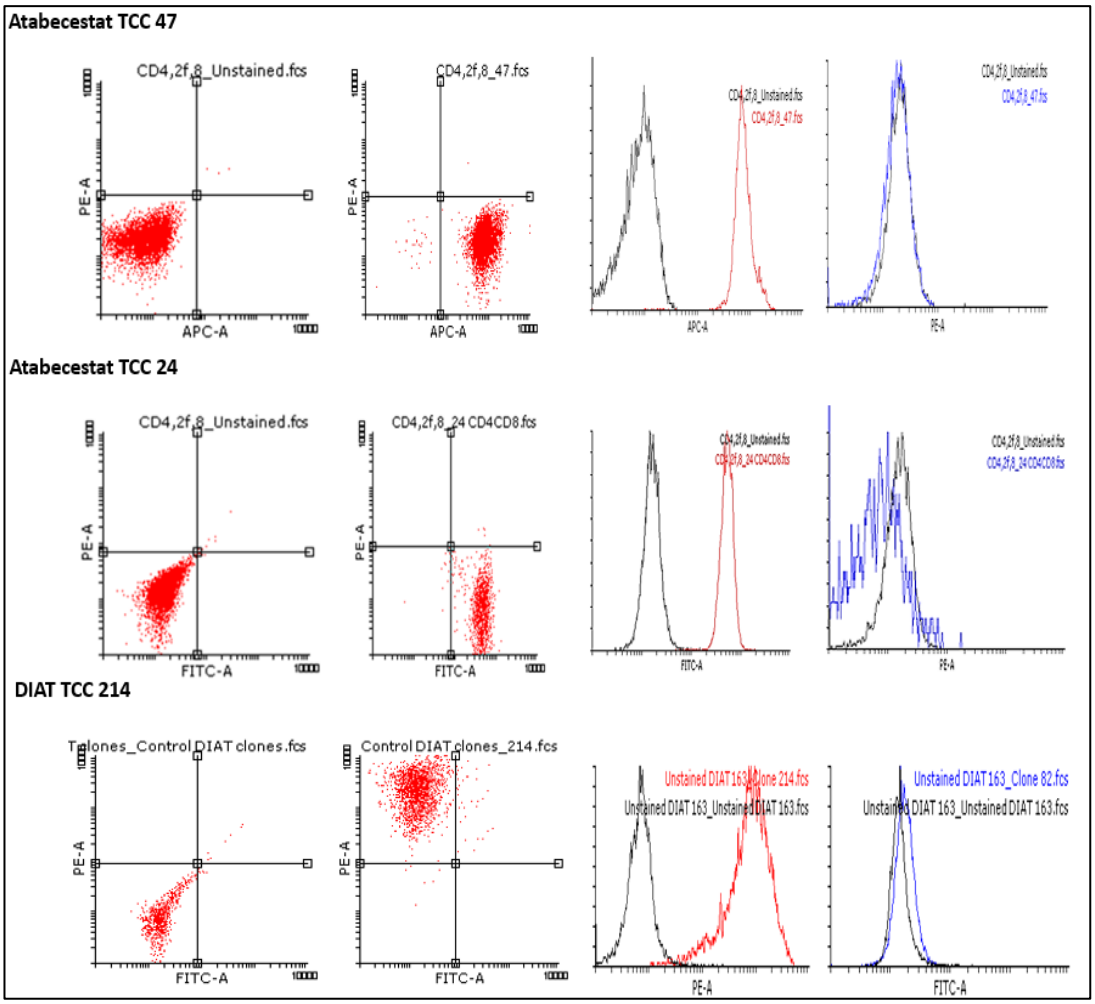



**Figure 4.5. CD4+/CD8+ phenotyping of atabecestat-(metabolite) responsive T-cell clones**. Cells (50µL) were stained with antibodies CD4-FITC/APC and CD8-PE and incubated for 20 minutes at 4°C. Cells were then washed and analyzed for phenotype using flow cytometry (BD FACSCANTO II). Stained cells shown as colored histograms (CD4-red, CD8-blue) and compared with unstained shown in black**.**

Across the T-cell clones tested, a varied expression of TCR-Vβ was detected to the parent drug and the DIAT metabolite, with expression of rare TCRs (not detected by the antibodies in the kit) being predominantly expressed. Chemokine receptor expression was assessed using six atabecestat- or DIAT-responsive clones expressing a single TCR. All tested T-cell clones expressed CXCR3, while the early activation marker CD69 and CCR4 were expressed on 5/7 clones. E-cadherin was expressed across some of the clones (Figure 4.6).

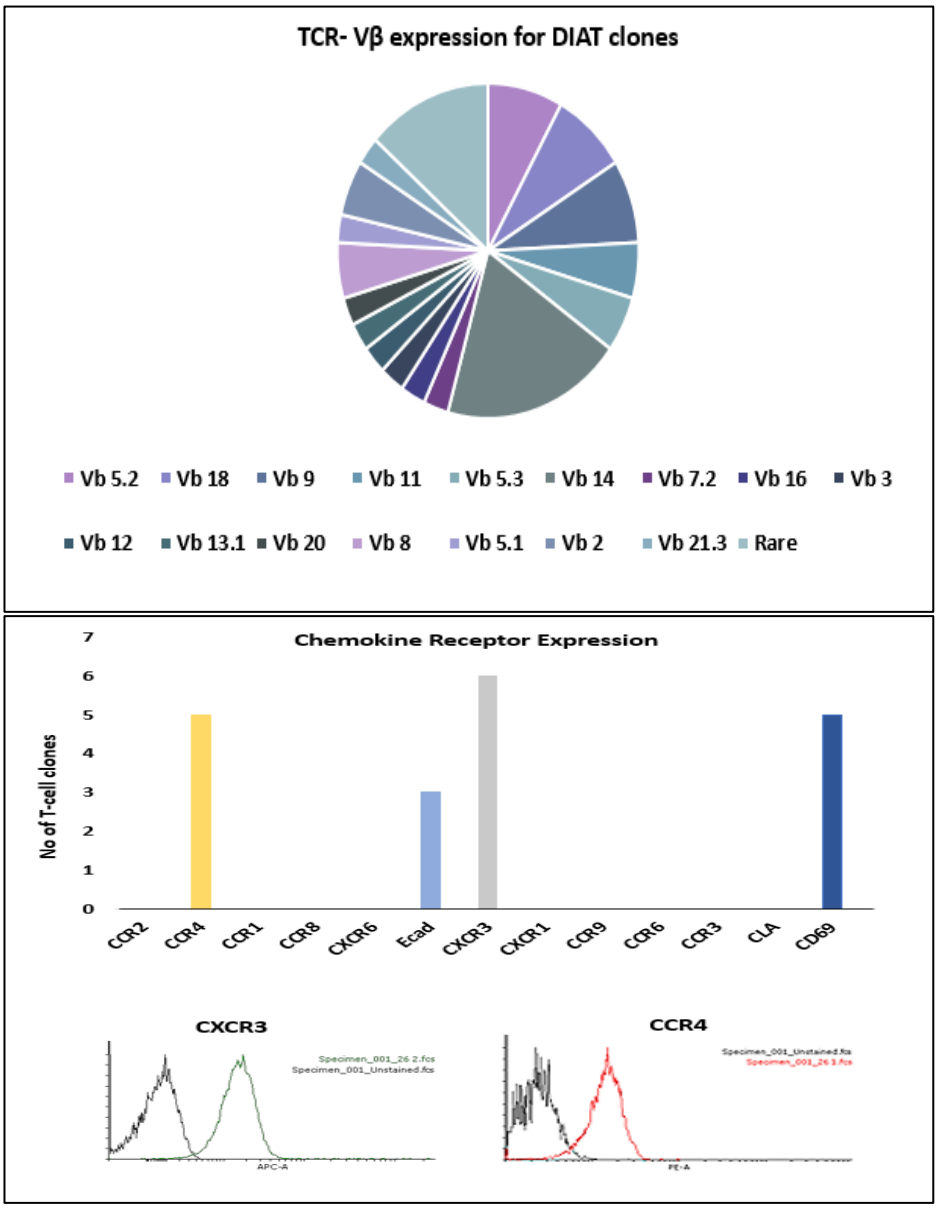
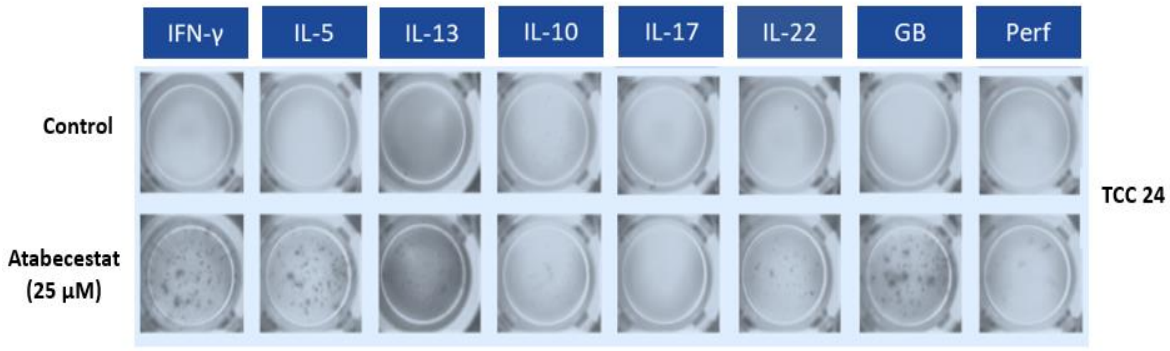



**Figure 4.6. Characterization of cellular surface markers of atabecestat-(metabolite) T-cell clones.** CD4/8 phenotype of T-cell clones. T-cells were stained with antibodies CD4-FITC/APC and CD8-PE and analysed for phenotype using flow cytometry. CD4/CD8/DP (double positive) clones expressed high levels of CD4 and CD8. (C) T-cells were stained with T-cell Vβ receptor antibodies. Cells were then washed and analysed for TCRVβ expression using flow cytometry. Rare refers to clones where V β receptor was not detected. (d) Cells were stained with antibodies CCR2, CXCR3, CCR1, CCR8, CCR9, CXCR1, CLA, CCR6, CXCR6, CD69, E-cadherin and CCR4. Cells were then washed and analysed for surface receptor expression using flow cytometry.

### 4.4.5. CHARACTERISATION OF CYTOKINE AND CYTOLYTIC MOLECULES IN RESPONSE TO ATABECESTAT-(METABOLITES).

Secretion of cytokines and cytolytic molecules from one atabecestat- and five DIAT-responsive T-cell clones was assayed by ELIspot. Secretion of cytokines IFN-γ and IL-13 were detected from all clones. Three clones secreted IL-10 and IL-5, while low levels of IL-22 was also detected in three clones. IL-17 secretion was not detected. Secretion of granzyme B and perforin was detected following study compound treatment (Figure 4.7).

**Atabecestat responsive clone**



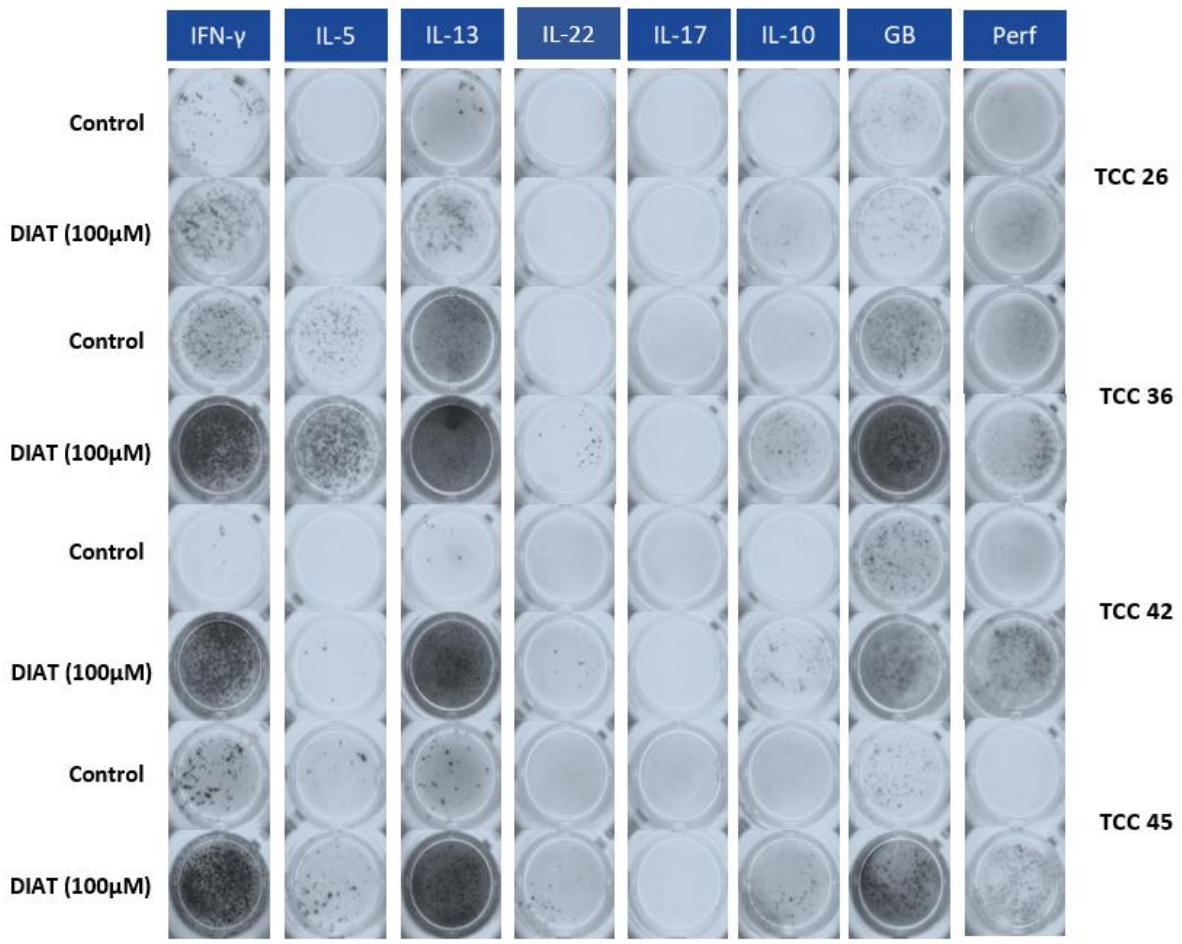



**Figure 4.7. Secretion of cytokines and cytolytic molecules from atabecestat-(metabolite) T-cell clones.** T-cell clones were co-incubated with autologous EBV-transformed antigen presenting cells in the presence and absence of atabecestat (25µM) or DIAT (100µM) in an ELIspot plate coated with antibodies for IFN-γ, granzyme B (GB), IL-5, IL-13, IL-10, IL-17, IL-22 and perforin for 48 hours. Following incubation, the ELIspot plates were developed for the respective cytokines.

### 4.4.6. ATABECESTAT- (METABOLITE) SPECIFIC CD4+ T-CELLS INTERACT WITH DRUG IN THE CONTEXT OF HLA-DR.

To assess HLA dependency and class restriction of the CD4+ T-cell clone responses, HLA blocking studies were conducted. All CD4+ clones responsive to atabecestat and DIAT demonstrated MHC class II HLA-DR dependency for T-cell activation (Figure 4.8).

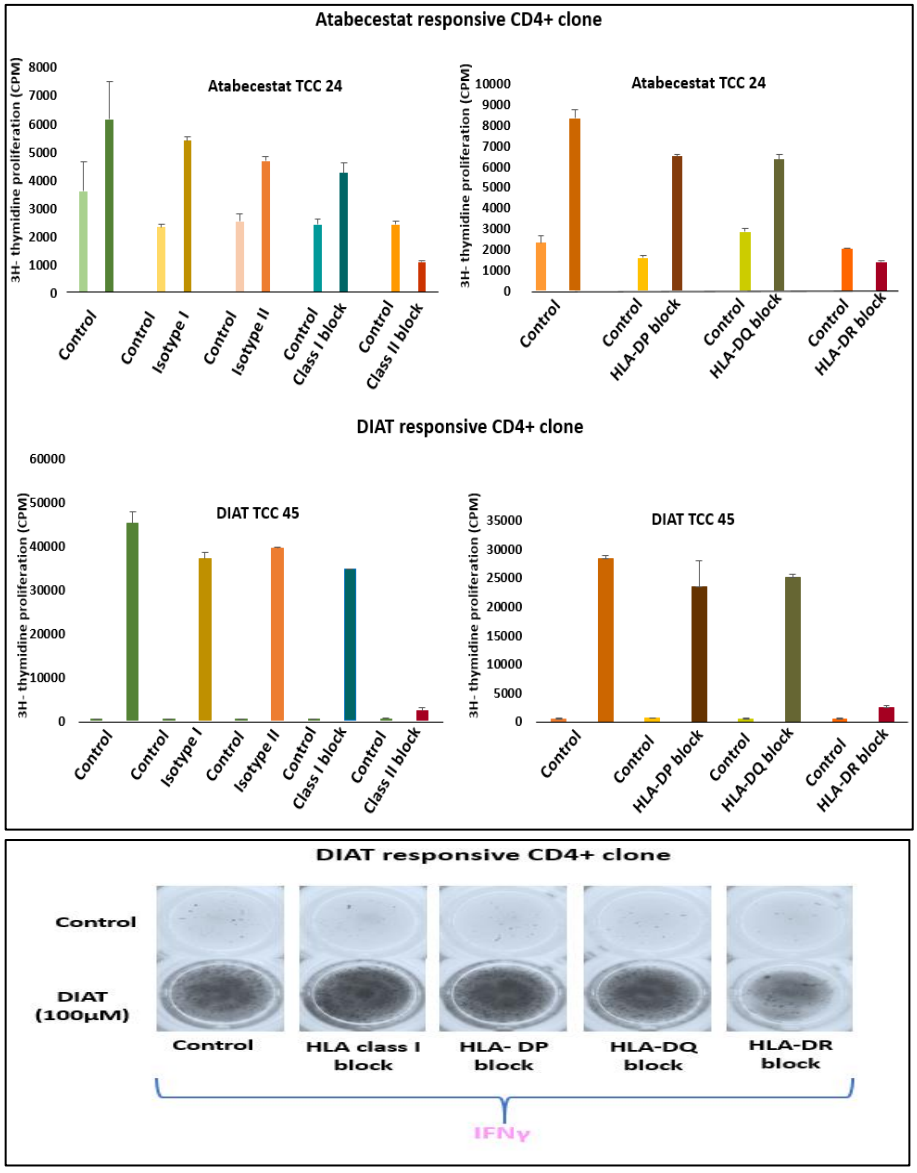


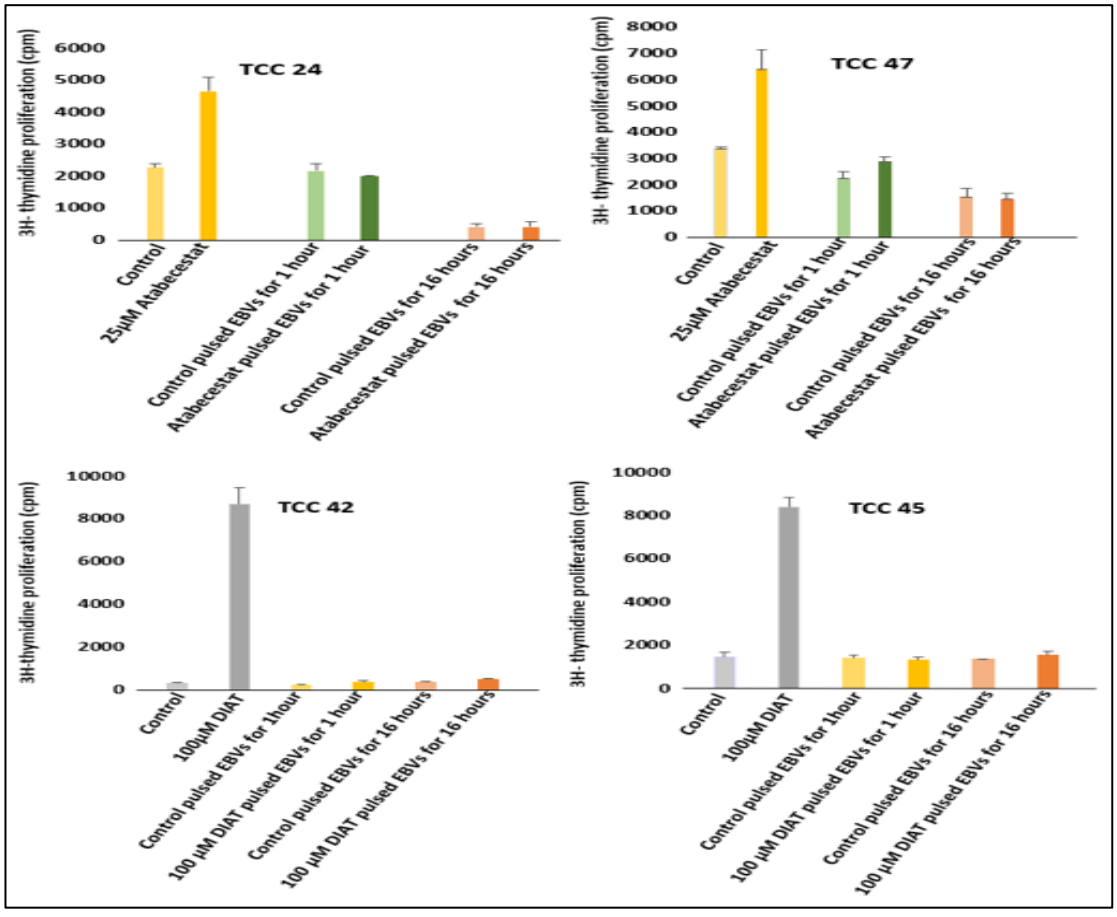

**Figure 4.8. HLA restriction of atabecestat-(metabolite) T-cell clones.** T-cell clones were co-incubated with autologous EBV-transformed antigen presenting cells in the presence and absence of atabecestat (25µM) or DIAT (100µM) for 48 hours prior to addition of the drugs, blocking antibodies; Isotype I, Isotype II, HLA Class I and HLA Class II were co-incubated with antigen presenting cells and T-cell clones for 1 hour. The assay was also conducted using HLA Class II sub-class-specific antibodies HLA-DP, DQ and DR. T-cell responses were quantified via

analysis of **(A)** proliferation using [$^{3}$H] thymidine and scintillation counting or **(B)** IFN-γ secretion by way of ELIspot.

### 4.4.7 ASSESSMENT OF PATHWAYS OF ANTIGEN PRESENTATION DURING THE ACTIVATION OF ATABECESTAT AND ATABECESTAT METABOLITE T CELL CLONES.

The pathway of atabecestat- and DIAT-specific T-cell activation was assessed using antigen presenting cell pulsing and glutaraldehyde fixation assays. T-cell clones exhibited no T-cell activation in the presence of antigen presenting cells pulsed with study compounds for 1 or 16 hours. The small increase in proliferation observed with antigen presenting cells pulsed with atabecestat for 1 hour was not significant. Conversely, T-cell activity was observed, in a reduced capacity, to atabecestat and DIAT in the presence of glutaraldehyde-fixed antigen presenting cells, but not in the absence of antigen presenting cells (Figure 4.9).

**A**



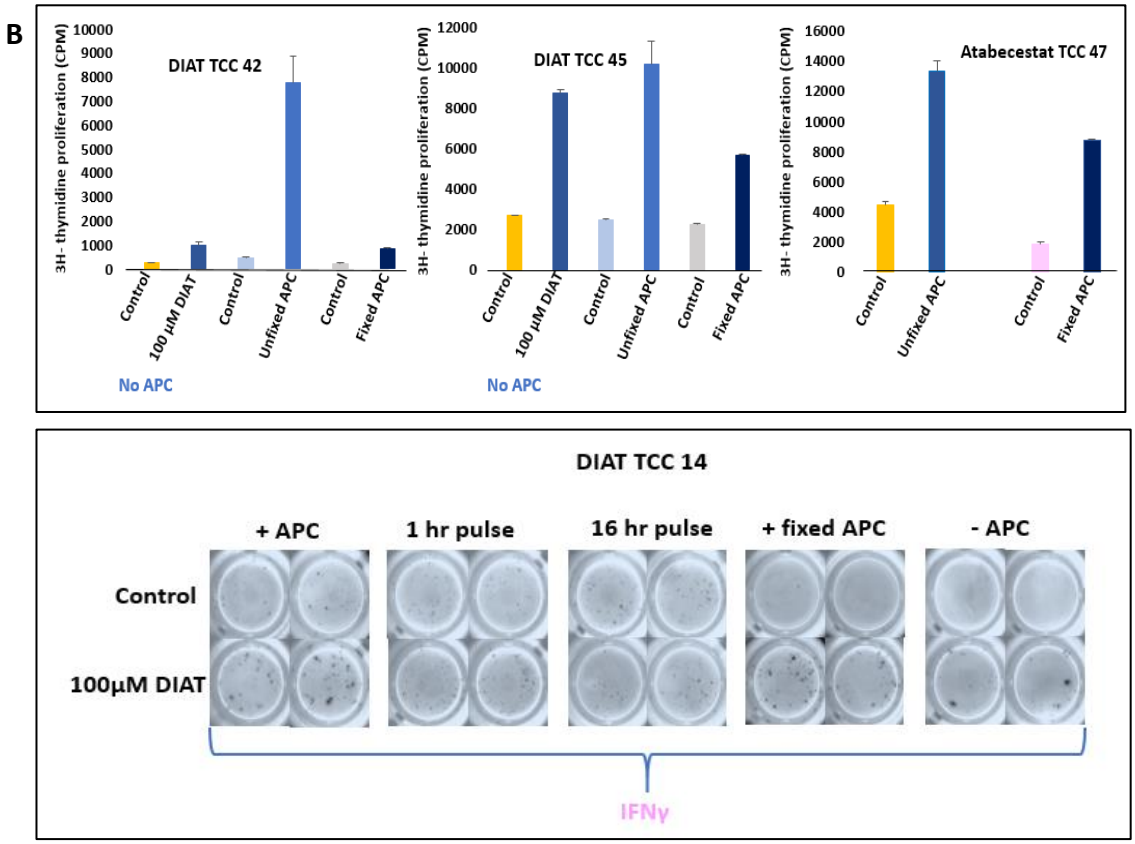



**Figure 4.9. Mechanistic studies of antigen presentation with atabecestat-(metabolite) responsive T-cells. A)** T-cell clones were co-incubated with autologous EBV-transformed B-cells pre-pulsed with 25 µM atabecestat (green and brown columns) or 100 µM DIAT (yellow and brown columns) for 1 or 16 hours. The study compound-pulsed EBV-transformed B-cells were washed repeatedly to remove free compound prior to culturing with T-cells. Atabecestat and DIAT were used as appositive control (yellow and grey columns respectively. **B)** T-cell clones were incubated with atabecestat (25µM) or DIAT (100µM) in the presence or absence of autologous EBV-transformed B-cells (APC) fixed with glutaraldehyde to prevent protein processing. T-cell responses were measured either via analysis of proliferation through addition of [$^3$H] thymidine and scintillation counting or through secretion of IFN-γ via ELIspot. A students' t test was used to compare treated wells with control wells and accepting $P < .05$ as significant.

### 4.4.8. ATABECESTAT-(METABOLITE) SPECIFIC T-CELL CLONES DISPLAY DISTINCT PATTERNS OF CROSS- REACTIVITY.

The cross-reactivity profiles of T cell clones were measured via co-incubation with EBV-transformed B-cells and the 4 study compounds and assessment of proliferative responses or IFN-γ release. All T cell clones generated to atabecestat yielded no

cross-reactivity with DIAT, while 3/4 clones displayed proliferative responses with acetyl DIAT. A weak proliferative response was observed with atabecestat epoxide in one clone (result not shown). DIAT-responsive T cell clones exhibited no cross-reactivity with atabecestat or atabecestat epoxide. However, 3/6 DIAT-responsive T-cell clones from various patients exhibited cross-reactivity towards acetyl DIAT. Figures 4.10 and 4.11 shows representative clones displaying the different cross-reactivity profiles (atabecestat-specific, DIAT-specific and cross-reactive). All three acetyl DIAT-responsive clones exhibited strong cross-reactivity towards the DIAT metabolite. One of these clones also cross-reacted with atabecestat (Figures 4.10 and 4.11).

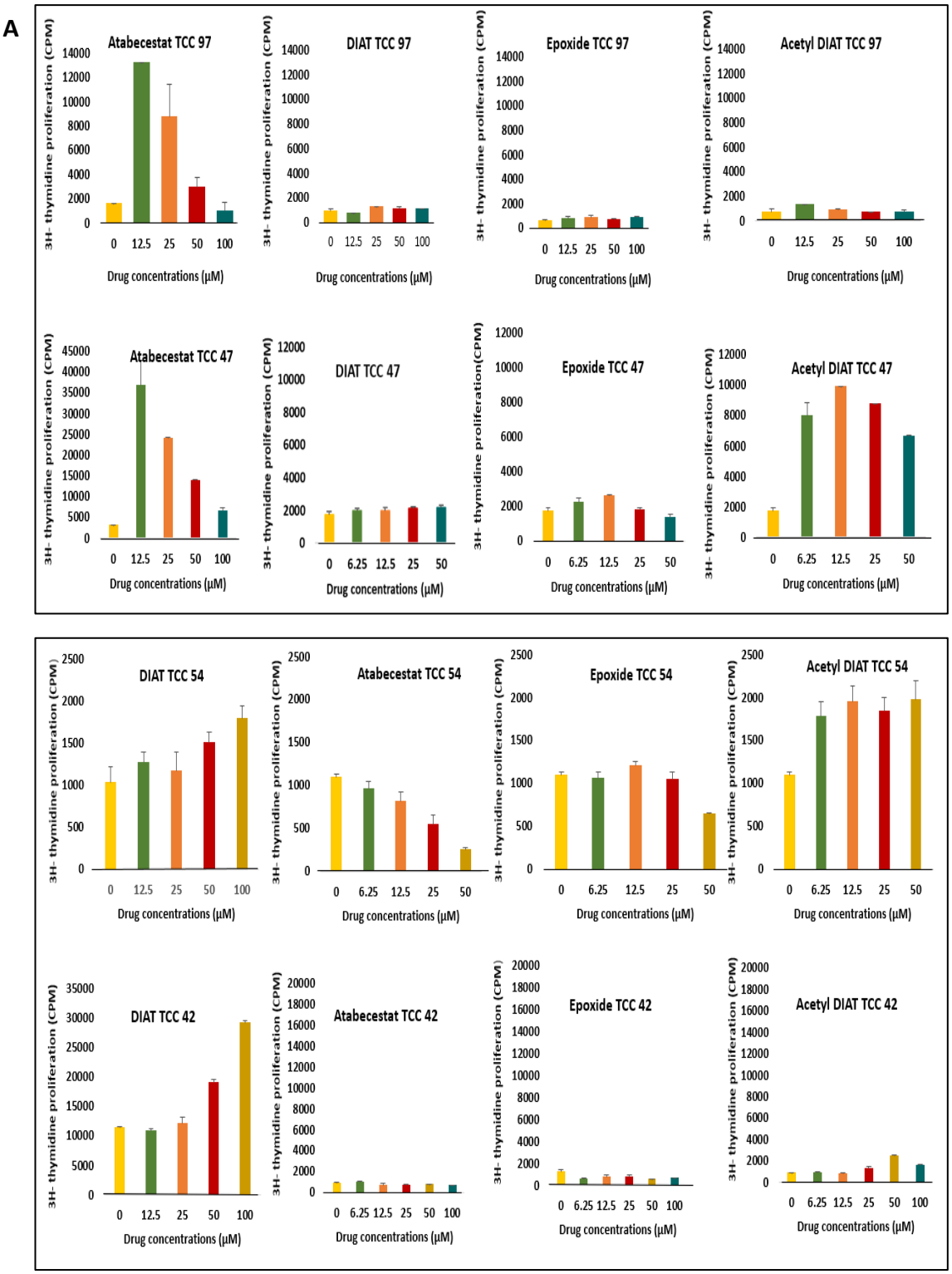



**Figure 4.10. Cross-reactivity profiles of atabecestat-(metabolite) specific T-cells. DIAT responsive Acetyl DIAT cross- reactive. A)** T-cell clones were co-incubated with autologous EBV-transformed B-cells in the presence and absence atabecestat, DIAT, atabecestat epoxide and acetyl DIAT for 48 hours. [$^{3}$H] thymidine was added for an additional 16 hours and proliferation was measured via scintillation counting. Error bars denote ± SEM.

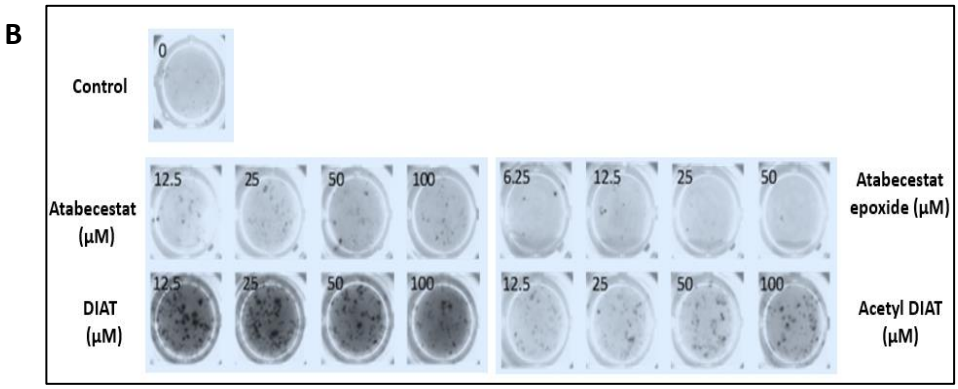



**Figure 4.11. Cross-reactivity profiles of atabecestat-(metabolite) specific T-cells. Acetyl DIAT- responsive T-cell clone.** IFN-γ secretion from study compound stimulated T-cell clones. T-cell clones were co-incubated with autologous EBV-transformed B-cells in the presence and absence of atabecestat, DIAT, atabecestat epoxide and acetyl DIAT in ELIspot plate for 48 hours. Following incubation, the ELIspot was developed for IFN-γ.

## 4.5 DISCUSSION

The use of T-cell diagnostic assays and T-cell cloning have successfully identified the role of T cells in the mediation of hypersensitivity reactions to a host of therapeutics including abacavir, carbamazepine, allopurinol, flucloxacillin (Bell et al., 2013, Lichtenfels et al., 2014, Yaseen et al., 2015) and as shown in chapter 3, naproxen. Indeed, in the previous chapter, we successfully characterised the role of the desmethyl metabolite of naproxen in driving the T cell responses observed in naproxen-induced DILI cases, contrary to the FDA warning pertaining to acyl-glucoridation of NSAIDs (Miyashita et al., 2014).

In this chapter, we focused on the BACE inhibitor atabecestat, which functioned to reduce the synthesis of amyloid plaques, a significant feature of AD. Phase I studies were conducted to atabecestat in amyloid positive Caucasian and Japanese patients. Four-week treatment with atabecestat resulted in a mean reduction of 67% in cerebrospinal fluid-Aβ (Sanabria-Castro et al., 2017, Burkhart et al., 2001) in both the Caucasian and Japanese patients, with a reduction of up to 90% in some patients (Timmers et al., 2018). Following this, phase 2/3 studies were conducted, with a plan to enrol up to 1650 patients at risk of dementia with a primary endpoint being the slowing of cognitive decline in those patients receiving either 10 mg (decreased to 5mg after 11 participants received 10 mg to increase safety margin between low and high doses) or 25 mg of the drug compared to placebo. However, in May 2018 the atabecestat program was discontinued based on the detection of elevated liver enzymes. Of the 475 patients exposed to atabecestat, approximately 24% had ALT/AST of >1.5 x ULN, and 11% had ALT elevations >3x ULN. The evolution of these elevations was heterogeneous and some elevations were transient while patients

remained on treatment. The patients with liver injury showed no signs of skin rash and/or eosinophilia. Interestingly, the dose of atabecestat (10-25 mg) is relatively low for a DILI causing drug and a genetic association with HLA has not been identified. Furthermore, the time to onset of raised liver enzymes (32-344 days; Table 4.1) is significantly longer than that associated with skin reactions such as maculopapular exanthema.

To investigate the involvement of the immune system in subjects who experienced elevated liver enzymes whilst receiving atabecestat, PBMC samples were collected, and T cells were cloned from atabecestat- and atabecestat metabolite-exposed PBMC lines for phenotypic and functional assessment. On the whole, diagnostic assays conducted on the PBMC of patients were negative by way of proliferation and secretion of cytokines and effector molecules. One exception was patient 6, which noted a subtle increase in secretion of granzyme B in response to the primary metabolite of atabecestat; DIAT (Figure 4.2). Patient 6 showed persistent liver enzyme elevations even one month after cessation of atabecestat treatment. Furthermore, a liver biopsy from this patient indicated a zone 3-predominant necro inflammatory process, with lymphocyte infiltration (De Jonghe et al., 2020).

Eight patient PBMC were subjected to T-cell cloning. Study compound-responsive clones were generated from five of these patients with the greatest number stemming from patient 6. Generation of atabecestat-responsive T cell clones were much more uncommon than seen with the DIAT metabolite, which may suggest a lower T-cell precursor frequency. Furthermore, no confirmed T cell clones were generated to the minor epoxide metabolite of atabecestat suggesting that if

atabecestat epoxide-responsive clones exist, their precursor frequency must be very low. In total atabecestat-responsive clones were generated from two of the patients PBMC to undergo T cell cloning. One of the atabecestat-responsive clones exhibited a CD8+ phenotype in contrast with all other generated clones which were CD4+. DIAT-responsive clones were detected in 5 patients, 8 responsive clones displayed a CD4+ phenotype, while 3 responsive clones shown CD8+, and one responsive clone presented mixture of CD4+ and CD8+.

Atabecestat- and DIAT-responsive clones exhibited a varied expression of TCR-Vβ with the majority expressing a rare TCR not detected by the antibody repertoire. Given the differential reactivity profiles of the clones it is unlikely that TCR expression is conserved across the clones. For example, two DIAT responsive T cell clones expressing rare TCR-Vβs exhibited cross-reactivity towards other study compounds, while two additional clones from the same patient exhibited no cross-reactivity.

Stimulation of clones with atabecestat or DIAT resulted in the secretion of the Th1 cytokine IFN-γ and Th2 cytokines IL-5 and IL-13. Furthermore, IL-10 and IL-22 were secreted from a more restricted number of study compound activated clones. Secretion of Th1 and Th2 cytokines, alongside IL-22 is a common feature for drug-responsive clones isolated from patients presenting with skin and liver conditions (Meng et al., 2017, Zhao et al., 2019, Kim et al., 2015). IL-10 secretion is observed less frequently. IL-10 is predominantly immunoregulatory, ameliorating excessive and potentially pathogenic Th1 and CD8+ T cell responses (Couper et al., 2008). The activated clones also secreted effector molecules perforin and granzyme B. These molecules act together, with perforin inserting pores into neighbouring cells to

permit the delivery of granzyme B, which cleaves caspases triggering the apoptotic cascade (Demers et al., 2013).

All tested atabecestat- and DIAT-responsive CD4+ clones were activated in a manner restricted to MHC Class II protein, namely HLA-DR. Antigen presenting cell pulsing experiments were conducted to characterise pathway(s) of atabecestat and DIAT-specific CD4+ T-cell activation. Antigen presenting cells were pulsed with study compounds for 1 or 16 hours prior to repeated washing to remove soluble and weakly associated compound prior to culturing the antigen presenting cells with T cells. Antigen presenting cells pulsed with protein-reactive compounds (e.g., flucloxacillin, nitroso sulfamethoxazole (Kim et al., 2014, Castrejon et al., 2010, Wuillemin et al., 2013) or compounds that associate strongly with HLA (e.g., abacavir (Adam et al., 2012, Illing et al., 2012, Ostrov et al., 2012) activate T cell responses, while compounds that associate weakly with HLA via reversible binding interactions (e.g., carbamazepine (Castrejon et al., 2010, Burkhart et al., 2001, Wu et al., 2006a, Schnyder et al., 2000b) do not. All atabecestat- or DIAT-responsive clones were not activated with study compound-pulsed antigen presenting cells. This indicates that the clones are stimulated with atabecestat and DIAT via a non-covalent pharmacological binding interaction with HLA-DR molecules. Activation of T-cells in the presence of glutaraldehyde fixed antigen presenting cells was also observed, which is indicative of a direct interaction of atabecestat and DIAT with HLA-DR molecules in a manner independent of antigen processing. Fixation blocks protein processing and inhibits T cell responses to protein antigens, but not antigenic peptides that bind directly to surface HLA molecules (Schnyder et al., 2000b, Wu et al., 2006a, Burkhart et al., 2001, Yaseen et al., 2015, Zanni et al., 1998, Nassif et al.,

2004). Collectively, these findings are in agreement with the p-I concept proposed by Pichler (Pichler, 2019). In essence, atabecestat and its metabolites have an undesirable (off-target) pharmacological interaction with immune receptors. The p-I concept also argues that certain drugs may bind initially to highly variable TCRs and that HLA proteins provide additional signals for T-cell activation.

One interesting finding was the cross-reactivity profile of all clones which demonstrated strong T cell activity towards the acetyl DIAT metabolite and only weak to no T-cell reactivity towards the other metabolites. Based on these findings, clones were generated from PBMC cultured with acetyl DIAT for further assessment of cross-reactivity. Interestingly, acetyl DIAT responsive clones displayed cross-reactivity with DIAT and in one clone atabecestat and DIAT (Figure 4.10). This further emphasizes the likelihood of T cell reactions being mediated by a metabolite of atabecestat.

In this chapter we successfully identified and characterised the role of T-cells in the DILI reactions observed in patients exposed to the BACE inhibitor atabecestat. Furthermore, using our established T cell characterization methods we show atabecestat metabolites are the likely primary drivers of the immune response without excluding the parent drug. While our findings are taken from a small number of clones, we conclude that the DILI reactions experienced in trial patients are at least in part mediated by a CD4+ immune response. Similar to data presented in the previous chapter, clones from patients with DILI were preferentially activated with phase I metabolite of the parent drug. This highlights the need to study all available derivatives of the drug to define mechanisms of the adverse event and in these cases pathways of drug-specific T cell activation.

# CHAPTER 5: EXPLORATION OF WHETHER DRUG SPECIFIC T CELL RESPONSES ARE DETECTABLE IN PBMCs FROM PATIENTS WITH LIVER INJURY SELECTED FROM THE DILIN AND DILIGEN NETWORKS.

## CONTENTS.

## 5.1 INTRODUCTION

### 5.1 DRUG-INDUCED LIVER INJURY AND THE DILIN NETWORK.

Certain forms of drug-induced liver injury can be classified as a form of drug allergy, especially when drug-specific activation of the immune system is detected. The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) established the Drug-Induced Liver Injury Network (DILIN) in 2003 to advance understanding and research into liver injury by selecting five clinical sites and a data coordinating centre that had submitted competitive grant applications. The aim of the prospective DILIN study was to identify a large number of patients with DILI that allowed for collection of epidemiological data and biological samples for future mechanistic studies. The network created a registry and tissue bank from people who had experienced liver injury due to prescription and over-the-counter drugs, as well as herbal products. Since the history of DILI is not well understood, all of the DILI cases were re-evaluated 6 months after enrolment.

Professor Naisbitt (Liverpool, UK) and Professor Bonkovsky (USA) applied to the DILIN Network in 2016 to acquire cryopreserved PBMC samples from patients with suspected minocycline-, isoniazid-, sulfamethoxazole- and amoxicillin-induced liver injury to explore whether drug-responsive T-cells were detectable, and if so, characterise the cellular pathophysiology of the reactions. This chapter provides an overview of the pharmacology and adverse events associated with the four test compounds and the results of the cellular immunological investigations.

## 5.2 CHAPTER AIMS

The aim of this chapter was to explore whether drug-specific T-cell responses are detectable in any or all cases of drug-induced liver injury using PBMCs collected through DILIN. T-cells were cloned to characterise the drug-specific cellular response in terms of phenotype and function and for mechanistic studies to define HLA-restriction and cross-reactivity.

## 5.3 METHODS

### 5.3.1 REPEATED METHODS

The following methods have been used in this chapter which are prescribed in more detail in the materials and methods chapter.

- TOXICITY TEST.
- LYMPHOCYTE TRANSFORMATION TEST (2.7.4).
- T-CELL CLONING (2.7.6).
- QUANTITIVE AND QUALITITIVE ASSESSMENT OF CLONE RESPONSE AND REACTIVITY TO DRUG (2.7.6.1 and 2.7.6.2).
- ELISPOT ASSAY (2.7.10).
- FLOWCYTOMETRIC PHENOTYPING OF T-CELLS (2.7.11).
- ASSESSMENT OF MHC RESTRICTION OF ANTIGEN PRESENTING CELLS (2.7.15).
- INVISTIGATION OF ANTIGEN PROCESSING OF T-CELLS (2.7.14.1, 2.7.14.2, 2.7.15).

## 5.4 RESULTS

### 5.4.1 TOXICITY TEST.

A PBMC toxicity assay was performed on healthy HLA- B*35:02 positive donors to determine the toxic concentration of minocycline. Minocycline concentrations were diluted on a log scale between 0.1 - 3000 µM. Cellular toxicity was analysed via measurement of inhibition of mitogen-induced T-cell proliferation. Cells tolerate minocycline at up to 1.5µM.

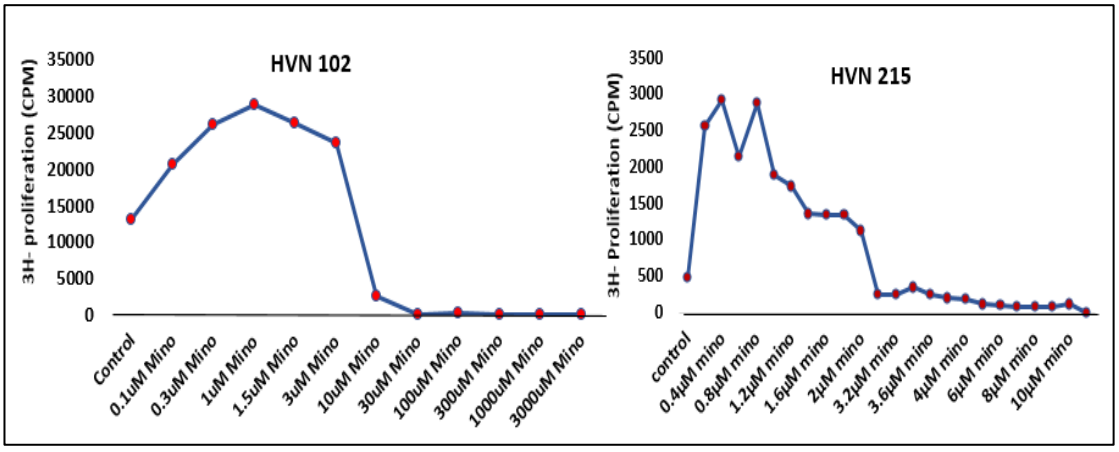



**Figure 5.1. Minocycline (Mino) Toxicity of PBMC from HLA-B*35:02 positive healthy donors.** PBMCS were plated at 150,000 cells/well and incubated with drug at a range of concentrations (0.1 µM-3000 µM) for 4 days. On day 4, PHA was added to all wells to stimulate the cells. [3H]-thymidine (0.5 µCi) was added for the final 16 hours before harvesting and analysis.

### 5.4.2 LYMPHOCYTE TRANSFORMATION TEST (LTT) WITH HLA- B*35:02 POSITIVE HEALTHY DONOR PBMC AND WITH PBMC FROM DILIN PATIENTS.

Nineteen donor PBMCs were identified through the DILIN network, one donor PBMC from healthy volunteers who expressed HLA B*35:02 were also used in the analysis, and one donor PBMCs were identified from DILIGEN. Of these, 11 donors were identified as minocycline DILI cases. The minocycline concentrations used in the lymphocyte transformation test were chosen based on the results of toxicity tests

(0.5-2.5 µM Minocycline). Three patients developed bactrim (combination of trimethoprim and sulfamethoxazole)-induced DILI, three developed isoniazid-induced DILI and three developed co-amoxiclav-induced DILI. Concentrations of these drugs to be used in the lymphocyte transformation test had previously been defined. All DILIN patient PBMCs were defrosted, counted and due to low cell viability numbers, lymphocyte transformation tests were conducted with between 30,000 and 150,000 calls per well (Table 5.1).

Drug-specific PBMC showed proliferative response with DILIN minocycline patient 10 and with DILIGEN minocycline patient 11. Also, drug specific PBMC proliferation was detected with DILIN sulfamethoxazole nitroso patients 1 and 2, and DILIN isoniazid patients 1 and 2. With certain patients a SI of 2 or above was only detected at one drug concentration (Figure 5.2). All other DILI patients including the 3 co-amoxiclav PBMC patients displayed no proliferation in the presence of the studied compounds. The proliferative responses of PBMC from DILI patients and healthy volunteers who expressed HLA B*35:02 positive are summarised in table 5.2. Secretion of IFN-γ and IL13 was not detected when PBMC from the DILI patients were cultured with suspect drugs in Elispot (Table 5.2). IFN-γ and IL13 secretion was detected with the positive control PHA. These largely negative data may be due to the viability of the cryopreserved PBMC, the length of storage or a very low drug-specific T cell precursor frequency. Thus, to investigate whether drug-specific T-cells were detectable, PBMC bulk cultures were serially diluted, and T cell clones were generated and tested for drug specificity.

**Table 5.1: Viability of PBMCs from DILIN patient samples.**

| Minocycline | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Patient ID | K0110981 | K106793 | K38970 | K44757 | K72380 | K73893 | K75883 | K84859 | K88468 | K96681 |
| Viable cell count | $2.4 \times 10^6$ | $3.14 \times 10^6$ | $4.42 \times 10^6$ | $4.54 \times 10^6$ | $2.68 \times 10^6$ | $2.5 \times 10^6$ | $2.04 \times 10^6$ | $4.92 \times 10^6$ | $2.72 \times 10^6$ | $1.98 \times 10^6$ |
| % Viability | 82% | 85% | 80% | 84% | 91% | 78% | 84% | 85% | 83% | 77% |
| Cloning possibility | Unlikely | Unlikely | Possible | Possible | Possible | Unlikely | Unlikely | Possible | Unlikely | Unlikely |

| SMX-NO | P1 | P2 | P3 | Isoniazid | P1 | P2 | P3 | Co-amoxiclav | P1 | P2 | P3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient ID | K74259 | K75357 | K86456 | Patient ID | K17546 | K15579 | K855420 | Patient ID | K44848 | K17508 | K15124 |
| Viable Cell Count | $7 \times 10^6$ | $4.5 \times 10^6$ | $2.5 \times 10^6$ | Viable Cell Count | $3.58 \times 10^6$ | $2.75 \times 10^6$ | $1.89 \times 10^6$ | Viable Cell Count | $6.5 \times 10^6$ | $4.40 \times 10^6$ | $2.25 \times 10^6$ |
| % viability | 86.5% | 84% | 84% | % viability | 85% | 82% | 79% | % viability | 90% | 83% | 80% |
| Cloning Possibility | Yes | Possible | Unlikely | Cloning Possibility | Possible | Unlikely | Unlikely | Cloning Possibility | Yes | Possible | Unlikely |

**Table 5.2: Results for diagnostic tests for all patients in the study.**

| Patient ID | Drug | PBMC proliferation assay | PBMC Elispot assay | | Patient ID | Drug | PBMC proliferation assay | PBMC Elispot assay | |
|---|---|---|---|---|---|---|---|---|---|
| | | | IFNγ | IL13 | | | | IFNγ | IL13 |
| Patient 1 | Minocycline | -ve | n/a | n/a | Patient 1 | Bactrim | +ve | n/a | n/a |
| Patient 2 | Minocycline | -ve | n/a | n/a | Patient 2 | Bactrim | +ve | n/a | n/a |
| Patient 3 | Minocycline | -ve | n/a | n/a | Patient 3 | Bactrim | -ve | n/a | n/a |
| Patient 4 | Minocycline | -ve | -ve | n/a | Patient 1 | Isoniazid | +ve | n/a | n/a |
| Patient 5 | Minocycline | -ve | -ve | n/a | Patient 2 | Isoniazid | +ve | n/a | n/a |
| Patient 6 | Minocycline | -ve | n/a | n/a | Patient 3 | Isoniazid | -ve | n/a | n/a |
| Patient 7 | Minocycline | -ve | n/a | n/a | Patient 1 | CO-AMOXCILAV | -ve | -ve | -ve |
| Patient 8 | Minocycline | -ve | n/a | n/a | Patient 2 | CO-AMOXCILAV | -ve | -ve | -ve |
| Patient 9 | Minocycline | -ve | -ve | n/a | Patient 3 | CO-AMOXCILAV | -ve | -ve | -ve |
| Patient 10 | Minocycline | +ve | -ve | n/a | | | | | |
| Patient 11 | Minocycline | +ve | -ve | -ve | | | | | |
| HVN 220 | Minocycline | -ve | -ve | -ve | | | | | |

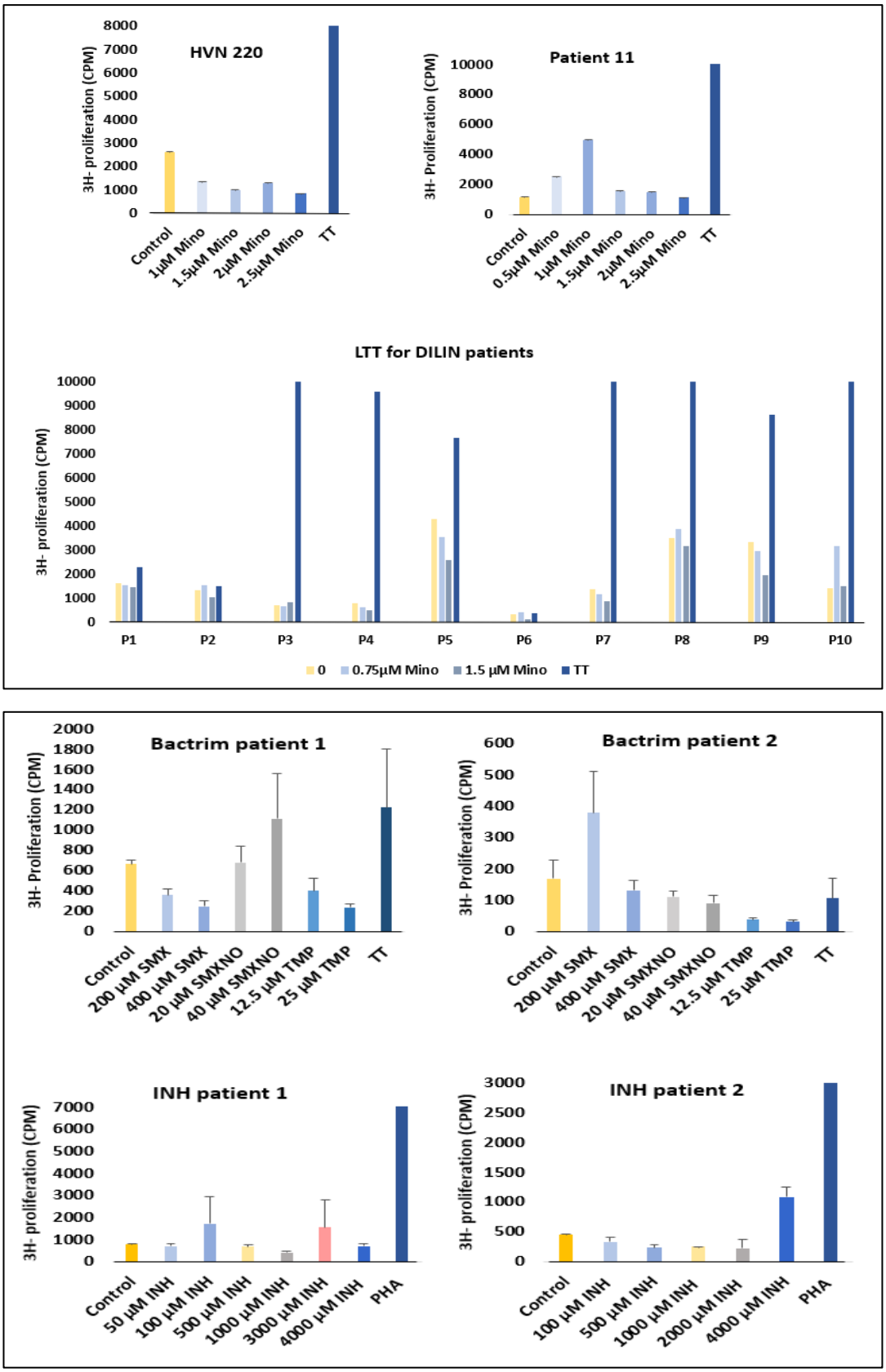



**Figure 5.2. Drug-specific activation of PBMCs from a healthy HLA-B*35:02 positive donor and minocycline (Mino), a combination of trimethoprim/sulfamethoxazole (TMP/SMX), isoniazid (INH) DILIN patients.** Lymphocyte transformation tests were performed for PBMCs

of twenty-one patients. PBMCs were cultured in triplicate (1.5x $10^5$ cells per well/100 µl) and incubated with graded concentrations of Mino (0.1 µM-3000µM), SMX (200 µM-400 µM), SMX-NO (20 µM-40 µM), TMP (12.5 µM-25µM, I added for a cross reactivity), and INH (50 µM-4000 µM). TT or PHA were used as positive controls (10 µg/ml**).** After a 6 days incubation, thymidine (0.5µCi 3H) was added and the cells were cultured for a further 16 hours. T cell proliferation was measured by scintillation counting. Statistical analysis was performed using The Mann-Whitney test to compare treated conditions with the untreated control at the same time point.

### 5.4.3 GENERATION OF DRUG SPECIFIC T- CELL CLONES.

Results to T-cell cloning are summarised in table 5.3. A total of 17, 54, 9, 57 and 6 T cell clones were generated to sulfamethoxazole nitroso, amoxicillin, clavulanic acid, minocycline and isoniazid respectively in patients. Six T-cell clones were generated to minocycline in a healthy volunteer. These clones were then subjected to the dose-response and mechanistic studies detailed below. The results of all patients tested are summarised in (Table 5.3). Figure 5.3 shows representative data from 6 patients.

**Table 5.3: Summary of specificity of T cell clones.**

| T –cell cloning to Minocycline | | | | |
|---|---|---|---|---|
| Patient ID | Tested number of clones | Number of drug responsive clones (1st test) | Number of drug specific clones (2nd test) | Percentage of responding clones (%) |
| Patient 4 | 482 | 24 | 4 | 0.83% |
| Patient 5 | 166 | 5 | 0 | - |
| Patient 8 | 397 | 13 | 1 | 0.25% |
| Patient 11 | 133 | 1 | 0 | - |
| Healthy donor | 386 | 14 | 4 | 1.04% |

| T –cell cloning to INH | | | | |
|---|---|---|---|---|
| Patient ID | Tested number of clones | Number of drug responsive clones (1st test) | Number of drug specific clones (2nd test) | Percentage of responding clones (%) |
| Patient 1 | 161 | 6 | 0 | - |
| Patient 2 | 11 | 0 | 0 | - |

| T –cell cloning to SMX-NO | | | | |
|---|---|---|---|---|
| Patient ID | Tested number of clones | Number of drug responsive clones (1st test) | Number of drug specific clones (2nd test) | Percentage of responding clones (%) |
| Patient 1 | 354 | 7 | 1 | 0.3 |
| Patient 11 | 148 | 10 | 0 | - |

| T –cell cloning to CO-AMOXICLAV | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Patient ID | Tested number of clones | | Number of drug responsive clones (1st test) | | Number of drug specific clones (2nd test) | | Percentage of responding clones (%) | |
| | Amox | Clav | Amox | Clav | Amox | Clav | Amox | Clav |
| Patient 1 | 209 | 17 | 44 | 4 | 20 | 1 | 20.4% | 23.5% |
| Patient 2 | 36 | 45 | 10 | 5 | 1 | 0 | 28.6% | 10.9% |
| Patient 3 | 32 | 11 | 0 | 0 | 0 | 0 | - | - |

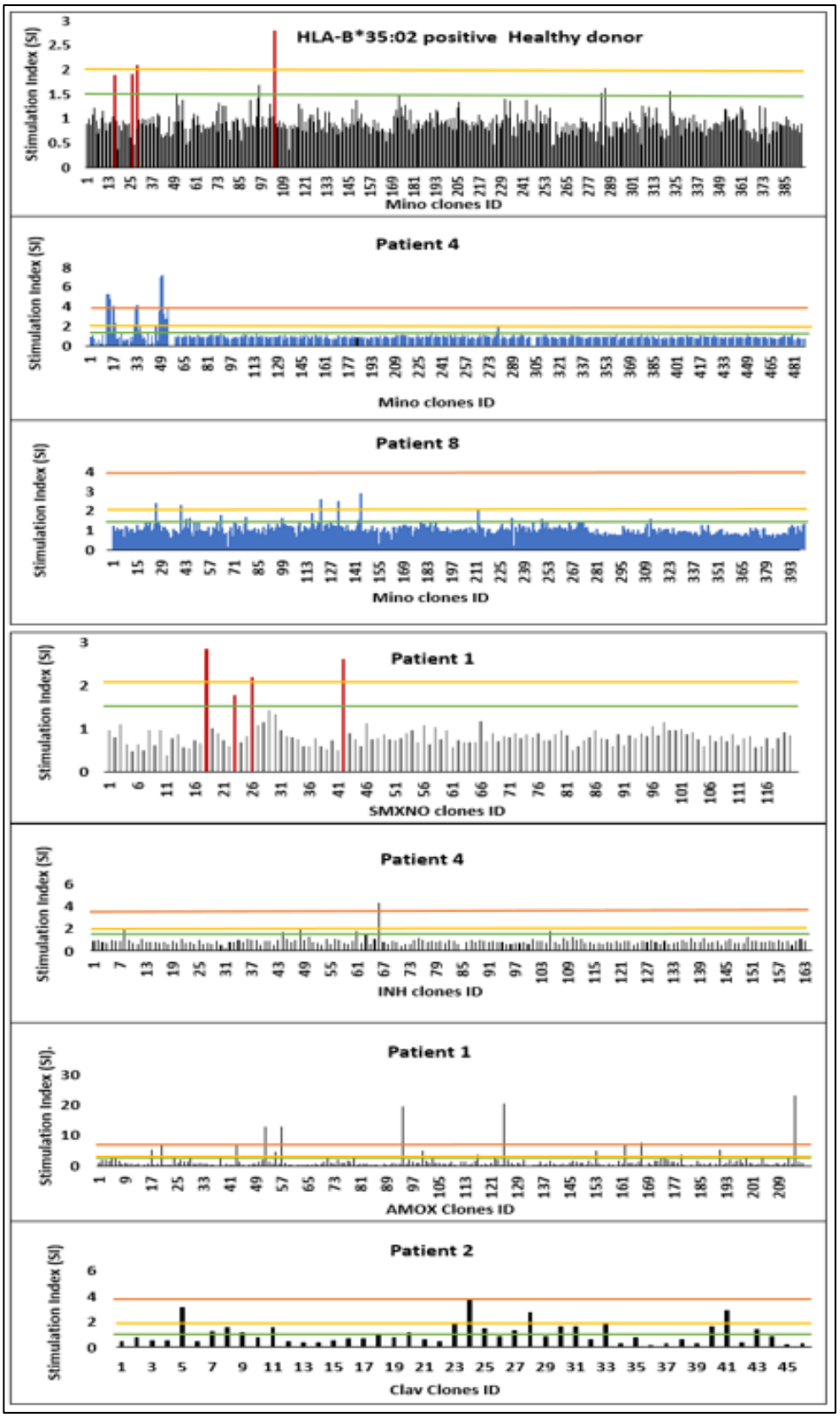



**Figure 5.3. Generation of minocycline (Mino), sulphamethoxasole nitroso (SMXNO), isoniazid (INH), and co-amoxiclav (Amox/Clav) specific T cell clones.** T cell clones ($5x10^4$cells per well/50µL) were cultured with irradiated EBVs ($1x10^4$cells per well/ 50µL) in the presence and absence of (1.5µM Mino), SMX-NO (40 µM), INH (4 mM), Amox (1mM) and Clav (1mM)

in a U-bottomed 96 well microplate for 48hrs (37°C; 5% $CO_2$). [3H]- thymidine incorporation (0.5µCi) was then added for the final 16 hours before harvesting to facilitate T cell proliferation. After harvesting, T cell proliferation was assessed using scintillation counting. Clones were analysed as stimulation index (SI) by dividing mean with study compounds by mean responses in control wells. An SI of 1.5 or above was considered as an antigen-reactive T-cell response (green, yellow and red lines) and was expanded for further study.

### 5.4.4 ANTIGEN SPECIFICITY OF T- CELL CLONES.

All T cell clones responsive in the first test were tested to the respective drugs in a dose dependent manner. A total of 1, 21, 1 and 9 T cell clones were confirmed responsive to sulfamethoxazole nitroso, amoxicillin, clavulanic acid and minocycline respectively. No T cell clones were confirmed responsive to isoniazid (Figure 5.4 A and B). Five T cell clones from DILI patients that were activated with amoxicillin in a dose-dependent manner are shown in Figure 5.5.

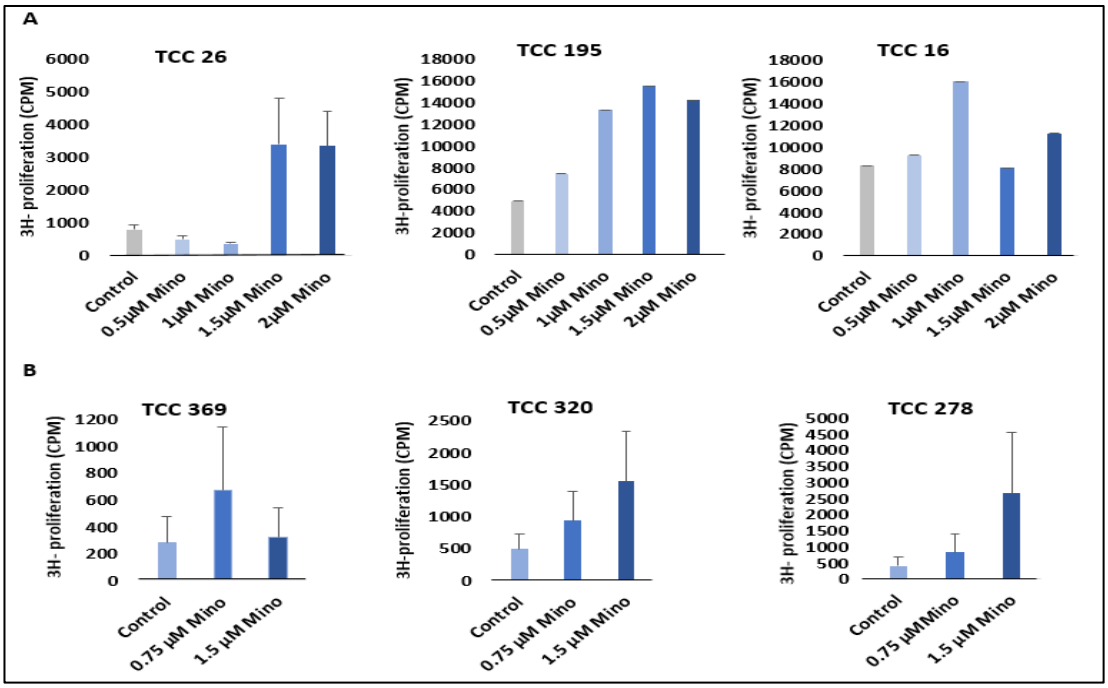



**Figure 5.4. Minocycline (Mino) activates T-cell clones in a drug concentrations dependent manner from (A) healthy volunteer who is HLA B*35:02 positive, and (B) T cell clones from DILIN network patients.** T cell clones ($5\times10^4$ cells/50 µL) were co-cultured with irradiated autologous EBVs ($1x10^4$ cells/50 µL) with graded Mino concentrations (0.5- 2µM) using a 96-well U-bottom microplate. Cell culture media was used as a negative control. The plates were then incubated for 48 hr in 37°C and 5% CO2. [3H] thymidine (0.5 µCi) was added for 16 hours

and T cell proliferation was measured using scintillation counting. Statistical analysis was performed using the students' t test to compare treated conditions with the untreated control at the same time point. A p-value < 0.05 was considered significant.

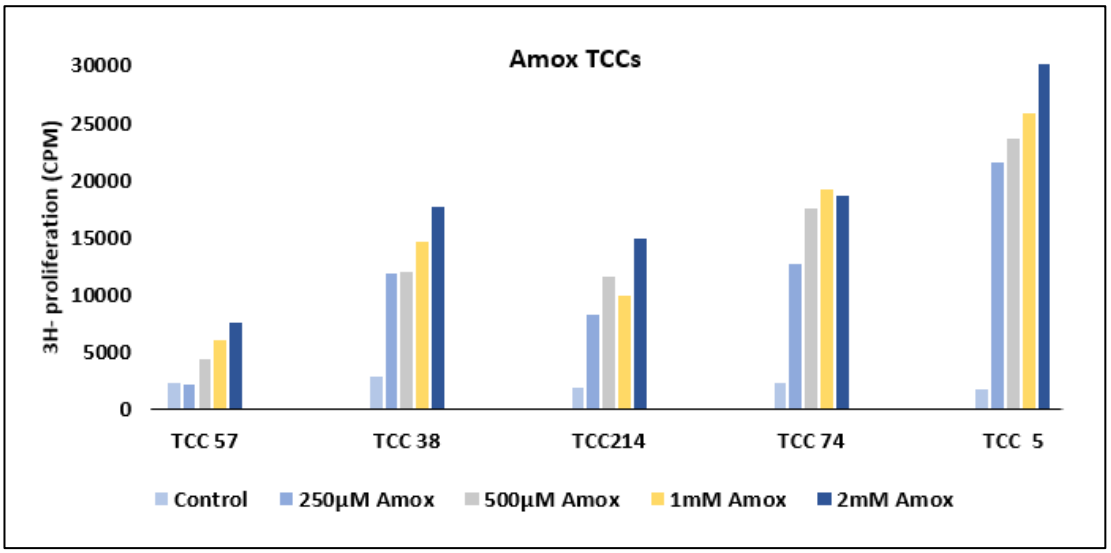



**Figure 5.5. Amoxicillin (Amox) activates T-cell clones in a drug concentrations dependent manner.**

### 5.4.5 ANTIGEN PRESENTING CELL DEPENDENT ACTIVATION OF RESPONSIVE T CELL CLONES.

The dependence of the activation of drug specific T cell clones on antigen-presenting cells was evaluated by incubation of T cell clones in the absence of EBV transformed B-cells (EBVs) with ranged concentrations of study drugs. The one available sulfamethoxazole nitroso-responsive clone was not activated with sulfamethoxazole nitroso in the absence of antigen presenting cells. In contrast all five amoxicillin clones tested displayed proliferation with and without EBVs and the strength of the induced response was similar i.e. these T cell clones can self-present amoxicillin to each other (Figure 5.6).

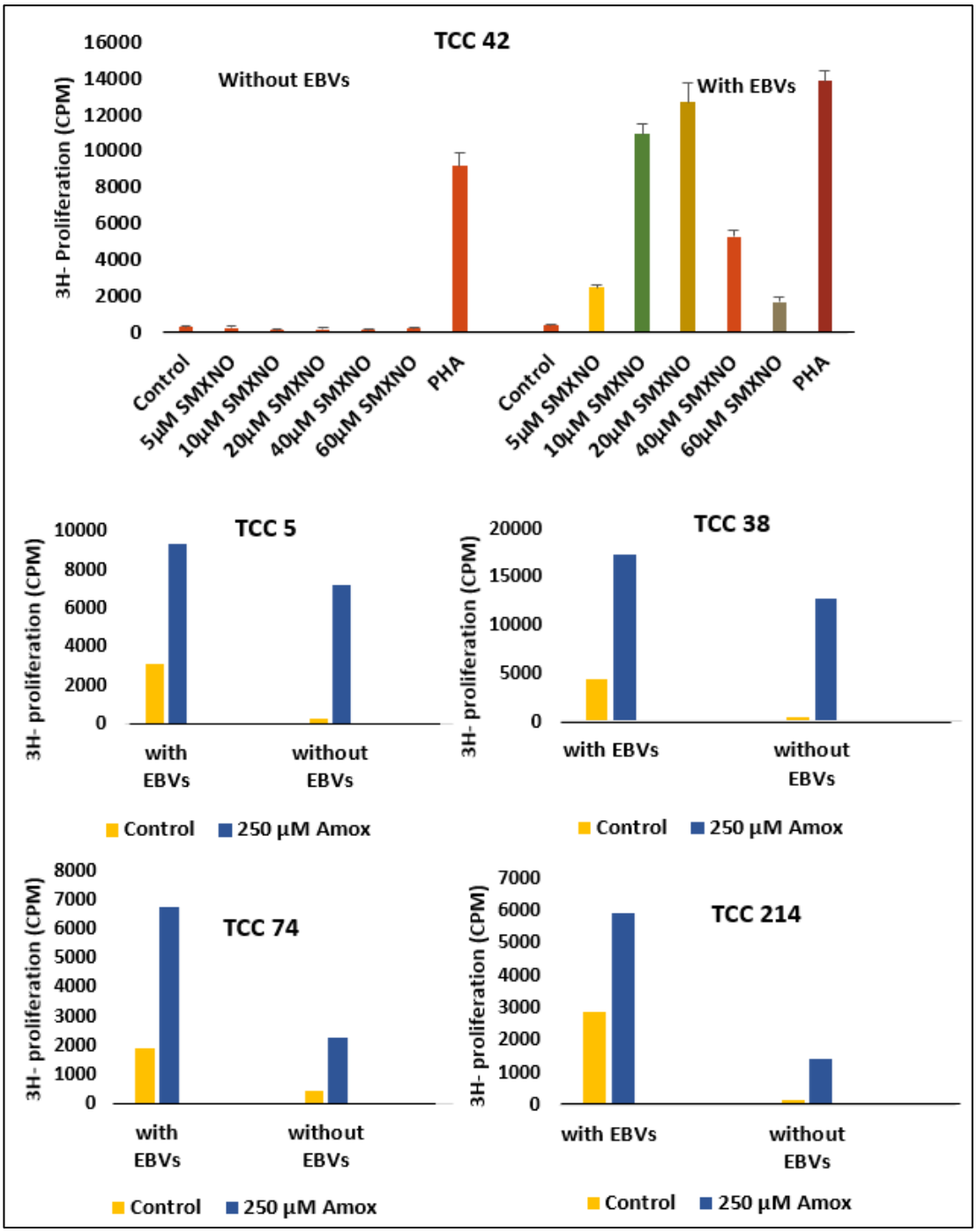



**Figure 5.6. The role of the antigen-presenting cell in the activation of sulfamethoxazole nitroso (SMXNO), amoxicillin (Amox)-responsive T cell clones.** The role of the antigen-presenting cells in the activation of SMXNO and Amox was studied by omitting EBVs from the proliferation assay. T cell clones (5x10$^4$cells per well/ 50µl) were cultured in the presence or absence of autologous irradiated EBV-transformed B-cells (1x10$^4$cell per well/ 50µl) and the study compound SMXNO (5µM-60µM) and Amox (250 µM-2mM) for 48 hours at 37°C, 5% $CO_2$. The negative control was T-lymphocyte culture medium. Proliferative responses were measured by adding [3H] - thymidine for the last 16 hours of the incubation to measure drug-specific proliferative responses. The proliferation of T cells was evaluated using scintillation counting.

### 5.4.6 CELLULAR PHENOTYPING OF T CELLS USING FLOW CYTOMETRY.

The majority of T cell clones generated from HLA-B*35:02 positive healthy donor, and minocycline and sulfamethoxazole nitroso DILIN patient samples expressed CD4+ cell surface protein. Only two clones expressed CD8+ (Figure 5.7). The amoxicillin-responsive T cell clones obtained from two DILIN patients expressed CD8+ (50%), or CD4+ (25%), while 25% expressed both CD4+ and CD8+ receptors. To elucidate the properties of CD4+ and CD8+ amoxicillin and sulfamethoxazole nitroso responsive T cell clones, several functional studies were conducted. TCR Vβ Repertoire Kit (IOTest® Beta Mark) was used to detect the distribution of TCR Vβ T cells expression by flow cytometric analysis. Clones from all healthy donors and DILIN patients expressed single but different TCR Vβ chains (Figure 5.8). No specific T cell receptor was expressed in high numbers on drug-responsive cells from a single donor or when clones responsive towards each drug was explored.

The other diagnostic markers was used to assess the adverse drug reaction is chemokine, as its important as a chemotactic factor that control the migratory patterns and locations of immune cells (Rollins, 1997). The immune cells Th1, Th2, Th17 and Th22 expression of receptor CXCR3, CCR9, CCR6. All Amoxicillin responsive T cell clones displayed high levels of CXCR3, CCR9, CCR6 where all predominantly expressed by Th1, Th2 and Th17 and Th22 (Saiman and Friedman, 2012, Gregor et al., 2017, Miyagawa and Asada, 2021, Groom et al., 2012).Three Amoxicillin responsive T cell clones expressed moderate level of E-cadherin was investigated to evaluate the cytotoxic mechanism (Jhang et al., 2021) Figure 5.9. Depending on the density of signal emitted at various wavelengths, thousands of single cells can be analysed one by one for various features.

The expression values quoted as mean fluorescence intensity index (MFI) of the whole population of each clone (Fluorescence intensity of stained cells/ Fluorescence intensity of unstained cells).

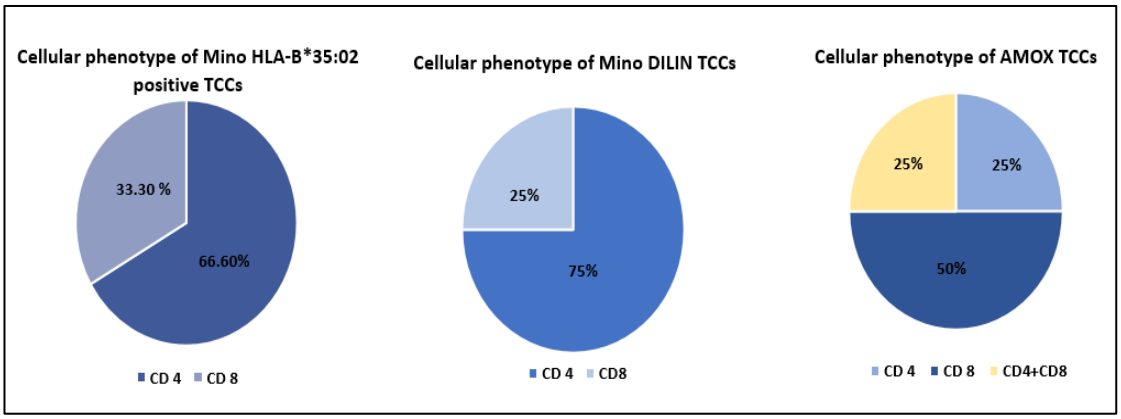



**Figure 5.7. Cell phenotyping for CD4 and CD8 protein expression on surface of minocycline (Mino) and amoxicillin (Amox) responsive T cell clones.** T cells ($5x10^4$ cells per well/50 µl) were incubated (20min) at 4°C with anti-CD4+/FITC and anti-CD8+/PE fluorophore conjugated antibodies. Cells were then washed and flow cytometry used to analyse cellular phenotype.

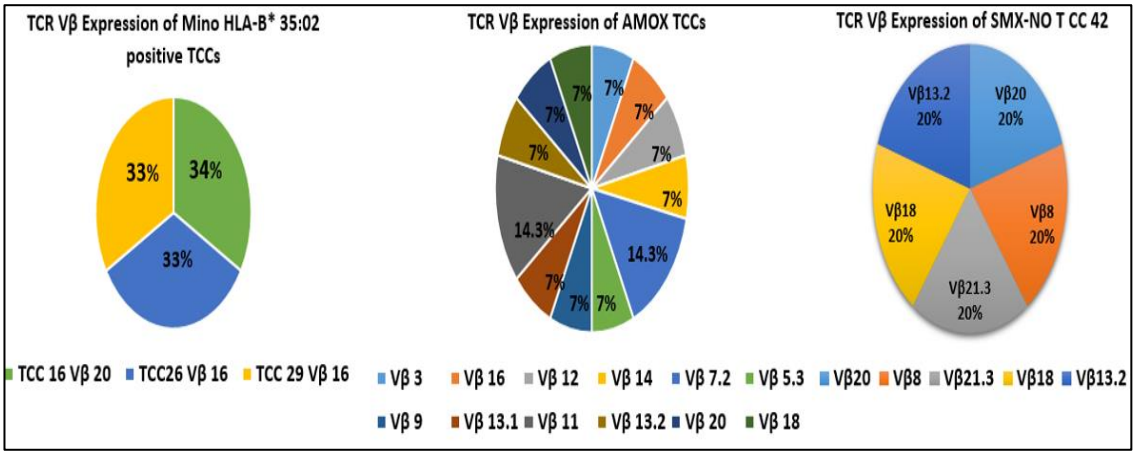



**Figure 5.8. TCR Vβ expression analysis**. Pie charts displaying the varied expression of TCR Vβ expression across minocycline (Mino) HLA-B*35:02, amoxicillin (Amox), and sulfamethoxazole nitroso (SMXNO) responsive T cell clones. T cell suspensions (100 µL) were incubated with various TCR Vβ antibodies and TCR Vβ usage determined using flow cytometry.

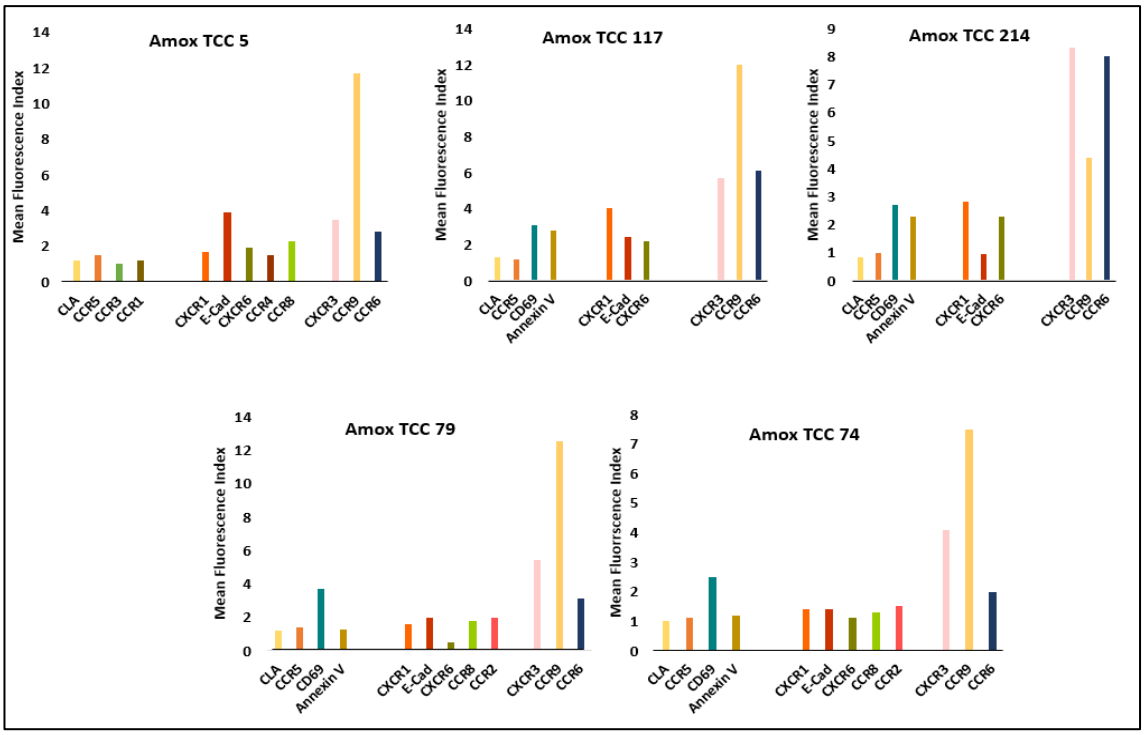
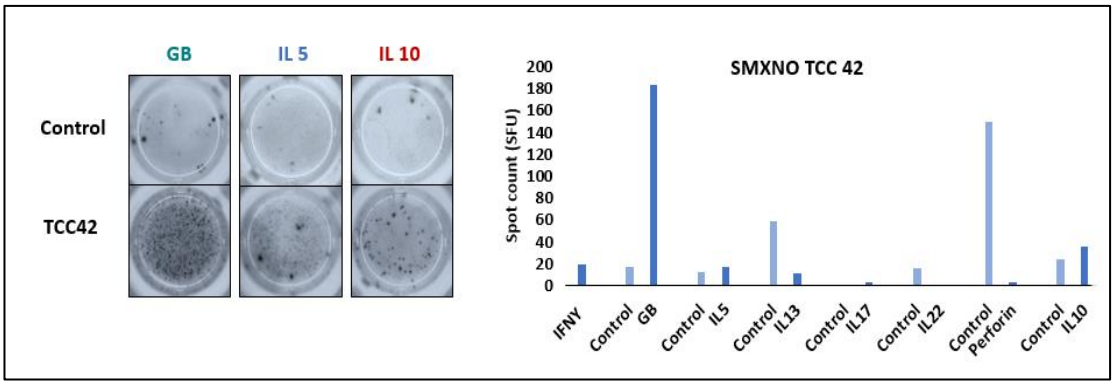


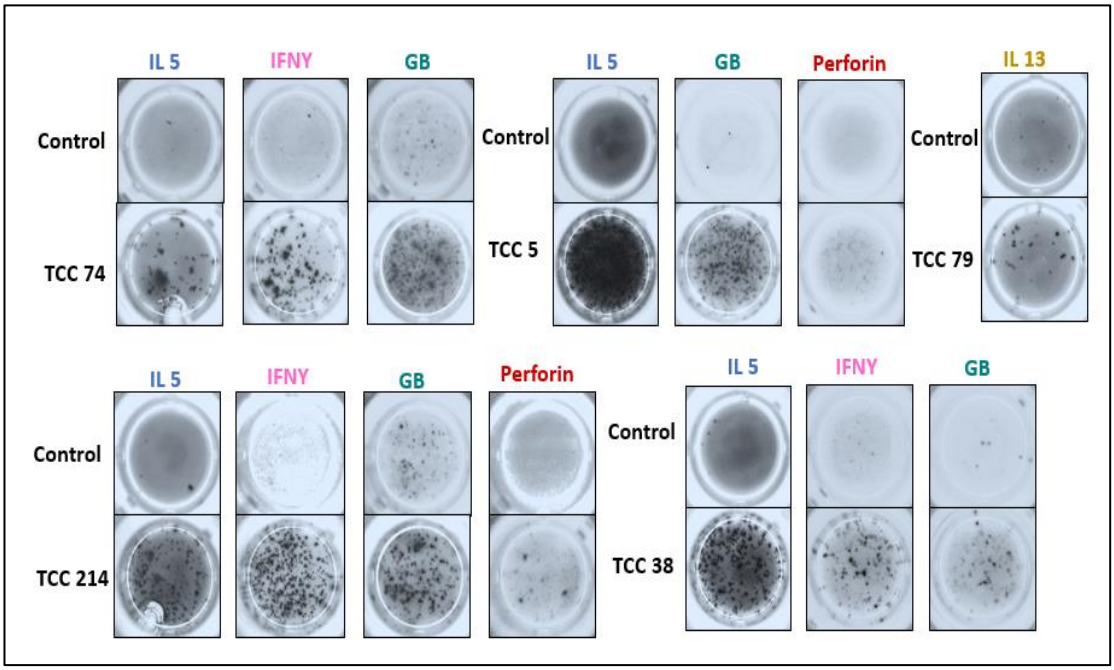

**Figure 5.9. Chemokine receptor expression on drug-responsive T cell clones.** T cell suspension (5x10$^4$cell per well/ 50 µL) were stained with antibodies CCR9, CCR6, CXCR3, CXCR6, E-cadherin, CXCR1, CCR8, CCR2, CLA, CD69, CCR3, CCR1, CCR4 and CCR5. Chemokine cell surface markers were analysed using flow cytometry. Results from 5 Amoxicillin-responsive clones are shown. Values quoted as mean fluorescence index.

### 5.4.7. CYTOKINES AND CYTOLYTIC PROFILE FOR AMOXICILLIN AND SULFAMETHOXAZOLE NITROSO RESPONSIVE T CELL CLONES.

To assess cytokines that are secreted from Th1, Th2, Th17 and Th22. Th1 behave as proinflammatory mediators, by involving in the immune-mediated hepatotoxicity (Dong, 2021, Abbas et al., 2019). Th1 cells secrets IFN-γ cytokine. Th2 are implicated in the pathogenesis of certain skin diseases and secrete cytokines IL-5, IL-10, IL-13 (Duhen et al., 2009, Kagami et al., 2010). Th17 cells participate in various autoimmune diseases such as systemic lupus erythematosus and the cytokine itself is defined as proinflammatory and implicated in acute and chronic liver diseases (Bettelli et al., 2007, Wang et al., 2015)**,** whereas Th22 cells are identified by the production of interleukin IL-22 and are implicated in skin diseases such as psoriasis,

infections, autoimmune diseases, hepatitis and pancreatitis (Eyerich et al., 2009). Five amoxicillin specific T cell clones were selected to assess cytokines (IL5, IFNY, IL13, IL10, IL22, and IL17 and cytolytic molecule (granzyme B, Perforin) secretion profiles using ELIspot. Similarly, cytokine secretion test was carried out for one sulfamethoxazole nitroso responsive T cell clone that was sufficiently expanded. Unfortunately, the isoniazid clones could not be expanded for these experiments. Clones secreted IFNy, granzyme B and perforin following amoxicillin exposure (Figure 5.10). IL5 was secreted from 4 clones, whereas the other cytokines were secreted at lower levels.



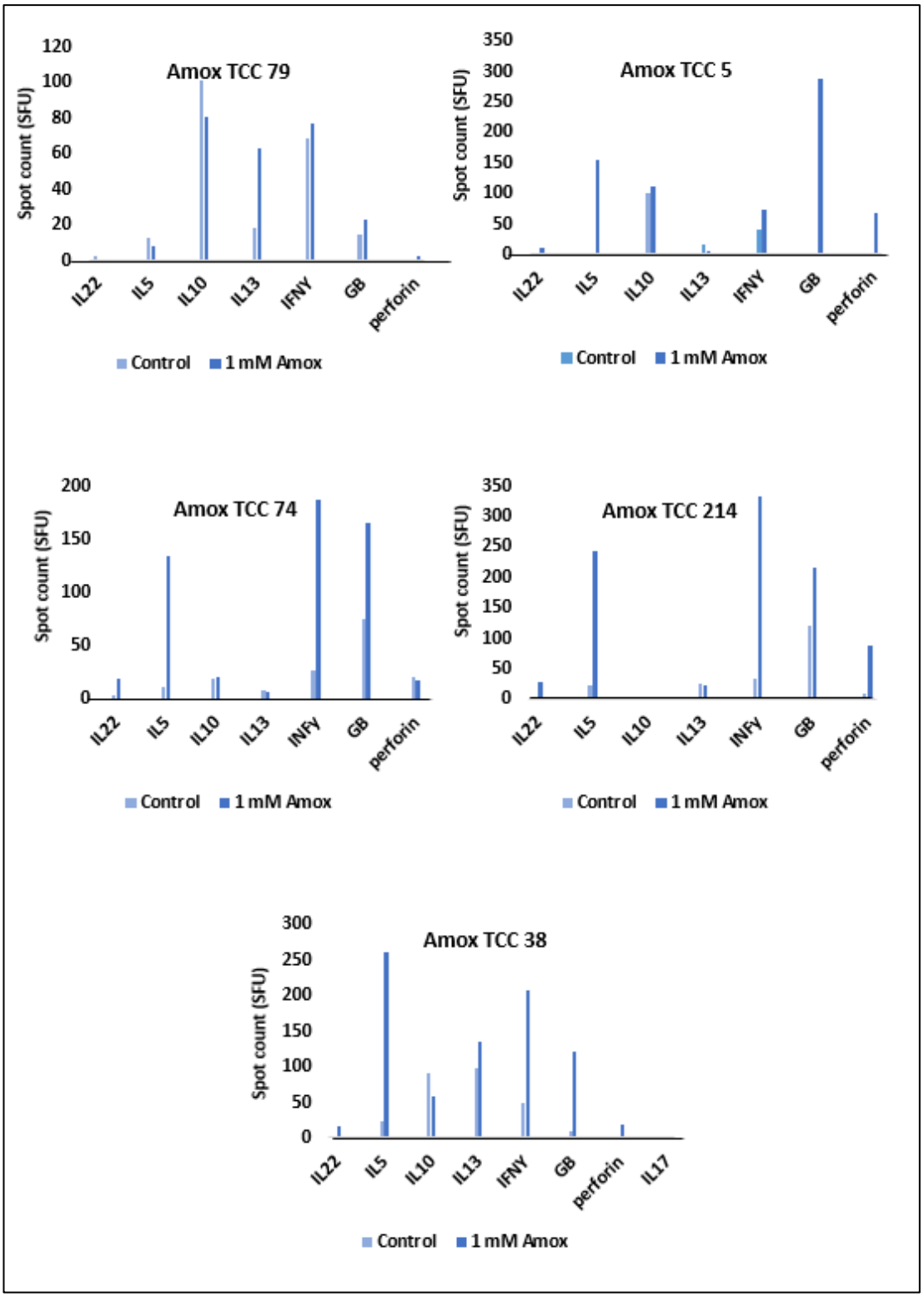



**Figure 5.10. Cytokine and cytolytic secretion profiles from sulfamethoxazole nitroso (SMXNO), amoxicillin (Amox), responsive T cell clones.** ELIspot plate membranes were pre-coated with human IFN-γ, IL13, IL22, IL 10, IL 5, IL 17, perforin, and GB capture antibodies and incubated at 4°C overnight. T cell clones ($5x10^4$cells per well/ 50µL) were co-incubated with irradiated autologous EBV- transformed B cells ($1x10^4$cells per well / 50µL) in the presence and absence of Amox (1mM) or SMXNO (40µM) for 48 hours at 37°C; 5% $CO_2$. Following the incubation, the ELIspot plate was developed according to the manufacturer's instructions. An AID ELIspot reader was used to measure cytokine release.

### 5.4.8 ANTIGEN SPECIFICITY AND CROSS-REACTIVITY TEST OF T-CELL CLONES.

The cross reactivity of the sulfamethoxazole nitroso specific T-cell clone was carried out with 2 concentrations of three sulphonamides; sulfadiazine, sulfamerazine, and sulfachloropyridazine, (Figure 5.11). Likewise, the clones were exposed to two DILI drugs and their metabolites; (1) naproxen, desmethyl naproxen, naproxen acyl glucuronide, (2) dapsone and nitroso dapsone, minocycline, amoxicillin and clavulanic acid. The clone was highly specific and was not stimulated to proliferate in the presence of these structurally-related or unrelated compounds (Figure 5.12).

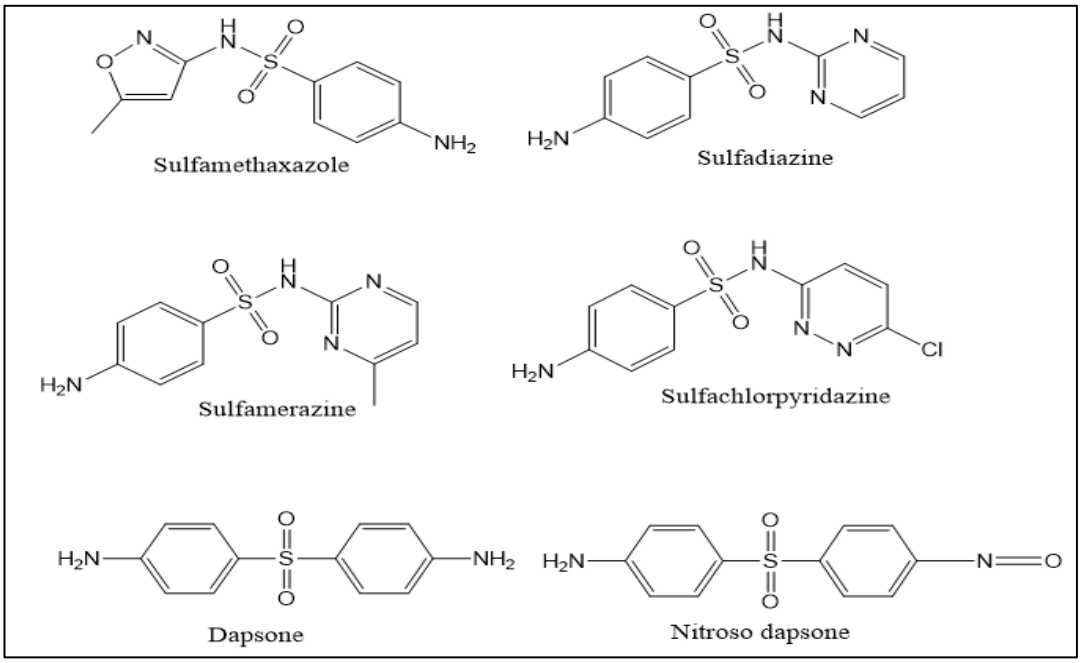



**Figure 5.11. Chemical structure of sulfamethoxazole (SMX), sulfadiazine (SFD), sulfamerazine (SFM), sulfachloropyridazine (SCHP), dapsone (DDS), and nitroso dapsone (DDSNO).**

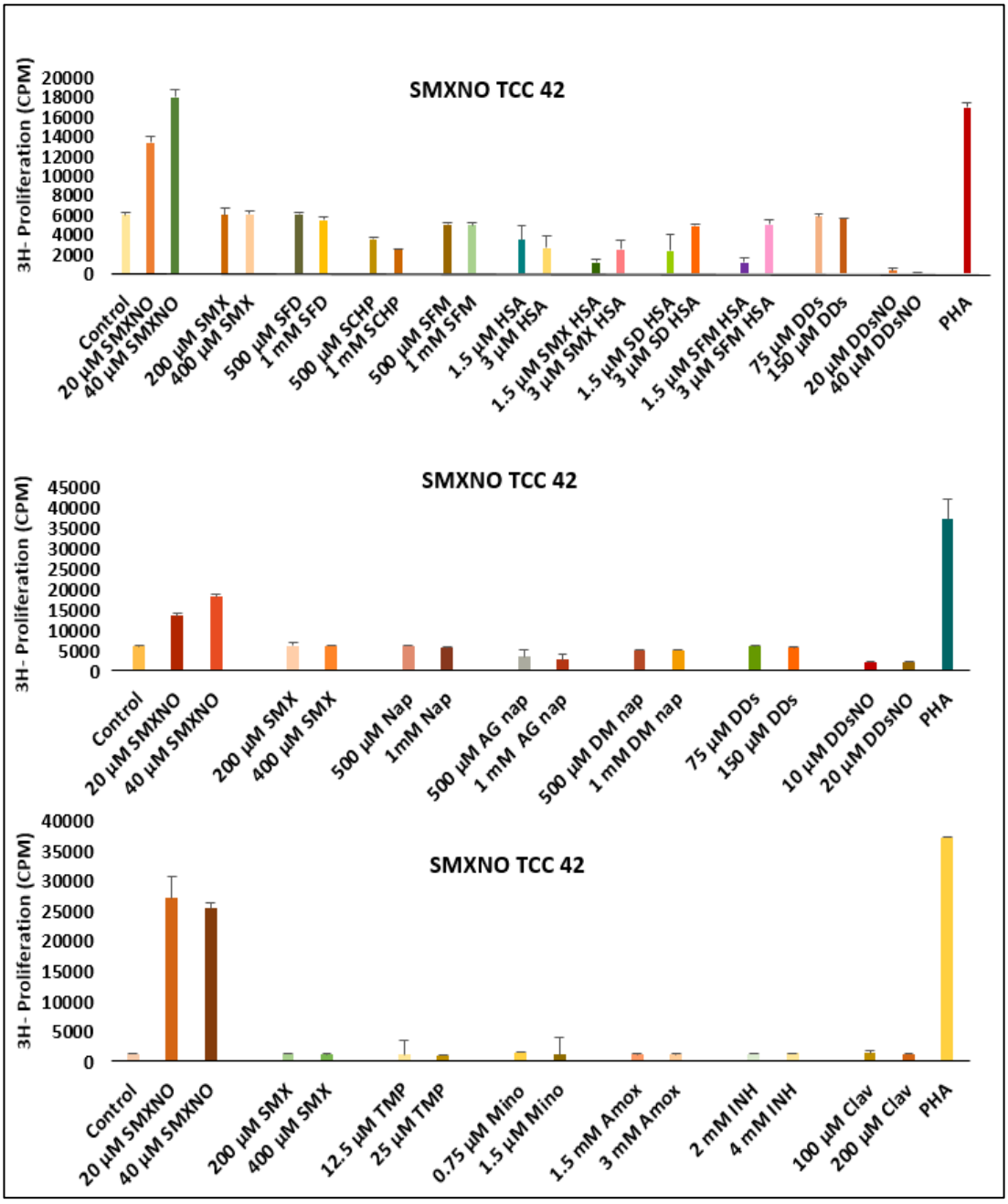



**Figure 5.12. Cross-reactivity of sulfamethoxazole nitroso (SMXNO) reactive T cell clone.** SMXNO reactive T cell clone ($5x10^4$cells per well) was incubated with autologous irradiated EBVs-transformed B-cell ($1x10^4$cells per well) in the presence and absence of two concentrations of different drugs; SMX (200 µM-400 µM), SFD (500 µM-1mM), SFM (500 µM-1mM), SCHP (500 µM-1mM), human serum albumin (HSA) with sulphonamides at two concentrations of each compound (1.5 µM- 3 µM), Nap, DMNAP, AGNAP (500µM-1mM), DDs (75µM- 150µM) and DDsNO (10µM- 20µM), SMX (200µM-400µM), TMP (12.5µM-25µM), Mino (0.75µM-1.5 µM), Amox (1.5mM- 3mM), Clav (100µM-200µM) and INH (2mM-4mM) for 48 hours in (37°C; 5% $CO_2$). [3H]-Thymidine (0.5µCi) was added for the final 16 hours to evaluate T cell proliferative responses before harvesting. T cell proliferation was analysed as stimulation Index (SI). A Student t-test was used to compare treated wells with untreated wells.

### 5.4.9 CROSS REACTIVITY OF AMOXICILLIN T CELL RESPONSIVE CLONES WITH β-LACTAM ANTIBIOTICS.

A cross reactivity study was conducted on 3 amoxicillin responsive T cell clones. T cell clones were incubated with EBVs and β- lactam antibiotics (penicillin G, piperacillin, flucloxacillin, phenoxy methyl penicillin, carbenicillin), and the third generation of cephalosporin (ceftriaxone) (Figure 5.13). T cell clones displayed a strong proliferative response to amoxicillin, while one cross-reacted with Phenoxy methyl penicillin. No cross-reactivity was detected with other antibiotics (Figure 5.14).

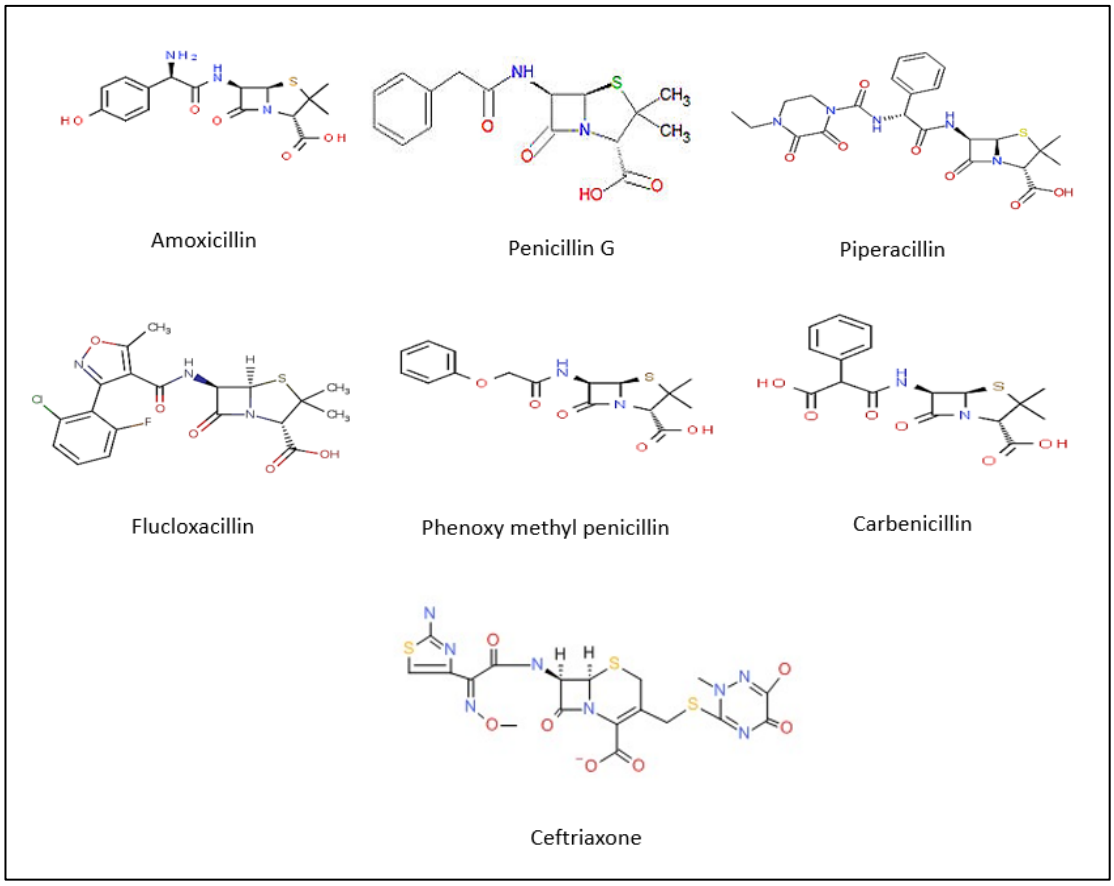



**Figure 5.13. Chemical structure of beta lactam antibiotics and the third generation of cephalosporin.**

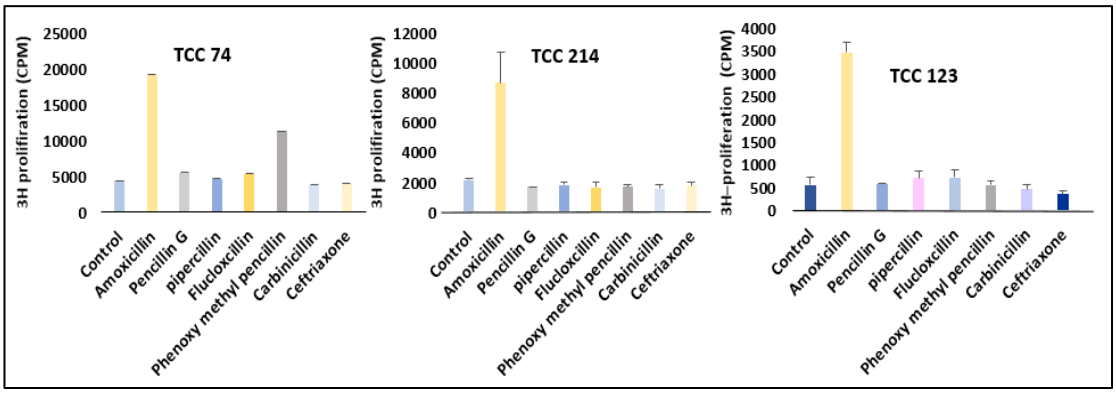



**Figure 5.14. Stimulation of amoxicillin (Amox) responsive T cell clones with different β-lactams antibiotics and Ceftriaxone.** Amox responsive T cell clones were cultured with irradiated EBV-transformed B-cell, soluble Amox, β-lactam antibiotics, and ceftriaxone. Each antibiotic was used in this proliferation assay at a concentration of (1mM). Plates were incubated for 48 hours at 37$^{o}$C; 5% $CO_2$, and 3H-thymidine was added for the final 16 hours to measure the proliferation.

### 5.4.10. ASSESSMENT OF T CELL RESPONSES IN THE PRESENCE OF PULSED AND FIXED ANTIGEN PRESENTING CELLS.

The importance of the pulsing test is to determine whether covalent protein binding is involved in the activation of the T cells. The sulfamethoxazole nitroso responsive clone was activated with autologous EBV-transformed B-cells pulsed with sulfamethoxazole nitroso for 0.5-16 hours (figure 5.15A). A significant proliferative response was detected in time period of 3 hours. Fixation of EBV transformed B- cells with glutaraldehyde prevented the activation of the clone (Figure 5.15B). T cell activation with pulsed EBVs is indicative of a hapten mechanism. Similar experiments were conducted with 4 amoxicillin-responsive T cell clones. The clones were stimulated with amoxicillin-pulsed EBVs, indicating again that the clones can be activated by protein adducts formed within APC or cell culture medium (Figure 5.16). However, fixation of EBVs only prevented T cell activation with one clone. This is because these clones self-present amoxicillin (see above).

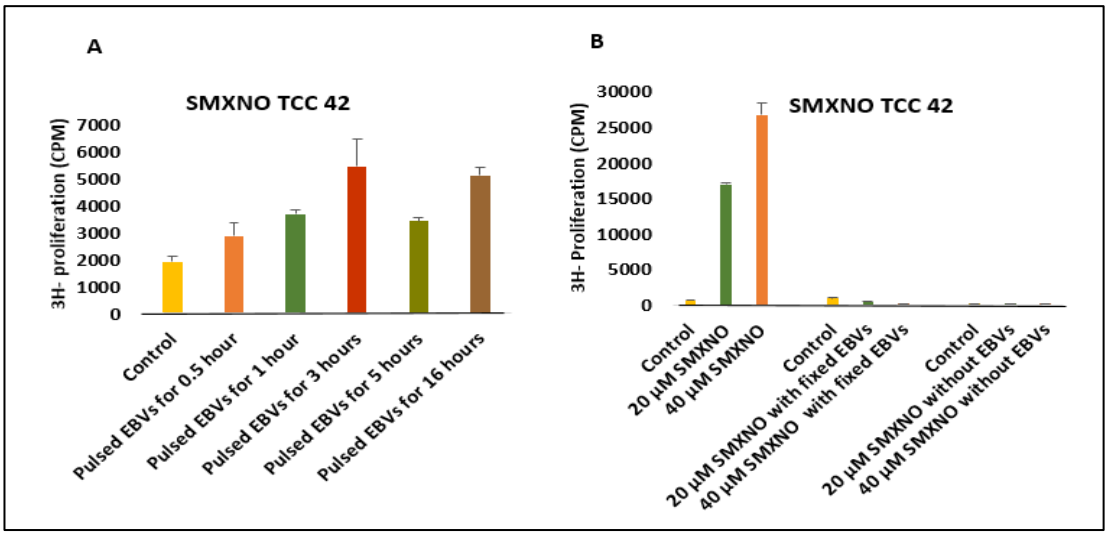



**Figure 5.15. Activation of the sulfamethoxazole nitroso (SMXNO)-responsive clone via a hapten mechanism involving adduct formation and antigen processing within antigen presenting cells. (A)** SMXNO responsive T cell clone was co-incubated for 48 hours with irradiated EBV-transformed B-cells pulsed with SMXNO for 0.5 hr, 1 hr, 3hr, 5hr, 16 hr. Experiments were conducted in triplicate using culture medium as a negative control. **(B)** SMXNO responsive T cell clone was co-incubated with SMXNO in the presence of either irradiated or glutaraldehyde fixed EBV-transformed B-cells ($1x10^4$cell per well) in triplicate cultures for 48 hrs. T cell proliferative responses were assessed through the addition of [3H] thymidine.

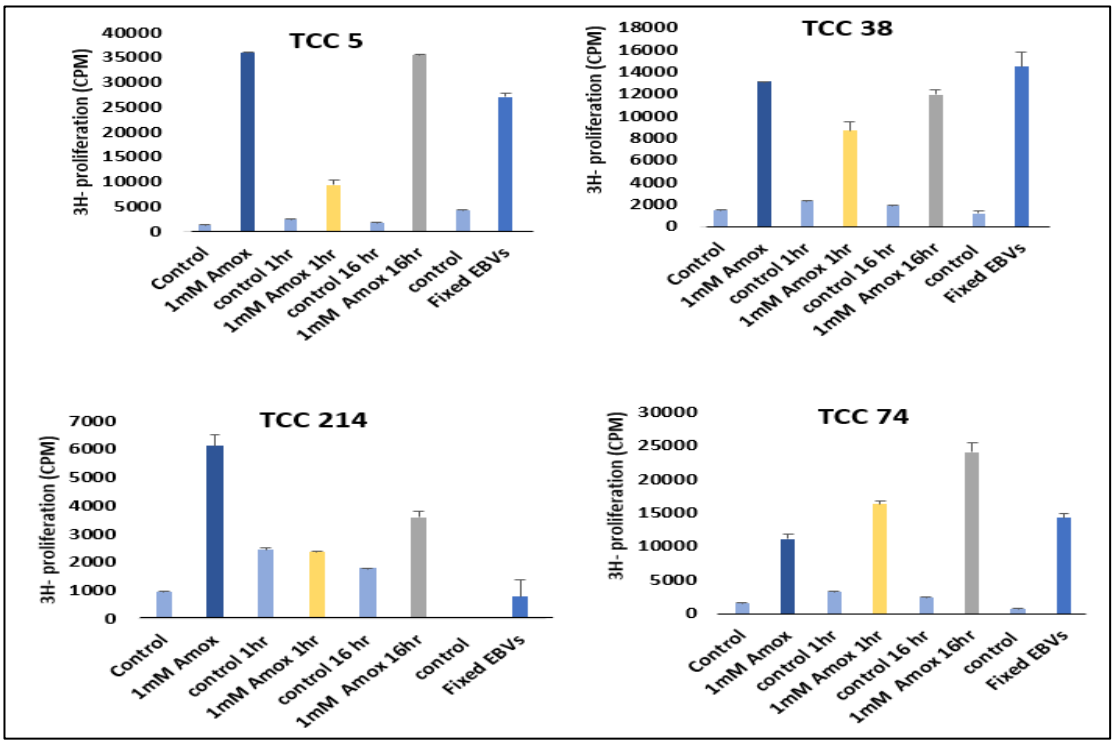



**Figure 5.16. Activation of amoxicillin (Amox)-responsive clones via a hapten mechanism involving adduct formation.** Amox-responsive T cell clones were co-incubated for 48 hours with irradiated EBV-transformed B-cells pulsed with Amox for 1 hour or 16 hours. Experiments were conducted in triplicate using culture medium as a negative control. The

same Amoxicillin-responsive T cell clones were co-incubated with Amoxicillin in the presence of either irradiated or glutaraldehyde fixed EBV-transformed B-cells ($1x10^4$cell per well) in triplicate cultures for 48 hrs. T cell proliferative responses were assessed through the addition of [3H] thymidine. Statistical analysis was performed using the students' t test to compare treated conditions with the untreated control for each desired statistical measure, accepting $P < .05$ as significant.

### 5.4.11 QUENCHING EXPERIMENT FOR SMXNO CLONE.

Antigen presenting cells were pulsed with sulfamethoxazole nitroso for 24 hours in the presence and absence of the nucleophiles N-acetyl lysine, N-acetyl tyrosine, and glutathione (all 1 mM). EBVs were washed repeatedly before addition the sulfamethoxazole nitroso-responsive T cell clone. This assay was conducted to investigate whether sulfamethoxazole nitroso binding to the nucleophiles prevented protein binding and activation of the clone. The strength of the proliferative response of the T cell clone to sulfamethoxazole nitroso was significantly reduced in the presence of N-acetyl lysine, N-acetyl tyrosine, and glutathione (Figure 5.17).

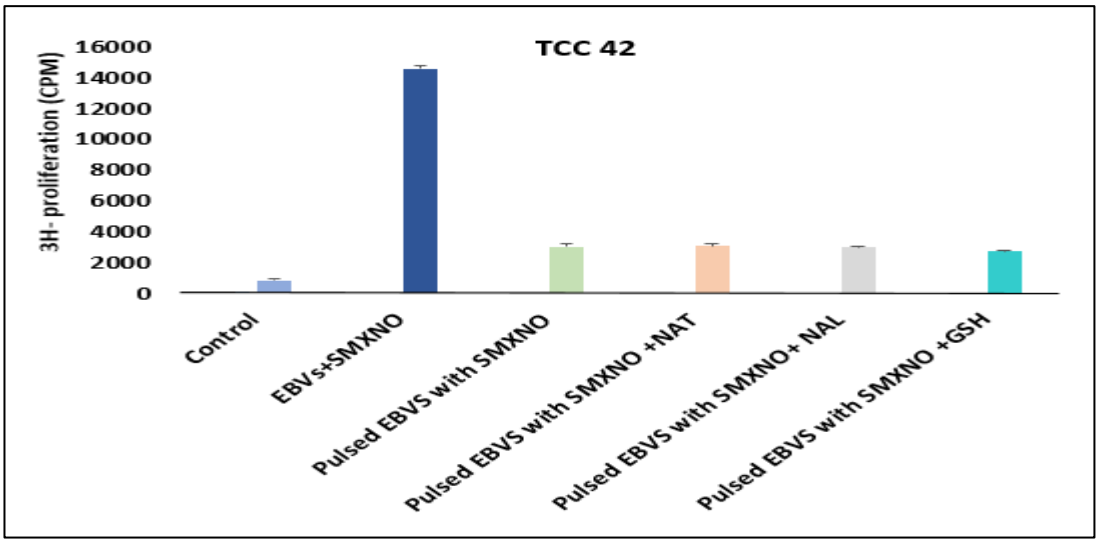



**Figure 5.17. N-acetyl lysine (NAL), N-acetyl tyrosine (NAT), and glutathione (GSH) inhibit the activation of the sulfamethoxazole nitroso (SMXNO)-responsive T cell clone.** Autologous EBV-transformed B-cells were prepared and transferred into 6 well plates. One well contained autologous EBVs treated with SMX, four wells treated with SMXNO at the optimal concentrations, and a culture medium in the sixth well. I ml of N-acetyl lysine, N-acetyl tyrosine, and glutathione were individually added into three SMXNO wells. The plate was incubated overnight at 37°C, 5% $CO_2$. Autologous EBVs were prepared and irradiated with pulsed EBVs were subsequently added with responsive T cell clone into 96 well U-

bottomed plates in triplicate, in the presence and absence of soluble drug per condition. The plate was incubated for 48 hrs. T cell proliferative responses were assessed through the addition of [3H]-thymidine.

### 5.4.12. MHC RESTRICTION OF THE DRUG-SPECIFIC T CELL CLONE RESPONSE.

The activation of sulfamethoxazole nitroso responsive T cell clones was dependent on the presence of antigen-presenting cells. Antigen-presenting cells express the two primary MHC I and II molecules. Anti-human HLA-ABC and HLA-DR/DQ/DP monoclonal antibodies were used to inhibit the MHC-T cell interaction by binding with MHCI and /or MHCII. Activation of the sulfamethoxazole nitroso-specific proliferative response was completely inhibited with the MHC class II blocking antibody (Figure 5.18). Similar experiments using MHC class II sub-type blocking antibodies demonstrated that sulfamethoxazole nitroso interacts with HLA DR molecules to stimulate the T cell clone. Due to self-presentation by amoxicillin-responsive T cell clones similar MHC blocking experiments could not be conducted.

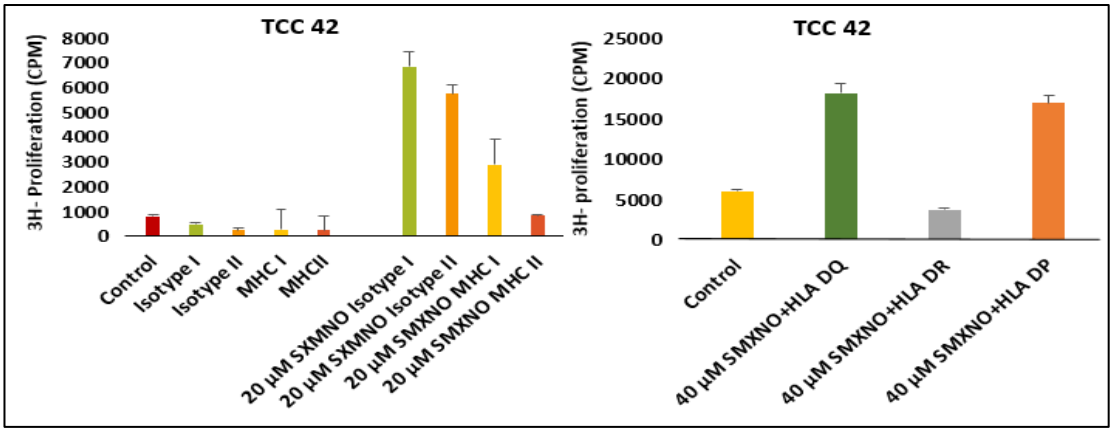



**Figure 5.18. MHC restriction of sulfamethoxazole nitroso (SMXNO) responsive T cell clone. (A)** The graph illustrates Anti-human HLA Class I and II blocking antibodies expression on T-cell clone 42. SMXNO was cultured with irradiated autologous EBV-transformed B-cells and (20µM SMXNO) in triplicate in the presence of and anti-HLA class I and II blocking antibodies and their respective isotype controls for 48 hours. 3H-Thymidine was used for 16 hours to determine T cell proliferation. T cell clone 42 was HLA class II more depleted**. (B)** T cell responsive clone was incubated with irradiated antigen-presenting cells, and (40µM SMXNO) in triplicate in the absence and presence of HLA-DQ/DR/DP blocking antibodies for 48hrs. 3H- proliferation was added for the final 16 hrs to assess drug-specific proliferative responses.

## 5.5 DISCUSSION

Idiosyncratic adverse reactions to drugs can affect any organ in the human body. The most common targets are the liver, kidney and skin (Park et al., 2001). Understanding of the pathophysiological basis of idiosyncratic reactions is important as it may benefit Pharmaceutical industries in development of safe new drugs and health service professionals exploring safer patient dosing regimens (Pichler et al., 2006). Idiosyncratic reactions targeting skin have been extensively studied. Reactions with a delayed onset often involve the drug-specific activation of naïve T-cells, which when expanded and activated release effector molecules that are directly involved in the tissue injury (Naisbitt et al., 2020). T-cells are activated by parent drugs and stable drug metabolites through a reversible interaction with HLA molecules expressed on antigen presenting cells and specific T-cell receptors. Furthermore, T-cells are activated with peptides derived from drug (metabolite) protein adducts. Interestingly, in the few sulfamethoxazole patients studied for drug and metabolite-specific T-cell responses, T-cells in the same individuals have been shown to be activated with the parent drug and metabolites (Castrejon et al., 2010). Since the liver is the main organ involved in drug metabolism, where the cytochrome P450 system works as a catalyst, this organ is exposed to high levels of stable and reactive metabolites. As such, it is important to explore whether T-cell responses in patients with drug-induced liver injury are activated with drugs and/or drug metabolites. In contrast to skin reactions, the role of the adaptive immune system in patients with liver injury is less well defined. Recent studies have identified drug responsive T-cells in patients with flucloxacillin- (Monshi et al., 2013), co-amoxiclav-(Kim et al., 2015) and isoniazid- (Usui et al., 2017a) induced liver injury. Earlier in this this thesis similar

T-cell culture methods were used to explore naproxen and atabecestat-induced liver injury and in each case T-cells were primarily activated with a stable hepatic metabolite. Thus, in this chapter PBMC samples from patients recruited through the DILIN network were used to attempt to characterise T-cell responses to 4 drugs, sulfamethoxazole, isoniazid, minocycline and co-amoxiclav, each associated with a high incidence of liver injury.

PBMC were present in much lower numbers than anticipated when cryopreserved vials were defrosted and viability was also often reduced. This significantly restricted number and size of experiments that could be conducted, which may explain why the vast majority of diagnostic assays yielded negative results (lymphocyte transformation test and ELIspot assays to measure cytokine release). Nevertheless, EBV-transformed B-cells were generated from patients with each form of drug-induced liver injury and PBMC cultures were established with the drugs to enrich the number of drug-specific T-cells prior to serial dilution experiments to establish T-cell clones to test for drug specificity. In initial experiments, when the number of cells is limiting, drug specificity was tested with a medium control and an optimal drug concentration in duplicate cultures. All 5 study drugs (minocycline, nitroso sulfamethoxazole, isoniazid, amoxicillin and clavulanic acid) stimulated several patient clones to proliferate in this initial test. Due to the limited scope of this initial experiment, all responsive clones were expanded further over a 2-week period and re-tested using at least 2 concentrations of the study compounds. Clones responsive toward minocycline, amoxicillin and nitroso sulfamethoxazole were detected in this experiment. These data are the first to suggest that drug-responsive T-cells may be

involved in the pathogenesis of minocycline- and sulfamethoxazole-induced liver injury. Unfortunately, it was not possible to expand the minocycline-responsive clones further and as such additional experiments were not possible. In particular, we were unable to explore whether the clones were activated with antigen presenting cells expressing HLA-B*35:02, the HLA alleles identified as a liver injury risk factor in genome-wide association studies (Urban et al., 2017). Clones responsive towards nitroso sulfamethoxazole and amoxicillin were expanded in much larger numbers and these were subjected to functional and mechanistic investigations and these are discussed below.

50% of amoxicillin-responsive T cell clones were CD8+, while 25% expressed the CD4+ protein and 25% were identified as double-positive expressing CD4+ and CD8+ receptors. With the exception of abacavir which universally gives a CD8+ T cell response, a mixture of CD4 and CD8 T-cell clones were detected when cloning to a number of drugs including flucloxacillin, carbamazepine and atabecestat. In this context our results were consistent with previous studies as T cell clones were CD4+ or CD8. Thus, MHC class II and MHC class I molecules display the drugs to cytotoxic T cells (CD8+) helper T cells (CD4+), respectively, which is to be expected from basic immunology dogma. Within our T-cell cloning assays we aim to clone one responsive T cell to a drug of interest. In our experiments up to 3 cells are added per well at the start of the incubation period. If one responsive T-cell expands, the other 2 usually die out, however in some instances we see drug-responsive T cell clones generated where we see two distinct populations, one CD4 and one CD8, as observed with T cell clones 214, 5 and 38. While true DP T cell clones individually are not commonly observed outside the thymus, several studies indicate that T cells bearing the αβ TcR

with dual expression of CD4 and CD8 co-receptors do circulate in the peripheral blood of target organs of patients suffering from various diseases such as cancer and chronic inflammatory disorders (Parel and Chizzolini, 2004, Bohner et al., 2019). Also, mature T cells bearing dual TcR have been documented in human peripheral blood, so it is likely that T cells bearing two TcR may exist in addition to being CD4CD8 DP (Padovan et al., 1993, Hinz et al., 2001). Due to the structures of αβ TcR and the formation of MHC I antigen-binding cleft α1 and α2 domains and the formation of MHC II antigen binding cleft α1 and β1 domains, DP T cells bearing TcR with appropriate affinity for MHC peptide complexes (Germain, 2002). Interestingly we observed by way of the MHC block that both populations were responsive to the drug as we see the response disappear when MHC I and II were blocked, which is something seen very rarely. The clones expressed single, but differing TCR, indicating that they derived from a single pre-curser cell. Amoxicillin treatment of the clones was associated with secretion of IFNγ and cytolytic molecules such as granzyme B and perforin, indicating that the have the capacity to kill autologous target cells. While no method is perfect, the generation of DP Tcell clones is rare in these assays as per usually a mixture of CD4 or CD8 single cell clones are generated making these methods still the best to study T-cell responses to drugs in vitro.

Amoxicillin-specific secretion of the regulatory cytokine IL10 and the tissue signalling cytokine IL22 was not detected. Clones were highly specific in terms of drug structure. Only one clone displayed a proliferative response in the presence of phenoxy methyl penicillin. Phenoxy methyl penicillin has a similar structure to amoxicillin and both drugs are used to treat bacterial infections. The amoxicillin-responsive clones were activated in the presence and absence of EBV-transformed B-cells; thus, it was

difficult to define the HLA molecules involved in T-cell activation. To activate the T-cells amoxicillin might bind directly to HLA molecules or to non-HLA associated protein forming a hapten protein conjugate. The conjugate would be processed by antigen presenting cells and the liberated peptides would bind to HLA proteins for presentation on the surface of antigen presenting cells (Horton et al., 1998). Antigen presenting cell pulsing and glutaraldehyde fixation experiments revealed that amoxicillin-responsive T cell clones were activated with the amoxicillin hapten bound either directly to HLA molecules expressed on antigen presenting cells or through the formation of a covalent bond with other cellular proteins. This is because antigen presenting cells pulsed with amoxicillin (non-covalently-bound drug washed away) activated the clones indicating a requirement for the formation of a covalent bond between the antigen presenting cell and the drug. The time required for optimal T-cell activation was 16hours, which has previously been shown to be the time needed for generation of high levels of β-lactam haptens in cell culture (Meng et al., 2017). However, the glutaraldehyde antigen presenting cell fixation experiments couldn't delineate the requirement for antigen processing. This is because as discussed above, the clones were able to self-present the amoxicillin antigen.

The single nitroso sulfamethoxazole-responsive clone that was expanded for mechanistic studies required antigen presenting cells for T-cell activation. The clone was activated with the drug antigen bound to HLA-DR molecules. Antigen presenting cell pulsing and fixation experiments revealed that this clone was activated via a hapten mechanism involving formation of a drug metabolite protein adduct and processing by antigen presenting cells. Antigen presenting cells pulsed with nitroso sulfamethoxazole for 0.5 – 1 hour stimulated the clone to proliferate; this is because

the nitroso moiety binds rapidly to cysteine and lysine residues on proteins (Naisbitt et al., 1996, Tailor et al., 2019). Similar to the amoxicillin-responsive clones, the nitroso sulfamethoxazole-responsive clone secreted IFN-gamma and the cytolytic molecule granzyme B. Furthermore, the nitroso sulfamethoxazole-specific response was highly specific and the clone was not stimulated to proliferate with structurally-related drugs or alternative drug structures associated with a high incidence of liver injury. Glutathione is the source of cellular thiols and works as an intracellular anti-oxidant. It can protect the cellular components from stress caused by free radicals and electrophiles either by conjugation or by reduction reactions (Attia, 2010, Naisbitt et al., 1996). Glutathione and other nucleophiles blocked the activation of the sulfamethoxazole nitroso -responsive clone when added to culture medium before the drug.

Collectively, these studies add sulfamethoxazole and minocycline to the increasing number of drug-induced liver reactions where drug-specific T-cells have been detected in patients with liver injury. Subsequent studies should attempt to define the role of T-cells in the liver pathology through development of co-culture systems and also define further the HLA molecules involved in drug antigen presentation. The former is complicated by the need for a fully autologous system with liver and immune cells from the same donor. In the case of minocycline, it would also be interesting to investigate whether an increased number of T-cells are activated with stable and/or reactive drug metabolites.

# CHAPTER 6. GENERAL DISCUSSION

Drug-induced liver injury is a life-threatening manifestation of drug exposure resulting in several drug-induced deaths and the withdrawal of otherwise effective therapeutic agents. The adverse event presents clinically in several different forms (Andrade et al., 2019). For example, paracetamol-induced liver injury occurs following drug overdose, when metabolic detoxification pathways become saturated leading to a build-up of a reactive metabolite that induces oxidative stress and binds covalently to endogenous proteins essential for normal cell function (Weaver et al., 2020). Since the liver is the primary site of paracetamol metabolism, the organ is exposed to the highest levels of formed reactive metabolite, which explains the organ-selective toxicity. Paracetamol-induced liver injury is predictable with a knowledge of the chemistry of the metabolic process and similar reactions are observed in all patients in overdose (Park et al., 2005).

Of much greater concern are the unpredictable reactions observed in a small percent of patients exposed to a drug a therapeutic concentration. There are many examples of drugs associated with such reactions and this thesis focused on 6 of these: atabecestat, naproxen, sulfamethoxazole, co-amoxiclav, isoniazid and minocycline. For each of these drugs, reactions have a delayed onset on initial exposure, while in the small number of patients rechallenged after recovering from the initial event, clinical symptoms appear rapidly (Mosedale and Watkins, 2017). For several drugs that cause unpredictable DILI, including sulfamethoxazole, co-amoxiclav and minocycline, the development of an adverse event has been associated with expression of a single DILI allele, or a specific HLA haplotype (Lucena et al., 2011,

Urban et al., 2017, Li et al., 2021). Each of these features outlined above are indicative of an immune pathogenesis. First, the delayed onset of clinical presentation may relate to the time needed to initiate a primary adaptive immune response against the drug (Demoly et al., 2014). Second, the rapid onset on re-exposure may relate to reactivation of the pre-primed immune cells. Finally, HLA alleles encode HLA proteins that present antigenic determinants, normally small peptide fragments, to T-lymphocytes (Wieczorek et al., 2017).

The discovery of DILI HLA alleles associations suggest that drug antigens interact preferentially with the HLA proteins to stimulate a drug-specific T-cell response (Kim and Naisbitt, 2016) . Studies for the most part exploring cutaneous hypersensitivity reactions have shown that drugs and metabolites stimulate patient T-cells through (1) a direct reversible interaction with HLA proteins and/or HLA binding peptides (Pichler, 2019) and (2) binding irreversibly to non-HLA associated protein generating a hapten protein conjugate that is processed and broken down into fragments within antigen presenting cells. These peptides associate with HLA class I and HLA class II molecules for presentation to CD8+ and CD4+ T-cells, respectively. It is assumed that peptides containing a covalently bound drug molecule activate the patient T-cells, but the nature of the immunogenic moieties is yet to be defined (Adair et al., 2021).

Until recently, the role of the adaptive immune system and in particular drug-specific T-cells in DILI was ill-defined. An early investigation by (Maria and Victorino, 1997b) detected drug-responsive PBMC proliferation in certain patients with liver injury, but the phenotype and function of the activated cells was not defined. More recently, cytotoxic T-cell infiltrations have been detected in biopsy samples of patients with

sulfasalazine, flucloxacillin and atabecestat induced liver injury (Wuillemin et al., 2014, Mennicke et al., 2009, De Jonghe et al., 2021). These data alongside the sparsity of effector T-cells in healthy liver indicate that drug-exposure in susceptible patients initiates events that lead to the recruitment of T-cells with the potential to cause tissue injury directly through the release of effector molecules such as perforin, granzymes and FasL. These pathophysiological studies alongside the discovery of DILI HLA associations resulted in a renewed interest in the investigation of potential immune mechanisms of DILI. Drug-responsive T-cells have been detected in patients with flucloxacillin-, co-amoxiclav- and TB medication-induced liver injury. In each case, T-cells were activated in a dose-dependent manner and the T-cell response was highly specific (i.e., related drug structures did not activate the T-cells) (Monshi et al., 2013, Kim et al., 2015, Usui et al., 2017a). Through serial dilution experiments T-cell clones were generated and classified in terms of cellular phenotype and function, and HLA-restriction.

The primary objective of this thesis was to explore additional drugs associated with a high prevalence of hepatic adverse events to determine whether drug-responsive T-cells were detectable in blood, and if so explore the primary drug-derived structure that activates T-cells and characterise the nature of the induced response. The primary hypothesis being tested was “drug-responsive T cells are the drivers of adverse events in patients presenting with liver injury”. The availability of synthetic drug metabolites for our in vitro studies allowed us to address the secondary hypothesis “T cell activation in patients with DILI is driven by metabolites of parent drug”. Studies described in chapters 3 and 4 focused on the drugs atabecestat and naproxen. Development of atabecestat was stopped due to the appearance of

several cases of liver injury, while naproxen is a widely used drug with known DILI liabilities. Although the drugs have dissimilar structures and pharmacological targets, they have defined metabolic profiles and metabolites were available alongside the parent compounds for T-cell studies. Antigen-specific T-cells were detected in both patient cohorts either through diagnostic assays such as the lymphocyte transformation test and/or IFN-gamma PBMC ELIspot or through the generation and assessment of T-cell clones. For atabecestat, the T-cell response was directed mainly against a primary diaminothiazine (phase I) metabolite. The diaminothiazine-responsive clones were CD4+ and expressed different TCR sequences. The drug metabolite was found to interact directly with HLA-DR (or peptides embedded in the binding cleft) molecules expressed on antigen presenting cells to stimulate a T-cell response, but the actual alleles involved in drug metabolite presentation likely differs from patient to patient, which partly explains why atabecestat-induced liver has not yet been associated with expression of a specific HLA allele. For naproxen, both phase I and phase II metabolites were available for T-cell studies. Of particular interest was the acyl glucuronide metabolite of naproxen, which is known to bind covalently to protein and as such has been implicated in the pathogenesis of naproxen-induced liver injury, without direct experimental evidence. In patients with naproxen-induced liver injury T-cells were again mainly activated with a stable phase I metabolite, desmethyl naproxen. The drug metabolite responsive T-cells were CD4+ and activated to proliferate and secrete cytokines in a dose-dependent manner. However, desmethyl naproxen interacted with HLA-DQ to stimulate the T-cell response. Interestingly, the desmethyl naproxen-responsive cloned T-cells were activated with antigen presenting cells pulsed with the drug metabolite for 1-16

hours, while, activation was not detected when the T-cells were cultured with the drug metabolite and glutaraldehyde-fixed antigen presenting cells. The pulsed antigen presenting cells are subjected to several washing steps; thus, the desmethyl naproxen must bind strongly to the antigen presenting cells for presentation to occur. Fixation of antigen presenting cells blocks antigen processing; thus, the activation of T-cells is dependent on the generation of newly formed HLA associated peptides. These data indicate that the desmethyl naproxen-responsive T-cells are likely activated via a hapten mechanism as described with β-lactam antibiotics (Meng et al., 2017, Kim et al., 2015, Monshi et al., 2013). However, further research is required to define the nature of the drug metabolite antigen presenting cell binding interaction and indeed whether naproxen-modified peptides are displayed by HLA class II molecules displayed on the cell surface. Despite intensive investigations, we found no evidence that naproxen acyl glucuronide activates patient T-cells. These data have important implications for the Pharmaceutical industry that considers acyl glucuronides to be an important undesired structural alert for new chemical entities.

Chapter 5 of the thesis involved a similar methodological approach using PBMC isolated from patients with minocycline-, sulfamethoxazole-, isoniazid- and co-amoxiclav-induced liver injury. Minocycline-, nitroso sulfamethoxazole- and amoxicillin-responsive T-cell clones were generated from the patient PBMC. As described previously (Castrejon et al., 2010, Meng et al., 2017), nitroso sulfamethoxazole and amoxicillin-responsive T-cells were activated via a mechanism dependent on the formation of a covalent bond between the drug (metabolite) and the antigen presenting cells. Minocycline-responsive T-cells could not be expanded sufficiently to explore the nature of the T-cell response and the pathway of T-cell

activation, which was unfortunate given that minocycline forms a reactive metabolite and protein adducts through myeloperoxidase and cytochrome p450 catalysed reactions (Mannargudi et al., 2009). Furthermore, minocycline-induced liver injury is strongly associated with expression of HLA-B*35:02 and therefore these reactions would be an ideal candidate to study pathways of drug and metabolite-specific T-cell activation in the same individuals.

Collectively the data generated in this thesis indicates that drug-specific T-cell activation is a common feature in patients with drug-induced liver injury. The preferential activation of T-cells from certain patients with primary drug metabolites indicates the importance of drug distribution and metabolism in the disease pathogenesis, which has important implications for in vitro diagnostics that routinely used the parent drug as the source of antigenic material. Positive responses in assays such as the lymphocyte transformation test are informative and allow clinicians to diagnose the causative drug susceptible patients; however, a negative result should be regarded with caution. T-cells may be detectable in these patients, but the immune response may be directed against a different form of the drug molecule that is not generated in the culture conditions. The classical example of this is allopurinol-induced hypersensitivity syndromes, where oxypurinol – a primary metabolite – interacts directly and immediately with the HLA protein HLA-B*58:01 to activate T-cells (Yun et al., 2014b, Yun et al., 2013).

With the knowledge that drugs selectively activate T-cells in patients with DILI it is now important to (1) define precisely the drug immune cell interaction and (2) determine the factors that predispose individual susceptibility. Studies published

during the work described herein have shown that cell membrane transporters facilitate the accumulation of hepatocellular flucloxacillin protein adducts. Flucloxacillin was found to bind to proteins localized in the bile canaliculi regions, which may provide a local source of antigen for HLA presentation in the liver (Waddington et al., 2020). Flucloxacillin-induced liver injury is strongly associated with expression of HLA-B*57:01 and recent studies identified flucloxacillin haptenated HLA-B*57:01 ligands naturally eluted off the surface of antigen presenting cells (Waddington et al., 2020, Puig et al., 2020). Ogese and his colleagues characterised β-lactam antibiotic protein adducts formed in primary human hepatocytes and demonstrated that these adducts are transported to antigen presenting cells encapsulated within exosomes (Ogese et al., 2019). An amoxicillin-modified 9-mer peptide derived from an exosomal protein was shown to activate naïve T-cells from healthy human donors. Similarly, (Tailor et al., 2020) found that amoxicillin-modified peptides designed to interact with HLA-DRB1*15:01-DQB1*06:02 selectively activated T-cells from patients with co-amoxiclav-induced liver injury. These data indicate that drug hapten protein interactions are important for the activation of T-cells in patients with DILI; however, detailed structural analyses combined with functional assessments of T-cell responses are need to delineate precise drug-peptide HLA binding epitopes and to determine the extent of cross reactivity seen with parent drugs, metabolites and protein adducts. In recent years, the immunoinformatic tool called ScanProsite was established to identify proteins across the whole proteome to help with the engineering of T-cells for the functional killing of tumours (Cameron et al., 2013). In a similar manner, the key motifs from

peptides should be incorporated into the study of drug hypersensitivity filtering the search for peptides derived from specific organs.

The heritable epigenetic expression of HLA genes has recently been shown to strongly influence disease outcome. Epigenetic evaluation of HLA molecules and their relative expression in disease states has shown and these genes can be differentially regulated. The process of antigen presentation is also highly inefficient with only 1 in 1000 processed proteins being presented on as few as 50-100 MHC molecules per cell. If we consider this, a small change in the epigenetic expression of MHC may have huge positive impact on the outcome of a reaction (Demotz et al., 1990, Yewdell et al., 2003). MHC antigens are also excreted by cells; thus, soluble MHC may have important immunoregulatory properties and play a role in determining susceptibility to drug hypersensitivity. In diseases such as viral infections, inflammation, autoimmunity and cancer, the level of soluble MHC molecules and their activity have been shown to differ and this translates into different abilities to regulate disease. Therefore, research is required to investigate how soluble MHC molecules impact on susceptibility towards drug hypersensitivity (Bakela and Athanassakis, 2018).

Finally, one needs to consider the patient-specific factors that overlay HLA epitopes and determine which individuals develop an adverse reaction following drug exposure. This is important because (1) it is known that all tolerant and hypersensitive individuals are exposed to drugs and protein adducts at similar levels (Meng et al., 2017); (2) all individuals have a T-cell repertoire for drug-specific T-cell activation, but most individuals safely tolerate drugs – this is evidenced through T-cell priming studies with drugs and reactive metabolites (Ogese et al., 2020, Faulkner

et al., 2016); and (3) even in individuals expressing the HLA risk allele predisposing them to abacavir hypersensitivity, only 50% go on to develop hypersensitivity when exposed to the drug (Mallal et al., 2008) this is despite 100% of HLA-B*57:01+ individuals possessing abacavir-responsive T-cells(Schnyder et al., 2013). These data suggest that patients must overcome a threshold of resistance that is not directly related to drug exposure to develop a pathogenic T-cell response and hypersensitivity. Clinical studies in the immune oncology field suggest that immune regulatory receptors may play an important role in maintaining tolerance to drugs. There is a growing body of evidence that patients administered immune checkpoint inhibitor therapy (anti PD-1, PD-L1, CTLA-4) are significantly more susceptible to drug hypersensitivity reactions by concomitant drugs (Naisbitt et al., 2020, Vocanson et al., 2020). Drugs such as sulfasalazine are simply not tolerable with a background of immune dysregulation (Ford et al., 2018). In the laboratory, naïve T-cells have been shown to be more easily primed in vitro with drugs in the presence of immune checkpoint inhibition(Gibson et al., 2014a, Gibson et al., 2017), while a recent case study has described T-cell-mediated hypersensitivity to previously tolerated iodinated contract media precipitated by introduction of the immune checkpoint inhibiter atezolizumab (Hammond et al., 2021). In animal models, liver injury to drugs such as amodiaquine and nevirapine and herbal products such as green tea is exaggerated when immune tolerance is impaired with checkpoint blockade (Mak and Uetrecht, 2015b, Mak and Uetrecht, 2015a, Metushi et al., 2015, Cho et al., 2021). Collectively, these data indicate that susceptibility to drug hypersensitivity is dependent not only on the drug structure and HLA peptide binding, but also on patient immune regulatory pathways. Since expression and activity of these

pathways varies across individuals and within the same individual throughout their life it will remain incredibly difficult to predict the outcome of drug exposure; ignorance, tolerance or a hypersensitivity reaction.

To conclude, the data generated in this thesis addresses our primary hypothesis and shows that drug-responsive T cells are selectively activated in certain patients with DILI. Furthermore, we were able to explore our secondary hypothesis and show that for drugs naproxen and atabecestat T cells are preferentially activated by a phase I metabolite of the parent drug. These data have important implications for pharma attempting to develop safer and more effective therapeutics and healthcare professional managing patients that develop adverse events such as DILI.

# 7. BIBLIOGRAPHY


ABAJO, F. 1988. Phenytoin interaction with rifampicin. *BMJ (Clinical research ed.),* 297**,** 1048.

ABBAS, A. K., LICHTMAN, A. H. & PILLAI, S. 2019. *Basic immunology e-book: functions and disorders of the immune system*, Elsevier Health Sciences.

ABE, M., FURUKAWA, S., TAKAYAMA, S., MLCHITAKA, K., MLNAMI, H., YAMAMOTO, K., HORIIKE, N. & ONJI, M. 2003a. Drug-induced Hepatitis with AutoimmuneFeatures during Minocycline Therapy. *Internal medicine,* 42**,** 48-52.

ABE, R., SHIMIZU, T., SHIBAKI, A., NAKAMURA, H., WATANABE, H. & SHIMIZU, H. 2003b. Toxic epidermal necrolysis and Stevens-Johnson syndrome are induced by soluble Fas ligand. *The American journal of pathology,* 162**,** 1515-1520.

ADAIR, K., MENG, X. & NAISBITT, D. J. 2021. Drug hapten-specific T-cell activation: Current status and unanswered questions. *Proteomics***,** 2000267.

ADAM, J., ERIKSSON, K. K., SCHNYDER, B., FONTANA, S., PICHLER, W. J. & YERLY, D. 2012. Avidity determines T-cell reactivity in abacavir hypersensitivity. *European journal of immunology,* 42**,** 1706-1716.

ADELMAN, D. C., CASALE, T. B. & CORREN, J. 2002. *Manual of Allergy and Immunology*, Lippincott Williams & Wilkins.

ADEREM, A. & UNDERHILL, D. M. 1999. Mechanisms of phagocytosis in macrophages. *Annual review of immunology,* 17**,** 593-623.

ADKINSON JR, N. F. 1984. Risk factors for drug allergy. *Journal of Allergy and Clinical Immunology,* 74**,** 567-572.

AGERBERTH, B. & GUÐMUNDSSON, G. 2006. Host antimicrobial defence peptides in human disease. *Antimicrobial Peptides and Human Disease***,** 67-90.

AIHARA, M., SUGITA, Y., TAKAHASHI, S., NAGATANI, T., ARATA, S., TAKEUCHI, K. & IKEZAWA, Z. 2001. Anticonvulsant hypersensitivity syndrome associated with reactivation of cytomegalovirus. *British Journal of Dermatology,* 144**,** 1231-1234.

ALARCÓN, G. S. 2000. Tetracyclines for the treatment of rheumatoid arthritis. *Expert opinion on investigational drugs,* 9**,** 1491-1498.

ALBERTS, B., JOHNSON, A., LEWIS, J., RAFF, M., ROBERTS, K. & WALTER, P. 2002. Integrins. *Molecular Biology of the Cell. 4th edition.* Garland Science.

ALI, S., PIMENTEL, J. D. & MA, C. 2011. Naproxen-induced liver injury. *Hepatobiliary Pancreat Dis Int,* 10**,** 552-6.

ALMAZROO, O. A., MIAH, M. K. & VENKATARAMANAN, R. 2017. Drug metabolism in the liver. *Clinics in liver disease,* 21**,** 1-20.

ALOMAR, M. J. 2014. Factors affecting the development of adverse drug reactions. *Saudi pharmaceutical journal,* 22**,** 83-94.

ALZAHRANI, A., OGESE, M., MENG, X., WADDINGTON, J. C., TAILOR, A., FARRELL, J., MAGGS, J. L., BETTS, C., PARK, B. K. & NAISBITT, D. 2017. Dapsone and Nitroso Dapsone Activation of Naïve T-Cells from Healthy Donors. *Chemical research in toxicology,* 30**,** 2174-2186.

AMACHER, D. E. 2012. The primary role of hepatic metabolism in idiosyncratic drug-induced liver injury. *Expert opinion on drug metabolism & toxicology,* 8**,** 335-347.

AMACHER, D. E. 2014. Female gender as a susceptibility factor for drug-induced liver injury. *Human & experimental toxicology,* 33**,** 928-939.

AMALI, M. O., SULLIVAN, A., JENKINS, R. E., FARRELL, J., MENG, X., FAULKNER, L., WHITAKER, P., PECKHAM, D., PARK, B. K. & NAISBITT, D. J. 2017. Detection of drug-responsive B lymphocytes and antidrug IgG in patients with β-lactam hypersensitivity. *Allergy,* 72**,** 896-907.

ANDRADE, R. J., AITHAL, G. P., BJÖRNSSON, E. S., KAPLOWITZ, N., KULLAK-UBLICK, G. A., LARREY, D., KARLSEN, T. H. & LIVER, E. A. F. T. S. O. T. 2019. EASL clinical practice guidelines: drug-induced liver injury. *Journal of hepatology,* 70**,** 1222-1261.

ANDRADE, R. J., LUCENA, M. I., ALONSO, A., GARCÍA-CORŕES, M., GARCÍA-RUIZ, E., BENITEZ, R., FERNÁNDEZ, M. C., PELAEZ, G., ROMERO, M. & CORPAS, R. 2004. HLA class II genotype influences the type of liver injury in drug-induced idiosyncratic liver disease. *Hepatology,* 39**,** 1603-1612.

ANDRADE, R. J., LUCENA, M. I., FERNÁNDEZ, M. C., PELAEZ, G., PACHKORIA, K., GARCÍA-RUIZ, E., GARCÍA-MUÑOZ, B., GONZÁLEZ-GRANDE, R., PIZARRO, A. & DURÁN, J. A. 2005. Drug-induced liver injury: an analysis of 461 incidences submitted to the Spanish registry over a 10-year period. *Gastroenterology,* 129**,** 512-521.

ARDERN-JONES, M. R. & FRIEDMANN, P. S. 2011. Skin manifestations of drug allergy. *British journal of clinical pharmacology,* 71**,** 672-683.

ARDERN-JONES, M. R. & FRIEDMANN, P. S. 2011. Skin manifestations of drug allergy. *British journal of clinical pharmacology,* 71**,** 672-683.

ARNAIZ, J. A., CARNÉ, X., RIBA, N., CODINA, C., RIBAS, J. & TRILLA, A. 2001. The use of evidence in pharmacovigilance. *European Journal of Clinical Pharmacology,* 57**,** 89-91.

ARONSON, J. K. 2009. *Meyler's side effects of cardiovascular drugs*, Elsevier.

ARULMANI, R., RAJENDRAN, S. & SURESH, B. 2008. Adverse drug reaction monitoring in a secondary care hospital in South India. *British journal of clinical pharmacology,* 65**,** 210-216.

ATTIA, S. M. 2010. Deleterious effects of reactive metabolites. *Oxidative medicine and cellular longevity,* 3**,** 238-253.

AVERY, A. J., ANDERSON, C., BOND, C. M., FORTNUM, H., GIFFORD, A., HANNAFORD, P. C., HAZELL, L., KRSKA, J., LEE, A. J., MCLERNON, D. J., MURPHY, E., SHAKIR, S. & WATSON, M. C. 2011. Evaluation of patient reporting of adverse drug reactions to the UK 'Yellow Card Scheme': literature review, descriptive and qualitative analyses, and questionnaire surveys. *Health Technol Assess,* 15**,** 1-234, iii-iv.

AVORN, J. 2012. Two Centuries of Assessing Drug Risks. *New England Journal of Medicine,* 367**,** 193-197.

AZAZ-LIVSHITS, T., LEVY, M., SADAN, B., SHALIT, M., GEISSLINGER, G. & BRUNE, K. 1998. Computerized surveillance of adverse drug reactions in hospital: pilot study. *British journal of clinical pharmacology,* 45**,** 309-314.

AZOURY, M., FILÌ, L., BECHARA, R., SCORNET, N., DE CHAISEMARTIN, L., WEAVER, R., CLAUDE, N., MAILLERE, B., PARRONCHI, P. & JOSEPH, D. 2018. Identification of T-cell epitopes from benzylpenicillin conjugated to human serum albumin and implication in penicillin allergy. *Allergy,* 73**,** 1662-1672.

BAILEY, M. J. & DICKINSON, R. G. 1999. Limitations of hepatocytes and liver homogenates in modelling in vivo formation of acyl glucuronide-derived drug-protein adducts. *Journal of pharmacological and toxicological methods,* 41**,** 27-32.

BAKELA, K. & ATHANASSAKIS, I. 2018. Soluble major histocompatibility complex molecules in immune regulation: highlighting class II antigens. *Immunology,* 153**,** 315-324.

BALA, S., WEAVER, J. & HASTINGS, K. L. 2005. Clinical relevance of preclinical testing for allergic side effects. *Toxicology,* 209**,** 195-200.

BALLENTINE, C. 1981. Taste of raspberries, taste of death: the 1937 elixir sulfanilamide incident. *FDA Consumer magazine,* 15.

BANCHEREAU, J. & STEINMAN, R. M. 1998. Dendritic cells and the control of immunity. *Nature,* 392**,** 245-252.

BARRERAS, A. & GURK-TURNER, C. 2003. Angiotensin II receptor blockers. *Proceedings (Baylor University. Medical Center),* 16**,** 123-126.

BARRINGER, W., SHULTZ, W., SIEGER, G. & NASH, R. 1974. Minocycline hydrochloride and its relationship to other tetracycline antibiotics. *American Journal of Pharmacy,* 146**,** 179-191.

BASS, B. H. 1974. Letter: Jaundice associated with naproxen. *Lancet,* 1**,** 998.

BASTUJI-GARIN, S., RZANY, B., STERN, R. S., SHEAR, N. H., NALDI, L. & ROUJEAU, J.-C. 1993. Clinical classification of cases of toxic epidermal necrolysis, Stevens-Johnson syndrome, and erythema multiforme. *Archives of dermatology,* 129**,** 92-96.

BATES, D. W., CULLEN, D. J., LAIRD, N., PETERSEN, L. A., SMALL, S. D., SERVI, D., LAFFEL, G., SWEITZER, B. J., SHEA, B. F., HALLISEY, R. & ET AL. 1995. Incidence of adverse drug events and potential adverse drug events. Implications for prevention. ADE Prevention Study Group. *Jama,* 274**,** 29-34.

BEELER, A., ENGLER, O., GERBER, B. O. & PICHLER, W. J. 2006. Long-lasting reactivity and high frequency of drug-specific T cells after severe systemic drug hypersensitivity reactions. *Journal of Allergy and Clinical Immunology,* 117**,** 455-462.

BEELER, A. & PICHLER, W. J. 2006. In vitro tests of T cell-mediated drug hypersensitivity. *Expert review of clinical immunology,* 2**,** 887-900.

BELL, C. C., FAULKNER, L., MARTINSSON, K., FARRELL, J., ALFIREVIC, A., TUGWOOD, J., PIRMOHAMED, M., NAISBITT, D. J. & PARK, B. K. 2013. T-cells from HLA-B* 57: 01+ human subjects are activated with abacavir through two independent pathways and induce cell death by multiple mechanisms. *Chemical research in toxicology,* 26**,** 759-766.

BENET, L. Z., SPAHN-LANGGUTH, H., IWAKAWA, S., VOLLAND, C., MIZUMA, T., MAYER, S., MUTSCHLER, E. & LIN, E. T. 1993. Predictability of the covalent binding of acidic drugs in man. *Life sciences,* 53**,** PL141-PL146.

BERLIN, J. A., GLASSER, S. C. & ELLENBERG, S. S. 2008. Adverse event detection in drug development: recommendations and obligations beyond phase 3. *American journal of public health,* 98**,** 1366-1371.

BETTELLI, E., OUKKA, M. & KUCHROO, V. K. 2007. TH-17 cells in the circle of immunity and autoimmunity. *Nature immunology,* 8**,** 345-350.

BHARADWAJ, M., ILLING, P., THEODOSSIS, A., PURCELL, A. W., ROSSJOHN, J. & MCCLUSKEY, J. 2012. Drug hypersensitivity and human leukocyte antigens of the major histocompatibility complex. *Annual review of pharmacology and toxicology,* 52**,** 401-431.

BHAT, G., JORDAN JR, J., SOKALSKI, S., BAJAJ, V., MARSHALL, R. & BERKELHAMMER, C. 1998. Minocycline-induced hepatitis with autoimmune features and neutropenia. *Journal of clinical gastroenterology,* 27**,** 74-75.

BISCHER, A., ZIA-AMIRHOSSEINI, P., IWAKI, M., MCDONAGH, A. F. & BENET, L. Z. 1995. Stereoselective binding properties of naproxen glucuronide diastereomers to proteins. *Journal of Pharmacokinetics and Biopharmaceutics,* 23**,** 379-395.

BJORKMAN, P. J. & PARHAM, P. 1990. Structure, function, and diversity of class I major histocompatibility complex molecules. *Annual review of biochemistry,* 59**,** 253-288.

BJÖRNSSON, E. 2010. drug-induced liver injury in clinical practice. *Alimentary pharmacology & therapeutics,* 32**,** 3-13.

BJÖRNSSON, E. & DAVIDSDOTTIR, L. 2009. The long-term follow-up after idiosyncratic drug-induced liver injury with jaundice. *Journal of hepatology,* 50**,** 511-517.

BLACK, M., MITCHELL, J. R., ZIMMERMAN, H. J., ISHAK, K. G. & EPLER, G. R. 1975. Isoniazid-associated hepatitis in 114 patients. *Gastroenterology,* 69**,** 289-302.

BLEIBEL, W., KIM, S., D'SILVA, K. & LEMMER, E. R. 2007. Drug-induced liver injury. *Digestive diseases and sciences,* 52**,** 2463-2471.

BLENKINSOPP, A., WILKIE, P., WANG, M. & ROUTLEDGE, P. A. 2007. Patient reporting of suspected adverse drug reactions: a review of published literature and international experience. *Br J Clin Pharmacol,* 63**,** 148-56.

BLUM, J. S., WEARSCH, P. A. & CRESSWELL, P. 2013. Pathways of antigen processing. *Annual review of immunology,* 31**,** 443-473.

BÖCKER, R., PETER, R., MACHBERT, G. & BAUER, W. 1991. Identification and determination of the two principal metabolites of minocycline in humans. *Journal of Chromatography B: Biomedical Sciences and Applications,* 568**,** 363-374.

BOCQUET, H., BAGOT, M. & ROUJEAU, J. C. Drug-induced pseudolymphoma and drug hypersensitivity syndrome (Drug Rash with Eosinophilia and Systemic Symptoms: DRESS). Seminars in cutaneous medicine and surgery, 1996. 250-257.

BODMER, W. 1987. The HLA system: structure and function. *Journal of clinical pathology,* 40**,** 948-958.

BODMER, W. F. 1997. HLA: what's in a name? *Tissue Antigens,* 49**,** 293-296.

BODMER, W. F., ALBERT, E., BODMER, J. G., DUPONT, B., MACH, B., MAYR, W., SASAZUKI, T., SCHREUDER, G. T., SVEJGAARD, A. & TERASAKI, P. 1989. Nomenclature for factors of the HLA system, 1987. *Immunobiology of HLA.* Springer.

BÖHM, R., PROKSCH, E., SCHWARZ, T. & CASCORBI, I. 2018. Drug Hypersensitivity: Diagnosis, Genetics, and Prevention. *Deutsches Aerzteblatt International,* 115**,** 501.

BOHNER, P., CHEVALIER, M. F., CESSON, V., RODRIGUES-DIAS, S.-C., DARTIGUENAVE, F., BURRUNI, R., TAWADROS, T., VALERIO, M., LUCCA, I. & NARDELLI-HAEFLIGER, D. 2019. Double positive CD4+ CD8+ T cells are enriched in urological cancers and favor T helper-2 polarization. *Frontiers in immunology,* 10**,** 622.

BORDET, R., GAUTIER, S., LE LOUET, H., DUPUIS, B. & CARON, J. 2001. Analysis of the direct cost of adverse drug reactions in hospitalised patients. *European journal of clinical pharmacology,* 56**,** 935-941.

BOUGIE, D. & ASTER, R. 2001. Immune thrombocytopenia resulting from sensitivity to metabolites of naproxen and acetaminophen. *Blood, The Journal of the American Society of Hematology,* 97**,** 3846-3850.

BOWALGAHA, K., ELLIOT, D. J., MACKENZIE, P. I., KNIGHTS, K. M., SWEDMARK, S. & MINERS, J. O. 2005. S-Naproxen and desmethylnaproxen glucuronidation by human liver microsomes and recombinant human UDP-glucuronosyltransferases (UGT): role of UGT2B7 in the elimination of naproxen. *Br J Clin Pharmacol,* 60**,** 423-33.

BRACKETT, C. C., SINGH, H. & BLOCK, J. H. 2004. Likelihood and mechanisms of cross-allergenicity between sulfonamide antibiotics and other drugs containing a sulfonamide functional group. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy,* 24**,** 856-870.

BRECKENRIDGE, A. 2015. The burden of adverse drug events. *British journal of clinical pharmacology,* 80**,** 785-787.

BRITT, R. B. & BROWN, J. N. 2018. Characterizing the severe reactions of parenteral vitamin K1. *Clinical and Applied Thrombosis/Hemostasis,* 24**,** 5-12.

BROCKOW, K., ROMANO, A., BLANCA, M., RING, J., PICHLER, W. & DEMOLY, P. 2002. General considerations for skin test procedures in the diagnosis of drug hypersensitivity. *Allergy,* 57**,** 45-51.

BROGDEN, R., SPEIGHT, T. & AVERY, G. 1975. Minocycline: a review of its antibacterial and pharmacokinetic properties and therapeutic use. *Drugs,* 9**,** 251-291.

BRUCE, R. D., ALTICE, F. L. & FRIEDLAND, G. H. 2008. Pharmacokinetic drug interactions between drugs of abuse and antiretroviral medications: implications and management for clinical practice. *Expert review of clinical pharmacology,* 1**,** 115-127.

BUHL, R., HOLROYD, K., MASTRANGELI, A., CANTIN, A., JAFFE, H. A., WELLS, F., SALTINI, C. & CRYSTAL, R. 1989. Systemic glutathione deficiency in symptom-free HIV-seropositive individuals. *The Lancet,* 334**,** 1294-1298.

BURKHART, C., VON GREYERZ, S., DEPTA, J. P., NAISBITT, D. J., BRITSCHGI, M., PARK, K. B. & PICHLER, W. J. 2001. Influence of reduced glutathione on the proliferative response

of sulfamethoxazole-specific and sulfamethoxazole-metabolite-specific human CD4+ T-cells. *British journal of pharmacology,* 132**,** 623-630.

BUXTON, I. & BENET, L. 2011. Goodman and Gilman's The Pharmacological Basis of Therapeutics 12th ed. ed LL Bruton, BA Chabner and BC Knollmann. New York: McGraw-Hill.

CAMERON, B. J., GERRY, A. B., DUKES, J., HARPER, J. V., KANNAN, V., BIANCHI, F. C., GRAND, F., BREWER, J. E., GUPTA, M. & PLESA, G. 2013. Identification of a Titin-derived HLA-A1–presented peptide as a cross-reactive target for engineered MAGE A3–directed T cells. *Science translational medicine,* 5**,** 197ra103-197ra103.

CARDONE, M., GARCIA, K., TILAHUN, M. E., BOYD, L. F., GEBREYOHANNES, S., YANO, M., RODERIQUEZ, G., AKUE, A. D., JUENGST, L. & MATTSON, E. 2018. A transgenic mouse model for HLA-B* 57: 01–linked abacavir drug tolerance and reactivity. *The Journal of clinical investigation,* 128**,** 2819-2832.

CASTREJON, J. L., BERRY, N., EL-GHAIESH, S., GERBER, B., PICHLER, W. J., PARK, B. K. & NAISBITT, D. J. 2010. Stimulation of human T cells with sulfonamides and sulfonamide metabolites. *Journal of Allergy and Clinical Immunology,* 125**,** 411-418. e4.

CELL, P. & SILVERSTEIN, A. M. 1962. Delayed hypersensitivity to hapten-protein conjugates: I. The effect of carrier protein and site of attachment to hapten. *The Journal of experimental medicine,* 115**,** 1037-1051.

CHALASANI, N., BONKOVSKY, H. L., FONTANA, R., LEE, W., STOLZ, A., TALWALKAR, J., REDDY, K. R., WATKINS, P. B., NAVARRO, V. & BARNHART, H. 2015. Features and outcomes of 899 patients with drug-induced liver injury: the DILIN prospective study. *Gastroenterology,* 148**,** 1340-1352. e7.

CHALASANI, N., FONTANA, R. J., BONKOVSKY, H. L., WATKINS, P. B., DAVERN, T., SERRANO, J., YANG, H., ROCHON, J. & NETWORK, D. I. L. I. 2008. Causes, clinical features, and outcomes from a prospective study of drug-induced liver injury in the United States. *Gastroenterology,* 135**,** 1924-1934. e4.

CHALASANI, N. P., HAYASHI, P. H., BONKOVSKY, H. L., NAVARRO, V. J., LEE, W. M., FONTANA, R. J. & GASTROENTEROLOGY, P. P. C. O. T. A. C. O. 2014. ACG Clinical Guideline: the diagnosis and management of idiosyncratic drug-induced liver injury. *American Journal of Gastroenterology,* 109**,** 950-966.

CHAVEZ-GALAN, L., ARENAS-DEL ANGEL, M., ZENTENO, E., CHAVEZ, R. & LASCURAIN, R. 2009. Cell death mechanisms induced by cytotoxic lymphocytes. *Cellular & molecular immunology,* 6**,** 15-25.

CHEN, P., LIN, J.-J., LU, C.-S., ONG, C.-T., HSIEH, P. F., YANG, C.-C., TAI, C.-T., WU, S.-L., LU, C.-H. & HSU, Y.-C. 2011. Carbamazepine-induced toxic effects and HLA-B* 1502 screening in Taiwan. *New England Journal of Medicine,* 364**,** 1126-1133.

CHESSMAN, D., KOSTENKO, L., LETHBORG, T., PURCELL, A. W., WILLIAMSON, N. A., CHEN, Z., KJER-NIELSEN, L., MIFSUD, N. A., TAIT, B. D. & HOLDSWORTH, R. 2008. Human leukocyte antigen class I-restricted activation of CD8+ T cells provides the immunogenetic basis of a systemic drug hypersensitivity. *Immunity,* 28**,** 822-832.

CHO, T., WANG, X., YEUNG, K., CAO, Y. & UETRECHT, J. 2021. Liver Injury Caused by Green Tea Extract in PD-1–/–Mice: An Impaired Immune Tolerance Model for Idiosyncratic Drug-Induced Liver Injury. *Chemical Research in Toxicology,* 34**,** 849-856.

CHOONARA, I. & HARRIS, F. 1984. Adverse drug reactions in medical inpatients. *Archives of disease in childhood,* 59**,** 578-580.

CHOQUET-KASTYLEVSKY, G., INTRATOR, L., CHENAL, C., BOCQUET, H., REVUZ, J. & ROUJEAU, J. 1998. Increased levels of interleukin 5 are associated with the generation of eosinophilia in drug-induced hypersensitivity syndrome. *The British journal of dermatology,* 139**,** 1026-1032.

CHOUDHARY, S., MCLEOD, M., TORCHIA, D. & ROMANELLI, P. 2013. Drug reaction with eosinophilia and systemic symptoms (DRESS) syndrome. *The Journal of clinical and aesthetic dermatology,* 6**,** 31.

CHUNG, W., HUNG, S. & HONG, H. Medical genetics: a markerfor Stevens-Johnsonsyndrome. *Nature2004,* 428**,** 486.

CHUNG, W. H., PAN, R. Y., CHU, M. T., CHIN, S. W., HUANG, Y. L., WANG, W. C., CHANG, J. Y. & HUNG, S. I. 2015. Oxypurinol-Specific T Cells Possess Preferential TCR Clonotypes and Express Granulysin in Allopurinol-Induced Severe Cutaneous Adverse Reactions. *J Invest Dermatol,* 135**,** 2237-2248.

CHUNG, W. H., WANG, C. W. & DAO, R. L. 2016. Severe cutaneous adverse drug reactions. *The Journal of Dermatology,* 43**,** 758-766.

CLASSEN, D. C., PESTOTNIK, S. L., EVANS, R. S., LLOYD, J. F. & BURKE, J. P. 1997. Adverse drug events in hospitalized patients: excess length of stay, extra costs, and attributable mortality. *Jama,* 277**,** 301-306.

CLAY, K. D., HANSON, J. S., POPE, S. D., RISSMILLER, R. W., PURDUM III, P. P. & BANKS, P. M. 2006. Brief communication: severe hepatotoxicity of telithromycin: three case reports and literature review. *Annals of internal medicine,* 144**,** 415-420.

CLUFF, L. E., THORNTON, G. F. & SEIDL, L. G. 1964. STUDIES ON THE EPIDEMIOLOGY OF ADVERSE DRUG REACTIONS. I. METHODS OF SURVEILLANCE. *Jama,* 188**,** 976-83.

COICO, R. 2021. *Immunology: a short course*, John Wiley & Sons.

COLEMAN, J. J., FERNER, R. E. & EVANS, S. J. W. 2006. Monitoring for adverse drug reactions. *British journal of clinical pharmacology,* 61**,** 371-378.

COOPER, M. A., FEHNIGER, T. A., FUCHS, A., COLONNA, M. & CALIGIURI, M. A. 2004. NK cell and DC interactions. *Trends in immunology,* 25**,** 47-52.

COUPER, K. N., BLOUNT, D. G. & RILEY, E. M. 2008. IL-10: the master regulator of immunity to infection. *The Journal of Immunology,* 180**,** 5771-5777.

COX, J. H., FERRARI, G. & JANETZKI, S. 2006. Measurement of cytokine release at the single cell level using the ELISPOT assay. *Methods,* 38**,** 274-282.

CRIADO, P. R., CRIADO, R. F. J., AVANCINI, J. D. M. & SANTI, C. G. 2012. Drug reaction with eosinophilia and systemic symptoms (DRESS)/drug-induced hypersensitivity syndrome (DIHS): a review of current concepts. *Anais brasileiros de dermatologia,* 87**,** 435-449.

CRIBB, A. E. & SPIELBERG, S. P. 1990. Hepatic microsomal metabolism of sulfamethoxazole to the hydroxylamine. *Drug metabolism and disposition,* 18**,** 784-787.

CRIBB, A. E. & SPIELBERG, S. P. 1992. Sulfamethoxazole is metabolized to the hydroxylamine in humans. *Clinical Pharmacology & Therapeutics,* 51**,** 522-526.

CROSSON, J. & STILLMAN, M. T. 1997. Minocycline-related lupus erythematosus with associated liver disease. *Journal of the American Academy of Dermatology,* 36**,** 867-868.

D'AMORE, F., AGOSTINO, A. & SANTORO, A. 1996. Drug-induced cholestasis. Clinical contribution. *Minerva Gastroenterol Dietol,* 42**,** 215-9.

DALY, A. K. 2012. Using genome-wide association studies to identify genes important in serious adverse drug reactions. *Annual review of pharmacology and toxicology,* 52**,** 21-35.

DALY, A. K. 2013. Pharmacogenomics of adverse drug reactions. *Genome medicine,* 5**,** 1-12.

DALY, A. K., DONALDSON, P. T., BHATNAGAR, P., SHEN, Y., PE'ER, I., FLORATOS, A., DALY, M. J., GOLDSTEIN, D. B., JOHN, S. & NELSON, M. R. 2009. HLA-B* 5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nature genetics,* 41**,** 816-819.

DALY, M. J., RIOUX, J. D., SCHAFFNER, S. F., HUDSON, T. J. & LANDER, E. S. 2001. High-resolution haplotype structure in the human genome. *Nature genetics,* 29**,** 229-232.

DAUSSET, J. 1958. Iso-Leuko-Antibodies*. *Vox Sanguinis,* 3**,** 40-41.

DAVIES, D. M. 1977. *Textbook of adverse drug reactions*, Oxford University Press.
DAVIES, N. M. & ANDERSON, K. E. 1997. Clinical pharmacokinetics of naproxen. *Clin Pharmacokinet,* 32**,** 268-93.
DE JONGHE, S., WEINSTOCK, D., ALIGO, J., WASHINGTON, K. & NAISBITT, D. 2020. Biopsy pathology and immunohistochemistry of a case of immune-mediated drug-induced liver injury with Atabecestat. *Hepatology*.
DE JONGHE, S., WEINSTOCK, D., ALIGO, J., WASHINGTON, K. & NAISBITT, D. 2021. Biopsy pathology and immunohistochemistry of a case of immune-mediated drug-induced liver injury with Atabecestat. *Hepatology,* 73**,** 452-455.
DE JONGHE, S., WEINSTOCK, D., ALIGO, J., WASHINGTON, K. & NAISBITT, D. 2020. Biopsy pathology and immunohistochemistry of a case of immune-mediated drug-induced liver injury with Atabecestat. *Hepatology,* n/a.
DEARMOND, B., FRANCISCO, C. A., LIN, J.-S., HUANG, F.-Y., HALLADAY, S., BARTIZEK, R. D. & SKARE, K. L. 1995. Safety profile of over-the-counter naproxen sodium. *Clinical Therapeutics,* 17**,** 587-601.
DELEMOS, A. S., GHABRIL, M., ROCKEY, D. C., GU, J., BARNHART, H. X., FONTANA, R. J., KLEINER, D. E., BONKOVSKY, H. L. & NETWORK, D.-I. L. I. 2016. Amoxicillin–clavulanate-induced liver injury. *Digestive diseases and sciences,* 61**,** 2406-2416.
DEMERS, K. R., REUTER, M. A. & BETTS, M. R. 2013. CD 8+ T-cell effector function and transcriptional regulation during HIV pathogenesis. *Immunological reviews,* 254**,** 190-206.
DEMIRAG, M. D., OZENIRLER, S., GOKER, B., POYRAZ, A., HAZNEDAROGLU, S. & OZTURK, M. A. 2007. Idiosyncratic toxic hepatitis secondary to single dose of naproxen. *Acta Gastroenterol Belg,* 70**,** 247-8.
DEMOLY, P., ADKINSON, N. F., BROCKOW, K., CASTELLS, M., CHIRIAC, A. M., GREENBERGER, P. A., KHAN, D. A., LANG, D. M., PARK, H. S. & PICHLER, W. 2014. International Con sensus on drug allergy. *Allergy,* 69**,** 420-437.
DEMOTZ, S., GREY, H. M. & SETTE, A. 1990. The minimal number of class II MHC-antigen complexes needed for T cell activation. *Science,* 249**,** 1028-1030.
DESCAMPS, V., MAHE, E., HOUHOU, N., ABRAMOWITZ, L., ROZENBERG, F., RANGER-ROGEZ, S. & CRICKX, B. 2003. Drug-induced hypersensitivity syndrome associated with Epstein–Barr virus infection. *British Journal of Dermatology,* 148**,** 1032-1034.
DONALDSON, P. T., DALY, A. K., HENDERSON, J., GRAHAM, J., PIRMOHAMED, M., BERNAL, W., DAY, C. P. & AITHAL, G. P. 2010. Human leucocyte antigen class II genotype in susceptibility and resistance to co-amoxiclav-induced liver injury. *Journal of hepatology,* 53**,** 1049-1053.
DONG, C. 2021. Cytokine regulation and function in T cells. *Annual Review of Immunology,* 39**,** 51-76.
DORMANN, H., CRIEGEE-RIECK, M., NEUBERT, A., EGGER, T., LEVY, M., HAHN, E. & BRUNE, K. 2004. Implementation of a computer-assisted monitoring system for the detection of adverse drug reactions in gastroenterology. *Alimentary pharmacology & therapeutics,* 19**,** 303-309.
DOWNEY, A., JACKSON, C., HARUN, N. & COOPER, A. 2012. Toxic epidermal necrolysis: review of pathogenesis and management. *Journal of the American Academy of Dermatology,* 66**,** 995-1003.
DUHEN, T., GEIGER, R., JARROSSAY, D., LANZAVECCHIA, A. & SALLUSTO, F. 2009. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nature immunology,* 10**,** 857-863.
EDWARDS, I. R. & ARONSON, J. K. 2000. Adverse drug reactions: definitions, diagnosis, and management. *Lancet,* 356**,** 1255-9.

EMMERSON, B. T., HAZELTON, R. A. & FRAZER, I. H. 1988. Some adverse reactions to allopurinol may be mediated by lymphocyte reactivity to oxypurinol. *Arthritis Rheum,* 31**,** 436-40.

ERNST, W. A., THOMA-USZYNSKI, S., TEITELBAUM, R., KO, C., HANSON, D. A., CLAYBERGER, C., KRENSKY, A. M., LEIPPE, M., BLOOM, B. R. & GANZ, T. 2000. Granulysin, a T cell product, kills bacteria by altering membrane permeability. *The Journal of Immunology,* 165**,** 7102-7108.

ESMON, C. T., VIGANO-D'ANGELO, S., D'ANGELO, A. & COMP, P. C. 1987. Anticoagulation Proteins C and S. *In:* WESSLER, S., BECKER, C. G. & NEMERSON, Y. (eds.) *The New Dimensions of Warfarin Prophylaxis.* Boston, MA: Springer US.

ETEBU, E. & ARIKEKPAR, I. 2016. Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. *Int. J. Appl. Microbiol. Biotechnol. Res,* 4**,** 90-101.

EVANS, B. J. 2012. The ethics of postmarketing observational studies of drug safety under section 505 (o)(3) of the Food, Drug, and Cosmetic Act. *American Journal of Law & Medicine,* 38**,** 577-606.

EVANS, R. S., PESTOTNIK, S. L., CLASSEN, D. C., HORN, S. D., BASS, S. B. & BURKE, J. P. 1994. Preventing adverse drug events in hospitalized patients. *Annals of Pharmacotherapy,* 28**,** 523-527.

EVANS, W. E. & RELLING, M. V. 1999. Pharmacogenomics: translating functional genomics into rational therapeutics. *science,* 286**,** 487-491.

EVIN, G., BARAKAT, A. & MASTERS, C. L. 2010. BACE: Therapeutic target and potential biomarker for Alzheimer's disease. *The International Journal of Biochemistry & Cell Biology,* 42**,** 1923-1926.

EWER, S. & LIPPMAN, S. M. 2005. Type II chemotherapy-related cardiac dysfunction: Time to recognize a new entity by Michael. *Journal of Clinical Oncology,* 23**,** 2900-2902.

EYERICH, S., EYERICH, K., PENNINO, D., CARBONE, T., NASORRI, F., PALLOTTA, S., CIANFARANI, F., ODORISIO, T., TRAIDL-HOFFMANN, C. & BEHRENDT, H. 2009. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *The Journal of clinical investigation,* 119**,** 3573-3585.

FALANY, C. N., STROM, P. & SWEDMARK, S. 2005a. Sulphation of o-desmethylnaproxen and related compounds by human cytosolic sulfotransferases. *Br J Clin Pharmacol,* 60**,** 632-40.

FALANY, C. N., STRÖM, P. & SWEDMARK, S. 2005b. Sulphation of o-desmethylnaproxen and related compounds by human cytosolic sulfotransferases. *British Journal of Clinical Pharmacology,* 60**,** 632-640.

FAN, W.-L., SHIAO, M.-S., HUI, R. C.-Y., SU, S.-C., WANG, C.-W., CHANG, Y.-C. & CHUNG, W.-H. 2017. HLA association with drug-induced adverse reactions. *Journal of immunology research,* 2017.

FARRELL, J., NAISBITT, D. J., DRUMMOND, N. S., DEPTA, J. P., VILAR, F. J., PIRMOHAMED, M. & PARK, B. K. 2003. Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and humans. *Journal of Pharmacology and Experimental Therapeutics,* 306**,** 229-237.

FAULKNER, L., GIBSON, A., SULLIVAN, A., TAILOR, A., USUI, T., ALFIREVIC, A., PIRMOHAMED, M., NAISBITT, D. J. & KEVIN PARK, B. 2016. Detection of primary T cell responses to drugs and chemicals in HLA-typed volunteers: implications for the prediction of drug immunogenicity. *Toxicological Sciences,* 154**,** 416-429.

FERNER, R. E. & ARONSON, J. K. 1999. Errors in prescribing, preparing, and giving medicines: definition, classification, and prevention. *Side effects of drugs annual.* Elsevier.

FERREIRA, J. P. S., ALBUQUERQUE, H. M. T., CARDOSO, S. M., SILVA, A. M. S. & SILVA, V. L. M. 2021. Dual-target compounds for Alzheimer's disease: Natural and synthetic AChE

and BACE-1 dual-inhibitors and their structure-activity relationship (SAR). *European Journal of Medicinal Chemistry,* 221**,** 113492.

FIELD, T. S., GILMAN, B. H., SUBRAMANIAN, S., FULLER, J. C., BATES, D. W. & GURWITZ, J. H. 2005. The costs associated with adverse drug events among older adults in the ambulatory setting. *Medical care***,** 1171-1176.

FOGARI, R., ZOPPI, A., MUGELLINI, A., DESTRO, M., LAZZARI, P. & DEROSA, G. 2011. Difference in incidence of cough induced by imidapril and ramipril: Role of prostaglandin synthesis inhibition. *Biomedicine & Aging Pathology,* 1**,** 73-77.

FONTANA, R. J., SHAKIL, A. O., GREENSON, J. K., BOYD, I. & LEE, W. M. 2005. Acute liver failure due to amoxicillin and amoxicillin/clavulanate. *Digestive diseases and sciences,* 50**,** 1785-1790.

FRENGER, W. & MORBACH, H. J. 1973. Klinische Untersuchung Von Naproxen, Vor Allem Mit Bezug Auf Seine Verträglichkeit. *Scandinavian Journal of Rheumatology,* 2**,** 137-139.

GARCIA, K. C. & ADAMS, E. J. 2005. How the T cell receptor sees antigen—a structural view. *Cell,* 122**,** 333-336.

GARNER, S. E., EADY, A., BENNETT, C., NEWTON, J. N., THOMAS, K. & POPESCU, C. M. 2012. Minocycline for acne vulgaris: efficacy and safety. *Cochrane Database of Systematic Reviews*.

GERMAIN, R. N. 2002. T-cell development and the CD4–CD8 lineage decision. *Nature reviews immunology,* 2**,** 309-322.

GIBSON, A., FAULKNER, L., LICHTENFELS, M., OGESE, M., AL-ATTAR, Z., ALFIREVIC, A., ESSER, P. R., MARTIN, S. F., PIRMOHAMED, M. & PARK, B. K. 2017. The effect of inhibitory signals on the priming of drug hapten–specific T cells that express distinct Vβ receptors. *The Journal of Immunology,* 199**,** 1223-1237.

GIBSON, A., HAMMOND, S., JARUTHAMSOPHON, K., ROTH, S., MOSEDALE, M. & NAISBITT, D. J. 2020. Tolvaptan-and Tolvaptan-Metabolite-Responsive T Cells in Patients with Drug-Induced Liver Injury. *Chemical research in toxicology,* 33**,** 2745-2748.

GIBSON, A., OGESE, M., SULLIVAN, A., WANG, E., SAIDE, K., WHITAKER, P., PECKHAM, D., FAULKNER, L., PARK, B. K. & NAISBITT, D. J. 2014a. Negative regulation by PD-L1 during drug-specific priming of IL-22-secreting T cells and the influence of PD-1 on effector T cell function. *Journal of immunology (Baltimore, Md. : 1950),* 192**,** 2611-2621.

GIBSON, A., OGESE, M., SULLIVAN, A., WANG, E., SAIDE, K., WHITAKER, P., PECKHAM, D., FAULKNER, L., PARK, B. K. & NAISBITT, D. J. 2014b. Negative Regulation by PD-L1 during Drug-Specific Priming of IL-22–Secreting T Cells and the Influence of PD-1 on Effector T Cell Function. *The Journal of Immunology,* 192**,** 2611-2621.

GIORGINI, S., MARTINELLI, C., TOGNETTI, L., CAROCCI, A., GIUNTINI, R., MASTRONARDI, V., TORRICELLI, F., LEONCINI, F. & LOTTI, T. 2011. Use of patch testing for the diagnosis of abacavir-related hypersensitivity reaction in HIV patients. *Dermatologic therapy,* 24**,** 591-594.

GORER, P. 1936. The detection of a hereditary antigenic difference in the blood of mice by means of human group A serum. *Journal of Genetics,* 32**,** 17-31.

GOUGH, A., CHAPMAN, S., WAGSTAFF, K., EMERY, P. & ELIAS, E. 1996. Minocycline induced autoimmune hepatitis and systemic lupus erythematosus-like syndrome. *Bmj,* 312**,** 169-172.

GREENBERGER, P. A. 2006. 8. Drug allergy. *J Allergy Clin Immunol,* 117**,** S464-70.

GREGOR, C. E., FOENG, J., COMERFORD, I. & MCCOLL, S. R. 2017. Chemokine-driven CD4+ T cell homing: new concepts and recent advances. *Advances in immunology,* 135**,** 119-181.

GREIG, S. L. & SCOTT, L. J. 2016. Intravenous minocycline: a review in Acinetobacter infections. *Drugs,* 76**,** 1467-1476.

GROOM, J. R., RICHMOND, J., MUROOKA, T. T., SORENSEN, E. W., SUNG, J. H., BANKERT, K., VON ANDRIAN, U. H., MOON, J. J., MEMPEL, T. R. & LUSTER, A. D. 2012. CXCR3 chemokine receptor-ligand interactions in the lymph node optimize CD4+ T helper 1 cell differentiation. *Immunity,* 37**,** 1091-1103.

GUILLON, J.-M., JOLY, P., AUTRAN, B., DENIS, M., AKOUN, G., DEBRÉ, P. & MAYAUD, C. 1992. Minocycline-induced cell-mediated hypersensitivity pneumonitis. *Annals of internal medicine,* 117**,** 476-481.

HAGLEY, M. T., HULISZ, D. T. & BURNS, C. M. 1993. Hepatotoxicity associated with angiotensin-converting enzyme inhibitors. *Ann Pharmacother,* 27**,** 228-31.

HALEVI, A., BEN-AMITAI, D. & GARTY, B. Z. 2000. Toxic Epidermal Necrolysis Associated with Acetaminophen Ingestion. *Annals of Pharmacotherapy,* 34**,** 32-34.

HALEVY, S., GHISLAIN, P.-D., MOCKENHAUPT, M., FAGOT, J.-P., BOUWES BAVINCK, J. N., SIDOROFF, A., NALDI, L., DUNANT, A., VIBOUD, C. & ROUJEAU, J.-C. 2008. Allopurinol is the most common cause of Stevens-Johnson syndrome and toxic epidermal necrolysis in Europe and Israel. *Journal of the American Academy of Dermatology,* 58**,** 25-32.

HAMILTON, R. G. 2010. Laboratory diagnosis and management of human allergic disease. *Pediatric Allergy: Principles and Practices, 2nd ed. Elsevier, Edinburgh***,** 240-249.

HAMILTON, R. G. 2019. Assessment of human allergic diseases. *Clinical immunology.* Elsevier.

HAMMOND, S., THOMSON, P., MENG, X. & NAISBITT, D. 2021. In-Vitro Approaches to Predict and Study T-Cell Mediated Hypersensitivity to Drugs. *Frontiers in Immunology,* 12**,** 1065.

HAMMOND, T. G., MENG, X., JENKINS, R. E., MAGGS, J. L., CASTELAZO, A. S., REGAN, S. L., BENNETT, S. N., EARNSHAW, C. J., AITHAL, G. P., PANDE, I., KENNA, J. G., STACHULSKI, A. V., PARK, B. K. & WILLIAMS, D. P. 2014. Mass spectrometric characterization of circulating covalent protein adducts derived from a drug acyl glucuronide metabolite: multiple albumin adductions in diclofenac patients. *J Pharmacol Exp Ther,* 350**,** 387-402.

HAN, T. H., JIN, P., REN, J., SLEZAK, S., MARINCOLA, F. M. & STRONCEK, D. F. 2009. Evaluation of three clinical dendritic cell maturation protocols containing lipopolysaccharide and interferon-gamma. *Journal of immunotherapy (Hagerstown, Md.: 1997),* 32**,** 399.

HARDING, C. V. & UNANUE, E. R. 1990. Cellular mechanisms of antigen processing and the function of class I and II major histocompatibility complex molecules. *Cell regulation,* 1**,** 499-509.

HARI, Y., FRUTIG-SCHNYDER, K., HURNI, M., YAWALKAR, N., ZANNI, M., SCHNYDER, B., KAPPELER, A., VON GREYERZ, S., BRAATHEN, L. & PICHLER, W. 2001. T cell involvement in cutaneous drug eruptions. *Clinical & Experimental Allergy,* 31**,** 1398-1408.

HÄRMARK, L. & VAN GROOTHEEST, A. 2008. Pharmacovigilance: methods, recent developments and future perspectives. *European journal of clinical pharmacology,* 64**,** 743-752.

HARR, T. & FRENCH, L. E. 2010. Toxic epidermal necrolysis and Stevens-Johnson syndrome. *Orphanet journal of rare diseases,* 5**,** 39.

HARTIGAN-GO, K. Y. & WONG, J. Q. 2000. Inclusion of therapeutic failures as adverse drug reactions. *Side Effects of Drugs annual.* Elsevier.

HARTY, J. T., TVINNEREIM, A. R. & WHITE, D. W. 2000. CD8+ T cell effector mechanisms in resistance to infection. *Annual review of immunology,* 18**,** 275-308.

HASTINGS, K. L. 2001. Pre-clinical methods for detecting the hypersensitivity potential of pharmaceuticals: regulatory considerations. *Toxicology,* 158**,** 85-89.

HAUTEKEETE, M. L., HORSMANS, Y., VAN WAEYENBERGE, C., DEMANET, C., HENRION, J., VERBIST, L., BRENARD, R., SEMPOUX, C., MICHIELSEN, P. P. & YAP, P. S. 1999. HLA

association of amoxicillin-clavulanate–induced hepatitis. *Gastroenterology,* 117**,** 1181-1186.

HAYASHI, P. H. & BJORNSSON, E. S. 2018. Long-term outcomes after drug-induced liver injury. *Current hepatology reports,* 17**,** 292-299.

HEESEMANN, J. 1993. Mechanisms of resistance to beta-lactam antibiotics. *Infection,* 21**,** S4.

HENLEY, D., RAGHAVAN, N., SPERLING, R., AISEN, P., RAMAN, R. & ROMANO, G. 2019. Preliminary Results of a Trial of Atabecestat in Preclinical Alzheimer's Disease. *The New England journal of medicine,* 380**,** 1483-1485.

HENNECKE, J., CARFI, A. & WILEY, D. C. 2000. Structure of a covalently stabilized complex of a human αβ T-cell receptor, influenza HA peptide and MHC class II molecule, HLA-DR1. *The EMBO journal,* 19**,** 5611-5624.

HINZ, T., WEIDMANN, E. & KABELITZ, D. 2001. Dual TCR-expressing T lymphocytes in health and disease. *International archives of allergy and immunology,* 125**,** 16-20.

HIRASAWA, M., HAGIHARA, K., ABE, K., ANDO, O. & HIRAYAMA, N. 2017. In silico and in vitro analysis of interaction between ximelagatran and human leukocyte antigen (HLA)-DRB1* 07: 01. *International journal of molecular sciences,* 18**,** 694.

HIRATA, K., TAKAGI, H., YAMAMOTO, M., MATSUMOTO, T., NISHIYA, T., MORI, K., SHIMIZU, S., MASUMOTO, H. & OKUTANI, Y. 2008. Ticlopidine-induced hepatotoxicity is associated with specific human leukocyte antigen genomic subtypes in Japanese patients: a preliminary case–control study. *The pharmacogenomics journal,* 8**,** 29-33.

HORTON, H., WESTON, S. & HEWITT, C. 1998. Allergy to antibiotics: T-cell recognition of amoxicillin is HLA-DR restricted and does not require antigen processing. *Allergy,* 53**,** 83-88.

HOWLAND, M. 2006. Antidotes in Depth. Vitamin K1. *Flomenbaum NE, Goldfrank LR, Hoffman RS, Howland MA, Lewin NA, Nelson LS. Goldfrank's Toxicologic Emergencies. 8th ed. New York: McGraw-Hill*.

HSIAO, C.-C., ROMBOUTS, F. & GIJSEN, H. J. M. 2019. New evolutions in the BACE1 inhibitor field from 2014 to 2018. *Bioorganic & Medicinal Chemistry Letters,* 29**,** 761-777.

HUBO, M., TRINSCHEK, B., KRYCZANOWSKY, F., TUETTENBERG, A., STEINBRINK, K. & JONULEIT, H. 2013. Costimulatory molecules on immunogenic versus tolerogenic human dendritic cells. *Frontiers in immunology,* 4**,** 82.

HUGHES, C. A., FOISY, M. M., DEWHURST, N., HIGGINS, N., ROBINSON, L., KELLY, D. V. & LECHELT, K. E. 2008. Abacavir hypersensitivity reaction: an update. *Annals of Pharmacotherapy,* 42**,** 387-396.

HUGHES, S. 2004. Pharmacovigilance . Edited by Ron Mann and Elizabeth Andrews. John Wiley & Sons: Chichester, 2002. Pages: 545. ISBN 0-471-49441-0. *Human Psychopharmacology: Clinical & Experimental,* 19**,** 141.

HUNG, S.-I., CHUNG, W.-H., LIOU, L.-B., CHU, C.-C., LIN, M., HUANG, H.-P., LIN, Y.-L., LAN, J.-L., YANG, L.-C. & HONG, H.-S. 2005. HLA-B* 5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. *Proceedings of the National Academy of Sciences,* 102**,** 4134-4139.

HUNZIKER, T., KÜNZI, U. P., BRAUNSCHWEIG, S., ZEHNDER, D. & HOIGNE, R. 1997. Comprehensive hospital drug monitoring (CHDM): adverse skin reactions, a 20-year survey. *Allergy,* 52**,** 388-393.

HURLEY, C. K. 2020. Naming HLA diversity: a review of HLA nomenclature. *Human immunology*.

HURWITZ, N. & WADE, O. L. 1969. Intensive hospital monitoring of adverse reactions to drugs. *British medical journal,* 1**,** 531-536.

HUSSAINI, S. H. & FARRINGTON, E. A. 2014a. Idiosyncratic drug-induced liver injury: an update on the 2007 overview. *Expert opinion on drug safety,* 13**,** 67-81.

HUSSAINI, S. H. & FARRINGTON, E. A. 2014b. Idiosyncratic drug-induced liver injury: an update on the 2007 overview. *Expert Opin Drug Saf,* 13**,** 67-81.

IARC 2000. Vitamin K substances. *In: Some Antiviral and Antineoplastic Drugs and Other Pharmaceutical Agents. World Health Organization (WHO), International Agency for Research on Cancer (IARC); Lyon, France. IARC Monographs on the Evaluation of Carcinogen Risks to Humans, Vol,* 76**,** 417-486.

ILLING, P. T., PURCELL, A. W. & MCCLUSKEY, J. 2017. The role of HLA genes in pharmacogenomics: unravelling HLA associated adverse drug reactions. *Immunogenetics,* 69**,** 617-630.

ILLING, P. T., VIVIAN, J. P., DUDEK, N. L., KOSTENKO, L., CHEN, Z., BHARADWAJ, M., MILES, J. J., KJER-NIELSEN, L., GRAS, S. & WILLIAMSON, N. A. 2012. Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. *Nature,* 486**,** 554-558.

IMPICCIATORE, P., CHOONARA, I., CLARKSON, A., PROVASI, D., PANDOLFINI, C. & BONATI, M. 2001. Incidence of adverse drug reactions in paediatric in/out-patients: a systematic review and meta-analysis of prospective studies. *British journal of clinical pharmacology,* 52**,** 77-83.

IWAKI, M., OGISO, T., INAGAWA, S. & KAKEHI, K. 1999. In Vitro Regioselective Stability of β-1-O- and 2-O-Acyl Glucuronides of Naproxen and Their Covalent Binding to Human Serum Albumin. *Journal of Pharmaceutical Sciences,* 88**,** 52-57.

IWAMURA, A., ITO, M., MITSUI, H., HASEGAWA, J., KOSAKA, K., KINO, I., TSUDA, M., NAKAJIMA, M., YOKOI, T. & KUME, T. 2015. Toxicological evaluation of acyl glucuronides utilizing half-lives, peptide adducts, and immunostimulation assays. *Toxicology in Vitro,* 30**,** 241-249.

IWASAKI, A. & MEDZHITOV, R. 2015. Control of adaptive immunity by the innate immune system. *Nature immunology,* 16**,** 343-353.

IWASAWA, T. & KIDO, T. 1969. Clinical and experimental studies on minocycline. *The Japanese journal of antibiotics,* 22**,** 511.

JANEWAY, C., MURPHY, K. P., TRAVERS, P. & WALPORT, M. 2008. *Janeway's immuno biology*.

JANEWAY, C. A. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor symposia on quantitative biology, 1989. Cold Spring Harbor Laboratory Press, 1-13.

JANEWAY JR, C. A., TRAVERS, P., WALPORT, M. & SHLOMCHIK, M. J. 2001. The components of the immune system. *Immunobiology: The Immune System in Health and Disease. 5th edition.* Garland Science.

JENKINS, R. T., ROONEY, P. J., JONES, D. B., BIENENSTOCK, J. & GOODACRE, R. L. 1987. INCREASED INTESTINAL PERMEABILITY IN PATIENTS WITH RHEUMATOID ARTHRITIS: A SIDE-EFFECT OF ORAL NONSTEROIDAL ANTI-INFLAMMATORY DRUG THERAPY? *Rheumatology,* 26**,** 103-107.

JHANG, J. F., LIN, T. Y., HO, H. C., JIANG, Y. H., HSU, Y. H., BIRDER, L. A. & KUO, H. C. 2021. Deficits of urothelial cell proliferation, cytoskeleton, and barrier function protein expressions in patients with recurrent and persistent urinary tract infections. *LUTS: Lower Urinary Tract Symptoms,* 13**,** 203-209.

JONAS, M. & CUNHA, B. A. 1982. Minocycline. *Ther Drug Monit,* 4**,** 137-45.

JONES, J. K. & KINGERY, E. 2014. History of pharmacovigilance. *Mann's pharmacovigilance***,** 11-24.

JOSEPHINE, H. R., KUMAR, I. & PRATT, R. 2004. The perfect penicillin? Inhibition of a bacterial DD-peptidase by peptidoglycan-mimetic β-lactams. *Journal of the American Chemical Society,* 126**,** 8122-8123.

KAGAMI, S., RIZZO, H. L., LEE, J. J., KOGUCHI, Y. & BLAUVELT, A. 2010. Circulating Th17, Th22, and Th1 cells are increased in psoriasis. *Journal of Investigative Dermatology,* 130**,** 1373-1383.

KALISCH, L. M., CAUGHEY, G. E., ROUGHEAD, E. E. & GILBERT, A. L. 2011. The prescribing cascade.

KANNANKERIL, P., RODEN, D. M. & DARBAR, D. 2010. Drug-induced long QT syndrome. *Pharmacological reviews,* 62**,** 760-781.

KANO, Y., HIRAHARA, K., MITSUYAMA, Y., TAKAHASHI, R. & SHIOHARA, T. 2007. Utility of the lymphocyte transformation test in the diagnosis of drug sensitivity: dependence on its timing and the type of drug eruption. *Allergy,* 62**,** 1439-1444.

KAPSENBERG, M. L. 2003. Dendritic-cell control of pathogen-driven T-cell polarization. *Nature Reviews Immunology,* 3**,** 984-993.

KAWAI, T. & AKIRA, S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol,* 11**,** 373-84.

KELLY, J. G., KINNEY, C. D., DEVANE, J. G., MULLIGAN, S. & COLGAN, B. V. 1989. Pharmacokinetic properties and clinical efficacy of once-daily sustained-release naproxen. *European Journal of Clinical Pharmacology,* 36**,** 383-388.

KELLY, R. & KANEGIS, L. 1967. Metabolism and tissue distribution of radioisotopically labeled minocycline. *Toxicology and applied pharmacology,* 11**,** 171-183.

KHAZEN, W., M'BIKA, J.-P., TOMKIEWICZ, C., BENELLI, C., CHANY, C., ACHOUR, A. & FOREST, C. 2005. Expression of macrophage-selective markers in human and rodent adipocytes. *FEBS letters,* 579**,** 5631-5634.

KIM, B.-H., YI, S., KIM, J., LIM, K. S., KIM, K.-P., LEE, B., SHIN, S.-G., JANG, I.-J. & YU, K.-S. 2009. Influence of alcohol on the hemodynamic effects and pharmacokinetic properties of mirodenafil: a single-dose, randomized-sequence, open-label, crossover study in healthy male volunteers in Korea. *Clinical therapeutics,* 31**,** 1234-1243.

KIM, J. H. & SCIALLI, A. R. 2011. Thalidomide: the tragedy of birth defects and the effective treatment of disease. *Toxicological sciences,* 122**,** 1-6.

KIM, S.-H. & NAISBITT, D. J. 2016. Update on advances in research on idiosyncratic drug-induced liver injury. *Allergy, asthma & immunology research,* 8**,** 3-11.

KIM, S.-H., SAIDE, K., YUAN, Y., DALY, A., ALFIREVIC, A., PIRMOHAMED, M., PARK, K. & NAISBITT, D. 2014. Characterization of amoxicillin and clavulanic-acid-responsive CD4+ And CD8+ T-cells in patients with co-amoxiclav-induced liver injury. *Clinical and Translational Allergy,* 4**,** 1-1.

KIM, S. H., SAIDE, K., FARRELL, J., FAULKNER, L., TAILOR, A., OGESE, M., DALY, A. K., PIRMOHAMED, M., PARK, B. K. & NAISBITT, D. J. 2015. Characterization of amoxicillin-and clavulanic acid-specific T cells in patients with amoxicillin-clavulanate–induced liver injury. *Hepatology,* 62**,** 887-899.

KNOWLES, S., SHAPIRO, L. & SHEAR, N. H. 2001. Should celecoxib be contraindicated in patients who are allergic to sulfonamides? *Drug safety,* 24**,** 239-247.

KO, J.-W., PARK, S.-H., SHIN, N.-R., SHIN, J.-Y., KIM, J.-W., SHIN, I.-S., MOON, C., HEO, J.-D., KIM, J.-C. & LEE, I.-C. 2017. Protective effect and mechanism of action of diallyl disulfide against acetaminophen-induced acute hepatotoxicity. *Food and Chemical Toxicology,* 109**,** 28-37.

KO, T.-M., CHUNG, W.-H., WEI, C.-Y., SHIH, H.-Y., CHEN, J.-K., LIN, C.-H., CHEN, Y.-T. & HUNG, S.-I. 2011. Shared and restricted T-cell receptor use is crucial for carbamazepine-induced Stevens-Johnson syndrome. *Journal of Allergy and Clinical Immunology,* 128**,** 1266-1276. e11.

KONVINSE, K. C., TRUBIANO, J. A., PAVLOS, R., JAMES, I., SHAFFER, C. M., BEJAN, C. A., SCHUTTE, R. J., OSTROV, D. A., PILKINTON, M. A. & ROSENBACH, M. 2019. HLA-A* 32: 01 is strongly associated with vancomycin-induced drug reaction with eosinophilia and systemic symptoms. *Journal of Allergy and Clinical Immunology,* 144**,** 183-192.

KOOPMANS, P. P., VAN DER VEN, A. J., VREE, T. B. & VAN DER MEER, J. W. 1995. Pathogenesis of hypersensitivity reactions to drugs in patients with HIV infection: allergic or toxic? *Aids,* 9**,** 217-222.

KORIYAMA, Y., HORI, A., ITO, H., YONEZAWA, S., BABA, Y., TANIMOTO, N., UENO, T., YAMAMOTO, S., YAMAMOTO, T., ASADA, N., MORIMOTO, K., EINARU, S., SAKAI, K.,

KANAZU, T., MATSUDA, A., YAMAGUCHI, Y., OGUMA, T., TIMMERS, M., TRITSMANS, L., KUSAKABE, K.-I., KATO, A. & SAKAGUCHI, G. 2021. Discovery of Atabecestat (JNJ-54861911): A Thiazine-Based β-Amyloid Precursor Protein Cleaving Enzyme 1 Inhibitor Advanced to the Phase 2b/3 EARLY Clinical Trial. *Journal of Medicinal Chemistry,* 64**,** 1873-1888.

KRUPSKI, A., CAMPBELL, K., JOESCH, J. M., LUCENKO, B. A. & ROY-BYRNE, P. 2009. Impact of access to recovery services on alcohol/drug treatment outcomes. *Journal of Substance Abuse Treatment,* 37**,** 435-442.

KUMAR, H., KAWAI, T. & AKIRA, S. 2009. Pathogen recognition in the innate immune response. *Biochemical Journal,* 420**,** 1-16.

LABBÉ, L., SIROIS, C., PILOTE, S., ARSENEAULT, M., ROBITAILLE, N. M., TURGEON, J. & HAMELIN, B. A. 2000. Effect of gender, sex hormones, time variables and physiological urinary pH on apparent CYP2D6 activity as assessed by metabolic ratios of marker substrates. *Pharmacogenetics and Genomics,* 10**,** 425-438.

LAMMERT, C., EINARSSON, S., SAHA, C., NIKLASSON, A., BJORNSSON, E. & CHALASANI, N. 2008. Relationship between daily dose of oral medications and idiosyncratic drug-induced liver injury: search for signals. *Hepatology,* 47**,** 2003-2009.

LAW, I. P. & KNIGHT, H. 1976. Jaundice associated with naproxen. *The New England journal of medicine,* 295**,** 1201.

LAWRENSON, R. A., SEAMAN, H. E., SUNDSTRÖM, A., WILLIAMS, T. J. & FARMER, R. D. 2000. Liver damage associated with minocycline use in acne. *Drug Safety,* 23**,** 333-349.

LAZAREVIC, V. & FLYNN, J. 2002. CD8+ T cells in tuberculosis. *American journal of respiratory and critical care medicine,* 166**,** 1116-1121.

LE CLEACH, L., DELAIRE, S., BOUMSELL, L., BAGOT, M., BOURGAULT-VILLADA, I., BENSUSSAN, A. & ROUJEAU, J. 2000. Blister fluid T lymphocytes during toxic epidermal necrolysis are functional cytotoxic cells which express human natural killer (NK) inhibitory receptors. *Clinical & Experimental Immunology,* 119**,** 225-230.

LEAPE, L. L., BRENNAN, T. A., LAIRD, N., LAWTHERS, A. G., LOCALIO, A. R., BARNES, B. A., HEBERT, L., NEWHOUSE, J. P., WEILER, P. C. & HIATT, H. 1991. The nature of adverse events in hospitalized patients. Results of the Harvard Medical Practice Study II. *N Engl J Med,* 324**,** 377-84.

LEE, A.-Y. 2000. Fixed drug eruptions. *American journal of clinical dermatology,* 1**,** 277-285.

LEE, W. M. & SENIOR, J. R. 2005. Recognizing drug-induced liver injury: current problems, possible solutions. *Toxicologic pathology,* 33**,** 155-164.

LEHMANN, P. V. & ZHANG, W. 2012. Unique strengths of ELISPOT for T cell diagnostics. *Handbook of ELISPOT***,** 3-23.

LEONE, P., SHIN, E.-C., PEROSA, F., VACCA, A., DAMMACCO, F. & RACANELLI, V. 2013. MHC class I antigen processing and presenting machinery: organization, function, and defects in tumor cells. *Journal of the National Cancer Institute,* 105**,** 1172-1187.

LEUNG, L., KALGUTKAR, A. S. & OBACH, R. S. 2012. Metabolic activation in drug-induced liver injury. *Drug metabolism reviews,* 44**,** 18-33.

LEVINE, B. B. 1966. Immunologic mechanisms of penicillin allergy: a haptenic model system for the study of allergic diseases of man. *New England Journal of Medicine,* 275**,** 1115-1125.

LEVY, M., AZAZ-LIVSHITS, T., SADAN, B., SHALIT, M., GEISSLINGER, G. & BRUNE, K. 1999. Computerized surveillance of adverse drug reactions in hospital: implementation. *European journal of clinical pharmacology,* 54**,** 887-892.

LI, Y. J., PHILLIPS, E., DELLINGER, A., NICOLETTI, P., SCHUTTE, R., LI, D., OSTROV, D. A., FONTANA, R. J., WATKINS, P. B. & STOLZ, A. 2020. HLA-B* 14: 01 and HLA-B* 35: 01 are associated with trimethoprim-sulfamethoxazole induced liver injury. *Hepatology*.

LI, Y. J., PHILLIPS, E. J., DELLINGER, A., NICOLETTI, P., SCHUTTE, R., LI, D., OSTROV, D. A., FONTANA, R. J., WATKINS, P. B. & STOLZ, A. 2021. Human Leukocyte Antigen B* 14: 01 and B* 35: 01 Are Associated With Trimethoprim-Sulfamethoxazole Induced Liver Injury. *Hepatology,* 73**,** 268-281.

LICHTENFELS, M., FARRELL, J., OGESE, M. O., BELL, C. C., ECKLE, S., MCCLUSKEY, J., PARK, B. K., ALFIREVIC, A., NAISBITT, D. J. & PIRMOHAMED, M. 2014. HLA restriction of carbamazepine-specific T-Cell clones from an HLA-A* 31: 01-positive hypersensitive patient. *Chemical research in toxicology,* 27**,** 175-177.

LINDQUIST, M. 2007. The need for definitions in pharmacovigilance. *Drug Saf,* 30**,** 825-30.

LIU YIN, J. A. & TOBAL, K. 1999. Detection of minimal residual disease in acute myeloid leukaemia: methodologies, clinical and biological significance. *British journal of haematology,* 106**,** 578-590.

LOPEZ, S., BLANCA-LOPEZ, N., CORNEJO-GARCIA, J. A., CANTO, G., TORRES, M. J., MAYORGA, C. & BLANCA, M. 2007. Nonimmediate reactions to betalactams. *Current opinion in allergy and clinical immunology,* 7**,** 310-316.

LUCENA, M. I., ANDRADE, R. J., KAPLOWITZ, N., GARCÍA-CORTES, M., FERNÁNDEZ, M. C., ROMERO-GOMEZ, M., BRUGUERA, M., HALLAL, H., ROBLES-DIAZ, M. & RODRIGUEZ-GONZÁLEZ, J. F. 2009. Phenotypic characterization of idiosyncratic drug-induced liver injury: the influence of age and sex. *Hepatology,* 49**,** 2001-2009.

LUCENA, M. I., MOLOKHIA, M., SHEN, Y., URBAN, T. J., AITHAL, G. P., ANDRADE, R. J., DAY, C. P., RUIZ–CABELLO, F., DONALDSON, P. T. & STEPHENS, C. 2011. Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple HLA class I and II alleles. *Gastroenterology,* 141**,** 338-347.

LUCKHEERAM, R. V., ZHOU, R., VERMA, A. D. & XIA, B. 2012. CD4+ T cells: differentiation and functions. *Clinical and developmental immunology,* 2012.

LUQUE, I., LEYVA, L., JOSE TORRES, M., ROSAL, M., MAYORGA, C., SEGURA, J., BLANCA, M. & JUAREZ, C. 2001. In vitro T-cell responses to β-lactam drugs in immediate and nonimmediate allergic reactions. *Allergy,* 56**,** 611-618.

MACDONALD, H., KELLY, R. G., ALLEN, E. S., NOBLE, J. F. & KANEGIS, L. A. 1973. Pharmacokinetic studies on minocycline in man. *Clinical Pharmacology & Therapeutics,* 14**,** 852-861.

MACEDO, A. F. P. A. D. 2004. *Desenvolvimento e validação de um instrumento de avaliação da imputabilidade de medicamentos a reacções adversas.*

MADDREY, W. C. & BOITNOTT, J. K. 1973. Isoniazid hepatitis. *Annals of Internal Medicine,* 79**,** 1-12.

MAHDI, B. M. 2013. A glow of HLA typing in organ transplantation. *Clinical and translational medicine,* 2**,** 1-5.

MAK, A. & UETRECHT, J. 2015a. The combination of anti-CTLA-4 and PD1–/–mice unmasks the potential of isoniazid and nevirapine to cause liver injury. *Chemical research in toxicology,* 28**,** 2287-2291.

MAK, A. & UETRECHT, J. 2015b. The role of CD8 T Cells in amodiaquine-induced liver injury in PD1–/–mice cotreated with anti-CTLA-4. *Chemical research in toxicology,* 28**,** 1567-1573.

MALHAME, I., CORMIER, M., SUGARMAN, J. & SCHWARTZMAN, K. 2016. Latent tuberculosis in pregnancy: a systematic review. *PLoS One,* 11**,** e0154825.

MALLAL, S., NOLAN, D., WITT, C., MASEL, G., MARTIN, A., MOORE, C., SAYER, D., CASTLEY, A., MAMOTTE, C. & MAXWELL, D. 2002a. Association between presence of HLA-B* 5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. *The Lancet,* 359**,** 727-732.

MALLAL, S., NOLAN, D., WITT, C., MASEL, G., MARTIN, A. M., MOORE, C., SAYER, D., CASTLEY, A., MAMOTTE, C., MAXWELL, D., JAMES, I. & CHRISTIANSEN, F. T. 2002b. Association

between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. *The Lancet,* 359**,** 727-732.

MALLAL, S., PHILLIPS, E., CAROSI, G., MOLINA, J.-M., WORKMAN, C., TOMAŽIČ, J., JÄGEL-GUEDES, E., RUGINA, S., KOZYREV, O. & CID, J. F. 2008. HLA-B* 5701 screening for hypersensitivity to abacavir. *New England Journal of Medicine,* 358**,** 568-579.

MAMOSHINA, P., RODRIGUEZ, B. & BUENO-OROVIO, A. 2021. Toward a broader view of mechanisms of drug cardiotoxicity. *Cell Reports Medicine,* 2**,** 100216.

MANNARGUDI, B., MCNALLY, D., REYNOLDS, W. & UETRECHT, J. 2009. Bioactivation of minocycline to reactive intermediates by myeloperoxidase, horseradish peroxidase, and hepatic microsomes: implications for minocycline-induced lupus and hepatitis. *Drug metabolism and disposition,* 37**,** 1806-1818.

MARIA, V. & VICTORINO, R. 1997a. Development and validation of a clinical scale for the diagnosis of drug-induced hepatitis. *Hepatology,* 26**,** 664-669.

MARIA, V. & VICTORINO, R. 1997b. Diagnostic value of specific T cell reactivity to drugs in 95 cases of drug induced liver injury. *Gut,* 41**,** 534-540.

MARSH, S. G., ALBERT, E., BODMER, W., BONTROP, R., DUPONT, B., ERLICH, H., FERNÁNDEZ-VIÑA, M., GERAGHTY, D., HOLDSWORTH, R. & HURLEY, C. 2010. Nomenclature for factors of the HLA system, 2010. *Tissue antigens,* 75**,** 291.

MASSON, C., CHEVAILLER, A., PASCARETTI, C., LEGRAND, E., BREGEON, C. & AUDRAN, M. 1996. Minocycline related lupus. *The Journal of Rheumatology,* 23**,** 2160-2161.

MATZARAKI, V., KUMAR, V., WIJMENGA, C. & ZHERNAKOVA, A. 2017. The MHC locus and genetic susceptibility to autoimmune and infectious diseases. *Genome biology,* 18**,** 1-21.

MAYNE, K., WHITE, J. A., MCMURRAN, C. E., RIVERA, F. J. & DE LA FUENTE, A. G. 2020. Aging and Neurodegenerative Disease: Is the Adaptive Immune System a Friend or Foe? *Frontiers in Aging Neuroscience,* 12.

MCBRIDE, W. G. 1961. Thalidomide and congenital abnormalities. *Lancet,* 2**,** 90927-8.

MCHEYZER-WILLIAMS, L. J., MALHERBE, L. P. & MCHEYZER-WILLIAMS, M. G. 2006. Helper T cell-regulated B cell immunity. *Curr Top Microbiol Immunol,* 311**,** 59-83.

MCLERNON, D. J., BOND, C. M., HANNAFORD, P. C., WATSON, M. C., LEE, A. J., HAZELL, L. & AVERY, A. 2010. Adverse drug reaction reporting in the UK: a retrospective observational comparison of yellow card reports submitted by patients and healthcare professionals. *Drug Saf,* 33**,** 775-88.

MEDICINE, I. O. 2012. *Ethical and Scientific Issues in Studying the Safety of Approved Drugs,* Washington, DC, The National Academies Press.

MEDZHITOV, R. & JANEWAY JR, C. A. 1997. Innate immunity: impact on the adaptive immune response. *Current opinion in immunology,* 9**,** 4-9.

MELLMAN, I. 2013. Dendritic cells: master regulators of the immune response. *Cancer immunology research,* 1**,** 145-149.

MENG, X., AL-ATTAR, Z., YASEEN, F. S., JENKINS, R., EARNSHAW, C., WHITAKER, P., PECKHAM, D., FRENCH, N. S., NAISBITT, D. J. & PARK, B. K. 2017. Definition of the nature and hapten threshold of the β-lactam antigen required for T cell activation in vitro and in patients. *The Journal of Immunology,* 198**,** 4217-4227.

MENG, X., LAWRENSON, A. S., BERRY, N. G., MAGGS, J. L., FRENCH, N. S., BACK, D. J., KHOO, S. H., NAISBITT, D. J. & PARK, B. K. 2014. Abacavir forms novel cross-linking abacavir protein adducts in patients. *Chemical research in toxicology,* 27**,** 524-535.

MENNICKE, M., ZAWODNIAK, A., KELLER, M., WILKENS, L., YAWALKAR, N., STICKEL, F., KEOGH, A., INDERBITZIN, D., CANDINAS, D. & PICHLER, W. J. 2009. Fulminant liver failure after vancomycin in a sulfasalazine-induced DRESS syndrome: fatal recurrence after liver transplantation. *American journal of transplantation,* 9**,** 2197-2202.

METUSHI, I., CAI, P., ZHU, X., NAKAGAWA, T. & UETRECHT, J. 2011. A fresh look at the mechanism of isoniazid-induced hepatotoxicity. *Clinical Pharmacology & Therapeutics,* 89**,** 911-914.

METUSHI, I. G., HAYES, M. A. & UETRECHT, J. 2015. Treatment of PD-1−/− mice with amodiaquine and anti-CTLA4 leads to liver injury similar to idiosyncratic liver injury in patients. *Hepatology,* 61**,** 1332-1342.

MILLER, J. & OSOBA, D. 1967. Current concepts of the immunological function of the thymus. *Physiological Reviews,* 47**,** 437-520.

MINUTH, J., HOLMES, T. & MUSHER, D. 1974. Activity of tetracycline, doxycycline, and minocycline against methicillin-susceptible and-resistant staphylococci. *Antimicrobial agents and chemotherapy,* 6**,** 411-414.

MIYAGAWA, F. & ASADA, H. 2021. Chemokines in Severe Cutaneous Adverse Reactions (SCARs). *Biomolecules,* 11**,** 847.

MIYASHITA, T., KIMURA, K., FUKAMI, T., NAKAJIMA, M. & YOKOI, T. 2014. Evaluation and mechanistic analysis of the cytotoxicity of the acyl glucuronide of nonsteroidal anti-inflammatory drugs. *Drug Metab Dispos,* 42**,** 1-8.

MOCKENHAUPT, M. 2009. Severe drug-induced skin reactions: clinical pattern, diagnostics and therapy. *JDDG: Journal der Deutschen Dermatologischen Gesellschaft,* 7**,** 142-162.

MOMBAERTS, P., CLARKE, A. R., RUDNICKI, M. A., IACOMINI, J., ITOHARA, S., LAFAILLE, J. J., WANG, L., ICHIKAWA, Y., JAENISCH, R. & HOOPER, M. L. 1992. Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. *Nature,* 360**,** 225-231.

MONSHI, M. M., FAULKNER, L., GIBSON, A., JENKINS, R. E., FARRELL, J., EARNSHAW, C. J., ALFIREVIC, A., CEDERBRANT, K., DALY, A. K. & FRENCH, N. 2013. Human leukocyte antigen (HLA)-B* 57: 01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury. *Hepatology,* 57**,** 727-739.

MORIDE, Y., BÉGAUD, B., PHARMACOVIGILANCE, A. & ARME, P. 1993. *Methodological approaches in pharmacoepidemiology application to spontaneous reporting,* Amsterdam, Elsevier.

MOSEDALE, M. & WATKINS, P. B. 2017. Drug-induced liver injury: advances in mechanistic understanding that will inform risk management. *Clinical Pharmacology & Therapeutics,* 101**,** 469-480.

NAISBITT, D. J., FARRELL, J., GORDON, S. F., MAGGS, J. L., BURKHART, C., PICHLER, W. J., PIRMOHAMED, M. & PARK, B. K. 2002. Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. *Molecular pharmacology,* 62**,** 628-637.

NAISBITT, D. J., GORDON, S. F., PIRMOHAMED, M., BURKHART, C., CRIBB, A. E., PICHLER, W. J. & PARK, B. K. 2001. Antigenicity and immunogenicity of sulphamethoxazole: demonstration of metabolism-dependent haptenation and T-cell proliferation in vivo. *British journal of pharmacology,* 133**,** 295-305.

NAISBITT, D. J., GORDON, S. F., PIRMOHAMED, M. & PARK, B. K. 2000. Immunological principles of adverse drug reactions. *Drug safety,* 23**,** 483-507.

NAISBITT, D. J., HOUGH, S. J., GILL, H. J., PIRMOHAMED, M., KITTERINGHAM, N. R. & PARK, B. K. 1999. Cellular disposition of sulphamethoxazole and its metabolites: implications for hypersensitivity. *British journal of pharmacology,* 126**,** 1393-1407.

NAISBITT, D. J., O'NEILL, P. M., PIRMOHAMED, M. & PARK, B. K. 1996. Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles: implications for immune mediated toxicity. *Bioorganic & Medicinal Chemistry Letters,* 6**,** 1511-1516.

NAISBITT, D. J., OLSSON-BROWN, A., GIBSON, A., MENG, X., OGESE, M. O., TAILOR, A. & THOMSON, P. 2020. Immune dysregulation increases the incidence of delayed-type drug hypersensitivity reactions. *Allergy,* 75**,** 781-797.

NAISBITT, D. J., YANG, E. L., ALHAIDARI, M., BERRY, N. G., LAWRENSON, A. S., FARRELL, J., MARTIN, P., STREBEL, K., OWEN, A. & PYE, M. 2015. Towards depersonalized abacavir therapy: chemical modification eliminates HLA-B∗ 57: 01-restricted CD8+ T-cell activation. *Aids,* 29**,** 2385-2395.

NASSIF, A., BENSUSSAN, A., BOUMSELL, L., DENIAUD, A., MOSLEHI, H., WOLKENSTEIN, P., BAGOT, M. & ROUJEAU, J.-C. 2004. Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells. *Journal of Allergy and Clinical Immunology,* 114**,** 1209-1215.

NEBERT, D. W. 1999. Pharmacogenetics and pharmacogenomics: why is this relevant to the clinical geneticist? *Clinical genetics,* 56**,** 247-258.

NELIS, H. & DE LEENHEER, A. 1982. Metabolism of minocycline in humans. *Drug metabolism and disposition,* 10**,** 142-146.

NICOLETTI, P., AITHAL, G. P., BJORNSSON, E. S., ANDRADE, R. J., SAWLE, A., ARRESE, M., BARNHART, H. X., BONDON-GUITTON, E., HAYASHI, P. H., BESSONE, F., CARVAJAL, A., CASCORBI, I., CIRULLI, E. T., CHALASANI, N., CONFORTI, A., COULTHARD, S. A., DALY, M. J., DAY, C. P., DILLON, J. F., FONTANA, R. J., GROVE, J. I., HALLBERG, P., HERNÁNDEZ, N., IBÁÑEZ, L., KULLAK-UBLICK, G. A., LAITINEN, T., LARREY, D., LUCENA, M. I., MAITLAND-VAN DER ZEE, A. H., MARTIN, J. H., MOLOKHIA, M., PIRMOHAMED, M., POWELL, E. E., QIN, S., SERRANO, J., STEPHENS, C., STOLZ, A., WADELIUS, M., WATKINS, P. B., FLORATOS, A., SHEN, Y., NELSON, M. R., URBAN, T. J. & DALY, A. K. 2017. Association of Liver Injury From Specific Drugs, or Groups of Drugs, With Polymorphisms in HLA and Other Genes in a Genome-Wide Association Study. *Gastroenterology,* 152**,** 1078-1089.

NICOLETTI, P., WERK, A. N., SAWLE, A., SHEN, Y., URBAN, T. J., COULTHARD, S. A., BJORNSSON, E. S., CASCORBI, I., FLORATOS, A. & STAMMSCHULTE, T. 2016. HLA-DRB1* 16: 01-DQB1* 05: 02 is a novel genetic risk factor for flupirtine-induced liver injury. *Pharmacogenetics and genomics,* 26**,** 218-224.

NOBLE, J., KANEGIS, L. & HALLESY, D. 1967. Short-term toxicity and observations on certain aspects of the pharmacology of a unique tetracycline-minocycline. *Toxicology and applied pharmacology,* 11**,** 128-149.

NORCROSS, M. A., LUO, S., LU, L., BOYNE, M. T., GOMARTELI, M., RENNELS, A. D., WOODCOCK, J., MARGULIES, D. H., MCMURTREY, C. & VERNON, S. 2012. Abacavir induces loading of novel self-peptides into HLA-B* 57: 01: an autoimmune model for HLA-associated drug hypersensitivity. *AIDS (London, England),* 26**,** F21.

NOVAK, G., STREFFER, J. R., TIMMERS, M., HENLEY, D., BRASHEAR, H. R., BOGERT, J., RUSSU, A., JANSSENS, L., TESSEUR, I. & TRITSMANS, L. 2020. Long-term safety and tolerability of atabecestat (JNJ-54861911), an oral BACE1 inhibitor, in early Alzheimer's disease spectrum patients: A randomized, double-blind, placebo-controlled study and a two-period extension study. *Alzheimer's research & therapy,* 12**,** 1-16.

NYFELER, B. & PICHLER, W. 1997. The lymphocyte transformation test for the diagnosis of drug allergy: sensitivity and specificity. *Clinical & Experimental Allergy,* 27**,** 175-181.

OCHSENDORF, F. 2010. Minocycline in acne vulgaris. *American journal of clinical dermatology,* 11**,** 327-341.

OGESE, M. O., JENKINS, R. E., ADAIR, K., TAILOR, A., MENG, X., FAULKNER, L., ENYINDAH, B. O., SCHOFIELD, A., DIAZ-NIETO, R. & RESSEL, L. 2019. Exosomal transport of hepatocyte-derived drug-modified proteins to the immune system. *Hepatology,* 70**,** 1732-1749.

OGESE, M. O., WATKINSON, J., LISTER, A., FAULKNER, L., GIBSON, A., HILLEGAS, A., SAKATIS, M. Z., PARK, B. K. & NAISBITT, D. J. 2020. Development of an improved T-cell assay to assess the intrinsic immunogenicity of haptenic compounds. *Toxicological Sciences,* 175**,** 266-278.

OGILVIE, R. I. & RUEDY, J. 1967. Adverse drug reactions during hospitalization. *Canadian Medical Association journal,* 97**,** 1450-1457.

OKADA, S., LI, Q., WHITIN, J. C., CLAYBERGER, C. & KRENSKY, A. M. 2003. Intracellular mediators of granulysin-induced cell death. *The Journal of Immunology,* 171**,** 2556-2562.

OPLATEK, A., BROWN, K., SEN, S., HALERZ, M., SUPPLE, K. & GAMELLI, R. L. 2006. Long-term follow-up of patients treated for toxic epidermal necrolysis. *Journal of burn care & research,* 27**,** 26-33.

ORGANIZATION, W. H. 1972. *International drug monitoring: the role of national centres, report of a WHO meeting [held in Geneva from 20 to 25 September 1971]*, World Health Organization.

ORGANIZATION, W. H. 2000. Consumer reporting of adverse drug reactions. *WHO Drug Information,* 14**,** 211-5.

OSTROV, D. A., GRANT, B. J., POMPEU, Y. A., SIDNEY, J., HARNDAHL, M., SOUTHWOOD, S., OSEROFF, C., LU, S., JAKONCIC, J. & DE OLIVEIRA, C. A. F. 2012. Drug hypersensitivity caused by alteration of the MHC-presented self-peptide repertoire. *Proceedings of the National Academy of Sciences,* 109**,** 9959-9964.

OVERGAARD, N. H., JUNG, J. W., STEPTOE, R. J. & WELLS, J. W. 2015. CD4+/CD8+ double-positive T cells: more than just a developmental stage? *Journal of leukocyte biology,* 97**,** 31-38.

PADOVAN, E., BAUER, T., TONGIO, M. M., KALBACHER, H. & WELTZIEN, H. U. 1997. Penicilloyl peptides are recognized as T cell antigenic determinants in penicillin allergy. *European journal of immunology,* 27**,** 1303-1307.

PADOVAN, E., CASORATI, G., DELLABONA, P., MEYER, S., BROCKHAUS, M. & LANZAVECCHIA, A. 1993. Expression of two T cell receptor alpha chains: dual receptor T cells. *Science,* 262**,** 422-424.

PANZA, F., LOZUPONE, M., LOGROSCINO, G. & IMBIMBO, B. P. 2019. A critical appraisal of amyloid-β-targeting therapies for Alzheimer disease. *Nature Reviews Neurology,* 15**,** 73-88.

PANZA, F., LOZUPONE, M., SOLFRIZZI, V., SARDONE, R., PICCININNI, C., DIBELLO, V., STALLONE, R., GIANNELLI, G., BELLOMO, A. & GRECO, A. 2018. BACE inhibitors in clinical development for the treatment of Alzheimer's disease. *Expert review of neurotherapeutics,* 18**,** 847-857.

PAREL, Y. & CHIZZOLINI, C. 2004. CD4+ CD8+ double positive (DP) T cells in health and disease. *Autoimmunity reviews,* 3**,** 215-220.

PARK, B. 1986. Metabolic basis of adverse drug reactions. *Journal of the Royal College of Physicians of London,* 20**,** 195.

PARK, B., NAISBITT, D., GORDON, S., KITTERINGHAM, N. & PIRMOHAMED, M. 2001. Metabolic activation in drug allergies. *Toxicology,* 158**,** 11-23.

PARK, B. K., PIRMOHAMED, M. & KITTERINGHAM, N. R. 1998. Role of drug disposition in drug hypersensitivity: a chemical, molecular, and clinical perspective. *Chemical research in toxicology,* 11**,** 969-988.

PARK, K., WILLIAMS, D. P., NAISBITT, D. J., KITTERINGHAM, N. R. & PIRMOHAMED, M. 2005. Investigation of toxic metabolites during drug development. *Toxicology and applied pharmacology,* 207**,** 425-434.

PATEL, D. S., SHARMA, N., PATEL, M. C., PATEL, B. N., SHRIVASTAV, P. S. & SANYAL, M. 2011. Analysis of a second-generation tetracycline antibiotic minocycline in human plasma by LC–MS/MS. *Bioanalysis,* 3**,** 2177-2194.

PAVLOS, R., MALLAL, S. & PHILLIPS, E. 2012. HLA and pharmacogenetics of drug hypersensitivity. *Pharmacogenomics,* 13**,** 1285-1306.

PERUCCA, E. 2006. Clinically relevant drug interactions with antiepileptic drugs. *British journal of clinical pharmacology,* 61**,** 246-255.

PERUCCA, P. & GILLIAM, F. G. 2012. Adverse effects of antiepileptic drugs. *The Lancet Neurology,* 11**,** 792-802.

PEYRIERE, H., DEREURE, O., BRETON, H., DEMOLY, P., COCIGLIO, M., BLAYAC, J. P., HILLAIRE-BUYS, D. & CENTERS, N. O. T. F. P. 2006. Variability in the clinical pattern of cutaneous side-effects of drugs with systemic symptoms: does a DRESS syndrome really exist? *British Journal of Dermatology,* 155**,** 422-428.

PHILLIPS, E. J. & MALLAL, S. A. 2010. Pharmacogenetics of drug hypersensitivity. *Pharmacogenomics,* 11**,** 973-987.

PHILLIPS, K. A., VEENSTRA, D. L., OREN, E., LEE, J. K. & SADEE, W. 2001. Potential role of pharmacogenomics in reducing adverse drug reactions: a systematic review. *Jama,* 286**,** 2270-2279.

PICHLER, W. A. & TILCH, J. 2004. The lymphocyte transformation test in the diagnosis of drug hypersensitivity. *Allergy,* 59**,** 809-820.

PICHLER, W. J. 2002. Pharmacological interaction of drugs with antigen-specific immune receptors: the pi concept. *Current opinion in allergy and clinical immunology,* 2**,** 301-305.

PICHLER, W. J. 2003. Delayed drug hypersensitivity reactions. *Annals of internal medicine,* 139**,** 683-693.

PICHLER, W. J. 2005. Direct T-cell stimulations by drugs—bypassing the innate immune system. *Toxicology,* 209**,** 95-100.

PICHLER, W. J. 2014. Lymphocyte transformation test. *Encyclopedia of Immunology,* 10**,** 978-3.

PICHLER, W. J. 2019. Immune pathomechanism and classification of drug hypersensitivity. *Allergy,* 74**,** 1457-1471.

PICHLER, W. J. 2021. The important role of non-covalent drug-protein interactions in drug hypersensitivity reactions. *Allergy*.

PICHLER, W. J., BEELER, A., KELLER, M., LERCH, M., POSADAS, S., SCHMID, D., SPANOU, Z., ZAWODNIAK, A. & GERBER, B. 2006. Pharmacological interaction of drugs with immune receptors: the pi concept. *Allergology International,* 55**,** 17-25.

PIRMOHAMED, M. 2001. Pharmacogenetics and pharmacogenomics. *British journal of clinical pharmacology,* 52**,** 345-347.

PIRMOHAMED, M., ATUAH, K. N., DODOO, A. N. & WINSTANLEY, P. 2007. Pharmacovigilance in developing countries. *Bmj,* 335**,** 462.

PIRMOHAMED, M., BRECKENRIDGE, A. M., KITTERINGHAM, N. R. & PARK, B. K. 1998. Adverse drug reactions. *Bmj,* 316**,** 1295-1298.

PIRMOHAMED, M., JAMES, S., MEAKIN, S., GREEN, C., SCOTT, A. K., WALLEY, T. J., FARRAR, K., PARK, B. K. & BRECKENRIDGE, A. M. 2004. Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. *Bmj,* 329**,** 15-19.

PIRMOHAMED, M., KITTERINGHAM, N. R. & PARK, B. K. 1994. The role of active metabolites in drug toxicity. *Drug safety,* 11**,** 114-144.

PLOTT, R. T. & WORTZMAN, M. S. 2006. Key bioavailability features of a new extended-release formulation of minocycline hydrochloride tablets. *Cutis,* 78**,** 6-10.

POIREL, L., BRINAS, L., VERLINDE, A., IDE, L. & NORDMANN, P. 2005. BEL-1, a novel clavulanic acid-inhibited extended-spectrum β-lactamase, and the class 1 integron In120 in Pseudomonas aeruginosa. *Antimicrobial Agents and Chemotherapy,* 49**,** 3743-3748.

POSADAS, S. & PICHLER, W. J. 2007. Delayed drug hypersensitivity reactions–new concepts. *Clinical & Experimental Allergy,* 37**,** 989-999.

PRESLE, N., LAPICQUE, F., FOURNEL-GIGLEUX, S., MAGDALOU, J. & NETTER, P. 1996. Stereoselective irreversible binding of ketoprofen glucuronides to albumin. Characterization of the site and the mechanism. *Drug Metabolism and Disposition,* 24**,** 1050.

PROUDFOOT, L., WATSON, K., HIGGINS, E. & CREAMER, J. 2009. Twenty cases of DRESS syndrome: culprits, clinical features, treatment and outcome: O-10. *British Journal of Dermatology,* 161.

PUIG, M., ANANTHULA, S., VENNA, R., POLUMURI, S. K., MATTSON, E., WALKER, L. M., CARDONE, M., TAKAHASHI, M., SU, S. & BOYD, L. F. 2020. Alterations in the HLA-B* 57: 01 immunopeptidome by flucloxacillin and immunogenicity of drug-haptenated peptides. *Frontiers in immunology,* 11.

QING-PING, S., XIAO-DONG, J., FENG, D., YAN, L., MEI-LING, Y., JIN-XIU, Z. & SHU-QIANG, Z. 2014. Consequences, measurement, and evaluation of the costs associated with adverse drug reactions among hospitalized patients in China. *BMC health services research,* 14**,** 73.

QIU, Y., BURLINGAME, A. L. & BENET, L. Z. 1998. Mechanisms for Covalent Binding of Benoxaprofen Glucuronide to Human Serum Albumin. *Drug Metabolism and Disposition,* 26**,** 246.

QUAH, B. J., WARREN, H. S. & PARISH, C. R. 2007. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nature protocols,* 2**,** 2049.

RADOJA, S., FREY, A. B. & VUKMANOVIC, S. 2006. T-cell receptor signaling events triggering granule exocytosis. *Critical Reviews™ in Immunology,* 26.

RAMMENSEE, H.-G. 1995. Chemistry of peptides associated with MHC class I and class II molecules. *Current opinion in immunology,* 7**,** 85-96.

RAULET, D. H. 2004. Interplay of natural killer cells and their receptors with the adaptive immune response. *Nature immunology,* 5**,** 996-1002.

RAWLINS, M. 1977. Pathogenesis of adverse drug reactions. *Text book of adverse drug reactions*.

RAWLINS, M. & THOMPSON, J. 1991. 3. Mechanisms of adverse drug reactions.

REMPE, S., HAYDEN, J., ROBBINS, R. & HOYT, J. 2007. Tetracyclines and pulmonary inflammation. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders),* 7**,** 232-236.

RESCIGNO, M., MARTINO, M., SUTHERLAND, C. L., GOLD, M. R. & RICCIARDI-CASTAGNOLI, P. 1998. Dendritic cell survival and maturation are regulated by different signaling pathways. *The Journal of experimental medicine,* 188**,** 2175-2180.

RIEDL, M. A. & CASILLAS, A. M. 2003. Adverse drug reactions: types and treatment options. *American family physician,* 68**,** 1781-1790.

RILEY, A. L. & KOHUT, S. 2010. Drug Toxicity. *In:* STOLERMAN, I. P. (ed.) *Encyclopedia of Psychopharmacology.* Berlin, Heidelberg: Springer Berlin Heidelberg.

RIVE, C. M., BOURKE, J. & PHILLIPS, E. J. 2013. Testing for drug hypersensitivity syndromes. *The Clinical Biochemist Reviews,* 34**,** 15.

ROBINSON, J. H. & DELVIG, A. A. 2002. Diversity in MHC class II antigen presentation. *Immunology,* 105**,** 252-262.

ROCHE, P. A. & FURUTA, K. 2015. The ins and outs of MHC class II-mediated antigen processing and presentation. *Nature Reviews Immunology,* 15**,** 203-216.

RODEN, D. M. 2004. Drug-induced prolongation of the QT interval. *New England Journal of Medicine,* 350**,** 1013-1022.

RODRÍGUEZ, L. A. G., STRICKER, B. H. & ZIMMERMAN, H. J. 1996. Risk of acute liver injury associated with the combination of amoxicillin and clavulanic acid. *Archives of internal medicine,* 156**,** 1327-1332.

ROEDERER, M., STAAL, F. J., OSADA, H., HERZENBERG, L. A. & HERZENBERG, L. A. 1991. CD4 and CD8 T cells with high intracellular glutathione levels are selectively lost as the HIV infection progresses. *International Immunology,* 3**,** 933-937.

ROHILLA, A. & YADAV, S. 2013. Adverse drug reactions: an overview. *Int J Pharmacol Res,* 3**,** 10-2.

ROLLINS, B. J. 1997. Chemokines. *Blood, The Journal of the American Society of Hematology,* 90**,** 909-928.

ROMANO, A., BLANCA, M., TORRES, M., BIRCHER, A., ABERER, W., BROCKOW, K., PICHLER, W., DEMOLY, P., ENDA & HYPERSENSITIVITY, T. E. I. G. O. D. 2004. Diagnosis of nonimmediate reactions to β-lactam antibiotics. *Allergy,* 59**,** 1153-1160.

ROTH, L., ADLER, M., JAIN, T. & BEMPONG, D. 2018. Monographs for medicines on WHO's Model List of Essential Medicines. *Bulletin of the World Health Organization,* 96**,** 378-385.

ROUJEAU, J.-C. 2005. Clinical heterogeneity of drug hypersensitivity. *Toxicology,* 209**,** 123-129.

ROYER, R. J. 1997. Mechanism of action of adverse drug reactions: an overview. *Pharmacoepidemiology and drug safety,* 6**,** S43.

RUBIO, M., BOUSQUET, P.-J. & DEMOLY, P. 2010. Update in drug allergy: novel drugs with novel reaction patterns. *Current opinion in allergy and clinical immunology,* 10**,** 457-462.

RUDOLPH, M. G., STANFIELD, R. L. & WILSON, I. A. 2006. How TCRs bind MHCs, peptides, and coreceptors. *Annu. Rev. Immunol.,* 24**,** 419-466.

RZANY, B., MOCKENHAUPT, M., BAUR, S., SCHRODER, W., STOCKER, U., MUELLER, J., HOLLANDER, N., BRUPPACHER, R. & SCHÖPF, E. 1996. Epidemiology of rare serious cutaneous adverse reactions. Results of the population based registry for erythema exsudativum multiforme with mucosal involvement (EEMM), Stevens-Johnson-syndrome (SJS) and toxic epidermal necrolysis (TEN) in Ge/r/many (1990–1992). *J Clin Epidemiol,* 49**,** 769-73.

SAIMAN, Y. & FRIEDMAN, S. L. 2012. The role of chemokines in acute liver injury. *Frontiers in physiology,* 3**,** 213.

SALAZAR-PARAMO, M., RUBIN, R. L. & GARCIA-DE LA TORRE, I. 1992. Systemic lupus erythematosus induced by isoniazid. *Annals of the rheumatic diseases,* 51**,** 1085-1087.

SALAZAR, T. A. 1989. End Organ Toxicities of Adverse Drug Reactions. *Journal of Pharmacy Practice,* 2**,** 245-250.

SALVO, F., POLIMENI, G., MORETTI, U., CONFORTI, A., LEONE, R., LEONI, O., MOTOLA, D., DUSI, G. & CAPUTI, A. P. 2007. Adverse drug reactions related to amoxicillin alone and in association with clavulanic acid: data from spontaneous reporting in Italy. *Journal of antimicrobial chemotherapy,* 60**,** 121-126.

SANABRIA-CASTRO, A., ALVARADO-ECHEVERRÍA, I. & MONGE-BONILLA, C. 2017. Molecular pathogenesis of Alzheimer's disease: an update. *Annals of neurosciences,* 24**,** 46-54.

SATHYA, M., PREMKUMAR, P., KARTHICK, C., MOORTHI, P., JAYACHANDRAN, K. S. & ANUSUYADEVI, M. 2012. BACE1 in Alzheimer's disease. *Clin Chim Acta,* 414**,** 171-8.

SCHNEIDER, C. & DE WECK, A. 1965. A New Chemical Aspect of Penicillin Allergy: The Direct Reaction of Penicillin with ε-Amino-Groups. *Nature,* 208**,** 57-59.

SCHNYDER, B., ADAM, J., RAUCH, A., THURNHEER, M. C. & PICHLER, W. J. 2013. HLA-B* 57: 01+ abacavir-naive individuals have specific T cells but no patch test reactivity. *Journal of allergy and clinical immunology,* 132**,** 756.

SCHNYDER, B., BURKHART, C., SCHNYDER-FRUTIG, K., VON GREYERZ, S., NAISBITT, D. J., PIRMOHAMED, M., PARK, B. K. & PICHLER, W. J. 2000a. Recognition of Sulfamethoxazole and Its Reactive Metabolites by Drug-Specific CD4<sup>+</sup> T Cells from Allergic Individuals. *The Journal of Immunology,* 164**,** 6647.

SCHNYDER, B., BURKHART, C., SCHNYDER-FRUTIG, K., VON GREYERZ, S., NAISBITT, D. J., PIRMOHAMED, M., PARK, B. K. & PICHLER, W. J. 2000b. Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals. *The Journal of Immunology,* 164**,** 6647-6654.

SCHNYDER, B., MAURI-HELLWEG, D., ZANNI, M., BETTENS, F. & PICHLER, W. J. 1997. Direct, MHC-dependent presentation of the drug sulfamethoxazole to human alphabeta T cell clones. *The Journal of clinical investigation,* 100**,** 136-141.

SCHUMOCK, G. & THORNTON, J. 1992. Focusing on the preventability of adverse drug reactions. *Hospital pharmacy,* 27**,** 538-538.

SEDDON, C. 2006. *Reporting adverse drug reactions A guide for healthcare professionals BMA May 2006*.

SEEGER, J. D., SCHUMOCK, G. T. & KONG, S. X. 1996. Estimating the rate of adverse drug reactions with capture-recapture analysis. *American journal of health-system pharmacy: AJHP: official journal of the American Society of Health-System Pharmacists,* 53**,** 178-181.

SEITZ, S., KRETZ-ROMMEL, A., OUDE ELFERINK, R. P. J. & BOELSTERLI, U. A. 1998. Selective Protein Adduct Formation of Diclofenac Glucuronide Is Critically Dependent on the Rat Canalicular Conjugate Export Pump (Mrp2). *Chemical Research in Toxicology,* 11**,** 513-519.

SEXTON, J. B., THOMAS, E. J. & HELMREICH, R. L. 2000. Error, stress, and teamwork in medicine and aviation: cross sectional surveys. *Bmj,* 320**,** 745-749.

SHAKIR, S. & WILTON, L. 2000. BOOK REVIEW: Detection of New Adverse Drug Reactions, 4th edition. Edited by M. D. B. Stephens, J. C. C. Talbot and P. A. Routledge. Macmillan Reference Ltd, Basingstoke. *Pharmacoepidemiology and Drug Safety,* 9**,** 435-435.

SHARMA, V. K., SETHURAMAN, G. & MINZ, A. 2008. Stevens Johnson syndrome, toxic epidermal necrolysis and SJS-TEN overlap: a retrospective study of causative drugs and clinical outcome. *Indian J Dermatol Venereol Leprol,* 74**,** 238-40.

SHERMAN, E. M., WORLEY, M. V., UNGER, N. R., GAUTHIER, T. P. & SCHAFER, J. J. 2015. Cobicistat: review of a pharmacokinetic enhancer for HIV infection. *Clinical therapeutics,* 37**,** 1876-1893.

SHIINA, T., INOKO, H. & KULSKI, J. 2004. An update of the HLA genomic region, locus information and disease associations: 2004. *Tissue antigens,* 64**,** 631-649.

SHIOHARA, T. 2007. The diagnosis of a DRESS syndrome has been sufficiently established on the basis of typical clinical features and viral reactivations. *Br J Dermatol,* 156**,** 1083-1084.

SHIOHARA, T. 2009. Fixed drug eruption: pathogenesis and diagnostic tests. *Current opinion in allergy and clinical immunology,* 9**,** 316-321.

SHIOHARA, T., KANO, Y., TAKAHASHI, R., ISHIDA, T. & MIZUKAWA, Y. 2012. Drug-induced hypersensitivity syndrome: recent advances in the diagnosis, pathogenesis and management. *Adverse cutaneous drug eruptions,* 97**,** 122-138.

SHIPKOVA, M. & WIELAND, E. 2012. Surface markers of lymphocyte activation and markers of cell proliferation. *Clinica chimica acta,* 413**,** 1338-1349.

SHU, P. 1966. Oxidative preparation of 9-hydroxytetracyclines. *Journal of the American Chemical Society,* 88**,** 4529-4530.

SIBBALD, B. 2004. Rofecoxib (Vioxx) voluntarily withdrawn from market. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne,* 171**,** 1027-1028.

SINGER, J. B., LEWITZKY, S., LEROY, E., YANG, F., ZHAO, X., KLICKSTEIN, L., WRIGHT, T. M., MEYER, J. & PAULDING, C. A. 2010. A genome-wide study identifies HLA alleles associated with lumiracoxib-related liver injury. *Nature genetics,* 42**,** 711-714.

SINGH, R., MANJUNATHA, U., BOSHOFF, H. I., HA, Y. H., NIYOMRATTANAKIT, P., LEDWIDGE, R., DOWD, C. S., LEE, I. Y., KIM, P. & ZHANG, L. 2008. PA-824 kills nonreplicating Mycobacterium tuberculosis by intracellular NO release. *Science,* 322**,** 1392-1395.

SKONBERG, C., OLSEN, J., MADSEN, K. G., HANSEN, S. H. & GRILLO, M. P. 2008. Metabolic activation of carboxylic acids. *Expert opinion on drug metabolism & toxicology,* 4**,** 425-438.

SONAWANE, K. B., CHENG, N. & HANSEN, R. A. 2018. Serious Adverse Drug Events Reported to the FDA: Analysis of the FDA Adverse Event Reporting System 2006-2014 Database. *J Manag Care Spec Pharm,* 24**,** 682-690.

SPAHN-LANGGUTH, H. & BENET, L. Z. 1992. Acyl glucuronides revisited: Is the glucuronidation process a toxification as well as detoxification mechanism? *Drug Metab Rev,* 24**,** 5-47.

STEIGBIGEL, N., REED, C. & FINLAND, M. 1968. Susceptibility of common pathogenic bacteria to seven tetracycline antibiotics in vitro. *American Journal of Medical Sciences,* 255**,** 179-95.

STEINKE, D. T. 2019. Essentials of Pharmacoepidemiology. *Clinical Pharmacy Education, Practice and Research.* Elsevier.

STEINMAN, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annual review of immunology,* 9**,** 271-296.

STRAUSS, J. S., KROWCHUK, D. P., LEYDEN, J. J., LUCKY, A. W., SHALITA, A. R., SIEGFRIED, E. C., THIBOUTOT, D. M., VAN VOORHEES, A. S., BEUTNER, K. A. & SIECK, C. K. 2007. Guidelines of care for acne vulgaris management. *Journal of the American Academy of Dermatology,* 56**,** 651-663.

STROM, B. L., SCHINNAR, R., APTER, A. J., MARGOLIS, D. J., LAUTENBACH, E., HENNESSY, S., BILKER, W. B. & PETTITT, D. 2003. Absence of cross-reactivity between sulfonamide antibiotics and sulfonamide nonantibiotics. *New England Journal of Medicine,* 349**,** 1628-1635.

STUART, M. C., KOUIMTZI, M. & HILL, S. R. 2009. *WHO model formulary 2008*, World Health Organization.

SUNDARARAMAN, S., KARULIN, A. Y., ANSARI, T., BENHAMOUDA, N., GOTTWEIN, J., LAXMANAN, S., LEVINE, S. M., LOFFREDO, J. T., MCARDLE, S. & NEUDOERFL, C. 2015. High reproducibility of ELISPOT counts from nine different laboratories. *Cells,* 4**,** 21-39.

SURESH, E., MORRIS, I. M. & MATTINGLY, P. C. 2004. Use of minocycline in rheumatoid arthritis: a district general hospital experience. *Annals of the Rheumatic Diseases,* 63**,** 1354.

SWEETMAN, S. C. 2009. *Martindale: the complete drug reference*, Pharmaceutical press London.

TAILOR, A., MENG, X., ADAIR, K., FARRELL, J., WADDINGTON, J. C., DALY, A., PIRMOHAMED, M., DEAR, G., PARK, B. K. & NAISBITT, D. J. 2020. HLA DRB1* 15: 01-DQB1* 06: 02-Restricted Human CD4+ T Cells Are Selectively Activated With Amoxicillin-Peptide Adducts. *Toxicological Sciences,* 178**,** 115-126.

TAILOR, A., WADDINGTON, J. C., HAMLETT, J., MAGGS, J., KAFU, L., FARRELL, J., DEAR, G. J., WHITAKER, P., NAISBITT, D. J. & PARK, K. 2019. Definition of haptens derived from sulfamethoxazole: in vitro and in vivo. *Chemical research in toxicology,* 32**,** 2095-2106.

TAIT, B. D. 2011. The ever-expanding list of HLA alleles: changing HLA nomenclature and its relevance to clinical transplantation. *Transplantation Reviews,* 25**,** 1-8.

TAKEUCHI, O. & AKIRA, S. 2010. Pattern recognition receptors and inflammation. *Cell,* 140**,** 805-820.

TALBOT, J. & ARONSON, J. K. 2012. *Stephen's detection and evaluation of adverse drug reactions*, Wiley Online Library.

TANGUAY, S. & KILLION, J. J. 1994. Direct comparison of ELISPOT and ELISA-based assays for detection of individual cytokine-secreting cells. *Lymphokine and cytokine research,* 13**,** 259-263.

TAPIA, B., PADIAL, A., SÁNCHEZ-SABATÉ, E., ALVAREZ-FERREIRA, J., MOREL, E., BLANCA, M. & BELLÓN, T. 2004. Involvement of CCL27-CCR10 interactions in drug-induced cutaneous reactions. *Journal of Allergy and Clinical Immunology,* 114**,** 335-340.

TEN BROEKE, T., WUBBOLTS, R. & STOORVOGEL, W. 2013. MHC class II antigen presentation by dendritic cells regulated through endosomal sorting. *Cold Spring Harbor perspectives in biology,* 5**,** a016873.

TENG, M., SMOLYAR, A., TSE, A., LIU, J., LIU, J., HUSSEY, R., NATHENSON, S., CHANG, H., REINHERZ, E. & WANG, J. 1998. Identification of a common docking topology with substantial variation among different TCR–peptide–MHC complexes. *Current biology,* 8**,** 409-414.

THIEN, F. C. 2006. 3. Drug hypersensitivity. *Medical journal of Australia,* 185**,** 333.

THOMAS, E. J. & BRENNAN, T. A. 2000. Incidence and types of preventable adverse events in elderly patients: population based review of medical records. *Bmj,* 320**,** 741-4.

TIMMERS, M., STREFFER, J. R., RUSSU, A., TOMINAGA, Y., SHIMIZU, H., SHIRAISHI, A., TATIKOLA, K., SMEKENS, P., BÖRJESSON-HANSON, A. & ANDREASEN, N. 2018. Pharmacodynamics of atabecestat (JNJ-54861911), an oral BACE1 inhibitor in patients with early Alzheimer's disease: randomized, double-blind, placebo-controlled study. *Alzheimer's research & therapy,* 10**,** 1-18.

TORPET, L. A., KRAGELUND, C., REIBEL, J. & NAUNTOFTE, B. 2004. Oral adverse drug reactions to cardiovascular drugs. *Critical reviews in oral biology & medicine,* 15**,** 28-46.

TORRES, M. A. & MORAES, M. E. H. 2011. Nomenclature for factors of the HLA system. *Einstein (São Paulo),* 9**,** 249-251.

TROWSDALE, J. 2011. The MHC, disease and selection. *Immunology letters,* 137**,** 1-8.

UDENFRIEND, S., CLARK, C. T., AXELROD, J. & BRODIE, B. B. 1954. Ascorbic acid in aromatic hydroxylation. *J Biol Chem,* 208**,** 731-738.

UETRECHT, J. 2007. Idiosyncratic drug reactions: current understanding. *Annu. Rev. Pharmacol. Toxicol.,* 47**,** 513-539.

UETRECHT, J. & NAISBITT, D. J. 2013. Idiosyncratic adverse drug reactions: current concepts. *Pharmacological reviews,* 65**,** 779-808.

UETRECHT, J. P. 1999. New concepts in immunology relevant to idiosyncratic drug reactions: the "danger hypothesis" and innate immune system. *Chemical research in toxicology,* 12**,** 387-395.

URBAN, T. J., NICOLETTI, P., CHALASANI, N., SERRANO, J., STOLZ, A., DALY, A. K., AITHAL, G. P., DILLON, J., NAVARRO, V. & ODIN, J. 2017. Minocycline hepatotoxicity: clinical characterization and identification of HLA-B∗ 35: 02 as a risk factor. *Journal of hepatology,* 67**,** 137-144.

USUI, T., MENG, X., SAIDE, K., FARRELL, J., THOMSON, P., WHITAKER, P., WATSON, J., FRENCH, N. S., KEVIN PARK, B. & NAISBITT, D. J. 2017a. From the Cover: Characterization of Isoniazid-Specific T-Cell Clones in Patients with anti-Tuberculosis Drug-Related Liver and Skin Injury. *Toxicol Sci,* 155**,** 420-431.

USUI, T., MENG, X., SAIDE, K., FARRELL, J., THOMSON, P., WHITAKER, P., WATSON, J., FRENCH, N. S., KEVIN PARK, B. & NAISBITT, D. J. 2017b. From the cover: characterization of isoniazid-specific T-cell clones in patients with anti-tuberculosis drug-related liver and skin injury. *Toxicological Sciences,* 155**,** 420-431.

USUI, T. & NAISBITT, D. J. 2017. Human leukocyte antigen and idiosyncratic adverse drug reactions. *Drug metabolism and pharmacokinetics,* 32**,** 21-30.

VALEYRIE-ALLANORE, L., SASSOLAS, B. & ROUJEAU, J.-C. 2007. Drug-induced skin, nail and hair disorders. *Drug safety,* 30**,** 1011-1030.

VAN DER VEN, A. A., KOOPMANS, P., VAN DER MEER, J. W. & VREE, T. 1991. Adverse reactions to co-trimoxazole in HIV infection. *The Lancet,* 338**,** 431-433.

VAN EEDEN, S. F., KLUT, M. E., WALKER, B. A. M. & HOGG, J. C. 1999. The use of flow cytometry to measure neutrophil function. *Journal of Immunological Methods,* 232**,** 23-43.

VAN NORMAN, G. A. 2016. Drugs, devices, and the FDA: part 1: an overview of approval processes for drugs. *JACC: Basic to Translational Science,* 1**,** 170-179.

VAN VLEET, T. R., LIU, H., LEE, A. & BLOMME, E. A. G. 2017. Acyl glucuronide metabolites: Implications for drug safety assessment. *Toxicol Lett,* 272**,** 1-7.

VANDIEDONCK, C. & KNIGHT, J. C. 2009. The human Major Histocompatibility Complex as a paradigm in genomics research. *Briefings in Functional Genomics and Proteomics,* 8**,** 379-394.

VARDJAN, N., GABRIJEL, M., POTOKAR, M., ŠVAJGER, U., KREFT, M., JERAS, M., DE PABLO, Y., FAIZ, M., PEKNY, M. & ZOREC, R. 2012. IFN-γ-induced increase in the mobility of MHC class II compartments in astrocytes depends on intermediate filaments. *Journal of neuroinflammation,* 9**,** 1-13.

VASSAR, R. 2005. beta-Secretase, APP and Abeta in Alzheimer's disease. *Subcell Biochem,* 38**,** 79-103.

VERDEL, B. M., SOUVEREIN, P. C., EGBERTS, A. C. & LEUFKENS, H. G. 2006. Difference in risks of allergic reaction to sulfonamide drugs based on chemical structure. *Annals of Pharmacotherapy,* 40**,** 1040-1046.

VERMA, R., VASUDEVAN, B. & PRAGASAM, V. 2013. Severe cutaneous adverse drug reactions. *medical journal armed forces india,* 69**,** 375-383.

VERMEER, C. & SCHURGERS, L. J. 2000. A comprehensive review of vitamin K and vitamin K antagonists. *Hematology/oncology clinics of North America,* 14**,** 339-353.

VICTORINO, R. M., SILVEIRA, J. C., BAPTISTA, A. & DE MOURA, M. C. 1980. Jaundice associated with naproxen. *Postgraduate medical journal,* 56**,** 368-370.

VINCENT, C., NEALE, G. & WOLOSHYNOWYCH, M. 2001. Adverse events in British hospitals: preliminary retrospective record review. *BMJ (Clinical research ed.),* 322**,** 517-519.

VOCANSON, M., NAISBITT, D. J. & NICOLAS, J.-F. 2020. Current perspective of the etiopathogenesis of delayed-type, and T-cell–mediated drug-related skin diseases. *Journal of Allergy and Clinical Immunology,* 145**,** 1142-1144.

VON GREYERZ, S., BÜLTEMANN, G., SCHNYDER, K., BURKHART, C., LOTTI, B., HARI, Y. & PICHLER, W. J. 2001. Degeneracy and additional alloreactivity of drug-specific human αβ+ T cell clones. *International immunology,* 13**,** 877-885.

VREE, T., VAN DEN BIGGELAAR-MARTEA, M., VERWEY-VAN WISSEN, C., VREE, M. & GUELEN, P. 1993. The pharmacokinetics of naproxen, its metabolite O-desmethylnaproxen, and their acyl glucuronides in humans. Effect of cimetidine. *British journal of clinical pharmacology,* 35**,** 467-472.

WADDINGTON, J. C., MENG, X., ILLING, P. T., TAILOR, A., ADAIR, K., WHITAKER, P., HAMLETT, J., JENKINS, R. E., FARRELL, J. & BERRY, N. 2020. Identification of flucloxacillin-haptenated HLA-B* 57: 01 ligands: evidence of antigen processing and presentation. *Toxicological Sciences,* 177**,** 454-465.

WALKER, C., KRISTENSEN, F., BETTENS, F. & DEWECK, A. 1983. Lymphokine regulation of activated (G1) lymphocytes. I. Prostaglandin E2-induced inhibition of interleukin 2 production. *The Journal of Immunology,* 130**,** 1770-1773.

WALSH, S. & CREAMER, D. 2011. Drug reaction with eosinophilia and systemic symptoms (DRESS): a clinical update and review of current thinking. *Clinical and Experimental Dermatology: Clinical dermatology,* 36**,** 6-11.

WANG, P., PRADHAN, K., ZHONG, X.-B. & MA, X. 2016. Isoniazid metabolism and hepatotoxicity. *Acta pharmaceutica sinica B,* 6**,** 384-392.

WANG, X., ZHANG, L. & JIANG, Z. 2015. T-helper cell-mediated factors in drug-induced liver injury. *Journal of Applied Toxicology,* 35**,** 695-700.

WARD, K. E., ARCHAMBAULT, R. & MERSFELDER, T. L. 2010. Severe adverse skin reactions to nonsteroidal antiinflammatory drugs: A review of the literature. *American Journal of Health-System Pharmacy,* 67**,** 206-213.

WATKINS, S. L. & PICHLER, W. J. 2013. Activating interactions of sulfanilamides with T cell receptors. *Open Journal of Immunology,* 3**,** 139-157.

WAX, P. M. 1995. Elixirs, diluents, and the passage of the 1938 Federal Food, Drug and Cosmetic Act. *Annals of internal medicine,* 122**,** 456-461.

WAXMAN, D. J. & HOLLOWAY, M. G. 2009. Sex differences in the expression of hepatic drug metabolizing enzymes. *Molecular pharmacology,* 76**,** 215-228.

WEAVER, R. J., BLOMME, E. A., CHADWICK, A. E., COPPLE, I. M., GERETS, H. H., GOLDRING, C. E., GUILLOUZO, A., HEWITT, P. G., INGELMAN-SUNDBERG, M. & JENSEN, K. G. 2020. Managing the challenge of drug-induced liver injury: a roadmap for the development and deployment of preclinical predictive models. *Nature Reviews Drug Discovery,* 19**,** 131-148.

WEAVER, R. J. & VALENTIN, J.-P. 2019. Today's challenges to de-risk and predict drug safety in human "mind-the-gap". *Toxicological Sciences,* 167**,** 307-321.

WEI, C.-Y., CHUNG, W.-H., HUANG, H.-W., CHEN, Y.-T. & HUNG, S.-I. 2012a. Direct interaction between HLA-B and carbamazepine activates T cells in patients with Stevens-Johnson syndrome. *Journal of Allergy and Clinical Immunology,* 129**,** 1562-1569. e5.

WEI, C. Y., CHUNG, W. H., HUANG, H. W., CHEN, Y. T. & HUNG, S. I. 2012b. Direct interaction between HLA-B and carbamazepine activates T cells in patients with Stevens-Johnson syndrome. *J Allergy Clin Immunol,* 129**,** 1562-9 e5.

WELTZIEN, H. U., MOULON, C., MARTIN, S., PADOVAN, E., HARTMANN, U. & KOHLER, J. 1996. T cell immune responses to haptens. Structural models for allergic and autoimmune reactions. *Toxicology,* 107**,** 141-151.

WELTZIEN, H. U. & PADOVAN, E. 1998. Molecular features of penicillin allergy. *Journal of investigative dermatology,* 110**,** 203-206.

WHRITENOUR, J., KO, M., ZONG, Q., WANG, J., TARTARO, K., SCHNEIDER, P., OLSON, E., VAN VOLKENBURG, M., SERRANO, J. & HAYASHI, P. 2017. Development of a modified lymphocyte transformation test for diagnosing drug-induced liver injury associated with an adaptive immune response. *Journal of immunotoxicology,* 14**,** 31-38.

WIECZOREK, M., ABUALROUS, E. T., STICHT, J., ÁLVARO-BENITO, M., STOLZENBERG, S., NOÉ, F. & FREUND, C. 2017. Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation. *Frontiers in Immunology,* 8.

WIFFEN, P. 2002. Adverse drug reactions in hospital patients-A systematic review of the prospective and retrospective studies. *Bandolier*.

WILLIAMS, T. M. 2001. Human leukocyte antigen gene polymorphism and the histocompatibility laboratory. *The journal of molecular diagnostics,* 3**,** 98-104.

WU, Y., SANDERSON, J. P., FARRELL, J., DRUMMOND, N. S., HANSON, A., BOWKETT, E., BERRY, N., STACHULSKI, A. V., CLARKE, S. E. & PICHLER, W. J. 2006a. Activation of T cells by carbamazepine and carbamazepine metabolites. *Journal of allergy and clinical immunology,* 118**,** 233-241.

WU, Y., SANDERSON, J. P., FARRELL, J., DRUMMOND, N. S., HANSON, A., BOWKETT, E., BERRY, N., STACHULSKI, A. V., CLARKE, S. E., PICHLER, W. J., PIRMOHAMED, M., PARK, B. K. & NAISBITT, D. J. 2006b. Activation of T cells by carbamazepine and carbamazepine metabolites. *J Allergy Clin Immunol,* 118**,** 233-41.

WUILLEMIN, N., ADAM, J., FONTANA, S., KRÄHENBÜHL, S., PICHLER, W. J. & YERLY, D. 2013. HLA haplotype determines hapten or pi T cell reactivity to flucloxacillin. *The Journal of Immunology,* 190**,** 4956-4964.

WUILLEMIN, N., TERRACCIANO, L., BELTRAMINELLI, H., SCHLAPBACH, C., FONTANA, S., KRÄHENBÜHL, S., PICHLER, W. J. & YERLY, D. 2014. T cells infiltrate the liver and kill hepatocytes in HLA-B∗ 57: 01-associated floxacillin-induced liver injury. *The American journal of pathology,* 184**,** 1677-1682.

WULF, N. R. & MATUSZEWSKI, K. A. 2013. Sulfonamide cross-reactivity: is there evidence to support broad cross-allergenicity? *American journal of health-system pharmacy,* 70**,** 1483-1494.

XIE, T., ROWEN, L., AGUADO, B., AHEARN, M. E., MADAN, A., QIN, S., CAMPBELL, R. D. & HOOD, L. 2003. Analysis of the gene-dense major histocompatibility complex class III region and its comparison to mouse. *Genome research,* 13**,** 2621-2636.

YANG, L., YU, H., DONG, S., ZHONG, Y. & HU, S. 2017. Recognizing and managing on toxicities in cancer immunotherapy. *Tumor Biology,* 39**,** 1010428317694542.

YASEEN, F. S., SAIDE, K., KIM, S.-H., MONSHI, M., TAILOR, A., WOOD, S., MENG, X., JENKINS, R., FAULKNER, L. & DALY, A. K. 2015. Promiscuous T-cell responses to drugs and drug-haptens. *Journal of Allergy and Clinical Immunology,* 136**,** 474-476. e8.

YAWALKAR, N., EGLI, F., HARI, Y., NIEVERGELT, H., BRAATHEN, L. R. & PICHLER, W. J. 2000a. Infiltration of cytotoxic T cells in drug-induced cutaneous eruptions. *Clin Exp Allergy,* 30**,** 847-55.

YAWALKAR, N., HARI, Y., FRUTIG, K., EGLI, F., WENDLAND, T., BRAATHEN, L. R. & PICHLER, W. J. 2000b. T cells isolated from positive epicutaneous test reactions to amoxicillin and ceftriaxone are drug specific and cytotoxic. *Journal of investigative dermatology,* 115**,** 647-652.

YAWALKAR, N., SHRIKHANDE, M., HARI, Y., NIEVERGELT, H., BRAATHEN, L. R. & PICHLER, W. J. 2000c. Evidence for a role for IL-5 and eotaxin in activating and recruiting eosinophils in drug-induced cutaneous eruptions. *Journal of Allergy and Clinical Immunology,* 106**,** 1171-1176.

YEWDELL, J. W., REITS, E. & NEEFJES, J. 2003. Making sense of mass destruction: quantitating MHC class I antigen presentation. *Nature Reviews Immunology,* 3**,** 952-961.

YOSHIE, O. & MATSUSHIMA, K. 2015. CCR4 and its ligands: from bench to bedside. *International immunology,* 27**,** 11-20.

YOUNG, N. S. & MACIEJEWSKI, J. 1997. The pathophysiology of acquired aplastic anemia. *New England journal of medicine,* 336**,** 1365-1372.

YUN, J., MARCAIDA, M. J., ERIKSSON, K. K., JAMIN, H., FONTANA, S., PICHLER, W. J. & YERLY, D. 2014a. Oxypurinol directly and immediately activates the drug-specific T cells via the preferential use of HLA-B*58:01. *J Immunol,* 192**,** 2984-93.

YUN, J., MARCAIDA, M. J., ERIKSSON, K. K., JAMIN, H., FONTANA, S., PICHLER, W. J. & YERLY, D. 2014b. Oxypurinol directly and immediately activates the drug-specific T cells via the preferential use of HLA-B* 58: 01. *The Journal of Immunology,* 192**,** 2984-2993.

YUN, J., MATTSSON, J., SCHNYDER, K., FONTANA, S., LARGIADÈR, C. R., PICHLER, W. J. & YERLY, D. 2013. Allopurinol hypersensitivity is primarily mediated by dose-dependent oxypurinol-specific T cell response. *Clinical & Experimental Allergy,* 43**,** 1246-1255.

ZANNI, M. P., VON GREYERZ, S., SCHNYDER, B., BRANDER, K. A., FRUTIG, K., HARI, Y., VALITUTTI, S. & PICHLER, W. J. 1998. HLA-restricted, processing-and metabolism-independent pathway of drug recognition by human alpha beta T lymphocytes. *The Journal of clinical investigation,* 102**,** 1591-1598.

ZHANG, F.-R., LIU, H., IRWANTO, A., FU, X.-A., LI, Y., YU, G.-Q., YU, Y.-X., CHEN, M.-F., LOW, H.-Q. & LI, J.-H. 2013. HLA-B* 13: 01 and the dapsone hypersensitivity syndrome. *New England Journal of Medicine,* 369**,** 1620-1628.

ZHAO, Q., ALHILALI, K., ALZAHRANI, A., ALMUTAIRI, M., AMJAD, J., LIU, H., SUN, Y., SUN, L., ZHANG, H. & MENG, X. 2019. Dapsone-and nitroso dapsone-specific activation of T cells from hypersensitive patients expressing the risk allele HLA-B* 13: 01. *Allergy,* 74**,** 1533-1548.

ZHOU, F. 2009. Molecular mechanisms of IFN-γ to up-regulate MHC class I antigen processing and presentation. *International reviews of immunology,* 28**,** 239-260.

ZHU, J., YAMANE, H. & PAUL, W. E. 2009. Differentiation of effector CD4 T cell populations. *Annual review of immunology,* 28**,** 445-489.