# Investigation of immunogenetic risk factors for carbamazepine-induced hypersensitivity reactions

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

by

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# Declaration

I hereby declare that this thesis is the result of my own work and has not been submitted for any other degree.

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# Acknowledgement

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# Table of Contents

# Abbreviations

| | |
|---|---|
| AA | Amino acid |
| ABC | Abacavir |
| ADR | Adverse drug reaction |
| AGEP | Acute generalized exanthematous pustulosis |
| APC | Antigen-presenting cell |
| β2m | beta 2 microglobulin |
| BCIP/NBT | 5-bromo-4-chloro-3-indolyl-phosphate/ nitro blue tetrazolium |
| B-LCL | B-lymphoblastoid cell line |
| CBZ | Carbamazepine |
| CD | Cluster of differentiation |
| CDR | Complementarity determining region |
| Ci | Curie |
| CLIP | Class II-associated invariant chain peptide |
| cpm | counts per minute |
| CSA | Cyclosporine A |
| CYP | Cytochrome P450 enzyme |
| DC | Dendritic cell |
| DILI | Drug-induced liver injury |
| DMSO | Diemethyl sulfoxide |
| DNA | Dioxyribonucleic acid |
| DRESS | Drug reaction with eosinophilia and systemic symptoms |
| EBV | Epstein-Barr virus |
| ELISpot | Enzyme-linked immunospot |
| ER | Endoplasmic reticulum |
| FasL | Fas Ligand |

| | |
|---|---|
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GWAS | Genome wide association study |
| HBSS | Hanks Balanced Salt Solution |
| HEPES | Hydroxyethyl piperazineethanesulfonic acid |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| HPLC | High performance liquid chromatography |
| HSR | Hypersensitivity reaction |
| HSS | Hypersensitivity syndrome |
| iADR | idiosyncratic adverse drug reaction |
| ICS | Intracellular cytokine staining assay |
| IFN-γ | Interferon gamma |
| Ig | Immunoglobulin |
| IL | Interleukin |
| LD | Linkage disequilibrium |
| LTT | Lymphocyte transformation test |
| MHC | Major histocompatibility complex |
| MPE | Maculopapular exanthema |
| MS | Mass spectrometry |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PHA | Phytohemagglutinin |
| PLC | Peptide loading complex |
| pMHC | Peptide-MHC complex |

| | |
|---|---|
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| RPMI | Roswell Park Memorial Institute |
| RT | Room temperature |
| SEM | Standard error of the mean |
| SFC | Spot forming cell |
| SI | Stimulation index |
| SJS | Stevens-Johnson syndrome |
| SMX | Sulfamethoxazole |
| SMX-NO | Nitroso Sulfamethoxazole |
| SN | Supernatant |
| SNP | Single nucleotide polymorphism |
| TAP | Transporter associated with antigen processing |
| TCC | T-cell clone |
| TCR | T-cell receptor |
| TEN | Toxic epidermal necrolysis |
| TGF | Transforming growth factor |
| TNF-α | Tumour necrosis factor alpha |
| TT | Tetanus toxoid |
| UK | United Kingdom |
| WHO | World Health Organisation |

# Publications

## Published papers

**Lichtenfels M**., Farrell J., Ogese M. O., Bell C. C., Eckle S., McCluskey J., Park B. K., Alfirevic A., Naisbitt D. J., Pirmohamed M.: HLA Restriction of Carbamazepine-Specific T-Cell Clones from an HLA-A*31:01-Positive Hypersensitive Patient. *Chemical Research in Toxicology* **2014**, *27*, 175- 177.

Farrell J., **Lichtenfels M**., Sullivan A., Elliott E. C., Alfirevic A., Stachulski A. V., Pirmohamed M., Naisbitt D. J., Park B. K.: Activation of carbamazepine-responsive T-cell clones with metabolically inert halogenated derivatives. *J Allergy Clin Immunol* **2013**, *132*, 493- 495.

Alfirevic A., Gonzalez-Galarza F., Bell C., Martinsson K., Platt V., Bretland G., Evely J., **Lichtenfels M**., Cederbrant K., French N., Naisbitt D., Park B. K., Jones A. R., Pirmohamed M.: In silico analysis of HLA associations with drug-induced liver injury: use of a HLA-genotyped cell archive from healthy volunteers. *Genome Med* **2012**, *4*, 51.

## Abstracts

**M. Lichtenfels**, A. Alfirevic, B. K. Park, D. J. Naisbitt, M. Pirmohamed: Investigating the functional role of the HLA-A*31:01 allele in Carbamazepine-induced hypersensitivity reactions. Poster (no. 179) presented at the Drug Hypersensitivity Meeting 5, 11- 14 April 2012, Munich, Germany.

**M. Lichtenfels**, A. Alfirevic, D. J. Naisbitt, B. K. Park, M. Pirmohamed: *In-vitro* reactivity of drug-specific T-cells from a HLA-A*31:01 positive carbamazepine hypersensitive patient. Poster (no. 126) presented at the 11th European Association for Clinical Pharmacology and Therapeutics congress, 28- 31 August 2013, Geneva, Switzerland.

# Abstract

T-cell mediated hypersensitivity reactions (HSRs) to carbamazepine (CBZ), a commonly used anti-epileptic drug, occur only in a small proportion of patients, but can often be severe in nature. As the underlying pathomechanisms are not fully understood, it has proven difficult to predict who may be at risk of developing CBZ-induced HSRs. Recently, specific human leukocyte antigen (HLA) alleles have been identified as susceptibility factors for CBZ hypersensitivity in diverse populations, indicating that HLA molecules may be functionally involved in CBZ-induced T-cell activation. *HLA-A*31:01* represents the latest example and has been implicated in CBZ-induced HSRs in Caucasian patients. Thus, the aim of this work was to explore the molecular interactions of CBZ with HLA-A*31:01 and drug-specific T-cells.

The HLA restriction pattern of CBZ-reactive T-cells from a patient expressing HLA-A*31:01 was investigated. It was shown that $CD8^+$ T-cells were activated in a HLA-A*31:01 dependent way. Further, HLA-DRB1*04:04 was found to be responsible for the stimulation of $CD4^+$ T-cells, suggesting a common HLA haplotype may be involved in mediating T-cell responses to CBZ in Europeans.

Next, *in vitro* priming of drug-naïve T-cells from *HLA-A*31:01*+ healthy volunteers against CBZ was attempted. Weak responses to CBZ could be detected in some but not all volunteers, indicating factors additional to *HLA-A*31:01* are required to induce a primary stimulation of T-cells to CBZ. Besides, the removal of T-regulatory cells and use of dendritic cells as antigen-presenting cells seemed to generally improve priming conditions.

In order to investigate whether CBZ affected the peptide-binding specificity of HLA-A*31:01, *in silico* and *in vitro* analysis were performed. In silico modelling provided a possible binding site for CBZ within the HLA peptide-binding cleft. A peptide elution study provided a preliminary indication that binding of CBZ to HLA-A*31:01 may alter the peptide repertoire presented by the allele, which could potentially result in T-cell activation.

Most recently, it has been suggested that the T-cell receptor (TCR) may represent an additional predisposing factor for CBZ-induced HSRs. Accordingly, a protocol for the analysis of the TCR Vβ repertoire of drug-reactive T-cells by flow cytometry as well as CDR3 spectratyping was set up using T-cells from healthy donors primed against the model antigen SMX-NO. Both methods showed that antigen stimulation resulted in skewing of common TCR Vβ subtypes among the donors. Combined, the optimised methods will allow assessment of whether specific TCR clonotypes may be implicated in *HLA-A*31:01* associated HSRs to CBZ.

In summary, the data presented in this thesis provide initial evidence that CBZ is able to interact directly, through a non-covalent binding mechanism, with HLA-A*31:01 causing T-cell activation. However, it cannot be excluded that the stimulation of T-cells *in vivo* requires the formation of a hapten complex. Further work is needed to define other factors that are involved in predisposing an individual to CBZ hypersensitivity.

# Chapter I

## General Introduction

## Contents

## 1.1 Introduction

Hypersensitivity reactions (HSRs) to drugs occur only in a few susceptible patients but can often be severe in nature. Specific human leukocyte antigen (HLA) alleles have recently been identified as risk factors in some forms of drug-induced hypersensitivity. It is known that drug-specific T-cells are involved in many of these reactions. However, the contribution of HLA to the development of drug-specific T-cell responses has not been fully understood.

## 1.2 Adverse Drug Reactions

### 1.2.1 Definitions

Besides producing a therapeutic effect, any drug that is administered to a patient can cause adverse reactions. The World Health Organisation (WHO) has defined an adverse drug reaction (ADR) as "a response to a drug which is noxious and unintended, and which occurs at doses normally used in man for the prophylaxis, diagnosis, or therapy of disease, or for the modification of physiological function" (WHO 1972). Another commonly used definition for ADRs was proposed by Edwards and Aronson: "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, I. R. and Aronson 2000). This clearly separates ADRs from adverse events, which comprehend any harmful event that happens to a patient during drug therapy but does not have to be caused by the drug itself.

Adverse drug reactions are a major burden to health care systems and considerably increase patients' morbidity and mortality. They also present a significant problem for drug development in the pharmaceutical industry, as

potentially effective drugs might have to be withdrawn from the market due to unforeseen ADRs arising during the post-marketing period (Lasser *et al.* 2002).

### 1.2.2 Incidence

A study by Pirmohamed *et al.* (2004) investigated the percentage of hospital admissions attributed to ADRs in two UK hospitals and found this to be as high as 6.5%. ADRs led to death in 0.15% of all patients admitted to hospital during the monitoring period and the costs of ADR treatment were estimated to amount to approximately £466m per year for the UK National Health Service (NHS). ADRs leading to hospital admission were seen more often in elderly patients and were more prevalent in women (Pirmohamed *et al.* 2004). This is in line with a meta-analysis performed by Lazarou and colleagues, which estimated the incidence of ADRs in the United States was 6.7% of all patients hospitalized and a rate of fatal ADRs of up to 0.32% of all patients admitted to hospital (Lazarou *et al.* 1998). A more recent study by Miguel *et al*. (2012) stated ADRs could occur in up to 16% of hospitalized patients, but significant heterogeneity between the studies analysed hampered the authors in drawing firm conclusions. The true proportion of ADRs within a population will most likely remain unknown until the definition of ADRs has been standardised as well as the methods used for ADR monitoring (Miguel *et al.* 2012). Determining the incidence of ADRs in outpatients is even more difficult as there are only limited reporting systems in place and most ADRs therefore remain undetected.

### 1.2.3 Classification

ADRs can be classified according to their severity, time to reaction, or localisation. But most frequently ADR are classified by their pharmacological characteristics.

Initially, ADRs were grouped into either type A or type B reactions based on the classification proposed by Rawlins and Thompson (Rawlins 1981).

Type A reactions, also known as augmented reactions, can be predicted from the known pharmacological action of the drug. They often represent an exaggerated effect of the drug and are generally dose-dependent. The majority of ADRs are type A reactions.

Type B reactions, referred to as bizarre or idiosyncratic reactions, are not related to the known pharmacology of the drug and are usually not directly dose-dependent. Type B reactions are rare, but often more severe in nature than type A reactions. Reactions of this type occur only in susceptible patients and the predisposing risk factors are mostly still unknown.

In order to allow a more distinguished classification of the different types of ADRs, more types were introduced accordingly (Edwards, I. R. and Aronson 2000). An overview of the types of ADR proposed in the literature is given in Table 1.1.

A more comprehensive system to classify ADRs has been introduced by Aronson and Ferner (2003) which takes into consideration the dose relatedness, timing and patient susceptibility (DoTS) of an adverse reaction. It has been proposed that the three-dimensional DoTS classification could improve the monitoring and treatment of ADRs and assist drug development and regulation (Aronson and Ferner 2003).

The focus of this thesis will be on type B reactions, particularly hypersensitivity reactions, which are covered later in the chapter. Initially, I will give an overview of the immune system and its principal cellular and molecular components.

**Table 1.1**: Classification of adverse drug reactions (adapted from (Park *et al.* 1998; Edwards, I. R. and Aronson 2000).

| Type of reaction | Mechanism | Example |
|---|---|---|
| **A (augmented)** | Predictable from the known pharmacology of the drug; dose-dependent effect | Haemorrhage with anticoagulants |
| **B (bizarre or idiosyncratic)** | Not predictable from pharmacological action of the drug; no direct dose relationship | Penicillin hypersensitivity |
| **C (chemical or chronic)** | Related to the chemical structure or a metabolite of the drug | Paracetamol hepatotoxicity |
| **D (delayed)** | Develop years after drug therapy | Secondary tumour after chemotherapy |
| **E (end of treatment)** | Appear after drug withdrawal, especially when stopped abruptly | Withdrawal syndrome after opiate therapy |
| **F (failure)** | Often caused by drug interactions | Reduced level of oral contraceptive during antibiotic therapy |

## 1.3 The Immune System

The immune system developed alongside human evolution to protect the human body against disease. The mechanisms involve elimination of existing pathogens and protection against new infections. One important aspect in this process is the differentiation between “foreign” and “self” molecules.

The immune system can be divided into two main components: the innate and the adaptive immune systems.

### 1.3.1 Innate immunity

The innate immune system is thought to be the initial form of protection against pathogenic structures in humans, and it also exists in a lower form in all animals and some plants (Murphy *et al.* 2008). Its components are functional straight after birth and always present within the body to clear away infectious pathogens. Activation of innate responses occurs rapidly, as the recognition of pathogenic molecules is non-specific, and no immunological memory is formed. The main purpose of innate immunity is the prevention of an infection or to reduce its spreading.

Epithelial membranes are considered a first barrier displaying a physical and chemical impediment to entering pathogens. If infectious agents circumvent these barriers, phagocytic cells, i.e. macrophages, monocytes and neutrophils, come into effect. These cells can recognise pathogen-specific molecules not expressed on human cells, e.g. mannose; and this results in the uptake and subsequent digestion of pathogens. The most important phagocytic cells are dendritic cells (DCs), which constantly ingest extracellular antigens. When immature DCs encounter pathogens they are activated and turn into so called antigen-presenting cells (APCs)(Delves and Roitt 2000). APCs are important for the initiation of the adaptive immune response, and DCs therefore form a crucial link between the innate and adaptive immunity.

Other cellular components of innate immunity include cells releasing inflammatory mediators, such as mast cells, basophils and eosinophils, and Natural Killer (NK) cells (Delves and Roitt 2000). NK cells recognise abnormal cells, such as those infected by viruses or malignant cells, and kill these through induction of apoptosis.

Protein components of the innate immune response include cytokines and acute-phase proteins. These cause local inflammation at the site of infection which attracts phagocytic cells and also recruits cells of the adaptive immune system (Murphy *et al.* 2008). The complement system, a group of soluble plasma proteins, works as an additional pathway in the early and unspecific removal of infections. Complement can envelop infectious agents and these complexes are recognised by phagocytes and eliminated (Murphy *et al.* 2008).

### 1.3.2 Adaptive immunity

The adaptive immune system recognises pathogens in an antigen specific manner through the use of specialised receptors (Murphy *et al.* 2008). Key components of adaptive immunity are B- and T-lymphocytes, which carry specific receptors on their surface each recognising different antigenic structures. Binding of antigens to these receptors results in lymphocyte activation.

B-cells encounter native antigens via their B-cell receptors, which are membrane-bound antibody molecules. Following antigen recognition, B-cells differentiate into plasma cells and start to secrete soluble antibodies. The secreted antibodies are specific for the encountered antigen and antibody-labelled antigens are taken up by phagocytic cells and destroyed. This is known as the humoral immune response.

T-cells are unable to recognise antigens directly but require the help of APCs. APCs can cleave antigens through proteolysis into short peptide fragments, also known as epitopes, and present these on human leukocyte antigen (HLA) molecules on their surface. T-cells recognise antigenic epitopes through T-cell

receptors (TCRs) expressed on their surface. After an interaction between APC, epitope and TCR takes place, T-cells differentiate into effector T-lymphocytes. This pathway is called the cell-mediated immune response.

An important feature of the adaptive immune response is the generation of an immunological memory population. After an infection has been successfully eliminated, most activated B- and T-cells die. However, a few B- and T-cells with high affinity to their specific antigens are transformed into memory cells. Upon re-exposure to a pathogen, these cells can be rapidly activated allowing a faster and more effective immune response (Murphy *et al.* 2008).

### 1.3.3 Antigen processing and presentation

The main role of APCs is the uptake and processing of antigens at the site of infection followed by the migration of the cells to draining lymph nodes, where they display antigenic peptides to T-cells. The most specialised APCs are DCs, but to a lesser extent macrophages and B-cells can also function as APCs.

Antigenic peptides generated within APCs generally originate from two different sources, extracellular or intracellular proteins. The process of antigen degradation into peptide fragments is called *antigen processing* and is depicted in Figure 1.1.

Antigenic proteins formed in the cell cytosol get digested by the proteasome. The resulting peptide fractions are transferred into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). In the ER, peptides get loaded onto newly synthesized HLA class I molecules by the peptide loading complex (PLC). The stable complexes of peptide and HLA I molecules are released from the ER and travel through the Golgi apparatus to the cell surface (Blum *et al.* 2013).

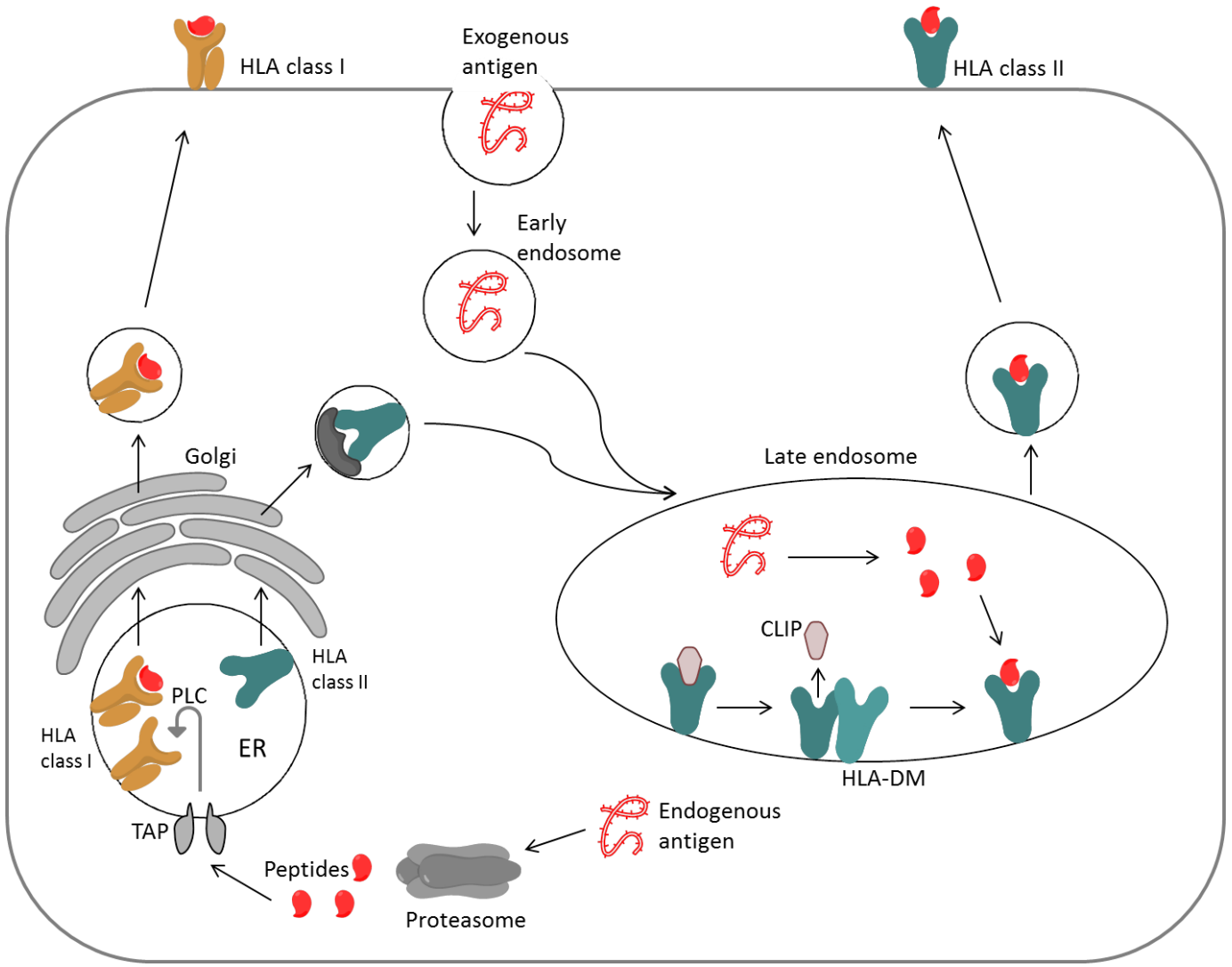



**Figure 1.1**: Antigen processing pathways(adapted from (Blum *et al.* 2013)):

Cytosolic antigens are degraded by the proteasome. The peptides are transported into the ER by TAP where they bind to HLA class I molecules. Extracellular antigens are internalised by endocytosis. In late endosomes, antigens are lysed into peptides and loaded onto HLA class II molecules. The resulting peptide-HLA complexes are transported to the cell surface for presentation to T-lymphocytes.

(CLIP= Class II-associated invariant chain peptide; ER= Endoplasmic reticulum; PLC= Peptide-loading complex; TAP= Transporter associated with antigen processing)

Extracellular antigens are taken up by phagocytic cells and captured in endosomes. Over time the endosomes become increasingly acidic which induces proteases to lyse the contained proteins into peptide fragments. These late endosomes fuse with vesicles containing HLA class II molecules, and the peptides are loaded onto HLA II proteins (Blum *et al.* 2013). The resulting peptide-HLA complexes are then transported to the cell surface. HLA class II molecules are formed in the ER and combined with an invariant chain. The invariant chain protects the HLA binding grove and guides the HLA class II molecules to endosomes. The proteolytic conditions in the endosomes degrade the invariant chain until only the short peptide CLIP, class II-associated invariant chain peptide, is left. CLIP is removed by HLA-DM, which enables binding of antigenic peptides to HLA class II molecules (Blum *et al.* 2013).

The display of peptide-HLA complexes on the surface of APCs is known as antigen presentation, and they are recognised by the corresponding TCRs on T-cells.

T-lymphocytes can be grouped into two subtypes according to their cluster of differentiation (CD) markers, CD4 and CD8. These cell-surface proteins are able to specifically recognise HLA molecules and assist in stabilising peptide-HLA complexes; they are therefore also termed co-receptors (Murphy *et al.* 2008). T-cells expressing the CD4 co-receptor will only interact with HLA class II complexes. $CD8^+$ T-cells will only recognize peptides presented on HLA class I molecules (Murphy *et al.* 2008). Interactions occurring between APCs and T-cells during antigen presentation and recognition are illustrated in Figure 1.2.

When a TCR engages an antigenic peptide, it forms contact with both the HLA molecule and the peptide. However, for T-lymphocytes to become fully activated additional co-stimulatory signals are needed. This includes the interaction between the co-receptors CD28 on T-cells, CD80/CD86 on APCs and the secretion of co-stimulatory cytokines (Murphy *et al.* 2008). Once T-cells are successfully activated they turn into effector T-cells.

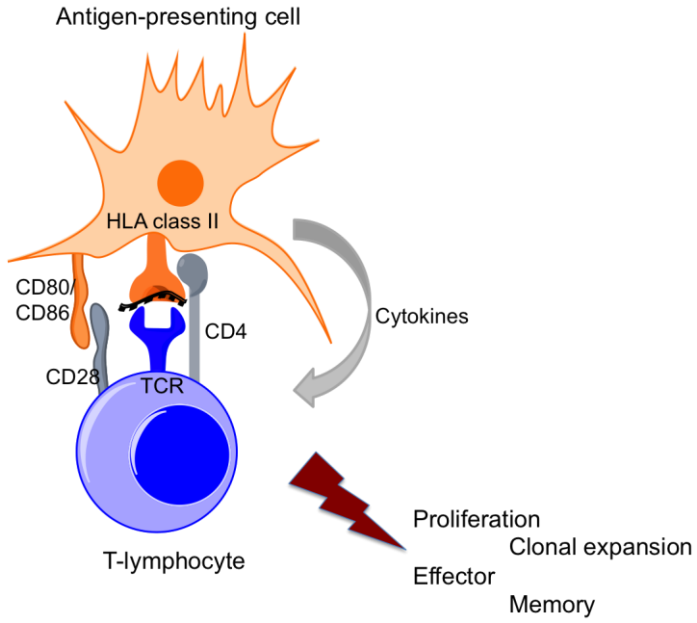



**Figure 1.2**: Interactions between APC and T-cell during antigen recognition (adapted from Janeway's Immunobiology (Murphy *et al.* 2008)).

There are three main types of effector T-lymphocytes: cytotoxic T-cells, helper T-cells and regulatory T-cells (Murphy *et al.* 2008). Cytotoxic T-cells are able to directly kill infected cells through the secretion of cytolytic molecules, which induce apoptosis in the target cells. Helper T-cells (Th cells) aid the activation of other immune cells through the release of cytokines. Depending on the cytokines secreted, they can be further divided into Th1, Th2 and Th17 cells. Th1 cells release mainly interferon (IFN) γ and tumour necrosis factor (TNF) α which promote macrophage activity. Th2 cells enhance the activation of B-cells by release of interleukin (IL) 4, IL-5 and IL-13. Th17 cells assist in neutrophil recruitment through secretion of IL-17 and IL-6. Regulatory T-cells help control immune responses through the release of inhibitory cytokines, such as IL-10 and transforming growth factor (TGF) β, which suppress T-cell activation and thereby limit immune responses. The effector T-cell subtypes and their phenotypic characteristics are summarised in Table 1.2.

**Table 1.2**: Effector T-cell subsets and their secreted effector molecules (taken from Janeway's Immunobiology (Murphy *et al.* 2008)).

| Cell type | **CD8 Cytotoxic T-cells** | **CD4 Th1 cells** | **CD4 Th2 cells** | **CD4 Th17 cells** | **CD4 Regulatory T-cells** |
|---|---|---|---|---|---|
| Effector molecules released | Perforin<br>Granzyme B<br>Granulysin<br>FasLigand<br><br>IFN-γ | IFN-γ<br>TNF-α<br><br>FasLigand | IL-4<br>IL-5<br>IL-13 | IL-17A<br>IL-17F<br>IL-6 | IL-10<br>TGF-β |
| Effector function | Cytotoxicity | Activation of macrophages | Activation of mucosal immunity | Recruitment of neutrophils | Immuno-suppression |

## 1.4 Major Histocompatibility Complex

The major histocompatibility complex (MHC) was first discovered in mice by Peter Gorer (Gorer 1936); he identified antigenic molecules in the blood of the animals to be responsible for the rejection of transplanted tumours. In the 1950s, Jean Dausset was able to prove that a similar antigenic system is expressed on the cell surface of human leukocytes (Dausset 1958). The gene products of the human MHC are therefore also referred to as human leukocyte antigens. Initially, HLA molecules were studied because of their clinical relevance in transplant medicine (Chinen and Buckley 2010). But their main biological role in the human immune system is the presentation of pathogen-derived peptide antigens to T-lymphocytes.

In the last decades extensive research of the MHC determined this genetic region to be the most polymorphic in the human genome; and its genetic variation has been shown to contribute to various diseases, especially autoimmune disorders (Trowsdale 2011).

### 1.4.1 Genetic organisation

In humans, the MHC is located on the short arm of chromosome 6 (6p21). Through the joint efforts of the MHC sequencing consortium, it was one of the first genomic regions to be fully sequenced. It spans a region of 3.6 megabase pairs (Mb) containing 224 gene loci of which about 60% are thought to be expressed and about 40% proposed to be implicated in immunity (The MHC sequencing consortium 1999).

The MHC can be divided into three major regions: MHC class I at the telomeric end, MHC class III, and MHC class II at the centromere. MHC class I genes encode the heavy chain of the glycoproteins HLA-A, -B and –C, also known as classical HLA class I molecules. These molecules present peptide antigens to $CD8^+$ T-cells. The non-classical HLA molecules (HLA-E, -F and –G) and the MHC class I chain like (MIC) proteins complete this gene cluster. The MHC class II cluster contains the genes coding for the α- and β-chain of the classical HLA-DR, -DQ and -DP heterodimer proteins, which present antigens to $CD4^+$ T-cells. Also encoded in this region are the non-classical HLA class II molecules, HLA-DM and -DO, which assist in antigen loading of HLA class II molecules. The genes encoding TAP and tapasin, proteins important for loading of HLA class I molecules, are also located in this cluster. Genes of the MHC class III region cover proteins of the complement system, heat shock proteins (HSP), and many cytokines (e.g. TNF-α). A schematic gene map of the three MHC gene clusters is shown in Figure 1.3.

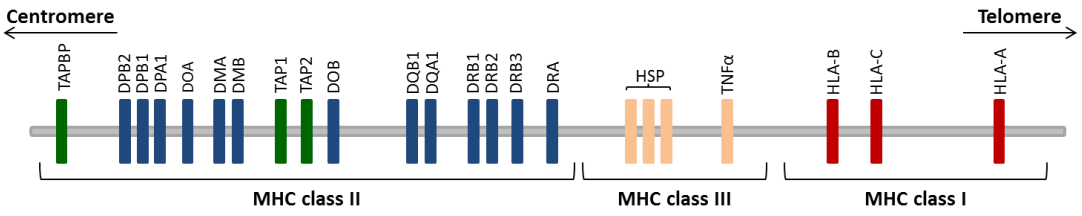



**Figure 1.3**: Gene organisation of the classical major histocompatibility complex (adapted from (Klein and Sato 2000)).

Further research in the field and advances in sequencing techniques have shown that genes related to the MHC extend beyond the region originally defined as MHC and the term extended MHC was coined, which covers about 7.6 Mb and comprises around 421 gene loci (Horton *et al.* 2004).

### 1.4.2 Polymorphisms

HLA genes are highly polymorphic with the HLA-B locus being the most polymorphic gene in the human genome (Vandiedonck and Knight 2009). It is believed that HLA polymorphisms evolved as a result of a constant evolutionary pressure to fight infections and protect the human body against diverse pathogens. Single nucleotide polymorphisms (SNPs) are the most common form of genetic variation found in HLA genes (Vandiedonck and Knight 2009). For HLA class I alleles, the majority of SNPs are located in exon two and three of the genes, forming the antigen-binding groove. Similarly, most SNPs in HLA class II alleles are found in the second exon. The only exception is the HLA-DRA locus, which shows no functional polymorphisms in the peptide-binding region. Many SNPs in the MHC region are non-synonymous leading to changes in the amino acid sequence of the peptide-binding groove. This in turn increases the likelihood of pathogenic antigens being recognised and eliminated (Vandiedonck and Knight 2009). Until today, more than 9,000 HLA alleles have been discovered and the numbers are growing continually. A complete list can be found on the IMGT/HLA database (www.ebi.ac.uk/ipd/imgt/hla) (Robinson *et al.* 2013).

A high degree of linkage disequilibrium (LD) is seen across the entire MHC region and HLA alleles are generally inherited in haplotype blocks. The frequency of HLA haplotypes are highly variable between different ethnic populations; and this has partly been attributed to differences in pathogen exposure in different geographic environments (Choo 2007).

### 1.4.3 Nomenclature

The numerous HLA polymorphisms and resulting high number of HLA molecules called for a systematic nomenclature.

Initially, HLA typing was only possible at the serological level using alloantibodies directed against HLA expressed on the cell surface (McCluskey *et al.* 2003). The HLA alleles defined through this process were numbered, but in a non-systematic way. When molecular DNA-based typing methods became available, the number of detectable HLA alleles grew exponentially. At the same time, different genotyping methods used different nomenclature systems for the HLA alleles detected. A standardised nomenclature was established through the work of the International Histocompatibility Working Group (IHW) (Gourraud *et al.* 2007), and this system is used today. The nomenclature is maintained and regularly updated by the WHO HLA Nomenclature Committee for Factors of the HLA system (Marsh *et al.* 2010).

The naming of individual HLA alleles follows a general structure: the name of the gene locus, e.g. HLA-A, is succeeded by an asterisk, and a number identifying the gene variant. An example is given in Figure 1.4.

The numbering system used to determine each HLA variant consists of up to four fields, and each field is separated by a colon. The first two digits identify the type, which in most cases corresponds to the serological phenotype. The next numeric field signifies the subtype representing the DNA genotype. These numbers are assigned in sequential order to allow newly discovered alleles to be integrated at any time. The third and fourth set of numbers can be included to describe synonymous mutations and mutations in non-coding regions respectively. Optional letters may be added at the end to indicate the expression status of the allele:

- **N** =“null” allele (not expressed at the cell surface)
- **L** =allele is expressed at low levels
- **S** = “soluble” allele (protein is secreted)
- **C** = allele is only expressed in the cytosol
- **A** = “aberrant” allele (doubts to whether the allele is expressed)

- **Q** =expression of the allele is "questionable" (mutation known to affect expression levels)

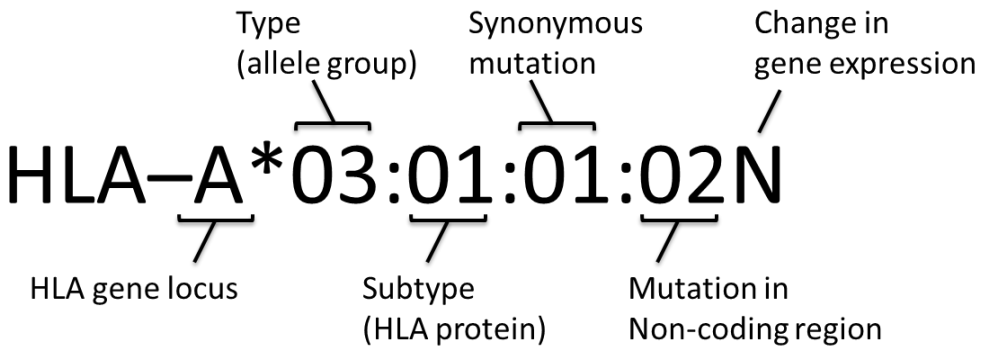



**Figure 1.4**: Nomenclature of HLA alleles.

Regular meetings are held to collect information on confirmed new HLA alleles, and any errors or corrections needed concerning already published sequences. The reports are published on a monthly basis in the journals Tissue Antigens, Human Immunology and the International Journal of Immunogenetics.

### 1.4.4 Structure and function

Although HLA class I and class II alleles play a similar role in the immune system, their structures are quite different.

HLA class I proteins consist of a heavy α chain encoded by the HLA class I genes and a non-covalently bound $\beta_2$-microglobulin ($\beta_2$m) chain (see Figure 1.5). Human $\beta_2$m is encoded by a gene located on chromosome 15 and its protein product is invariant. The glycosylated α chain is built up of five domains: the peptide-binding region (α1 and α2), an immunoglobulin-like constant domain (α3), a transmembrane region and a cytoplasmic tail (Klein and Sato 2000). The α1 and α2 domains create a distinct structure forming the peptide-binding groove. It comprises a platform of eight antiparallel β strands and two walls of antiparallel α-helices. The two α-helices assemble closely, which allows only

short peptide fragments between seven to fifteen amino acids to be bound (Klein and Sato 2000). The α3 domain assembles with $\beta_2$m to form a structure resembling the constant region of immunoglobulins.

HLA class I molecules are expressed on almost all nucleated cells and antigens presented in the peptide-binding groove are recognised by the TCR of $CD8^+$ T-lymphocytes.

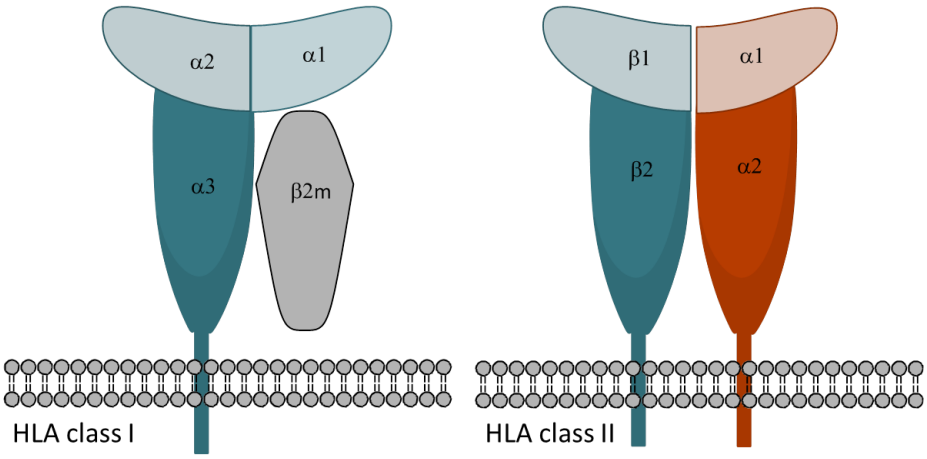



**Figure 1.5**: Schematic structure of HLA class I and class II molecules (adapted from (Klein and Sato 2000)).

The HLA class II genes encode the α- and β-chain of HLA class II proteins. The two polypeptide chains form heterodimers through non-covalent binding and consist of four domains each (Klein and Sato 2000). The α1 and β1 domain form the peptide-binding region. The α2 domain together with the β2 domain makes up the immunoglobulin-like region. Both chains are anchored in the cell membrane through a short transmembrane domain and a cytoplasmic tail. A schematic structure is depicted in Figure 1.5. Due to the heterodimer structure, the peptide-binding groove of HLA class II molecules is open, and longer peptides of up to twenty-five amino acid length can be presented (Rammensee, Hans-Georg 1995).

In contrast to HLA class I, HLA class II molecules are normally only expressed on antigen-presenting cells. However, when T-cells become activated, this can induce the expression of class II molecules on their cell surface. The secretion of IFN-γ can also lead to expression of HLA class II on other cells (Murphy *et al.* 2008). Peptides presented by HLA class II molecules are usually recognised by the TCR of $CD4^+$ T-cells.

### 1.4.5 HLA associated diseases

Due to their key function in initiating T-cell mediated immune responses, many HLA alleles have been linked to autoimmunity, infectious diseases and cancer (Trowsdale 2011). In most cases it is not yet known how the HLA protein contributes to the development of the disease. It is generally difficult to prove whether the reported genes are causal variants or merely an effect of extensive LD across the MHC (Trowsdale 2011). This is further complicated by the fact that most diseases are multifactorial, involving both environmental and genetic factors. A summary of diseases associated with specific HLA alleles or haplotypes are presented in Table 1.3.

Furthermore, some forms of drug-induced hypersensitivity reactions have been linked to specific HLA class I and class II alleles. These will be discussed separately in sections 1.6.4 and 1.6.5 of this chapter.

**Table 1.3**: Examples of HLA-associated diseases reported in the literature.

| Disease | HLA association(s) | Reference |
|---|---|---|
| **Infectious diseases** | | |
| **HIV** | B*27, B*57→ improved HIV control; B*35→ lack of HIV control | (Kaslow *et al.* 1996); (Carrington *et al.* 1999) |
| **Malaria** | B*35, DRB1*13:02-DQB1*05:01 → reduced malaria risk | (Hill *et al.* 1991) |
| **Hepatitis C** | C*04→ virus persistence; DRB1*11:01-DQB1*03:01 → spontaneous virus clearance | (Thio *et al.* 2002); (Hong *et al.* 2005) |
| **Cancer** | | |
| **Cervical carcinoma** | DRB1*15:01-DQB1*06:02 (risk alleles in Hispanics); B*07 (risk allele in Chinese) | (Apple *et al.* 1994); (Qiu *et al.* 2011) |
| **Hodgkin's lymphoma** | HLA-DPB1*03:01 (risk allele in Caucasians) | (Oza *et al.* 1994) |
| **Autoimmune diseases** | | |
| **Ankylosing spondylitis** | B27 | (Brewerton *et al.* 1973) |
| **Celiac disease** | DQ2 (DQA1*05-DQB1*02) and DQ8 (DQA*03-DQB1*03:02) haplotypes | (Megiorni and Pizzuti 2012) |
| **Multiple sclerosis** | DRB5*01:01-DRB1*15:01-DQA1*01:02-DQB1*06:02 haplotype | (Fogdell *et al.* 1995) |
| **Narcolepsy** | DRB1*15:01-DQA1*01:02- DQB1*06:02 haplotype | (Mignot *et al.* 2001) |
| **Rheumatoid arthritis** | DRB1*04 and DRB1*01 subtypes | (Wordsworth *et al.* 1989) |
| **Systemic lupus erythematosus** | DRB1*15:01-DQB1*06:02 haplotype, DRB1*03:01-DQB1*02:01 haplotype | (Graham *et al.* 2007) |
| **Type 1 diabetes** | DRB1*03:01-DQA1*05:01-**DQB1*02:01** ("DR3" haplotype), DRB1*04-DQA1*03:01-**DQB1*03:02** ("DR4" haplotype) → strongly predisposing; B*3906→ risk allele, DRB1*15:01-DQA1*01:02-**DQB1*06:02** ("DR2" haplotype) → highly protective | (Erlich *et al.* 2008); (Noble *et al.* 2010) |

## 1.5 T-cell Receptor

The T-cell receptor (TCR) is a highly variable polypeptide on the cell surface of T-lymphocytes which specifically recognises antigenic peptides in complex with an MHC molecule. As each TCR is restricted to recognise only a few structurally similar antigens, a broad TCR repertoire is needed to guarantee recognition of any invading pathogen. After binding of the TCR to a specific peptide-MHC (pMHC) complex, intracellular signalling cascades are induced which lead to T-cell proliferation and activation of their effector functions.

### 1.5.1 Structure and function

The TCR protein is composed of two polypeptide chains, either α and β, or γ and δ, which form disulphide-linked heterodimers. The vast majority of T-cells express α:β heterodimers, whereas only a minority are γ:δ T-cells. Only little is known so far about the functions and antigen-binding mode of γ:δ TCRs. It has been suggested that γ:δ TCRs specialise on infections caused by parasites and mycobacteria (Rudolph *et al.* 2006). They are able to especially recognise small non-peptide antigens that contain phosphate (Rudolph *et al.* 2006). Although structurally similar to α:β TCRs, it appears that γ:δ TCRs are not restricted by classical MHC class I or class II molecules. For reasons of simplification this chapter will focus on α:β TCRs.

The general molecular structure of TCRs resembles the antigen-binding fragment of immunoglobulins and is illustrated in Figure 1.6. Each TCR chain consists of four domains: an N-terminal variable region, a constant region followed by a short cysteine-containing stalk segment which forms the interchain bond, a hydrophobic transmembrane region and a cytoplasmic tail (Rudolph *et al.* 2006). The variable (V) region forms three loops which are known as the complementarity determining regions (CDRs). The CDR regions of the α- and β-chain together form the TCR binding site and are responsible for antigen recognition. The CDR1 and CDR2 regions each contact the side chains of the MHC binding groove, while the CDR3 loops interact with the peptide ligand

and therefore determine the TCR specificity (Garcia and Adams 2005). The constant (C) region associates with the CD3 co-receptors, and this complex transmits any signal generated at the antigen-recognition site into the cell, leading to the activation of downstream signalling pathways. The crystal structures solved to date demonstrate that the docking mode of TCRs with a pMHC complex is largely preserved. The TCR heterodimer binds diagonally to the long side of the pMHC complex (Morris and Allen 2012). The Vα domain is hereby located above the N-terminal peptide half and the Vβ domain comes to lie above the C-terminus of the peptide (Gras *et al.* 2008).

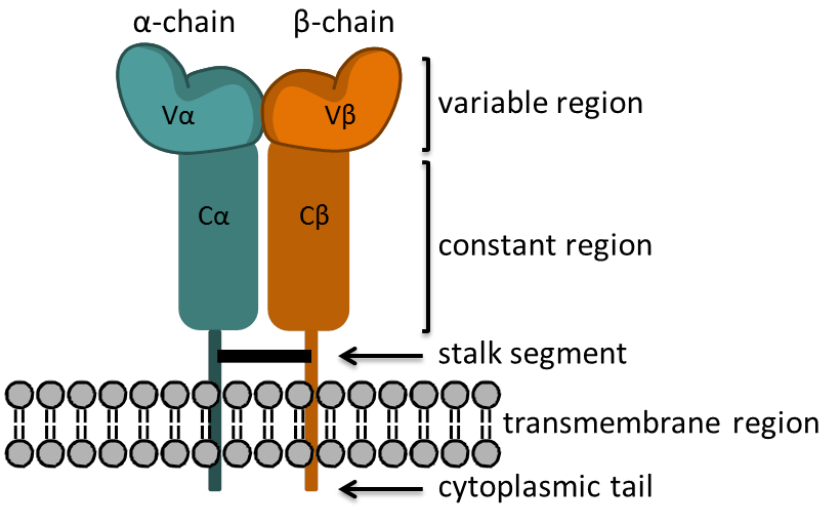



**Figure 1.6**: Schematic structure of the T-cell receptor (adapted from Janeway's Immunobiology (Murphy *et al.* 2008)).

### 1.5.2 Generation of the TCR repertoire

The variety of antigens that can be recognised by the TCR repertoire is determined by the V region, especially the CDR3 loops. As it would not be possible to encode all receptor chains present in an individual at full length in the genome, a complex genetic mechanism for generating the TCR repertoire evolved. This mechanism is termed gene rearrangement.

The TCR genes are divided into a multitude of interchangeable gene segments and spread over a large DNA section. The human TCR α genes are located on chromosome 14 and comprise 43 variable (Vα), 58 junctional (Jα) gene segments and a single Cα gene. The human TCR β genes are located on chromosome 7 and consist of 42 variable (Vβ), 2 diversity (Dβ) and 12 junctional (Jβ) gene segments, as well as two Cβ genes (Gras *et al.* 2008). The V, D and J gene segments are randomly rearranged by events of somatic recombination to form a complete TCR-V region. The TCR-V region is then spliced together with a particular C region and together they shape a TCR α- or β-chain (Murphy *et al.* 2008). The two chains are then paired to form an α:β heterodimer expressed on the cell surface. The genetic processes involved in the formation of T-cell receptors are illustrated in Figure 1.7.

The CDR3 loops, as the site of antigen recognition, are shaped by the D and J segments of the α- and β-chains. The variation in the CDR3 region is formed through juxtaposition of the different V, D and J segments after somatic recombination (Turner *et al.* 2006). And the TCR repertoire is further diversified through the addition of non-template encoded nucleotides at the junctions of V, D and J segments.

The range of TCRs generated through the above described mechanism represents the TCR repertoire of immature T-cells. Selection processes in the thymus guarantee that only T-cells expressing TCRs which are able to interact with self-MHC molecules but are not autoreactive undergo maturation (Delves and Roitt 2000). During positive selection, early T-lymphocytes, expressing both co-receptors CD4 and CD8, get to screen self-peptides presented on self-MHC molecules. Those T-cells able to engage a self-pMHC complex are rescued from programmed cell death and mature into single-positive $CD4^+$ or $CD8^+$ T-cells. To avoid autoimmune reactions, T-cells interacting too strongly with self-peptides get deleted through induction of apoptosis. This process is known as negative selection. Through this process the TCR repertoire is shaped to encompass receptors that recognise foreign antigens in a self-MHC restricted manner. At the same time, T-cells that could induce autoimmune reactions are removed (Turner *et al.* 2006).

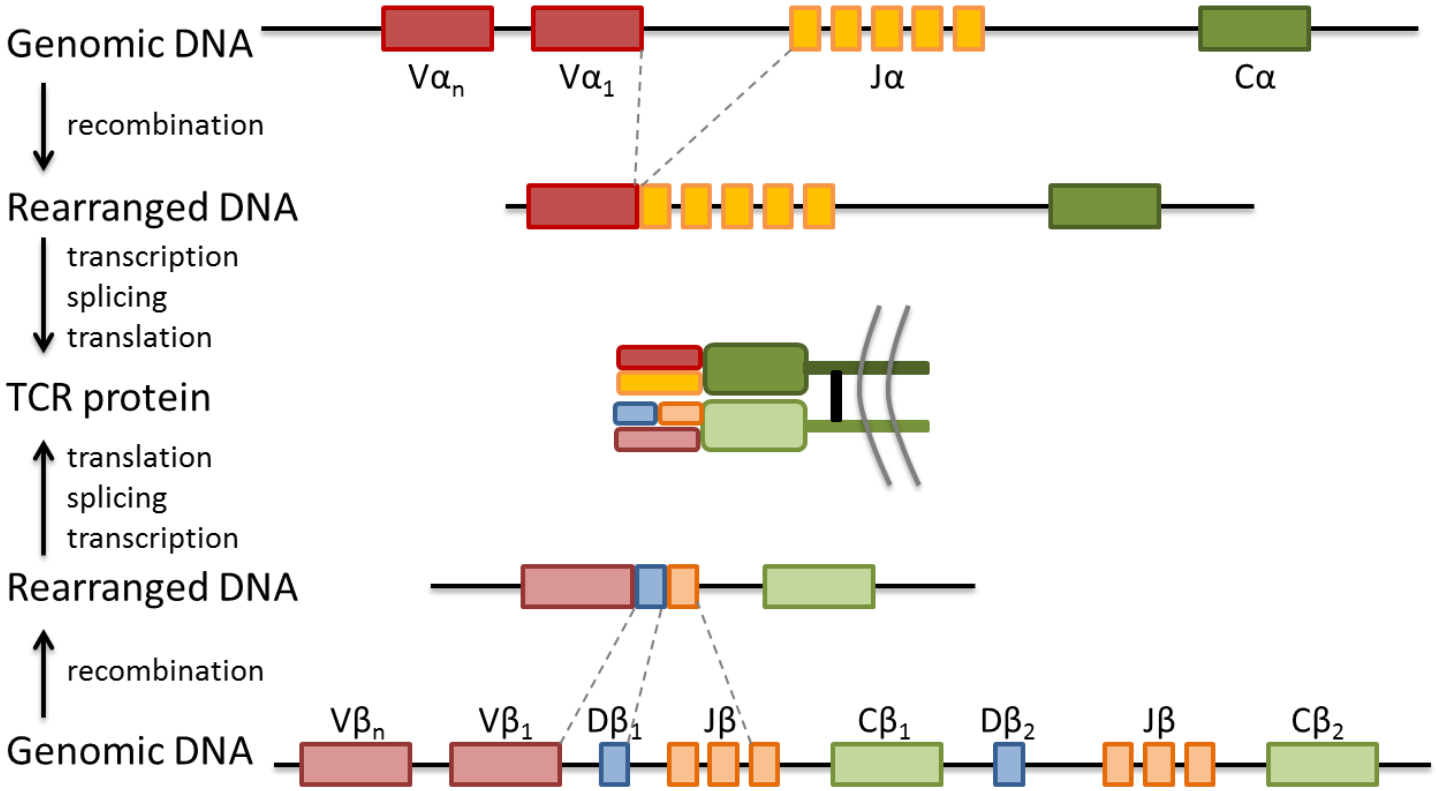



**Figure 1.7**: Gene rearrangement and assembly of T-cell receptors (taken from Janeway's Immunobiology (Murphy *et al.* 2008)).

### 1.5.3 T-cell receptor signalling

T-cell receptors do not have the ability to activate intrinsic signalling cascades on their own. In order to gain full functionality, the TCR associates with CD3 receptors to what is known as the TCR complex. CD3 proteins consist of γ, δ and ε chains forming heterodimers or ζ:ζ homodimers. In the TCR complex, the TCR α-chain associates with a CD3ζ homodimer and a CD3δ:CD3ε heterodimer, whereas the TCR β-chain is thought to interact with a CD3γ:CD3ε heterodimer (Murphy *et al.* 2008). The complex is maintained through hydrophobic interactions between the receptor chains. In addition to enabling intracellular signalling, the CD3 proteins assist in transporting the TCR to the cell membrane and stabilise the TCR at the cell surface. The CD3 receptors have an immunoglobulin-like extracellular domain, with the exception of the CD3ζ homodimer, and a cytosolic domain containing immunoreceptor tyrosine-based activation motifs (ITAMs). ITAMs are the elements responsible for transforming extracellular signals received from the TCR into intracellular signalling events.

Signalling cascades within T-cells are activated by phosphorylation of the ITAMs following TCR engagement. The pathways involved in TCR signalling are summarised in Figure 1.8.

Phosphorylated ITAMs at the CD3ζ chain recruit ZAP-70 (Zeta-chain-associated protein kinase 70), which in turn gets phosphorylated by Lck (lymphocyte-specific protein tyrosine kinase). Phosphorylated ZAP-70 can then activate adaptor proteins, such as the linker for the activation of T-cells (LAT) (Gorentla and Zhong 2012). Phosphorylated LAT leads to the recruitment of phospholipase C-γ (PLC-γ). Activation of PLC-γ enables hydrolysis of $PIP_2$ (phosphatidylinositol-4,5-bisphospahte) into two second messenger molecules, diacylglycerol (DAG) and inositol triphosphate ($IP_3$).

$IP_3$ triggers an increase in intracellular $Ca^{2+}$ levels, and this leads to the activation of the transcription factor NFAT (nuclear factor of activated T-cells). NFAT translocates to the nucleus and induces gene transcription leading to cell proliferation and differentiation.

DAG can activate the transcription factor NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), and at the same time induce the Ras-MAP kinase pathway, both causing transcription of target genes related to T-cell proliferation and effector function.

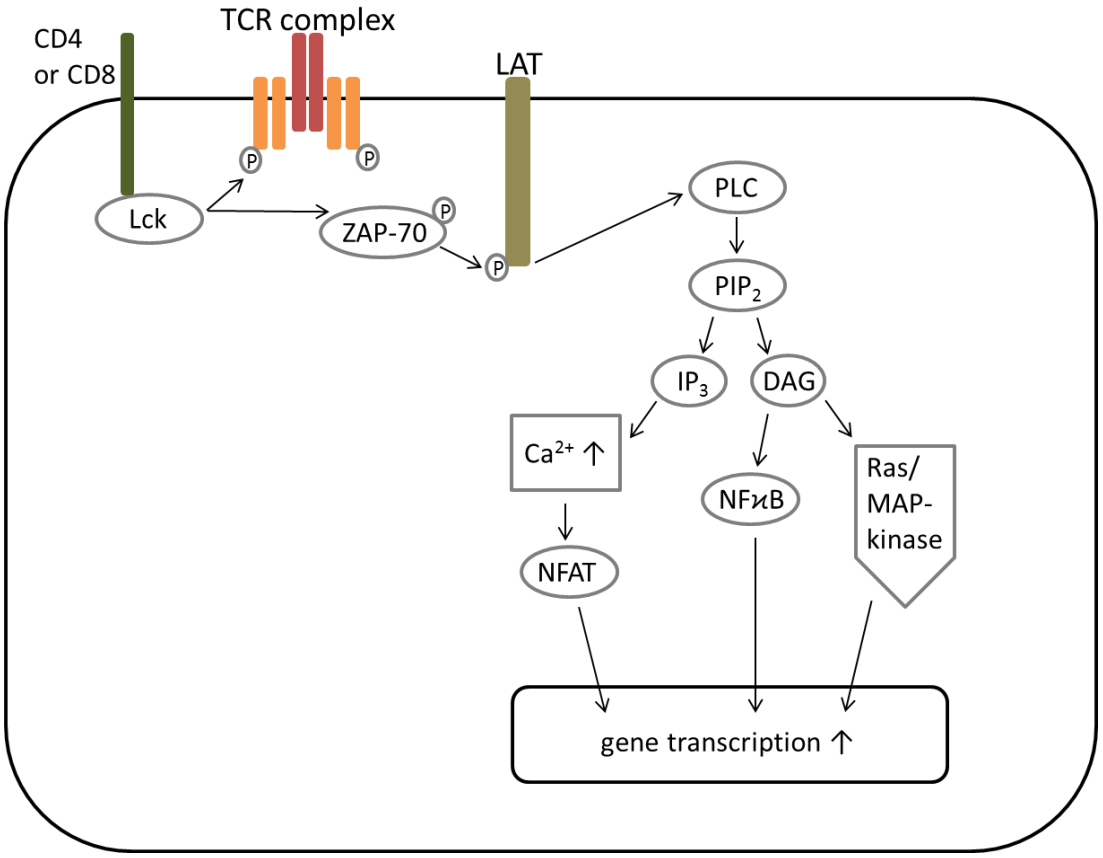



**Figure 1.8**: Summary of the intracellular TCR signalling pathways (adapted from (Morris and Allen 2012)).

Recognition of an antigen by the TCR leads to phosphorylation of the TCR complex and activation of downstream signalling cascades. These involve $Ca^{2+}$ influx, activation of Ras-MAP kinase pathway and recruitment of transcription factors like NFκB. The activated transcription factors induce T-cell development and effector functions.

(Lck= Lymphocyte-specific protein tyrosine kinase; ZAP-70= Zeta-chain-associated protein kinase 70; LAT= Linker for the activation of T-cells; PLC= Phospholipase C; $PIP_2$= Phosphatidylinositol-4,5-bisphospahte; $IP_3$= Inositol triphosphate; DAG= Diacylglycerol; NFAT= Nuclear factor of activated T-cells; NFκB= Nuclear factor kappa-light-chain-enhancer of activated B cells; MAP kinase= mitogen-activated protein kinase)

## 1.6 Drug Hypersensitivity Reactions

Hypersensitivity reactions (HSRs) to drugs are a form of idiosyncratic adverse drug reaction (iADR) that involves one or more immunological mechanisms (Pichler 2003). In most cases, however, the exact process through which a drug causes an immune response is not known. These reactions are also commonly referred to as drug allergy.

Drug-induced HSRs are thought to make up about one third of all ADRs (Gomes and Demoly 2005). But the exact percentage is hard to determine as there are currently no epidemiological studies focusing on HSRs. Also, definitions of iADRs and HSRs are not clear cut and are often used interchangeably (Uetrecht 2007).

Hypersensitivity reactions can manifest themselves in a diverse range of diseases, affecting almost any organ of the human body. The most commonly affected organ is the skin, followed by the liver and haematological system (Uetrecht and Naisbitt 2013). Some HSRs are limited to one organ, but often drug allergies show multi-organ involvement. Antibiotics and anticonvulsants are drugs known to cause hypersensitivity reactions most frequently.

Clinical characteristics of most HSRs include a delayed onset of disease symptoms, ranging from days to several weeks or even months after the start of drug therapy (Uetrecht 2007). Upon rechallenge symptoms usually appear more quickly. Both factors strengthen the assumption of an underlying immune-mediated mechanism. Also, the risk of developing drug-induced HSRs does not seem to be correlated with the therapeutic dose of the drug received, but in general are rarely seen at doses below 10mg per day (Uetrecht 2007).

### 1.6.1 Classification

HSRs are generally classified into four subtypes following the system described by Gell and Coombs. This classification system has proven useful in clinical practice to relate the clinical symptoms to the fundamental immune response

(Pichler 2003). However, many drugs are able to elicit several forms of hypersensitivity, suggesting different immune mechanisms can be activated by a single drug (Park *et al.* 1998).

Type I reactions, also known as immediate-type hypersensitivity, are caused by the formation of immunoglobulin (Ig) E against drug hapten complexes leading to mast cell activation. Type-I reactions depend on covalent binding of a drug hapten to an endogenous protein and recognition of the antigenic structure by the humoral immune system. A typical example is anaphylaxis as a form of penicillin hypersensitivity.

Type II hypersensitivity is a result of a drug binding to the surface of mainly blood cells (e.g. erythrocytes or platelets), and subsequent generation of anti-drug IgG antibodies which cause cellular lysis. Haemolytic anaemia caused by cephalosporins falls into this subgroup.

Type III reactions are mediated though excess formation of immune complexes of soluble drug antigen and IgG antibodies. Serum sickness is a classic example of this type of reaction.

Type IV hypersensitivity reactions are also referred to as delayed-type hypersensitivity and are mediated by antigen-specific T-cells. Effector T-cells involved in delayed-type HSRs have been shown to be rather heterogeneous. Type IV reactions have therefore been divided further into Type IVa- IVd reactions (Posadas and Pichler 2007). Table 1.4 summarizes the different effector T-cells of Type IV reactions and their functions. Most delayed-type hypersensitivity reactions show an overlap in effector T-cells and the resulting inflammatory response.

Delayed-type hypersensitivity reactions will be the focus of the following chapters and this thesis.

**Table 1.4**: Characteristics of Type IV hypersensitivity reactions (adapted from (Posadas and Pichler 2007)).

| | Type IV a | Type IV b | Type IV c | Type IV d |
|---|---|---|---|---|
| **T-cell type** | Th1-type reaction | Th2-type reaction | Cytotoxic T-cells | T-cells |
| **Secreted cytokines** | IFN-γ, TNF-α | IL-4, IL-13, IL-5 | Perforin, granzyme B, FasL | IL-8 |
| **Effector mechanism** | Macrophage activation; Stimulation of proinflammatory responses | B-cell production; macrophage deactivation; eosinophilic inflammation | Killing of tissue cells (keratinocytes, hepatocytes) | Neutrophilic inflammation |
| **Clinical phenotype** | Eczema | Maculopapular exanthema (MPE) | Bullous exanthema (SJS, TEN), MPE | Pustular exanthema (e.g. AGEP) |

### 1.6.2 Clinical phenotypes

The most frequent delayed-type HSRs are cutaneous manifestations, some of which include systemic organ involvement. The different syndromes each bear distinct clinical and histopathological characteristics (Hausmann *et al.* 2010).

***Maculopapular rash***

Maculopapular exanthema (MPE) is the most common form of delayed HSRs, making up over 90% of all drug-induced skin rashes. Among the drugs most commonly causing MPE are β-lactam antibiotics, sulfamethoxazole, quinolones, antiepileptic drugs and diuretics (Hausmann *et al.* 2010). Symptoms typically arise one to two weeks after start of drug treatment and often resolve spontaneously despite continuation of drug therapy (Uetrecht and Naisbitt 2013).

$CD4^+$ cytotoxic T-cells have been found to be the dominant cell type in these reactions, present in both the dermis and blood of patients. The $CD4^+$ T-cells can

kill activated keratinocytes through the release of perforin and granzyme B (Pichler 2003). Apoptosis of keratinocytes might be less severe due to the limited expression of HLA class II on normal skin cells. Cytokines released by the drug-specific $CD4^+$ T-cells include type 1 as well as type 2 cytokines (Hausmann *et al.* 2010).

### *Hypersensitivity syndrome*

Hypersensitivity syndrome (HSS) was initially associated with aromatic anticonvulsants, but it is also with other drugs, such as allopurinol, sulfonamides, and anti-retrovirals, including nevirapine and abacavir (Uetrecht and Naisbitt 2013). Other terms used to describe this phenotype are drug-induced hypersensitivity syndrome (DIHS) or drug reaction with eosinophilia and systemic symptoms (DRESS) (Pirmohamed *et al.* 2011). Clinical symptoms usually occur two to six weeks after commencement of drug therapy and comprise rash, fever and internal organ involvement, such as hepatitis, nephritis, carditis, pneumonitis and lymphadenopathy (Uetrecht and Naisbitt 2013). Hematologic abnormalities are common, with eosinophilia seen most frequently. It should be noted that the symptoms can persist for several weeks or even reoccur despite drug withdrawal (Hausmann *et al.* 2010). Mortality has been estimated to be around 10% and is most often due to liver failure.

In patients with HSS, a high number of activated T-cells, secreting large amounts of IFN-γ and IL-5, can be detected in the circulation (Hausmann *et al.* 2010). It has also been shown that many patients experience a reactivation of herpes viruses during HSS, but the implication of this association has not yet been clarified (Descamps *et al.* 2001; Picard *et al.* 2010).

### *Acute Generalized Exanthematous Pustulosis*

Acute generalized exanthematous pustulosis (AGEP) is a rare disease estimated to occur in approximately 1: 100,000 patients with a mortality rate of 2-4% (Hausmann *et al.* 2010). The typical symptoms include a non-infectious pustular skin rash, fever and leucocytosis (Pichler 2003). The time to onset is a relatively

short period of three to five days after treatment (Hausmann *et al.* 2010). The pustules are infiltrated by neutrophilic granulocytes and activated $CD4^+$ and $CD8^+$ T-cells (Pichler 2003). Antibiotics are the causative drugs in most cases of AGEP (Uetrecht and Naisbitt 2013).

***Stevens-Johnson syndrome and Toxic Epidermal Necrolysis***

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are the most severe types of delayed-type HSRs with an estimated incidence of 1: 100,000 and 1:1,000,000 patients, respectively (Hausmann *et al.* 2010). The syndromes are considered to be variants of the same disease and are classified by the extent of skin detachment. SJS involves less than 10%, TEN more than 30%, and SJS/TEN overlap 10- 30% of skin detachment. Both conditions are associated with high mortality rates of about 10% and 30%, respectively (Hausmann *et al.* 2010). The reactions usually occur after two weeks of treatment with the first signs being a sudden onset of fever and malaise. This is followed by a quickly spreading, painful rash and the development of blisters (Uetrecht and Naisbitt 2013). Mucous membranes of the mouth, eyes, genitals and intestine can also be affected.

Immunohistology shows widespread apoptosis of keratinocytes which is mediated by $CD8^+$ T-cells secreting cytolytic molecules, such as Fas Ligand (FasL) and granulysin (Nassif *et al.* 2004; Chung and Hung 2010).

***Drug-induced liver injury***

The liver is the most frequently involved extra-cutaneous organ in drug-induced HSRs, either in combination with other organs or as an isolated syndrome in form of drug-induced liver injury (DILI) (Zhang, X. *et al.* 2011a).

Most drugs that cause DILI can be metabolised in the liver to reactive metabolites, which are thought to induce liver injury. The mechanisms through which DILI is caused are largely unknown, but for many drugs there is evidence that the immune system is involved (Zhang, X. *et al.* 2011a). In general,

symptoms occur with a delay of one to three months after the start of treatment. Anti-drug antibodies or autoantibodies have been detected in several cases of DILI, but their function in the pathogenesis of liver injury has not yet been elucidated. Liver histology frequently shows infiltration of lymphocytes and eosinophils, similar to viral hepatitis. Recently, strong HLA associations have been found with several drugs causing DILI, the prime example being flucloxacillin-induced liver injury and *HLA-B*57:01* (Daly *et al.* 2009).

### 1.6.3 Mechanistic hypotheses

The involvement of T-cells in delayed-type HSRs was clearly demonstrated by the successful isolation of drug-specific T-cells from blood and tissue cells of patients suffering from a diverse range of drug-induced HSRs (Mauri-Hellweg *et al.* 1995; Hashizume *et al.* 2002; Naisbitt *et al.* 2003b). This strengthened the presumption of an underlying T-cell mediated pathomechanism. Due to the heterogeneity of the clinical syndromes of HSRs and the lack of adequate animal models, studies trying to unravel the mechanisms behind drug-induced T-cell activation focused on *in vitro* analysis of drug-specific T-cells isolated from hypersensitive patients. Several hypotheses have been put forward to explain how drugs might stimulate an immune response.

#### *Hapten hypothesis*

Initial models were built on the hapten hypothesis, first introduced by Landsteiner and Jacobs in 1935. It is based on the fact that drugs of low molecular weight as such are not immunogenic, but have to covalently bind to endogenous proteins in order to elicit an immune response (Landsteiner and Jacobs 1935). Such chemically reactive molecules are referred to as haptens. Haptenization of different proteins can lead to the formation of diverse immunogenic structures resulting in a broad range of immune responses (Adam *et al.* 2011). Modification of soluble or cell-bound proteins can lead to the formation of anti-drug antibodies. On the other hand, haptenized proteins can

be taken up by antigen-presenting cells (APCs), processed into drug-modified peptides and presented on HLA molecules to naïve T-cells (Adam *et al.* 2011). A classic example are penicillins, which are known to irreversibly bind to lysine residues on serum albumin (Uetrecht and Naisbitt 2013), and both anti-penicillin antibodies and T-cells specific to penicillin-conjugates can be detected in hypersensitive patients. The hapten hypothesis is illustrated in Figure 1.9.

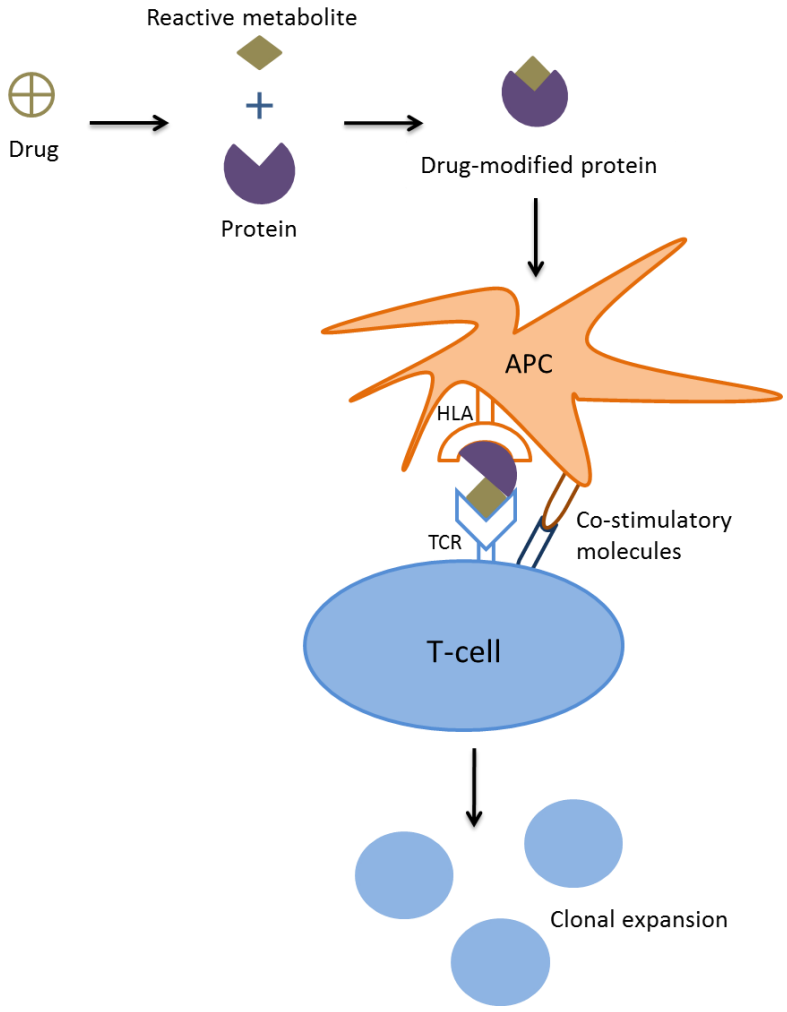



**Figure 1.9**: Hapten hypothesis (adapted from (Uetrecht 2007)).

The drug or its reactive metabolite binds to an endogenous protein. APCs take up the modified protein, process it and display drug-modified peptides in complex with HLA to T-cells.

Drugs that are chemically inert may become reactive through metabolism, and lead to metabolites acting as haptens. In this case the parent drug is referred to as a pro-hapten. Bioactivation predominantly takes place in the liver through cytochrome P450 (CYP) enzymes, but it can also occur in the skin, albeit at a lower rate (Posadas and Pichler 2007). If not eliminated from the body, reactive metabolites can cause direct cellular damage, activating the innate immune system, and at the same time form protein adducts that are able to elicit an adaptive immune response (Uetrecht and Naisbitt 2013). Sulfamethoxazole (SMX) is a typical example of a pro-hapten. SMX is metabolised to a hydroxylamine intermediate which can be spontaneously oxidized to nitroso-SMX. The nitroso metabolite is highly reactive and able to modify cysteine residues of various proteins (Uetrecht and Naisbitt 2013).

***Danger hypothesis***

The danger hypothesis takes into account that activation of T-cells only occurs if at least two signals are present. Signal one represents the interaction between TCR and peptide-HLA complex. Signal two refers to the expression of costimulatory molecules on APCs interacting with CD28 receptors on T-cells (see Figure 1.2). Polly Matzinger proposed that T-cell mediated immune responses are initiated by so called danger signals rather than recognition of non-self epitopes (Matzinger 1994). These "danger signals" are released upon cellular damage leading to the activation of APCs and upregulation of costimulatory molecules on the cell surface. In case of HSRs, reactive drugs or metabolites could induce cellular stress causing the release of danger signals, and at the same time function as antigenic stimuli (Pirmohamed *et al.* 2002). Depending on the affected tissue, this could determine the localisation and nature of the hypersensitivity reaction seen *in vivo* (Uetrecht 2007). Figure 1.10 represents a schematic model of the danger hypothesis.

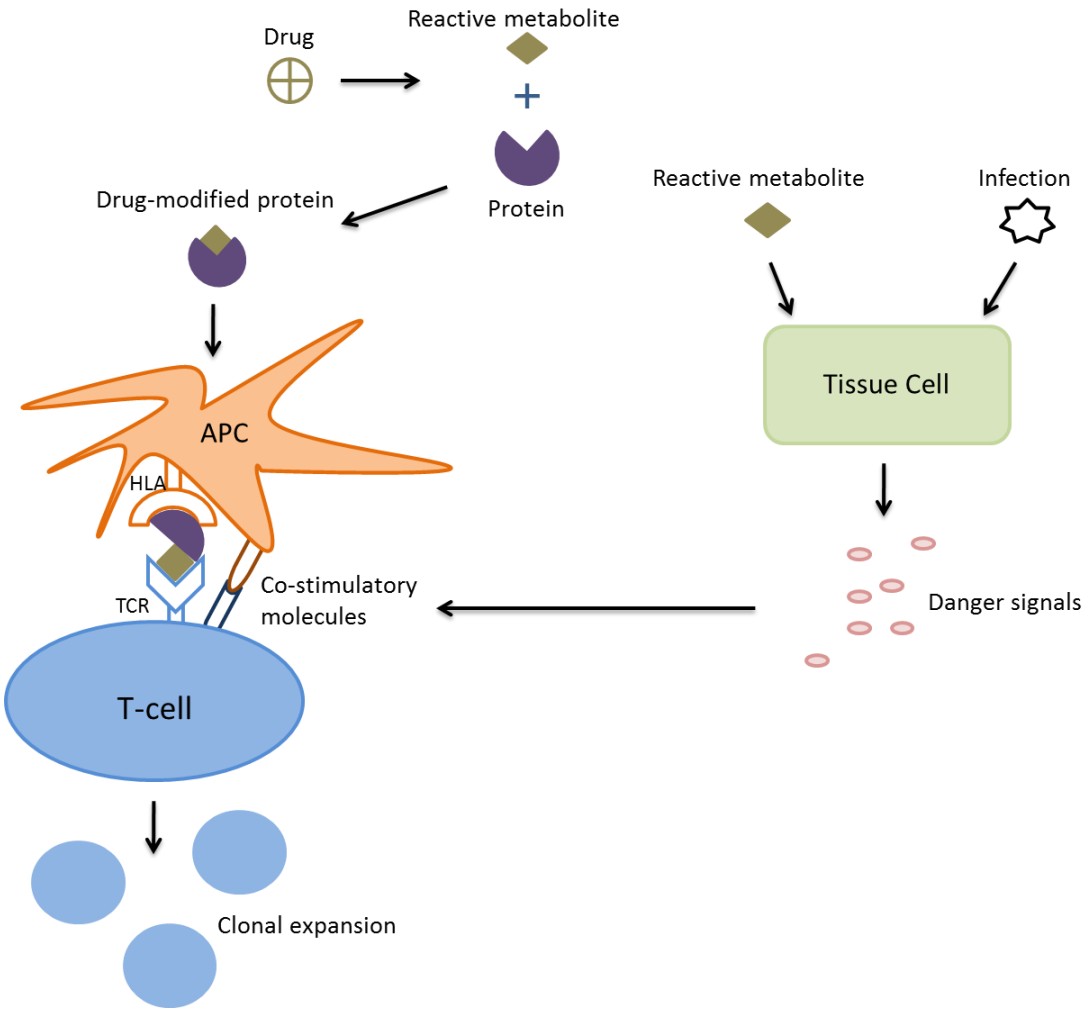



**Figure 1.10**: Danger hypothesis (adapted from (Uetrecht 2007)).

Danger signals are produced by stressed cells, which activate APCs and lead to the up-regulation of costimulatory molecules. This signal, in addition to the interaction between HLA and TCR, is required for the initiation of a T-cell response. The absence of this signal results in tolerance.

The danger hypothesis may explain why some chemically reactive drugs or metabolites do not cause hypersensitivity, as these might not cause cellular damage (Uetrecht 2007). Other factors such as viral infections could also trigger the release of danger signals and thereby increase the risk of HSRs, as seen for some drugs. On the other hand, not every drug known to cause cell damage is associated with hypersensitivity (Pirmohamed *et al.* 2002).

***Pharmacological interaction of drugs with immune receptors (p-i concept)***

Based on the observation that T-cell clones generated from SMX hypersensitive patients could be stimulated by SMX in the absence of metabolism, Pichler developed the so called p-i concept (Pichler 2002). According to this hypothesis, drugs that are chemically inert can activate T-cells through direct interaction with either the TCR or HLA molecule given that a sufficient binding affinity is generated (Adam *et al.* 2011). The interaction is reversible and seems to be independent of a specific peptide ligand present in the HLA binding groove. This resembles the binding of superantigens to HLA-TCR complexes (Uetrecht 2007). T-cells stimulated by drugs through the p-i mechanism are thought to derive from previously primed effector cells and memory T-cells (Adam *et al.* 2011). In comparison to naïve T-cells, these cells have a lower activation threshold and therefore may not require co-stimulatory signals from APCs (Adam *et al.* 2011). Drugs reported to stimulate T-cell clones *in vitro* via a p-i mechanism include lamotrigine, lidocaine and carbamazepine (Zanni *et al.* 1999; Naisbitt *et al.* 2003b; Wu *et al.* 2007). The p-i concept is illustrated in Figure 1.11 A.

***Altered peptide hypothesis***

A novel mechanism of drug-induced T-cell activation has been proposed recently for HSRs displaying strong associations with specific HLA alleles.

Several independent research groups were able to show that abacavir can bind non-covalently to specific residues within the HLA binding groove of HLA-B*57:01 which leads to changes in the peptide repertoire presented by the HLA allele. The novel self-peptides displayed by HLA-B*57:01 could subsequently trigger T-cell activation (Illing *et al.* 2012; Norcross *et al.* 2012; Ostrov *et al.* 2012). This new concept became known as the altered peptide repertoire model and is depicted in Figure 1.11 B.

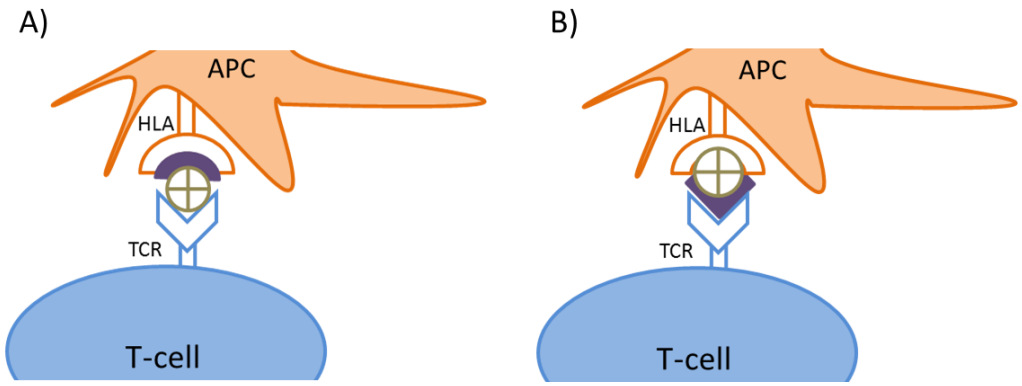



**Figure 1.11**: Schematic representation of the p-i concept (A) and altered peptide model (B) (adapted from (Bharadwaj *et al.* 2012))

The p-i concept states that a drug (in green) can bind directly to either HLA or TCR molecule and is not dependent on the presence of a specific peptide (in purple) in the HLA binding-groove. In the altered peptide model, a drug (green shape) binds to the empty HLA molecule changing its physicochemical properties and allowing novel self-peptides (purple shape) to be presented to T-cells.

### 1.6.4 Risk factors

Hypersensitivity reactions are known to be multi-factorial diseases, involving both environmental and genetic risk factors.

Genetic factors have been hypothesised to play a role in predisposing individuals to HSRs for a long time. Family case studies describing similar reactions to a drug given to members of the same family have provided some early evidence (Pirmohamed 2006). In the past, most genetic studies focused on two main aspects, genes involved in drug metabolism and immune-related genes, especially the MHC region due to its essential functions in the immune system.

To date, genetic polymorphisms in drug metabolising enzymes were found to contribute only a minor relative risk to HSRs and most studies proved negative (Uetrecht 2007). One example is hydralazine-induced systemic lupus erythematosus which occurs more frequently in patients who are slow

acetylators due to a deficiency in the N-acetyl-transferase 2 enzyme (Naisbitt *et al.* 2003c).

Early studies looking into polymorphisms of MHC genes turned out negative, and this was mainly a result of small sample sizes, ill-defined clinical phenotypes and insensitive serotyping methods (Pirmohamed 2006). This changed when high-resolution HLA genotyping became available, and several strong associations between specific HLA alleles and certain drug hypersensitivity phenotypes have already been identified. Recent genome-wide association studies (GWAS) have added further evidence for the involvement of HLA alleles in drug hypersensitivity. An overview of drug-induced HSRs known to be associated with specific HLA alleles is presented in Table 1.5. Three examples of HLA-associated HSRs are described in more detail below.

Environmental factors predisposing individuals to HSRs include viral infections. For example patients suffering from Epstein-Barr virus-induced infectious mononucleosis and treated with amoxicillin have a strongly increased risk of developing skin rash (Pirmohamed 2006). Another example is the increased frequency of hypersensitivity to SMX among HIV patients, which increases from 3% in HIV-negative to 30% in HIV-positive patients (Pirmohamed 2006).

Other risk factors predisposing individuals are related to the treatment regimen. For instance intermittent or repeated drug administration has been shown to increase the frequency of HSRs (Gomes and Demoly 2005). Also, parenteral administration of a drug appears to be more immunogenic than oral administration of the same drug (Gomes and Demoly 2005). Host-related factors, such as age, sex and weight, have been found to be risk factors in a few forms of HSRs. Women for instance have an increased risk of developing halothane-induced hepatitis or clozapine-induced agranulocytosis compared to men (Uetrecht 2007).

**Table 1.5**: Summary of well-characterised HLA-associated drug hypersensitivity reactions.

| Drug (Indication) | HLA allele | Syndrome | Odds ratio | Reference |
|---|---|---|---|---|
| **Abacavir** (Antiretroviral) | B*57:01 | HSS | 23.4; 117 | (Hetherington *et al.* 2002; Mallal *et al.* 2002) |
| **Allopurinol** (Gout) | B*58:01 | SJS/TEN, HSS | 580.3; 96.6 | (Hung *et al.* 2005b; Somkrua *et al.* 2011) |
| **Carbamazepine** (Antiepileptic) | B*15:02 (Han Chinese) | SJS/TEN | 2504 | (Chung *et al.* 2004) |
| | A*31:01 (Caucasian, Japanese) | MPE, HSS, SJS/TEN | 12.1; 9.5 | (McCormack *et al.* 2011; Ozeki *et al.* 2011) |
| **Co-amoxiclav** (Antibiotic) | A*02:01-B*07:02-DRB1*15:01-DQB1*06:02, | DILI | 13.0 | (Lucena *et al.* 2011) |
| | A*02:01-B*18:01 (Spanish) | | 6.4 | |
| **Dapsone** (Antibiotic) | B*13:01 | HSS | 20.5 | (Zhang, F. R. *et al.* 2013) |
| **Efavirenz** (Antiretroviral) | DRB1*01 (French) | Skin rash | (p= 0.004) | (Vitezica *et al.* 2008) |
| **Flucloxacillin** (Antibiotic) | B*57:01 | DILI | 80.6 | (Daly *et al.* 2009) |
| **Lamotrigine** (Antiepileptic) | B*15:02 (Han Chinese) | SJS/TEN | 5.1 | (Hung *et al.* 2010) |
| | A*30:01, B*13:02 (Han Chinese) | MPE | (p= 0.013) | (Li, Li-Juan *et al.* 2013) |

DILI= Drug-induced liver injury; HSS= Hypersensitivity syndrome; MPE= Maculopapular exanthema; NSAID= Non-steroidal anti-inflammatory drug; SJS= Stevens-Johnson syndrome; TEN= Toxic epidermal necrolysis

**Table 1.5 (*continued*)**: Summary of well-characterised HLA-associated drug hypersensitivity reactions.

| Drug (Indication) | HLA allele | Syndrome | Odds ratio | Reference |
|---|---|---|---|---|
| **Lapatinib** (Breast cancer) | DRB1*07:01-DQA1*02:01-DQB1*02:02 | DILI | 2.2 | (Spraggs *et al.* 2011) |
| **Lumiracoxib** (NSAID) | DRB1*15:01-DQB1*06:02-DRB5*01:01-DQA1*0102 | DILI | 5.0 | (Singer *et al.* 2010) |
| **Methazolamide** (Glaucoma) | B*59:01, C*02:01 (Korean) | SJS/TEN | 249.8 and 22.1 | (Kim *et al.* 2010) |
| **Nevirapine** (Antiretroviral) | DRB1*01:01 | Hepatitis with rash | 4.8 | (Martin *et al.* 2005) |
| | B*58:01, DRB1*01:02 (South Africans) | Hepatotoxicity | (p= 0.03) | (Phillips, E. *et al.* 2013) |
| | C*04:01 (Malawian) | SJS/TEN | 17.5 | (Carr *et al.* 2013) |
| | C*8-B*14 (Sardinians)<br>C*8 (Japanese)<br>DRB1*01 (French)<br>C*4-B*35:05 (Thai)<br>C*4-DRB1*15 (Han Chinese) | Cutaneous reactions | (p= 0.004)<br>(p= 0.03)<br>(p= 0.004)<br>18.9<br>3.6 | (Littera *et al.* 2006; Gatanaga *et al.* 2007; Vitezica *et al.* 2008; Chantarangsu *et al.* 2009; Gao *et al.* 2012) |
| **Phenytoin** (Antiepileptic) | B*15:02 (Thai and Han Chinese) | SJS/TEN | (p= 0.005); 5.1 | (Locharernkul *et al.* 2008; Hung *et al.* 2010) |
| **Ticlopidine** (Antiplatelet) | A*33:03 (Japanese) | DILI | 13.0 | (Hirata *et al.* 2008) |
| **Ximelagatran** (Anticoagulant) | DRB1*07-DQA1*02 | DILI | 4.4 | (Kindmark *et al.* 2008) |

DILI= Drug-induced liver injury; SJS= Stevens-Johnson syndrome; TEN= Toxic epidermal necrolysis

### 1.6.5 HLA associated drug hypersensitivity reactions

The growing number of HLA associations with various drug hypersensitivity reactions implies that a causal relationship between the HLA molecules and hypersensitivity might exist (Wei, C. Y. *et al.* 2012b). However, the underlying mechanism for most HLA-associated reactions has not been resolved yet. This is further complicated by the fact that the majority of HLA associations are phenotype specific and the association can vary between populations of different ethnicity (Wei, C. Y. *et al.* 2012b).

Three examples of HLA-associated hypersensitivity reactions will be discussed here in more detail: abacavir hypersensitivity and *HLA-B*57:01*, flucloxacillin-induced liver injury and *HLA-B*57:01*, and allopurinol and *HLA-B*58:01*.

#### *Abacavir and HLA-B*57:01*

Abacavir (ABC) is an antiretroviral drug used to treat human immunodeficiency virus (HIV) infections. ABC can cause hypersensitivity syndrome in 5-8% of patients, with symptoms of fever, gastrointestinal manifestations and internal organ involvement (Pavlos *et al.* 2012). In up to 70% of hypersensitive patients a mild to moderate skin rash can develop. In 2002, two independent research groups reported a strong association between HLA-B*57:01 and ABC hypersensitivity (Hetherington *et al.* 2002; Mallal *et al.* 2002). Subsequent clinical trials showed that 100% of patch-test positive patients carried HLA-B*57:01 and demonstrated a 100% negative-predictive value of HLA-B*57:01 for ABC hypersensitivity (Pavlos *et al.* 2012). $CD8^+$ T-cells have been found in the skin of ABC hypersensitive patients (Phillips, E. J. *et al.* 2002) and drug-specific T-cell responses could be induced in patients as well as ABC-naïve, HLA-B*57:01+ healthy donors (Chessman *et al.* 2008). T-cell responses were restricted by HLA-B*57:01 and closely related HLA allotypes were unable to elicit a response (Chessman *et al.* 2008). Most recently, three individual studies showed that ABC can bind non-covalently within the F-pocket of HLA-B*57:01 and thereby change the properties of the peptide-binding cleft, allowing an

altered set of self-peptides to be presented by HLA-B*57:01 (Illing *et al.* 2012; Norcross *et al.* 2012; Ostrov *et al.* 2012).

It is important to note that 45% of HLA-B*57:01 positive patients are able to tolerate ABC, indicating additional factors are required for the development of a reaction (Pavlos *et al.* 2012). However, screening for HLA-B*57:01 prior to ABC therapy has been recommended by both the US Food and Drug Administration (FDA) and European Medicines Agency (EMA). Pharmacogenetic testing has strongly reduced the incidence of ABC hypersensitivity and has proven to be cost effective (Phillips, E. and Mallal 2009).

***Flucloxacillin and HLA-B*57:01***

Flucloxacillin is a β-lactam antibiotic used in the treatment of staphylococcal infections. The drug is associated with a risk of cholestatic hepatitis occurring in about 8 out of 100,000 patients (Russmann *et al.* 2005). A GWAS has recently identified HLA-B*57:01 as a risk factor for flucloxacillin-induced liver injury (Daly *et al.* 2009). The HLA allele was present in 85% of patients compared to 5% of the general population. The detection of drug-specific T-cells in patients together with the HLA association suggests an underlying immune-mediated mechanism. Recently, two separate groups of researchers were able to generate drug-specific T-cells from HLA-B*57:01 positive patients and healthy volunteers. In the study by Monshi *et al*., drug-responsive $CD8^+$ T-cell clones were shown to secrete cytolytic molecules upon flucloxacillin stimulation. The clones were shown to express chemokine receptors linked to the migration of immune cells to the liver (Monshi *et al.* 2013). T-cell activation was observed in the presence of APCs expressing HLA-B*57:01 or the closely related HLA-B*58:01 allele (Monshi *et al.* 2013). Wuillemin *et al.* reported that HLA-$B*57:01^+$ T-cells were able to recognise flucloxacillin as a hapten complex, but the majority of T-cells were stimulated via a direct interaction as explained by the p-i concept (Wuillemin *et al.* 2013). In contrast to abacavir, no changes in the peptide repertoire of a HLA-B*57:01 expressing cell lines were seen following flucloxacillin treatment (Norcross *et al.* 2012).

It remains unclear why HLA-B*57:01 is only associated with flucloxacillin-induced liver injury. The molecular pathomechanism through which flucloxacillin is able to specifically kill liver cells has yet to be resolved.

***Allopurinol and HLA-B*58:01***

Allopurinol is used for the prevention of gout and hyperuricemia, and is associated with the occurrence of HSS and SJS/TEN syndromes. The first study linking HLA-B*58:01 with allopurinol-induced serious cutaneous reactions was conducted in Han Chinese patients, showing that HLA-B*58:01 was present in 100% of patients and only 15% of tolerant controls (Hung *et al.* 2005b). This has meanwhile been replicated in several Asian populations, including Japanese, Thai and Koreans (Pavlos *et al.* 2012). In Caucasians the association between HLA-B*58:01 and allopurinol hypersensitivity was found to be lower but still present with about 50% of patients carrying the risk allele (Lonjou *et al.* 2008). This might be due to the lower allele frequency of HLA-B*58:01 in Caucasians compared to Asians (Pavlos *et al.* 2012). The mechanism through which this HLA allele might contribute to the development of allopurinol HSRs remains to be elucidated. Additional risk factors have been found to influence the occurrence of allopurinol hypersensitivity, namely renal failure and administered daily dose (Yun *et al.* 2012). Yun *et al*. hypothesized that both factors could lead to the accumulation of allopurinol, or its metabolite oxypurinol, which may be presented by HLA-B*58:01 to drug-specific T-cells; and if the TCR avidity is high enough this might result in T-cell activation (Yun *et al.* 2012).

## 1.7 Carbamazepine

### 1.7.1 Indication

Carbamazepine (CBZ) is an anticonvulsant which is primarily used in the treatment of epilepsy for the prevention of partial and secondarily generalized tonic-clonic seizures (Engel and Pedley 2008). It was first introduced to the market in the 1960s, and has since then become one of the most often prescribed drugs in neurologic disorders due to its proven clinical efficacy, inexpensiveness and wide availability (Engel and Pedley 2008). CBZ is also effectively used in the treatment of neuropathic pain syndromes, such as trigeminal neuralgia and diabetic neuropathy (Spina and Perugi 2004). In 2004, CBZ was approved as mood stabilizer for the treatment of manic and mixed episodes of bipolar disorders (Ettinger and Argoff 2007). Its efficacy is comparable to lithium and many patients intolerant to lithium can be successfully treated with CBZ (Grunze 2010).

### 1.7.2 Mechanism of action

The mechanism of action is still not completely understood. CBZ is known to block voltage-dependent sodium channels in the neuronal membrane promoting their inactivation; and this leads to reduced firing of high-frequency action potentials and stabilisation of hyperexcited neurones (Rogawski and Loscher 2004). The inhibitory effect is voltage- and use-dependent, so that only hyperreactive nerves are affected. The reduced firing of action potentials is followed by a reduced amount of neurotransmitters being released into the synaptic cleft (Rogawski and Loscher 2004).

### 1.7.3 Pharmacokinetics and Metabolism

Carbamazepine is slowly absorbed from the gastrointestinal tract and has a bioavailability of about 80 to 90%. The drug is quickly distributed to various organs and readily transported across the blood-brain barrier (Wyllie *et al.*

2006). CBZ is metabolized extensively and almost entirely in the human liver, involving steps of oxidation, hydroxylation and conjugation. Only about 5% of the parent drug is excreted unchanged in the urine (Thorn *et al.* 2011), alongside more than 30 different metabolites (Lertratanangkoon and Horning 1982). The main metabolic pathways are shown in Figure 1.12.

CBZ is oxidized by cytochrome P-450 (CYP) enzymes, mainly CYP3A4 and to some extent CYP2C8 and CYP3A5, to CBZ-10,11-epoxide (Thorn *et al.* 2011). The epoxide is further hydrolysed by microsomal epoxide hydrolase 1 (EPHX1) to CBZ-10,11-trans-diol, which can be excreted in the urine directly or as a conjugate after glucuronidation (Maggs *et al.* 1997). The epoxide-diol pathway is the major route of metabolism. Minor metabolites include hydroxylation at variable sides of the aromatic rings, presumably via arene oxide intermediates. These reactions are catalysed by various CYP enzymes, including CYP 3A4, CYP2B6, CYP2E1, CYP2A6 and CYP1A2 (Pearce *et al.* 2002). Subsequent metabolism leads to the formation of reactive quinone structures. These can be conjugated with glucuronic acid and excreted into the urine (Lu and Uetrecht 2008).

CBZ can enhance the transcription of several metabolising enzymes, including autoinduction of CYP3A4 and CYP2B6 (Thorn *et al.* 2011). This can cause various forms of drug interactions, for example decreased plasma concentrations of concomitantly administered anticonvulsants. This process is known to appear about 2 to 6 weeks after treatment initiation and requires dose adjustment (Wyllie *et al.* 2006).

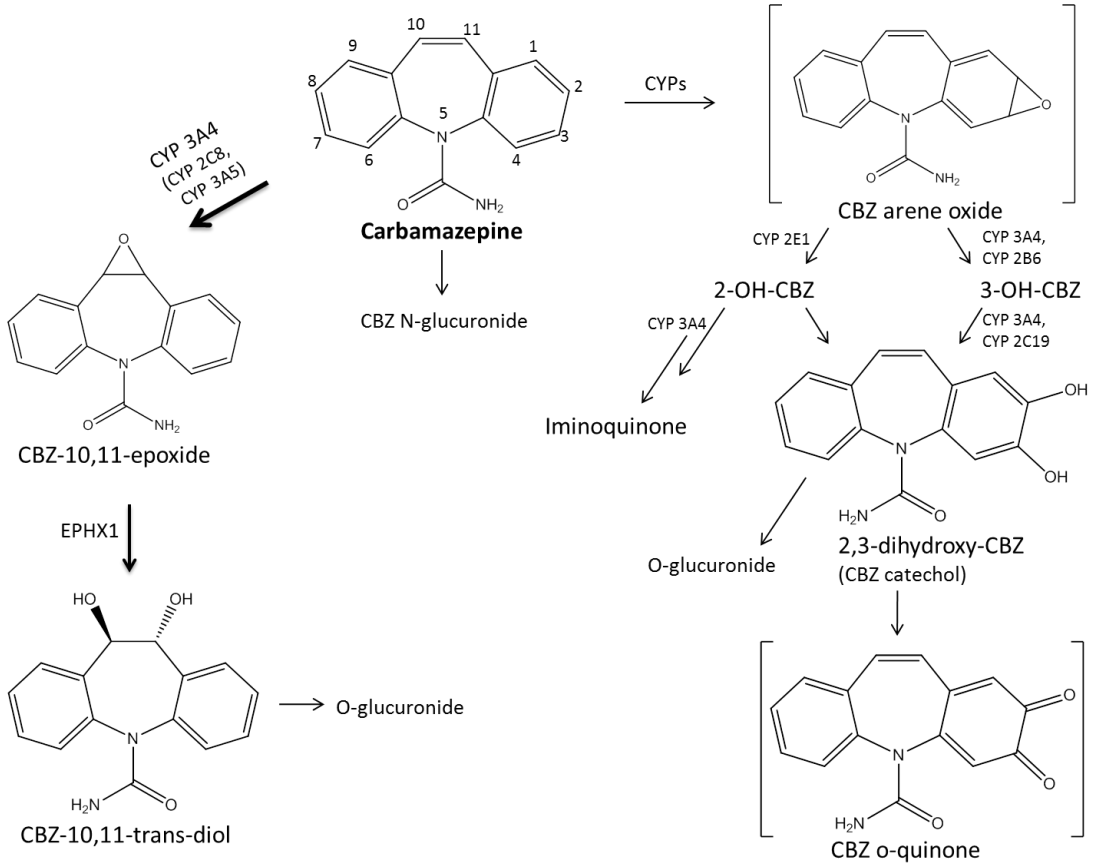



**Figure 1.12**: Metabolic pathways of carbamazepine bioactivation and detoxification (adapted from (Pearce *et al.* 2002; Lu and Uetrecht 2008; Pearce *et al.* 2008)). Structures in brackets were deduced from products.

## 1.8 Carbamazepine-induced Hypersensitivity

Carbamazepine is usually well tolerated but it can cause cutaneous ADRs in up to 10% of patients treated with the drug (Marson *et al.* 2007). Most commonly, a mild erythematous skin rash develops several days after treatment initiation, which can resolve spontaneously without further intervention. More severe reactions include hypersensitivity syndrome (HSS) which usually occurs within the first eight weeks of CBZ therapy and is associated with rash, fever, eosinophilia and internal organ involvement, mostly hepatitis (Knowles *et al.* 1999). CBZ-induced HSS occurs at an estimated frequency of 4 in 10,000 exposures (Gogtay *et al.* 2005). Cases of SJS and TEN have also been reported but are rare in Caucasians, with an estimated prevalence of 1: 5,000 patients (Yip, V. *et al.* 2013). In contrast, CBZ-induced SJS is known to occur at an approximately ten-fold higher frequency in Han Chinese (Hung *et al.* 2005a).

### 1.8.1 Pharmacogenetics

Predisposition to CBZ hypersensitivity has long been thought to have a genetic basis. This was supported by case reports of hypersensitivity reactions in members of the same family, as well as monozygotic twins (Edwards, S. G. *et al.* 1999). Since chemically reactive metabolites of CBZ, such as arene oxides, were suspected to cause direct cellular toxicity and potentially stimulate the immune system, early investigations concentrated on genetic defects in detoxification mechanisms (Knowles *et al.* 1999). However, studies on polymorphisms in genes of metabolising enzymes, such as microsomal epoxide hydroxylase (Green *et al.* 1995), failed to find any significant association. Autoantibodies directed against CYP isoenzymes have been detected in some patients, suggesting CBZ or a metabolite may form adducts with CYP enzymes leading to the formation of neoantigens (Leeder *et al.* 1992). However, whether these antibodies are immunogenic has not yet been demonstrated.

A major advancement was the discovery that specific HLA alleles are strongly associated with defined clinical phenotypes of CBZ hypersensitivity. In 2004, Chung *et al.* reported that HLA-B*15:02 was found to be present in 100% of Han Chinese patients suffering from CBZ-induced SJS/TEN, compared to 3% of CBZ-tolerant controls (Chung *et al.* 2004). This association has been replicated in several populations of Southeast Asian ancestry, including Thai, Malaysians and Indians (Locharernkul *et al.* 2008; Mehta *et al.* 2009; Then *et al.* 2011). However, HLA-B*15:02 is not associated with CBZ hypersensitivity in Caucasian populations (Alfirevic *et al.* 2006; Lonjou *et al.* 2006) or Japanese (Kaniwa *et al.* 2008). This is possibly due to the low frequency of HLA-B*15:02 in Europeans and Japanese (both < 1%; reported on www.allelefrequencies.net) compared to Chinese populations (up to 35%; see www.allelefrequencies.net). Genetic testing for HLA-B*15:02 prior to start of CBZ therapy has therefore been recommended by the FDA in patients of Asian descent only (Ferrell and McLeod 2008).

More recently, two independent GWAS have identified an association between HLA-A*31:01 and all phenotypes of CBZ hypersensitivity in Japanese (Ozeki *et al.* 2011) and in Caucasians (McCormack *et al.* 2011), with a prevalence of 60% and 26% respectively. The HLA-A*31:01 allele had previously been reported in association with CBZ-induced MPE in Han Chinese (Hung *et al.* 2006).

### 1.8.2 Functional studies

The clinical features of CBZ hypersensitivity suggest an immune-mediated aetiology, and the presence of drug-specific T-cells in hypersensitive patients supports this assumption (Mauri-Hellweg *et al.* 1995; Wu *et al.* 2007). The strong associations with specific HLA alleles indicate a direct role for HLA in the presentation of CBZ and subsequent initiation of a T-cell mediated immune response.

*In vitro* studies using CBZ-specific T-cell clones have shown that T-cells are stimulated by CBZ in the absence of drug metabolism and antigen processing (Wu *et al.* 2006; Wu *et al.* 2007). These observations suggest that T-cell

activation occurs through direct interaction between the drug, HLA and/ or TCR, as proposed in the p-i concept. Peptide elution studies using HLA-B*15:02-transfected cell lines cultured in the presence of the drug were unable to detected any CBZ-modified peptides (Yang *et al.* 2007). A study by Wei *et al*. demonstrated that CBZ binds directly to the binding groove of HLA-B*15:02 and subsequently presented to cytotoxic T-cells (Wei, C. Y. *et al.* 2012a). However, CBZ-specific T-cell activation was only possible if endogenous peptides were present to stabilize the HLA complex. Moreover, *in silico* modelling showed that CBZ preferably bound in the B-pocket of HLA-B*15:02 (Wei, C. Y. *et al.* 2012a). In a recent study by Illing *et al.* (2012), CBZ was found to bind non-covalently to HLA-B*15:02 and induce a modest shift in the peptide repertoire to slightly more hydrophobic peptides. These findings suggest that the altered peptide hypothesis might be implicated in the pathogenesis of CBZ hypersensitivity.

Nonetheless, it is important to note that many patients who are carriers of CBZ-associated risk alleles are able to tolerate the drug. This indicates that additional factors are important in predisposing individuals to develop hypersensitivity. Recently, Ko *et al.* (2011) reported that CBZ-responsive $CD8^+$ T-cells isolated from HLA-B*15:02+ individuals used a common restricted TCR repertoire. The predominant TCR clonotypes could be detected in both blister fluid cells and unstimulated peripheral blood mononuclear cells (PBMCs) of patients suffering from SJS. The oligoclonal T-cells can also be found in HLA-B*15:02+ healthy donors, and when stimulated with CBZ *in vitro* this results in a robust immune response to the drug (Ko *et al.* 2011). This study indicates that the expression of both the correct TCR and the predisposing HLA molecule are essential in eliciting hypersensitivity reactions to CBZ.

## 1.9 Sulfamethoxazole

Sulfamethoxazole (SMX) is an antibiotic agent used in combination with trimethoprim to treat various infections. SMX is known to cause cutaneous HSRs in approximately 3-8% of patients (Castrejon *et al.* 2010). Clinical manifestations range from immediate-type reactions, such as anaphylaxis, to delayed-type reactions, including mild exanthematous rash and blistering skin reactions like SJS and TEN. Many studies investigating the mechanisms behind drug hypersensitivity have used SMX as a model drug (Pirmohamed *et al.* 2002; Engler *et al.* 2004), mainly because of its well characterised metabolic pathways.

SMX is metabolised by N-acetyltransferases in the liver and the nontoxic products are quickly excreted. A fraction of the drug can be converted to a hydroxylamine intermediate (SMX-NHOH) via CYP 2C9 (Cribb, A. E. *et al.* 1995). The hydroxylamine is rather stable, but spontaneous oxidation can lead to the formation of a highly reactive nitroso metabolite (SMX-NO) (Cribb, Alastair E. and Spielberg 1992). SMX-NO is known to modify cysteine residues on multiple proteins (Naisbitt *et al.* 2002; Callan *et al.* 2009).

SMX-NO has been shown to be highly immunogenic, stimulating the innate immune system by activating dendritic cells (Sanderson *et al.* 2007) and inducing an adaptive immune response through generation of drug-modified peptides (Castrejon *et al.* 2010). In animal models, treatment with SMX-NO stimulates the activation of T-cells, which specifically recognise SMX-NO-modified peptides in a TCR dependent, MHC restricted manner (Naisbitt *et al.* 2002; Farrell *et al.* 2003). However, SMX itself is unable to stimulate these T-cells. Studies of T-cell clones (TCCs) generated from hypersensitive patients show a slightly more complex picture. Most TCCs are activated by SXM-NO and the response is dependent on covalent binding and antigen processing (Castrejon *et al.* 2010). At the same time it has been reported that TCCs can also recognise SMX directly, independent of covalent binding and without the need

for antigen processing (Schnyder *et al.* 1997). Whether SMX represents the original immunogen in these cases remains unclear.

*In vitro* studies on T-cells isolated from drug-naïve individuals demonstrated that SMX-NO can elicit a T-cell response in nearly 100% of cases (Engler *et al.* 2004). Recently, it has been shown that SMX-NO priming induces a phenotypic change of naïve T-cells to a memory T-cell population (Faulkner *et al.* 2012).

## 1.10 Aims

There is substantial evidence that hypersensitivity reactions to carbamazepine are mediated by the adaptive immune system and drug-specific T-cells have been identified as key players in the pathogenesis of these reactions (Mauri-Hellweg *et al.* 1995; Leeder 1998; Roujeau 2006). The isolation of CBZ-reactive T-cells from patients and *in vitro* stimulation of the cells with CBZ has made it possible to study their phenotype and effector functions in detail (Naisbitt *et al.* 2003a; Wu *et al.* 2006), as discussed earlier in this chapter. However, the molecular mechanisms through which CBZ causes T-cell activation still remain largely unknown.

The numerous associations between specific HLA alleles and drug-induced HSRs imply that HLA alleles may have a functional role in mediating T-cell responses to drugs. A mechanistic basis for some HLA-associated drug hypersensitivity reactions, such as abacavir and HLA-B*57:01 (Chessman *et al.* 2008) or carbamazepine and HLA-B*15:02 (Wei, C. Y. *et al.* 2012a), is beginning to be uncovered. At the same time, *in vitro* studies investigating the interaction between HLA-B*15:02 and CBZ-specific T-cells have demonstrated that specific T-cell receptors may present additional susceptibility factors in the development of CBZ-induced HSRs (Ko and Chen 2012).

HLA-A*31:01 represents the first HLA allele associated with CBZ-induced HSRs in Caucasian patients (McCormack *et al.* 2011), indicating CBZ may selectively bind to HLA-A*31:01 and trigger T-cell activation in this population. However, studies investigating whether any direct interactions between CBZ, HLA-A*31:01 and drug-responsive T-cells occur have not yet been performed. Thus, I wanted to explore whether HLA-A*31:01 is functionally involved in mediating carbamazepine hypersensitivity in patients of European ancestry.

The aims of this thesis were therefore to:

1) Determine the pattern of HLA restriction of drug-specific T-cells isolated from a HLA-A*31:01 positive, carbamazepine hypersensitive patient.

2) Generate a primary immune response to carbamazepine *in vitro* using T-cells from HLA-A*31:01 positive, drug-naïve individuals.

3) Characterise the peptide-binding specificity of HLA-A*31:01 in comparison to HLA-B*15:02 using *in silico* and *in vitro* approaches.

4) Establish a method for T-cell receptor spectratyping using the model drug antigen nitroso sulfamethoxazole as a first step. At a later stage, this method will then be used to study the T-cell receptor usage in carbamazepine hypersensitivity.

# Chapter II

# HLA restriction of drug-specific T-cells from a HLA-A*31:01 positive carbamazepine hypersensitive patient

## Contents

## 2.1 Introduction

Carbamazepine (CBZ) is known to cause hypersensitivity reactions of varying severity in a small proportion of patients treated with the drug. The reactions are thought to have an underlying immune-mediated aetiology, and drug-reactive T-lymphocytes of CD4 and CD8 phenotype have been isolated from patients' blood and skin (Mauri-Hellweg *et al.* 1995; Leyva *et al.* 2000). Genetic factors predisposing individuals to CBZ hypersensitivity have long been postulated to be important, and recent genetic studies revealed strong associations with specific human leukocyte antigen (HLA) alleles.

*HLA-B*15:02*, the first CBZ-associated HLA allele reported, was detected in almost all cases of CBZ-induced Stevens-Johnson syndrome (SJS) in patients of Han Chinese or Southeast Asian ancestry (Chung *et al.* 2004; Tassaneeyakul *et al.* 2010; Chang *et al.* 2011; Zhang, Y. *et al.* 2011b). Its functional role in development of CBZ-induced SJS has been well characterised. T-cell responses in these patients are predominantly mediated by cytotoxic $CD8^+$ T-cells (Nassif *et al.* 2004), which are restricted by HLA-B*15:02 (Wei, C. Y. *et al.* 2012a). The drug-specific effector T-cells have been shown to mediate cytotoxicity through the use of shared restricted T-cell receptors (TCRs) (Ko and Chen 2012).

*HLA-A*31:01* represents the latest example, and two independent genome-wide association studies (GWAS) have found it to be associated with all clinical phenotypes of CBZ hypersensitivity in Caucasian and Japanese populations (McCormack *et al.* 2011; Ozeki *et al.* 2011). A study by Niihara *et al*. (2012) aimed to determine whether lymphocyte activation was correlated to HLA-A31 status by comparing lymphocyte responses in HLA-A31 positive to A31 negative CBZ hypersensitive patients, but no significant difference was detected (Niihara *et al.* 2012). However, functional studies investigating whether T-cell responses to CBZ are dependent on the drug interacting specifically with HLA-A*31:01 have not been performed.

The main aim of the work presented in this chapter was to characterise in detail the HLA restriction of CBZ-reactive T-cell clones (TCCs) generated from a CBZ

hypersensitive patient carrying HLA-A*31:01. I focused on the $CD8^+$ as well as $CD4^+$ T-cell populations, as both lymphocyte types have been shown to be involved in CBZ hypersensitivity (Wu *et al.* 2007). In contrast to SJS, $CD4^+$ T-cell have been shown to be the prevalent cell type in mild maculopapular rashes and are also involved in the elicitation of DRESS (see chapter 1.6.2). Thus, it was of interest to explore whether CBZ-specific TCCs of both CD4 and CD8 phenotypes were activated in a HLA restricted manner.

## 2.2 Materials & Methods

### 2.2.1 Patient Characteristics

The carbamazepine hypersensitive patient was recruited retrospectively at the Royal Liverpool University Hospital. The study was approved by the local ethics committee and informed consent obtained from the patient. The patient was a 74-year old female who had suffered a hypersensitivity reaction to carbamazepine 22 years ago. She had developed a hypersensitivity syndrome to CBZ six days after treatment initiation and the clinical details are summarised in Table 2.1. Previously performed HLA genotyping had shown that the patient was a carrier of *HLA-A*31:01*.

### 2.2.2 Chemicals

Carbamazepine (Sigma-Aldrich, UK) was prepared as stock solution (10mg/ml) in T-cell medium containing 10% dimethyl sulfoxide (DMSO; Sigma, UK). The stock solutions was made up fresh and diluted to the appropriate concentration directly before use. Tetanus toxoid was obtained from the Statens Serum Institut in Denmark. All other reagents were purchased from Sigma-Aldrich, UK, unless stated otherwise.

**Table 2.1**: Clinical data of the CBZ hypersensitive patient.

| | |
|---|---|
| **Gender** | Female |
| **Age (years)** | 74 |
| **Type of reaction** | Generalized maculopapular rash with fever, eosinophilia and lymphocytosis |
| **Time to reaction (days)** | 6 |
| **Time since reaction (years)** | 22 |
| **Rechallenge** | No |
| **HLA genotype** | A*11:01/31:01<br>B*27:05/40:01<br>C*01:02/03:04<br>DRB1*03:01/04:04<br>DQB1*02:01/03:02 |

### 2.2.3 Cell culture medium

T-cell culture medium consisted of RPMI 1640 medium supplemented with 10% pooled human AB serum (Innovative Research, USA), 25mM HEPES buffer, 2mM L-glutamine, 100μg/ml streptomycin, 100U/ml penicillin and 25μg/ml transferrin. To maintain T-cells in long-term culture, the medium was supplemented with 200U/ml IL-2 (Preprotech, UK).

B-cell medium comprised of RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS; Invitrogen, UK), 25mM HEPES buffer, 100μg/ml streptomycin and 100U/ml penicillin.

### 2.2.4 Lymphocyte isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of the patient by density gradient centrifugation. The blood was carefully layered on an equal volume of Lymphoprep (Axis-Shield, UK) and spun down at 2000rpm for 25min. The layer of PBMCs was extracted with a Pasteur pipette

and washed twice in Hanks balanced salt solution (HBSS) to remove any remaining Lymphoprep. Isolated lymphocytes were resuspended in T-cell culture medium.

### 2.2.5 Generation of antigen-presenting cell lines

Epstein-Barr virus (EBV) transformed B-lymphoblastoid cell lines (B-LCLs) were used as antigen-presenting cells. B-LCLs were generated by transformation of PBMCs with supernatant (SN) from the EBV-producing B95-8 cell line (Neitzel 1986). PBMCs (5x $10^6$) were incubated overnight with 5ml of filtered (0.45µm syringe filter) EBV-containing B95-8 SN at 37°C. Cyclosporine A (CSA) was added at 1µg/ml to inhibit T-cell proliferation. On the next day, cells were spun down (1500rpm, 5min), resuspended in B-cell medium supplemented with 1µg/ml CSA and plated into a 24-well plate (2.5- 10x $10^5$ cells; 2ml). Medium was exchanged twice weekly. CSA treatment was stopped after 2-3 weeks and cells transferred to tissue culture flasks.

### 2.2.6 Lymphocyte transformation test

The lymphocyte transformation test (LTT) represents an *in vitro* method to determine whether drug-specific lymphocytes are present in the blood of hypersensitive patients (Pichler and Tilch 2004).

Isolated PBMCs (1.5x $10^5$; 100ul) were cultured with CBZ (1-100µg/ml; 100ul) in a 96-well U bottom plate in triplicate for six days. Tetanus toxoid (TT; 1µg/ml) was used as positive control. [$^3$H]-thymidine (Moravek, USA) was added for the final 16 hours of incubation. Cells were harvested and proliferation determined by [$^3$H]-thymidine incorporation using a MicroBeta TriLux β-counter (Perkin Elmer, USA). Proliferative responses were expressed as stimulation index (SI) using the following formula: SI= cpm of drug-treated cultures/ cpm of control. An SI value above 2 was considered a positive response (Nyfeler and Pichler 1997).

### 2.2.7 T-cell enrichment culture

Drug-specific T-cells were enriched by stimulating lymphocytes with CBZ repeatedly over four weeks as described previously (Naisbitt *et al.* 2003a). PBMCs (5x $10^6$, 1ml) were cultured with CBZ (25μg/ml) in a 24-well plate for 7 days. IL-2 containing T-cell medium was added on day 3. On day 7, cells were restimulated with autologous irradiated PBMCs (1x $10^6$) and CBZ (25μg/ml). Three cycles of restimulation were performed, and wells were split when necessary to support proliferation.

### 2.2.8 $^{51}$Cr-release cytotoxicity assay

The cytotoxic activity of T-cell enriched PBMC cultures was assessed *in vitro* in a $^{51}$Cr-release assay using previously established methods (Brunner *et al.* 1968; Dunkley *et al.* 1974).

Autologous B-LCLs (1.5x $10^6$) were incubated with $^{51}$Chromium (50μCi; PerkinElmer, UK) for 1 hour and then washed three times to remove any free $^{51}$Cr. Radioactive labelled B-LCLs (2.5x $10^3$) were mixed with PBMCs (1- 10x $10^4$) and incubated for 4 hours at 37°C in the presence and absence of CBZ (10-50μg/ml). T-cell mediated killing of B-LCLs was determined by the amount of $^{51}$Cr released into the supernatant and measured by liquid scintillation counting. The specific lysis was calculated using the following formula:

(cpm CBZ treatment – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release) x 100.

### 2.2.9 Generation of drug-specific T-cell clones

Initially, PBMCs (1x $10^6$, 1ml) were cultured in T-cell medium containing 25μg/ml CBZ for 14 days in a 48-well plate at 37°C. On day 6 and day 9, IL-2 supplemented medium was added to the cultures. On day 14, T-cells were cloned by serial dilution using established methodology (Naisbitt *et al.* 2003a). Cells were seeded into 96-well U bottom plates at concentrations of 0.5, 1.0 and 3.0 cells per well. Allogeneic irradiated PBMCs (5x $10^5$), phytohemagglutinin

(PHA, 5ug/ml) and IL-2 (200U/ml) were added to stimulate T-cell growth. If necessary a further restimulation cycle was performed after two weeks. Well-growing clones were expanded in new plates and split when necessary. Drug specificity of TCCs was assessed by T-cell proliferation assay.

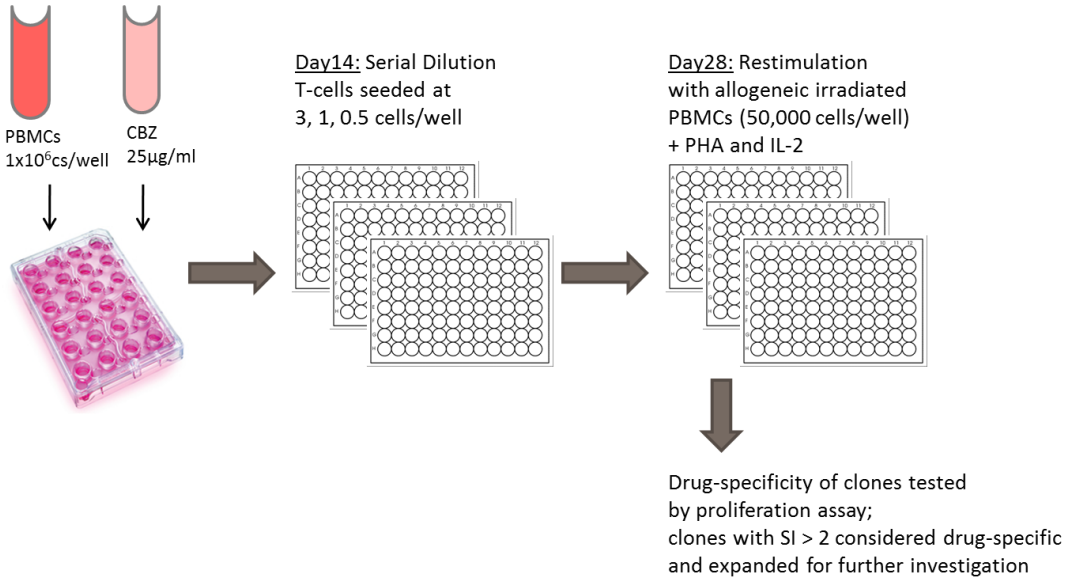



**Figure 2.1**: T-cell cloning procedure.

PBMCs were cultured for 14 days with drug, then serial diluted at cell densities of 3, 1 and 0.5 cells per well. T-cell clones were restimulated with allogeneic irradiated APCs after another 14 days if necessary. Well-growing clones were picked into new plates and expanded. Drug-specificity was tested by proliferation assay 2 weeks later.

Drug-specific clones were restimulated every 2-3 weeks as described above in order to maintain T-cell proliferation.

In subsequent cloning experiments, T-cell bulks were separated into $CD4^+$ and $CD8^+$ T-cells prior to serial dilution using magnetic cell sorting beads (Miltenyi Biotech, UK). T-cells were incubated with CD8 Multisort microbeads and separated over a magnetic column. The flow through contained the unlabelled $CD4^+$ T-cells. After removal of the column from the magnetic field, $CD8^+$ T-cells were flushed out using a plunger.

### 2.2.10 T-cell proliferation assay

The T-cell proliferation assay was used to determine the drug-specificity and functionality of the T-cell clones. T-cells (5x $10^4$) were incubated with autologous, irradiated B-LCLs (1x $10^4$) and CBZ (10- 50µg/ml) in a 96-well U bottom plate. After 48 hours, [$^3$H]-thymidine (0.5µCi) was added and proliferation measured by scintillation counting 16 hours later as described in 2.2.6. TCCs with an SI above 2 were considered to be drug-specific.

To test for HLA restriction the protocol was modified in two different ways. First, autologous B-LCLs were incubated with human anti-HLA blocking antibodies (anti-MHC I, anti-MHC II, anti-HLA DR, anti-HLA DQ - all BD Bioscience, UK; anti-HLA DP and anti-HLA A30/A31 - Abcam, UK) before being added to the proliferation assay.

Second, T-cell clones were cultured with CBZ in the presence of allogeneic APCs expressing HLA alleles of interest. PBMCs of HLA-typed healthy volunteers were used to generate allogeneic B-LCLs via the method described in 2.2.5. The healthy volunteers were all part of the HLA-typed cell archive established at the University (Alfirevic *et al.* 2012) and their respective HLA genotypes are shown in Table 2.2. In another experimental set-up, HLA-DRB1*04:04-transfected T2-cell lines (kindly donated by the laboratory of Prof. James McCluskey, University of Melbourne, Australia) were used as APCs. Wild type T2 cells are a B-lymphoblast cell line that only expresses HLA-A2 but no HLA class II alleles.

### 2.2.11 CD phenotyping

CD phenotype of clones was determined by flow cytometry. TCCs were stained with phosphatidylethanolamine (PE) labelled anti-CD4 and fluorescein isothiocyanate (FITC) labelled anti-CD8 antibodies for 20min at 4°C. Clones were washed and resuspended in HBSS containing 1% FBS and 0.02% sodium azide. Fluorescence was measured on a BD FACS Canto II flow cytometer recording a minimum of 10,000 events, and data analysed using Cyflogic software (CyFlo Ltd., Finland).

**Table 2.2**: HLA genotypes of healthy volunteers selected for this study.

| ID | HLA-A | HLA-B | HLA-C | HLA-DRB1 | HLA-DQB1 |
|---|---|---|---|---|---|
| **HV2** | 02:01/31:01 | 15:01/44:02 | 03:03/05:01 | 01:01/08:01 | 05:01/06:02 |
| **HV4** | 03:01/11:01 | 27:05/47:01 | 02:02/06:02 | 03:01/04:04 | 02:01/03:02 |
| **HV5** | 02:01/31:01 | 15:05/40:06 | 03:03/15:02 | 04:04/14:04 | 03:02/05:03 |
| **HV6** | 02:01/24:02 | 08:01/15:07 | 03:03/07:01 | 03:01/04:04 | 02:01/03:02 |
| **HV7** | 24:02/31:01 | 14:02/40:01 | 02:02/03:04 | 01:02/04:04 | 03:02/05:01 |
| **HV8** | 02:01/03:01 | 07:02/44:02 | 07:04/07:02 | 03:01/04:04 | 02:01/03:02 |
| **HV13** | 24:02/31:01 | 55:01/56:01 | 01:02/03:03 | 04:01/12:01 | 03:02/03:01 |
| **HV16** | 01:01/02:01 | 44:02/57:01 | 05:01/06:02 | 01:01/04:04 | 03:03/05:01 |
| **HV17** | 02:01/03:01 | 07:02/57:01 | 06:02/07:02 | 15:01/07:01 | 03:03/06:02 |
| **HV20** | 29:02/31:01 | 07:02/15:01 | 03:03/07:02 | 13:01/14:01 | 05:03/06:03 |
| **HV23** | 02:02/31:01 | 27:05/51:01 | 02:02/15:02 | 16:01/13:01 | 05:02/06:03 |
| **HV24** | 11:01/31:01 | 40:01/51:01 | 03:04/15:02 | 04:04/04:08 | 03:02/03:01 |
| **HV28** | 02:01/33:01 | 14:02/52:01 | 08:02/15:02 | 01:02/04:04 | 03:02/05:01 |
| **HV29** | 02:01/25:01 | 07:02/18:01 | 07:02/12:03 | 15:01/13:01 | 06:02/06:03 |
| **HV34** | 01:01/26:01 | 08:01/35:01 | 04:01/07:01 | 01:03/03:01 | 02:01/05:01 |
| **HV36** | 01:01/02:01 | 08:01/44:04 | 07:01/16:01 | 03:01/11:01 | 02:01/03:01 |
| **HV38** | 01:01/26:01 | 40:01/57:01 | 03:04/06:02 | 01:01/13:01 | 05:01/06:03 |

### 2.2.12 Enzyme-linked immunospot assay

Cytokine secretion was determined by enzyme-linked immunospot (ELISpot) assay according to the manufacturer's protocol. IFN-γ, IL-13, granzyme B and perforin kits were purchased from Mabtech, Sweden; and FasL kit was obtained from Abcam, UK.

MultiscreenHTS filter plates (Millipore, UK) were coated with cytokine-specific capture antibody and incubated overnight at 4°C. Wells were washed the next day with sterile HBSS and blocked with T-cell medium for 30min (for FasL assay blocking was performed for 2 hours using 2% skimmed milk). T-cells (5x $10^4$, 50µl) together with autologous irradiated B-LCLs (1x $10^4$, 50µl) and CBZ (25 and 50µg/ml, 100µl) were added to the wells and incubated for 48 hours at 37°C. The plate was emptied and then washed five times with phosphate buffered saline (PBS) (for FasL assay 0.1% Tween-20 was added to PBS). Biotin-labelled detection antibody was added to the wells and incubated for 2 hours at room temperature (RT). After another washing step, streptavidin-alkaline phosphatase was added and incubated for one hour at RT. Spots were developed by addition of BCIP/NBT substrate until distinct spots were visible. An outline of the protocol is depicted in Figure 2.2. Colour development was stopped by washing under tap water. When plates had dried completely, spots were counted on the AID ELISpot reader.

### 2.2.13 Statistical analysis

Statistical analysis was performed using Mann-Whitney U test to compare T-cell responses of independent samples and Wilcoxon test for paired samples. Kruskal-Wallis H test was used for the assessment of multiple groups. Non-parametric tests were used due to small sample size which prevented a clear assessment whether the data follows the normal distribution. A p-value $< 0.05$ was considered significant.

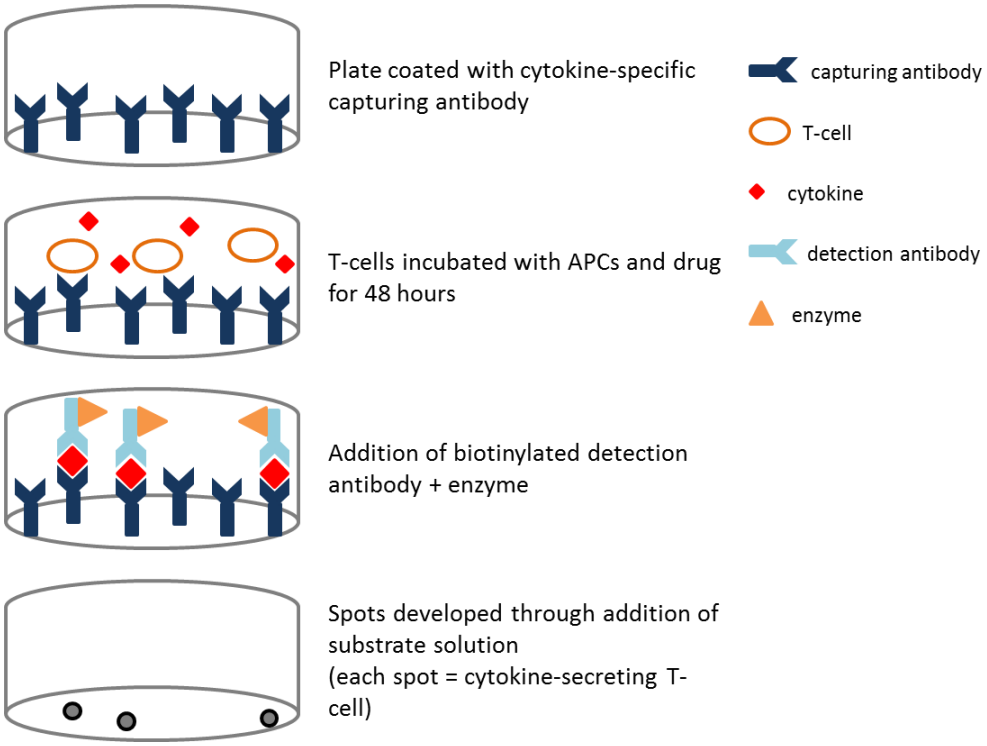



**Figure 2.2**: Schematic illustration of the ELISpot assay.

## 2.3 Results

### 2.3.1 *In vitro* response of PBMCs to carbamazepine

In order to confirm that a sensitisation of T-cells to CBZ had taken place earlier, and reactive T-cells were still present in the patient's circulation, the lymphocyte transformation test (LTT) was performed. Isolated PBMCs from the patient proliferated strongly in the presence of the drug, with a maximal stimulation index of 15.9 recorded at a concentration of 50µg/ml CBZ (Figure 2.3).

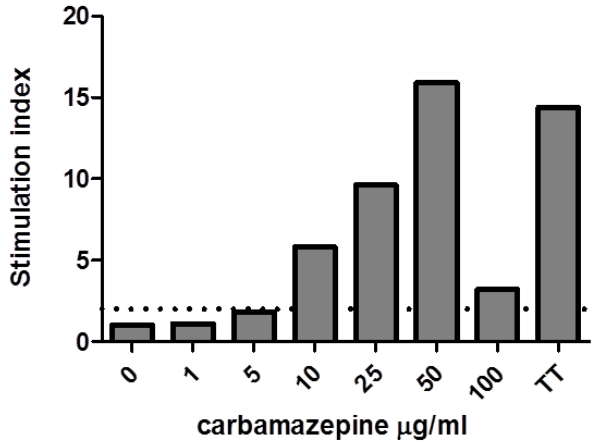
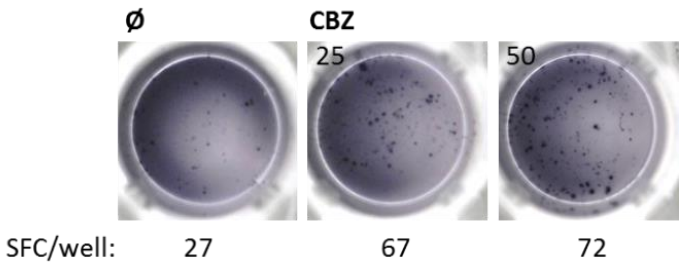



**Figure 2.3**: Lymphocyte transformation test.

PBMCs were incubated with CBZ (1-100µg/ml) in triplicate for 6 days, and [$^3$H]-thymidine added in the final 16 hours of the experiment. Proliferation was determined by scintillation counting. A stimulation index above 2 (dashed line) was considered a positive response. (TT= tetanus toxoid)

Drug-specific lymphocytes were enriched by weekly stimulation of PBMCs with CBZ over four weeks' time. A subsequently performed IFN-γ ELISpot assay showed that CBZ was able to stimulate an increase in IFN-γ secretion in the PBMC culture (Figure 2.4), indicating a successful expansion of drug-specific T-cells.

**Figure 2.4**: CBZ-specific secretion of IFN-γ after T-cell enrichment culture.




T-cell enriched PBMCs were incubated with CBZ and autologous B-LCLs. Cytokine secretion of drug-specific T-cells was visualised in an IFN-γ ELISpot assay following the manufacturer's protocol. (SFC= spot-forming cell)

The T-cell enriched PBMCs showed weak cytotoxic activity against autologous B-LCLs (Figure 2.5). T-cell mediated killing of $^{51}Cr$ labelled B-LCL was induced at CBZ concentrations above 25µg/ml and at an effector: target cell ratio of 40:1.

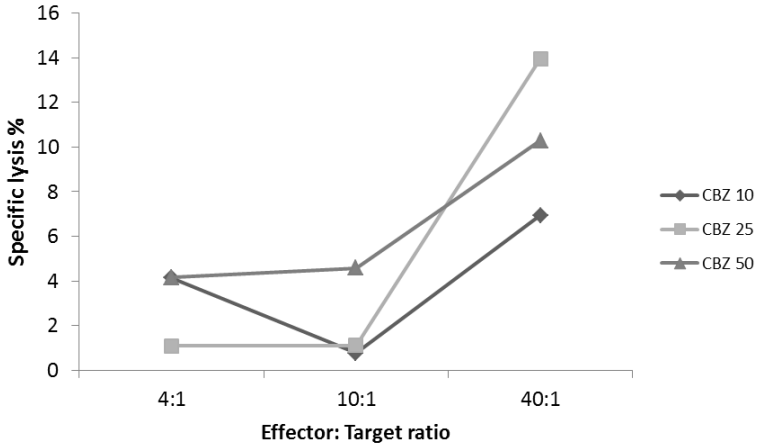



**Figure 2.5**: Cytotoxic activity of T-cell enriched PBMCs after three cycles of restimulation.

T-cell enriched PBMCs were incubated with CBZ (10- 50µg/ml) and $^{51}Cr$ loaded B-LCLs at the indicated effector: target cell ratios for 4 hours. Specific lysis was calculated as (cpm CBZ treatment – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release) x 100.

## 2.3.2 Characterisation of carbamazepine-specific T-cell clones

T-cell clones were generated by the serial dilution method as described above. In total, three rounds of T-cell cloning were carried out producing 947 clones. The third cloning attempt was performed after a magnetic cell separation of the CBZ-stimulated T-cell bulks into $CD4^+$ and $CD8^+$ T-cell populations. Only cloning of the $CD8^+$ population was performed in order to increase the number of available $CD8^+$ TCCs for further characterisation. Reactivity of the clones to CBZ was tested by 48-hour proliferation assay. Sixty-seven TCCs displayed robust proliferative responses to CBZ (control: 5,525.8 ± 18,928cpm; CBZ 25µg/ml: 34,418.8 ± 43,632.5cpm) and were therefore classified as drug-specific (Table

2.3). Drug-specific clones were phenotyped for CD expression by flow cytometry and almost equal numbers of $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ TCCs were detected (35%, 41% and 24% respectively; Table 2.3).

**Table 2.3**: Specificity and phenotype of drug-specific T-cell clones.

| | Clones tested (n) | Specific clones (n) | Proliferation (cpm) | | CD phenotype (%) | | |
|---|---|---|---|---|---|---|---|
| | | | control | CBZ (25µg/ml) | CD4+ | CD8+ | CD4+ CD8+ |
| **total** | **947** | **67** | **5,525.8 ±18,928.0** | **34,418.8 ±43,632.5** | **35** | **41** | **24** |
| **1** | 35 | 10 | 8,020.2 ±12,132.7 | 50,582.9 ±66,184.1 | 100 | 0 | 0 |
| **2** | 600 | 26 | 7,348.5 ±29,381.1 | 39,783.7 ±49,183.2 | 38 | 62 | 0 |
| **3** | 312 | 31 | 3,175.7 ±1,800.2 | 24,721.7 ±23,604.5 | -‡ | 40 | 60 |

Proliferation data presented as mean cpm ± SD. ‡) only CD8+ T-cell population was cloned.

T-cell clones proliferated to CBZ in a dose-dependent manner irrespective of their CD phenotype (Figure 2.6). However, proliferative responses were strongest in the $CD4^+$ population reaching a maximum of approximately 60,000cpm (25ug/ml CBZ; Figure 2.6 A) compared to about 20,000cpm for $CD8^+$ and $CD4^+CD8^+$ TCCs (Figure 2.6 B+C). This is in line with previous studies which have found $CD8^+$ TCCs to proliferate weakly upon CBZ stimulation compared to $CD4^+$ clones but instead show strong cytotoxic responses (Wu *et al.* 2007). Eleven well-growing $CD4^+$, five $CD8^+$, and ten $CD4^+CD8^+$ clones were selected for more detailed analyses.

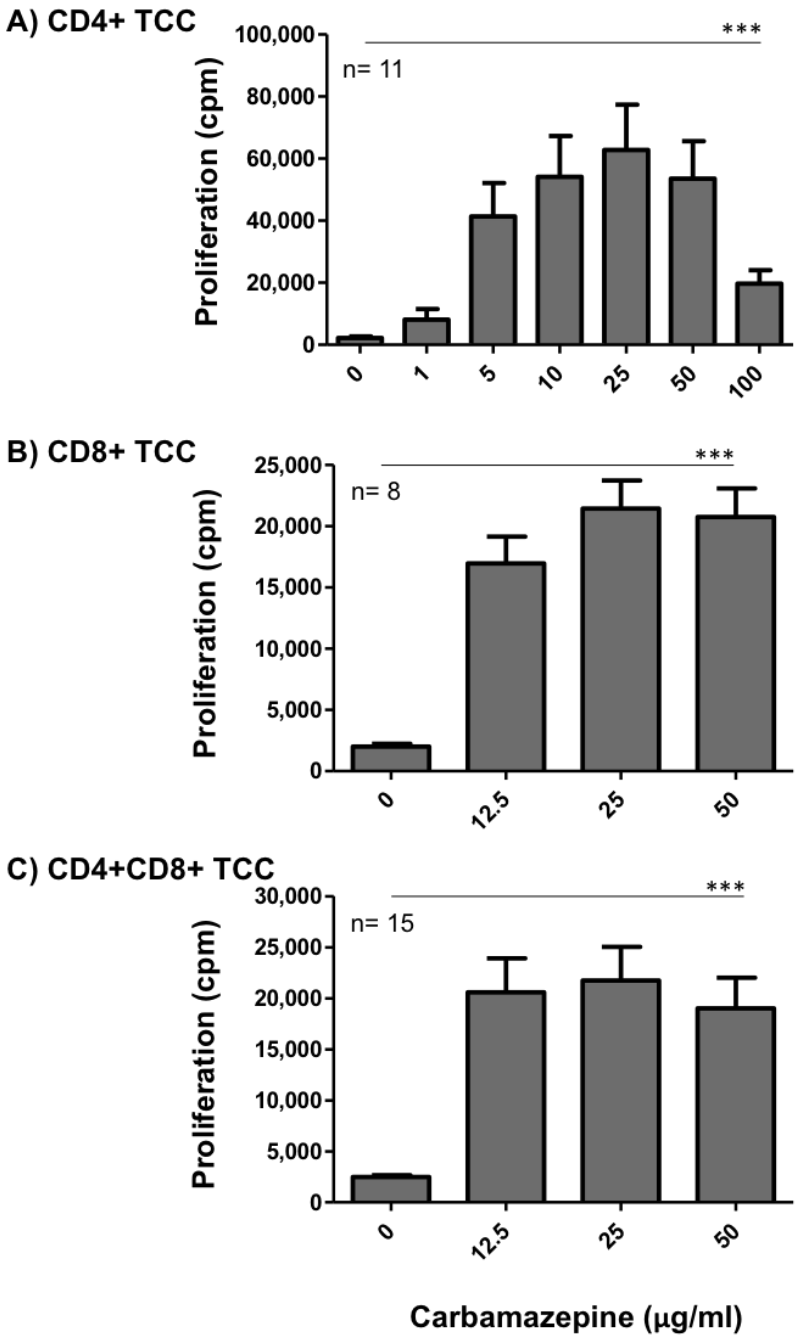



**Figure 2.6**: CBZ-dependent proliferation of drug-specific $CD4^+$ (A), $CD8^+$ (B), and $CD4^+CD8^+$ (C) T-cell clones.

T-cell clones were incubated with autologous irradiated B-LCLs and carbamazepine at indicated concentrations for 48 hours. Proliferation was determined by [$^3$H]-thymidine uptake. Data represents mean cpm ± SEM of n clones. Statistical analysis was performed using Kruskal-Wallis H test for comparison of multiple doses (***$p < 0.001$).

To assess the functionality of the different phenotypic clones, the secretion of cytokines and cytolytic molecules was determined by the ELISpot technique (Figure 2.7). $CD4^+$ TCCs secreted significant amounts of type 1 and type 2 cytokines, i.e. IFN-γ ($p= 0.02$) and IL-13 ($p= 0.02$) respectively, suggesting a mixed Th1/Th2 type effector population (Figure 2.7 A). $CD4^+$ clones displayed cytotoxic effector function by secretion of FasL ($p= 0.02$), and some clones also exhibited cytotoxicity through the release of granzyme B and perforin. $CD8^+$ T-cell responses were associated with strong secretion of IFN-γ ($p= 0.02$), and high levels of cytotoxicity through the release of granzyme B, perforin and FasL ($p=0.05$, 0.01, and 0.004 respectively; Figure 2.6 B). The effector phenotype of $CD4^+CD8^+$ TCCs resembled the $CD8^+$ population. $CD4^+CD8^+$ TCCs secreted high levels of IFN-γ ($p= 0.004$), and only a few clones secreted low amounts of IL-13. Cytotoxic activity was demonstrated by high levels of FasL and perforin secretion ($p= 0.006$ and 0.005 respectively; Figure 2.6 C). Granzyme B secretion did not increase significantly because of high background levels.

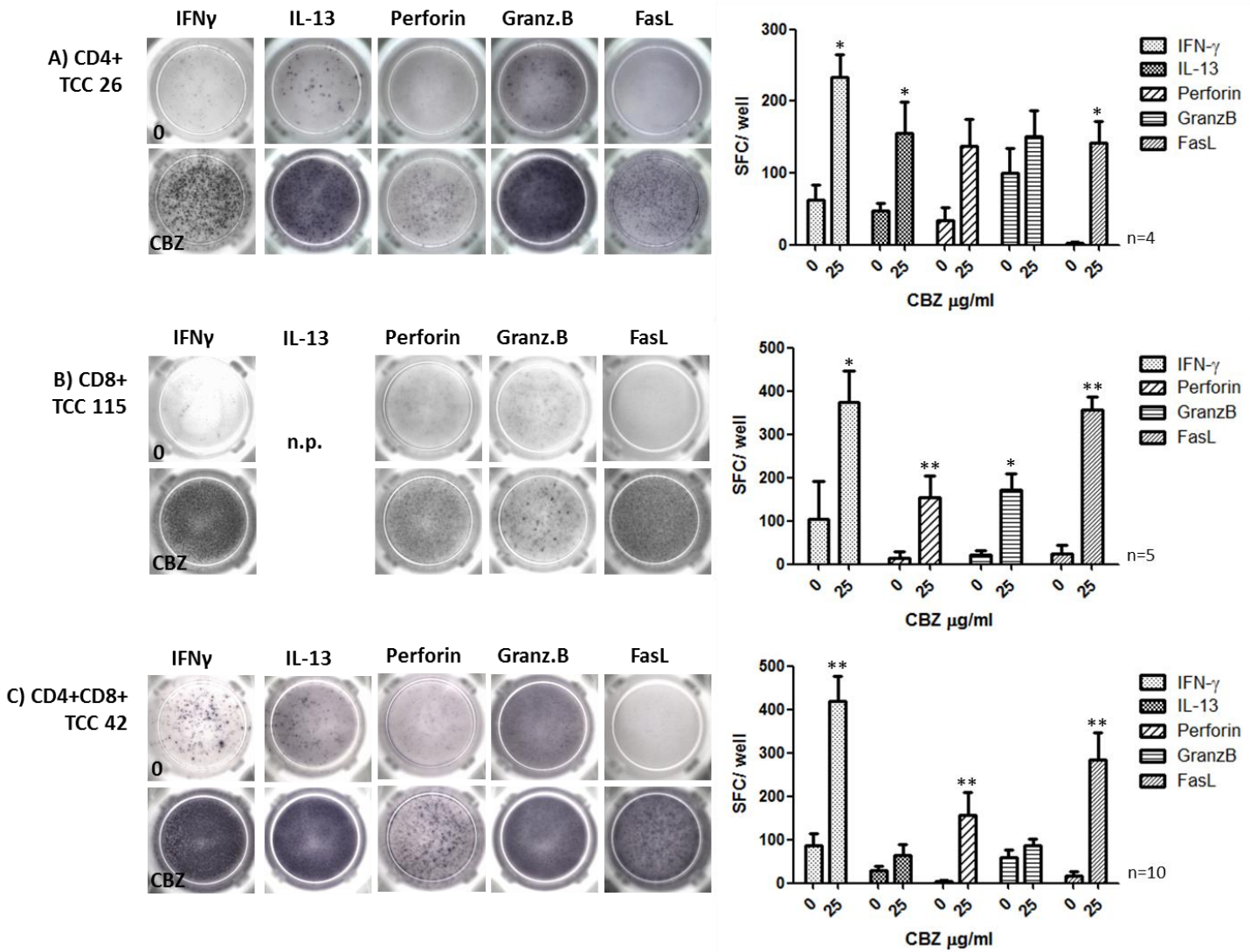



**Figure 2.7**: Secretion of cytokines and cytolytic molecules from $CD4^+$ (A), $CD8^+$ (B), and $CD4^+CD8^+$ (C) TCCs after CBZ stimulation using ELISpot assay.

Well images show cytokine profile of a representative TCC for each phenotype. Graphs represent mean SFC per well ± SEM of n clones. Statistical analysis was performed by comparing SFC between drug treated and untreated wells using Mann-Whitney U test (*p< 0.05, **p< 0.01).

(SFC= spot forming cells; n.p. = not performed)

### 2.3.3 HLA restriction of carbamazepine-specific T-cell clones

For the detailed investigation whether recognition of CBZ by drug-specific TCCs was HLA restricted further analysis concentrated on the $CD4^+$ and $CD8^+$ clones.

In a first step restriction by MHC class I and class II was tested. T-cell clones were stimulated with CBZ in the presence of autologous B-LCLs which had been pre-treated with human anti-MHC antibodies to inhibit presentation of the drug by MHC molecules expressed on the cell surface. $CD4^+$ T-cell responses to CBZ were clearly MHC class II restricted, as addition of anti-MHC II antibody, but not anti-MHC I, inhibited proliferation (Figure 2.8 A). The response of $CD8^+$ clones was restricted by MHC class I (Figure 2.8 B). However, as the $CD8^+$ clones could be stimulated by CBZ in the absence of B-LCLs, an effect known as self-presentation, the results were not as pronounced as for $CD4^+$ clones. When MHC blocking antibodies were added to both B-LCLs and TCCs, minor proliferative responses could still be detected.

The fine-specificity of the HLA class II restriction observed in $CD4^+$ clones was determined using blocking antibodies against HLA-DR, -DQ and ,-DP respectively. CBZ-induced proliferation of $CD4^+$ TCCs could be suppressed by antibodies against HLA-DR and –DP (Figure 2.9). With a few exceptions, blocking of HLA-DQ had a minor effect on T-cell proliferation of $CD4^+$ clones.

As there are currently no commercial antibodies available to test for HLA-A, -B, and-C restriction, a blocking antibody specific for HLA-A30/A31 was used instead to test recognition of CBZ by $CD8^+$ clones. Proliferation of CBZ-specific $CD8^+$ clones was suppressed in the presence of anti-HLA-A30/A31 antibody ($p= 0.003$, Figure 2.10). These results suggest a HLA-A31 restriction, since the patient was a carrier of HLA-A*31:01, but not A*30 (see Table 2.1). However, proliferative responses could not be abrogated completely, which was partially due to strong self-presentation of $CD8^+$ TCCs (see Appendix F1) and required a suboptimal CBZ concentration to be used in the assay. Also, proliferation of some $CD8^+$ TCCs was inhibited more strongly by blocking antibodies against MHC class I, suggesting that other factors may also be involved in the presentation of CBZ.

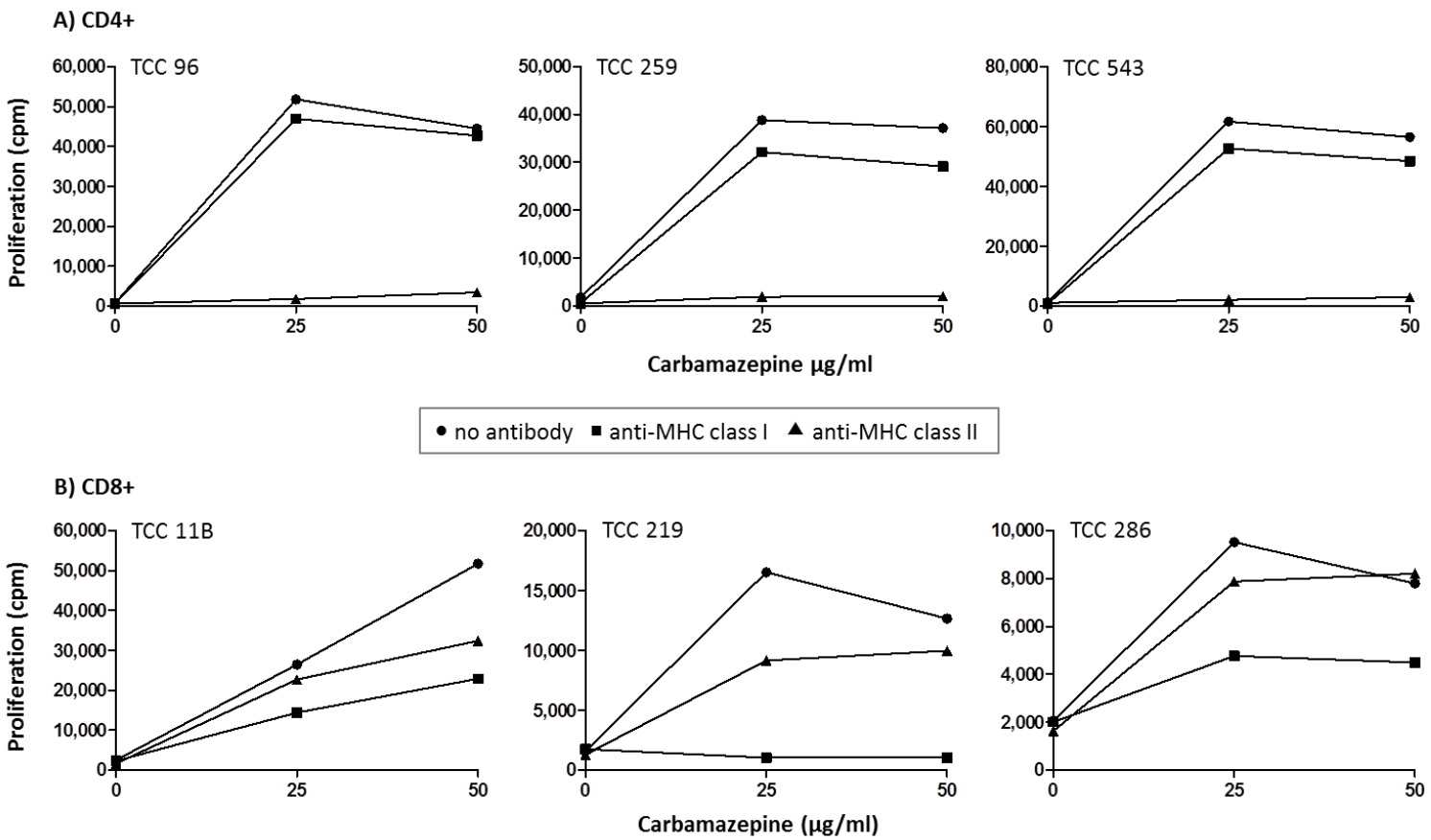



**Figure 2.8**: MHC restriction of CBZ-specific CD4+ (A) and CD8+ (B) TCCs.

Autologous irradiated B-LCLs were incubated with antibodies against MHC class I and class II respectively. Pre-treated B-LCLs were then added to TCCs and CBZ in a 48 hour proliferation assay. Results are given as mean cpm of duplicate cultures.

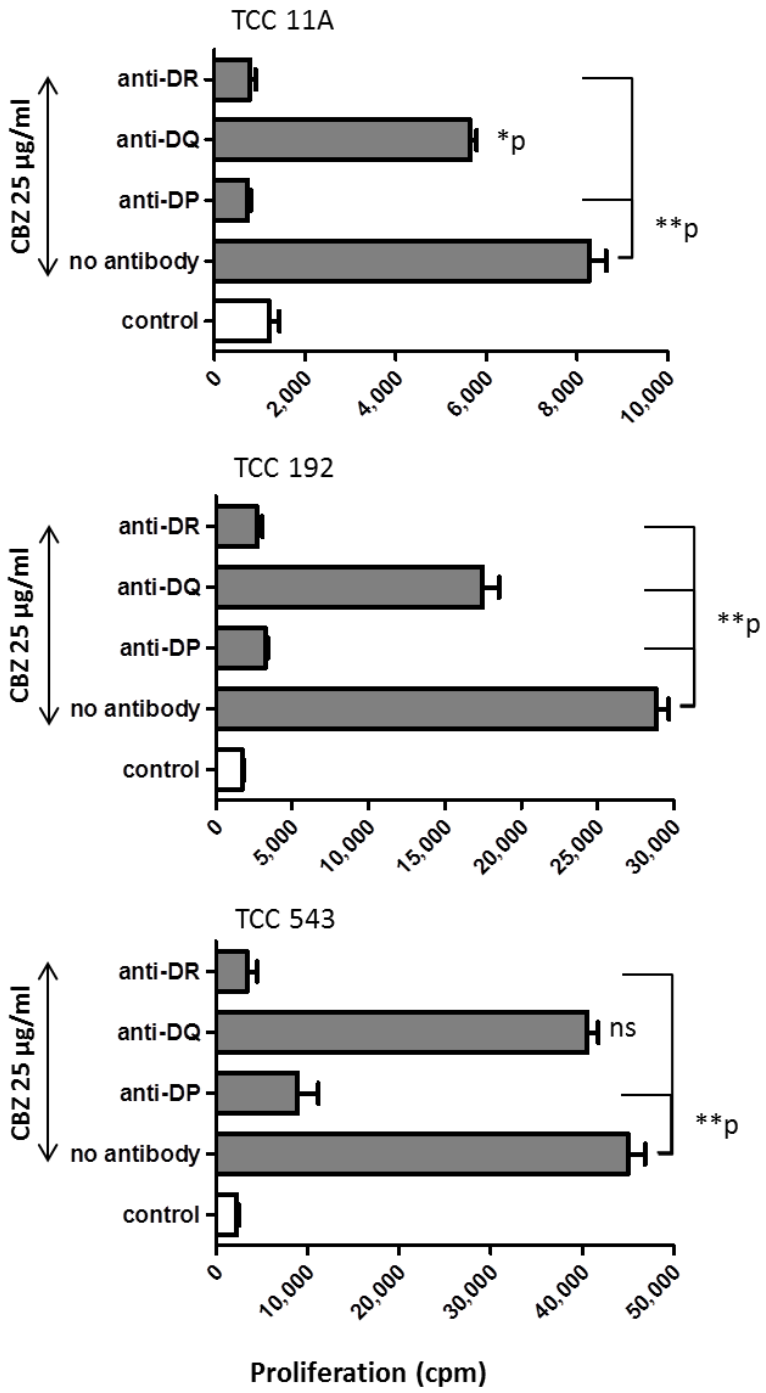



**Figure 2.9**: Restriction of $CD4^+$ clones by HLA class II subtypes.

TCCs were cultured with CBZ and autologous irradiated B-LCLs which had been pre-incubated with blocking antibodies against HLA-DR, -DQ, and –DP respectively. Proliferation assay was used as read-out. Data represent mean cpm ± SEM of triplicate cultures. Statistical analysis was performed comparing cultures without antibody to those treated with HLA blocking antibody (*p< 0.05, **p< 0.01, ns=not significant).

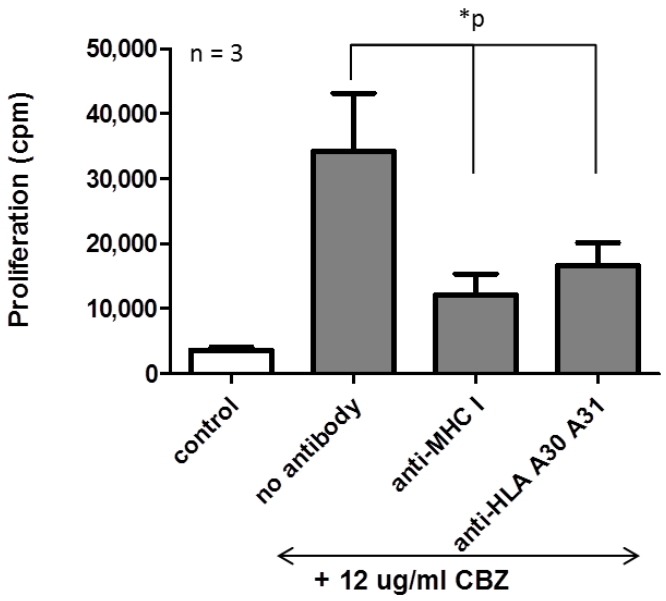



**Figure 2.10**: Recognition of CBZ by $CD8^+$ clones in the presence of HLA-A30/A31 blocking antibody.

Autologous irradiated B-LCLs were treated with antibodies against either MHC class I or HLA-A30/A31. TCCs were incubated with B-LCLs and CBZ, which was added at a suboptimal concentration of 12µg/ml to avoid self-presentation. Proliferation was determined by [$^3$H]-thymidine incorporation. Results are presented as mean cpm ± SEM of n clones. Statistical analysis was performed using Wilcoxon test for the comparison of paired samples (*$p < 0.05$).

As a next step, allogeneic HLA-matched B-LCLs were used to further assess the role of HLA-A*31:01 on the activation of $CD8^+$ TCCs by CBZ. B-LCLs were generated from PBMCs of HLA-typed healthy volunteers which are part of an in-house cell bank specifically set up to investigate HLA-associated ADRs (Alfirevic *et al.* 2012). Healthy volunteers expressing either HLA-A*31:01 or control HLA-A alleles, which are common in Caucasians and not reported in association with CBZ hypersensitivity (HLA-A*01:01, -A*02:01, or -A*03:01), were selected for the generation of B-cell lines. The complete HLA genotypes of the different B-LCLs are shown in Table 2.2.

CD8+ clones were strongly activated by CBZ in the presence of autologous B-LCLs and allogeneic *HLA-A*31:01*+ B-LCLs (Figure 2.11). CBZ-specific responses in cultures containing B-LCLs expressing other HLA-A alleles were generally weaker, and in some cases below the threshold of self-presentation which were therefore considered to be negative.

Interestingly, when some $CD4^+$ clones were incubated with allogeneic *HLA-A*31:01*+ B-LCLs, these clones also started to proliferate upon CBZ stimulation (data not shown). Since $CD4^+$ TCCs had previously been shown to be HLA class II restricted, the complete HLA haplotype of the antigen-presenting cell lines was taken into consideration and analysed for any overlap in HLA class II. The *HLA-DRB1*04:04* allele was found to be present in all *HLA-A*31:01*+ B-cell lines which induced $CD4^+$ T-cell proliferation. This corresponded with the HLA class II restriction data showing preferential HLA-DR restriction. After consulting Allele Frequency Net Database (www.allelefrequencies.net, (Gonzalez-Galarza *et al.* 2011)), *HLA-DRB1*04:04* was confirmed to be part of a common haplotype with *HLA-A*31:01* in Caucasians.

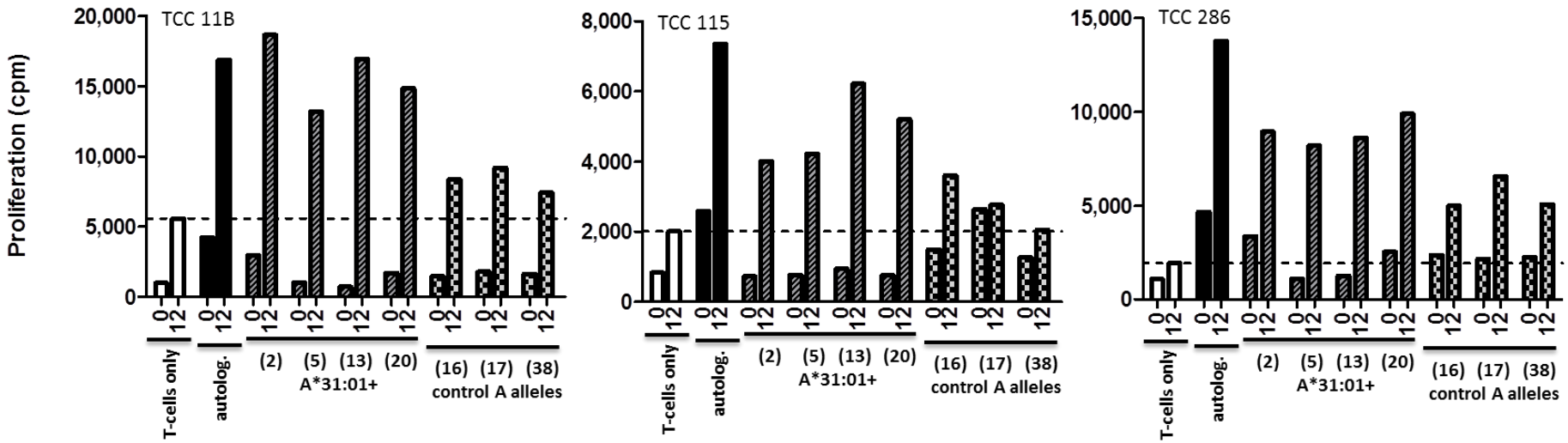



**Figure 2.11**: Reactivity of $CD8^+$ TCCs to CBZ presented on allogeneic HLA-matched B-LCLs.

T-cells were incubated with CBZ (12μg/ml) and partially HLA-matched B-LCLs, and proliferation was determined in a 48-hour proliferation assay. To assess the strength of self-presentation, T-cells were also cultured in the absence of B-LCLs (white bars). Proliferation above counts of self-presentation (dashed line) was considered a positive response. Results are shown as mean cpm of duplicate cultures.

In order to investigate whether CBZ-specific CD4+ T-cell responses were HLA-DRB1*04:04-restricted, studies using partially HLA-matched B-LCLs were performed. CD4+ clones were incubated with CBZ and allogeneic B-LCLs expressing either HLA-A*31:01, or HLA-DRB1*04:04, or both alleles. Antigen-presenting cells negative for both alleles were used as controls.

CBZ-specific CD4+ clones were stimulated to proliferate in the presence of B-LCLs expressing HLA-A*31:01 and HLA-DRB1*04:04, but also *HLA-DRB1*04:04*+ B-LCLs lacking HLA-A*31:01 (Figure 2.12). No CBZ-induced T-cell proliferation was seen in cultures containing B-LCLs expressing only HLA-A*31:01 or in the *HLA-A*31:01* negative and *DRB1*04:04* negative controls. However, some antigen-presenting cell lines serving as controls were able to stimulate a non-specific proliferative response in CD4+ clones, which was independent of CBZ as it could also be detected in drug-free cultures. The antigenic stimulus causing these responses is currently unknown.

HLA-DRB1*04:04 restriction of CD4+ clones could be further validated through the use of B-cell lines which had been specifically transfected with *HLA-DRB1*04:04* (Figure 2.13). CD4+ clones proliferated vigorously when stimulated with CBZ in the presence of *HLA-DRB1*04:04*-transfected T2-cells. However, T-cell activation was only seen in cultures containing antigen-presenting cells that also expressed HLA-DM (Figure 2.13), indicating that the CLIP peptide prevents CBZ from binding to HLA-DR and being presented to T-cells.

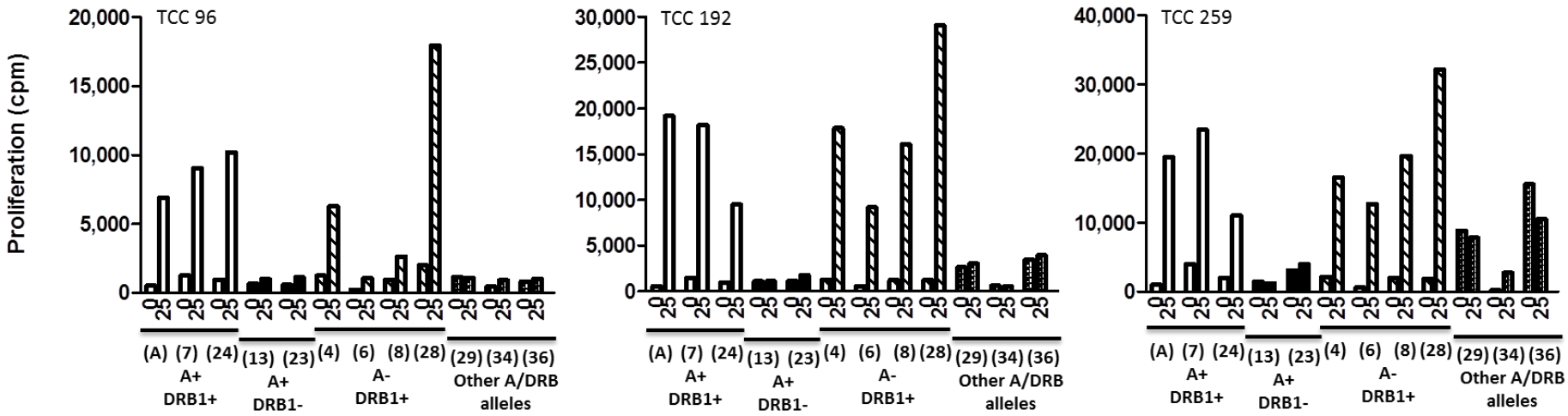



**Figure 2.12**: CBZ-specific activation of $CD4^+$ T-cell clones with allogeneic HLA-matched B-LCLs.

T-cells were incubated with CBZ (25µg/ml) and partially HLA-matched B-LCLs. Proliferation was measured by $[^3H]$-thymidine incorporation. Results are shown as mean cpm of duplicate cultures. (A+ = positive for HLA-A*31:01, DRB1+ = positive for HLA-DRB1*04:04)

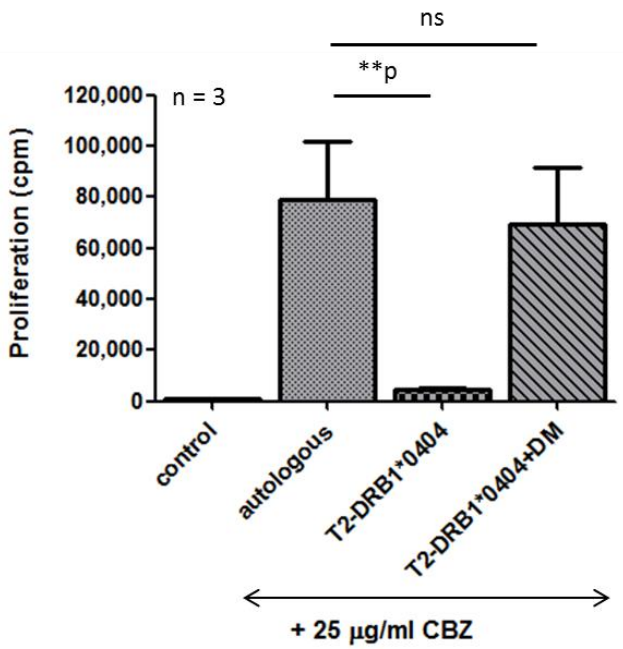



**Figure 2.13**: CBZ-specific proliferation of $CD4^+$ clones in the presence of HLA-DRB1*04:04 transfected B-cells.

T-cells were incubated with CBZ and HLA-DRB1*04:04 transfected T2 cell lines. Proliferation was determined by [$^3$H]-thymidine uptake. Data represent mean cpm ± SEM of n clones. Statistical analysis was performed using Mann-Whitney U test (**p< 0.01, ns= not significant).

## 2.4 Discussion

In this chapter, carbamazepine-specific T-cells were isolated from a CBZ hypersensitive patient expressing HLA-A*31:01 and subsequently used to explore whether drug-specific T-cells displayed HLA restriction and if HLA-A*31:01 was functionally involved in T-cell activation.

A positive lymphocyte transformation test with a maximal SI of 15.9 confirmed a hypersensitivity reaction to carbamazepine *in vitro* and that drug-specific T-cells were still present in the patient's blood even many years after resolution of clinical symptoms. Isolated T-lymphocytes displayed effector functions when

stimulated with CBZ, which was demonstrated by drug-induced IFN-γ secretion and killing of autologous B-LCLs.

Drug-specific T-cell clones were generated using established *in vitro* cloning methods. However, it has previously been shown that this leads to the preferred expansion of $CD4^+$ clones, i.e. approximately 90% of TCCs express the CD4 co-receptor (Naisbitt *et al.* 2003a; Wu *et al.* 2007). The reasons are not entirely clear, but might be explained in part by the reduced proliferation capacity of $CD8^+$ T-cells resulting in decreased T-cell expansion rate (Wu *et al.* 2007). A revised cloning protocol, including magnetic cell separation of the different T-cell phenotypes prior to serial dilution, was therefore used in order to increase the number of $CD8^+$ clones available for functional studies. As a result, almost equal numbers of CBZ-specific $CD4^+$ and $CD8^+$ clones (35% and 41% respectively) were obtained. The clones displayed a concentration-dependent proliferation profile and cytotoxic effector functions. Similar to previous studies (Wu *et al.* 2007), $CD4^+$ T-cells proliferated vigorously when stimulated with CBZ but showed limited cytotoxicity, whereas $CD8^+$ T-cell activation was characterised by high levels of cytotoxic molecules and low proliferation rates.

Stimulation of CBZ-specific $CD4^+$ and $CD8^+$ T-cells was restricted by MHC class II and MHC class I respectively. However, the results for $CD8^+$ clones were partially obscured by self-presentation. Drug presentation between T-cells without the need for APCs has been reported previously (von Greyerz *et al.* 2001), but the origin and function of self-presenting T-cells has not yet been resolved. Attempts to overcome self-presentation by blocking MHC on both T-cells and B-LCLs were only marginally successful (data not shown), which might be due to up-regulation of MHC following T-cell activation (Van Den Elsen *et al.* 2004).

HLA restriction experiments using an antibody directed against HLA-A30/A31 demonstrated for the first time that drug-specific $CD8^+$ T-cells isolated from an *HLA-A*31:01* positive individual respond to CBZ in a HLA-A31 restricted manner *in vitro*. For some clones blocking of MHC class I seemed to inhibit proliferation more effectively. This might be due to a higher binding affinity of

the antibody or alternatively indicate that additional factors might facilitate presentation of CBZ to $CD8^+$ T-cells.

Studies with HLA-matched B-LCLs support a functional role of *HLA-A*31:01* in activating CBZ-specific $CD8^+$ clones. CBZ-induced proliferation of $CD8^+$ clones was strongest when *HLA-A*3101*+ B-LCLs were present. However, to a lesser extend T-cell activation was also seen with B-LCLs expressing other HLA-A alleles, which again suggests CBZ might not be exclusively recognized in the context of HLA-A*31:01.

Responses of $CD4^+$ clones were restricted mainly by HLA-DR and –DP. Blocking of HLA-DQ affected $CD4^+$ T-cell proliferation to a minor extent and was only observed in a few clones. Previous studies had shown that the majority of drug-specific $CD4^+$ TCCs respond to CBZ in a HLA-DR restricted manner, despite a few clones which had been restricted by HLA-DQ (Naisbitt *et al.* 2003a). However, no restriction by HLA-DP had been observed. The function of HLA-DP in autoimmune disorders has been far less studied than that of other HLA class II subtypes and HLA-DP is mostly omitted from HLA genotyping. This could explain why hardly any HLA-DP associations, beryllium hypersensitivity representing a rare exception (Dai *et al.* 2013), have been detected to date. Due to this knowledge gap, further experiments concentrated on investigating the role of HLA-DR in $CD4^+$ T-cell activation. However, it cannot be excluded that HLA-DP might be involved in CBZ presentation to $CD4^+$ T-cells.

Knowing that the patient is a carrier of *HLA-DRB1*04:04* and that this allele forms a common haplotype with *HLA-A*31:01* in Caucasians, HLA mismatch studies were performed with APCs expressing HLA-DRB1*04:04 and/or HLA-A*31:01 or control DRB1 alleles.

$CD4^+$ clones recognized CBZ presented by *HLA-A*31:01*+ *DRB1*04:04*+ B-LCLs, but also with B-LCLs expressing only HLA-DRB1*04:04 suggesting a functional role for HLA-DRB1*04:04. It is important to note that some B-LCLs induced non-specific proliferation of $CD4^+$ clones, which was observed in the absence of drug stimulation. This might be due to cross-reactivity of the drug-specific TCCs. As the generation of B-LCLs involves transformation with Epstein-Barr virus,

viral peptides may be produced by B-LCLs and presented in the context of MHC. If the TCR expressed on drug-specific T-cells is able to cross-recognise the viral peptides, this may lead to T-cell activation. This has been observed previously in some DRESS patients with reactions to drugs including carbamazepine, sulfamethoxazole and allopurinol (Picard *et al.* 2010). Another explanation might be that some clones display HLA alloreactivity, i.e. clones recognise allogeneic HLA molecules as antigenic structures and become activated. This has been reported in cases of Graft-versus-host disease where virus-specific lymphocytes could be isolated from patients showing cross reactivity against allogeneic HLA molecules (Amir *et al.* 2010). Virus specificity and HLA alloreactivity were mediated by the same TCR.

*HLA-DRB1*04:04* restricted recognition of CBZ by $CD4^+$ clones was further substantiated when HLA-transfected T2 cell lines were used as APCs. T2 wildtype cells lack HLA class II expression and only display HLA-A2 on their cell surface. In the presence of *HLA-DRB1*04:04* transfected T2 cells, $CD4^+$ clones proliferated strongly when stimulated with CBZ. This finding strengthens the assumption that HLA-DRB1*04:04 may be functionally relevant in CBZ-specific $CD4^+$ T-cell responses. Interestingly, T-cell proliferation was only observed if HLA-DM was expressed at the same time. As HLA-DM is responsible for the removal of the class II invariant chain peptide (CLIP) prior to antigen loading of HLA class II molecules, binding of CBZ to HLA-DRB1*04:04 seems to be hindered in the presence of CLIP. This might be due to insufficient binding affinity between CBZ and CLIP preventing the formation of a stable drug-peptide-MHC complex. Alternatively, CBZ may only bind to an empty HLA binding groove, thereby inducing a change to the peptide repertoire, which in turn causes T-cell activation. Further studies are needed in order to fully understand the implication of this observation.

In conclusion, CBZ-specific $CD4^+$ and $CD8^+$ T-cell clones could be generated from isolated PBMCs of a *HLA-A*31:01* positive hypersensitive patient. A HLA-A*31:01-dependent activation of $CD8^+$ clones could be demonstrated for the first time, implying a functional relevance of the genetic association. Additionally, a HLA class II allele, i.e. *HLA-DRB1*04:04*, was shown to be

functionally involved in $CD4^+$ T-cell activation. Genetic data from genome-wide association studies and next-generation sequencing studies have shown that *HLA-DRB1*04:04* was more prevalent in hypersensitive patients than in controls, but this does not reach statistical significance (unpublished data). The strong linkage disequilibrium between *HLA-A*31:01* and *DRB1*04:04* suggests a common haplotype may contribute to the multi-clonal response seen in Caucasian carbamazepine hypersensitive patients. The significance of the results presented in this chapter is strongly limited by the fact that the data is based on only one patient. Therefore further studies involving a larger patient cohort are needed in order to confirm the observations described here.

# Chapter III

## Stimulation of a primary immune response to carbamazepine in healthy volunteers

## Contents

## 3.1 Introduction

An array of *in vitro* assays have been developed to assist in the diagnosis of drug-induced hypersensitivity reactions (HSRs), alongside *in vivo* provocation and skin patch tests. *In vitro* tests are a safe alternative for patients by avoiding resensitisation or exacerbation of the reaction (Porebski *et al.* 2011). Additionally, *in vitro* assays allow testing of several drugs in parallel, which may help identify the causative drug and assess potential cross-reactivity to alternative medication. Furthermore, the assays can be used to gain further insight into the pathomechanisms implicated in the development of HSRs (Porebski *et al.* 2011).

The lymphocyte transformation test (LTT) represents the most common *in vitro* test in the diagnosis of HSRs (Nyfeler and Pichler 1997). It measures the proliferative response of T-cells isolated from peripheral blood of patients upon stimulation with a specific drug *in vitro*. The LTT shows high specificity (85-100%) in detecting the causative drug, but its sensitivity varies depending on the drug and clinical phenotype (Nyfeler and Pichler 1997). In severe forms of HSRs, such as Stevens-Johnson syndrome and toxic epidermal necrolysis, the LTT often fails to show a positive response (Kano *et al.* 2007; Tang *et al.* 2012). A more sensitive method to detect drug-specific T-cells *in vitro* involves the measurement of drug-induced cytokine release using enzyme-linked immunoassays, such as ELISA or ELISpot (Porebski *et al.* 2011). In a study by Rozieres and colleagues, increased secretion of IFN-γ could be detected in T-cells isolated from amoxicillin-hypersensitive patients, but not in control patients (Rozieres *et al.* 2009). The analysis of IL-5 levels in combination with IFN-γ, IL-13 or IL-2, following stimulation of T-cells with drug *in vitro*, has been proposed a valuable diagnostic tool in HSRs diagnosis, as the release of these cytokines seems to be independent of the class of drug and clinical phenotype (Lochmatter *et al.* 2009). Furthermore, up-regulation of cell surface markers, e.g. CD69, has been demonstrated to be a suitable method for the detection of drug-specific T-cells *in vitro* (Beeler *et al.* 2008).

However, all of the above described methods assess the reactivity of drug-specific memory T-cells which have been originally primed *in vivo*. In order to study immune responses to drugs using naive T-cells from healthy volunteers, a different approach is required. Naïve T-cells have to be primed *in vitro* with the aim of generating a memory T-cell population expressing a similar phenotype to T-cells primed *in vivo*.

Two different strategies have been used in order to induce a primary immune response in cells from healthy volunteers. Engler and co-workers were able to induce a primary T-cell response to sulfamethoxazole (SMX) and its reactive nitroso metabolite SMX-NO by stimulating peripheral blood mononuclear cells (PBMCs) repeatedly over five weeks with the drug in the presence of autologous antigen-presenting cells (APCs) (Engler *et al.* 2004). This approach has been successfully translated to prime PBMCs from *HLA-B*57:01*+ and *HLA-B*15:02*+ drug-naïve volunteers to abacavir (ABC) and carbamazepine (CBZ) respectively (Chessman *et al.* 2008; Ko *et al.* 2011). The second *in vitro* priming method involves culturing of purified naïve T-cells with dendritic cells as professional APCs and the drug antigen of interest. This system has been well established for priming naïve T-cells against chemicals contact sensitizers (Dietz *et al.* 2010). Recently, a similar assay has been developed to induce drug-specific responses in naïve T-cells from healthy volunteers (Faulkner *et al.* 2012). The method was shown to be successful in generating a primary immune response against the model allergen SMX-NO, and its application for other drugs associated with HSRs is currently under investigation (Faulkner *et al.* 2012; Monshi *et al.* 2013).

Given that HSRs generally occur at low frequency and are unpredictable in nature, prospective studies in humans are not feasible. At the same time, valid animal models are lacking, as drug-induced HSRs are also idiosyncratic in animals and mechanisms seem to differ for different drugs as well as between species (Uetrecht 2007). Moreover, the discovery that some reactions only develop in individuals expressing specific HLA alleles, e.g. ABC causing hypersensitivity exclusively in *HLA-B*57:01*+ patients, has highlighted the need to refine existing models to study HSRs *in vitro*. This has resulted in a growing interest to use genetically characterised immune cells from drug-naïve healthy

donors for functional studies attempting to characterise the mechanisms involved in HSRs, and predict the potential of a drug to cause an immune response (Alfirevic *et al.* 2012).

In this chapter, the above described *in vitro* priming methods were used to explore the possibility of inducing a primary immune response to carbamazepine in *HLA-A*31:01*+ healthy volunteers. *HLA-A*31:01* is the first allele which has been shown to be strongly associated with CBZ-induced HSRs in Caucasian populations (McCormack *et al.* 2011). Generally, the frequency of *HLA-A*31:01* in Europeans is relatively low, ranging between 2-6% (derived from www.allelefrequencies.net). However, the presence of *HLA-A*31:01* in patients treated with CBZ increases the risk to develop CBZ-induced HSRs from 5% to 26 %, indicating it could be a clinically relevant marker for the prediction of CBZ-induced HSRs in Europeans (McCormack *et al.* 2011). The local cell bank of HLA-typed lymphocytes from 400 healthy volunteers (Alfirevic *et al.* 2012) was used as resource to test the propensity of CBZ to induce drug-specific T-cell activation in non-sensitised *HLA-A*31:01*+ individuals.

## 3.2 Methods

### 3.2.1 Volunteer characteristics

PBMCs from eight *HLA-A*31:01*+ healthy volunteers, five *HLA-A*31:01*- donors, and three *HLA-B*15:02*+ donors were used in this study. The samples were part of our HLA-typed cell archive from 385 healthy individuals, comprising 23 *HLA-A*31:01+* and 7 *HLA-B*15:02+* donors in all (Alfirevic *et al.* 2012).

The healthy volunteers had been recruited from the North West of England between August 2009 and April 2010, and each volunteer initially donated 100ml blood. DNA was isolated from 10ml blood to determine the HLA genotype of each individual, whereas the remaining 90ml of blood was used to isolate PBMCs for functional *in vitro* studies. The isolated PBMCs (96x $10^6$ cells

± 40x $10^6$ per donor) were stored at -150°C until needed. Individual donors were approached for a second blood donation by the responsible study nurse, if more cells were required. The healthy volunteers could withdraw their consent from participating in the study at any time.

The complete HLA-genotypes of the 16 samples included in this study are shown in Table 3.1.

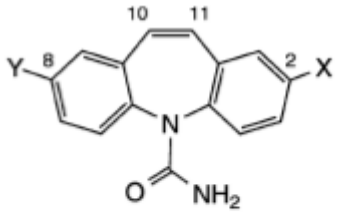
**Table 3.1**: HLA-genotypes of healthy volunteer samples included in this study.

| ID | HLA-A | HLA-B | HLA-C | HLA-DRB | HLA-DQB |
|---|---|---|---|---|---|
| **HV3** | 03:01/31:01 | 07:02/07:02 | 07:02/07:02 | 15:01/08:01 | 04:02/06:02 |
| **HV5** | 02:01/31:01 | 15:05/40:06 | 03:03/15:02 | 04:04/14:04 | 03:02/05:03 |
| **HV9** | 01:01/31:01 | 08:01/39:01 | 07:01/12:03 | 16:01/03:01 | 02:01/05:02 |
| **HV11** | 31:01/66:01 | 27:05/40:01 | 02:02/03:04 | 01:01/04:01 | 03:02/05:01 |
| **HV13** | 24:02/31:01 | 55:01/56:01 | 01:02/03:03 | 04:01/12:01 | 03:02/03:01 |
| **HV14** | 24:02/33:03 | 15:02/44:03 | 07:01/08:01 | 07:01/12:02 | 02:01/03:01 |
| **HV15** | 31:01/33:03 | 40:01/50:01 | 03:04/06:02 | 03:01/04:04 | 02:01/03:02 |
| **HV19** | 02:01/02:01 | 40:01/50:01 | 03:04/06:02 | 15:01/07:01 | 02:01/06:02 |
| **HV20** | 29:02/31:01 | 07:02/15:01 | 03:03/07:02 | 13:01/14:01 | 05:03/06:03 |
| **HV21** | 01:01/31:01 | 07:02/37:01 | 06:02/07:02 | 15:01/15:01 | 06:02/06:02 |
| **HV22** | 01:06/29:02 | 27:05/44:03 | 02:02/16:01 | 04:01/07:01 | 02:01/03:02 |
| **HV25** | 11:01/11:01 | 15:02/15:25 | 04:03/08:01 | 11:06/12:02 | 03:01/03:01 |
| **HV26** | 01:01/25:01 | 07:02/40:01 | 03:04/07:02 | 01:01/15:01 | 05:01/06:02 |
| **HV31** | 02:01/24:02 | 40:01/40:02 | 03:03/07:02 | 15:01/14:05 | 05:03/06:02 |
| **HV35** | 11:01/24:02 | 15:02/15:02 | 08:01/08:01 | 15:01/12:02 | 03:01/06:01 |
| **HV39** | 01:01/24:02 | 08:01/44:02 | 05:01/07:01 | 11:01/04:04 | 03:02/03:01 |

### 3.2.2 Chemicals

Carbamazepine (Sigma-Aldrich, UK) was prepared as stock solution (10µg/ml) in T-cell medium comprising 10% DMSO (Sigma-Aldrich, UK). The stock solution was made up fresh before each experiment and diluted to the appropriate concentration immediately before use. Unless stated otherwise, all other reagents were purchased from Sigma-Aldrich.

Chemical analogues of carbamazepine, namely 2-monohalo and 2,8-dihalo derivatives (Figure 3.1), were synthesized by the Department of Chemistry (Elliott *et al.* 2012).




**CBZ**: X= Y= H
**2-F CBZ**: X= H, Y= F
**2-Cl CBZ**: X= H, Y= Cl
**2-Br CBZ**: X= H, Y= Br
**2,8-F CBZ**: X= Y= F
**2,8-Cl CBZ**: X= Y= Cl
**2,8-Br CBZ**: X= Y= Br

**Figure 3.1**: Structures of halogenated derivatives of carbamazepine

### 3.2.3 Cell culture media

T-cell and B-cell lines were cultured in their respective cell culture medium as described previously in chapter 2.2.3. T-cell medium was used to culture monocyte-derived dendritic cells.

For the work performed in the Department of Microbiology & Immunology, University of Melbourne, RF10 medium was used to culture both the T- and B-cell lines. RF10 medium consisted of RPMI 1640 medium (Gibco, USA) supplemented with 10% FBS (Bovogen Biologicals, Australia), 7.5mM HEPES (MP Biomedicals, Australia), 2mM L-glutamine (MP Biomedicals, Australia), 150µg/ml streptomycin, 150U/ml benzylpenicillin (CSL, Australia), 150µM non-essential amino acids (Gibco, USA), and 76µM β-mercaptoethanol.

### 3.2.4 Lymphocyte transformation test

The lymphocyte transformation test was performed as described earlier in chapter 2.2.6 in order to confirm that the healthy donors had not been previously sensitised to CBZ.

### 3.2.5 4-week induction culture

Lymphocytes were repeatedly stimulated with CBZ *in vitro* over four weeks to induce a primary immune response to the drug using previously described methodology (Engler *et al.* 2004).

Freshly thawed PBMCs (5x $10^6$, 2ml) from each volunteer were cultured in T-cell medium containing 25µg/ml CBZ in a 24-well plate. On day 3, fresh medium supplemented with IL-2 (200U/ml; Preprotech, UK) was added to the cultures. On day 7, lymphocytes were restimulated with CBZ (25µg/ml) in the presence of autologous, irradiated PBMCs (1x $10^6$) as feeder cells. Restimulation was repeated every week, and wells were split when necessary to sustain proliferation of T-cell lines.

### 3.2.6 *In vitro* T-cell priming assay

An alternative *in vitro* method to stimulate primary responses to drugs in naïve T-cells from healthy individuals was recently developed within the Department (Faulkner *et al.* 2012); and the procedure outlined below was carried out by Dr. Lee Faulkner.

***Isolation of PBMCs and magnetic cell separation***

PBMCs were isolated from 100ml fresh whole blood of the selected healthy donors using the density gradient centrifugation method as described previously in chapter 2.2.4. Monocytes were isolated by magnetic cell separation using CD14 microbeads (Miltenyi Biotec, UK). T-cells were isolated from non-CD14 cells by negative selection using the Pan T isolation kit II

(Miltenyi Biotec, UK). Next, regulatory T-cells were removed using CD25 microbeads (Miltenyi Biotec, UK). The remaining T-cells were further separated into naïve and memory populations using CD45RO microbeads (Miltenyi Biotec, UK). Magnetic cell separation was carried out according to the manufacturer's instructions. The phenotype and purity of the isolated cells was assessed by flow cytometry using CD14-FITC, CD3-APC, CD25-PE, CD45RA-FITC, CD45RO-Cy5, CD4-FITC, CD8-PE antibodies (all BD Bioscience, UK; except CD25-PE, Miltenyi Biotec, UK). The purity of the separated cell populations exceeded 95%. All cells were frozen down and stored at -150°C until needed.

***Generation of monocyte-derived dendritic cells***

$CD14^+$ monocytes (6 $x10^6$, 6ml) were differentiated into dendritic cells (DCs) for 7-8 days in 6-well plates using T-cell medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF; 800U/ml; Preprotech, UK) and IL-4 (800U/ml; Preprotech, UK). Fresh medium (3ml) supplemented with GM-CSF and IL-4 was added every 2 days.

***T-cell and monocyte-derived DC co-culture***

Freshly thawed naïve T-cells (2 $x10^6$, 1ml) were cultured with immature monocyte-derived DCs (8 $x10^5$, 500µl) and CBZ (100µg/ml, 500µl) in a 24-well plate for 7-8 days.

### 3.2.7 Generation of antigen-presenting cell lines

EBV-transformed B-lymphoblastoid cell lines (B-LCL) were used as antigen-presenting cells (APCs) for read-outs following 4-week induction cultures. B-LCLs were generated for each of the healthy volunteers used in this study as described previously in chapter 2.2.5.

PHA blasts were used as APCs in the intracellular cytokine staining (ICS) assay, which was performed at the Department of Microbiology & Immunology, University of Melbourne. PHA blasts were generated by culturing PBMCs (2 $x10^6$) from the selected volunteers with phytohemagglutinin (PHA; 20µg/ml) in a 24-well plate for 7 days. On day 3, fresh IL-2 supplemented medium was

added to the cultures. On day 7, PHA blasts were washed and expanded into tissue culture flasks. Proliferation was maintained by adding fresh IL-2 containing medium every 2-3 days.

### 3.2.8 T-cell proliferation assay

CBZ-specific proliferation of T-lymphocytes was assessed by [$^3$H]-thymidine incorporation, which has been described in detail in chapter 2.2.10.

### 3.2.9 Enzyme-linked immunospot assay

CBZ-induced secretion of cytokines was determined by enzyme-linked immunospot (ELISpot) assay using the same protocol as described in chapter 2.2.12.

### 3.2.10 Intracellular cytokine staining (ICS) assay

T-cells (2 x$10^5$) were incubated with PHA blasts (1 x$10^5$) and CBZ (25µg/ml) in a 96-well U-bottom plate (final volume 200µl). Anti-CD3/anti-CD28 monoclonal antibodies and phorbol 12-myristate 13-acetate (PMA)/ ionomycin were used as positive controls. After 2 hours, Brefeldin A (10µl/ml) was added to the cultures to prevent cytokine secretion, and incubation was continued overnight.

The next day, cells were spun down and the supernatant removed. The cells were surface stained (30min on ice) with fluorescent-conjugated antibodies CD3-PerCP (BD Pharmingen, USA), CD4-APC-Cy7 (Biolegend, USA), and CD8-FITC (BD Pharmingen, USA) in the dark for 30min on ice. This was followed by a brief washing step with PBS (120µl). Next, cells were fixed for 30min using 1% para-formaldehyde (ProSciTech, Australia), and then washed with PBS. In the last step, cells were permeabilised using 0.3% saponin and intracellular cytokines were stained using fluorescent-labelled antibodies, including TNFα-PE-Cy7 (BD Pharmingen, USA), IFNγ-Pacific Blue (Biolegend, USA), granzyme B-Alexa Fluor 700 (BD Pharmingen, USA), and IL4-APC (eBioscience, USA) (see

Appendix T1 for more details). Samples were run on a BD FACS LSRII flow cytometer and data analysed using FlowJo software (Tree Star Inc., USA).

### 3.2.11 T-cell cloning

T-cell lines derived from the 4-week induction cultures or T-cell priming assays were used to generate drug-specific T-cell clones. The protocol for T-cell cloning has been described in detail in chapter 2.2.9.

### 3.2.12 Statistical analysis

One-way ANOVA was performed on the proliferation data. ELISpot data were analysed by Student's t-test, applying Bonferroni correction for multiple testing if necessary. ICS were therefore analysed using the non-parametric Mann-Whitney U test, as low sample numbers prevented an assessment for normal distribution.

## 3.3 Results

### 3.3.1 *In vitro* stimulation of PBMCs

PBMCs from *HLA-A*31:01* positive and *A*31:01* negative healthy volunteers were tested in a lymphocyte transformation test to exclude the possibility of prior sensitisation to CBZ. Proliferative responses to CBZ were negative in all healthy volunteers tested (Figure 3.2). In contrast, lymphocytes from *HLA-A*31:01*+ volunteers proliferated markedly when stimulated with tetanus toxoid (TT), with SI values ranging between 4 and 22. However, responses to TT were negative in all *HLA-A*31:01*- volunteers.

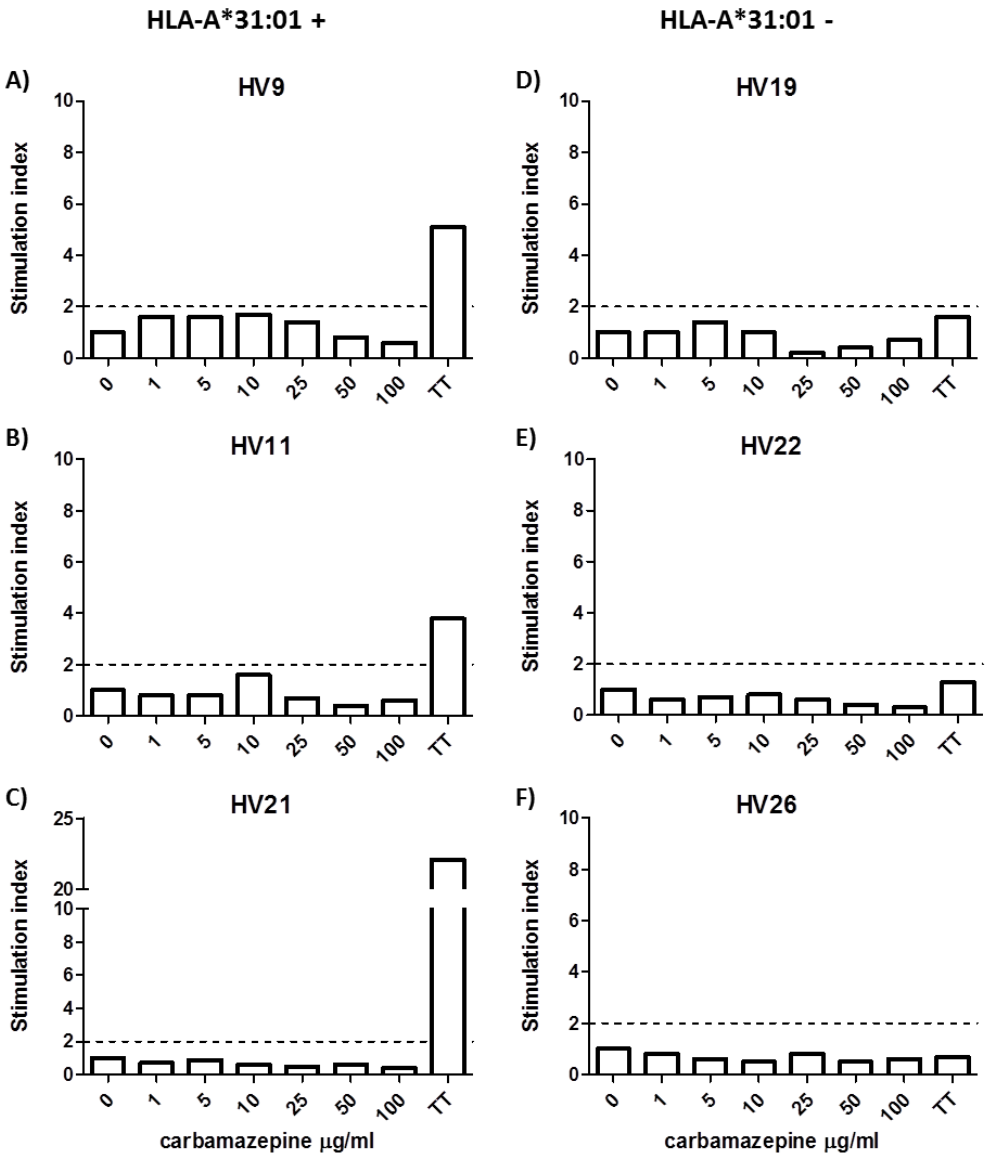



**Figure 3.2**: Lymphocyte transformation test of PBMCs from *HLA-A*31:01*+ (A-C) and *A*31:01*- (D-F) healthy donors.

PBMCs were cultured for 6 days in presence of CBZ (25μg/ml), and [$^3$H]-thymidine was added for the final 16h of the assay. Proliferation was determined by scintillation counting. A stimulation index of >2 (dashed line) was considered a positive response.

Additionally, an IFN-γ ELISpot assay was carried out to confirm the results from the LTT, which is considered a less sensitive assay and might fail to detect weak responses. CBZ did not induce IFN-γ secretion in lymphocytes from any volunteer, whereas PHA lead to a robust release of IFN-γ in all donors tested (Figure 3.3). The consistent response to PHA indicated that the cells isolated from the volunteers were still functional, and suggested that the missing response to TT in the LTT of some volunteers might be due to incomplete vaccination.

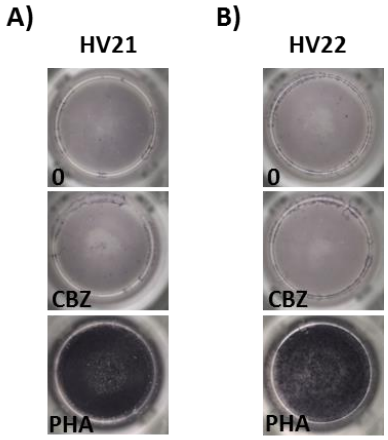



**Figure 3.3**: IFN-γ secretion of PBMCs from healthy volunteers stimulated with CBZ or PHA (positive control). Representative assay from *HLA-A*31:01*+ (A) and *HLA-A*31:01*- (B) volunteer cells are shown.

PBMCs (1x $10^6$) were incubated with CBZ (25µg/ml) for 2 days, and spots developed according to the manufacturer's protocol.

### 3.3.2 4-week induction cultures

Following the protocol by Engler and colleagues (Engler *et al.* 2004), PBMCs from three non-sensitised individuals expressing *HLA-A*31:01* (HV9, HV11, and HV15) were repeatedly stimulated with CBZ over four weeks with the aim of eliciting a primary immune response to the drug. In parallel, T-cell cultures from three *HLA-A*31:01*- (HV19, HV22, and HV31) and two *HLA-B*15:02*+ (HV25 and HV35) healthy donors were set up to be used as negative and positive controls respectively.

After 5 weeks, reactivity of T-lymphocytes to CBZ was tested by proliferation assay (Figure 3.4) and IFN-γ ELISpot (Figure 3.5) in order to assess whether sensitisation to CBZ had been induced.

A weak but significant increase in proliferation to different concentrations of CBZ was only observed in T-cells from volunteer HV15 (Figure 3.4 C). The statistically significant decrease in proliferation of T-cells from volunteer HV9, seen at the highest concentration of CBZ used in this assay, was attributed to the cytotoxic effect the drug is known to cause at doses of 100µg/ml CBZ and above (Figure 3.4 B). In contrast, there was no CBZ-induced proliferation in T-cells from any HLA-A*31:01- volunteer, but stimulation with PHA activated T-cells significantly (Figure 3.4 D). Furthermore, CBZ was unable to induce a proliferative response in T-cells from HLA-B*15:02+ volunteers (Figure 3.4 E). However, the majority of CBZ-reactive T-lymphocytes in HLA-B*15:02+ individuals have previously been shown to be $CD8^+$ T-cells (Ko *et al.* 2011), which might not be detected in the proliferation assay due to their low proliferation rate.

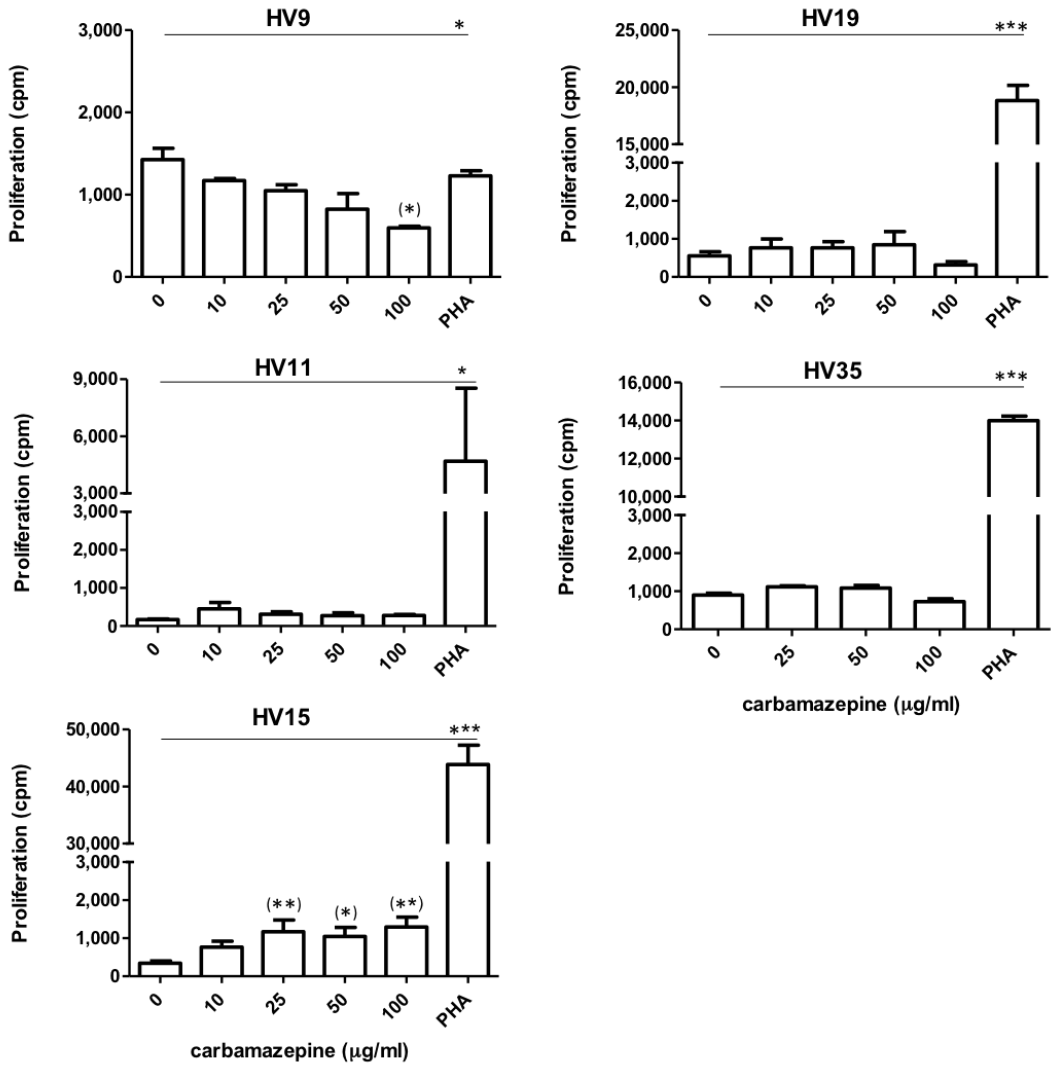



**Figure 3.4**: CBZ-specific proliferation of T-cells isolated from healthy volunteers following 4-week induction culture. (A-C) T-cell responses from *HLA-A*31:01*+ volunteer cultures; (D+E) results of representative T-cell cultures from a *HLA-A*31:01*- and *HLA-B*15:02*+ volunteer respectively.

T-cells were incubated with CBZ (10- 100μg/ml) in the presence of autologous irradiated B-LCLs for 48h, and proliferation was determined by [$^3$H]-thymidine incorporation. Data represent mean cpm ± SEM of triplicate cultures. Statistical analysis was performed using one-way ANOVA (indicated as line above graph) with Tukey post-hoc test (noted in brackets) (*p< 0.05, **p< 0.01, ***p< 0.001).

The release of IFN-γ, measured by ELISpot assay, was used as a second read-out to detect CBZ-specific T-cell activation (Figure 3.5). *HLA-A*31:01*+ T-cell cultures secreted high levels of IFN-γ at baseline, thus, no drug-induced increase in cytokine secretion could be observed (Figure 3.5 A). In contrast, no IFN-γ release was seen in T-cells isolated from *HLA-A*31:01*- donors, neither before nor after stimulation with CBZ (Figure 3.5 B). However, stimulation of T-cells from *HLA-B*15:02*+ volunteers with CBZ led to a two-fold increase in IFN-γ secretion (Figure 3.5 C).

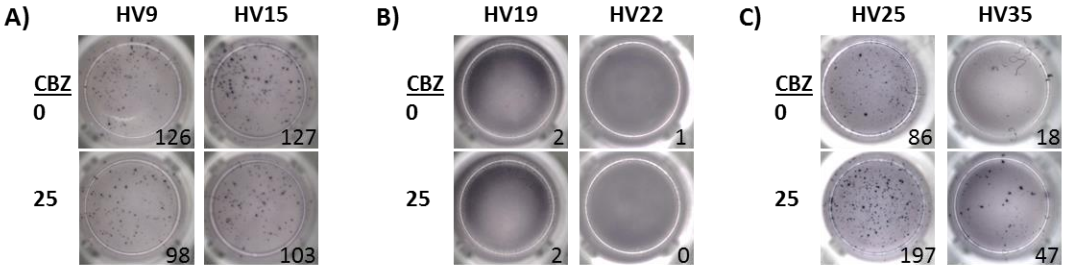



**Figure 3.5**: CBZ-induced IFN-γ secretion of T-cells from *HLA-A*31:01*+ (A), *HLA-A*31:01*- (B) and *HLA-B*15:02*+ (C) donors after 4-week induction culture.

T-cells (5x $10^5$) were incubated with CBZ (25μg/ml) and autologous irradiated B-LCLs (1x $10^5$) for 2 days, and spots visualised following the manufacturer's instructions. Number of spot-forming cells is presented in the lower right corner of each image.

Successful priming of drug-naïve T-cells from healthy donors against ABC had been reported previously. After a 14-day priming period of PBMCs from healthy donors with ABC, Chessman and co-workers were able to detect a drug-induced increase in IFN-γ and TNF-α levels in $CD8^+$ T-cells by ICS (Chessman *et al.* 2008). Thus, in a second attempt to sensitise drug-naïve T-cells isolated from *HLA-A*31:01*+ volunteers, the ICS assay was used as alternative detection method to identify CBZ-reactive T-cells following the 4-week induction culture.

Similar to the studies by Chessman *et al*., CBZ-primed T-cells from three healthy donors (HV3, HV5, and HV20) were stained for IFN-γ and TNF-α after a four week restimulation period. No increase in IFN-γ producing T-cells was

observed for any of the *HLA-A*31:01+* healthy volunteers tested. One volunteer, i.e. HV3, showed a marked increase in TNF-α producing $CD4^+$ T-cells (from 12 % to 19% of total CD4+ T-cells) when stimulated with CBZ (Figure 3.6 A); however, this was not statistically significant.

As mentioned previously, IL-5 and cytotoxic molecules, such as granzyme B, had been reported to be robust markers for the *in vitro* detection of drug-specific T-cells from hypersensitive patients. Thus, *in vitro* primed volunteer T-cell cultures were subjected to a second ICS assay which covered additional cytokines, including granzyme B, IL-4 and IL-5. No expansion of T-cells expressing IL-4 or IL-5 could be detected in any of the volunteers tested. However, a strong increase in granzyme B producing CD8+ T-cells (from 28% to 75% of total CD8+ T-cells) was observed for one of the healthy volunteer, i.e. HV5, following CBZ stimulation (Figure 3.6 B). Due to the high standard deviation, the increase was not statistically significant. Increased expression of granzyme B was also seen in the CD4+ T-cell population of this volunteer, although to a lesser extent.

### 3.3.3 DC-T-cell co-cultures

An alternative strategy to prime drug-naïve T-cells *in vitro* has been developed recently using monocyte-derived DCs (Faulkner *et al.* 2012). As attempts to generate CBZ-specific T-cells from healthy volunteers using the 4-week induction protocol were largely unsuccessful, we decided to apply this novel experimental system instead.

Naïve T-cells from two *HLA-A*31:01*+ healthy donors (HV3 and HV13) were cultured with autologous monocyte-derived DCs and CBZ for 8 days, and subsequently proliferation and cytokine secretion was assessed by [$^3$H]-thymidine uptake and ELISpot method respectively. Likewise, priming of naïve T-cells isolated from a *HLA-B*15:02*+ healthy volunteer (HV14) was performed.

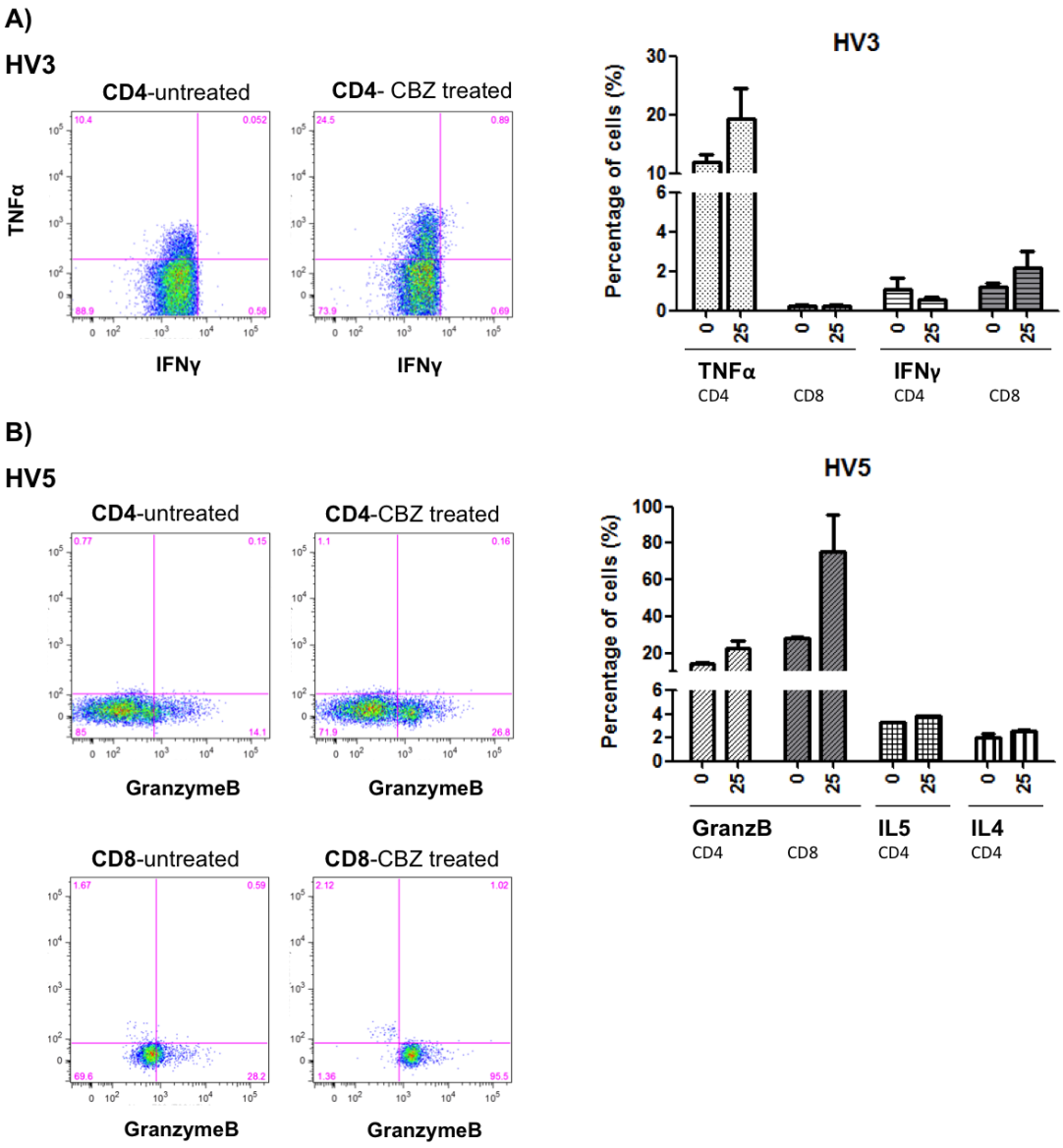



**Figure 3.6**: Cytokine expression of T-cells from two *HLA-A*31:01*+ volunteers (A+B) upon CBZ stimulation (after 4-week induction culture).

T-cells were stimulated with CBZ (25µg/ml) and autologous, irradiated B-LCLs overnight: brefeldin A was added after 2h to inhibit cytokine release. Cells were permeabilised the next day and stained for intracellular cytokines as well as surface markers CD4 and CD8. Graphs represent mean ± SEM of duplicate cultures. Statistical analysis was performed using Mann-Whitney U-test.

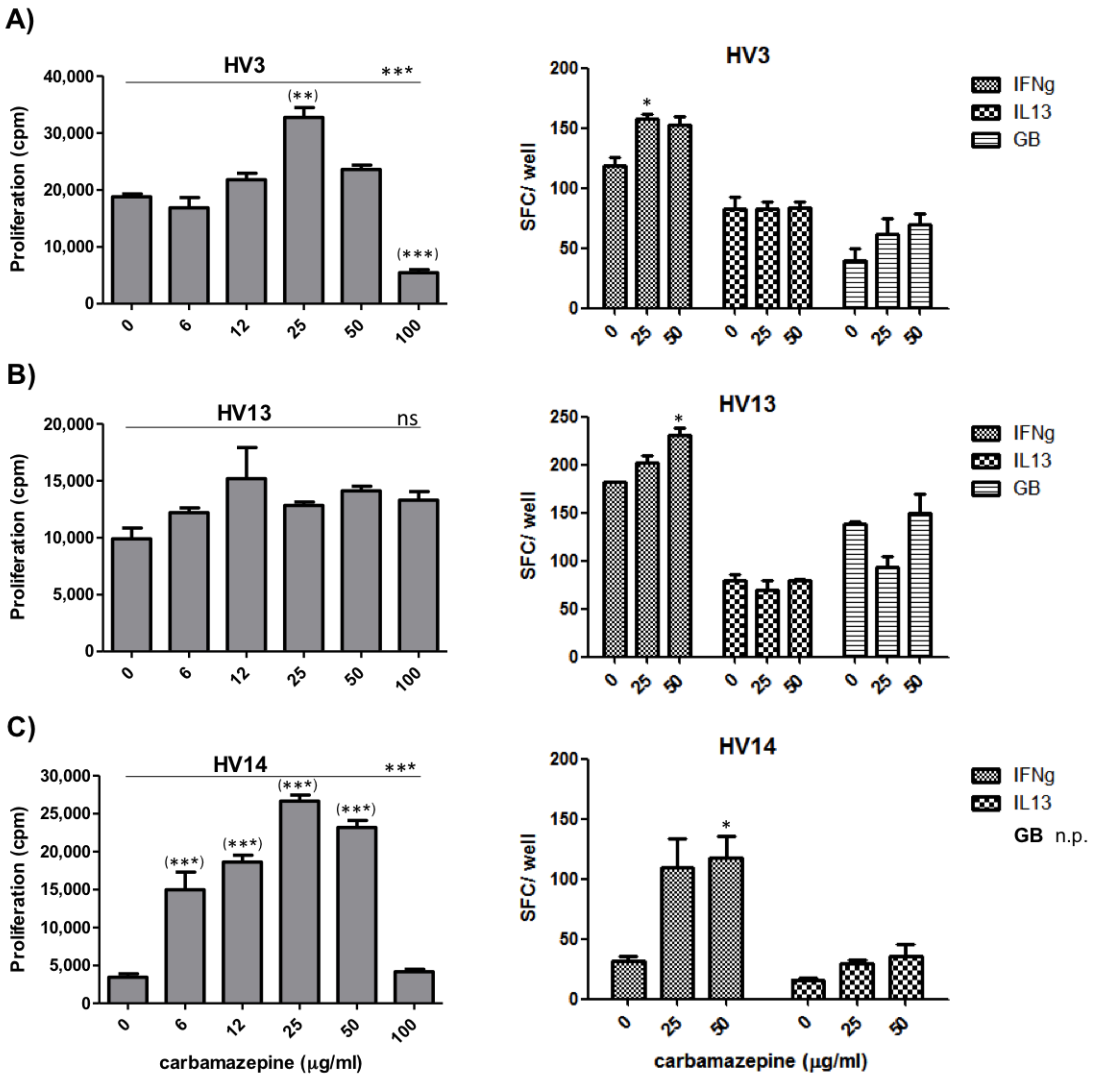



**Figure 3.7**: CBZ-specific proliferation and cytokine secretion of T-cells from *HLA-A*31:01*+ volunteers (A+B) and a *HLA-B*15:02*+ volunteer (C) after DC T-cell co-culture.

Purified, naïve T-cells were cultured with CBZ and autologous monocyte-derived DCs for 8 days. T-cells (1x $10^5$) were restimulated with CBZ (6- 100µg/ml) in the presence of autologous monocyte-derived DCs (4x $10^3$). Proliferation was determined by [$^3$H]-thymidine uptake, with graphs showing mean cpm ± SEM of triplicate cultures. Cytokine release was measured by ELISpot assay, and data are given as mean SFC ± SEM of duplicate cultures. For proliferation data, statistical analysis was performed using one-way ANOVA (with Tukey post-hoc test; **p< 0.01, ***p< 0.001). Student's t-test was used to analyse ELISpot results (*p< 0.03). (SFC= spot-forming cells)

A weak but significant proliferative response to CBZ could be detected in one of the *HLA-A*31:01*+ donors (Figure 3.7 A), whereas T-cells from the second *HLA-A*31:01*+ donor did not show a significant increase in proliferation when stimulated with CBZ (Figure 3.7 B). Conversely, CBZ stimulated a strong and highly significant response of T-cells isolated from the HLA-B*15:02+ volunteer (Figure 3.7 C). Moreover, proliferation levels of T-cells in the absence of CBZ differed greatly between *HLA-A*31:01*+ and *HLA-B*15:02*+ donors, showing a two- to four-fold increase in background proliferation rates of T-cells from *HLA-A*31:01*+ volunteers compared to the *HLA-B*15:02*+ donor.

Release of cytokines, including INF-γ, IL-13 and granzyme B, was the second endpoint examined. CBZ was able to stimulate a weak significant increase in IFN-γ secretion of T-cells from all three volunteers (Figure 3.7 A-C). However, baseline levels of INF-γ released by T-cells expressing HLA-A*31:01 was increased two-to three-fold compared to T-cells expressing HLA-B*15:02. No significant increase in IL-13 or granzyme B secretion was observed for any of the volunteers.

### 3.3.4 T-cell cloning

T-cells from two *HLA-A*31:01*+ volunteers (HV5 and HV15) and two *HLA-B*15:02*+ donors (HV14 and HV35), all of which had shown a CBZ-specific response in one of the above described read-outs following *in vitro* priming, were cloned by serial dilution in order to confirm a primary immune response to CBZ had been induced. Furthermore, T-cell cloning would allow a more detailed analysis of the phenotypic and functional characteristics of *in vitro* sensitised T-cells. Two individuals lacking the CBZ-associated HLA risk alleles (HV31 and HV39), and one *HLA-A*31:01*+ donor (HV20) with a negative response in the ICS after T-cell priming, served as controls. The cloning results for all seven volunteers are summarised in Table 3.2.

**Table 3.2**: Specificity and phenotype of CBZ-specific T-cell clones generated from healthy volunteers.

| Volunteer ID | Clones tested (n) | Specific clones (n) [mean SI] | Dose-response (cpm) CBZ 0 | 25µg/ml | CD phenotype (%) CD4 | CD8 |
|---|---|---|---|---|---|---|
| **HLA-A*31:01+** | | | | | | |
| HV5 | 91 | 7 [2.7] | 8,170 ±6,161 | 20,415 ±13,539 | n.d. | n.d. |
| HV15 | 64 | 3 [2.2] | 333 ±262 | 760 ±508 | n.d. | n.d. |
| HV20 | 360 | 16 [8.4] | 5,135 ±6,359 | 22,788 ±17,479 | 50 | 30 |
| **HLA-B*15:02+** | | | | | | |
| HV14 | 312 | 4 [2.2] | 747 ±287 | 1,696 ±722 | n.d. | n.d. |
| HV35 | 240 | 44 [9.2] | 4,488 ±5,077 | 18,695 ±14,280 | 14 | 67 |
| **Controls (A*31:01- B*15:02-)** | | | | | | |
| HV31 | 96 | 5 [4.4] | n.s. | n.s. | n.d. | n.d. |
| HV39 | 336 | 4 [2.5] | n.s. | n.s. | n.d. | n.d. |

n.d. = not determined; n.s. = not specific

Only a few CBZ-specific clones, with very low SI values, could be generated from *HLA-A*31:01*+ volunteers HV5 and HV15 (Table 3.2). For donor HV15, the low proliferation counts (< 1000cpm) in presence and absence of CBZ indicated that identified drug-specific clones represented false positive results. The seven clones generated from volunteer HV5 lost their specificity within about one month, precluding any detailed analysis. In contrast, sixteen CBZ-reactive clones could be generated from volunteer HV20, of which 50% were $CD4^+$ clones and 30% expressed CD8 (Table 3.2). The remaining 20% of T-cell clones comprised $CD4^+$ as well as $CD8^+$ T-cells, and therefore resembled T-cell lines rather than clones. Upon CBZ stimulation, the clones displayed a drug concentration-dependent proliferation profile and secreted significant amounts of IFN-γ (Figure 3.8 A). These results were rather surprising, given that earlier attempts to prime T-cells from this volunteer *in vitro* had been unsuccessful.

Disparate numbers of drug-specific clones were also detected among the *HLA-B*15:02*+ volunteers. Forty-four CBZ-responsive clones could be generated from donor HV35 (Table 3.2). Phenotyping for CD expression revealed two-thirds of the clones were $CD8^+$ and one-third $CD4^+$. T-cell clones (TCC) proliferated to CBZ in a dose-dependent manner, and high levels of IFN-γ secretion were detected in presence of the drug (Figure 3.8 B). From the second *HLA-B*15:02*+ volunteer, i.e. HV31, only four TCC could be generated, all of which displayed very weak proliferation counts and SI values (Table 3.2). Within a few weeks these clones no longer responded to CBZ stimulation.

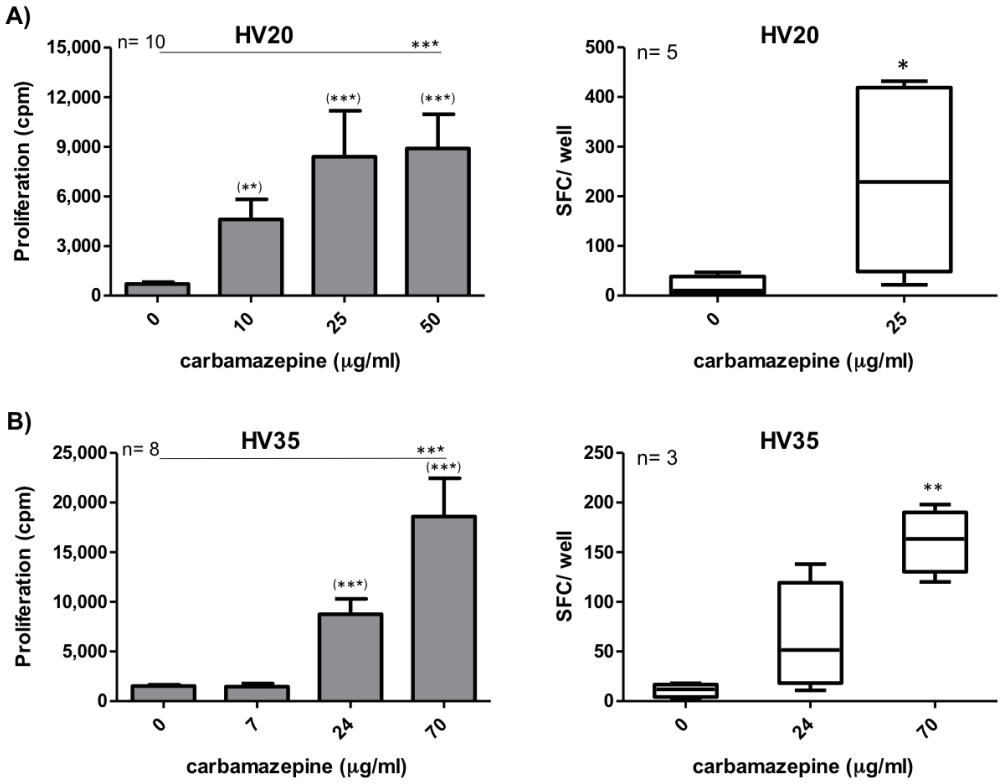



**Figure 3.8**: CBZ-specific proliferation and IFN-γ secretion of TCC from a *HLA-A*31:01*+ (A) and a *HLA-B*15:02*+ volunteer (B).

TCC were incubated with CBZ (7- 70μg/ml) and autologous irradiated B-LCLs for 48 hours. Proliferation was determined by [$^3$H]-thymidine incorporation; and data are shown as mean cpm ± SEM of n clones. IFN-γ secretion was measured by ELISpot assay, and graphs represent mean SFC ± SEM of n clones. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc test (proliferation) and Student's t-test (ELISpot) (*p< 0.05, **p< 0.01, ***p< 0.001).

From the volunteers selected as controls, nine clones were initially considered to be drug-specific from the calculated SI values (Table 3.2). However, when the proliferation assay was repeated two weeks later all nine clones failed to show a drug-specific response.

Although it would have been interesting to examine the HLA restriction profile of the CBZ-specific clones generated from the HLA-B*15:02+ volunteer (HV35), in a similar way as reported in the study by Wei and colleagues (Wei, C. Y. *et al.* 2012a), this was not possible due to the underrepresentation of cells expressing relevant HLA-B alleles in our HLA-typed cell archive, which would have been needed to generate HLA-matched APCs. Instead, structural aspects of CBZ presentation to the T-cell clones was explored using a series of halogenated CBZ derivatives recently synthesized by the Department of Chemistry. It had been shown that halogenation at the aromatic ring inhibited or reduced (2-fluoro CBZ) hydroxylation, and thus formation of reactive arene oxide intermediates *in vitro* (Elliott *et al.* 2012), indicating that the halogenated CBZ analogues are metabolically inert while largely retaining the same chemical properties as CBZ. Increasing evidence suggests that the parent drug CBZ can function as antigen causing T-cell activation, requiring neither metabolism nor formation of haptenated peptides (Wu *et al.* 2006; Yang *et al.* 2007; Wei, C. Y. *et al.* 2012a). The use of halogenated analogues therefore served as an alternative strategy to explore whether CBZ-reactive T-cells can be activated by the drug without the need for metabolism.

When the clones from the HLA-B*15:02+ volunteer were stimulated with the halogenated derivatives, different patterns of cross-reactivity could be observed. Although the maximal response was lower with the derivatives, significant proliferative responses could be detected for almost all CBZ analogues (Fig. 3.9). All six clones were activated by at least 3 of the analogues. However, only two clones could be stimulated with 2,8-dibromo CBZ, which is likely due to steric effects preventing adequate binding of the drug to the HLA-peptide complex.

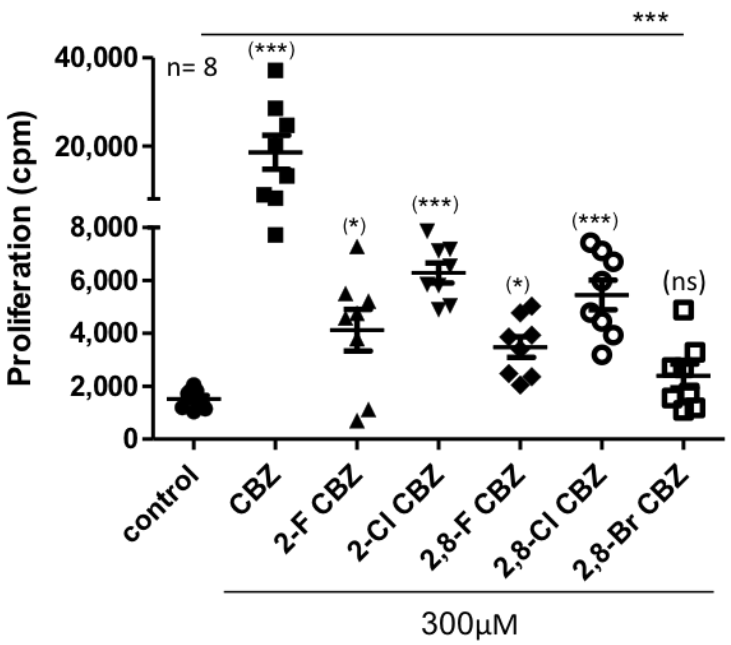



**Figure 3.9**: Activation of CBZ-primed T-cell clones from a HLA-B*15:02+ drug-naive individual with halogenated derivatives of CBZ.

TCC were incubated with CBZ or CBZ analogues (300μM) and autologous B-LCLs for 48 hours. Proliferation was measured by addition for [$^3$H]-thymidine and subsequent scintillation counting. Data are presented as mean cpm ± SEM of n clones. One-way ANOVA with Tukey post-hoc test was used to compare responses in the presence and absence of drug (ns= not significant, *p< 0.05, ***p< 0.001). (2-Br CBZ was not available for this study)

## 3.4 Discussion

*In vitro* priming of T-cells from healthy volunteers represents a promising new tool to study the pathomechanisms implicated in drug-induced HSRs and predict the risk of a drug to cause hypersensitivity. Several methods to stimulate a primary T-cell immune response from non-sensitised individuals have been developed recently using the well-characterised drug antigens SMX and SMX-NO. Furthermore, successful priming of T-cells from drug-naïve individuals expressing HLA-B*57:01 and B*15:02 has been accomplished with ABC and CBZ respectively (Chessman *et al.* 2008; Ko *et al.* 2011).

In this study, we attempted to elicit a primary immune response to CBZ in healthy individuals expressing HLA-A*31:01 using the same methods. In addition, priming of T-cells from *HLA-B*15:02*+ healthy donors was performed in order to compare T-cell responses to those observed in *HLA-A*31:01*+ volunteers.

The first attempt to prime T-cells from *HLA-A*31:01*+ volunteers involved repeated stimulation of lymphocytes with CBZ over four weeks. Three different endpoints- T-cell proliferation, IFN-γ secretion, and production of intracellular cytokines- were investigated in order to detect drug-specific responses. In contrast to the findings for ABC (Chessman *et al.* 2008), *in vitro* stimulation of drug-naïve T-cells with CBZ did not result in a primary immune response in all volunteers expressing HLA-A*31:01.

In the proliferation assay, only one out of three volunteers showed a weak to moderate response to CBZ. However, measuring proliferative responses may not be sensitive enough if priming occurred predominantly in $CD8^+$ T-cells, which would be expected in context of HLA class I alleles and has been shown to be the case for *in vitro* primed T-cells from *HLA-B*15:02*+ donors (Ko *et al.* 2011). Furthermore, the strength of the detectable T-cell response is limited by the low frequency of antigen-specific precursor T-cells within the PBMC cultures (Esser *et al.* 2014). When the more sensitive ELISpot assay was utilised as a read-out, no CBZ-induced increase in IFN-γ secretion could be detected for any of the *HLA-A*31:01*+ volunteers. This may be partially due to the high background levels of IFN-γ released in the absence of drug, indicating the presence of pre-activated T-cells. Similar observations have been reported previously (Engler *et al.* 2004), and it has been suggested that the addition of high amounts of IL-2 during the 4-week induction cultures may be responsible for the strong, but non-specific T-cell stimulation. Conversely, no increased baseline levels of IFN-γ were observed in T-cells from *HLA-B*15:02*+ donors, and stimulation with CBZ led to a clear increase in IFN-γ release. This suggests that other, currently unknown factors, must be involved in causing non-specific T-cell activation in *HLA-A*31:01*+ volunteers. Finally, analysis of intracellular cytokine production did not reveal a CBZ-specific increase of cytokines in T-

cells from all three *HLA-A*31:01*+ volunteers. Instead, two *HLA-A*31:01*+ individuals showed increased TNF-α and granzyme B expression in CBZ-stimulated T-cells respectively. Whether the weak T-cell responses seen in some of the HLA-A*31:01+ individuals represent true positive results cannot be determined at this stage. A repetition of the assays would be required to confirm that a primary T-cell stimulation to CBZ can be induced in these volunteers.

In a second approach to sensitise naïve T-cells to CBZ, the *in vitro* T-cell priming assay recently developed by Faulkner and colleagues was applied (Faulkner *et al.* 2012). Highly purified naïve T-cells from *HLA-A*31:01*+ volunteers were cultured with CBZ in the presence of autologous DCs for one week. It was anticipated that the removal of regulatory T-cells (Treg) and the use of monocyte-derived DCs as highly specialised antigen-presenting cells would improve *in vitro* priming conditions, leading to the induction of more robust CBZ-specific T-cell responses. For one of the *HLA-A*31:01*+ volunteers, a weak but significant CBZ-specific T-cell response could be detected in both the proliferation assay as well as the IFN-γ ELISpot. However, T-cells from the second HLA-A*31:01+ volunteer only responded in the IFN- γ ELISpot. At the same time, baseline levels of proliferation and IFN-γ secretion were found to be strongly increased in both samples, indicating the presence of pre-activated T-cells. As observed previously, this was not the case for T-cells from the *HLA-B*15:02*+ donor. Here, stimulation with CBZ led to an increased release of IFN-γ and a strong proliferative response, which was highly significant. From the preliminary data presented here, it appears that the DC-T-cell priming method may be more efficient in inducing a primary T-cell stimulation. However, a larger number of volunteers should be tested before a firm conclusion is drawn.

Because the two preceding *in vitro* priming methods produced conflicting results depending on the detection method used to determine primary T-cell stimulation, generation of CBZ-specific T-cell clones was attempted. T-cell cloning was performed from three *HLA-A*31:01*+ volunteers in total, of which two had earlier displayed a positive response. Varying numbers of clones were isolated from the three *HLA-A*31:01*+ donors, and most clones were not found

to be CBZ-specific. Moreover, CBZ-reactive clones from the formerly negative *HLA-A*31:01*+ volunteer showed the strongest responses, whereas CBZ-responsive clones from the formerly positive volunteers quickly lost specificity. A similar situation was observed when T-cell cloning from two *HLA-B*15:02*+ volunteers was performed. Although both samples had previously responded in the priming assay, only minimal numbers of CBZ-specific clones could be isolated from one of the volunteers, and after a short culture period, they became unresponsive to CBZ treatment. T-cell clones from the second HLA-B*15:02+ volunteer, in contrast, displayed strong and stable proliferative responses. These clones were used in a substudy examining the influence of CBZ metabolism on T-cell activation. Halogenated derivatives of CBZ, which have been shown to be resistant to metabolism *in vitro*, could clearly stimulate CBZ-responsive clones to proliferate, suggesting T-cell activation was mediated by the parent drug in a metabolism-independent process. However, whether the primary stimulation of drug-naïve T-cells from the *HLA-B*15:02+* healthy volunteer to CBZ was induced by the parent drug, a reactive metabolite, or a drug protein adduct, remains to be demonstrated.

Overall, T-cell responses to CBZ varied greatly among the *HLA-A*31:01*+ individuals tested in this study, clearly indicating that factors additional to HLA-A*31:01 are needed in order to induce a primary immune response to CBZ. This is not surprising given that the positive predictive value of *HLA-A*31:01* in Caucasians was determined to be 43% (Yip, V. L. *et al.* 2012), i.e. 57% of individuals carrying the allele do not develop hypersensitivity when treated with CBZ.

In context of *HLA-B*15:02*, Ko *et al.* reported that primary stimulation of T-cells from *HLA-B*15:02*+ healthy donors to CBZ could only be achieved if T-cells expressing a specific TCR were present. In absence of the specific T-cell clonotypes no CBZ-specific response could be detected (Ko *et al.* 2011). Whether specific TCR clonotypes may be required for eliciting CBZ-specific responses in *HLA-A*31:01*+ individuals has not been investigated yet.

Other factors that have been reported to affect *in vitro* priming efficiency include inadequate antigen presentation, lack of co-stimulatory cytokines, and the presence of Treg suppressing an immune response (Jedema *et al.* 2011). The removal of Treg prior to the DC-based priming assay may explain why primary T-cell stimulation might have been slightly more successful in this part of the study. With regards to the cytokine milieu, addition of IL-7 has been proposed to specifically stimulate the growth of naïve T-cells without inducing background proliferation, and may substitute IL-2 treatment (Fry and Mackall 2005). However, one of the most critical factors for the successful stimulation of primary T-cell responses to drugs relates to the presentation of drug antigen. For many drugs causing hypersensitivity, including CBZ, the exact antigenic structure has not yet been identified. Thus, we cannot be sure that sufficient amounts of drug antigen are present during the *in vitro* priming process. Further studies examining the mechanisms of drug antigen formation are therefore needed in order to advance our understanding of how drug-specific immune responses develop.

Taken together, there are many variables which may affect the process to stimulate primary T-cell responses to drugs *in vitro*. Identifying these variables and subsequent adaptation of current priming protocols to control for them should lead to enhanced *in vitro* T-cell stimulation and give more robust responses.

# Chapter IV

## *In silico* and *in vitro* characterisation of the HLA-A*31:01 binding specificity

## Contents

## 4.1 Introduction

Carbamazepine (CBZ) is known to cause a variety of hypersensitivity reactions (HSRs), most commonly affecting the skin, ranging from mild maculopapular rashes to severe reactions, such as Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) (Zaccara *et al.* 2007). Two distinct HLA class I alleles, i.e. *HLA-B*15:02* and *HLA-A*31:01*, have recently been found to be associated with an increased risk for the development of CBZ hypersensitivity. However, the two alleles have been linked to different clinical phenotypes of CBZ hypersensitivity and also act as predisposing factors for different ethnic groups (Yip, V. L. *et al.* 2012). *HLA-B*15:02* is strongly associated with CBZ-induced SJS and TEN in Southeast Asian populations (Chung *et al.* 2004; Locharernkul *et al.* 2008; Mehta *et al.* 2009; Chang *et al.* 2011), whereas *HLA-A*31:01* occurs at increased frequency in Japanese, Korean and Caucasian patients across all phenotypes of CBZ hypersensitivity (Kim *et al.* 2011; McCormack *et al.* 2011; Ozeki *et al.* 2011).

Drug-specific T-cells isolated from hypersensitive patients have been shown to respond to CBZ *in vitro*, and presentation of CBZ to drug-specific T-cells was demonstrated to be MHC-dependent, but did not require intracellular metabolism or antigen processing (Wu *et al.* 2007). These observations support the p-i concept, proposing that a direct non-covalent binding of the drug to HLA or T-cell receptor (TCR) can cause T-cell activation and ultimately result in development of HSRs. However, the molecular mechanism through which CBZ activates T-cells and more specifically how the drug interacts with HLA alleles to stimulate T-cells remains largely unknown.

A fundamental step in the activation of antigen-specific T-cells is the recognition of a peptide-MHC complex (pMHC) by the corresponding TCR. This interaction can be described as one of the crucial events in adaptive immune responses, taking place in two highly selective steps (Lundegaard *et al.* 2010). First, the antigenic structure has to bind to the HLA molecule. Second, pMHC has to be recognised by the TCR of T-cells.

Individual HLA molecules can only bind a restricted range of epitopes, which is mainly defined by the physicochemical properties of the amino acids forming the HLA binding groove. The amino acid sequence of peptides which are able to bind to a specific HLA allele create the so-called peptide binding motif of the HLA allele (Gowthaman and Agrewala 2009).

Traditionally, MHC peptide elution studies were used to define the binding motif of individual HLA alleles (Gowthaman and Agrewala 2009). In order to store and analyse the vast amount of data generated during these experiments, several bioinformatic tools have been developed over the past years. Advances in bioinformatics and increased availability of computational models have enabled the study of immunological processes *in silico*. This gave rise to the field of computational immunology or immunoinformatics (Petrovsky and Brusic 2002; Tomar and De 2010); and it is now possible to predict HLA binding specificities and identify new epitopes using computational methods.

Immunoinformatic resources comprise immunological databases, sequence alignment tools, epitope prediction algorithms, structural modelling and 3D simulations, as well as various mathematical models (Yan 2010). The methods are aimed at reducing time-consuming and expensive experimental procedures, thereby assisting in testing research hypotheses and guiding experimental research processes (Yan 2010). Immunoinformatic analyses are usually conducted in a systematic way starting at the DNA level comparing genetic sequence information, followed by correlating the genetic structure to the functional core, and finally using three-dimensional modelling to depict any structure-function relationship (Yan 2010). Experimental assays can then be used to test the structure-function associations and prove *in silico* findings.

In this chapter the individual binding characteristics of *HLA-A*31:01* and *HLA–B*15:02* were explored using publicly accessible bioinformatic tools. A systematic *in silico* analysis was conducted to investigate whether any similarities exist between *HLA-A*31:01* and *–B*15:02* with regards to their structure and/ or epitope binding specificity, which might provide a basis for their respective association with CBZ-induced HSRs.

Moreover, it has recently been proposed that CBZ might be able to stimulate T-cell responses in a similar way to abacavir, binding directly to the susceptible HLA allele and thereby altering the peptide repertoire (Illing *et al.* 2012).

For *HLA-B*15:02*, initial experimental evidence seems to support this mechanistic hypothesis (Illing *et al.* 2012). However, studies investigating the influence of CBZ on the peptide repertoire of *HLA-A*31:01* have not yet been performed. Therefore, a conventional MHC peptide elution study using a HLA-A*31:01-transfected C1R cell line was carried out, aiming to establish whether the standard peptide binding motif of *HLA-A*31:01* can be modified by CBZ treatment.

## 4.2 Methods

HLA *in silico* analysis was conducted in collaboration with Dr. Andrew Jones and Dr. Faviel Gonzalez Galarza from the Institute of Integrative Biology, University of Liverpool.

### 4.2.1 HLA sequence alignment

Amino acid sequence alignment was performed using CLUSTAL W2 (available at http://www.ebi.ac.uk/Tools/msa/clustalw2/) to determine differences and similarities between selected alleles. Sequences were extracted in FASTA file format from dbMHC database (see Table 4.1) and entered into CLUSTAL W2 for analysis.

### 4.2.2 Peptide binding motifs

Peptide binding motifs published for *HLA-A*31:01* and *-B*15:02* were obtained from MHC Motif Viewer and SYFPEITHI databases (Table 4.1).

Motifs were compared manually by eye to determine the preferred amino acids at each position of nonamer peptide ligands.

### 4.2.3 3D modelling

The three-dimensional model structures of HLA-A*31:01 and HLA-B*15:02 were generated using MODELLER 9.9 software (Sali and Blundell 1993), and structures of HLA-A*11:01 (PDB ID 1Q94) and HLA-B*15:01 (PBD ID 1XR8) published on the RCSB Protein Data Bank (PDB) were used as templates respectively. The selected HLA template structures both represented the closest matching alleles for which a crystal structure was available on the Protein Data Bank. After screening Immune Epitope Database (IEDB) and SYFPEITHI database (Table 4.1), a common epitope reported to bind to both HLA-A*31:01 and HLA-B*15:02 was modelled into the HLA binding groove.

Theoretical 3D structures of HLA-A*31:01 and HLA-B*15:02 were sent to RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) for validation of the three-dimensional spacing of amino acids by Ramachandran plot. Valid 3D structures were visualised in PyMol (DeLano 2002).

**Table 4.1**: Bioinformatic resources and databases used in this study

| Name | Description | URL | Reference |
|---|---|---|---|
| **IMGT/HLA** | Database on HLA sequences and analysis tools | http://www.ebi.ac.uk/ipd/imgt/hla/ | (Robinson *et al.* 2013) |
| **dbMHC** | Database comprising DNA and clinical data related to MHC | http://www.ncbi.nlm.nih.gov/projects/gv/mhc/ | (Wheeler *et al.* 2004) |
| **CLUSTAL W 2.0** | Program for multiple sequence alignments of nucleotide or amino acid sequences | http://www.ebi.ac.uk/Tools/msa/clustalw2/ | (Larkin *et al.* 2007) |
| **IEDB** | Database on B- and T-cell epitopes, including search interface | http://www.iedb.org/ | (Peters *et al.* 2005) |
| **SYFPEITHI** | Database of MHC ligands and peptide binding motifs | http://www.syfpeithi.de/ | (Rammensee, H. *et al.* 1999) |
| **MHC Motif Viewer** | Repository of MHC binding motifs | http://www.cbs.dtu.dk/biotools/MHCMotifViewer/Home.html | (Rapin *et al.* 2008) |
| **RSCB Protein Data Bank** | Archive of crystal structures of biological macromolecules | http://www.rcsb.org/pdb/home/home.do | (Berman *et al.* 2000) |
| **RAMPAGE** | Program assessing Ramachandran plots of protein structures | http://mordred.bioc.cam.ac.uk/~rapper/rampage.php | (Lovell *et al.* 2003) |

The methods outlined below have been described previously (Illing *et al.* 2012) and the work was carried out in the laboratories of Prof. James McCluskey, Department of Microbiology& Immunology, University of Melbourne, Australia and Prof. Anthony Purcell, Department of Biochemistry, Monash University, Melbourne, Australia.

### 4.2.4 Transfection of C1R cell lines

*This part of the work was performed by Patricia Illing, Department of Microbiology& Immunology, University of Melbourne, Australia*.

C1R cells were transfected with either *HLA-A*31:01* or one of two control alleles, *HLA-A*03:01* or *HLA-A*31:04* (cDNA clones for each were purchased from GenScript, USA), using a murine stem cell virus-based retroviral expression system (Szymczak *et al.* 2004; Holst *et al.* 2006). The B-lymphoblast cell line C1R is considered a HLA-A,B negative mutant cell line which only expresses HLA-C*04 and low amounts of HLA-B*35 on its surface (Zemmour *et al.* 1992).

Briefly, cDNA constructs were ligated into the MSCV-IRES-GFP retroviral expression vector (pMIG; Clonotech laboratories, USA) and transformed into *E.coli*. Vectors containing the correct insert were introduced into 293T HEK cells by retroviral transduction for replication. C1R cell lines were subsequently transfected with viral vectors produced by 293T cells. Successfully transfected C1R cell lines were screened for surface HLA expression by flow cytometry followed by single cell sorting of highly HLA expressing cells.

### 4.2.5 C1R cultures

HLA-transfected C1R cell lines were cultured in 100ml RF10 medium (see chapter 3.2.3) in T175 flasks at 37°C. C1R cell lines were grown in duplicate cultures, one left untreated and one treated with 25µg/ml CBZ, until a cell density of approximately $1x10^8$ cells/culture was reached. The culture medium was changed every 2-3 days to maintain proliferation. C1R cells were harvested

by centrifugation (2400rpm, 15min, 4°C), washed twice in cold PBS, then pelleted in 50ml falcon tubes at 5-10x$10^7$ cells and frozen immediately on dry ice. Cell pellets were stored at -80°C until needed.

In a second experimental set-up, HLA-A*31:01 transfected C1R cells were grown to high density in T175 flasks in presence and absence of CBZ. Cultures were then transferred into 2L roller bottles and cultured at 37°C under constant rolling. 300ml RF10 medium (+/- 25µg/ml CBZ) was added every 2-3 days until 1-1.2L of dense cell cultures were obtained. Cells were harvested as described above and frozen at 5-10x$10^8$ cells.

During the culture period, HLA surface expression was monitored regularly using flow cytometry.

### 4.2.6 Preparation of w6/32 cross-linked Protein A resin

W6/32 hybridoma cells (Barnstable *et al.* 1978) were cultured in 800ml RF3 (RF10 with only 3% FBS) medium in roller bottles at 37°C under constant rolling. When cell density was considered high enough, a 500ml cell suspension was spun down (2400rpm, 20min, 4°C) and supernatant was filtered through Nalgene® Rapid Flow 0.2µm aPES vacuum filter (Thermo Scientific, USA).

*The subsequent steps were carried out by Rochelle Ayala.*

W6/32 anti-MHC I antibody was purified by Affinity Chromatography Profina Purification System (Bio-Rad, Australia). The antibody was eluted into PBS according to the manufacturer's protocol.

2ml Protein A Sepharose Fast Flow (PAS; Amersham, GE Healthcare, UK) was applied to a Poly-Prep Chromatography column (Bio-Rad, Australia), washed with PBS and resuspended in PBS supplemented with 20mg purified w6/32 antibody. PAS mix was incubated at 4°C for 30min slowly rotating end over end. The mixture was poured into a Poly-Prep Chromatography column and the flow-through passed over the column a second time. W6/32 bound resin was washed with filtered 50mM $H_3BO_3$, 50mM KCl (Ajax Finechem, Australia), 4mM

NaOH pH 8.0 (ChemSupply, Australia). Resin was equilibrated in 0.2M Triethanolamine pH 8.2 (Ajax Finechem, Australia). W6/32 was cross-linked to PAS by incubation in 40mM Dimethylpimelimidate (DMP; Sigma-Aldrich, USA) in 0.2M Triethanolamine pH 8.2. Cross-linking reaction was terminated using ice-cold 0.2M Tris pH 8.0, and any un-cross-linked w6/32 was removed with 0.1M citric acid (Ajax Finechem, Australia). Resin was equilibrated into PBS and stored at 4°C for max. 1 week until used. Directly before use the w6/32 affinity column was run into wash buffer 1 (see Table 4.2).

### 4.2.7 Elution and Purification of MHC bound peptides

Frozen C1R pellets were ground in a Retsch Mixer Mill MM 400 (Retsch, Germany) under cryogenic conditions and resuspended in 20ml lysis buffer containing 0.5% IGEPAL (Sigma-Aldrich, USA), 50mM Tris pH 8.0, 150mM NaCl (Merck-Millipore, Germany) and protease inhibitors (Complete Protease Inhibitor Cocktail Tablet; Roche Molecular Biochemicals, Switzerland). Cell lysates were incubated for 45min at 4°C rotating slowly end over end. Lysates of $1x10^8$ cells were spun down at 13,000rpm for 20min. For $1x10^9$ cell pellets, lysates were cleared by ultracentrifugation at 45,000rpm. Any remaining particles were removed by passing the lysates through a Pre-Prep Chromatography column containing only PAS, which was pre-washed with wash buffer 4 and equilibrated into wash buffer 1 prior to use. Lysates were then passed through 1ml of w6/32 resin in a Pre-Prep Chromatography column (prepared as described above) to capture MHC class I bound peptides. Any unspecific bound material was removed by washing with 20ml of wash buffers 1-4 sequentially (see Table 4.2). Peptide-MHC complexes were eluted off the column using 4ml of 10% acetic acid. Peptides were separated from the protein components (MHC class I heavy chain and β2m) using a 4.6mm internal diameter x 50mm long monolithic reversed-phase (RP) C18 High Performance Liquid Chromatography (HPLC) column (Chromolith Speed Rod, Merck-Millipore, Germany) on an ÄKTAmicro™ HPLC system (GE Healthcare, UK) operated by UNICORN™ software (GE Healthcare, UK). The mobile phase consisted of 0.1% trifluoroacetic acid (TFA, Thermo Scientific, USA) [buffer A]

and 80% acetonitrile (Fisher Scientific, USA)/ 0.1% TFA [buffer B]. The column was pre-equilibrated into 2% buffer B (98 % buffer A) at a flow rate of 1ml/min, and then loaded with the eluted peptide/protein mix via a 5ml injection loop. The separation of peptides from the protein components was facilitated by an increasing gradient of buffer B. Explicitly, after re-equilibration of the column into 2% buffer B, the concentration of buffer B increased linearly to 40% over 4ml, to 45% over the subsequent 4ml (to resolve peptides), then to 100% over the next 2ml. The flow rate was maintained at 1ml/min throughout and UV absorbance of eluted material was monitored at 215nm. Fractions of 500µl volume expected to contain peptides were collected in 1.7ml Protein LoBind Eppendorf tubes.

**Table 4.2**: Composition of wash buffers for peptide elutions.

| Wash buffer | components |
|---|---|
| **1** | 0.005% IGEPAL, 50mM Tris pH 8.0, 150mM NaCl, 5mM EDTA, 100uM PMSF, 1µg/ml Pepstatin A in MilliQ $H_2O$ |
| **2** | 50mM Tris pH 8.0, 150mM NaCl in MilliQ $H_2O$ |
| **3** | 50mM Tris pH 8.0, 450mM NaCl in MilliQ $H_2O$ |
| **4** | 50mM Tris pH 8.0 in MilliQ $H_2O$ |

### 4.2.8 Peptide identification by mass spectrometry

*This part of the work was undertaken by Patricia Illing at the Department of Biochemistry, Monash University, Australia.*

Peptide containing fractions were concentrated using a speed vacuum concentration system (LABCONCO, USA) and loaded onto a microfluidic trap column packed with ChromXP C18-CL 3µm particles (300Å nominal pore size; AB SCIEX, USA), pre-equilibrated in 0.1% formic acid (AnalaR, Australia), 5% acetonitrile at 5µl/min using an Eksigent NanoUltra cHiPLC system (AB SCIEX,

USA). An analytical (15 cm x 75 μm ChromXP C18-CL 3) microfluidic column (AB SCIEX, USA) was switched in line and peptides separated using a linear gradient of 0-64% acetonitrile over 55min at a flow rate of 300nl/min. Separated peptides were analysed using an AB SCIEX 5600 TripleTOF mass spectrometer equipped with a Nanospray III ion source and accumulating up to 20 MS/MS spectra per second. Acquired data was analysed with Protein Pilot™ software version 4.5 (AB SCIEX, USA) searching against the human proteome, and identified peptides subjected to strict bioinformatic criteria assigning confidence values to each peptide. A false discovery rate was calculated by use of a decoy database.

### 4.2.9 Generation of peptide binding motifs

The peptide binding motif of HLA-A*31:01 was generated through analysis of the mass spectrometry data using excel analysis files designed by Patricia Illing and Nathan Croft at the Department of Biochemistry, Monash University, Australia.

The distribution of peptides of specific length was calculated, as well as the frequency with which amino acids appeared at a specific position within the peptides.

## 4.3 Results

### 4.3.1 In silico modelling

***HLA sequence alignment***

Initially, amino acid (AA) signatures of *HLA-A*31:01* and *HLA-B*15:02* were compared to members of the respective serotype and supertype family. The alignment was performed for the AA sequence of the α1 and α2 domain (exon two and three) forming the binding cleft of HLA class I molecules. AA changes highlighted by the programme CLUSTAL W2 were extracted and residues located in the binding pockets A- F of HLA class I molecules, previously described by Saper and colleagues (Saper *et al.* 1991), were analysed further.

When *HLA-A*31:01* was compared to members of the HLA A31 serotype, as defined by the WHO and listed on the IMGT/HLA database, no AA residue was found to occur exclusively in *HLA-A*31:01* (Table 4.3). Unique AA residues could be observed in isolated alleles of the A31 serotype but not in *HLA-A*31:01*. In total, only 12 out of 182 residues differed between A31 serotype members, eight of which were located in binding pockets (Table 4.3).

**Table 4.3**: Summary of HLA A31 serotype sequence alignment.

| Residue (Pocket) Physicochem | 66 (A/B) s | 73 (C) n | 77 (F) s | 80 (F) n | 81 (F) n | 97 (C/E) n | 114 (D/E) s | 167 (A) n |
|---|---|---|---|---|---|---|---|---|
| **A*31:02** | K | I | D | T | L | M | Q | W |
| **A*31:12** | N | I | D | T | L | K | Q | W |
| **A*31:01** | N | I | D | T | L | M | Q | W |
| **A*31:04** | N | I | D | T | L | I | R | W |
| **A*31:05** | N | I | D | T | L | M | Q | G |
| **A*31:10** | N | T | N | I | A | M | Q | W |

Physicochemical properties of amino acid residues: s= strong similarity; n= not conserved



**Table 4.4**: Summary of HLA A03 supertype sequence alignment.

| Residue (Pocket) Physicochem | 9 (B/C) n | 63 (A/B) s | 70 (B/C) s | 73 (C) n | 97 (C/E) n | 114 (D/E) s | 152 (E) n | 156 (D/E) n | 163 (A) n | 171 (A) s |
|---|---|---|---|---|---|---|---|---|---|---|
| **A*33:01** | T | N | H | I | M | Q | V | L | T | H |
| **A*33:03** | T | N | H | I | M | Q | V | L | T | Y |
| **A*31:01** | T | E | H | I | M | Q | V | L | T | Y |
| **A*74:01** | F | E | H | T | M | Q | V | L | T | Y |
| **A*66:01** | Y | N | Q | T | R | Q | E | W | R | Y |
| **A*68:01** | Y | N | Q | T | M | R | V | W | T | Y |
| **A*03:01** | F | E | Q | T | I | R | E | L | T | Y |
| **A*11:01** | Y | E | Q | T | I | R | A | Q | R | Y |

A second sequence alignment was performed on the HLA A03 supertype family as defined in the paper by Sidney *et al.* (2008). HLA supertypes represent groups of HLA molecules which show an overlap in peptide specificity and therefore display similar peptide binding motifs (Sidney *et al.* 2008). As expected the variation in AA sequences was broader for the HLA A03 supertype than the A31 serotype. Sequences varied in 22 of the 182 residues forming the binding cleft, with ten AAs located in HLA class I binding pockets. AA changes were detected in all binding pockets apart from the F-pocket, which was found to be fully conserved across the A03 supertype. But no AA residue was specific to *HLA-A*31:01* (Table 4.4).

In parallel, *HLA-B*15:02* was analysed in comparison to HLA alleles of the HLA B75 serotype and B62 supertype respectively (Table 4.5 and 4.6). Six residues were found to vary among the alleles of the HLA B75 serotype, and five of the residues were positioned in binding pockets. No alterations were observed in the A and C pockets. The only variable residue of the F pocket, residue 95, fluctuated between leucine and isoleucine (Table 4.5). Most altered residues were defined by two different amino acids, with the exception of residue 67 which was characterised by a different amino acid for nearly every allele. However, none of the AA residues were solely expressed by *HLA-B*15:02* (Table 4.5). For the HLA B62 supertype, AA sequences differed in 25 residues in total. Sixteen residues were determined to be part of the binding pockets A to F (Table 4.6). Most variations affected only a single HLA allele but not *HLA-B*15:02*. Limited sequence variations which affected *HLA-B*15:02* alongside *HLA-B*15:13* were observed for residues 63 (Glu63Asn) and 113 (His113Tyr). However, the different amino acids in both positions were determined to be of strong similarity regarding their physicochemical properties.

**Table 4.5**: Summary of HLA B75 serotype sequence alignment.

| Residue (Pocket) Physicochem | 67 (B) n | 95 (F) s | 113 (D) s | 152 (E) n | 156 (D/E) n |
|---|---|---|---|---|---|
| **B*15:02** | S | I | Y | E | L |
| **B*15:21** | C | I | Y | E | L |
| **B*15:31** | S | L | Y | V | L |
| **B*15:08** | F | L | H | E | W |
| **B*15:11** | Y | L | H | E | W |



**Table 4.6**: Summary of HLA B62 supertype sequence alignment.

| Residue (Pocket) Physicochem | 63 (A/B) s | 66 (A/B) n | 67 (B) n | 70 (B/C) n | 74 (C) n | 77 (F) w | 80 (F) n | 81 (F) n | 95 (F) n | 97 (C/E) n | 113 (D) s | 114 (D/E) s | 116 (F) n | 156 (D/E) n | 167 (A) n | 171 (A) s |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **B*15:01** | E | I | S | T | Y | S | N | L | L | R | H | D | S | W | W | Y |
| **B*46:01** | E | K | Y | R | D | S | N | L | L | R | H | D | S | W | W | Y |
| **B*15:12** | E | I | S | T | Y | S | N | L | L | R | H | D | S | W | G | Y |
| **B*15:02** | N | I | S | T | Y | S | N | L | I | R | Y | D | S | L | W | Y |
| **B*15:13** | N | I | S | T | Y | N | I | A | I | R | Y | D | S | L | W | Y |
| **B*52:01** | E | I | S | T | Y | N | I | A | W | T | H | N | Y | L | W | H |

Physicochemical properties of amino acid residues: s= strong similarity; w= weak similarity; n= not conserved

Next, an alignment of *HLA-A*31:01* and *B*15:02* was performed, including *A*03:01* and *B*46:01* as control alleles (Table 4.7). *HLA-A*03:01* and *HLA-B*46:01* were selected as control alleles due to their high frequency in Caucasian and Asian populations respectively, and therefore assumed not to be a risk factor for CBZ hypersensitivity. Additionally, both control alleles are part of the respective HLA supertype. Especially modifications in the B- and F-pocket were considered to be of interest, as these pockets are known to interact with anchor residues P2 and PΩ of binding peptide epitopes (Sidney *et al.* 2008). Furthermore, the F-pocket of the *HLA-B*57:01* allele had previously been shown to be the major binding site for abacavir, an antiretroviral drug known to cause hypersensitivity syndrome in patients carrying *HLA-B*57:01* (Chessman *et al.* 2008; Illing *et al.* 2012).

The AA sequences of the four alleles varied in 37 residues, of which 13 were located in binding pockets (Table 4.7). Although the alignment shows the highest variety between AA sequences in comparison to afore described alignments, almost 80% of the AA residues of the α1 and α2 domains were fully conserved. Three residues of the F pocket (77, 80, 116) were found to be modified, clearly separating the HLA-A alleles from HLA-B (Table 4.7). In the B pocket, alterations in five residues (9, 63, 66, 67, 70) were observed; and the physicochemical properties of two of these residues (63, 70) were characterised as strongly similar across the four alleles. However, none of the 13 altered amino acids were the same for *HLA-A*31:01* and *B*15:02*.

**Table 4.7**: Differences in amino acid sequence between *HLA-A*31:01* and *HLA-B*15:02* and two control alleles.

| Residue (Pocket) Physicochem | 9 (B) n | 63 (B/A) s | 66 (B) n | 67 (B) n | 70 (B) s | 73 (C) n | 74 (C) n | 77 (F) w | 80 (F) w | 97 (C/E) n | 114 (D/E) n | 116 (F) w | 152 (E) n |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A*03:01** | F | E | N | V | Q | T | D | D | T | I | R | D | E |
| **A*31:01** | T | E | N | V | H | I | D | D | T | M | Q | D | V |
| **B*15:02** | Y | N | I | S | N | T | Y | S | N | R | D | S | E |
| **B*46:01** | Y | E | K | Y | R | T | D | S | N | R | D | S | E |

Physicochemical properties of amino acid residues: s= strong similarity; w= weak similarity; n= not conserved

***Peptide binding motifs***

Following the analysis of the AA sequence characteristics of *HLA-A*31:01* and *B*15:02*, the nature of binding peptide epitopes was examined.

First, published binding motifs of the two HLA alleles were obtained from the MHC Motif viewer website (Figure 4.1). The most outstanding feature for both *HLA-A*31:01* and *B*15:02* is the very dominant P9 anchor residue indicating that the binding specificity of both alleles is strongly determined by the C-terminal residue of nonamer peptides. The binding contribution of this AA residue is far more prominent in *HLA-A*31:01* and *B*15:02* than in common, classical HLA class I alleles, e.g. *HLA-A*02:01* and *HLA-B*08:01* (Figure 4.1). Furthermore, the C-terminal binding site of *HLA-A*31:01* shows a strong preference for peptides with a basic arginine residue, whereas *HLA-B*15:02* prefers neutral to hydrophobic amino acids at the P9 position. Generally, the main anchor residues of HLA class I binding peptides are the positions P2 and P9, but this is not the case for *HLA-A*31:01* or *B*15:02*. When comparing the binding motifs of the two alleles to motifs of classic HLA class I alleles, such as *HLA-A*02:01*, the P2 residue does not seem to act as an anchor residue for either *HLA-A*31:01* or *B*15:02* (Figure 4.1). Nor does any other peptide residue appear to function as a substitute for the missing anchor residue.

These observations are largely consistent with the data available from the SYFPEITHI database (Table 4.8). However, the peptide binding motif of *HLA-B*15:02* published on SYFPEITHI displays position P2 as anchor residue. For *HLA-A*31:01*, peptide positions P2, P3 and P6 are classified as auxiliary anchor residues. When the preferred amino acids in positions P1 to P9 were compared between *HLA-A*31:01* and *B*15:02*, a considerable overlap could be detected in position P2, P3 and P4 (Table 4.8). Nonetheless, *HLA-A*31:01* seemed to accommodate a wider range of amino acids in its' binding pockets which suggests that *HLA-A*31:01* exhibits weaker binding specificity in preferred amino acids with the exception of anchor position P9.

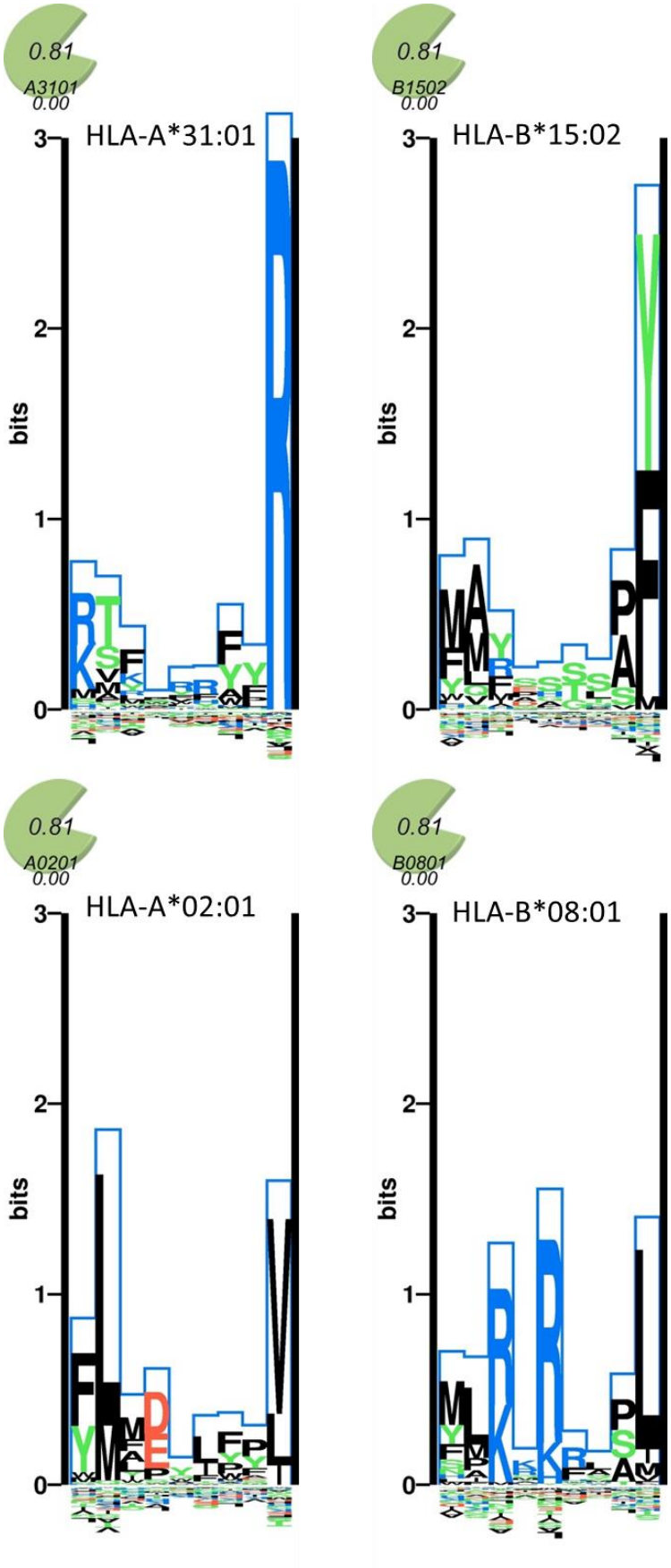



**Figure 4.1**: Peptide binding motifs of *HLA-A*31:01, HLA-B*15:02* and two control HLA alleles. Logo-plots were extracted from the MHC Motif Viewer (Rapin *et al.* 2008).

Binding motifs are plotted for nonamer peptides. Amino acids with a positive binding effect are mapped on the positive y-axis; negative amino acids with a negative binding effect are shown on the negative y-axis. The height of each amino acid represents the relative contribution to the binding specificity.

The pie chart represents the reliability index (upper number) for the binding motif, which is determined by Pearson correlation calculating the pseudo sequence distance (lower number) to the nearest neighbouring allele with a well-characterised binding specificity.

**Table 4.8**: Comparison of HLA-A*31:01 and HLA-B*15:02 peptide binding motifs derived from SYFPEITHI database (Rammensee, H. *et al.* 1999).

| | P1 | | P2 | | P3 | | P4 | | P5 | | P6 | | P7 | | P8 | | P9 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***A*** | ***B*** | ***A*** | ***B*** | ***A*** | ***B*** | ***A*** | ***B*** | ***A*** | ***B*** | ***A*** | ***B*** | ***A*** | ***B*** | ***A*** | ***B*** | ***A*** | ***B*** |
| (auxil.) **anchor** | | | L<br>V<br>Y<br>F | **Q**<br>**L**<br>**V**<br>**P** | F<br>L<br>Y<br>W | | | | | | L<br>F<br>V<br>I | | | | | | **R** | **F**<br>**Y**<br>**M** |
| preferred | K<br>R | | T<br>Q | | K<br>N | Y<br>R<br>F<br>K<br>N<br>W | P<br>D<br>E<br>G<br>S<br>V<br>T | P<br>E<br>D | P<br>I<br>V<br>F<br>L<br>Y<br>W | P<br>E<br>D | T<br>N<br>D<br>E<br>R | D | N<br>V<br>R<br>F<br>T<br>H<br>L<br>Y | V | L<br>R<br>N<br>Q | | | |

P= position of amino acid in nonamer; A= HLA-A*31:01; B= HLA-B*15:02

***3D structural modelling***

As no crystal structures for either *HLA-A*31:01* nor *HLA-B*15:02* were available on the RSCB Protein Data Bank, three-dimensional model structures were generated *in silico* using MODELLER 9.9 software. The structures of *HLA-A*11:01* (Li, Lenong and Bouvier 2004) and *HLA-B*15:01* (Roder *et al.* 2006) were determined to be the closest neighbouring alleles for which a crystal structure had been deposited on PBD, and thus were used as template structures. *HLA-A*31:01* differs in 18 AA residues from *HLA-A*11:01*, with eight amino acids (9, 70, 73, 97, 114, 152, 156, 163) located in binding pockets. Five amino acid residues vary between *HLA-B*15:02* and *HLA-B*15:01*, of which four amino acids (63, 95, 113, 156) form parts of binding pockets.

The 3D models of HLA-A*31:01 and HLA-B*15:02 comprised each of the HLA alleles in complex with the *Escherichia coli* peptide KLAEIFQPF, as HLA molecules without embedded peptide are considered to be unstable and might show a different three-dimensional architecture. KLAEIFQPF had been identified as a common binder of both *HLA-A*31:01* and *B*15:02*, after comparing published peptide epitopes for each of the HLA alleles on IEDB and SYFPEITHI database. Peptide epitopes were cross-matched for common peptide

binders between *HLA-A*31:01* and *B*15:02* which were not present in the previously selected control alleles, i.e. *HLA-A*03:01* and *HLA-B*46:01*.

Spatial arrangement of amino acids in the generated 3D structures was validated by Ramachandran plot via RAMPAGE (see Appendix F2). Both models satisfied the evaluation criteria of the Ramachandran analysis (Table 4.9). In each model only 1 amino acid was considered as an outlier, however, in both cases the amino acid in question did not fall in the HLA binding region and was therefore considered to be unimportant.

**Table 4.9**: RAMPAGE results for 3D models of *HLA-A*31:01* and *HLA–B*15:02*.

| Ramachandran plot Evaluation | HLA-A*31:01 | HLA-B*15:02 |
|---|---|---|
| Residues in favoured region (98% expected) | 730 (97.9%) | 371 (98.1%) |
| Residues in allowed region (2% expected) | 7 (1.9%) | 6 (1.6%) |
| Residues in outlier region | 1 (0.3%) | 1 (0.3) |

To assess the structural characteristics of the peptide binding cleft of HLA-A*31:01 and B*15:02, the 3D models were visualised in PyMol and the binding cleft of each allele was selected for display. Further analysis concentrated on the spatial conformation of altered amino acids between template and model structures which were located in HLA class I binding pockets.

The binding cleft of HLA-A*31:01 displayed modifications in eight amino acids in comparison to HLA-A*11:01 (Figure 4.2). When the AA sequence of *HLA-A*03:01* was taken into consideration as additional control allele, only four AA residues remained specific to HLA-A*31:01. The modified residues Gln70His, Thr73Ile, Ile97Met and Arg114Gln displayed an altered spatial conformation which could affect peptide binding, and thus might demarcate the binding specificity of HLA-A*31:01 (Figure 4.2). The altered residues were located across binding pockets B to E (70-B/C, 73-C, 97-C/E, 114-D/E).

The three-dimensional model of HLA-B*15:02 showed changes in only four binding pocket residues when compared to HLA-B*15:01 (Figure 4.3). The altered amino acids Glu63Asn, Leu95Ile, His113Tyr and Trp156Leu were located in binding pockets A/B, F, D and D/E respectively. Importantly, the sequences of *HLA-B*15:01* and *B*46:01* are identical in these four positions. In conjunction with a change in physiochemical properties, the structural conformation of AA residues 63 and 113 might mark the binding specificity of HLA-B*15:02 (Figure 4.3).

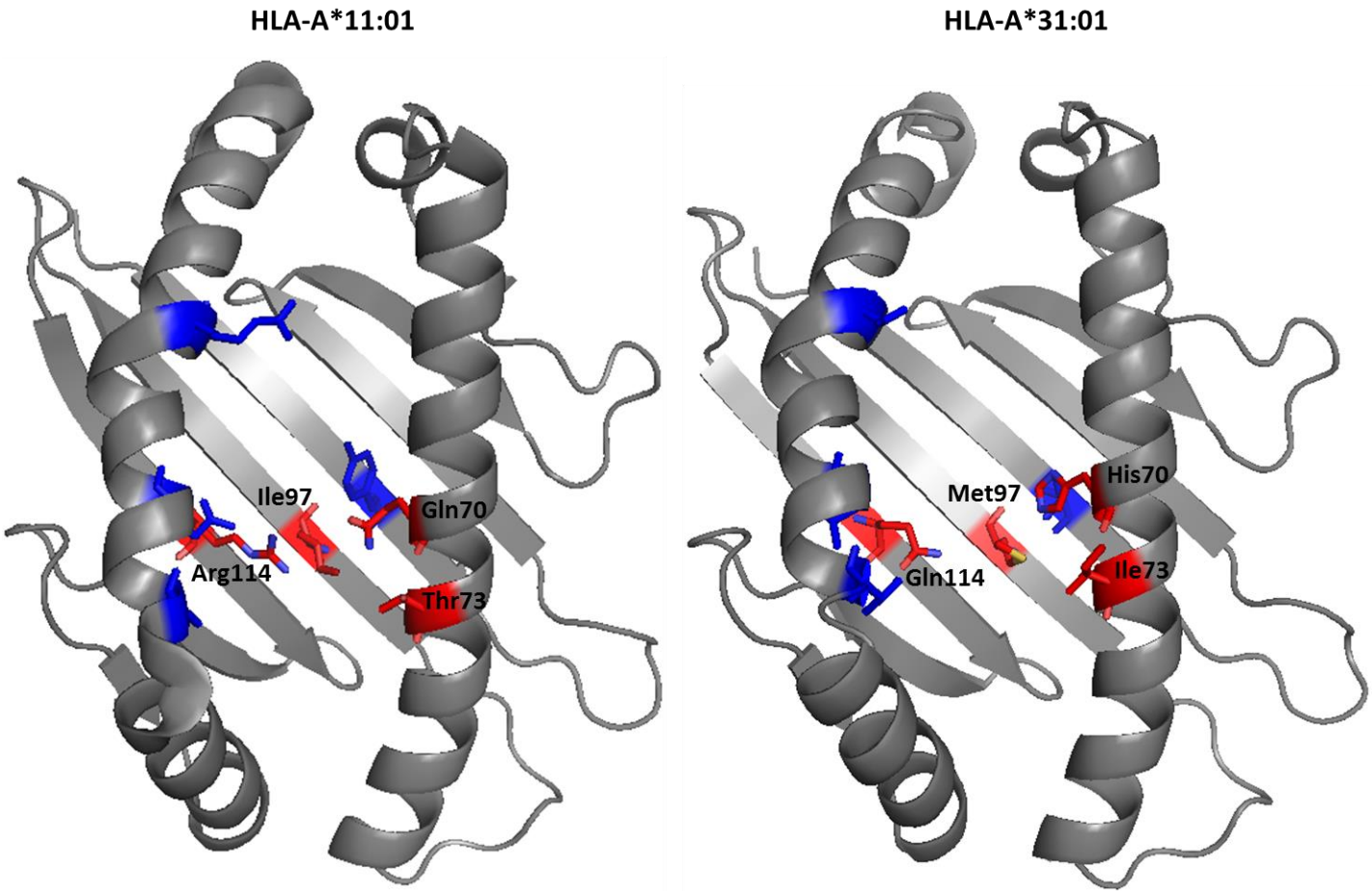



**Figure 4.2**: Binding cleft of HLA-A*31:01 in comparison to HLA-A*11:01.

The graphics represent the three-dimensional ribbon structure of the HLA binding cleft (grey). Altered amino acid residues which are located in binding pockets are highlighted as stick structures.

The red sticks depict amino acids which are characteristic to HLA-A*31:01 when residues were compared to sequences of HLA-A*11:01 and A*03:01. The blue sticks illustrate amino acids which vary across all three HLA-A alleles.

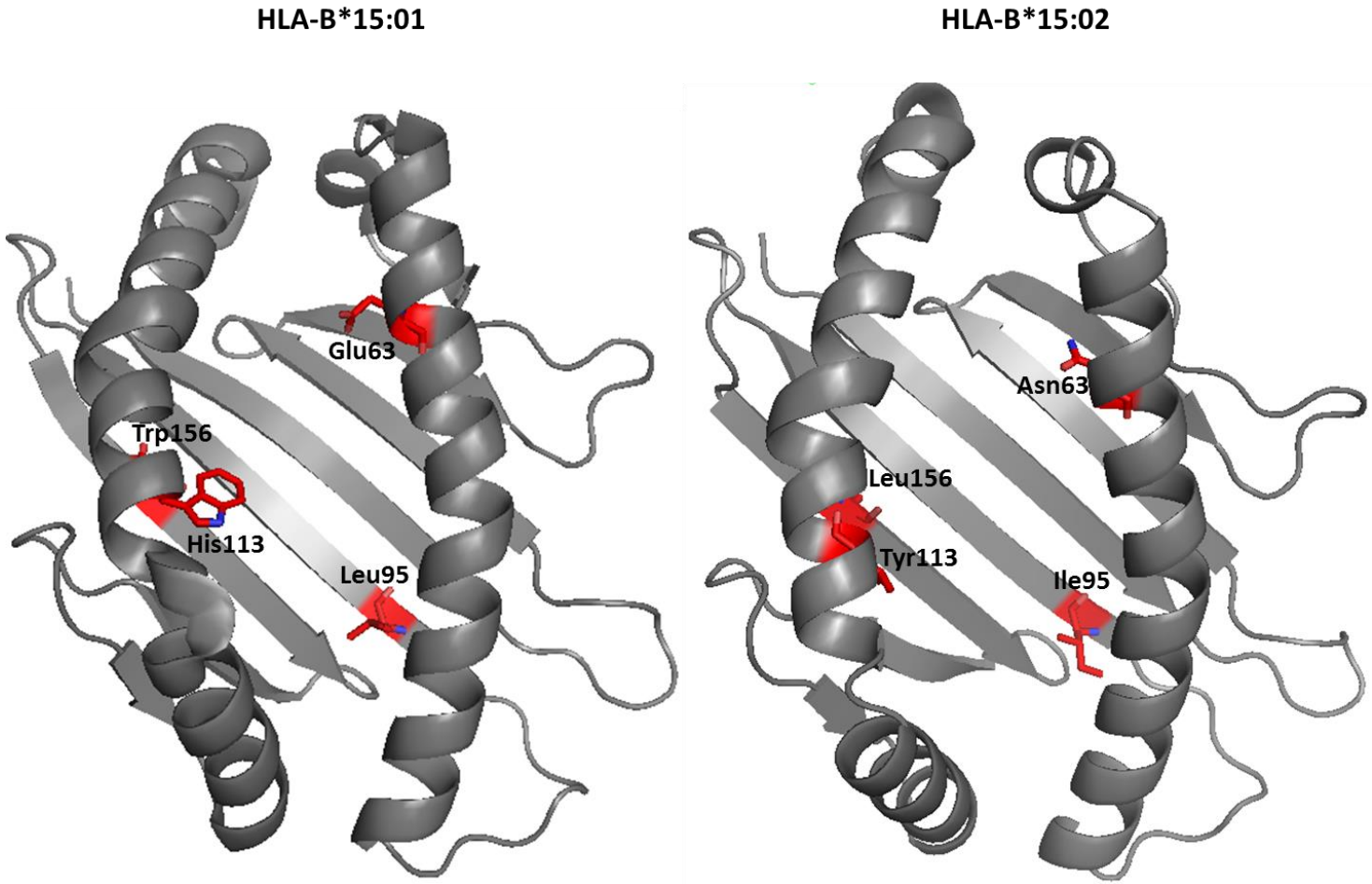



**Figure 4.3**: HLA-B*15:02 binding cleft in relation to HLA-B*15:01.

The HLA binding cleft (grey) is depicted in ribbon configuration and modified amino acids located in binding pockets are highlighted as stick structures.

Red amino acids are unique to HLA-B*15:02 in comparison to amino acid sequences of HLA-B*15:01 and B*46:01.

### 4.3.2 Peptide elution studies

In order to characterise the peptide binding motif of *HLA-A*31:01 in vitro* and to test whether CBZ is able to modify the *HLA-A*31:01* peptide repertoire, the B-lymphoblastoid cell line C1R was transfected with *HLA-A*31:01* or a control HLA-A allele, i.e. *HLA-A*03:01* or *HLA-A*31:04*. C1R cells are considered to lack HLA class I expression, except for HLA-C*04 and minor amounts of HLA-B*35, and are a widely used tool for functional studies of individual HLA class I alleles (Zemmour *et al.* 1992). *HLA-A*03:01* and *HLA-A*31:04* were selected as control alleles, on the grounds that *HLA-A*03:01* was absent in CBZ hypersensitive patients in our GWAS (McCormack *et al.* 2011) and *HLA-A*31:04* shows a high similarity to *HLA-A*31:01* with only two changes in AA sequence.

HLA-transfected C1R cells were cultured in the presence and absence of CBZ, and HLA-bound peptides isolated by affinity chromatography using the anti-HLA class I specific antibody w6/32. The purified peptide-HLA complexes were eluted with acetic acid, which also facilitates dissociation of peptide and HLA, and separated by RP-HPLC. When the chromatograms of CBZ-treated cultures were compared to untreated cultures, no peak signifying the accumulation of CBZ could be detected in the UV trace (Figure 4.4). The peptide-containing fractions were subsequently analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Unmodified CBZ could be clearly detected in all drug-treated samples (Figure 4.5). However, CBZ levels were no higher for HLA-A*31:01 than for the control alleles, suggesting binding of CBZ to HLA-A alleles was non-specific (data not shown). No CBZ metabolites could be observed in the MS spectra of the peptide fractions.

Next, the peptide repertoire presented by HLA-A*31:01 in the presence and absence of CBZ was assessed in more detail.

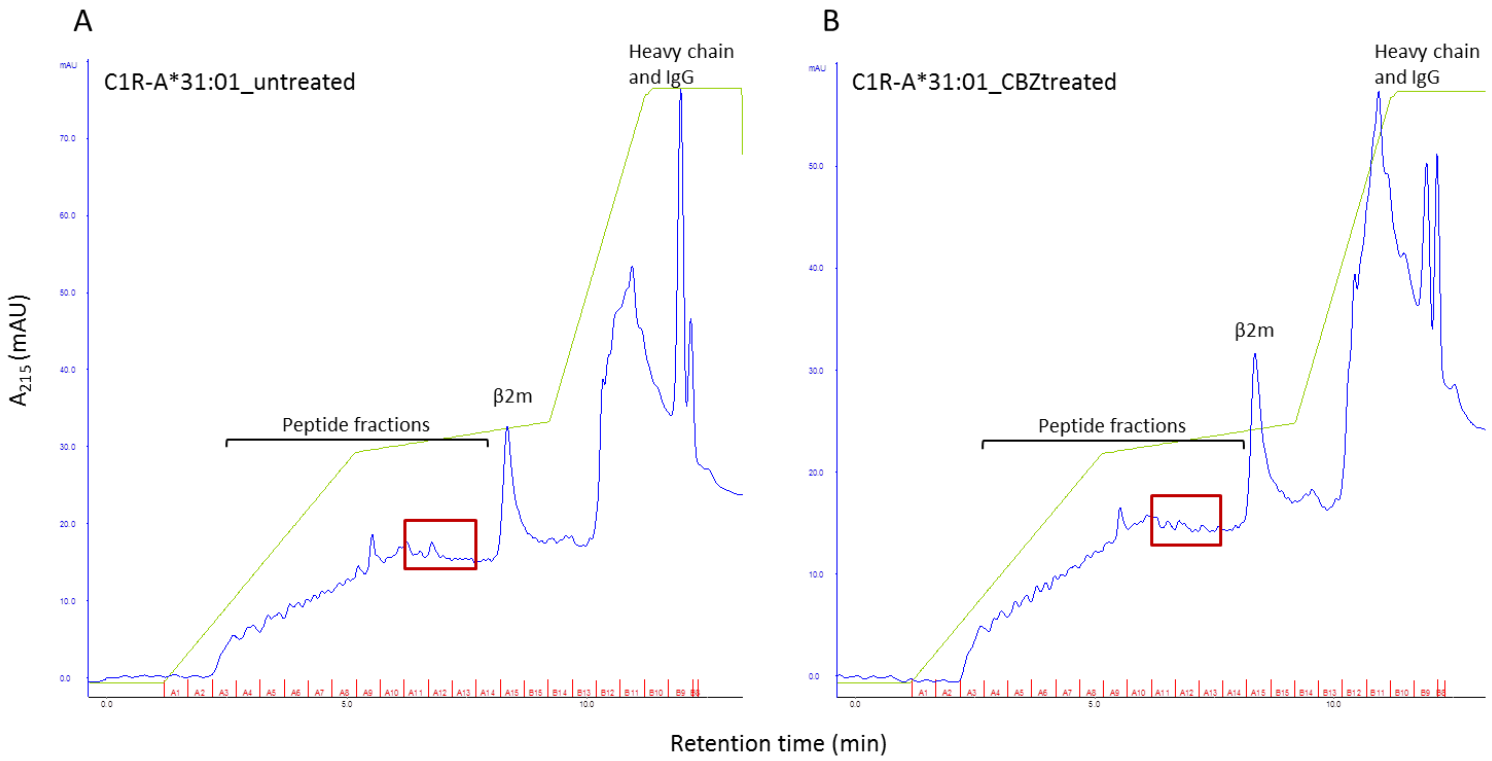



**Figure 4.4**: Chromatograms of RP-HPLC separation of material eluted from HLA-A*31:01-transfected C1R cells in the absence (A) and presence (B) of CBZ.

Eluted peptides from HLA-A*31:01 were separated from β2m and HLA heavy chain by RP-HPLC. The blue line shows the UV traces of the eluted material, and the green line represents the buffer gradient. The red box indicates the retention time interval containing CBZ.

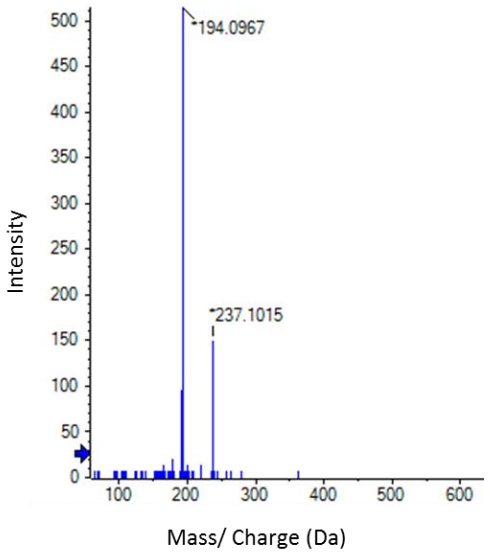



**Figure 4.5**: Detection of CBZ by LC-MS/MS.

CBZ-containing fractions were analysed by LC-MS/MS and CBZ was detected in all drug-treated cultures, as exemplified by this spectrum (predicted mass $MH^+$= 237.1022 Da).

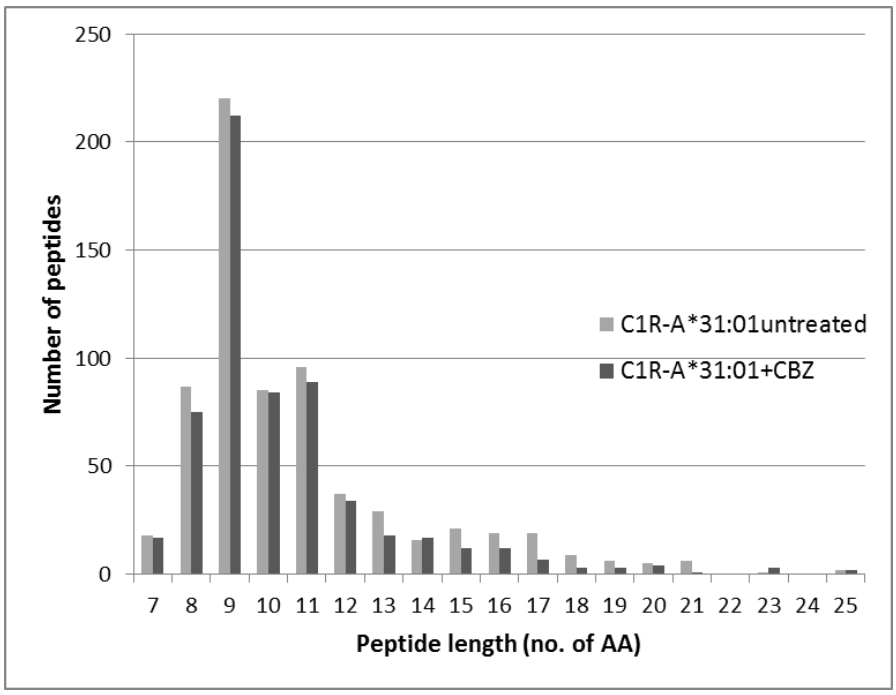



**Figure 4.6**: Length distribution of peptides eluted from HLA-A*31:01-transfected C1R cells in absence and presence of CBZ.

A total of 3329 and 2667 peptides were identified from the untreated and CBZ-treated HLA-A*31:01-transfected C1R cultures respectively. After removal of peptides specific for HLA-C*04 and HLA-B*35 using an in-house built decoy database, only 693 and 605 peptides remained that were determined to be presented by HLA-A*31:01 in the absence and presence of CBZ respectively.

In a first step, the distribution of peptide lengths was evaluated, as depicted in Figure 4.6. Most peptides were eight to eleven amino acids long, with nonamers being most abundant. Overall, there was no change in peptide length distribution between drug-treated and untreated cell lines (Figure 4.6).

Next, the binding motif of nonamer peptides was generated by calculating the relative frequency of each amino acid at each residue of the peptide. The resulting HLA-A*31:01 binding motif displayed a dominant arginine residue at position P9, which was the only obvious anchor residue (Table 4.10 A). This is characteristic for *HLA-A*31:01* and concordant with previously published motifs (see Figure 4.1). Furthermore, treatment of *HLA-A*31:01* transfectants with CBZ did not seem to induce any substantial changes in preferred AA residues within the HLA-A*31:01 peptide binding motif (Table 4.10 B).

This was examined in more detail by extracting the nonamer peptides unique to the untreated or CBZ-treated C1R cultures (99 and 91 peptides respectively), and comparing the AA frequencies at positions P1 to P9 (Table 4.11). AA frequencies were classified as increased or decreased if a difference of more than five percentage points was observed. In general, only minor differences were detected. In the presence of CBZ, hydrophobicity decreased marginally at positions P2, P3, P5 and P9, whereas it increased slightly at P6. Furthermore, a small shift in preferred amino acids at P7 and P8 towards charged residues could be observed (Table 4.11).

**Table 4.10**: Peptide binding motif of *HLA-A*31:01* in absence (A) and presence (B) of CBZ.

A) HLA-A*31:01 - untreated

| 9mers (n=220) | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 |
|---|---|---|---|---|---|---|---|---|---|
| **Dominant (≥40%)** | | | | | | | | | R |
| **Strong (≥20%)** | | F | D | | | | | | L |
| **Moderate (≥10%)** | R F | Y P | L | E D | I | L | V | L | F |
| **Increased (≥5%)** | V I K A S L T | L V T | F I | G L S V P | V L R T F K A | F E V D S A I P | I T A R F E S | S F E Y N K P G | |

B) HLA-A*31:01 - CBZ treated

| 9mers (n=212) | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 |
|---|---|---|---|---|---|---|---|---|---|
| **Dominant (≥40%)** | | | | | | | | | R |
| **Strong (≥20%)** | | F | D | | | | | | L |
| **Moderate (≥10%)** | R F | Y | L | E D | | L V | V T | L | F |
| **Increased (≥5%)** | K I L V M S T | P V L T | N | L P S G V T | I F V S A L R T K P | F E I R P D G | R E F I H S | E F S Y T K N P | M |

**Table 4.11**: CBZ-induced shift in preferred amino acid residues of nonamer peptides specific for *HLA-A*31:01*.

| AA Frequency | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 |
|---|---|---|---|---|---|---|---|---|---|
| Increased ↑ (>5PPT, <10PPT) | M L | Y | | | S | V | E R | E | |
| Decreased ↓ (>5PPT, <10PPT) | V | F P | F | | I | S | V A | G S | L |

PPT= percentage points

At the same time, however, it became apparent the binding motifs still contained considerable amounts of HLA-C*04 specific peptides. This was most evident in the anchor residues P2 and P9 which showed a strong preference for amino acids Phe and Leu/Phe respectively; and was further supported by the strong Asp residue in position P3 (Table 4.10). These amino acid residues are typical for peptides presented by HLA-C*04 in wild-type C1R cells (Schittenhelm *et al.* 2014).

As a consequence, the peptide sets were screened again manually and all peptides exhibiting a classic HLA-C*04 motif were removed. This resulted in a decrease of HLA-A*31:01 specific nonamer peptides to 140 and 133 peptides for untreated and CBZ-treated C1R cells respectively, of which 55 and 58 peptides were unique to each of the data sets. These peptide numbers were determined to be too low to generate a reliable binding motif and prevented any further analysis at this point.

## 4.4 Discussion

In the first part of this chapter, *in silico* analyses were performed in order to explore whether the two distinct HLA class I alleles associated with CBZ hypersensitivity, i.e. *HLA-A*31:01* and *HLA-B*15:02*, bear any similarities in their structure or binding specificity which might explain their individual correlation with CBZ-induced hypersensitivity.

When comparing the sequences of HLA-B75 serotype alleles, which comprises *HLA-B*15:02*, no relevant changes in amino acids forming the A-, C- and F-pockets could be observed. Alignment of the alleles forming the HLA-B62 supertype indicated that alterations in amino acid residues 63, 113 and 156, which are located in the B- and D-pocket, distinguish *HLA-B*15:02* to some extent from the other HLA-B alleles. Analysis of the 3D structure of HLA-B*15:02 implied that residues 63N and 113Y could delineate the binding specificity of HLA-B*15:02 from other HLA-B alleles, and therefore CBZ may be interacting primarily with these residues.

A recent study by Wei and colleagues confirmed most of the *in silico* findings described above (Wei, C. Y. *et al.* 2012a). They demonstrated that CBZ was able to bind directly to HLA-B*15:02 interacting with amino acid residues 63N, 95I and 156L within the HLA binding groove (Wei, C. Y. *et al.* 2012a). Computational docking of CBZ to HLA-B*15:02 showed that the energetically most favoured binding positions of CBZ were all predicted to be located in the B-pocket near residue 62R (Wei, C. Y. *et al.* 2012a).

Amino acid sequence alignment of *HLA-A*31:01* to other HLA-A31 serotype alleles did not reveal any AA residue or binding pocket which might permit specific binding of CBZ to HLA-A*31:01. When the sequence of *HLA-A*31:01* was compared to members of the HLA-A03 supertype, no differences were observed in amino acids of the F-pocket, making it unlikely to be a preferred binding site for CBZ. The 3D model structure revealed that the binding specificity of HLA-A*31:01 might be characterised by AA residues 70Q, 73T, 97I and 114R. If the findings on HLA-B*15:02 are taken into consideration, these observations

suggest that binding of CBZ to HLA-A*31:01 might occur in close proximity to these residues. However, in contrast to HLA-B*15:02, no *in vitro* studies investigating the binding mode of CBZ to HLA-A*31:01 have been published yet. Further studies are therefore needed in order to verify the hypothesis presented here. This would involve the application of computational docking programmes, as well as *in vitro* assays using antigen-presenting cells expressing HLA-A*31:01 and closely related HLA-A alleles to define the exact binding site of CBZ.

Direct comparison of the *HLA-A*31:01* sequence to *HLA-B*15:02* showed no overlap in AA residues in addition to those which also exist in the control alleles *HLA-A*03:01* and *B*46:01*. Comparable observations have been reported recently in a study by Niihara *et al.*, leading them to the conclusion that the two HLA alleles show no similarities in presentation of antigens (Niihara *et al.* 2012). However, the data presented in this chapter show that the physicochemical properties of AA residues 63 and 70, both located in the B-pocket, can be considered to be strongly alike, suggesting some indirect structural similarities between the two alleles may exist. As a result, CBZ may bind at slightly different locations within the HLA binding groove based on the unique amino acid microenvironment of the individual HLA alleles.

Previous *in vitro* studies established that CBZ binding to HLA occurred directly in the absence of metabolism and antigen processing, suggesting CBZ-specific T-cells are activated through a p-i like mechanism (Wu *et al.* 2007; Wei, C. Y. *et al.* 2012a). This was further supported by a peptide elution study of HLA-B*15:02-transfected cell lines which failed to detect any CBZ-modified peptides (Yang *et al.* 2007). The altered peptide repertoire model, recently published in the context of abacavir hypersensitivity (Illing *et al.* 2012; Norcross *et al.* 2012; Ostrov *et al.* 2012), could provide an alternative mechanism explaining the HLA-restricted activation of T-cells by CBZ. The second part of this chapter therefore focused on the peptide repertoire presented by HLA-A*31:01. A classic peptide elution study was carried out in order to determine whether CBZ

treatment leads to changes in peptides presented by HLA-A*31:01 and subsequently might induce T-cell activation.

Free CBZ was detected by mass spectrometry in peptide fractions originating from drug-treated HLA-A*31:01-transfected C1R cells, suggesting CBZ bound non-covalently to pMHC, and dissociated after treatment with acetic acid prior to HPLC purification. However, CBZ was not found to be enriched in *HLA-A*31:01* transfectants compared to C1R cells transfected with control HLA-A alleles, i.e. *HLA-A*03:01* and *A*31:04*, indicating that CBZ does not bind preferentially to HLA-A*31:01. This is in stark contrast to the findings published for abacavir and *HLA-B*57:01*, and CBZ and *HLA-B*15:02* (Illing *et al.* 2012). Furthermore, no additional peaks or ions with mass shift indicating metabolites of CBZ could be detected in the mass spectra. However, the *in vitro* system used in this study may not mirror the *in vivo* situation, as lymphocytes only show limited metabolic activity (Raucy *et al.* 1999).

As expected, the length of peptides recovered from *HLA-A*31:01* transfectants were typical of peptides presented by HLA class I alleles (Rammensee, Hans-Georg 1995; Burrows *et al.* 2006). As for abacavir and HLA-B*57:01, the distribution of peptide lengths was not affected by CBZ treatment (Illing *et al.* 2012). Sequencing of eluted peptides from HLA-A*31:01 in the presence and absence of CBZ using mass spectrometry allowed the generation of HLA-A*31:01 specific binding motifs. The binding motif of untreated HLA-A*31:01 generally agreed with previously published motifs on MHC motif viewer (Rapin *et al.* 2008) and SYFPEITHI database (Rammensee, H. *et al.* 1999), showing only one anchor residue at position P9 with a dominant preference for arginine. Only minor shifts in AA preferences across nonamer peptides could be observed after CBZ treatment. However, it needs to be noted that both peptide motifs were clouded by remnant *HLA-C*04* specific peptides. This was deduced from the occurrence of amino acids Phe, Pro and Leu/Phe as strong residues at positions P2, P3 and P9 respectively, which is typical for *HLA-C*04* peptides in C1R cells (Schittenhelm *et al.* 2014). Contamination of the binding motif of HLA-A*31:01 by HLA-C*04 peptides could be explained in parts by loss of HLA-A*31:01 expression on C1R cells. Regular monitoring of HLA surface expression

of HLA-transfected C1R cell lines showed a clear decrease in HLA-A*31:01 expression over time (data not shown). Overall, this effect results in a relative reduction of HLA-A*31:01 specific peptides and a corresponding increase in HLA-C*04 specific peptides with regards to total peptide numbers presented on C1R cells. As a consequence, in order to ascertain whether CBZ is able to induce a change in peptides presented by HLA-A*31:01, this study would need to be repeated using a new HLA-transfected cell line, showing a higher, more stable expression of HLA-A*31:01.

In conclusion, the structural binding specificity of HLA-B*15:02 and HLA-A*31:01 was analysed *in silico*, and potential binding sites for CBZ derived through consolidated AA sequence comparison and three-dimensional structure modelling. The research findings published by Wei *et al.* have provided evidence supporting the *in silico* data presented for HLA-B*15:02 in this study, and at the same time substantiate the *in silico* observations presented for HLA-A*31:01. However, defined *in vitro* studies are warranted to confirm the binding position of CBZ within the HLA-A*31:01 binding groove. The peptide elution study performed on HLA-A*31:01-transfected cell lines provided some very preliminary evidence that CBZ may be able to alter the peptide repertoire presented by HLA-A*31:01. MS analysis revealed the presence of unmodified CBZ, which adds to the existing evidence that CBZ may induce drug-specific T-cell responses through a non-covalent binding mechanism to HLA. At this stage, however, it cannot be excluded that *in vivo* a hapten mechanism might be important in the development of CBZ-induced HSRs.

# Chapter V

## T-cell receptor Vβ analysis of naïve and SMX-NO specific T-cell populations

## Contents

## 5.1 Introduction

It has been well-established that T-lymphocytes play a central role in delayed-type drug-induced hypersensitivity reactions (HSRs). The pathomechanisms involved in HSRs are thought to include the presentation of a drug antigen in context of HLA to T-cells and their respective T-cell receptor (TCR). The drug may either bind covalently to a peptide presented on a HLA molecule, as defined by the hapten hypothesis (Landsteiner and Jacobs 1935), or directly interact with either the HLA or TCR molecule through a non-covalent interaction, as proposed by the p-i concept (Pichler 2002). Ultimately, recognition of a drug antigen by a specific TCR, particularly its complementarity-determining region 3 (CDR3), results in T-cell activation and clonal expansion of effector T-cells. In recent years, specific HLA alleles have been found to be associated with certain drug-induced HSRs (see chapter 1.6.4), and in some cases their function in drug antigen presentation is beginning to be uncovered (Chessman *et al.* 2008; Illing *et al.* 2012; Norcross *et al.* 2012; Ostrov *et al.* 2012; Wei, C. Y. *et al.* 2012a; Monshi *et al.* 2013). However, the impact of the TCR in the pathogenesis of HSRs remains largely unknown.

In several autoimmune and infectious disease settings, such as multiple sclerosis, psoriasis, influenza A or HIV (Pantaleo *et al.* 1994; Lehner *et al.* 1995; Musette *et al.* 1996; Hwang *et al.* 2003), analysis of the TCR repertoire has shown that specific TCR subtypes are responsible for antigen recognition and initiation of an immune response. This suggests that the presence or absence of specific TCRs may influence the magnitude of an immune response to a specific antigen.

With regards to drug hypersensitivity, only a few studies have investigated whether drug-specific T-cells show predominant expression of specific TCRs. Generally, heterogeneous TCR Vβ profiles have been observed among individual patients hypersensitive to a specific drug, and only a few commonly used TCR Vβ subtypes could be detected (Cederbrant *et al.* 2000; Hashizume *et al.* 2002). Most recently, Ko and colleagues investigated the TCR usage in Han Chinese

patients carrying the risk allele *HLA-B*15:02* and suffering from carbamazepine-induced Stevens-Johnson syndrome (SJS) (Ko *et al.* 2011). They were able to show that only patients expressing a specific TCR Vβ in combination with the risk allele HLA-B*15:02 developed CBZ-induced SJS, providing initial evidence that specific TCRs may function as an additional risk factor in drug-induced HSRs (Ko *et al.* 2011). This finding may also explain why some patients carrying the HLA risk allele do not develop hypersensitivity. Whether specific TCR Vβs may play a pathogenic role in other populations and other phenotypes of CBZ hypersensitivity remains to be established.

Several methods have been developed over the last few years to measure the TCR diversity of T-cell populations. A panel of fluorescent-labelled monoclonal antibodies targeting the variable (V) region of the TCR β-chain can be used to examine TCR protein expression levels. This method follows a standard flow cytometry staining protocol, can be easily implemented, and delivers rapid results. A more refined technique is the CDR3 spectratyping method, also known as the immunoscope approach, first described by Pannetier *et al.* (1995). This method determines the lengths of the CDR3 region of the different TCRs used by antigen-specific T-cells by polymerase chain reaction (PCR) and subsequently separates the PCR products by electrophoresis (Pannetier *et al.* 1995). With this approach, T-cell responses to a specific antigen can be classified into polyclonal, oligoclonal, or monoclonal, depending on the CDR3 length profile. More detailed profiles of the T-cell repertoire implicated in an antigen-specific immune response can be acquired by sequencing of the CDR3 region; and this can be performed on polyclonal T-cell populations as well as on a single-cell level (Turner *et al.* 2006). Analysis of the TCR repertoire is often limited to testing TCR Vβ expression for two main reasons. First, the majority of T-cells (≥90%) are known to express α:β TCRs (Davis and Bjorkman 1988). Second, a single α:β T-cell can display up to two TCRs which consist of either one of two α-chains but the same β-chain (Padovan *et al.* 1993).

Until now, the T-cell receptor repertoire and its potential influence on the susceptibility of an individual to develop a drug-specific immune response have largely been ignored. With the findings by Ko *et al* (2011), however, it has

become evident that further research into TCR diversity in context of drug-induced HSRs is needed in order to fully understand the mechanisms leading to drug-specific T-cell activation. This may also assist in determining more accurately who may be at risk of developing a reaction when administered a drug. In the case of flucloxacillin, for example, it has been demonstrated that drug-protein conjugates are present in the plasma of all patients treated with the antibiotic (Jenkins *et al.* 2009), but only a small subset of patients develop hypersensitivity. One reason for this could be that particular TCR subtypes which are able to recognize the drug antigen need to be present in an individual in order to develop flucloxacillin hypersensitivity.

The main aim of this chapter was to establish a protocol for CDR3 spectratyping allowing us to characterise the TCR Vβ repertoire of different drug-specific T-cell populations. For the work presented in this study, naïve T-cells from healthy donors were primed to nitroso sulfamethoxazole (SMX-NO), a commonly used model drug antigen. The TCR Vβ usage of SMX-NO specific T-cells was then analysed and compared to the TCR diversity of naïve T-cells. Protein and mRNA expression levels of 24 TCR Vβ subtypes were assessed using flow cytometry and CDR3 spectratyping respectively.

## 5.2 Methods

### 5.2.1 Healthy volunteers

100ml blood was taken from six randomly selected healthy volunteers after informed consent was obtained. The study protocol was approved by the University of Liverpool research ethics committee. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation as described in chapter 2.2.4.

### 5.2.2 Chemicals

Nitroso sulfamethoxazole (SMX-NO; Dalton Pharma Services, Canada) was prepared as stock solution (50mM) in DMSO (Sigma-Aldrich, UK), and diluted in T-cell medium to a concentration of 200μM before use. All other reagents were purchased from Sigma-Aldrich, if not stated otherwise.

### 5.2.3 Cell culture media

T-cells and monocyte-derived dendritic cells (DCs) were cultured in T-cell medium as specified in chapter 2.2.3. For the culture of naïve T-cells, the culture medium was prepared without penicillin and streptomycin.

### 5.2.4 *In vitro* T-cell priming assay

The individual steps of the *in vitro* T-cell priming assay, including magnetic cell separation, generation of monocyte-derived DCs and DC-T-cell co-cultures, were carried out by Dr. Lee Faulkner according to the protocol described in detail in chapter 3.2.6. For this study, SMX-NO (50μM) was used as the drug antigen.

2x $10^6$ freshly isolated naïve T-cells from each volunteer were frozen down in RNAlater (stored at -80°C) to be used for TCR Vβ CDR3 spectratyping analysis. After the *in vitro* T-cell priming culture, T-cells were restimulated with fresh monocyte-derived DCs (DC:T-cell ratio 1:25) and SMX-NO (50μM) in a 24-well plate for three days. Memory T-cells were then isolated by magnetic cell separation using CD45RO microbeads (Miltenyi Biotech, UK), and 2x $10^6$ $CD45RO^+$ T-cells were immediately frozen down in RNAlater for CDR3 spectratyping analysis. The remaining memory T-cells were used for TCR Vβ FACS analysis.

### 5.2.5 Detection of SMX-NO specific T-cell responses

After the week-long DC-T-cell co-culture, SMX-NO specificity of primed T-cells was assessed by a proliferation assay.

T-cells (1x $10^5$) were incubated with monocyte-derived DCs (4x $10^3$) and SMX-NO (1.5- 25μM) in triplicate for three days in a 96-well U bottom plate. Phytohemagglutinin (PHA; 20μg/ml) was used as a positive control. [$^3$H]-thymidine (0.5μCI/well) was added for the final 16h of the culture period. Cells were harvested and proliferation was determined by scintillation counting using a MicroBeta TriLux β-counter (Perkin-Elmer, USA).

### 5.2.6 T-cell receptor Vβ analysis by flow cytometry

*This part of the study was performed by Dr. Lee Faulkner.*

TCR Vβ protein expression was determined using the IOTest Beta Mark TCR Vβ repertoire kit (Immunotech, Beckman Coulter, UK). Aliquots of T-cells (50μl) were stained with 24 different fluorescence-conjugated TCR Vβ antibodies (as mixtures of 8 x 3 antibodies, 4μl each) for 20min in the dark at RT. At the same time, cells were stained with an APC-conjugated anti-CD3 monoclonal antibody (BD Bioscience, UK) to enable gating on T-cells during FACS analysis. T-cells were washed with PBS (500μl), spun down (1500rpm, 5min), and resuspended in HBSS containing 10% FBS (300μl). The samples were run on a BD FACS Canto II flow cytometer recording a minimum of 50,000 events, and data was analysed using Cyflogic software (CyFlo Ltd., Finland).

### 5.2.7 RNA Extraction and Reverse Transcription

Total RNA was extracted from naïve and SMX-NO primed memory T-cells (2x$10^6$) using the RNeasy mini kit (Qiagen, UK), following the manufacturer's protocol. After the RNA was eluted from the spin column using RNase-free water (30μl), the eluate was passed over the column a second time in order to increase the RNA concentration. Total RNA was quantified using the NanoDrop

N-8000 spectrophotometer (Thermo Scientific, UK), and RNA integrity assessed using the Agilent Bioanalyser (Agilent, UK).

Total RNA (0.4µg) was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad, UK), as recommended by the manufacturer.

### 5.2.8 T-cell receptor Vβ CDR3 spectratyping

As described previously by Pannetier and colleagues, analysis of the CDR3 sizes within the 24 TCR Vβ chains was performed in a two-step polymerase chain reaction (PCR) (Pannetier *et al.* 1998). The primers are listed in Appendix T2.

CDR3 spectratyping was performed in the laboratory of Prof. Stefan Martin, Department of Dermatology, University Medical Centre Freiburg, Germany.

***PCR amplification of cDNA***

For each sample, aliquots of cDNA (20ng) were amplified with 1 of 24 Vβ forward primers (10µM; metabion, Germany) and a Cβ reverse primer (10µM; metabion, Germany) on the thermal cycler Bio-Rad iCycler (Bio-Rad, Germany) using the HotStartTaq Master Mix kit (Qiagen, Germany). The final reaction mix contained 12.5µl master mix, 1.3µl Vβ forward primer, 1.3µl reverse primer, 1µl cDNA and 8.9µl RNase-free water. The thermal cycling conditions were as follows: heat activation of Taq polymerase at 95°C for 5min, followed by 40 cycles of denaturation at 95°C for 45s, annealing at 60°C for 45s, extension at 72°C for 1min, and a final extension step at 72°C for 10min.

***Run-off PCR***

PCR products from the first PCR were submitted to three cycles of an elongation run-off reaction with a FAM-labelled Cβ primer (10µM; metabion, Germany). The final reaction mix contained 0.1µl run-off primer, 0.1µl Pfu polymerase (Promega, Germany), 1µl dNTPs (30µM; Promega, Germany), 1µl 10x Pfu buffer (Promega, Germany), 1µl PCR product, and 6.8µl RNase-free water. The PCR was performed on the thermal cycler Bio-Rad iCycler (Bio-Rad, Germany) under the following conditions: 94°C for 2min, 5 cycles of 94°C for 25s, 60°C for 45s, and 72°C for 45s, followed by a final elongation step of 72°C for 10min.

***CDR3 spectratyping by GeneScan***

Run-off PCR product (2µl) was added to a mix of Hi-Di formamide (10µl; Applied Biosystems, Germany) and 500 LIZ size standard (1µl; Applied Biosystems, Germany) in a Thermo-Fast 96 PCR Detection plate (Applied Biosystems, Germany). The plate was sealed with a 96-well Plate Septa (Applied Biosystems, Germany) and the samples denaturated at 94°C for 2min. CDR3 size was determined on the ABI PRISM 3300 Genetic Analyzer, and data analysed using GeneMapper 4.0 software (Applied Biosystems, USA).

### 5.2.9 Statistical analysis

Proliferative responses of SMX-NO primed T-cell lines were analysed by Student's test using Bonferroni correction for multiple testing. A P-value of < 0.02 was considered significant.

## 5.3 Results

### 5.3.1 SMX-NO specific T-cell responses

Purified naïve T-cells from six healthy donors were cultured with 50 µM SMX-NO in the presence of autologous monocyte-derived DCs for eight days. Following the priming period, T-cells were restimulated with monocyte-derived DCs and SMX-NO for 3 days, and proliferative responses to SMX-NO were assessed by [$^3$H]-thymidine uptake.

In five out of six healthy volunteers, SMX-NO was able to induce significant proliferation of T-cells (Figure 5.1). Maximal responses were observed at a concentration of 25µM SMX-NO, apart from donor S5 (max. at 12µM; Figure 5.1). These results demonstrated that SMX-NO reactive T-cells had been generated during the priming cultures. However, the maximum proliferative response of T-cells from donor S1 only showed a stimulation index of 1.5 (at

25μM SMX-NO), which was not statistically significant (Figure 5.1). This suggested that priming to SMX-NO had not been successful in this volunteer.

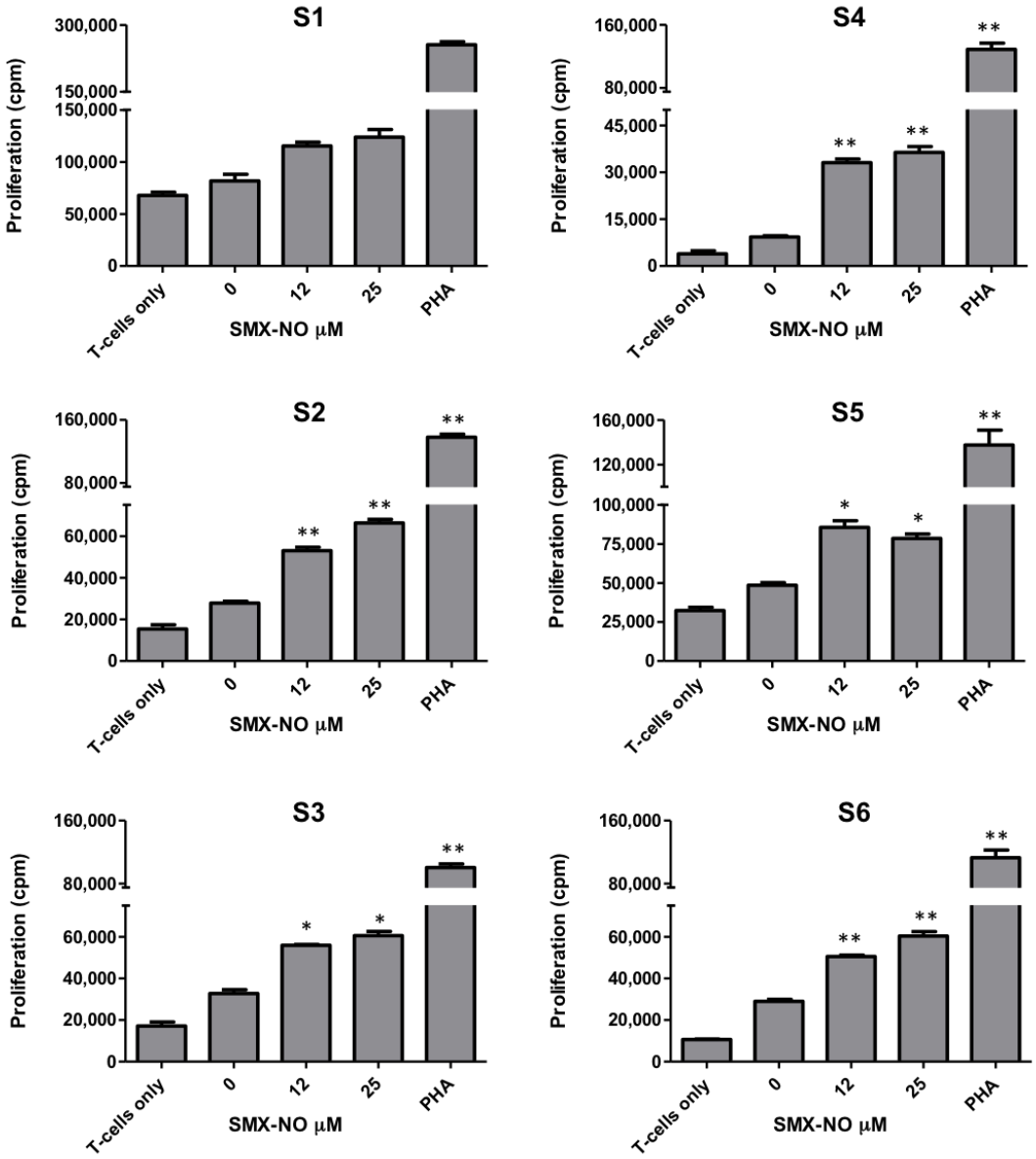



**Figure 5.1**: Proliferative response of T-cells from healthy donors to SMX-NO after *in vitro* T-cell priming assay.

Naïve T-cells were cultured in the presence of autologous monocyte-derived DCs and SMX-NO for 8 days. T-cells (1x $10^5$) were then restimulated with SMX-NO (12+ 25 μM) and autologous monocyte-derived DCs (4x $10^3$) for 72 hours, and [$^3$H]-thymidine was added for the final 16 hours. Proliferation was determined by scintillation counting. Data represent mean cpm ± SEM of triplicate cultures. (*p< 0.02, **p< 0.003; Student's t test).

### 5.3.2 TCR Vβ usage of SMX-NO responsive T-cells

Using flow cytometry, we next analysed the TCR Vβ repertoire expressed by SMX-NO primed memory T-cells, and then compared the TCR Vβ usage of SMX-NO responsive T-cells from each donor to the corresponding naïve T-cell population.

An expansion of the TCR Vβ repertoire covered by the monoclonal antibodies was observed in T-cells from five donors following *in vitro* priming to SMX-NO, indicating clonal expansion of drug-specific T-cells had occurred (Table 5.1). Only a minor increase in TCR Vβ coverage was detected for T-cells from donor S1, supporting the assumption that *in vitro* priming had been unsuccessful. The sample was consequently excluded from further analysis. In naïve T-cells all 24 TCR Vβs could be detected, with mean expression levels ranging from 0.18% for Vβ 9 up to 6.98% for Vβ 2 (see Appendix T3). After *in vitro* priming, an increase in expression levels of up to 16/24 TCR Vβs was seen in SMX-NO specific T-cells from the five positive donors (Figure 5.2). Skewing of individual TCR Vβ subtypes was examined as described by Hashizume *et al*. (2002) defining skewed TCR Vβ usage as the percentage of a particular TCR Vβ which is above the mean percentage + 3 SD of the same TCR Vβ in normal cells (in this study: naïve T-cells) (Hashizume *et al.* 2002). Skewed usage of TCR Vβ 4 and 9 was detected in SMX-NO reactive T-cells from all five individuals (Figure 5.2 & Table 5.2). Usage of TCR Vβ 11, 13.6, 14 and 18 was found to be skewed in four donors respectively. Further, preferential expression of TCR Vβ 5.2 and 5.3 was observed in three of the five donors (Figure 5.2 & Table 5.2). In contrast, expression of TCR Vβ 1, 2 and 5.1 was substantially decreased (< mean %- 3SD of TCR Vβ in naïve cells) in three of the five donors (Appendix T3).

**Table 5.1**: Coverage of the TCR Vβ repertoire of T-cells before (naïve) and after priming to SMX-NO (memory) using flow cytometry.

| TCR Vβ coverage (%) | **S1** | **S2** | **S3** | **S4** | **S5** | **S6** |
|---|---|---|---|---|---|---|
| Naïve | 61 | 56 | 52 | 57 | 53 | 50 |
| Memory | 65 | 83 | 78 | 71 | 76 | 65 |

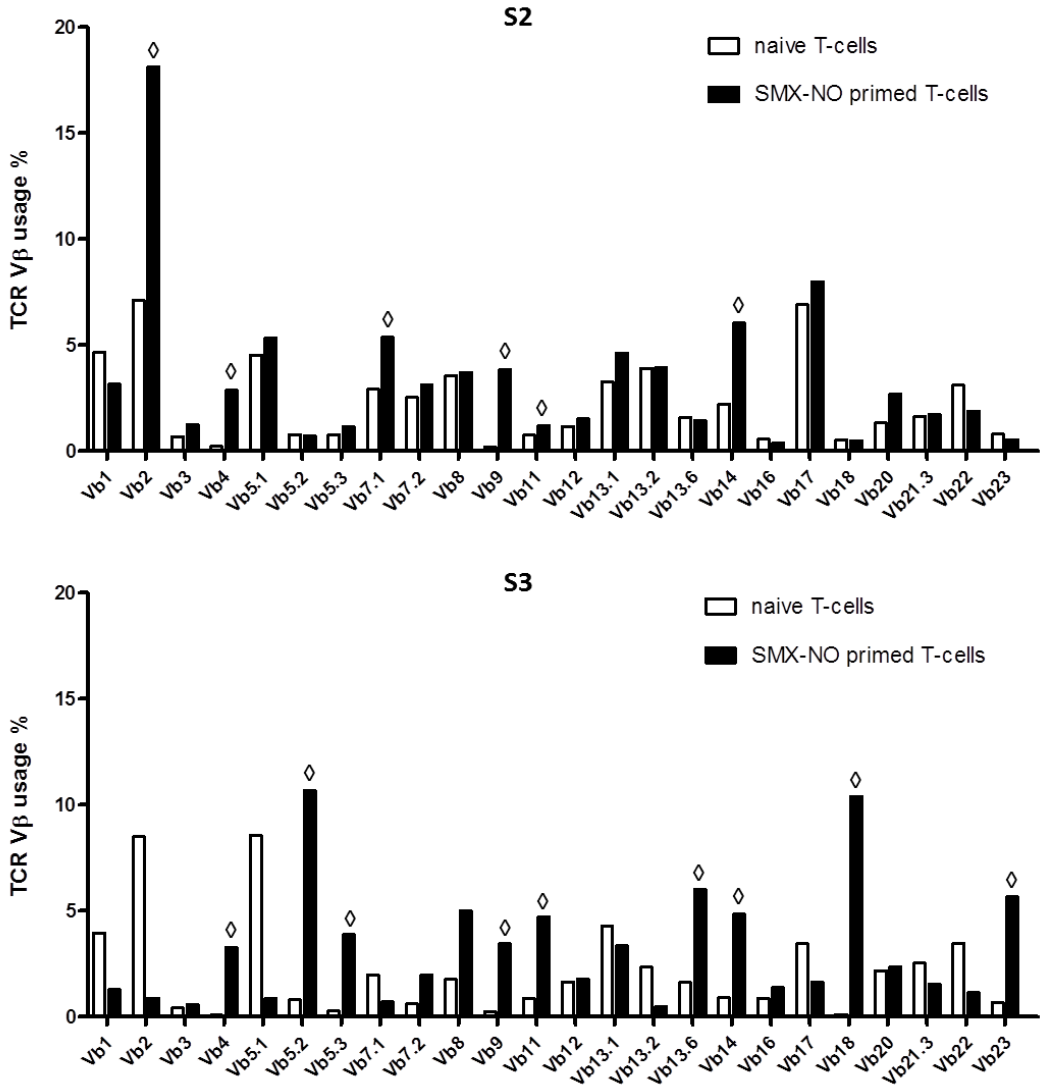



**Figure 5.2**: Clonogram of naïve and SMX-NO primed memory T-cells from five healthy donors.

TCR Vβ repertoire in naïve (white bars) and SMX-NO primed memory (black bars) $CD3^+$ T-cells was measured by flow cytometry using fluorescence conjugated monoclonal antibodies against 24 TCR Vβ. Data represent mean percentages of T-cells expressing the individual TCR Vβ subtypes. (◊ = skewed TCR Vβ usage, defined as percentage above mean value +3SD of TCR Vβ in naïve T-cells)

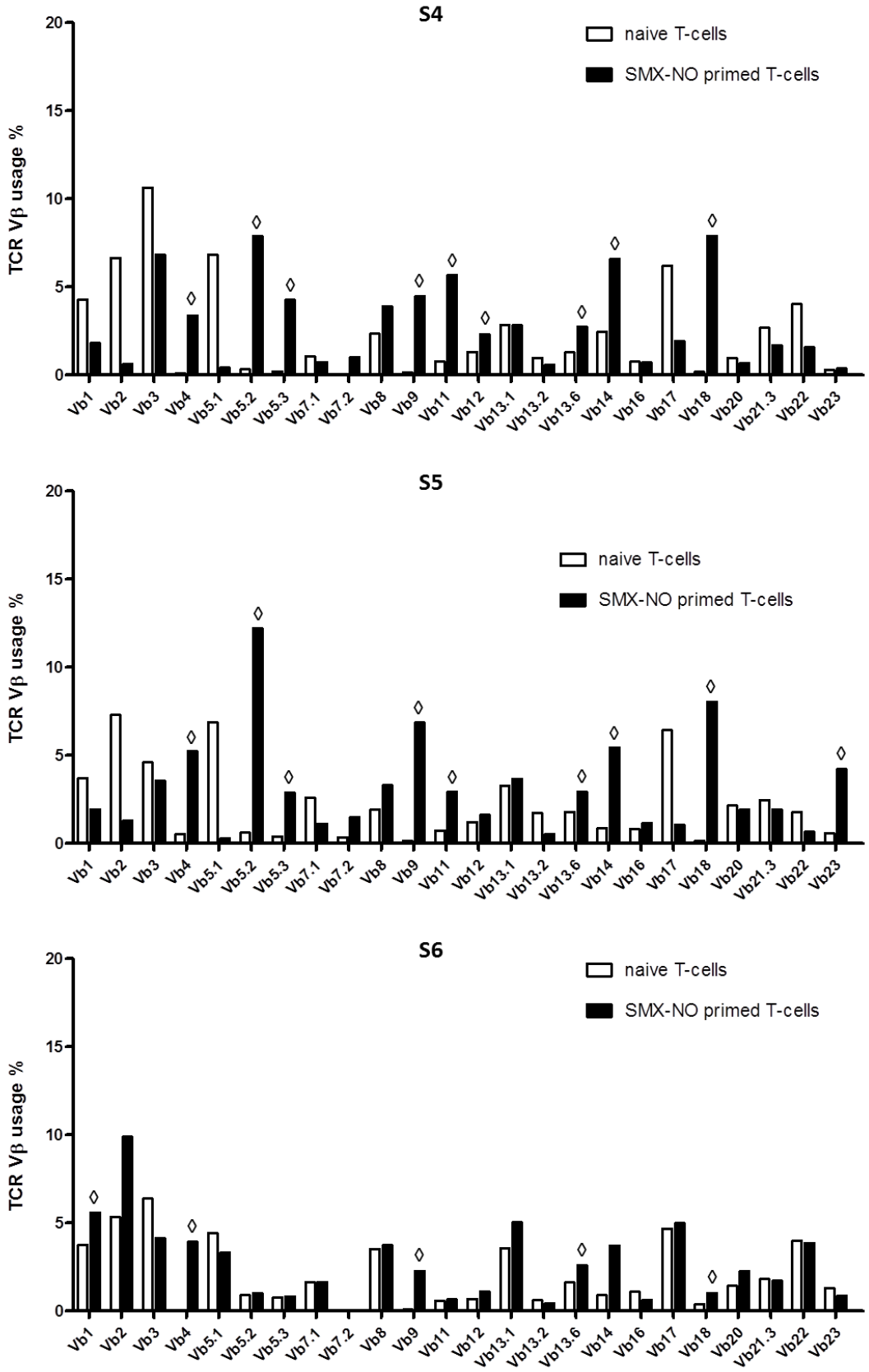



**Figure 5.2 (*continued*)**: Clonogram of naïve and SMX-NO primed memory T-cells from five healthy donors.

**Table 5.2**: Summary of common skewed TCR Vβ usage (= mean % of TCR Vβ in naïve T-cells +3SD) of SMX-NO responsive T-cells from five healthy donors as detected by FACS analysis.

| TCR Vβ | 4 | 5.2 | 5.3 | 9 | 11 | 13.6 | 14 | 18 |
|---|---|---|---|---|---|---|---|---|
| S2 | ● | | | ● | ● | | ● | |
| S3 | ● | ● | ● | ● | ● | ● | ● | ● |
| S4 | ● | ● | ● | ● | ● | ● | ● | ● |
| S5 | ● | ● | ● | ● | ● | ● | ● | ● |
| S6 | ● | | | ● | | ● | | ● |

### 5.3.3 CDR3 spectratyping analysis of TCR Vβ diversity

The TCR Vβ repertoire of naïve T-cells and memory T-cells reactive to SMX-NO was analysed in more detail by CDR3 spectratyping.

Total RNA from naïve and memory T-cells was reverse transcribed to cDNA, and TCR Vβ chain transcripts amplified with 24 Vβ gene-specific primers and a Cβ primer. PCR products were subsequently amplified in a run-off reaction using a fluorescently labelled Cβ primer, and products analysed on an automated sequencer regarding the CDR3 size pattern of each TCR Vβ subtype. In the absence of antigenic stimulation CDR3 size profiles of individual TCR Vβs show a Gaussian-like distribution. A distortion of the CDR3 profiles characterised by the emergence of single or multiple dominant peaks within the TCR Vβ spectratyping profiles signifies antigen specific T-cell stimulation leading to clonal expansion.

Nineteen of the 24 TCR Vβ subtypes could be detected in naïve T-cells from all five healthy volunteers. As expected, the majority of the TCR Vβ spectratyping profiles of naïve T-cells (52% and above) displayed a polyclonal distribution of CDR3 lengths (defined as quasi-Gaussian profile with ≥ 5 peaks) in all donors (see Appendix T3). Transcripts of TCR Vβ 2, 4, 11, 20 and 21 were present in some of the donors, but absent in others. For volunteers S3 and S5, no

spectratyping profile could be established for TCR Vβ 6 and TCR Vβ 8, 11, 17, and 23 respectively, as the genetic analyser failed to detect the size standard (no sizing data available).

In SMX-NO primed memory T-cells, 19/24 TCR Vβ chains were present in all five donors responsive to SMX-NO (Appendix T4). TCR Vβ 2, 3, 4, 20 and 21 could only be detected in some of the donors (Table 5.3). Due to missing sizing data, spectratyping failed for TCR Vβ 5, 6 and 24, and TCR Vβ 3, 17 and 23 in donors S2 and S4 respectively (Table 5.3). After SMX-NO priming, up to 80% of TCR Vβ subtypes showed an oligoclonal distribution of CDR3 lengths (skewed Gaussian profile with at least one dominant peak). Skewing of CDR3 profiles from poly- to oligoclonal distribution could be observed for between six and fifteen TCR Vβs (Table 5.3), indicating the expansion of a clonal T-cell population reactive to SMX-NO. Representative CDR3 spectratyping profiles of skewed TCR Vβs are shown in Figure 5.3.

The TCR Vβs presenting skewed CDR3 profiles were compared among the five SMX-NO responsive donors to determine whether SMX-NO stimulation induced common skewing of specific TCR Vβ subtypes. SMX-NO specific memory T-cells from all five volunteers showed oligoclonal expression of TCR Vβ 18 (Table 5.3). Skewed usage of TCR Vβ 13B was observed in four donors, and TCR Vβ 1, 5, 9, 13A, and 14 displayed a skewed CDR3 pattern in three individuals respectively (Table 5.3). These results suggest that T-cell responses to SMX-NO derived antigens may be in part controlled by public TCRs, which are present in all individuals responsive to SMX-NO treatment, alongside private TCR repertoires specific to each individual.

**Table 5.3**: TCR Vβ subtypes with oligoclonal distribution of CDR3 sizes following SMX-NO priming.

| TCR Vβ | 1 | 2 | 3 | 4 | 5 | 6 A | 6 BC | 7 | 8 | 9 | 11 | 12 | 13 A | 13 B | 14 | 15 | 16 | 17 | 18 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S2 | ● | x | | x | | | ● | ● | | ● | | ● | | | | | | | ● | | | | | |
| S3 | | | | x | s | s | s | | ● | | | | | ● | ● | | | ● | ● | ● | | ● | | s |
| S4 | | | | x | ● | | | | | ● | | ● | ● | ● | ● | ● | | | ● | x | x | | | |
| S5 | ● | x | s | | ● | | | ● | s | | s | | ● | ● | | | ● | s | ● | | x | | s | |
| S6 | ● | x | ● | x | ● | ● | ● | | | ● | x | | ● | ● | ● | | ● | ● | ● | | x | ● | ● | ● |

● = oligoclonal expansion; x = not detected; s = no sizing data

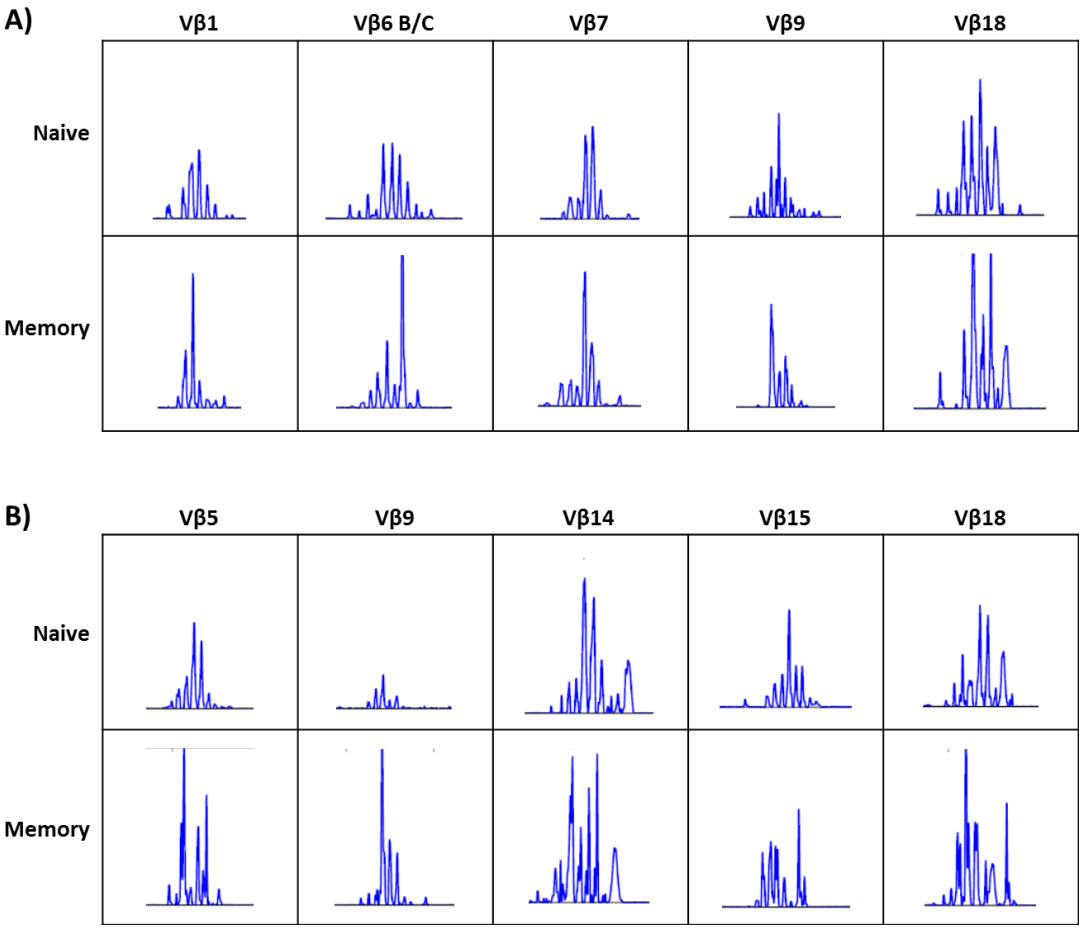



**Figure 5.3**: Representative TCR Vβ spectratyping profiles of T-cells from healthy donors S2 (A) and S4 (B) showing skewed CDR3 lengths distribution after *in vitro* priming of naïve T-cells to SMX-NO.

### 5.3.4 Comparison of flow cytometric TCR Vβ analysis and CDR3 spectratyping

When skewed TCR Vβ subtypes of SMX-NO responsive memory T-cells identified by flow cytometry were compared to those observed in CDR3 spectratyping, the results overlapped in large parts (Table 5.4). However, skewing of some TCR Vβs was only detected in the CDR3 spectratyping analysis (e.g. Vβ 1 in S2 and S5; Table 5.4). To some extent this was anticipated, as CDR3 spectratyping is a more sensitive detection method with which even subtle changes in frequencies of clonal T-cell populations can be distinguished. On the other hand, preferential usage of some TCR Vβ subtypes was only observed in the FACS analysis (e.g. Vβ 4 and 11; Table 5.4). One reason for this could be that the specific TCR Vβ transcript could not be amplified due to inadequate binding specificity of the corresponding Vβ primer, which might have been the case for TCR Vβ 4. Alternatively, non-specific binding of monoclonal antibodies from the TCR repertoire kit might have caused false positive results, which could explain the events seen for TCR Vβ 11. Previous studies using both flow cytometry and CDR3 spectratyping to determine TCR Vβ usage have generally reported good correlation between the results obtained by flow cytometry and CDR3 spectratyping (Pantaleo *et al.* 1994; Ortonne *et al.* 2006; Okajima *et al.* 2009). However, a comparison between the respective studies is difficult, as different antibodies and primers were used in each study, and strategies for data analysis, including criteria for determining clonal expansion, varied greatly.

**Table 5.4**: Skewed TCR Vβ usage of SMX-NO responsive T-cells as detected by flow cytometry (a) and CDR3 spectratyping (b).

| TCR Vβ | 1 | | 4 | | 5 (=5.1-3) | | 9 | | 11 | | 13A (=13.6) | | 13B | | 14 | | 18 | | 23 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | a | b | a | b | a | b | a | b | a | b | a | b | a‡ | b | a | b | a | b | a | b |
| **S2** | | ● | ● | **x** | | | ● | ● | ● | | | | ǂ | | ● | | | ● | | |
| **S3** | | | ● | **x** | ● | **s** | ● | | ● | | ● | | ǂ | ● | ● | ● | ● | ● | ● | |
| **S4** | | | ● | **x** | ● | ● | ● | ● | ● | | ● | ● | ǂ | ● | ● | ● | ● | ● | | |
| **S5** | | ● | ● | **s** | ● | ● | ● | | ● | **s** | ● | ● | ǂ | ● | ● | | ● | ● | ● | **s** |
| **S6** | ● | ● | ● | **x** | | ● | ● | ● | | | ● | ● | ǂ | ● | | ● | ● | ● | | ● |

a= flow cytometric analysis; b= CDR3 spectratyping analysis; ●= skewing; **x**= not detected; **s**= no sizing data; ǂ= no antibody available. Nomenclature of TCR Vβ monoclonal antibodies largely corresponds to the nomenclature of the TCR Vβ primer, with a few exceptions as indicated in brackets (for more details see Appendix T5).

## 5.4 Discussion

There is a large body of evidence demonstrating that drug-specific T-cells are the essential effector cells in delayed-type drug hypersensitivity reactions. It has been shown that the recognition of drug antigens by T-cells requires the interaction of the drug with HLA and TCR. Most studies investigating the molecular mechanisms involved in drug-induced HSRs have focused on the interaction of drug antigens with HLA expressed on the surface of APCs, whereas the role of the T-cell receptor in mediating drug hypersensitivity has been left largely unexplored. However, the recent study by Ko et al. (2011) has illustrated that specific T-cell receptors, similar to HLA alleles, may function as susceptibility factors for certain forms of drug-induced HSRs. Thus, the aim of this study was to establish a robust protocol which would allow a comprehensive characterisation of the TCR repertoire of T-cells from drug hypersensitive patients and healthy controls.

Although various methods exist to determine the TCR repertoire diversity in individuals, we decided to concentrate on flow cytometric analysis and molecular CDR3 spectratyping, as both techniques have been widely used across different disease settings (Ortonne *et al.* 2006; Okajima *et al.* 2009; Hsieh *et al.* 2013; Tzifi *et al.* 2013). Naïve T-cells from healthy volunteers primed against SMX-NO were utilised to set up the different methods for TCR Vβ typing for several reasons. First, large amounts of T-lymphocytes were needed to optimize each of the assays, which excluded the use of patient samples. Also, the naïve T-cell population could be used as a baseline for TCR diversity in an individual. Third, *in vitro* priming of T-cells against SMX-NO has repeatedly been shown to be successful, producing stable drug-specific T-cell responses (Engler *et al.* 2004; Faulkner *et al.* 2012).

In contrast to a previous study by Faulkner et al. (2012), priming of naïve T-cells against SMX-NO was only successful in five of the six healthy volunteers. As the *in vitro* priming assay used in both studies is rather novel, and only a small number of samples have been processed, it seems more than likely that priming

will not be effective in 100% of samples. Although using a different *in vitro* priming protocol, a study by Engler *et al.* (2004) demonstrated that SMX-NO specific responses could be induced in nine out of ten drug-naïve donors. However, proliferative responses to SMX-NO were only detected in six donors, whereas nine individuals showed drug-specific cytotoxicity in a standard chromium release assay (Engler *et al.* 2004). Thus, it may be useful to include a more sensitive method, e.g. ELISpot assay, to test for drug specificity of *in vitro* primed T-cells.

The flow cytometric analysis of TCR Vβ expression on drug-naïve T-cells showed that all 24 TCR Vβs were present in the five proliferation positive donors, with mean expression levels ranging from 0.18% for Vβ 4 to 6.98% for Vβ 2, and an average TCR Vβ coverage of 53.47%. Following SMX-NO *in vitro* priming, TCR Vβ coverage increased to a mean value of 74.58%, suggesting drug-specific clonal expansion had occurred. This is in line with previous studies which generally describe coverage of ≥ 70% of the total TCR repertoire (van den Beemd *et al.* 2000; McLean-Tooke *et al.* 2008; Tzifi *et al.* 2013), and it also matches the reference value of the TCR Vβ antibody kit reported to cover about 70% of the normal TCR Vβ repertoire. However, only a minor increase in TCR coverage (from 60% to 65%) was detected for T-cells from donor S1, confirming that *in vitro* priming against SMX-NO had been ineffective in this case. SMX-NO primed memory T-cells from the five drug-responsive volunteers displayed broad TCR Vβ usage, with increased expression levels in up to 16 TCR Vβs. TCR skewing was observed for 9 different Vβ subtypes across the five donors. Together these results suggest that multiple TCRs may be involved in drug antigen recognition. Similar observations have been made in T-cells responsive to abacavir, in which case the extensive TCR usage is thought to be responsible for the recognition of diverse novel self peptides presented on HLA-B*57:01 in the presence of abacavir (Illing *et al.* 2012). Given that a range of SMX-NO protein adducts known to stimulate T-cells from hypersensitive patients have been reported in the literature (Schnyder *et al.* 2000; Farrell *et al.* 2003; Callan *et al.* 2009; Castrejon *et al.* 2010), this result was somewhat anticipated. Further, common skewing of particular TCR Vβ subtypes (Vβ 4 and

Vβ 9) in all five drug-responsive individuals indicates that a public TCR repertoire may be involved in recognition of SMX-NO derived antigens.

In order to gain a more complete picture of the clonal expansion occurring as a result of *in vitro* priming of T-cells against SMX-NO, CDR3 spectratyping analysis was performed. Eleven TCR Vβ subtypes were present in naïve as well as SMX-NO specific T-cells from all five volunteers, whereas six TCR Vβs (2, 3, 4, 11, 20, and 21) were only detected in some of the donors. Spectratyping of 8 TCR Vβs (3, 5, 6, 8, 11, 17, 23, and 24) failed in some cases due to missing sizing data. In most cases this technical issue can be solved by repeating the amplification and GeneScan run for the specific TCR Vβ in question. However, due to shortage of reagents and time constraints, we were unable to re-run the samples in this study. In naive T-cells most TCRs displayed polyclonal CDR3 patterns, whereas CDR3 profiles of SMX-NO specific T-cells showed oligoclonal expansions across several TCR Vβ subtypes. For the individual volunteers, skewing of TCR Vβ profiles from polyclonal to oligoclonal distribution of CDR3 sizes was detected in up to 16 different Vβ subtypes. Again, this may be explained by the various antigenic structures formed by SMX-NO, which are then recognised by different TCR Vβ subtypes. Further, common skewing of particular TCR Vβs was observed among the five donors, including TCR Vβ 18 in 5/5 donors, TCR Vβ 13B in 4/5 donors, and TCR Vβ 1, 5, 9, 13A, and 14 in 3/5 volunteers respectively. These findings strengthen the concept that a public TCR repertoire may be implicated in SMX-NO specific T-cell responses.

Skewed TCR Vβ usage of SMX-NO responsive memory T-cells identified by CDR3 spectratyping only partially match the results obtained by flow cytometry. This was somewhat anticipated, as the definition and assessment of TCR Vβ skewing differs greatly between the two methods. The assessment of clonality of T-cells and definition of cut-off values is critical to obtain accurate results. For this reason, the use of a mathematical scoring system, e.g. as described by Okajima et al. (2008), might improve the evaluation of the TCR Vβ repertoire usage of different T-cell populations.

Another factor that might have affected our analysis is the fact that TCR Vβ usage of naïve T-cells, representing the control population, was analysed directly without undergoing an *in vitro* culture period. In contrast, analysis of SMX-NO specific memory T-cells was performed after 11 days of *in vitro* culture. Thus, any skewing of TCR Vβ usage due to *in vitro* culture conditions was not taken into account, and this could explain some of the discrepancies observed between the results from TCR Vβ spectratyping and flow cytometric analysis.

Overall, we have been successful in testing the TCR Vβ usage of drug-specific T-cells using both the CDR3 spectratyping method as well as TCR Vβ flow cytometric analysis. Further optimisation of the experimental set-up and data analysis strategy is however needed in order to achieve consistent and robust results, characterising the full scope of TCR Vβ expression patterns of drug-reactive T-cells.

# Chapter VI

## Final Discussion

Drug-induced hypersensitivity reactions (HSRs) represent a diverse group of adverse reactions which occur only in a minority of patients and cannot be predicted from the pharmacological mechanism of the drug. Extensive research in the field has shown that specific components of the immune system are involved in most, if not all, of these reactions. However, the detailed pathomechanisms leading to drug hypersensitivity have not yet been fully uncovered.

Drug-specific T-cells have been identified as the key components in delayed-type HSRs, and T-cells isolated from hypersensitive patients have been characterised extensively *in vitro* regarding their cellular phenotype and effector functions (Mauri-Hellweg *et al.* 1995; Zanni *et al.* 1999; Schnyder *et al.* 2000; Naisbitt *et al.* 2003a; Naisbitt *et al.* 2003b; Nassif *et al.* 2004). This has helped considerably to define the different phenotypes of delayed-type HSRs and also explain the variety of clinical manifestations.

The discovery that certain forms of drug hypersensitivity are strongly associated with specific HLA alleles has provided a new indication to the molecular mechanisms underlying these reactions. Furthermore, this has opened up the possibility of predicting patients at risk of developing drug hypersensitivity. A prime example is *HLA-B*57:01* associated abacavir (ABC) hypersensitivity (see chapter 1.6.5).

Similar achievements have been made in regards to carbamazepine-induced Stevens-Johnson syndrome (SJS) associated with *HLA-B*15:02*. Carbamazepine (CBZ) was found to specifically interact with HLA-B*15:02 resulting in $CD8^+$ T-cell stimulation (Wei, C. Y. *et al.* 2012a). However, as HLA-B*15:02 is expressed almost exclusively in South-east Asian populations, pharmacogenetic testing has been restricted to patients of Asian ancestry (Ferrell and McLeod 2008).

In Caucasian patients, genetic studies have recently revealed a strong association between CBZ-induced HSRs and *HLA-A*31:01*, suggesting *HLA-A*31:01* may be a molecular target for CBZ, and the allele may therefore represent a possible genetic marker for CBZ hypersensitivity in Europeans (McCormack *et al.* 2011).

Thus, following the examples of CBZ and *HLA-B*15:02* as well as ABC and *HLA-B*57:01*, the relationship between HLA-A*31:01 expression and CBZ-induced T-cell activation was explored *in vitro* using isolated PBMCs from a *HLA-A*31:01*+ CBZ hypersensitive patient (chapter 2).

$CD8^+$ T-cell clones from the patient were shown to be HLA-A31 restricted, and strong proliferative responses to CBZ were observed in the presence of allogeneic *HLA-A*31:01*+ APCs. The data present the first tentative proof that *HLA-A*31:01* is functionally involved in mediating CBZ-specific T-cell responses. However, some $CD8^+$ clones could be activated if APCs expressing other HLA-A were present, suggesting that recognition of CBZ may not be defined solely by *HLA-A*31:01*. A comprehensive study using APCs expressing HLA-A allotypes closely related to *HLA-A*31:01* should facilitate the characterisation of the binding capacity of CBZ to HLA-A and determine the amino acid residues involved in CBZ presentation, as has been accomplished for CBZ in association with *HLA-B*15:02* and for ABC (Chessman *et al.* 2008; Wei, C. Y. *et al.* 2012a). Ideally, this would involve the use of HLA transfected cell lines expressing a single HLA allele rather than EBV-transfected B-cells from healthy donors expressing the full range of HLA class I and class II alleles in order to avoid any HLA cross-reactivity.

In chapter 2, I also investigated the HLA restriction profile of $CD4^+$ T-cells isolated from the CBZ hypersensitive patient. In contrast to the predominant $CD8^+$ T-cell response in CBZ-induced SJS patients, drug-specific $CD4^+$ T-cells are commonly found in patients with CBZ-induced hypersensitivity syndrome or maculopapular rash. It was therefore of interest to see whether $CD4^+$ T-cells from the patient also displayed specific HLA restriction. Activation of $CD4^+$ clones was restricted by HLA-DP and DR simultaneously. In other autoimmune diseases, such as narcolepsy and type I diabetes, it has previously been reported that HLA class II alleles may co-operatively confer immune recognition (Temajo and Howard 2009; Han *et al.* 2012). Although it would have been interesting to explore further how each of the two HLA class II loci affects the stimulation of $CD4^+$ T-cells by CBZ, the lack of information on HLA-DP prevented further analysis. Instead, I focused on HLA-DR, and in particular *HLA-DRB1*04:04*. This

allele has been shown to be part of a common haplotype with *HLA-A*31:01* in Caucasians, and the patient expressed both HLA alleles. In accordance with this, it was demonstrated that $CD4^+$ clones from the patient responded to CBZ in a *HLA-DRB1*04:04* restricted manner. This suggests that CBZ is able to interact with several HLA molecules, which may lead to the phenotypically diverse T-cell responses seen in Caucasian patients. Nevertheless, whether the HLA restriction profile observed in the patient is universally applicable needs to be confirmed in a larger cohort of patients.

Due to the low incidence of drug-induced HSRs, the recruitment of drug hypersensitive patients to clinical studies has always been a large obstacle. Furthermore, *in vitro* studies investigating the immunological mechanisms have so far relied on the use of patient derived drug-specific memory T-cells, which does not allow the prediction of the allergic potential of a drug. As a result, several research groups have recently attempted to generate primary T-cell responses to drugs using cells from drug-naïve healthy volunteers (Engler *et al.* 2004; Chessman *et al.* 2008; Ko *et al.* 2011; Faulkner *et al.* 2012; Monshi *et al.* 2013; Wuillemin *et al.* 2013).

Using the *in vitro* priming methods reported in the literature, I tried to generate a primary immune response to CBZ in drug-naïve T-cells isolated from *HLA-A*31:01*+ healthy volunteers (chapter 3). A weak CBZ specific T-cell response could be induced in five out of eight *HLA-A*31:01*+ donors. However, activation of T-cells was not observed consistently across all read-out methods applied, but instead was limited to individual assays. Ideally, the drug specificity of the individual T-cell cultures would have been evaluated using the complete panel of read-outs available, however, the amount of T-cells that could be recovered after *in vitro* priming were in most cases insufficient to test all end-points. For this reason, it was difficult to determine in which of the samples a genuine T-cell response to CBZ had been elicited and which, if any, might have represented an artefact. At the same time, the persistently elevated background levels of IFN-γ in T-cells from *HLA-A*31:01*+ volunteers suggested that these T-cells may

be more prone to bystander T-cell activation compared to those from *HLA-B*15:02*+ donors, which in turn may obscure a response caused by CBZ. Further investigations are needed in order to understand the differences in bystander stimulation of T-cells from *HLA-B*15:02*+ compared to *HLA-A*31:01*+ donors. However, the data in chapter 3 clearly indicate that the expression of *HLA-A*31:01* on its own is not sufficient to stimulate a primary T-cell response to CBZ. The analysis of the extended HLA haplotype did not reveal any common pattern between the samples tested, making it unlikely that *HLA-DRB1*04:04* might play a role, as reported earlier for the *HLA-A*31:01*+ hypersensitive patient.

Multiple factors, both environmental and genetic, are thought to contribute to the risk of an individual to develop a hypersensitivity reaction. Most recently, after specific T-cell clonotypes had been identified to play a role in mediating T-cell responses to CBZ in *HLA-B*15:02*+ patients (Ko *et al.* 2011), the T-cell receptor has been brought forward as a possible susceptibility factor in drug-induced hypersensitivity.

In order to examine whether a specific TCR may be involved in CBZ-induced HSRs in *HLA-A*31:01*+ patients, a protocol for the comprehensive analysis of the TCR Vβ repertoire was established (chapter 5).

Methods for the examination of TCR Vβ expression by flow cytometry as well as molecular CDR3 spectratyping were set up individually. The protocols were tested using naïve T-cells from healthy donors that were primed against the model drug antigen SMX-NO, as patient samples were considered too valuable. The TCR Vβ usage of the different T-cell subsets, i.e. naïve and SMX-NO specific memory T-cells, could be determined successfully on both protein and mRNA level. However, the data analysis process requires further optimisation in order to improve the coherence between the two read-outs, and thus allow a detailed characterisation of the TCR repertoire expressed by drug-specific T-cells.

In an earlier attempt to characterise the TCR Vβ usage of a few selected T-cell clones from the *HLA-A*31:01*+ hypersensitive patient, flow cytometric analysis

had produced inconsistent results (data not shown). Expression of a single TCR Vβ could only be detected on two clones, whereas for the majority of CBZ-specific clones no definite TCR Vβ usage could be determined. It may be that the TCR expressed by these particular clones was not covered by the antibody kit. In some cases, however, it seemed that non-specific binding of the monoclonal antibodies had occurred which masked the true TCR expression levels of the clones. For this reason, it was considered necessary to set up the more sensitive CDR3 spectratyping method, which encompasses a slightly broader range of TCR Vβs, and has already been used successfully in the study by Ko et al. (2011).

Using the optimised spectratyping protocol, it should be possible to screen drug-specific T-cells from *HLA-A*31:01*+ CBZ hypersensitive patients for TCR Vβ expression, and determine whether particular TCR Vβs predominantly occur in patients compared to tolerant controls. If this were the case, this may explain why some carriers of the HLA risk allele remain tolerant to CBZ. Likewise, this could also explain why in some cases *in vitro* priming of drug-naïve T-cells from HLA-A*31:01+ volunteers had failed.

Besides, if the corresponding TCR Vα chain could be identified, this would provide an opportunity to generate TCR transfected cell lines that could be used as an alternative to T-cell clones derived from patients, thereby avoiding the laborious process of T-cell cloning and the problems associated with it (e.g. loss of drug specificity). In previous studies it has been demonstrated that TCR transfected hybridoma cells show the same specificity and drug recognition pattern as the T-cell clones they were derived from originally, and therefore provide a valuable tool for the investigation of drug-TCR interactions (Schmid *et al.* 2006).

In addition, CDR3 spectratyping would allow to assess whether T-cell response to CBZ in *HLA-A*31:01*+ patients are poly-, oligo- or monoclonal in nature, and hence give an indication of the scope of CBZ-derived antigens that may be involved in T-cell activation.

Until now, the drug antigen(s) causing CBZ hypersensitivity remains elusive. Most evidence gained from *in vitro* studies so far points towards CBZ, the parent

drug, inducing the activation of T-cells. First, fixation of APCs with aldehyde, which inhibits the processing of proteins, did not suppress T-cell stimulation by CBZ, suggesting that the formation of a haptenated protein is not required (Wu *et al.* 2006; Wu *et al.* 2007; Wei, C. Y. *et al.* 2012a). Also, mass spectrometry analysis was unable to detect any CBZ-modified peptides bound by HLA-B*15:02 (Yang *et al.* 2007; Illing *et al.* 2012). Second, addition of glutathione and other neutralising agents, which capture reactive metabolites, did not inhibit T-cell responses to CBZ, signifying a metabolism-independent process (Wu *et al.* 2006). Third, CBZ-pulsed APCs did not induce the activation of T-cells, suggesting a non-covalent binding mechanism (Wu *et al.* 2006; Wei, C. Y. *et al.* 2012a).

According to current hypotheses, presentation of CBZ as drug antigen could take place in either one of two ways: CBZ may bind non-covalently to a peptide-HLA complex or directly to the TCR (p-i mechanism), or CBZ may be loaded into an empty HLA binding groove leading to a change of the peptide repertoire presented by the HLA allele (altered peptide model). The strong associations with *HLA-B*15:02* and *A*31:01* suggest that CBZ binding occurs at the HLA rather than the TCR interface.

The data presented in chapter 3 and chapter 4 adds to the existing evidence that CBZ binds directly to the HLA molecule and subsequently induces T-cell activation.

In a novel approach to assess the effect of metabolic activation of CBZ on T-cell stimulation, it was shown that halogenated derivatives of CBZ, which are stable against metabolic transformation, stimulated a proliferative response in T-cell clones from a *HLA-B*15:02*+ volunteer (chapter 3). Similarly, CBZ-specific clones isolated from a *HLA-A*31:01*+ patient could also be activated by the halogenated derivatives (Farrell *et al.* 2013). These findings further strengthen the concept that the metabolic activation of CBZ is not needed in order to form an antigenic structure that is presented to T-cells.

Mass spectrometry analysis of peptides eluted from a HLA-A*31:01 expressing cell line treated with CBZ *in vitro* did not exhibit any CBZ-modified peptides

(chapter 4). Instead, unbound CBZ could be detected in the peptide fractions. These results support a non-covalent binding mechanism of CBZ to HLA-A*31:01. However, whether the interaction between CBZ and HLA molecule occurred within in the HLA binding groove and affected the peptide binding specificity of the HLA allele could not be determined conclusively, as too low numbers of HLA-A*31:01 specific peptides were identified to perform a detailed comparison of peptide binding in absence and presence of drug.

In chapter 4, I performed *in silico* analysis of the binding specificity of HLA-A*31:01 in comparison to B*15:02; this suggested that CBZ may bind in close proximity to the B-pocket of the HLA-A*31:01 molecule, but in a slightly shifted location compared to HLA-B*15:02. Earlier computational modelling studies have indicated that CBZ is most likely to bind to HLA-B*15:02 within the B-pocket of the peptide binding cleft, underneath the peptide residues P4/P6 (Illing *et al.* 2012; Wei, C. Y. *et al.* 2012a). *In vitro* studies involving the refolding of the individual HLA molecules in complex with a self-peptide and CBZ, and subsequent crystallisation of the drug-peptide-HLA complexes may provide proof of the *in silico* observations described in the literature and in this thesis.

Taken together, the preliminary results from the *in silico* modelling and peptide elution study indicate that the altered peptide model may apply to interactions occurring between CBZ and HLA risk alleles and consequently cause T-cell activation. However, these findings need to be confirmed in further studies before a firm conclusion is drawn.

Another question that remains to be answered in this context is: where do the T-cells responding to drug antigens or altered peptides originate from?

In relation to this, it has been hypothesised that a subpopulation of memory T-cells, which have been primed to a different antigen earlier, may be able to cross-react with the drug and/or altered peptide (Adam *et al.* 2011). Given that memory T-cells have a lower activation threshold than naïve T-cells, this could explain why the HLA-TCR interaction may be sufficient to cause T-cell activation. It has further been proposed that these pre-activated memory T-cells may be derived through heterologous immune responses induced by viral

infections, which have been associated with an increased risk of developing hypersensitivity (Adam *et al.* 2011; Ostrov *et al.* 2012).

In case of CBZ, however, it seems that pre-activated memory T-cells are not a prerequisite to stimulate a primary T-cell response, as highly purified naïve T-cells from *HLA-B*15:02*+ healthy volunteers could be successfully primed against CBZ (chapter 3). Thus, primary T-cell responses against CBZ and other drugs may be induced through different processes of drug antigen presentation, which may also occur concurrently (Pichler 2013).

As a matter of fact, we have recently shown that a reactive metabolite of CBZ is able to form stable adducts with human serum albumin (HSA) *in vitro*, and the equivalent metabolite-conjugated HSA peptides could be identified in patients on regular CBZ treatment (data unpublished). However, at this stage, it is unclear whether these haptenated peptides may have antigenic potential and may induce T-cell responses in CBZ susceptible individuals. Following the *in vitro* studies performed with the contact sensitizer 2,4-dinitrobenzene sulfonic acid (Dietz *et al.* 2010), it may be possible to prepare synthetic HSA peptides haptenated with the CBZ metabolite and test these initially in T-cells from hypersensitive patients to assess their T-cell activation potential. Subsequently, priming of naïve T-cells from *HLA-B*15:02*+ donors against the synthetic CBZ-modified HSA peptides could be attempted to evaluate the role of CBZ-modified peptides in generating primary T-cell responses.

Overall, it is difficult to link T-cell responses observed *in vitro* to the conditions occurring *in vivo*. The majority of mechanistic studies has relied on the use of T-cells derived from hypersensitive patients, and can therefore only describe the processes involved in the secondary stimulation of T-cells *in vitro*. The steps leading to the initial sensitisation of T-cells against a drug *in vivo* however remain unclear. Although it has been possible to induce a primary T-cell response *in vitro* against a few drugs (including SMX-NO, ABC and CBZ), the formation of the respective antigenic complex and its structure has not yet been elucidated. It can therefore not be excluded that the stimulation of a primary

immune response against a drug, both *in vivo* and *in vitro*, requires the formation of drug-modified protein adducts, which may have the ability to activate the innate as well as the adaptive immune system.

In conclusion, a number of questions remain to be answered in order to fully understand the individual molecular processes involved in drug-induced HSRs. These relate especially to the mechanism of initial sensitisation to a drug, the formation of the drug antigen complex, as well as the function of the TCR in drug antigen recognition. Future research efforts aiming to resolve these ambiguities may permit to predict the risk of a drug to cause hypersensitivity, and ultimately assist in the development of safer drugs in future.

# Appendix

## Figures



## Tables

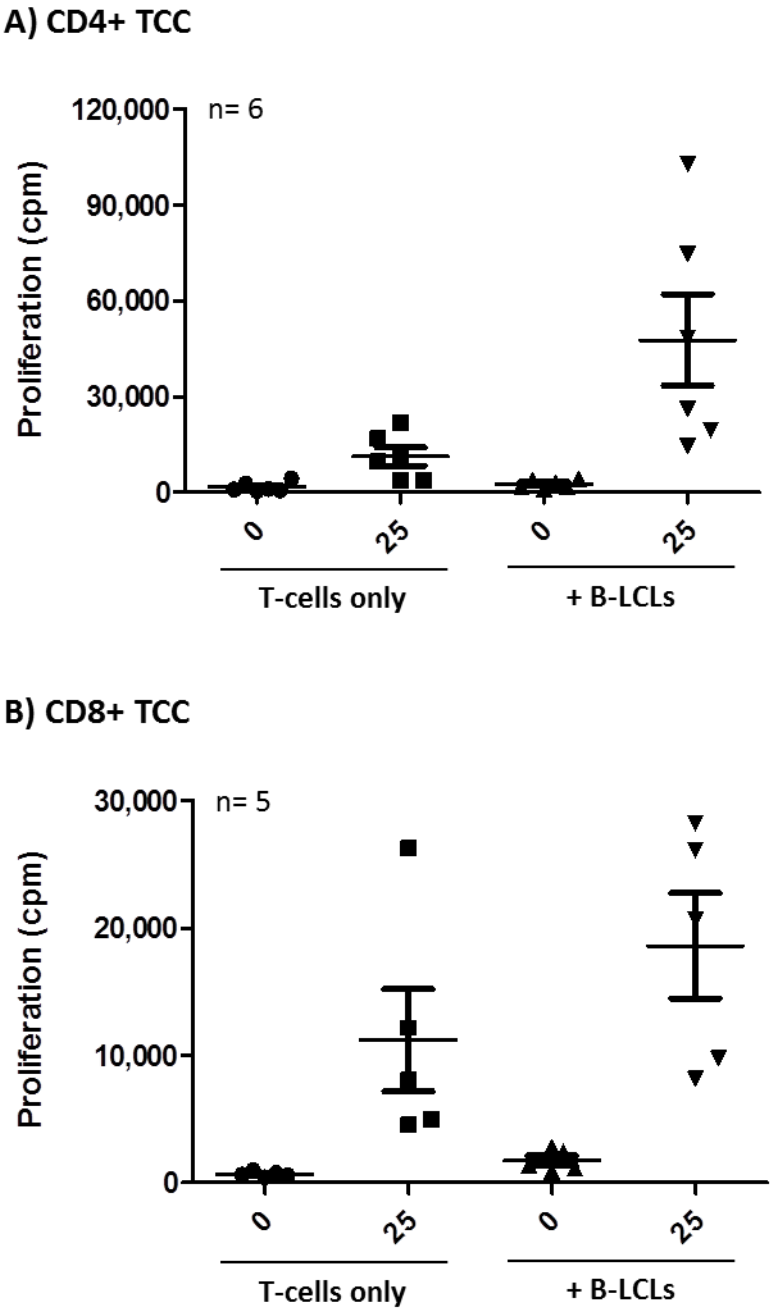



**Figure F1**: Self-presenting CD4+ (A) and CD8+ (B) T-cell clones reactive to CBZ.

T-cell clones were cultured with CBZ (25µg/ml) in the absence and presence of autologous B-LCL. Proliferation was determined by [3H]-thymidine uptake. Data represent mean cpm ± SEM of n clones.

## A) HLA-A*31:01

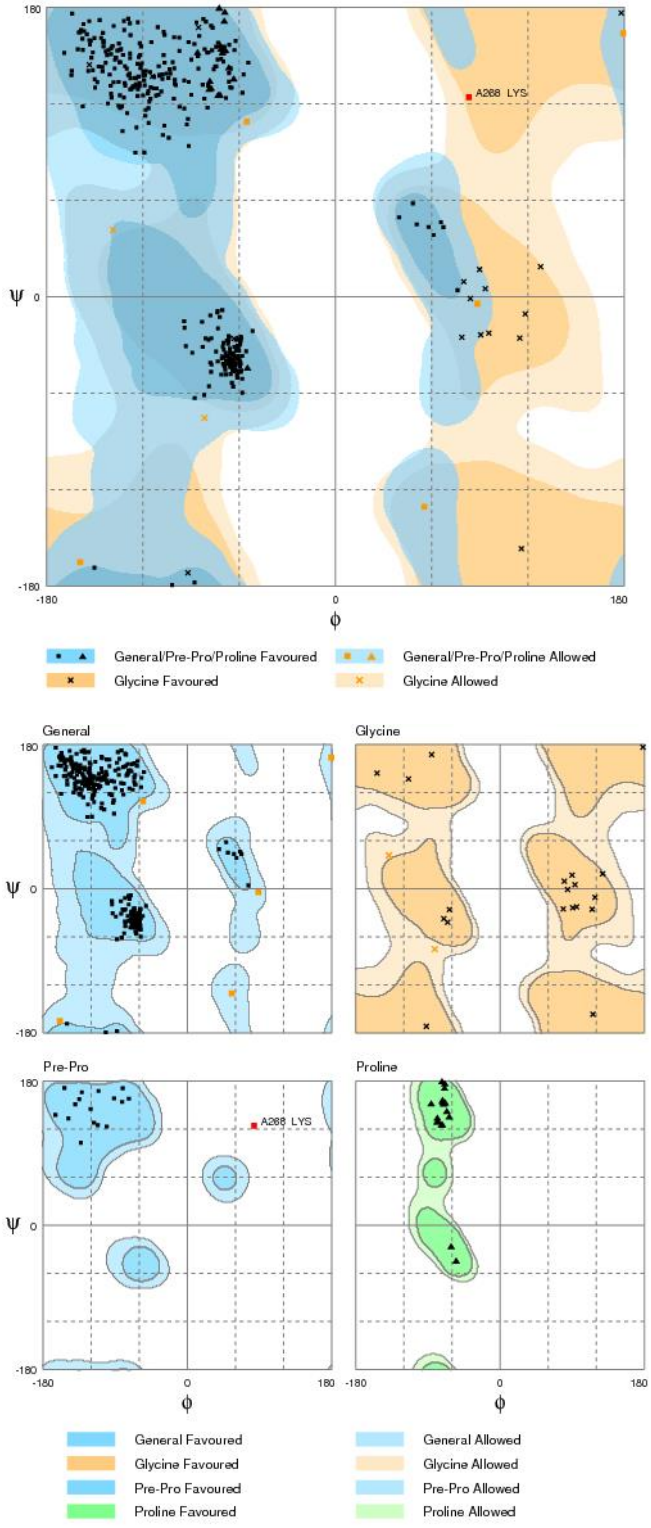



**Figure F2:** Ramachandran plots of 3D-model structures of HLA-A*31:01 (A) and HLA-B*15:02 (B) generated by RAMPAGE.

**B) HLA-B*15:02**

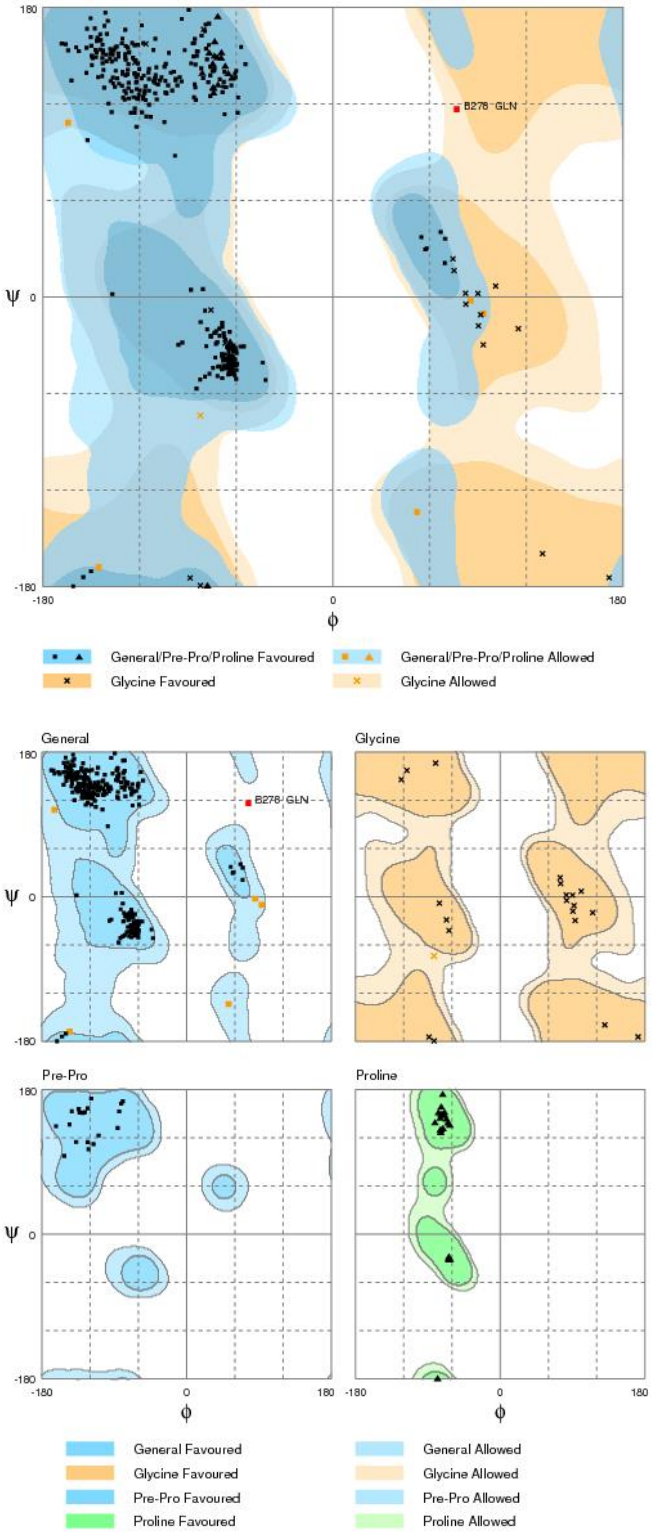



**Figure F2 (*continued*):** Ramachandran plots of 3D-model structures of HLA-A*31:01 (A) and HLA-B*15:02 (B) generated by RAMPAGE.

**Table T1:** Summary of monoclonal antibodies used in FACS analysis.

| Monoclonal antibody | Fluorochrome | Clone ID | Supplier |
|---|---|---|---|
| CD phenotyping | | | |
| **anti-CD4** | PE | RPA-T4 | BD Pharmingen |
| **anti-CD8** | FITC | HIT8a | BD Pharmingen |
| Intracellular cytokine staining assay | | | |
| **anti-CD3** | PerCP | SP34-2 | BD Pharmingen |
| **anti-CD4** | APC-Cy7 | RPA-T4 | BD Pharmingen |
| **anti-TNF α** | PE-Cy7 | MAb11 | BD Pharmingen |
| **anti-IFN γ** | Pacific Blue | 4S.B3 | Biolegend |
| **anti-granzyme B** | Alexa Fluor 700 | GB11 | BD Pharmingen |
| **anti-IL 4** | APC | 8D4-8 | eBioscience |
| *In vitro* T-cell priming assay | | | |
| **anti-CD14** | FITC | M5E2 | BD Pharmingen |
| **anti-CD3** | APC | UCHT1 | BD Pharmingen |
| **anti-CD25** | PE | 4E3 | Miltenyi Biotech |
| **anti-CD45RA** | FITC | HI100 | BD Pharmingen |
| **anti-CD45RO** | Cy5 | UCHL1 | BD Pharmingen |
| **anti-CD4** | FITC | RPA-T4 | BD Pharmingen |
| **anti-CD8** | PE | RPA-T8 | BD Pharmingen |

APC= Allophycocyanin; Cy= cyanine; FITC= Fluorescein isocyanate; PE= Phosphatidylethanolamine; PerCP= Peridinin chlorophyll protein

**Table T2**: List of primers used in TCR Vβ CDR3 spectratyping analysis

| Sequence Name | Sequence ('5-'3) |
|---|---|
| **humTCR-VB1** | CCG CAC AAC AGT TCC CTG ACT TGC |
| **humTCR-VB2** | ACA TAC GAG CAA GGC GTC GA |
| **humTCR-VB3** | CGC TTC TCC CTG ATT CTG GAG TCC |
| **humTCR-VB4** | CAT CAG CCG CCC AAA CCT AA |
| **humTCR-VB5** | GAT CAA AAC GAG AGG ACA GC |
| **humTCR-VB6 A** | GAT CCA ATT TCA GGT CAT ACT G |
| **humTCR-VB6 B** | CAG GGS CCA GAG TTT CTG AC |
| **humTCR-VB6 C** | CAG GGT TCA GAG GTT CTG AC |
| **humTCR-VB7** | CAA GTC GCT TCT CAC CTG AAT GC |
| **humTCR-VB8** | GGT ACA GAC AGA CCA TGA TGC |
| **humTCR-VB9** | TTC CCT GGA GCT TGG TGA CTC TGC |
| **humTCR-VB11** | GTC AAC AGT CTC CAG AAT AAG |
| **humTCR-VB12** | TCC TCC TCA CTC TGG AGT C |
| **humTCR-VB13 A** | GGT ATC GAC AAG ACC CAG GCA |
| **humTCR-VB13 B** | AGG CTC ATC CAT TAT TCA AAT AC |
| **humTCR-VB14** | GGG CTG GGC TTA AGG CAG ATC TAC |
| **humTCR-VB15** | CAG GCA CAG GCT AAA TTC TCC CTG |
| **humTCR-VB16** | GCC TGC AGA ACT GGA GGA TTC TGG |
| **humTCR-VB17** | TGT GAC ATC GGC CCA AAA GAA |
| **humTCR-VB18** | CAA AGA GGG CCC CAG CAT |
| **humTCR-VB20** | TGC CCC AGA ATC TCT CAG CCT CCA |
| **humTCR-VB21** | GGA GTA GAC TCC ACT CTC AAG |
| **humTCR-VB22** | GAT CCG GTC CAC AAA GCT GG |
| **humTCR-VB23** | ATT CTG AAC TGA ACA TGA GCT CCT |
| **humTCR-VB24** | GAC ATC CGC TCA CCA GGC CTG |
| | |
| **humTCR-CB1 (use with TCR-VB 5, 6B/C, 20)** | GGG TGT GGG AGA TCT CTG C |
| **humTCR-CB2RO (run-off PCR)** | FAM-ACA CAG CGA CCT CGG GTG GG |
| **humTCR-CB3** | CCT TTT GGG TGT GGG AGA TCT C |

**Table T3**: Flow cytometric analysis of TCR Vβ usage on naïve and SMX-NO specific memory T-cells.

| | Day 0- % naïve T-cells | | | | | | | | | | Day 11- % SMX-NO spec. memory T-cells | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **TCR** | **S1** | **S2** | **S3** | **S4** | **S5** | **S6** | **Mean day0 (exclud.S1)** | **SD** | **mean -3SD** | **mean +3SD** | **S1** | **S2** | **S3** | **S4** | **S5** | **S6** |
| **Vb 1** | 4.06 | 4.66 | 3.96 | 4.28 | 3.69 | 3.76 | 4.07 | 0.40 | 2.87 | 5.27 | 6.40 | 3.17 | **1.28** | **1.82** | **1.93** | **5.59** |
| **Vb 2** | 6.94 | 7.12 | 8.49 | 6.65 | 7.29 | 5.34 | 6.98 | 1.14 | 3.57 | 10.39 | 10.66 | **18.12** | **0.88** | **0.62** | **1.30** | 9.89 |
| **Vb 3** | 4.19 | 0.67 | 0.43 | 10.64 | 4.60 | 6.39 | 4.55 | 4.26 | -8.23 | 17.32 | 3.21 | 1.24 | 0.57 | 6.81 | 3.57 | 4.11 |
| **Vb 4** | 0.94 | 0.21 | 0.07 | 0.09 | 0.53 | 0.03 | 0.18 | 0.20 | -0.42 | 0.79 | 0.10 | **2.85** | **3.27** | **3.37** | **5.24** | **3.92** |
| **Vb 5.1** | 11.67 | 4.53 | 8.54 | 6.82 | 6.88 | 4.44 | 6.24 | 1.75 | 1.01 | 11.48 | 9.15 | 5.34 | **0.86** | **0.41** | **0.30** | 3.32 |
| **Vb 5.2** | 0.98 | 0.77 | 0.80 | 0.33 | 0.61 | 0.91 | 0.68 | 0.23 | 0.01 | 1.36 | 0.11 | 0.73 | **10.65** | **7.88** | **12.22** | 1.00 |
| **Vb 5.3** | 2.08 | 0.78 | 0.26 | 0.21 | 0.40 | 0.77 | 0.48 | 0.27 | -0.33 | 1.30 | 0.16 | 1.16 | **3.88** | **4.25** | **2.88** | 0.82 |
| **Vb 7.1** | 3.47 | 2.91 | 1.95 | 1.04 | 2.59 | 1.62 | 2.02 | 0.75 | -0.22 | 4.26 | 3.56 | **5.36** | 0.70 | 0.73 | 1.12 | 1.64 |
| **Vb 7.2** | 0.90 | 2.53 | 0.60 | 0.06 | 0.35 | 0.03 | 0.71 | 1.04 | -2.42 | 3.85 | 0.14 | 3.11 | 1.98 | 1.00 | 1.47 | 0.00 |
| **Vb 8** | 1.17 | 3.55 | 1.76 | 2.34 | 1.92 | 3.52 | 2.62 | 0.86 | 0.03 | 5.21 | 3.02 | 3.72 | 5.00 | 3.89 | 3.30 | 3.74 |
| **Vb 9** | 1.35 | 0.18 | 0.22 | 0.15 | 0.15 | 0.07 | 0.15 | 0.05 | -0.01 | 0.32 | 0.43 | **3.84** | **3.45** | **4.47** | **6.85** | **2.27** |
| **Vb 11** | 0.78 | 0.75 | 0.87 | 0.77 | 0.73 | 0.58 | 0.74 | 0.10 | 0.43 | 1.05 | 0.09 | **1.18** | **4.69** | **5.66** | **2.92** | 0.65 |
| **Vb 12** | 1.08 | 1.17 | 1.64 | 1.32 | 1.22 | 0.68 | 1.21 | 0.34 | 0.18 | 2.24 | 1.20 | 1.52 | 1.78 | **2.32** | 1.61 | 1.10 |
| **Vb 13.1** | 2.36 | 3.27 | 4.27 | 2.84 | 3.27 | 3.57 | 3.45 | 0.53 | 1.86 | 5.03 | 5.20 | 4.62 | 3.35 | 2.81 | 3.66 | 5.03 |
| **Vb 13.2** | 1.49 | 3.91 | 2.34 | 0.97 | 1.73 | 0.60 | 1.91 | 1.31 | -2.01 | 5.83 | 1.37 | 3.92 | 0.49 | 0.57 | 0.51 | 0.42 |
| **Vb 13.6** | 1.88 | 1.57 | 1.62 | 1.29 | 1.79 | 1.61 | 1.58 | 0.18 | 1.03 | 2.13 | 1.02 | 1.45 | **6.02** | **2.75** | **2.93** | **2.59** |
| **Vb 14** | 0.38 | 2.19 | 0.89 | 2.47 | 0.85 | 0.89 | 1.46 | 0.80 | -0.95 | 3.86 | 3.66 | **6.05** | **4.83** | **6.59** | **5.44** | 3.70 |
| **Vb 16** | 1.07 | 0.55 | 0.86 | 0.75 | 0.82 | 1.09 | 0.81 | 0.19 | 0.23 | 1.39 | 0.17 | 0.38 | 1.37 | 0.69 | 1.15 | 0.62 |
| **Vb 17** | 1.76 | 6.90 | 3.45 | 6.20 | 6.44 | 4.68 | 5.54 | 1.43 | 1.24 | 9.83 | 2.03 | 8.00 | 1.64 | 1.91 | **1.06** | 4.97 |
| **Vb 18** | 1.26 | 0.52 | 0.11 | 0.17 | 0.12 | 0.39 | 0.26 | 0.18 | -0.28 | 0.81 | 0.31 | 0.49 | **10.40** | **7.89** | **8.05** | **1.02** |
| **Vb 20** | 5.16 | 1.33 | 2.18 | 0.96 | 2.14 | 1.43 | 1.61 | 0.54 | 0.00 | 3.22 | 2.05 | 2.68 | 2.35 | 0.67 | 1.93 | 2.25 |
| **Vb 21.3** | 1.86 | 1.63 | 2.54 | 2.67 | 2.46 | 1.82 | 2.22 | 0.47 | 0.82 | 3.63 | 1.10 | 1.72 | 1.55 | 1.69 | 1.93 | 1.70 |
| **Vb 22** | 2.02 | 3.10 | 3.47 | 4.02 | 1.79 | 4.00 | 3.27 | 0.92 | 0.52 | 6.03 | 6.40 | 1.88 | 1.14 | 1.56 | 0.65 | 3.87 |
| **Vb 23** | 1.72 | 0.79 | 0.67 | 0.29 | 0.57 | 1.30 | 0.72 | 0.37 | -0.39 | 1.84 | 3.81 | 0.54 | **5.65** | 0.37 | **4.21** | 0.85 |
| **total Vb** | 60.60 | 55.59 | 51.95 | 57.33 | 52.95 | 49.52 | | | | | 65.33 | 83.08 | 77.79 | 70.72 | 76.23 | 65.09 |

**Table T4**: CDR3 size distribution of TCR Vβ subtypes on naïve and SMX-NO specific memory T-cells.

| TCR Vβ | S1 | | S2 | | S3 | | S4 | | S5 | | S6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Naive | Memory | Naive | Memory | Naive | Memory | Naive | Memory | Naive | Memory | Naive | Memory |
| **1** | P | O | P | O | P | P | P | P | P | O | P | O |
| **2** | X | X | X | X | P | P | S/P | P | X | X | X | X |
| **3** | P | O | X | P | P | P | X | O | P | S | P | O |
| **4** | X | X | X | X | X | X | X | X | X | S | S/P | X |
| **5** | O | O | P | P | P | S | P | O | P | O | P | O |
| **6A** | P | O | O | O | S | O | P | P | P | P | P | O |
| **6B/C** | P | O | P | O | S | S | P | S/P | P | P | P | O |
| **7** | P | O | P | O | P | P | O | O | P | O | P | P |
| **8** | O | O | O | O | P | O | O | O | S | O | O | O |
| **9** | P | O | P | O | P | P | P | O | P | P | S/P | O |
| **11** | O | O | O | O | O | O | O | O | S | O | S/O | X |
| **12** | P | X | P | S/O | P | P | S/P | O | P | P | S/P | P |
| **13A** | P | O | P | P | P | P | S/P | O | P | O | S/P | O |
| **13B** | O | O | O | S/O | P | O | P | S/O | P | O | P | O |
| **14** | O | O | P | P | P | P | P | O | O | O | P | O |
| **15** | P | O | P | P | P | P | P | O | P | P | P | P |
| **16** | O | O | P | P | P | P | O | O | P | O | P | O |
| **17** | O | O | O | O | P | O | O | O | S | S | P | O |
| **18** | P | O | P | O | P | O | P | O | P | O | P | O |
| **20** | P | O | O | O | P | O | P | S/X | P | P | S/X | P |
| **21** | S/P | P | S/O | O | P | P | X | X | X | X | X | X |
| **22** | P | O | O | O | P | O | P | P | P | P | P | O |
| **23** | S/P | O | S/O | O | P | P | S/O | S/O | S | S | P | O |
| **24** | O | O | O | P | P | S | O | O | O | S | P | O |

P= polyclonal (Gaussian-like distribution, ≥ 5 peaks); O= oligoclonal (skewed Gaussian profile, at least 1 dominant peak); X= undetectable (fluorescence intensity of profile below 250); S= no sizing data (no spectratype profile shown)

**Table T5:** Correspondence between the nomenclatures of TCR Vβ monoclonal antibodies and TCR Vβ primers.

| TCR Vβ primers (Pannetier *et al.* 1998) | IMGT nomenclature (Lefranc *et al.* 1999) | TCR Vβ mAB (Wei, S. *et al.* 1994) |
|---|---|---|
| **1** | 9 | **1** |
| **2** | 20 | **2** |
| **3** | 28 | **3** |
| **4** | 29 | **4** |
| **5** | 5(1,3-8) | **5.1, 5.2, 5.3** |
| **6A** | 7(2-3) | **-** |
| **6B/C** | 7(4,6,8,9) | **-** |
| **7** | 4(1-3) | **7.1, 7.2** |
| **8** | 12(3-5) | **8** |
| **9** | 3(1-2) | **9** |
| **11** | 25 | **11** |
| **12** | 10-3 | **12** |
| **13A** | 6(1-3,5-9) | **13.1, 13.2, 13.6** |
| **13B** | 6-4 | **-** |
| **14** | 27 | **14** |
| **15** | 24-1 | **-** |
| **16** | 14 | **16** |
| **17** | 19 | **17** |
| **18** | 18 | **18** |
| **20** | 30 | **20** |
| **21** | 11(1-3) | **21.3** |
| **22** | 2 | **22** |
| **23** | 13 | **23** |
| **24** | 15 | **-** |

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