

**The Phenotype and Function of Hapten specific T-Cell isolated from
Hypersensitive patients and healthy human donors**

Thesis submitted in accordance with the requirement of the University of Liverpool for the degree of

Doctor in Philosophy by

Eryi Wang

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Declaration

I declare that the work presented in this thesis is my own work and has not been submitted previously.

Eryi Wang

Eryi Wang (B.Sc.)

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Firstly, I will thank my parents. I am so indebted to the love they have given me. Besides huge financial support, they have missed me every day for five years. No language can express my gratitude to them.

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Abbreviations

APC	Allophycocyanin
APC	Antigen presenting cells
CCR	Chemokine receptor (C-C) motif
CD	Cluster of differentiation
cpm	Counts per minute
CSA	Cyclosporin
CTL	Cytotoxic T lymphocyte
CYP	Cytochrome P450 enzyme
DC	Dendritic cell
DHR	Drug hypersensitivity reaction
DILI	Drug-induced liver injury
DMSO	Dimethyl sulfoxide
DNA	Dioxyribonucleic acid
DNCB	Dinitrochlorobenzene
DRESS	Drug reaction with eosinophilia and systemic symptoms
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme-linked immunospot
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSH	Reduced glutathione
HBSS	Hanks balanced salt solution
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HLA	Human leukocyte antigen
IFN- γ	Interferon-gamma

IgE	Immunoglobulin E
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motifs
LAT	Transmembrane adapter protein linker for the activation of T-cells
LPS	Lipopolysaccharide
LTT	Lymphocyte transformation test
MHC	Major Histocompatibility complex
Mins	Minutes
Mo-DC	Monocyte-derived dendritic cells
NHS	National Health Service
NK	Natural killer
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
pH	Power of hydrogen
pi	Pharmacological interaction
RIPA	Radioimmunoprecipitation assay
RPMI	Roswell Park Memorial Institute
SFC	Spot forming cell
SI	Stimulation index
SJS	Stevens-Johnson syndrome
SMX	Sulfamethoxazole
SMX-NOH	Sulfamethoxazole hydroxylamine
SMX-NO	Nitroso sulfamethoxazole
STAT	Signal Transducer and Activator of Transcription
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TEN	Toxic epidermal necrolysis

Th1	T helper type 1 cell
Th2	T helper type 2 cell
Th17	T helper type 17 cell
Th22	T helper type 22 cell
TNF- α	Tumor necrosis factor- α
Tregs	Regulatory T-cells
TT	Tetanus toxoid
UK	United Kingdom
w/v	weight/volume
WHO	World Health Organization

Publication

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Abstract

Drug hypersensitivity reactions are an important problem for pharmaceutical industry, especially when reactions are observed in late phase clinical trials. Furthermore, management of patients with reactions leads to personal suffering and financial burden on the NHS. Reactions are almost impossible to predict as there is no simple relationship between the dose of drug administered and the development of hypersensitivity. In recent years, pharmacogenetic studies identified strong associations between the expression of specific HLA alleles and susceptibility to different forms of hypersensitivity, which would explain why only a small number of drug-exposed patients develop hypersensitivity. Studies utilizing peripheral blood mononuclear cells have detected drug-specific T-cells in patients with hypersensitivity, but not drug-exposed tolerant controls, suggesting that the adaptive immune system plays an important role in the disease pathogenesis. Despite this, there remains a need to further understand mechanisms as more detailed knowledge will assist the development of diagnostic and predictive assays.

Data described herein utilized hypersensitivity reactions to the β -lactam antibiotic piperacillin as a model to investigate the phenotype and function of drug-specific T-cells in blood and skin, focusing specifically on the profile of cytokines secreted. PBMC from hypersensitive patients were activated to proliferate *in vitro* with piperacillin. T-cell clones responsive to the drug were generated from blood of all patients studied. CD4⁺ clones were stimulated to proliferate with piperacillin in a concentration-dependent manner and the proliferative response was associated with secretion of Th1 and Th2 cytokines alongside IL-22. In contrast, IL-17 was not secreted from piperacillin-specific clones. Piperacillin-specific CD4⁺ clones were also isolated from inflamed skins of 2 piperacillin hypersensitive patients. Activation of these clones was associated

with secretion of Th1, Th2 cytokines and IL-22, in the absence of IL-17. Finally, CD4⁺ nitroso sulfamethoxazole (SMX-NO)-responsive clones were isolated from sulfamethoxazole hypersensitive patients and a similar cytokine secretion profile was observed, which suggests that IL-22 secretion might be a common feature of drug hypersensitivity.

Evolution of T-cell culture methods means it is now possible to prime naïve T-cells from healthy donors to antigens, including drugs, which they have not previously been exposed to. Piperacillin and SMX-NO were found to prime naïve CD4⁺ and CD8⁺ T-cells from healthy donors, when the drug-derived antigens were presented in the context of autologous dendritic cells. Cloned drug-specific T-cells secreted a similar panel of cytokines to that observed with patient cells. Of particular importance was the detection of IL-22 in the absence of IL-17.

The final component of the project utilized cloned T-cells with specificity for SMX-NO, piperacillin and flucloxacillin to explore mechanisms of drug-specific T-cell activation and potential cross-reactivity. Clones responsive against all 3 drugs were activated via a hapten mechanism involving (1) formation of protein adducts, (2) antigen processing and (3) presentation of derived peptides in an MHC-restricted manner. No cross-reactivity was observed with the 3 drugs.

Collectively, these data showed that drug-haptens activate T-cells from patients with clinically divergent forms of hypersensitivity. T-cells secrete a similar profile of cytokines including the tissue-specific cytokine IL-22 following stimulation through the T-cell receptor. Furthermore, it is possible to prime naïve T-cells with a similar function against drugs using peripheral blood mononuclear cells from healthy donors.

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Chapter 1: General introduction

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1.1 Adverse drug reactions

Edwards and Aronson, (2000) define adverse drug reactions (ADRs) as: “An appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product.”

Several studies have estimated the proportion of patients hospitalized due to the development of ADRs. A study by Lazarou et al (1998) in the USA used four electronic databases and showed that between 1966 and 1976 the total percentage of ADRs was 15.5%. The percentage of the patients with severe ADRs was 6.7% with 0.37% of reactions resulting in fatality. According to a study by Pirmohamed et al (2004) which looked at 18,829 patients hospitalized in the two large hospitals in the UK, 1225 of these were classified as ADRs according to the Edwards and Aronson definition. The total percentage of ADRs was 6.25% with fatality rates of 0.15%. ADRs also extend the length of stay in hospital and enhancing the costs of hospitalization (Suh et al, 2000). ADRs can be classified in terms of clinical and chemical characteristics (Park et al, 1998).

Type A (augmented): The vast majority (95%) of adverse drug reactions are classified as this type. They are dose-dependent and predictable from the knowledge of pharmacology of the drug, thus they can be prevented. When administration of the drug stops the reaction disappears. Examples of type A reactions include (1) gastrointestinal bleeding induced by the treatment of drug combinations of aspirin and warfarin; (2) gastrointestinal bleeding induced by NSAIDs; (3) diuretic-induced renal impairment; and (4) β -blocker-induced heart block or hypertension (Pirmohamed et al, 2004).

Type B (bizarre): These reactions cannot be predicted by knowledge of the drug's

pharmacology. They display individual susceptibility and are non-dose-dependent, although this has been questioned (Uetrecht 2001). Such reactions are also referred to as idiosyncratic reactions as mechanisms have not been clearly defined. The reactions are believed to be related to drug metabolite and immunological components that maybe key to individual susceptibility. Because of this, reactions occur in a small percentage of patients. Despite this, type B reactions are extremely important because they are often severe and can lead to death (Pirmohamed et al., 2004). Type B reactions can involve any organ, and may cause anaphylaxis, and severe skin inflammation such as hypersensitivity-syndromes or drug induced lupus. Exemplar drugs that can induce this type of reaction include antibiotics, such as amoxicillin and flucloxacillin; sulfonamides, such as sulfamethoxazole; non-steroidal anti-inflammatory drugs and anticonvulsants.

Type C (chemical): These reactions can be explained by the chemical structure of the drug or drug metabolite. Paracetamol-induced hepatotoxicity is a well-defined example. The mechanism of tissue injury involves the conversion of the drug by metabolizing enzymes to a reactive quinoneimine intermediate, which (1) induces oxidative stress and (2) binds covalently to proteins. Eventually both of these processes lead to hepatotoxicity.

Type D (delayed): These reactions include delayed effects such as carcinogenicity and teratogenicity after drug administration.

Type E (end of treatment reactions): Onset of clinical symptoms of the reaction develops after removal of the drug, especially following a sudden removal. A well-known example is after

withdrawal of anxiolytics.

1.2 Drug Hypersensitivity

Type B ADRs are mostly dominated by antigen-specific immune responses induced following drug exposure. This form of reaction is often termed drug hypersensitivity.

Drug hypersensitivity reactions involve the drug initiating an immune reaction that causes tissue damage in the patient. These reactions can be defined simply as “a *serious adverse drug reaction with an immunological aetiology, to an otherwise safe and effective therapeutic agent*”.

Alternatively, drug hypersensitivity has been defined by the World Allergy Organization as "an immunologically mediated drug adverse reaction of which the mechanism is IgE or non-IgG mediated and with T-cell mediated reaction largely presented in the latter (Johansson et al., 2004).

1.2.1 Epidemiological investigation of drug hypersensitivity

According to the definitions of drug hypersensitivity presented above, several studies tried to investigate the incidence of drug hypersensitivity. In France, a 6-month prospective study has been carried out, in which each individual inpatient was examined physically by a dermatologist and reviewed by a pharmacologist. The prevalence of cutaneous drug hypersensitive reactions was 3.6 per 1000 hospitalized patients (Fiszenson-albala et al., 2003). Among these patients, 57% presented with maculopapular exanthema, 8% with erythroderma and 2% with severe conditions such as Stevens Johnson syndrome or toxic epidermal necrolysis. In Mexico in 2006, a 10-month prospective study showed 7 per 1000 hospitalized patients developed cutaneous hypersensitivity reactions (Hernandez-Salazar et al., 2006). Similar studies were carried in

Singapore (Thong et al., 2003) and in Korea (Park et al., 2008). In these studies, 4.2 and 20 per 1000 hospitalized patients, respectively, developed hypersensitivity reactions.

Risk factors of drug hypersensitivity include: gender; female: male ratio of drug hypersensitivity has been estimated to be approximately 2:1 (Impicciatore et al., 2001), age; the rate of drug hypersensitivity is more frequent in elderly people than in children. Concomitant disease may predispose individuals to drug hypersensitivity through altering metabolic pathways and the immune response to the suspect drug. For example, patients with HIV develop an increased number of drug hypersensitivity reactions when compared with control subjects exposed to the same treatment regime. In particular, reactions to the drug sulfamethoxazole are 10 times more common in patients with HIV infection. Environmental factors such as disease, alcohol consumption, smoking and diet may also be important in an individuals' susceptibility to ADRs. Furthermore, environment factors may interact with genetic factors and either increase or decrease the risk of an ADR (Pirmohamed et al., 2001). Patients infected with virus such as human herpes virus (HHV) 6 have an increased likelihood of developing hypersensitivity reactions and it has been reported that the pathogenesis of certain drug hypersensitivity syndrome actually involve drug-specific reactivation of the latent virus infection (Ozcan et al., 2010). Patients with cystic fibrosis have ten times higher rates of piperacillin hypersensitivity compared with normal people (Whitaker et al., 2012).

The study of medical genetics has been focused on the associations between HLA genotypes and drug hypersensitivity. This part will be discussed in detail in the following section.

1.2.2 The symptoms of drug hypersensitivity

The term of drug hypersensitivity encompasses clinical conditions varying in severity from mild skin rashes to severe reactions such as drug reaction with eosinophilia and systemic symptoms

(DRESS), Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). Hypersensitivity reactions also develop in other organs, with the most common being drug-induced liver injury (DILI) (Pavlos et al., 2012).

Skin rashes are the most common manifest of drug hypersensitivity. This may be because skin injury is more visible than other manifestations such as liver injury. Thus, even mild skin conditions are often reported. Furthermore, the skin is a very immunologically active organ (Utrecht and Naisbitt, 2013). Skin expresses specialized antigen presenting cells such as Langerhans cells and cutaneous dendritic cells that are constantly surveying skin for new and potentially dangerous antigenic determinants. Finally, skin is rich in resident T-cells, which following priming will respond rapidly to antigen encounter. The following skin details the most common cutaneous manifestations of drug hypersensitivity.

Maculopapular exanthema (MPE). MPE are the most common type of skin rash that develops following drug exposure. The reactions account for more than 90% of drug-induced immune-mediated skin rashes (Hunziker et al., 1997). The time to onset is typically 1-2 weeks after treatment (Valeyrie-Allanore et al., 2007). This manifestation alone is not severe and patients commonly recover fully after drug withdrawal. Furthermore, it is also relatively safe to rechallenge patients suspected of developing a drug-induced maculopapular drug eruption (P-Codrea Tigaran et al., 2005). The phenotype of T-cells that dominate in MPE are CD4⁺ T-cells that display cytolytic activity (Pichlar 2003). In comparison with the most severe drug induced skin reaction, toxic epidermal necrolysis, which dominated by CD8⁺ cytotoxic T-cells, MPE is mild as most cells in the inflamed site do not express MHC-II molecules that present the drug-derived antigen to CD4⁺ T-cells. In contrast, cell ubiquitously express MHC-I that presents antigens to CD8⁺ T-cells. Finally, although CD4⁺ and CD8⁺ T-cells secrete the same cytolytic

molecules, CD8⁺ T-cells display significantly higher levels of cytotoxicity when activated by the same antigen.

Urticaria is the second most common manifestation of drug-induced skin rash (Hunziker et al., 1997). It is an IgE-mediated reactions. Penicillins are the most common inducers of urticarial reactions. Urticaria is characterized by relatively large, raised, pruritic skin lesions, which will last for several days. As with other types of drug-induced skin reactions, urticaria reaction appear very quickly after inadvertent rechallenge. Clinical signs of a reaction often appear minutes to hours after drug exposure.

A fixed drug eruption is a type of drug-induced skin reaction with lesions that always develop at the same site every time when a drug is administered. When the drug is removed, the lesion usually recovers with a fade hyperpigmentation, which make it easy to define the affected area. This reaction is thought to be mediated by cutaneous CD8⁺ T-cells that are limited to the site of inflammation (Shiohara, 2009). Fixed drug eruptions are commonly mild, but it can be more serious when combined with other systemic manifestations such as fever and arthralgia.

Drug reaction with eosinophilia and systemic symptoms (DRESS) and drug-induced hypersensitivity syndrome (DIHS). Although these two nomenclature are not in totally agreement, they are used to describe drug-induced symptoms with characteristics including an acute onset of rash, fever, and involvement of at least one of the following: lymphadenopathy, hepatitis, nephritis, pneumonitis, carditis, thyroiditis, and hematologic abnormalities (eosinophilia, atypical lymphocytes, thrombocytopenia, or leukopenia) (Peyriere et al., 2006; Um et al., 2010; Walsh and Creamer, 2011). The most common drugs that lead to DRESS/DIHS are carbamazepine and other aromatic anticonvulsants, allopurinol and several anti-HIV drugs such

as abacavir and nevirapine. The onset of symptoms is commonly associated with herpes virus reactivation (Descamps et al., 1997). The reason for the virus association is not known but DRESS is associated with some specific HLA genotypes which will be discussed in detail later.

Acute generalized exanthematous pustulosis (AGEP). AGEP is a type of drug-induced skin reaction with characteristics including an acute onset of a non-infectious pustular skin reaction, commonly affecting the face, neck, groin and axillae and manifestations of fever and neutrophilia (Roujeau et al., 1991; Choi et al., 2010). The main drugs that initiate AGEP are antibiotics. AGEP is thought to be mediated by Th17 cells that have been found in the PBMCs and inflamed skin (Fili et al., 2014).

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). TEN is the most severe type of skin rash with a fatality rate of 30% (Pereira et al., 2007; Downey et al., 2012). SJS is a severe skin rash that is milder than TEN with a fatality rate of 10%. Overlap of SJS/TEN has the fatality rate between 10% and 30%. SJS and TEN are characterized by large scale of skin detachment between epidermal layer and dermal layer. On histological examination, widespread apoptosis of keratinocytes between dermis and epidermis and mononuclear infiltration can be seen. The time to onset is usually 1-2 weeks, but the time is decreased if the patient is re-exposed to the drug (Roujeau, 2005). SJS-TEN are clearly immune mediated reactions as drug-specific T-cells have been detected in skin in the acute phase of reactions (Nassif et al., 2002). Furthermore, HLA allele associations have been described for certain drugs. However, the lymphocyte transformation test, a simple assay for diagnosis based on the drug-specific proliferation of patient lymphocytes, is typically negative for SJS/TEN (Tang et al., 2012). It has been reported that the skin rash is mediated by cytotoxic CD8⁺ T-cells, (Nassif et al., 2004; Wei et al., 2012)

and keratinocytes apoptosis is mediated by the release of Fas ligand (Downey et al., 2012) and tumor necrosis factor related apoptosis-inducing ligand (TRAIL) (de Araujo et al., 2011). Recent studies have identified granulysin as another important cytolytic mediator in patients with TEN (Chung et al., 2008).

Drug induced liver injury

Liver injury is of another major manifestation of drug hypersensitivity and is the major cause of drug withdrawal or black box warnings. Drug induced liver injury (DILI) can be divided into two types, hepatocellular and cholestatic. Specifically, if the ratio of alanine transaminase/alkaline phosphatase is less than two, it is considered cholestatic liver injury; if the ratio is greater than 5, it is considered hepatocellular liver injury; if the ratio is between 2 to 5, it is considered an overlap of the two types of liver injury (Danan G, Benichou C, 1993).

Hepatocellular liver injury is characterized by death of hepatocytes. Histologically, infiltration of mononuclear cells and eosinophils can be seen (Zimmerman, 1999). Cholestatic liver injury is characterized by a great increase of alkaline phosphatase and bilirubin relative to alanine transaminase. Although hepatocellular liver injury more commonly leads to liver failure (Chalasanani et al., 2008), cholestatic liver injury requires more time to recover, usually more than a month (Hussaini and Farrington, 2007).

The time to onset of DILI is usually 1-3 months; however sometimes the time between drug administration and appearance of DILI can be up to a year (Bjornsson, 2010). In certain instances, DILI appears very rapidly after the culprit drug is rechallenged, potentially indicating an immunological mechanism (Maddrey and Boitnott, 1973).

The association between HLA genotype and susceptibility of DILI has been reported for several drugs, e.g., flucloxacillin [B*57:01](Daly et al., 2009), ximelagatran [DRB1*07:01 and HLA-DQA1*02] (Kindmark et al., 2008), lapatinib [HLA-DRB1*0701-DQA1*0202/DQB1*0203](Spraggs et al., 2011), lumiracoxib [DRB1*15:01] (Kindmark et al., 2008), anti-tuberculosis drugs [HLA-DQB1*0502] (Chen et al., 2015), and isoniazid [HLA-DRB1*03], rifampin [HLA-DQA1*0102], and ethambutol [HLA-DQB1*0201] (Sharma et al., 2002).

1.3 The types of hypersensitivity

The adaptive immune response is an important component of host defense against infection. However, sometimes the adaptive immune system over-reacts to innocuous agents such as pollen, food and drugs. This type of reaction is named hypersensitivity and is harmful and may cause serious tissue damage. Hypersensitivity reactions have been classified into 4 broad types by Gell and Coombs as shown in Table 1.1 (Gell and Coombs., 1963).

I. Type I hypersensitivity: is also called immediate hypersensitivity. It is mediated by IgE and occurs within minutes after challenge or re-exposure to the innocuous antigen. IgE is produced by plasma cells, rather than other types of Ig accumulating in the serum or tissue. IgE tightly binds to the surface of mast cells and basophils via the high the affinity IgE receptor, FcεRI. Antigen binds to IgE and cross-links the receptors, leading to the release of pharmacological active agents such as histamine, prostaglandins, and leukotrienes, which increase vascular permeability and contraction of smooth muscle, inducing clinical manifestations, including rhinitis, asthma, and, in severe cases, anaphylaxis.

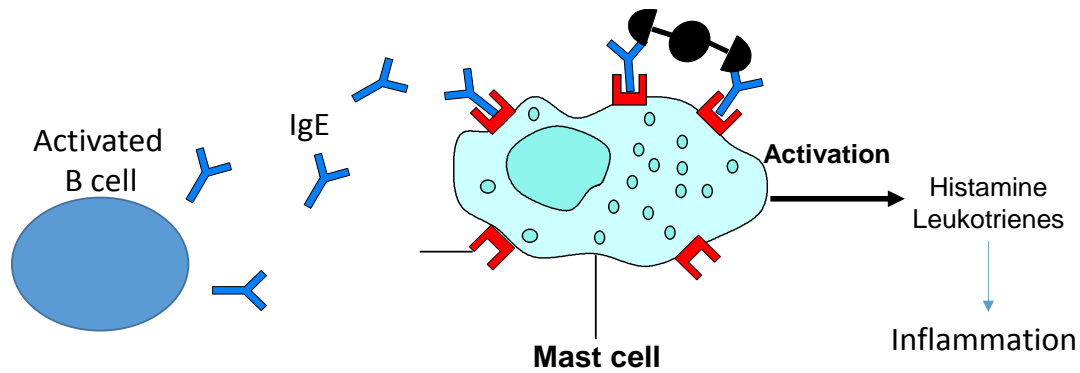


Figure 1.1A The mechanism of Type I hypersensitivity. Activated B cells secrete large amount of IgE antibody, which binds to the $Fc\epsilon R$ expressed on the surface of mast cells. When an allergen binds it cross-links two IgE on the cell surface, the mast cell degranulates and releases histamine and leukotrienes, thereby inducing inflammation.

II. Type II hypersensitivity is induced by the binding of IgM or IgG antibodies to antigen on the surface of cells and thereafter activating the complement cascade, which causes the massive death of the cells. For example, IgG or IgM antibody-induced destruction of red blood cells or platelets can be induced by some drugs, such as penicillin and cephalosporin. Drugs bind to cell surfaces and become a target of anti-drug IgG antibodies, which in turn activate the complement cascade and lead to cell damage.

III. Type III hypersensitivity: reactions are caused by soluble antigens. The reaction occurs when the complex of IgM or IgG and antigen accumulate in the circulation or in the tissue and activates complement system. The large immune complexes can be removed by monocyte-phagocyte system, whereas small complexes can escape and deposit in blood vessel walls, where they can ligate Fc receptors on leukocytes, generating local inflammatory response and increase vascular permeability. Fluid and cells then enter the site of inflammation from local blood vessels. IgG forms immune complexes by binding $Fc\gamma RIII$, triggering the complement cascade by activating complement fragment $C5a$ and in turn causing tissue damage.

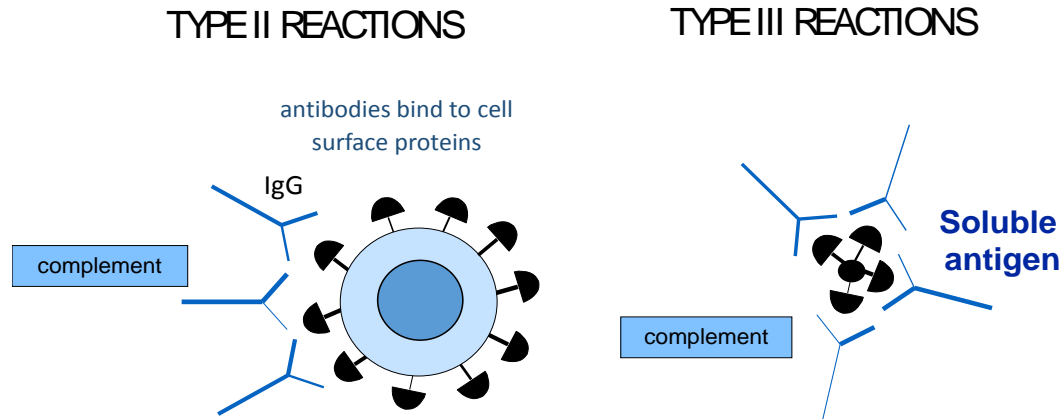


Figure 1.1B The mechanism of type II and type III hypersensitivity. Type II and type III hypersensitivity are induced by IgG and IgM antibodies. In type II, Ig antibodies bind to the surface of innocent cells. Then the complement system activates and induces cytotoxicity. Type III hypersensitivity is due to the accumulation of antigen-complex in blood vessels or tissue. The complexes activate the complement system which then causes massive tissue damage.

IV. Type IV hypersensitivity is mediated by antigen-specific effector T-cells. The response can take many weeks to develop but symptoms appear rapidly, after the re-challenge with the antigen. This type of hypersensitivity is also called delayed-type hypersensitivity. When activated, T-cells secrete cytokines which induce infiltration of inflammatory cells and the release of cytokines which in turn causes local tissue damage. Type IV hypersensitivity is further classified into four subsets depending on the different cytokines secreted by T-cells.

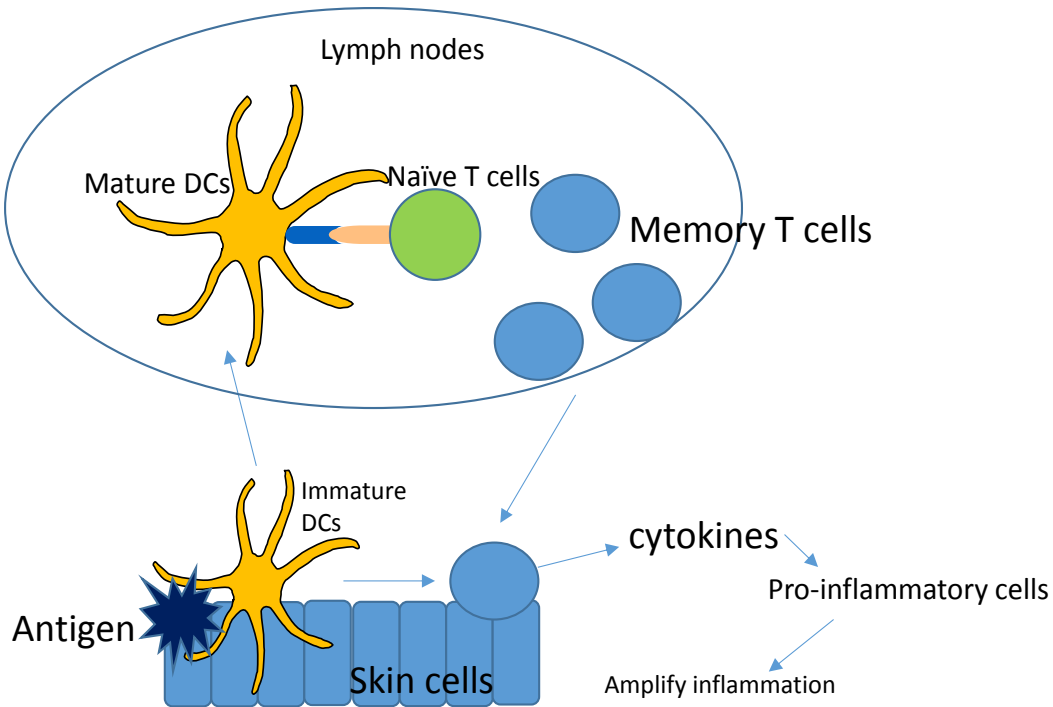


Figure 1.1C The mechanism of type IV hypersensitivity. Antigen penetrates the skin and is confronted with dendritic cells (DCs). DCs uptake the antigen, travel to lymph nodes where they mature and efficiently prime naïve T-cells. The naïve T-cells then proliferate and turn into memory T-cells which then travel into the blood and back to inflammatory site. There they secrete cytokines, promoting the recruitment of other inflammatory cells, thereby amplifying skin inflammation.

Type IVa hypersensitivity is induced by Th1-type T-cell responses. Th1-type T-cells secrete IFN- γ and TNF- α . These cytokines stimulate the expression of adhesion molecules on endothelial cells and increase the vessel permeability which allows plasma and inflammatory cells to the inflammatory site. Moreover, IFN- γ promotes macrophages to secrete TNF- α and IL-12, which stimulates NK cells. As macrophages contain TNF- α receptors on self-surface, they can be auto-activated by TNF- α and then generate more TNF- α and IL-12, forming a positive feedback chain and thereby amplifying the inflammatory response. An example of type IVa hypersensitivity is tuberculin skin test (Sinigaglia et al., 1985). Type IVb correlates to Th2 type immune responses. Th2 secreting T-cells secrete IL-4, IL-5 and IL-13 cytokines, which stimulate B-cells to secrete

antibodies such as IgE and IgG4. IgE in turn activate mast cells and IgG4 is associated with allergy, which suggest a link with type I hypersensitivity. IL-5 secretion leads to an eosinophilic inflammation, which is the characteristic of many drug hypersensitivity reactions (Pichler et al., 2003, Yawalkar et al., 2000).

Type IVc reactions involve cytotoxic T-cell migration into the tissue and direct cytotoxicity to tissue cells by release of cytolytic molecules such as perforin/ granzyme B. Alternatively, T-cells may secrete FasL and induce apoptosis through triggering the Fas receptor. Cytotoxic T-cells play a role in the drug hypersensitivity reactions such as maculopapular or bullous skin diseases, neutrophilic inflammation and in contact dermatitis. Type IVc reactions appear to be predominant in severe drug hypersensitivity reactions especially SJS/TEN syndrome, in which keratinocytes are killed by CD8 cytotoxic T-cells (Yawalkar et al., 2000, Schnyder et al., 1998). In general, CD8 T cell-mediated cytotoxic skin reactions are more severe than CD4 T-cell mediated cytotoxicity, as MHC I is ubiquitously expressed in keratinocytes as well as other skin cells, leading them being potential targets of CD8⁺ cytotoxic T-cells. In contrast, CD4 cytotoxic T-cells only recognize fewer cells expressing MHC II molecules, mainly antigen presenting cells. Hence, the massive presence of CD8 cytotoxic T-cells found in the blister fluid of SJS/TEN is a characteristic of the symptom.

Type IVd reactions involve the recruitment of neutrophils through the release of IL-8 and CXCL8 from T-cells. The neutrophils play an active role in the induction of pustular inflammation. Symptoms include pustular exanthema and acute-generalized exanthematous pustulosis (AGEP) (Pichler 2003). Kabashima et al., (2011) found a significantly higher percentage of Th17 cells in the patients suffering from AGEP. As IL-17 and IL-22 cooperatively stimulate keratinocytes to secrete IL-8, Th17 cells are considered an important T-cell subset in

the recruitment of neutrophils. Cytokines play a fundamental role in pathogenesis of drug hypersensitivity and their function is summarized in table 1.2.

Table 1.1 Extended Coombs and Gell Classification Adapted from (Pichler 2003)

Classification	Type of Immune responses	Pathologic characteristics	Clinical systems	Cell types
Type I	IgE	Mast-cell degranulation	Urticaria anaphylaxis	B cells/Ig
Type II	Ig G and IgM	FcR-dependent cell destruction	Blood cell dyscrasia	B cells/Ig
Type III	Ig G, IgM and complement	Immune complex deposition	Vasculitis	B cells/Ig
Type Iva	Th1 (IFN- γ , TNF- α)	Monocyte activation	Ec zema	Th1 cells
Type IVb	Th2 (IL-5 and IL-4)	Eosinophilic inflammation	Maculopapular exanthema, bullous exanthema	Th2 cells
Type IVc	CTL (perforin and granzyme B and fasl)	CD4-or CD8-mediated killing of cells	Maculopapular exanthema, eczema, bullous, exanthema, pustular exanthema	Cytotoxic T-cells
Type IVd	T-cells (IL-8)	Neutrophil recruitment and activation	Pustular exanthema	T-cells

Table 1.2 Brief summary of cytokines and their functions (Adopted from Janeway 2012 Immunobiology)

Family	Cytokine	Function	Producer cells
Interleukins	IL-1 α	Fever, T-cell activation, macrophage activation	Macrophages, epithelial cells
	IL-1 β	Th17 differentiation, Fever, T-cell activation, macrophage activation	Macrophages, epithelial cells
	IL-2	T-cell proliferation	Th1
	IL-3	Synergistic action in early haematopoiesis	Th1, Th2
	IL-4	B cell activation, IgE switch, induce differentiation into Th2 cells	T-cells, mast cells
	IL-5	Eosinophil growth, differentiation	T-cells, mast cells
	IL-6	T- and B-cell growth and differentiation, Th17 differentiation, fever	T-cells, macrophages
	IL-8	Chemo attractant for neutrophils and T-cells	Macrophages
	IL-10	Potent suppressant of macrophage functions	Monocytes
	IL-12	Activates NK cells, induces CD4 T-cell differentiation into Th1 cells	Macrophages, dendritic cells
	IL-13	B-cell growth and differentiation, inhibits Th1 cells	T-cells
	IL-17A	Induces cytokine production by epithelia, endothelia, and fibroblasts, proinflammatory	Th17 cells
	IL-17F	Induces cytokine production by epithelia, endothelia, and fibroblasts, proinflammatory	Th17 cells, monocytes
	IL-21	Induces proliferation of B, T and NK cells, promotes Th17 differentiation	Th2
	IL-22	Epithelial barrier, pro-inflammatory agents	NK cells, Th17 cells, Th22 cells
	IL-23	Induces proliferation of Th17	Dendritic cells
	IFN- γ	Macrophage activation, increased expression of MHC molecules and antigen processing components, Ig class switching, suppresses Th2	T-cells, NK cells
Colony-stimulating factors	G-CSF	Stimulates neutrophil development and differentiation	Fibroblasts and monocytes
	GM-CSF	Stimulates growth and differentiation of dendritic cells	Macrophages, T-cells
TNF family	TNF- α	Promotes inflammation, promotes differentiation into Th22 cells	Macrophages, NK cells, T-cells
	Fas Ligand	Apoptosis	T-cells
Transforming growth factor beta (TGF- β) superfamily	TGF- β 1	Anti-inflammation, induces Th17 and Tregs differentiation	T-cells, monocytes
	TGF- β 3	Promotes Th17 differentiation, promotes inflammation	T-cells, monocytes

1.4 Immune response

The immune system is designed to help the body to remove harmful microorganisms or viruses that enter it. Antigen presenting cells (APCs) can interact with other cells of the immune system, to achieve this.

Dendritic cells (DCs) are the most powerful antigen presenting cell as they are the only type of APCs that can stimulate naïve T-cells. When they are activated, they present peptide antigens on the cell surface using MHC class I and MHC Class II molecules (See Figure 1.2.1). They travel out of the inflammatory site to the local lymph nodes where they meet naïve T-cells and prime them through an interaction of the MHC-peptide complex with the T-cell receptor (TCR) and by interactions between the costimulatory molecules. Thereafter, primed T-cells become antigen-specific effector T-cells that circulate the body and can return to inflammatory sites to help resolve the infection via cytokine secretion and recruitment of pro-inflammatory cells.

1.4.1 The mechanisms of antigen presentation.

When antigens penetrate the skin and enter the body, they may encounter APCs, such as DCs or macrophages. The antigen may be taken up by the APCs via phagocytosis and chopped up into small pieces by the proteasome, or cleaved within the endosome. The resulting peptides are presented on the cell surface on MHC class I and MHC class II molecules. The peptides displayed by MHC class I molecules are usually 8-10 amino acid residues in length and can activate CD8 T-cells, whereas the peptides displayed by MHC II molecules are usually 12-20 amino acid residues in length and can activate CD4 T-cells (Janeway et al, 2012).

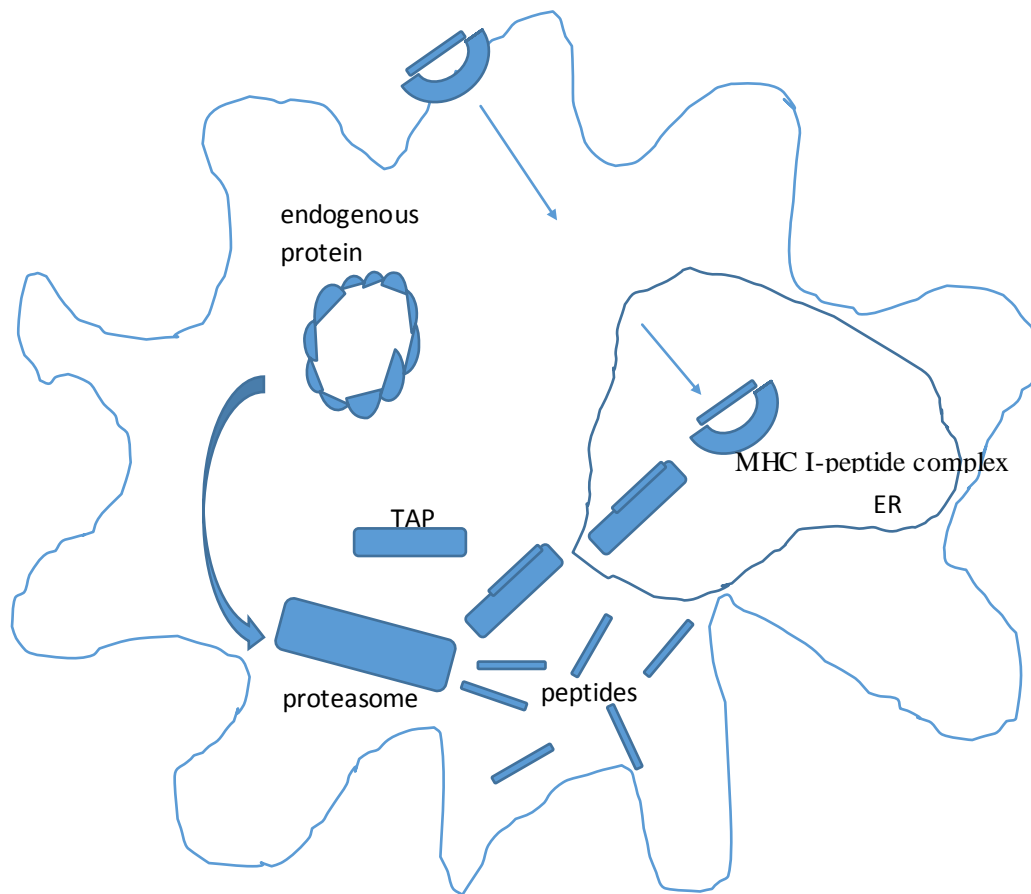


Figure 1.2.1 Class I MHC molecules display peptides to CD8 T-cells. Endogenous protein is cut into small peptides by the proteasome. These peptides are then carried by TAP (The transporter associated with antigen processing) and transported into endoplasmic reticulum (ER), where they will meet up with MHC I molecules. MHC I molecules then bind with the peptides, forming MHC I-peptide complexes. The complexes are then transported to surface of the cell and displayed to CD8 T-cells.

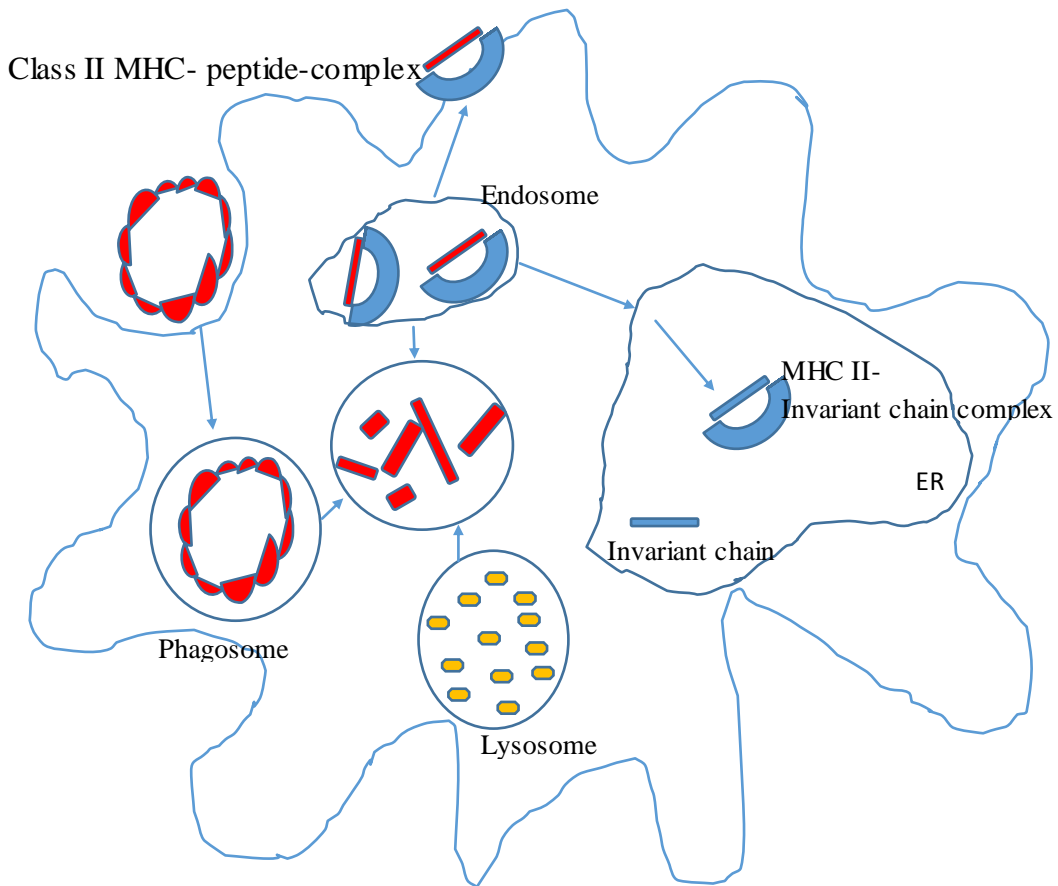


Figure 1.2.2 Class II MHC molecules display peptides derived from non-self-proteins, especially bacterial proteins. When pathogen is swallowed by the antigen presenting cells, they are trapped into the phagosome. They meet chemicals and enzymes from lysosomes and in turn are digested into small peptides. This process is termed phagocytosis. MHC II molecules do not load peptides in the ER. Rather, in the ER their binding grooves are occupied by invariant chain peptides, protected from the binding of endogenous proteins. MHC II-invariant chain complexes are transported from ER and enter endosomes, where the chain peptides are replaced with pathogen peptides digested through phagocytosis. The MHC II-peptide complexes are then transported to the cell surface and displayed to CD4 T-cells.

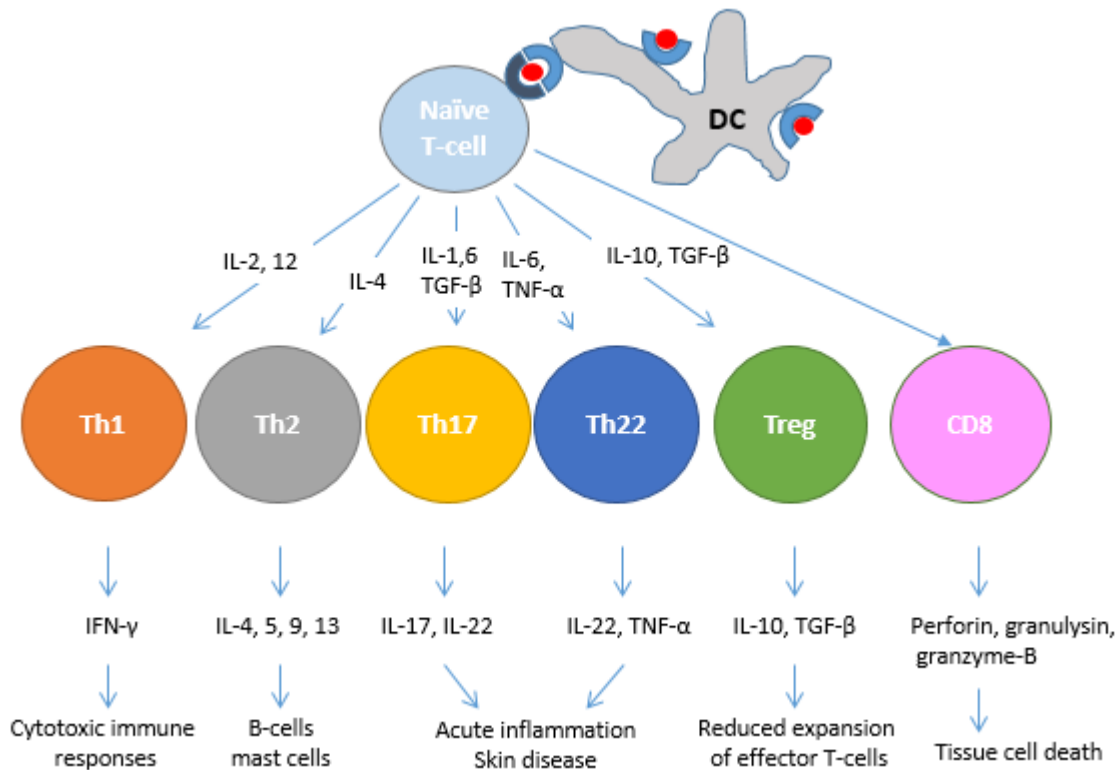


Figure 1.3 After contacting of TCR with antigenic-peptide-MHC complex that displayed on the surface of dendritic cells, naïve T-cells are activated and expanded. During priming, micro-milieu cytokines determine the fate of the antigen specific T-cells and consequently the role they play in the immune response.

1.4.2 T-cell subsets

During priming, naïve T-cells, which have been exposed to an antigen, are activated. They differentiate into antigen specific effector cells over the course of 1-2 weeks. During this activation period, three signals are required, 1) appropriate contact with antigenic peptide presented by MHC molecules that are displayed on the surface of antigen presenting cells; 2) costimulatory molecule binding, such as the binding of B7.1 and B7.2 on the surface of T-cells and CD40 of APCs; 3) cytokines that are in the milieu at the time of naïve T-cell priming. These

signals determine the nature of the T-cells that are activated and their subsequent phenotype.

T-cells are subdivided into CD4 and CD8 T-cells according to the expression of the co-receptors CD4 and CD8, respectively. These co-receptors interact and stabilize MHC-peptide-T cell receptor complex when T-cell receptors recognize antigen. After drug priming, of the cytokine micro-environment determines the nature of the T-cell response. CD4+ T helper cells can differentiate into various subsets such as Th1, Th2, Th17, Th22, Th9 secreting cells. Each subset is dominant in a specific type of inflammation. In the context of drug hypersensitivity, Th1 and Th2 secreting T-cells are known to be involved in different forms of tissue injury. However, the newer subsets have not been investigated.

1.4.2.1 CD8+ T-cell

CD8+ T-cells have been found in SJS/TEN skin lesions and these cells are also the dominant T-cell population in blister fluid (Nassif et al., 2004). Moreover, CD8+ T-cells have been shown induce to drug antigen-specific cytotoxicity both in vivo and in vitro (Wu et al., 2006; Rozieres et al., 2010). In carbamazepine-induced drug hypersensitivity, drug specific CD8+ T-cells have been cloned and these clones show a strong toxicity against target cells in vitro. CD8+ T-cells isolated from patients with abacavir-induced hypersensitivity reactions also kill target cells by recognizing drug-peptide-MHC I complexes displayed on surface of antigen presenting cells (Chessman et al., 2008).

1.4.2.2 CD4+ T-cell and phenotypes

As described above, CD4+ T-cells play an important role in delayed type drug hypersensitivity and reactions have been classified into 4 sub-types based on the effects of different CD4+ T-cell subsets (cytokine secretion and subsequently inflammatory cell recruitment).

CD4+ helper T-cells play important roles for host defense and immune-mediated disease by their

ability to differentiate into specialized subsets. These effector T-cells are defined by the expression of a restricted panel of cytokines and the expression of specific master regulator transcription factors (Szabo et al., 2003). Initially, the understanding of distinctive populations of differentiated CD4⁺ T-cells came from analysis of T-cell clones isolated from mice (Mosmann, Coffman 1989). Subsequently, (Bottomly et al., 1989) identified the key cytokines of each T-cell subset and the T-cell populations were named Th1 and Th2 secreting cells, in which T helper 1 (Th1) cells express T-bet and selectively produce interferon (IFN)- γ , while Th2 cells express Gata3 and produce cytokines such as IL-4 and IL-13 (Nakayamada et al., 2012). To understand the mechanisms of drug hypersensitivity, it is important to define the T-cell subsets involved.

The picture is complicated by the fact that in certain circumstances, IFN- γ secreting T-cells also secrete Th2 cytokines. However, T-cell subset classification holds its value in certain circumstances: first, stable Th1 and Th2 lineage with typical cytokine expression can be obtained by cytokine polarization during naïve T-cells expansion. Second, in terms of host defense, each T-cell subset plays a particular role in pathogen eradication, e.g. intracellular bacteria for Th1 cells and helminths for Th2 cells. Third, these subsets express stable key regulators, T-bet for Th1 and GATA3 for Th2. Finally, the classification of T helper cell subsets renders a great therapeutic value in allergic and autoimmune diseases, generally Th2 cells induce allergic diseases, whereas Th1 and Th17 (discussed in detail below) cells induce autoimmune diseases.

Th1 cells

Th1 cells secrete IFN- γ , IL-2 and TNF- α , inducing cell-mediated immunity and phagocytic inflammation. Th1 cells eradicate intracellular pathogens such as bacteria, virus and protozoa. IL-12 and IFN- γ suppresses formation of Th2 cells, increase MHC class I and MHC Class II

expression and promote antigen presenting cell function. IL-2 functions as a growth factor promoting T-cell proliferation. Th1 cells may also mediate local inflammation at sites of infection. Th1 cells express the CCR5 homing receptor as a character of this T-cell subset. T-bet is a major transcription factor involved in the inducing the release of IFN- γ and Th1 cell differentiation (Szabo et al., 2000). IFN- γ responses to *Leishmania major* are significantly decreased in T-bet knockout mouse (Szabo et al., 2002).

Th2 cells

Previously, it was thought that a main function of Th2 secreting T-cells was helping B cell antibody generation. However, recent research found that follicular B helper T-cells (Tfh) are the main subsets that mediate this process and Th2 cell just play a regulatory role in the antibody generation. Th2 cells produce IL-4, IL-5, and IL-13 which are important in the elimination of parasites, such as helminthes. IL-4 induces Th2 differentiation and inhibits Th1 differentiation together with IL-13. Th2 cells may affect eosinophils, mast cells, and basophils by cytokine release i.e. IL-4, IL-5, and IL-13. Th2 cells express the CCR3 homing receptor. Th2 polarization requires the addition of IL-4 (Le Gros et al., 1990). GATA3 is the master regulator of Th2 (Zheng et al., 1997). GATA3 is also critical for the development of CD4⁺ T-cell responses (Ho et al., 2009). GATA3 expression is up-regulated or down-regulated during Th2 and Th1 polarization, respectively (Zhang et al., 1997). Moreover, Th2 differentiation is completely blocked in vivo and in vitro in the absence of GATA3 (Zhu et al., 2004).

Th17 cells

Dysregulation of Th1 responses has been associated with an autoimmune response. For example, IFN- γ expression in the target tissue is associated with clinical signs of experimental autoimmune encephalomyelitis (EAE). Furthermore, based on the evidence of blocking one of the heterodimers of IL-12p40, EAE got alleviated. However, blockage of the other chain of IL-12 did not protect from EAE, but made the condition more severe (Krakowski et al., 1996; Tran et al., 2000; Gran et al., 2002; Zhang et al., 2003; Gutcher et al., 2006). This confusion did not get resolved until the discovery of IL-23, an important inducer of Th17 secreting cells. These cells share a p40 chain with IL-12 and have a unique heterodimer chain of p19 (Oppmann et al., 2000). This data implies blocking p40 blocks both Th1 and Th17 signaling and thus, the previous conclusion that EAE is solely Th1 induced autoimmune disorders has to be questioned.

Depletion of p19 of IL-23 but not p35 of IL-12 was shown to block EAE, which confirms that EAE is dominated by Th17 but not Th1 cells (Cua et al., 2003; Langrish et al., 2005).

Th17 cells were named after one of the specific cytokines they secrete (i.e., IL-17). Th17 cells do not express GATA3 or T-bet (Park et al., 2005, Harrington et al., 2005), instead, they express high level of ROR γ t (Ivanov et al., 2006). ROR γ t has been shown to be critical for the release of IL-17, as ROR γ t deficient mice show an impaired Th17 differentiation (Lee et al., 2009). Th17 cells were established as an independent T-cell subset due to its unique differentiation factors. IL-23, IL-6 and TGF- β are all required to induce Th17 differentiation (Veldhoen et al., 2006; Betteli et al., 2006; Mangan et al., 2006). ROR γ t is the master transcription factor of Th17 cells, maintaining Th17 character for a long duration of time (Ivanov et al., 2006; He et al., 1998). ROR γ t is regulated by its upper signaling molecule, STAT3. STAT3 is activated by Th17 inducer cytokine IL-23 and IL-6, forming a positive feedback loop in Th17 differentiation (Zhou et al.,

2007; Yang et al., 2007).

Th17 cells are critical in host defense. They also play important roles in immune disorders such as psoriasis (Krueger et al., 2007), rheumatoid arthritis (Kirkham et al., 2006), multiple sclerosis (Matusevicius et al., 1999), inflammatory bowel disease (Sarra et al., 2006), asthma (Molet et al., 2001; Barczyk et al., 2003) and EAE (as discussed above).

In host defense, Th17 cells can be induced by pathogen-associated molecular patterns (PAMP) which are external stimuli derived from extracellular pathogens such as bacteria (Chtanova et al., 2004) and fungi.

In psoriasis, Th17 cells are the major subset of T-cells isolated from skin lesions (Pane et al., 2008) and with CCL20/CCR signaling being important for infiltration of inflammatory cell to the skin. Moreover, the blockage of p40 has been shown to reduce the psoriatic skin area (Krueger et al., 2007).

In patients with rheumatoid arthritis, IL-17, IL-1 and TNF have been shown to play a direct role in joint destruction (Kirkham et al., 2006). Furthermore, the molecule of RANKL expressed on the surface of Th17 induces cartilage and bone destruction directly (Kotake et al., 1999; Miranda-Carus et al., 2006; Sato et al., 2006).

Evidence of involvement of Th17 cells drug hypersensitivity is lacking. Most importantly, of the role of Th17 cells in drug-induced mild skin reactions such as MPE has not been studied. However, it has been suggested that Th17 cells may play a role in neutrophilic infiltrated drug hypersensitivity reactions such as AGEP and SJS-TEN. These studies are discussed in more detail below.

Th22 cells

The phenomenon that some T helper cells secrete more than one signature cytokine is called plasticity. A T-cell population was found as they did not secrete signature cytokines such as IFN- γ , IL-4 and IL-17, but IL-22 solely (Eyerich et al., 2009). Therefore, this population is named after the cytokine IL-22, Th22. The specific microenvironment for differentiating Th22 cells is composed of TNF- α and IL-6. Skin DCs have also been shown to play a critical role in Th22 differentiation (Duhén et al., 2009). Once differentiated, the Th22 phenotype remains stable and does not convert to other cell types (Eyerich et al., 2009). The aryl-hydrocarbon-receptor (AHR) is thought to be the master transcriptional regulator for Th22 cells (Trifari et al., 2009). Th22 express characteristic chemokine receptors such as CCR4 and CCR10 (Duhén et al., 2009), which suggest that they reside in normal skin and inflammatory skin, migrating following exposure to CCL27, a ligand of CCR10. The IL-22 receptor is expressed exclusively on tissue cells, mostly in epithelial cells. In contrast to many cytokine receptors, the receptor for IL-22 is not expressed on immune cells (Wolk et al., 2004). Therefore, tissue cells are the major target of IL-22. Accordingly, IL-22 secreting lymphocytes are strongly enriched in peripheral tissue (Eyerich et al., 2009; Anmmziato et al., 2007). In terms of host defense, both Th17 cells and Th22 cells are involved in protection from bacterial infection. Th17 cells play a role in bacteria eradication (Lin et al., 2009), whereas Th22 have a protective effect on epithelial cells, especially in epithelial cell regeneration, proliferation and enhancement of migration (Nogales et al., 2008; Wolk et al., 2006).

The protective characteristic of IL-22 cells induces epithelial cell expansion. However, in psoriasis IL-22 is directly involved in the disease pathogenesis. Psoriasis is a chronic inflammatory disease with characteristic of keratinocytes over-proliferation and impaired

differentiation that leads to considerable thickening and scaling of the epidermis (Nestle et al., 2009; Perera et al., 2012). Together with IL-17 and TNF- α , IL-22 induces hyper-proliferation of keratinocytes, leading to the maintenance of acanthosis which is a hallmark of psoriasis (Wolk et al., 2009; Boniface et al., 2005; Delle et al., 2007).

Th22 cells also play a pathological role in inflammatory bowel disease and rheumatoid arthritis (Zenewicz et al., 2008; Kim et al., 2012). Moreover, serum IL-22 was reported to be elevated in patients with Crohn's disease. IL-22 elevation was thought to be induced by the activation of IL-23/Th17 signaling (Schmechel et al., 2008).

The involvement of Th17 and Th22 cells in drug hypersensitivity.

Similar to Th17 cells, there is very limited evidence to support a role for Th22 cells in drug hypersensitivity. Th17 cells might play a role in AGEF and SJS/TEN, whereas IL-22 secretion has only been detected in patients with AGEF.

In carbamazepine induced AGEF, drug specific IL-17 secreting T- cell clones have been shown to co-secrete either IL-4, IL-5 or IFN- γ . The Th1/ Th17 or Th2/Th17 populations may explain the characteristic eosinophil infiltration that induced by IL-4, keratinocyte apoptosis mediated by IFN- γ , and neutrophilic infiltration by IL-17. It is known that IL-17 and IL-22 secreting cells play pathological roles in psoriasis. It has been considered AGEF have a similar neutrophilic inflammatory processes to psoriasis since neutrophil-recruiting CXCL8/ IL-8 producing drug-specific T-cells are found in the circulation. Furthermore, a remarkable increase in IL-17 and IL-22 secreting cells has been described. IL-17 and IL-22 have been shown to promote keratinocytes to release IL-8, which is a well-known neutrophil recruitment mediator (Nakamizo et al., 2010).

In SJS/TEN, it has also been shown that the proportion of circulating Th17 cells is elevated among the CD4⁺ T-cell population in blisters and this proportion is decreased following improvement of the disease. In contrast, this phenomenon is not observed with Th1 or Th2 cells (Watanabe et al., 2011). It is not hard to understand the involvement of Th17 cells since Caproni et al (2006) has reported that neutrophils play a pathologic role in SJS/TEN by releasing reactive oxygen species and lysosomal enzymes and Th17 cells promote neutrophils recruitment.

1.4.3 TCR signaling and V β receptor

The TCR is a multiprotein transmembrane complex comprising TCR $\alpha\beta$ (or TCR $\gamma\zeta$), CD3 $\epsilon\gamma$, and CD3 $\zeta\zeta$ dimers (Alarcon et al. 2003; Kuhns and Davis 2012).

TCR are similar to immunoglobulin, in terms of both structure and genes. As for immunoglobulins, the TCR $\alpha\beta$ dimers comprise both variable (V) and constant (C) regions which form domains that interact with antigen presented by MHC molecules on the surface of APCs. This interaction forms 3 complementary determining regions (CDR1, 2, and 3) on V regions. CD3 molecules are tightly associated with TCR $\alpha\beta$ on T-cell surface. However, CD3 molecules do not bind to antigen. When antigen binds to $\alpha\beta$ chain of TCR, cell signaling will be transmitted into the cell by phosphorylation of immune-receptor tyrosine-based activation motif (ITAM) that is located on the cytoplasmic tail of CD3. Phosphorylation is the critical event in initiating downstream signaling cascades, during which, phosphorylation of phospholipase C γ (PLC γ) and consequently induce calcium influx into cytosol, resulting in the activation of T-cells.

CDR3 majorly determines antigen recognition by TCR. Hyper-variability of this region enables the TCR to recognize a large number of antigens. The nature of this variability is the somatic recombination of non-contiguous V α and J α segments of α -chain and variable (V β) and diversity

(D β), and joining (J β) segments of the b-chain (Hughes et al., 2003), among which, V β has 25 genes, D β , J β , V α , and J α have gene numbers of 2, 12, 70, 50, respectively. One particular gene from each group enables the generation of 10^{15-20} variable TCRs that allow for recognition of almost all the antigens in the universe (Davis, Bjorkman 1988). TCR diversity is often associated with potency of antigen and is often tested by V β receptor distributions (Kimber et al., 2012a, b). The activated TCR repertoire can be determined by dependent on the pathway of T-cell activation (Currier et al., 1996). PBMCs stimulated by mitogen (PHA), super-antigen (TSST-1), or normal antigen (tetanus toxoid) show diverse TCR repertoires, in which both fresh blood and PHA stimulated PBMCs showed a normal spread distribution, whereas tetanus toxoid stimulated PBMCs showed a restricted profile. Finally, super-antigen stimulation resulted in a unique pattern of diversity (Currier et al., 1996). To explain the relationship between TCR diversity and potency of antigen, Moon and colleagues suggest that high TCR repertoire diversity could be induced by strong antigen with multiple antigen determinants and verse vice (Moon et al., 2007).

In drug hypersensitivity, V β receptors have been found to be expressed in a restricted panel. For example, some V β receptors are expressed in a wide distribution panel. In the study by Ko et al., (2011), patient with genotype of HLA-B*15:02 and with carbamazepine induced SJS/TEN were recruited to analyze the drug-specific T-cell receptor repertoire. 16 out of 19 patients with carbamazepine induced SJS/TEN expressed a single clonotype of VB-11-1SGSY and this clonotype was not expressed in all drug-tolerant patients. Carbamazepine specific CD8+ T-cells with this clonotype showed a strong cytotoxicity and this cytotoxicity was inhibited by the addition of anti VB-11-1SGSY antibodies.

When testing the T-cell repertoire of abacavir specificity which is also mediated by CD8+ T-cell

responses and is restricted by a single HLA allele association (HLA-B*57:01), drug-specific T-cells displayed a random distribution of V β receptors (Illing et al., 2012). These findings are in stark contrast to the work of Ko et al exploring carbamazepine-induced SJS.

In my thesis, piperacillin specific T-cells displayed a random distribution of V β receptor repertoire. In our lab, nitroso-sulfamethoxazole (SMX-NO) T-cell clones isolated from patients with sulfamethoxazole hypersensitivity also displayed a random TCR V β repertoire (unpublished data). It is known that SMX-NO is a strong hapten that is capable of binding to both intracellular protein and the proteins in the serum, and wide V β receptor distribution suggests that SMX-NO protein binding generates a large number of peptide epitopes, which subsequently interact with multiple TCRs.

1.4.4 Tissue homing

Cellular tissue homing was dominated by the interaction between chemokines and their receptor (Campbell & Butcher 2000). As reviewed by Charo & Ransohoff (2006), chemokines are divided into two groups, CC chemokines and CXC chemokines. CC chemokines have two adjacent cysteine residues near the amino terminus whereas in CXC chemokines, two cysteine residues are separated by a single amino acid. CC chemokines tend to induce the migration of monocytes and CXC cytokines tend to induce the migration of neutrophils. For example, CCL2 is a chemokine that stimulates monocytes to migrate from the bloodstream to the tissue. Another example, CXCL8, however, attracts neutrophils out of the blood and migrates into the peripheral tissue.

Chemokines have two main roles. Firstly, they act on the lymphocytes rolling along the endothelial cells at sites of inflammation and convert this rolling status into steady binding to the

endothelial cells, via changing the conformation of adhesion molecules on lymphocytes and thereafter binding to their ligands on endothelial cells. Next the lymphocytes squeeze between the endothelial cells, going out of the vessel to the tissue. Secondly, chemokines direct the migration of lymphocytes along a gradient of chemokine molecules bound to the extracellular matrix and the surface of endothelial cells. This gradient increases in concentration toward the site of infection.

T-cell priming occurs in lymphoid tissue but effector cells are needed in peripheral tissue at the original site of infection. T-cells express specific chemokine receptors i.e., CCR4, so that release of CCL17 (or TARC) and its binding to the receptor causes T-cells to migrate; following a chemokine diffusion gradient and accumulation in the inflamed site.

In immunological conditions targeting skin, antigens are transported to the draining lymph node by dendritic cells. Antigen specific T-cells must then be transported from the lymph node back to skin. When T-cells encounter antigen in the skin, they become activated and may release effector molecules i.e., cytolytic molecules. These cause damage to skin cells, including keratinocytes. Migrating immune cells reach the inflamed skin initially through a series of selectin and integrin contacts, including cutaneous lymphocyte antigen (CLA). CLAs function as an integrin, which binds to the ligand E-selectin expressed in the endothelium thereby, inducing T-cells to move through the endothelium into the tissue (Rossiter et al., 1994).

Skin-infiltrating lymphocytes in patients suffering from psoriasis and allergic contact dermatitis express CCR10 and on the other hand, the ligand of CCR10, CCL27, is expressed by keratinocytes which orchestrate the migration of CCR10⁺ T-cells to the skin (Homey et al., 2002). In vivo, the neutralization of CCL27-CCR10 interactions dampens lymphocyte recruitment to the skin leading to the suppression of skin inflammation. The results suggest an

important role for CCR10 in the skin inflammation. CCR4 was also demonstrated to be important in skin inflammation. CCR4⁺ T-cells were only observed in memory skin homing T-cells and not in naïve T-cells and intestine homing T-cells. In chronic cutaneous disease, CLA⁺CCR4⁺ T-cells migrate following exposure to the ligands of CCR4, TARC and MDC (Campbell et al., 1999).

Several studies have investigated the role of chemokines and chemokine receptors in drug hypersensitivity. CLA expression on T-cells, isolated from skin and blood of hypersensitive patients, correlated with disease severity (Leyva et al., 2000). Increased expression of CCR4, CCR8, and CCR10 has been implicated in allergic reactions in the skin, such as contact dermatitis, atopic dermatitis and psoriasis (Hudak et al., 2002, Moed et al., 2004, Vestergaard et al., 1999). In patients with dermatitis, CCR4 and CCR10 are important in T-cell migration to the inflamed skin whereas CCR8 was important in homing of memory T-cells to healthy skin (Vestergaard et al., 2003, Schaerli et al., 2004).

In patients with delayed-type drug hypersensitivity, drug-specific CCR6⁺ T-cells initiate reactions by secreting TNF- α and IFN- γ . This leads to an acute phase response and induction of inflammatory chemokines such as CXCL8 or CCL20, the ligands of CCR6 in keratinocytes (Schaerli et al., 2004).

Most T-cells in the skin are CCR6 T-cells; they enter the skin and secrete more pro-inflammatory cytokines, thereby amplifying the immunological reaction.

1.5 HLA associations with drug hypersensitivity

Over the recent years, there has been a significant increase in the volume of publications associating various HLA alleles with different forms of drug hypersensitivity reaction. Genetic association studies of both drug metabolizing genes and immune-related genes have helped to expand our knowledge and improve our understanding of drug hypersensitivity. HLA alleles with high levels of polymorphism are critical in immune surveillance because every variant molecule interacts with different peptides. Only a small proportion of them will stimulate T-cells. Some of the HLA proteins protect from disease, whereas others function as predisposing factors for disease (Temajo et al., 2009; Han et al., 2012), including T cell-mediated drug hypersensitivity. For example, individuals expressing the HLA-B*57:01 allele are susceptible to abacavir hypersensitivity (Mallal et al., 2002) and flucloxacillin-induced hepatitis (Daly et al., 2009). In Han Chinese, carbamazepine-induced SJS and TEN are associated with expression of HLA-B*15:02 (Chung et al., 2004). However, in Caucasians, carbamazepine-induced hypersensitivity reactions have recently been associated with HLA-A*31:01 (McCormack et al., 2011). Allopurinol induces severe cutaneous allergic reactions (SCAR) in Han Chinese patients with HLA-B*58:01 (Hung et al., 2005). Pre-description screening of related HLA alleles has significantly decreased the occurrence of hypersensitivity to abacavir and carbamazepine (Chen et al., 2011; Mallal et al., 2008). The strong association with HLA indicates that the drug derived antigens interact with high level of restriction to HLA molecules to stimulate T-cells. The nature of this interaction has been defined for a limited number of drugs. For example, abacavir interacts with the F-pocket in the peptide-binding groove of the HLA-B*57:01 molecule (Chessman et al., 2008). A similar mechanism was observed in the binding of carbamazepine with HLA-B*1502 (Illing et al., 2012). The observation that HLA-DQ A*02:01 is associated

with lapatinib-induced liver injury indicates MHC I and II molecules can be involved in different forms of hypersensitivity (Spraggs et al., 2011).

1.6 Mechanisms of drug hypersensitivity

There are three main theories as to how pharmaceutical drugs can act as antigens and generate an immune response (See Figure 1.2).

1.6.1 Hapten and Prohapten theory

The basis of hapten theory was built up by the early research from Landsteiner and Jacobs (1935). They sensitized guinea pigs to the low molecular weight, chemically reactive compound, dinitrochlorobenzene (DNCB). The authors showed that the immune reaction resulted from the formation of a covalent protein adduct in skin through modification of specific nucleophilic residues. This leads to the hypothesis that chemicals and drugs are too small to function as antigens and activate an immune response directly. The hypothesis states that they bind to proteins forming an intact antigen to trigger an adaptive immune response; β -lactams are common causes of both type I and type IV hypersensitivity.

Many more studies support a role for the binding of haptenic substances to protein in the effector phase of immune responses to drugs and chemicals. Certain haptens bind spontaneously to protein via formation of a covalent bond with specific amino acid residues. For example, penicillins may induce hypersensitivity by binding directly to lysine residues on protein (see below). Antigen specific IgE isolated from patients with penicillin-induced anaphylaxis patients were found not against penicillin but against penicillin-haptenic structures formed by drug-

protein conjugation. Furthermore, certain penicillin-specific T-cells are activated specifically with drug-protein adducts. Penicillins contain a 6-aminopenicillanic acid nucleus which includes a β -lactam ring and a five numbered ring. When drug-protein conjugates form, the β -lactam ring opens up spontaneously and acylates with lysine residue of the binding protein. A similar profile of binding happens with a range of antibiotics, including piperacillin and flucloxacillin, which are discussed in detail in the sections below. In contrast to the penicillins, other compounds only bind to protein after drug metabolism and the liberation of reactive species. For example, urushiol, an allergen contained in ivy and poison oak induces a severe contact dermatitis in both human and mouse models. Urushiols are oxidised into reactive quinone species and the reactive species binds covalently to amino acid residues on protein (Kalergis et al., 1997). Some of drugs might also be metabolized into reactive intermediates prior binding to proteins. This indirect process is known as the pro-hapten hypothesis. A classical example is the drug halothane, which induces immunological hepatotoxicity in a small percentage of the population. Halothane is metabolized by P4502E1 (Kharasch et al., 1996) into a reactive trifluoroacetyl chloride intermediate, which binds covalently to lysine residues in proteins forming an antigen (Kenna et al., 1988). Tienilic acid an urisuric diuretic used in the treatment of hypertension was withdrawn from the market in 1980 due to its hepatotoxic potential. Tienilic acid is metabolized by human P4502C9 enzymes to yield a reactive metabolite S-oxide which binds covalently with nucleophilic groups on the enzyme forming an antigen. Anti-P450 antibodies have been detected in the serum of patients with hepatotoxicity. The most well characterized pro-hapten drug is sulfamethoxazole which will be discussed in the following section.

1.6.2 Pharmacological interaction of drugs with immune receptors: p.i. concept.

This theory suggests that parent drugs can trigger an immune response by direct interaction with

MHC molecules and TCRs. The theory is based on evidence that drugs can stimulate drug specific T-cells in an MHC processing independent way when APCs were fixed with glutaraldehyde (Pichler et al., 2002).

When a drug stimulates T-cells immediately, as shown by intracellular calcium release, then this response is too fast to require antigen processing (Pichler et al., 2002). Pulsing experiments show that T-cell responses can be inhibited by washing the antigen presenting cells, which removes the non-covalently bound drug. This shows that the drug is binding directly to immune receptors and activating T-cells by a pharmacological mechanism. Drugs that activate T-cells via a p.i. mechanism include carbamazepine, lidocaine, lamotrigine, and sulfamethoxazole (Farrell et al., 2003, Zanni et al., 1998, Schnyder et al., 1998).

1.6.3 Altered self-peptide repertoire model

Certain drug hypersensitivity syndromes are highly associated with particular human leukocyte alleles (Mallal et al., 2002, Yun et al., 2013, Chessman et al. 2008, Mallal et al. 2008, Bharadwaj et al. 2012). Abacavir hypersensitivity syndrome is associated with HLA-B*57:01 and investigations into this syndrome have resulted in the evolution of the altered self-peptide hypothesis to explain how this drug interacts with the HLA molecule to activate T-cells (Mallal et al., 2002, Martin et al. 2005, Mallal et al. 2008). The in vitro study of abacavir-induced T-cell activation showed that the ability of abacavir-treated cells to stimulate T-cells could be completely abrogated by a single amino acid residue change at position 116 (Ser116), which lines an anchor pocket in the peptide-binding groove of the HLA-B*57:01 molecule. Collectively, the authors propose that the drug occupies specific area in the cleft of the MHC class I molecule, which in turn alters the configuration of the peptide that sits in the groove. This

altered self-peptide-MHC complex only displayed on the surface of APC in the presence of the drug activates CD8⁺ T-cells which cause tissue damage (Figure 1.4).

Figure 1.4 Different pathways of T-cell activation in drug hypersensitivity.

Three mechanisms of drug hypersensitivity have been proposed, which are hapten/prohapten theory, p.i. concept and peptide alteration theory.

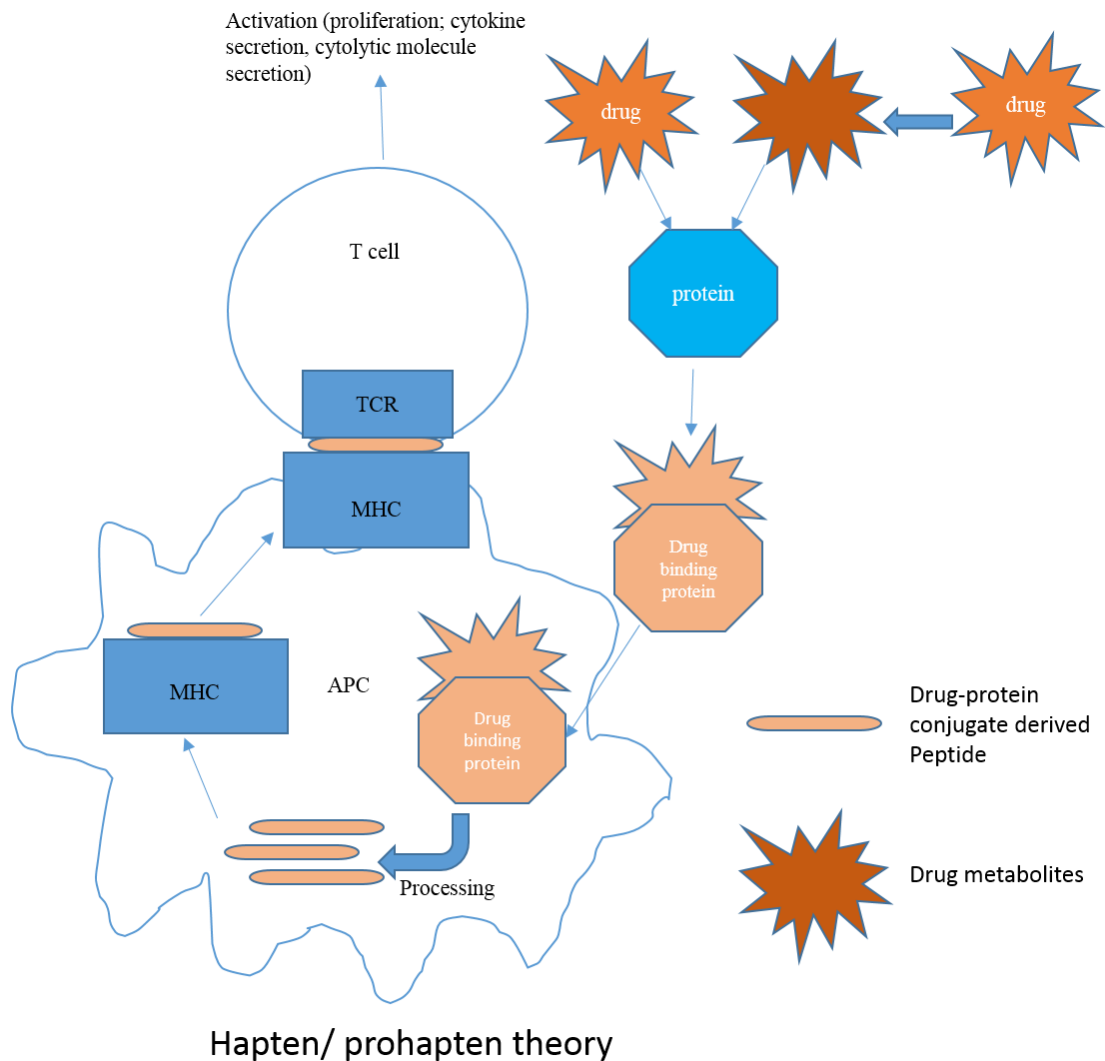


Figure 1.4 A illustrates the hapten/prohapten hypothesis; in which drug or drug metabolites bind covalently to protein to activate an immune response. Drug-protein conjugates are then taken up and processed by APC, cutting the protein into peptides which are then displayed on the cell surface by MHC molecule.

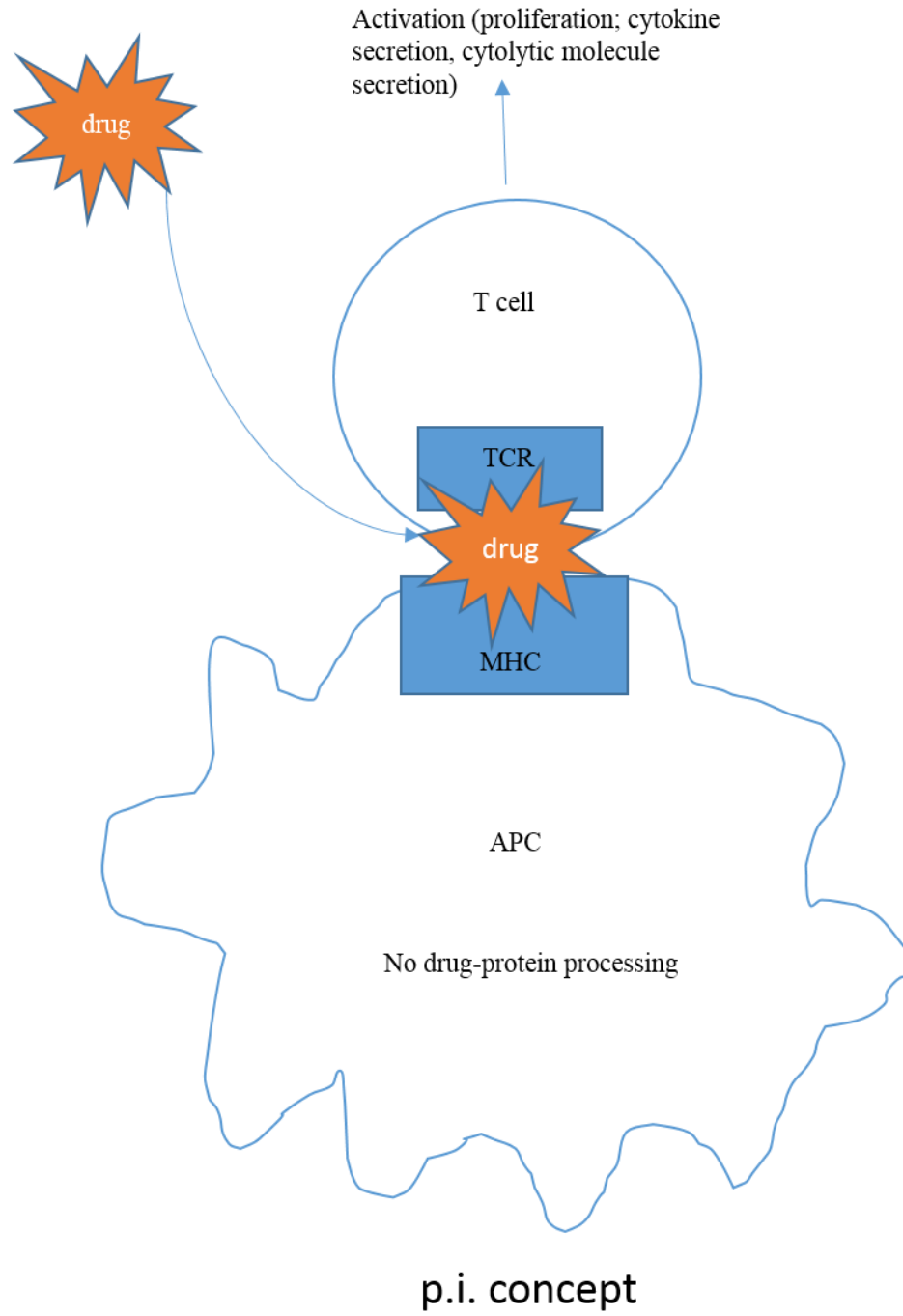


Figure 1.4 B illustrates p.i. concept. Here a drug may activate TCRs through a directly non-covalent binding against TCR and MHC molecules. Protein processing is not required.

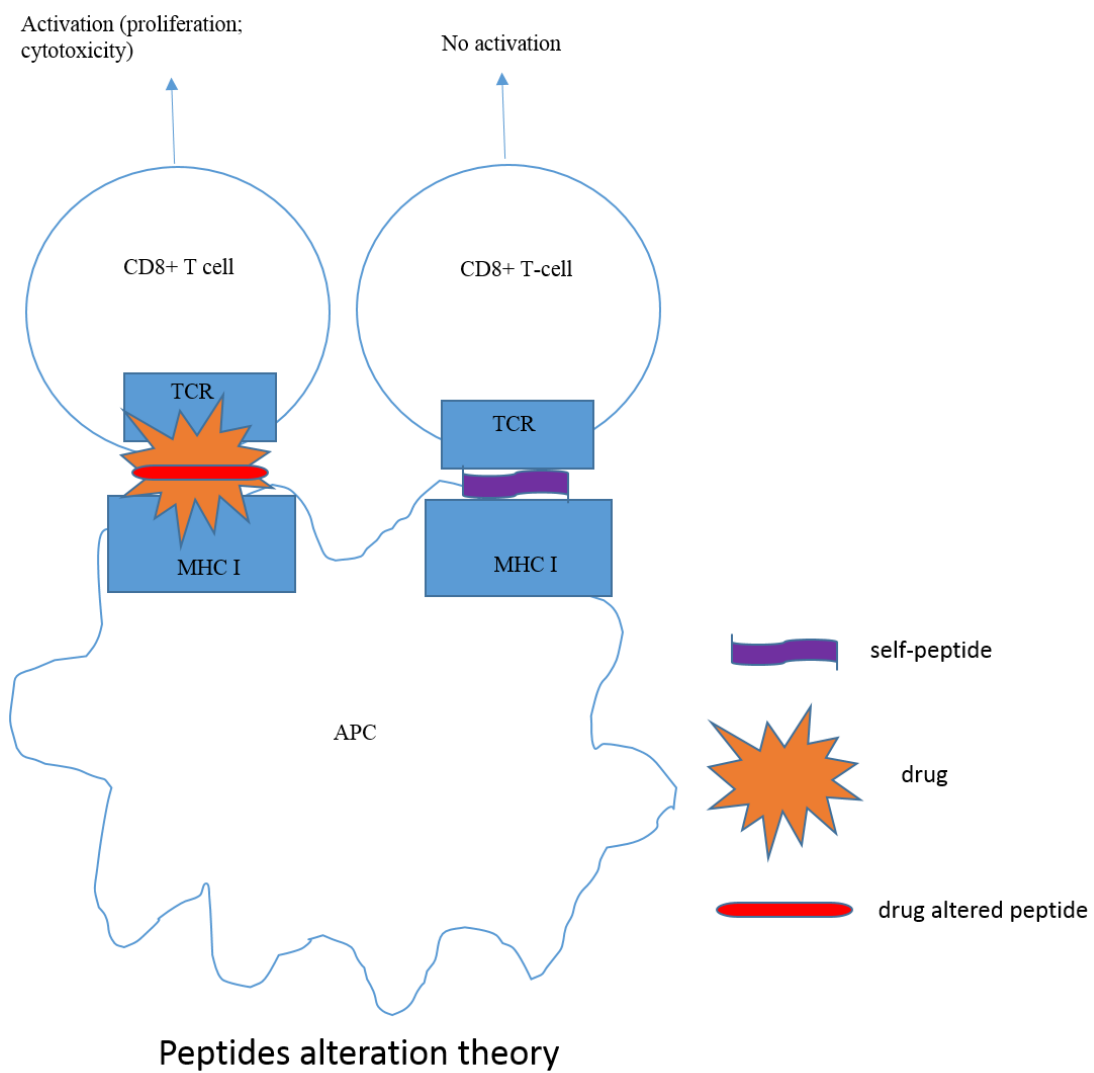


Figure 1.4 C shows peptide alteration theory. When certain peptides are displayed to T-cells, they do not generate immune response. However, when peptides load into the groove of MHC molecules in the presence of drugs, such as abacavir, they may induce immune reactions, due to the drug altering the configuration of the MHC peptide-binding groove, rendering the peptides immunogenic.

1.6.4 Drug metabolism and the activation of T-cells

SMX has been used as a model to study the role of metabolism in drug hypersensitivity for several reasons. Firstly its metabolism is well-defined. Secondly, stable and readily metabolites have been synthesized and are available for functional studies. Thirdly, patient samples are readily available for functional studies. Most of SMX is detoxicated in the liver. The drug is metabolized by hepatic N-acetyltransferase enzymes to an acetylated derivative which is easily eliminated from the body. However, a small amount of SMX is converted to a hydroxylamine intermediate. A reaction is catalyzed by CYP2C9 (Cribb et al., 1995). SMX hydroxylamine is stable and eliminated in urine (Gill et al., 1999). This suggests that most tissues are exposed to the hydroxylamine after SMX administration. The hydroxylamine either undergoes reduction back to SMX, a reaction by catalyzing by NADH cytochrome b5 reductase and CYP3A4 or is spontaneously oxidized to nitroso SMX (SMX-NO). SMX-NO has been shown to bind and modify selective cysteine residues expressed on both cellular and protein serum (Naisbitt et al., 1999, Naisbitt et al., 2001). Modification of cell surface proteins occurs quickly and then these protein conjugates are internalized through caveolae-dependent endocytosis (Elsheikh et al., 2010). Therefore, it is possible for the transport of intermediates (i.e. the hydroxylamine) out of the liver, which is rich in detoxification, to remote areas (i.e. the skin) where it is converted to SMX-NO, which generates protein adducts and ultimately hypersensitivity. The high number of SMX hypersensitivity reactions in patients with HIV and cystic fibrosis might be related to the fact that the redox balance is tipped in favor of a pro-oxidative environment by the disease process (van der Ven et al., 1997, Walmsley et al., 1997).

SMX-NO activates dendritic cells (DCs), enhancing expression of the co-stimulatory molecule CD40 (Sanderson et al., 2007) and generates potential antigens by binding to cysteine residues in

proteins. In rodent models, SMX-NO primed naïve CD4 and CD8 T-cells and the T-cell activation was antigen processing dependent (Farrell et al., 2003, Naisbitt et al., 2001, Naisbitt et al., 2002, Castrejon et al., 2010). On the contrary, administration of SMX does not activate immune cells. In vitro studies using PBMCs from drug naïve volunteers showed that SMX-NO generates T-cell responses in almost 100% of the volunteers (Engler et al., 2004). Application of a DC T-cell priming assay (Faulkner et al., 2012), using naïve T-cells from healthy volunteers who have never exposed to SMX, demonstrated that SMX-NO readily activates naïve T-cells. The newly generated memory T-cells were drug antigen specific. SMX-NO treatment resulted in proliferative responses and cytokine release. Several studies have shown that T-cells from blood and skin of all SMX hypersensitive patients are activated by SMX-NO, which suggests that SMX metabolites and the SMX-NO modified proteins are involved in the development of clinical symptoms in hypersensitive patients (Elsheikh et al., 2010, Schnyder et al., 2000, Burkhart et al., 2001, Nassif et al., 2004). Moreover, it has been shown that SMX-NO stimulates the majority of the drug responsive T-cell clones generated from patients with SMX hypersensitivity (Castrejon et al., 2010).

Skin cells are known to express different CYP enzymes from the CYPs in the liver. To clarify the possibility that SMX (or SMX NHOH) can travel to the skin and generate SMX-NO and in turn induce skin inflammation, several studies (Reilly et al., 2000; Vyas et al., 2006) have shown that sulfonamides are metabolized by flavin-containing monooxygenase 3 and peroxidases expressed in human epidermal keratinocytes/cutaneous dendritic cells into SMX-NO that binds covalently to cellular protein. Exposure of keratinocytes to SMX promoted the secretion of pro-inflammatory cytokines and increased expression of heat shock protein 70 (Khan et al., 2007). SMX-NO covalently binds to cellular proteins, which therefore may act as a source of the

antigen (Naisbitt et al., 2001). A study of SMX hypersensitive patients shows that SMX-NO selectively binds to a single amino acid residue of human albumin (HSA), cysteine 34. It has also been shown that (1) SMX-NO and SMX-NO modification of protein can induce cell death when the levels of binding exceeds a threshold; and 2) SMX-NO metabolite modified necrotic cells provide a strong maturation signal to DCs (Naisbitt et al., 2002, El-Ghaiesh et al., 2012). Based on this discussion, it is clear that, SMX metabolites are generated in the skin and may provide activation signals to DCs.

1.6.5 SMX and SMX-NO specific T-cell clones

In addition to the above discussion, SMX specific T-cell clones have been isolated from blood of patients with different types of skin eruptions (Schnyder et al., 1998, Brander et al., 1995). T-cells express CD4 or CD8 or both and upon stimulation by drugs, they secrete high levels of IL-5 and perforin which induces the killing of keratinocytes (Schnyder et al., 1998). Furthermore, drug-specific CD8 T-cells have been isolated from patients with TEN, the most severe form of cutaneous hypersensitivity, and fully characterized (Yawalkar et al., 2000, Nassif et al., 2004). Stimulation of T-cells with SMX follows the p.i. concept. T-cells from hypersensitive patients stimulated by SMX bound directly to MHC and T-cell receptor in a non-covalent fashion (Schnyder et al., 1998, Schnyder et al., 2000, Burkhart et al., 2001, Nassif et al., 2004). The threshold period of drug incubation for a T-cell clone response varies from 0.1 to 4 h (Zanni et al., 1998) which is incompatible with the time that is required for antigen presentation. The response is MHC restricted, although not all of the response is restricted to a specific HLA allele (Sanderson et al., 2007).

1.7 Drugs and drug antigens

In this thesis I have investigated drug hypersensitivity reactions to the pro-hapten to sulfamethoxazole (SMX) and its metabolites nitroso-sulfamethoxazole (SMX-NO) and the β -lactam antibiotics: piperacillin and flucloxacillin.

1.7.1 Sulfonamides

In 1940, Woods showed that sulfonamides prevented bacteria from growing. Sulfonamides prevent bacteria using para-aminobenzoic acid for folate biosynthesis, which is crucial for the synthesis of thymidine, purines and bacterial DNA (Woods, 1940). In 1960, the combination of sulfonamides and trimethoprim was developed based on the recognition that they both targeted the same pathway and double inhibition of this pathway is more effective than using a single drug (Masters, 2003).

Trimethoprim-SMX is available in oral and intravenous preparations. Clinically, it is used as one part trimethoprim to five parts SMX. SMX is primarily metabolized in the liver, with approximately 30% being excreted unchanged in urine. SMX is metabolized by CYP2C9 in human liver to a pro-reactive hydroxylamine metabolite SMX hydroxylamine then automatically oxidizes into nitroso sulfamethoxazole which may either bind covalently to cellular proteins, forming drug antigen, or may be reduced by non-protein thiols (i.e., glutathione) back to the hydroxylamine form (Cribb et al., 1995, Gill et al., 1999, Naisbitt et al., 2002) (See Figure 1.3).

Skin reactions occur in 3-4% of the general population treated with SMX. Numerous skin reactions have been described, including maculopapular rash, urticaria, diffuse erythema, morbilliform lesions, erythema multiform and purpura, and photosensitivity. Severe skin reactions related to SMX include SJS and TEN (Kocak et al., 2006). Disorders of the blood and internal organs (e.g., liver) are occasionally reported.

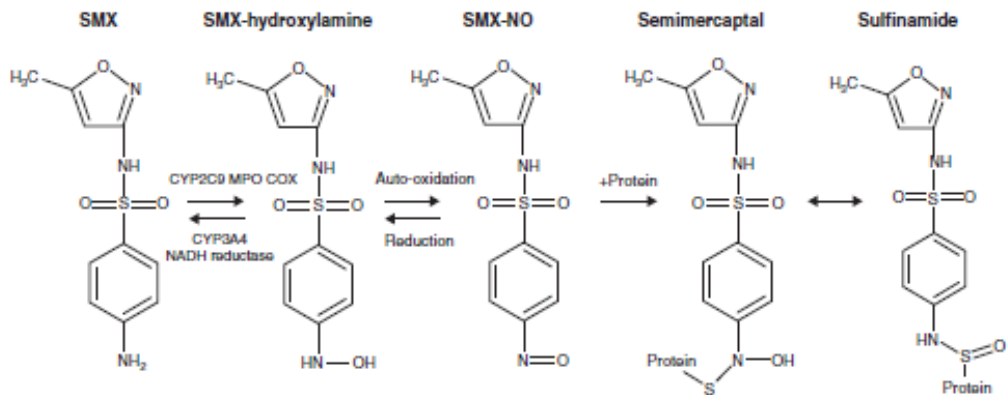


Figure 1.5 Metabolism of SMX and protein conjugate formation (Sullivan et al., 2015)

1.7.2 β -lactam antibiotics

Piperacillin was developed at the end of the 1970s. It is a wide-spectrum antibacterial agent developed for microbes resistant to other β -lactams, such as *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Jones et al., 1977). Combined with tazobactam, a B-lactamase, the anti-bacterial effect of piperacillin has been improved especially against B-lactamase producing bacteria (e.g. staphylococci, Escherichia coli, Haemophilus influenza) (Speich et al., 1998). Piperacillin is administered via intramuscular or intravenous injection as a sodium salt because it is poorly absorbed by the intestine.

Piperacillin is commonly prescribed for patients with cystic fibrosis (CF). Cystic fibrosis is the most common lethal autosomal recessive condition in Caucasians. Recurrent infections lead to airway destruction, bronchiectasis, and respiratory failure. Therefore, antibiotics, including piperacillin are required to maintain patients' health. Although antibiotic therapy is necessary to reduce the deterioration of lung function, it results in high incidence of adverse drug reactions. The incidence of ADRs in patients with cystic fibrosis is 26%-50% compared with 1-10% in the

general population (Brock & Roach, 1984; Moss et al., 1984).

For the formation of protein conjugates, the β -lactam ring is broken by nucleophilic lysine residues, leading to binding of the penicilloyl group (Batchelor et al., 1965). The penicilloyl antigen can also be formed by binding of the reactive degradation product penicillenic acid (Levine et al., 1960). Recent advances in mass spectrometry has allowed researchers to use piperacillin as a model to precisely characterize the nature of the piperacillin binding interaction with protein (Figure 1.4.1). As for all β -lactam antibiotics, piperacillin selectively interacts with specific lysine residues on serum proteins such as human serum albumin (Figure 1.4.2). The binding interactions are dependent on the dose and incubation time (Meng et al., 2011, Whitaker et al., 2011). Moreover, as T-cells from hypersensitive patients are activated with piperacillin HSA adducts, it was possible to investigate the minimum level of modification of the drug that can stimulate drug-specific T-cells. At low drug concentration, only Lys541 modification was observed, whereas at higher concentration, up to 13 lysine modifications were detected, four of which (Lys 190, 195, 432, and 541) were detected in patients' plasma (El-Ghaiesh et al., 2012, Whitaker et al., 2011). A synthetic β -lactam-protein conjugate mimicking the drug antigen found in the patients was found to stimulate PBMCs and 100% β -lactam specific T-cell clones. Whereas T-cell response to drug conjugates is dampened when antigen processing is inhibited, which suggests that the antigenic peptides are derived from drug-protein conjugates.

Flucloxacillin is a narrow-spectrum β -lactam antibiotic. It is used to treat infections caused by susceptible Gram-positive bacteria. Unlike other penicillins, flucloxacillin is effective against beta-lactamase-producing organisms such as *Staphylococcus aureus* as it is β -lactamase stable.

Flucloxacillin is a common cause of drug-induced liver injury in Europe, affecting about 8.5 in

every 100,000 first time users of the drug. (Andrews et al., 2008). The liver injury is predominantly cholestatic in nature. It has now been discovered that flucloxacillin-induced liver injury is associated with the expression of HLA-B*57:01 (Daly AK., et al 2009) and CD8 T-cells play a role in liver injury (Monshi M et al., 2013). The risk of flucloxacillin induced liver injury is increased in females, with a high daily dosage and with increasing age (Elise Andrews et al., 2008).

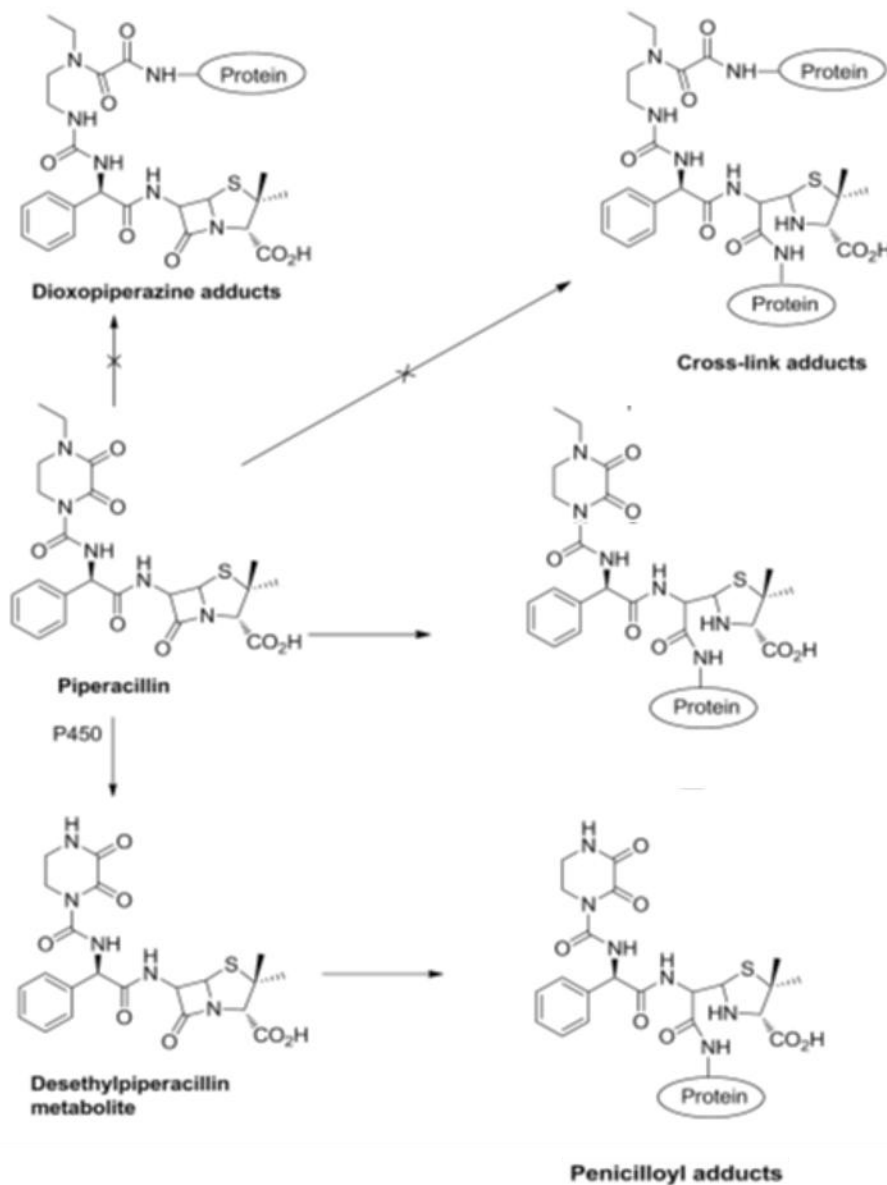


Figure 1.6.1 Formation of piperacillin-protein conjugated antigen (adapt from Whitaker, Meng et al., 2011). Piperacillin can be metabolized into desethylpiperacillin by P450 or be reduced into dioxopiperazine. Piperacillin conjugated antigen can be formed either with piperacillin, via its β -lactam ring opening and binding covalently to albumin protein forming an intact antigen; or with its desethyl metabolite also via β -lactam ring opening process; but not with dioxopiperacillin via 2,3-dioxopiperazine ring opening; nor cross-linking adduct.

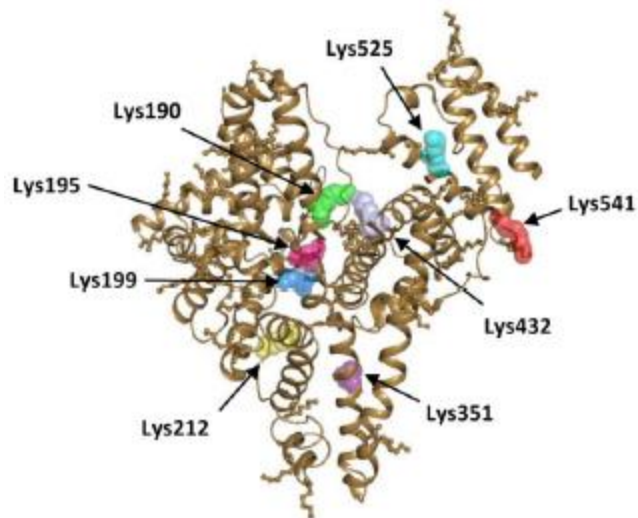


Figure 1.6.2 Binding site of piperacillin to HSA protein, which are the modifications on lysine residues identified by mass spectrometry. Adapted from Whitaker, Meng et al., (2011)
 Model of albumin showing piperacillin binding sites at positions Lys190, Lys195, Lys199, Lys212, Lys351, Lys432, Lys525 and Lys541.

1.8 Aim of the thesis

The primary aim of this study is to characterize the phenotype and function of piperacillin specific T-cells isolated from hypersensitive patients' PBMC (chapter 3) and skin (chapter 4). The involvement of Th17 and Th22 cells in piperacillin hypersensitivity was also studied using a recently established in vitro DC T-cell priming assay (chapter 5). Finally, the priming assay was applied to study the mechanism(s) of drug antigen presentation and T-cell cross reactivity using three haptenic drugs, SMX-NO, piperacillin and flucloxacillin.

The characterization of drug specific T-cells isolated from hypersensitive patient PBMC has been conducted previously; however, the involvement of new T-cell subsets, Th17 and Th22 in drug hypersensitivity reactions has not been investigated. Furthermore, a comparison of drug-specific T-cells isolated from PBMC and inflamed tissue has not been performed. These studies are

important because Th17 and Th22-secreting T-cells are known to play a critical role in several resilient and chronic immune-mediated diseases, such as psoriasis, Crohn's disease, asthma, multiple sclerosis, and inflammatory bowel disease.

The data presented in this thesis aims to answer the following questions:

1. Are Th17 and Th22 involved in the drug hypersensitivity? And if so, which drug-specific cytokines do they secrete?
2. Are there phenotypic and/or functional differences between drug-specific T-cells isolated from skin and PBMC?
3. Is it possible to generate drug specific Th17 cells or Th22 cells under Th17 and Th22 under polarization conditions in vitro?
4. Do APCs activate drug primed naïve T-cells via a hapten/pro-hapten pathway or a p.i. mechanism?

Chapter 2

Materials and Methods

- 2.1 Medium for cell culture.
 - 2.1.1 Preparation of the medium for T-cell cloning and culturing peripheral blood mononuclear cells (PBMCs)
 - 2.1.2 Preparation of Epstein-Bar virus (EBV)-transformed B-cells culturing medium
- 2.2 Preparation and the use of drugs.
- 2.3 Isolation of PBMCs
- 2.4 Lymphocyte transformation test (LTT)
- 2.5 Generation of T-cell lines and T-cell clones
- 2.6 Generation of EBV transformed B-cell lines
- 2.7 Analysis the phenotype of T-cell clones by flow cytometry
- 2.8 Testing drug specific T-cell proliferation
- 2.9 Testing T-cell clones for HLA restriction
- 2.10 Testing T-cell clones for antigen processing
- 2.11 EBV transformed B-cells cell lysate preparation.
- 2.12 Bradford assay.
- 2.13 Western blotting
- 2.14 ELISpot assay
- 2.15 Priming Assay
 - 2.15.1 CD14 selection.
 - 2.15.2 T-cell selections.
 - 2.15.3 DC culture from CD14 cells.
 - 2.15.4 Co-culture of DCs and naïve T-cells.
 - 2.15.5 Testing the drug antigen specificity of the primed cells.
- 2.16 Human memory T-cell Th subsets differentiation, including Th1, Th2, Th17 and Th22.
- 2.17 T-cell isolation from skin biopsies.
- 2.18 Glutaraldehyde APC fixation.
- 2.19 Glutathione and NAL blocking assay
- 2.20 T-cell receptor V β expression
- 2.21 Statistics analysis

Chapter 2:

Materials and Methods

2.1 Medium for cell culture.

2.1.1 Preparation of the medium for T-cell cloning and culturing peripheral blood mononuclear cells (PBMCs) (R9 medium)

R9 was used as the basic cell culture medium for all cell culture assays and for the culture of PBMCs and T-cells. Medium consisted of RPMI 1640 supplemented with 10% pooled heat-inactivated human AB serum (v/v), HEPES buffer (25 mM), L-glutamine (2 mM), transferrin (25 µg/ml), streptomycin (100 µg/ml), and penicillin (100 U/ml).

2.1.2 Preparation of Epstein-Bar virus (EBV)-transformed B-lymphoblastoid cell lines (B-LCL) culturing medium (F1 medium)

F1 medium was used to culture B-LCL cell lines, but was not used in cell culture assays. consists of RPMI 1640 supplemented with 10% pooled heat-inactivated foetal bovine serum (FBS), HEPES buffer (25 mM), L-glutamine (2 mM), streptomycin (100 µg/ml), and penicillin (100 U/ml).

Human AB serum was purchased from Innovative Research (Michigan, USA). FBS was bought from Invitrogen, Paisley, UK. HEPES buffer, L-glutamine, transferrin, streptomycin, and penicillin were purchased from Sigma-Aldrich (Dorset, UK).

Freeze medium: 20% DMSO (v/v) with 80% of FBS (v/v).

2.2 Preparation and the use of drugs

The drug metabolite nitroso-sulfamethoxazole (SMX-NO) was freshly prepared at a stock concentration of 50mM in dimethyl sulfoxide (DMSO), and then diluted into antibiotic-free R9

medium at a final concentration of 50 μ M. Piperacillin and flucloxacillin were diluted directly into antibiotic-free R9 medium at a range of concentrations between 0.5-2 mM.

SMX-NO was purchased from Dalton chemical laboratories Inc. (Toronto, Canada).

Both piperacillin and flucloxacillin were purchased from Sigma Chemical Co. (Poole, Dorset, UK). DMSO was purchased from Sigma-Aldrich (Dorset, UK). Drug chemical structures are shown below:

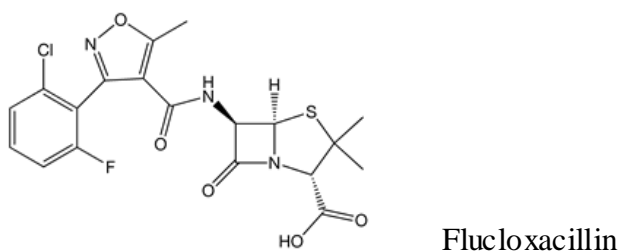
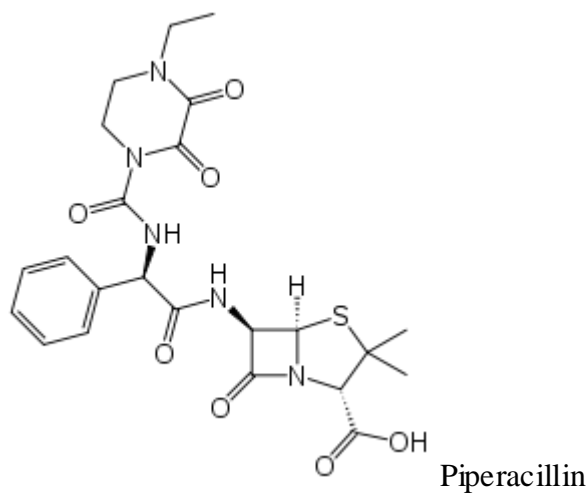
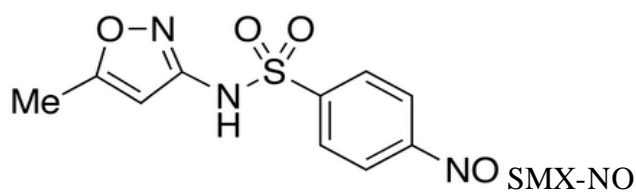


Figure 2.1 Chemical structures of the focus drugs in the thesis. Although these three drugs are thought to activate T-

cells via covalent protein binding, variation of their chemical structures seems to determine variations in drug hypersensitivity (Figures adapted from Wikipedia).

2.3 Isolation of PBMCs

Blood was taken from patients and healthy volunteers under informed consent. All protocols were approved by the Local Research Ethics Committee. PBMCs were isolated from fresh blood collected in 9 ml heparinised Vacuette tubes (Greiner Bio-One Ltd, Stonehouse, UK) using Lymphoprep density gradient separation media (Axis Shield, Dundee, UK). Blood was layered onto an equal volume of Lymphoprep and tubes were spun at 2000 rpm for 25 mins with low acceleration and no brake. The cloudy layer containing PBMCs was carefully aspirated using a sterile Pasteur pipette. The PBMCs were washed in HBSS buffer at 1800 rpm for 15 min at room temperature to remove the excess Lymphoprep. After discarding the supernatant, cells were re-suspended in 50 mL Hanks buffer and centrifuged at 1500 rpm for 10 minutes. The cells were then re-suspended in culture medium and counted using a Neubauer haemo-cytometer (Sigma) under a Wilovert Microscope (Will Wertzlar, Germany). Cells were diluted with 10 μ l 2% trypan blue stain and 10 μ l of the cell solution was placed on the haemo-cytometer and counted. The cell viability was calculated using the following equation: % viability = viable cells/total number of cells*100%. Normally the viability was found to be higher than 95%.

2.4 Lymphocyte transformation test (LTT)

100 μ l/well of isolated PBMCs at 1.5×10^6 cells/ml was aliquoted in 96-well U-bottomed tissue culture plates. 100 μ l/well of medium, 10 μ g/ml of Tetanus Toxoid or drug (piperacillin 0.5 mM, 1 mM, and 2 mM) were added and incubated for 6 days at atmosphere of 37 $^{\circ}$ C 5% CO₂. Proliferation was measured by adding 0.5 μ Ci/well [³H]-thymidine (5 Ci/mmol, Morovek Biochemicals Ltd, Brea, CA, USA) for 16 hours. Plates were harvested onto glass-fibre filter

mats using a TomTec Harvester 96 (Receptor Technologies, Leamington Spa, UK). After drying, the filter mats were fused with wax scintillant MeltiLex A and counted in a MicroBeta TriLux 1450 LSC β -counter (Perkin Elmer, Cambridge, UK). T-cell proliferation was given as cpm (counting per minute). The results were analyzed using Stimulation Index (SI, mean counting per minute (cpm) with drug/mean cpm without drug). An SI>2 was considered positive.

2.5 Generation of T-cell lines and T-cell clones

10^6 PBMCs from the hypersensitive patients were incubated in 48-well tissue culture plates with culprit drug (37 °C, 5% CO₂). 330 μ l of fresh medium supplemented with 500U/ml IL-2 was added on Day 6 and Day 9 to maintain T-cell proliferation. On Day 14, the cultured cells were washed with medium and counted by trypan blue dye exclusion and thereafter were seeded in triplicate in 96-well U-bottomed plates at different cell concentrations (0.3, 1 and 3 cells/well; in a total volume 100 μ l). Irradiated PBMCs (45 Gy) were used as feeder cells (provide stimulatory molecules, mainly CD28 molecules, which are required signal for T-cell activation) for 10^4 cells/well and added to each well in culture medium supplemented with 200 U/ml IL-2 (maintaining T-cell survival) and 5 μ g/ml PHA (stimulate TCR via CD3 molecule, thereby promoting T-cell activation). On Day 5, T-cells were fed with medium supplemented with 60 U/ml IL-2 and afterwards the IL-2 supplemented culture medium was fed every 48h.

T-cell cloning cultures were re-stimulated using 45 Gy irradiated allogenic lymphocytes as 5×10^4 cells/well as feeder cells. 500 U/ml IL-2 and 10 μ g/ml PHA were added to maintain the T-cell expansion. Wells which showed large pellets were expanded into 4 wells and fed every 2 days. The clones were then tested for drug specific T-cell proliferation. About 5×10^4 T-cell clones were cultured in duplicate, in the presence or absence of drug, in the addition of 60 Gy irradiated

autologous B-LCLs (10^4 /well, 0.2ml) as antigen presenting cells (APCs). The cultures were incubated for 3 days at a temperature of 37 °C and an atmosphere of 5% CO₂. [³H] thymidine was added at 0.5 µCi/well for the final 16h of the incubation. T-cell proliferation was measured using a β-counter and SI>2 were considered positive. Drug specific clones were further re-stimulated using the medium containing 45Gy irradiated allogenic 10⁶ PBMCs, 500U/ml IL-2 and 10 µg/ml PHA in the total volume of 0.66 ml. Cultures were maintained at 37 °C, 5% CO₂ and were fed with culture medium containing 60 U/ml IL-2 every two days after the second day.

2.6 Generation of EBV transformed B-cell lines

EBV-transformed B-cell lines (B-LCL) are an immortalized B-cell line used as a source of autologous antigen presenting cells. B-LCLs were generated by incubating isolated PBMCs from the donors with supernatant from the Epstein-Barr virus producing cell line B9-58 (Beatty et al., 1998). Donor PBMCs were isolated and co-cultured with 0.2 µm filtered B9-58 supernatant supplemented with 1 µg/ml cyclosporine A (CSA) and incubated overnight at 37 °C in an atmosphere of 5% CO₂. The next day, the supernatant was discarded and PBMCs were cultured in F1 medium supplemented with 1 µg/ml CSA every 3 days in 24 well plates for 3 weeks. Cells were then grown in F1 medium alone.

2.7 Analysis the phenotype of T-cell clones by flow cytometry

5×10^5 T-cell clones were incubated with anti-human CD4-FITC, CD8-PE, CXCR3-APC, CCR1-FITC, CCR6-APC, CCR4-PE, CLA-FITC, CCR10-PE, CCR5-FITC, CCR2-PE, CCR3-FITC, CCR8-PE, CCR9-APC, CXCR1-APC, CXCR6-PE, and E-cad-PE antibodies (purchased from BD Biosciences, Oxford, UK) for 20 min on ice in the dark. Afterwards, the cells were washed

with 1 ml of FACS buffer (phosphate buffered saline [PBS] + 10% (v/v) FCS + 0.02% (v/v) [sodium azide]) by centrifuging at 1500 rpm for 5 min. The cells were re-suspended in 150 μ l FACS buffer and analyzed by flow cytometry on a FACS Canto II (BD Biosciences).

2.8 Testing drug specific T-cell proliferation

5×10^4 T-cell clones were plated in duplicate, 10^4 autologous B-LCLs per well were added as well as medium in the presence or absence of the drug to a final volume of 200 μ l/well. The drug was added at the following concentrations (piperacillin 0.5 mM, 1 mM, 2 mM, flucloxacillin 0.5 mM, 1 mM and 2 mM, SMX-NO 12.5 μ g/ml, 25 μ g/ml, 50 μ g/ml and PHA 5 μ g/ml). The proliferation was measured by [3 H]-thymidine incorporation as described in 2.5.

2.9 Testing T-cell clones for HLA restriction

5 μ g/ml MHC class I or 5 μ g/ml MHC class II blocking antibodies (from BD Bioscience Oxford, UK) were incubated with 10^4 cells/well of autologous B-LCLs for 30 min at 37 $^{\circ}$ C and atmosphere of 5% CO₂. 5×10^4 T-cell clones/well and medium or drug solution (piperacillin 2 mM, flucloxacillin 2 mM, SMX-NO 50 μ M) was then placed in each well, making up to 200 μ l/well. Proliferation was measured by [3 H]-thymidine incorporation as described in 2.5.

2.10 Testing T-cell-clones for antigen processing

APCs were incubated with piperacillin (2mM), flucloxacillin (2 mM) and SMX-NO (50 μ M) for 1h, 4h, 16h and then extensively washed (3 times) to discard the free drug, also known as pulsing the cells. 5×10^4 T-cell clones/well were cultured with drug pulsed B-LCLs (1×10^4 cells/well) and drugs in duplicate in 96 well plates for 3 days at 37 $^{\circ}$ C in an atmosphere of 5% CO₂. Proliferation

was measured by [³H]-thymidine incorporation as described in 2.5.

2.11 EBV transformed B-cells cell lysate preparation.

To test the formation of drug antigen in the medium, EBV transformed B-cell lines were incubated with either SMX-NO or piperacillin or flucloxacillin in a 12 well culture plate at 37 °C in an atmosphere of 5% CO₂ for 16 hours. The cells were washed 3 times with Hanks buffer (in the centrifugation of 1500rpm for 5 minutes) to remove free drugs. Cell pellets were suspended in 200 µl RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 10% (w/v) Glycerol, 1% (w/v) Triton X-100, 1 mM Na₃VO₄, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1mM PMSF, 0.1% (w/v) SDS, and 0.5% (w/v) Na deoxycholate) and placed on ice for 30 minutes to lyse. Cells were given three bursts of sonication for 20 seconds each whilst on ice. The cell suspensions were then centrifuged at 14000 g for 10 minutes at 4 °C. Supernatants were then collected and protein concentration was determined by Bradford assay (Bradford 1976).

2.12 Bradford assay.

A standard calibration curve was prepared using BSA (0-2000 µg/ml). Briefly, 10 µl of BSA standard solution/ sample solution was plated into a 96-well flat-bottom microplate. 200 µl Bradford reagent (from BIO-RAD Hempstead, UK) was added to each well prior to being read at 570 nm using a microplate reader (Dynex, Technologies, Billingham, West Sussex). The protein concentration of each sample was obtained using the standard curve generated from BSA. Protein lysates were then standardized to 250 µg/ml.

2.13 Western blotting

The following buffers were used - TST buffer: 150 mM NaCl, 50 mM Tris-HCL, 0.05% Tween-

20, pH 7.6 and Laemmli buffer: 63 mM Tris HCl, 10% Glycerol, 2% SDS, 0.0025% bromophenol blue pH 6.8. Protein lysates were added at 10 µl/lane on a 10% SDS-polyacrylamide gel and were ran at 300 V, 60 mA for 1 hour. Separated proteins were then transferred from the gel onto a nitrocellulose membrane which was pre-wetted by 20% methanol, at 300 V, 250 mA for 1 hour. The membrane was then blocked by using TST containing 2.5% (w/v) non-fat dry milk and subsequently incubated with blocking buffer containing 1:1000 (v/v) dilution of rabbit anti-SMX-NO (Panigen, Blanchard Ville, USA) overnight at 4 °C. (For anti-piperacillin western blotting, mouse primary anti-penicillin antibody [ABCAM, UK] was diluted in blocking buffer in 1: 20,000 [v/v]; and for anti-flucloxacillin western-blotting, rabbit primary antibody, [personal gift from Frank Von Pelt, National University of Ireland] was diluted in blocking buffer in 1:5000 [v/v]).

The next day, the membrane was washed five times for 5 min with TST to remove any free antibody. The membrane was then incubated with a 1:10,000 (v/v) dilution of secondary antibody: mouse anti-rabbit alkaline phosphatase-conjugated antibody (Sigma-Aldrich, Gillingham, UK) at room temperature for 2 hours. [For piperacillin, goat anti-mouse HRP [P0447, DAKO, Ontario, Canada] was diluted in blocking buffer in 1:10,000 (v/v). For flucloxacillin, goat anti-rabbit IgG [P0448, DAKO, Ontario, Canada] were used as the secondary antibody, diluted in blocking buffer in 1: 2,000 [v/v]).

Afterwards, the membrane was washed five times with TST. The signal was then developed using enhanced chemical luminescence (Western Lightning; PerkinElmer Life and Analytical Sciences, Waltham, MA) by developing autoradiography film in a dark room and GS800 calibrated scanning densitometer (Bio-Rad Laboratories, Hemel Hempstead, UK).

2.14 ELISpot assay

Polyvinylidene fluoride (PVDF) membrane ELISpot plates were pre-wetted with 35% ethanol for 30 seconds prior to washing with 250 μ l sterile ELGA distilled water 5 times. ELISpot plates were then coated with 100 μ l/well of IFN- γ capture antibody (15 μ g/ml) and incubated overnight at 4 $^{\circ}$ C. The following day, wells were washed 5 times with sterile phosphate-buffered saline (PBS) to remove the free antibody and were then blocked with 200 μ l/well of R9 medium for 30 minutes at room temperature. Drug specific T-cell clones (5×10^4 cells, 50 μ l) were put into the wells alongside with autologous 60 Gy irradiated EBV-transformed B cells (1×10^4 cells, 50 μ l), with the addition of 100 μ l of R9 medium, SMX-NO (50 μ M), piperacillin (2 mM) and flucloxacillin (2 mM). The cells were cultured at 37 $^{\circ}$ C for 48 hours.

After 48 hours, the cells were discarded and then the wells were washed for 5 times with 250 μ l/well PBS. Biotin conjugated detection antibody (1 μ g/ml) was diluted in 0.5% FBS/ PBS and 100 μ l was added to each well. Plates were incubated at room temperature for 2 hours, then wells were then washed 5 times with PBS. 100 μ l/ well streptavidin-ALP (1:1000) in 0.5% FBS/PBS was added to the wells and incubated at room temperature for 1 hour. Plates were then washed with PBS for 5 times and 100 μ l 0.45 μ m filtered BCIP/NBT substrate was added to each well. Plates were incubated for 10-15 minutes in the dark at room temperature before the substrate was washed off with tap water. Plates were left to dry overnight prior to counting by an AID ELISpot reader (Cadama Medical, Stourbridge, UK).

2.15 T-cell priming assay

PBMCs were isolated from 100 ml of blood as described in 2.3. Cell populations required for the dendritic cell priming of naïve t-cells were isolated from PBMCs using magnetic beads,

according to the manufacturers' instructions (Miltenyi Biotec Ltd, Bisley, UK). Firstly, CD14 cells were isolated by positive selection. Secondly, T-cells were isolated from the CD14 negative population by negative selection and finally Tregs and memory T-cells were removed by positive selection leaving the naïve T-cell population.

2.15.1 Isolation of CD14+ cells by positive selection.

PBMCs were counted and 800 µl Macs buffer and 200 µl CD14 microbeads were added per 10^8 cells. The beads were vortexed before use. The cells were incubated in the fridge for 15 minutes at 4 °C. 15 ml of Macs buffer was added and the cells were spun at 1500 rpm for 10 minutes at 4 °C. After pipetting off the supernatant completely, cells were resuspended in 500 µl Macs buffer per 10^8 cells. An LS column was put on magnet and the column was washed with 3 ml of MACs buffer. The cells were then added to the column and washed 3 times with 3 ml Macs buffer. Non-CD14 cells were collected as the flow through and positively collected cells were removed from the column by adding 5ml Macs buffer to the column and immediately applying the plunger firmly to the column washing the cells off. The cells were counted. CD14 were used to culture dendritic cells or were frozen at $6-8 \times 10^6$ cells/vial in freezing medium. The non-CD14 cells were spun down and used for T-cell selection.

2.15.2 Naïve T-cell selections.

T-cells were selected using the Pan-T isolation kit as follows. The non-CD14 cells were resuspended in 400 µl Macs buffer and 100 µl biotin-antibody cocktail added per 10^8 cells and incubated for 10 minutes in the fridge at 4 °C. Then 300 µl Macs buffer and 200 µl anti-biotin microbeads was added per 10^7 cells and incubated for 15 minutes in the fridge. Then 15 ml Macs buffer was added and the cells spun at 1500 rpm for 10 minutes at 4 °C. The cells were resuspended in 500 µl Macs buffer and put through an LS column as previously described. The

flow through from the column contained the T-cells and non-T-cells were recovered from the column. Tregs and memory T-cells were then removed using CD25 and CD45RO microbeads as described for the CD14 cells, leaving the naïve T-cells as flow through from the column. CD14⁻CD3⁻ cells (for generation of B-LCL), CD14⁻CD3⁺CD45RO⁻CD25⁻ cells (naïve T-cells), and CD14⁻CD3⁺CD45RO⁺CD25⁻ (memory T-cells) were frozen down at 10^7 per vial in freezing medium.

2.15.3 DC culture from CD14 cells.

$6-8 \times 10^6$ CD14 cells were diluted in 6 ml of R9 medium supplemented with 800 U/ml GM-CSF and IL-4 and aliquoted 3 ml/well in a 6 well plate. The cells were fed every 2 days with 3 ml/well R9 medium containing 800 U/ml GM-CSF, IL4. On day 6, the cells were matured with 1 µg/ml LPS and 25 ng/ml TNF- α . On day 7, the mature DCs were scraped from the bottom of wells. After being spun at 1500 rpm, DCs were resuspended in 2 ml medium and counted.

2.15.4 Co-culture of DCs and naïve T-cells.

DCs were prepared at 1.6×10^5 cells/ml in R9 medium and 0.5 ml/well added to 24-well plates. Naïve T-cells were thawed and washed. The cells were counted and made up 2.5×10^6 cells/ml in R9 medium, and 1 ml/well added to 24 well plates. The drugs were added at 500 µl/well to a final concentration of 2 mM piperacillin, 2 mM flucloxacillin, and 50 µM SMX-NO. The co-culture cells were incubated in the incubator in 5%CO₂ at 37 °C for 7 days. During this time, new DCs were cultured from CD14 cells as described previously.

2.15.5 Testing the drug antigen specificity of the primed cells.

On Day 14, the co-cultured cells and DCs were harvested. The drug primed T-cells were tested by ELISpot and proliferation assay using primed T-cells at 10^5 cells/well and autologous DCs at 4×10^3 cells/well with various drug concentrations as described previously.

2.16 Human memory T-cell Th subsets differentiation, including Th1, Th2, Th17 and Th22.

10 µg/ml of CD3 antibody was diluted in HBSS solution and pre-coated 300 µl/well in 48 sterile well plates over night at 4 °C. On the next day, excess CD3 antibody was washed with HBSS for 3 times. 5×10^5 memory T-cells were then added onto the CD3 coated wells with 500 µl of R9 medium supplemented with 5 µg/ml of anti-CD28 antibody, with or without Th17 differentiation cytokines including TGF-β (1 ng/ml), IL-1β (10 ng/ml), IL-6 (10 ng/ml), IL-23 (10 ng/ml). Th22 differentiation cytokines were 50 ng/ml TNF-α, 20 ng/ml IL-6, 5 µg/ml anti IL-4 and 5 µg/ml anti IL-12. When differentiated into Th1 cells, the secreted cytokines were anti-IL-4 (5 µg/ml) and 25 ng/ml IL-12; when differentiated into Th2 cells, the secreted cytokines were 25 ng/ml IL-4, 5 µg/ml anti-IL-12, and 5 µg/ml anti-IFN-γ. The cells were incubated at atmosphere of 37 °C/5%CO₂ for 5 days. On Day 6, polarized memory T-cells cytokine secretion was tested by ELISpot in the stimulation of PHA (5 µg/ml).

2.17 T-cell isolation from skin biopsies.

Skin biopsies were transported to the laboratory in R9 medium + 100 U/ml IL2 and on arrival were then removed from the R9 medium using a Pasteur pipette and spun at 1500 rpm for 10 minutes to pellet any cells that may have migrated from the sample during transportation. A scalpel was used to mince the sample as finely as possible in a petri dish. The cell pellet was then re-suspended in 2 ml of R9-IL2 and plated in 7 ml of R9-IL2 for a combined volume of 9 ml. 3 ml of the solution was transferred into each of the three wells on a 12 well cell culture plate and placed into a 37°C, 5% CO₂ incubator. Skin biopsies were prone to the formation of a fat layer at the surface of the well. If necessary, a Pasteur pipette was used to remove the fat and the volume

removed was replaced with a similar amount of R9-IL2 before mixing. After 3-5 days the wells were aspirated and the contents passed through a 50 micron cell strainer into a 50 ml tube to remove any debris. The strainer was flushed with several washes of sterile PBS/Hanks and then spun at 1500 rpm to obtain a cell pellet. The pellet was re-suspended in 1 ml of R9 and 330 μ l was placed into each of 3 wells on a 48 well plate. 330 μ l of stimulation cocktail which consisted of 5U of IL2, 10 μ g of PHA and 0.5×10^6 irradiated PBMC was added to each of the wells. Cells were fed by removing 330 μ l of medium from each well on Days 5 and 9 and replaced with an equal volume of R9 + 100 μ g/ml IL2. On the day 14, the wells were mixed and the contents aspirated into a tube and spun at 1500 RPM for 10 minutes to obtain a pellet.

The cell pellet was re-suspended in 1 ml of freezing medium and immediately transferred to a cryovial, which was placed in a -80 °C freezer. After 24 hours the cryovial was removed and stored in either a -150 °C freezer.

2.18 Glutaraldehyde APC fixation.

Autologous B-LCLs were washed twice and re-suspended in 1 ml HBSS. Glutaraldehyde (25%, 1 ml) was purchased from Sigma. It was then added and the cells were gently mixed for 30 s. Cells were then washed 3 times to remove glutaraldehyde and were re-suspended in T-cell culture medium. 5×10^4 T-cell clones were cultured with 10^4 glutaraldehyde fixed B-LCLs in presence or absence of drugs (50 μ M SMX-NO, or piperacillin 2mM, or flucloxacillin 2 mM) in duplicate in 96 well plates for 3 days at 37 °C in an atmosphere of 5% CO₂. Proliferation was measured by [³H]-thymidine incorporation as described in 2.5.

2.19 Glutathione and NAL blocking assay

N α -Acetyl-lysine (Sigma) was diluted in final concentration 1 mM and cultured with 1×10^4 autologous B-LCLs and 5×10^4 T-cell clones with or without 50 μ l of drug solution (piperacillin or flucloxacillin or SMX-NO) in a total volume of 200 μ l in 96 well plates. Proliferation was measured by [3 H]-thymidine incorporation as described in 2.5.

Glutathione (Sigma) was prepared and tested in the same way as N α -Acetyl-lysine.

2.20 T-cell receptor V β expression

TCR V β expression of individual clones can be typed using the IOtest kit. At least 5×10^4 T-cell clones were added into 9 FACs tubes in suspensions of 50 μ l of T-cell culture medium. Tube 1 did not contain antibodies, and the tube was used to gate the T-lymphocyte population using flow cytometry. TCR V β antibodies (5 μ l) labelled A-H were then added into another 8 tubes (from 2 to 9). Each TCR V β antibody cocktail was used to investigate three TCRs, twenty-four in total. Tubes were incubated at room temperature for 20 minutes. Unbound antibodies were washed with FACS buffer (1 ml), 1500 rpm for 5 minutes at room temperature. Finally, T-cell clones were resuspended in FACS buffer (200 μ l) and samples were analyzed by flow cytometry.

2.21 Statistics analysis

Candidate screening for drug-specific clones was performed by expanding clones which had a stimulation index greater than 2. A number of statistical tests were used to further this analysis depending on the experiment. T-cell clones were tested in triplicate with multiple doses or treatments. For multiple comparisons between treatments within a single clone population the parametric, one-way ANOVA test was applied. This method was also used to analyze chemokine

receptor data. Even within the same donor, there was a great variability in stimulation between drug-specific T-cell clones and thus the data did not follow a normal distribution. Therefore, for comparisons between T-cell clones, the non-parametric, Mann-Whitney test was employed. $P < 0.05$ was considered statistically significant.

Chapter 3

Characterization of peripheral T-cells in piperacillin hypersensitivity.

3.1. Introduction

3.2. Methods

3.3. Results

3.3.1 Lymphocyte transformation test (LTT) of patients with piperacillin hypersensitivity

3.3.2 Generation of piperacillin specific T-cell clones from patients' PBMCs.

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3.3.4 The cytokines and cytolytic molecule profile of the piperacillin-specific T-cell clones.

3.3.5 The surface markers of the T-cell clones.

3.3.6 V β expression of piperacillin specific T-cell clones.

3.4. Discussion

3.1. Introduction

Piperacillin hypersensitivity usually involves skin inflammation such as maculopapular exanthema (MPE), or delayed onset urticaria. It has been established that T-cells play an important role in piperacillin-induced skin inflammation (Schnyder et al., 1998; Pichler, 2002; Naisbitt et al., 2007; Martin et al., 2010). Cystic fibrosis (CF) patients were chosen for this study because of the high frequency of hypersensitivity reactions. CF patients live with a life-long recurrent bacterial infection and piperacillin is commonly prescribed. Unfortunately, piperacillin induced 10 times higher rates of drug hypersensitivity in CF patients compared with normal population, 26%-50% compared with 1-10% (Moss et al., 1984). Thus the CF patients' treatment with antibiotics has been restricted by drug hypersensitivity.

Hypersensitivity reactions such as MPE involve drug-specific cytotoxic CD4⁺T cells. In contrast, the presence of drug-specific CD8⁺T cells, which mediate severe drug hypersensitivity reactions such as SJS-TEN, are much more restricted. However, the role of CD8⁺T cells in MPE cannot be ruled out. Drug-specific T-cell clones have been used routinely in mechanistic studies to define the cellular pathophysiology of drug hypersensitivity reactions. The characterization includes T-cell cytokine secretion, cytotoxicity, cell surface molecules such as CD4/CD8 and chemokine receptors, and drug specific TCR diversity.

It is known that, piperacillin specific T-cell clones can be isolated from hypersensitive patients' blood. Drug stimulation of the PBMC results in the secretion of IFN- γ , IL-5, IL-13 but not IL-10, which indicates a mixed panel of Th1 and Th2 cells, but not regulatory T-cells. However the involvement of the newly discovered CD4⁺ T-cell subsets Th17 and Th22 has not been investigated. Th17 and Th22 play important role in skin reactions and they are named after the

particular cytokines they secrete; IL-17 and IL-22, respectively (Infante-Duarte, et al 2000; Eyerich et al., 2009). Th17 cells are critical in autoimmune diseases, such as psoriasis (Kagami et al., 2010), Crohn's disease (Holta et al., 2008), and rheumatoid arthritis (Hirota et al. 2007). In psoriasis, IL-17 promotes chemokine secretion by keratinocytes and amplifies inflammation by recruiting more inflammatory cells (Harper et al., 2009). In an *in vitro* skin injury model, IL-22 promoted the proliferation of keratinocytes and enhanced wound healing (Eyerich et al., 2009), whereas in auto-immune disease such as the very late phase of psoriasis, Th22 promotes the inflammatory response (Kagami et al., 2010; Michalak-Stoma et al., 2013). Thus, it is important to analyze the cytokine profile that T-cells secrete in drug hypersensitivity.

Chemokine receptor expression directs T-cell migration and dictates the final destination of T-cells. Cutaneous lymphocyte antigen (CLA) expressed on the T-cell surface is a hallmark of skin migration. CLA is an integrin that slows down T-cells rolling along the vesicular endothelium near the inflamed site. Subsequently, chemokines released by inflamed skin bind to chemokine receptors on T-cell surfaces such as CCR4, CCR8 and CCR10. T-cells then migrate following a chemokine diffusion gradient and accumulate at the site of inflammation. To study tissue-homing of drug specific T-cells, the profile of chemokine receptors has been tested including skin- and gut-oriented CXCR3.

The aim of this chapter was to analyze the phenotypic and functional abilities of peripheral blood T-cells isolated from hypersensitive patients with cystic fibrosis. The data generated are used in chapter 4 to compare the drug-specific T-cell response induced in blood and inflamed skin of the same hypersensitive patients.

3.2 Methods

Three patients with CF and piperacillin hypersensitivity were recruited in 2011 for this study and the clinical details are shown in Table 3.1. Three normal volunteers were also recruited as controls. Although these numbers are low and not representative of the general hypersensitive population, cloning is very labor intensive procedure. Cloning T-cells from three patients and detailed characterization of the clones takes approximately 12 months. Thus, analysis of additional patients was not possible.

Patient	Age / Gender	Drug	Reaction	Reaction Time ⁺	Time*	Skin Prick Test	Intradermal skin test
P1	20/M	piperacillin	MPE/fever	2	0.5	-	-
P2	29/F	piperacillin	Fever/arthralgia	9	6	-	-
P3	29/M	piperacillin	MPE	5	2	-	+ at 48 hours

Table 3.1 The clinical history of hypersensitive patients with cystic fibrosis. (Age in years, Reaction Time+: time from treatment to reaction in days, Time*: time since reaction in years, M: male, F: female, MPE: maculopapular exanthema, P: patient)

PBMCs from patients and healthy volunteers were used to perform a lymphocyte transformation test (LTT) using piperacillin at 0.5 mM to 4 mM. PBMCs were then used to set up bulk cultures with piperacillin which were used for isolation of drug-specific T-cell clones. Irradiated, autologous EBV transformed B cells were used as antigen presenting cells (APCs) in assays with the T-cell clones. Clones displaying a stimulation index (cpm in drug-treated cultures/cpm in medium control cultures) of 2 or above were used in all experiments. When available multiple clones from different donors were used in all mechanistic studies. However, clones have a very short life-span. They very rapidly become anergic to drug stimulation. Thus, several experiments

were limited to 3 clones. Although this number was low, it allowed us to conduct statistical analysis of the data.

The phenotype and functional abilities of the T-cell clones was then assessed: proliferation was measured by thymidine incorporation, release of cytokines and cytolytic molecules was measured by ELISpot, and the surface phenotype was assessed by flow cytometry looking at CD4, CD8, TCR V β and chemokine receptor expression.

3.3 Results

Three piperacillin hypersensitive patients were recruited. They had a similar reactions to piperacillin, which is maculopapular exanthema (MPE). Patient 1 and 2 also showed fever and patient 2 showed arthralgia as side effects (Table 3.1). The time of onset of hypersensitivity reaction to the drug administration was a minimum 2 days. Skin prick tests were taken and all the patients were negative. Intradermal test result showed patient 1 and 2 were negative. Patient 3 had a reaction to the intradermal test in 2 hours. The tests above suggest piperacillin reaction is delayed-type hypersensitivity. Characterization of the function of piperacillin-reactive T-cells was conducted in subsequent sections using peripheral blood from the hypersensitive patients.

3.3.1 Lymphocyte transformation test (LTT) of patients with piperacillin hypersensitivity

LTT results presented in figure 3.2 are measured by SI. All 3 patients with CF showed a positive LTT when PBMCs were incubated *in vitro* with piperacillin. The response was dose dependent with a maximal response at 1-2mM. PBMCs from healthy volunteers (n=3) proliferated in response to tetanus toxoid (5 μ g/ml) but not to piperacillin.

	cpm in control	Piperacillin (mM)				TT (5µg/ml)
		0.5	1	2	4	
P1	905	1.8	2.6	4.8	2.2	16
P2	2333	25.4	32.8	30	19.4	3.6
P3	137	3.7	3.9	23.5	4.7	52.8
V1	1121	1	0.8	1.3	1.3	84.1
V2	3683	0.6	0.5	0.5	0.6	26.7
V3	1352	0.7	0.5	0.5	0.7	7.2

Table 3.2 Piperacillin-specific lymphocyte transformation test (LTT). LTT of the piperacillin hypersensitive patients and normal volunteers was performed using PBMCs at 1.5×10^5 cells/well. Cells were incubated with piperacillin or tetanus toxoid (TT) as a control for 6 days and proliferation was measured by ^3H -thymidine incorporation. Drug specificity of PBMCs were shown by SI (stimulation index).

3.3.3 Piperacillin specific T-cell responses of the patients.

After serial dilution and drug specificity test, 45 T-cell clones of patient 1, 18 clones of patient 2, and 15 clones of patient 3 were picked out for more detailed analysis (Table 3.3A, B and C). The proliferation of clones against piperacillin was dose-dependent between 0.5-2mM piperacillin.

Table 3.3A Piperacillin-specific proliferation (SI) of T-cell clones from patient 1 (45 clones)

Clones	cpm in control	Drug (mM)		
		0.5	1	2
12*	4088	3.0	3.0	2.8
15	3889	3.5	5.2	3.5
19	4338	11.7	6.5	5.3
21	4804	2.4	2.3	2.4
22	5323	4.0	4.9	4.9
24	2911	6.5	7.0	7.8
41	5101	8.4	8.0	8.1
56	3583	10.7	13.0	10.9
67	3714	3.3	5.9	4.7
76	3794	2.8	6.4	5.3
79	3836	7.8	10.3	10.5
82	4778	3.2	4.8	4.1
114	4270	6.9	6.5	8.9

120	5186	4.8	4.6	5.1
122	4379	5.1	3.9	10.0
125	9799	4.3	3.7	4.6
126	4526	10.0	8.1	9.9
128	5059	4.2	3.3	4.3
142	3645	4.1	5.6	5.3
143	4370	2.6	3.3	3.4
145	3302	4.7	8.8	7.8
148	8642	9.7	9.3	9.4
149	5869	6.1	6.7	6.4
150	4037	8.3	8.2	7.3
154	3838	2.9	4.9	5.8
157	4105	5.2	8.3	7.8
158	8442	3.4	3.9	4.3
165	6171	4.1	5.2	5.3
176	7293	23.3	27.8	31.2
180	8702	4.7	7.3	6.6
183	15548	5.4	4.8	5.2
187	4511	5.3	6.3	5.2
188	8247	5.0	5.0	6.9
190	9131	5.7	8.4	7.3
195	6405	16.7	27.4	27.4
196	11283	4.9	6.8	6.2
198	3596	2.4	4.9	3.8
199	2848	8.3	12.3	12.0
202	3455	23.7	28.4	25.8
211	3680	10.4	17.3	14.8
215	2887	67.1	71.2	81.0
219	3798	19.7	21.2	23.5
258	4594	2.5	2.5	2.4
259	5571	8.7	13.2	9.6
269	5160	8.2	6.8	6.3

*Number assigned to T-cell clones throughout chapter 1 (including ELISpot test, chemokine receptor, and V β receptor).

Table 3.3B Piperacillin-specific proliferation of T-cell clones from patient 2 (18 clones)

Clone number	cpm in control	Drug (mM)		
		0.5	1	2
9*	2467	29.0	44.6	45.7
23	2547	33.7	44.3	40.9
29	3256	4.6	5.0	5.2
39	4012	3.6	3.9	4.6
55	3290	8.1	13.9	11.1
73	3181	20.6	19.1	13.4
77	4908	11.3	11.7	11.3
88	2757	11.4	10.4	9.7
106	45242	2.0	2.3	2.2
109	6117	3.1	2.9	3.3
119	4503	2.9	5.4	4.3
150	3077	6.0	5.9	5.8
153	3958	10.2	10.5	10.6
154	9422	2.5	3.3	2.7
168	10211	2.5	2.1	2.3
235	3441	14.9	12.1	11.9
237	4256	10.1	8.6	10.4
266	3948	8.6	9.0	6.7

*Number assigned to T-cell clones throughout chapter 1 (including ELISpot test, chemokine receptor, and V β receptor).

Table 3.3C Piperacillin-specific proliferation of T-cell clones from patient 3 (15 clones)

Clone number	cpm in control	Drug (mM)		
		0.5	1	2
14*	4180	17.9	17.3	19.5
27	3764	7.4	8.9	9.4
146	3668	16.4	25.7	20.5
160	3271	6.4	8.9	9.5
171	6888	5.7	9.6	8.8
174	5541	6.7	9.0	4.5
182	6693	4.3	8.7	7.6
220	3655	2.9	2.7	0.8
222	3471	4.1	3.3	1.3
231	7991	3.3	3.3	2.0
239	2555	41.0	62.0	71.6
246	2169	10.8	17.5	20.1

247	2257	10.7	14.1	16.0
250	2417	25.8	32.4	36.3
251	2791	2.9	5.2	5.4

*Number assigned to T-cell clones throughout chapter 1 (including ELISpot test, chemokine receptor, and V β receptor).

Table 3.3 T-cell clones from 3 patients are reactive to piperacillin in a dose dependent manner. 5×10^4 T-cell clones were placed duplicate wells. 1×10^4 Epstein-Barr virus (EBV) transformed B-cell were added in the presence of either medium or piperacillin to a final volume of 200 μ l/well. The drug was added at the concentrations indicated. 78 clones from 3 patients were tested. Proliferation was measured by 3 H-thymidine incorporation for the final 16 hours. Data is presented as SI.

3.3.4 The cytokines and cytolytic molecules secreted by piperacillin-specific T-cell clones.

Twenty four well-growing piperacillin-specific T-cell clones were randomly selected with SI from 3 to 40 for the assessment of cytokine secretion. 3 million cells of a T-cell clone were required for a full scale experiment. Thus, it was not possible to conduct cytokine profiling experiments on all clones.

ELISpot image results presented in Table 3.4A and Figure 3.1A-G and the result summary (Table 3.4 B-D) show that of the 24 clones, 14 clones secreted IFN- γ , 20 clones secreted IL-5 and 18 clones secreted IL-13 (Figure 3.1 A, B, C). Furthermore, 21 clones secreted IL-22, but no IL-17A secretion observed with any of these clones (Figure 3.1 D, E). 20 clones secreted the cytolytic molecule, granzyme B and 14 clones secreted FasL (Figure 3.1 F, G). This data suggests IL-22 may be an important feature in hypersensitivity to piperacillin, whereas, IL-17 does not seem to be involved. Th1 secreting T-cells secreted mainly IFN- γ whereas Th2 T-cells secreted cytokines such as IL-5 and IL-13. The cytokine profiles showed that both Th1 and Th2 T-cell clones activated with piperacillin were generated from patients with CF. However, Th1 and Th2 cytokine secretion in T-cell clones is not always clearly defined in that some T-cells secreted a mixture of Th1 and Th2 cytokines. For example, clones 176, 215, 14, and 39 express IFN- γ which is a Th1 cytokine, as well as IL-5 and IL-13, which are Th2 cytokines. In addition T-cell clones also secreted a range of cytolytic molecules such as granzyme B and Fas-ligand.

To characterize whether drug-specific proliferation and cytokine release after drug stimulation were synergistic, an attempt has been made to find a correlation between the drug specific secretions of Th2 cytokines, IL-13 and IL-5, by 24 clones (Figure 3.1 H). However, no significant correlation ($r^2 = 0.056$, and $r^2 > 0.5$) was observed. Similarly, the correlation between IL-22 and IFN- γ , and the proliferative response of these clones was studied, but still, no correlation was observed (Appendix data).

	Piperacillin exposure					
	0	0.5mM	0	0.5mM	0	0.5mM
Layout of T-cell clones (Black = Patient 1) (Red = Patient 2) (Blue=Patient 3)	56	56	250	250	39	39
	126	126	266	266	55	55
	176	176	235	235	73	73
	199	199	77	77	119	119
	202	202	182	182	146	146
	211	211	231	231	160	160
	215	215	14	14	171	171
	239	239	27	27	174	174

Table 3.4 A. The layout of T-cell clones on ELISpot plates.

Figure 3.1 A

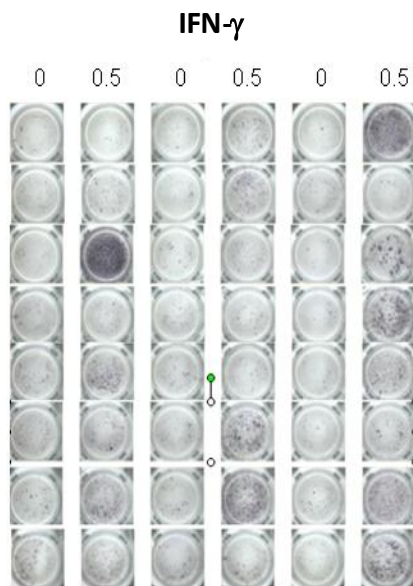


Figure 3.1 B

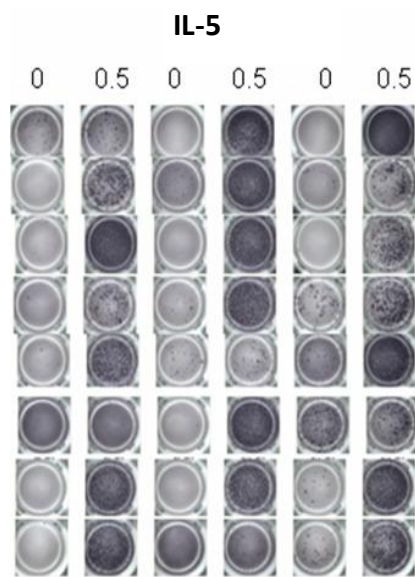


Figure 3.1 C

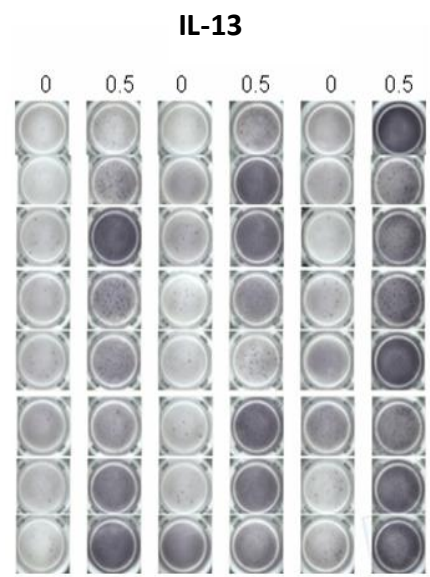


Figure 3.1 D

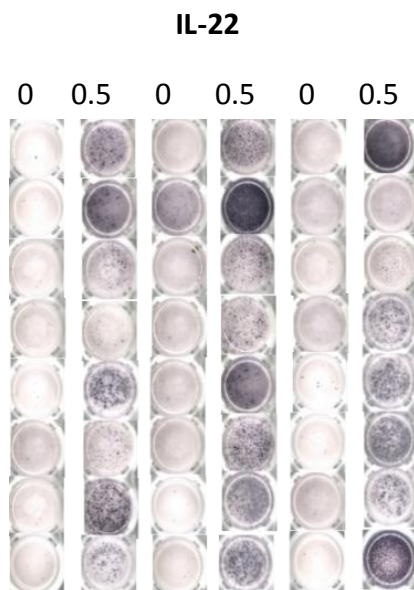


Figure 3.1 E

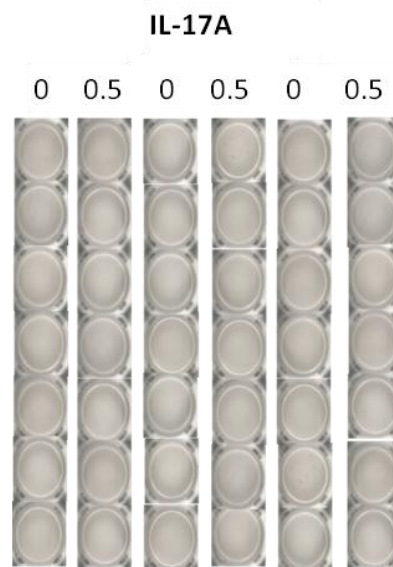


Figure 3.1 F

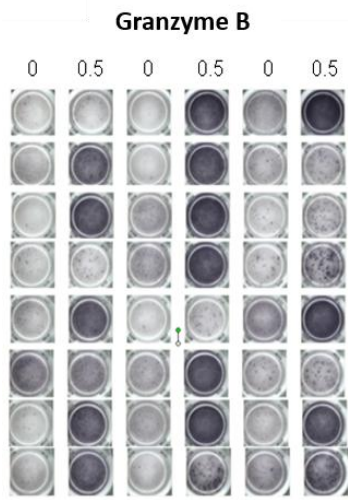


Figure 3.1 G

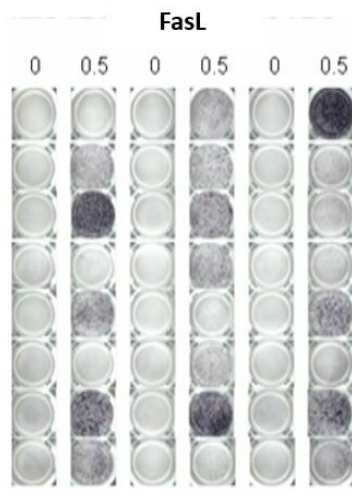


Figure 3.1 H

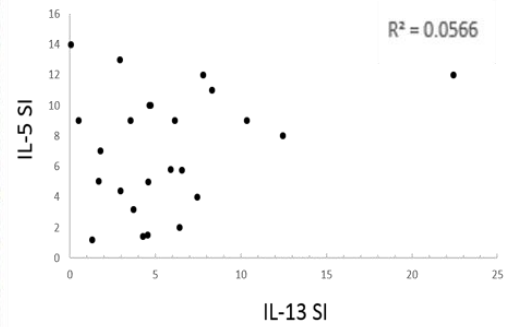


Figure 3.1 Cytokine and cytolytic molecule profile of the piperacillin-specific T-cell clones generated from hypersensitive patients. (A) IFN- γ ELISpot image. (B) IL-5 ELISpot image (C) IL-13 ELISpot image. (D) IL-22 ELISpot image and (E) IL-17A ELISpot image. (F) granzyme B ELISpot image. (G) FasL ELISpot image. T-cell clones from 3 patients were plated at 5×10^4 cells/well with 1×10^4 cells/well irradiated autologous B cell APCs in the presence of 0.5 mM of piperacillin for 2 days prior to the plate developing according to manufacturer's instruction. The layout of the T-cell clones in the ELISpot image is shown in Table 3.3A. (H) The correlation between piperacillin specific IL-5 and IL-13SI.

Table 3.3B

Patient 1							
Clone	IFN- γ	IL-5	IL-13	IL-17	IL-22	Granzyme-B	FasL
56	-	-	-	-	+++	-	-
126	-	++	+	-	+++	-	++
176	+++	+++	+++	-	++	+++	+++
199	-	++	+	-	-	-	-
202	++	+++	+	-	+++	++	++
211	-	-	-	-	+	-	-
215	+	+++	+	-	+++	+++	+++
239	-	+++	++	-	++	++	+++
250	++	+++	+	-	+++	+++	+++
266	++	+++	++	-	+++	+++	+++

Table 3.3 C

Patient 2							
Clone	IFN- γ	IL-5	IL-13	IL-17	IL-22	Granzyme-B	FasL
235	-	+++	+	-	++	+++	++
77	-	+++	+	-	++	+++	++
39	++	+++	++	-	+++	+++	+++
55	-	++	+	-	-	-	+
73	++	+++	+	-	+	+	+
119	++	+++	+	-	++	++	+

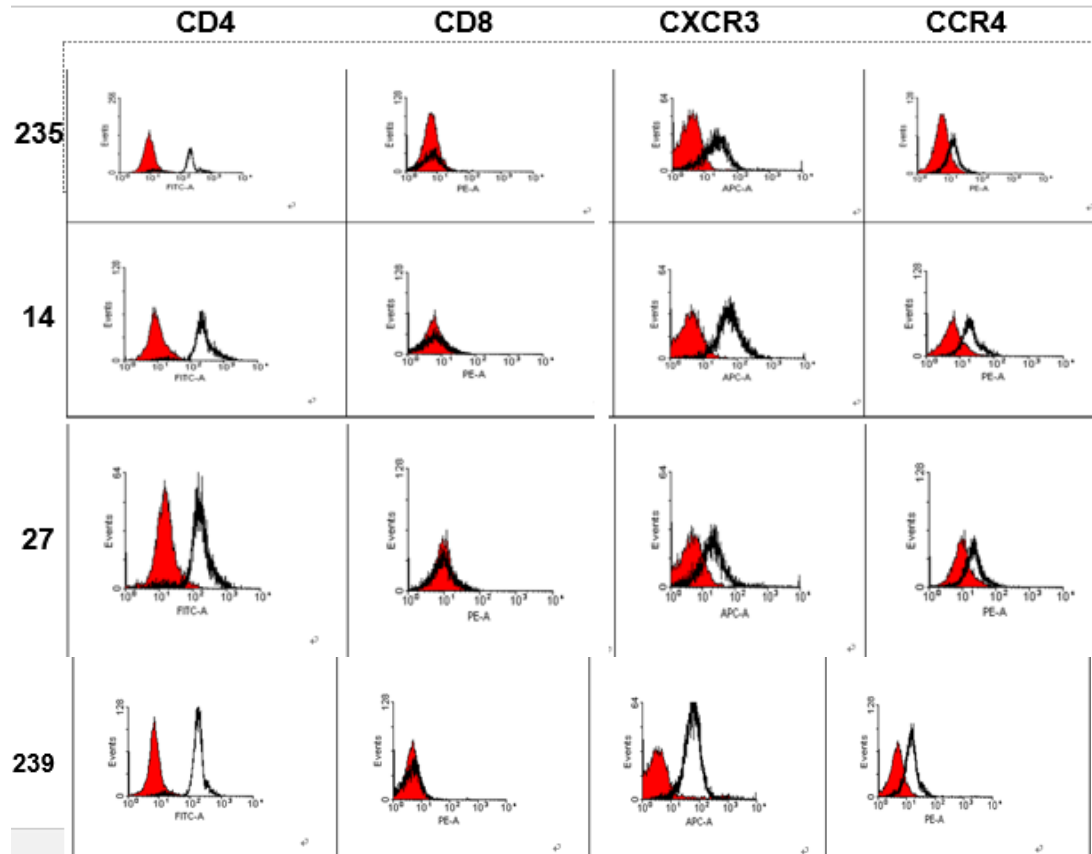
Table 3.3 D

Patient 3							
Clone	IFN- γ	IL-5	IL-13	IL-17	IL-22	Granzyme-B	Fas-ligand
182	-	-	+	-	++	+	-
231	+	++	+	-	+++	++	+
14	+++	++	++	-	+++	++	+++
27	-	-	+	-	+++	++	-
146	++	+	-	-	++	++	++
160	-	-	+	-	+++	-	-
171	++	++	+	-	++	++	+++
174	+	++	-	-	+++	++	-

Table 3.4 B-D. A summary of the ELISpot results of Figure 3.3. Cytokine and cytolytic molecule profile of the piperacillin-specific T-cell clones generated from the 3 patients. (B) the summary result for 10 T-cell clones from patient 1. (B) the summary result for 6 T-cell clones from patient 2. (C) the summary result for 8 T-cell clones from patient 3. The ELISpot results were semi quantified as follow: the numbers of spots in the non-stimulated control as -. +: 40 spots more than the control. ++: 80 spots more than the control. +++: 120 spots more than the control.

3.3.5 Surface markers expressed on piperacillin-specific T-cell clones

The expression of CD markers on the T-cell clones was determined by flow cytometry (Figure 3.2 and Table 3.5). Among the 78 clones isolated from the 3 patients, 77 clones were CD4⁺ and the remaining clone was CD4⁺CD8⁺ (Table 3.5). Further analysis of 12 piperacillin-specific T-cell clones showed that all clones expressed the chemokine receptor CXCR3, 8 clones expressed CCR4, 3 clones expressed CCR10 and one clones expressed CCR8 (Table 3.5 and Figure 3.2). However, CLA was only expressed at low levels.



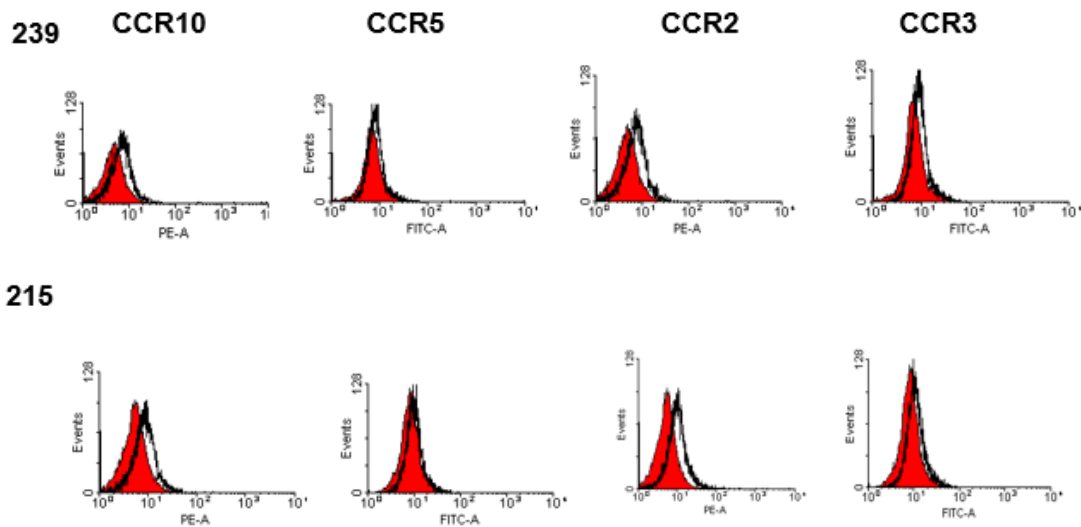


Figure 3.2 T-cell surface molecule identification on piperacillin-specific T-cell clones. The cell surface molecule expression on specific T-cell clones was measured by flow cytometry. The figure shows representative clones. Red shaded area represents isotype control. Black line shows antibody-stained cells.

Clone	Mean Fluorescence Index									
	CD4	CD8	CXCR3	CCR4	CLA	CCR10	CCR5	CCR2	CCR3	CCR8
211	6.4	5.5	4.1	2.0	-	-	-	-	-	-
215	11.3	-	11.0	2.1	-	-	-	-	-	-
239	11.5	-	12.0	2.5	-	-	-	-	-	-
235	10.8	-	5.9	2.0	-	-	-	-	-	-
14	11.7	-	10.2	2.6	-	-	-	-	-	-
27	6.2	-	3.6	1.9	-	-	-	-	-	-
55	10.6	-	7.5	2.2	-	-	-	-	-	-
171	11.6	-	13.5	2.5	-	-	-	-	-	-
231	90.9	-	3.9	-	-	-	-	-	-	-
202	111.1	-	5.9	-	-	2.4	-	-	-	-
266	38.8	-	1.9	-	-	2.0	-	-	-	2.1
182	95.8	-	8.6	-	-	2.0	-	-	-	-

Table 3.5 Expression of T-cell surface molecules. 5×10^5 T-cell clones were incubated with anti-human CD4-FITC, CD8-PE, CXCR3-APC, CCR4-PE, CLA-FITC, CCR10-PE, CCR5-FITC, CCR2-PE, CCR3-FITC, CCR8-PE antibodies (for 20 min on ice in the dark). The cells were then washed and re-suspended in 150 μ l FACS buffer and analyzed by flow cytometry on a FACS Canto II. Data is given as mean fluorescence index (mean fluorescence of test antibody/mean fluorescence of isotype control antibody). An index of <2.0 was regarded as no expression.

3.3.6 TCR V β expression of piperacillin specific T-cell clones.

Sixteen clones were assessed for TCR V β expression by flow cytometry. These analyses were conducted on the best growing clones randomly selected from the three patients. The panel of 24 antibodies represents most of the common V β types and covers 85% of all V β s. Two T-cell clones (clone 27 and 199) were negative for all the TCR V β tested. The remaining 14 clones expressed 9 different TCR V β s, with V β 5.2, V β 13.1 and V β 9 being the most common (Figure 3.3 and 3.4). T-cell clone 174 was in fact a mixture of 2 different clones with 2 different TCR V β , namely V β 9 and V β 17 and should therefore be referred to as a drug-responsive cell line (Figure 3.4).

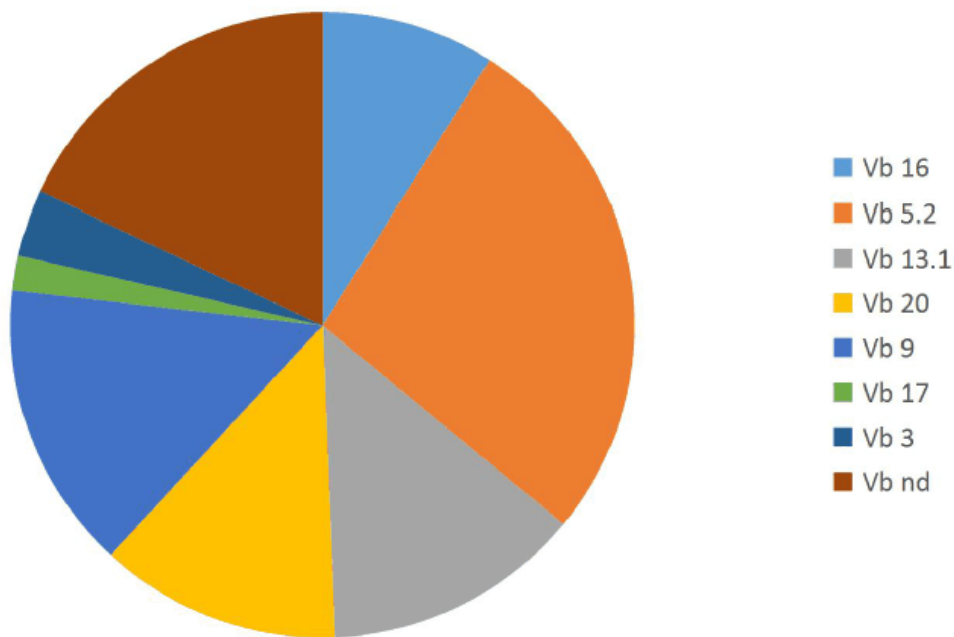


Figure 3.3 TCR V β Expression on piperacillin-specific T-cell clones from hypersensitive patients with CF.

16 piperacillin-specific T-cell clones were tested. Minimum of 5×10^4 cells were stained with $5 \mu\text{l}$ V β detecting antibody for 20 minutes. These cells were washed and the stained and analyzed using a FACS Canto II.

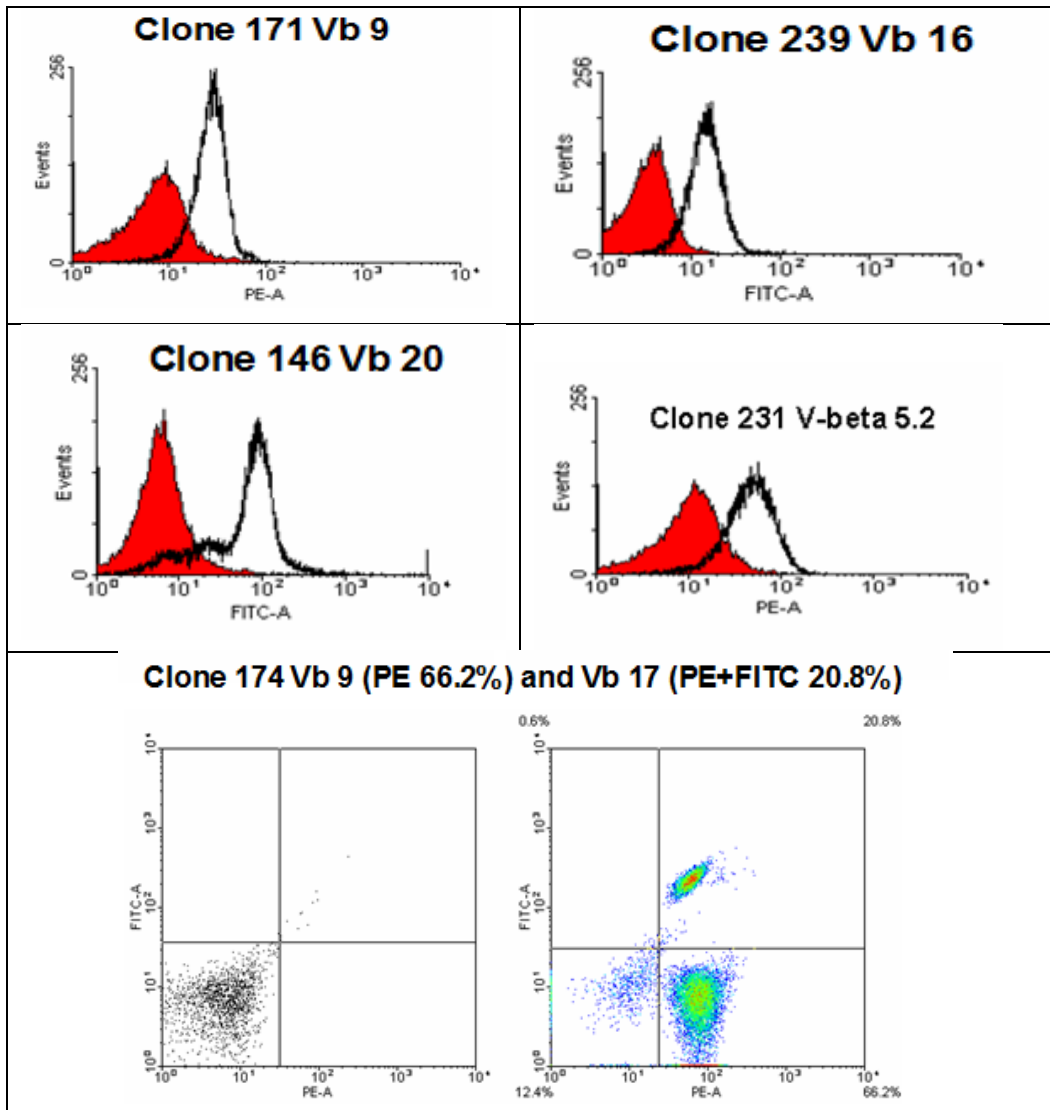


Figure 3.4. Flow cytometry assessment of TCR V β expression on T-cell clones. These graphs show an example of V β receptor expression on the tested clones. Flow cytometry profiles of 4 T-cell clones expressing single V β receptors, and the profile of one clone, clone 174, which was a mixed cell population expressing V β 9 and V β 17.

3.4 Discussion

T-cell responses to piperacillin were examined in 3 patients with CF. Lymphocytes from all 3 hypersensitive patients were stimulated to proliferate in the presence of piperacillin in a dose-

dependent manner. In contrast, lymphocytes from healthy controls were not stimulated to proliferate. Collectively, these data indicate the presence of piperacillin-responsive T-cells in the peripheral circulation of the hypersensitive patients (Pichler et al., 2004).

To analyze the phenotype and function of these drug-specific responses in detail, drug-responsive T-cell lines were generated and individual T-cells were cloned. The T-cell response to piperacillin was dominated by CD4⁺ T-cells because 77 out of 78 clones expressed the TCR co-receptor CD4. CD4⁺ T-cells dominate in mild skin rashes such as maculopapular exanthema, whereas CD8⁺ T-cells tended to dominate in severe drug hypersensitivity reactions such as Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) (Correia et al., 1993).

Following piperacillin treatment, the majority of clones (21 out of 24 clones) secreted IL-22 but not IL-17. These data suggest that Th22, but not Th17, cells play a role in piperacillin hypersensitivity. Th22 secreting cells promote the proliferation of keratinocytes and play a role in healing wounds in a skin injury model, which suggests Th22 cells may be protective in skin injury (Eyerich et al., 2009). In contrast, Th22 cells have also been shown to promote inflammation. For example, in the very late phase of psoriasis, Th22 cells play an important role in mediating the inflammatory condition (Kagami et al., 2009; Michalak-Stoma et al., 2013). The role of IL-22 in the pathogenesis of piperacillin hypersensitivity is not known. Thus, in next chapter patient T-cells derived from inflamed skin will be isolated to further characterize the role of Th17 and Th22 cells in drug hypersensitivity.

Both Th1 and Th2 cells contribute to the pathology of drug hypersensitivity. The T-cell clones identified in this study were a mixture of Th1 and Th2 cytokine secreting cells as shown by their cytokine profiles. Correlation between drug specific secretion of Th2 cytokines, IFN- γ , or IL-22 and drug-specific proliferation was studied. However, significant correlation was not observed,

highlighting the significant variability of individual clones. These data are consistent with a previous study of maculopapular reactions caused by a variety of drugs in that the majority of clones expressed low/moderate levels of IFN- γ and high levels of IL-5 and IL-13 (Lochmatter et al., 2009). Lochmatter et al (2009) showed that IFN- γ and IL-13 are more sensitive marker for drug sensitization than IL-5. IL-5 is able to stimulate eosinophils and thereby amplify inflammatory conditions. IL-13 promotes B-cell IgE antibody and polarizes the alternatively activated macrophages, which are important in tissue remodeling and allergy. IFN- γ plays a role in inducing inflammatory cytokine production and inflammation. It also promotes keratinocyte killing by up-regulating MHC expression of the tissue cells. Some of the piperacillin-reactive clones may also be capable of cytotoxic activity as shown by secretion of FasL and granzyme B. Therefore, upon drug stimulation the piperacillin-specific T-cell clones can act as pro-inflammatory cells. If these T-cells were present in the skin of patients with CF they could cause tissue damage, including the keratinocytes apoptosis (Schnyder et al., 2000).

The homing of human T-cells in piperacillin hypersensitivity has not been previously studied. Chemokine receptors can regulate T-cell migration from the lymph nodes to the inflamed site. Once there, the T-cells may increase inflammation by secreting cytokines and cytolytic molecules. In this study I have analyzed the chemokine receptors expressed on 12 piperacillin-specific T-cell clones. The T-cell clones expressed high levels of CXCR3 and CCR4, and low levels of CCR8 and CCR10. In patients with dermatitis, CCR4 and CCR10 were classified as important mediators of T-cell migration to the inflamed tissue (Reiss et al., 2001), whereas CCR8 was important in homing of memory T-cells to healthy skin (Schaerl et al., 2004). CXCR3 is expressed primarily on activated T-lymphocytes and plays a role in the recruitment of inflammatory cells. The T-cell clones did not express CLA which is known to be important in

patients with drug hypersensitivity associated with exposure to carbamazepine (CBZ) (Wu et al., 2007). Besides CLA, CBZ-reactive T-cell clones also expressed chemokine receptors such as CXCR4, CCR4, CCR5, and CCR8 (Wu Y et al., 2007), which suggested mechanistic differences between the two types of hypersensitivity.

Piperacillin reactive T-cells express a mixed pattern of TCR V β receptors. If T-cell clones express the same V β receptor, it is believed that the cells may be generated from a single cell ie a pure clone. Furthermore, since multiple receptors were expressed, the data suggests that the piperacillin haptenic antigen is a strong antigen as it activates T-cells expressing multiple TCRs. This is likely due to the drug hapten binding to multiple sites on proteins such as HSA. For carbamazepine (CBZ) hypersensitivity, the CBZ specific T-cell TCRs were dominated with V β -11-ISGSY, and the CD8⁺ T-cells isolated from the inflamed site secreted granulysin, inducing the killing of keratinocytes (Ko TM et al., 2007). For abacavir (ABC) hypersensitivity, the majority of ABC specific T-cell clones were CD8⁺ and they expressed broad spectrum of TCR V β receptors (Chessman D et al., 2008). Fourteen T-cell clones from piperacillin hypersensitive patients show a mixed pattern of TCR V β expression usage which is more similar to the situation with abacavir reactive clones. However, the importance of this similarity is limited since the mechanisms of T-cell activation by the two drugs differ. For example, abacavir hypersensitivity is associated with a specific HLA risk allele and CD8⁺ T-cells whereas piperacillin hypersensitivity is not associated with a HLA risk allele and involves CD4⁺ cells.

Drug hypersensitivity can also be induced by other penicillins, such as amoxicillin and flucloxacillin. Susceptibility to these drugs is associated with specific HLA alleles. These differences suggest that different mechanisms may be involved in the hypersensitivity reaction of piperacillin compared to amoxicillin and flucloxacillin. Clinically, piperacillin induces milder

reactions compared with flucloxacillin and amoxicillin. Piperacillin-specific T-cells induce CD4-dependent drug hypersensitivity, whereas CD8⁺ T-cells play a crucial role in the reaction to amoxicillin and flucloxacillin (Monshi et al 2013, Kim et al., 2015). Flucloxacillin-specific CD8⁺ T-cells isolated from hypersensitive patients with liver injury are activated via a hapten pathway, in need of protein processing and presentation by antigen presenting cells, which is restricted to HLA-B*57:01 (Monshi et al., 2013). Amoxicillin specific T-cells isolated from patients with liver injury are also predominantly CD8 positive and the activation is again dependent on antigen processing (Kim et al., 2015). The hypersensitivity reaction with a greater clinical similarity to piperacillin is sulfamethoxazole (SMX), which is not β -lactam antibiotic. Both SMX and piperacillin generate similar clinical symptoms of skin rash, and the SMX inflammatory T-cells are mainly a CD4⁺ phenotype (Schnyder et al., 2000).

The main limitation of this study is the small number of patients studied (n=3). It can be difficult to obtain the patient samples. Experiments were also lost due to microbial contamination. Finally experiments failed due to poor growth of the cells. A larger study size might show greater patient variation. However, the validity of my study is supported by 1) the data being consistent with published work showing a mixed Th1 and Th2 cytokine profile of individual clones, and 2) the limited variation in T-cell clones isolated from individual patients.

In conclusion, piperacillin specific T-cells isolated from patients with cystic fibrosis were found to be mainly CD4⁺ T-cells; secreting a mixed cytokine profile of Th1, Th2 and Th22 cytokines, but IL-17. Expression of chemokine receptors of CXCR3, CCR4, CCR8 or CCR10 was detected on the clones indicating that they may migrate from blood to inflamed skin. The varied TCR V β receptor profile suggested that piperacillin activates a variety of clones expressing different receptors.

Chapter 4

The role of IL-17 and IL-22 producing cells in the skin from piperacillin hypersensitive patients with Cystic fibrosis

4.1 Introduction

4.2 Methods

4.3 Results

4.3.1 Characteristics of two piperacillin hypersensitive patients with CF.

4.3.2 Lymphocyte transformation test (LTT) of peripheral blood lymphocyte cells from piperacillin hypersensitive patients.

4.3.3 IFN- γ , IL-13, IL-17A, and IL-22 secretory profile of the peripheral T-cells.

4.3.4 Characteristics of T-cell clones generated from skin biopsy of hypersensitive patients.

4.3.5 IFN- γ , IL-13, IL-17A, and IL-22 secretory profile of skin T-cell clones.

4.3.6 Chemokine receptor profile in piperacillin specific T-cell clones.

4.4 Discussion.

4.1 Introduction

MPE is the mildest but the most common symptom among the immune mediated skin reactions, comprising more than 90% of reactions (Hunziker et al., 1997). MPE usually occurs in 1-2 weeks after drug administration (Valeyre-Allanore et al., 2007). Piperacillin is one of the drugs that commonly induce MPE in patients with cystic fibrosis.

T-cells are thought to play a critical role in inducing these skin reactions. Immuno-histochemical staining of the skin and functional studies with patient blood lymphocytes reveal the presence of drug-specific T-cells that induce cytotoxicity and inflammation in targeted tissues when activated. Yawalkar N et al., (2000a) demonstrated that MPE skin is infiltrated with both CD4⁺ T-cell and CD8⁺ T-cells and CD4⁺ T-cells dominant in the inflamed tissue. Both types of T-cells secrete cytolytic molecules such as perforin and granzyme B and thus have the capacity to cause cytotoxicity.

Secretion of IL-12 and IFN- γ suggest that cytotoxic Th1 cells play an important role in these reactions. Yawalkar N et al., (2000b) also found IL-5 and exotoxin secreting in T-cells in MPE skin biopsies, which were capable of attracting and promoting eosinophil-mediated inflammation, suggesting that MPE is mediated by drug-specific T-cells that secrete Th1 and Th2 cytokines.

In the previous chapter, drug specific T-cells isolated from PBMCs were characterized as cytotoxic CD4⁺ T-cells that secrete Th1, Th2 and Th22 cytokine signatures. Furthermore, some of the clones had a tendency to migratory towards skin since they expressed skin-homing chemokine receptors CCR4 and CCR10.

In this chapter, T-cells isolated from piperacillin-induced skin lesions have been studied with the aim of giving a complete characterization of piperacillin-specific T-cells that participate in the

drug hypersensitivity reaction.

Skin biopsies were obtained following piperacillin skin testing to isolate T-cells and generate clones.

Chemokines play a central role in the immune system by orchestrating the migration of immune cells. As introduced in previous chapter, CLA and the chemokine receptors CCR4, CCR8 and CCR10 are key mediators in skin homing. Gut homing receptors such as CCR9, CXCR3 and CXCR6 were also been tested in our study to explore whether the drug-specific T-cells preferentially express receptors involved in migration towards skin. Finally, CD69 a marker of activated T-cells (Moretta et al., 1991) and E-cadherin, which mediates the connection between epithelial cells and T-cells were measured (Cepek et al., 1994).

The roles of Th17 and Th22 secreting T-cells that reside in the MPE skin biopsy of drug hypersensitive patients have not been studied. In this study, we characterized the T-cells in inflamed skin biopsies and compared the finding to the results presented in chapter 3 characterizing drug-specific T-cells isolated from blood of piperacillin hypersensitive patients with CF. Importantly the two patients studied were also included in the analysis in chapter 3.

IL-17 is the founding member of the IL-17 family of cytokines. Six IL-17 family members has been identified, which includes IL-17A (also called IL-17), IL-17B, IL-17C, IL-17D, IL-17E and IL-17F (Korn et al., 2009). IL-17A has the closest sequence homology (58% at the protein level) with IL-17F and they have a similar biological effect (Hurst et al., 2002). To test IL-17A secretion in the assay because IL-17A are secreted by T-cells (Korn et al., 2009) whereas IL-17F can be secreted by both Th17 and monocytes (McAllister et al., 2005). Therefore, in this study IL-17A secretion was chosen as a signature of drug specific Th17 cells.

4.2 Methods

To characterize drug specific T-cells in inflamed skin and peripheral blood from the same patients with CF, T-cells were isolated from patients' blood and skin biopsies (described in section 2.17), and cloned (described in 2.5). The T-cells were stimulated with Epstein-Barr virus (EBV) transformed B cells (described in 2.4) as antigen presenting cells (APCs). T-cell cytokine secretion was measured by ELISpot (described in 2.14). T-cell cellular surface molecule expression, such as CD4, CD8 and chemokine receptors, was determined by flow cytometry (described in 2.7). The methods for PBMC isolation and T-cell cloning are described in section 2.3 and 2.5, respectively). Drug specificity of the T-cell response was measured using a proliferation assay (described in 2.8).

Statistics

Unless stated otherwise, the ANOVA test was applied to multiple comparisons. A p value < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Characteristics of two piperacillin hypersensitive patients with CF.

Patient	Age / Gender	Drug	Reaction	Reaction Time ⁺	Time*	Skin Prick Test	Intradermal skin test
P1	23/M	piperacillin	MPE/fever	2	0.5	-	+ at 48 hours
P2	32/M	piperacillin	MPE	5	2	-	+ at 48 hours

Table 4.1 Clinical history of patients with cystic fibrosis. The table shows the age, gender and clinical information of the hypersensitive patients. (Age in years, Reaction Time⁺ = time from treatment to reaction in days, Time* = time since reaction in years, M: male, F: female, MPE: maculopapular exanthema, P: patient).

4.3.2 Lymphocyte transformation test (LTT) of peripheral blood lymphocyte cells from piperacillin hypersensitive patients.

In order to determine whether piperacillin-specific T-cells circulate in blood and to explore differences between the T-cells in the circulation and inflamed skin tissue, I firstly performed a LTT using PBMC from the patients. Lymphocytes from both piperacillin hypersensitive patients with CF proliferated when PBMCs were incubated *in vitro* with the drug. The response was dose-dependent in both patients with a maximal response observed at 2mM (Table 4.2). The LTT results are similar to the results we conducted on the same patients three years earlier (Table 3.2).

	cpm in control	Piperacillin ($\mu\text{g/ml}$)					TT ($5\mu\text{g/ml}$)
		0.25	0.5	1	2	4	
P1	370	5.3	8.4	16.8	28.0	14.6	60.8
P2	1436	15.5	21.3	26.4	30.2	28.0	18.3

Table 4.2 Piperacillin-specific lymphocyte transformation test (LTT). The LTT was performed using PBMCs at 1.5×10^5 cells/well. PBMC were exposed to piperacillin 0.125-4 mM for 6 days with TT as control. Cellular proliferation was measured by ^3H -thymidine incorporation for the final 16 hours. Drug specificity is shown by SI.

4.3.3 Characteristics of T-cell clones generated from blood of hypersensitive patients.

Piperacillin-responsive lymphocytes were investigated for drug specific cytokine secretion profiles using ELISpot analysis. Piperacillin stimulation markedly enhanced IFN- γ , IL-13 and IL-22 secretion in both samples compared with control cultures without piperacillin (Figure 4.2). In contrast, piperacillin-treatment did not result in the secretion of IL-17A. In the PBMCs samples without piperacillin challenge, only low levels of IFN- γ were detected.

In all cases, phytohaemagglutinin (PHA; positive control) activation of PBMCs resulted in cytokine secretion of cytokines including IL-17A (Lindahl-Kiessling & Book, 1964).

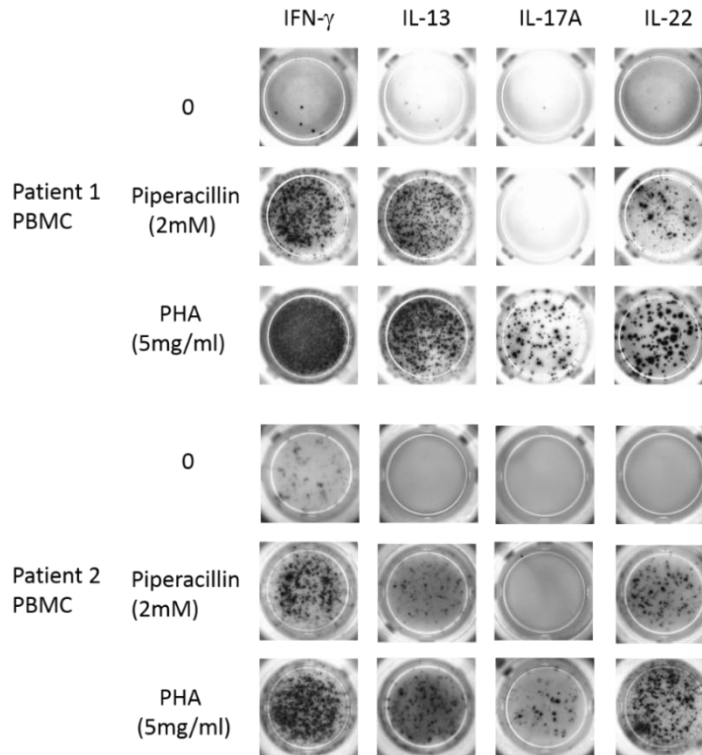


Figure 4.1. Piperacillin-specific cytokine secretion from PBMC of hypersensitive patients detected by ELISpot. Piperacillin hypersensitive patients' PBMCs 1.5×10^6 cells/ml were cultured in 96-well U-bottomed tissue culture plates with or without 5mg/ml of PHA or piperacillin (2mM) for 6 days at 37 °C, 5% CO₂. Antigen-specific cytokine production was measured by IFN- γ , IL-13, IL-17A and IL-22 ELISpot.

4.3.4 Characteristics of T-cell clones generated from skin biopsies of hypersensitive patients.

To phenotypically characterize the T-cells in skin of the two drug hypersensitive patients, piperacillin-specific T-cell clones were generated. The skin biopsy samples were obtained through 3mm puncturing, after a positive dermal prick test. T-cells residing in the skin were isolated according to the protocol described in chapter 2 (section 2.17). Flow cytometric analysis was carried out on T-cell clones obtained from hypersensitive patient skin. In total, 690 CD4⁺ or CD8⁺ T-cell clones were isolated from inflamed skin of the 2 piperacillin hypersensitive patients with CF. Ninety six of the clones tested were piperacillin responsive (Table 4.3). 89% of the piperacillin responsive clones from patient 1 and 82% from patient 2 were CD4 positive T-cells.

All other clones were CD8⁺ T-cells.

Patient ID	Tested	Piperacillin specific	% CD4 ⁺	% CD8 ⁺
Patient 1	354	48	89	11
Patient 2	336	48	82	18

Table 4.3. Phenotypic characteristics of T-cell clones obtained from skin of hypersensitive patient 1 and 2. T-cells were isolated from skin biopsies as described in the Material and Methods (2.17) and piperacillin-specific T-cells were cloned. The phenotype of the - cells (CD4⁺ or CD8⁺) was further characterized by flow cytometry.

4.3.5 IFN- γ , IL-13, IL-17A, and IL-22 secretory profile of skin-derived T-cell clones.

In total, 96 piperacillin specific clones were generated from a total of 690 T-cell clones tested with the majority expressing the CD4⁺ receptor. Drug-specific CD8⁺ T-cell clones were observed at lower numbers. Piperacillin specific cytokine and cytotoxic molecule secretion in 3 T-cell clones from patient 1 and 6 clones from the patient 2 was measured by ELISpot. As shown in the figure 4.3a-b and Table 4.3 A-B, a similar pattern of drug-specific cytokine and cytotoxic protein secretion was observed with all the clones from the two patients. High levels of IFN- γ and IL-22 secretion was detected after the clones were cultured with antigen presenting cells and piperacillin. IL-13 was detected to a lesser extent when drug-treated and control wells were compared. Production of cytolytic molecules such as Fas ligand, granzyme B and perforin was also observed with clones from patient 2, while only granzyme B was detected with the clones derived from patient 1. IL-17 was not secreted from the piperacillin-specific clones.

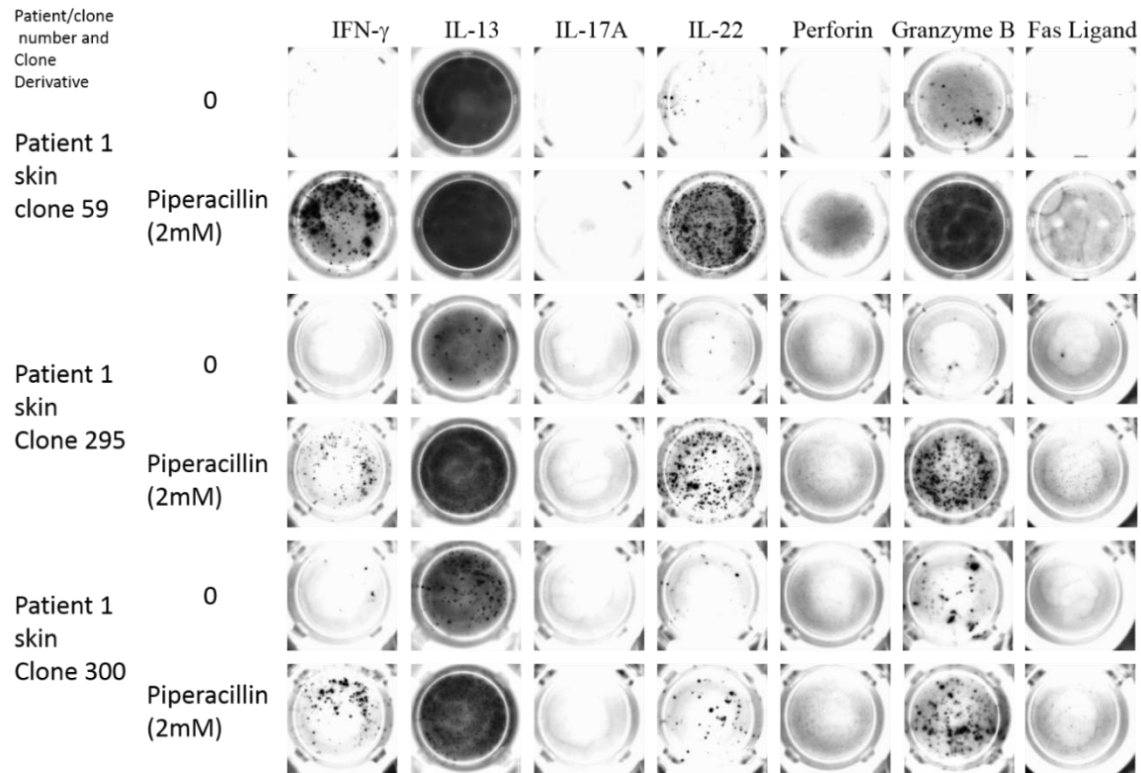


Figure 4.2a Cytokine and cytolytic protein secretion from piperacillin specific T-cell clones (clones 59, 295 and 300) generated from inflamed skin of drug hypersensitive patient 1. 5×10^4 T-cell clones were placed duplicate wells. 1×10^4 autologous APCs per well were added as well as medium or 2 mM piperacillin. The cultures were incubated for 6 days in an atmosphere of 37 °C 5% CO₂. The production of IFN- γ , IL-13, IL-17A, IL-22, perforin, granzyme B, and fas-ligand were determined by ELISpot.

Patient 1							
Clone	IFN- γ	IL-13	IL-17A	IL-22	Perforin	Granzyme B	FasL
59	+++	-	-	+++	-	++	-
295	++	+	-	+++	-	+++	-
300	++	-	-	+	-	++	-

Table 4.4 A. Cytokine and cytolytic protein secretion from piperacillin specific T-cell clones.

The ELISpot results were quantified as follow: the numbers of spots in the non-stimulated control as -. +: 40 spots more than the control. ++: 80 spots more than the control. +++: 120 spots more than the control.

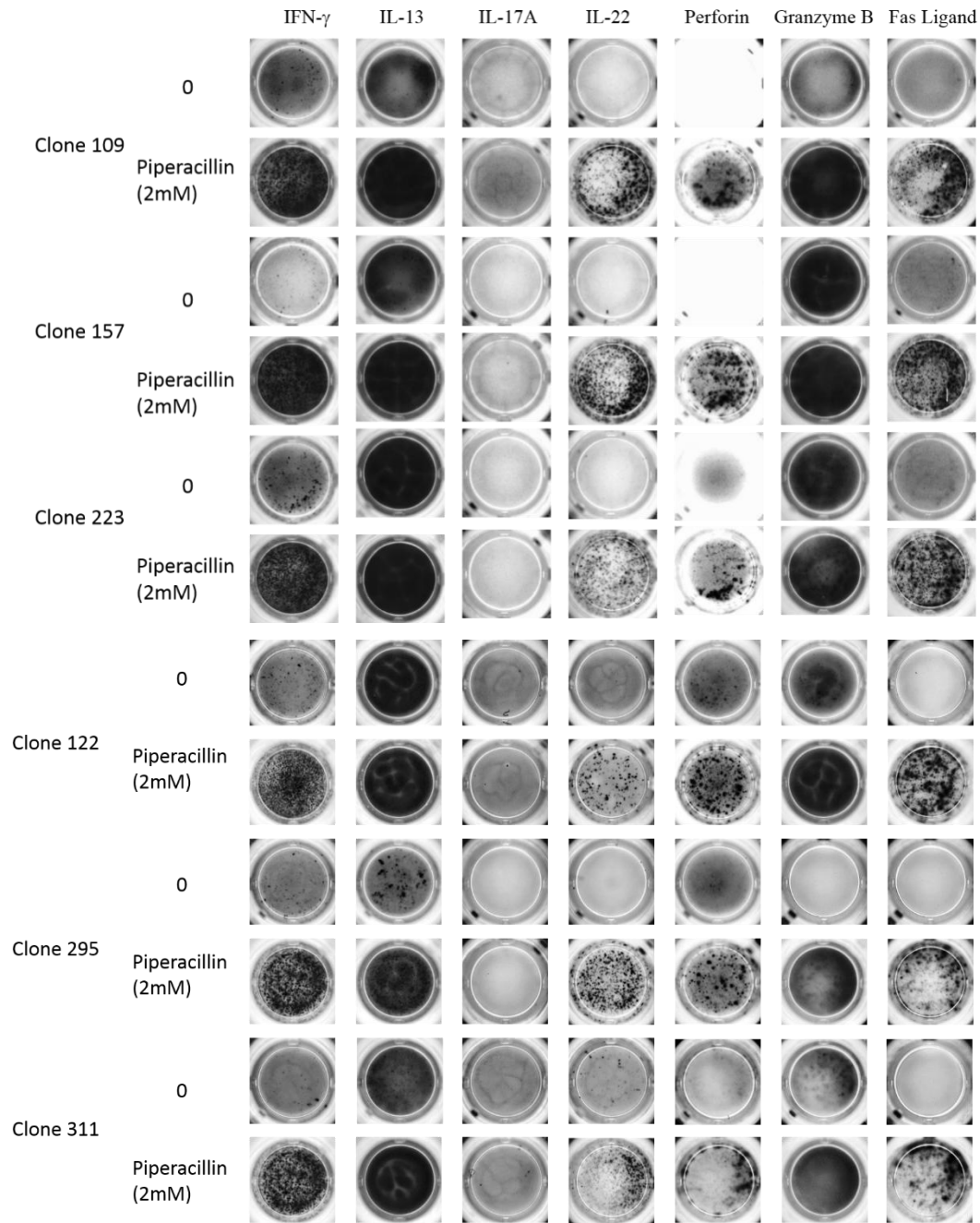


Figure 4.2b Cytokine and cytolytic protein secretion from piperacillin specific skin T-cell clones (clones 122, 295, 311, 109, 157 and 223) isolated from inflamed skin of drug hypersensitive patient 2 with CF. As described in Figure 4.2a, 5×10^4 T-cell clones were placed duplicate each well in 2 wells, 10^4 autologous B cell APCs per well were added as well as medium +/- 2mM piperacillin to a final volume of 200 μ l/well. The cultures were incubated for 6 days at an atmosphere of 37 $^{\circ}$ C 5% CO_2 . ELISpot was applied to measure the productions of IFN- γ , IL-13, IL-17A, IL-22, perforin, granzyme B, and Fas-ligand.

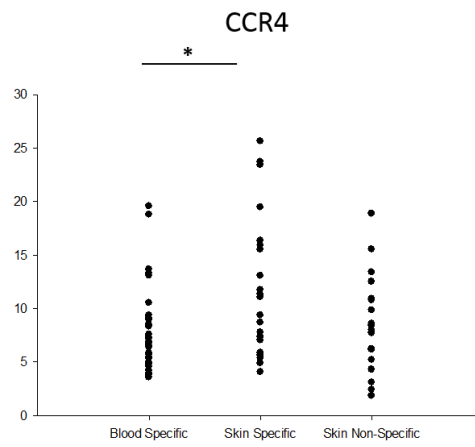
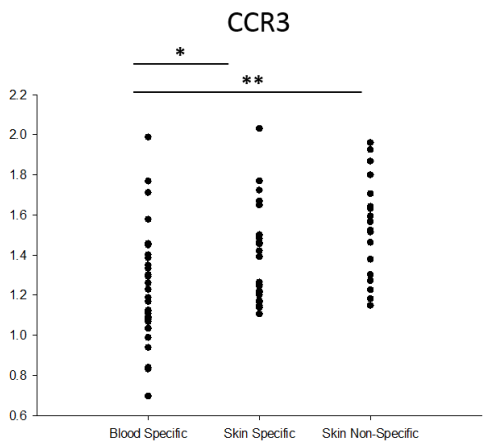
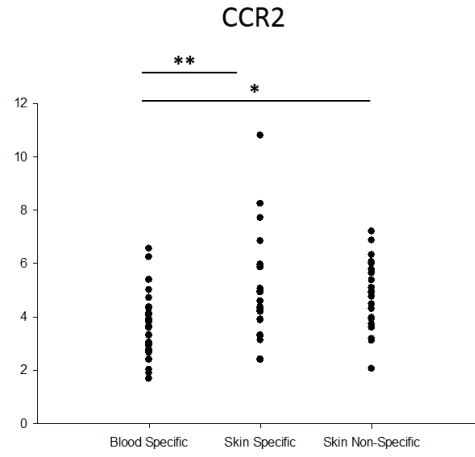
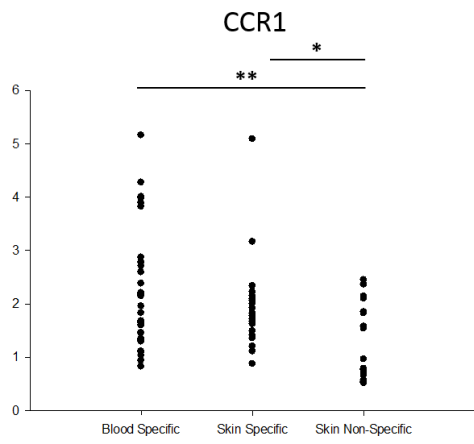
Patient 2							
Clone	IFN- γ	IL-13	IL-17A	IL-22	Perforin	Granzyme B	FasL
109	+++	++	-	+++	++	+	+++
157	+++	+	-	+++	+++	++	+++
223	+++	+	-	+++	++	++	+++
122	+++	+	-	+++	+++	-	+++
295	+++	++	-	+++	++	+	+++
311	+++	+	-	++	+	-	+++

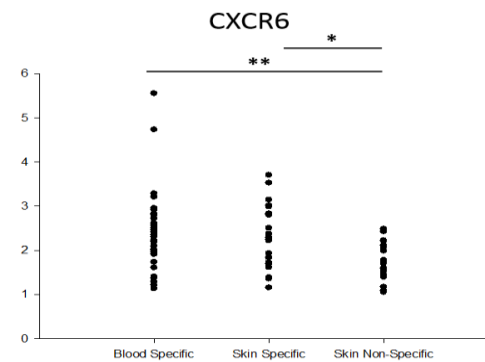
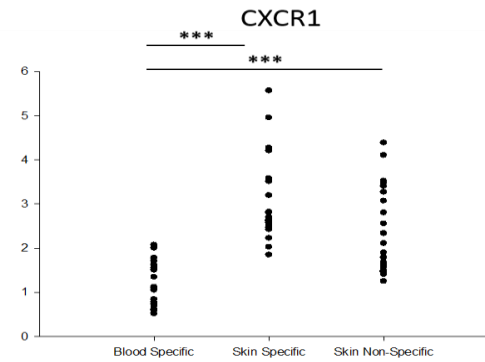
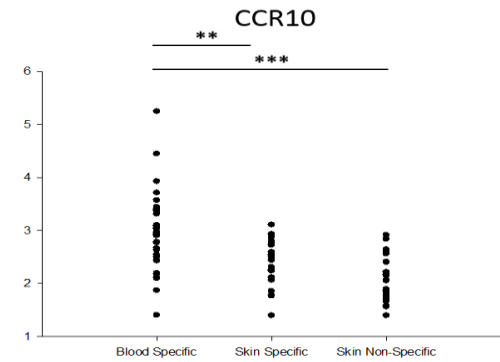
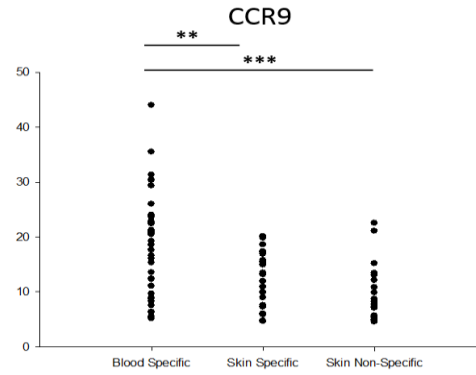
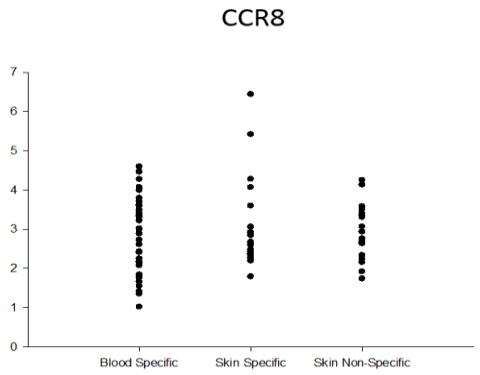
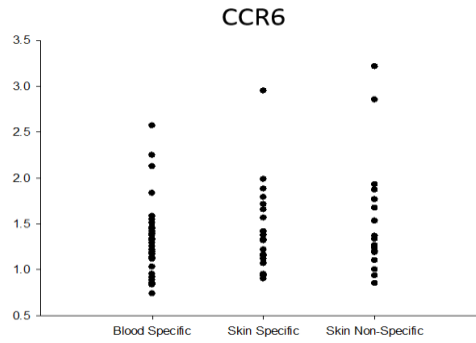
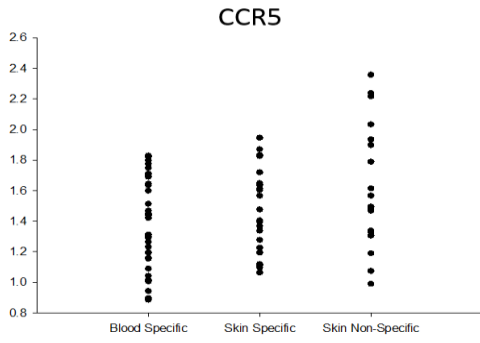
Table 4.4 B. Cytokine and cytolytic protein secretion from piperacillin specific T-cell clones.

The ELISpot results were semi quantified as follow: the numbers of spots in the non-stimulated control as -, +: 40 spots more than the control. ++: 80 spots more than the control. +++: 120 spots more than the control.

4.3.6 Chemokine receptors expressed on piperacillin-specific T-cell clones.

To further characterize the drug specific T-cells isolated from skin of hypersensitive patients, chemokine receptor expression was measured using flow cytometry. Comparisons were made between blood- and skin-derived clones responsive towards piperacillin, along with piperacillin non-specific skin-derived clones (Figure 4.4). Significant differences in the expression of receptors were observed for multiple chemokines. Blood derived T-cell clones were shown to express high level of CCR1, CCR9, CCR10, CXCR6, CD69 and CLA when compared with piperacillin non-specific skin-derived T-cell clones, and with significant expression of CCR9, CCR10 and CD69 when compared with piperacillin-specific skin-derived clones. Piperacillin-specific skin clones showed significant expression of CCR1, CXCR6 and CLA when compared to non-specific skin-derived clones, whereas in comparison to T-cell clones isolated from PBMCs, piperacillin-specific clones expressed significantly higher levels of CCR2, CCR3, CCR4, CXCR1, CLA and E-cadherin.





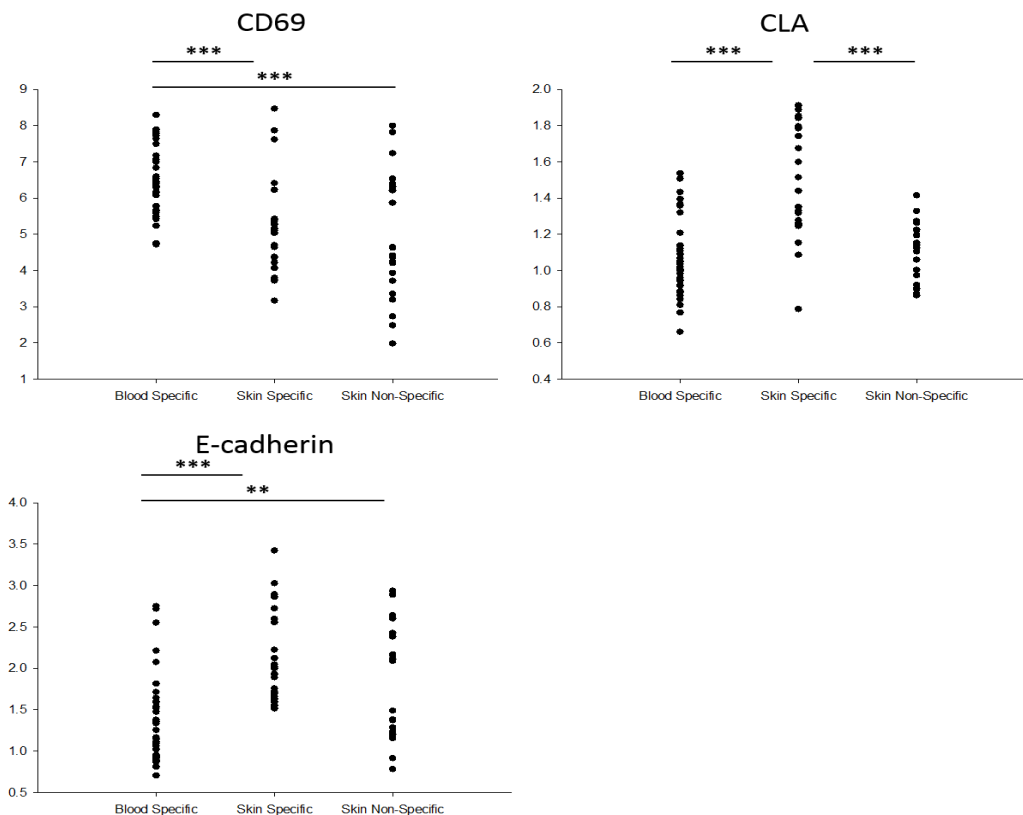


Figure 4.3 Comparison of the chemokine receptors expressed on piperacillin-specific blood- and skin-derived T-cell clones. T-cell clones were also compared with skin-derived clones that were not-responsive to piperacillin. 5×10^5 T-cell clones were incubated with anti-human CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CD9, CCR10, CXCR1, CXCR3, CXCR6, CLA, CD69 and E-cadherin antibodies labelled with either APC, FITC or PE for 20 min on ice in the dark. The cells were washed and re-suspended in $150\mu\text{l}$ FACS buffer and analyzed by flow cytometry on a FACS Canto II. The expression of surface molecules is presented as mean fluorescence index, calculated as mean fluorescence of test antibody/ mean fluorescence of isotype control antibody. The average MFI was used to compare the expression of chemokine receptors for each population of T-cell clones. ANOVA tests were used to compare variables between groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.4 Discussion

In order to study whether piperacillin-specific T-cell responses are detectable several years after an adverse event, two of the piperacillin hypersensitive patients recruited in 2010 (Chapter 3) were re-recruited in 2013 in this study. For patient 1 was intradermal skin test negative at the earlier time-point, but positive three years later. The LTT and ELISpot results suggest that there is no marked difference in terms of the T-cell proliferation, key cytokine profile and cytotoxic molecules produced when PBMC were cultured with the drug. This suggests that memory T-cells

with drug specificity circulated in the blood of hypersensitive patients for several years.

In order to compare the drug-specific T-cells in inflamed skin biopsies and in the peripheral blood of the same piperacillin hypersensitive patients, T-cell clones were generated and analyzed in terms of cellular phenotype, function and the involvement of Th17 and Th22 cytokines and cytolytic molecules. Skin samples were taken from patients following a positive dermal test, mimicking the pathogenesis of drug induced MPE which is the most commonly observed symptom induced by piperacillin. T-cell clones generated from patient PBMCs as well as non-drug specific skin-derived clones were studied for comparisons.

The drug-specific T-cell clones isolated from skin were generally CD4⁺ T-cells. This observation is consistent with the immuno-chemical staining of MPE biopsies, showing that the majority of MPE infiltrated T-cells are CD4⁺. However, CD8⁺ T-cell clones displaying piperacillin specificity were also isolated from inflamed skin; hence, their role in the disease pathogenesis cannot be excluded. The profile of piperacillin-specific cytokine secretion from T-cell clones isolated from skin was generally consistent with the blood-derived drug-specific clones described earlier in chapter 3.

The clones secreted a mixed profile of Th1 and Th2 cytokines, with high levels of IFN- γ and IL-13 detected when activated with piperacillin. However, the newly discovered T-cell subsets (Th17 and Th22) render this classification incomplete. Utilizing ELISpot, drug-specific IL-22 secretion was detected from patient PBMCs and skin-derived T-cell clones. The nature and function of the IL-22 secreting T-cells in piperacillin hyperactive is less understood and warrants further investigation. In contrast, IL-17 secretion was not observed, suggesting that Th17 cells are less important in piperacillin hypersensitivity. A similar cytokine profile between piperacillin-

specific T-cells isolated from PBMCs and inflamed skin are consistent with recent research which suggests that every memory T-cell clone generated in the skin bears an identical TCR that was presented in the lymph node and they are considered to be derived from a common naïve T-cell after immunization (Gaide et al., 2015).

Chemokine receptors are critically involved in the drug hypersensitivity reactions. To understand how chemokine receptor expression effects the migration of piperacillin-specific T-cells into the inflamed skin, cell surface chemokine receptor expression was measured by flow cytometry. Three populations of clones were selected: drug specific and non-drug specific T-cell clones from inflamed skin of piperacillin hypersensitive patients along with drug-specific T-cell clones isolated from patient PBMCs.

Naïve T-cells take 12-24 hours for DC priming in the lymph nodes (von Andrian & Mempel, 2003). Those cells that recognize the specific antigen become activated and expand. One naïve T-cell grows up to tens of thousands of progeny (Tubo et al., 2013, von Andrian et al., 2000). Although these T-cells that derived from a single naïve T-cell have the same TCR, the expanded cells are different in terms of their chemokine receptor expression (Liu et al., 2006). Thus, in consideration of variations of these cells, the expression of chemokine receptors in each T-cell clone was compared. Piperacillin specific T-cell clones from skin expressed significant levels of CLA, CCR2, CCR4, CXCR1 and E-cadherin when compared to the other two types of clone. In contrast, piperacillin specific T-cell clones isolated from PBMCs expressed higher level of CCR1, CCR9, CCR10 and CXCR6. Finally non-drug reactive skin-derived T-cell clones expressed higher levels of CCR3 than the other two groups.

Cutaneous lymphocyte antigen (CLA), binds selectively to the vascular lectin endothelial cell-

leukocyte adhesion molecule 1 (ELAM-1), acting as a skin homing receptor (Berg et al., 1991). In this study, only piperacillin specific T-cell clones isolated from inflamed skin expressed high level of CLA but neither circulating clones nor non-drug specific clones expressed CLA, which suggests that CLA is required for the migration of drug-specific T-cells from peripheral blood to inflamed skin, which may play a critical role in piperacillin-induced MPE. CCR4 also mediates migration of T-cells to skin (Campbell et al., 1999); CXCR1 mediates T-cell homing to inflamed tissue (Hess et al., 2004). Thus each of these chemokine receptors may be involved in drug specific T-cell homing to the site of MPE.

When naïve T-cells get primed against antigen, they become effector T-cells or memory T-cells and go into the blood, traveling to the inflamed site (e.g. skin) or remain in the circulation. After the inflammation resolves, the majority of effector cells die and the survivors are memory T-cells that can live for decades. Furthermore, when skin located memory T-cells have not been exposed to this antigen for a long time, the number decreases, and migrate into the blood (Watanabe et al., 2015, Rosa et al., 2015).

Memory T-cells provide rapid and highly effective immunity when an individual is re-exposed to an antigen. They are classified into three subsets, effector memory T-cells, central memory T-cells and resident memory T-cells (Chang & Kupper, 2015). Central memory T-cells express lymph node homing chemokine receptor CCR7 that enable them to enter lymph nodes from blood. Effector memory T-cells express low level of CCR7 but highly express CLA, which allows them access to the skin. Resident memory T-cells can be activated rapidly by antigens at inflamed sites independent of recruitment of T-cells from the blood (Jiang et al., 2012, Clark et al., 2015).

In a recent study, four populations of CD4 resident antigen specific memory T-cells have been identified, CCR7⁺ with or without CD62L expression that are transient skin homing T-cells; CD69⁺ with or without CD103 expression are true resident memory T-cells (Watanabe et al., 2015). Significant expression of CD69 expression was detected on piperacillin-specific T-cells isolated from skin suggesting they are long term skin resident cells that play a critical role in drug hypersensitivity.

When effector T-cells infiltrate into the skin, they do not only accumulate at the inflamed site, they spread throughout the skin (Jiang et al., 2012, Gaide et al., 2015). Non-inflamed skin contains post-capillary venues that express low levels of E-selectin, chemokines and intracellular adhesion molecules that allow skin-homing T-cells escape from blood vessels and locate to non-inflamed skin (Chong et al., 2004).

Drug specific T-cell clones isolated from circulating PBMCs expressed CCR9 and CCR10. CCR9 mediates migration of T-cells to lamina propria of the intestine, while CCR10 promotes T-cell skin migration (Agace 2006). This suggests these circulating T-cells may migrate to various sites in the body.

To conclude, skin resident drug specific T-cells are considered primary mediators of the drug hypersensitivity reaction. Drug specific T-cells from both peripheral blood and MPE inflamed skin were found to secrete IFN- γ , IL-22 and IL-13, but IL-17 secretion was not detected. T-cell cloning suggests that drug-specific T-cells induce inflammation either through cell killing by granzyme B/perforin or through inducing Fas-ligand dependent cell apoptosis directly.

Chapter 5

Priming and characterization of drug-specific T-cells from naive CD4⁺ T-cells

5.1 Introduction

5.2 Methods.

5.2.1 Isolation and priming of naive T-cells from healthy donor PBMCs.

5.2.2 Memory T-cell polarization

5.2.3 Drug-specific T-cell priming and Th17, Th22 differentiation from naive T-cells.

5.2.4 Characterization of drug-specific T-cell clone from SMX-NO and piperacillin specific T-cells from patients or normal volunteers.

5.3 Results:

5.3.1 The polarization of Human memory T-cells.

5.3.2 Priming of drug specific T-cells from naive T-cells

5.3.3 SMX-NO selectively polarizes drug specific IL-22 but not IL-17 producing cells from naive T-cells

5.3.4 Characterization of SMX-NO-specific T-cell clones isolated from SMX hypersensitive patients and SMX-NO primed naive T-cell of normal donors

5.3.5 Cytokine profile of the SMX-NO specific T-cell clones isolated from patients or drug primed T-cell populations

5.4 Discussion

5.1 Introduction

Drug hypersensitivity is a major clinical problem. Reactions are unpredictable in nature and when they develop they tend to be severe. In the last decade, genome-wide association studies have identified the expression of particular HLA alleles as risk factors for certain reactions (Phillips et al., 2011; Daly et al., 2012). Since drug-antigen specific T-cells have been detected in blood/tissue of patients with mild and severe skin reactions (Nassif et al., 2002) and liver injury (Monshi et al., 2013), they are believed to play an important role in the disease pathogenesis. Even though certain HLA alleles are associated with drug hypersensitivity, only a limited number of individuals with the allele develop hypersensitivity. Thus, additional approaches are required to compensate HLA screening to predict why certain individuals develop hypersensitivity to a particular drug. Naïve T-cell priming assays using blood from HLA typed donors have been used as a highly valuable resource to enhance drug hypersensitivity investigations. (Faulkner et al., 2012, 2016).

In this study, we investigated the possible immune mechanism by which drugs like piperacillin and nitroso-sulfamethoxazole (SMX-NO) induce hypersensitivity *in vitro*. SMX-NO is a metabolite of sulfamethoxazole that binds to the cellular proteins at the cysteine residues (Naisbitt et al., 2001). Piperacillin is associated with a high incidence of skin rash (El-Ghaiesh et al., 2012). As discussed in chapter 3, piperacillin bind to the lysine residues on serum proteins to form an antigen that activates T-cells. Both compounds activate T-cells via a hapten mechanism involving adduct formation and processing of the derived adduct by APCs.

How drugs induce hypersensitivity is not fully understood. Furthermore, how to modulate and control the drug-mediated pathogenic response is an important clinical issue. Current evidence suggests that T-cells play a pivotal role in allergy, including drug allergy. Originally, Th1 and

Th2 cells are critically involved in the pathogenesis and modulation of allergic diseases. Recently, Th17 cells have been implicated in the pathogenesis of asthma and other inflammatory diseases, such as psoriasis (Nestle FO et al., 2009), Crohn's disease (Kobayashi et al., 2008; Andoh et al., 2005).

Th22 cells are a CD4⁺ T-cell population found in humans and secrete IL-22 but not IL-17. IL-22 is a cytokine that mediates tissue response by stimulation of epithelial barrier tissues such as gut, lungs and skin, promoting the antimicrobial defense and epithelial barrier integrity. Also, IL-22 secreting cells have been found in patients with allergic contact dermatitis (Dyringanderson et al., 2013).

Th17 cells can produce both IL-17 and IL-22 which have been implicated in tissue inflammation (Kolls et al., 2004, Xie et al., 2000). However, there exist functional differences between cytokines. IL-17 is more pro-inflammatory and destructive, whereas IL-22 alone often functions in a protective way that promotes the proliferation of fibroblasts in tissue repair (Eyerich et al., 2010). When IL-22 acts synergistically with IL-17, TNF- α and IFN- γ , it might be pro-inflammatory (Michalak-Stoma et al., 2013). Since the microenvironment determines T-cell differentiation which in turn induces different types of inflammation, the possibility that drugs induce T-cell differentiation to Th1, Th2, Th17 and Th22 secreting cells was tested, especially Th17 and Th22 polarization, to ascertain their involvement of in drug hypersensitivity.

The aim of this chapter was to further elucidate the underlying mechanism by which Th17 and Th22 subsets are involved in the cutaneous reactions following drug exposure. We focused on how drug initiates a drug-specific T-cell response and the involvement of T-cell subsets and related cytokines, in particular the Th17 and Th22 cytokines, in drug hypersensitivity using an

established *in vitro* T-cell priming assay.

5.2 Methods.

5.2.1 Isolation and priming of naïve T-cells from normal donor PBMCs.

PBMCs isolation and the purification of naïve T-cells (CD14⁻CD3⁺CD45RO⁻CD25⁻) was performed according to the protocols described in chapter 2. PBMCs were isolated from peripheral blood of healthy volunteers. Different populations of cell were selected by magnetic bead separation. These populations include naïve T-cells (CD3⁺CD45RO⁻CD14⁻), monocytes (CD14⁺), and memory T-cells (CD3⁺CD45RO⁺CD14⁻). Naïve T-cells were then cultured with both drug antigens, in the presence of mature monocyte-derived DCs as APCs. After 7 days culture under an atmosphere of 37 °C 5% CO₂, a number of the naïve T-cells turn into drug specific memory effector T-cells and can be tested for antigen specificity when re-exposed to the drug. This assay has been applied to prime naïve T-cells against a number of drug haptens, including nitroso sulfamethoxazole and β-lactam antibiotics.

5.2.2 Memory T-cell polarization

T-cell activation requires three signals which are TCR activation, costimulatory molecule signalling and the cytokine micro-environment. Therefore, we used an anti-CD3 antibody to activate TCR, and an anti-CD28 antibody to stimulate costimulatory signalling. Finally we explored whether culturing the naïve and memory T-cells with desired cytokine cocktails induced differentiation of T-cells into different cytokine producing populations. If these cytokine combinations were found to induce T-cell differentiation, the aim was to apply them to study the effect of the cytokine microenvironment on the priming of naïve T-cells to drugs.

Memory T-cells (CD14⁻CD3⁺CD45RO⁺CD25⁻) were isolated as described in 2.15. 10 µg/ml of

anti-CD3 antibody was diluted in HBSS solution and pre-coated (300 μ l/well) in sterile ELISpot plates over night at 4 $^{\circ}$ C. The next day, excessive anti-CD3 antibody was washed by HBSS for 3 times. Then 1.5×10^5 memory T-cells were added into anti-CD3 coated wells with 200 μ l of R9 medium which was supplemented with 5 μ g/ml of anti-CD28 antibody and different cytokine cocktail for Th cell polarizations. Th17 differentiation cytokines were TGF- β (1 ng/ml), IL-1 β (10 ng/ml), IL-6 (10 ng/ml), IL-23 (10 ng/ml). Th22 differentiation cytokines were 50 ng/ml TNF- α , 20 ng/ml IL-6, 5 μ g/ml anti-IL-4 and 5 μ g/ml anti-IL-12. When differentiated into Th1 cells, the cytokines were anti-IL-4 (5 μ g/ml) and 25 ng/ml IL-12; when differentiated into Th2 cells, the cytokines are 25 ng/ml IL-4, 5 μ g/ml anti-IL-12, and 5 μ g/ml anti-IFN- γ . The cells were incubated at atmosphere of 37 $^{\circ}$ C/5%CO₂ for 5 days. On day 6, polarized memory T-cells cytokine secretion was tested by ELISpot.

5.2.3 Na ν e T-cell priming and Th17/Th22 differentiation from na ν e T-cells.

The T-cell priming assay is described in detail in 2.15. Na ν e T-cells (2×10^6) and mature DCs (8000) were cultured with Th17 polarizing cytokines (IL-1 β , IL-6, TGF- β), 10 ng/ml alongside SMX-NO (50 μ M). Additional cytokines were added on days 4 and 9. Polarization of na ν e T-cells was then determined by ELISpot.

5.2.4 Characterization of SMX-NO-specific T-cells from hypersensitive patients and normal volunteers.

PBMCs from drug hypersensitive patients were isolated and T-cell clones were generated as described in chapter 2.5. The patients' clinical information is presented in Table 5.1.

Patient	Age / Gender	Drug	Reaction	Reaction Time ⁺	Time*
P1	30/F	SMX-NO	MPE	2	7
P2	25/F	SMX-NO	MPE	4	5
P3	34/F	SMX-NO	MPE	6	8
P4	23/M	SMX-NO	MPE	10	20

Table 5.1 The clinical history of patients with sulfamethoxazole hypersensitive patients with cystic fibrosis. (Age in years, Reaction Time⁺ = time from treatment to reaction in days, Time* = time since reaction in years, M: male, F: female, MPE: maculopapular exanthema, P: patient)

5.2.5 Statistics analysis

Experiments were performed in duplicate or triplicate, based on the availability of cells. Mean values and SDs were calculated and statistical analysis was performed by paired *t* test or ANOVA when appropriate.

5.3 Results:

5.3.1 The polarization of human memory T-cells.

The results in Chapter 3 and 4 shows that T-cells isolated from piperacillin hypersensitivity patients can respond to the drug antigen when re-challenge *in vitro*, suggesting the presence of piperacillin-memory T-cells in the patients. It will be therapeutically important to know how to modulate the drug-specific T-cells. To address the aim of this chapter, we investigated if memory T-cells can be further polarized into different Th cell subsets.

Memory T-cells secreted IFN- γ and IL-13 and a low level of IL-22 under Th1 polarization conditions. The same cells secreted IFN- γ and IL-13 under Th2 polarization. Upon culturing memory T-cells with Th17 polarizing cytokines, they secreted IL-17A, IFN- γ , and IL-22. Under Th22 polarization, memory T-cells secreted IFN- γ , IL-13 and IL-22 (figure 5.1). The controls wells without the addition of antibodies or cytokines did not secrete cytokines. These data suggests that memory T-cells can be further polarized into different Th subsets depending on cytokine environment.

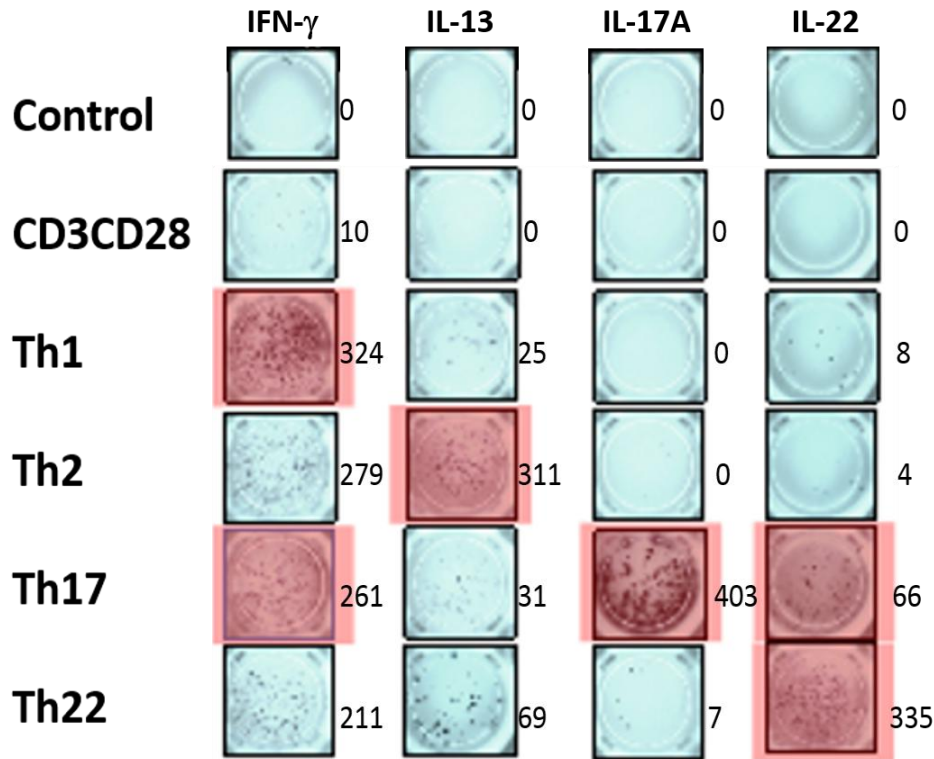


Figure 5.1 Memory T-cell polarization. Memory T-cells were isolated from normal individuals and then 5×10^5 memory T-cells were stimulated with anti-CD3, anti-CD28 antibody and different cytokine cocktail for Th cell polarization. The cells were incubated for 6 days at an atmosphere of 37°C 5% CO_2 . On day seven, upon the stimulation of PHA, the production of IFN- γ , IL-13, IL-17A and IL-22 were determined by ELISpot. Red shaded squares indicate cytokine polarization. Spots counting was shown.

5.3.2 Priming of drug-specific T-cells from naïve precursors

In chapter 4, piperacillin-specific T-cell clones isolated from the blood and skin of hypersensitive patients were found to secrete mainly IFN- γ , IL-13 and IL-22, but not IL-17A. Thus, to investigate the mechanism of drug specific IL-22 secretion and further determine the potential of drug-specific secretion of IL-17A, priming assays were applied to test the origin of drug-specific T-cells. SMX-NO and piperacillin were used as a model drug haptens in the T-cell priming assay. In initial experiments, naïve CD3^+ T-cells were cultured with dendritic cells and SMX-NO or piperacillin in the absence of polarizing cytokines. These cells were then harvested, re-stimulated

with the drugs and assayed for IFN- γ , IL-13, IL-17 and IL-22 secretion. As showed in Figure 5.2A, SMX-NO-specific T-cells were polarized, and secreted IFN- γ , IL-13 and IL-22. However, IL-17 release was not detected (Figure 5.2A). Piperacillin (Figure 5.2B) primed naïve T-cell population showed a similar pattern, secreting IFN- γ , IL-13 and IL-22 but not IL-17A.

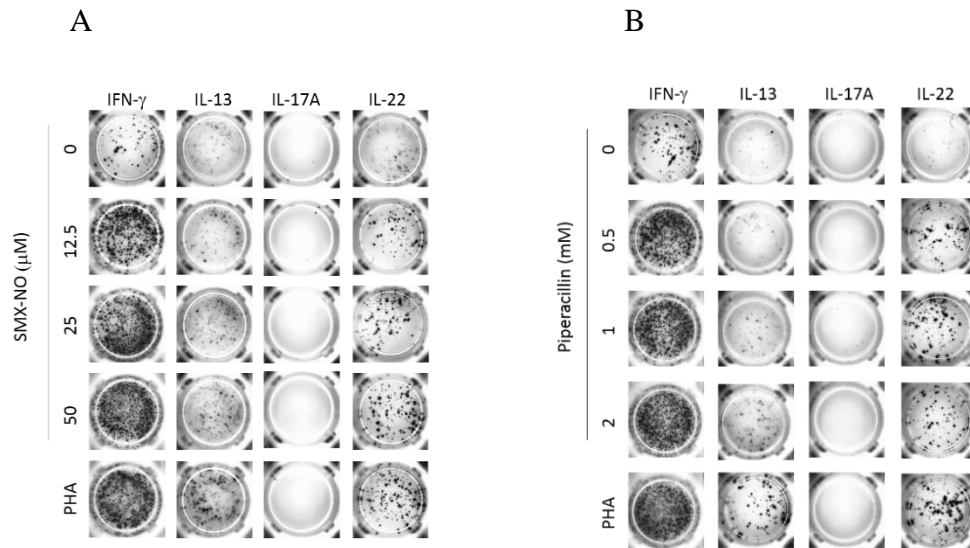


Figure 5.2 Drug-specific cytokine secretion from SMX-NO- or piperacillin-primed naïve T-cells from normal donors. CD3⁺ naïve T-cells from normal volunteers were co-cultured with dendritic cells in the presence of different drug concentrations, (A) SMX-NO (12.5 μ M to 50 μ M) and (B) piperacillin (0.5mM to 2mM) ,in an atmosphere of 37 °C/5% CO₂ for 7 days. Drug-specific cytokine secretion was measured by ELISpot.

5.3.3 SMX-NO selectively polarizes drug specific IL-22 producing, but not IL-17 producing, T-cells from naïve precursors

To investigate the polarization of naïve T cells further, we cultured naïve T-cells with SMX-NO under Th22 and Th17 polarization conditions. When the priming assay was performed under Th22 polarizing conditions, a drug specific IL-22 secretion was observed (Figure 5.3 B). In contrast, SMX-NO-specific IL-17 secretion was not detected (Figure 5.3 A). When the naïve T-cells were primed with SMX-NO under Th17 polarizing conditions drug-specific IL-17 or IL-22 secretion was not detected. (Figure 5.3 A, B).

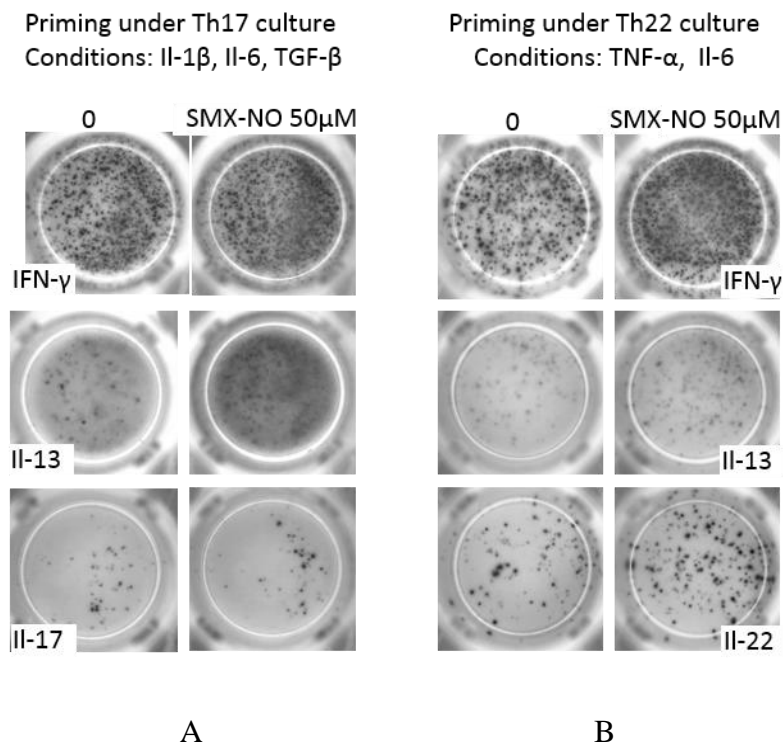


Figure 5.3 Cytokine secretion by T-cells primed to SMX-NO under Th17 and Th22 polarizing conditions. Naïve CD3⁺ T-cells were co-cultured with SMX-NO and dendritic cells at a ratio of 25:1 in the presence or absence of Th17 or Th22 polarizing cytokines for 8 days. The cultures were then plated and re-stimulated with fresh dendritic cells and SMX-NO. Antigen-specific T-cell responses were measured by IFN-γ, IL-13, IL-17 and IL-22 ELISpot. Cells were incubated for 48h and spots developed by ELISpot according to manufacturer's instruction.

5.3.4 Characterization of SMX-NO specific T-cell clones isolated from SMX hypersensitive patients PBMC and normal donors following T-cell priming.

SMX-NO-specific TCCs were generated from 4 SMX hypersensitive patients and 1 drug-naïve donor. We next characterized and compared the drug specific T-cell clones generated from hypersensitive patients to those generated from normal donors using the thymidine proliferation assay. All the clones tested showed a significant proliferation when exposed to SMX-NO (Figure 5.4 a). The number of clones generated from hypersensitive patients, their CD phenotype and the SMX-NO-specific proliferative response are summarized in Table 5.2. All the clones generated expressed the CD4 cell surface marker. Two hundred and eighty three T-cell clones were generated from drug-naïve donors following SMX-NO priming. SMX-NO responsive clones secreted high levels of IFN- γ , and to lesser extent, IL-5 and IL-13 following activation (Figure 5.4 b). There was no major difference in the drug-specific cytokine secretion profile when clones isolated from patients and volunteers were compared. The drug-specific proliferation of 68 T-cell clones from 4 patients and of 10 clones from naïve volunteers is shown in Figure 5.4.

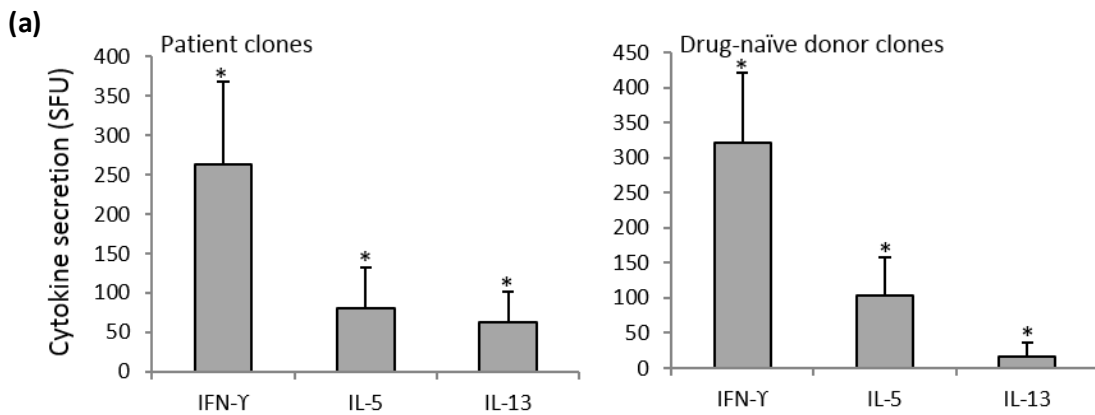
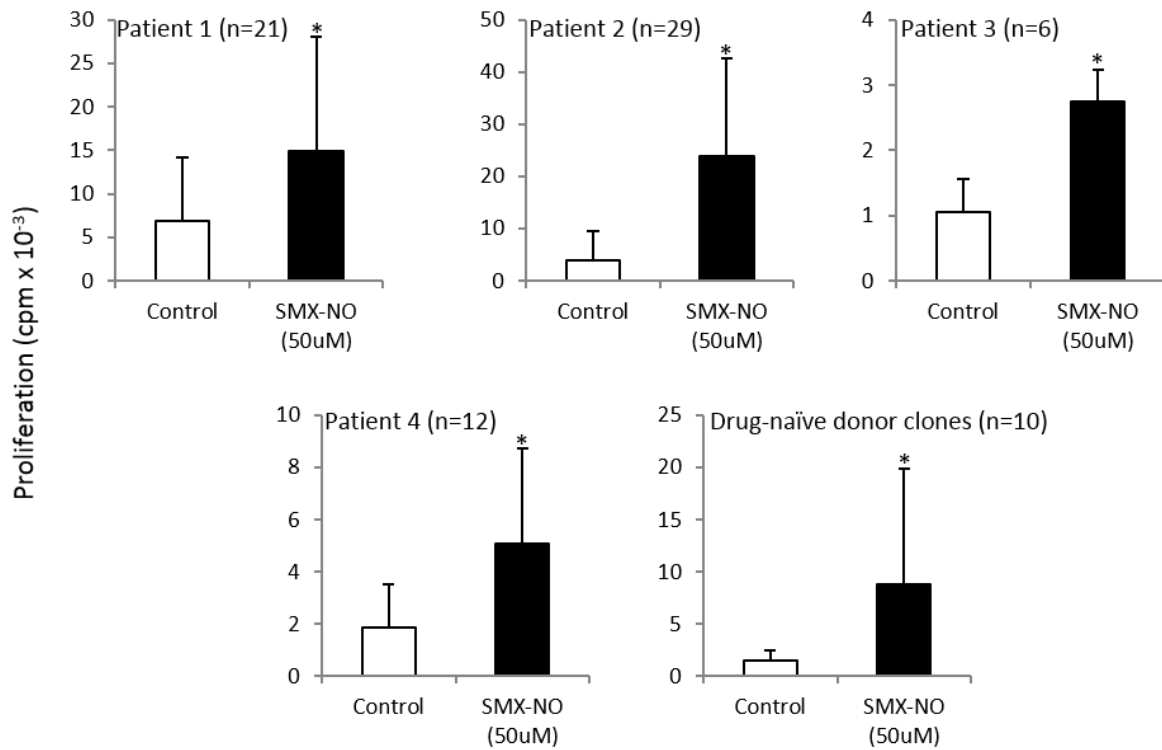


Figure 5.4 Activation of SMX-NO-responsive CD4⁺ clones generated from drug naïve donors (n = 1) after in vitro priming and from hypersensitive patients (n=4). The Antigen-specific responses were measured by [³H]-thymidine proliferation (a) and IFN-γ, IL-13, IL-17 and IL-22 ELISpot (b). The data shows mean and the standard deviation of triplicate cultures and paired t test has been used to perform comparisons. *p<0.05 was considered positive.

(b)

Patient ID	Clone tested (n)	SMX-NO specific clones	Phenotype (%) CD4 ⁺	Phenotype (%) CD8 ⁺	Proliferation (cpm)	
					Control	SMX-NO
1	336	21	100	-	6840 ±7406	14837 ±13224
2	394	29	100	-	3899 ±5522	23970 ±18651
3	216	6	100	-	1058 ±203	2740 ±469
4	240	2	100	-	1584 ±493	7613 ±5294

Table 5.2. The origin, phenotype and specificity of T-cell clones generated from PBMCs of the 4 SMX hypersensitive patients.

5.3.5 Cytokine profile of SMX-NO specific T-cell clones isolated from patients or drug primed T-cell populations

Since hypersensitive patient clones and clones isolated from *in vitro* priming secreted similar Th1 and Th2 cytokines a panel of 17 clones (from multiple donors) with a strong growth pattern were selected to study their cytokine secretion profile. While approximately all the clones produced IFN- γ , IL-5 and IL-13, about 50% of clones were found to secrete IL-22 following exposure to SMX-NO. In contrast, IL-17 secretion was only detected with 1 clone (Figure 5.5 A). IL-22 secreting low and high clones were isolated from PBMC of hypersensitive patients and following *in vitro* priming (Figure 5.5 B). Importantly, the isolation of SMX-NO responsive, IL-22 secreting clones from the priming assay was not dependent on the presence of Th22 polarizing cytokines and clones were not maintained under Th22 polarizing conditions. Most of the clones also produced granzyme B and FasL but not the perforin (Figure 5.5).

A. Cytokine secretion from 17 SMX-NO specific CD4+ T-cell clones

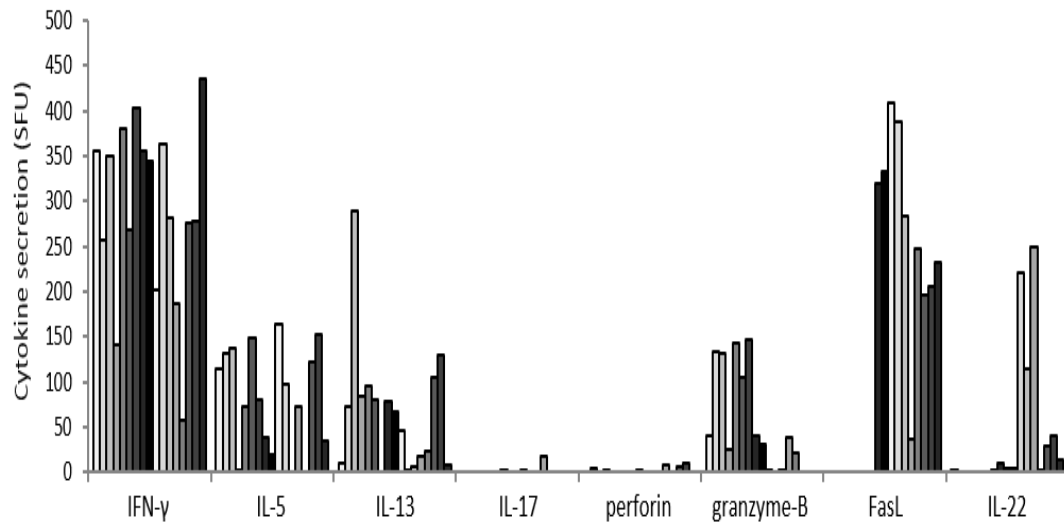


Figure 5.5 A Cytokine secretion by SMX-NO responsive CD4⁺T cell clones. Analysis of SMX-NO-specific cytokine and cytolytic molecule secretion from m 17 clones using the ELISpot

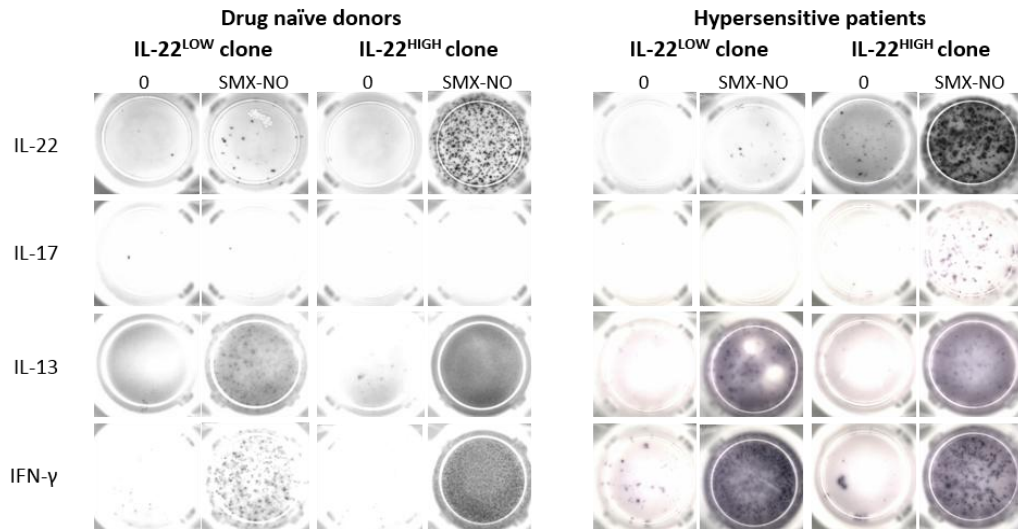


Figure 5.5 B Representative T-cell clones with IL-22 high or low secretion, generated from SMX-NO primed naïve T-cell population and drug hypersensitive patients. Cytokine profile of representative SMX-NO-responsive IL-22^{high}- and IL-22^{low}-secreting clones generated from drug-naive donors after *in vitro* priming and hypersensitive patients.

5.4 Discussion

Previous immuno-histological studies have characterized the phenotype of T-cells infiltrating inflamed skin of patients with maculopapular rashes, and have reported a larger numbers of CD4⁺ T-cells and lower numbers of CD8⁺ T-cells (Pichler 2003; Pichler et al., 2002). Studies focusing on SMX-specific T-cell response indicate that CD4⁺ and CD8⁺ T-cells secrete cytolytic molecules in response to culprit drug and keratinocytes can be specifically killed by CD4⁺ T-cells (Schnyder et al., 1998). Consistent with these findings, several studies have demonstrated that most SMX-NO-specific T-cells isolated from hypersensitive patients are CD4⁺, and secrete mixed panel of Th1/Th2 cytokines including IFN- γ , IL-5 and IL-13 (Schnyder et al., 1998; Elsheikh et al., 2011; Schnyder et al., 2000). However, the discovery of new T-cell populations, such as Th17, Th22 renders this classification obsolete, and the importance of these new Th cell subsets in drug hypersensitivity is still unknown. For this reason, we characterized SMX-NO specific CD4⁺ T-cells in terms of cytokine profiling from hypersensitive patients and healthy donors following priming. Following antigen re-challenge, the SMX-NO primed T-cells from healthy donors and drug hypersensitive patients were found to secrete IFN- γ , IL-13 and IL-22, but IL-17 secretion was not detected. CD4⁺ clones isolated from the priming assay also secreted drug-specific IFN- γ , IL-13 and IL-2, but not IL-17.

IL-22 secretion was detected from approximately 50% of the clones from patients (Figure 5.5A). A similar pattern of cytokine secretion was seen with drug-specific T-cell clones (IFN- γ ^{high} IL-5^{high} IL-13^{high} IL-22^{low} and IFN- γ ^{high} IL-5^{high} IL-13^{high} IL-22^{high}) isolated from SMX hypersensitive patients (Figure 5.5B). IL-22 is a cytokine that modulates tissue epithelia responses as expression of the IL-22 receptor is restricted to the non-haematopoietic cells. In skin, the IL-22 receptor is expressed at high levels on keratinocytes and IL-22 has been found to

enhance keratinocyte proliferation and inhibit terminal differentiation (Boniface et al., 2005). Furthermore, IL-22 has been shown to mediate inflammatory responses in patients with psoriasis and IL-22 secreting cells have been identified in patients with allergic contact dermatitis (Cavani et al., 2012; Eyerich et al., 2010). Thus, IL-22 is a pleotropic cytokine and its functions are likely context-dependent. Our data is, however, the first to demonstrate the production of IL-22 alongside IFN- γ by antigen-specific T-cells from drug hypersensitive patients. More works are required to reveal the physiological and pathological function of IL-22 in drug hypersensitivity. In particular, it will be interesting to know if the detection of IL-22 is dependent on the different cytokine-specific transcription factors or signaling pathways.

However, there was only one IL-17 secreting clone being detected out of 17 clones. Therefore these result of my project indicate that IL-17 may not play an important in drug induced MPE. Typically, IL-17 play important roles in chronic or/and autoimmune disease with a recruitment of neutrophils by stimulating keratinocytes to produce neutrophilia chemokines such as IL-8/CXCR8 and CXCR1 (Schroder et al., 1992, Nograles et al., 2008). It has been shown that IL-17 secreting cells are involved in severe drug hypersensitivity such as carbamazepine induced AGEP (Kabashima et al., 2011) and SJS/TEN (Kang et al., 2011). Our results are consistent with the signature of MPE, in which there is a mild skin rash with less infiltration of neutrophils and a quick recovery after drug withdrawal. Polarization condition has been known to determine the fate of T-cells from naïve T-cells. In my study, although strong signal of Th17 polarization were exerted, drug specific IL-17 secretion was not detected, which indicates this drug-antigen-MHC-complex displayed by DCs may not tend to stimulate a Th17 response.

The low ratio of IL-17 secretion was also consistent with Lochmatter study in 2009 in which they showed IFN- γ and IL-13 are sensitive markers of drug hypersensitivity but not IL-17. They

cultured PBMCs of 6 patient with MPE with culprit drug for 72 hours, all the patients showed high fold (dozens to hundreds) of drug specific cytokine secretion of IL-13 and IFN- γ but only 3 patients showed drug specific IL-17 secretion, with low fold of 6 to 8 (Lochmatter et al., 2009).

To summarize, drugs such as SMX-NO and piperacillin can polarize drug-specific T-cells *in vitro* and *in vivo* in the presence of antigen-presenting cells. These T-cells express drug specific cytokines, in particular IL-22, which suggests that IL-22 may play a critical role in drug hypersensitivity.

Chapter 6

Priming na ν e T-cells to drug haptens and the characterization of drug-specific antigen presentation

6.1 Introduction

6.2 Methods

6.3 Results

6.3.1 Priming of Na ν e T-cells with SMX-NO, piperacillin and flucloxacillin

6.3.2 Mechanism of activation of drug-specific T-cell clones

6.4 Discussion

6.1 Introduction

Delayed-type drug hypersensitivity is mediated by T-cells, has an onset time of more than one hour after drug administration (Bousquet et al., 2007), and is considered pharmacologically unpredictable and unrelated to the concentration of the drug administered. These adverse reactions can target a wide range of organs and therefore pose a serious threat to patient health as they can induce potentially fatal responses including drug-induced liver injury (DILI) and severe cutaneous reactions such as toxic epidermal necrolysis. A range of drugs are known to induce this type of reaction including the penicillin antibiotics such as piperacillin and flucloxacillin, and sulfonamides such as sulfamethoxazole (SMX). The mechanism of drug antigen presentation of the exemplar drugs, SMX (Schnyder et al., 2000, Naisbitt et al., 1999), piperacillin (Whitaker et al., 2011) and flucloxacillin (Monshi et al., 2013), are thought to be similar and follow the pathway outlined by hapten hypothesis. However, it has also been reported that drug specific memory T-cells isolated from patients can respond to certain drug antigens in the absence of antigen presentation, indicating the potential for drugs to stimulate T-cells via the p.i concept; directly and without the need for processing (Schnyder et al., 2000, Wullemin et al., 2013, El-Ghaiesh et al., 2010). These before mentioned studies have focused on responses using allergic patient T-cells, allowing for the determination of the memory T-cell response to drug antigens. However, the use of patient T-cells provides little insight into the priming of naïve T-cells at the moment a susceptible individual is sensitized. Thus, to determine the mechanism of naïve T-cells activation by SMX-NO, piperacillin or flucloxacillin we utilized an established *in vitro* priming assay, whereby drugs are cultured with naïve T-cells and autologous mature dendritic cells isolated from healthy donors before secondary stimulation of the primed T-cells to perform a battery of readouts assays to determine antigen responsiveness. Furthermore, previous patient

studies have also reported that T-cell responses to piperacillin are highly specific in that piperacillin-specific T-cells do not cross-react with other antibiotics with similar chemical structures (El-Ghaiesh et al., 2010). Other studies report similar findings for SMX-NO specific T-cells, detailing a lack of cross-reactivity with alternative antibiotics (Schnyder et al., 2000). In contrast, flucloxacillin specific T-cells have previously been shown to cross react with several antibiotics including amoxicillin and piperacillin (Monshi et al., 2013). Therefore, using the naïve T-cell priming assay, we also investigate drug antigen cross-reactivity to determine whether highly structurally specific T-cells are readily primed *in vitro*.

Several methods are available to investigate the mechanisms by which drugs activate T-cells (i.e., hapten mechanism or direct binding to MHC molecules). First, antigen presenting cell (APC) pulsing assays can be performed whereby cells are cultured with drugs for different durations. The drug-treated APCs are then washed repeatedly to remove free drug, reconstituted in drug-free medium and added to T-cells as a source of antigen. If T-cells respond, they must be activated by a drug protein adduct. Secondly, APCs can be fixed with glutaraldehyde, which blocks protein processing, before pulsing with antigen. If T-cells are subsequently stimulated with the drug, the response is likely through a direct interaction of the drug with surface MHC molecules. Thirdly, T-cell assays can be performed with MHC class I and/or class II blocking antibodies to confirm that the drug-derived antigen is presented in the context of MHC. Finally, Glutathione (GSH) can be utilized to block SMX-NO protein binding to cysteine, and N-acetyllysine (NAL) can be used to reduce the likelihood of piperacillin and flucloxacillin binding protein at lysine residues, thereby reducing the formation of drug-protein adducts. Thus glutathione or N-acetyl lysine can be added to T-cell assays to potentially block T-cell responses

to drug haptens. We have utilized each of these techniques to investigate the mechanism of SMX-NO, piperacillin and flucloxacillin-specific naïve T-cell priming using cells from healthy donors.

6.2 Methods

To determine the propensity of drug antigen to prime naïve T-cells, we used different concentrations of SMX-NO, piperacillin and flucloxacillin. Western blotting was used to study whether SMX-NO could alter piperacillin or flucloxacillin protein binding when these three drug antigens are cultured with dendritic cells and naïve T-cells in one environment. T-cell clones were then generated from the initial priming cultures to assess whether priming to one drug antigen allows for cross reactivity to alternate drug antigens. To investigate the mechanisms of antigen presentation using the priming assay, several experiments were performed: APC fixation and pulsing assays were performed with T-cell clones to investigate whether the activation of the T-cells requires adduct formation and antigen processing. MHC blocking assays were used to determine whether T-cell activation was HLA restricted.

6.2.1 T-cell priming assay. The priming assay model is described in 2.15.

6.2.2 ELISpot. To test the specificity of drug primed T-cell clones ELISpot was applied following the protocol described in 2.14.

6.2.3 T-cell cloning. To study the drug specificity and drug antigen presentation, drug specific T-cells were generated using the protocol described in 2.5.

6.2.4 T-cell proliferation assay. The concentration-dependent T-cell response was determined using [³H]-thymidine to measure proliferation as described in section 2.8.

6.2.5 Western blotting. To test the drug protein binding in the serum and/or cells, western

blotting was applied following the protocol described in 2.13.

6.2.6 APC fixation was applied following the protocol introduced in 2.18.

6.2.7 SMX-NO, piperacillin, and flucloxacillin cross reactivity. SMX-NO, piperacillin and flucloxacillin specific activation of T-cell clones was tested following the protocol described in section 2.8.

6.2.8 APC pulsing for proliferation assay was to study the mechanism of drug antigen presentation. The protocol is described in 2.10.

6.2.9 MHC blockage experiment using blocking antibody was used to study the MHC restriction in drug specific T-cell response. The procedure is described in 2.9.

6.2.10 Glutathione and N-acetyl lysine blocking assay was used to ascertain the drug-protein binding site in priming assay.

6.2.11 Varied responses by T-cell clones are common due to a multitude of factors such as varied growth conditions, differing drug-antigen specificity or TCR affinity. Subsequently, Mann-Whitney tests were performed to show the significance of data in comparison with control. $P < 0.05$ was considered statistically significant.

6.3 Results.

6.3.1 Healthy donor naïve T-cell priming to SMX-NO, piperacillin, and flucloxacillin

Healthy donor naïve ($CD3^+$, $CD45RO^-$) T-cells were cultured with autologous mature DCs and SMX-NO (50 μ M), piperacillin (2mM), or flucloxacillin (2mM) for 8 days before restimulation of T-cells using fresh mature autologous DCs and drug antigen. T-cell antigen-specificity and cross-reactivity was determined by analyzing T-cell proliferation and IFN- γ secretion in response to all three drug antigens irrespective of the drug-antigen present during initial priming culture.

As flucloxacillin-induced liver injury is strongly associated with the HLA-B*57:01 genotype (Daly, Donaldson et al., 2009), donors who were HLA-B*57:01⁺ were recruited in this study to assess comparative responses to those who were HLA-B*57:01⁻. Analysis of IFN- γ secretion using ELISpot showed that naïve T-cells from all four donors were successfully primed to SMX-NO and piperacillin. These SMX-NO or piperacillin-primed T-cells showed no cross-reactivity in response to culture with piperacillin or SMX-NO, respectively, or the third alternate drug antigen flucloxacillin. Flucloxacillin failed to prime naïve T-cells from healthy donors, irrespective of the presence of HLA-B*57:01, as determined by cytokine secretion (Figure 6.1 and Table 6.1). Due to higher cell recovery from certain donors after priming, proliferative responses were able to be additionally measured in response to drug antigen by thymidine incorporation. Similar to ELISpot, proliferative studies revealed the successful priming of naïve T-cells from all drug-naïve healthy donors to SMX-NO, and for 6/7 donors to piperacillin. In contrast to measuring cytokine secretion, naïve T-cells from 2/6 donors were successfully, albeit weakly, primed to flucloxacillin. As before, none of the drug-antigen primed T-cells proliferated upon re-challenge with SMX-NO, piperacillin or flucloxacillin (Figure 6.2). In total 16 priming assays were conducted. The rate of positive priming tested by proliferation assay and ELISpot is summarized in Table 6.1 and Table 6.2. Figure 6.1 and 6.2 show representative data from donors who displayed positive responses to the different drug antigens.

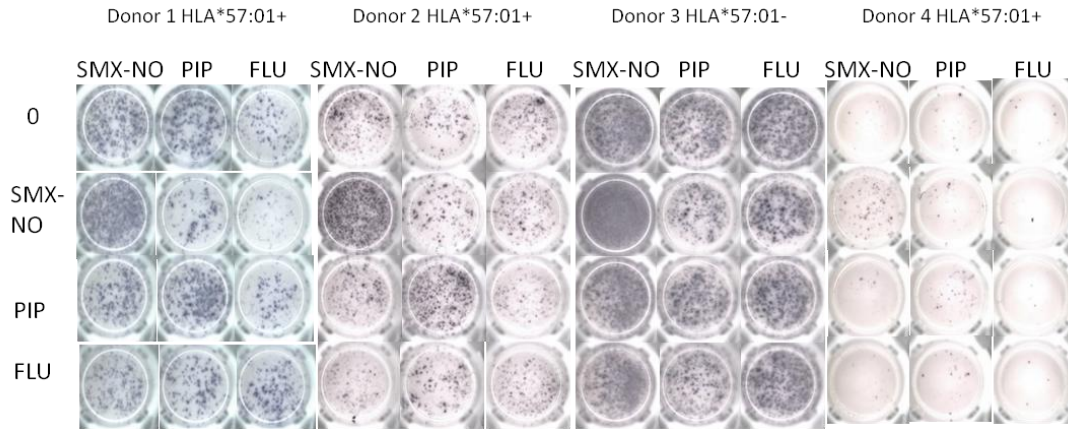


Figure 6.1 Specificity of drug primed naïve T-cells from HLA-B*57:01 positive or negative donor tested by ELISpot. Naïve CD3 T-cells were co-cultured with DCs at a ratio of 25:1 in the presence of either SMX-NO (50 μ M), piperacillin (2 mM), or flucloxacillin (2 mM) for 8 days in a 24-well plate. 1×10^5 T-cells were then restimulated with 4×10^3 fresh DCs and SMX-NO (50 μ M) or piperacillin (2 mM), or flucloxacillin (250 mg/ml) in an ELISpot plate pre-coated with IFN- γ capture antibody. Cells were cultured at 37 $^{\circ}$ C under an atmosphere of 5% CO₂ for 2 days and then developed according to manufacturer's instructions and spots visualized under an AID ELISpot reader.

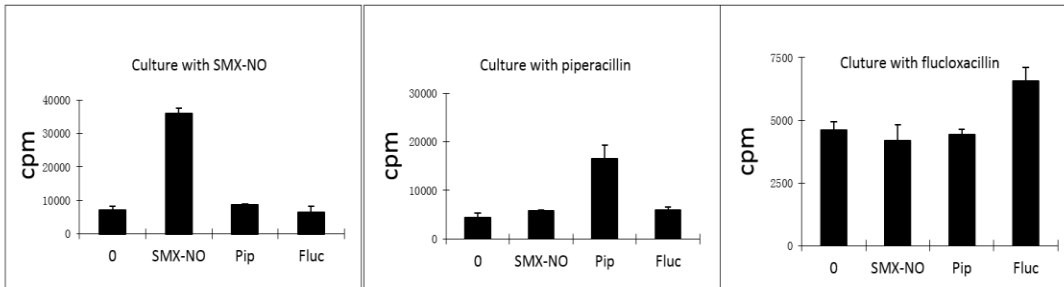
	Donor 1 HLA*B 57:01+			Donor 2 HLA*B 57:01+			Donor 3 HLA*B 57:01-			Donor 4 HLA*B 5701+		
Culture with	SMX-NO	PIP	FLU	SMX-NO	PIP	FLU	SMX-NO	PIP	FLU	SMX-NO	PIP	FLU
0	-	-	-	-	-	-	-	-	-	-	-	-
SMX-NO	++	-	-	+++	-	-	+++	-	-	++	-	-
PIP	-	+	-	-	++	-	-	+	-	-	+	-
FLU	-	-	-	-	-	-	-	-	-	-	-	-

Table 6.1 The summary table for the ELISpot results in the Figure 6.1.

The ELISpot results were semi quantified as follow: the numbers of spots in the non-stimulated control as -, +: 40 spots more than the control. ++: 80 spots more than the control. +++: 120 spots more than the control.

Overall, SMX-NO and piperacillin were much more effective at priming naïve T-cells than flucloxacillin (table 6.2). These data highlight that measurement of proliferative response is a more sensitive technique to ascertain antigen-specific T-cell priming than IFN- γ secretion.

Donor 1



Donor 2

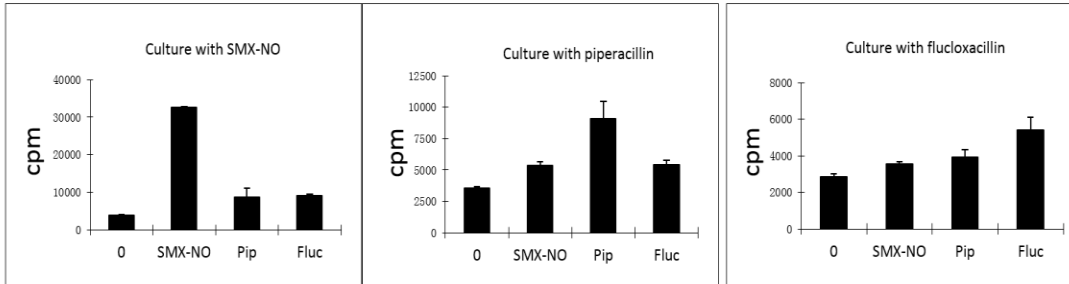


Figure 6.2 Drug specific induction of T-cell proliferation by SMX-NO and piperacillin but not flucloxacillin.

The drug primed T-cells were challenged with particular drug. Donor 1 and donor 2 primed naive T-cells were used for the proliferation assay. Drug primed naive T cells (5×10^4) were co-cultured with DCs (4×10^3) with either SMX-NO (50 μ M), piperacillin (2mM) or flucloxacillin (2mM) in a similar manner as described in the ELISpot assay above in an U-bottomed 96-well plate. The plate was cultured for 2 days and [3 H]-thymidine (0.5Ci/well) was added to each well in the final 16 hours of culture. T-cell proliferation was measured by scintillation counting. The data shows mean and the standard deviation of triplicates. The SI>2 can be observed, indicating a drug specific response.

Number of priming assay (numbers)	SMX-NO	piperacillin	Flucloxacillin
IFN- γ ELISpot	7/16	6/16	0/16
Positive/Total			
Proliferation	7/7	6/7	2/6
Positive/Total			

Table 6.2. Summary table showing the rate of positive priming assays. Drug reaction of primed T-cells was tested by proliferation assay and IFN- γ ELISpot. The positive rate of priming assays is shown as: the number of positive assays / total assays.

Further analysis of drug-responsive T-cell phenotype and function warranted the generation of T-cell clones. As previously stated, due to the association of HLA-B*57:01 with flucloxacillin-induced hepatitis, two HLA-B*57:01 positive donors were recruited for priming and the subsequent generation of drug-specific T-cell clones. These two donors produced only weakly positive responses to flucloxacillin after priming, with proliferative SI of 1.8 and 1.2. In contrast, a third donor selected for T-cell cloning was HLA-B*57:01 negative. From the original priming cultures, a serial dilution was performed and after mitogen-driven expansion, T-cell clones were generated. Well growing T-cell clones were selected for further investigation of drug-antigen specificity and to assess the mechanisms of drug antigen presentation.

Table 6.3 shows 1) the number of the SMX-NO, piperacillin and flucloxacillin-specific clones generated from 3 priming assays; 2) the phenotype of these drug specific T-cell clones; 3) the drug reaction of these clones. SMX-NO-, piperacillin- and flucloxacillin-specific T-cell clones were generated from all three donors irrespective of the HLA type. As has been previously described, the major phenotype of SMX-NO-specific T-cell clones were CD4⁺ T-cells, whereas for piperacillin-specific clones, the majority were CD8⁺. Flucloxacillin-responsive CD4⁺ and CD8⁺ T-cell clones were generated from all three donors.

Donor genotype & T-cell clone specificity		Total clones (n)	Reactive clones (n)	CD4 (%)	CD8 (%)	Mixture of CD4 and CD8 T-cell	Proliferation assay (cpm)	
Donor ID and genotype	Drug specificity						Control	Drug
Donor 1 HLA-B*57:01+	SMX-NO	480	68	48%	40%	12%	6519 ±7063	13797 ±9703
	PIP	426	49	15%	80%	5%	5682 ±3792	23419 ±16374
	FLU	322	8	37%	63%	0	2351 ±504	9372 ±1635
Donor 2 HLA-B*57:01+	SMX-NO	546	57	55%	36%	9%	4638 ±6917	16279 ±9028
	PIP	280	25	17%	79%	4%	5627 ±4826	19063 ±17352
	FLU	246	3	33%	67%	N/A	3621 ±418	8904 ±1395
Donor 3 HLA-B*57:01-	SMX-NO	208	26	64%	28%	8%	3947 ±763	16872 ±11372
	PIP	162	18	12%	84%	4%	3592 ±473	8496 ±4281
	FLU	156	2	50%	50%	N/A	2950 ±316	5742 ±407

Table 6.3. Generation of drug-specific T-cell clones and phenotypic characterization. The number of the total and the SMX-NO, piperacillin and flucloxacillin-specific clones generated from 3 priming assays were further

characterized. PIP: piperacillin, FLU: flucloxacillin.

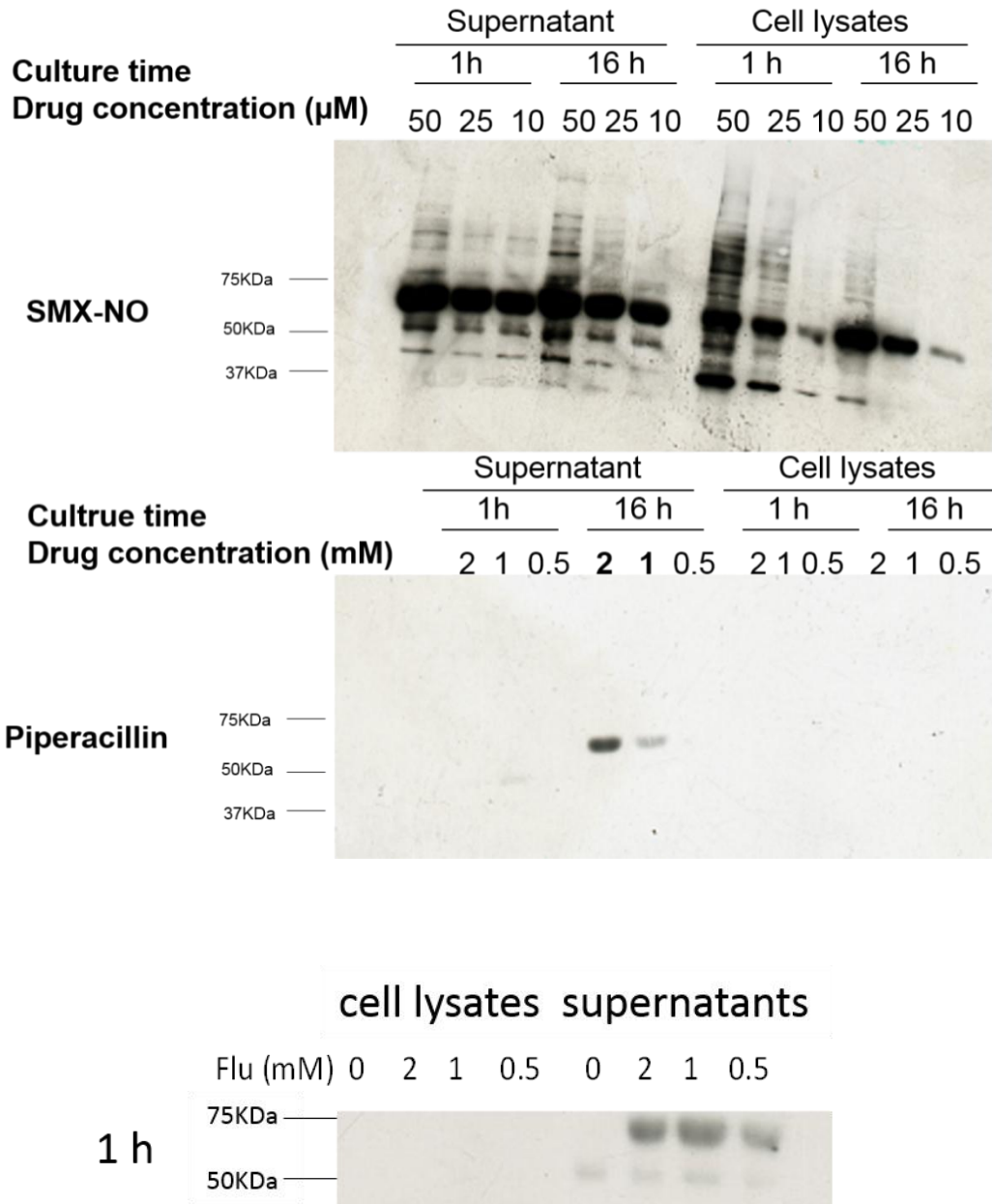


Figure 6.3. Analysis of the time- and concentration- dependent binding of SMX-NO, piperacillin and flucloxacillin to intracellular and extracellular proteins by western blotting. Various concentrations of piperacillin and flucloxacillin, from 0.5 mM to 2 mM and SMX-NO, from 10 μM to 50 μM were co-cultured with antigen presenting cells in serum containing medium for 1 hour or 16 hours under an atmosphere of 37°C/5% CO₂. The proteins were separated by SDS-polyacrylamide gel and transferred onto nitrocellulose membrane and incubated with anti-SMX, anti-piperacillin or anti-flucloxacillin antibodies. Western blotting performed in triplicate after a 16-hour culture of flucloxacillin was not published due to overwhelming non-specific binding, including in the control. The protocol of flucloxacillin western blotting was described in section 2.13. However, our previous result detailing that flucloxacillin only binds to supernatant protein rather than to cell lysate protein was repeatable in the lab. Therefore, together with the 1h culture results, the result described above was reliable.

To determine whether any drug-protein complex is located in the serum and/or cells, western blotting was performed using cell culture supernatant or cell lysates, respectively. The drug-protein complex was identified by the enhanced molecular weight (MW) of the drugs in the Western blotting assay. The MW of SMX-NO, piperacillin and flucloxacillin is 267.26 Da, 517.55 Da, and 453.87 Da, respectively. Our data show that for all three drugs, the drug-protein MW is approximately 50K Da to 75K Da. We also show that while piperacillin and flucloxacillin bind selectively to protein in the serum, SMX-NO binds to both serum and cellular proteins (Figure 6.3). The binding of drug antigens to extracellular protein was found to be both time and concentration dependent. In contrast, while the binding of SMX-NO to B-cell APC lysate is also concentration dependent, it was not time dependent (Figure 6.3). In detail, our data highlights comparable signals between a culture length of 1 hour and 16 hours, but a marked difference between 10 μ M and 50 μ M SMX-NO when using cell lysates.

To further study the mechanisms of drug-specific T-cell activation antigen specificity was assessed using thymidine incorporation assays to measure antigen-specific proliferation. T-cell clones were found to proliferate significantly in the presence of the drug antigen to which they were originally primed, but displayed a complete lack of cross-reactivity to the two alternate drug antigens (Figure 6.4). These results mirror those obtained direct from the priming cultures.

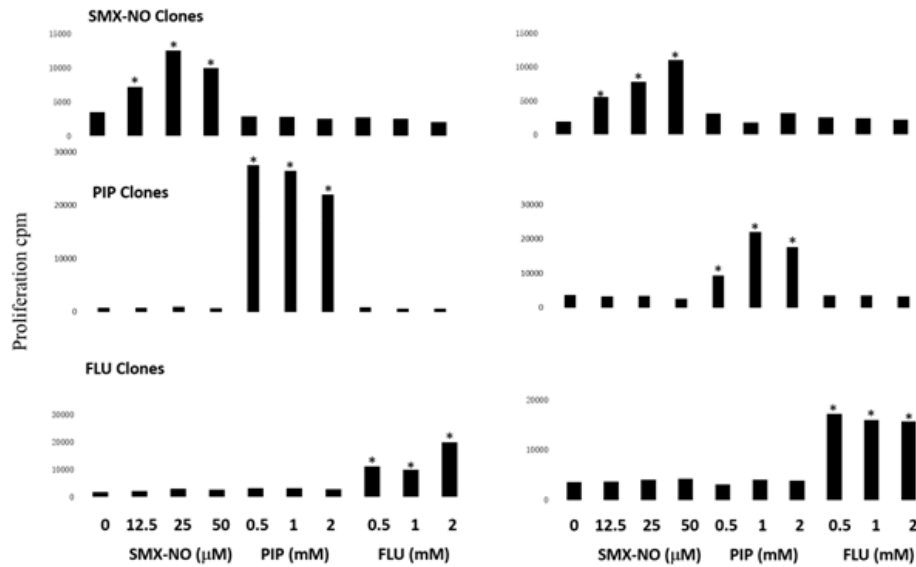


Figure 6.4 T-cell clones that are reactive to SMX-NO, piperacillin or flucloxacillin do not show cross reactivity against the other two drugs. 5×10^4 T-cell clones were co-cultured with autologous B-cell APCs (1×10^4) in the presence of SMX-NO (from 12.5 mM to 50 mM) or piperacillin (from 0.5 mM to 2 mM) or flucloxacillin (from 0.5 to 2 mM) under an atmosphere of 5% CO_2 at 37 °C for 3 days and [^3H] thymidine was added in the final 16 hours. Proliferation was measured by scintillation counting. The data shows mean of triplicate or duplicate wells depend on cells availability. Mann-Whitney test was applied. * $p < 0.05$ compared with control.

6.3.2 Mechanism of activation of drug-specific T-cell clones

There are three main theories describing how drug-antigens can stimulate a TCR, namely the hapten theory, p.i. concept, and the more recently described altered peptide hypothesis. To determine the relevant mechanism of TCR activation for individual drug antigens we performed a series of assays. First, we assessed the requirement for antigen processing to stimulate antigen-specific responses by comparing T-cell stimulation when using standard APCs in comparison to those pre-cultured in the fixative glutaraldehyde. While fixation renders the cell intrinsic antigen-processing machinery non-functional, cell surface molecules including MHC remain intact and thus the cells maintain the ability to facilitate direct TCR-MHC interactions. SMX-NO, piperacillin and flucloxacillin-specific T-cell clones generated from drug-naïve donors were cultured with normal or glutaraldehyde-fixed APCs. T-cell proliferation in response to all three drug-antigens was abolished when using the fixed APCs compared with the irradiated APCs used as control (Figure 6.5). These data indicate that the activation of T-cells in response to SMX-NO, piperacillin and flucloxacillin-derived antigens requires the processing and subsequent presentation of antigen by functional APCs.

Second, HLA restriction was performed. EBV-transformed B-cells which we utilize as immortalized APCs to assess antigen-specificity of T-cell clones express both MHC class I and II molecules at the cell surface which, if they respond to antigen, stimulate CD8⁺ and CD4⁺ T-cells, respectively. To explore the functional relevance of the MHC we cultured EBV-transformed B-cells with MHC I or MHC II blocking antibodies for 30 minutes, so preventing their binding to antigen, before subsequent culture with antigen-responsive T-cell clones and assessment of proliferation in response to drug. As expected CD4⁺ T-cell clone responses were all inhibited by the presence of anti-MHC II antibodies, shown in Figure 6.6 and 6.7 where 2 representative

SMX-NO-specific T cell clones, 2 piperacillin-specific T-cell clones and 2 flucloxacillin-specific T-cell clones are shown. Two CD8⁺ flucloxacillin-specific T-cell clones were also studied which both showed MHC class I molecule restricted activation (Figure 6.7).

Third, to further study the mechanisms of antigen presentation, drug antigen pulsing assays were performed. Pulsing in this context refers to the exposure of APCs to drug antigen for a defined time period, allowing any potential antigen uptake to occur, before performing multiple washing steps to remove free antigen prior to culture with T-cell clones. Any subsequent antigen-specific T-cell response, due to the lack of any additional free antigen added during the culture with T-cell clones, must be induced by antigen that has been taken up and processed by the APCs. Furthermore, to determine the length of culture period required for sufficient antigen uptake and processing, separate batches of APCs were co-cultured with drug antigen for 1 hour, 4 hours and 16 hours. After an extensive washing procedure with HBSS, the now antigen pulsed APCs were cultured with T-cell clones for 3 days and proliferation measured using thymidine incorporation. Our data, displayed in figure 6.8, shows that all T-cell clones responsive to SMX-NO, piperacillin, or flucloxacillin proliferated in the presence of drug-antigen pulsed APCs without the presence of soluble antigen. For SMX-NO-specific clones, a highly proliferative response was seen when APCs had been cultured with SMX-NO for as little as 1 hour, suggesting that protein binding occurs rapidly (Figure 6.8). The response to flucloxacillin-specific T-cell clones was more varied. While some clones produced a weak response upon stimulation with pulsed APCs, other clones reached the maximum response when APCs were pulsed with flucloxacillin for 1 hour again indicating the potential for rapid protein binding and neoantigen formation (Figure 6.8). On the other hand, although piperacillin-specific T-cell clones produced responses when cultured with APCs that had been pulsed with antigen for 1 hour, longer pulses led to

increased proliferative responses with a pulse of up to 16 hours to reach the maximum response (Figure 6.9).

Lastly, to form a drug antigen SMX-NO is known to bind to the cysteine residues of protein, whereas piperacillin and flucloxacillin bind to the lysine residues. To further characterize and confirm the propensity for SMX-NO, piperacillin and flucloxacillin to form protein conjugates before stimulating a drug antigen-specific response, we used glutathione and N α -acetyl-L-lysine to block protein binding before culture with T-cell clones. Specifically, glutathione (GSH) which contains a reactive cysteine residue was utilized to block SMX-NO protein binding. Separately, N α -Acetyl-L-lysine (NAL) was used to competitively bind to the β -lactam antibiotics thereby blocking protein binding. Our data shows that SMX-NO-mediated T-cell responses were totally inhibited by GSH but not NAL confirming a requirement for the binding of SMX-NO to cysteine residues in proteins before T-cell stimulation (Figure 6.10). In stark contrast, piperacillin responses were down regulated by NAL but not GSH indicating a requirement for piperacillin-protein conjugates to form at lysine residues (Figure 6.11). For flucloxacillin-specific clones, the antigen-induced T-cell response was decreased by NAL (Figure 6.12).

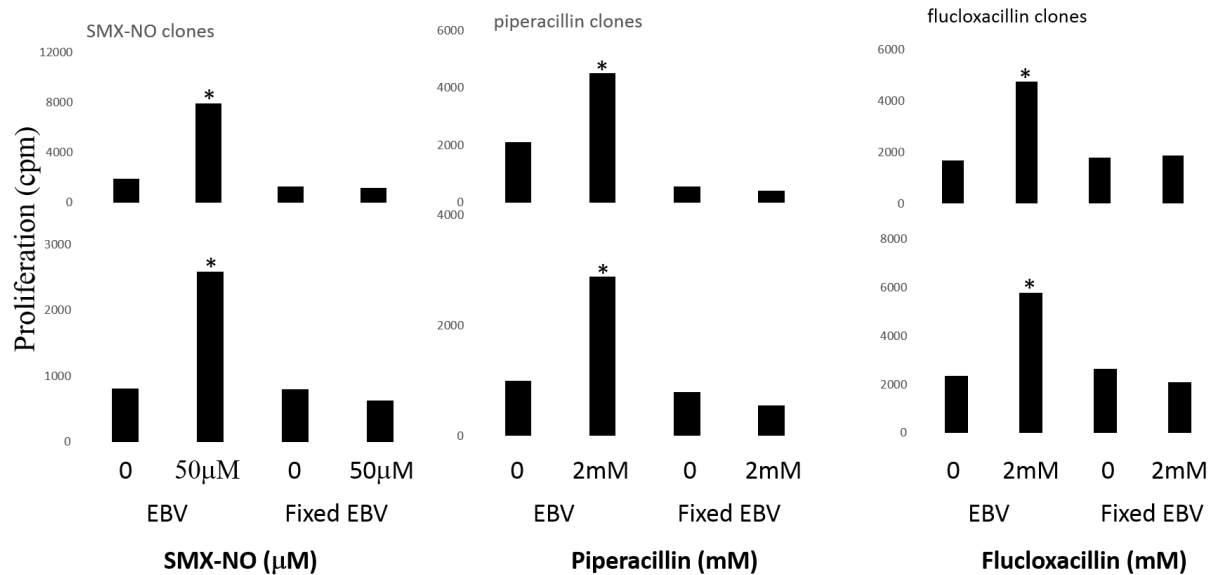


Figure 6.5 SMX-NO, piperacillin and flucloxacillin primed naïve T-cells do not proliferate when exposed to drug antigen in the presence of glutaraldehyde fixed APCs. Autologous EBV-transformed B-cells (2×10^6) were washed twice in HBSS and resuspended in 1ml HBSS. Glutaraldehyde (12.5 μ l, 2%) was then added and the cells agitated for 30 seconds. Glycine (1ml, 1M) was quickly added and cells were agitated for a further 45 seconds. The cells were resuspended in 10ml T-cell medium after extensive washing (3 times, T-cell medium). Glutaraldehyde-fixed B-cell APCs (1×10^4 , 50 μ l) were then added to T-cell clones (5×10^4) in the presence or absence SMX-NO (50mM), piperacillin (2mM) or flucloxacillin (2mM). Control APCs were the irradiated EBV-transformed B-cells. Cells were cultured for 3 days at 37°C with [3 H] thymidine added for the final 16h. Proliferation was determined by scintillation counting. The data shows mean of triplicate or duplicate wells depend on cells availability. Mann-Whitney test was applied. *p<0.05 compared with control.

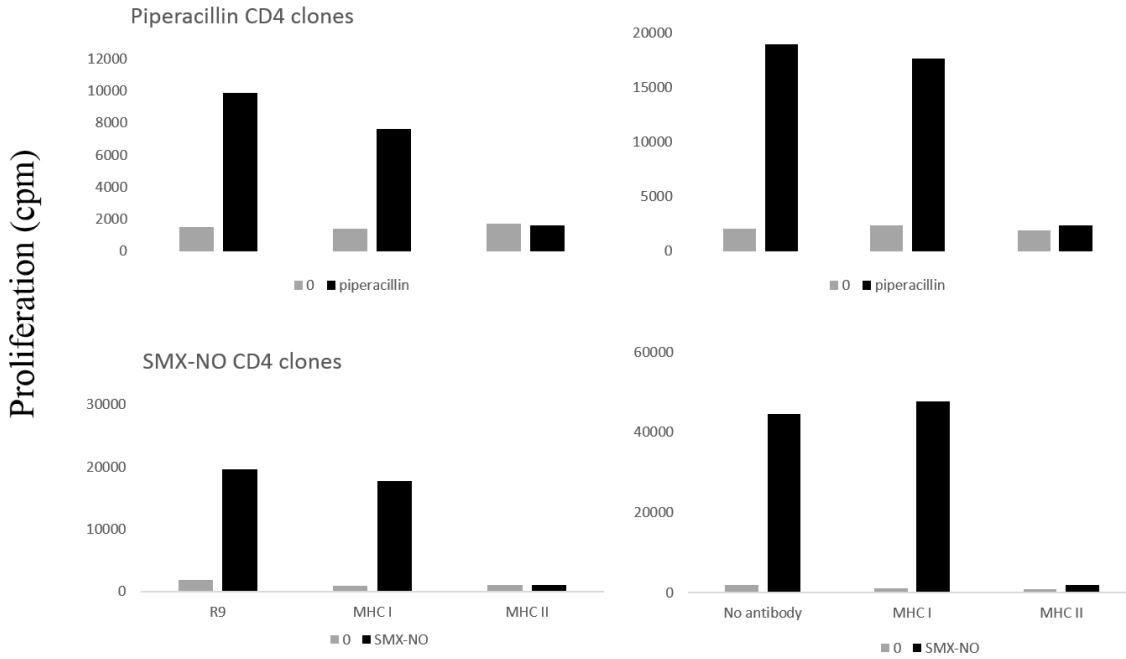


Figure 6.6 **Activation of piperacillin and SMX-NO specific T-cell clones were HLA class II restricted.** MHC class I/II blocking antibodies (5 µg/ml) were incubated with 10⁴ cells/well of autologous APCs for 30 min at 37 °C in an atmosphere of 5% CO₂. 5 x 10⁴ T-cell clones/well and medium or drug solution (piperacillin 2 mM, SMX-NO 50 µM) was then placed in each well. The cultures were incubated under atmosphere of 5% CO₂ at 37°C for 3 days and [³H] thymidine was added in the final 16 hours. Proliferation results were measured by scintillation counting. The data shows mean of triplicate or duplicate wells depend on cells availability.

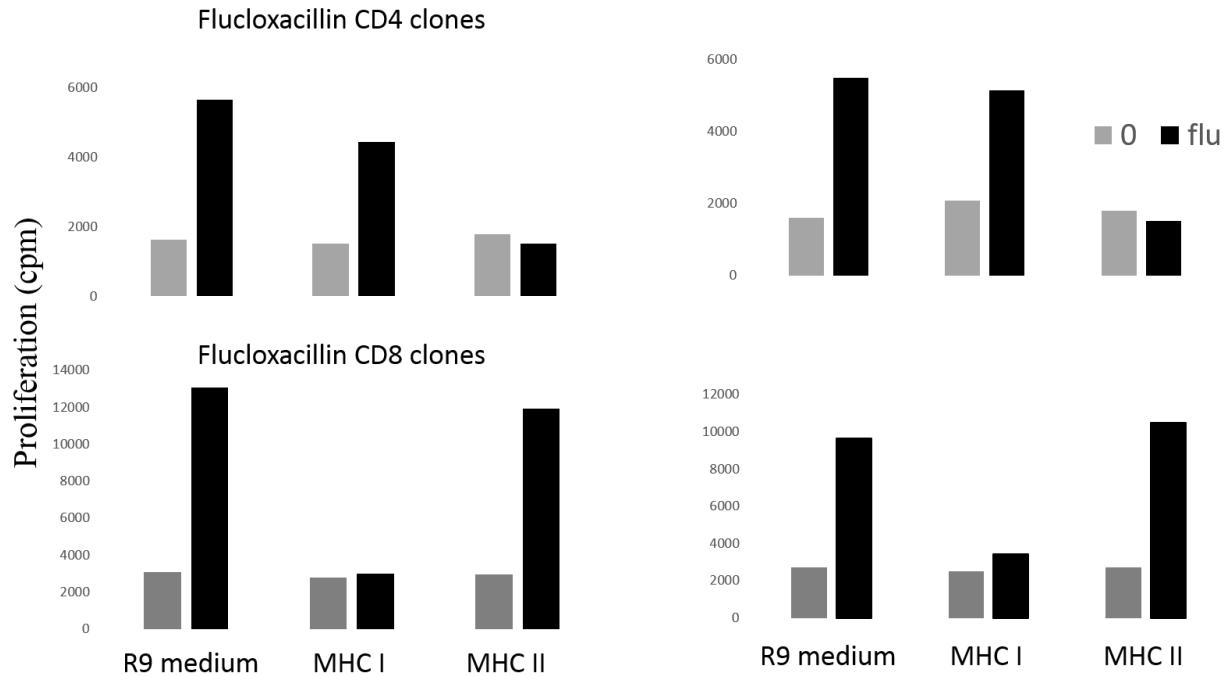


Figure 6.7 CD4⁺ and CD8⁺ flucloxacillin specific T-cell clones are MHC class II and I restricted, respectively. MHC class I/II blocking antibodies (5µg/ml) were incubated with 10⁴ cells/well of autologous APCs for 30 min at 37 °C with atmosphere of 5% CO₂. 5 x 10⁴ T-cell clones/well and medium or drug solution flucloxacillin (2mM) was then placed each well. The cultures were incubated under atmosphere of 5% CO₂ at 37°C for 3 days and [³H] thymidine was added in the final 16 hours. Proliferation results were measured by scintillation counting. The data shows mean of triplicate or duplicate wells depend on cells availability.

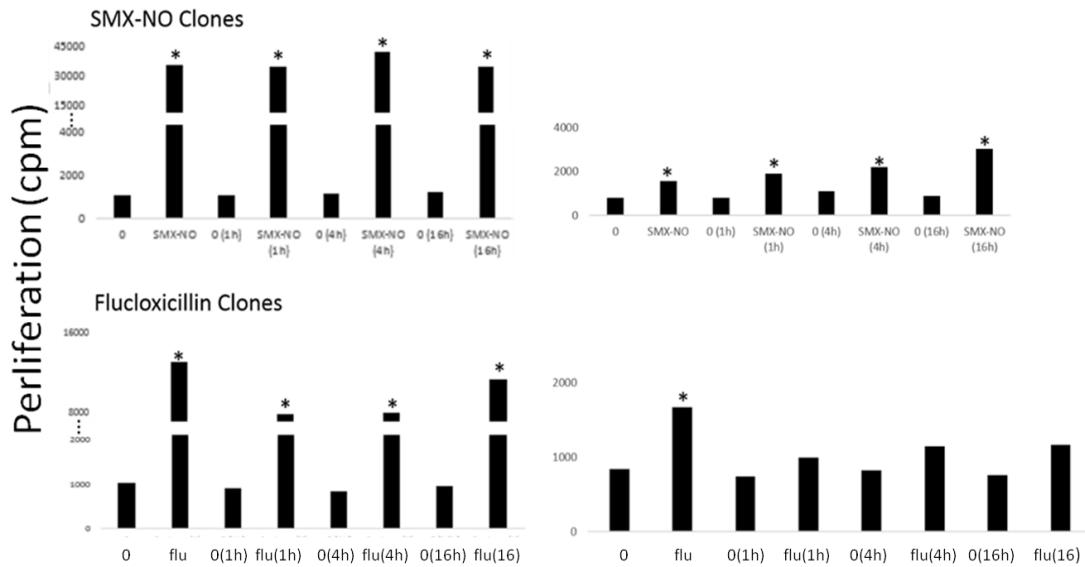


Figure 6.8 APCs pulsed with SMX-NO or flucloxacillin are able to generate strong proliferative responses in a time-dependent manner. APCs were incubated with flucloxacillin (2mM) and SMX-NO (50 μ M) for 1h, 4h, 16h and then extensively washed (3 times) to remove free drug. This is called pulsing the APCs. 5×10^4 T-cell clones/well were cultured with drug pulsed APCs (1×10^4 cells/well) and drugs in duplicate in 96 well plates for 3 days at 37 $^{\circ}$ C in an atmosphere of 5% CO₂. Proliferation was measured by [³H]-thymidine incorporation. The data shows mean of triplicate or duplicate wells depend on cells availability. Mann-Whitney test was applied. *p<0.05 compared with control.

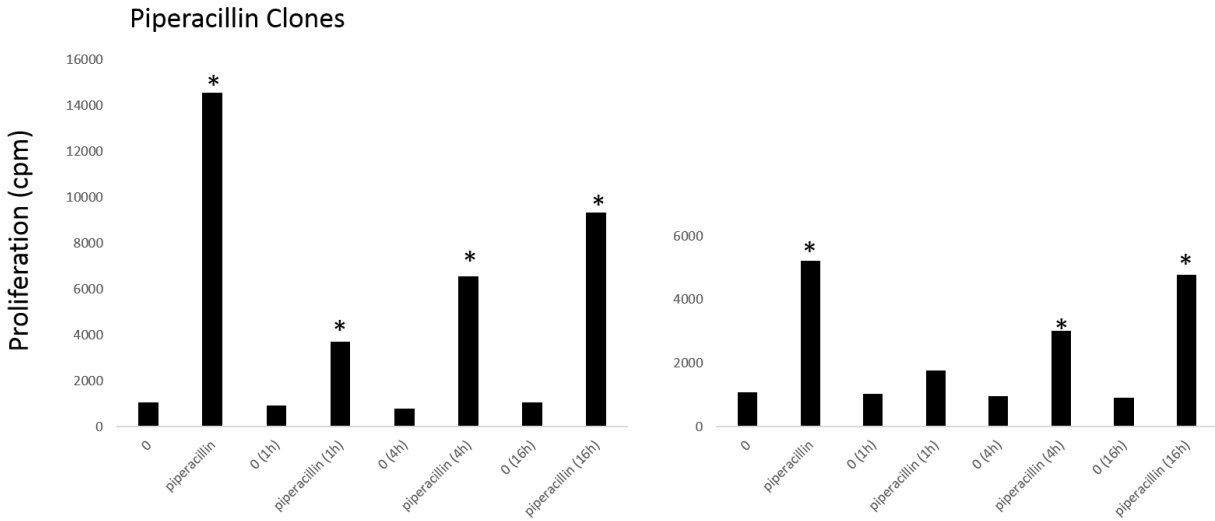


Figure 6.9 APCs pulsed with piperacillin for 16 hours are able to trigger antigen specific T-cell responses that are almost comparable with soluble drug. APCs were incubated with piperacillin (2mM) for 1h, 4h, 16h and then extensively washed (3 times) to remove the free drug. 5×10^4 T-cell clones/well were cultured with drug pulsed APCs (1×10^4 cells/well) and drugs in duplicate in 96 well plates for 3 days at 37 °C in an atmosphere of 5% CO₂. Proliferation was measured by [³H]-thymidine incorporation. The data shows mean of triplicate or duplicate wells depend on cells availability. Mann-Whitney test was applied. *p<0.05 compared with control.

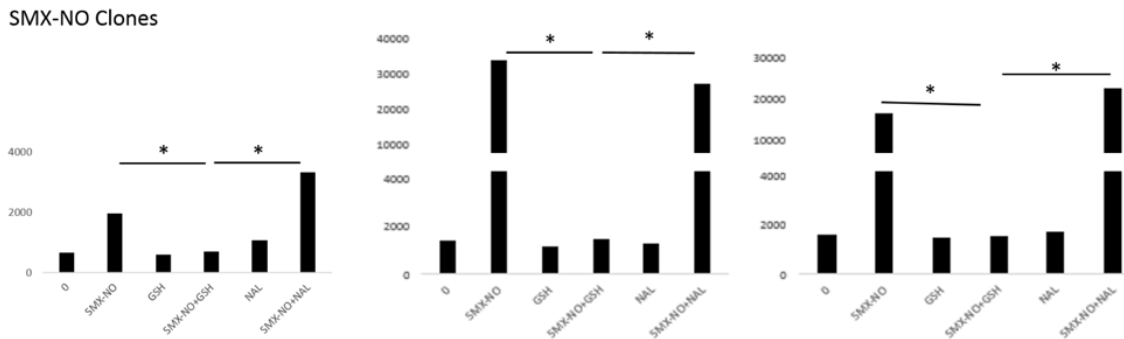


Figure 6.10 SMX-NO specific T-cell activation was blocked by GSH but not by NAL. T-cell clones (5×10^4 cells, 50 μ l) isolated from priming assay were cultured with EBV-transformed B-cell APCs (1×10^4 cells, 50 μ l) in the presence of GSH and NAL under the atmosphere of 5% CO₂ for 3 days and [³H]-thymidine (0.5Ci/well) was added to each well in the final 16 hours of culture. T-cell proliferation was measured by scintillation counting. The data shows mean of triplicate or duplicate wells depend on cells availability. Mann-Whitney test was applied. *p<0.05 compared with control.

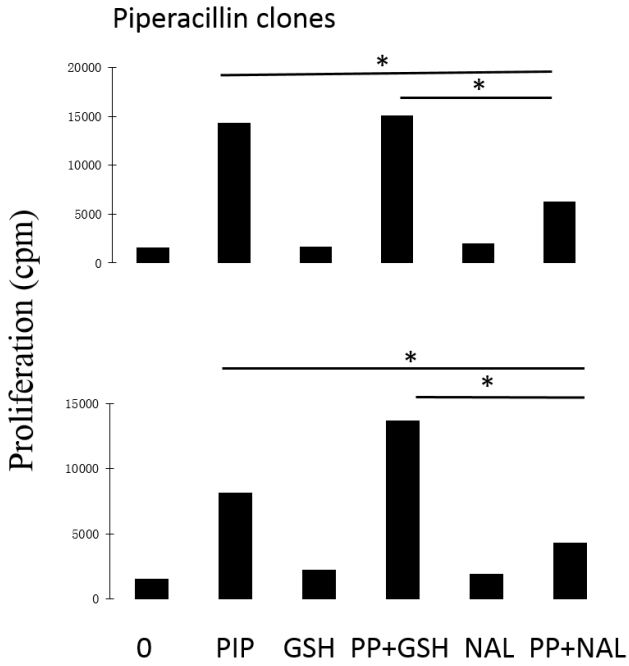


Figure 6.11 Piperacillin specific T-cell activation was blocked by NAL but not by GSH. T-cell clones (5×10^4 cells, $50 \mu\text{l}$) isolated from priming assay were cultured with EBV-transformed B-cell APCs (1×10^4 cells, $50 \mu\text{l}$) in the presence of GSH and NAL under the atmosphere of $5\% \text{ CO}_2$ for 3 days and [^3H]-thymidine (0.5Ci/well) was added to each well in the final 16 hours of culture. T-cell proliferation was measured by scintillation counting. The data shows mean of triplicate or duplicate wells depend on cells availability. Mann-Whitney test was applied. * $p < 0.05$ compared with control.

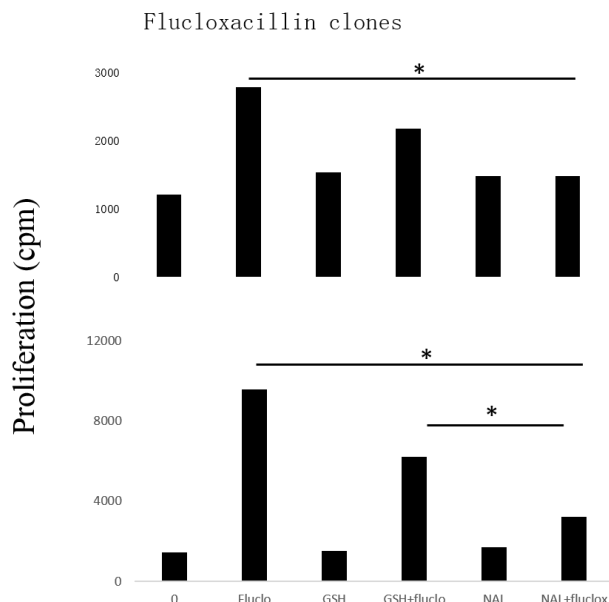


Figure 6.12 Flucloxacillin specific T-cell activation was blocked by NAL but not by GSH. Flucloxacillin specific T-cell clones (5×10^4 cells, $50 \mu\text{l}$) isolated from priming assay were cultured with EBV-transformed B-cell APCs (1×10^4 cells, $50 \mu\text{l}$) in the presence of GSH and NAL under the atmosphere of 5% CO_2 for 3 days and [^3H]-thymidine (0.5 Ci/well) was added to each well in the final 16 hours of culture. T-cell proliferation was measured by scintillation counting. The data shows mean of triplicate or duplicate wells depend on cells availability. Mann-Whitney test was applied and $*p < 0.05$ compared with control.

6.4 Discussion

Currently, it is almost impossible to predict whether a novel chemical entity will cause hypersensitivity during the drug development process. This is because validated *in vitro* methods using lymphocytes from human donors to characterize the drug immunogenicity do not exist. Furthermore, animals do not develop hypersensitivity reactions that mimic the human condition. Thus, *in vivo* experiments provide very little information regarding a compounds sensitization potential. We have recently developed an *in vitro* T-cell priming assay using PBMC from healthy human donors, and SMX-NO as a model drug metabolite immunogen, to investigate naïve T-cell

responses to drug antigens (Faulkner, Martinsson et al. 2012, Monshi, Faulkner et al. 2013, Gibson, Ogeese et al. 2014). The assay relies on the presentation of the drug antigen by autologous dendritic cells as these are the only professional APCs thought able to prime naïve T-cells. These dendritic cells are generated through a week-long culture of CD14⁺ monocytes isolated from peripheral blood with GM-CSF and IL4-supplemented R9 medium. 24 hours prior to harvesting, the dendritic cells are additionally cultured with lipopolysaccharide from E.Coli and TNF in order to promote the establishment of a mature phenotype leading to an increased ability to engulf antigen and the upregulation of cell surface co-stimulatory molecules to promote T-cell stimulation. Therefore, this enforced maturation stage allows this *in vitro* assay to bypass the role played by danger signaling *in vivo*. Once primed, the T-cells can then be restimulated *in vitro* and T-cell responses measured using readouts for proliferation and cytokine release. Furthermore, individual T-cells can be expanded and tested for drug antigen specificity. This assay allows us to investigate mechanisms of drug antigen presentation using T-cells from healthy drug-naïve donors and determine how these naïve T-cell responses induced *in vitro* relate to responses seen in hypersensitive patients. In this study we focused on three drug-antigens, SMX-NO, piperacillin, and flucloxacillin, which are strongly linked with hypersensitivity reactions to explore mechanisms of T-cell activation and TCR cross-reactivity.

Drug hapten-specific T-cell activation is presumed to occur via a classical hapten mechanism where the drug must first form a protein adduct. The drug antigen-protein conjugate is then presumed to be taken up by APCs and processed into peptide fragments. To activate T-cells, the peptide fragments must bind to endogenous MHC molecules before they are transported to the cell surface where they can be presented to passing T-cells. T-cells from patients with several forms of chemical sensitization and drug hypersensitivity have been shown to be activated via a

hapten mechanism (Brander, Mauri-Hellweg et al. 1995, Pickard, Smith et al. 2007, Castrejon, Berry et al. 2010, Jenkinson, Jenkins et al. 2010, El-Ghaiesh, Monshi et al. 2012). However, T-cells may also be activated by drugs and chemicals through a direct interaction with surface MHC molecules via the pharmacological interaction (PI) pathway that does not require antigen processing (Schnyder, Mauri-Hellweg et al. 1997, Schnyder, Burkhart et al. 2000, Wu, Sanderson et al. 2006, Ko, Chung et al. 2011, Adam, Eriksson et al. 2012). This is the proposed mechanism of T-cell activation by sulfamethoxazole as it is able to do so in the presence of fixed APCs, which are incapable of processing antigen (Elsheikh et al., 2011). In recent years, some have argued that haptenic drugs might preferentially activate T-cells via this mechanism (Sieben, Kawakubo et al. 2002, Wullemin, Adam et al. 2013, Wullemin, Terracciano et al. 2014, Yaseen, Saide et al. 2015) but also that the PI pathway is likely more associated with the activation of memory rather than naïve T-cells as this mechanism suits responses dependent upon lower activation thresholds, i.e. those cells that have been previously stimulated (Adam et al 2011, Pichler et al 2011). Therefore the mechanism of TCR activation reported by the studies mentioned above, which refer to those in patients and thus memory T-cell responses, may be different to those during naïve T-cell priming. Thus, to investigate the mechanisms involved in naïve T-cell activation, T-cell priming assays were conducted using SMX-NO, piperacillin, and fluxcloxacillin, each of which has been shown to covalently modify proteins (Callan, Jenkins et al. 2009, Jenkins, Meng et al. 2009, Whitaker, Meng et al. 2011, Ogese, Jenkins et al. 2015) and induce T-cell responses in hypersensitive patients (Castrejon, Berry et al. 2010, Elsheikh, Castrejon et al. 2011, El-Ghaiesh, Monshi et al. 2012, Monshi, Faulkner et al. 2013) and so are ideal candidate antigens to investigate SMX-NO and piperacillin effectively primed naïve T-cells from healthy drug-naïve donors in 7/7 and 6/7 donors, respectively, as determined by measuring

proliferation after antigen-specific restimulation of T-cells. While the secretion of IFN- γ was also assessed using a higher number of donors, a much lower proportion of donors showed antigen-specific cytokine secretion after naive T-cell priming indicating a known lack of sensitivity of the ELISpot compared to thymidine incorporation (Porebski et al 2011). Unlike piperacillin hypersensitivity, flucloxacillin-induced liver injury is associated with the expression of HLA-B*57:01 (Daly, Donaldson et al. 2009) and a recent study from our lab has shown the ability to prime naive T-cells from healthy donors expressing this allele (Monshi, Faulkner et al., 2013). Despite this association, HLA-B*57:01 is clearly not the only predisposing factor as only 1 in 500-1000 individuals who express this risk allele go on to develop flucloxacillin-induced hypersensitivity after drug administration (Daly et al., 2009). Nonetheless, T-cell priming with flucloxacillin was attempted using cells from both HLA-B*57:01 positive and negative donors. While no IFN- γ secretion was detected, similarly weak proliferative responses were detected in two donors after naive T-cell priming. Interestingly, these responses occurred irrespective of the presence of HLA-B*57:01. Importantly, the lack of a flucloxacillin-specific T-cell response was not related to inadequate protein adduct formation as flucloxacillin-modified proteins were detected in our binding study. In order to characterize the phenotype and function of antigen-responsive T-cells we subsequently generated T-cell clones from the priming cultures of two HLA-B*57:01 positive and one negative donor, allowing us to extract and utilize just the antigen-responsive cells. Both CD4⁺ and CD8⁺ T-cell clones were generated that responded to SMX-NO, piperacillin, or flucloxacillin from all three donors. However, those clones that were beta-lactam specific formed a predominantly CD8⁺ population, reflecting the majority phenotype of T-cell isolated from the blood of beta lactam allergic patients (Hertl et al, 1993). A plethora of published articles are available that detail the cross-reactivity, or lack of, between

drugs that bind directly to the MHC molecules (Pichler 2003, Pichler 2005, Schnyder and Pichler 2009, Adam *et al* 2011). In the case of β -lactam antibiotics, Mauri-Hellweg and colleagues found that while some T-cell clones from allergic patients were cross reactive with other beta-lactams, a second subset were highly specific (Mauri-hellweg *et al*, 1996). More recently, Monshi *et al* detailed similar variability regarding cross reactivity in patient clones where some flucloxacillin-specific T-cell clones proliferated strongly in response to piperacillin exposure (Monshi *et al*, 2013). In our study using cells from healthy donor naïve T-cell priming, all clones were found to be highly specific with a distinct lack of cross reactivity between panels of SMX-NO, piperacillin, and flucloxacillin-responsive clones.

In order to determine the mechanism of naïve TCR activation for each drug antigen, we first had to establish the propensity for each drug antigen in question to form a hapten by binding to either extracellular or intracellular protein. We found that SMX-NO was able to form haptens by binding to protein from either the cell lysates or the supernatant, while hapten formation regarding flucloxacillin or piperacillin was restricted to serum proteins. The formation of hapten intermediates by beta lactam antibiotics *in vitro* mirrors the scene depicted *in vivo* by early experiments in the 1960s detailing that the serum of allergic patients contained antibodies responsive to benzylpenicilloyl-modified protein structures (Levine and Price, 1964; Siegel and Levine, 1964). Attempts to identify these drug-protein conjugates as the culprit antigens that trigger the immune response were made. If the conjugates induced a T-cell response, mass spectrometry could then be employed to characterize the exact identity of the processed antigens that are formed during the priming assay, thus providing direct evidence of hapten-induced T-cell activation *in vitro*. However, when using established piperacillin-albumin conjugate synthesized

in our lab to stimulate piperacillin-specific T-cells, we were unable to maintain the T-cell clones due to either a time-dependent loss of antigenicity or poor growth. We found that primed piperacillin-specific T-cell clones may lose their antigen-specificity in just one and a half months. Further work will be required to utilize this strategy.

A battery of established assays were then utilized to determine the nature of the drug-specific T-cell response including; the use of glutaraldehyde-fixed APCs to assess the requirement for antigen processing, HLA restriction using MHC class I and II blocking antibodies, antigen-pulsing to determine the ability of free drug antigen to activate T-cells but also the length of exposure time required for sufficient processing of antigen by APCs, and the use of GSH and NAL to identify the specific binding of drug antigen to either protein cysteine or lysine residues, respectively. We found that all three drug antigen required uptake and processing by APCs in order to stimulate T-cells as no response could be detected in the presence of fixed APCs, and stimulation of CD4⁺ and CD8⁺ T-cells was MHC class II and I restricted, respectively. Interestingly, the SMX-NO responsive clones were activated with APCs pulsed with the drug for as little as 1 hour, which coincides with the previously reported almost instantaneous binding of the drug metabolite to protein (Naisbitt, Hough et al. 1999, Naisbitt, Farrell et al. 2002, Callan, Jenkins et al. 2009). In contrast, β -lactam antibiotics bind to protein in a time-dependent manner (Whitaker, Meng et al. 2011, El-Ghaiesh, Monshi et al. 2012) and in general, a longer (4-16 hour) APC pulse was required to activate T-cells. Collectively these mechanistic studies indicate that T-cells responsive to all three drug antigens were activated by a hapten mechanism and that hapten formation was able to readily activate naïve T-cells *in vitro*. Hence, hapten formation in patients should be considered an important risk factor. However, this leads to the intriguing

puzzle as to why exposure to drugs such as paracetamol is not associated with a high incidence of hypersensitivity reactions. Paracetamol is metabolized in the liver to a reactive quinoneimine intermediate, which binds irreversibly to hepatic proteins and in overdose causes acute liver failure. However, these haptenated proteins are not “recognized” by the adaptive immune system and hypersensitivity reactions are reported infrequently.

In summary, we show that highly structurally-specific T-cells are readily primed *in vitro* when autologous functionally mature dendritic cells present antigenic determinants, derived from haptenic structures bound covalently to protein, to naïve T-cells. The T-cell priming assays allows us to effectively determine the functional mechanisms by which distinct drug antigens activate the immune system by utilizing T-cells from healthy donors. This bypasses the difficulty of obtaining access to patient samples, and importantly allows for the investigation of the naïve T-cell response rather than the memory T-cell responses we detect in pre-sensitized responsive individuals. Furthermore, the availability of such an *in vitro* assay, once developed further to incorporate several donors on a single test plate, might represent an effective strategy for pharmaceutical companies to screen the immunogenicity of novel chemical entities thus enhancing the safety profile of future compounds.

Chapter 7

7.1 Final discussion.

Drug hypersensitivity represents an impediment to the drug development process and a burden on national health services. Although many reactions are mild and self-limiting, i.e., symptoms resolve after drug withdrawal, a limited number of patients develop serious and sometimes life-threatening conditions. Drug hypersensitivity is described as an idiosyncratic or type B hypersensitivity reaction as mechanisms generally remain unresolved. In most cases, no simple relationship is apparent between the dose of drug administered and the development of clinical symptoms, due to the curve of therapeutic drug doses and the curve of drug doses induce hypersensitivity are not in the same range. Despite this, it should be noted that most drugs associated with a high incidence of hypersensitivity are administered at high mass doses.

Immuno-histological studies of inflamed tissue of hypersensitive patients show an infiltration of T-cells. The T-cells have been isolated from the tissue, expanded in vitro, and shown to display reactivity against the drug the patient was exposed to at the time of the reaction. (Schnyder, Frutig et al. 1998, Yawalkar, Hari et al. 2000, Yawalkar, Shrikhande et al. 2000, Britschgi, Steiner et al. 2001, Nassif, Bensussan et al. 2002, Nassif, Bensussan et al. 2004, Ko, Chung et al. 2011) In mild conditions, CD4⁺ T-cells are believed to be the primary mediators of tissue injury, potentially inducing cell death indirectly through the release of cytokines that recruit phagocytes, or directly through the production of apoptosis-inducing cytolytic molecules. In more severe conditions, i.e., Stevens-Johnson syndrome and toxic epidermal necrolysis, granulysin-secreting cytotoxic CD8⁺ T-cells predominate. Phenotypic and functional characterization of drug-specific T-cells from hypersensitive patients led to the development of an expanded Coombs and Gell classification of hypersensitivity (Pichler, Yawalkar et al. 2002, Pichler 2003, Pichler 2003).

Therefore, it was possible to describe different clinical conditions according to the CD phenotype of the drug-specific T-cells and the cytokines/effector molecules they secreted. Importantly, this classification is somewhat obsolete as it does not encompass the new populations of T-cell that have been discovered over the last decade.

Identification of HLA alleles as important susceptibility factors for many forms of hypersensitivity has led to a renewed interest in understanding the nature of the drug interaction with immune receptors, in particular MHC molecules and specific T-cell receptors. The most progress has been made exploring whether HLA class I associations with particular forms of hypersensitivity relate to a specific fit of the drug-derived antigen within MHC class I molecules. For example, we now know that antigens derived from abacavir (HLA-B*57:01), (Mallal, Nolan et al. 2002) allopurinol (HLA-B*58:01), (Hung, Chung et al. 2005) carbamazepine (HLA-B*15:02 & HLA-A*31:01) (Chung, Hung et al. 2004, McCormack, Alfirevic et al. 2011) and flucloxacillin (HLA-B*57:01) (Daly, Donaldson et al. 2009) interact with a degree of selectivity with the HLA risk allele to activate T-cells from hypersensitive patients. (Chessman, Kostenko et al. 2008, Ko, Chung et al. 2011, Monshi, Faulkner et al. 2013, Yun, Mattsson et al. 2013, Lichtenfels, Farrell et al. 2014) Furthermore, for each of the drugs highlighted above, it is possible to activate T-cells from healthy drug-naïve donors with the drug if they carry the risk allele. (Chessman, Kostenko et al. 2008, Farrell, Lichtenfels et al. 2013, Monshi, Faulkner et al. 2013, Yun, Marcaida et al. 2014) Although genetic studies have identified several HLA class II associations with drug hypersensitivity e.g., amoxicillin-clavulanate, (Donaldson, Daly et al. 2010) lapatinib, (Spraggs, Budde et al. 2011, Spraggs, Parham et al. 2012) ximelagatran (Kindmark, Jawaid et al. 2008), as yet functional studies have not as yet been able to relate the activation of T-cells to restriction of the fit of the drug-derived antigen within the MHC molecule

encoded by the HLA risk allele.

Does the association between specific hypersensitivity reactions and MHC proteins provide mechanistic information on how T-cells are likely to be triggered? The simple answer is no. Traditionally, drugs are thought to activate T-cells via 2 pathways. Pathway 1 (i.e., hapten hypothesis) involves the formation of drug-protein adducts as the initiation step. This is followed by protein processing and release of drug-modified peptides that are believed to bind directly to MHC molecules prior to triggering T-cells (Brander, Mauri-Hellweg et al. 1995, Padovan, Bauer et al. 1997). Pathway 2 (i.e., PI concept) involves direct binding of drugs to the peptide-loaded MHC molecules expressed on the surface of antigen presenting cells. The drug-MHC binding interaction is reversible, but sufficiently stable to trigger T-cell responses (Zanni, von Greyerz et al. 1998, Schnyder, Burkhart et al. 2000). From this brief discussion one can see that the species interacting with T-cell receptors is similar for both pathways (i.e., a MHC peptide drug complex). The main differences are (1) the nature of the drug peptide binding interaction and (2) the way in which the drug is transported to the MHC peptide binding groove.

Studies using cells from healthy donors and hypersensitive patients who carry specific HLA risk alleles has enhanced our understanding of drug MHC binding interactions and gone some way to address the suppositions associated with the hapten hypothesis and PI concept. Carbamazepine and allopurinol (or more precisely the metabolite oxypurinol) bind directly to surface MHC molecules and activate T-cells via a PI mechanism (Lichtenfels, Farrell et al. 2014, Yun, Marcaida et al. 2014). Flucloxacillin, a β -lactam antibiotic that forms adducts with lysine residues on protein directly, activates T-cells from patients with liver injury via a classical hapten mechanism (Monshi, Faulkner et al. 2013). However, it should be noted that T-cells from healthy donors expressing HLA-B*57:01 are activated in vitro under conditions of excess antigen by

both hapten and PI pathways (Wuillemin, Adam et al. 2013, Yaseen, Saide et al. 2015). Finally, ground-breaking studies from 3 independent research groups exploring abacavir HLA binding devised a new pathway of drug-dependent T-cell activation. (Illing, Vivian et al. 2012, Norcross, Luo et al. 2012, Ostrov, Grant et al. 2012) The authors demonstrated that abacavir binds directly to endogenous HLA-B*57:01 (within antigen presenting cells) and alters the structure of the peptide binding groove. As such, with time, an altered repertoire of HLA binding self-peptides is displayed on the surface of the antigen presenting cell. It is assumed that a portion of these peptides cross-react with pathogen-derived peptides and activate pre-existing memory T-cells in susceptible patients and ultimately the clinical condition of abacavir hypersensitivity. Initially, it was assumed that many drugs might activate T-cells via this pathway; however, despite intensive studies as yet a second example has not been forthcoming. This is not so surprising as for most forms of drug hypersensitivity, even those with an HLA allele association, patients expressing multiple HLA alleles go on to develop hypersensitivity indicating that the presence of the risk allele increases the likelihood of developing hypersensitivity, but is not the sole predisposing factor.

Notwithstanding the above discussion several forms of drug hypersensitivity are not linked to expression of a particular HLA allele. Most forms of β -lactam and sulphonamide hypersensitivity reactions can be included in this list. The sulphonamides have been studied most extensively and to date HLA risk alleles have not been identified. (Alfirevic, Vilar et al. 2009) One explanation for this might be the high incidence of reactions seen in patients exposed to these classes of drug. For example, sulfonamide reactions are seen in 30-50% of patient with HIV infection, (Pirmohamed and Park 2001) whereas β -lactam reactions are observed in up to 30% of patients with cystic fibrosis (Whitaker, Naisbitt et al. 2012). In both instances patients are exposed to

high doses of drug, often for a prolonged duration.

In this thesis I focused on three drugs associated with a high incidence of hypersensitivity reaction: the sulfonamide sulfamethoxazole and the β -lactam antibiotics piperacillin and flucloxacillin. Sulfamethoxazole is a pro-hapten that requires metabolism to generate a hapten. It is metabolized preferentially by CYP2A9 to a hydroxylamine intermediate. (Gill, Tjia et al. 1999) This metabolite circulates unchanged in the periphery. However, under pro-oxidative conditions it is spontaneously oxidized to nitroso sulfamethoxazole (SMX-NO), (Naisbitt, O'Neill et al. 1996, Naisbitt, Hough et al. 1999) which binds covalently to cysteine residues on protein. (Callan, Jenkins et al. 2009, Ogeese, Jenkins et al. 2015) Fortunately, SMX-NO can be synthesized (Naisbitt, O'Neill et al. 1996) and when added to aqueous buffers has a half-life of 5-10mins. (Naisbitt, Hough et al. 1999, Naisbitt, Gordon et al. 2001, Castrejon, Lavergne et al. 2010) This timeframe is sufficient to generate drug protein adducts that activate dendritic cells, (Sanderson, Naisbitt et al. 2007, Elsheikh, Lavergne et al. 2010) and T-cells from (1) hypersensitive patients, (Schnyder, Burkhart et al. 2000, Castrejon, Berry et al. 2010) (2) drug naïve donors (following priming) (Engler, Strasser et al. 2004, Faulkner, Martinsson et al. 2012) and (3) in animal models of immunogenicity. (Naisbitt, Gordon et al. 2001, Naisbitt, Farrell et al. 2002, Farrell, Naisbitt et al. 2003) The β -lactam antibiotics piperacillin and flucloxacillin both form adducts through a direct interaction with lysine residues on protein; however, they differ in terms of (1) the nature of the hypersensitivity reaction: flucloxacillin, liver injury; piperacillin, skin injury, (2) the dominant phenotype of drug-specific T-cell: flucloxacillin, CD8⁺; (Monshi, Faulkner et al. 2013) piperacillin, CD4⁺ (Whitaker, Meng et al. 2011, El-Ghaiesh, Monshi et al. 2012) and (3) whether reactions are associated with an HLA risk allele: flucloxacillin, HLA-B*57:01; (Daly, Donaldson et al. 2009) piperacillin, no known associations (unpublished data). T-

cell clones responsive to the drugs were generated from blood and skin of hypersensitive patients to study the functionality of T-cells, focussing specifically on the cytokines IL-17 and IL-22. PBMC from healthy donors were subjected to drug-specific dendritic cell T-cell priming to assess whether T-cells with a similar phenotype can be generated *in vitro*. Finally, clones responsive to the 3 drugs were used to assess T-cell cross reactivity and mechanisms of drug-specific T-cell activation.

In initial studies, piperacillin-responsive T-cells were cloned from peripheral blood of hypersensitive patients. In agreement with published data, the vast majority of piperacillin-responsive clones were CD4⁺. Clones proliferated in concentration-dependent manner following drug stimulation, with concentrations above 4 mM inhibiting the response due to cytotoxicity (results not shown). ELISpot was used to visualize the cytokines released by piperacillin-specific clones. T-cell activation resulted in secretion of a mixed panel of Th1 and Th2 cytokines with IFN- γ , IL-5 and IL-13 secreted by individual clones. Importantly, the availability of antibodies for IL-17 and IL-22 allowed us to investigate for the first time whether these cytokines are secreted by drug-specific T-cells. Many piperacillin-specific clones were found to secrete IL-22 alongside Th1 and Th2 cytokines. However, IL-17 was not detected.

One question that is frequently asked by researchers in the field of drug hypersensitivity is whether clones isolated from blood accurately reflect what occurs in skin at the time of the hypersensitivity reaction. To address this issue, through a close collaboration with the clinical team at the Leeds cystic fibrosis unit, skin biopsies were obtained from two patients following a positive piperacillin skin prick challenge. The hypothesis we were testing is that T-cells that migrate into the inflamed skin are the primary mediators of the hypersensitivity reaction. These biopsies were transferred to Liverpool to isolate and characterize the infiltrating T-cells. Skin was

digested and T-cells were allowed to migrate from the tissue to culture supernatant containing IL-2. The T-cells were stimulated non-specifically with the mitogen PHA and cloned directly without any requirement for a round of drug-specific expansion. Piperacillin-specific CD4⁺ clones were successfully isolated from both biopsy specimens and subjected to the same analysis as the blood-derived clones. Importantly, the cytokine profile of the skin-derived and blood-derived piperacillin-specific clones was similar, with Th1 and Th2 cytokines detected alongside IL-22. These data are in agreement with the elegant studies of (Gaide, Emerson et al. 2015) who found that for every abundant skin resident memory T-cell generated from a naïve precursor, an abundant central memory T-cell bearing the same T-cell receptor can be found. They suggested that the tissue resident cells respond rapidly following antigen exposure, whereas the central memory cells produce a delayed response that extend the duration of the allergic reaction. The discovery of IL-22 as an important mediator of piperacillin hypersensitivity might explain why patients exposed to piperacillin rarely develop severe forms of hypersensitivity reaction as IL-22 has been shown to promote keratinocyte proliferation and wound repair in several forms of skin disease.(Eyerich, Eyerich et al. 2010, Cavani, Pennino et al. 2012, Avitabile, Odorisio et al. 2015)

Based on these findings, in the next component of the thesis I choose to explore whether it was possible to prime naïve T-cells from healthy donors to piperacillin and if so explore the nature of the induced response. To fulfil this objective, a recently developed dendritic cell T-cell priming assay was utilized. The assay relies on culture of highly purified naïve T-cells with autologous monocyte-derived dendritic cells and piperacillin for 8 days to prime the T-cells. Drug-antigen responses are then measured through assessment of proliferation and/or cytokine secretion by restimulating the now primed T-cells with a second batch of autologous dendritic cells and

piperacillin. Piperacillin effectively activated naïve T-cells from the healthy donors. Through the cloning of T-cells from the priming assay it was possible to show that piperacillin-specific T-cell activation was associated with secretion of Th1 and Th2 cytokines alongside IL-22, which mirrored the responses observed in hypersensitive patient skin. The one intriguing difference between healthy donor and patient T-cells was that many healthy donor T-cells expressed the CD8 co-receptor. This may relate to differences in the provision of piperacillin antigens *in vitro* and in patient skin; however, further investigation in this area was beyond the scope of this thesis.

All of the experiments investigating piperacillin-specific T-cell priming utilized SMX-NO as a positive control. Hence, the cytokines secreted from SMX-NO-responsive T-cells was also profiled and related to T-cells from sulfamethoxazole hypersensitive patients. Similar to piperacillin, SMX-NO-responsive clones secreted Th1 and Th2 cytokines and IL-22, but IL-17 was not detected. These data lead to the possibility that IL-22 secretion from drug-specific T-cells is a common feature of drug hypersensitivity reactions in patients. Obviously further experiments using T-cells from patients with mild and severe forms of hypersensitivity are needed to confirm this possibility.

The ability of CD4⁺ T-cells to initiate hypersensitivity is often questioned. The clones generated from piperacillin and sulfamethoxazole hypersensitive patients secreted IFN- γ following drug stimulation and as such will have the capacity to recruit and activate phagocytes; thus, providing an indirect pathway to tissue injury. Furthermore, several clones secreted cytolytic molecules including perforin, granzyme B and FasL. Hence, the T-cells might also damage keratinocytes directly through induction of the apoptotic cascade.

The final objective of my project was to explore mechanisms of drug-hapten-specific T-cell

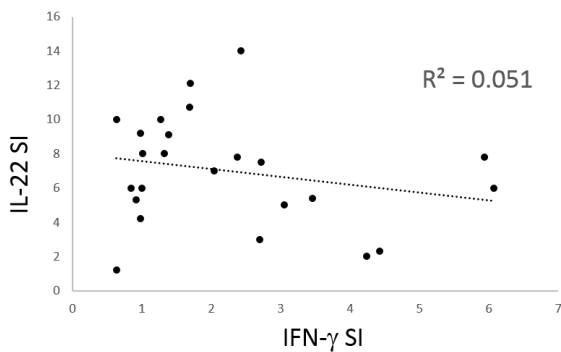
activation and cross-reactivity between 3 different drug antigens: piperacillin, flucloxacillin and SMX-NO. Clones responsive to all 3 drugs were activated via a hapten mechanism. Several pieces of evidence support this conclusion. First, drug-pulsed antigen presenting cells activated all of the clones. In these experiments, the antigen presenting cells are cultured with drug for different durations. This is followed by repeated washing with drug-free medium. Finally, the antigen presenting cells are added to the T-cell assay in the absence of soluble drug. Second, fixation of antigen presenting cells with glutaraldehyde blocked the activation of the T-cell clones. Glutaraldehyde inhibits antigen processing but not the presentation of drug bound directly to MHC expressed on the surface of antigen presenting cells. Third, addition of exogenous glutathione (SMX-NO) and *N*-acetyl lysine (piperacillin and flucloxacillin) to culture medium reduced the strength of the drug-specific T-cell response. These mediators reduce drug hapten protein binding but will not alter the ability of the drug to bind directly to surface MHC. (Schnyder, Burkhart et al. 2000, Burkhart, von Greyerz et al. 2001, Jenkins, Meng et al. 2009, Meng, Jenkins et al. 2011) Finally, MHC blocking antibodies inhibited the drug specific T-cell response. Collectively, these data indicate that clones responsive against all 3 drugs are activated via a pathway involving formation of drug protein adducts, uptake of the adducted proteins by antigen presenting cells, protein processing and binding of the derived peptides to MHC.

The availability of hapten-responsive clones with specificity for 3 drugs provided the unique opportunity to study T-cell cross reactivity. To date, several studies have addressed T-cell cross reactivity with parent drugs that bind directly to MHC, but knowledge of the drug-hapten-specific response was limited. Clones responsive against SMX-NO, piperacillin and flucloxacillin haptens were not activated with the alternative compounds, which suggests that the hapten structure is important for the T-cell receptor binding interaction and triggering of the T-

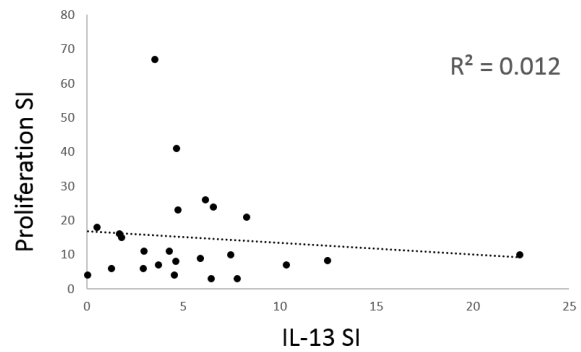
cell response.

In summary, this thesis describes a series of investigations that explore the way in which T-cells are activated by drug haptens and the nature of the induced response. Two areas of interest deriving from these studies: specifically, (1) the role of IL-22 in drug hypersensitivity reactions and (2) the preferential generation of piperacillin-responsive CD4⁺ and CD8⁺ T-cells in patients skin and in vitro, respectively, warrant further investigation.

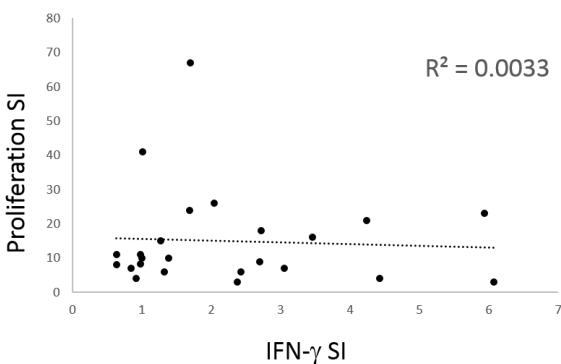
Appendix



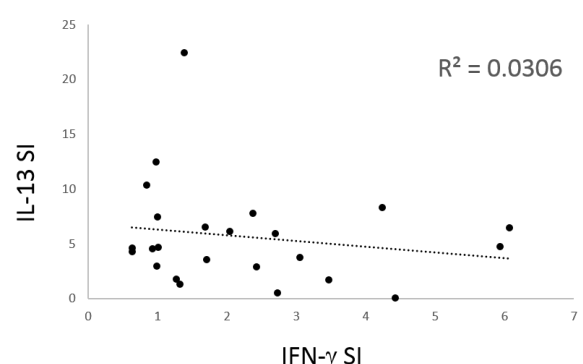
Appendix Figure A



Appendix Figure B



Appendix Figure C



Appendix Figure D

Appendix Figures showed the piperacillin specific cytokine correlations in chapter 3, which includes (A) Drug specific IL-22 secretion (shown by stimulatory index) and drug specific IFN- γ , (B) Drug specific T-cell proliferation and IL-13, (C) Drug specific T-cell proliferation and IFN- γ , and (D) Drug specific IFN- γ and IL-13.

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