Drug Immunogenicity and Co-Stimulatory Signalling: Evidence for Formation of a Functional Antigen through Immune Cell Metabolism

Thesis submitted in accordance with the requirements of

the University of Liverpool for the degree of Doctor in Philosophy

by

Ayman A. Elsheikh

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DECLARATION

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

Ayman A. Elsheikh

(MB.ChB, M.Sc. in Pharmacology, Tanta University, Egypt)

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Dedicated to

My Parents, My Wife and My Children

(Rawan & Kareem)

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CONTENTS

LIST OF ABBREVIATIONS

1-ABT	1-aminobenzotriazole
ADRs	Adverse drug reactions
AGEP	Acute generalised exanthematous pustulosis
APCs	Antigen presenting cells
AP-1	Activator protein 1
B-cells	Bone cells
BCR	B-cell receptor
BLCLs	B-lymphoblastic cell lines
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDRs	Complementarity-determining regions
CF	Cystic fibrosis
COX	Cyclooxygenase
cpm	Counts per minute
Cr^{51}	Chromium
CSA	Cyclosporin A
CTL	Cytotoxic lymphocyte
CYPs	Cytochrome P450
DAMPs	Damage or danger-associated molecular patterns
DC	Dendritic cell
DC-LAMP	Dendritic cell–lysosome-associated membrane protein
DHP	Dihydropterase enzyme
DHS, DiHS	Drug (induced) hypersensitivity syndrome
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNCB	2,4-dinitro-chlorobenzene
DRESS	Drug reaction with eosinophilia and systemic symptoms
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbant assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate-conjugated
FMOs	Flavin-containing monooxygenases
FOXP3	Forkhead box p3
GATA-3	GATA binding protein 3
GM-CSF	Granulocyte macrophage colony stimulating factor

GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidised form)
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HEPES	N-2-Hydroxyethyl piperazine-N-2-ethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMGB1	High mobility group box-1
HPLC	High pressure liquid chromatography
HSA	Human serum albumin
HSPs	Heat shock proteins
ICOS	Inducible co-stimulator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INS	Infectious-non-self model
ITAMs	Immunoreceptor tyrosine-based activation motifs
JNK	C-Jun N-terminal kinases
LAT	Linker for the activation of T-cell
LC-MS	Liquid chromatography-mass spectrometry
LPS	Lipopolysaccharide
LTT	Lymphocyte transformation test
mAb	Monoclonal antibody
MCP-1	Monocyte chemotactic protein-1
MDRs or MRPs	Multi-drug resistance associated proteins
Meth	Methimazole
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP-1	Macrophage inflammatory protein-1
MPE	Maculopapular exanthema
MPO	Myeloperoxidase
NADP (H)	Nicotinamide adenine dinucleotide phosphate (reduced)
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-κ light chain-enhancer of activated B cell
NK-T	Natural killer T cells
NMR	Nuclear magnetic resonance
OD	Optical density
PABA	Para-aminobenzoic acid
PAMPs/MAMPs	Pathogens (microbes)-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline

PE-	Phycoerythrin conjugated
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PHA	Phytohemagglutinin
p-i concept	Pharmacological interaction concept
PI	Propidium iodide
PKC	Protein kinase C
5-PL	Five-parametric logistic regression
PRRs	Pattern recognition receptors
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell park memorial institute
SA-PE	Streptaviden- Phycoerythrin conjugated
SD	Standered deviation
SI	Stimulation index
SJS	Stevens-johnson syndrome
SMX	Sulfamethoxazole
SMX-HA	Sulfamethoxazole hydroxylamine
SMX-NO	Sulfamethoxazole nitroso
SNS	Self/Non-self model
Src family	Sarcoma family of kinases
STAT	Signal transducer and activator of transcription
T regs	Regulatory T cells
TAP	Transporter associated with antigen processing
T-bet	T-box expressed in T cells
Tc	Cytotoxic T cells
T-cells	Thymus cells
TCR	T-cell receptors
TEN	Toxic epidermal necrolysis
TGF	Transforming growth factor
Th	T helper cells
THF	Tetrahydrofolate
TLC	Thin layer chromatography
TLR	Toll like receptors
TMP	Trimethoprim
TNF	Tumor necrosis factor
TT	Tetanus toxoid
WHO	World Health Organization
ZAP-70	Zeta-chain-associated protein kinase 70

LIST OF PUBLICATIONS

Published papers

Elsheikh A, Lavergne S, Castrejon J, Farrell J, Wang H, Sathish J, Pichler WJ, Kevin Park B and Naisbitt DJ **(2010 Oct 27)** Drug antigenicity, immunogenicity and co-stimulatory signalling: evidence for formation of a functional antigen through immune cell metabolism. *Journal of Immunology (Epub ahead of print).*

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ABSTRACT

Sulfamethoxazole (SMX) is metabolised to a nitroso intermediate (SMX-NO), which has been implicated in the pathogenesis of hypersensitivity reactions. Exposure of patients with cystic fibrosis to sulfonamides is associated with a high incidence of hypersensitivity. However, the reason for this has not been defined. The aim of this thesis was to use a mouse model to characterise functional antigens in immune cells and define the relationship between adduct formation, cell death, co-stimulatory signalling and stimulation of a T-cell response. In addition, the phenotype and function of T-cells from SMX hypersensitive patients with and without cystic fibrosis were characterised.

In the animal experiments, formation of SMX-derived adducts was dose- and time-dependent, detectable at non-toxic concentrations and blocked by enzyme inhibition. Adduct formation above a threshold induced necrotic cell death and dendritic cell co-stimulatory signalling and cytokine secretion. Antigen presenting cells (APCs) cultured with SMX for 16 h, the time needed for metabolism, stimulated T-cells from sensitised mice. The T-cell response with SMX was blocked by enzyme inhibition. Dendritic cells cultured with SMX and adoptively transferred to recipient mice initiated an immune response; however, T-cells were stimulated with SMX-NO and SMX-derived metabolites.

In human experiments, SMX and SMX-NO-responsive T-cells were cloned from six patients and characterised in terms of phenotype and function. Antigen specificity, mechanisms of antigen presentation, and cross-reactivity of specific clones were then explored. SMX-derived protein adducts were detected in APCs from patients with and without cystic fibrosis. Adduct formation was decreased with enzyme inhibitors. A significantly higher quantity of adducts were detected with cells from patients with cystic fibrosis. Over 500 $CD4^+$ or $CD8^+$ T-cell clones were generated and shown to proliferate, kill target cells and secrete an array of cytokines following antigen stimulation. Clones from patients with cystic fibrosis secreted higher levels of IFN-γ, IL-6 and IL-10. Three patterns of MHC-restricted reactivity (SMX-responsive, SMX-NO responsive and cross-reactive) were observed with clones from patients without cystic fibrosis. From patients with cystic fibrosis, SMX-NO and cross-reactive, but not SMX-responsive clones were observed. The response of the cross-reactive clones to SMX was dependent on APCs drug metabolism and the generation of drug protein adducts. The response was blocked by glutathione and enzyme inhibitors.

In conclusion, APCs were shown to metabolise SMX; subsequent protein binding generates a functional T-cell antigen in human and mouse models. Adduct formation above a threshold stimulated necrotic cell death, which provides a maturation signal for dendritic cells. SMX metabolism and protein adduct formation is critical for the stimulation of T-cells from patients with cystic fibrosis.

Chapter 1

General Introduction

Contents

1.1 Adverse drug reactions

Since the incident of foetal malformation induced by thalidomide in the 1960s, drug safety science represents a vital research area to improve our mechanistic understanding of adverse drug reactions (ADRs) and explore how to build up the design, tailoring and choice of drugs. ADRs have been reported following human exposure to almost every commercially available drug. Reactions of varying severity can target almost every organ/tissue.

The objective of this chapter is to describe the different forms of ADRs, focussing mainly on immune-mediated adverse reactions. The chapter highlights the different immunological processes involved in drug-specific adverse reactions and the way in which drugs interact specifically with immunological receptors to stimulate a pathogenic immune response in susceptible patients. Of particular importance is the section on drug metabolism and the ability of intracellular metabolites generated in antigen presenting cells (APCs) to stimulate immune cells following covalent modification of endogenous protein. The chapter concludes with a list of objectives that were generated to test the following primary thesis hypothesis; immune cell drug metabolism generates protein-reactive intermediates that are involved in drug immunogenicity and co-stimulatory signalling.

1.1.1 Definition of adverse drug reactions

The World Health Organization (WHO) defined ADRs for more than 40 years ago as "Any response to a drug which is noxious and unintended, and which occurs at

doses normally used in man for the prophylaxis, diagnosis or therapy of disease" (WHO, 1966). In 1972, the following phrase was added to the end of the definition "or for modification of physiological function" (WHO, 1972). Although this definition is still widely used by WHO (WHO, 1975; WHO, 1992; WHO, 2002), it has some defects related to the words (noxious) and (normally) because some reactions are inconvenient (rather than noxious), and some can occur at test doses (rather than therapeutic doses) (Aronson and Ferner, 2005).

Several other definitions of ADRs have been described, including those of Karch and Lasagna (Karch and Lasagna, 1975), Inman (Inman, 1977), Venning (Venning, 1983), and Laurence and Carpenter (Laurence and Carpenter, 1998). All of these definitions and others exclude reactions to inactive ingredients (in formulations), or contaminants (in herbal medications) in addition to excluding medication errors as a source of ADRs (Ferner and Aronson, 1999; Edwards and Aronson, 2000). These definitions are listed below.

Karch and Lasagna: Any response to a drug that is noxious and unintended, and that occurs at doses used in humans for prophylaxis, diagnosis, or therapy, excluding failure to accomplish the intended purpose.

Inman: Noxious and unintended event occurring after the administration of a drug in dosages normally recommended for therapeutic purposes.

Venning: Any clinical event or illness resulting from an unwanted action of a drug.

Laurence and Carpenter: A harmful or significantly unpleasant effect caused by a drug at doses intended for therapeutic effect (or prophylaxis or

diagnosis), which warrants reduction of dose or withdrawal of the drug and/or foretells hazard from future administration.

More recent broader definitions have been proposed by Edward and Aronson (Edwards and Aronson, 2000) and modified slightly by Aronson and Ferner (Aronson and Ferner, 2005). Both definitions have been used as an alternative to the WHO's definition and other definitions that excludes failure of the drug to produce its desired effect and excludes any effects of drugs taken in overdose.

Aronson and Ferner: An appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product; adverse effects usually predict hazard from future administration and warrant prevention, or specific treatment, or alteration of the dosage regimen, or withdrawal of the product.

1.1.2 Impact of adverse drug reactions

ADRs pose a major health concern due to their impact on all personnel involved in health care e.g. physicians, pharmacists, patients, pharmaceutical industry, and drug regulatory agencies, in addition to their impact on the health care system including hospital admissions and health care costs. ADRs are among the most important causes of fatality in several countries. A recent study placed ADRs as the seventh most common cause of death (Wester et al., 2008).

The involvement of ADRs in hospital admissions has been investigated in several studies performed in different countries (e.g. US, France, Switzerland, Germany, UK, Italy, Netherland and UAE). Each study showed extensive variability (Raschetti et al., 1999; Fattinger et al., 2000; Senst et al., 2001; Dormann et al.,

2004; Al-Tajir and Kelly, 2005; Nebeker et al., 2005; Bond and Raehl, 2006; Lugardon et al., 2006; Van Der Hooft et al., 2006; Davies et al., 2009). This heterogenicity may be attributed to many factors such as the population's characteristics, types of hospitals, type of health care system, design of the study (e.g. prospective, or retrospective), change in medical practice over years, or techniques used to identify ADRs (e.g. medical and pharmaceutical visits, patient charts, or computerised databases). A meta-analysis performed by Lazarou et al. (1998) in the US demonstrated that severe ADRs account for 6.7% (of which 4.7% caused admission to hospital and 2.1% occurred in in-patient after hospital admission). Furthermore, 10.9% of in-patients suffered ADRs of all severities. A prospective study conducted in two National Health Service (NHS) hospitals in the UK (Pirmohamed et al., 2004), showed that the rate of ADR-related hospital admissions is 6.5%. In another recent larger-scale prospective study in UK on in-patients, Davies et al. (2009) found that 14.7% of patients suffered ADRs, the majority of which were avoidable. A systematic literature review conducted on studies published during the last 10 years found that the percentage of patients with ADRs ranged from 1.6 to 41.4 (for in-patients) and from 1.7 to 51.8 (for hospital admissions (Cano and Rozenfeld, 2009).

ADRs contribute to health care costs as a result of prolonged hospital bed occupancy or additional intervention. A recent study in the US (Bates et al., 1997) estimated the hospital burden from adverse drug events as $8000 per hospital bed per year and that due to ADRs in mainland Europe to be £4700 per hospital bed per year (Moore et al., 1998). A systematic review by Davies et al. (2009)

estimated that in the NHS in England, the figure was comparable with annual cost of £637 million or £5000 per hospital bed per year.

Drug failure, withdrawal from the market, or generating a "black box" warning is attributed to problems in the pharmacokinetics, the drug efficacy, or the drug toxicity. The later represents a particular problem. Of greatest concern are the rare, unpredictable, and poorly understood ADRs. They are unlikely to be identified until the late phase of testing or even during post-marketing, which increases the costs.

ADRs have been accounted for the withdrawal of 4% of all drugs licensed in the UK between 1974 and 1994 (Jefferys et al., 1998), and over 10% of the drugs approved by the FDA from 1975 to 2000 (Lasser et al., 2002). The cost of drug development exceeds US$ 800 million for each new drug introduced onto the market (DiMasi et al., 2003; Adams and Van Brantner, 2006). Thus, each drug withdrawal represents a significant financial burden to the pharmaceutical industry.

Although the true picture is uncertain due to hidden costs, ADRs are a noteworthy health problem, and over half are potentially avoidable, so any steps and strategies taken to reduce their impact will be valuable throughout the health care system.

1.1.3 Classifications of adverse drug reactions

ADRs were first classified into predictable and unpredictable effects (Wayne, 1985), then into dose-related and non-dose-related (Levine, 1973; Wade and Beeley, 1976). Alphabetic classification was first proposed and characterised into

type A and B at the end of the 1970s (Rawlins and Thompson, 1977; Rawlins and Thompson, 1981; Rawlins, 1981) (table 1.1). Other types, from C to G, were proposed subsequently (Grahame-Smith and Aronson, 1984; Laurence and Bennett, 1992; Park et al., 1992; Hartigan-Go and Wong, 2000; Aronson, 2002) (table 1.2).

Another classification of ADRs (DoTS classification) was developed based on the dose (Do) at which reactions occur, the time course (T) of the reaction, and the susceptibility (S) of the patient (Laurence and Bennett, 1992). In addition, ADRs can be classified based on the need to change the offending drug dosage regimen and the treatability of the reaction (Aronson and Ferner, 2005) (table 1.3). Recently, ADRs are classified into 'on-target' and 'off-target'. A predictable reaction from basic drug pharmacology with dose-dependency is an on-target reaction, whereas an unpredictable reaction is an off-target one. The latter is uncommon, serious and thought to be due to reactive drug metabolites (Park et al., 2010). Unpredictable reactions might be idiosyncratic, drug intolerance, or might have an immunologic basis.

Table 1.1: Features of type A and type B reactions

Feature	Type A (Augmented)	Type B (Bizarre)
Frequency	Common	Uncommon
Dose dependency	Dose-dependent	More or less dose independent
Predictability	Yes	No
Severity	Usually mild	More serious
Morbidity	High	High
Mortality	Low	High
Examples	Digoxin toxicity	*Hypersensitivity (Allergy) (immune mediated)* e.g. penicillin
	Opioids constipation	*Drug intolerance* (at therapeutic or subtherapeutic doses of drug)
	NSAIDs intestinal bleeding	*Idiosyncratic reactions* (patient-specific pharmocokinetic or genetic characteristics e.g. malignant hyperthermia)

Table 1.2: Types C-G in alphabetic classification of adverse drug reactions

Type	Features	Example
C : Chronic	Dose and time related	Osteoporosis with steroids
D : Delayed	Time related, usually dose-related	Carcinogenicity, teratogenicity
E : End therapy	Drug withdrawal	Opiate withdrawal syndrome
F : Failure	Dose related, often due to drug interactions	Inadequate dose of an oral contraceptive particularly when used with enzyme inducers
G : Genetic	Dose related, often due to irreversible genetic damage.	Genotoxins, carcinogens, and teratogens

Table 1.3: Classification of adverse drug reactions based on reaction intensity

	Grade 1	Grade 2	Grade 3
Change drug dosage	Not required	Required or desirable	withdraw drug
Treatability of the reaction	A. No treatment required B. Relieved or partly relieved by treatment C. Not relieved by treatment		

1.1.4 Hypersensitivity reactions

1.1.4.1 Subtypes of hypersensitivity reactions

Hypersensitivity reactions (Immune-mediated or allergic drug reactions) are off-target drug reactions that account for 15–20% of all ADRs (Lazarou et al., 1998). They can be classified according to Gell and Coombs (Gell and Coombs, 1963) into reactions that are mediated by drug-specific IgE antibodies such as urticaria, asthma, and anaphylaxis (type I, mostly immediate-type), cytotoxic reactions such as acute haemolytic anaemia mediated by drug specific IgG antibodies (type II), immune complexes reactions such as vasculitis and serum sickness mediated by drug specific IgM antibodies (type III), or T-cell mediated reactions (type IV, mostly delayed-type).

Delayed hypersensitivity reactions (type IV) were further subclassified into four subtypes IVa-IVd based on cytokine production by T-cells, cytotoxic activity of both $CD4^+$ and $CD8^+$ T cells, and other effector cells (monocytes in type IVa, eosinophils in IVb, T-cell in IVc, and neutrophils in IVd) (Pichler, 2003) (table 1.4).

Diagnosis of allergic reactions relies mainly on good history taking, clinical observations (e.g. edema, vesicles, bullae, organ affected), and choice of the diagnostic test (e.g. basophil activation test for type I and lymphocyte transformation or skin tests for type IV) (Bircher, 2005).

Table 1.4: Subtypes of hypersensitivity reactions

	Immune response	Pathophysiology	Examples
IVa	⇨ Th1 (IFN-γ)	Monocytic inflammation	Eczema
IVb	⇨ Th2 (IL-5, IL-4)	Eosinophilic inflammation	MPE, BE
IVc	⇨ CTL (Perforin/ Granzyme B)	Keratinocyte death mediated by $CD4^+$ or $CD8^+$ T cells	MPE, BE, PE
IVd	⇨ T-cells (CXCL-8, IL-8)	Neutrophilic inflammation	AGEP

MPE: Maculo-papular exanthema *BE: Bullous exanthema*
AGEP: Acute generalised exanthematous pustulosis *PE: Pustular exanthema*

1.1.4.2 Major targets of hypersensitivity reactions

Drug induced hypersensitivity reactions can target liver, skin, heart, kidney, lungs, and other organs. Although the liver is the most important target for dose-dependent ADRs since it has a major role in pharmacokinetics and a unique anatomic and physiologic structure (e.g. portal circulation), it is only rarely involved in drug hypersensitivity. Skin is affected much more commonly due to several reasons including large surface area, rich vascularisation, and dense network of APCs and T cells ready to elicit an immune response (Keller et al.,

2005; Clark et al., 2006) (figure 1.1). Furthermore, skin contains many resident cells express metabolising enzyme (see below).

Drugs can induce different forms of cutaneous hypersensitivity reactions including maculopapular exanthema (MPE), acute generalized exanthematous pustulosis (AGEP), drug rash/reaction with eosinophilia and systemic symptoms (DRESS), Stevens-Johnson Syndrome [SJS], toxic epidermal necrolysis [TEN], and other reactions (e.g. allergic contact dermatitis). The pathophysiology and clinical patterns of some of these reactions are discussed below.

These reactions differ mainly in the effector cells and cytokine profile of activated T cells. $CD4^+$ T cells are thought to be dominant in MPE and AGEP, while $CD8^+$ cells dominate in TEN. Studies on skin has shown that IL-5 is the predominant cytokine in MPE due to its ability to recruit eosinophils to the site of inflammation (Pichler et al., 1997). IL-8 and IFN-γ are thought to take part in AGEP (Britschgi et al., 2001; Roujeau, 2006) due to the ability of IL-8 to recruit neutrophils to the site of inflammation, and IFN-γ to upregulate MHC class II on keratinocytes rendering them more susceptible to T-cell mediated killing (Friedmann et al., 1994; Roujeau, 2006). IFN-γ and TNF-α were found at high levels in the blister fluid of patients with SJS/TEN (Nassif et al., 2004b), TNF-α can induce keratinocyte apoptosis (Paquet et al., 1994; Groves et al., 1995), while TNF-α and IFN-γ enhance keratinocyte cytotoxicity (Friedmann et al., 1994).

MPE is the most frequent, mild (mostly) drug hypersensitivity reaction. The pathophysiology includes (a) keratinocyte death with cytotoxic $CD4^+$ and/or $CD8^+$ releasing perforin and granzymeB and (b) eosinophilic inflammation due to a

Th2-type immune response with the secretion of IL-5 (Schnyder et al., 1998; Yawalkar et al., 2000; Hari et al., 2001; Pichler, 2003).

AGEP is a rare disease characterised by acute febrile sterile pustular eruptions within 5 days of drug administration. The reaction is accompanied by neutrophilia, sometimes eosinophilia, with no blisters. T cells kill keratinocytes leading to formation of vesicles and release of IL-8 from both types of cells (under the effect of IFN-γ/TNF-α). This leads to recruitment of neutrophils into the vesicles, which then change into pustules (Britschgi et al., 2001). Causative drugs include macrolide antibiotic pristinamycine, amoxicillin, quinolones, hydroxyl chloroquine, sulfonamides, terbinafine, and diltiazem (Sidoroff et al., 2007).

DRESS, also known as drug [induced] hypersensitivity syndrome (DHS, DiHS) is a syndrome presented clinically (as the name refers) by exanthema with transition into a generalised epidermal necrolysis, internal organs involvement (liver, lymph node, kidney, lung, pancreas), and eosinophilia. In addition, it has been reported that viral activation (e.g. herpes simplex virus 6, cytomegalovirus, Epstein-Barr virus) is associated with DRESS (Hashimoto et al., 2003; Shiohara et al., 2007). Drug-specific T-cells that secrete large amounts of IL-5 and IFN-γ are detected in the blood of patients with DRESS (Naisbitt et al., 2003b). Common causative drugs include carbamazepine, phenytoin, sulfonamides, and allopurinol (Allam et al., 2004).

SJS and TEN are the most severe form of cutaneous hypersensitivity reaction. They are bullous conditions characterised by severe necrosis and epidermal

detachment, followed by erosive lesions in the skin and mucous membrane of the mouth and genitals. Other manifestations are fever and internal organ involvement (e.g., liver, kidney or gastrointestinal tract) (Greenberger, 2006). The underlying mechanism of these reactions involves immune-mediated cytotoxicity. Two main pathways have been reported to demonstrate the role of cytotoxic T-lymphocytes in keratinocytes necrolysis and subsequent blister formation; (a) Fas-mediated pathway (particularly in TEN) (Viard et al., 1998; Abe et al., 2003; French, 2006; Alanore and Roujeau, 2007) and (b) perforin/granzymeB-mediated pathway (particularly in drug exanthema) (Nassif et al., 2002; Nassif et al., 2004a). Dermal and epidermal inflammatory infiltration with predominant $CD8^+$ T lymphocytes in TEN and SJS has been reported (Miyauchi et al., 1991; Hertl et al., 1993). These CD8+ T cells were isolated from epidermal skin lesions in penicillin-induced bullous drug-reactions (Hertl et al., 1993; Hertl and Merk, 1995) and from fluid extracted from blisters (Nassif et al., 2002; Nassif et al., 2004a). Characterisation of these $CD8^+$ clones revealed cytotoxic properties against B lymphocytes and keratinocytes (Hertl et al., 1993) and against EBV-transformed lymphocytes (Nassif et al., 2002; Nassif et al., 2004a) as well as secretion of cytokines including IL-2 and IFN-γ, but not IL-4 (Merk and Hertl, 1996). Moreover, signs of necrolysis were detected microscopically in keratinocytes close to $CD8^+$ lymphocytes (Paul et al., 1996). Drugs which are most often associated with SJS and TEN are allopurinol carbamazepine, cotrimoxazole, nevirapine, phenobarbital, phenytoin, and lamotrigine (Halevy et al., 2008). Recently, HLA-associations with severe $CD8^+$ T cell-mediated drug hypersensitivity reactions has

been reported with drugs including carbamazepine, allopurinol, and abacavir (Mallal et al., 2002; Chung et al., 2004; Hung et al., 2005).

Other hypersensitivity cutaneous reactions include (a) allergic contact dermatitis, mostly presented at the site of contact with the allergen and requires a prior sensitisation to the drug e.g. neomycin, gentamicin, chloramphenicol (Gehrig and Warshaw, 2008) and (b) photoallergic/phototoxic reactions, due to the interaction of drug with UV light leading to formation of highly protein reactive derivatives or radicals as in drugs like tetracycline and sulfonamide (Gould et al., 1995).

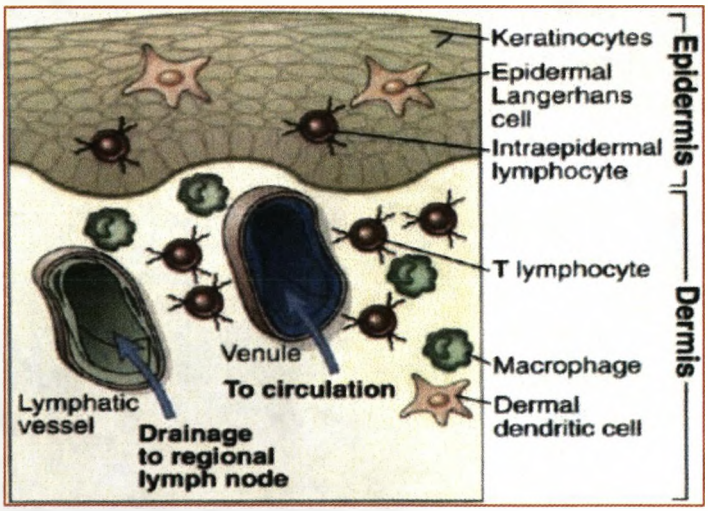

Figure 1.1: Network of immune cells in dermal and epidermal layers of skin (Abbas et al., 2007)

1.2 Drug metabolism

1.2.1 Major sites of drug metabolism

Lipophilic drugs, in the absence of metabolism, would not be eliminated and would accumulate in the body, eventually causing toxicity. For most drugs, the main aim of metabolism is detoxification. Metabolising enzymes convert lipophilic compounds into more water soluble derivatives that are more readily excreted. For a limited number of drugs, this process results in formation of toxic/reactive metabolites that can disturb cellular functions, interact with distinct cellular proteins, lipids, or nucleic acids, disrupt the ionic (e.g calcium) gradient, and interfere with metabolic or signalling pathways leading to cellular impairment, death, and possibly organ failure.

The principal site of drug metabolism is the liver because of its large size, the high content of many metabolising enzymes, and because it is the first organ exposed to drugs absorbed from the gastrointestinal tract. Another important site of metabolism is the skin (Williams, 2008) due to large surface area, vascularisation, and expression of individual drug metabolising enzymes by many cell types including keratinocytes, fibroblasts, langerhans cells and melanocytes (table 1.5). Skin cells express the most important hepatic cytohrome P450 (CYPs) including CYPs 1A1, 1B1, 2B6, 2C9, and 3A4 as well as other CYPs that have never been identified in the liver (e.g. CYP2 members) (Du et al., 2004). In addition to the liver and skin, drug metabolising enzymes have been shown to be expressed by cells of the immune system such as lymphocytes, neutrophils, and APCs (table 1.5). Many drugs have been shown to be metabolised by immune cells such as carbamazepine (Furst and Uetrecht, 1993), dapsone (Uetrecht et al., 1993), and

sulfamethoxazole (Cribb et al., 1990; Uetrecht et al., 1993; Sanderson et al., 2007). Other sites of drug metabolism include the gastrointestinal tract, kidney, lung, brain, and plasma (Williams, 1987).

Table 1.5: Local (extrahepatic) metabolism of drugs in skin and immune cells

Cells	Enzyme(s)	References
Keratinocytes	CYP COX FMO	(Kanekura et al., 1998; Rys-Sikora et al., 2000; Baron et al., 2001; Saeki et al., 2002; Du et al., 2004; Roychowdhury et al., 2005; Vyas et al., 2006)
Fibroblasts	CYP	(Saeki et al., 2002; Roychowdhury et al., 2007a)
Melanocytes	CYP	(Saeki et al., 2002)
Langerhans cells	CYP	(Saeki et al., 2002)
Lymphocytes	CYP COX MPO	(Spencer et al., 1999; Starkel et al., 1999; Krovat et al., 2000; Dey et al., 2001; Siest et al., 2008)
Neutrophils	MPO	(Cribb et al., 1990; Furst and Uetrecht, 1993; Hofstra and Uetrecht, 1993; Uetrecht et al., 1993; Lai et al., 1999)
Dendritic cells	CYP COX MPO FMO	(Sieben et al., 1999; Norgauer et al., 2003; Roychowdhury et al., 2007b; Sanderson et al., 2007; Lavergne et al., 2009)

MPO: myeloperoxidase, COX: cyclooxygenase, CYP: cytochrome P450, FMO: flavin-containing monooxygenases

1.2.2 The multi-phase metabolic system

Metabolism can be divided into three phases; phase I, II and II. Phase I reactions usually precede phase II; though this is not always the case (e.g. isoniazid is metabolised in the reverse order).

Phase I biotransformations result in more polar metabolites by adding or exposing the polar functional groups (e.g. -OH, -COOH, -SH, -O-, or NH2) permitting the products to serve as substrates for phase II conjugating enzymes or sometimes for excretion. Phase I reactions include oxidation, reduction, and hydrolysis, of which only oxidation is of importance for this review.

Phase II reactions involve conjugation of the polar functional groups of phase I products by the addition of a large, polar moiety (sulfate, methyl, acetyl, glutathione and glucuronic acid) producing a metabolite with improved water solubility and increased molecular weight, thereby facilitating drug elimination. For few cases, phase II reactions have been associated with the formation of reactive metabolites; this may be one of the mechanisms involved in the hepatotoxicity seen with diclofenac (Kenny et al., 2004).

Phase III metabolism involves a series of pump systems or efflux transporters, which provide a gradient to eject the metabolites from aqueous areas of cells relatively quickly into the interstitial fluid between cells and to the blood to be removed by kidneys thus diminishing any effects the drug might exert on the target tissue. Many efflux transporters have been identified including P-glycoprotein and multi-drug resistance associated proteins (MDRs or MRPs)(Borst et al., 1999; Baron et al., 2001; Leslie et al., 2001).

1.2.3 Major Phase 1 oxidative reactions

The cytochrome P450 monooxygenases (CYP) or oxido-reductases are a group of enzymes belonging to a family of proteins containing a heme co-factor

(hemoproteins). Its activity depends on generation of water from the heme moiety. The heme iron binds oxygen in the active site, where oxidation of substrates occurs (Waszkowycz et al., 1994) as represented in figure 1.2.

Figure 1.2: Cytochrome P450 (CYP) oxidative reaction using a substrate (RH), an oxygen molecule (O_2), two hydrogens (H), and two electrons (*e*) (supplied by NADPH and NADPH cytochrome P450 oxidoreductase) to generate a hydroxyl group (OH) and one molecule of water (H_2O).

There are 18 families of human CYPs and 43 subfamilies (Nelson, 2003). CYPs are named depending on cellular (cyto) location and spectrophotometric characteristics (chrome) with the root "CYP" followed by a number indicating the family, a letter denoting the subfamily, and a second number designating the CYP isoform. P450 is the most efficient nanometre wavelength of light at which CYPs absorb light when reduced and bound to carbon monoxide. Thus, CYP 2C9 indicate the ninth member of subfamily C of the family 2. Amino acid structure with a sequence identity of more than 40% indicates members of the same family, while sequence identity of more than 55% indicates the same subfamily (Nelson et al., 1996). Most of CYPs responsible for drug metabolism in humans fall within families CYP1-4. The most important CYPs for drug metabolism are CYP3A4, CYP1A2, CYP2A6, CYP2C9, CYP2C8, CYP2D6, and CYP2E1 (Shimada et al., 1994; Mutch and Williams, 2006; Sanderson et al., 2007).

The CYP enzymes account for as much as 60% of the biotransformation of FDA-approved drugs (Venkatakrishnan et al., 2001). They are located mainly in liver

but extrahepatic locations such as intestine are important for first pass metabolism. CYPs are also found in the skin and immune cells as discussed previously. CYPs are membrane-associated proteins located either in the inner membrane of mitochondria or in the endoplasmic reticulum of cells (Guengerich, 2004). Many reactions are carried out by CYPs including aromatic hydroxylation, rearrangement reactions (dealkylation, deamination, dehalogenation) as well as N-oxidation, S-oxidation, and primary amine oxidation. CYPs are involved in metabolism of dietary agents, as well as synthesis of hormones, cholesterol, and vitamin D metabolism. They are the major enzyme system involved in drug metabolism and bioactivation, accounting for about 75% of the total metabolism (Guengerich, 2008).

Due to the nature of substrate binding sites in CYPs, a single compound can be metabolised by multiple CYPs or by multiple positions in the same CYP as well as the ability of a single CYP to metabolise structurally dissimilar chemicals. Changes in CYP enzyme activity (induction or inhibition) are a major source of ADRs as they can affect the clearance of different drugs. It is therefore vital to find out which CYPs metabolise a drug, and to avoid co-administering drugs that are metabolised by the same CYP. Enzyme inhibitors often inhibit their own metabolism and metabolism of co-administered drugs such as azoles e.g. the antifungal ketoconazole, which is a potent CYP inhibitor (Thomson et al., 1988). Administration of ketoconazole with the anti-HIV protease inhibitors increases their risk of toxicity (Treluyer et al., 2003). On the other hand, enzyme inducers may induce metabolism of co-administered drugs such as steroids (e.g. prednisolone) that induce hepatic CYP3A4, increasing the metabolism of many

drugs (Kishi et al., 2004). Other factors affecting CYP enzyme activity are diet and genetic polymorphisms.

Flavin-containing monooxygenases (FMOs) are another family of phase I oxidative enzymes that are expressed at high levels in the liver and are localised to the endoplasmic reticulum. The general reaction utilises NADPH and oxygen to oxidise nucleophilic centres in drugs and to form their respective oxidative products as represented in figure 1.3.

Figure 1.3: Flavin monooxygenase (FMO) oxidative reaction using a substrate (RH), an oxygen molecule (O_2), hydrogen (H), and NADPH to generate a hydroxyl group (OH) and one molecule of water (H_2O).

Five isoforms of FMO are expressed in humans (Mitchell, 2008), of which FMO 3 is highly expressed in human liver. FMO 1 is expressed in kidney (Phillips et al., 1995) at levels higher than that of CYPs and is thought to be responsible for renal biotransformation of certain drugs. FMOs generally produce harmless metabolites. They metabolise drugs such as nicotine, cimetidine, clozapine, and itopride. However, in some cases FMOs have been reported to mediate the bioactivation of various chemical moeities to toxic intermediates. It has been shown that FMO is involved in metabolism of drugs in the skin and immune cells (e.g. keratinocytes, dendritic cells) (Vyas et al., 2006; Lavergne et al., 2009). Unlike CYPs, FMOs are less involved in drug-drug interactions because they are not induced or inhibited by any clinically used drugs (Cashman and Zhang, 2006). In addition, FMOs catalyse oxidation of the nucleophilic centres (rather than the

carbon atoms in CYPs-catalysed oxidative reactions) (Eswaramoorthy et al., 2006).

Animal heme-containing peroxidises are a large family of peroxidases that play an important role in cell biology, host defence against infection (antimicrobial effect), and hormone synthesis. This family includes heme-containing enzymes such as myeloperoxidase (MPO), eosinophil peroxidase, lactoperoxidase, thyroid peroxidase, and cyclooxygenase (COX) (Kimura and Ikeda-Saito, 1988; Furtmuller et al., 2006). MPO is the best studied and is a main interest in this study. It is largely found in neutrophils and leukocytes and its heme pigment causes a green colour in neutrophil-rich secretions.

Peroxidases can react with hydroperoxides (mostly hydrogen peroxide [H_2O_2]) through a multistep reaction. The cycle starts with a reaction between the ferrous form of the peroxidase and hydrogen peroxide to form intermediate compounds known as compound I and compound II. These reactions result in the formation of oxidising agents that act either directly or indirectly in the presence of halide ions (e.g. Cl^-, I^-, thiocyanate ions) leading to formation of another oxidising agents such as hypochlorous acid (Furtmuller et al., 2000) (figure 1.4).

$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} HOCL + H_2O$$

Figure 1.4: Myeloperoxidase (MPO) oxidative reaction using hydrogen peroxide (H_2O_2), chloride ion (Cl^-), and hydrogens (H^+) to generate hypochlorous acid (HOCL, oxidising agent) and one molecule of water (H_2O).

Several studies have shown the role of peroxidase enzymes including MPO (Cribb et al., 1990; Zhao Chao and Uetrecht, 1995), COX (Vogel, 2000), lactoperoxidase (Goodwin et al., 1996) and thyroid peroxidase (Doerge et al., 1997) in xenobiotic metabolism and certain metabolites have been shown to irreversibly modify protein generating potential antigenic determinants for immune cells.

1.2.4 Role of drug metabolism in drug hypersensitivity

Drugs are referred to by Landsteiner and Jacobs (1935) as incomplete antigens due to their low molecular mass (<1KD). They were considered too small to interact directly with immunological receptors. Thus, the hapten theory was proposed to explain the way in which drugs and sensitising chemicals interact with the immune system. A hapten is defined as any low molecular weight compound that has a propensity to bind irreversibly to protein, generating a hapten-protein complex.

Drug metabolism can, in some cases, induce toxicity through the generation of chemically reactive, electrophilic metabolites that bind covalently to cellular macromolecules inhibiting critical cellular functions (Park et al., 2005a; Park et al., 2005c; Baillie, 2006; Park et al., 2006; Uetrecht, 2006; Uetrecht, 2007; Uetrecht, 2008). The metabolite formed through metabolism is referred to as a hapten. It is thought that even a low level of hapten, often undetectable using state of-the-art bioanalytical methods, could be involved in an immune response due to the serial triggering character of T-cell receptors and the small number of these receptors needed for activation of T-cells (Schodin et al., 1996; Wei et al., 1999;

Labrecque et al., 2001; Chakraborty, 2002). Serial triggering is the concept applied to the stimulation of multiple T-cell receptors by a single antigenic ligand. The tendency for a xenobiotic to form a reactive intermediate is based on its structure with special "alerts" identified, that are particularly susceptible to metabolic activation (Park et al., 2005a). Several studies have demonstrated that the majority of marketed drugs associated with a high incidence of ADRs are able to form reactive metabolites which have the capacity to bind covalently to proteins (Kalgutkar et al., 2005; Kalgutkar and Soglia, 2005; Zhou et al., 2005; Boelsterli et al., 2006).

Due to the importance of metabolic activation in drug development, many pharmaceutical companies accepted a new approach to deal with the reactive intermediates (Nassar and Lopez-Anaya, 2004; Kalgutkar et al., 2005; Kalgutkar and Soglia, 2005; Erve, 2006; Baillie, 2008). This approach is based on assessment of the quantity and quality of the potential reactive metabolite. Qualitative identification is concerned with the recognition of the structural alert involved in the undesirable metabolic pathway and blocking it chemically or medically.

Reactive metabolites can be classified into two categories (Williams et al., 2002); electrophiles and free radicals. Electrophiles, the most common reactive metabolites, are electron deficient species (e.g. epoxides and quinoids). They have, at their electrophilic center, a high positive charge density (hard electrophiles) or a low positive charge density (soft electrophiles). Free radicals contain an unpaired electron that is able to propagate a chain of reactions leading

to lipid peroxidation, oxidative stress, or modification of other types of biological molecules including protein (Uetrecht, 1995). Both phases of drug metabolism are able to induce reactive metabolite formation, but phase 1 CYPs are the most important enzymes involved in this process.

To clarify the role of reactive metabolites in drug hypersensitivity reactions, it is critical that future investigations are designed to shed light on some features including the site of adduct formation, the nature and extent of drug metabolite-specific protein modification, the role of cytotoxicity in the activation of the innate immune system and mechanisms of drug metabolite-specific T-cell activation. Furthermore, it is important to define whether genetic polymorphisms in drug metabolising enzymes contribute toward susceptibility.

It has been shown, both *in vivo* and *in vitro*, that binding of drug metabolites to protein is involved in immune mediated ADRs, thus providing support for the hapten concept (Meekins et al., 1994; Worrall and Dickinson, 1995; Gruchalla et al., 1998; Lavergne et al., 2006a; Aithal and Day, 2007; Cheng et al., 2008). Nevertheless, some authors have reported that this is not always the case. In certain circumstances, drug-protein adducts may not induce ADRs (Carey and Van Pelt, 2005; Pichler et al., 2006; Cheng et al., 2008) or may even protect against ADRs (Zhou et al., 2005) due to the absence of another signal (e.g. danger signal).

Cytotoxicity associated with drug metabolites is thought to supply a danger signal to stimulate an innate immune response. The Mechanisms of metabolite cytotoxicity are still not fully defined, but may relate to oxidative stress (Vyas et

al., 2005; Lavergne et al., 2006b; Aycicek and Iscan, 2007), modification of vital proteins (Leeder et al., 1991), or mitochondrial injury (Bort et al., 1999). Cytotoxicity has been demonstrated for many drugs such as amodiaquine (Naisbitt et al., 1998), SMX (Carr et al., 1993; Rieder et al., 1995), diclofenac (Bort et al., 1999), carbamazepine (Valentine et al., 1996), and clindamycin (Wijsman et al., 2005).

Drug metabolites may interact directly with innate cells. Studies on dendritic cells have shown that drugs/chemicals as amoxicillin (Rodriguez-Pena et al., 2006), SMX (Sanderson et al., 2007), contact sensitisers (Aeby et al., 2004; Toebak et al., 2006; Coulter et al., 2007), and abacavir (Martin et al., 2007), can induce activation and cellular maturation signals, thought to be critical for the activation of a primary immune response. Danger signalling is discussed in detail below.

Although the interaction between drug metabolites and the adaptive immune response has been intensively studied, a defined mechanism is yet to be identified. Metabolite-specific T-cells were detected in hypersensitive patients (Schnyder et al., 2000; Sachs et al., 2001; Naisbitt et al., 2003b; Nassif et al., 2004a; Wu et al., 2006; Tsuge et al., 2007; Castrejon et al., 2010) and animal models of drug immunogenicity (Farrell et al., 2003; Naisbitt et al., 2007). However, whether this interaction is critical for the initiation of an immune response is yet to be defined. This issue will also be discussed in detail later in this chapter.

1.3 Immune system

Immunity is a term that describes all host mechanisms which confer defence or resistance to foreign substances, such as infectious agents and biological invasion without damage to the host. These mechanisms are typically divided into two categories: innate (using non-clonally distributed receptors) and adaptive (using clonally distributed receptors). The two branches of immunity were largely separated till 1989 when Janeway proposed a new concept that adaptive immunity is initiated and controlled by the stimulation of pattern recognition receptors expressed on cells of the innate immune system (Janeway Jr, 1989).

1.3.1 Components of the immune system

The innate (non specific) component is the front line of defence present since birth, has no immunological memory (i.e. the response remains the same following repeated infections), and acts immediately or within hours of an antigen's appearance in the body. It involves a diverse collection of molecules and mechanisms that include; (a) physicochemical barriers such as epithelial layers, and chemical substances e.g. definsins, lysosyme and phospholipase, (b) humoral barriers such as the complement system, coagulation system, cytokines, and inflammatory mediators and (c) cellular barriers such as phagocytes (neutrophils, macrophages, dendritic cells), eosinophils, and natural killer cells. Some of these elements are working at all times, such as in the epithelia, others work in response to microbes (Abbas et al., 2007). These non specific natural mechanisms have two main functions; (1) immediate defensive to prevent, control, or eliminate infection early and (2) a warning function to allow effective and selective subsequent

adaptive immune response (Abbas et al., 2007; Lee and Iwasaki, 2007). Cells of the innate immune system such as macrophages, and dendritic cells express receptors known as pattern recognition receptors which act as immune sensors (discussed later in detail).

A second layer of protection is provided by the adaptive (acquired or specific) immune system. In contrast to innate immunity, the adaptive immune system has improved recognition of pathogens through specific diverse clonally distributed receptors (B-cell and T-cell receptors) leading to precise recognition of the specific epitopes giving an antigen specific response. Adaptive immunity also includes immunological memory that allows faster and stronger responses upon subsequent encounters with pathogens (Pancer and Cooper, 2006). The adaptive immune system is comprised of two components which may carry out their protective function independently; (1) the cellular component (T-lymphocytes [helper cells, effector cells, and regulatory cells]) and (2) the humoral component (soluble signalling molecules e.g. antibodies secreted from B-lymphocytes).

1.3.2 Cells of the adaptive immune response

1.3.2.1 Dendritic cells

Cells of the immune system continually inspect their environment for the existence of invading pathogens or self proteins. This scanning is instructed and regulated by the APCs including dendritic cells, B lymphocytes, and macrophages. APCs have the capacity to induce a tolerogenic or immunogenic response and hence sieving the molecular surroundings for both self and non self proteins. Dendritic cells are unique APCs due to their capacity to stimulate a

primary immune response and hence creating immunological memory in addition to taking part in maintaining T cell tolerance to self (Banchereau and Steinman, 1998).

Although the heterogenicity of dendritic cells has its impact on the outcome of immune responses, each subset of dendritic cell has the ability to stimulate T cell responses. Dendritic cells are distinguished in different ways. One way is to classify them into two major subsets; plasmacytoid and conventional. According to lineage, conventionl dendritic cells are divided into myeloid and lymphoid. Another way to classify dendritic cells is according to the tissue distribution of dendritic cells (tissue-derived [interstitial] and blood-derived [lymphoid-resident]). Tissue derived dendritic cells are found in peripheral tissue such as the skin and mucosal sites and migrate upon activation, via lymphatic vessels, to the secondary lymphoid organs to prime T cell responses. In contrast, lymphoid-resident dendritic cells are found in blood and enter secondary lymphoid organs such as the spleen and lymph nodes directly (Shortman and Liu, 2002).

In steady-state laboratory mice, five dendritic cells subtypes are found (table 1.6). Spleen contains three of these cell subtypes; the $CD8^+$, the $CD4^+$ and the double negative dendritic cells (Vremec et al., 2000). Lymph nodes contain two more double negative dendritic cells subtypes; $CD205^+CD11b^+$ in all lymph nodes and $CD205^{high}CD11b^+$ in skin-draining lymph nodes (Anjuere et al., 1999; Henri et al., 2001). In thymus, $CD8^+$ dendritic cells are the dominant subtype.

Table 1.6: Subsets of dendritic cells in mice

	Subset	CD4	CD8	CD205	CD11b	
1	Lymphoid	-	+	+	-	Spleen, lymph node , thymus
2	Myeloid	+	-	-	+	Spleen, lymph node
3	Myeloid	-	-	-	+	Spleen, lymph node
4	Myeloid	-	-	+	+	lymph node
5	Langerhans	-	low	high	+	Skin-draining lymph node

Blood is the only available source of primary dendritic cells in humans in spite of few recent studies on lymphoid tissue dendritic cells (spleen, tonsil, and thymus). Human dendritic cells lack the expression of CD8. Three main subsets of dendritic cells were identified: myeloid, plasmacytoid, and monocyte-derived. The latter is a subset that is often generated in vitro by culturing monocytes in the presence of GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). Myeloid dendritic cells can be generated from human umbilical cord blood under the effect of GM-CSF and TNF-α (Caux et al., 1996; Caux et al., 1997). They are similar in morphology to monocytes and they secrete IL-12 cytokine (table 1.7). Plasmacytoid dendritic cells are similar to plasma cells in morphology, derived from a lymphoid lineage, located in lymph nodes, found in low levels in peripheral blood, and they secrete IFN-γ (Grouard et al., 1997; Colonna et al., 2004) (table 1.7). Both myeloid and plasmacytoid cells are derived from haematopoietic progenitor $CD34^+/Lin^-/CD38^-$ cells from bone marrow (Miller et al., 1999).

Dendritic cells perform an important role in determining the equilibrium between two contrasting states; immune tolerance and immune reactivity, through the

provision of receptor ligand interactions and cytokine secretion (Novak and Bieber, 2008).

Table 1.7: Subsets of human dendritic cells

	Myeloid	Plasmacytoid
Morphology	Monocytic morphology with irregular outline	Plasma cells like with large amounts of rough ER
Expression	**Express** CD11c, **Express** myeloid markers such as CD13 and CD33, **Low expression** of CD123 (O'Doherty et al., 1994; Olweus et al., 1997; Borras et al., 2001)	**Do not express** CD11c, **Do not express** CD13, CD33, **High expression** of CD123 (alpha chain of the IL-3R) and MHC II (Grouard et al., 1997; Robinson et al., 1999; Guermonprez et al., 2002).
Function	**Capture antigens** in the periphery by phagocytosis and macropinocytosis (Robinson et al., 1999).	**Poor antigen-capture capacity**. They are thought to be involved in recognition of self antigens or viruses due to their location within T cell areas of lymphoid tissues (Grouard et al., 1997; Robinson et al., 1999).

The antigen presenting capacity of dendritic cells is enhanced in response to toll-like receptor signalling (e.g., LPS, CpG oligodeoxynucleotides) (De Smedt et al., 1996; Akiba et al., 2004) and molecules released from necrotic (e.g., uric acid, high-mobility group box 1 protein) (Shi et al., 2003; Bianchi and Manfredi, 2007) or oxidatively stressed cells (Becker et al., 2003). Dendritic cells respond to these signals by secreting polarising cytokines, and increased expression of co-stimulatory receptors and MHC class II.

The change of dendritic cells from immature antigen capturing cells to mature antigen presenting cells is associated with several events such as changes in endocytic capacity (Sallusto et al., 1995), MHC class II expression (Landmann et al., 2001), co-stimulatory molecules expression (Sallusto et al., 1995; Carreno and Collins, 2002), lysosomal compartments (CD68, dendritic cell–lysosome-associated membrane protein DC-LAMP), cytokine secretion (e.g. IL-12 from mature cells) (Cella et al., 1996) and chemokines receptor expression (e.g. CCR7, CCR1, and CCR5) (Sallusto et al., 1998). These events are summarised in figure 1.5. Once activated, mature dendritic cells migrate to secondary lymphoid organs to activate specific lymphocytes (Steinman, 1991).

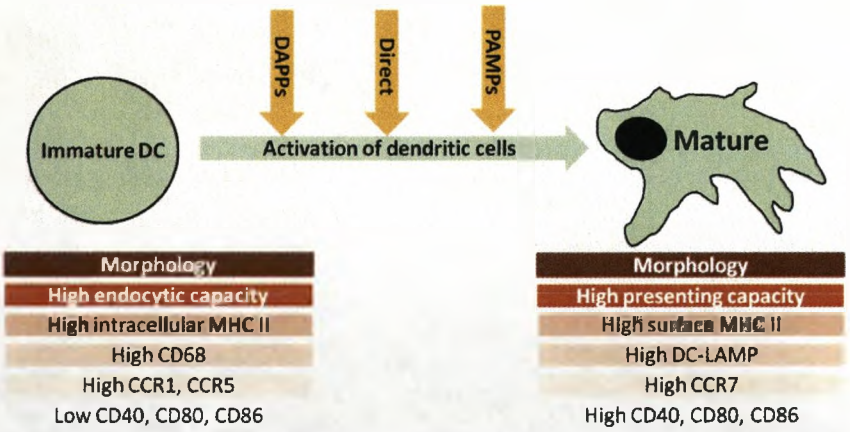

Figure 1.5: Events associated with dendritic cell activation include changes in morphology, endocytic capacity, presenting capacity, MHC class II expression, co-stimulatory molecules expression, lysosomal compartments, cytokine secretion, and chemokine expression.

1.3.2.2 T-lymphocytes

Lymphocytes are white blood cells that constitute the major cellular components of the adaptive immune response. They include large granular innate immune

cells (natural killer cells), small cells involved in cell-mediated immunity (Thymus cells, T-cells), and small cells involved in humoral immunity (Bone cells, B-cells).

Lymphocytes originate from haematopoietic stem cells in the bone marrow from a common lymphoid precursor, and then differentiate following various pathways into their distinct lymphocyte subsets. B cells mature in the bone marrow into B lymphocytes. CD20 is expressed during almost all stages of B cell development. T cells migrate to and mature in the thymus. Immature thymocytes are double-negative ($CD4^-CD8^-$) cells which can be subdivided into four populations according to expression of CD25 and CD44. They then become double-positive ($CD4^+CD8^+$), and finally mature to single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) thymocytes (Bhandoola and Sambandam, 2006; Rolink et al., 2006). Before leaving the thymus or bone marrow, most lymphocytes (>95%) are destroyed due to auto-reactivity against self antigens and/or MHC affinity (negative and positive selection) (Romagnani, 2006). The remaining mature lymphocytes enter the circulation and peripheral lymphoid organs (e.g. spleen and lymph nodes) where they survey peptides derived from invading pathogens and presented in the context of MHC molecules expressed on dendritic cells.

B and T cells differentiate further after exposure to an antigen to form effector and memory lymphocytes. Effector lymphocytes eliminate the antigen by a) releasing antibodies (IgG, IgA, IgM, IgD and IgE) that recognise the antigen, enabling macrophages and natural killer cells to target and attack easier, b) signalling to other immune cells, or c) releasing cytotoxic granules. Memory cells stay in the

peripheral tissues and circulation for an extended period (often the life-time of the individual) ready to counter the same antigen upon future encounters.

An important difference between B cells and T-cells is the way each lymphocyte distinguishes its antigen. B cells recognise their cognate antigen in its whole natural soluble state in the blood or lymph using their B-cell receptors or membrane bound immunoglobulin, enabling macrophages and natural killer cells to target and attack easier. On the contrary, T cells recognise their cognate antigen as short peptide fragments resulting from the intracellular processing of protein antigens presented to the T cell receptor by MHC molecules on the surface of APCs. T lymphocytes are the mediators of cell-mediated immunity and are the focal point of my thesis.

Classification of T lymphocytes as helper and cytotoxic is obsolete as it has been confirmed that both subsets can cause cytotoxic cell death (Berke, 1995). **$CD4^+$ T cells, previously known as helper (Th)** assist other white blood cells in immunologic processes such as maturation of B cells into plasma cells and activation of cytotoxic T cells and macrophages. They become activated by recognition of an antigen associated with MHC class II molecules expressed on the surface of APCs (De Jong et al., 2006). Once activated, they divide rapidly into functional subsets and secrete cytokines. Th1 cells secrete predominantly IL-2, IFN-γ, and TNF-α that support cell-mediated immune responses against pathogens (intracellular), while Th2 cells release IL-4, IL-5, IL-10, IL-9, IL-13 and TGF-β that are important in sustaining a humoral immune response against pathogens (Constant and Bottomly, 1997; Mowen and Glimcher, 2004;

Romagnani, 2004). Several studies have demonstrated that the cytokine IL-12 secreted mainly by APCs, the transcription factor T-bet (T-box expressed in T cells), and STAT1,4 signalling (the signal transducer and activator of transcription 1 and 4) are essential for the promotion of Th1 cell differentiation and the suppression of Th2 subtypes (Thierfelder et al., 1996; Szabo et al., 2000; Afkarian et al., 2002; Lund et al., 2003; Sundrud et al., 2003; Szabo et al., 2003; Athie-Morales et al., 2004; Lund et al., 2004; Romagnani, 2004). In contrast, GATA3 and STAT6 are important in Th2 cell differentiation (Takeda et al., 1996; Ouyang et al., 1998; Usui et al., 2003; Skapenko et al., 2004; Tahvanainen et al., 2006; Lund et al., 2007). T helper 17 (Th17) cells are IL-17 producing T cells that are thought to play a role in autoimmune diseases such as multiple sclerosis and Type 1 diabetes (Steinman, 2007). Differentiation of this subset is promoted by exposure to IL-6, TGF-β, IL-21, and IL-23 and inhibited by Th1 and Th2 cytokines (Stockinger and Veldhoen, 2007; Dong, 2008; Manel et al., 2008). Th17 cells are not associated with T bet and GATA-3 expression which are crucial molecular mechanisms of Th1 and Th2 responses respectively (Park et al., 2005b; Veldhoen et al., 2006). The role of Th17 cells in drug hypersensitivity has not been defined.

$CD8^+$ T cells known as cytotoxic (Tc cells, or CTLs) destroy virally infected cells and tumour cells, and are implicated in transplant rejection. They are thought to play a role in many forms of delayed type drug hypersensitivity reactions and allergic contact dermatitis (Kalish and Askenase, 1999). $CD8^+$ T cells become activated by recognition of an antigen associated with MHC class I molecules on the surface of nearly every cell of the body. Upon activation, they divide into

functional subsets and secrete cytokines. $CD8^+$ T cells are divided into Tc1 and Tc2 cells based on their cytokine profiles with Tc1 and Tc2 being similar to Th1 and Th2 secreting T cells (Noble et al., 1995). Tc1 cells initiate targeted killing, while the role of Tc2 cells in the immune response is largely unknown, although their presence in chronic infections, cancer and autoimmune diseases is associated with increased severity and disease progression (Iezzi et al., 2006). To be ready to respond upon future exposure to the same antigen, **memory cells ($CD4^+$ or $CD8^+$)** persist in the peripheral tissues and circulation for an extended period after the infection fades.

$CD4^+$ regulatory (Treg) cells were known previously as suppressor T cells. They effectively regulate immune response and control auto-reactive T cells. There are two major classes of $CD4^+$ Treg; naturally occurring ($CD25^+FoxP3^+$), and adaptive (Tr1 or Th3). Naturally occurring Treg cells arise in thymus and express $CD25^+$ and the characteristic marker $FoxP3^+$ (Sakaguchi, 2004). Disruption of the Foxp3 gene induces block of natural Treg development or dysfunctional Treg leading to hyperactivation of auto-reactive T-cells causing autoimmune and allergic responses (Sakaguchi et al., 2006). Adaptive Treg cells are thought to originate during a normal immune response in conditions of suboptimal antigen exposure and/or co-stimulation (Maggi et al., 2005). Th3/Tr1 cells have the ability to inhibit the proliferation of cells mainly by the secretion of cytokines IL-10 and TGF-β. CD127 (IL-7 receptor) expression has been described as an excellent cell surface marker for Treg in human blood. $CD4+CD25+FoxP3^{high}CD127^{low}$ Treg represent a small population of circulating $CD4^+$ T cells in peripheral blood (Liu et al., 2006).

Natural killer T cells (NKT cells) can recognise lipid antigens presented by CD1 molecules. Once activated, they can perform functions attributed to both Th and Tc cells (i.e., cytokine production and release of cytolytic molecules). They are also able to fight pathogens rich in lipids as mycobacteria (Moody and Porcelli, 2003; Brigl and Brenner, 2004; Watts, 2004). **Gamma delta T cells** have distinct T cell receptor made up of one γ-chain and one δ-chain (rather than αβ T cell receptor), they are found at their highest abundance in the gut mucosa. The response to antigen is not MHC restricted (Abbas et al., 2007).

1.3.3 Immunological models

Three main models were proposed over the last 60 years to figure out immunological mechanisms; the self/non-self model, the infectious/non-self model (both are based on recognition of foreignness), and the most recent danger model (based on recognition of potential danger).

1.3.3.1 Self/Non-self (SNS) model and co-stimulation

Medawar and his colleagues (Billingham et al., 1953) opened the way for this model by showing the experimental aspects of acquired immunological tolerance and other types of immunological non-reactivity. Later on, this was formulated theoretically by Burnet in 1961 (Burnet, 1961) by emphasising the importance of self recognition in immunology. From that moment, the SNS model was considered the most important basic theory of immunology. SNS model suggested that the immune system functions by discriminating SNS using lymphocytes to accept self and attack non-self. Lymphocytes are programmed early in life

through negative selection. The latter term stands for deletion of inappropriate and immature lymphocytes that have high affinity for self molecules in the thymus (self-reactive lymphocytes) thus allowing the system to detect non-self antigens without mistakenly detecting self-antigens. Since the discrimination of SNS by the effector cells needs signals from stimulator cells, the concept of co-stimulation was added to refine the SNS model.

Co-stimulatory pathways (signal 2) transmit signals from the APCs to the T-cell through a range of ligand-receptor interactions between molecules expressed on the membrane of activated APCs and T-cells. Absence of these signals renders the immune system tolerant to the antigen (Uetrecht, 1999). Here I discuss the three most common co-stimulatory signalling pathways; (a) CD80/86:CD28/CTLA-4, (b) ICOS-L:ICOS (inducible co-stimulator), and (c) CD40: CD154

The CD28 pathway is generally known as the main T-cell co-stimulatory pathway (Bour-Jordan and Bluestone, 2002; Song et al., 2008). Mature dendritic cells express the highest levels of co-stimulatory molecules among all potential APCs so they are the most potent stimulators of naive T- cells (Reis E Sousa, 2006). CD80 (B7.1) and CD86 (B7.2) are two molecules of the B7 family present on the surface of APCs. They can interact with the structurally related T-cell surface receptors CD28 and CTLA-4 (Chambers et al., 2001; Salomon and Bluestone, 2001). CD28 is constantly expressed on T cell surfaces and the engagement with its ligands leads to T-cell activation, stimulation of cytokine production (June et al., 1987; Subudhi et al., 2005), promotion of cell cycle progression (Song et al., 2008), promotion of cell survival signals to T cells by enhancing the expression of

Bcl-xL (Boise et al., 1995), and enhancing memory T-cell development (Muller et al., 2008). In contrast, CTLA-4 is not constantly expressed and its expression is increased following T cell activation to inhibit a potentially excessive immune response through competitive inhibition with CD28 for CD80/CD86 molecules (Saito and Yamasaki, 2003). In addition, CTLA-4 prevents progression of the cell cycle and inhibits the production of IL-2 through inhibition of CD28 activity (Walunas et al., 1996)

The ICOS receptor is another member of the CD28 family expressed on activated T cells and binds exclusively to ICOS ligand on APCs to augment T cell proliferation and cytokine production (IL-4, IL-5, IL-10, IFN-γ and TNF-α) (Greenwald et al., 2005; Van Berkel et al., 2005). ICOS synergises with CD28 to activate T-cells especially during the effector stage of pathogenic responses (Watanabe et al., 2006), while CD28 is essential during the initiation of the response (Van Berkel et al., 2005).

CD40 is a cell surface receptor belonging to the tumour necrosis factor family. It is a co-stimulatory protein expressed on all APCs and is required for their activation. CD40 binds to its ligand CD154 (CD40L) on activated T cells (Van Kooten and Banchereau, 2000; Quezada et al., 2004). This interaction stimulates activation of APCs as defined by increased surface expression of MHC, co-stimulatory receptors and adhesion molecules (O'Sullivan and Thomas, 2003). This process enhances expression of B7-1 and B7-2 on the APCs (Van Gool et al., 1996). In addition, CD40:CD40 L interaction induces a variety of downstream effects such as induction of secretion of inflammatory cytokines that promotes T

cell differentiation and Th1/Th2 polarisation. Interaction between CD40 and its ligand has been shown to supply an important stimulation for sustained activation of the NF-κB pathway, which also plays a role in prolonged up-regulation of co-stimulatory molecules (CD40, CD80 & CD86), immuno-stimulatory cytokines such as IL-12 & TNFα (Yoshimura et al., 2001) and prolonged APC survival (Banchereau and Steinman, 1998).

1.3.3.2 Infectious/non-self (INS) model

This model was proposed in 1989 by Janeway (Janeway Jr, 1989). The basic idea for this model is that APCs are not antigen specific (they capture self and non-self) and do not co-stimulate till activated via encoded pattern recognition receptors. These receptors allow APCs to discriminate between "infectious non-self" and "non-infectious self" by the recognition of conserved pathogens (microbes)-associated molecular patterns (PAMPs or MAMPs) on bacteria (Janeway Jr, 1989; Janeway Jr, 1992). Activation of APCs is accompanied with upregulation of co-stimulatory molecules (signal 2) on their cell surface, which in addition to the antigen-MHC II complex (signal 1) induces T-cell activation and differentiation.

PAMPs are a highly conserved signatures expressed by large families of microbes such as bacterial carbohydrates (LPS, mannose), nucleic acids (bacterial or viral DNA or RNA), and bacterial peptides (flagellin) (Martinon and Tschopp, 2005; Medzhitov, 2007). On the basis of function, PRRs can be classified into three families; endocytic, soluble, and signalling. According to location, PRRs can be divided into cell-associated and soluble (table 1.8) (Abbas et al., 2007).

Both the SNS and INS models are based on non-self recognition, but they could not explain several features of the immune system such as autoimmunity, and cases of exposure to non-self antigen without triggering an immune response (e.g. tumours, changed tissue in aging).

Table1.8: Classification of pattern recognition receptors based on location

Example	Location	PAMP ligands
A. Cell associated pattern recognition receptors		
TLR 1-9 (Toll-like receptors)	Plasma membrane and endosomal membranes of Dendritic cells, phagocytes, others	Various bacterial and viral molecules
Mannose receptor (C-type lectins)	Plasma membrane of phagocytes	Microbial surface carbohydrates with terminal mannose and fructose
CD36 (Scavenger receptor)	Plasma membrane of phagocytes	Mmicrobial diacylglycerides
Nod1, 2, NALP3 (Nod-like receptors)	Cytoplasm of phagocytes and other cells	Bacterial peptidoglycans
FPR and FPRL 1 (*N-formyl* Met-Leu-Phe Receptors)	Plasma membrane of phagocytes	Peptides containing N-formylmethionyl residues
B. Soluble recognition molecules		
C reactive protein (Pentraxins)	Plasma	Microbial phosphorylcholine /phosphatidylethanolamine
Mannose-binding lectin (Collectins)	Plasma	Carbohydrates with terminal mannose and fructose
Surfactant proteins (Collectins)	Alveoli	Various microbial structures
Ficolin (Ficolins)	Plasma	N-acetylglucosamine and lipoteichoic acid of cell walls of gram-positive bacteria

1.3.3.3 Danger model

This model was proposed in 1994 by Polly Matzinger (Matzinger, 1994) on the basis of potential danger (from cell damage) rather than foreignness (self/non-self) as a determinant for an immune response. It is thought that APCs detect and process alarm signals from cell injury (due to pathogens, toxins, stress, necrosis, etc) leading to their activation and upregulation of co-stimulatory molecules. This model depends on cell damage or stress to trigger an immune response, so it does not matter whether the stimulus is an exogenous chemical/pathogen, or an endogenous intracellular molecule.

Alarmins are molecules produced by damaged or stressed cells that have the potential to act as danger signals (Bianchi, 2007; Oppenheim et al., 2007; Kono and Rock, 2008). Examples of these molecules are (a) high mobility group box 1 (HMGB1) (Scaffidi et al., 2002), (b) heat shock proteins (HSPs) (Panayi et al., 2004), (c) S100 proteins (Foell et al., 2007), (d) ATP (Bours et al., 2006), (e) uric acid (Shi et al., 2003) and (f) DNA (Farkas et al., 2007). Because endogenous alarmins and exogenous PAMPs transmit a similar message and trigger similar responses; they were collectively called Damage (or danger)-associated molecular patterns (Bianchi, 2007). The list of DAMPs is growing rapidly, thus Polly Matzinger has proposed that any hydrophobic surface ("Hyppo", or Hydrophobic protein part) might act as a DAMP (Seong and Matzinger, 2004). The response to DAMPs is under the control of toll-like receptors and nod-like receptors (Miller et al., 2003; Apetoh et al., 2007).

It is thought that the immune response is initiated by exogenous pathogen-associated molecular patterns or endogenous alarmins and that both groups are not mutually exclusive since PAMPs appear to act on many of the same receptors as DAMPs (Seong and Matzinger, 2004). Based on this concept, pathogens should have similar effects on APCs. This may explain the increased risk of hypersensitivity in the presence of risk factors (that act as danger signal) such as infection or surgical operations as demonstrated in the following published articles: (a) skin rashes appear almost always in patients with mononucleosis when given ampicillin or amoxicillin (Pullen et al., 1967), (b) increased chance of hypersensitivity reactions in AIDS patients to 50% when given sulfamethoxazole (Fischl et al., 1988), (c) increased risk of agranulocytosis in patients after open-heart surgery when given procainamide (Ellrodt et al., 1984; Uetrecht, 1999) and (d) increased incidence of allergic reactions to β-lactam in patients with cystic fibrosis (Koch et al., 1991; Pleasants et al., 1994)

In addition, involvement of danger signals in the pathogenesis of ADRs has been proposed experimentally. Tienilic acid-induced liver toxicity is supported by some observations that include: (1) the presence of antibodies against CYP2C9 (tienilic metabolic enzyme in human) in patients who were treated with tienilic acid and developed liver toxicity (Lecoeur et al., 1996), (2) the changes in mRNA expression was consistent with danger signals released early from hepatocytes after administration of tienilic acid in rats (Pacitto et al., 2007), (3) the ability of the reactive metabolite of tienilic acid to bind proteins other than CYP (Koenigs et al., 1999). Similarly, changes in gene expression induced by carbamazepine and phenytoin were consistent with oxidative stress and hence danger signals (Lu and

Uetrecht, 2008). In contrast, danger signal related-gene expression (e.g. inflammation, oxidative stress) was downregulated when SMX was given (Pacitto et al., 2007). However, such inhibition of mRNA expression might be a marker of cell stress (Li and Uetrecht, 2010).

The danger model has important implications for vaccination, autoimmunity, and foetal/maternal immunity. In vaccination, modification of the vaccine by infection or adjuvant that is able to induce some cellular injury may increase the effectiveness of the vaccine (Schirrmaker, 2003; Bergmann-Leitner and Leitner, 2004). In autoimmunity, it is suggested that damaged cells (rather than the immune system), in the presence of risk factors, might direct the immune reactions towards self damage (Matzinger, 2007). Finally, the danger model proposes that foetal attack by the maternal immune system depends on the health status of foetus (Bonney, 2007) rather than immunosuppressive molecules released during pregnancy to induce a state of tolerance (Gorczynski et al., 2002; Power et al., 2002; Le Bouteiller and Tabiasco, 2006), which may explain cases like preeclampsia.

1.3.4 Recognition of antigens by T lymphocytes

1.3.4.1 Major histocompatibility complex

Antigens are usually displayed in two forms; soluble and cell-associated. Unlike B lymphocytes, T cells can only recognise cell-associated antigens. Antigen display is a function of the major histocompatibility complex (MHC) expressed on APCs.

The MHC complex is a genomic region called human leukocyte antigen (HLA) on chromosome number 6 which is about 3.6 Mb and containing 140 genes (Horton et al., 2004). MHC molecules have an important role in immune response because antigen recognition and presentation to immune cells (e.g. T cells or natural killer cells) occur in their context. This leads to effective interaction or cell-mediated killing of infected cells. MHC polymorphisms are essential to protect against a diverse range of proteins and pathogens. During embryogenesis, effector cells are trained to ignore self antigens within MHC molecules and react against non self. Failure of this process leads to autoimmunity or immunodeficiency. There are three classes of MHC molecules; (1) class I expressed on all nucleated cells and present endogenous antigens to $CD8^+$ T cells, (2) class II expressed on APCs and present exogenous antigens to $CD4^+$ T cells and (3) class III composed of soluble complement molecules that do not present antigens. These molecules are encoded by many different genes. The class I genes include classic molecules involved in antigen prresentation (A, B, and C), and non classic molecules not involved in antigen presentation (E, F, and G). The class II genes include classic molecules involved in antigen prresentation (DP, DQ, and DR), and non classic molecules (DM, and DO) (Abbas et al., 2007).

Although both MHC class I and class II molecules share the function of binding peptide antigens and presenting them to T cells, they are distinguishable on the basis of structure, tissue distribution and the method of peptide presentation. Both classes are consisted of an extracellular peptide binding cleft, an immunoglobulin-like region, a transmembrane region, and a cytoplasmic region. MHC class I molecules are made up of one α chain (with three domains α1, α2, α3) and β2

microglobulin whereas MHC class II molecules are made up of one α chain, and one β chain (each has 2 domains; α1, α2, β1, β2). The immunoglobulin-like region is the site of MHC-T cell interaction where the α3 domain of MHC class I molecules bind CD8 co-receptor, and β2 subunit of MHC class II molecules bind the CD4 co-receptor. The peptide binding cleft or groove is the site of MHC-peptide interaction. This groove is located between α1 and α2 subunits of MHC I molecule and between α1 and β1 subunits of MHC class II molecule (Bjorkman et al., 1987; Engelhard, 1994; Klein and Sato, 2000; Spencer et al., 2010).

1.3.4.2 Antigen processing and presentation to T lymphocytes

For antigens (exogenous or endogenous) to be recognised by T lymphocytes they should be first converted from the protein form into MHC-bound fragments or peptides (antigen processing). The derived peptides are subsequently displayed in the context of MHC molecules to lymphocytes (antigen presentation). The generation of peptide-MHC complexes does not have a mechanism to discriminate self from non self antigens, so only small number of antigens presented through MHC complexes will able to activate T cell response.

The antigen recognised by T lymphocytes should have several common features including: (1) being protein in nature, (2) has a specific amino acid sequence, (3) cell-associated, (4) displayed by MHC and (5) restricted to the MHC molecule and to the same individual ($CD4^+$ T cells recognise MHC II-bound peptides while CDB^+T cells recognise MHC I-bound peptides) (Abbas et al., 2007). Below, I discuss the pathways of antigen processing and MHC-restricted presentation.

Cytosolic antigens are derived from intracellular proteins generated due to infection, tumour, or protein degradation. They are considered a problem to the immune system as phagocytes or immunoglobulins cannot deal with them. The class I antigen-processing pathway (figure 1.7) consists of sequence of events enabling the cytosolic antigen to be recognised by T cells. First, the antigen is degraded in the proteasome (enzyme complex in cytoplasm of most cells) to generate peptides. Secondly, the peptides are transported from the cytoplasm to the endoplasmic reticulum (ER) by transporter located in ER called TAP (transporter associated with antigen processing) where binding to a newly synthesised MHC molecule takes place. The peptide binds to the binding groove in MHC molecules which accommodates biding of 8-11 amino acid residues. Thirdly, the stable MHC-peptide complexes leave the endoplasmic reticulum through the Golgi to be expressed on the cell surface. Finally, MHC class I molecules present the peptide to $CD8^+$ T lymphocytes through the binding of T cell co-receptor CD8 to α3 domain in the MHC molecule (Tanaka, 1994; Pamer and Cresswell, 1998; Rock and Goldberg, 1999; Hewitt, 2003; Kloetzel, 2004; Rock et al., 2004; Yewdell and Haeryfar, 2005; Abbas et al., 2007).

APCs such as B cells, macrophages, and dendritic cells are adapted to acquire various forms of antigens in the extracellular environment for subsequent processing and presentation to $CD4^+$ T lymphocytes in the context of class II molecules. This pathway (figure 1.7) involves multiple steps. First, antigens are captured by APCs and internalised into lysosomes or endosomes (vesicles with proteolytic activity and acidic pH). Secondly, the proteins are degraded enzymatically (e.g. cathepsin proteases) inside vesicles into peptide fragments.

Thirdly, the peptides are transported to newly synthesised MHC class II molecules associated with protein (invariant chain) from ER through Golgi to endosomes. The invariant chain occupies the peptide binding groove to prevent peptides binding in ER as well as to guide MHC molecule to the endosome. In the endosomes, the antigenic peptide binds to MHC molecules by replacing the peptide in the binding groove. MHC II molecules accommodate binding of peptides of more than 30 amino acid residues. Fourthly, the stable MHC-peptide complex is transported to the cell surface to be expressed on APCs. Finally, MHC class II molecules present the peptide to $CD4^+$ T lymphocytes through the binding of T cell co-receptor CD4 to β2 subunit of MHC molecule (Emoto et al., 2000; Bryant et al., 2002; Sercarz and Maverakis, 2003; Watts, 2004; Stern et al., 2006; Abbas et al., 2007).

One of the most important features of the interaction of MHC molecules and antigenic peptides is the dependency of binding on specific binding motifs. These motifs are based on specific anchor residues at the C- and N-termini of the peptide chain (figure 1.6). Although MHC molecules can bind peptides depending on specific structural motifs and MHC molecule can only bind one peptide at a time, they are able to accommodate any peptide sharing common anchor residues. Once the peptide binds to the MHC binding groove, the dissociation rate is low, allowing enough time for T-cell recognition (Fairchild, 1998; Rock and Goldberg, 1999; Abbas et al., 2007).

In some cases, APCs can process and present exogenous antigens to $CD8^+$ T cells in the context of MHC class I molecules (cross presentation) (Carbone and Bevan,

1990; Kovacsovics-Bankowski and Rock, 1995) leading to $CD8^+$ T cells priming (Heath, 1999; Bevan, 2006). The cellular mechanisms of cross-presentation are still not defined, and multiple pathways are proposed such as access of T cells to the cytoplasm (Kovacsovics-Bankowski and Rock, 1995; Ackerman and Cresswell, 2004), and involvement of transporter that transport proteins out of ER to the cytoplasm (Guermonprez et al., 2003; Imai et al., 2005; Burgdorf et al., 2008). Cross priming is required to fight tumors and viruses that do not infect APCs (Heath and Carbone, 2001; Burgdorf et al., 2007) and to induce cytotoxic immunity by tumor vaccination with protein antigens (Melief, 2003).

Lipid presentation occurs through CD1 molecules (non classical class I-like MHC molecules) expressed on APCs. These molecules form complexes with the endocytosed exogenous lipid antigens to be displayed to non MHC-restricted T-cells or NK-T cells (Abbas et al., 2007). This pathway is important to fight pathogens rich in lipids such as mycobacteria (Moody and Porcelli, 2003; Brigl and Brenner, 2004; Watts, 2004).

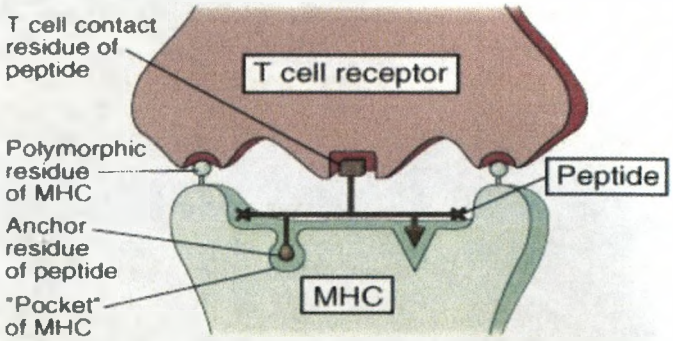

Figure 1.6: The interaction of T cell receptor with MHC-peptide complex showing the peptide anchor residues and the polymorphic residue of MHC (Abbas et al., 2007).

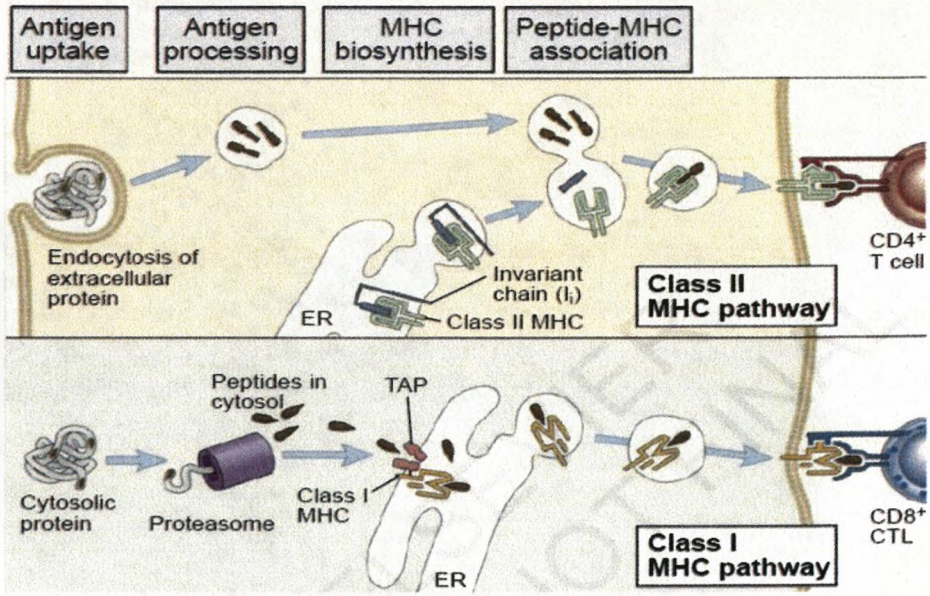

Figure 1.7: Class I and class II MHC antigen presentation pathways for exogenous and cytosolic antigens showing antigen uptake, processing, and association with MHC to be displayed to T lymphocytes (Abbas et al., 2007).

1.3.4.3 T cell membrane molecules involved in antigen recognition and initiation of functional responses

The response of T lymphocytes to the displayed peptide-MHC complex need several molecules and receptors that function or assist in functions related to antigen recognition, APC-T cell adhesion, and transduction of signals from the T cell surface to the nucleus. These molecules include: (1) the T cell receptor complex (formed by T cell receptors linked non-covalently to CD3 and ζ) involved in antigen recognition and signalling and (2) the accessory molecules involved in stable cell adhesion and signalling such as the co-stimulatory molecules. These molecules and receptors form with their ligands in the APC a transient structure called the immunological synapse which is important in

regulation of signalling (Abbas et al., 2007). Here I discuss these molecules and their role in antigen recognition and signalling.

T-cell receptors (TCR) are of two types; TCR-2 which is the most common and recognises only peptides, and TCR-1 which is less common and recognises lipid antigens. Structurally, they are composed of two transmembrane protein chains (two dimers) with disulfide bonds. Four dimmers are well known and are named alpha (α), beta (β) (both constitute TCR-2), gamma (γ) and delta (δ) (both constitute TCR-1). As a member of the immunoglobulin family, each TCR chain is formed of an inward C-terminal region facing the cell membrane called the constant region, and an outward N-terminal region to interact with MHC molecules called the variable region. In addition, three small regions extend from the constant region (short hinge region, transmembrane region, and short cytoplasmic tail) (Call and Wucherpfennig, 2005; Kuhns et al., 2006). The variable region is characterised by having hypervariable domains known as complementarity-determining regions (CDRs) that determine TCR contact with the MHC-peptide complex (e.g. CDR1 is thought to interact with the antigenic peptide, CDR2 recognises MHC, CDR3 recognises processed antigens, and CDR4 recognises superantigens) (Van Der Merwe and Davis, 2003). Functionally, both types of TCR differ in terms of antigen recognition and presentation. Because of limited diversity and limited ligands to TCR-1, they recognise antigen via a similar pathway to antibodies (recognise intact protein and non protein antigens). In contrast, TCR-2 has a big diversity and unique TCR complex that enables recognition of membrane-bound antigens in the context of MHC molecules (Casetti and Martino, 2008).

The TCR Complex is a functional structure involved in T-cell activation and signal transduction from TCR into the cell (Kuhns et al., 2006). It is formed of a TCR (has a positively charged transmembrane region and short cytoplasmic tail), an associated molecule CD3, and the ζ chain (have a negatively charged transmembrane region and longer cytoplasmic tail). CD3 has three distinct chains (γ, δ, ε) (Call and Wucherpfennig, 2005). The cytoplasmic tail of such chains has a characteristic signature of aminoacids (tyrosine separated from a leucine or isoleucine by another two amino acids) known as immunoreceptor tyrosine-based activation motif (ITAM) which is important in signal transduction (Abbas et al., 2007).

CD4 and CD8 are co-receptors expressed on the cell surface of T cells to assist TCR complex function in signal transduction at the time of antigen recognition through ensuring antigen specificity and prolonged APC-T cell engagement. CD4 is expressed on T helper cells and its N-terminal immunoglobulin-like domains interacts with β2 subunit of MHC class II, while CD8 expressed on cytotoxic T cells and its single immunoglobulin-like domain binds the α3 domain of MHC class I molecule (Singer and Bosselut, 2004; Rudolph et al., 2006).

1.3.5 T-cell activation

Recognition of antigenic peptides bound to MHC by T lymphocytes is the primary trigger for the initiation of a T cell response. This process involves association of TCR complexes with specific peptide-MHC protein complexes on the surface of an APC (signal 1) and expression of co-stimulators on APCs (signal 2) leading to T cells proliferation and differentiation into effector and memory cells as well as

cytokine synthesis. Effector cells either mediate cell-mediated immune response through $CD4^+$ cells or kill infected cells through $CD8^+$ cells. In previous sections, TCR complex and co-stimulators were described. This section below describes the signalling pathways involved in T cell activation.

These signals move through a junction between the APC and the T cell known as immunological synapse which is important to ensure specific and tight communication (Call and Wucherpfennig, 2005; Dustin, 2005; Mossman et al., 2005). This synapse is formed during T cell activation by coalescence of lipid rafts (specialised membranes formed by accumulation of signalling molecules in T cell plasma membranes).

After TCR activation by the interaction between TCR complex/CD28 and MHC complex/B7, the pathway of signal transduction from T cell surface into the cell induces (1) membrane events include activation of Src family protein tyrosine kinases (LcK and Fyn) leading to the phosphorylation of the ITAMs and the recruitment of protein tyrosine kinases especially ZAP-70 (Zeta-chain-associated protein kinase 70), and adapter proteins such as LAT (linker for the activation of T-cell) (Zhang et al., 1998; Zhang et al., 1999; Smith-Garvin et al., 2009), (2) cytoplasmic signalling pathways leading to the activation of effector enzymes, such as the kinases ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinases), and PKC (protein kinase C), and the phosphatase calcineurin and (3) production of transcription factors such as NFAT (nuclear factor of activated T-cells), AP-l (activator protein 1), STAT3 (signal transducer and activator of transcription 3) and NF-κB (nuclear factor kappa-light-chain-enhancer

of activated B cells), which function to enhance gene expression in antigen-stimulated T cells (Macian, 2005; Vallabhapurapu and Karin, 2009).

1.3.5.1 Drug-specific T cell activation

Hypersensitivity reactions can be explained based on two main pathways; the hapten/prohapten concept and the pharmacological interactions of drugs with immune receptors (p-i) concept.

Several compounds including drugs and metal ions comply with the hapten concept (Park et al., 1998; Uetrecht, 1999; Naisbitt et al., 2001; Park et al., 2001). These compounds are of small molecular mass and they need to bind strongly to protein in order to offer a new antigenic determinant for the adaptive immune system (Landsteiner and Jacobs, 1935; Cavani et al., 1995; Padovan et al., 1997; Cavani et al., 1998; Griem et al., 1998; Aiba et al., 2003). Some drugs seem to comply with the prohapten concept. They are not directly protein reactive and need to be metabolised (bioactivated) in liver cells, keratinocytes, or dendritic cells (Kimber and Cumberbatch, 1992; Reilly et al., 2000; Janmohamed et al., 2001; Swanson, 2004; Roychowdhury et al., 2005) to generate a hapten that binds to protein (Griem et al., 1998; Naisbitt et al., 2000a; Aiba et al., 2003). The hapten-protein interaction is either extracellular (membrane) or intracellular (Lavergne et al., 2009). As discussed previously, the immune system deals with the exogenous and endogenous antigens in different ways; exogenous antigens are presented through MHC class II to $CD4^+$ T cells whereas cytosolic antigens are presented through MHC class I to $CD8^+$ T cells (Kalish and Askenase, 1999).

Whether cellular distribution of drug protein adduct formation relates to the selective activation of $CD4^+$ and $CD8^+$ T cells has not been explored.

Although the hapten/prohapten pathway of drug specific T-cell activation is widely cited and perceived to be the primary pathway involved in the activation of pathogenic T-cells, direct evidence showing that drug-protein conjugates stimulate primary T-cells is largely lacking.

In fact, lymphocytes from patients hypersensitive to a range of drugs including SMX (Schnyder et al., 1997; Zanni et al., 1998), carbamazepine (Naisbitt et al., 2003a), lamotrigine (Naisbitt et al., 2003b), lidocaine (Zanni et al., 1997; Zanni et al., 1999), mepivacaine (Zanni et al., 1999), p-phenylendiamine (Sieben et al., 2002), and radio contrast media (Christiansen et al., 2000; Christiansen, 2002) have been shown to be stimulated in vitro with the parent drug even if APCs were fixed (to exclude processing) (Schnyder et al., 1997; Zanni et al., 1998; Zanni et al., 1999; Naisbitt et al., 2003a; Naisbitt et al., 2003b). Moreover, pulsing APCs with drugs as lidocaine, lamotrigine, carbamazepine, ciproxin or SMX for 1 h followed by washing the drug resulted in no stimulation of T cells (Zanni et al., 1998; Naisbitt et al., 2003a; Naisbitt et al., 2003b; Schmid et al., 2006). These data lead Pichler and co-workers to investigate further the way in which non-covalently bound drugs might cross-link MHC molecules and specific T-cell receptors to stimulate a T-cell response.

The p-i concept proposes the stimulation of specific T-cells through the interaction between TCR and a drug (in its native form) in the presence of the APC and appropriate MHC molecules without processing or carrier binding

(Pichler, 2003; Posadas and Pichler, 2007). The exact mechanism of this pathway is not yet defined, but it appears to bypass the innate immune system (Pichler, 2005) through mechanisms depending on (a) the lower activation threshold of memory T-cells than naive T-cells allowing low signals to stimulate TCR-drug reactions (Croft, 1994; Rogers et al., 2000), (b) the peptide specificity of TCR (Depta et al., 2004) as shown by transfection of TCR with different antigens into a mouse hybridoma T cell line (Schmid et al., 2006), and (c) the cross reactivity of these peptide-specific memory T cells. These features are important as they partly explain (a) drug induced allergic reactions without previous exposure as in neuromuscular blockers, iodinated contrast media, and cetuximb (Kvedariene et al., 2006; Harboe et al., 2007; Chung et al., 2008), (b) the higher incidence of hypersensitivity reactions in patients with autoimmune disease or infection (Pichler et al., 2006), and (c) the faster secondary memory response by the immune system.

Unlike hapten/prohapten pathway, the p-i conept is not dependent on bioactivation or protein binding and hence it is not confined to the site of drug application or metabolism. This may explain the predominance of this pathway in hypersensitivity reactions linked to systemic drug application (Posadas and Pichler, 2007). In addition, p-i concept may be the predominant pathway in drug reactions involving skin due to the presence of dense network of T cells (mainly memory) in close contact with dendritic cells (expressing MHC) (Schaerli and Moser, 2005; Clark et al., 2006).

Generally, p-i concept supplements the hapten/prohapten concept as some reactions can be described clearly based on one pathway (e.g. penicillin based on a hapten mechanism) (Padovan et al., 1997), while others are thought to involve both pathways as seems to be the case with SMX and quinolones (Schnyder et al., 2000; Manfredi et al., 2004; Schmid et al., 2006).

Recently, studies have focussed on the role of environmental and genetic factors in hypersensitivity reactions. The association of HLA alleles with drug hypersensitivity reactions has been reported with some drugs including carbamazepine (HLA-B*1502 allele), allopurinol (HLA-B*5801), and abacavir (HLA-B*5701) (Mallal et al., 2002; Chung et al., 2004; Martin et al., 2004; Hung et al., 2005). Most of these studies have reported the importance of other factors located in HLA region of human chromosome 6 such as HSP 70. In addition, it has been shown that certain drugs have the potential to act on cells of the innate immune system (e.g. dendritic ells) leading to their activation, supplying danger signals, and driving the pathogenic immune response in hypersensitivity. These drugs include abacavir (Martin et al., 2007), SMX (Sanderson et al., 2007), and amoxicillin (Rodriguez-Pena et al., 2006). Thus, the big qestion remains; which of these signals are vital in hypersensitivity reactions; MHC, TCR, HLA, and/or co-stimulatory molecules?

1.4 Sulfamethoxazole: a model drug antigen to study mechanisms of drug hypersensitivity

Sulfonamides were originally demonstrated as antibacterial agents by the German Nobel-prized physician Gerhard Domagk who was the first to demonstrate the

selective antibacterial action of a drug (Domagk, 1935). Since that time, many sulfonamide derivatives have appeared in the clinical use with identical microbiology and different pharmacokinetics. In spite of diminished clinical importance of sulfonamide in recent years due to the introduction of penicillin and other antibiotics, a high frequency of hypersensitivity reactions (Gordin et al., 1984; Carr et al., 1994; Walmsley et al., 1998), and rapid resistance development, the drug is still considered in certain therapeutic fields. Furthermore, the general increase of β-lactam resistance and the improvement in dealing with allergic side effects, might force sulfonamides back into clinical practice as efficient inexpensive antibacterial drugs (Kong et al., 2010).

Sulfonamides are structural analogues of para-aminobenzoic acid (PABA) and exert their bacteriostatic effect by the competitive inhibition of the enzyme dihydropterase (DHP) synthetase which is involved in the formation of folic acid in bacteria.

4-amino-N-(5-methyl-1,2-oxazol-3-yl) benzenesulfonamide known as sulfamethoxazole (SMX, $C_{10}H_{11}N_3O_3S$, MW253.3, figure 1.9) is an antibacterial drug belonging to the sulfonamide group. SMX is most often used in combination with trimethroprim (SMX-trimethoprim) in the ratio of 5:1. However, as the volume of distribution of trimethoprim is much higher than that of SMX, their plasma ratio becomes 20:1, the optimal ratio for synergistic activity. This well-recognised combination blocks the metabolic pathway of tetrahydrofolate (THF) synthesis in microorganism at two different points with a net bactericidal effect;

the first point is dihydropterase synthetase (target of SMX), while the second point is dihydrofolate reductase (target of trimethoprim) (figure 1.8).

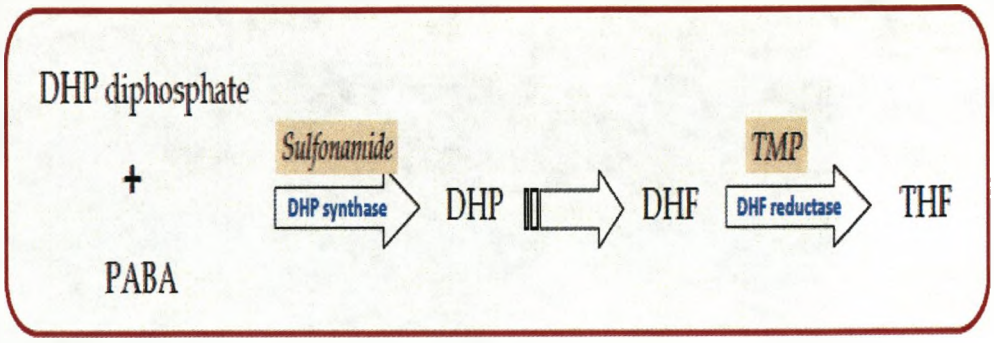

Figure 1.8: Tetrahydrofolate (THF) synthesis pathway in microorganisms with the sites of action of SMX and trimethoprim. PABA: para-aminobenzoic acid, DHP: dihydropterase, DHF: dihydrofolate.

1.4.1 Sulfamethoxazole metabolism

The acetylated metabolites of SMX are not cytotoxic and can be cleared rapidly by renal excretion. However, slow acetylation of SMX favours the oxidative pathway of metabolism (Shear et al., 1986). Oxidative metabolism of SMX has been shown to be mediated via phase I enzyme systems such as CYPs (CYP2C9, CYP2C8) and/or MPO. The unstable metabolite hydroxylamine (SMX-HA) in humans and other species is the primary product of SMX oxidative metabolism (Cribb et al., 1990; Cribb and Spielberg, 1990; Cribb and Spielberg, 1992; Cribb et al., 1995; Gill et al., 1997; Roychowdhury et al., 2007b; Sanderson et al., 2007). SMX-HA is usually eliminated by phase II sulphates or glucuronides. Furthermore, reduction of SMX-HA yields the parent SMX through a cytochrome b5/b5R pathway (Kurian et al., 2004). Auto-oxidation route leads to formation of a highly unstable metabolite (nitroso SMX; SMX-NO) that can bind to cell

surface proteins of lymphocytes and keratinocytes (Naisbitt et al., 1999; Reilly et al., 2000; Naisbitt et al., 2002), and the model extracellular protein human serum albumin (Callan et al., 2009). Thus, SMX-NO protein adducts have been implicated in the pathogenesis of drug hypersensitivity reaction (Shear and Spielberg, 1985; Rieder et al., 1988).

It has been postulated that antioxidant and detoxification enzymes play a central role in determining the level of tissue exposure to the electrophilic highly reactive nitroso-intermediate and hence drug haptenization, drug immunogenicity, and risk of SMX hypersensitivity reactions. Antioxidants such as ascorbate, glutathione and cysteine can abolish toxicity and immunogenicity of SMX-NO by non-enzymatic reduction to SMX-HA (Cribb et al., 1991; Gill et al., 1997; Naisbitt et al., 1999; Naisbitt et al., 2000b; Trepanier et al., 2004). It was found that SMX-NO adducts in lymphoid cells in vitro were decreased by ascorbate and glutathione (Manchanda et al., 2002; Farrell et al., 2003; Lavergne et al., 2009). Disturbance of the balance between bioactivation and bioinactivation in some cases due to environmental or genetic factors leads to increased levels of the reactive metabolites. In support, decreased capacity for SMX-NO reduction was shown in patients with HIV infection due to deficient redox regulation (i.e low thiol and ascorbate levels in cells and plasma) (Naisbitt et al., 2000b; Trepanier et al., 2004). Degradation of SMX-NO in solution gives nitro SMX (by oxidation), SMX-HA (by reduction), and azo/azoxy adducts (by dimerisation) (Naisbitt et al., 2002).

In chapter 3, the issue of SMX metabolism will be discussed in detail including aspects such as SMX metabolism by immune cells, SMX-metabolising enzymes, as well as the involvement of protein adducts and cytotoxicity of SMX reactive metabolites in the development of an immune response.

1.4.2 Sulfamethoxazole adverse reactions

Adverse effects of SMX-trimethoprim combination include mainly skin rash of varying severity, gastrointestinal tract effects, and disorders of the hematopoietic system. Among these adverse effects, hypersensitivity reactions are the main interest of this thesis. Generalised maculopapular rash is the most common sulfonamide skin reaction. Symptoms may be accompanied by fever and internal organ toxicity. Other rare reactions include anaphylaxis and blistering skin conditions such as SJS/TEN.

1.4.3 Sulfamethoxazole immune response

Although hypersensitivity reactions involving antibiotics such as sulfonamides and penicillin have been studied extensively, the chemical mechanism associated with the development of a cellular immune response is not yet fully understood. This is largely because reactive metabolites are generally not available for *in vitro* assay, and when they are, they are often toxic to immune cells. It has been found that human exposure to almost any primary aromatic amine (e.g. SMX or dapsone) at a dose of 50 mg/day or higher is associated with a high incidence of adverse reactions independent of the remaining structure (Uetrecht, 2002). This is

due to their ability to form reactive metabolites such as hydroxylamine and nitroso compounds.

Most studies exploring the sulfonamide immune response and the underlying mechanisms of sulfonamide hypersensitivity have focused on SMX due to evident hypersensitivity reactions, known pharmacokinetics of the drug, availability of SMX synthetic metabolites with known protein reactivity, and its importance in treatment of infection in immunocompromised patients with HIV and in patients with cystic fibrosis.

SMX/metabolites have the ability to initiate immunological reactions including both adaptive and innate immune responses. Cells of the innate immune system (e.g. dendritic cells) have an important role in SMX immune response as shown by Sanderson et al. (2007), who found that *in vitro* exposure of dendritic cells to SMX and its metabolite nitroso SMX was associated with partial dendritic cell activation. The response to SMX was dependent on metabolism and formation of protein adducts with cellular protein. In addition, it was thought that infection and cell injury could contribute to the great rise of incidence of SMX hypersensitivity through the innate immune cell response mediated by recognition of PAMPs or DAMPs (Peitsch et al., 1988; Asea et al., 2002; Biragyn et al., 2002; Janeway Jr and Medzhitov, 2002; Termeer et al., 2002; Wallin et al., 2002; Shi et al., 2003; Seong and Matzinger, 2004). In this respect, Lavergne et al. (2009) demonstrated that cellular activators and cytokines, which stimulate dendritic cell maturation, significantly enhance SMX metabolism.

On the other hand, adaptive immunity with its two arms; cellular and humoral plays a central role in SMX immune response as evident by detection of drug-specific T cells and antibodies. Drug or metabolite-specific T-cells were characterised in patients with SMX hypersensitivity (Schnyder et al., 2000; Castrejon et al., 2010; Lavergne et al., 2010), while animal experiments showed that T-cells recognise only the metabolite when the animals were immunised with the reactive metabolite SMX-NO (Farrell et al., 2003). Although most of sulfonamide hypersensitivity reactions are delayed type (type IV), some appear to be IgE mediated as indicated by detection of both anti-drug and anti-tissue antibodies in SMX allergic individuals (Cribb and Spielberg, 1992; Cribb et al., 1997; Lavergne et al., 2008a). Such antibodies are thought to be due to neoantigens created in response to drug-protein adduct formation and are able to elicit an immune reaction against both; the adducts and the neoantigens.

Controversy regarding immune responses towards the SMX arises from the way by which the drug can stimulate T-cells. Metabolite-specific T-cell clones were identified from SMX hypersensitive patients (Farrell et al., 2003; Nassif et al., 2004a; Castrejon et al., 2010). On the other hand, T-cells from hypersensitive patients can also be stimulated with the parent drug through a direct interaction with MHC and T-cell receptors without prior processing by APCs (Schnyder et al., 1997; Burkhart et al., 2001). Recent study conducted by Castrejon et al. (2010) on SMX hypersensitive patients demonstrated that SMX-NO provided a potent antigenic determinant for T-cells and that the response against SMX was detected infrequently and may be dependent on the formation of densely coated,

but weakly associated SMX-MHC peptide complexes that cross-react with the haptenic immunogen.

1.4.4 Sulfamethoxazole use in patients with HIV infection

SMX is used in treatment of a wide variety of infections such as urinary tract infections, sinusitis (alternative to amoxicillin-based antibiotics), ulcerative colitis and toxoplasmosis. Furthermore, SMX is an important drug in prophylaxis and treatment of pneumocystic carinii pneumonia which is a common opportunistic infection in immunocompromised patients with HIV infection (Furrer et al., 1999; Rabaud et al., 2001; Dirienzo et al., 2002; Goldie et al., 2002; Grimwade and Swingler, 2003). However, SMX hypersensitivity is also an important clinical problem in this population as the risk of hypersensitivity reactions is greatly increased with reported incidence of 25–65% (Gordin et al., 1984; Medina et al., 1990; Walmsley et al., 1998; Pirmohamed and Park, 2001) compared to HIV-negative patients (3%) (Koch-Weser et al., 1971; Jick, 1982). Underlying mechanisms may include infection, reduced levels of thiols and ascorbate, and a decreased capacity to reduce the metabolites back to the parent drug (Walmsley et al., 1997; Naisbitt et al., 2000b; Trepanier et al., 2004). However, to date, the reasons for the enhanced susceptibility is not fully defined.

1.4.5 Sulfamethoxazole use in patients with cystic fibrosis

Antimicrobial therapy is important to preserve pulmonary function in patients with cystic fibrosis. Unfortunately, this is restricted by drug-induced hypersensitivity reactions (Koch et al., 1991; Pleasants et al., 1994; Lavergne et

al., 2010). The underlying mechanism responsible for these reactions is unknown but recurrent respiratory infection is likely to play a central role in changing the immune status through the secretion of cytokines such as IL-6, IL-8, and IL-17 (Dubin et al., 2007; Nembrini et al., 2009), or the generation of high levels of reactive oxygen species (Nembrini et al., 2009). In chapter 6, I investigated the involvement of drug antigen-specific T-cells from patients with cystic fibrosis in the pathogenesis of delayed hypersensitivity reactions.

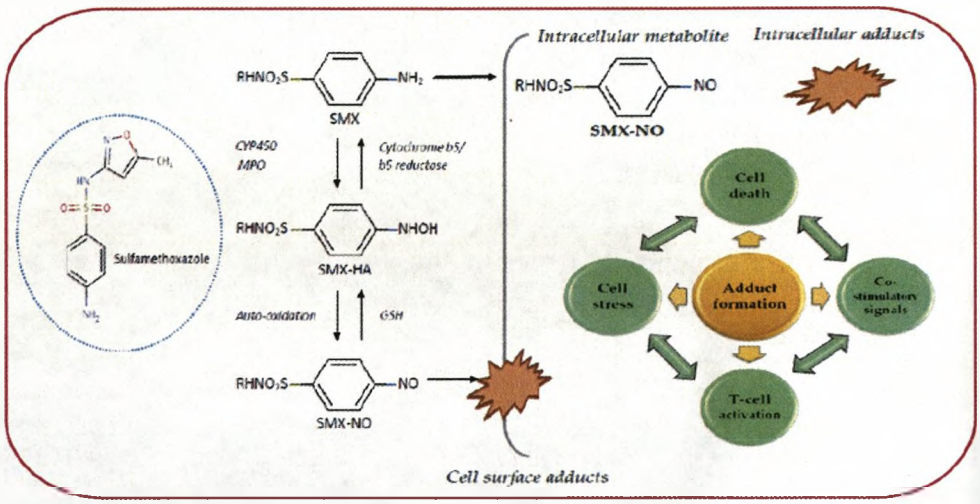

Figure 1.9: SMX metabolism and the possible involvement of protein adducts in cell stress, cell death, co-stimulatory signalling, and the development of antigen-specific T cell response.

1.5 Aim of the thesis

The overall aim of this study was to investigate the role of drug metabolism by immune cells in the formation of functional antigens using SMX as a defined drug antigen. Also, the availability of cells from hypersensitive patients and animal models of immunogenicity allowed us to define the relationship between adduct formation, cell death, co-stimulatory signalling and T-cell stimulation.

Chapter 3 explores the metabolism of SMX by mouse APCs and the generation of metabolism-derived protein adducts. The direct and indirect effects of SMX and its reactive metabolites on the maturation status of mouse dendritic cells are investigated in chapter 4. The ability of adducts formed as a consequence of SMX metabolism in APC is studied in mouse and human systems in chapters 5 and 6 respectively. In addition, chapter 6 compares the phenotype and function of T-cells from SMX hypersensitive patients with and without cystic fibrosis.

Specific research questions addressed by the data presented within this thesis include:

1. Do APCs metabolise SMX to a protein reactive intermediate?
2. Are SMX metabolites generated in APCs able to provide maturation signals to dendritic cells?
3. Do SMX metabolites generated in APCs form functional antigens for T-cells?
4. Does SMX metabolism differ in APCs from patients with and without cystic fibrosis and does this alter T-cell immunogenicity in hypersensitive patients?

Chapter 2

Materials and Methods

Contents

2.1 Materials and methods for animal experiments

2.1.1 Mice

Female Balb/c mice (6-10 weeks of age) were purchased from Charles River (Margate, Kent, UK) and maintained under specific pathogen-free conditions in standard plastic laboratory cages with a wire mesh lid in an air conditioned, sound-proof room at an ambient temperature of 24.0 ± 0.5°C. All animals were allowed to habituate to the animal laboratory conditions for at least one week before starting the experiments. The animals had access to standard lab chow and tap water ad libitum. A twelve hour light/dark cycle was maintained throughout the experiments. All experiments were carried out under the provisions of the United Kingdom Animals (Scientific Procedures) Act, 1986. Drug/naive animals were used to obtain bone-marrow-derived dendritic cells and/or splenocytes using aseptic techniques, unless stated otherwise.

2.1.2 Culture medium

RPMI (Roswell Park Memorial Institute)-1640 medium, penicillin-streptomycin, L-glutamine, HEPES buffer (N-2-HydroxyEthylPiperazine-N-2-EthaneSulfonic acid), and 2-Mercaptoethanol were from Sigma-Aldrich (Gillingham, Dorset, UK). Heat-inactivated fetal bovine serum (FBS) was purchased from Gibco Invitrogen (Paisley, UK). Heat-inactivated Balb/c mouse serum (replaced FBS in certain dendritic cell experiments) was purchased from three different companies (Biosera, East Sussex, UK; Innovative Research, Michigan, USA; AbD Serotec, Oxford, UK).

Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was obtained from Peprotech (London, UK). The vial was centrifuged before opening and then reconstituted in water to a concentration of 10 μg/ml (5 x 10^4 units/ml). Aliquots were stored at -20 °C for future use at a concentration of 20 ng (2 μl aliquot, 100 units/ml). Isolated bone marrow cells were suspended in culture medium RPMI-1640 supplemented with heat-inactivated and filtered FBS (10% v/v), penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), HEPES (25 mM), 2-mercaptoethanol (50 μM) and recombinant murine GM-CSF (20 ng/ml). Isolated splenocytes were maintained in medium consisting of RPMI-1640 supplemented with heat-inactivated and filtered FBS (10% v/v), penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and HEPES (25 mM).

2.1.3 Chemicals, reagents and buffers

Sulfamethoxazole was obtained from Sigma-Aldrich (Gillingham, Dorset, UK). The oxidative metabolites of SMX (nitroso SMX [SMX-NO]; $C^{10}H^9N^3O^4S$; M.W. 267.26) was synthesised according to the method of Naisbitt et al (Naisbitt et al., 1996), and found to be >99% pure by liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR). Stock solutions of SMX were freshly prepared before use in a mixture of cell culture medium: dimethyl sulfoxide (10:1, v/v) and diluted as required.

Synthesis of SMX-NO involves three steps; step 1 involves conjugation of 3-amino-5-methylisoxazole and 4- nitrobenzenesulfonyl chloride to generate nitro SMX (SMX-NO_2), step 2 involves the catalytic reduction of SMX-NO_2 to

generate SMX-NHOH, and step 3 involves partial oxidation of SMX-NHOH to generate SMX-NO. Each of these steps is described in detail below.

SMX-NO_2 was prepared by the reaction of 3-amino-5-methylisoxazole (20 g; 203 mmol) with 4- nitrobenzenesulfonyl chloride (45 g; 203 mmol) in 75ml ice-cold pyridine. The reaction mixture was stirred on ice overnight, and a brown precipitate was produced by the addition of excess distilled water (dH_2O). This precipitate was filtered, washed with further dH_2O to remove remaining pyridine, recrystallised from an ethyl acetate:toluene mixture (1:3 v/v) and left overnight at 0°C. The final mixture was then collected by filtration and analysed for purity by LC-MS and NMR.

SMX-NHOH was synthesised from SMX-NO_2 by reduction using sodium phosphinite as a hydrogen donor and a two-phase solvent system (tetrahydrofuran/water) with a palladium/carbon catalyst; 5% palladium carbon catalyst (0.1 g) was added to a stirred solution containing sodium phosphinite (1.12 g, 11 mmol) and SMX-NO_2 (1 g, 3.53 mmol) in dH_2O (10 ml)/tetrahydrofuran (100 ml). The reaction mixture was monitored by thin-layer chromatography (TLC), using a mobile phase of dichloromethane:ethyl acetate (65:35), until nearly all of the starting material had reacted. This typically took 20 min. The product was filtered under vacuum to remove excess catalyst, extracted with ether and dried with $MgSO_4$ to give a pale orange oily product. This was further dried on a rotary evaporator (Rotovapor, BÄuchi, Switzerland) to give a pale yellow solid. This product was then recrystallised from chloroform, and the purity of the final product assessed by NMR and HPLC.

Oxidation of SMX-NHOH with iron (III) chloride was performed to generate SMX-NO. SMX-NHOH (200 mg, 0.743 mmol) in ethanol (30 ml) was added to a stirred solution of iron (III) chloride hexahydrate (1.5 g, 5.549 mmol) in dH_2O (40 ml) over a period of 10 min. This mixture was stirred at room temperature for 3 h, during which a yellow solid precipitated within the reaction mixture. This solid was filtered and dried under vacuum and the purity was analysed by NMR and HPLC.

Tritiated [^{3}H]-methyl thymidine (specific activity 5 Ci/mmol, concentration 1 mCi/ml; 48.5 µg/ml) was obtained from Moravek (California, USA). Thymidine was diluted in culture medium to adjust the concentration at 0.5 µCi per well in proliferation assays.

Multiplex assay kits (manufactured by Linco Research) for simultaneous detection of 11 mouse cytokines in mouse cell culture samples (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-15, IFN-γ, and TNF-α) were purchased from Millipore (Watford, Herts, UK). Kits were stored at 2-8 °C. All reagents were warmed to room temperature (20-25°C) prior to use.

Dimethyl sulfoxide (DMSO), hank's balanced salt solution (HBSS), alkaline phosphatase substrate, methimazole (meth), 1-aminobenzotriazole (1-ABT), tween 20, bovine serum albumin (BSA) powder, trypan blue 0.04%, phosphate-buffered saline (PBS), lipopolysaccharide (LPS, E.coli), reduced glutathione (GSH), and paraformaldehyde (PFA) were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). Lymphoprep was obtained from Axis-Shield PoC AS

(Oslo, Norway). The annexin V: FITC assay kit was obtained from AbD Serotec (Oxford, U.K.).

2.1.4 Antibodies

The following mouse antibodies were used for flow cytometric characterisation of dendritic cell surface receptor expression: PE-labelled CD11c (Caltag, Bucks, UK), FITC-labelled CD40, FITC-labelled CD86, PE-labelled I A/I-E (all BD Biosciences Pharmingen, Oxford, UK), FITC-labelled CD11b, PE-labelled Gr-1 (Miltenyi Biotec, Surrey, UK), FITC-labelled neutrophil, FITC-labelled CD19 and FITC-labelled CD204 (all AbD Serotec, Oxford, UK).

FITC-labelled anti-rabbit antibody and alkaline phosphatase conjugated anti-rabbit antibodies (Sigma-Aldrich; Gillingham, Dorset, UK) were used for immunochemical detection of SMX derived protein adducts. To generate an anti-SMX antibody, SMX was conjugated to human serum albumin (for use as a positive control for the validation of the ELISA protocol), and to keyhole limpet hemocyanin for use as a soluble antigen for rabbit immunisation, using a previously described procedure (Lavergne et al., 2006b). Drug-protein conjugation was confirmed by spectrophotometry, and by reactivity with rabbit anti-SMX antibody, kindly provided by Dr. Michael Rieder (London Ontario, Canada). The rabbit immunisation against SMX-keyhole limpet hemocyanin was approved by an Institutional Animal Care and Use Committee, under a USDA license (# 35-B-0097 and 36-R-0108) and a PHS accreditation (# A4284-01) (Panigen Inc, Branchardville WI) (Lavergne et al., 2006b). The reactivity of anti-sera against SMX-human serum albumin was confirmed by both ELISA and immunoblotting.

2.1.5 Immune cell preparation

2.1.5.1 Bone marrow-derived mouse dendritic cells

Bone marrow-derived mouse dendritic cells were generated according to the procedure of Lutz (Lutz et al., 1999), with slight modifications. Mice were sacrificed by raising the concentration of CO_2 followed by cervical dislocation. Femurs, tibiae and humeri were removed and purified from surrounding tissues using sterile forceps and scissors then rubbed with gauze. Intact bones were suspended in 70% ethanol for 5 min for disinfection and washed twice with iced PBS. The ends of each bone were removed with bone scissor and the marrow flushed out with PBS using a syringe with a 20-gauge needle. Clusters within the marrow suspension were disintegrated by pipetting and passed through a 40 μm nylon mesh to remove small pieces of bone and debris. Cell suspension was washed twice at 1500 rpm for 5 min, and counted. Bone marrow cells were suspended in complete culture medium and aliquoted in 100 mm sterile tissue culture petri dishes at an initial density of 2×10^5 cells/ml (10 ml/plate). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 10 days. Half of the medium was replaced with fresh complete medium on days 3, 6 and 8 to wash out the lymphocytes and granulocytes in the starting marrow preparation. Immature dendritic cells were ready for use on day 9. Activation of the dendritic cells was induced by the addition of LPS (1 μg/ml) on day 9 for 16 h.

2.1.5.2 Mouse splenocytes

Mice were sacrificed by raising the concentration of CO_2 followed by cervical dislocation and spleens were removed using aseptic technique. Mice were placed on their right sides and the fur was matted down with 70% ethanol. Two

successive incisions were created on the same orientation on the left side of each animal between the last rib and the hip joint. This procedure was followed by skin incision to expose the peritoneal wall, and peritoneal incision to expose the spleen. The spleen was drawn out of the body cavity using medium forceps, and dissected from connective tissue using fine forceps. The remainder of procedure was carried out in tissue culture hood. Spleens were placed in a 100 mm tissue culture dish containing 10 ml of HBSS and homogenised using flat end of a syringe plunger to release the cells. Thereafter, the cell suspension was mixed by gentle pipetting and passed through a 40 μm nylon mesh cell strainer (BD Biosciences) into a new 50-ml conical tube to remove any remaining tissue particle. Red blood cells were removed by density centrifugation using lymphoprep, and splenocytes were then suspended in culture medium. One spleen yielded approximately 1 $x10^8$ cells.

2.1.6 Analyais of SMX/metabolite-induced dendritc cells activation

2.1.6.1 Drug exposure

Cells ($1x10^6$ /ml; unless indicated otherwise) were incubated with SMX (0.05-2 mM) or SMX-NO (0.1-100 μM; DMSO end concentration < 0.5%). Certain experiments were conducted in the presence of the enzyme inhibitors methimazole or 1-aminobenzotriazole at a concentration that blocks CYP2C9 and myeloperoxidase-catalyzed SMX metabolism (both 1 mM). On completion of the incubation period, cells were then washed three times with HBSS prior to processing for detection of SMX and SMX-metabolite derived adduct formation (confocal microscopy and ELISA), cell viability, intracellular glutathione content, dendritic cell function and T-cell activation.

2.1.6.2. Dendritic cell surface receptor expression

Bone marrow derived dendritic cells were incubated with SMX, SMX metabolites or LPS (1 μg/ml) for 24 h. On completion of the incubation period, cells were harvested, washed twice in PBS, and stained with immunofluorescence labeled mAbs to CD40, CD86, MHC class II, CD11c, CD11b, Gr-1 and CD204 for 30 min at 4°C in the dark. Cells were washed and suspended in PBS to a final volume of 500 μl. Cells were then analysed on a Coulter Epics flow cytometer (Coulter Epics, XL software; Beckman Coulter). A minimum of 5000 events per sample were analysed. The appropriate mouse immunoglobulin isotypes were used as negative controls, and LPS-activated cells served as positive controls. Gating was performed for elimination of debris and dead cells. Data files were further processed for presentation using WinMDI software (version 2.9; Scripps Research Institute, California, U.S.A.).

2.1.6.3 Multiplex analysis of dendritic cell cytokine secretion

Levels of secreted cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-15, GM-CSF, IFN-γ, and TNF-α) were analysed using Millipore mouse cytokine 11-plex assay kit (Watford, Herts, UK) on Bio-Plex suspension array system Luminex (xMAP-based platform, Biorad Laboratories, Hercules, USA).

2.1.6.3.1 Principle and technology

The Bio-Plex system is based on three technologies:

- xMAP technology using colour-coded beads for simultaneous detection and quantitation of up to 100 different analytes (cytokines) in a single well of a 96-well microplate. The antibody specific to each cytokine is covalently

coupled to the internally dyed beads allowing capture and detection of specific analytes in samples; each antibody is coupled to a different bead uniquely labelled with a fluorescent dye mixture. After addition of detection antibody, a sandwich of antibodies will surround the cytokine.

- Flow cytometer using high-tech fluidics and two lasers to measure the molecules bound to the surface of the beads. A stream of suspended beads line up in a single file before passing through the detection chamber of the analyser which allows the particles to be measured discretely. Within the analyser, a red laser excites both the internal red and infrared dyes, allowing the proper classification of the bead to one of the 100 sets (i.e. classification channel reading), whereas the green laser excites any orange fluorescence associated with the binding of the analyte.
- High-speed digital processor: to manage the fluorescent data output.

2.1.6.3.2 Preparation of Antibody-immobilised beads

The antibody-bead for each cytokine was sonicated (30 sec), and vortexed (1 min). To a mixing bottle, 150 µl of each bead was added and the final volume was brought to 3 ml with assay buffer. Each well required 25 µl. Each bead has a number (bead region) from 1 to 100 according to the ratio of the two dyes contained. For correct data acquisition, the bead regions were used in Bio-Plex assay (as described in manufacturer's manual).

2.1.6.3.3 Preparation of cell culture supernatant samples

Bone marrow derived dendritic cells were incubated with SMX, SMX metabolites or LPS (1 µg/ml) for 24 h. On completion of the incubation period, supernatants

were collected and stored at -80 °C for measuring the levels of secreted cytokines. Samples were centrifuged to remove cell debris, and then kept on ice till use.

2.1.6.3.4 Preparation of controls and standards

Each mouse cytokine control was reconstituted with 250 µl water, mixed, vortexed, and allowed to set for 10 minutes before transfer into labelled microfuge tubes. Cytokine standards were reconstituted with 250 µl of water to give a stock concentration of 10,000 pg/ml. After 10 minutes, Standard concentrations (3.2-2000 pg/ml) were prepared by serial dilution of the stock concentration with the assay buffer and used within one hour of preparation (table 2.1). The 0 pg/ml standard (Background) was assay buffer alone.

Table 2.1: Dilution of standards for multiplex assay

Standard (pg/ml)	Deionized Water to add (µl)	Standard to add (µl)
10,000	250	0
Standard (pg/ml)	**Assay buffer to add (µl)**	**Standard to add (µl)**
2000	200	50 µl of 10,000 pg/ml
400	200	50 µl of 2000 pg/ml
80	200	50 µl of 400 pg/ml
16	200	50 µl of 80 pg/ml
3.2	200	50 µl of 16 pg/ml

2.1.6.3.5 Assay procedure

Analysis of supernatants was performed according to the manufacturers' protocols (Linco Research, Inc.) (figure 2.1). The filter-bottom, 96- well microplate was blocked with assay buffer (200 µl/well, 10 minutes incubation on plate shaker at room temperature) followed by removal of assay buffer by vacuum, and addition of the standard, control and samples in duplicate (25 µl/well) to the appropriate wells (cell culture media was added to the quality control wells). The sonicated

bead mixture (25 µl/well) was added followed by incubation of the sealed microplate with agitation for 2 h on a plate shaker at room temperature. Wells were then washed twice before incubation (25 µl/well, one hour, on plate shaker, at room temperature) with a PE-conjugated detection antibody. Samples were then incubated for another 30 minutes with streptavidin phycoerythrin (25 µl/well, on a plate shaker, at room temperature) before a final wash and resuspension in sheath fluid. The plate was covered and put on a plate shaker for 5 minutes before analysis using Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA).

2.1.6.3.6 Data analysis

Data was analysed and presented as fluorescence intensity (FI) and target concentration on Bio-Plex Manager™ software (version 3.0) with a five-parametric logistic (5-PL) curve fitting using a Bio-Plex Suspension Array System (model Luminex 100). The limit of detection (LOD), defined as the lowest concentration of analyte that can be detected, was 3.2 pg/ml. A standard curve (range of detection between 3.2 and 10,000 pg/ml) was generated from a single mixed standard and the concentration of each analyte in the sample was determined automatically and data is presented as pg/ml.

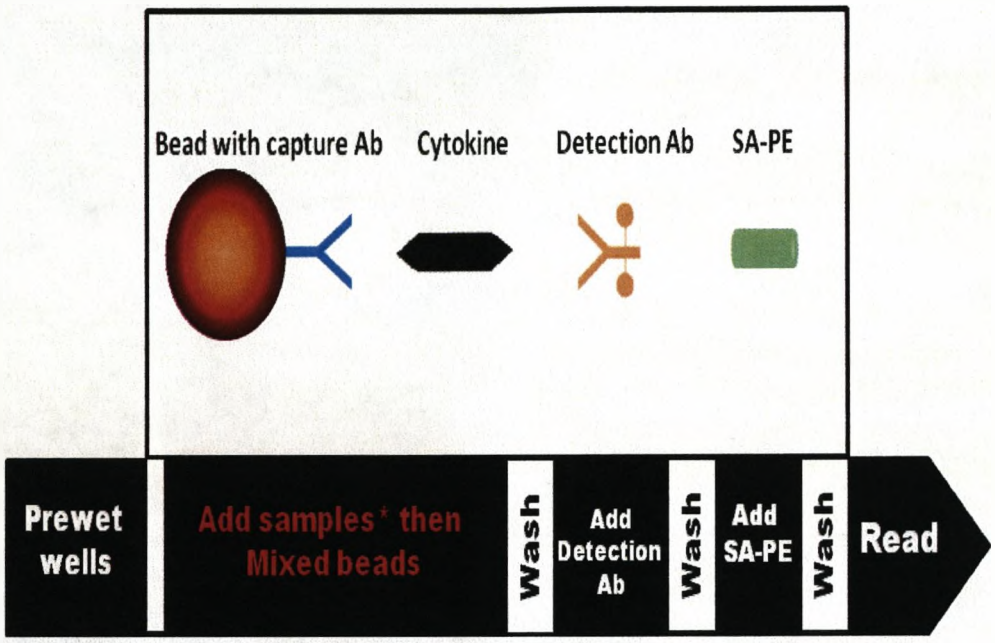

Figure 2.1: Assay principle and workflow of multiplex analysis of cytokine secretion. Colour-coded beads with unique spectral signature and coated with specific capture antibody to a particular bioassay, allowing the capture and detection of specific analytes from a sample.

2.1.6.4 Determination of protein quantity

Cells incubated with SMX or SMX metabolites were washed, suspended in distilled water and lysed by freeze-thawing. Following sonication, protein content was determined by the method of Bradford (Bradford, 1976) and standardised to 250 µg/ml. The Bradford assay relies on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution causing a shift in the absorption of the dye from 465 to 595 nm. The amount of absorption is proportional to the protein present. A standard curve of BSA (31.25-2000 µg/ml, 10 µl/well) was prepared as showed in table 2.2 and figure 2.2 using a stock solution of 2 mg/ml BSA in distilled water. In other wells, 10 µl of samples were added to each well of a flat-bottomed 96-well plate in duplicate. Bradford reagent was diluted 1:5 with distilled water and 200 µl/well added immediately prior to spectrophotometric analysis (MRX microplate reader running Revelation version 3.04 software, Dynex Technologies, Billinghurst, W. Sussex) at 570 nm. Blank wells were subtracted from all values, and linear standard curves were prepared of absorbance versus micrograms protein.

Table 2.2: Dilution of bovine serum albumin (BSA) standards for Bradford assay

Standard (µg/ml)	**Deionized water (µl)**	**BSA (µg)**
2000	1000	2000
Standard (µg/ml)	**Deionized water (µl)**	**Standard to add (µl)**
1000	100	100 µl of 2000 µg/ml
500	100	100 µl of 1000 µg/ml
250	100	100 µl of 500 µg/ml
125	100	100 µl of 250 µg/ml
62.5	100	100 µl of 125 µg/ml
31.25	100	100 µl of 62.5 µg/ml

2.1.6.5 Detection of sulfamethoxazole metabolite protein adduct formation

2.1.6.5.1 ELISA

Cells incubated with SMX or SMX-NO were washed, suspended in distilled water and lysed by repeated freeze-thawing cycles. Following sonication, the protein content was standardised to 250 μg/ml. One hundred microliter aliquots of cell suspension (25 μg/ml protein) were plated in duplicate in 96-well ELISA plates (high capacity protein-binding microtiter plate, Falcon, BD Bioscience, Oxford, UK), and allowed to adsorb for 16 h at 4°C. After washing with PBS-Tween 20 (0.001% v/v) to discard unbound antibody, plates were blocked for 1 h with irrelevant protein, such as skimmed dry milk (2.5% in PBS-Tween 20) at 4°C. Samples were then incubated with rabbit anti-SMX anti-sera (1:2000; overnight at 4°C). Following 2 h incubation with an alkaline phosphatase-conjugated anti-rabbit IgG (1:1000), plates were washed, and incubated for 30 min with alkaline phosphatise substrate (100 μl/well). This allows a colorimetric reaction to occur that can be analysed spectrophotometrically using microplate reader (MRX; Dynatech Laboratories Inc., Chantilly, VA, USA) running Revelation version 3.04 software (Dynex Technologies, Chantilly, VA, USA) with the resulting optical density (OD) at 405 nm relating directly to the amount of antigen present within the sample. Results are expressed as "ΔOD = sample OD – vehicle OD". To verify that the antibody binding truly reflects expression of SMX-derived adducts, hapten inhibition experiments were performed using soluble SMX (2 mM) in the presence of the primary anti-SMX antibody. Detection of SMX-derived protein adducts was completely inhibited by soluble SMX. Steps of ELISA are represented in figure 2.3.

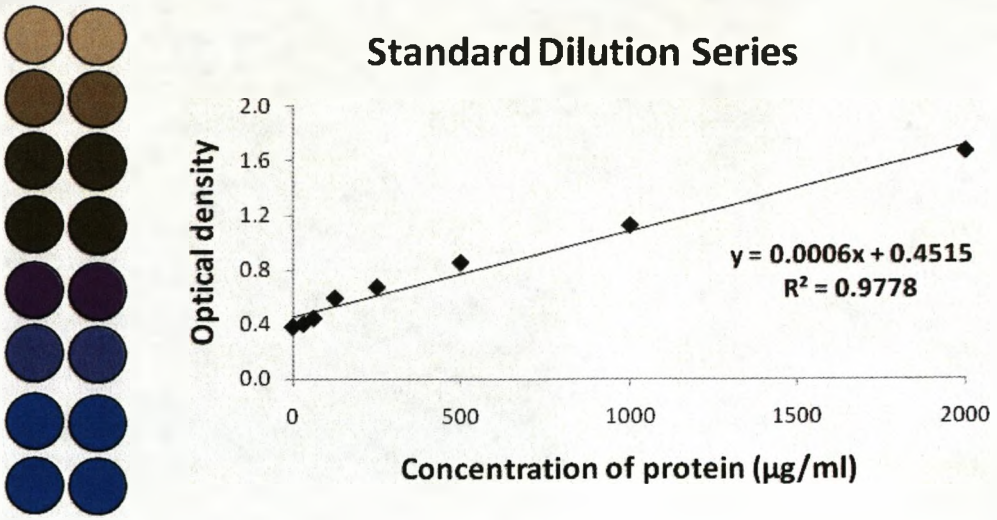

Figure 2.2: A standard curve of bovine serum albumin using a stock solution of 2 mg/ml.

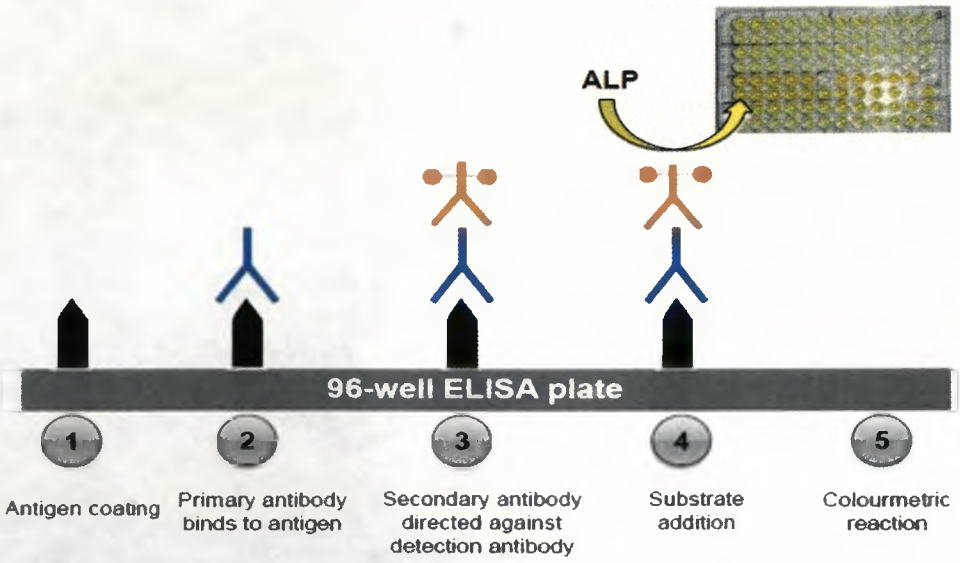

Figure 2.3: Diagramatic representation of ELISA. One hundred microliter aliquots were plated in duplicate in ELISA plates and left to adsorb for 16 h at 4°C. The wells were then washed, blocked with dry milk, and incubated with a rabbit anti-SMX Ab for 16 h. The wells were then washed and incubated with alkaline phosphatase-linked Ab for 2 h followed by washing and incubation for 30 min with alkaline phosphatase yellow substrate. The OD of the wells at 405 nm was then determined.

2.1.6.5.2 Confocal microscopy

To visualise SMX-derived protein adduct formation, cells were washed with HBSS and fixed with paraformaldehyde (4%; 30 min; room temperature; 500 µl/sample). After two washes, cells were permeabilised with NH_4Cl buffer (0.16 M; 10 min, room temperature, 500 µl/sample), followed by TritonX 0.1% - BSA 0.1% solution (500 µl/sample; 30 min), and blocked with RPMI supplemented with FBS (10% v/v; 1 h). Samples were then washed and incubated overnight with rabbit anti-SMX antibody (1:500) at 4 °C, before being washed again and incubated with FITC-linked goat anti-rabbit IgG (1:500) for 2 h at 4°C. Following further washes with HBSS, slides were finally sealed using Vectashield H-1200 (Vector Laboratories Inc., Peterborough,UK) and stored in foil at 4°C to be analysed by confocal microscopy within 3 days. This method was conducted by S. Lavergne who is a post-doctoral fellow in the Department of Pharmacology.

2.1.6.6 Analysis of cell yield and viability

Trypan blue (TB) exclusion with haemocytometer counting was used to evaluate the final yield of cells and their viability. It is based on the principle that viable cells will not take up certain dyes, whereas non-viable cells will. Cultured cells were washed and an aliquot volume mixed 1:1 in TB solution. Erythrocytes were excluded by size and shape, while TB negative large leukocytes were counted as viable under microscope using haemocytometer.

To analyse the percentage of apoptotic and necrotic cells of mouse dendritic cells and splenocytes incubated with SMX or SMX-NO, the Annexin-V FITC/propidium iodide double staining method originally described by Vermes et

al (Vermes et al., 1995) was used. Annexin V binds to phosphatidylserine residues expressed on apoptotic cell surfaces, whereas propidium iodide (PI) is a membrane-impermeable fluorescent dye that binds to DNA of cells killed by necrosis (figure 2.4A). A combination of these two characteristics permits simultaneous detection of viable, apoptotic, and necrotic cells. Cells were washed three times using PBS, and suspended in pre-diluted (1:4 dilution) binding buffer at 2-5 x 10^5 cells/ml. Cells were then stained with annexin-V for 10 min in the dark at room temperature. After an additional wash cycle, cells were incubated with PI (1 μg/ml) in binding buffer for 30 min at 4°C. A minimum of 5000 cells were then analysed using an EPICS-XL flow cytometer (Coulter Electronics, Luton, Beds., UK). A gated population (to exclude cellular debris) was assessed for annexin V on FL-1 and PI on FL-3. Proportions of viable cells (annexin negative/PI negative), apoptotic cells (annexin positive/PI negative) and necrotic cells (annexin positive/PI positive) were calculated using WinMDI software (version 2.9; Scripps Research Institute, California, USA). Cells incubated with formaldehyde (3%) or DMSO (10 %) served as a positive control. Negative controls included unstained viable cells and stained cells not exposed to the drug or its metabolite (figure 2.4B).

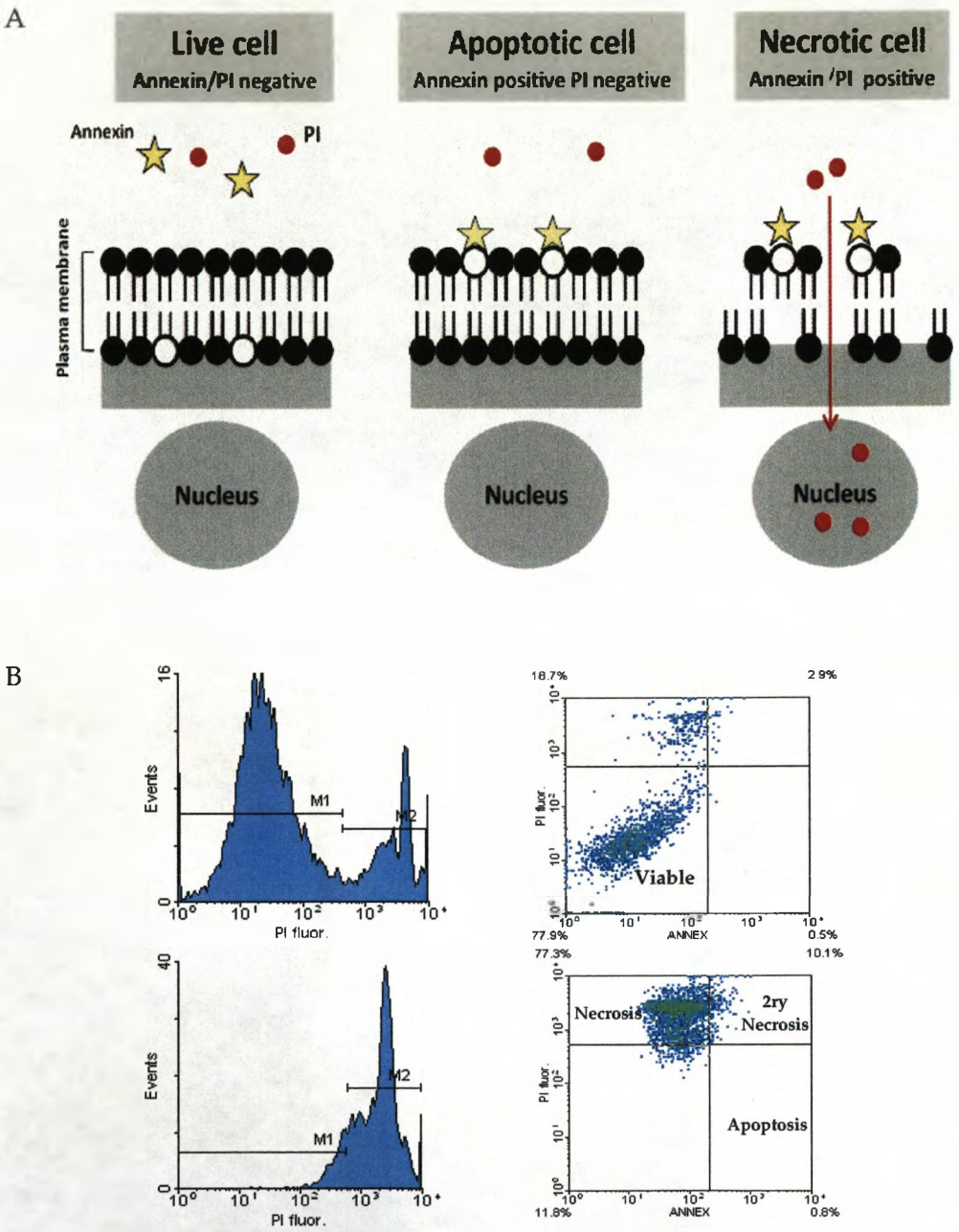

Figure 2.4: Annexin-V FITC/propidium iodide (PI) double staining method. Annexin V binds to phosphatidylserine residues expressed on apoptotic cell surfaces, whereas PI is a membrane-impermeable fluorescent dye that binds to DNA of cells killed by necrosis (A). This allows detection of viable (double negative), apoptotic (annexin positive/PI negative), and necrotic cells (double positive) as shown by dot plots and histograms (B).

2.1.6.7 Analysis of dendritic cell glutathione content

Glutathione content in SMX and SMX-metabolite treated bone marrow-derived mouse dendritic cells was measured using a microtitre plate assay according to the method of Vandeutte (Vandeputte et al., 1994). Dendritic cells were washed, lysed with HCl (10 mM) and protein precipitated with sulfasalicylic acid (6.6% w/v). Glutathione content was then measured spectrophotometrically (MRX microplate reader running Revelation version 3.04 software, Dynex Technologies, Chantilly, VA, USA) against a GSH standard curve (standards: 0.5-80 nmol/ml as GSH equivalent) using the enzyme glutathione reductase in the presence of 5,5'-dithiobis-2- nitrobenzoic acid (DTNB) and co-factor, NADPH. Formation of the reaction product, the DTNB GSH conjugate, was quantified kinetically at a wavelength of 405 nm. The function of the enzyme in the reaction was to convert all the glutathione present to the reduced form, GSH. Oxidised glutathione levels (GSSG) were quantified by the inclusion of a GSH derivatisation step using 2-vinylpyridine prior to analysis. Standards (0.5-8.0 nM/ml as GSH equivalents) were prepared by diluting a GSSG stock solution (400 mM/L) in 10 mM/L HCl containing 1.3% of 5-sulfosalicylic acid. GSH levels were determined by subtracting GSSG from the total glutathione, and all levels are expressed as nanomoles of glutathione per 10^6 cells.

2.1.6.8 Co-culture of dendritic cells with nitroso SMX-treated splenocytes

To explore the relationship between SMX metabolite-mediated cell death and dendritic cell activation, Splenocytes were incubated with SMX-NO (10-500 μM) for 16 h at 37 °C in 5% CO_2 incubator. After repeated washing to remove non-covalently bound drug, splenocytes (3 x 10^6 /ml) were cultured with mouse bone

marrow-derived dendritic cells (1 x 10^6 /ml) in 24 well cell culture plates (2 ml final volume). After 16 h, dendritic cells were assayed for cell viability, expression of cell surface receptors and cytokine secretion using methods described above.

2.1.7 Analysis of sulfamethoxazole/metabolite-specific T-cell responses

2.1.7.1 Intra-peritoneal injection of nitroso sulfamethoxazole

Female Balb/c strain mice were administered SMX-NO (5 mg/kg; n = 4) in DMSO (100 µl) via i.p. injection four times weekly for 2 weeks according to a previously described protocol (Naisbitt et al., 2001). On completion of this dosing regimen, animals were killed by raising concentration of CO_2 followed by cervical dislocation and the spleen was removed using aseptic technique for analysis of splenocyte proliferation (figure 2.5). Spleens were homogenised in HBSS (10 ml) and filtered to liberate a single cell suspension. Red cells were removed by density centrifugation with lymphoprep. A similar protocol was performed on non-immunized mice to obtain syngeneic naive splenocytes.

2.1.7.2 Adoptive transfer of mouse dendritic cells to naive recipient mice

Bone marrow-derived dendritic cells, cultured in medium supplemented with mouse serum to prevent T-cell responses against FBS-derived antigens were incubated for 16 h in culture medium with SMX (2 mM) or SMX-NO (100 µM). For immunization, dendritic cells were harvested, washed extensively, and injected via i.v. injection in the lateral tail vein of Balb/c strain mice. Each mouse received one injection of 0.5 x 10^6 viable dendritic cells in HBSS (200 µl). Control mice received unmodified dendritic cells or dendritic cells treated with

DMSO. After 21 days, mice were sacrificed and splenocytes isolated as described above for analysis of splenocyte proliferation (figure 2.5).

2.1.7.3 T-cell proliferative response from immunised or adopted mice

Freshly isolated splenocytes (3 x10^6/ml) from a SMX-NO–sensitised mice or adopted mice were aliquoted in 96-well U-bottomed cell culture plates and incubated (37 °C, 5% CO_2) with soluble drug (SMX; 100-1000 μM or SMX-NO; 10-100 μM) for 72 h (figure 2.5). Proliferation was measured by the addition of [^{3}H] thymidine (0.5 μCi/well) for the final 16 h of culture. Cells were harvested and incorporated radioactivity was measured as counts per minute on a beta counter (PerkinElmer Life Sciences, Cambridge, UK). Proliferative responses were represented as stimulation indices (SI; cpm in drug-treated cultures/cpm in cultures with solvent alone).

To evaluate the role of SMX metabolism by antigen presenting cells in drug-specific T-cell proliferation, Dendritic cells or splenocytes (used as antigen presenting cells in the proliferation assay) were incubated with SMX or SMX-NO for 16 h, prior to repeated washing to remove unbound drug, irradiation to prevent proliferation and co-incubation with freshly isolated splenocytes (3 x10^6/ml) from a SMX-NO–sensitized mice or adopted mice. Proliferation was measured using [^{3}H] thymidine incorporation as described above.

To confirm that SMX-derived protein adducts formation and T-cell proliferation is dependent on the enzymatic oxidation of SMX, certain experiments were conducted in the presence of metabolic enzyme inhibitors. T cells and irradiated APC were pre-incubated for 1 h with 1-aminobenzotriazole (a non-selective

inhibitor), or methimazole (an inhibitor of peroxidases and FMO) at a concentration that blocks SMX metabolism without causing cytotoxicity (both 1 mM) (Sanderson et al., 2007; Lavergne et al., 2009). After pre-incubation with inhibitors, cells were incubated with SMX or SMX-NO.

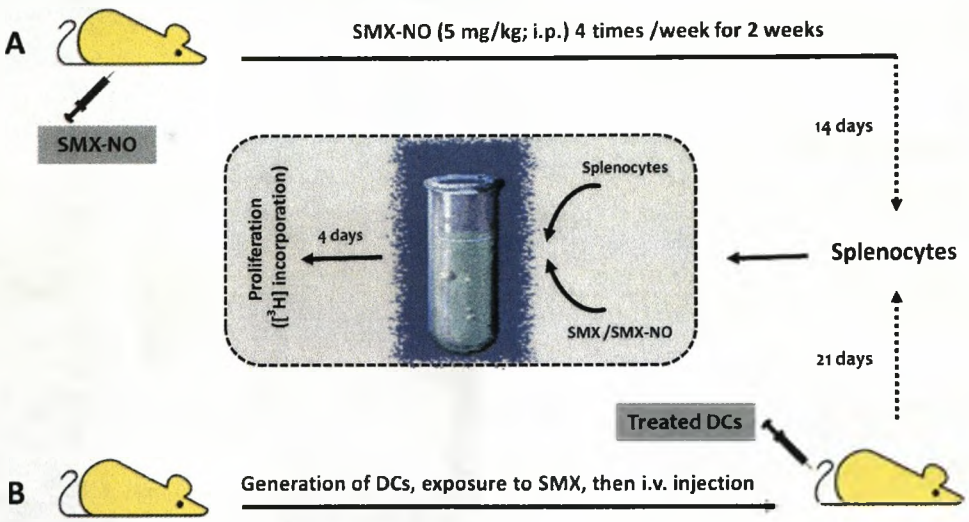

Figure 2.5: Analysis of sulfamethoxazole/metabolite-specific T-cell responses in mice. A) i.p. injection of mice with SMX-NO (5 mg/kg) four times weekly for 2 weeks followed by analysis of splenocyte proliferation against soluble drug or drug-pulsed antigen presenting cells, B) i.v. injection of mice with drug-treated dendritic cells (DCs). Three weeks after injection, splenocytes were isolated and stimulated with SMX or SMX-NO.

2.2 Materials and methods for human experiments

2.2.1 Donors characteristics

Blood was obtained and peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of six patients with history of hypersensitivity to SMX (three with cystic fibrosis) and three drug exposed volunteers. Patients were selected on the basis of lymphocyte transformation test (LTT) positivity and their readiness to donate blood on several occasions. Samples from patients with cystic fibrosis were kindly donated by Dr Paul Whitaker and Dr. Daniel Peckham (Regional Adult Cystic Fibrosis Unit, St James's Hospital, Leeds, UK), while cells from patients without cystic fibrosis were kindly donated by Prof. Werner Pichler (Division of Allergology, Clinic of Rheumatology and Clinical Immunology/Allergology, Bern, Switzerland). Approval for the study was obtained from Liverpool Local Research Ethics Committee and informed written consent was obtained from each donor (table 2.3).

Table 2.4: Clinical details of sulfamethoxazole hypersensitive patients

	Cystic fibrosis	Clinical details
1	No	Maculopapular exanthema and malaise
2	No	DRESS and erythrodema
3	No	SJS (exanthema, conjunctivitis and bullae)
4	Yes	Maculopapular exanthema
5	Yes	Maculopapular exanthema
6	Yes	Maculopapular exanthema

2.2.2 Culture medium

RPMI-1640, penicillin-streptomycin, L-glutamine, human holo-transferrin, HEPES buffer, and phytohemagglutinin (PHA) were from Sigma-Aldrich (Gillingham, Dorset, UK). Fetal bovine serum and pooled human AB serum were

purchased from Innovative Research (Michigan, USA). Human recombinant IL-2 was obtained from Peprotech (London, UK) to maintain T-cell growth and proliferation. IL-2 vial was centrifuged before opening and then reconstituted in 100 mM acetic acid to a concentration of 1 mg/ml (1 x 10^7 units/ml). This solution was diluted and stored at -20 °C for future use. APCs were maintained in medium consisting of RPMI-1640 supplemented with 10% FBS, HEPES buffer (25 mM), L-glutamine (2 mM), streptomycin (100 µg/ml) and penicillin (100 U/ml). T-cell clones were maintained in medium consisting of RPMI-1640 supplemented with pooled heat-inactivated human AB serum (10%, v/v), HEPES buffer (25 mM), L-glutamine (2 mM), holo-transferrin (25 µg/ml), streptomycin (100 µg/ml), penicillin (100 U/ml), and IL-2 (60 U/ml).

2.2.3 Chemicals, reagents and buffers

The chemicals, reagents and buffers used in human experiments were listed previously (section 2.1.3). Chromium-51 (^{51}Cr) as $Na_2{}^{51}CrO_4$ (specific activity 441.16 mCi/mg, concentration 1 mCi/ml) was obtained from PerkinElmer life and analytical sciences (Boston, MA). Cyclosporine A, glutaraldehyde solution (25%, to prepare 2% solution add 10µl to 115µl HBSS) and paraformaldehyde were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). L-Glycine (75.07 g/mol) was purchased from VWR Prolabo (Hunter Boulevard, Lutterworth, UK), 0.2 M glycine was prepared in HBSS (15 mg glycine + 1 ml HBSS). Multiplex assay kits for simultaneous detection of 17 human cytokines (IL-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, MCP-1, MIP-1β, IFN-γ, and TNF-α) were purchased from Bio-Rad Laboratories (Hemel Hempstead, UK). Kits were stored at 2-8°C and were warmed to room

temperature (20-25°C) prior use. B95-8 cell line (HPA Culture collections, Salisbury, UK) is an Epstein-Barr virus (EBV)-producing marmoset B-cell line used to provide a source of EBV containing supernatants for human B-cell transformation.

2.2.4 Antibodies

Antibodies used for flow cytometric phenotyping of T cells were FITC-labelled CD8 and PE-labelled CD4 (both from BD Biosciences Pharmingen, Oxford, UK). Antibodies used for immunochemical detection of SMX protein adducts were previously described in section 2.1.4. Specific anti–HLA blocking antibodies were used to assess the involvement of major histocompatibility complex (MHC) in drug presentation to T-cell clones. Anti–HLA class I mAb (anti-human HLA-A,B,C; 1 mg/ml) and anti–HLA class II mAb (anti-human HLA-DR, DP, DQ; 1 mg/ml) were obtained from BD Biosciences Pharmingen (Oxford, UK).

2.2.5 Processing of peripheral blood mononuclear cells (PBMCs)

2.2.5.1 Isolation of PBMCs

PBMCs were isolated from fresh venous blood by density gradient centrifugation on lymphoprep. This yields a distinct cloudy band of mononuclear cells (lymphocytes and monocytes) at the interface that has been depleted of red blood cells and most polymorphonuclear leukocytes or granulocytes (figure 2.6). Peripheral blood was drawn into heparinised vacutainer tubes. Tubes were opened in laminar flow hood, and blood was then layered carefully on the surface of lymphoprep (10 ml lymphoprep + 15 ml blood). After centrifugation for 20 minutes at 2000 rpm without brake, the PBMCs band was formed between the

plasma and the lymphoprep layers. This band was gently aspirated from the interface using a sterile Pasteur pipette, washed twice in HBSS to remove residual lymphoprep, and centrifuged at 1800 rpm and 1500 rpm, respectively. Cell yield was assessed using a haemocytometer after re-suspension of the cell pellet. Total number of cells was calculated as follows: number of cells per big square x 10,000 x total volume of cell suspension (in ml) x dilution factor (if required) (figure 2.6). Cell viability was determined by trypan blue dye staining. Trypan blue (10 µl, 0.2% w/v) was mixed with cell suspension (40 µl in HBSS) and left for 3-5 minutes at room temperature. 10 µl of the cell suspension / trypan blue mixture was placed on hemacytometer and cells [unstained (viable) and stained (non-viable)] were counted. The percentage of viable cells was calculated as follows: 100 x (number of viable cells/total number of cells). Cells found to be more than 95% viable were re-suspended in culture medium at the required concentration.

2.2.5.2 Cryopreservation and thawing of PBMCs

Isolated fresh PBMCs were re-suspended in a freezing mixture of DMSO 10% and FBS 90% at concentration of 1 x 10^7 cells/ml. Cells were dispensed into cryovials (1 ml/vial) on ice. Cryovials were placed in a Mr. Frosty-style freezing container filled with 70% isopropanol, and kept in –80°C freezer before being transferred to liquid nitrogen after 24 h. It is important for cell viability and cell recovery that the cells are thawed and processed quickly. RPMI was warmed in a 37 °C water bath before thawing. Cryovials were transferred from liquid nitrogen to a laminar flow cabinet. Cryovials caps were loosened to allow the nitrogen to escape during thawing. Warm RPMI was added into the cryovial containing the

cell suspension followed by transfer of the cell suspension to a sterile 50 ml tube containing warm RPMI. Cells were centrifuged for five minutes at 1500 rpm then re-suspended in the desired volume of warm RPMI. Cell number and viability were determined as described above.

2.2.6 Preparation of antigen presenting cells

The most useful source of autologous readily available APC is represented by EBV-transformed human B-lymphoblastic cell lines (B-LCLs). They were generated *in vitro* at the time of the blood isolation by transformation of freshly isolated PBMCs with supernatant of the EBV-producing cell line B95-8 according to established methods (Wu et al., 2007) divided into 5 steps as described below.

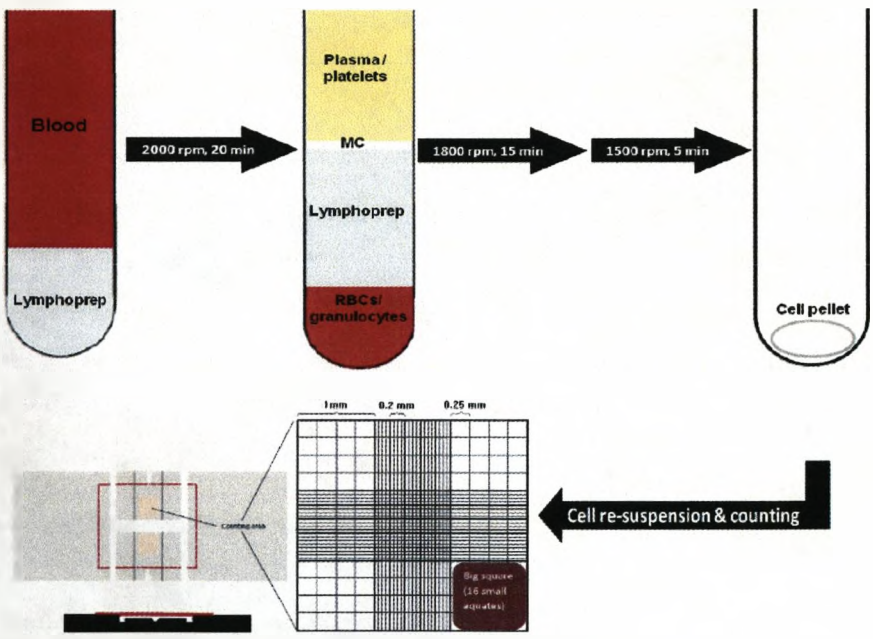

Figure 2.6: Isolation of peripheral blood mononuclear cells (PBMCs). Cells were isolated by density gradient centrifugation on lymphoprep followed by assessment of cell yield using haemocytometer.

2.2.6.1 Preparation of PBMCs

PBMCs were isolated and prepared as described above. Cell pellet was re-suspended in complete medium at 1 x 10^7 cells per ml.

2.2.6.2 Preparation of EBV-containing culture supernatants

B95-8 cells were grown and maintained in APC medium in flasks in a humidified 37°C, 5% CO_2 incubator. After 10 days without replacing the medium, the supernatant containing the cell-free EBV was harvested by centrifugation and filtered (0.2 μm).

2.2.6.3 Transformation of B-cells by EBV

Freshly isolated lymphocytes ($2x10^6$) were incubated overnight in 20 ml tube with 5 ml of undiluted B95-8 supernatant (37°C, 5% CO2) supplemented with cyclosporin A (CSA; 1 μg/ml) to prevent T-cell mediated suppression of B-cell infection, and to prevent EBV induced T cell growth.

2.2.6.4 Growth and maintenance of infected B cells

After overnight incubation, cells were washed, re-suspended at 1 x 10^6/ml, then dispensed into 24-well cell culture plates (1 ml per well). Cells were fed twice weekly with APC medium containing CSA (1 ug/ml) in the first 3-4 weeks until confluence is obtained. Confluent wells were expanded in the same plate then transferred to 25-cm^2-tissue-culture flasks and maintained in APC medium, replacing half of the culture every three days. B-LCL were expanded and used as APC by splitting 1:2-1:4, depending on the rate of growth. Cells were also frozen by aliquoting in freezing mixture (FBS with 10% DMSO) and kept as a stock in liquid nitrogen.

2.2.6.5 Irradiation of EBV-transformed B-cell lines

In order to be used as APCs, B-LCL cellular division was blocked by gamma-irradiation (4000 rads) with a cobalt source (Gammacell 1000 irradiator, Best Theratronics Ltd; Ontario, Canada) immediately before use. After irradiation, the cells were washed three times in order to remove possible toxic substances released during irradiation.

2.2.7 Lymphocyte transformation test

Proliferation and expansion of lymphocytes from SMX hypersensitive patients after stimulation with SMX/metabolite was measured using the lymphocyte transformation test (LTT) as described previously (Nyfeler and Pichler, 1997). Freshly isolated PBMCs (1.5×10^5; total volume 0.2ml) were cultured in triplicate for 5 days with SMX (50-400 µg/ml), SMX-NO (10-80 µM), or tetanus toxin (5 µg/ml) as a positive control, in 96-well U-bottomed tissue culture plates (37°C, 5% CO_2). Cell cultures containing lymphocytes in the absence of SMX/metabolite were taken as a negative control. Proliferation was measured by addition of [3H]-thymidine (0.5 µCi/well) for the final 16 h of incubation. Cells were harvested, and counted as cpm on a liquid scintillation counter (Wallac microbeta trilux, PerkinElmer, Cambridge, UK). Proliferative responses were calculated as mean ± SD stimulation index (SI; cpm in drug-treated cultures/cpm in cultures without drug). An SI ≥ 2 was considered a positive response. A novel indirect LTT was developed and conducted by S. Lavergne by the addition of an antigen-driven T-cell enrichment step prior to analysis of proliferation. Lymphocytes (4.5×10^6; 2 ml, 24 well culture plates) were incubated with SMX and SMX-NO for 11 days with IL-2 supplement on day 4. On day 11, lymphocytes were washed and

incubated in 96-well plates (1×10^5/well) with frozen autologous irradiated lymphocytes (0.25×10^5/well) as APCs in the presence of SMX and SMX-NO (Lavergne et al., 2010).

2.2.8 Generation of sulfamethoxazole/metabolite-specific T-cell clones

In theory, T cell cloning is a simple technique; PBMCs are isolated, seeded at one cell per well, and induced to proliferate using PHA. The antigen specificity of growing cells is then tested. In practice, the procedure is difficult and many problems can be encountered. In particular, the procedure requires a high level of expertise in aseptic technique and handling of long-term cultures. Furthermore, the frequency of antigen specific T-cells is incredibly low and as such several thousand separate cultures must be established to generate working numbers of T-cell clones. Generation of T-cell clones was conducted by limiting dilution using previously described methodology (Schnyder et al., 1997; Wu et al., 2006). The aim of this method was to obtain monoclonal populations of T cells that retain the required specificity and function, and survive in the *in vitro* culture for a reasonable time to be studied in depth.

2.2.8.1 Primary T cell culture

Freshly isolated PBMCs from SMX allergic patients were incubated at a cell density of 1 x 10^6 per well with SMX (50, 100 and 200 μg/ml), or SMX-NO (20, 40 and 80 μM) in T-cell culture medium in a 48-well tissue culture plate. T cell growth was associated with clumps of cells distributed onto the surface of the plate with a frequency dependent on the antigen dose. At day 6 and 9, IL-2 (60 U/ml) was added in complete medium to facilitate the proliferation of those cells

that, following antigen recognition on the surface of APC have the IL-2 receptor up-regulated. Microscopic inspection on day 10 showed clumps of cells and scattered cells out of clumps with blast morphology. At day 14, cell proliferation made the medium to turn yellow, indicating the acidification of the medium and suggesting the need to proceed with the cloning experiment (figure 2.8).

2.2.8.2 Limiting dilution

The aim of this procedure is to obtain a pool of cells deriving from antigen-activated cultures of T lymphocytes. On day 14 of the primary T-cell culture, treated cells were diluted to maximize the probability of obtaining individual T cells in any one well (Schnyder et al., 2000; Naisbitt et al., 2003a; Naisbitt et al., 2003b; Wu et al., 2006). Cells from bulk cultures were seeded in U-bottom 96-well plates at dilutions of 0.3, 1, and 3 cells per well with the stimulation cocktail (autologous irradiated PBMCs at 0.5 x 10^6 /ml plus 60 U/ml IL-2 plus 5 µg/ml PHA) in total volume of 200 µl per well (figure 2.7). Complete medium supplemented with IL-2 was added on day five of the limiting dilution and then on alternative days. Plates were discarded if (1) growing cells were not apparent after 21 days culture or (2) growing cultures develop in > 90% of wells.

2.2.8.3 Transfer of growing cells

After 14 days, plates were inspected and wells containing growing cells were marked, transferred to a new U-bottomed 96-well plates, and expanded in culture medium containing IL-2 by splitting into the neighbour wells. Growing cultures were re-stimulated using the stimulation mixture (see above) and remained in culture for a further two weeks (figure 2.8).

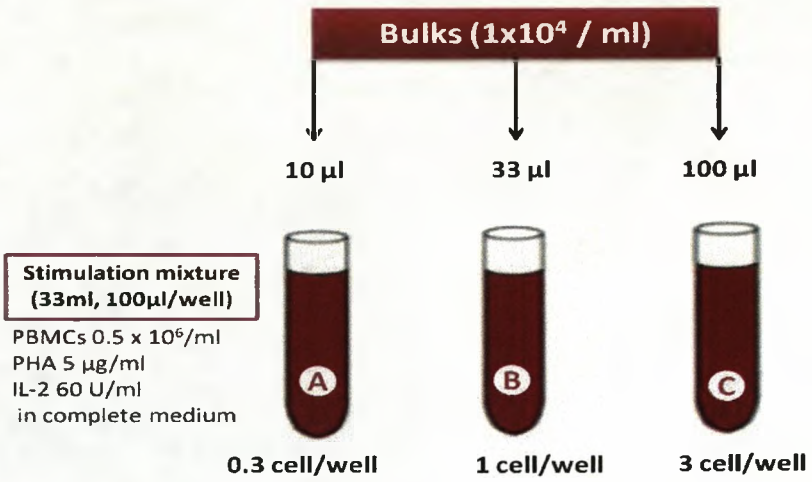

Figure 2.7: Limiting dilution method of generation of T-cell clones. Cells of bulk cultures were plated at different dilutions in a 96-well cell culture plates in a stimulation mixture.

2.2.8.4 Antigen specificity test

At the end of cell culture period, three cell populations were generated: resting T-cells, non-specifically activated T-cells and antigen-specific T-cells. An antigen specificity test was performed (not before 10 days from the re-stimulation) to select the antigen-specific T-cells among all the growing clones (figure 2.8). This test requires the availability of autologous APCs and the same antigen used for primary culture. T cell clones were collected, washed to remove IL-2, re-suspended with complete medium, and dispensed into a fresh 96 well plate in quadruplicate at density of $5x10^4$ per well. Autologous B-LCLs (1×10^4) were irradiated to block their proliferation which would overwhelm the response of T cell clones, and incubated with the wells previously filled with T-cells in the presence or absence of the drug (SMX, 200 µg/ml; SMX-NO 80 µM) in a final volume of 200 µl culture medium in U-bottom 96-well plates for 48 hours. For every clone to test, two wells contained T-cells plus APCs without antigen and

two wells with T-cell plus APCs and antigen. Proliferation was measured by the addition of [^{3}H] thymidine (section 2.2.7). Cell cultures with an SI $\geq$ 2 were considered antigen-specific and expanded further in 48 well cell culture plates.

2.2.8.5 Transfer of antigen-specific T-cells

Antigen-specific clones were identified in the culture plates then expanded in 48 well tissue culture plates using the stimulation mixture of complete culture medium, irradiated PBMCs (1 x 10^6), PHA (5 µg/ml), and IL-2 (60 U/ml). Clones were maintained in IL-2 enriched medium for at least 2 weeks before using them.

2.2.8.6 Re-stimulation of antigen-specific T-cell clones

Clones can be expanded for functional assays for long periods by re-stimulation every 15–20 days. There is no need to use the antigen and MHC compatible APC to re-stimulate them because they represent a single antigen-specific cell and not contaminated by other cells. Each clone was washed and re-suspended in 330 µl of culture medium in 48-well plate with an equal volume of re-stimulation mixture. Wells were fed after 24 h with 330 µl of culture medium supplemented with IL-2. Wells were checked every 2 days for growth and split 1:2–1:4, according to the cell density, in complete medium supplemented with IL-2. 10–15 days later, the growth rate declines with less splitting.

2.2.8.7 Freezing of T-cell clones

The most common problem in T-cell cloning is the loss of valuable cells. Thus, it is essential to freeze clones once adequate numbers are available. T-cells were frozen after 2 weeks of the last stimulation using the freezing mixture and method described previously.

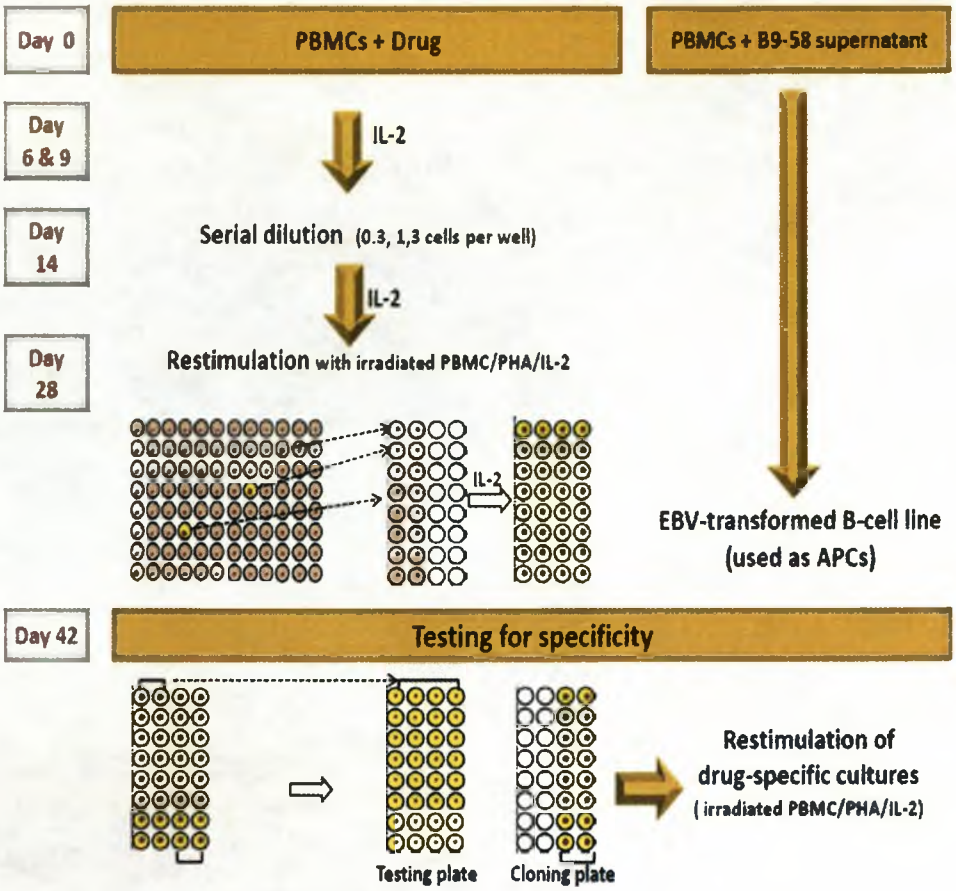

Figure 2.8: Generation of antigen-specific T-cell clones and the autologous antigen presenting cells. Freshly isolated PBMCs were incubated with the drug or its metabolites in T-cell medium in 48-well tissue culture plates. Cultures were supplemented with IL-2 on days 6 and 9 to maintain drug-specific proliferation. On day 14, T-cell clones were prepared by serial dilution by incubation of 0.3, 1 or 3 cells in 96-well round-bottomed tissue culture plates in T-cell medium, supplemented with allogenic irradiated lymphocytes, PHA and IL-2. T-cells were expanded in T-cell medium with IL-2 and re-stimulated every 14 days with irradiated allogenic lymphocytes and PHA. Specific T-cell clones were identified by proliferation assay using SMX/SMX-NO and B-LCLs that were generated at the time of the blood isolation. T-cell with a positive proliferative response were expanded and maintained in IL-2 containing medium.

2.2.9 Measurement of the proliferative response of T cell clones

The most frequently used method to measure proliferation *in vitro* is relying on the uptake of [^{3}H] thymidine. After 10-15 days of re-stimulation, T-cell clones from allergic patients ($5x10^4$ cells) were incubated in duplicate culture with irradiated autologous B-LCL (1×10^4) in the presence or absence of the drug in a total volume of 0.2 ml in U-bottom 96-well plate for 48 hours. It is recommended to use a range of drug concentrations to find the concentration that results in optimal proliferation of specific cells (SMX, 50–400 μg/ml; SMX-NO, 10-160 μM) (figure 2.9). [^{3}H]-thymidine was added at 0.5 μCi/well for the final 16 h of incubation. Cells were subsequently harvested using a multiwell harvester that aspirates cells, lyses cells, and transfers [^{3}H]-thymidine-labeled DNA onto a filter paper, while allowing non-incorporated [^{3}H]-thymidine in the medium to flow through. The incorporated radioactivity was measured using a liquid scintillation counter (Wallac microbeta trilux, PerkinElmer, Cambridge, UK) for beta particles as count per minutes (cpm). T-cell clones with a positive proliferative response (SI $\geq$ 2) were taken to be drug-specific and maintained in IL-2 containing medium. Antigen-induced T cell activation was also evaluated by measuring cytokine secretion using multiplex methods (see below). In this case, the culture supernatants were collected prior to the addition of [^{3}H]-thymidine, pooled for each condition and stored at -80 °C till the time of analysis.

2.2.10 Cross-reactivity of T cell clones with SMX and its metabolites

To analyse the cross-reactivity pattern of specific T-cells with SMX or its metabolite, SMX-specific T-cell clones (5×10^4 cells) were incubated with APCs (1×10^4 cells) and titrated concentrations of SMX-NO (20–80 μM) in a final

volume of 200 µl of T-cell culture medium. Similarly, T-cell clones with specificity for SMX-NO were incubated with APCs and different concentrations of the parent drug SMX (100–400 µg/ml). Proliferation was measured by incorporation of [^{3}H] thymidine as described above. Clones were defined as cross-reactive if they were stimulated to proliferate following incubation with both SMX and SMX metabolites.

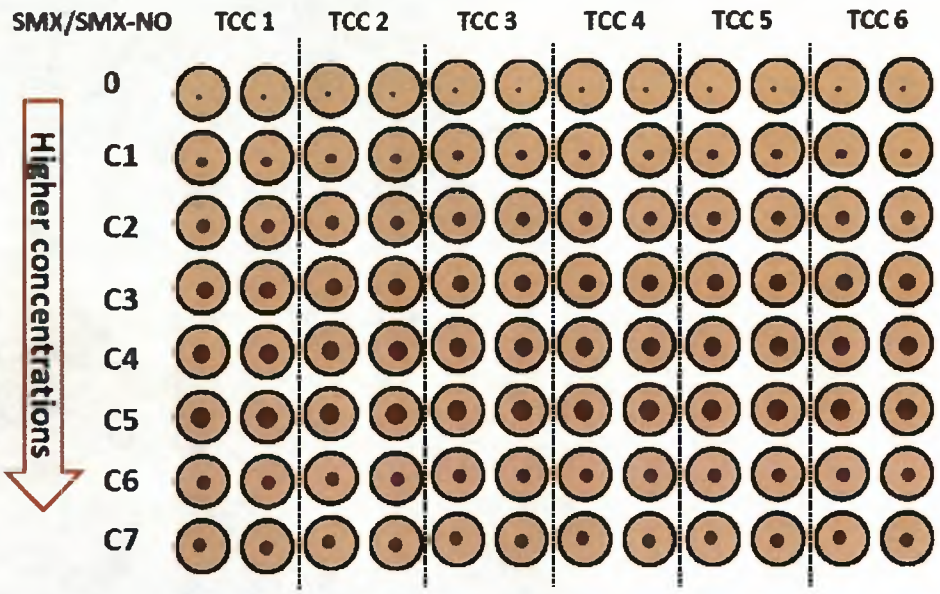

Figure 2.9: Plate map for the proliferation assay. T-cells were incubated with autologous irradiated B-LCLs and a range of SMX/SMX-NO concentrations in a 96-well round-bottomed tissue culture plate. After 48 h, [3H]-thymidine was added, and 16 h later proliferation was determined. T-cells with a positive proliferative response (SI > 2) were taken to be drug-specific and maintained in IL-2 containing medium.

2.2.11 Analysis of the phenotype of antigen-specific T cell clones

The phenotype of antigen-specific T-cell clones was determined using flow cytometer by dual staining with PE-labelled anti-CD4 and FITC-labelled anti-

CD8 which are both markers for blood T cells. All steps were carried out on ice. One million cells were washed with HBSS and the cell pellet was re-suspended in a saturating concentration of CD8 and CD4 antibodies for 30 min at 4°C in the dark. Following incubation, cells were washed twice with HBSS to remove unbound antibody and to pellet the cells (5 min, 1500 rpm, 4°C). The cell pellet was then re-suspended in 400 µl HBSS (4°C) and analysed using flow cytometry (Coulter Epics XL software; Beckman Coulter, Inc). The primary gate was set on lymphocyte-sized cells and analysed for the expression of CD4 versus CD8.

2.2.12 Assessment of the cytotoxic activity of T-cell clones

SMX (metabolite)-induced T-cell mediated cytotoxicity was tested at days 10–14 after re-stimulation through the use of a 4-hour [^{51}Cr] chromium-release assay. To measure immune-mediated killing, autologous B-LCLs were used as target cells in presence or absence of SMX or SMX-NO. The principle and workflow of the assay are illustrated in figure 2.10 and described below in 4 steps.

2.2.12.1 Preparation of target cells (B-LCL)

B-LCLs (1×10^6) were centrifuged at 1500 rpm for 5 min. The supernatant was removed and the cells were re-suspended in [^{51}Cr] chromium (50 µCi; 50 µl) and incubated for 90 min with gentle mixing every 15 min. The cells were then washed three times to remove free [^{51}Cr] chromium and re-suspended in 20 ml of complete medium to adjust cell concentration to 5×10^4 cells/ml.

2.2.12.2 Preparation of effector cells

T-cell clones were harvested, washed at 1500 rpm for 5 min, re-suspended in complete medium, and counted. Stock solutions of 2.5 x 10^6, 1 x 10^6, 0.5 x 10^6, and 0.25 x 10^6 cells/ml were prepared.

2.2.12.3 Preparation of the plate

Chromium loaded APCs (50 µl; 5 × 10^4 cells/ml) were incubated in duplicate in 96-well U-bottomed culture plate with T-cells (50 µl; 2.5 x 10^6, 1x10^6, 0.5x10^6 and 0.25x10^6 cells/ml) at effector:target (E:T) ratios of 50:1, 20:1, 10:1, and 5:1 respectively in presence or absence of drug (SMX, 200–400 µg/ml; SMX-NO, 40–80 µM). After 4 h incubation at 37°C in 5% CO_2, 100 µl of the supernatant was collected from each well and transferred to scintillation vials and topped up with 4 ml scintillation fluid. Spontaneous and maximum releases were determined by parallel incubations of labelled target cells with medium and methanol (to lyse remaining cells) respectively in the presence or absence of SMX/SMX-NO. Replicates of 8 wells were established for each condition of spontaneous or maximum release. Direct toxicity of SMX/SMX-NO was excluded by incubating the drug/metabolite with [^{51}Cr] chromium loaded B-LCLs for 4 h in the absence of T cells.

2.2.12.4 Analysis of Data

Radioactivity in supernatants was determined using Gamma-counter and expressed as cpm. Specific lysis of the target cells was then calculated from the formula:

$$\% \text{ Specific release} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

Experimental release = T cell - induced release
Maximum release = Methanol - induced release
Spontaneous release = Medium - induced release

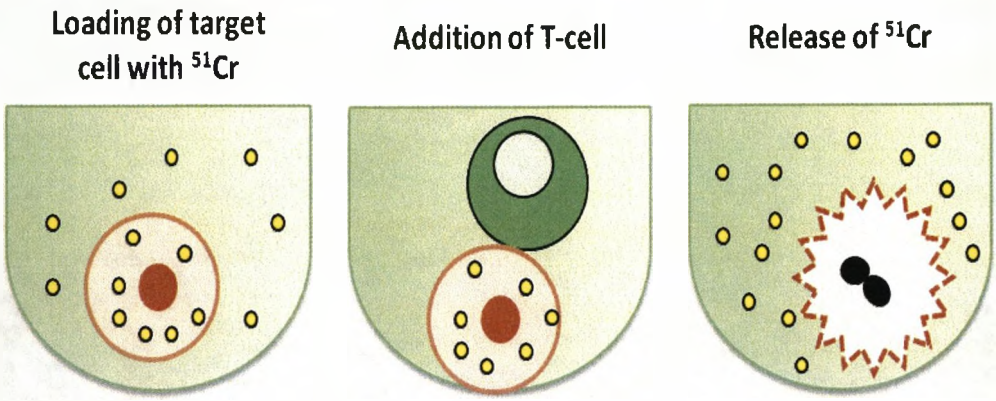

Figure 2.10: The principle of chromium release assay. The assay measures the killing of target cells by specific T cells. Target cells were labeled by [^{51}Cr] chromium. Effector cells were added to the target cells in different amounts (effector to target ratios) and incubated for 4 hours. An aliquot of the supernatant was analysed for radioactivity in a gamma counter. Radioactivity in the supernatant indicated the lysis of target cells.

2.2.13 Multiplex analysis of T-cell cytokine secretion

Levels of secreted cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCAF, MIP-1β, and TNF-α) were analysed using Bio-Plex 17-plex human cytokine assay kit (Bio-Rad Laboratories, Hercules, USA) on Bio-Plex suspension array system (xMAP-based

platform, Bio-Rad Laboratories, Hercules, USA). The assay procedure is similar to that described previously (section 2.1.6.3) with the following main differences:

- Each well needs 50 µl of beads instead of 25 µl in mouse experiment.
- Differences in the bead regions as illustrated in manufacture's manual.
- Eight standard concentrations were prepared instead of six in mouse assay.
- Incubation periods in human assay were: 30 min (for beads and samples), 30 min (for detection antibody), and 10 min (for SA-PE). In mouse experiments, it is 2h, 1h, and 30 min respectively.

2.2.14 Determination of the involvement of drug metabolism, protein adduct formation and antigen processing in the stimulation of T-cell clones

To evaluate mechanisms of drug (metabolite)-specific T-cell activation we implemented a stepwise approach. At each stage, the assay to measure antigen-specific proliferative responses was modified as follows: (1) Assays were conducted in the presence of glutathione (1 mM) – glutathione prevents SMX-NO protein binding (Burkhart et al., 2001; Naisbitt et al., 2001), (2) Assays were conducted in the presence of inhibitors of drug metabolising enzymes, specifically methimazole and 1-ABT, at a concentration that blocks SMX metabolism (both 1 mM) (Sanderson et al., 2007; Lavergne et al., 2009), (3) Assays were conducted with glutaraldehyde-fixed APCs– fixation blocks antigen processing (Zanni et al., 1998; Schnyder et al., 2000), (4) APCs were pulsed with SMX or SMX-NO for 1 & 16 h prior to repeated washing steps to remove unbound drug, and the addition of the pulsed APCs to the proliferation assay – 16 h is the time required for SMX metabolism and binding of derived metabolites to intracellular protein and (5) APCs and T-cells were pulsed together with SMX or SMX-NO for 16 h in the

presence of enzyme inhibitors, prior to repeated washing and culture of T-cells for the remainder of the assay in the absence of soluble antigen.

2.2.14.1 Addition of glutathione to the proliferation assay

T-cells (5 X 10^4) and irradiated B-LCLs (1 X 10^4) were incubated with GSH (1 mM) for 1 h prior to the addition of SMX (200 μg/ml) or SMX-NO (80 μM) to the standard proliferation assay. GSH binds covalently to SMX reactive metabolites and hence prevent protein complex formation. Proliferation was measured by [3H]-thymidine incorporation as described previously.

2.2.14.2 Enzyme inhibition experiments

The involvement of metabolism in T-cell activation by SMX was explored by the addition of 1-ABT or meth to the standard proliferation assay. 1-ABT is a potent non-selective inhibitor of a wide range of metabolic enzymes including cytochrome P450 isoforms (Ortiz de Montellano et al., 1984; Emoto et al., 2005; Sanderson et al., 2007; Lavergne et al., 2009), while methimazole is an inhibitor of peroxidases and FMO (Lavergne et al., 2009). T cell clones (5 x 10^4) and irradiated APCs (1 x 10^4) were incubated for 1 h in the presence of meth (1mM) or 1-ABT (1mM). After pre-incubation with inhibitors, cells were incubated for an additional 16 h with SMX or SMX-NO. The enzyme inhibitor at concentration of 1mM did not cause cytotoxicity.

2.2.14.3 Glutaraldehyde-fixation experiments

The involvement of processing in presentation of SMX and SMX metabolite to specific T-cell clones was evaluated by modifying the standard T-cell proliferation assay using chemically fixed APC. Fixation inhibits antigen

processing (prevents presentation of drug-hapten protein complexes) but does not alter MHC expression, presentation of antigenic peptides (pre-processed antigens or processed peptides) to T-cells or the direct drug-MHC interaction (Shimonkevitz et al., 1983; Zanni et al., 1997; Schnyder et al., 2000; Naisbitt et al., 2003b; 2005). B-LCLs (2 x 10^6; 0.5 ml HBSS) were fixed by addition of 12.5 µl glutaraldehyde 2% (end concentration 0.05%) for 30 seconds with vortexing at room temperature before the reaction was terminated by adding L-glycine (1ml, 0.2 M) for an additional 45 sec. Fixed B-LCLs were extensively washed, re-suspended in medium, and then added to the proliferation assay (with T cell clone and drug/metabolite). Incubation of non fixed B-LCLs with T cell clone and the soluble drug/metabolite was considered as a positive control. Certain wells contained fixed B-LCLs with T cell clone in absence of the drug or its metabolite.

2.2.14.4 Pulsing experiments

In APC-pulsing experiments, autologous B-LCLs were incubated in culture medium with SMX (200-400 µg/ml) or SMX-NO (80 µM) for 2 and 16 h; the optimal time for protein complex formation. Pulsed B-LCLs (used as a source of antigen) were washed repeatedly with HBSS to remove non-covalently bound drug before irradiated, adjusted at concentration of (1 x 10^4 cells/well), and co-incubated with different sets of antigen-specific T-cell clones (5 x 10^4) in a final volume of 0.2 ml in the absence of soluble antigen. T-cell incubation contained the non-pulsed irradiated B-LCLs and the soluble form of the drug was used as a positive control. Proliferation was measured using [^{3}H] thymidine incorporation as described above.

2.2.15 Detection of SMX metabolite protein adduct formation

Protein adduct formation in SMX and SMX-NO treated EBV-transformed B-cells from hypersensitive patients was measured at different concentration (SMX 50-400 μg/ml; SMX-NO 10-80 μM) and time (0.1, 2, 6, 16 h) points by ELISA and confocal microscopy using a specific anti-SMX-antibody (described previously in section 2.1.6.5). Certain experiments were conducted in the presence of various enzyme inhibitors added 1 h prior to SMX or SMX-NO.

2.2.16 Involvement of MHC molecules in T-cell activation

To assess the involvement of MHC in drug presentation to T-cell clones, Specific anti–HLA blocking antibodies (anti–HLA class I mAb (anti-human HLA-A,B,C; 1mg/ml) and anti–HLA class II mAb (anti-human HLA-DR, DP, DQ; 1mg/ml) were added to the proliferation assay to inhibit MHC-restricted stimulations of the $CD8^+$ and $CD4^+$ T cell clones. Anti–HLA blocking antibodies were added at concentration of 1 μg/ml, 30 minutes before addition of the drug. Certain incubations contained T-cell and drug in the absence of APCs. The monoclonal antibodies were tested in the same concentrations on related and unrelated clones without showing any toxicity on T cell clones.

2.3 Statistical analysis

Values to be compared were analysed for normality using the Shapiro-Wilks test. Normally distributed data were analysed using a Student T test. Non-normally distributed data were compared with the Mann-Whitney test for comparing two groups for continuous variables using the SPSS 16.0 software (SPSS Inc., Chicago, USA). In all cases, $p < 0.05$ was considered statistically significant.

Chapter 3

Sulfamethoxazole Metabolism by Mouse Immune Cells

Contents

3.1 Introduction

Drug hypersensitivity reactions are adverse drug reactions that represent one of the most feared adverse effects in both clinical practice and drug development process due to the off-target (unpredictable, dose independent) and serious nature. The pathogenesis of these reactions remains unidentified in spite of plentiful studies and investigations.

It is thought that drug hypersensitivity reactions are the consequences of an abnormal immune reaction triggered by a drug or its metabolites. The presence of drug-specific cytotoxic T-cells in the peripheral circulation and target organs of hypersensitive patients, but not in drug-exposed controls provides a robust case for the involvement of these T-cells in the pathogenesis of the reaction (Britschgi et al., 2001; Nassif et al., 2004; Beeler et al., 2006; Wu et al., 2006). To initiate an immune response, the drug antigen must be presented in the context of specific MHC molecules expressed on dendritic cells to specific T-cell receptors in a micro-environment rich in co-stimulatory signalling and cytokines, which are necessary for sustained T-cell expansion (Curtsinger et al., 1999; Gerner and Mescher, 2009).

Our understanding of the chemical basis of drug hypersensitivity reactions derives from the field of allergic contact dermatitis, where Landsteiner and Jacobs (Landsteiner K. and Jacobs, 1935) defined low molecular weight chemical allergens as "incomplete antigens" since the compounds themselves were not directly antigenic (i.e., do not bind with high affinity to immunological receptors)

and only gained immunogenic potential following covalent modification of a protein carrier.

The β-lactam antibiotics, which cause a high incidence of drug hypersensitivity reactions, also react spontaneously with protein (Padovan et al., 1996; Jenkins et al., 2009) and synthetically constructed penicillin protein adducts stimulate specific T-cells following processing (Brander et al., 1995). On this basis, the "hapten concept" (hapten referring to any substance that modifies protein to induce an immune response) is the hypothesis most commonly used to describe the interaction of drugs with immune cells. The picture is further complicated because most drugs are not directly protein-reactive. They may however gain protein reactivity through normal metabolic processes, generating a hapten with the potential to modify specific amino acid residues on protein (Naisbitt et al., 2000a; Park et al., 2001).

Several independent investigators have shown that the immune system is involved in the pathogenesis of sulfonamide-induced hypersensitivity reactions (Hertl et al., 1995; Mauri-Hellweg et al., 1995; Schnyder et al., 1997; Schnyder et al., 2000; Burkhart et al., 2001; Hari et al., 2001; Svensson et al., 2001; Roychowdhury and Svensson, 2005; Castrejon et al., 2010). Sulfamethoxazole (SMX) has been associated with reactions that range in severity from mild urticarial rashes to severe necrolytic skin lesions and hepatotoxicity (Cribb et al., 1996a; Cribb et al., 1996b; Barranco and Lopez-Serrano, 1998; Svensson et al., 2001; Naisbitt, 2004; Nassif et al., 2004; Kouklakis et al., 2007).

Drug metabolism and bioactivation occurs in organs and cells expressing drug-metabolising enzymes mainly liver (hepatocytes), and skin (keratinocytes, fibroblasts, langerhans cells, and melanocytes) (Wolkenstein et al., 1998; Kinobe et al., 2005; Roychowdhury et al., 2005; Bhaiya et al., 2006; Sanderson et al., 2006; Vyas et al., 2006a; Vyas et al., 2006b; Oesch et al., 2007; Roychowdhury et al., 2007a; Roychowdhury et al., 2007b). In addition, immune cells as lymphocytes, granulocytes, APCs can metabolise drugs (Sieben et al., 1999; Sanderson et al., 2007; Siest et al., 2008). Dendritic cells express multiple drug metabolising enzymes (Sieben et al., 1999; Tafazoli et al., 2005; Sanderson et al., 2007; Siest et al., 2008) that can generate drug-derived protein in increasing amounts when exposed to "danger" signals (Lavergne et al., 2009).

SMX can be metabolised by several phase 1 enzymes including CYPs (CYP2C9, CYP2C8) and/or MPO to a non protein-reactive hydroxylamine-metabolite which is unstable and can be rapidly auto-oxidised to an electrophilic nitroso-intermediate (Shear and Spielberg, 1985; Rieder et al., 1988; Cribb and Spielberg, 1990; Cribb et al., 1991; Cribb and Spielberg, 1992; Cribb et al., 1995; Cribb et al., 1996b; Gill et al., 1996; Naisbitt et al., 1996; Gill et al., 1997; Sanderson et al., 2007) (figure 1.9). The level of tissue exposure to SMX-NO is dependent on the ability to bioinactivate (detoxify) the reactive metabolites by glutathione, cysteine, and detoxification enzymes (Cribb et al., 1991; Gill et al., 1997; Naisbitt et al., 1999; Naisbitt et al., 2000b; Trepanier et al., 2004). Degradation of SMX-NO in solution gives nitro SMX (by oxidation), SMX-HA (by reduction), and azo/azoxy adducts (by dimerisation) (Naisbitt et al., 2002).

SMX metabolites are considered to take part in the pathogenesis of immune-mediated SMX-induced hypersensitivity reactions as evidenced by numerous studies shown in table 3.1. It is currently thought that immune cells play an essential role in SMX–induced immune-mediated hypersensitivity reactions through the formation of immunogenic conjugates. These conjugates are formed from the reaction of a drug reactive metabolite with cellular proteins (Park et al., 1998; Roychowdhury et al., 2005; Cheng et al., 2008; Lavergne et al., 2008b).

Synthetic SMX metabolites interact with MHC and T-cell receptors with sufficient affinity to stimulate blood and skin-derived T-cells from hypersensitive patients (Schnyder et al., 2000; Burkhart et al., 2001; Farrell et al., 2003; Nassif et al., 2004; Castrejon et al., 2010). These data confirm that hapten-specific T-cells are present in hypersensitive patients and that T-cell responses are directed against drug metabolite conjugated protein. However, critical experiments showing a causal relationship between compound distribution and metabolism in APCs and the development of T-cell responses have not been described.

In this investigation, we study SMX metabolism in mouse immune cells indirectly through the use of a hapten inhibitable anti-SMX antibody that detects SMX metabolites bound irreversibly to protein, and the effect of enzyme inhibitors on adduct formation. The formation of metabolite protein adducts in immune cells was investigated using mice splenocytes and bone marrow-derived dendritic cells. This is a step before studying the functional consequences of these adducts in the

context of exploration the complex relationship between metabolism, adduct formation, cell death and both dendritic cell and T-cell activation.

Table 3.1: Involvement of sulfamethoxazole metabolites and immune system in the drug induced hypersensitivity reactions through (A) protein reactivity, (B) cytotoxicity, and (C) immunogenicity.

(A) Findings indicate protein-reactivity of SMX metabolites	
Hepatic SMX metabolism resulted in the generation of covalently associated protein adducts (haptenation of tissue protein).	(Cribb et al., 1996b)
Antibodies to hepatic microsomal proteins were found in patients with sulfonamide hypersensitivity.	(Cribb et al., 1997)
Ability of SMX metabolites to haptenate cellular proteins on the surface of skin keratinocytes, lymphocytes, and splenocytes.	(Naisbitt et al., 1999; Reilly et al., 2000; Naisbitt et al., 2001)
Incubation of normal human keratinocytes with SMX/SMX metabolites gave rise to drug/metabolite-protein adducts.	(Reilly et al., 2000; Roychowdhury et al., 2005)
In vitro exposure of T lymphoblastic cells to SMX reactive metabolites resulted in the formation of drug-protein adducts.	(Manchanda et al., 2002)
Covalent binding of SMX-HA to lymphoid cellular proteins in cultured hystiocytic lymphoma cells.	(Summan and Cribb, 2002)
Activation of dendritic cells by SMX metabolites	(Sanderson et al., 2007)
Identification of SMX-NO-protein adducts in spleen and lymph nodes of mice treated with SMX-NO.	(Cheng et al., 2008)
Detection of meta-stable protein adducts when SMX-NO was incubated with HSA or glutathione S-transferase π.	(Callan et al., 2009)

(B) Direct toxicity of SMX metabolites to cells of the immune system in vitro	
In vitro cytotoxicity of SMX metabolites towards peripheral blood mononuclear cells.	(Rieder et al., 1988; 1989; Cribb et al., 1991; 1995; Rieder, 1997; Reilly et al., 1998; Hess et al., 1999; Naisbitt et al., 1999; 2002; Lavergne et al., 2006b)
In vitro cytotoxicity of SMX metabolites towards Skin cells.	(Reilly et al., 2000; Vyas et al., 2005)
In vitro toxicity is observed with methemoglobin formation in human red blood cells	(Reilly et al., 1999)
In vitro cytotoxicity of the SMX metabolites has been used as a marker of hypersensitivity especially in HIV-infected persons.	(Rieder et al., 1989; Carr et al., 1993; Wolkenstein et al., 1995; Neuman et al., 2000; Lavergne et al., 2006b)
(C) Binding provides an antigenic signal to antigen-specific T cells	
Identification of SMX-substituted serum proteins and anti-SMX antibodies in patient sera.	(Meekins et al., 1994) (Daftarian et al., 1995) (Gruchalla et al., 1998)
Proliferation of $CD8^+$ dermal T-cells from lesional skin when stimulated with SMX metabolites	(Hertl et al., 1995)
Identification of SMX-substituted hepatic protein	(Cribb et al., 1996b)
Administration of a mixture of SMX metabolites and Freund's adjuvant, led to SMX-NO specific but not SMX-specific, delayed-type hypersensitivity in mice in the form of foot pad edema.	(Choquet-Kastylevsky et al., 2001)

Incubation of rat APCs with SMX-NO, led to cell surface haptenation and specific T cells stimulation.	(Naisbitt et al., 2002)
Immunisation of different species of animals with SMX-NO, but not the parent drug, led to stimulation of a specific T-cell proliferative responses and production of anti-SMX antibodies.	(Gill et al., 1997; Naisbitt et al., 2001; Farrell et al., 2003; Cheng et al., 2008)
Proliferation of T cells cloned from peripheral blood of hypersensitive patients in response to the covalently bound SMX-NO	(Schnyder et al., 2000; Burkhart et al., 2001; Castrejon et al., 2010)
Stimulation of skin derived T-cells from SMX hypersensitive patients with the protein-reactive metabolite SMX-NO.	(Nassif et al., 2004)

3.2 Results

3.2.1 Detection of protein adducts in mouse antigen presenting cells exposed to sulfamethoxazole or its metabolites

Drug-protein adducts formed in splenocytes and dendritic cells (from naïve mice) were measured after 16 h with a range of concentrations of SMX and SMX-NO. Adduct formation was measured by both ELISA (see 2.1.6.5.1) and confocal microscopy (see 2.1.6.5.2) using a specific anti-SMX antibody.

By ELISA, dose dependent SMX-derived protein adducts in mouse splenocytes were detected (blanked control; 250μM: 0.6±0.1; 500μM: 0.9±0.4; 1000μM: 0.9±0.4; 2000μM: 1.9±0.8, $P<0.05$, figure 3.1). Adduct formation with SMX-NO was detected over the used range of drug concentrations (blanked control; 5μM:

2.4±0.1; 10μM: 2.7±0.2; 25μM: 2.8±0.1; 50μM: 2.7±0.3; 100μM: 2.8±0.2; 250μM: 2.8±0.2, $P<0.05$, figure 3.1).

Likewise, drug-protein complex formation in mouse bone marrow derived dendritic cells was explored by ELISA using a specific anti-SMX antibody after incubation with SMX, SMX-NO, or vehicle for 16 h. Binding of SMX to dendritic cells was concentration-dependent, with adducts detected at concentrations above 250 μM (blanked control; 500μM: 0.2±0.0; 1000μM: 0.3±0.1; 2000μM: 0.4±0.2, $P<0.05$, figure 3.2). SMX-NO treatment of dendritic cells was associated with significant adduct formation at a concentration of 50 μM and above (blanked control; 50μM: 0.4±0.2; 100μM: 0.9±0.0; 250μM: 2.1±0.8; 500μM: 2.7±0.1; 1000μM: 3.0±0.2, $P<0.05$, figure 3.2).

Hapten-protein complex formation in splenocytes exposed to SMX or non-toxic concentrations of its nitroso metabolite for 16 h was also visualized using immunofluorescence confocal microscopy using a specific anti-SMX antibody. Microscopic imaging showed that, SMX-NO (10 μM, 50 μM) and SMX (1 mM, 2 mM) form intracellular protein adducts. These adducts were not detected in controls without drug exposure (figure 3.3).

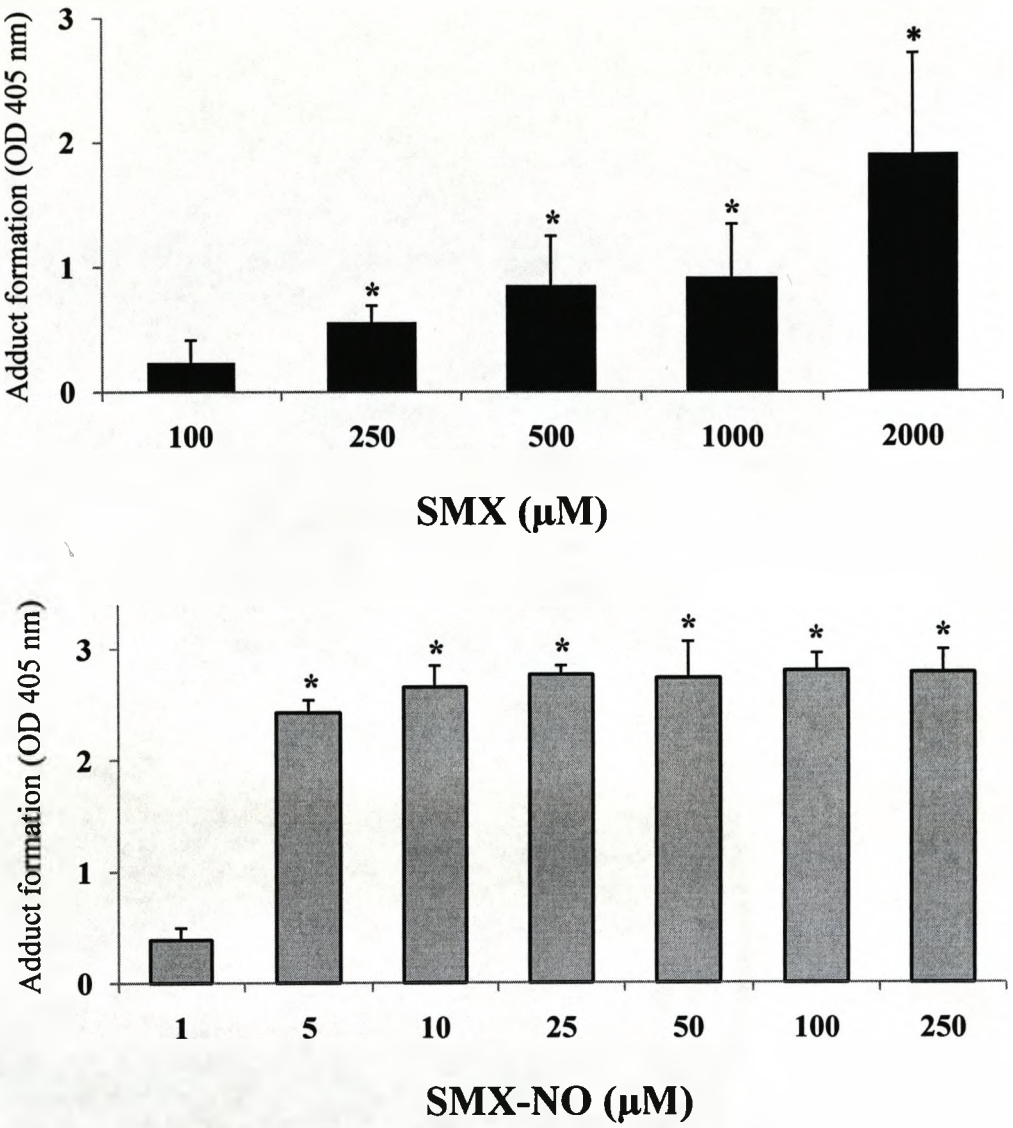

Figure 3.1: Sulfamethoxazole metabolism and protein adduct formation in mouse splenocytes. Cells were exposed to SMX (100–2000 μM) or SMX-NO (1–250 μM) for 16 h. Concentration-dependent protein adduct formation in SMX-treated cells was quantified by ELISA using a specific anti-SMX antibody. Data are presented as mean ± SD of at least three separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*, significantly different from untreated cells; $P<0.05$).

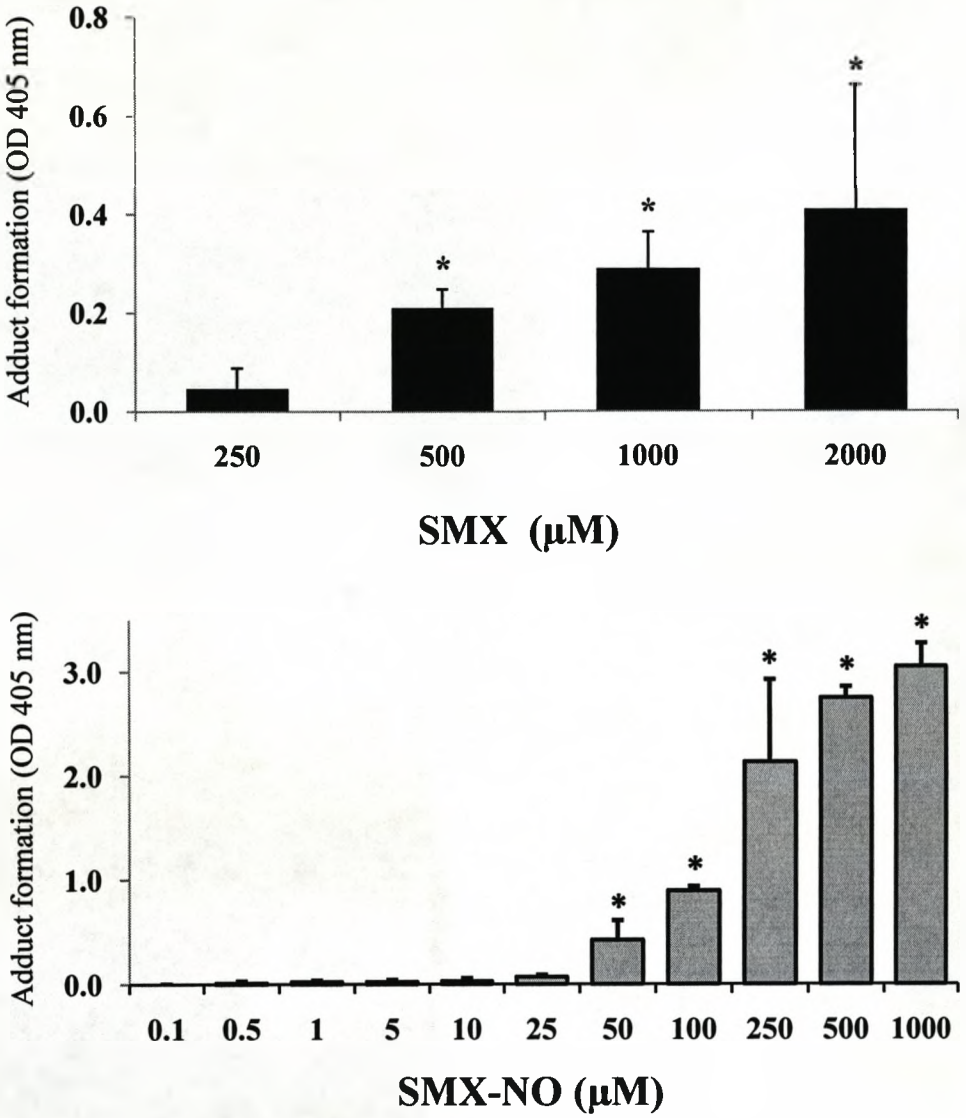

Figure 3.2: Sulfamethoxazole metabolism and protein adduct formation in mouse dendritic cells. Cells were incubated with SMX (250–2000 μM) or SMX-NO (0.1–1000 μM) for 16 h. Concentration-dependent protein adduct formation in SMX and SMX-NO-treated cells was quantified by ELISA using a specific anti-SMX antibody. Data are presented as mean ± SD of at least 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*, significantly different from untreated cells; $P<0.05$).

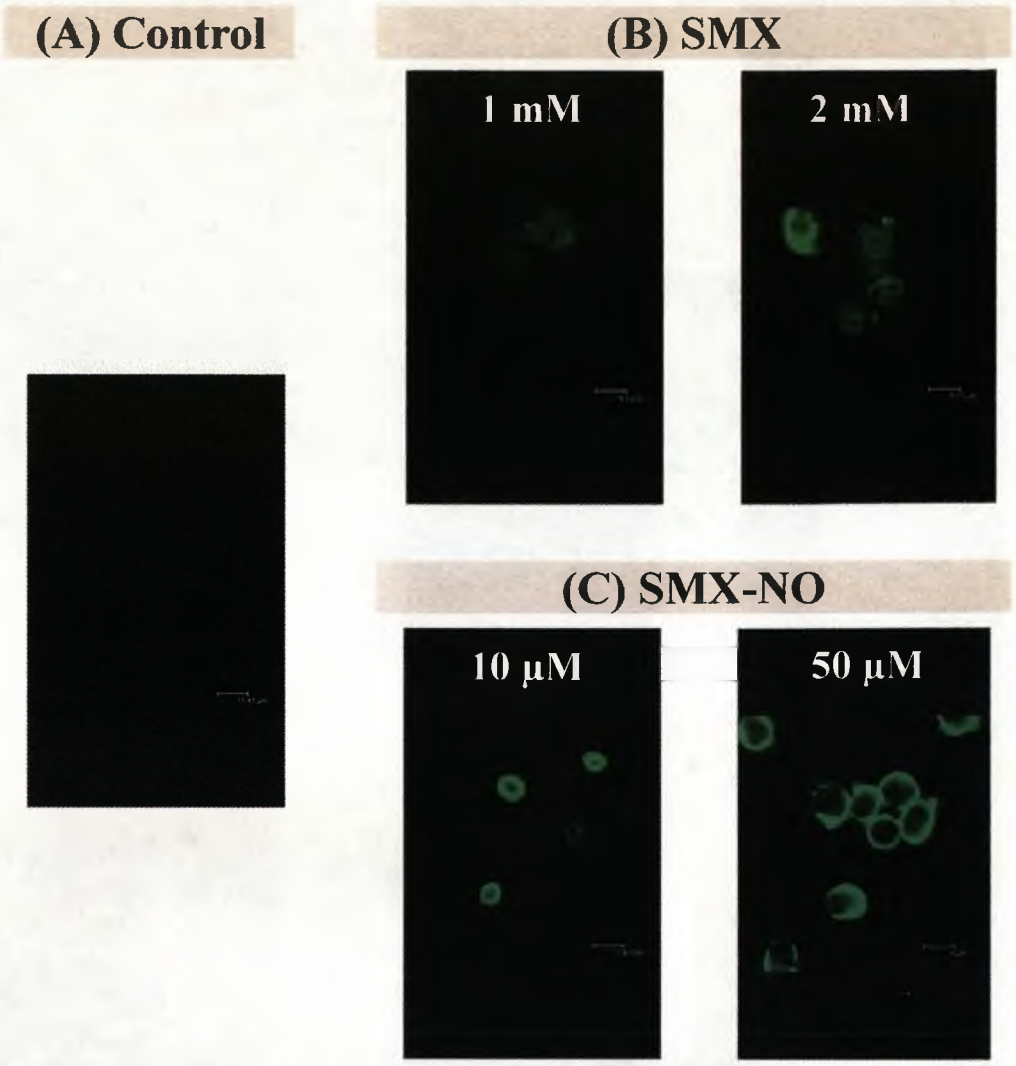

Figure 3.3: Confocal microscopy imaging of mouse splenocytes exposed to sulfamethoxazole or its metabolite. Cells were incubated with (A) DMSO 0.2% as a vehicle, (B) SMX (1 mM and 2 mM), or (C) the protein reactive metabolite SMX-NO (10 µM and 50 µM) for 16 h before being processed for confocal microscopy. Adducts were visualised in cells incubated with SMX or SMX-NO with no adducts in control samples.

3.2.2 Time dependent formation of sulfamethoxazole protein adducts in mouse antigen presenting cells

As described above, adducts were detected in splenocytes incubated with SMX or SMX-NO for 16 h. To assess time dependency of adduct formation; different time points (0.1–16 h) were examined. The levels of hapten-complex formation were significantly reduced with SMX when the culture period was reduced indicating time dependent binding and adduct formation (blanked control; 0.1h: 0.3±0.1; 2h: 0.4±0.2; 6h: 0.4±0.3; 16h: 1.2±0.6, $P<0.05$, figure 3.4). On the contrary, the level of SMX-NO adducts remained constant when the culture period was reduced (blanked control; 0.1h: 2.4±0.3; 2h: 2.6±0.2; 6h: 2.5±0.2; 16h: 2.6±0.2, figure 3.4).

Similar results were observed with mouse dendritic cells. Over a period of 16 h, SMX-protein adducts increased steadily with significant levels detected after 6 hours (blanked control; 6h: 0.3±0.2; 16h: 0.6±0.2, $P<0.05$, figure 3.5). The level of SMX-NO adducts remained constant when the culture period was reduced, with detected adducts early at 5 minutes (blanked control; 0.1h: 1.0±0.3; 2h: 1.2±0.3; 6h: 1.2±0.2; 16h: 1.3±0.2, figure 3.5).

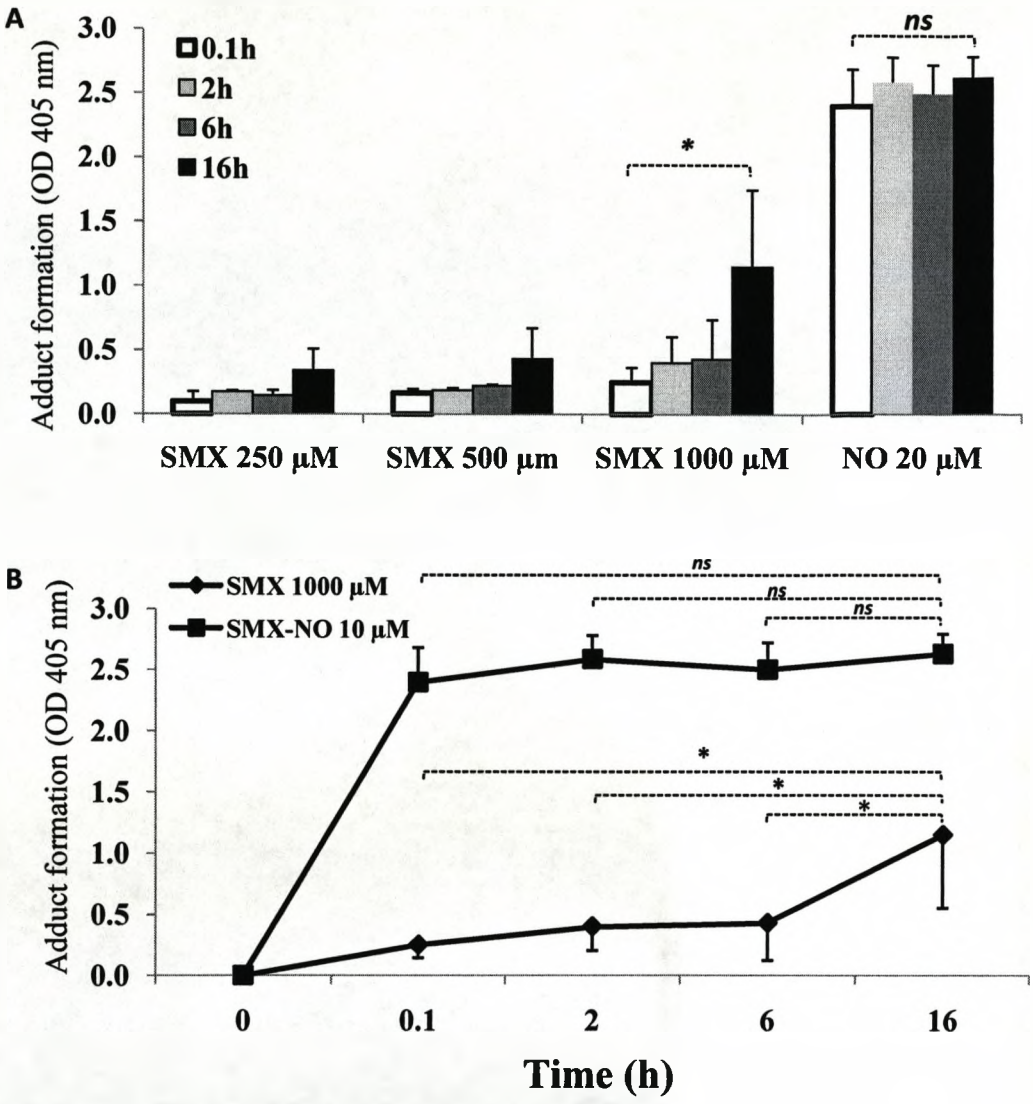

Figure 3.4: Time dependency of protein adducts formation in mouse splenocytes. Cells were incubated with SMX at different concentration points (A) or SMX-NO (10, 20 µM) for different time points (0.1-16 h). Time-dependent protein adduct formation in SMX (but not SMX-NO)-treated cells was quantified by ELISA using a specific anti-SMX antibody (A, B). Data are presented as mean ± SD of at least 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*, significant at $P<0.05$; *ns*, non significant).

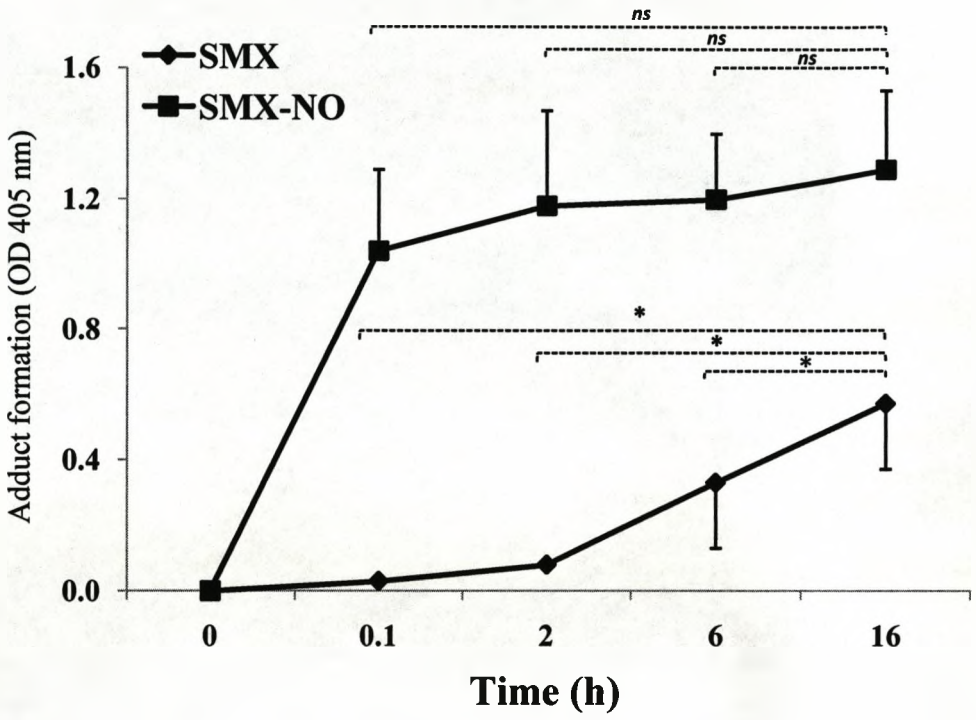

Figure 3.5: Time dependency of protein adduct formation in mouse bone marrow derived dendritic cells. Cells were incubated with SMX (1000 μM) or SMX-NO (100 μM) for different time points (0.1-16 h). Time-dependent protein adduct formation in SMX (but not SMX-NO)-treated cells was quantified by ELISA using a specific anti-SMX antibody. Data are presented as mean ± SD of at least 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*, significant at $P<0.05$; *ns*, non significant).

3.2.3 Inhibition of sulfamethoxazole-protein adduct formation by enzyme inhibitors in mouse antigen presenting cells

To assess the importance of SMX metabolism in generation of protein adducts, we evaluated the impact of inhibitors of metabolic enzymes (methimazole [meth] or 1-aminobenzotriazole [1-ABT]) on protein haptenation in APCs.

Mouse splenocytes were incubated with SMX (1 mM, 2mM) or SMX-NO (10 μM, 20 μM) in the presence or absence of enzyme inhibitors (1 mM) for 16 h, before being processed for ELISA. Inhibitors were added to the culture 1 h prior to the addition of drug. Data indicate that the levels of adduct formation were significantly reduced with SMX when meth or 1-ABT was added (blanked control; SMX: 1.5±0.4; SMX/meth: 0.4±0.3; SMX/1-ABT: 0.4±0.3, $P<0.05$, figure 3.6A). Enzyme inhibition was not observed with SMX-NO (blanked control; SMX-NO: 2.5±0.4; SMX-NO/meth: 2.6±0.3; SMX-NO/1-ABT: 2.5±0.3, figure 3.6B).

Similarly, 1-ABT and meth pre-treatment of mouse dendritic cells significantly decreased the formation of SMX-protein adducts (blanked control; SMX: 0.7±0.3; SMX/meth: 0.2±0.0; SMX/1-ABT: 0.2±0.1, $P<0.05$, figure 3.7A). Levels of hapten-complex formation were not significantly reduced with SMX-NO after addition of enzyme inhibitors (blanked control; SMX-NO: 1.2±0.4; SMX-NO/meth 1.2±0.3; SMX-NO/1-ABT: 1.3±0.4, figure 3.7B).

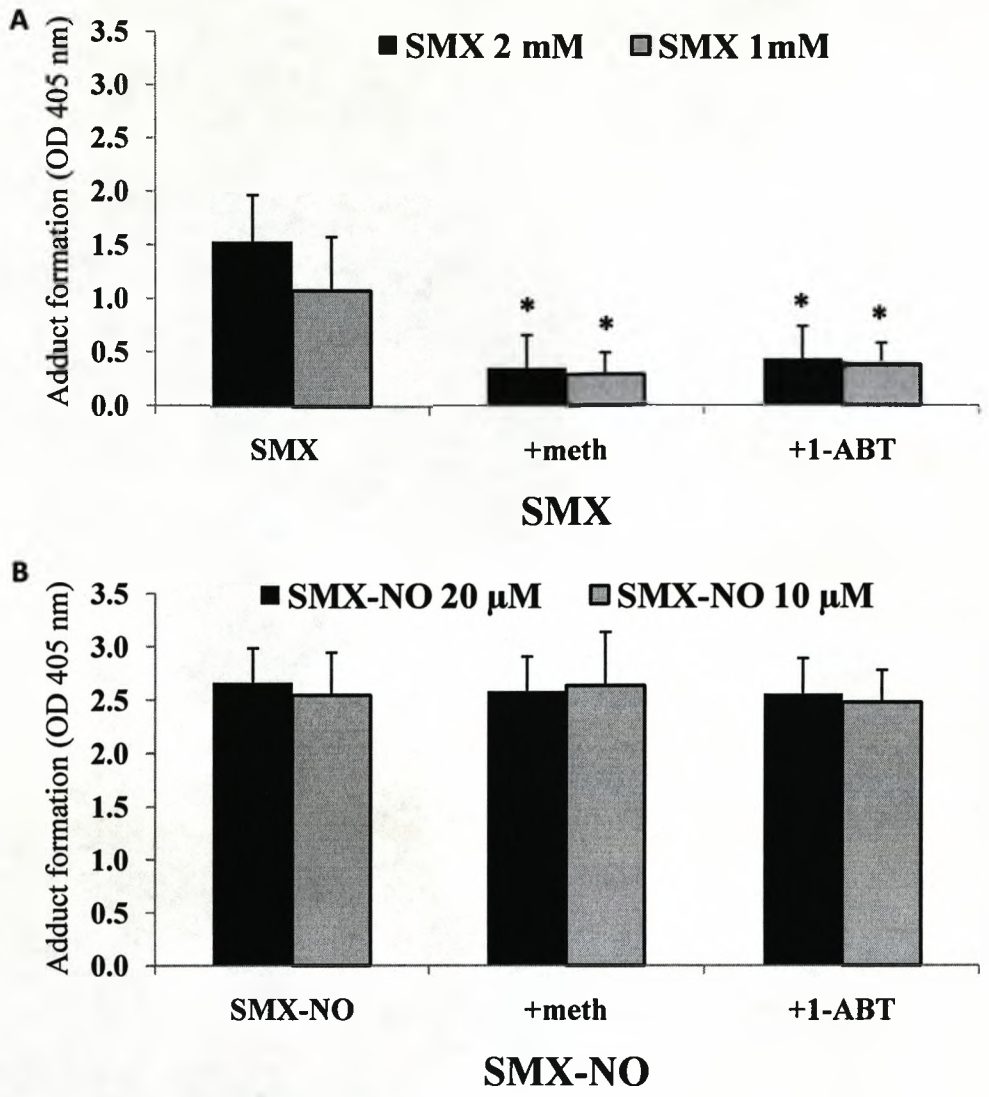

Figure 3.6: Inhibition of protein adduct formation in sulfamethoxazole-treated but not nitroso sulfamethoxazole-treated mouse splenocytes quantified by ELISA using a specific anti-SMX antibody. The inhibitors 1-aminobenzotriazole (1-ABT) and methimazole (meth) were incubated with cells for 1 h before the addition of SMX (A) or SMX-NO (B). Results are presented as mean ± SD of 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*significantly different from drug treated cells without inhibitors; $P < 0.05$)

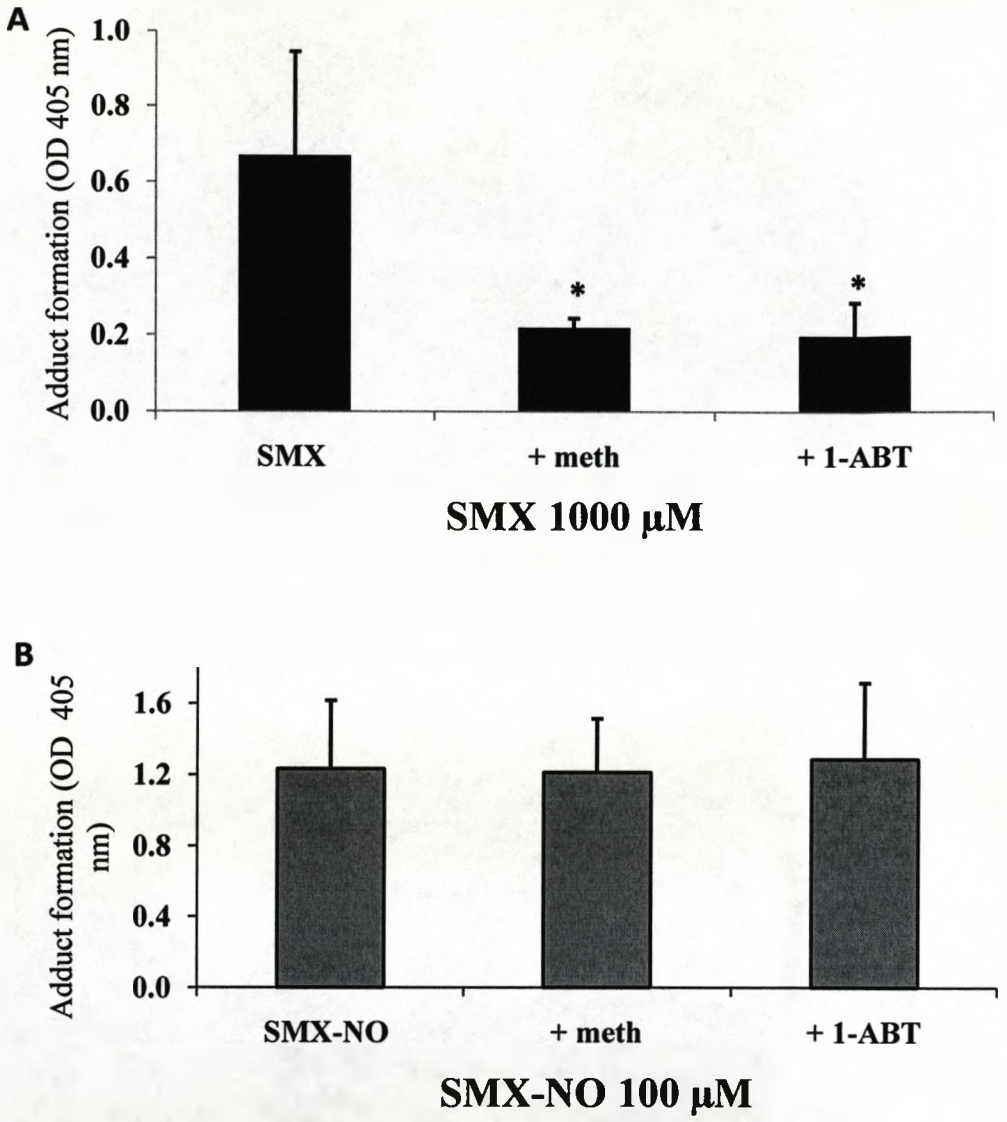

Figure 3.7: Inhibition of protein adduct formation in sulfamethoxazole-treated but not nitroso sulfamethoxazole-treated mouse dendritic cells quantified by ELISA using a specific anti-SMX antibody. The inhibitors 1-aminobenzotriazole (1-ABT) and methimazole (meth) were incubated with cells for 1 h before the addition of SMX (A) or SMX-NO (B). Results are presented as mean ± SD of 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*significantly different from drug treated cells without inhibitors; $P < 0.05$).

3.2.4 Intracellular glutathione levels in dendritic cells following sulfamethoxazole or nitroso sulfamethoxazole exposure

Mouse dendritic cells were incubated with varying concentrations of SMX or SMX-NO for 16 h, and intracellular glutathione levels were quantified using a colorimetric assay (Vandeputte et al., 1994). Oxidised glutathione (GSSG) levels were consistently at or below the level of detection. Consequently, total glutathione has been used as a surrogate for reduced glutathione (GSH) concentrations. A significant glutathione depletion was detected with SMX-NO at concentrations of 250 μM and above (nmol/10^6 cells, 0: 14±1.4; 250μM: 6.6±1.6, 500μM: 5.9±1.5, 1000μM: 3.8±1.8, $P<0.05$, figure 3.8A), whilst no significant glutathione depletion was seen with SMX at any concentration studied (nmol/10^6 cells, 0: 14±1.4; 50μM: 14.8±1.0; 100μM: 14.4±0.8; 250μM: 13.0±1.5; 500μM: 13.5±1.8; 1000μM: 13.2±1.9; 2000μM: 12.7±2.2, figure 3.8A).

In similar, glutathione depletion was detected when mouse splenoytes exposed to SMX-NO at a concentration above 50 μM for 16 h (nmol/10^6 cells, 0: 17.2±1.3; 50μM: 12.3±1.4, 100μM: 5.3±0.8, 250μM: 3.4±0.9; 500μM: 3.7±0.2; 1000μM: 2.5±0.5, $P<0.05$, figure 3.8B), while no significant depletion with SMX at any concentration (nmol/10^6 cells, 0: 17.2±1.3; 25μM: 16.9±1.4; 50μM: 17.1±1.1; 100μM: 17.0±1.5; 250μM: 16.9±1.7; 500μM: 16.3±1.0; 1000μM: 16.5±1.2; 2000μM: 15.7±1.6, figure 3.8B).

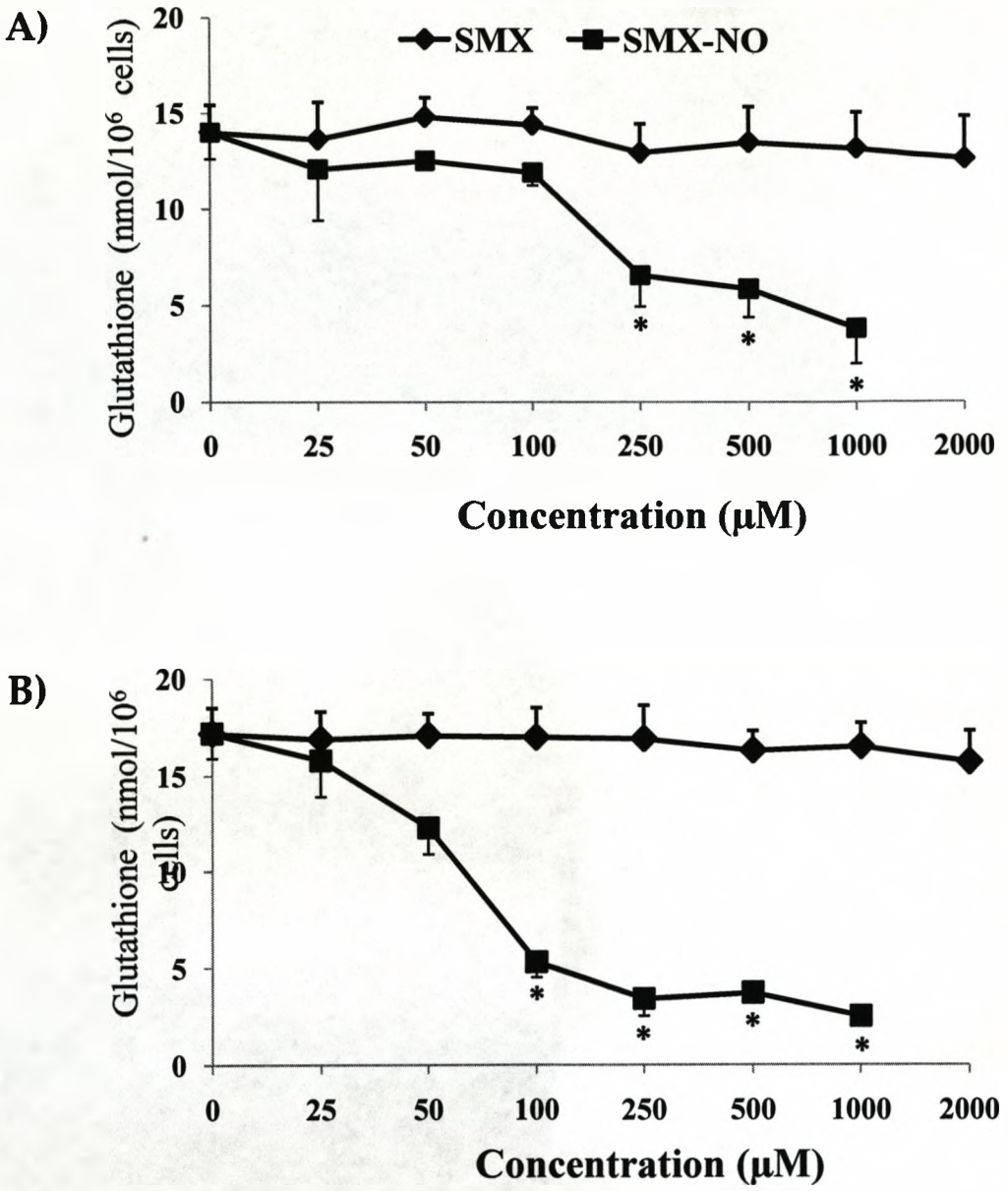

Figure 3.8: Intracellular glutathione levels in mouse dendritic cells (A) and splenocytes (B) exposed to sulfamethoxazole or nitroso sulfamethoxazole. Glutathione depletion was detected at SMX-NO 250 μM and above in mouse dendritic cells and above 50 μM in splenocytes with no significant glutathione depletion at any concentration of SMX. Data are presented as mean ± SD of 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*significantly different from untreated cells; $P < 0.05$).

3.2.5 Toxicity of SMX and its metabolites against antigen presenting cells

Initially, cells were counted, and cell recovery was determined using trypan blue staining. Necrotic and apoptotic cell death was quantified by flow cytometry using annexin V/PI double staining. Isolated splenocytes were incubated with varying concentrations of SMX/SMX-NO for 16 h, and then analysed for cell death.

There was no significant necrotic or apoptotic cell death when splenocytes were incubated with SMX at any concentration below 2 mM (% viable cells; 0: 92.3±4.7; 500μM: 91.2±4.1; 1000μM: 86.7±4.9; 2000μM: 86.7±4.2, % necrotic cells; 0: 5.7±3.1; 500μM: 5.3±2.3; 1000μM: 9.3±2.3, 2000μM: 8.7±2.3, figure 3.9A), whereas significant quantities of necrotic cells were observed with SMX-NO only at concentrations ≥ 50 μM (% viable cells; 0: 92.3±4.7; 50μM: 57.3±5.0; 100μM: 35.0±5.6; 250μM: 10.0±2.0, % necrotic cells; 0: 5.7±3.1; 50μM: 37.0±6.2; 100μM: 57.3±6.4; 250μM: 79.7±2.1, $P<0.05$, figure 3.9B). No significant increase in apoptotic cell death was observed at any concentration.

Similarly, mouse dendritic cells were incubated with SMX (25-2000 μM), or SMX-NO (1-1000 μM), for 16 h, before cell viability was determined. Dendritic cell death was detected at SMX-NO concentrations of 250 μM and above (% viable cells; 0: 82.7±2.2; 250μM: 67.3±1.1; 500μM: 55.0±3.2; 1000μM: 40.0±2.9, % necrotic cells; 0: 4.2±2.5; 250μM: 25.5±0.8; 500μM: 37.8±3.1; 1000μM: 60.1±3.6, $P<0.05$, figure 3.10B), whereas there was no significant cell death when cells were incubated with SMX at any tested concentration (% viable cells; 0: 83.4±2.5; 1000μM: 79.4±2.1; 2000μM: 79.1±1.7, % necrotic cells; 0: 5.6±2.8; 1000μM: 8.3±2.5; 2000μM: 9.7±2.2, figure 3.10A).

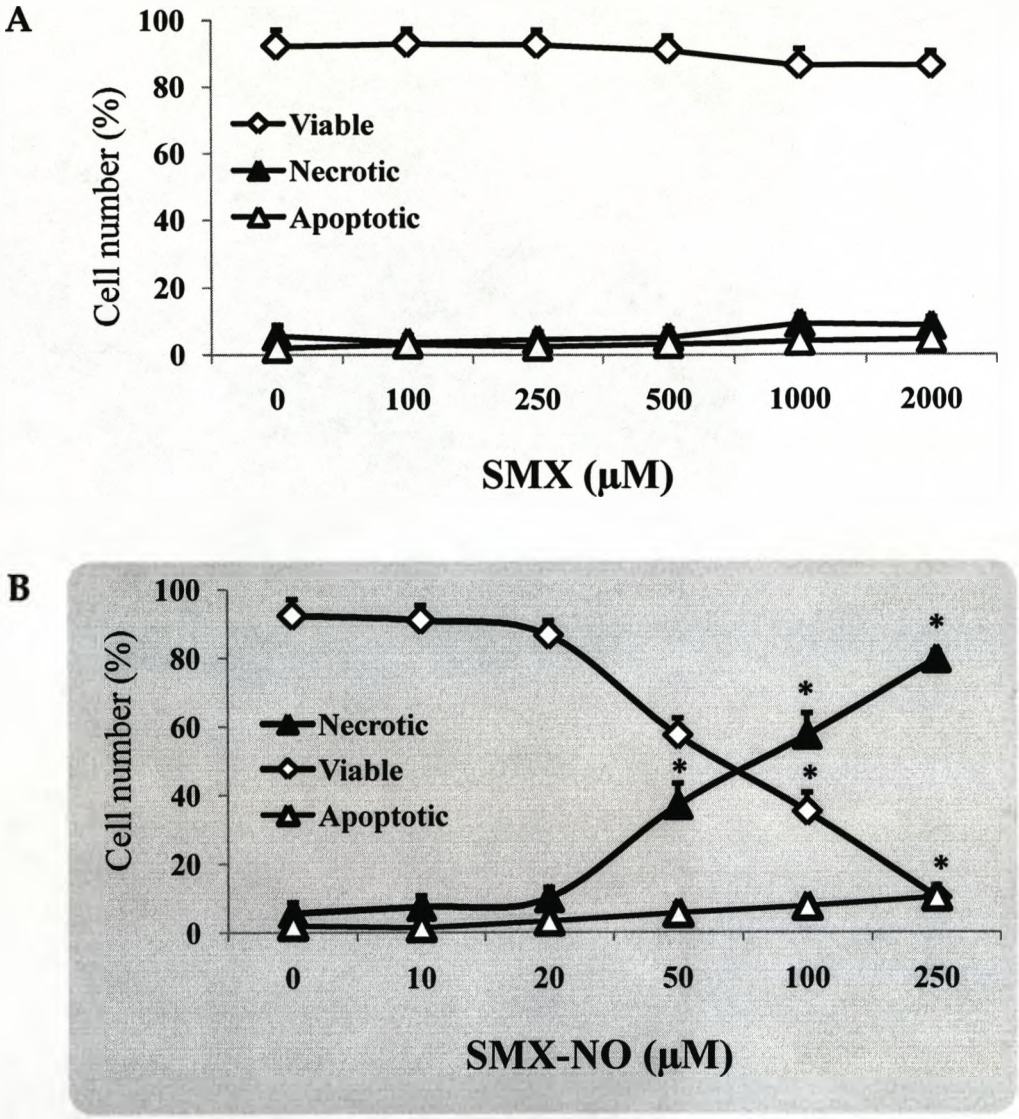

Figure 3.9: Viability of mouse splenocytes exposed to sulfamethoxazole or its nitroso metabolites. Cells were incubated with varying concentrations of SMX and its metabolite, and proportions of cells viable, necrotic and apoptotic was quantified by flow cytometry using Annexin V/PI staining. No cytotoxicity was seen with SMX up to 2000 μM (A), and SMX-NO below 50 μM (B). Data are presented as mean ± SD of 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*significantly different from untreated cells; $P < 0.05$).

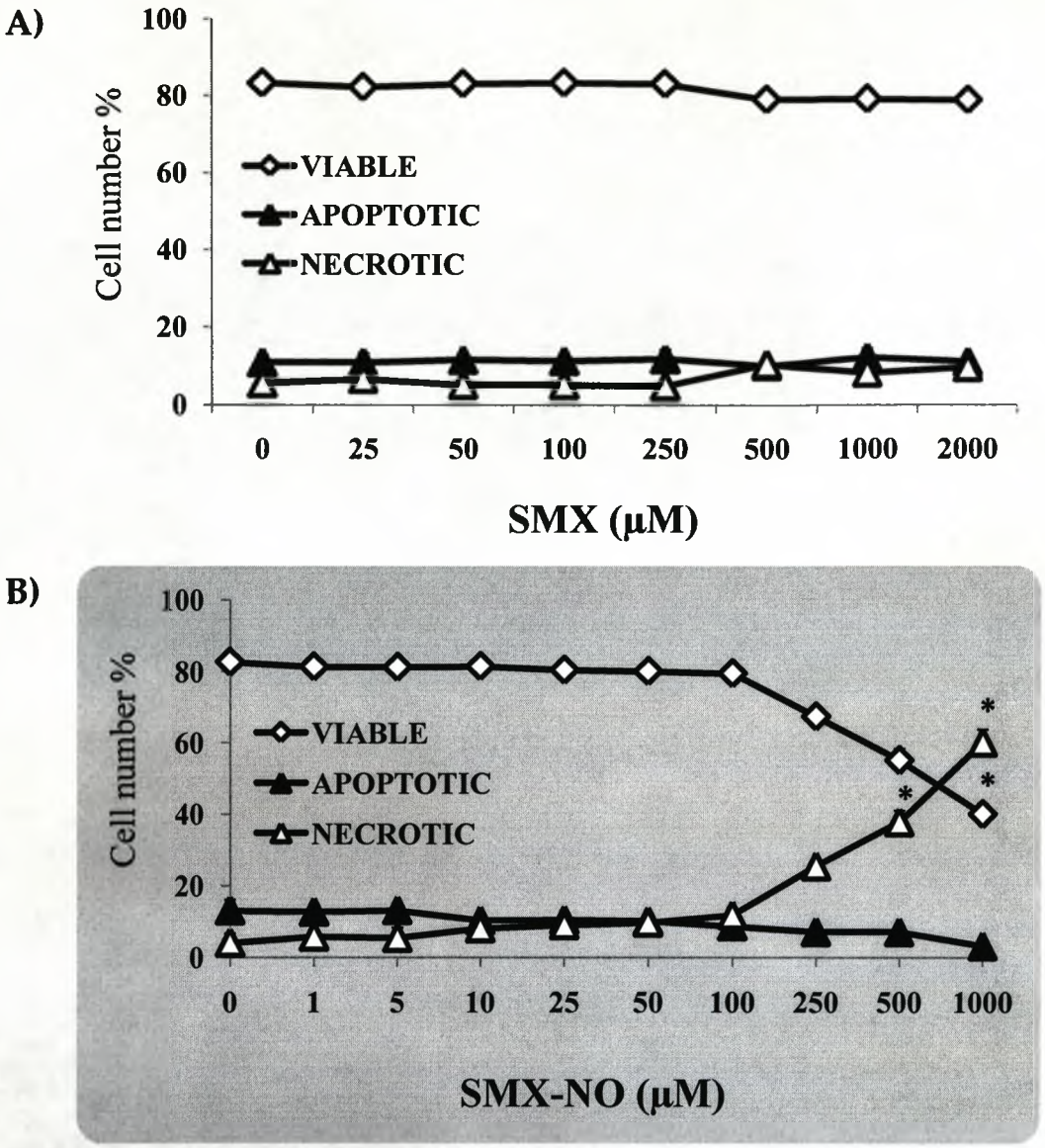

Figure 3.10: Viability of mouse dendritic cells exposed to sulfamethoxazole or its nitroso metabolites. Cells were incubated with varying concentrations of SMX and its metabolite, and proportions of viable, necrotic and apoptotic cells were quantified by flow cytometry using Annexin V/PI staining. No cytotoxicity was seen with SMX up to 2000 μM (A), and SMX-NO below 250 μM (B). Data are presented as mean ± SD of 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*significantly different from untreated cells; $P < 0.05$).

3.3 Discussion

The oxidative metabolite of SMX, SMX-NO is a potent immunogen in animal models providing antigenic signals through covalent modification of specific cysteine residues on protein (Naisbitt et al., 2001; Naisbitt et al., 2002; Farrell et al., 2003; Sanderson et al., 2007). Furthermore, SMX-NO stimulates skin and blood derived T-cells from all SMX-hypersensitive patients to proliferate, secrete cytokines and kill target cells (Schnyder et al., 2000; Farrell et al., 2003; Nassif et al., 2004; Castrejon et al., 2010).

Despite this, the role of metabolism in allergic drug reactions remains ill-defined, because (1) the direct addition of SMX-NO to cell culture systems generates an artificial cell surface adduct that is unlikely to be observed *in vivo* where SMX-NO is generated by intracellular enzymes and (2) cell culture systems that relate metabolite formation to immune function do not exist. Data presented in this chapter demonstrate that the intracellular drug protein adducts are generated as a consequence of metabolism in viable mouse APCs.

Metabolic drug activation has long been thought to play an important role in the pathogenesis of drug hypersensitivity, through the formation of reactive intermediates that bind to protein and activate immune cells (Park et al., 1998; Roychowdhury et al., 2005; Cheng et al., 2008; Lavergne et al., 2008b). This theory originates largely from the observation that drugs associated with a high incidence of hypersensitivity are metabolised to reactive intermediates in liver. However, these metabolites are unlikely to escape hepatic detoxification

mechanisms, diffuse around the body and bind to skin or immune cells. In this respect, we have embarked upon a series of experiments to address the hypothesis "APCs, which play an important role in the skin's defence against invading pathogens, metabolise drugs in sufficient quantities to form protein adducts that stimulate both dendritic cells and T-cells".

The immune response may arise directly through drug-protein adducts in viable cells or indirectly through inducing necrotic cell death and subsequent release of endogenous antigen and possibly "danger signals" (Matzinger, 1998; Park et al., 1998; Gallucci et al., 1999; Shi et al., 2003; Shi et al., 2006).

Previous studies attempting to identify the chemical nature of SMX-protein adducts revealed that SMX-NO conjugates with GSH in a non enzymatic reaction in *in vitro* to generate an unstable semimercaptal conjugate. This conjugate can isomerise to a stable sulfinamide in the presence of equimolar levels of GSH and SMX-NO at pH 7.4 or cleaved to SMX-HA at physiological GSH levels (Cribb et al., 1991; Naisbitt et al., 1996). Recently, Cheng et al. 2008 have detected SMX-NO adducts on albumin and IgG in an *in vivo* animal model (Cheng et al., 2008). In an *in vitro* study using mass spectrometry, Callan et al. 2009 demonstrated the selective binding of SMX-NO to the oxidised form of cysteine residues in GSH and model proteins HSA, and glutathione S-transferase π (Callan et al., 2009) to yield *N* hydroxysulfinamide and sulfonamide adducts (Callan et al., 2009; Lavergne et al., 2009). Moreover, enhanced intracellular SMX adduct formation under danger conditions (e.g. bacterial endotoxins LPS, flu viral proteins,

inflammatory molecules as PGE2, oxidants as H_2O_2, and hyperthermia) (Lavergne et al., 2009) may be correlated with enhanced cysteine residue oxidation under pathological conditions (Carballal et al., 2003; Saurin et al., 2004).

ELISA and confocal microscopy were used to detect and visualise the formation of immunogenic conjugates formed from the reaction of SMX-NO with cellular proteins. Data presented herein show the presence of intracellular protein adducts in viable mouse splenocytes and bone-marrow-derived dendritic cells incubated with SMX (figures 3.1-3.3). Importantly, adduct formation was time- and dose-dependent and not detectable immediately after SMX exposure (figure 3.4 and 3.5). Recent study performed on human APC (e.g. PBMCs, EBV-modified B lymphocytes, monocyte-derived dendritic cells) showed similar results of time- and concentration-dependent adduct formation using ELISA and confocal microscopy (Lavergne et al., 2009).

SMX is proposed to be metabolised by many enzymes, including *N*-acetyltransferase in the process of detoxification (Cribb et al., 1993), CYPs enzymes as CYP2C9 and CYP2C8 (Cribb et al., 1995; Sanderson et al., 2007), myeloperoxidase (Cribb et al., 1990; Roychowdhury et al., 2007b; Sanderson et al., 2007), cyclooxygenases (Goebel et al., 1999) and flavin-containing monooxygenases in normal human epidermal keratinocytes (Vyas et al., 2006a).

It has been previously reported that human monocyte-derived dendritic cells from 10 donors expressed consistently high levels of myeloperoxidase, but only low

levels of CYPs, leading to propose that a peroxidase is responsible for SMX metabolism in dendritic cells (Sanderson et al., 2007). More recently, it has been shown that pathogenic conditions, which potentially activate peroxidase enzymes, enhance the formation of SMX-derived protein adducts in immune cells (Lavergne et al., 2009). To substantiate a causal relationship between peroxidase-mediated SMX metabolism and T-cell immunogenicity, enzyme inhibitor studies were performed with 1-ABT, a non-selective suicide inhibitor of a wide range of metabolic enzymes (Ortiz de Montellano et al., 1984), and meth, an inhibitor of peroxidases and FMO (Nace et al., 1997). Both inhibitors blocked SMX-derived adduct formation in mouse splenocytes and mouse dendritic cells confirming the enzymatic dependency of the adduct formation (figure 3.6, 3.7). On the other hand, these inhibitors of metabolising enzymes had no inhibitory effect on the oxidative metabolite-derived adduct formation (figure 3.6, 3.7).

Drug-protein adducts were detected on viable mouse spenocytes and dendritic cells incubated with SMX-NO (figure 3.1 and 3.2). Increasing the concentration of SMX-NO (above 50 μM for splenocytes and above 250 μM for dendritic cells) was associated with depletion of intracellular glutathione and increased necrotic cell death (figures 3.8-3.10). SMX-derived adducts were detected in dendritic cells; however, glutathione depletion and necrotic cell death was not seen with SMX at any of the studied concentrations. The effect of adduct formation, glutathione depletion, and cell death on dendritic cell and T-cell activation is explained in detail in later chapters.

Previous studies exploring the importance of drug metabolism in the pathogenesis of hypersensitivity reactions have focussed on metabolism in liver (Cribb et al., 1995; Trepanier and Miller, 2000) and target organs such as the skin (Roychowdhury et al., 2005; Bhaiya et al., 2006), and produced largely negative results. This chapter shows that mouse APCs metabolise SMX and generate metabolism-derived protein adducts.

Chapter 4

Activation of Dendritic Cells by Sulfamethoxazole and Nitroso Sulfamethoxazole

Contents

4.1 Introduction

T cell clonal expansion and differentiation, and hence induction of an effective immune response to an antigen requires different signals: signal 1 (T-cell receptors), signal 2 (co-stimulatory receptors) and signal 3 (adjuvant environmental signals or cytokine-dependent signals e.g. IL-12 and IFN-α/β acting on CD8 T cells and IL-1 on CD4 T cells). Signals 1 and 2 are essential to initiate proliferation of naive T cells, whereas signal 3 is important for the productive response to an antigen through determining the danger/non danger conditions and supporting memory formation (i.e. signal 3 determines the outcome of signals 1 and 2; either tolerance or strong immune response) (Curtsinger and Mescher, 2010).

Dendritic cells are professional APCs that exist in three states; mature (activated), immature, and semi-immature (Lanzavecchia and Sallusto, 2001; Lutz and Schuler, 2002). They perform an important role in determining the equilibrium between immune tolerance and immune reactivity through the provision of receptor ligand interactions (co-stimulatory signalling) and cytokine secretion (Novak and Bieber, 2008). Dendritic cell activation is triggered through several mechanisms including PAMPs (Janeway Jr and Medzhitov, 2002; Kapsenberg, 2003), DAMPs (Gallucci et al., 1999; Kono and Rock, 2008), inflammatory cytokine (TNF-α, IL-1β or PGE2), or direct interaction of CD40L with the CD40 receptor found on dendritic cells (direct activation).

The potential of drugs to activate dendritic cells and supply co-stimulatory signals to T-cell has not been extensively investigated. Drugs including abacavir, amoxicillin and sulfamethoxazole have been shown to partially stimulate human dendritic cell co-stimulatory signalling (Rodriguez-Pena et al., 2006; Martin et al., 2007; Sanderson et al., 2007), which potentially drives pathogenic immune responses in hypersensitive patients. However, the mechanism of dendritic cell activation and the source of co-stimulatory signalling in response to drugs are still not clear. Drugs such as imiquimod (imidazoquinolene antiviral compound) and lenalidomide (thalidomide analogue) have been shown to stimulate dendritic cells directly (Hemmi et al., 2002; LeBlanc et al., 2004). Drugs may also stimulate dendritic cells by inducing oxidative stress and/or irreversible modification of critical stress response protein, as has been shown with chemical sensitisers (e.g. DNCB) (Arrighi et al., 2001; Aiba et al., 2003). Finally, it is possible that dendritic cells receive maturation signal from non drug-related sources (e.g. bacterial or viral infection) (Pirmohamed and Park, 2001; Roth et al., 2003). In support of this argument, patients with HIV or cystic fibrosis have an increased risk of developing drug hypersensitivity.

Dendritic cells express multiple drug metabolising enzymes (Sieben et al., 1999; Tafazoli et al., 2005; Sanderson et al., 2007; Siest et al., 2008; Lavergne et al., 2009) that can generate drug-derived protein adducts in increased amounts when exposed to "danger" signals (Lavergne et al., 2009). SMX-mediated dendritic cell signalling has been shown to be dependent on intracellular metabolism and protein-adduct formation (Sanderson et al., 2007). Despite this, the relationship

between drug-specific dendritic cell signalling and cytotoxicity remains controversial.

Therefore, in this study we investigated the direct and indirect effects of SMX and SMX-NO on the maturation status of mouse bone marrow-derived dendritic cells. Initially, SMX and SMX-NO were incubated with dendritic cells directly. In subsequent experiments, toxic and non-toxic concentrations of SMX-NO were incubated with autologous splenocytes and the modified cells were cultured with dendritic cells following repeated washing steps to remove non-covalently associated drug.

4.2 Results

4.2.1 Mouse bone marrow-derived dendritic cells, generated with high yields and purity, express increased levels of cell surface markers when cultured with lipopolysaccharide

Mouse bone marrow cells from female Balb/c mice, were cultured with murine GM-CSF and subsequently analysed for the presence of dendritic cells after ten days of culture. During the culture period, the cells altered morphology from rounded monocytes to a dendritic cell-like structure (figure 4.1). On day six, both small rounded cells and larger dendritic-like cells with an irregular shape were observed. Eight days after the culture was established, cells with branched projections (dendrites) had appeared. On day 10, two types of cells were visible; cells had an irregular shape with dendritic protrusions and larger cells with a more regular shape, possibly representing immature stages of dendritic cells. Ten days

after initiation and following LPS stimulation, the cultures were dominated (70-80%) by cells with numerous cytoplasmic protrusions (veils), a basic characteristic of mature dendritic cells in suspension.

Typically, a total of 0.8–1.2 x 10^8 primary bone-marrow cells from one Balb/c strain mouse were isolated. After ten days of culture with GM-CSF, the number of cells was 1.5–2.5 x 10^8 with overall cell viability of more than 90% using annexin/propidium iodide staining methodology.

Flow cytometric analysis of the dendritic cells harvested on day ten showed high expression of CD11c (a specific dendritic cell marker in mice; 85-95 %, figure 4.2). Cultures showed very low level of contamination with B cells, granulocytes, macrophages and neutrophils as shown by staining with CD19, Gr-1, CD204, and neutrophil specific antibody (figure 4.2).

In addition, histograms displayed a distinctly biphasic expression of MHC class II and CD86, consistent with the presence of both immature and relatively mature dendritic cells (figure 4.3). Upon addition of LPS to the culture one day before cell harvesting, the expression of MHC class II, CD86, and CD40 was strongly up-regulated on the cell surface (figure 4.3) as well as a significant increase in cytokine secretion (e.g. TNF-α, IL-1β, IL-6 and IL-12) (figure 4.6). Concomitantly, the level of mature cells in cultures derived from Balb/c reached 70-80% following LPS stimulation with no effect on the expression of CD11c, which remained positive (figure 4.3).

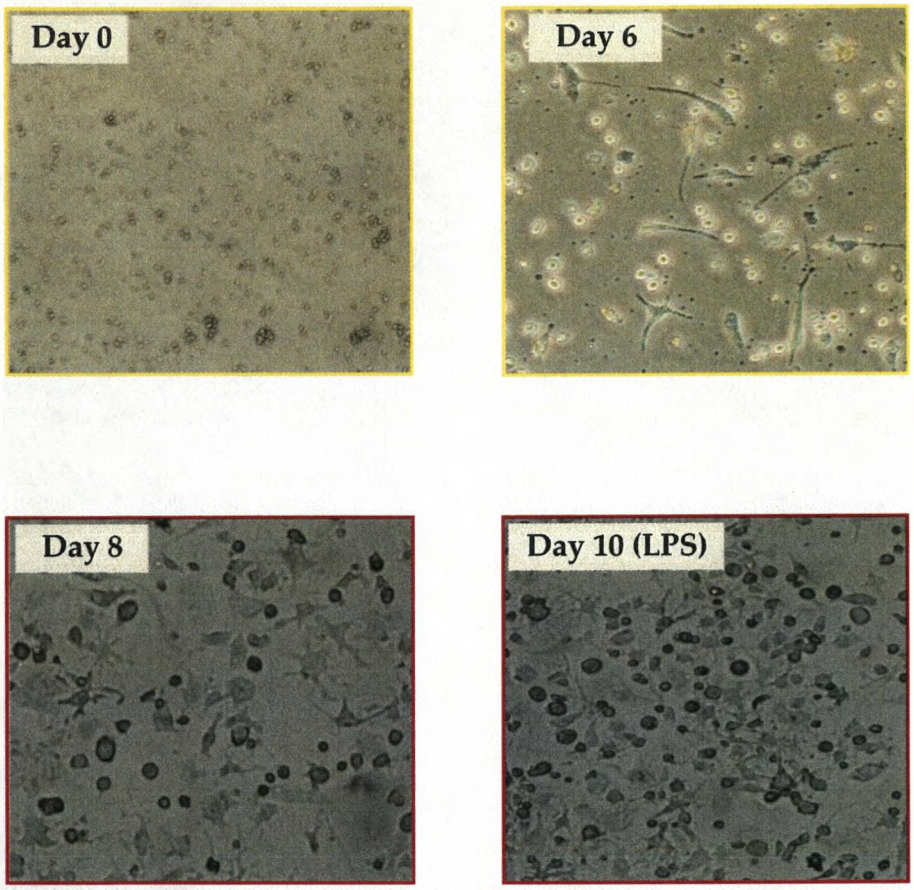

Figure 4.1: Morphology of bone marrow cells from Balb/c mouse cultured for 10 days in the presence of GM-CSF. LPS was added on day 9 to induce maturation of dendritic cells. Cells changed morphology from rounded monocytes to irregular structures with dendritic protrusions.

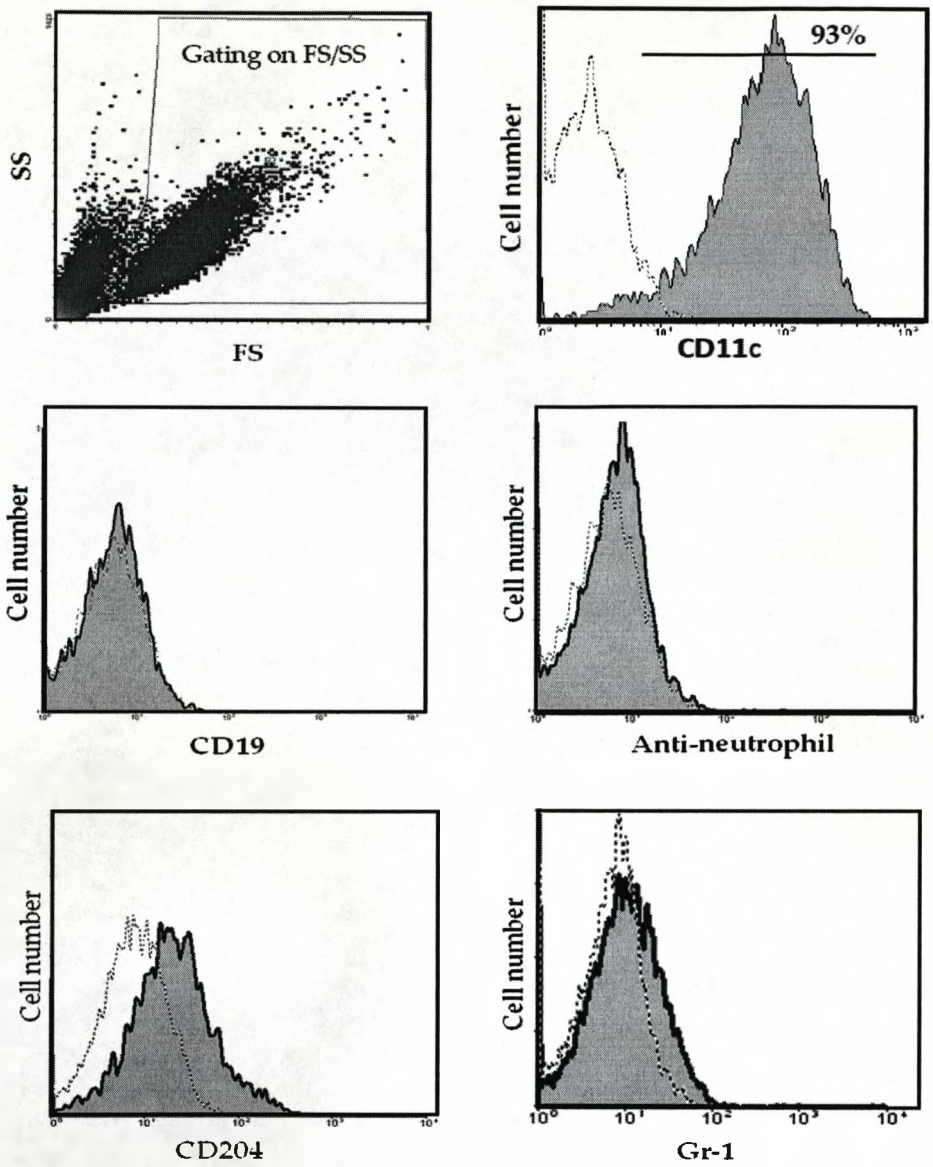

Figure 4.2: Phenotypic analysis of bone marrow-derived dendritic cells generated from Balb/c mice. The cells were stained with CD11c, CD19, Gr-1, CD204, and an anti-neutrophil antibody and subjected to flow cytometric analysis. Dot plot shows the gate employed. Cultures show low level of contamination with B cells, granulocytes, macrophages and neutrophils. Histograms illustrate the antibody stained cells as shaded profiles and the background fluorescence as dotted line. Results are representative of at least 3 separate experiments.

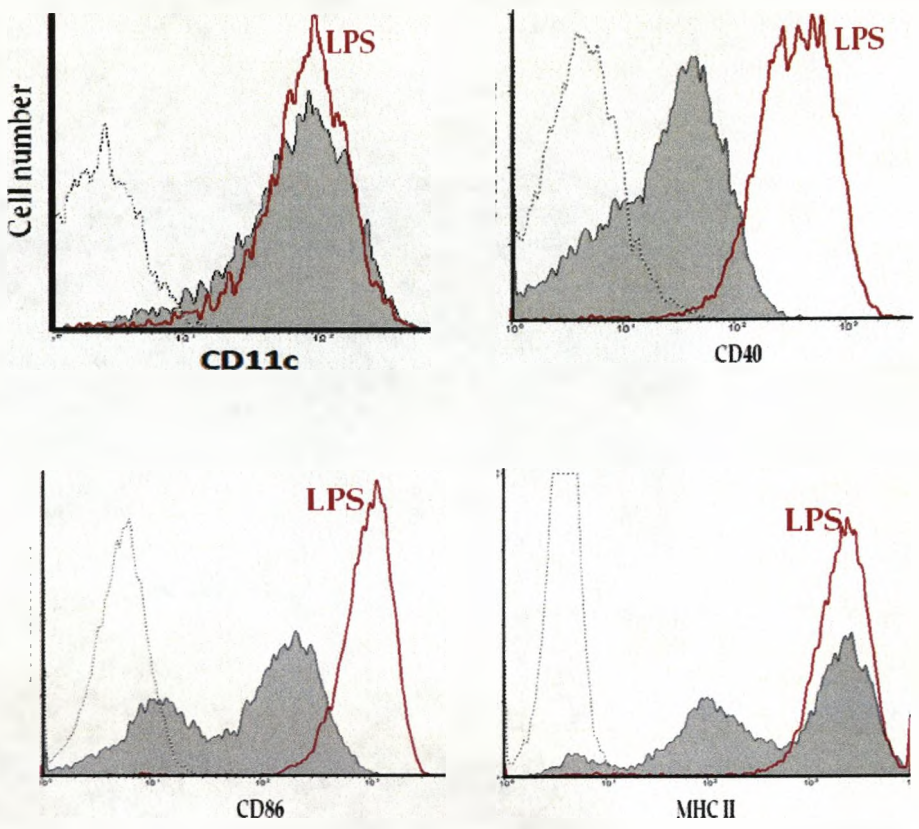

Figure 4.3: Expression of CD40, CD86 and MHC class II on the generated CD11c$^+$ mouse dendritic cells. Cells were stained and subjected to flow cytometric analysis. Up-regulation of CD40, CD86 and MHC class II expression was shown in response to LPS. Histograms illustrate antibody stained untreated cells as shaded profiles, the background fluorescence as dotted line, and antibody-stained LPS-treated cells as solid line (red). Results are representative of at least 3 separate experiments.

4.2.2 Sulfamethoxazole, intracellular sulfamethoxazole metabolites and nitroso sulfamethoxazole do not activate mouse dendritic cells directly

To study whether SMX (50–2000 μM) or the metabolite SMX-NO (0.1–500 μM) activate mouse dendritic cells directly, both compounds were cultured with dendritic cells for 16 h prior to analysing co-stimulatory receptor expression (using flow cytometry) and cytokine secretion (using Luminex methods). Dendritic cells stimulated with LPS displayed an increase in cell surface markers (CD40, CD86 and MHC class II) as described previously. However, SMX and SMX-NO exposure, at toxic and non-toxic concentrations, failed to stimulate dendritic cell maturation (figures 4.4 and 4.5).

Luminex analysis showed very low or undetectable amounts of cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-15, IFN-γ, and TNF-α) in supernatants of untreated mouse dendritic cells (data not shown). However, LPS-activation was associated with a significant increase in cytokine secretion (TNF-α, IL-1β, IL-6 and IL-12, figure 4.6). Treatment of mouse dendritic cells with a range of concentrations of SMX and SMX-NO was associated (at certain concentrations) with a small, but statistically significant decrease in secretion of IL-10 (expressed as pg/ml; 0: 23±11; SMX: 6±10; SMX-NO: 1±2, $P<0.05$, figure 4.6), an increase of IL-6 (pg/ml; 0: 40±70; SMX: 133±156; SMX-NO: 218± 54, $P<0.05$, figure 4.6) and finally, an increase in IFN-γ (pg/ml; 0: 7±6; SMX: 26±3; SMX-NO: 34±7, $P<0.05$, figure 4.6). The percentage of $CD11c^+$ cells was similarly high in all conditions (figure 4.1).

Similar results were obtained with the metabolite SMX hydroxylamine which forms SMX-NO in culture.

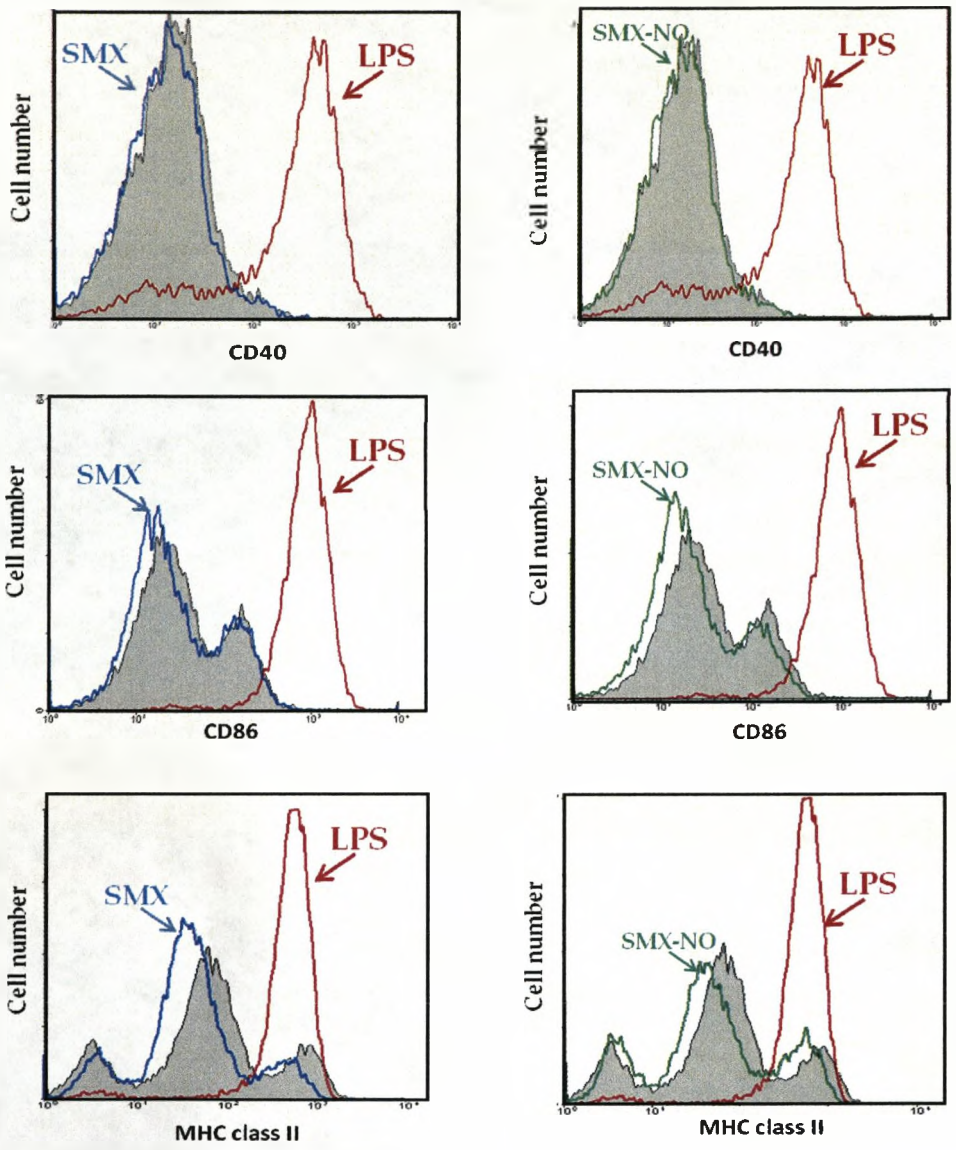

Figure 4.4: Sulfamethoxazole/metabolite does not increase mouse dendritic cell co-stimulatory expression. Dendritic cells were incubated with a range of concentrations of SMX (left panel) or SMX-NO (right panel) for 16 h prior to flow cytometric analysis of CD40, CD86 and MHC class II expression. Histograms illustrate antibody stained untreated cells as shaded profiles and antibody stained treated cells (drug or LPS) as solid line. Results are representative of at least 3 separate experiments.

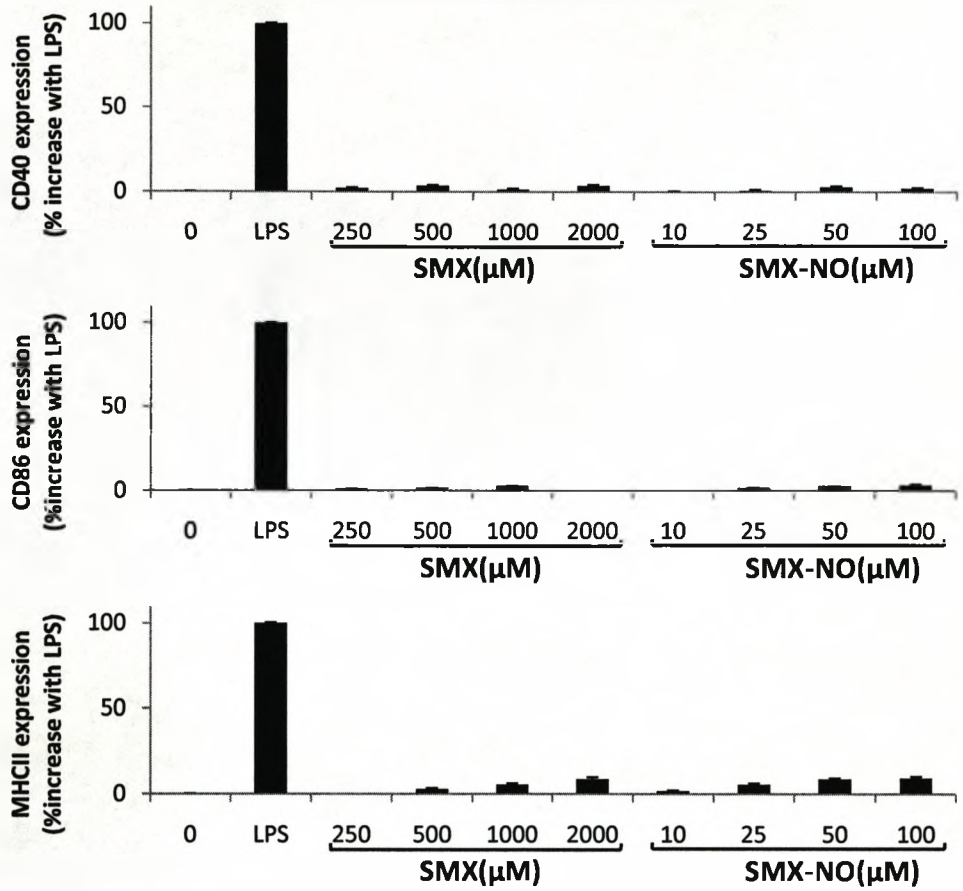

Figure 4.5: Sulfamethoxazole/metabolite does not increase mouse dendritic cell co-stimulatory expression. SMX at concentrations of 250-2000 μM or SMX-NO at concentrations of 10-100 μM failed to upregulate expression of CD40, CD86 and MHC class II on the surface of mouse dendritic cells, whereas LPS increased the expression of these co-stimulatory molecules. Results are expressed as % of increase with LPS of at least 3 separate experiments.

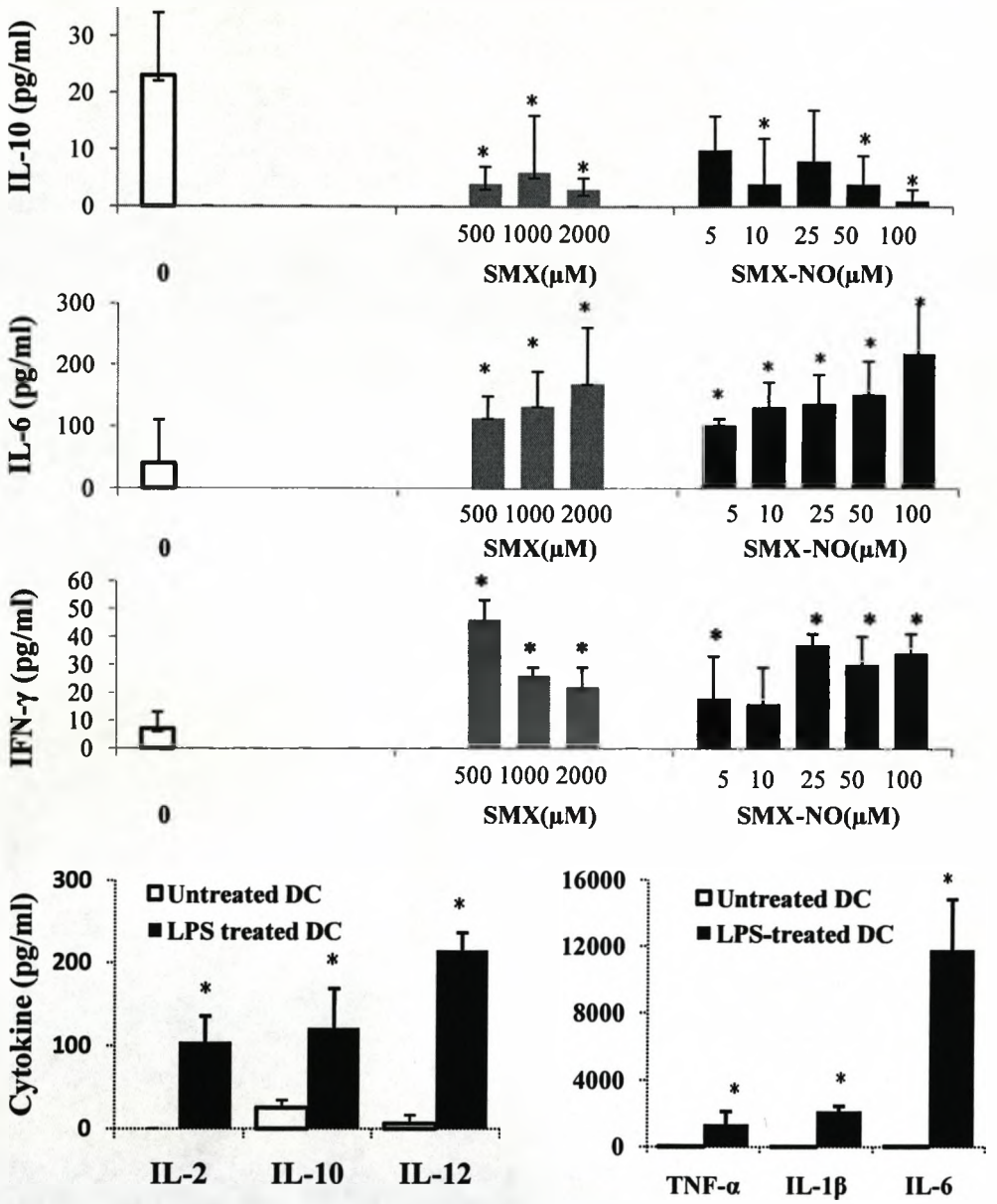

Figure 4.6: Sulfamethoxazole/metabolite slightly changes certain mouse dendritic cells cytokine secretion at certain doses. Cells were incubated with SMX, SMX-NO, or LPS (1 μg/ml) for 16 h. Cytokines were measured in supernatants using multiplex analysis. Data are presented as mean ± SD of at least 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*significantly different from untreated cells; $P < 0.05$).

4.2.3 Activation of dendritic cells by necrotic by-standers cells modified with nitroso sulfamethoxazole

To explore the relationship between SMX metabolite-mediated cell death and dendritic cell activation, SMX and SMX-NO treated splenocytes were cultured with dendritic cells prior to analysis of dendritic cell activation.

As described in chapter 3, SMX-NO binds rapidly to splenocytes (within 1 h) and necrotic cell death was detectable at concentrations of 50 μM and above. Adduct formation in splenocytes cultured with SMX was both time and concentration-dependent. However, adduct formation with SMX was not associated with an increase in either apoptotic or necrotic cell death, indicating that the number of modified proteins did not exceed a threshold that must be surmounted prior to the development of proportionate increases in cell death.

On day 10 of culture, dendritic cells were co-incubated with untreated splenocytes and splenocytes that had been rendered necrotic by exposure to SMX-NO (50-500 μM) for 16h. The addition of untreated mouse splenocytes to dendritic cells did not increase dendritic cell co-stimulatory receptor expression or cytokine secretion. In contrast, SMX-NO treated splenocytes stimulated dendritic cell maturation in a concentration-dependent manner. Dendritic cells showed a significant increase in expression of CD40 (% of increase with LPS; SMX-NO 100μM: 71±2; 250μM: 68±3; 500μM: 78±3, $P< 0.05$, figure 4.7), CD86 (% of increase with LPS; SMX-NO 100μM: 44±4; 250μM: 48±3; 500μM: 53±3, $P< 0.05$, figure 4.8), and MHC class II (% of increase with LPS; SMX-NO 100μM:

56±5; 250μM: 54±4; 500μM: 61±4, P< 0.05, figure 4.9). An analysis using Pearson's correlation coefficient indicated a statistically significant linear relationship between % of necrotic cell death and the upregulation of CD40, D86, and MHC class II (r^2 = 0.83, 0.91, and 0.96 respectively; P<0.05).

In addition to upregulation of MHC class II and co-stimulatory receptor expression, investigation of the cytokine secretion profiles of mouse dendritic cells revealed significant increase in the secretion of cytokines (TNF-α, IFN-γ, IL-1β, and IL-6). Dendritic cells co-incubated with SMX-NO modified necrotic splenocytes displayed a significant increase in secretion of TNF-α (expressed as pg/ml; 0: 4±11; SMX-NO 50μM: 612±81; 100μM: 668±13; 250μM: 649±20, P<0.05, figure 4.10A), an increase in IFN-γ (expressed as pg/ml; 0: 9±7; SMX-NO 50μM: 47±22; 100μM: 64±5; 250μM: 80±19, P<0.05, figure 4.10B), an increase in IL-6 (expressed as pg/ml; 0: 44±38; SMX-NO 50μM: 6079±207; 100μM: 8176±11; 250μM: 10436±161, P<0.05, figure 4.10C), and finally, an increase of IL-1β (expressed as pg/ml; 0: 2±8; SMX-NO 50μM: 25±6; 100μM: 26±3; 250μM: 27±7, P<0.05, figure 4.10D). The percentage of $CD11c^+$ cells was similarly high in all conditions. The number of activated dendritic cells was associated with increasing quantities of SMX-NO modified necrotic splenocytes. Dendritic cell activation was not detectable with splenocytes treated with non-toxic concentrations of SMX-NO (less than 50 μM). Furthermore, SMX (100-2000 μM) treatment of splenocytes was not associated with cell death or a concomitant increase in dendritic cell maturation.

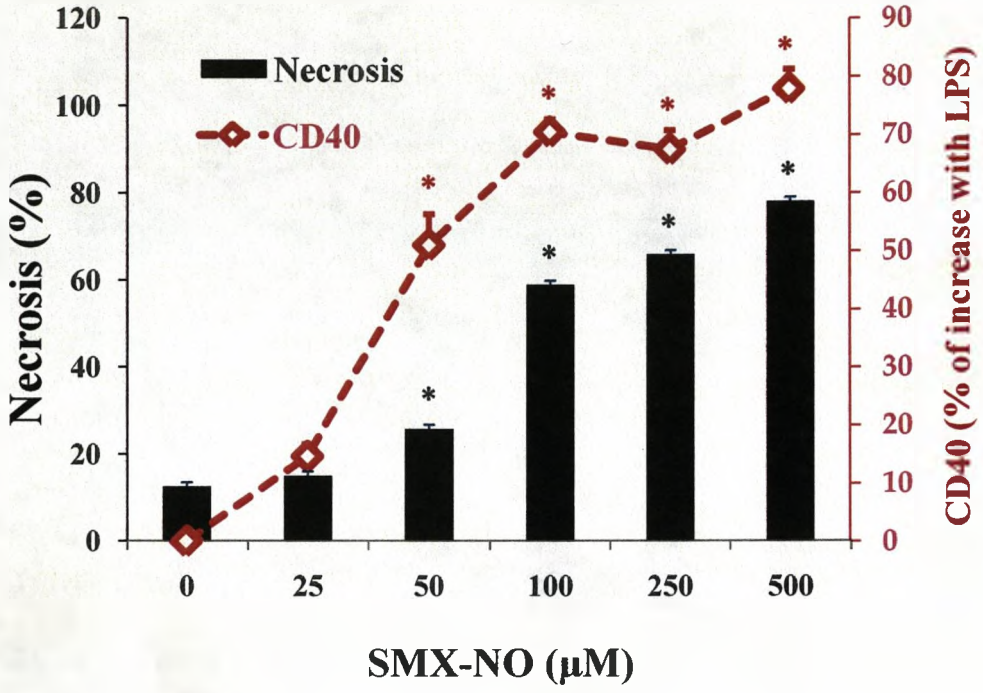

Figure 4.7: Sulfamethoxazole metabolite-mediated necrotic cell death provides a potent signal for dendritic cell co-stimulatory signalling. SMX-NO treated mouse splenocytes were incubated in a co-culture system with dendritic cells prior to flow cytometric analysis of CD40 expression on dendritic cells. Up-regulation of CD40 expression is presented as the percentage of the increase with LPS over control. Graphical representations compare SMX-NO mediated necrotic cell death with up-regulation of CD40 expression. Data are presented as mean ± SD of 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*significantly different from untreated cells; $P < 0.05$).

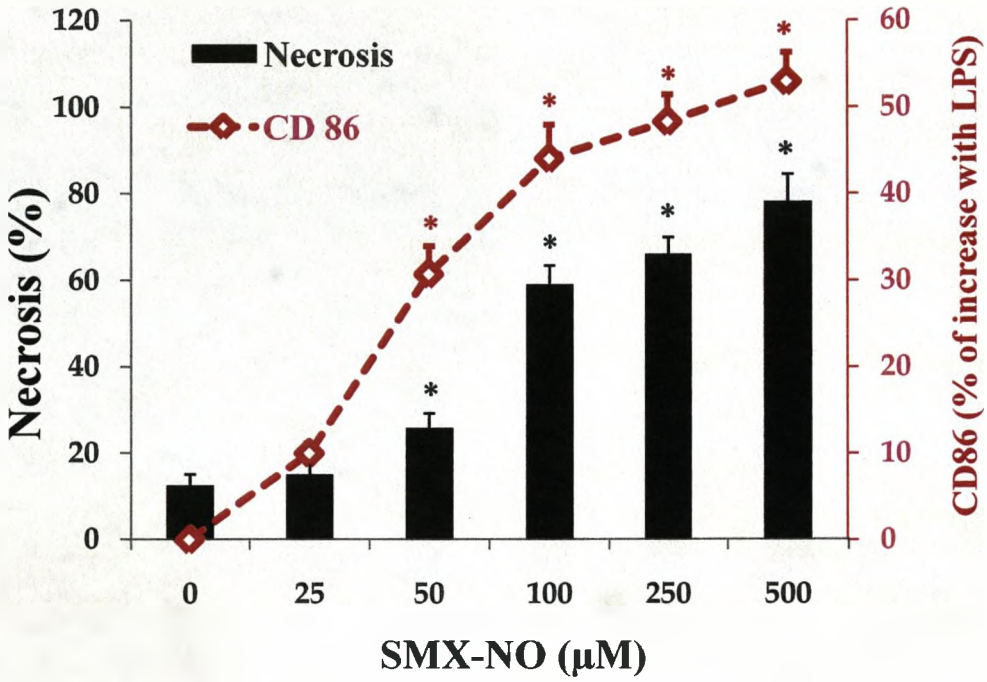

Figure 4.8: Sulfamethoxazole metabolite-mediated necrotic cell death provides a potent signal for dendritic cell co-stimulatory signalling. SMX-NO treated mouse splenocytes were incubated in a co-culture system with dendritic cells prior to flow cytometric analysis of CD86 expression on dendritic cells. Up-regulation of CD86 expression is presented as the percentage of the increase with LPS over control. Graphical representations compare SMX-NO mediated necrotic cell death with up-regulation of CD86 expression. Data are presented as mean ± SD of 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*significantly different from untreated cells; $P < 0.05$).

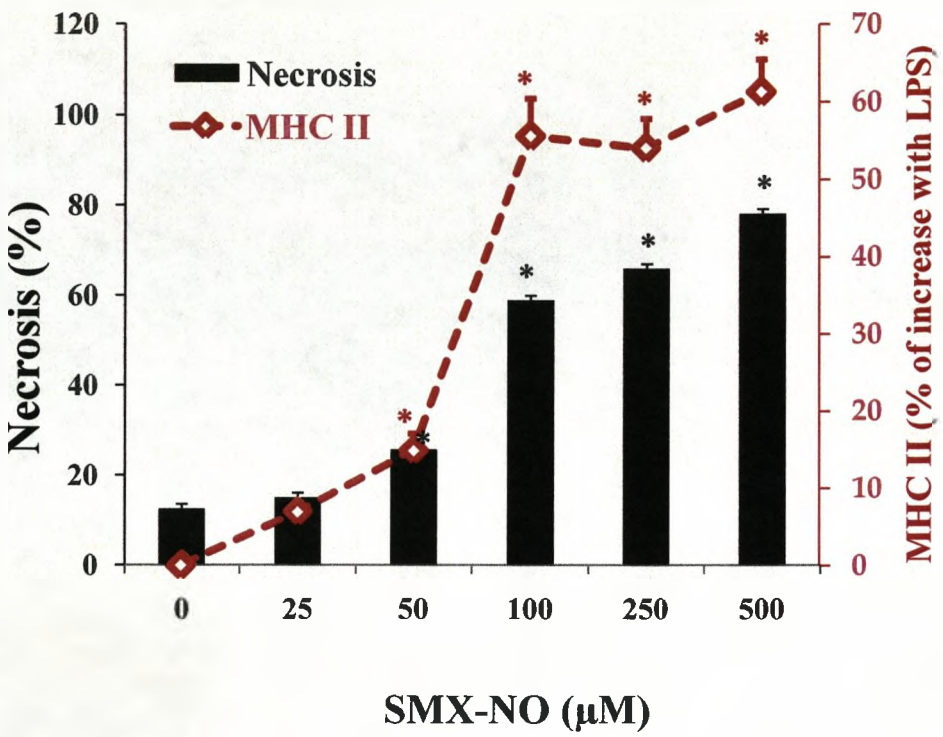

Figure 4.9: Sulfamethoxazole metabolite-mediated necrotic cell death provides a potent signal for dendritic cell co-stimulatory signalling. SMX-NO treated mouse splenocytes were incubated in a co-culture system with dendritic cells prior to flow cytometric analysis of MHC class II expression on dendritic cells. Up-regulation of MHC class II expression is presented as the percentage of the increase with LPS over control. Graphical representations compare SMX-NO mediated necrotic cell death with up-regulation of MHC class II expression. Data are presented as mean ± SD of 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*significantly different from untreated cells; $P < 0.05$).

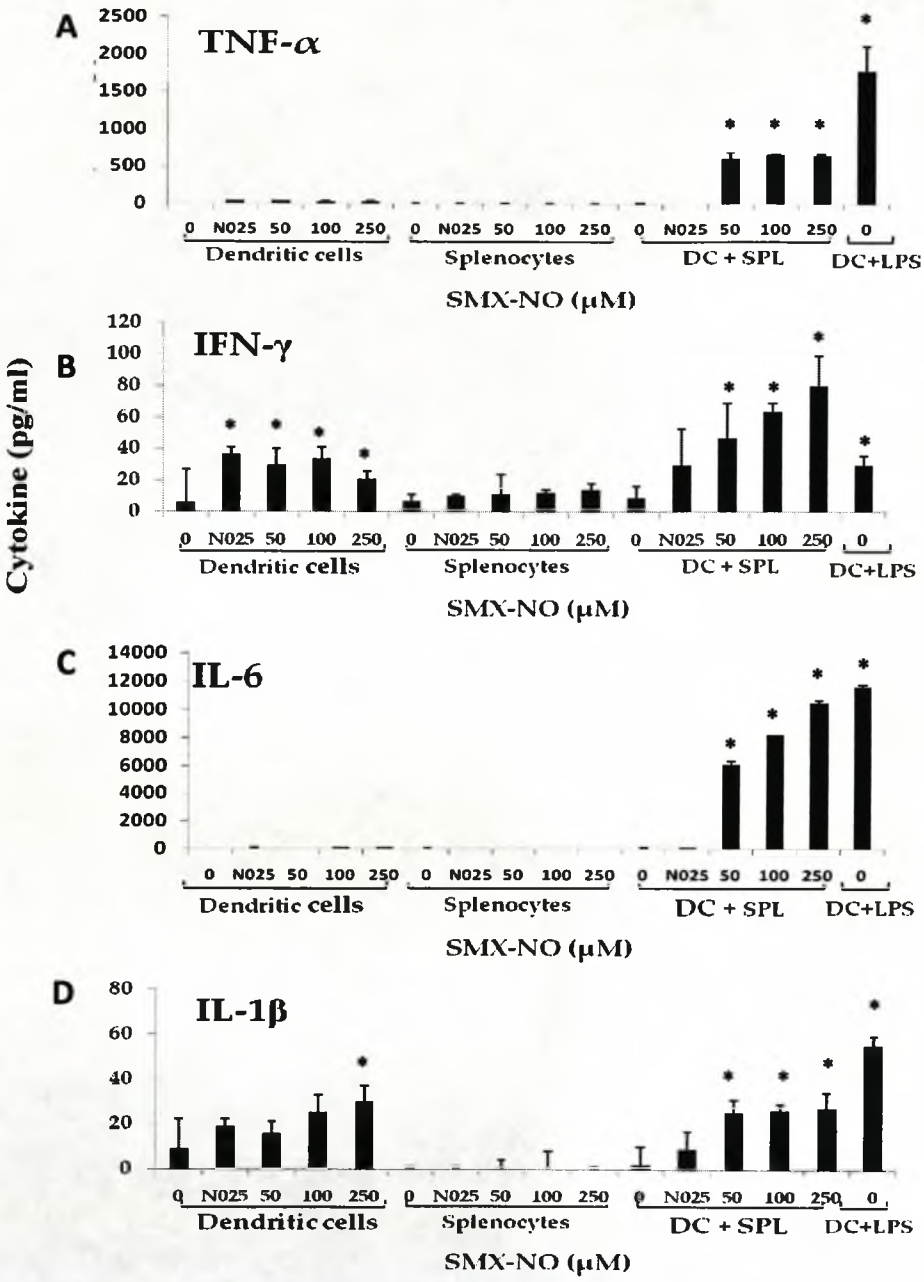

Figure 4.10: SMX metabolite-mediated necrotic cell death provides a signal for dendritic cell cytokine secretion. Cytokine secretion was measured using multiplex analysis following co-culture of dendritic cells with SMX-NO modified splenocytes. Data are presented as mean ± SD of 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*significantly different from untreated cells; $P < 0.05$).

4.3 Discussion

Dendritic cell maturation signals may arise directly through drug-protein adduction of viable cells or indirectly through inducing necrotic cell death and subsequent release of endogenous antigen and possibly "danger signals" (Matzinger, 1998; Park et al., 1998; Gallucci et al., 1999; Shi et al., 2003; Shi et al., 2006).

The antigen presenting capacity of dendritic cells is enhanced in response to TLR signalling (e.g., LPS, CpG oligodeoxynucleotides) (De Smedt et al., 1996; Akiba et al., 2004) and molecules released from necrotic (e.g., uric acid high-mobility group box 1 protein) (Shi et al., 2003; Bianchi and Manfredi, 2007) or oxidatively stressed cells (Becker et al., 2003). Dendritic cells respond to these signals by secreting polarising cytokines, increased expression of co-stimulatory receptors and enhanced expression of MHC class II.

Previously, it has been shown that exposure of human monocyte-derived dendritic cells to non-toxic concentrations of SMX selectively increased CD40 expression. Furthermore, CD40 expression was 5-fold higher on drug-treated cells from a hypersensitive patient compared with volunteers (Sanderson et al., 2007). Our experiments using mouse bone-marrow-derived dendritic cells did not reproduce these findings, indicating that drug-specific CD40 signalling might be restricted to human cells (figures 4.4 and 4.6).

In the previous chapter (chapter 3), Drug-protein adducts were detected on viable mouse dendritic cells incubated with SMX-NO (50 – 100 μM). Increasing the concentration of SMX-NO (250 – 1000 μM) was associated with depletion of intracellular glutathione and increased necrotic cell death. SMX-derived adducts were detected in dendritic cells; however, glutathione depletion and necrotic cell death were not seen with SMX.

Although no increase in co-stimulatory receptor expression, MHC class II or cytokine secretion were detected following the direct incubation of SMX or SMX-NO with dendritic cells, in a co-culture system consisting of SMX/SMX-NO treated splenocytes and dendritic cells, there was a strong association between SMX metabolite-mediated necrotic cell death and dendritic cell activation. Splenocytes incubated with SMX-NO (at concentrations of 50 μM and above) stimulated an increase in dendritic cell CD40, CD86 and MHC class II expression and cytokine secretion (figures 4.7-4.10).

Previously, it has been reported that SMX metabolite-derived adduct formation on splenocytes must surpass a critical threshold to induce necrosis (Naisbitt et al., 2002). The cells that became intensely haptenated were the same as those that underwent necrotic cell death. Presumably, the quantity of adduct formation in SMX-treated splenocytes did not reach the threshold to induce necrosis and therefore dendritic cell activation. It is however possible that this may occur in patients where environmental factors (bacterial endotoxins, flu viral proteins and cytokines) alter the oxidation state of cysteine residues on protein and increase

SMX-derived protein adduct formation (Lavergne et al., 2009). These observations may explain the increased *in vitro* drug-specific cell death observed in hypersensitive patients (Spielberg et al., 1981; Shear et al., 1986; Rieder et al., 1989; Pirmohamed et al., 1991), as patients administered SMX are carriers of such factors.

Collectively these data indicate that human patients receive at least three sources of maturation signals for dendritic cells. The first signal is detectable at non-toxic drug concentrations; it is restricted to human cells and results in partially matured dendritic cells. The second signal occurs at higher drug concentrations. By-stander cell death and presumably the release of endogenous molecules results in a potent maturation signal and maturation of dendritic cells. Finally, infections which are present in most patients exposed to SMX, may provide signals to activate dendritic cells directly and provide a positive feedback system to enhance SMX metabolism in immune cells.

Chapter 5

Activation of T-Cells by Sulfamethoxazole and Nitroso Sulfamethoxazole

Contents

5.1 Introduction

Animal models of drug immunogenicity represent an important tool in the armament of drug safety scientists to understand the reaction pathogenesis and to screen the potential of drugs to induce hypersensitivity reactions (mechanistic and predictive value). For example, as in our study, having an experimental approach to relate the drug metabolism to immune activation is important to define the role of metabolism in the development of a primary immune response. Antigen-specific T-cells from hypersensitive patients cannot be used to define antigenic determinants that prime T-cells since it is impossible to differentiate between cross-reactive drug antigens.

However, the relevance of animal experimentation in the field of drug hypersensitivity is often questioned as animals develop drug-specific immunity, but the immune response is not translated into an allergic reaction (Naisbitt et al., 2001; Naisbitt et al., 2002; Farrell et al., 2003). T-cells from allergic patients are stimulated with SMX-NO though a direct interaction with MHC/peptide complexes and via a hapten mechanism involving processing (Schnyder et al., 2000; Burkhart et al., 2001; Nassif et al., 2004a; Castrejon et al., 2010). Unlike animal models, patient T-cells can be stimulated with the parent drug itself. This latter phenomenon is thought to involve a cross-linkage between MHC and the T-cell receptor through reversible non-covalent interactions.

Using the antibacterial agent sulfamethoxazole as a model drug, it has been shown that the oxidative metabolite of SMX (SMX-NO) is a potent antigen and immunogen in animal models. First, incubations of rat APCs with SMX-NO, led

to cell surface protein haptenation and stimulation of SMX-NO-specific T cells (Naisbitt et al., 2002); second, immunisation of mice, rats and rabbits with SMX-NO led to stimulation of a specific T-cell proliferative responses and production of anti-SMX antibodies (Gill et al., 1997; Naisbitt et al., 2001; Farrell et al., 2003; Cheng et al., 2008); third, administration of a mixture of SMX and Freund's adjuvant, led to SMX-NO specific delayed-type hypersensitivity in mice in the form of foot pad edema (Choquet-Kastylevsky et al., 2001). Stimulation of a SMX metabolite-specific response in rodents exposed to the parent drug in adjuvant is particularly important. The data suggests that SMX is metabolised *in vivo* in sufficient quantities to generate an antigenic signal; however the quantities formed are insufficient to provide the additional co-stimulatory signals needed to induce a primary immune response. Data presented in previous chapters indicate that SMX is metabolised *in vitro* in APCs and that derived metabolites bind in a time and concentration dependent fashion to cellular protein. However, critical experiments showing the relationship between compound distribution and metabolism in APCs and the development of T-cell responses have not been described.

In this study we have utilised the drug SMX to develop an animal experimental system to explore the relationship between drug metabolism in dendritic cells and T-cell activation. We have found that protein adducts derived as a consequence of SMX metabolism in APCs represent functional antigens for SMX-NO sensitised mice. Furthermore, SMX-derived protein adduts are able to sensitise naive recipient mice.

5.2 Results

5.2.1 Sulfamethoxazole metabolism in antigen presenting cells generates a functional antigen for T-cells

Experiments were designed to analyse SMX/metabolite-specific T-cell responses in Balb/c strain mice through administration of SMX-NO (5 mg/kg; n = 4) via i.p. injection four times weekly for 2 weeks according to a previously described protocol (Naisbitt et al., 2001). On completion of this dosing regimen, animals were sacrificed and the spleen was removed using aseptic technique for analysis of splenocyte proliferation against the soluble drug/metabolite (SMX 100–2000 μM, SMX-NO 10–100 μM), or drug/metabolite-pulsed APCs (syngeneic naive irradiated splenocytes or dendritic cells).

SMX did not provoke proliferation of splenocytes from mice administered SMX-NO at any of the concentrations studied (cell proliferation expressed as cpm, 0: 268±123; SMX 100μM: 422±198; 250μM: 441±107; 500μM: 322±283; 1000μM: 204±131; 2000μM: 126±97, figure 5.1A). However, splenocytes from SMX-NO sensitised mice displayed a concentration-dependent proliferative response on in vitro stimulation with SMX-NO (10–50 μM) with a maximal response at 25-50 μM. Concentrations of SMX-NO above 50 μM inhibited the proliferative response (cell proliferation expressed as cpm, 0: 655±420; SMX-NO 10μM: 5214±1906; 25μM: 9318±887; 50μM: 7874±5110, $P<0.05$, figure 5.1B).

Additionally, splenocytes from sensitised mice were stimulated when cultured with SMX-NO modified (10–100 μM), irradiated splenocytes from naive

syngeneic mice. The proliferative response was dose-dependent with a maximal response at 100 μM (cell proliferation expressed as cpm, 0: 281±17; SMX-NO 10μM: 1312±1196; 25μM: 3737±3855; 50μM: 5005±767; 100μM: 6538±4609, $P<0.05$, figure 5.2B). Interestingly, unlike the soluble SMX, splenocytes from SMX-NO sensitised mice were stimulated to proliferate when incubated with irradiated splenocytes pulsed with SMX (0.1–2 mM) for 16 h. The response was concentration-dependent with a maximal proliferation at 0.1 mM (cell proliferation expressed as cpm, 0: 187±74; SMX 250μM: 573±174; 500μM: 959±490; 1000μM: 1196±723; 2000μM: 607±211, $P<0.05$, figure 5.2A).

Using mouse dendritic cells as APCs reproduced similar results. Sensitised splenocytes were stimulated to proliferate when cultured with SMX modified irradiated dendritic cells (cell proliferation expressed as cpm, 0: 1144±122; SMX 1000μM: 13660±1278; 2000μM: 17098±7742, $P<0.05$, figure 5.3A), and SMX-NO modified irradiated dendritic cells from naive syngeneic mice (cell proliferation expressed as cpm, 0: 3870±1261; SMX-NO 10μM: 26101±1435; 25μM: 29853±1811; 50μM: 35540±1817, $P<0.05$, figure 5.3B).

To investigate the relationship between the dynamics of SMX metabolism and the generation of a functional antigen for spleen cells, APCs were pulsed with SMX and SMX-NO at different concentration and time points in the presence or absence of the enzyme inhibitors methimazole (an inhibitor of peroxidases and FMO) and 1-aminobenzotriazole (a non-selective suicide inhibitor), at a

concentration that block CYP2C9 and peroxidase-catalysed SMX metabolism (both 1 mM) (Sanderson et al., 2007; Lavergne et al., 2009).

The proliferative response of splenocytes against SMX-pulsed APCs (naive splenocytes and dendritic cells) was time-dependent. Significant proliferation of sensitised splenocytes against SMX-pulsed APCs was detectable after 6–16 h, the time required for SMX metabolism and protein binding (cell proliferation expressed as cpm, 6h [0: 103±2; SMX: 335±64, $P<0.05$]; 16h [0: 92±29; SMX: 529±129, $P<0.05$], figure 5.4A), with no significant proliferation at earlier time points (cell proliferation expressed as cpm, 0.1h [0: 102±5; SMX: 146±33]; 2h [0: 99±28; SMX: 150±57], figure 5.4A). In contrast, 0.1-2 h pulse with SMX-NO was sufficient for adduct formation (chapter 3) and T-cell proliferation (cell proliferation expressed as cpm, 0.1h [0: 102±5; SMX-NO: 418±28, $P<0.05$]; 2h [0: 99±28; SMX-NO: 611±73, $P<0.05$], figure 5.4A). There was no significant time-dependent increase in cell proliferation against SMX-NO (figure 5.4A). Splenocytes from control mice did not proliferate after *ex-vivo* exposure to SMX, SMX-NO or SMX (metabolite)-pulsed cells (data not shown).

Similar results were reproduced when naive SMX-pulsed dendritic cells were used as APCs (cell proliferation expressed as cpm, 2h [0: 1144±122; SMX: 3764±441; SMX-NO: 7212±2048, $P<0.05$], 16h [0: 1023±92; SMX: 17098±7742, SMX-NO: 8944±4344, $P<0.05$], figure 5.4B),

Methimazole and 1-aminobenzotriazole pre-treatment of splenocytes eliminated the concentration-dependent T-cell response against APCs cells pulsed with SMX for 16 h (cell proliferation expressed as SI, SMX 250μM: 3.3±0.5; 250μM/meth: 0.9±0.4; 250μM/1-ABT: 1.5±0.5; SMX 500μM: 4.3±0.6; 500μM/meth: 0.9±0.6; 500μM/1-ABT: 1.3±0.1, SMX 1000μM: 4.7±0.5; 1000μM/meth: 1.1±0.5; 1000μM/1-ABT: 1.7±0.4, $P<0.05$, figure 5.5A). Antigen-specific proliferative responses to the directly reactive SMX-NO were unchanged by enzyme inhibition (cell proliferation expressed as SI, SMX-NO 10μM: 2.9±0.2; 10μM/meth: 2.3±0.7; 10μM/1-ABT: 2.9±0.3; 20μM: 3.2±0.8; 20μM/meth: 3.4±0.5; 20μM/1-ABT: 3.7±0.5, 50μM: 5.7±2.5; 50μM/meth: 4.9±0.9; 50μM/1-ABT: 4.6±0.9, $P>0.05$, figure 5.5B).

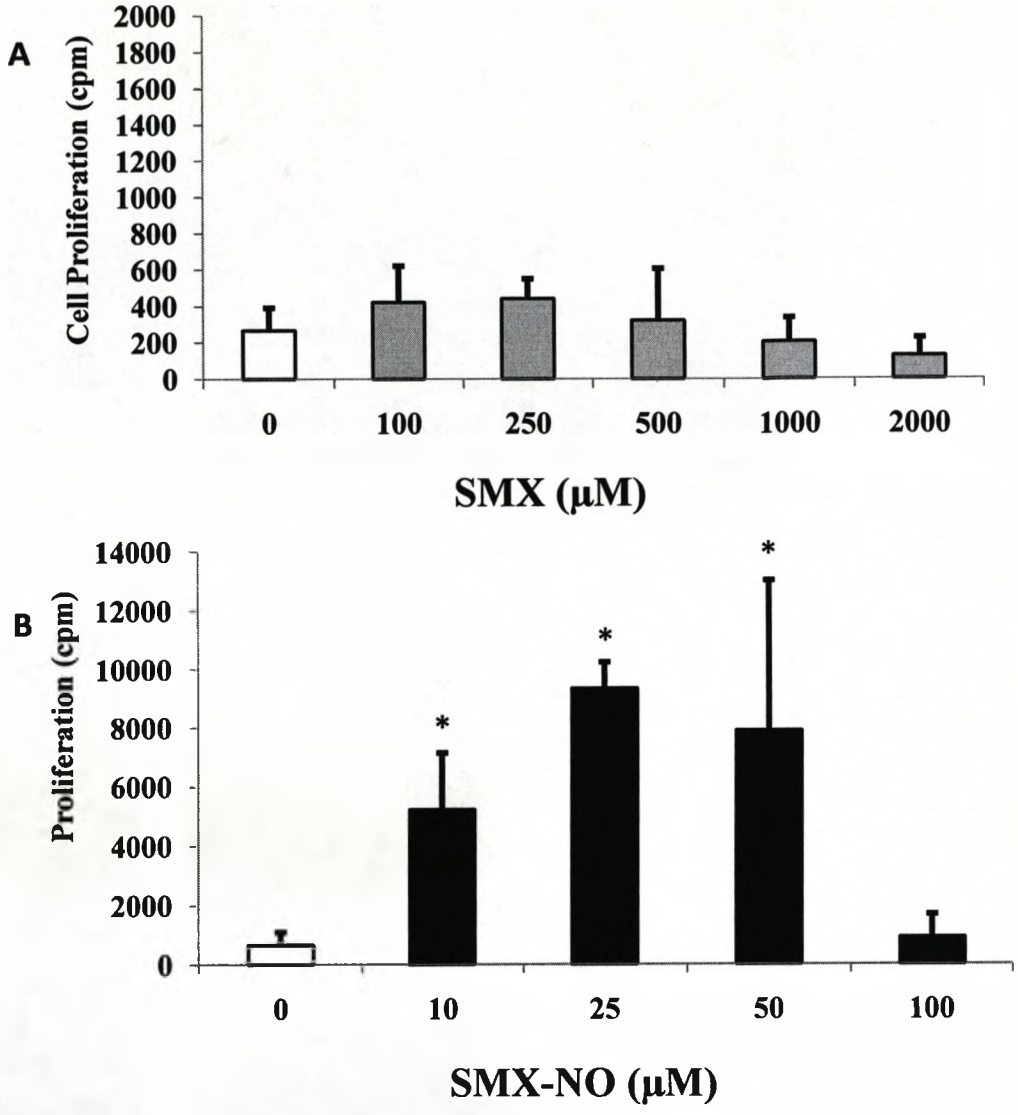

Figure 5.1: Proliferation of splenocytes from SMX-NO sensitised mice. Sensitised cells were proliferated when cultured with soluble SMX-NO (B) but not the parent drug (A). Proliferation was quantified by incorporation of [^{3}H] thymidine. Data presented as mean ± SD from 3 sensitised mice, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitised splenocytes after the addition of drug antigen and solvent alone. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*$P < 0.05$).

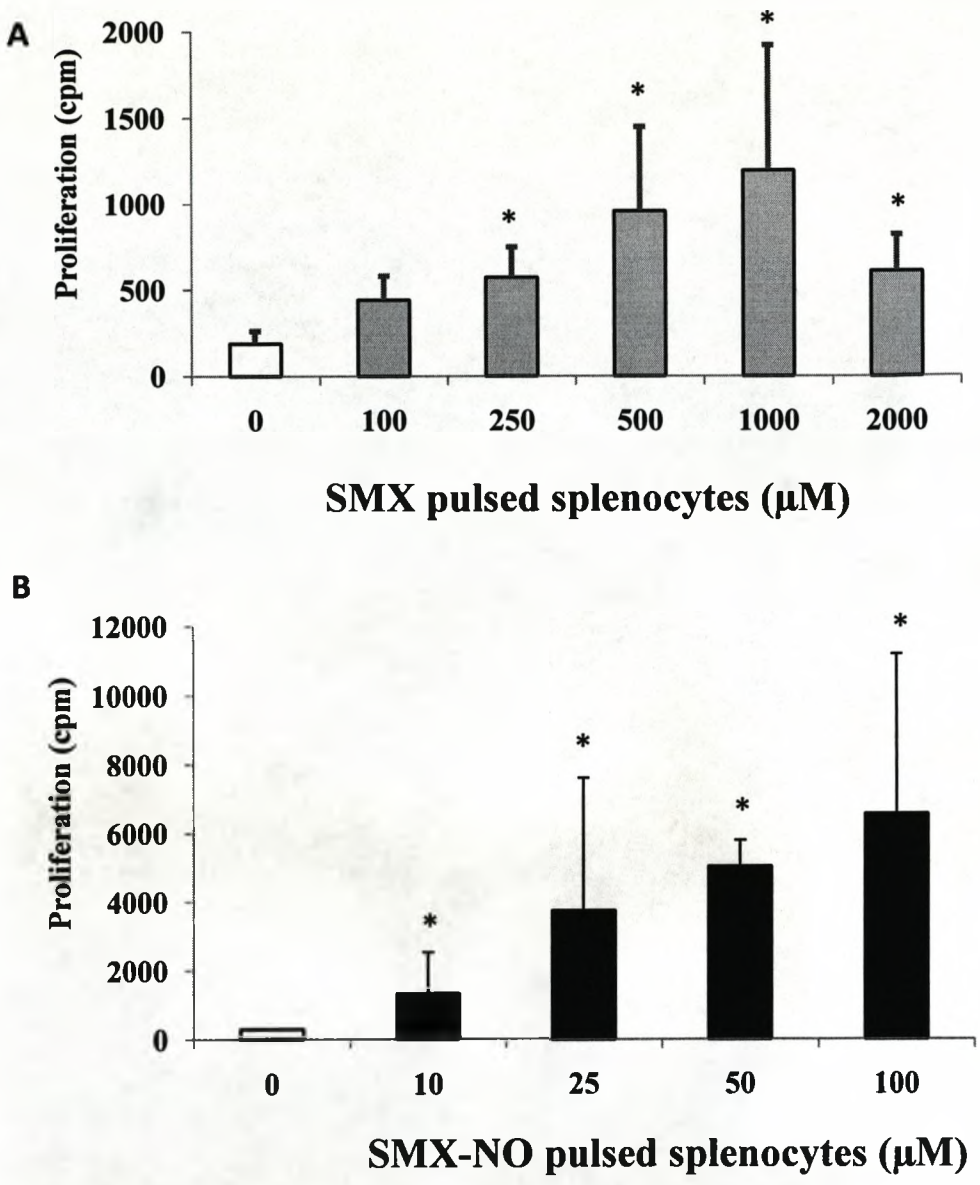

Figure 5.2: Proliferation of splenocytes from SMX-NO sensitised mice. Sensitised cells were proliferated when cultured with syngeneic naive irradiated splenocytes pulsed with SMX (A) or SMX-NO (B). Proliferation was quantified by incorporation of [^{3}H] thymidine. Data presented as mean ± SD from 3 sensitised mice, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitised splenocytes after the addition of drug antigen pulsed cells and solvent pulsed cells. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*$P < 0.05$).

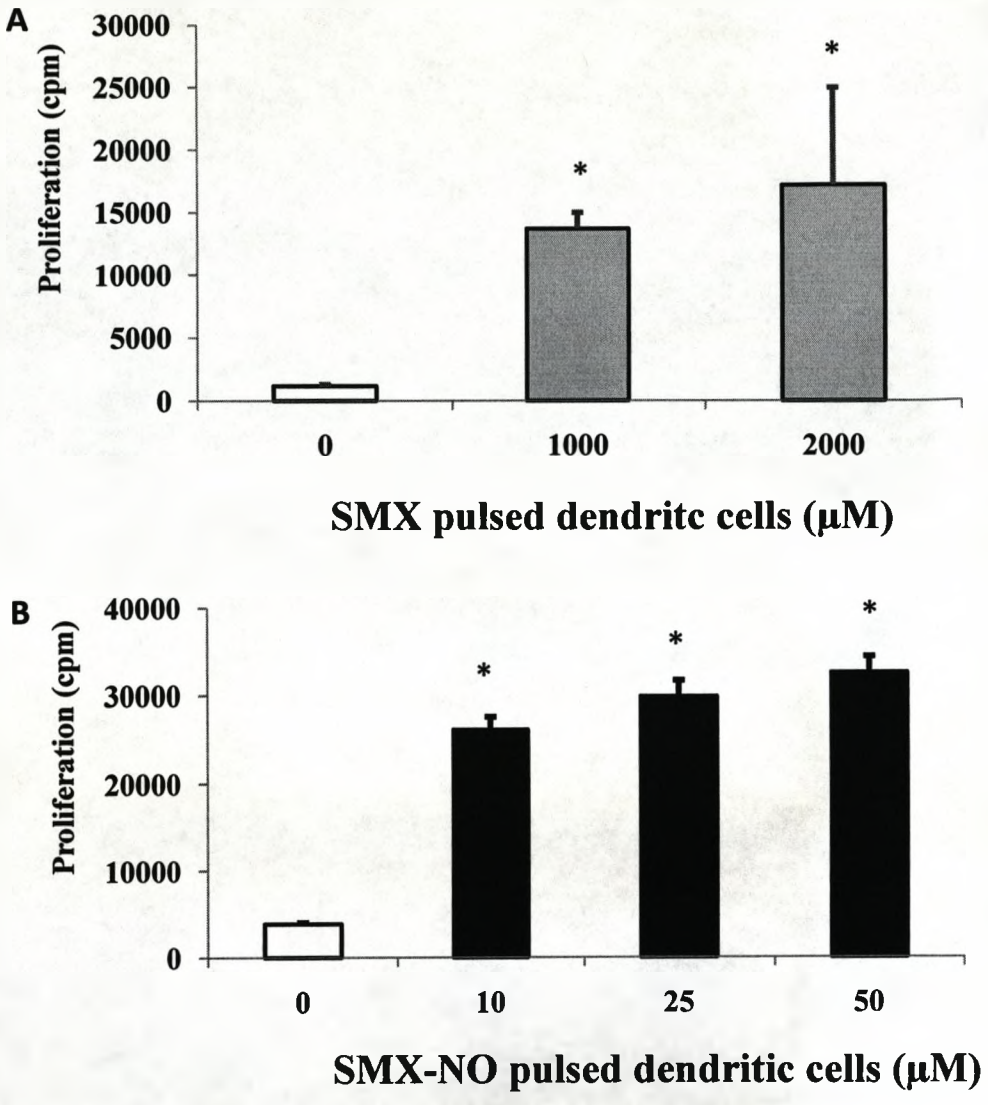

Figure 5.3: Proliferation of splenocytes from SMX-NO sensitised mice. Sensitised cells were proliferated when cultured with syngeneic naive irradiated dendritic cells pulsed with SMX (A) or SMX-NO (B). Proliferation was quantified by incorporation of [^{3}H] thymidine. Data presented as mean ± SD from three sensitised mice, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitised splenocytes after the addition of drug antigen pulsed cells and solvent pulsed cells. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*$P < 0.05$).

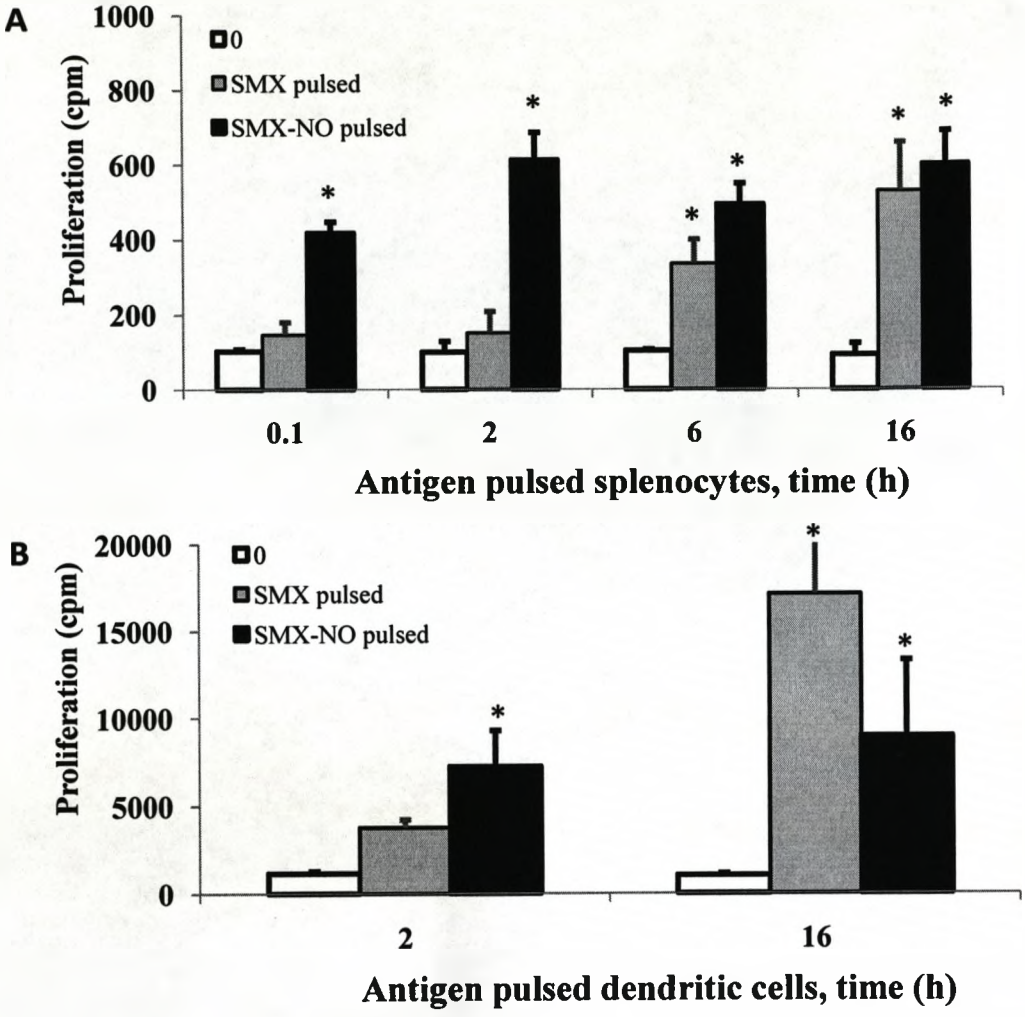

Figure 5.4: Time-dependent proliferative response of splenocytes from SMX-NO sensitised mice. Sensitised cells proliferated when cultured with syngeneic naive irradiated splenocytes pulsed with SMX-NO or SMX (0.1-16 h) (A) or dendritic cells pulsed with SMX-NO or SMX (1 and 16 h) (B). In these pulsing experiments, sensitised splenocytes were not exposed to soluble drug. Proliferation was quantified by incorporation of [^{3}H] thymidine. Data presented as mean ± SD from 3 sensitised mice, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitised splenocytes after the addition of drug antigen pulsed cells and solvent pulsed cells. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*$P < 0.05$).

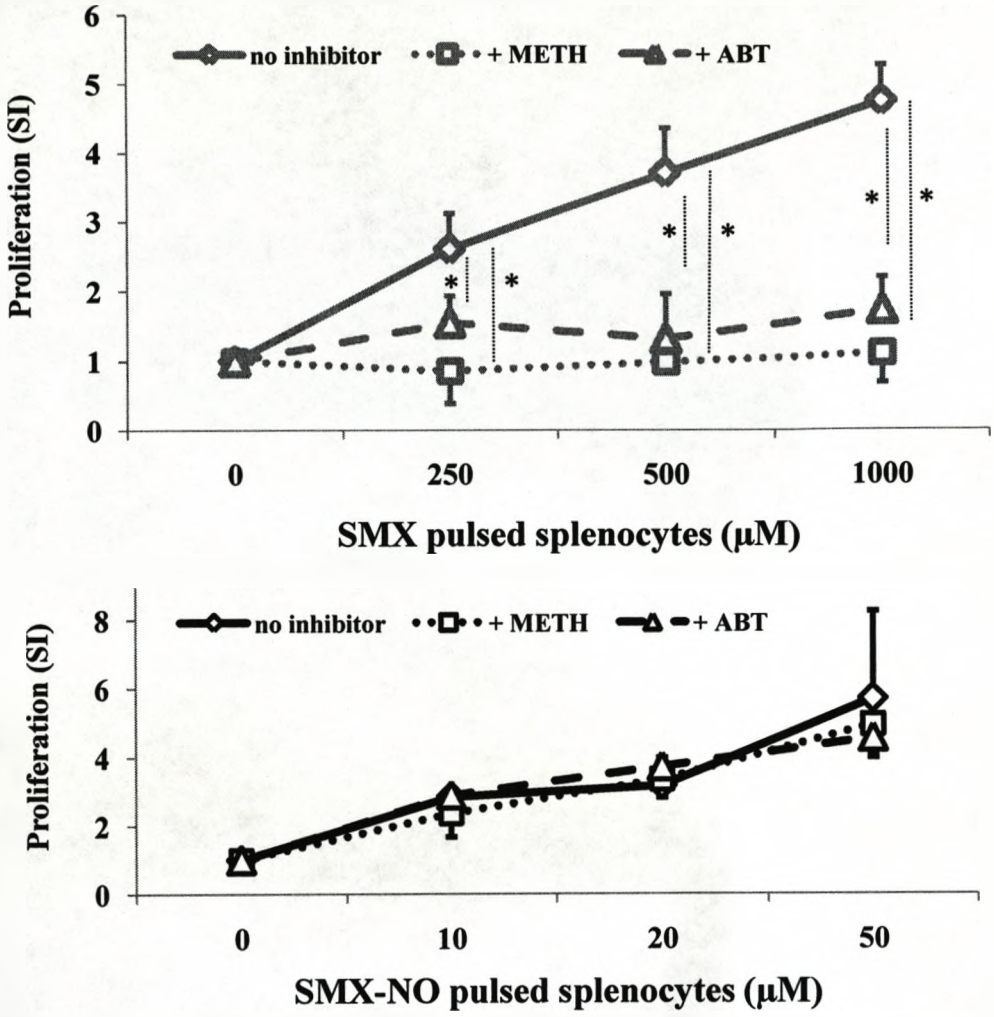

Figure 5.5: Inhibition of the antigen-specific proliferative response. The inhibitors methimazole (meth) and 1-minobenzotriazole (1-ABT) were incubated with cells for 1 h before the addition of drug. 1-ABT and meth inhibited proliferation of sensitised splenocytes cultured with naive irradiated splenocytes pulsed with SMX (16 h) without blocking the proliferative response of SMX-NO modified splenocytes. Proliferation was quantified by incorporation of [^{3}H] thymidine and expressed as SI. Data presented as mean ± SD from 3 sensitised mice, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitised splenocytes after the addition of drug antigen pulsed cells with and without the inhibitors. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*$P < 0.05$).

5.2.2 Adoptive dendritic cell transfer of SMX-derived functional antigen to recipient mice is associated with an immune response against nitroso SMX.

The data presented above show for the first time that SMX metabolism in APCs generates a functional antigen for T-cells. To test whether protein adducts derived from SMX metabolism in APCs can sensitise mice; bone marrow-derived dendritic cells were adoptively transferred to naive recipient mice. For immunisation, dendritic cells were incubated with SMX (2 mM) or SMX-NO (100 μM) in culture medium supplemented with mouse serum to prevent T-cell responses against FBS-derived antigens. Then, cells were washed extensively and administered to mice via i.v. injection in the lateral tail vein. Control mice received unmodified or DMSO-treated dendritic cells. After 21 days, mice were sacrificed and isolated splenocytes were stimulated in vitro with the soluble antigen or drug-pulsed APCs. Proliferation was quantified by incorporation of [3H] thymidine and the proliferative response of sensitised splenocytes after the addition of drug antigen was compared with that with the solvent alone. Cells from control mice did not display any significant immune response to SMX or its nitroso metabolite (figure 5.6), while SMX treatment of dendritic cells was associated with adduct formation (described previously in chapter 3) and a significant immune response (figures 5.7-5.9).

Splenocytes isolated 21 days after the adoptive transfer of mice with SMX-NO - treated dendritic cells were stimulated to proliferate *ex vivo*. The proliferative response was directed against soluble SMX-NO without significant proliferation

in response of the parent drug (cell proliferation expressed as cpm, 0: 344±42; SMX-NO 10μM: 1880±637; 20μM: 1433±229, $P<0.05$, SMX 1000μM: 228±126; 2000μM: 204±64, $P>0.05$, figure 5.7). in addition, the proliferative response of splenocytes isolated from mice sensitised with SMX-treated dendritic cells was directed against the soluble SMX-NO with no displayed proliferation against SMX (cell proliferation expressed as cpm, 0: 377±56; SMX-NO 10μM: 1309±215; 20μM: 1190±24, $P<0.05$, SMX 1000μM: 164±56, 2000μM: 160±31, $P>0.05$, figure 5.8).

Additionally, sensitised splenocytes showed a proliferative response against SMX-derived metabolites following *in vitro* stimulation by antigen-pulsed APCs. The proliferative response of splenocytes from mice sensitised with SMX-NO-treated dendritic cells was directed against both SMX and SMX-NO-pulsed APCs (cell proliferation expressed as cpm, 0: 160±43; SMX-NO 20μM: 545±61; 50μM: 607±93; SMX 1000μM: 452±58, $P<0.05$, figure 5.9A). Similarly, the response of splenocytes from mice sensitised with SMX-treated dendritic cells was directed against the SMX-derived metabolites (cell proliferation expressed as cpm, 0: 283±64; SMX-NO 10μM: 629±291; 20μM: 931±205; 50μM: 999±353; SMX 250μM: 822±95; 500μM: 778±76; 1000μM: 975±46, $P<0.05$, figure 5.9B). Splenocytes from mice immunised with DMSO-treated or unmodified dendritic cells showed no response against SMX and SMX-NO (figure 5.6).

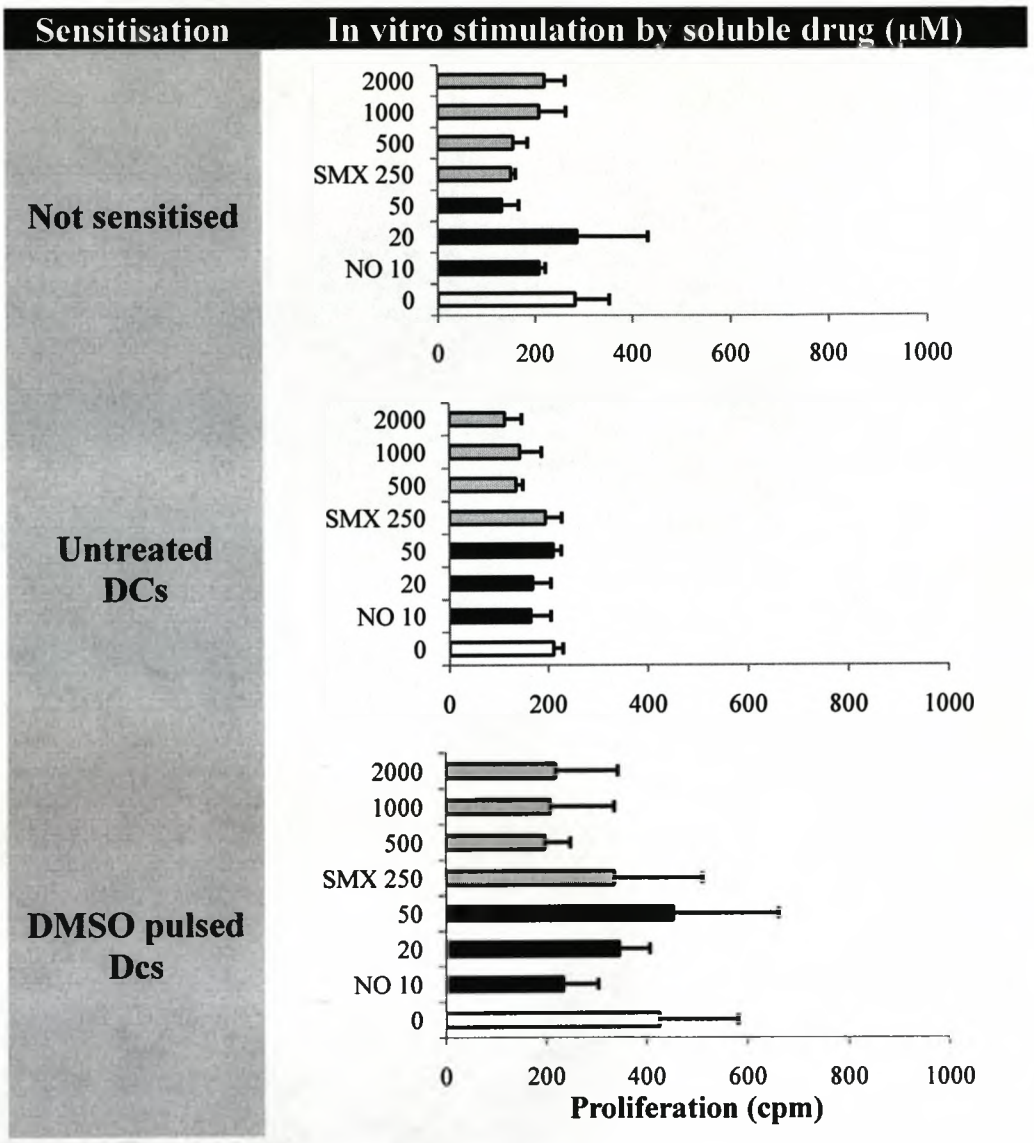

Figure 5.6: Adoptive transfer of dendritic cells. Splenocytes from mice immunised with DMSO-treated or unmodified dendritic cells showed no response against SMX and SMX-NO. For immunisation, dendritic cells were incubated with DMSO or left unmodified, washed extensively, and injected in the lateral tail vein. After 21 days, mice were sacrificed and splenocytes were stimulated *in vitro* with the soluble antigen (as shown) or drug-pulsed APCs (not shown). Proliferation was quantified by incorporation of [^{3}H] thymidine. Data presented as mean ± SD, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitised splenocytes after the addition of drug antigen and solvent alone.

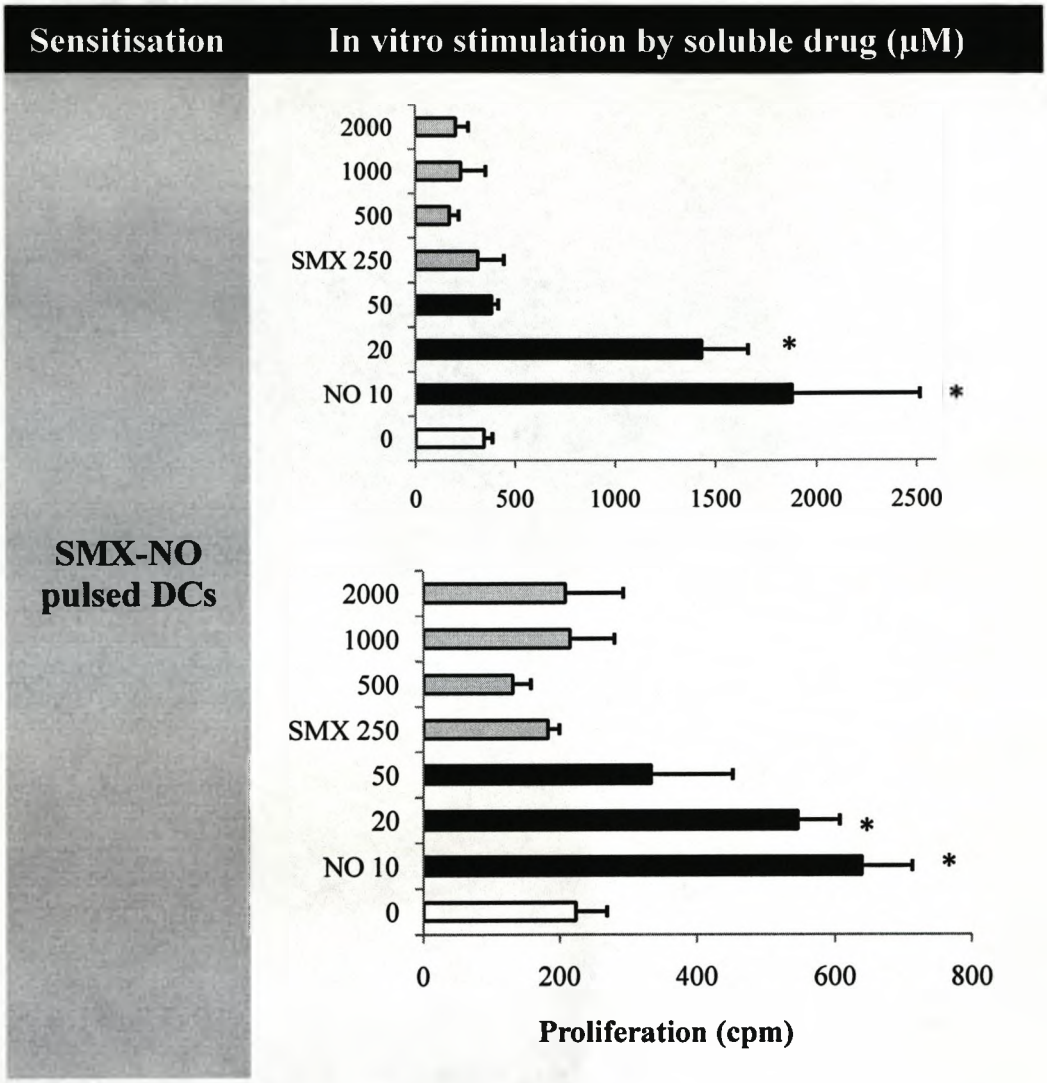

Figure 5.7: Stimulation of drug metabolite-specific T-cells following adoptive transfer of SMX-NO treated dendritic cells. The proliferative response of splenocytes was directed against SMX-NO, not the parent drug. For immunisation, dendritic cells were incubated for 16 h with SMX-NO, washed extensively, and injected in the lateral tail vein. After 21 days, mice were sacrificed and splenocytes were stimulated *in vitro* with the soluble antigen. Proliferation was quantified by incorporation of [^{3}H] thymidine. Data presented as mean ± SD, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitised splenocytes after the addition of drug antigen and solvent alone. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*$P < 0.05$).

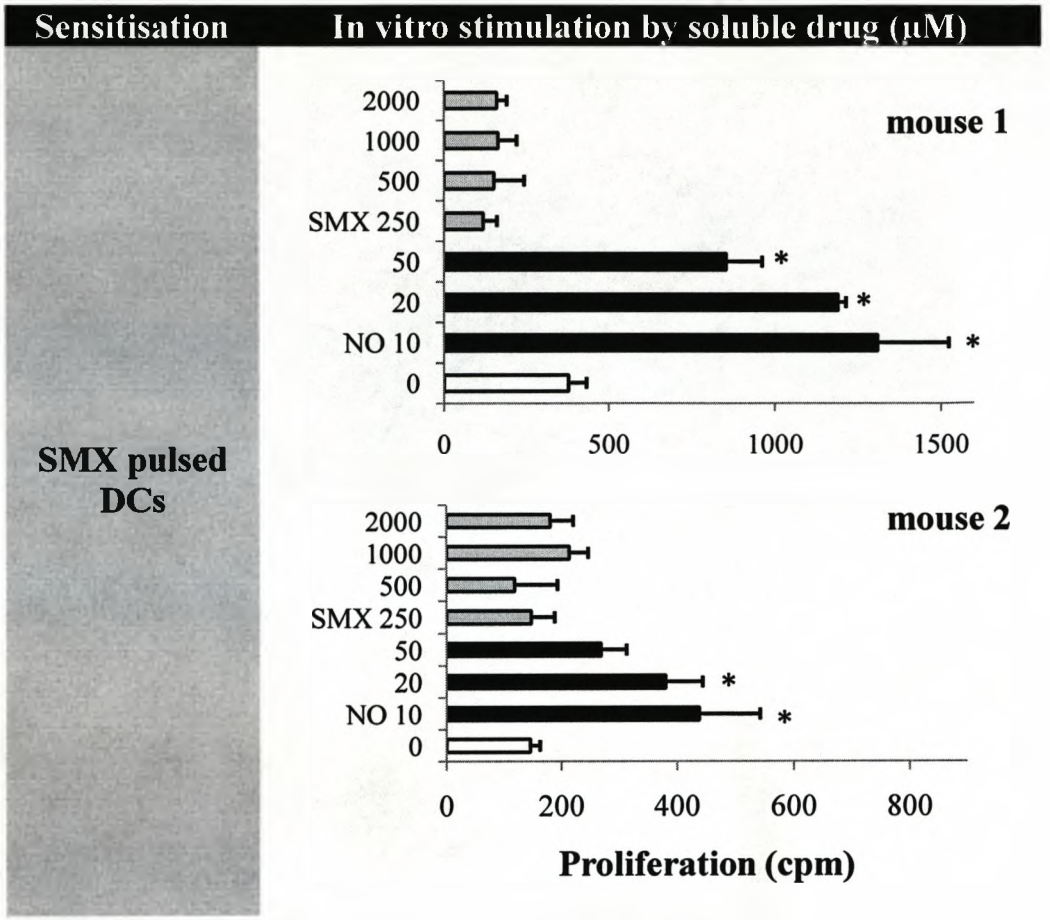

Figure 5.8: Stimulation of drug (metabolite)-specific T-cells following adoptive transfer of SMX- treated dendritic cells. The proliferative response of splenocytes was directed against SMX-NO. For immunisation, dendritic cells were incubated for 16 h in culture medium with SMX, washed extensively, and injected in the lateral tail vein. After 21 days, mice were sacrificed and splenocytes were stimulated *in vitro* with the soluble antigen. Proliferation was quantified by incorporation of [^{3}H] thymidine. Data presented as mean ± SD, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitised splenocytes after the addition of drug antigen and solvent alone. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*$P < 0.05$).

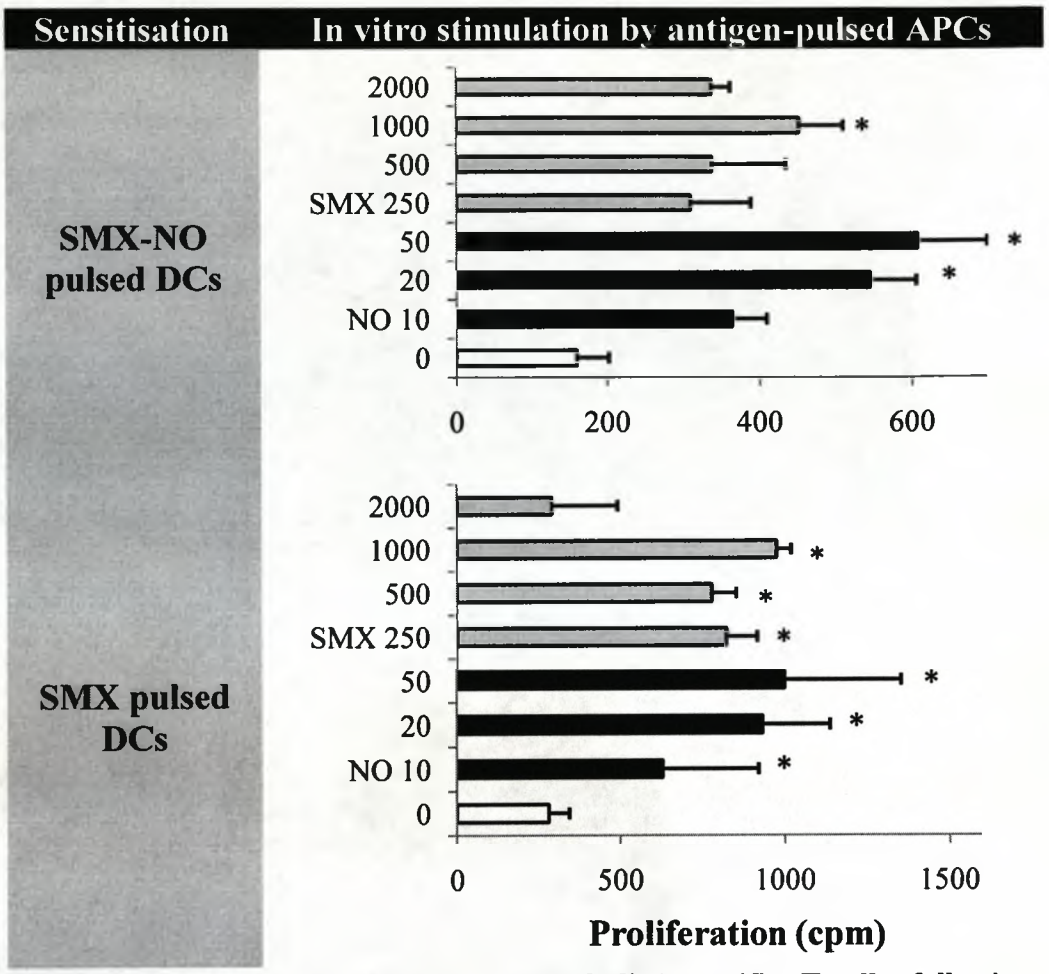

Figure 5.9: Stimulation of drug (metabolite)-specific T-cells following adoptive transfer of SMX and SMX-NO-treated dendritic cells. The proliferative response of splenocytes was directed against SMX and SMX-NO. For immunisation, dendritic cells were incubated for 16 h in culture medium with SMX or SMX-NO, washed extensively, and injected in the lateral tail vein. After 21 days, mice were sacrificed and splenocytes were stimulated *in vitro* with the drug-pulsed dendritic cells. Proliferation was quantified by incorporation of [^{3}H] thymidine. Data presented as mean ± SD, with incubations carried out in triplicate. Statistical analysis compares the proliferative response of sensitised splenocytes after the addition of drug antigen pulsed APCS and solvent pulsed APCs. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*$P < 0.05$).

5.3 Discussion

The nature of the drug antigen to which T-cells respond remains a question with no clear answer. Landsteiner and Jacobs (Landsteiner and Jacobs, 1935) defined low molecular weight chemical allergens as "incomplete antigens" since they were not directly antigenic and only gained immunogenic potential following conjugation with a protein carrier. On this basis, the "hapten concept" is the hypothesis most commonly used to describe the interaction of drugs with immune cells. The picture is further complicated because most drugs are not directly protein-reactive. They may however gain protein reactivity through normal metabolic processes, generating a hapten with the potential to modify specific amino acid residues on protein.

The role of metabolism in the development of a primary immune response is ill-defined as an experimental approach to relate metabolism to immune activation is lacking. Antigen-specific T-cells from hypersensitive patients cannot be used to define antigenic determinants that prime T-cells since it is impossible to differentiate between cross-reactive drug antigens. Thus, to evaluate whether SMX-derived protein adducts drive T-cell responses, two experimental mouse systems were used: (1) i.p. injection of mice with SMX-NO (5 mg/kg) four times weekly for 2 weeks, and (2) i.v. injection of mice with drug (metabolite)-treated dendritic cells followed by isolation of splenocytes for analysis of splenocyte proliferation against soluble antigen or antigen-pulsed APCs.

SMX is a relatively stable compound in *in vitro* cultures with most of the parent drug being recovered unchanged after 6 days in culture. However, human monocyte-derived dendritic cells (Sanderson et al., 2007) and dendritic cell-like cell lines (Roychowdhury et al., 2007b) have been shown to metabolise SMX to an unstable intermediate that forms intracellular protein adducts. In previous chapters (3 and 4), immune cells from Balb/c strain mice (splenocytes and dendritic cells) were isolated and characterised in terms of drug distribution, irreversible protein binding and immune reactivity. Incubation of viable mouse splenocytes and bone-marrow-derived dendritic cells with SMX was associated with the formation of intracellular protein adducts. In addition, SMX adduct formation was shown to be time- and dose-dependent and not detectable immediately after SMX exposure. Consistent with these findings, T-cell responses were only obtained with APCs pulsed with SMX for 6-16 h, the time needed for high levels of adduct formation (figure 5.4). In contrast, SMX-NO bound to cellular proteins instantly and derived adducts were able to stimulate a T-cell response (figure 5.4).

It has been previously reported that human monocyte-derived dendritic cells from 10 donors expressed consistently high levels of MPO, but only low levels of CYPs, leading us to propose that a peroxidase is responsible for SMX metabolism in dendritic cells (Sanderson et al., 2007). More recently, it has been shown that pathogenic conditions, which potentially activate peroxidase enzymes, enhance the formation of SMX-derived protein adducts in immune cells (Lavergne et al., 2009). To substantiate a causal relationship between peroxidase-mediated SMX

metabolism and T-cell immunogenicity, enzyme inhibitor studies were performed with 1-ABT, a non-selective suicide inhibitor, and meth, an inhibitor of peroxidases and FMO. In chapter 3, we demonstrated that both inhibitors blocked SMX-derived adduct formation in mouse splenocytes and dendritic cells. Consistently, in this chapter, incubation of the enzyme inhibitors with cells for 1 h before the addition of drug was associated with inhibited proliferation of sensitised splenocytes cultured with naive irradiated splenocytes pulsed with SMX (16 h) without blocking the proliferative response of SMX-NO modified splenocytes. These data confirm that SMX-derived protein adduct formation and T-cell proliferation are dependent on the enzymatic oxidation of SMX.

The detection of T-cells with reactivity to functional intracellular antigens arising as a consequence of cellular metabolism and extracellular antigens generated through direct conjugation of SMX-NO to cell surface proteins, implies that the antigen-specific response displays exquisite specificity for the drug hapten structure, rather than the peptide associated with MHC. SMX-derived adduct formation was 3 fold higher when splenocytes were compared with dendritic cells (chapter 3). Despite the lower quantity of adduct formed, dendritic cells stimulated a 10 fold stronger response (figures 5.2 and 5.3), which likely relates to their superior capacity to present endogenous antigens.

Although the standard FBS-supplemented culture medium is extensively used in protocols for the generation of mouse dendritic cells in *in vitro* studies (Lutz and Rossner, 2008), it affects the quality of the antigen-specific T cell response

induced by such dendritic cells when used in *in vivo* studies due to the deviation of immune response towards FBS derived peptides (non specific) rather than the specific antigenic peptide (Porgador and Gilboa, 1995; Toldbod et al., 2003; Haase et al., 2005; Kadri et al., 2007). Rossner et al. 2008 argued that this may only occur during the priming phase due to inability of FBS specific T cells to be re-stimulated and expanded in vivo (Rossner et al., 2008). Alternatively, protocols to generate mouse dendritic cells in syngeneic sera (normal mouse serum), or serum-free media have been developed in spite of the lower yields compared to FBS-cultured dendritic cells (Strobl et al., 1997; Muller et al., 2000; Haase et al., 2005; Wells et al., 2005; Warncke et al., 2006).

In this study, further evaluation of T-cell responses against SMX-derived protein adducts was performed by developing a mouse adoptive transfer system using syngeneic mouse serum to generate and culture the mouse dendritic cells. SMX and SMX-NO were cultured with bone-marrow derived dendritic cells and administered to syngeneic mice following quantification of adduct formation. After 21 days, mice were sacrificed and splenocytes were stimulated in vitro with the soluble antigen or drug-pulsed APCs. Splenocytes isolated from mice that received SMX-treated dendritic cells were stimulated *in vitro* with SMX-NO and SMX-derived intracellular metabolites, but not the parent drug (figures 5.8 and 5.9). These data provide unequivocal evidence that protein adducts generated by dendritic cell metabolism represent functional antigens that drive drug-specific immunity.

Characterisation of drug intracellular metabolism by APCs and the formation of functional antigens that stimulate T-cell response in mice have several implications regarding the use of drugs in *in vitro* experimental systems. An absence of on-line bioanalysis to measure compound distribution and metabolism makes it almost impossible to draw valid conclusions as to the nature of the chemical entity interacting with cellular proteins. Furthermore, to fully define the sensitivity and specificity of diagnostic tests for drug hypersensitivity, future validation studies must incorporate steps to relate the nature of the antigenic determinant(s) formed in the test to the biological readout. Chapter 6 explores the ability of SMX-derived cellular adducts to stimulate lymphocytes from hypersensitive patients.

Chapter 6

Enhanced Antigenicity Leads to Altered Immunogenicity in Sulfamethoxazole Hypersensitive Patients with Cystic Fibrosis

Contents

6.1 Introduction

Antibiotics provide the cornerstone of treatment and reduce the rate of decline in lung function in patients with cystic fibrosis, but their use is limited by a high frequency of delayed hypersensitivity reactions, when compared to the general population (Koch et al., 1991; Pleasants et al., 1994; Lavergne et al., 2010).

Differences in the occurrence of reactions in patients with cystic fibrosis may be related to prescribing practice (dose, frequency and duration of exposure) or route of administration. However, altered immune status associated with the development of recurrent respiratory infections, is likely to be the most important factor influencing susceptibility. In this respect, increased production of inflammatory cytokines such as IL-6 and IL-8 in patients with cystic fibrosis may lower the co-stimulatory threshold required to activate dendritic cells and initiate a T-cell response, while activation of IL-17 producing T-cells – also a common feature in patients with cystic fibrosis – may skew effector and regulatory mechanisms (Dubin et al., 2007; Nembrini et al., 2009). The redox balance is also perturbed in patients with cystic fibrosis. Antioxidant levels are lower and the enzyme MPO has been shown to produce higher levels of reactive oxygen species (Ntimbane et al., 2009).

In patients without cystic fibrosis drug antigen-specific T-cells are believed to be involved in the pathogenesis of most forms of delayed hypersensitivity reactions

(Schnyder et al., 2000; Nassif et al., 2004a; Wu et al., 2007; Rozieres et al., 2009a; Castrejon et al., 2010). However, it is important to recognise that the role of immune cells in drug hypersensitivity reactions in patients with cystic fibrosis has not been defined.

Using SMX as a model to study mechanisms of drug-specific T-cell activation, the parent drug and the protein-reactive metabolite SMX-NO have been shown to interact directly with MHC, via reversible and irreversible bonds respectively, and crosslink T-cell receptors to stimulate a T-cell response (Schnyder et al., 2000; Nassif et al., 2004a; Castrejon et al., 2010). In addition, SMX-NO modified protein stimulates T-cells via a classical hapten mechanism involving antigen processing and the evolution of antigenic peptides.

Pathological factors (viral infection, cytokines) that are a feature of cystic fibrosis significantly increase SMX-derived protein adduct formation in APCs (Lavergne et al., 2009). Accordingly, the aim of this study was to compare: (1) SMX metabolism in APC from patients with and without cystic fibrosis and (2) the phenotype and function of antigen-specific T-cells from SMX hypersensitive patients with and without cystic fibrosis and define mechanism(s) of antigen presentation.

6.2 Results

6.2.1 Lymphocytes stimulation with sulfamethoxazole and/or nitroso sulfamethoxazole

Lymphocytes from SMX hypersensitive patients without cystic fibrosis were stimulated *in vitro* to proliferate with SMX (maximum SI with direct proliferation assay: 8.7, 15.3, and 11.2) and SMX-NO (maximum SI with direct proliferation assay, 4.9, 2.1, and 24.8). Antigen-specific proliferative responses were also detected *in vitro* with lymphocytes from hypersensitive patients with cystic fibrosis, but only with SMX-NO (maximum SI with direct or indirect proliferation assay: 7.6, 33.1, and 10.2) (table 6.1 and figure 6.1). Lymphocyte responses were not detected with cells from drug-exposed volunteers (SI < 2).

Table 6.1: Details of hypersensitive patients and lymphocyte proliferation

	Patients Details		Direct LTT (maximum SI)		Indirect LTT (maximum SI)	
	Reactions	**CF**	**SMX**	**SMX-NO**	**SMX**	**SMX-NO**
1	MPE and malaise	No	8.7	4.9	NP	NP
2	DRESS and erythrodema	No	15.3	2.1	NP	NP
3	SJS	No	11.2	24.8	NP	NP
4	MPE	Yes	< 2	< 2	< 2	7.6
5	MPE	Yes	< 2	< 2	< 2	33.1
6	MPE	Yes	2.4	10.2	NP	NP

LTT: lymphocyte transformation test, **CF:** cystic fibrosis, **NP:** indirect proliferation assay was ***not performed*** if direct LTT generated positive results, **MPE:** Maculopapular exanthema, **SJS:** Stevens–Johnson syndrome, **DRESS:** Drug reaction with eosinophilia and systemic symptoms.

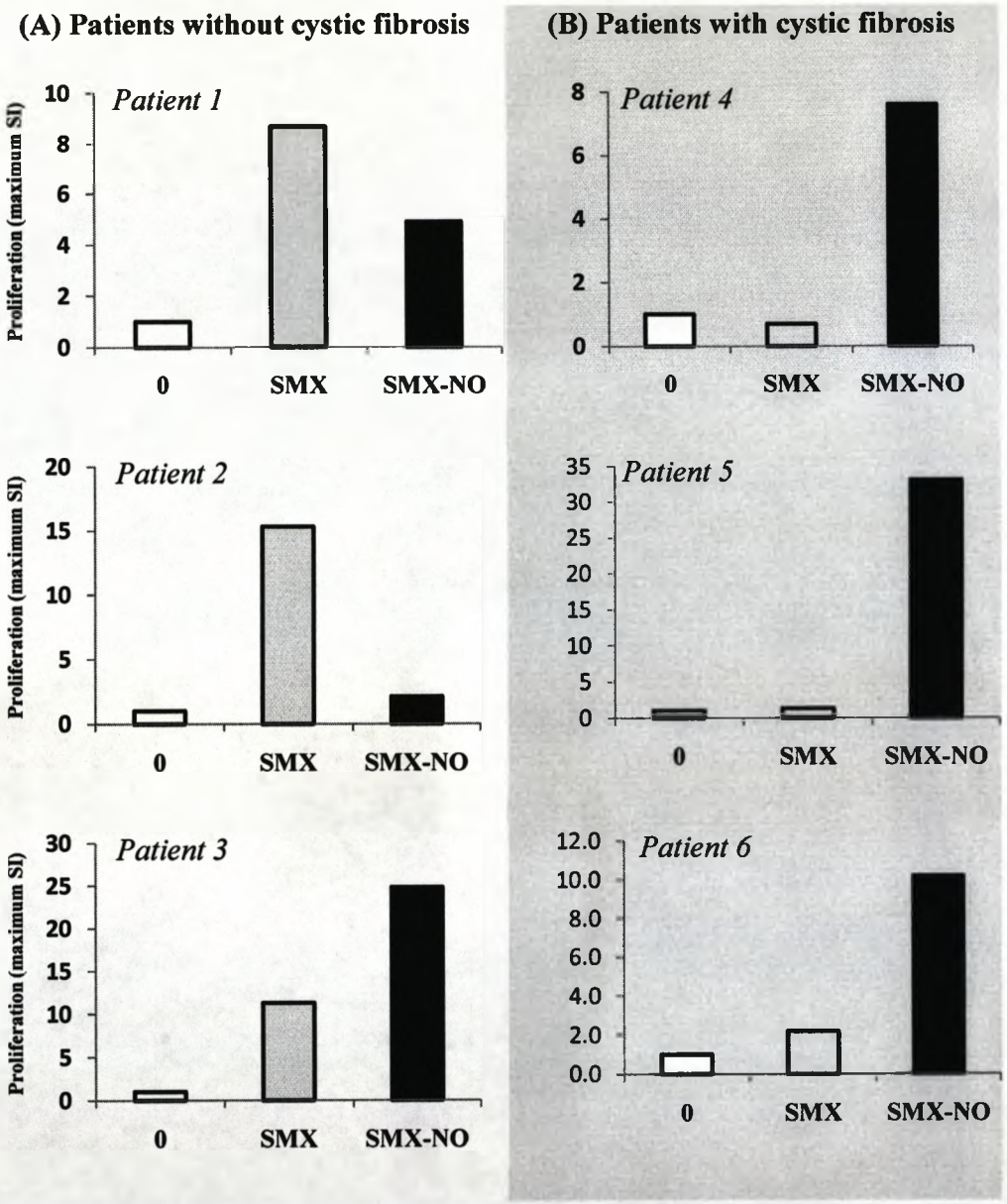

Figure 6.1: Antigen-specific stimulation of lymphocytes from hypersensitive patients. Lymphocytes were stimulated to proliferate with SMX and SMX-NO in patients without cystic fibrosis (A), but only with SMX-NO in cystic fibrosis patients (B). Proliferation was measured by [^{3}H] thymidine incorporation for the last 16h of incubation. Results are expressed as maximum SI (mean value of triplicate culture).

6.2.2 Generation of T-cell clones

A total of 293 antigen-specific T-cell clones were generated from the hypersensitive patients without cystic fibrosis. Of these, 137 were identified from SMX-stimulated lymphocytes, and 156 from SMX-NO-stimulated lymphocytes. From patients with cystic fibrosis, 241 antigen-specific clones were generated; 83 from SMX-stimulated lymphocytes and 158 from SMX-NO stimulated lymphocytes. The number of SMX and SMX-NO-responsive T-cell clones and their phenotype and cross reactivity profile is summarised in table 6.2.

Over 100 clones from patients with and without cystic fibrosis were characterised phenotypically ($CD4^+$ and $CD8^+$, table 6.2, figure 6.2) and subsequently characterised in terms of additional reactivity against SMX or SMX metabolites, cytolytic activity, the involvement of MHC molecules in antigen presentation and mechanism of antigen presentation. Clones expressing high levels of $CD8^+$ were detected from 5/6 patients presenting with different clinical signs (exanthema, DRESS and SJS).

As described previously, three patterns of reactivity (SMX-NO responsive, SMX responsive and cross-reactive) were seen with clones from each hypersensitive patient without cystic fibrosis (figure 6.3) (Castrejon et al., 2010). Somewhat surprisingly, SMX-NO responsive and cross-reactive clones, but not SMX-responsive clones, were identified from patients with cystic fibrosis (figure 6.3). It is important to note that these data refer simply to the antigen added to the culture

conditions and do not take into account APC drug metabolism. This is discussed in detail below.

Proliferation of $CD4^+$ and $CD8^+$ clones with the nitroso SMX (figure 6.4) and SMX (figure 6.5) was dose-dependent and inhibited with antibodies against either MHC class I or II (figure 6.6). Furthermore, certain clones from patients with and without cystic fibrosis displayed cytolytic activity against autologous target cells. Cytolytic activity was detected with $CD4^+$ and $CD8^+$ clones from patients with different clinical signs of hypersensitivity (exanthema, DRESS and SJS) (figure 6.7).

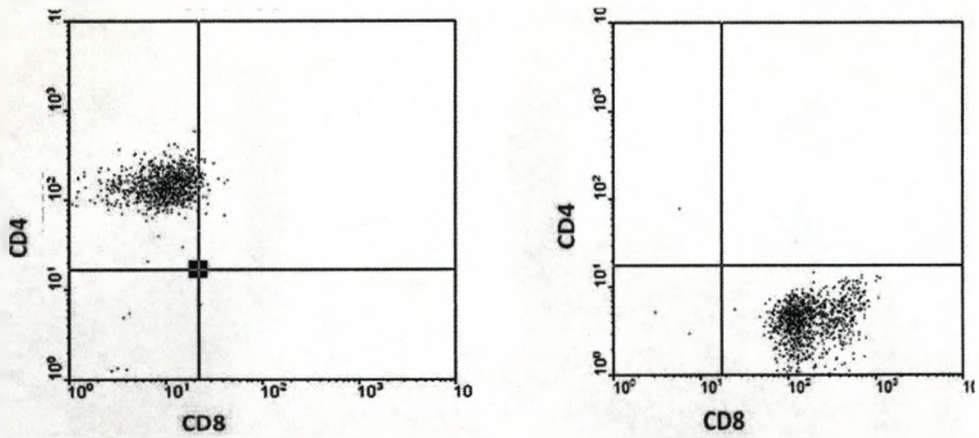

Figure 6.2: Phenotyping of the antigen responsive T-cell clones using flow cytometry. Clones expressing $CD4^+$ (left) were obtained from all patients while clones expressing high levels of $CD8^+$ (right) were detected from 5/6 patients presenting with different clinical signs.

Table 6.2: Origin, phenotype and specificity of T-cell clones from sulfamethoxazole hypersensitive patients.

	Exposure before cloning to	n	Proliferation (cpm)		Phenotype (%)		Cross-reactivity (%)	
			Control	Antigen	CD4	CD8	SMX	NO
Hypersensitive patients without cystic fibrosis								
1	SMX	31	6526 ± 6035	22669 ± 14960	69	31	-	0
	SMX-NO	14	4486 ± 1613	25345 ± 25964	71	29	0	-
2	SMX	92	2474 ± 1796	35134 ± 35834	83	17	-	0
	SMX-NO	51	2111 ± 787	16030 ± 18066	91	7	70	-
3	SMX	14	3123 ± 2628	21101 ± 33472	72	28	-	43
	SMX-NO	91	2111 ± 2033	14459 ± 11699	87	13	25	-
Hypersensitive patients with cystic fibrosis								
4	SMX	10	1140 ± 222	8045 ± 2450	67	33	-	100
	SMX-NO	12	1114 ± 363	13169 ± 12705	33	33	40	-
5	SMX	30	2042 ± 165	43310 ± 14818	100	0	-	100
	SMX-NO	67	2401 ± 1241	20024 ± 19494	100	0	40	-
6	SMX	43	1453 ± 1433	4859 ± 3706	73	27	-	100
	SMX-NO	79	1198 ± 805	3842 ± 2074	86	14	83	-

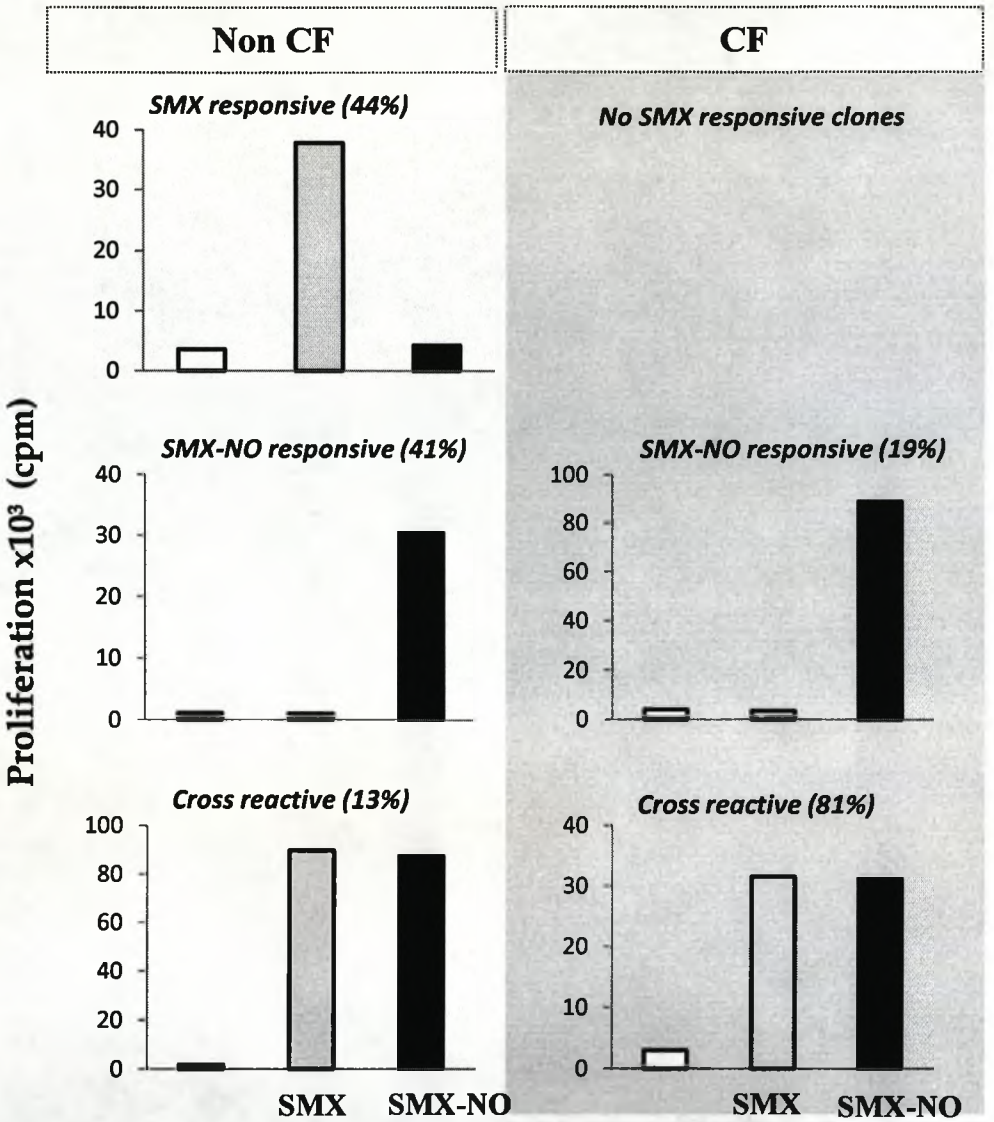

Figure 6.3. Patterns of reactivity of T-cell clones from hypersensitive patients without (left panel) and with cystic fibrosis (right panel). To measure proliferation, T cell clones were incubated with SMX or SMX-NO and irradiated autologous APCs for 48 h. Results are given as mean [^{3}H]thymidine incorporation. Differences in replicate cultures at each antigen concentration were less than 15%.

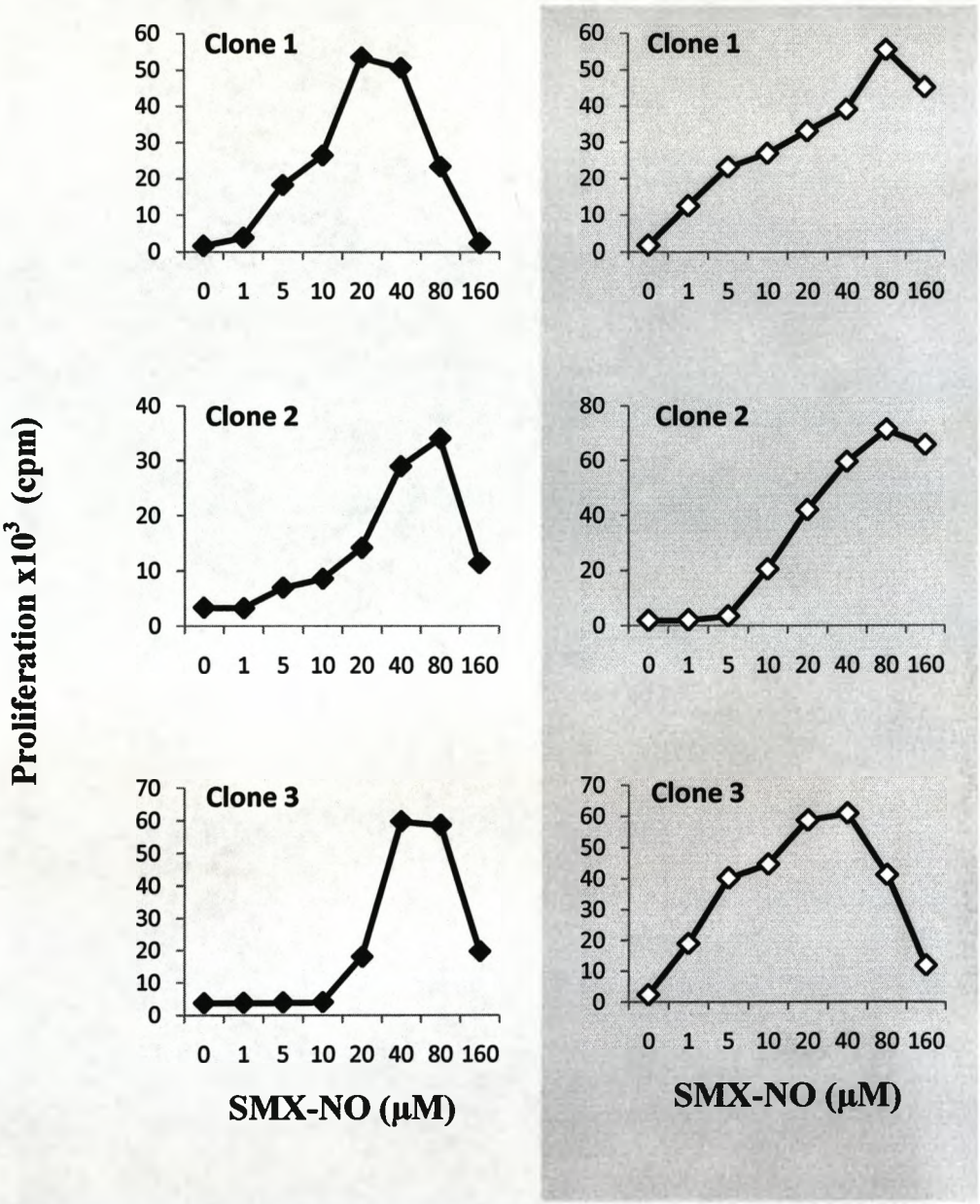

Figure 6.4: Concentration-dependent proliferation of SMX-NO responsive T-cell clones from hypersensitive patients without (left panel) and with (right panel) cystic fibrosis. Proliferation was determined by incubating T-cells with irradiated APCs and SMX-NO for 48 h, followed by [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean cpm of triplicate cultures.

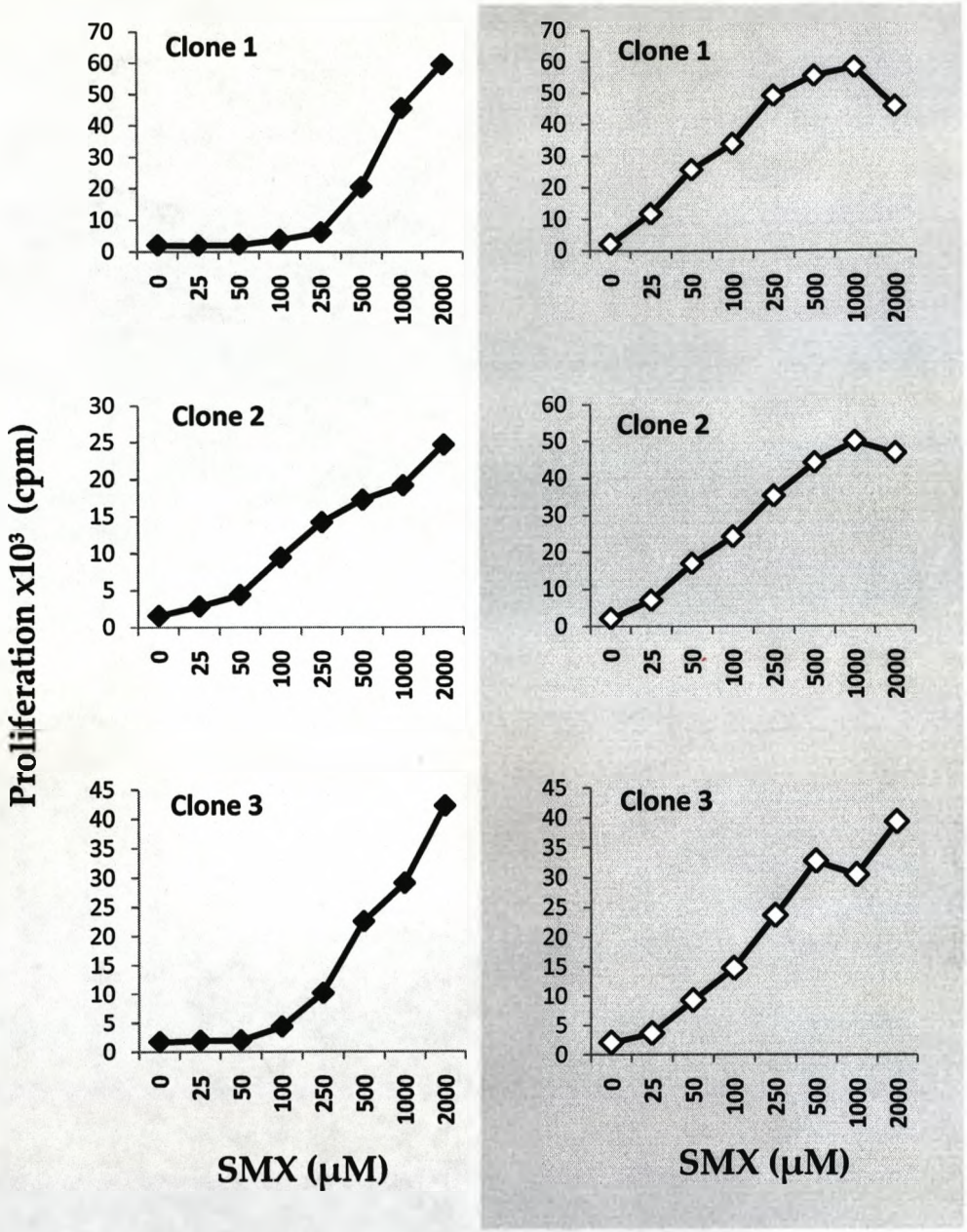

Figure 6.5: Concentration-dependent proliferation of SMX responsive T-cell clones from hypersensitive patients without (left panel) and with (right panel) cystic fibrosis. Proliferation was determined by incubating T-cells with irradiated APCs and SMX for 48 h, followed by [^{3}H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean cpm of triplicate cultures.

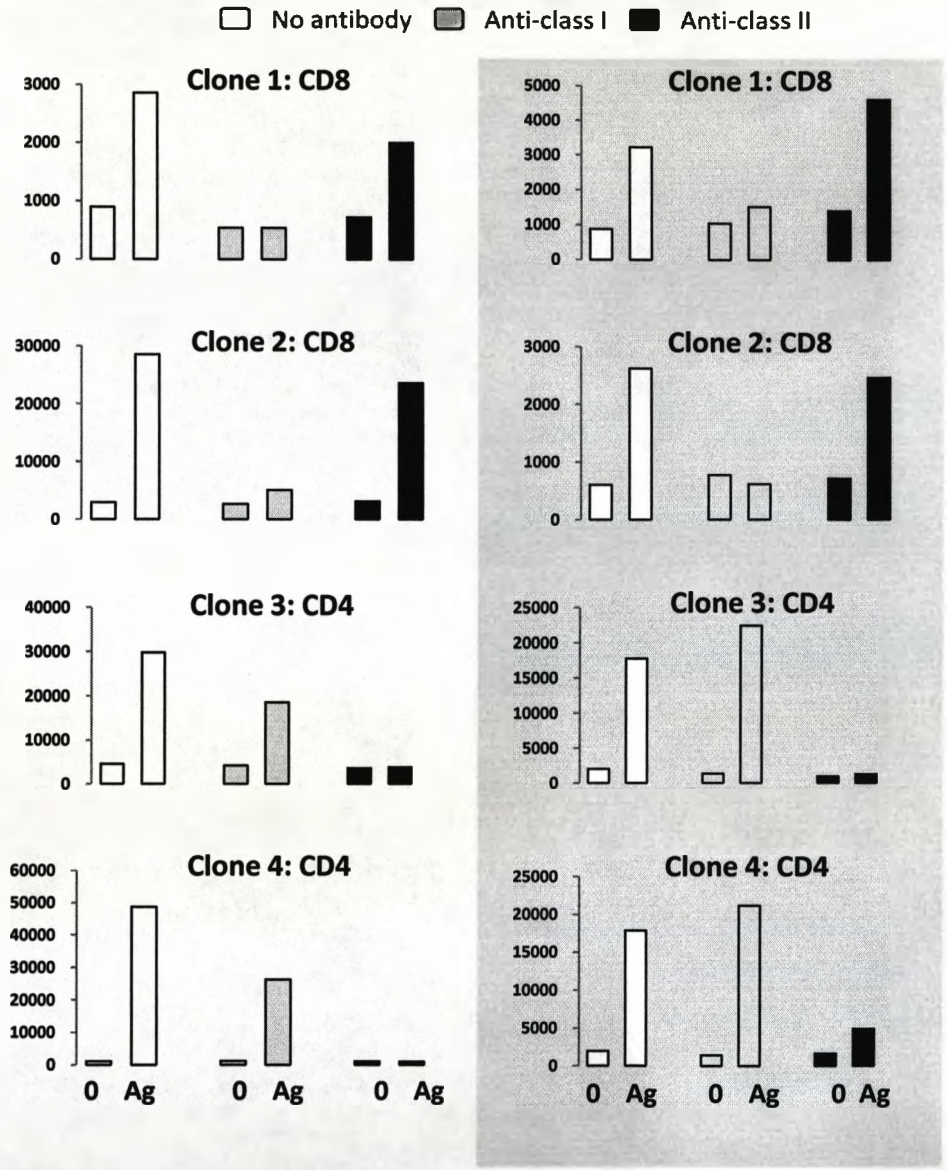

Figure 6.6: The involvement of MHC molecules in antigen presentation in both groups of patients (non CF on the left and CF on the right) was shown though the addition of anti-MHC class I and class II antibodies to the proliferation assay.

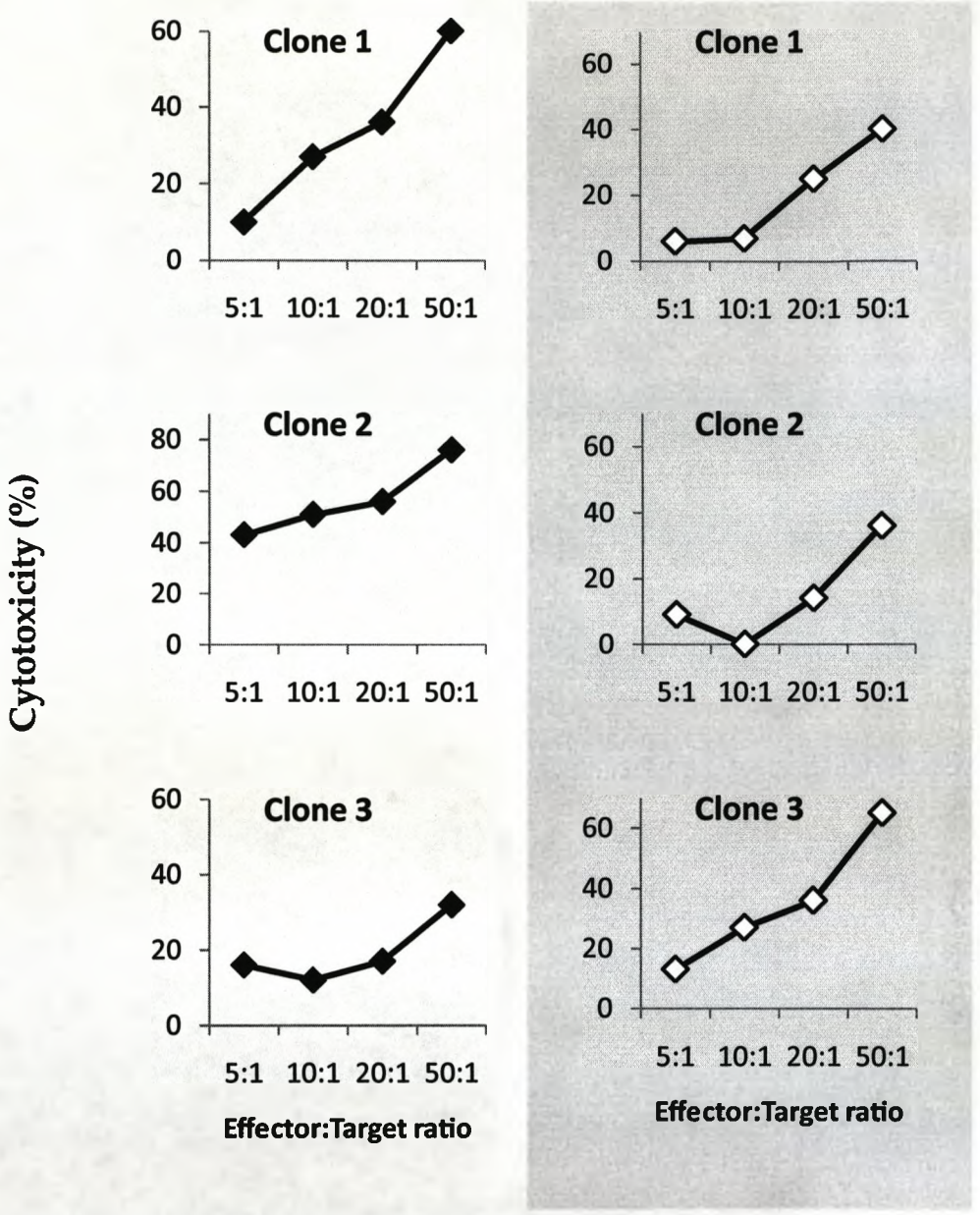

Figure 6.7: Cytolytic activity of SMX and SMX-NO responsive T-cell clones from hypersensitive patients without (left) and with (right) cystic fibrosis using the [^{51}Cr] chromium assay at effector:target ratio of 5:1-50:1.

6.2.3 Cytokine secretion from sulfamethoxazole and nitroso sulfamethoxazole stimulated T-cell clones

Levels of the cytokines secreted from stimulated T cells (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCAF, MIP-1β, and TNF-α) were assessed using Bio-Plex 17-plex human cytokine assay.

The cytokines IL-13, IL-17, IFN-γ, TNF-α and GM-CSF were secreted from greater than 95% of SMX or SMX-NO stimulated clones from hypersensitive patients with and without cystic fibrosis. Antigen stimulation was also associated with the secretion of IL-4, IL-5, IL-6, IL-8, and IL-10 from a limited number of clones (figure 6.8; table 6.3).

Although a similar profile of cytokines were secreted by T-cells from patients with and without cystic fibrosis, the two panels of clones display clear quantitative differences. Clones from patients with cystic fibrosis secreted significantly higher levels of IFN-γ, IL-6 and IL-10, but lower levels of IL-17 (figure 6.8).

Table 6.3: Cytokine secretion (pg/ml)[a] from drug/metabolite stimulated T-cell clones.

id	IL4	IL5	IL6	IL8	IL10	IL13	IL17	IFNγ	TNFα	GMCSF
Hypersensitive patients without cystic fibrosis										
1	-[b]	-	-	-	-	426	32	544	358	271
2	137[c]	>10000	-	25	-	>10000	62	33	149	273
3	136	373	13	59	-	>10000	71	1202	1967	991
4	-	-	56	1408	-	7375	57	8004	3346	1676
5	-	14	-	-	-	726	36	108	34	108
6	55	1791	323	56	25	>10000	77	817	2058	827
7	-	-	-	-	-	1012	37	441	953	441
8	-	-	11	56	-	978	35	326	855	326
9	16	12	186	295	-	7742	56	947	2499	947
Hypersensitive patients with cystic fibrosis										
1	-	-	124	58	147	553	39	3395	559	305
2	-	103	176	11	36	1758	34	4804	310	312
3	-	1565	306	122	18	>10000	-	2425	648	624
4	-	24	252	102	69	3872	51	5376	907	627
5	-	143	267	361	178	>10000	55	9346	1193	1092
6	-	117	209	317	317	2943	27	4618	1019	693
7	-	0	217	410	77	1569	20	6331	1081	502
8	-	238	486	733	84	>10000	41	7330	1887	696
9	-	-	357	517	113	4182	24	7224	1487	938
10	-	457	108	-	10	8908	22	1878	399	336
11	-	-	248	22	100	818	-	8121	342	165
12	16	442	120	319	331	1685	24	1188	900	354
13	211	221	-	247	226	>10000	34	4993	2227	1835
14	-	912	232	78	30	>10000	22	303	542	188
15	3381	>10000	223	84	141	>10000	15	-	323	289
16	14	662	32	-	-	>10000	46	>10000	766	2187
17	-	311	650	18	36	>10000	12	237	522	484
18	146	2712	154	338	392	>10000	52	1458	2130	1612
19	-	1934	86	12	-	>10000	11	1803	55	445

[a] No detectable drug-specific secretion of IL-1β, IL-7 & MCP-1.

[b] -; not detectable.

[c] Data represents mean of duplicate cultures with cytokine levels (less than 10pg/ml IL4, IL5, IL6, IL-8, IL-10, TNFα; less than 20pg/ml IFNγ; less than 50pg/ml IL13, IL17, GMCSF) in drug-free wells subtracted.

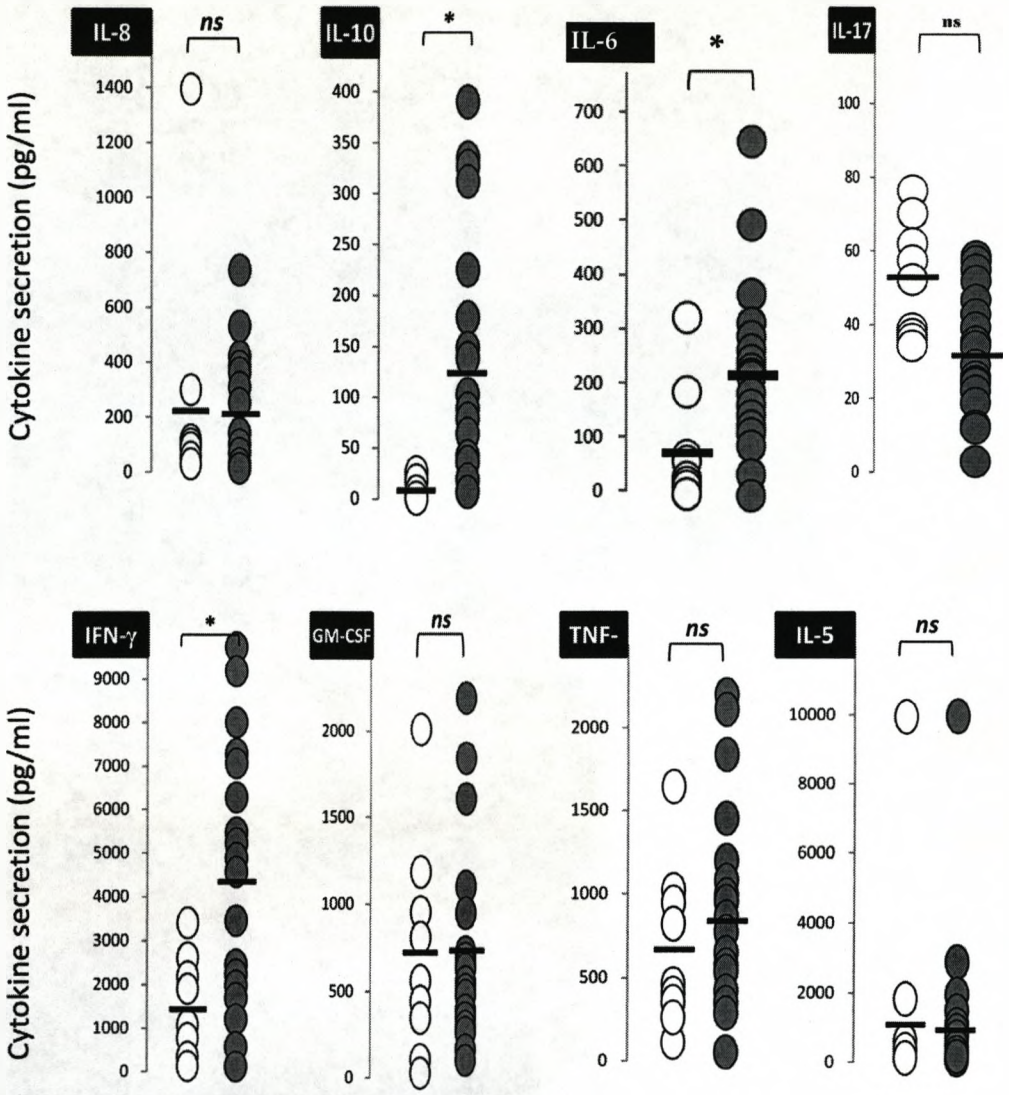

Figure 6.8: Cytokine secretion from drug antigen stimulated T-cell clones from hypersensitive patients with (grey circles) and without (white circles) cystic fibrosis. Cytokines were quantified using a multiplex assay kit. Each data point shows mean cytokine secretion from individual clones (*: significance at $P<0.05$, *ns*: non significant).

6.2.4 Definition of the contribution of drug metabolism in antigen presenting cells and covalent binding of derived metabolites to cellular protein towards sulfamethoxazole-specific T-cell responses in patients with and without cystic fibrosis.

SMX responsive clones from patients without cystic fibrosis were stimulated via two pathways: first, through a direct interaction of the parent drug with MHC molecules and specific T-cells; and secondly, through drug metabolism in APCs, irreversible binding of derived metabolites to cellular proteins and processing of the protein adducts to generate antigenic peptides. In contrast, SMX-responsive clones from patients with cystic fibrosis were stimulated *exclusively* via the pathway involving drug metabolism and the generation of drug protein adducts. These conclusions are based on the following experimental observations.

6.2.4.1 Adduct formation

SMX-derived protein adducts were detected in APCs from patients with and without cystic fibrosis. Adduct formation with SMX was concentration- (figure 6.9A) and time-dependent (figure 6.10A), while protein adducts were detected instantaneously with SMX-NO (figures 6.9B and 6.10A). Adduct formation was decreased by pre-incubating the APCs with the inhibitors of drug metabolizing enzymes methimazole and 1-aminobenzotriazole (figure 6.10B). APCs from SMX hypersensitive patients with cystic fibrosis displayed a significantly higher quantity of protein adducts when cultured with SMX for 16 h. These data were subsequently reproduced in 3 separate experiments (figure 6.9A).

6.2.4.2 Pulsing experiments

APCs pulsed with SMX for 16 h, the time required for SMX metabolism and high levels of protein adduct formation, stimulated 100% (n = 25) of T-cell clones from patients with cystic fibrosis (figure 6.11). These clones were stimulated with soluble and pulsed (1 and 16 h) SMX-NO.

In contrast, only 33% (n = 4/12) SMX-responsive T-cell clones from patients without cystic fibrosis were stimulated with SMX-pulsed APCs. The clones responding to SMX-pulsed APC were additionally stimulated with SMX-NO. Figure 6.12 shows APC pulsing data from representative SMX-specific, SMX-NO-specific and cross reactive T-cell clones from patients without cystic fibrosis.

6.2.4.3 Glutathione and enzyme inhibitors

SMX-specific responses of 11 out of 15 T-cell clones from patients with cystic fibrosis were blocked by glutathione and the inhibitors of SMX metabolism, methimazole and 1-aminobenzotriazole. In contrast, glutathione, methimazole and 1-aminobenzotriazole addition had no effect on the SMX-specific response of clones from hypersensitive patients without cystic fibrosis (figure 6.13). The response of T-cell clones from patients with and without cystic fibrosis to the synthetic metabolite SMX-NO was blocked with glutathione, but enzyme inhibition had no effect (figure 6.14).

Finally, the response of SMX-specific clones from patients with cystic fibrosis, where enzyme inhibition had no effect in the direct proliferation assay, could be

blocked by pulsing APCs and T-cell clones together with SMX and methimazole or 1-aminobenzotriazole for 16 h, prior to washing and reconstitution of the cells in drug-free medium for the remainder of the incubation period (No drug: 1543±256; SMX: 7176±604, $P<0.05$; SMX/meth: 3228±1836, $P>0.05$; SMX/1-ABT: 2397±645, $P>0.05$).

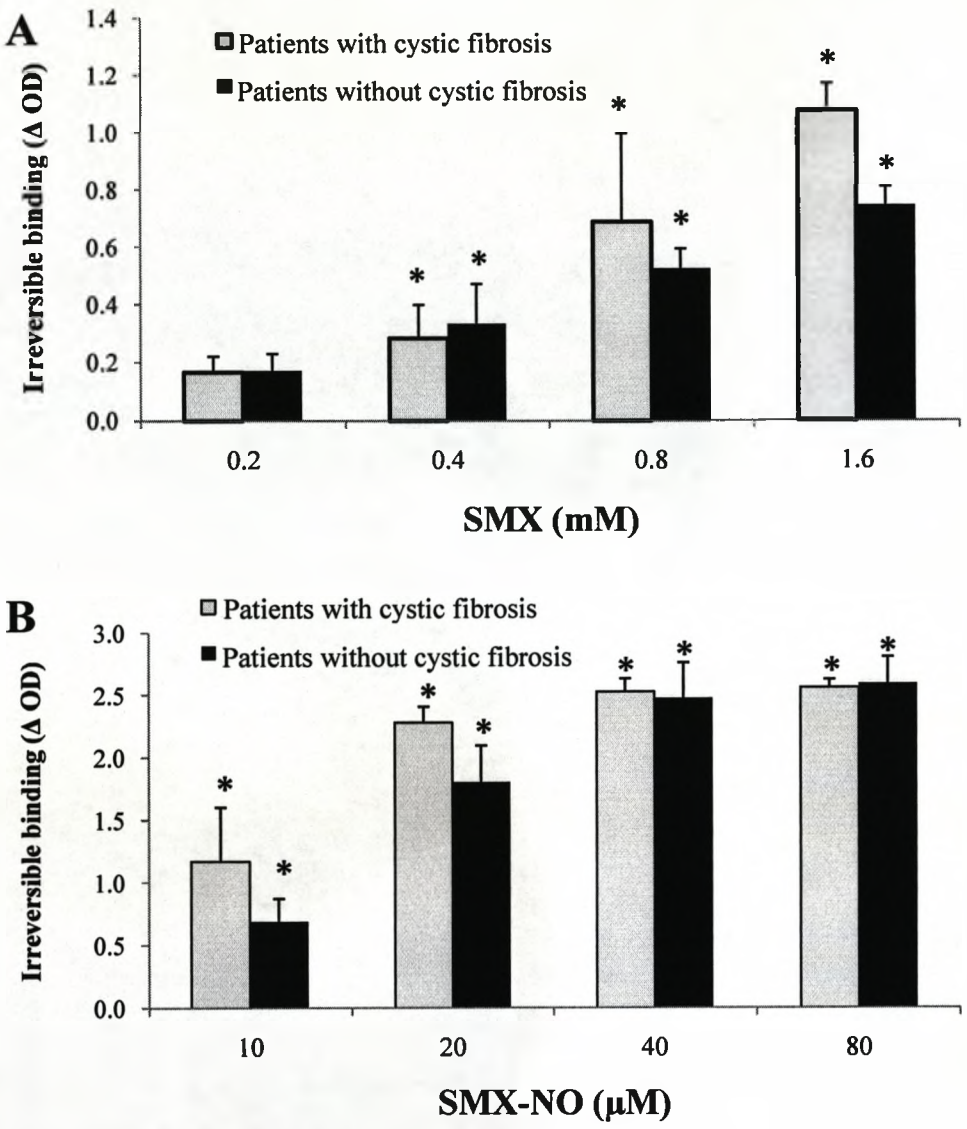

Figure 6.9: Sulfamethoxazole-derived protein adducts in antigen presenting cells quantified by ELISA using a specific anti-SMX antibody. Drug-protein adducts in SMX (A) and SMX-NO (B) treated APCs were detected from both groups of hypersensitive patients with enhanced SMX adduct formation in cystic fibrosis. Data presented as mean ± SD from 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*$P < 0.05$).

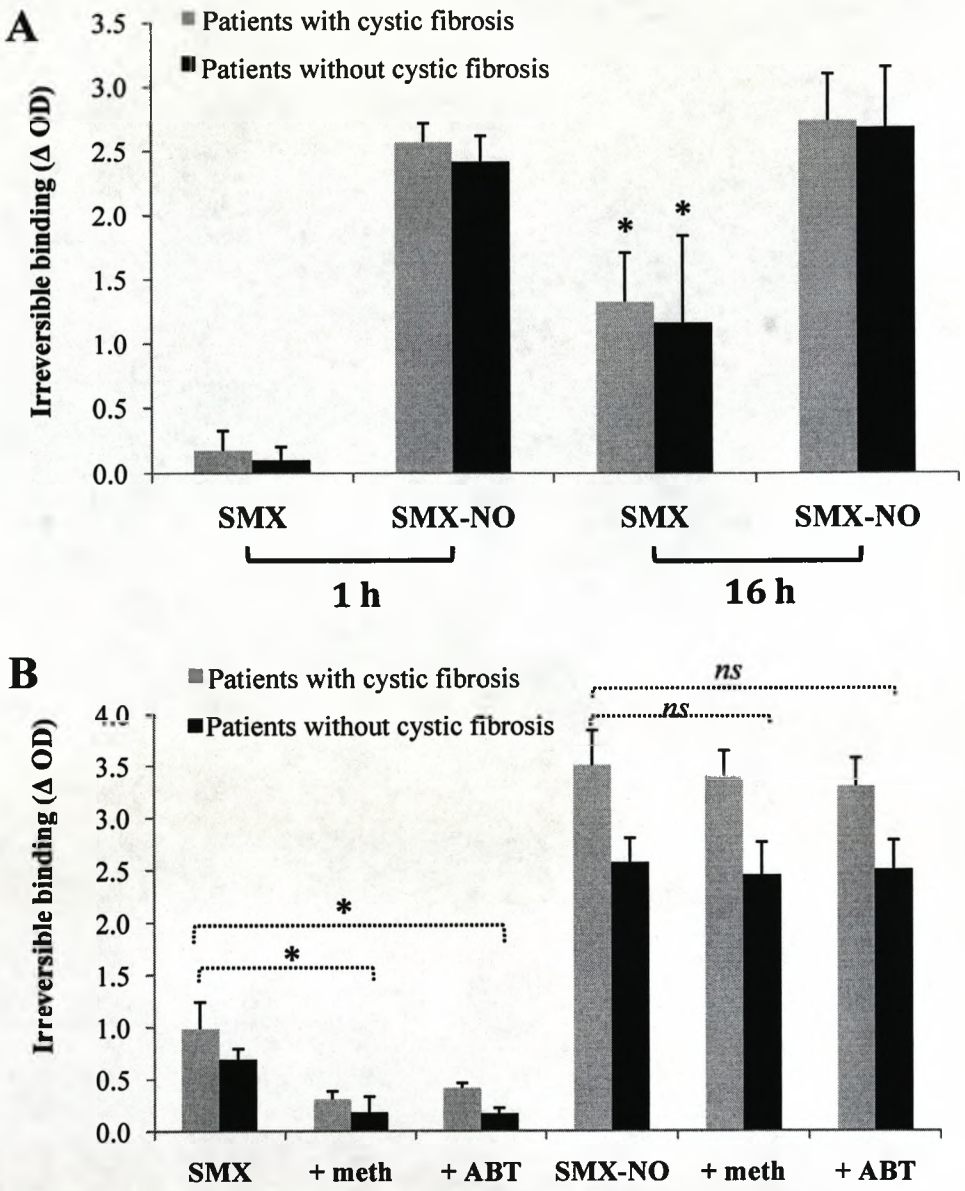

Figure 6.10: Sulfamethoxazole-derived protein adducts in antigen presenting cells quantified by ELISA using a specific anti-SMX antibody. Adduct formation with SMX was time-dependent, while protein adducts were detected instantaneously with SMX-NO (A). Enzyme inhibition with 1-ABT and methimazole decreased SMX-derived protein adducts (B). Data presented as mean ± SD from 3 separate experiments. Student T test (normally distributed data) and Mann-Whitney test (non-normally distributed data) were used to compare two groups for continuous variables (*$P < 0.05$).

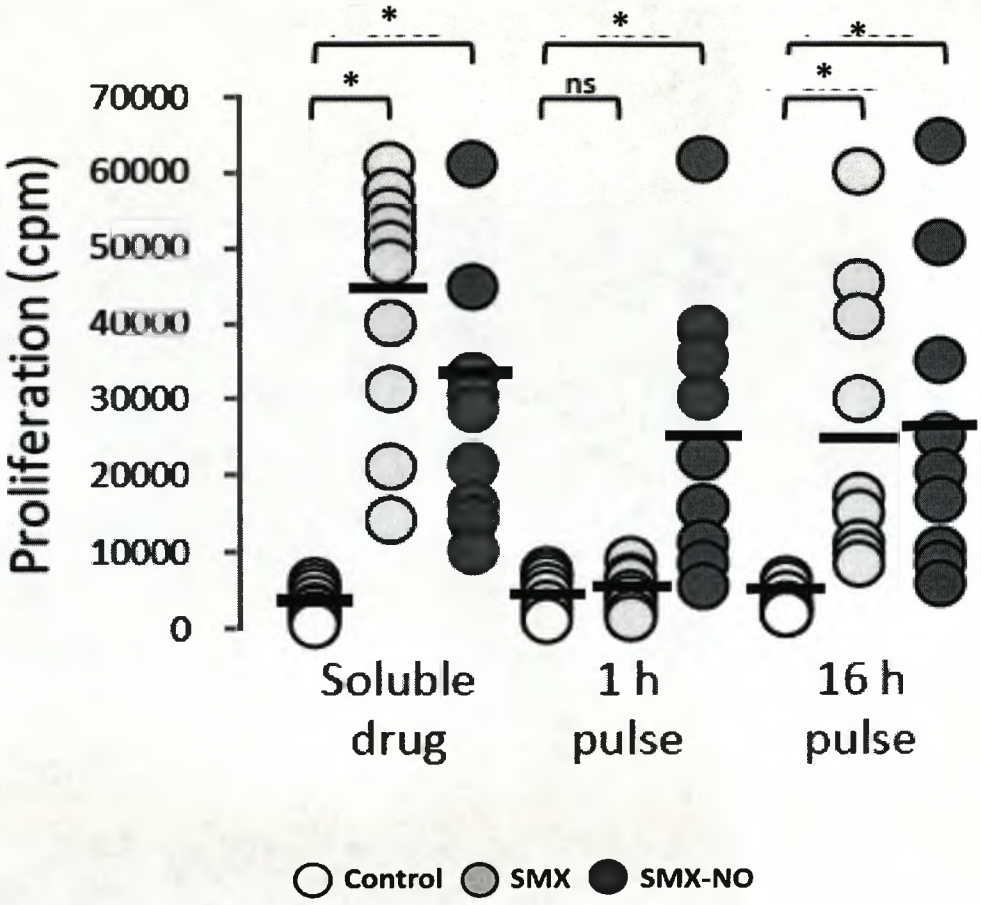

Figure 6.11: Stimulation of T-cells from hypersensitive patients with cystic fibrosis exclusively with SMX-derived metabolites. Antigen presenting cells pulsed with SMX for 16 h stimulates SMX-responsive T cell clones from hypersensitive patients with cystic fibrosis. Each of these clones was stimulated with soluble and pulsed (1 and 16 h) SMX-NO. Results are given as mean [3H]thymidine incorporation of individual clones (*P<0.05).

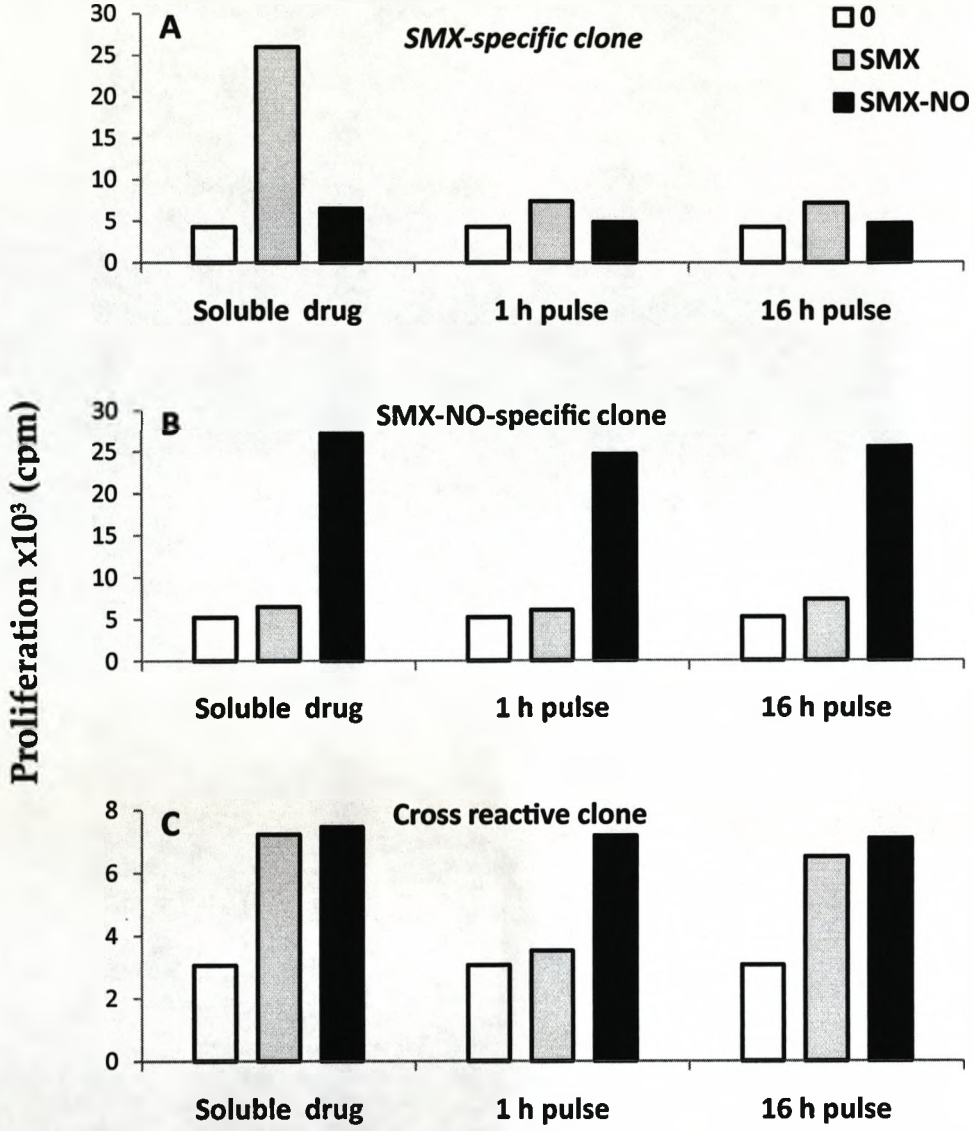

Figure 6.12: Stimulation of T-cells from hypersensitive patients without cystic fibrosis with SMX and SMX metabolites. SMX-responsive clones were not stimulated with SMX- or SMX-NO-pulsed APCs (A), whereas SMX-NO-responsive (B) and cross-reactive clones (C) were stimulated with APCs pulsed with SMX-NO for 1 and 16 h. Certain clones were stimulated with APCs pulsed with SMX (16 h). Results are given as mean [^{3}H]thymidine incorporation of individual clones (*$P < 0.05$).

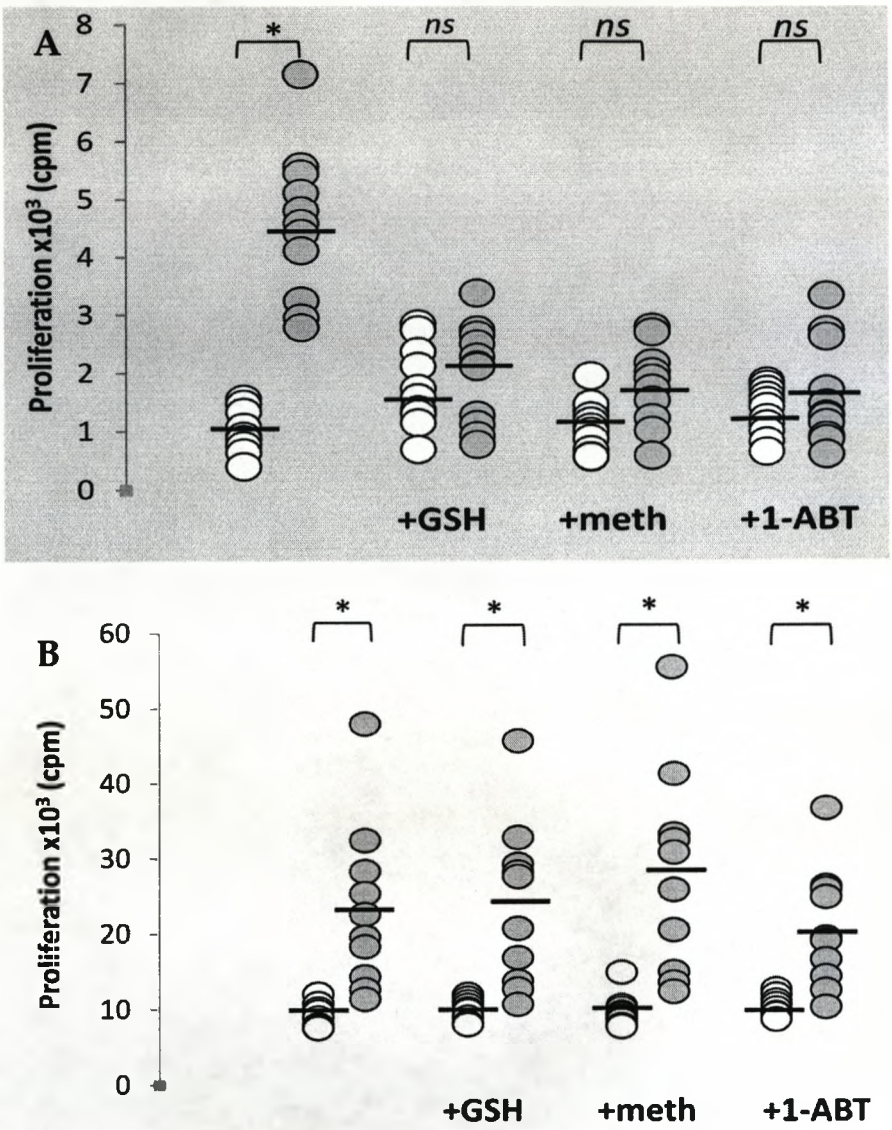

Figure 6.13: Effect of glutathione and enzyme inhibitors on the SMX-specific responses of T-cell clones from patients with and without cystic fibrosis. The response was blocked by glutathione (GSH) and the inhibitors of SMX metabolism, methimazole (meth) and 1-aminobenzotriazole (1-ABT) in patients with cystic fibrosis (A). In contrast, GSH, meth and 1-ABT addition had no effect on the SMX-specific response of clones from hypersensitive patients without cystic fibrosis (B). Results are given as mean [^{3}H]thymidine incorporation of individual clones (*$P < 0.05$).

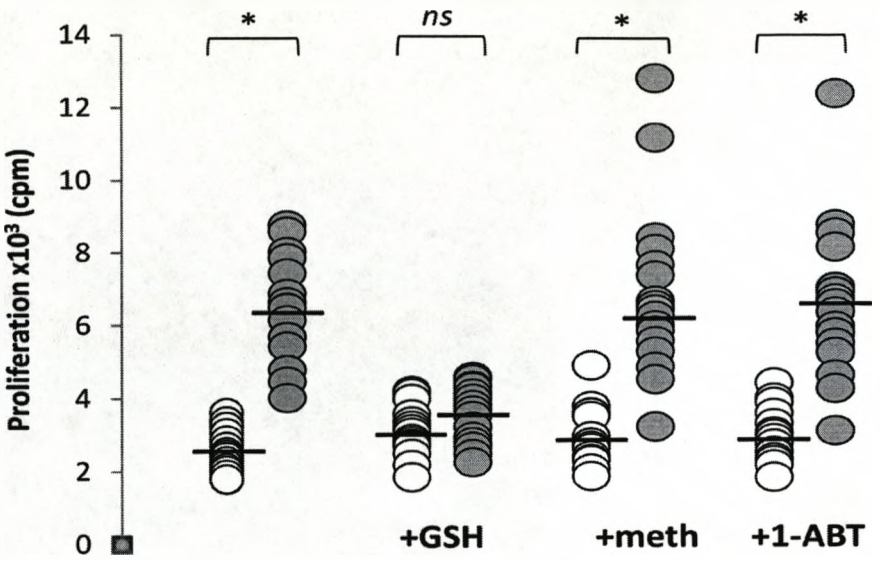

Figure 6.14: Effect of glutathione and enzyme inhibitors on the SMX-NO-specific responses of T-cell clones from patients with and without cystic fibrosis. The response of SMX-NO-responsive T-cell clones towards SMX-NO was blocked by glutathione, but not by enzyme inhibitors. Results are given as mean [^{3}H]thymidine incorporation of individual clones (*P < 0.05).

6.2.5 The involvement of processing in the presentation of sulfamethoxazole metabolites to T-cells

Glutaraldehyde fixation prevents antigen processing, but not the presentation of pre-processed antigen or antigens that bind directly to MHC molecules or MHC/peptide complexes (Zanni et al., 1998). Using glutaraldehyde fixation, SMX metabolite-responsive T-cell clones from patients with and without cystic fibrosis were shown to be stimulated via both processing-dependent and – independent pathways (figure 6.15).

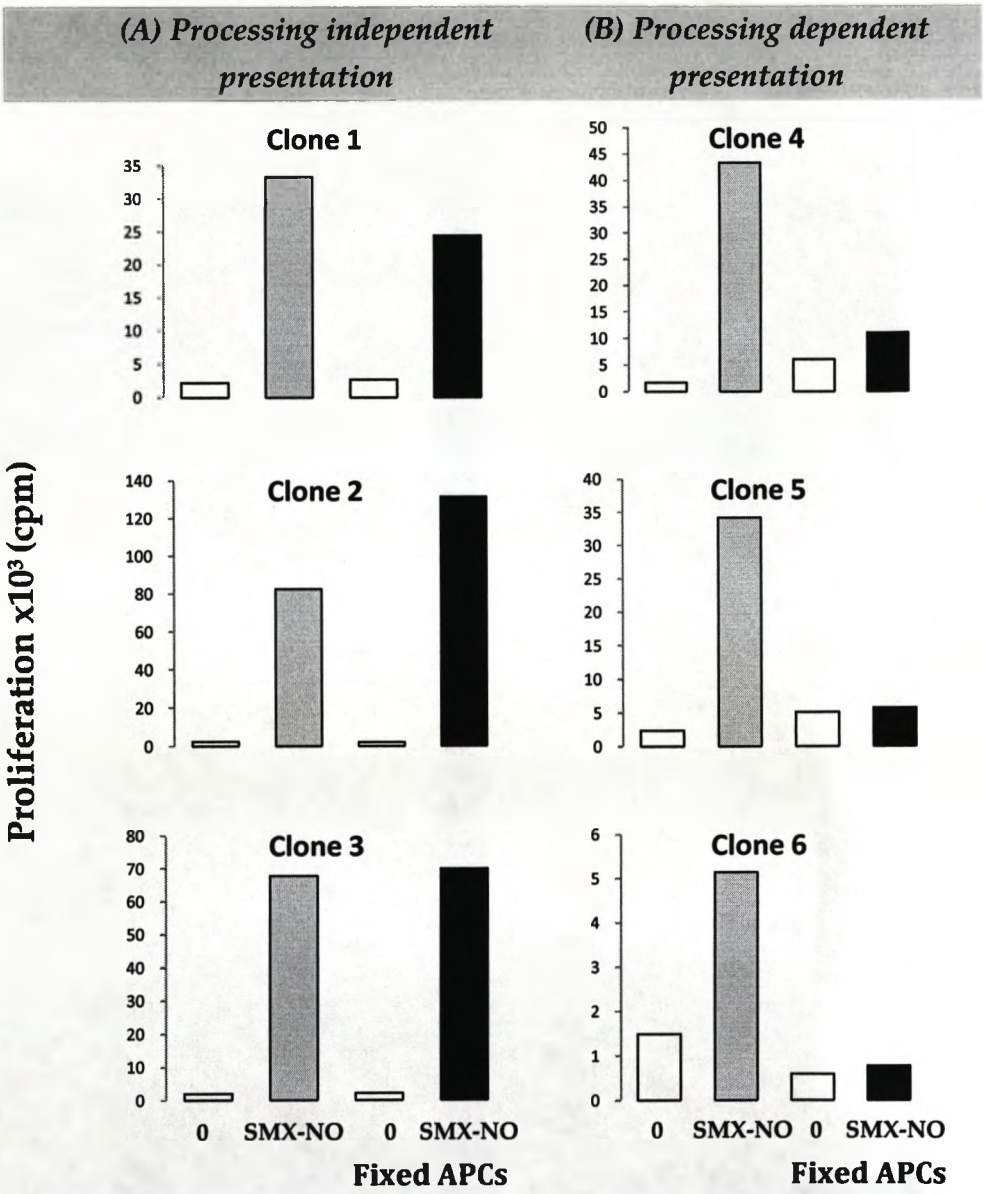

Figure 6.15: The involvement of processing in the presentation of sulfamethoxazole metabolites to T-cells. SMX-NO is presented to T-cell clones via both processing-independent (A) and dependent (B) pathways. T-cell clones were incubated with irradiated or fixed APCs and SMX-NO for 48 h, followed by [3H]-methyl-thymidine for the final 16 h of incubation. Results are expressed as the mean cpm of duplicate cultures.

6.3 Discussion

Factors that predispose to drug hypersensitivity have been explored and discussed in detail. A recent success has been the discovery of a strong association between the presence of HLA-B*5701 and abacavir hypersensitivity (Mallal et al., 2002) which resulted in the development of a genetic test and effectively the eradication of reactions to abacavir (Mallal et al., 2008). Abacavir has also been shown to stimulate specific T-cells when associated with B*5701, but not closely related HLA allotypes (Chessman et al., 2008).

Unfortunately, the picture is not so straightforward for most drugs associated with a high incidence of hypersensitivity reactions. In particular for the drug SMX, HLA polymorphisms are not major predisposing factors for hypersensitivity (Lonjou et al., 2008; Alfirevic et al., 2009). Furthermore, association analysis of drug metabolising enzyme gene polymorphisms have revealed that differences in drug metabolism are unlikely to be major predisposing factors in determining individual susceptibility (Pirmohamed et al., 2000; Alfirevic et al., 2003).

These data indicate that environmental factors, more than genetic factors, increase the risk of developing SMX hypersensitivity. In this respect, patients with HIV infection and cystic fibrosis develop SMX hypersensitivity reactions much more frequently than the general population (Carr and Cooper, 1995; Pirmohamed and Park, 2001; Lavergne et al., 2010).

The clinical picture in patients with cystic fibrosis is particularly intriguing since (1) both innate and adaptive immune responses are perturbed (Dubin et al., 2007; Nembrini et al., 2009), (2) the redox balance is modified in favour of an oxidative environment (Ntimbane et al., 2009; Wetmore et al., 2010), (3) peroxidase enzymes exist in a more activated state (Ntimbane et al., 2009) and (4) the disease itself is associated with the development of recurrent bacterial infections at the time of drug therapy, which potentially lower the drug-specific co-stimulatory threshold required to initiate an immune response and activate drug metabolising enzymes.

SMX is metabolised in the liver by cytochrome P450 enzymes to a hydroxylamine intermediate (Cribb and Spielberg, 1990; Cribb and Spielberg, 1992). Autoxidation of the hydroxylamine generates SMX-NO, which binds to thiol residues on serum and cellular protein (Cribb et al., 1991; Naisbitt et al., 1999; Manchanda et al., 2002; Naisbitt et al., 2002; Summan and Cribb, 2002; Callan et al., 2009). SMX-NO has been shown to stimulate T-cells from SMX hypersensitive patients, splenocytes from animal models of SMX immunogenicity and the partial maturation of monocyte-derived dendritic cells (Schnyder et al., 2000; Burkhart et al., 2001; Naisbitt et al., 2001; Farrell et al., 2003; Nassif et al., 2004a; Sanderson et al., 2007; Castrejon et al., 2009; Castrejon et al., 2010). Metabolites generated in liver are unlikely to be responsible for the majority of hypersensitivity reactions that develop in skin. In this respect, metabolism of SMX and formation of irreversibly bound cellular adducts have been shown to

occur in keratinocytes and APCs (Roychowdhury et al., 2005; Roychowdhury et al., 2007b; Sanderson et al., 2007).

On this basis, we have explored whether metabolism of SMX is enhanced in APCs from patients with cystic fibrosis. APC metabolism of SMX – quantified through measurement of SMX-derived protein adducts using a specific antibody – was time- and concentration-dependent and blocked with inhibitors of drug metabolising enzymes (methimazole and 1-aminobenzotriazole; Figures 6.9 and 6.10). Adduct formation was not detected when SMX was incubated with cells for 1 h, confirming that the antibody does not detect the weakly associated parent drug. Importantly, metabolite formation was found to be significantly higher, in 3 separate experiments, with cells from patients with cystic fibrosis

To explore whether the immune response to SMX differs in terms of mechanisms of antigen presentation or cellular phenotype, T-cells were cloned from 6 hypersensitive patients with or without cystic fibrosis. The hypersensitive patients with cystic fibrosis developed relatively mild maculopapular reactions which are typical clinical signs observed in the cystic fibrosis clinic. For comparison, the hypersensitive patients without cystic fibrosis developed a variety of cutaneous signs including SJS, DRESS and maculopapular reactions.

Over 500 antigen-specific T-cell clones were generated from the hypersensitive patients. From patients with and without cystic fibrosis, $CD4^+$ and $CD8^+$ T-cells were characterised as antigen-specific based on their ability to proliferate (figures

6.4 and 6.5) and/or kill autologous targets following antigen-stimulation (figure 6.7). Cytotoxic clones were generated from patients with mild and severe reactions indicating, as suggested recently (Rozieres et al., 2009b), that they play a role in the pathophysiology of most delayed drug hypersensitivity reactions. The response in both patient groups was MHC-restricted, with both SMX and SMX-NO presented to $CD8^+$ and $CD4^+$ T-cells in the context of MHC class I and II molecules, respectively (figure 6.6).

T-cells from hypersensitive patients without cystic fibrosis have been shown previously to be stimulated with the parent drug SMX and SMX-NO bound directly to MHC molecules and specific T-cell receptors. Both the parent drug and drug metabolite apparently contain the steric and electronic features required to ensure molecular interactions with both immunological receptors and the stimulation of a T-cell response. Additionally, SMX-NO binds to non-MHC associated protein generating a protein conjugate that stimulates T-cell receptors via a hapten mechanism involving processing of the conjugate and liberation of antigenic peptide fragments. Each pathway of drug stimulation was observed in the current study.

Notably, T-cells from hypersensitive patients with cystic fibrosis were stimulated with SMX-NO, bound directly to MHC molecules and via a hapten mechanism, but not the parent drug. There are several complementary pieces of evidence that support this conclusion. First, SMX-NO responsive and cross-reactive, but not SMX-responsive, T-cells were generated from all 3 patients with cystic fibrosis

(figure 6.3). Secondly, the response of the cross-reactive clones to SMX was blocked by glutathione, which binds to SMX-NO preventing protein binding (Naisbitt et al., 1999; Naisbitt et al., 2001) and inhibitors of drug metabolising enzymes (figure 6.13A). Thirdly, a 16 h pulse of APCs with SMX, the time required for metabolism and the formation of high levels of irreversibly bound protein adducts (figures 6.9A and 6.10), stimulates T-cells (figure 6.11). In contrast, the SMX-specific response of clones from hypersensitive patients without cystic fibrosis was not blocked with glutathione or enzyme inhibitors (figure 6.13B). Furthermore, pulsing APCs with SMX failed to stimulate a response (figure 6.12A).

T-cell clones from patients with cystic fibrosis were found to secrete significantly higher levels of IL-6 (without cystic fibrosis 65 pg/ml, with cystic fibrosis 224 pg/ml) and IFN-γ (without cystic fibrosis 1358 pg/ml, with cystic fibrosis 4254 pg/ml), when compared with equivalent clones from hypersensitive patients without cystic fibrosis (figure 6.8). The increased secretion of IFN-γ by antigen stimulated clones from patients with cystic fibrosis is particularly interesting. IFN-γ is a pro-inflammatory cytokine normally associated with bullous-skin diseases (TEN/SJS) and DRESS (Pichler et al., 2002; Naisbitt et al., 2003; Nassif et al., 2004b). In this respect, one would have expected T-cells from the patients with cystic fibrosis presenting with maculopapular drug eruptions to secrete lower levels of IFN-γ. Nevertheless, the data support work of Rozieres et al.(2009a) quantifying drug-specific T-cells in penicillin allergic patients presenting with maculopapular reactions through the use of IFN-γ ELIspot and suggest that IFN-γ

ELIspot may represent a sensitive biological tool for the diagnosis of most forms of delayed drug hypersensitivity reactions.

T-cells from patients with cystic fibrosis also secreted higher levels of the regulatory cytokine IL-10. In fact, little or no IL-10 was secreted by T-cells from hypersensitive patients without cystic fibrosis. These findings may be related to the relatively mild clinical signs observed in most hypersensitive patients with cystic fibrosis. This however obviously requires further investigation.

To conclude, we have found that T-cells from hypersensitive patients with cystic fibrosis are stimulated exclusively with the oxidative metabolites of SMX. The high incidence of hypersensitivity reactions attributed to SMX in this patient population may be due to the disease *per se*, which is associated with a cellular matrix rich in dendritic cell maturation signals that enhance drug metabolising enzyme activity.

Chapter 7

Final Discussion

The serious dilemma of adverse drug reactions affects patient health care through impediment of specific treatment, amendment of the dosage schedule, or abandonment of the drug. A recent study in Sweden has ranked ADRs within the first ten most common causes of death (Wester et al., 2008), while two recent UK studies have indicated that ADRs-related admissions account for ten 800-bed NHS hospitals and an annual £637 million financial burden (Pirmohamed et al., 2004; Davies et al., 2009). Although type B reactions are a less common type of ADRs accounting for 15–20% (Lazarou et al., 1998), they are generally more serious. In addition, mechanisms of most type B reactions are not defined due to unpredictable nature of the reactions and lack of relevant animal models.

Several lines of evidence indicated that drug hypersensitivity reactions are immune-mediated. Antigen-specific T-cells were detected in the peripheral circulation and target organs of hypersensitive patients, but not in drug-exposed controls (Britschgi et al., 2001; Nassif et al., 2004; Beeler et al., 2006; Wu et al., 2006). Moreover, $CD8^+$ dermal T-cells from lesional skin (Hertl et al., 1995), or blood and skin derived T-cells from hypersensitive patients were stimulated to

proliferate when cultured with relevant drug (Schnyder et al., 2000; Farrell et al., 2003; Nassif et al., 2004; Castrejon et al., 2010).

For the induction of an immune response to a drug, the drug antigen must be presented to T-cells through MHC molecules expressed on the APCs in the presence of co-stimulatory signalling and cytokines, which are necessary for sustained T-cell expansion (Curtsinger et al., 1999; Gerner and Mescher, 2009). Three concepts exist on how T-cells interact with the drug and become activated. The (pro)hapten concept indicates that binding of the drug to protein is a prerequisite for antigen formation. In contrast, the p-i concept suggests that drugs interact with immunological receptors directly via a weak and readily reversible interaction without metabolism or processing. Finally, the "danger model" originally proposed by Matzinger (1994) represents a complimentary addition to both hapten and p-i models. The danger model describes how drugs might interact with cells of the innate immune system, dendritic ells in particular, to provide the additional - non T-cell receptor - signals needed to initiate a primary immune response.

Sulfamethoxazole was used as a model of drug allergy in the studies described herein to investigate these concepts as it is a relatively stable compound; with most of the parent drug being recovered unchanged after 6 days in culture. Furthermore, SMX metabolism is well defined and synthetic stable and reactive metabolites are available for functional studies. The distribution of SMX-NO in *in vitro* culture is complex. Auto-oxidation generates nitro SMX, self-conjugation

generates azo and azoxy dimers and reduction through enzymatic reactions or glutathione dependent processes to liberate the hydroxylamine metabolite and the parent drug (figure 7.1) (Cribb et al., 1991; Naisbitt et al., 1996; Naisbitt et al., 2002; Kurian et al., 2004; Castrejon et al., 2009; Sacco and Trepanier, 2010). Although these reactions result in rapid (within 5 min) and complete degradation of SMX-NO; multiple meta-stable protein adducts that become internalised via a caveole-dependent mechanism (Manchanda et al., 2002; Sanderson et al., 2007; Callan et al., 2009) are readily detectable.

To explore the underlying mechanisms of drug hypersensitivity, an integrated approach was developed using both animal and human experimental systems to explore the complex relationship between drug metabolism, adduct formation, cell death and both dendritic cell and T-cell activation.

Data deriving from animal experiments (chapters 3, 4, and 5) demonstrated that drug protein adducts generated as a consequence of APC metabolism represent functional antigens for T-cells. In chapter 3, adducts were identified in viable mouse splenocytes and bone-marrow-derived dendritic cells exposed to SMX and its metabolite. Significantly, SMX adduct formation was dose dependent, time dependent (i.e. not detectable immediately after SMX exposure), and blocked by enzyme inhibitors. The functionality of these adducts were tested *in vitro* in chapter 4 against APCs. Adducts were unable to activate dendritic cells directly (upregulation of co-stimulatory receptor expression and MHC class II or stimulation of cytokine secretion). However, in a co-culture system containing

drug-treated necrotic cells, dendritic cells were activated in a concentration-dependent fashion. Activated dendritic cells expressed higher levels of co-stimulatory receptors and secreted high levels of cytokines. To explore the antigenicity of SMX-derived protein adducts *ex vivo*, two mouse models were used in chapter 5; (1) administration of SMX-NO (5 mg/kg, 100 µl) via i.p. injection to Balb/c mice four times weekly for 2 weeks according to a previously described protocol (Naisbitt et al., 2001) and (2) immunisation of mice with drug-treated dendritic cells (0.5×10^6, 200 µl) via i.v injection in the lateral tail vein followed by splenocyte isolation after 21 days of injection. Splenocytes isolated from treated mice were stimulated *in vitro* with SMX-NO and SMX-derived intracellular metabolites, but not the parent drug providing a clear evidence that protein adducts generated by dendritic cell metabolism represent functional antigens that drive drug-specific immunity.

Data deriving from experiments on human cells from hypersensitive patients (chapter 6) confirmed the data derived from mouse experiments in that APCs from hypersensitive patients were able to metabolise SMX and the derived intracellular adducts were able to stimulate T-cells. Formation of SMX-derived protein adducts was time dependent, dose dependent (i.e. increased drug concentration was associated with increased adduct levels), and blocked with inhibitors of drug metabolising enzymes. Noticeably, metabolism of SMX was enhanced in APCs from patients with cystic fibrosis and T-cells from hypersensitive patients were stimulated exclusively with SMX-NO or SMX-derived metabolites. In contrast, in patients without cystic fibrosis, three clonal

patterns of MHC-restricted reactivity were detected; SMX-responsive, SMX-metabolite responsive and cross-reactive (figure 7.2). These human studies represent the first direct evidence to support the (pro)hapten concept as a viable model to explain the chemical basis of drug hypersensitivity in humans.

Capsule summary

Antigen presenting cell drug metabolism and subsequent irreversible binding of derived SMX metabolites to cellular protein represents an important pathway for the generation of functional antigens for T-cells. When this pathway combined with specific individual factors (e.g. differences in drug metabolism or perturbed immunity) and/or disease factors (e.g. accompanying infection or redox imbalance), it might explain the increased risk of certain individuals to drug-induced hypersensitivity.

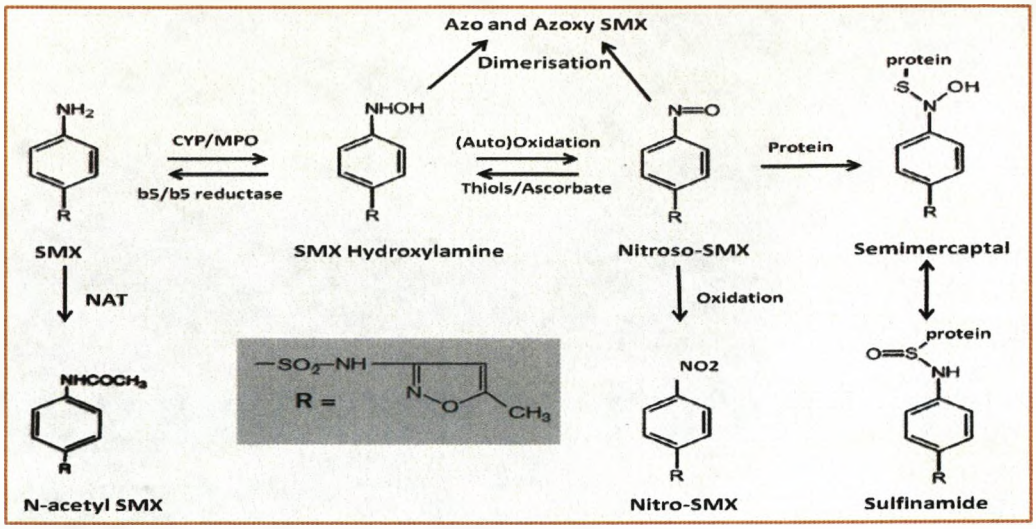

Figure 7.1: Schematic representation of SMX metabolism showing the detoxification and oxidative bioactivation pathways. CYP: cytochrome P450, MPO: myeloperoxidase, NAT: N-acetyltransferase, SMX: sulfamethoxazole.

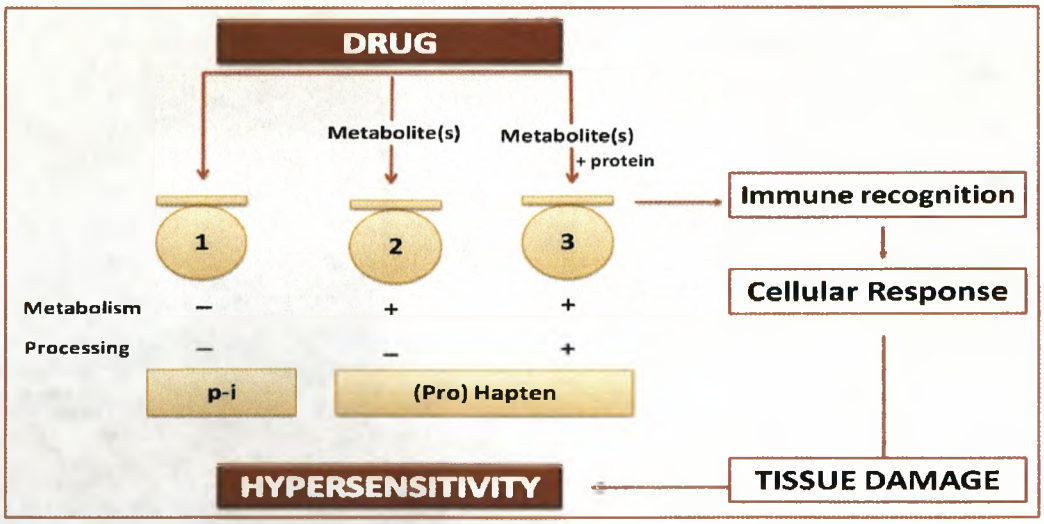

Figure 7.2: T-cell activation in SMX hypersensitive patients. Three pathways exist, (1) direct binding of SMX with MHC and specific TCR, (2) direct binding of SMX-NO with MHC and specific TCR and (3) binding of SMX-NO to a protein to form protein conjugate that undergoes processing and liberation of antigenic peptide fragments. The three pathways were observed in patients without CF, while pathways 2 and 3 are the exclusive ways for activation of T-cells in patients with CF.

References

Abbas A, Lichtman A and Pillai S (2007) Cellular and molecular immunology 6th ed., Saunders/Elsevier, Pennsylvania

Abe R, Shimizu T, Shibaki A, Nakamura H, Watanabe H and Shimizu H (2003) Toxic epidermal necrolysis and Stevens-Johnson syndrome are induced by soluble fas ligand. American Journal of Pathology 162:1515-1520.

Ackerman AL and Cresswell P (2004) Cellular mechanisms governing cross-presentation of exogenous antigens. Nature Immunology 5:678-684.

Adams CP and Van Brantner V (2006) Market watch : Estimating the cost of new drug development: Is it really $802 million? Health Affairs 25:420-428.

Aeby P, Wyss C, Beck H, Griem P, Scheffler H and Goebel G (2004) Characterization of the sensitizing potential of chemicals by in vitro analysis of dendritic cell activation and skin penetration. Journal of Investigative Dermatology 122:1154-1164.

Afkarian M, Sedy JR, Yang J, Jacobson NG, Cereb N, Yang SY, Murphy TL and Murphy KM (2002) T-bet is a STATI-induced regulator for IL-12R expression in naïve $CD4^+$ T cells. Nature Immunology 3:549-557.

Aiba S, Manome H, Nakagawa S, Mollah ZUA, Mizuashi M, Ohtani T, Yoshino Y and Tagami H (2003) p38 Mitogen-activated protein kinase and extracellular signal-regulated kinases play distinct roles in the activation of dendritic cells by two representative haptens, NiCl2 and 2,4-dinitrochlorobenzene. Journal of Investigative Dermatology 120:390-399.

Aithal GP and Day CP (2007) Nonsteroidal Anti-Inflammatory Drug-Induced Hepatotoxicity. Clinics in Liver Disease 11:563-575.

Akiba H, Satoh M, Iwatsuki K, Kaiserlian D, Nicolas JF and Kaneko F (2004) CpG immunostimulatory sequences enhance contact hypersensitivity responses in mice. Journal of Investigative Dermatology 123:488-493.

Alanore L and Roujeau J (2007) Clinic and pathogenesis of severe bullous skin reactions: Stevens-Johnson syndrome, toxic epidermal necrolysis. In: Pichler WJ (ed) Drug hypersensitivity. Karger, Basel, pp 267–277.

Alfirevic A, Stalford AC, Vilar FJ, Wilkins EGL, Park BK and Pirmohamed M (2003) Slow acetylator phenotype and genotype in HIV-positive patients with sulphamethoxazole hypersensitivity. British Journal of Clinical Pharmacology 55:158-165.

Alfirevic A, Vilar FJ, Alsbou M, Jawaid A, Thomson W, Ollier WER, Bowman CE, Delrieu O, Park BK and Pirmohamed M (2009) TNF, LTA, HSPA1L and HLA-DR gene polymorphisms in HIV-positive patients with hypersensitivity to cotrimoxazole. Pharmacogenomics 10:531-540.

Allam JP, Paus T, Reichel C, Bieber T and Novak N (2004) DRESS syndrome associated with carbamazepine and phenytoin. European Journal of Dermatology 14:339-342.

Al-Tajir GK and Kelly WN (2005) Epidemiology, comparative methods of detection, and preventability of adverse drug events. Annals of Pharmacotherapy 39:1169-1174.

Anjuere F, Martin P, Ferrero I, Fraga ML, Del Hoyo GMD, Wright N and Ardavin C (1999) Definition of dendritic cell subpopulations present in the spleen, Peyer's patches, lymph nodes, and skin of the mouse. Blood 93:590-598.

Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V, Clavel-Chapelon F, Bourhis J, André F, Delaloge S, Tursz T, Kroemer G and Zitvogel L (2007) Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nature Medicine 13:1050-1059.

Aronson J (2002) Evidence-based medicine and the principles of drug therapy. Chapter 2. In: Haslett C, Chilvers ER, Boon NA, et al., editors. 19th ed. Davidson's textbook of medicine. Edinburgh: Churchill Livingstone.

Aronson JK and Ferner RE (2005) Clarification of terminology in drug safety. Drug Safety 28:851-870.

Arrighi JF, Rebsamen M, Rousset F, Kindler V and Hauser C (2001) A critical role for p38 mitogen-activated protein kinase in the maturation of human

blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha and contact sensitizers. Journal of Immunology 166:3837-3845.

Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA and Calderwood SK (2002) Novel signal transduction pathway utilized by extracellular HSP70: Role of toll-like receptor (TLR) 2 and TLR4. Journal of Biological Chemistry 277:15028-15034.

Athie-Morales V, Smits HH, Cantrell DA and Hilkens CMU (2004) Sustained IL-12 Signaling Is Required for Th1 Development. Journal of Immunology 172:61-69.

Aycicek A and Iscan A (2007) The effects of carbamazepine, valproic acid and phenobarbital on the oxidative and antioxidative balance in epileptic children. European Neurology 57:65-69.

Baillie TA (2006) Future of toxicology-metabolic activation and drug design: Challenges and opportunities in chemical toxicology. Chemical Research in Toxicology 19:889-893.

Baillie TA (2008) Metabolism and toxicity of drugs. Two decades of progress in industrial drug metabolism. Chemical Research in Toxicology 21:129-137.

Banchereau J and Steinman RM (1998) Dendritic cells and the control of immunity. Nature 392:245-252.

Baron JM, Holler D, Schiffer R, Frankenberg S, Neis M, Merk HF and Jugert FK (2001) Expression of multiple cytochrome p450 enzymes and multidrug resistance-associated transport proteins in human skin keratinocytes. J Invest Dermatol 116:541-548.

Barranco P and Lopez-Serrano MC (1998) General and epidemiological aspects of allergic drug reactions. Clinical and Experimental Allergy, Supplement 28:61-62.

Bates DW, Spell N, Cullen DJ, Burdick E, Laird N, Petersen LA, Small SD, Sweitzer BJ and Leape LL (1997) The costs of adverse drug events in hospitalized patients. Journal of the American Medical Association 277:307-311.

Becker D, Valk E, Zahn S, Brand P and Knop J (2003) Coupling of contact sensitizers to thiol groups is a key event for the activation of monocytes

and monocyte-derived dendritic cells. Journal of Investigative Dermatology 120:233-238.

Beeler A, Engler O, Gerber BO and Pichler WJ (2006) Long-lasting reactivity and high frequency of drug-specific T cells after severe systemic drug hypersensitivity reactions. Journal of Allergy and Clinical Immunology 117:455-462.

Bergmann-Leitner ES and Leitner WW (2004) Danger, death and DNA vaccines. Microbes and Infection 6:319-327.

Berke G (1995) The CTL's kiss of death. Cell 81:9-12.

Bevan MJ (2006) Cross-priming. Nature Immunology 7:363-365.

Bhaiya P, Roychowdhury S, Vyas PM, Doll MA, Hein DW and Svensson CK (2006) Bioactivation, protein haptenation, and toxicity of sulfamethoxazole and dapsone in normal human dermal fibroblasts. Toxicology and Applied Pharmacology 215:158-167.

Bhandoola A and Sambandam A (2006) From stem cell to T cell: One route or many? Nature Reviews Immunology 6:117-126.

Bianchi ME (2007) DAMPs, PAMPs and alarmins: All we need to know about danger. Journal of Leukocyte Biology 81:1-5.

Bianchi ME and Manfredi AA (2007) High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. Immunological Reviews 220:35-46.

Billingham RE, Brent L and Medawar PB (1953) "Actively acquired tolerance" of foreign cells. Nature 172:603-606.

Biragyn A, Ruffini PA, Leifer CA, Klyushnenkova E, Shakhov A, Chertov O, Shirakawa AK, Farber JM, Segal DM, Oppenheim JJ and Kwak LW (2002) Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. Science 298:1025-1029.

Bircher AJ (2005) Symptoms and danger signs in acute drug hypersensitivity. Toxicology 209:201-207.

Bjorkman PJ, Saper MA and Samraoui B (1987) Structure of the human class I histocompatibility antigen, HLA-A2. Nature 329:506-512.

Boelsterli UA, Ho HK, Zhou S and Leow KY (2006) Bioactivation and hepatotoxicity of nitroaromatic drugs. Current Drug Metabolism 7:715-727.

Boise LH, Minn AJ, Noel PJ, June CH, Accavitti MA, Lindsten T and Thompson CB (1995) CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL.Immunity 3:87-98.

Bond CA and Raehl CL (2006) Clinical pharmacy services, pharmacy staffing, and adverse drug reactions in United States hospitals. Pharmacotherapy 26:735-747.

Bonney EA (2007) Preeclampsia: a view through the danger model. Journal of Reproductive Immunology 76:68-74.

Borràs FE, Matthews NC, Lowdell MW and Navarrete CV (2001) Identification of both myeloid CD11c+ and lymphoid CD11c- dendritic cell subsets in cord blood. British Journal of Haematology 113:925-931.

Borst P, Evers R, Kool M and Wijnholds J (1999) The multidrug resistance protein family. Biochimica et Biophysica Acta (BBA) - Biomembranes 1461:347-357.

Bort R, Ponsoda X, Jover R, Gómez-Lechón MJ and Castell JV (1999) Diclofenac toxicity to hepatocytes: A role for drug metabolism in cell toxicity. Journal of Pharmacology and Experimental Therapeutics 288:65-72.

Bour-Jordan H and Bluestone JA (2002) CD28 function: A balance of costimulatory and regulatory signals. Journal of Clinical Immunology 22:1-7.

Bours MJL, Swennen ELR, Di Virgilio F, Cronstein BN and Dagnelie PC (2006) Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacology and Therapeutics 112:358-404.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry 72:248-254.

Brander C, Mauri-Hellweg D, Bettens F, Rolli H, Goldman M and Pichler WJ (1995) Heterogeneous T cell responses to beta-lactam-modified self-

structures are observed in penicillin-allergic individuals. Journal of Immunology 155:2670-2678.

Brigl M and Brenner MB (2004) CD1: Antigen presentation and T cell function, in Annual Review of Immunology pp 817-890.

Britschgi M, Steiner UC, Schmid S, Depta JPH, Senti G, Bircher A, Burkhart C, Yawalkar N and Pichler WJ (2001) T-cell involvement in drug-induced acute generalized exanthematous pustulosis. Journal of Clinical Investigation 107:1433-1441.

Bryant PW, Lennon-Duménil AM, Fiebiger E, Lagaudrière-Gesbert C and Ploegh HL (2002) Proteolysis and antigen presentation by MHC class II molecules, in Advances in Immunology pp 71-114.

Burgdorf S, Kautz A, Böhnert V, Knolle PA and Kurts C (2007) Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. Science 316:612-616.

Burgdorf S, Schölz C, Kautz A, Tampé R and Kurts C (2008) Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. Nature Immunology 9:558-566.

Burkhart C, Von Greyerz S, Depta JPH, Naisbitt DJ, Britschgi M, Park KB and Pichler WJ (2001) Influence of reduced glutathione on the proliferative response of sulfamethoxazole-specific and sulfamethoxazole-metabolite-specific human $CD4^+$ T-cells. British Journal of Pharmacology 132:623-630.

Burnet FM (1961) Immunological recognition of self. Science 133:307-311.

Call ME and Wucherpfennig KW (2005) The T cell receptor: Critical role of the membrane environment in receptor assembly and function, in Annual Review of Immunology pp 101-125.

Callan HE, Jenkins RE, Maggs JL, Lavergne SN, Clarke SE, Naisbitt DJ and Kevin Park B (2009) Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues of peptides and proteins: Implications for hapten formation. Chemical Research in Toxicology 22:937-948.

Cano FG and Rozenfeld S (2009) Adverse drug events in hospitals: A systematic review. Eventos adversos a medicamentos em hospitais: Uma revisão sistemática 25, SUPPL. 3.

Carballal S, Radi R, Kirk MC, Barnes S, Freeman BA and Alvarez B (2003) Sulfenic acid formation in human serum albumin by hydrogen peroxide and peroxynitrite. Biochemistry 42:9906-9914.

Carbone FR and Bevan MJ (1990) Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. Journal of Experimental Medicine 171:377-387.

Carey MA and Van Pelt FNAM (2005) Immunochemical detection of flucloxacillin adduct formation in livers of treated rats. Toxicology 216:41-48.

Carr A and Cooper DA (1995) Pathogenesis and management of HIV-associated drug hypersensitivity. AIDS Clin Rev:65-97.

Carr A, Gross AS, Hoskins JM, Penny R and Cooper DA (1994) Acetylation phenotype and cutaneous hypersensitivity to trimethoprim-sulphamethoxazole in HIV-infected patients. AIDS 8:333-337.

Carr A, Tindall B, Penny R and Cooper DA (1993) In vitro cytotoxicity as a marker of hypersensitivity to sulphamethoxazole in patients with HIV. Clinical and Experimental Immunology 94:21-25.

Carreno BM and Collins M (2002) The B7 family of ligands and its receptors: New pathways for costimulation and inhibition of immune responses, in Annual Review of Immunology pp 29-53.

Casetti R and Martino A (2008) The plasticity of gamma delta T cells: innate immunity, antigen presentation and new immunotherapy. Cellular & molecular immunology 5:161-170.

Cashman JR and Zhang J (2006) Human flavin-containing monooxygenases, in Annual Review of Pharmacology and Toxicology pp 65-100.

Castrejon JL, Berry N, El-Ghaiesh S, Gerber B, Pichler WJ, Park BK and Naisbitt DJ (2010) Stimulation of human T cells with sulfonamides and sulfonamide metabolites. Journal of Allergy and Clinical Immunology 125:411-418.

Castrejon JL, Lavergne SN, El-Sheikh A, Farrell J, Maggs JL, Sabbani S, O'Neill PM, Kevin Park B and Naisbitt DJ (2009) Metabolic and chemical origins of cross-reactive immunological reactions to arylamine benzenesulfonamides: T-cell responses to hydroxylamine and nitroso derivatives. Chemical Research in Toxicology 23:184-192.

Caux C, Massacrier C, Vanbervliet B, Dubois B, De Saint-Vis B, Dezutter-Dambuyant C, Jacquet C, Schmitt D and Banchereau J (1997) CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha, in Advances in Experimental Medicine and Biology pp 21-25.

Caux C, Vanbervliet B, Massacrier C, Dezutter-Dambuyant C, De Saint-Vis B, Jacquet C, Yoneda K, Imamura S, Schmitt D and Banchereau J (1996) CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. Journal of Experimental Medicine 184:695-706.

Cavani A, Hackett CJ, Wilson KJ, Rothbard JB and Katz SI (1995) Characterization of epitopes recognized by hapten-specific CD4+T cells. Journal of Immunology 154:1232-1238.

Cavani A, Mei D, Guerra E, Corinti S, Giani M, Pirrotta L, Puddu P and Girolomoni G (1998) Patients with allergic contact dermatitis to nickel and nonallergic individuals display different nickel-specific T cell responses. Evidence for the presence of effector CD8+ and regulatory CD4+T cells. Journal of Investigative Dermatology 111:621-628.

Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A and Alber G (1996) Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. Journal of Experimental Medicine 184:747-752.

Chakraborty AK (2002) Lighting up TCR takes advantage of serial triggering. Nature Immunology 3:895-896.

Chambers CA, Kuhns MS, Egen JG and Allison JP (2001) CTLA-4-mediated inhibition in regulation of T cell responses: Mechanisms and manipulation in tumor immunotherapy, in Annual Review of Immunology pp 565-594.

Cheng L, Stewart BJ, You Q, Petersen DR, Ware JA, Piccotti JR, Kawabata TT and Ju C (2008) Covalent binding of the nitroso metabolite of sulfamethoxazole is important in induction of drug-specific T-cell responses in vivo. Molecular Pharmacology 73:1769-1775.

Chessman D, Kostenko L, Lethborg T, Purcell AW, Williamson NA, Chen Z, Kjer-Nielsen L, Mifsud NA, Tait BD, Holdsworth R, Almeida CA, Nolan D, Macdonald WA, Archbold JK, Kellerher AD, Marriott D, Mallal S, Bharadwaj M, Rossjohn J and McCluskey J (2008) Human Leukocyte Antigen Class I-Restricted Activation of CD8+ T Cells Provides the Immunogenetic Basis of a Systemic Drug Hypersensitivity. Immunity 28:822-832.

Choquet-Kastylevsky G, Santolaria N, Tedone R, Aujoulat M and Descotes J (2001) Induction of delayed-type hypersensitivity to sulfamethoxazole in mice: Role of metabolites. Toxicology Letters 119:183-192.

Christiansen C (2002) Late-onset allergy-like reactions to X-ray contrast media. Current Opinion in Allergy and Clinical Immunology 2:333-339.

Christiansen C, Pichler WJ and Skotland T (2000) Delayed allergy-like reactions to X-ray contrast media: Mechanistic considerations. European Radiology 10:1965-1975.

Chung CH, Mirakhur B, Chan E, Le QT, Berlin J, Morse M, Murphy BA, Satinover SM, Hosen J, Mauro D, Slebos RJ, Zhou Q, Gold D, Hatley T, Hicklin DJ and Platts-Mills TAE (2008) Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. New England Journal of Medicine 358:1109-1117.

Chung WH, Hung SI, Hong HS, Hsih MS, Yang LC, Ho HC, Wu JY and Chen YT (2004) Medical genetics: a marker for Stevens-Johnson syndrome. Nature 428:486.

Clark RA, Chong B, Mirchandani N, Brinster NK, Yamanaka KI, Dowgiert RK and Kupper TS (2006) The vast majority of CLA+T cells are resident in normal skin. Journal of Immunology 176:4431-4439.

Colonna M, Trinchieri G and Liu YJ (2004) Plasmacytoid dendritic cells in immunity. Nature Immunology 5:1219-1226.

Constant SL and Bottomly K (1997) Induction of Th1 and Th2 CD4+ T cell responses: The alternative approaches, in Annual Review of Immunology pp 297-322.

Coulter EM, Farrell J, Mathews KL, Maggs JL, Pease CK, Lockley DJ, Basketter DA, Park BK and Naisbitt DJ (2007) Activation of human dendritic cells by p-phenylenediamine. Journal of Pharmacology and Experimental Therapeutics 320:885-892.

Cribb AE and Spielberg SP (1990) Hepatic microsomal metabolism of sulfamethoxazole to the hydroxylamine. Drug Metabolism and Disposition 18:784-787.

Cribb AE and Spielberg SP (1992) Sulfamethoxazole is metabolized to the hydroxylamine in humans. Clinical Pharmacology and Therapeutics 51:522-526.

Cribb AE, Lee BL, Trepanier LA and Spielberg SP (1996a) Adverse reactions to sulphonamide and sulphonamide-trimethoprim antimicrobials: Clinical syndromes and pathogenesis. Adverse Drug Reactions and Toxicological Reviews 15:9-50.

Cribb AE, Miller M, Leeder JS, Hill J and Spielberg SP (1991) Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione: Implications for idiosyncratic toxicity. Drug Metabolism and Disposition 19:900-906.

Cribb AE, Miller M, Tesoro A and Spielberg SP (1990) Peroxidase-dependent oxidation of sulfonamides by monocytes and neutrophils from humans and dogs. Molecular Pharmacology 38:744-751.

Cribb AE, Nakamura H, Grant DM, Miller MA and Spielberg SP (1993) Role of polymorphic and monomorphic human arylamine N-acetyltransferase in determining sulfamethoxazole metabolism. Biochemical Pharmacology 45:1277-1282.

Cribb AE, Nuss CE, Alberts DW, Lamphere DB, Grant DM, Grossman SJ and Spielberg SP (1996b) Covalent binding of sulfamethoxazole reactive metabolites to human and rat liver subcellular fractions assessed by immunochemical detection. Chemical Research in Toxicology 9:500-507.

Cribb AE, Pohl LR, Spielberg SP and Leeder JS (1997) Patients with delayed-onset sulfonamide hypersensitivity reactions have antibodies recognizing endoplasmic reticulum luminal proteins. Journal of Pharmacology and Experimental Therapeutics 282:1064-1072.

Cribb AE, Spielberg SP and Griffin GP (1995) N4-hydroxylation of sulfamethoxazole by cytochrome P450 of the cytochrome P4502C subfamily and reduction of sulfamethoxazole hydroxylamine in human and rat hepatic microsomes. Drug Metabolism and Disposition 23:406-414.

Croft M (1994) Activation of naive, memory and effector T cells. Current Opinion in Immunology 6:431-437.

Curtsinger JM and Mescher MF (2010) Inflammatory cytokines as a third signal for T cell activation. Current Opinion in Immunology 22:333-340.

Curtsinger JM, Schmidt CS, Mondino A, Lins DC, Kedl RM, Jenkins MK and Mescher MF (1999) Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+T cells. Journal of Immunology 162:3256-3262.

Daftarian MP, Filion LG, Cameron W, Conway B, Roy R, Tropper F and Diaz-Mitoma F (1995) Immune response to sulfamethoxazole in patients with AIDS. Clinical and Diagnostic Laboratory Immunology 2:199-204.

Davies EC, Green CF, Taylor S, Williamson PR, Mottram DR and Pirmohamed M (2009) Adverse drug reactions in hospital in-patients: A prospective analysis of 3695 patient-episodes. PLoS ONE 4:e4439. Epub

De Jong JMH, Schuurhuis DH, Ioan-Facsinay A, Welling MM, Camps MGM, Van Der Voort EIH, Huizinga TWJ, Ossendorp F, Verbeek JS and Toes REM (2006) Dendritic cells, but not macrophages or B cells, activate major histocompatibility complex class II-restricted CD4+T cells upon immune-complex uptake in vivo. Immunology 119:499-506.

De Smedt T, Pajak B, Muraille E, Lespagnard L, Heinen E, De Baetselier P, Urbain J, Leo O and Moser M (1996) Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. Journal of Experimental Medicine 184:1413-1424.

Depta JPH, Altznauer F, Gamerdinger K, Burkhart C, Weltzien HU and Pichler WJ (2004) Drug interaction with T-cell receptors: T-cell receptor density determines degree of cross-reactivity. Journal of Allergy and Clinical Immunology 113:519-527.

Dey A, Parmar D, Dayal M, Dhawan A and Seth PK (2001) Cytochrome P450 1A1 (CYP1A1) in blood lymphocytes: Evidence for catalytic activity and mRNA expression. Life Sciences 69:383-393.

DiMasi JA, Hansen RW and Grabowski HG (2003) The price of innovation: New estimates of drug development costs. Journal of Health Economics 22:151-185.

Dirienzo AG, Van Der Horst C, Finkelstein DM, Frame P, Bozzette SA and Tashima KT (2002) Efficacy of trimethoprim-sulfamethoxazole for the prevention of bacterial infections in a randomized prophylaxis trial of Patients with advanced HIV infection. AIDS Research and Human Retroviruses 18:89-94.

Doerge DR, Divi RL, Deck J and Taurog A (1997) Mechanism for the anti-thyroid action of minocycline. Chemical Research in Toxicology 10:49-58.

Domagk G (1935) Beitrag zur Chemotherapie der bakteriellen Infektionen. Dtsch Med Wschr 7: 250–253.

Dong C (2008) TH17 cells in development: An updated view of their molecular identity and genetic programming. Nature Reviews Immunology 8:337-348.

Dormann H, Neubert A, Criegee-Rieck M, Egger T, Radespiel-Tröger M, Azaz-Livshits T, Levy M, Brune K and Hahn EG (2004) Readmissions and adverse drug reactions in internal medicine: The economic impact. Journal of Internal Medicine 255:653-663.

Du L, Hoffman SMG and Keeney DS (2004) Epidermal CYP2 family cytochromes P450. Toxicology and Applied Pharmacology 195:278-287.

Dubin PJ, McAllister F and Kolls JK (2007) Is cystic fibrosis a TH17 disease? Inflammation Research 56:221-227.

Dustin ML (2005) A dynamic view of the immunological synapse. Seminars in Immunology 17:400-410.

Edwards IR and Aronson JK (2000) Adverse drug reactions: Definitions, diagnosis, and management. Lancet 356:1255-1259.

Ellrodt AG, Murata GH and Riedinger MS (1984) Severe neutropenia associated with sustained-release procainamide. Annals of Internal Medicine 100:197-201.

Emoto C, Murase S, Sawada Y and Iwasaki K (2005) In vitro inhibitory effect of 1-aminobenzotriazole on drug oxidations in human liver microsomes: a comparison with SKF-525A. Drug metabolism and pharmacokinetics. 20:351-357.

Emoto M, Zerrahn J, Miyamoto M, Perarnau B and Kaufmann SHE (2000) Characterization of MHC class II-presented peptides generated from an antigen targeted to different endocytic compartments. European Journal of Immunology 30:2333-2343.

Engelhard VH (1994) Structure of peptides associated with class I and class II MHC molecules. Annual Review of Immunology 12:181-207.

Erve JCL (2006) Chemical toxicology: Reactive intermediates and their role in phrmacology and toxicology. Expert Opinion on Drug Metabolism and Toxicology 2:923-946.

Eswaramoorthy S, Bonanno JB, Burley SK and Swaminathan S (2006) Mechanism of action of a flavin-containing monooxygenase. Proceedings of the National Academy of Sciences of the United States of America 103:9832-9837.

Fairchild PJ (1998) Presentation of antigenic peptides by products of the major histocompatibility complex. Journal of Peptide Science 4:182-194.

Farkas AM, Kilgore TM and Lotze MT (2007) Detecting DNA: Getting and begetting cancer. Current Opinion in Investigational Drugs 8:981-986.

Farrell J, Naisbitt DJ, Drummond NS, Depta JPH, Vilar FJ, Pirmohamed M and Park BK (2003) Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and humans. Journal of Pharmacology and Experimental Therapeutics 306:229-237.

Fattinger K, Roos M, Vergères P, Holenstein C, Kind B, Masche U, Stocker DN, Braunschweig S, Kullak-Ublick GA, Galeazzi RL, Follath F, Gasser T and Meier PJ (2000) Epidemiology of drug exposure and adverse drug reactions in two Swiss departments of internal medicine. British Journal of Clinical Pharmacology 49:158-167.

Ferner RE and Aronson JK (1999) Errors in prescribing, preparing, and giving medicines: definition, classification, and prevention, in Side Effects of Drugs Annual 22: xxiii-xxxvi

Fischl MA, Dickinson GM and La Voie L (1988) Safety and efficacy of sulfamethoxazole and trimethoprim chemoprophylaxis for Pneumocystis carinii pneumonia in AIDS. Journal of the American Medical Association 259:1185-1189.

Foell D, Wittkowski H and Roth J (2007) Mechanisms of Disease: A 'DAMP' view of inflammatory arthritis. Nature Clinical Practice Rheumatology 3:382-390.

French LE (2006) Toxic epidermal necrolysis and Stevens Johnson syndrome: Our current understanding. Allergology International 55:9-16.

Friedmann PS, Strickland I, Pirmohamed M and Park BK (1994) Investigation of mechanisms in toxic epidermal necrolysis induced by carbamazepine. Archives of Dermatology 130:598-604.

Furrer H, Egger M, Opravil M, Bernasconi E, Hirschel B, Battegay M, Telenti A, Vernazza PL, Rickenbach M, Flepp M and Malinverni R (1999) Discontinuation of primary prophylaxis against Pneumocystis carinii pneumonia in HIV-1-infected adults treated with combination antiretroviral therapy. New England Journal of Medicine 340:1301-1306.

Furst SM and Uetrecht JP (1993) Carbamazepine metabolism to a reactive intermediate by the myeloperoxidase system of activated neutrophils. Biochemical Pharmacology 45:1267-1275.

Furtmuller PG, Burner U, Jantschko W, Regelsberger G and Obinger C (2000) The reactivity of myeloperoxides compound I formed with hypochlorous acid. Redox Report 5:173-178.

Furtmuller PG, Zederbauer M, Jantschko W, Helm J, Bogner M, Jakopitsch C and Obinger C (2006) Active site structure and catalytic mechanisms of human peroxidases. Archives of Biochemistry and Biophysics 445:199-213.

Gallucci S, Lolkema M and Matzinger P (1999) Natural adjuvants: Endogenous activators of dendritic cells. Nature Medicine 5:1249-1255.

Gehrig KA and Warshaw EM (2008) Allergic contact dermatitis to topical antibiotics: Epidemiology, responsible allergens, and management. Journal of the American Academy of Dermatology 58:1-21.

Gell P and Coombs R (1963) In: Clinical Aspects of Immunology. 1st ed. Oxford, England: Blackwell. .

Gerner MY and Mescher MF (2009) Antigen processing and MHC-II presentation by dermal and tumor-infiltrating dendritic cells. Journal of Immunology 182:2726-2737.

Gill HJ, Hough SJ, Naisbitt DJ, Maggs JL, Kitteringham NR, Pirmohamed M and Park BK (1997) The relationship between the disposition and immunogenicity of sulfamethoxazole in the rat. Journal of Pharmacology and Experimental Therapeutics 282:795-801.

Gill HJ, Maggs JL, Madden S, Pirmohamed M and Park BK (1996) The effect of fluconazole and ketoconazole on the metabolism of sulphamethoxazole. British Journal of Clinical Pharmacology 42:347-353.

Goebel G, Vogel C, Wulferink M, Mittmann S, Sachs B, Schraa S, Abel J, Degen G, Uetrecht J and Gleichmann E (1999) Procainamide, a drug causing lupus, induces prostaglandin H synthase-2 and formation of T cell-sensitizing drug metabolites in mouse macrophages. Chemical Research in Toxicology 12:488-500.

Goldie SJ, Kaplan JE, Losina E, Weinstein MC, Paltiel AD, Seage Iii GR, Craven DE, Kimmel AD, Zhang H, Cohen CJ and Freedberg KA (2002) Prophylaxis for human immunodeficiency virus-related Pneumocystis carinii pneumonia: Using simulation modeling to inform clinical guidelines. Archives of Internal Medicine 162:921-928.

Goodwin DC, Grover TA and Aust SD (1996) Redox mediation in the peroxidase-catalyzed oxidation of aminopyrine: Possible implications for drug-drug interactions. Chemical Research in Toxicology 9:476-483.

Gorczynski RM, Hadidi S, Yu G and Clark DA (2002) The same immunoregulatory molecules contribute to successful pregnancy and transplantation. American Journal of Reproductive Immunology 48:18-26.

Gordin FM, Simon GL, Wofsy CB and Mills J (1984) Adverse reactions to trimethoprim-sulfamethoxazole in patients with the acquired immunodeficiency syndrome. Annals of Internal Medicine 100:495-499.

Gould JW, Mercurio MG and Elmets CA (1995) Cutaneous photosensitivity diseases induced by exogenous agents. Journal of the American Academy of Dermatology 33:551-576.

Grahame-Smith D and Aronson J (1984) The Oxford textbook of clinical pharmacology and drug therapy. Oxford: Oxford University Press, 134.

Greenberger PA (2006) 8. Drug allergy. Journal of Allergy and Clinical Immunology 117.

Greenwald RJ, Freeman GJ and Sharpe AH (2005) The B7 family revisited, in Annual Review of Immunology pp 515-548.

Griem P, Wulferink M, Sachs B, González JB and Gleichmann E (1998) Allergic and autoimmune reactions to xenobiotics: How do they arise? Immunology Today 19:133-141.

Grimwade K and Swingler (2003) Cotrimoxazole prophylaxis for opportunistic infections in adults with HIV. Cochrane database of systematic reviews (Online).

Grouard G, Rissoan MC, Filgueira L, Durand I, Banchereau J and Liu YJ (1997) The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. Journal of Experimental Medicine 185:1101-1111.

Groves RW, Allen MH, Ross EL, Barker JNWN and MacDonald DM (1995) Tumour necrosis factor alpha is pro-inflammatory in normal human skin and modulates cutaneous adhesion molecule expression. British Journal of Dermatology 132:345-352.

Gruchalla RS, Pesenko RD, Do TT and Skiest DJ (1998) Sulfonamide-induced reactions in desensitized patients with AIDS-The role of covalent protein haptenation by sulfamethoxazole. Journal of Allergy and Clinical Immunology 101:371-378.

Guengerich FP (2004) Cytochrome P450: What have we learned and what are the future issues? Drug Metabolism Reviews 36:159-197.

Guengerich FP (2008) Cytochrome P450 and chemical toxicology. Chemical Research in Toxicology 21:70-83.

Guermonprez P, Saveanu L, Kleijmeer M, Davoust J, Van Endert P and Amigorena S (2003) ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature 425:397-402.

Guermonprez P, Valladeau J, Zitvogel L, Théry C and Amigorena S (2002) Antigen presentation and T cell stimulation by dendritic cells, in Annual Review of Immunology pp 621-667.

Haase C, Ejrnaes M, Juedes AE, Wolfe T, Markholst H and Von Herrath MG (2005) Immunomodulatory dendritic cells require autologous serum to circumvent nonspecific immunosuppressive activity in vivo. Blood 106:4225-4233.

Halevy S, Ghislain PD, Mockenhaupt M, Fagot JP, Bouwes Bavinck JN, Sidoroff A, Naldi L, Dunant A, Viboud C and Roujeau JC (2008) Allopurinol is the most common cause of Stevens-Johnson syndrome and toxic epidermal necrolysis in Europe and Israel. Journal of the American Academy of Dermatology 58:25-32.

Harboe T, Johansson SGO, Florvaag E and Öman H (2007) Pholcodine exposure raises serum IgE in patients with previous anaphylaxis to neuromuscular blocking agents. Allergy: European Journal of Allergy and Clinical Immunology 62:1445-1450.

Hari Y, Frutig-Schnyder K, Hurni M, Yawalkar N, Zanni MP, Schnyder B, Kappeler A, Von Greyerz S, Braathen LR and Pichler WJ (2001) T cell involvement in cutaneous drug eruptions. Clinical and Experimental Allergy 31:1398-1408.

Hartigan-Go K and Wong J (2000) Inclusion of therapeutic failures as adverse drug reactions. In: Aronson JK, editor. Side effects of drugs. Annual 23. Amsterdam: Elsevier, xxvii-xxxiii.

Hashimoto K, Yasukawa M and Tohyama M (2003) Human herpesvirus 6 and drug allergy. Current Opinion in Allergy and Clinical Immunology 3:255-260.

Heath WR (1999) Cytotoxic T lymphocyte activation by cross-priming. Current Opinion in Immunology 11:314-318.

Heath WR and Carbone FR (2001) Cross-presentation in viral immunity and self-tolerance. Nature Reviews Immunology 1:126-134.

Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K and Akira S (2002) Small-antiviral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nature Immunology 3:196-200.

Henri S, Vremec D, Kamath A, Waithman J, Williams S, Benoist C, Burnham K, Saeland S, Handman E and Shortman K (2001) The dendritic cell populations of mouse lymph nodes. Journal of Immunology 167:741-748.

Hertl M and Merk HF (1995) Lymphocyte activation in cutaneous drug reactions. Journal of Investigative Dermatology 105.

Hertl M, Bohlen H, Jugert F, Boecker C, Knaup R and Merk HF (1993) Predominance of epidermal CD8+ T lymphocytes in bullous cutaneous reactions caused by β-lactam antibiotics. Journal of Investigative Dermatology 101:794-799.

Hertl M, Jugert F and Merk HF (1995) CD8+ dermal T cells from a sulphamethoxazole-induced bullous exanthem proliferate in response to drug-modified liver microsomes. British Journal of Dermatology 132:215-220.

Hess DA, Sisson ME, Suria H, Wijsman J, Puvanesasingham R, Madrenas J and Rieder MJ (1999) Cytotoxicity of sulfonamide reactive metabolites: Apoptosis and selective toxicity of CD8+ cells by the hydroxylamine of sulfamethoxazole. FASEB Journal 13:1688-1698.

Hewitt EW (2003) The MHC class I antigen presentation pathway: Strategies for viral immune evasion. Immunology 110:163-169.

Hofstra AH and Uetrecht JP (1993) Myeloperoxidase-mediated activation of xenobiotics by human leukocytes. Toxicology 82:221-242.

Horton R, Wilming L, Rand V, Lovering RC, Bruford EA, Khodiyar VK, Lush MJ, Povey S, Talbot Jr CC, Wright MW, Wain HM, Trowsdale J, Ziegler A and Beck S (2004) Gene map of the extended human MHC. Nature Reviews Genetics 5:889-899.

Hung SL, Chung WH, Liou LB, Chu CC, Lin M, Huang HP, Lin YL, Lan JL, Yang LC, Hong HS, Chen MJ, Lai PC, Wu MS, Chu CY, Wang KH, Chen CH, Fann CSJ, Wu JY and Chen YT (2005) HLA-B*5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. Proceedings of the National Academy of Sciences of the United States of America 102:4134-4139.

Iezzi G, Boni A, Degl'Innocenti E, Grioni M, Bertilaccio MTS and Bellone M (2006) Type 2 cytotoxic T lymphocytes modulate the activity of dendritic cells toward type 2 immune responses. Journal of Immunology 177:2131-2137.

Imai J, Hasegawa H, Maruya M, Koyasu S and Yahara I (2005) Exogenous antigens are processed through the endoplasmic reticulum-associated degradation (ERAD) in cross-presentation by dendritic cells. International Immunology 17:45-53.

Inman W (1977) Detection and investigation of adverse drug reactions. In Davis DM (ed): Textbook of adverse drug reactions, Oxford U Pr, Oxford, Engl, 41-53.

Janeway Jr CA (1989) Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor Symposia on Quantitative Biology 54:1-13.

Janeway Jr CA (1992) The immune system evolved to discriminate infectious nonself from noninfectious self. Immunology Today 13:11-16.

Janeway Jr CA and Medzhitov R (2002) Innate immune recognition, in Annual Review of Immunology pp 197-216.

Janmohamed A, Dolphin CT, Phillips IR and Shephard EA (2001) Quantification and cellular localization of expression in human skin of genes encoding flavin-containing monooxygenases and cytochromes P450. Biochemical Pharmacology 62:777-786.

Jefferys DB, Leakey D, Lewis JA, Payne S and Rawlins MD (1998) New active substances authorized in the United Kingdom between 1972 and 1994. British Journal of Clinical Pharmacology 45:151-156.

Jenkins RE, Meng X, Elliott VL, Kitteringham NR, Pirmohamed M and Park BK (2009) Characterisation of flucloxacillin and 5-hydroxymethyl flucloxacillin haptenated HSA in vitro and in vivo. Proteomics - Clinical Applications 3:720-729.

Jick H (1982) Adverse reactions to trimethoprim-sulfamethoxazole in hospitalized patients. Reviews of Infectious Diseases 4:426-428.

June CH, Ledbetter JA, Gillespie MM, Lindsten T and Thompson CB (1987) T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. Molecular and Cellular Biology 7:4472-4481.

Kadri N, Potiron N, Ouary M, Jegou D, Gouin E, Bach JM and Lieubeau B (2007) Fetal calf serum-primed dendritic cells induce a strong anti-fetal calf serum immune response and diabetes protection in the non-obese diabetic mouse. Immunology Letters 108:129-136.

Kalgutkar AS and Soglia JR (2005) Minimising the potential for metabolic activation in drug discovery. Expert opinion on drug metabolism & toxicology 1:91-142.

Kalgutkar AS, Gardner I, Obach RS, Shaffer CL, Callegari E, Henne KR, Mutlib AE, Dalvie DK, Lee JS, Nakai Y, O'Donnell JP, Boer J and Harriman SP (2005) A comprehensive listing of bioactivation pathways of organic functional groups. Current Drug Metabolism 6:161-225.

Kalish RS and Askenase PW (1999) Molecular mechanisms of CD8+T cell-mediated delayed hypersensitivity: Implications for allergies, asthma, and autoimmunity. Journal of Allergy and Clinical Immunology 103:192-199.

Kanekura T, Laulederkind SJF, Kirtikara K, Goorha S and Ballou LR (1998) Cholecalciferol induces prostaglandin E2 biosynthesis and transglutaminase activity in human keratinocytes. Journal of Investigative Dermatology 111:634-639.

Kapsenberg ML (2003) Dendritic-cell control of pathogen-driven T-cell polarization. Nature Reviews Immunology 3:984-993.

Karch FE and Lasagna L (1975) Adverse drug reactions. A critical review. Journal of the American Medical Association 234:1236-1241.

Keller M, Spanou Z, Schaerli P, Britschgi M, Yawalkar N, Seitz M, Villiger PM and Pichler WJ (2005) T cell-regulated neutrophilic inflammation in autoinflammatory diseases. Journal of Immunology 175:7678-7686.

Kenny JR, Maggs JL, Meng X, Sinnott D, Clarke SE, Park BK and Stachulski AV (2004) Syntheses and characterization of the acyl glucuronide and hydroxy metabolites of diclofenac. Journal of Medicinal Chemistry 47:2816-2825.

Kimber I and Cumberbatch M (1992) Dendritic cells and cutaneous immune responses to chemical allergens. Toxicology and Applied Pharmacology 117:137-146.

Kimura S and Ikeda-Saito M (1988) Human myeloperoxidase and thyroid peroxidase, two enzymes with separate and distinct physiological functions, are evolutionarily related members of the same gene family. Proteins: Structure, Function and Genetics 3:113-120.

Kinobe RT, Parkinson OT, Mitchell DJ and Gillam EMJ (2005) P450 2C18 catalyzes the metabolic bioactivation of phenytoin. Chemical Research in Toxicology 18:1868-1875.

Kishi S, Yang W, Boureau B, Morand S, Das S, Chen P, Cook EH, Rosner GL, Schuetz E, Pui CH and Relling MV (2004) Effects of prednisone and genetic polymorphisms on etoposide disposition in children with acute lymphoblastic leukemia. Blood 103:67-72.

Klein JAN and Sato A (2000) The HLA system: First of two parts. New England Journal of Medicine 343:702-709.

Kloetzel PM (2004) The proteasome and MHC class I antigen processing. Biochimica et Biophysica Acta - Molecular Cell Research 1695:225-233.

Koch C, Hjelt K, Pedersen SS, Jensen ET, Jensen T, Lanng S, Valerius NH, Pedersen M and Hoiby N (1991) Retrospective clinical study of hypersensitivity reactions to aztreonam and six other beta-lactam antibiotics in cystic fibrosis patients receiving multiple treatment courses. Reviews of Infectious Diseases 13:Suppl 7: S608-611.

Koch-Weser J, Sidel VW, Dexter M, Parish C, Finer DC and Kanarek P (1971) Adverse reactions to sulfosoxazole, sulfamethoxazole, and nitrofurantoin. Manifestations and specific reaction rates during 2,118 courses of therapy. Archives of Internal Medicine 128:399-404.

Koenigs LL, Peter RM, Hunter AP, Haining RL, Rettie AE, Friedberg T, Pritchard MP, Shou M, Rushmore TH and Trager WF (1999) Electrospray ionization mass spectrometric analysis of intact cytochrome P450: Identification of tienilic acid adducts to P450 2C9. Biochemistry 38:2312-2319.

Kong KF, Schneper L and Mathee K (2010) Beta-lactam antibiotics: From antibiosis to resistance and bacteriology. APMIS 118:1-36.

Kono H and Rock KL (2008) How dying cells alert the immune system to danger. Nature Reviews Immunology 8:279-289.

Kouklakis G, Mpoumponaris A, Zezos P, Moschos J, Koulaouzidis A, Nakos A, Pehlivanidis A, Iosiphidis M, Molyvas E, Nikolaidis N and Koulaouzidis T (2007) Cholestatic hepatitis with severe systemic reactions induced by trimethoprim-sulfamethoxazole. Annals of Hepatology 6:63-65.

Kovacsovics-Bankowski M and Rock KL (1995) A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science 267:243-246.

Krovat BC, Tracy JH and Omiecinski CJ (2000) Fingerprinting of cytochrome P450 and microsomal epoxide hydrolase gene expression in human blood cells. Toxicological Sciences 55:352-360.

Kuhns MS, Davis MM and Garcia KC (2006) Deconstructing the form and function of the TCR/CD3 complex. Immunity 24:133-139.

Kurian JR, Bajad SU, Miller JL, Chin NA and Trepanier LA (2004) NADH cytochrome b5 reductase and cytochrome b5 catalyze the microsomal

reduction of xenobiotic hydroxylamines and amidoximes in humans. Journal of Pharmacology and Experimental Therapeutics 311:1171-1178.

Kvedariene V, Martins P, Rouanet L and Demoly P (2006) Diagnosis of iodinated contrast media hypersensitivity: Results of a 6-year period. Clinical and Experimental Allergy 36:1072-1077.

Labrecque N, Whitfield LS, Obst R, Waltzinger C, Benoist C and Mathis D (2001) How much TCR does a T cell need? Immunity 15:71-82.

Lai WG, Zahid N and Uetrecht JP (1999) Metabolism of trimethoprim to a reactive iminoquinone methide by activated human neutrophils and hepatic microsomes. Journal of Pharmacology and Experimental Therapeutics 291:292-299.

Landmann S, Mühlethaler-Mottet A, Bernasconi L, Suter T, Waldburger JM, Masternak K, Arrighi JF, Hauser C, Fontana A and Reith W (2001) Maturation of dendritic cells is accompanied by rapid transcriptional silencing of class II transactivator (CIITA) expression. Journal of Experimental Medicine 194:379-391.

Landsteiner K and Jacobs J (1935) Studies on the sensitization of animals with simple chemical compounds. Journal of Experimental Medicine 61:643-656.

Lanzavecchia A and Sallusto F (2001) The instructive role of dendritic cells on T cell responses: Lineages, plasticity and kinetics. Current Opinion in Immunology 13:291-298.

Lasser KE, Allen PD, Woolhandler SJ, Himmelstein DU, Wolfe SM and Bor DH (2002) Timing of new black box warnings and withdrawals for prescription medications. Journal of the American Medical Association 287:2215-2220.

Laurence D and Bennett P (1992) Clinical pharmacology. Edinburgh: Churchill Livingstone, 121-2.

Laurence D and Carpenter J (1998) A dictionary of pharmacology and allied topics, 2nd edn. Amsterdam: Elsevier, 8–9.

Lavergne SN, Danhof RS, Volkman EM and Trepanier LA (2006a) Association of drug-serum protein adducts and anti-drug antibodies in dogs with

sulphonamide hypersensitivity: A naturally occurring model of idiosyncratic drug toxicity. Clinical and Experimental Allergy 36:907-915.

Lavergne SN, Drescher NJ and Trepanier LA (2008a) Anti-myeloperoxidase and anti-cathepsin G antibodies in sulphonamide hypersensitivity. Clinical and Experimental Allergy 38:199-207.

Lavergne SN, Kurian JR, Bajad SU, Maki JE, Yoder AR, Guzinski MV, Graziano FM and Trepanier LA (2006b) Roles of endogenous ascorbate and glutathione in the cellular reduction and cytotoxicity of sulfamethoxazole-nitroso. Toxicology 222:25-36.

Lavergne SN, Park BK and Naisbitt DJ (2008b) The roles of drug metabolism in the pathogenesis of T-cell-mediated drug hypersensitivity. Current Opinion in Allergy and Clinical Immunology 8:299-307.

Lavergne SN, Wang H, Callan HE, Park BK and Naisbitt DJ (2009) "Danger" conditions increase sulfamethoxazole-protein adduct formation in human antigen-presenting cells. Journal of Pharmacology and Experimental Therapeutics 331:372-381.

Lavergne SN, Whitaker P, Peckham D, Conway S, Park BK and Naisbitt DJ (2010) Drug metabolite-specific lymphocyte responses in sulfamethoxazole allergic patients with cystic fibrosis. Chemical Research in Toxicology 23:1009-1011.

Lazarou J, Pomeranz BH and Corey PN (1998) Incidence of adverse drug reactions in hospitalized patients: A meta- analysis of prospective studies. Journal of the American Medical Association 279:1200-1205.

Le Bouteiller P and Tabiasco J (2006) Immunology of pregnancy: Renewed interest. Immunologie de la grossesse: Faits nouveaux 22:745-750.

LeBlanc R, Hideshima T, Catley LP, Shringarpure R, Burger R, Mitsiades N, Mitsiades C, Cheema P, Chauhan D, Richardson PG, Anderson KC and Munshi NC (2004) Immunomodulatory drug costimulates T cells via the B7-CD28 pathway. Blood 103:1787-1790.

Lecoeur S, Andre C and Beaune PH (1996) Tienilic acid-induced autoimmune hepatitis: Anti-liver and -kidney microsomal type 2 autoantibodies

recognize a three-site conformational epitope on cytochrome P4502C9. Molecular Pharmacology 50:326-333.

Lee HK and Iwasaki A (2007) Innate control of adaptive immunity: Dendritic cells and beyond. Seminars in Immunology 19:48-55.

Leeder JS, Dosch HM and Spielberg SP (1991) Cellular toxicity of sulfamethoxazole reactive metabolites - I. Inhibition of intracellular esterase activity prior to cell death. Biochemical Pharmacology 41:567-574.

Leslie EM, Deeley RG and Cole SPC (2001) Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. Toxicology 167:3-23.

Levine R (1973) Factors modifying the effects of drugs in individuals. In: Pharmacology: drug actions and reactions. Boston (MA): Little, Brown and Co, 261-91.

Li J and Uetrecht J (2010) The danger hypothesis applied to idiosyncratic drug reactions, In: Adverse Drug Reactions, Handbook of Experimental pharmacology 196, J. Uetrecht (ed.) Springer-Verlag Berlin Heidelberg.

Liebler DC and Guengerich FP (2005) Elucidating mechanisms of drug-induced toxicity. Nature Reviews Drug Discovery 4:410-420.

Liu W, Putnam AL, Xu-yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, De St. Groth BF, Clayberger C, Soper DM, Ziegler SF and Bluestone JA (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. Journal of Experimental Medicine 203:1701-1711.

Lonjou C, Borot N, Sekula P, Ledger N, Thomas L, Halevy S, Naldi L, Bouwes-Bavinck JN, Sidoroff A, De Toma C, Schumacher M, Roujeau JC, Hovnanian A and Mockenhaupt M (2008) A European study of HLA-B in Stevens-Johnson syndrome and toxic epidermal necrolysis related to five high-risk drugs. Pharmacogenetics and Genomics 18:99-107.

Lu W and Uetrecht JP (2008) Peroxidase-mediated bioactivation of hydroxylated metabolites of carbamazepine and phenytoin. Drug Metabolism and Disposition 36:1624-1636.

Lugardon S, Desboeuf K, Fernet P, Montastruc JL and Lapeyre-Mestre M (2006) Using a capture-recapture method to assess the frequency of adverse drug reactions in a French university hospital. British Journal of Clinical Pharmacology 62:225-231.

Lund RJ, Chen Z, Scheinin J and Lahesmaa R (2004) Early target genes of IL-12 and STAT4 signaling in Th cells. Journal of Immunology 172:6775-6782.

Lund RJ, Löytömäki M, Naumanen T, Dixon C, Chen Z, Ahlfors H, Tuomela S, Tahvanainen J, Scheinin J, Henttinen T, Rasool O and Lahesmaa R (2007) Genome-wide identification of novel genes involved in early Th1 and Th2 cell differentiation. Journal of Immunology 178:3648-3660.

Lund RJ, Ylikoski EK, Aittokallio T, Nevalainen O and Lahesmaa R (2003) Kinetics and STAT4- or STAT6-mediated regulation of genes involved in lymphocyte polarization to Th1 and Th2 cells. European Journal of Immunology 33:1105-1116.

Lutz MB and Rossner S (2008) Factors influencing the generation of murine dendritic cells from bone marrow: The special role of fetal calf serum. Immunobiology 212:855-862.

Lutz MB and Schuler G (2002) Immature, semi-mature and fully mature dendritic cells: Which signals induce tolerance or immunity? Trends in Immunology 23:445-449.

Lutz MB, Kukutsch N, Ogilvie ALJ, Rößner S, Koch F, Romani N and Schuler G (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. Journal of Immunological Methods 223:77-92.

Macian F (2005) NFAT proteins: Key regulators of T-cell development and function. Nature Reviews Immunology 5:472-484.

Maggi E, Cosmi L, Liotta F, Romagnani P, Romagnani S and Annunziato F (2005) Thymic regulatory T cells. Autoimmunity Reviews 4:579-586.

Mallal S, Nolan D, Witt C, Masel G, Martin AM, Moore C, Sayer D, Castley A, Mamotte C, Maxwell D, James I and Christiansen FT (2002) Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and

hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. Lancet 359:727-732.

Mallal S, Phillips E, Carosi G, Molina JM, Workman C, Tomažiè J, Jägel-Guedes E, Rugina S, Kozyrev O, Cid JF, Hay P, Nolan D, Hughes S, Hughes A, Ryan S, Fitch N, Thorborn D and Benbow A (2008) HLA-B*5701 screening for hypersensitivity to abacavir. New England Journal of Medicine 358:568-579.

Manchanda T, Hess D, Dale L, Ferguson SG and Rieder MJ (2002) Haptenation of sulfonamide reactive metabolites to cellular proteins. Molecular Pharmacology 62:1011-1026.

Manel N, Unutmaz D and Littman DR (2008) The differentiation of human TH-17 cells requires transforming growth factor-β and induction of the nuclear receptor RORγt. Nature Immunology 9:641-649.

Manfredi M, Severino M, Testi S, Macchia D, Ermini G, Pichler WJ and Campi P (2004) Detection of specific IgE to quinolones. Journal of Allergy and Clinical Immunology 113:155-160.

Martin AM, Almeida CA, Cameron P, Purcell AW, Nolan D, James I, McCluskey J, Phillips E, Landay A and Mallal S (2007) Immune responses to abacavir in antigen-presenting cells from hypersensitive patients. AIDS 21:1233-1244.

Martin AM, Nolan D, Gaudieri S, Almeida CA, Nolan R, James I, Carvalho F, Phillips E, Christiansen FT, Purcell AW, McCluskey J and Mallal S (2004) Predisposition to abacavir hypersensitivity conferred by HLA-B*5701 and a haplotypic Hsp70-Hom variant. Proceedings of the National Academy of Sciences of the United States of America 101:4180-4185.

Martinon F and Tschopp J (2005) NLRs join TLRs as innate sensors of pathogens. Trends in Immunology 26:447-454.

Matzinger P (1994) Tolerance, danger, and the extended family. Annual Review of Immunology 12:991-1045.

Matzinger P (1998) An innate sense of danger. Seminars in Immunology 10:399-415.

Matzinger P (2007) Friendly and dangerous signals: Is the tissue in control? Nature Immunology 8:11-13.

Mauri-Hellweg D, Bettens F, Mauri D, Brander C, Hunziker T and Pichler WJ (1995) Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. Journal of Immunology 155:462-472.

Medina I, Mills J, Leoung G, Hopewell PC, Lee B, Modin G, Benowitz N and Wofsy CB (1990) Oral therapy for Pneumocystis carinii pneumonia in the acquired immunodeficiency syndrome. A controlled trial of trimethoprim-sulfamethoxazole versus trimethoprim-dapsone. New England Journal of Medicine 323:776-782.

Medzhitov R (2007) Recognition of microorganisms and activation of the immune response. Nature 449:819-826.

Meekins CV, Sullivan TJ and Gruchalla RS (1994) Immunochemical analysis of sulfonamide drug allergy: Identification of sulfamethoxazole-substituted human serum proteins. Journal of Allergy and Clinical Immunology 94:1017-1024.

Melief CJM (2003) Regulation of cytotoxic T lymphocyte responses by dendritic cells: Peaceful coexistence of cross-priming and direct priming? European Journal of Immunology 33:2645-2654.

Merk HF and Hertl M (1996) Immunologic mechanisms of cutaneous drug reactions. Seminars in Cutaneous Medicine and Surgery 15:228-235.

Miller J, Carr A, Emery S, Law M, Mallal S, Baker D, Smith D, Kaldor J and Cooper DA (2003) HIV lipodystrophy: Prevalence, severity and correlates of risk in Australia. HIV Medicine 4:293-301.

Miller JS, McCullar V, Punzel M, Lemischka IR and Moore KA (1999) Single adult human CD34+/Lin-/CD38- progenitors give rise to natural killer cells, B-lineage cells, dendritic cells, and myeloid cells. Blood 93:96-106.

Mitchell SC (2008) Flavin mono-oxygenase (FMO) - The other oxidase. Current Drug Metabolism 9:280-284.

Miyauchi H, Hosokawa H, Akaeda T, Iba H and Asada Y (1991) T-cell subsets in drug-induced toxic epidermal necrolysis: Possible pathogenic mechanism induced by CD8-positive T cells. Archives of Dermatology 127:851-855.

Moody DB and Porcelli SA (2003) Intracellular pathways of CD1 antigen presentation. Nature Reviews Immunology 3:11-22.

Moore N, Lecointre D, Noblet C and Mabille M (1998) Frequency and cost of serious adverse drug reactions in a department of general medicine. British Journal of Clinical Pharmacology 45:301-308.

Mossman KD, Campi G, Groves JT and Dustin ML (2005) Immunology: Altered TCR signaling from geometrically repatterned immunological synapses. Science 310:1191-1193.

Mowen KA and Glimcher LH (2004) Signaling pathways in Th2 development. Immunological Reviews 202:203-222.

Muller G, Muller A, Jonuleit H, Steinbrink K, Szalma C, Paragnik L, Lingnau K, Schmidt E, Knop J and Enk AH (2000) Fetal calf serum-free generation of functionally active murine dendritic cells suitable for in vivo therapeutic approaches. Journal of Investigative Dermatology 114:142-148.

Muller N, Van Den Brandt J, Odoardi F, Tischner D, Herath J, Flugel A and Reichardt HM (2008) A CD28 superagonistic antibody elicits 2 functionally distinct waves of T cell activation in rats. Journal of Clinical Investigation 118:1405-1416.

Mutch E and Williams FM (2006) Diazinon, chlorpyrifos and parathion are metabolised by multiple cytochromes P450 in human liver. Toxicology 224:22-32.

Nace CG, Genter MB, Sayre LM and Crofton KM (1997) Effect of methimazole, an FMO substrate and competitive inhibitor, on the neurotoxicity of 3,3'-iminodipropionitrile in male rats. Fundamental and Applied Toxicology 37:131-140.

Naisbitt DJ (2004) Drug hypersensitivity reactions in skin: Understanding mechanisms and the development of diagnostic and predictive tests. Toxicology 194:179-196.

Naisbitt DJ, Britschgi M, Wong G, Farrell J, Depta JPH, Chadwick DW, Pichler WJ, Pirmohamed M and Park BK (2003a) Hypersensitivity reactions to carbamazepine: Characterization of the specificity, phenotype, and cytokine profile of drug-specific T cell clones. Molecular Pharmacology 63:732-741.

Naisbitt DJ, Farrell J, Chamberlain PJ, Hopkins JE, Berry NG, Pirmohamed M and Park BK (2005) Characterization of the T-cell response in a patient with phenindione hypersensitivity. Journal of Pharmacology and Experimental Therapeutics 313:1058-1065.

Naisbitt DJ, Farrell J, Gordon SF, Maggs JL, Burkhart C, Pichler WJ, Pirmohamed M and Park BK (2002) Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. Molecular Pharmacology 62:628-637.

Naisbitt DJ, Farrell J, Wong G, Depta JPH, Dodd CC, Hopkins JE, Gibney CA, Chadwick DW, Pichler WJ, Pirmohamed M and Park BK (2003b) Characterization of drug-specific T cells in lamotrigine hypersensitivity. Journal of Allergy and Clinical Immunology 111:1393-1403.

Naisbitt DJ, Gordon SF, Pirmohamed M and Park BK (2000a) Immunological principles of adverse drug reactions: The initiation and propagation of immune responses elicited by drug treatment. Drug Safety 23:483-507.

Naisbitt DJ, Gordon SF, Pirmohamed M, Burkhart C, Cribb AE, Pichler WJ and Park BK (2001) Antigenicity and immunogenicity of sulphamethoxazole: Demonstration of metabolism-dependent haptenation and T-cell proliferation in vivo. British Journal of Pharmacology 133:295-305.

Naisbitt DJ, Hough SJ, Gill HJ, Pirmohamed M, Kitteringham NR and Park BK (1999) Cellular disposition of sulphamethoxazole and its metabolites: Implications for hypersensitivity. British Journal of Pharmacology 126:1393-1407.

Naisbitt DJ, Neill PM, Pirmohamed M and Park BK (1996) Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles:

Implications for immune mediated toxicity. Bioorganic and Medicinal Chemistry Letters 6:1511-1516.

Naisbitt DJ, Sanderson LS, Meng X, Stachulski AV, Clarke SE and Park BK (2007) Investigation of the immunogenicity of diclofenac and diclofenac metabolites. Toxicology Letters 168:45-50.

Naisbitt DJ, Vilar FJ, Stalford AC, Wilkins EGL, Pirmohamed M and Park BK (2000b) Plasma cysteine deficiency and decreased reduction of nitrososulfamethoxazole with HIV infection. AIDS Research and Human Retroviruses 16:1929-1938.

Naisbitt DJ, Williams DP, O'Neill PM, Maggs JL, Willock DJ, Pirmohamed M and Kevin Park B (1998) Metabolism-dependent neutrophil cytotoxicity of amodiaquine: A comparison with pyronaridine and related antimalarial drugs. Chemical Research in Toxicology 11:1586-1595.

Nassar AEF and Lopez-Anaya A (2004) Strategies for dealing with reactive intermediates in drug discovery and development. Current Opinion in Drug Discovery and Development 7:126-136.

Nassif A, Bensussan A, Boumsell L, Deniaud A, Moslehi H, Wolkenstein P, Bagot M and Roujeau JC (2004a) Toxic epidermal necrolysis: Effector cells are drug-specific cytotoxic T cells. Journal of Allergy and Clinical Immunology 114:1209-1215.

Nassif A, Bensussan A, Dorothée G, Mami-Chouaib F, Bachot N, Bagot M, Boumsell L and Roujeau JC (2002) Drug specific cytotoxic T-cells in the skin lesions of a patient with toxic epidermal necrolysis. Journal of Investigative Dermatology 118:728-733.

Nassif A, Moslehi H, Le Gouvello S, Bagot M, Lyonnet L, Michel L, Boumsell L, Bensussan A and Roujeau JC (2004b) Evaluation of the potential role of cytokines in toxic epidermal necrolysis. Journal of Investigative Dermatology 123:850-855.

Nebeker JR, Hoffman JM, Weir CR, Bennett CL and Hurdle JF (2005) High rates of adverse drug events in a highly computerized hospital. Archives of Internal Medicine 165:1111-1116.

Nelson D (2003) Cytochromes P450 in humans. [http://drnelson.utmem.edu/P450lect.html]:Retrieved May 9, 2005

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC and Nebert DW (1996) P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 6:1-42.

Nembrini C, Marsland BJ and Kopf M (2009) IL-17-producing T cells in lung immunity and inflammation. Journal of Allergy and Clinical Immunology 123:986-994.

Neuman MG, Malkiewicz IM and Shear NH (2000) A novel lymphocyte toxicity assay to assess drug hypersensitivity syndromes. Clinical Biochemistry 33:517-524.

Noble A, Macary PA and Kemeny DM (1995) IFN-γ and IL-4 regulate the growth and differentiation of CD8+ T cells into subpopulations with distinct cytokine profiles. Journal of Immunology 155:2928-2937.

Norgauer J, Ibig Y, Gmeiner D, Herouy Y and Fiebich BL (2003) Prostaglandin E2 synthesis in human monocyte-derived dendritic cells. International journal of molecular medicine 12:83-86.

Novak N and Bieber T (2008) 2. Dendritic cells as regulators of immunity and tolerance. Journal of Allergy and Clinical Immunology 121.

Ntimbane T, Comte B, Mailhot G, Berthiaume Y, Poitout V, Prentki M, Rabasa-Lhoret R and Levy E (2009) Cystic fibrosis-related diabetes: from CFTR dysfunction to oxidative stress. Clin Biochem Rev 30:153-177.

Nyfeler B and Pichler WJ (1997) The lymphocyte transformation test for the diagnosis of drug allergy: Sensitivity and specificity. Clinical and Experimental Allergy 27:175-181.

O'Doherty U, Peng M, Gezelter S, Swiggard WJ, Betjes M, Bhardwaj N and Steinman RM (1994) Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. Immunology 82:487-493.

Oesch F, Fabian E, Oesch-Bartlomowicz B, Werner C and Landsiedel R (2007) Drug-metabolizing enzymes in the skin of man, rat, and pig. Drug Metabolism Reviews 39:659-698.

Olweus J, BitMansour A, Warnke R, Thompson PA, Carballido J, Picker LJ and Lund-Johansen F (1997) Dendritic cell ontogeny: A human dendritic cell lineage of myeloid origin. Proceedings of the National Academy of Sciences of the United States of America 94:12551-12556.

Oppenheim JJ, Tewary P, de la Rosa G and Yang D (2007) Alarmins initiate host defense. Advances in Experimental Medicine and Biology 601:185-194.

Ortiz de Montellano PR, Mathews JM and Langry KC (1984) Autocatalytic inactivation of cytochrome p-450 and chloroperoxidase by 1-aminobenzotriazole and other aryne precursors. Tetrahedron 40:511-519.

O'Sullivan B and Thomas R (2003) CD40 and dendritic cell function. Critical Reviews in Immunology 23:83-107.

Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC and Murphy KM (1998) Inhibition of Th1 development mediated by GATA-3 through an IL-4- independent mechanism. Immunity 9:745-755.

Pacitto SR, Uetrecht JP, Boutros PC and Popovic M (2007) Changes in gene expression induced by tienilic acid and sulfamethoxazole: Testing the danger hypothesis. Journal of Immunotoxicology 4:253-266.

Padovan E, Bauer T, Tongio MM, Kalbacher H and Weltzien HU (1997) Penicilloyl peptides are recognized as T cell antigenic determinants in penicillin allergy. European Journal of Immunology 27:1303-1307.

Padovan E, Mauri-Hellweg D, Pichler WJ and Weltzien HU (1996) T cell recognition of penicillin G: Structural features determining antigenic specificity. European Journal of Immunology 26:42-48.

Pamer E and Cresswell P (1998) Mechanisms of MHC class I-restricted antigen processing, in Annual Review of Immunology pp 323-358.

Panayi GS, Corrigall VM and Henderson B (2004) Stress cytokines: Pivotal proteins in immune regulatory networks. Opinion. Current Opinion in Immunology 16:531-534.

Pancer Z and Cooper MD (2006) The evolution of adaptive immunity, in Annual Review of Immunology pp 497-518.

Paquet P, Nikkels A, Arrese JE, Vanderkelen A and Pierard GE (1994) Macrophages and tumor necrosis factor α in toxic epidermal necrolysis. Archives of Dermatology 130:605-608.

Park BK, Kitteringham NR, Maggs JL, Pirmohamed M and Williams DP (2005a) The role of metabolic activation in drug-induced hepatotoxicity, in Annual Review of Pharmacology and Toxicology pp 177-202.

Park BK, Laverty H, Srivastava A, Antoine DJ, Naisbitt D and Williams DP (2010) Drug bioactivation and protein adduct formation in the pathogenesis of drug-induced toxicity. Chem Biol Interact in press.

Park BK, Naisbitt DJ, Gordon SF, Kitteringham NR and Pirmohamed M (2001) Metabolic activation in drug allergies. Toxicology 158:11-23.

Park BK, Pirmohamed M and Kitteringham NR (1992) Idiosyncratic drug reactions: A mechanistic evaluation of risk factors. British Journal of Clinical Pharmacology 34:377-395.

Park BK, Pirmohamed M and Kitteringham NR (1998) Role of drug disposition in drug hypersensitivity: A chemical, molecular, and clinical perspective. Chemical Research in Toxicology 11:969-988.

Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q and Dong C (2005b) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nature Immunology 6:1133-1141.

Park K, Williams DP, Naisbitt DJ, Kitteringham NR and Pirmohamed M (2005c) Investigation of toxic metabolites during drug development. Toxicology and Applied Pharmacology 207: S425-S434.

Park KB, Dalton-Brown E, Hirst C and Williams DP (2006) Selection of new chemical entities with decreased potential for adverse drug reactions. European Journal of Pharmacology 549:1-8.

Paul C, Wolkenstein P, Adle H, Wechsler J, Garchon HJ, Revuz J and Roujeau JC (1996) Apoptosis as a mechanism of keratinocyte death in toxic epidermal necrolysis. British Journal of Dermatology 134:710-714.

Peitsch MC, Tschopp J, Kress A and Isliker H (1988) Antibody-independent activation of the complement system by mitochondria is mediated by cardiolipin. Biochemical Journal 249:495-500.

Phillips IR, Dolphin CT, Clair P, Hadley MR, Hutt AJ, McCombie RR, Smith RL and Shephard EA (1995) The molecular biology of the flavin-containing monooxygenases of man. Chemico-Biological Interactions 96:17-32.

Pichler WJ (2003) Delayed Drug Hypersensitivity Reactions. Annals of Internal Medicine 139:683-693+I646.

Pichler WJ (2005) Direct T-cell stimulations by drugs - Bypassing the innate immune system. Toxicology 209:95-100.

Pichler WJ, Beeler A, Keller M, Lerch M, Posadas S, Schmid D, Spanou Z, Zawodniak A and Gerber B (2006) Pharmacological interaction of drugs with immune receptors: The p-i concept. Allergology International 55:17-25.

Pichler WJ, Yawalkar N, Britschgi M, Depta J, Strasser I, Schmid S, Kuechler P and Naisbitt D (2002) Cellular and molecular pathophysiology of cutaneous drug reactions. American Journal of Clinical Dermatology 3:229-238.

Pichler WJ, Zanni M, Von Greyerz S, Schnyder B, Mauri-Hellweg D and Wendland T (1997) High IL-5 production by human drug-specific T cell clones. International Archives of Allergy and Immunology 113:177-180.

Pirmohamed M and Park BK (2001) HIV and drug allergy. Current Opinion in Allergy and Clinical Immunology 1:311-316.

Pirmohamed M, Alfirevic A, Vilar J, Stalford A, Wilkins EGL, Sim E and Park BK (2000) Association analysis of drug metabolizing enzyme gene polymorphisms in HIV-positive patients with co-trimoxazole hypersensitivity. Pharmacogenetics 10:705-713.

Pirmohamed M, Graham A, Roberts P, Smith D, Chadwick D, Breckenridge AM and Park BK (1991) Carbamazepine-hypersensitivity: Assessment of clinical and in vitro chemical cross-reactivity with phenytoin and oxcarbazepine. British Journal of Clinical Pharmacology 32:741-749.

Pirmohamed M, James S, Meakin S, Green C, Scott AK, Walley TJ, Farrar K, Park BK and Breckenridge AM (2004) Adverse drug reactions as cause of admission to hospital: Prospective analysis of 18 820 patients. British Medical Journal 329:15-19.

Pleasants RA, Walker TR and Samuelson WM (1994) Allergic reactions to parenteral beta-lactam antibiotics in patients with cystic fibrosis. Chest 106:1124-1128.

Porgador A and Gilboa E (1995) Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. Journal of Experimental Medicine 182:255-260.

Posadas SJ and Pichler WJ (2007) Delayed drug hypersensitivity reactions - New concepts. Clinical and Experimental Allergy 37:989-999.

Power LL, Popplewell EJ, Holloway JA, Diaper ND, Warner JO and Jones CA (2002) Immunoregulatory molecules during pregnancy and at birth. Journal of Reproductive Immunology 56:19-28.

Pullen H, Wright N and Murdoch JM (1967) Hypersensitivity reactions to antibacterial drugs in infectious mononucleosis. Lancet 2:1176-1178.

Quezada SA, Jarvinen LZ, Lind EF and Noelle RJ (2004) CD40/CD154 interactions at the interface of tolerance and immunity, in Annual Review of Immunology pp 307-328.

Rabaud C, Charreau I, Izard S, Raffi F, Meiffredy V, Leport C, Guillemin F, Yeni P and Aboulker JP (2001) Adverse reactions to cortrimoxazole in HIV-infected patients: Predictive factors an subsequent HIV disease progression. Scandinavian Journal of Infectious Diseases 33:759-764.

Raschetti R, Morgutti M, Menniti-Ippolito F, Belisari A, Rossignoli A, Longhini P and La Guidara C (1999) Suspected adverse drug events requiring emergency department visits or hospital admissions. European Journal of Clinical Pharmacology 54:959-963.

Rawlins M and Thompson J (1977) Pathogenesis of adverse drug reactions. In: Davies DM, ed. Textbook of adverse drug reactions. Oxford: Oxford University Press, 10.

Rawlins M and Thompson J (1981) Pathogenesis of adverse drug reactions. In: Davies DM, editor. Textbook of adverse drug reactions. 2nd ed. Oxford: Oxford University Press, 11.

Rawlins MD (1981) Clinical pharmacology. Adverse reactions to drugs. British Medical Journal 282:974-976.

Reilly TP, Bellevue Iii FH, Woster PM and Svensson CK (1998) Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. Biochemical Pharmacology 55:803-810.

Reilly TP, Lash LH, Doll MA, Hein DW, Woster PM and Svensson CK (2000) A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. Journal of Investigative Dermatology 114:1164-1173.

Reilly TP, Woster PM and Svensson CK (1999) Methemoglobin formation by hydroxylamine metabolites of sulfamethoxazole and dapsone: Implications for differences in adverse drug reactions. Journal of Pharmacology and Experimental Therapeutics 288:951-959.

Reis E Sousa C (2006) Dendritic cells in a mature age. Nature Reviews Immunology 6:476-483.

Rieder MJ (1997) In vivo and in vitro testing for adverse drug reactions. Pediatric Clinics of North America 44:93-111.

Rieder MJ, Krause R and Bird IA (1995) Time-course of toxicity of reactive sulfonamide metabolites. Toxicology 95:141-146.

Rieder MJ, Uetrecht J, Shear NH and Spielberg SP (1988) Synthesis and in vitro toxicity of hydroxylamine metabolites of sulfonamides. Journal of Pharmacology and Experimental Therapeutics 244:724-728.

Rieder MJ, Uetrecht J, Shear NH, Cannon M, Miller M and Spielberg SP (1989) Diagnosis of sulfonamide hypersensitivity reactions by in-vitro 'rechallenge' with hydroxylamine metabolites. Annals of Internal Medicine 110:286-289.

Robinson SP, Patterson S, English N, Davies D, Knight SC and Reid CDL (1999) Human peripheral blood contains two distinct lineages of dendritic cells. European Journal of Immunology 29:2769-2778.

Rock KL and Goldberg AL (1999) Degradation of cell proteins and the generation of MHC class I-presented peptides, in Annual Review of Immunology pp 739-779.

Rock KL, York IA and Goldberg AL (2004) Post-proteasomal antigen processing for major histocompatibility complex class I presentation. Nature Immunology 5:670-677.

Rodriguez-Pena R, Lopez S, Mayorga C, Antunez C, Fernandez TD, Torres MJ and Blanca M (2006) Potential involvement of dendritic cells in delayed-type hypersensitivity reactions to beta-lactams. Journal of Allergy and Clinical Immunology 118:949-956.

Rogers PR, Dubey C and Swain SL (2000) Qualitative changes accompany memory T cell generation: Faster, more effective, responses at lower doses of antigen. Journal of Immunology 164:2338-2346.

Rolink AG, Massa S, Balciunaite G and Ceredig R (2006) Early lymphocyte development in bone marrow and thymus. Swiss Medical Weekly 136:679-683.

Romagnani S (2004) Immunologic influences on allergy and the TH1/TH2 balance. Journal of Allergy and Clinical Immunology 113:395-400.

Romagnani S (2006) Regulation of the T cell response. Clinical and Experimental Allergy 36:1357-1366.

Rossner S, Zinser E, Menges M, Wiethe C, Littmann L, Hänig J., Steinkasserer A and Lutz MB (2008) Minor role of bystander tolerance to fetal calf serum in a peptide-specific dendritic cell vaccine model against autoimmunity comparison with serum-free cultures. Journal of Immunotherapy 31:656-664.

Roth RA, Luyendyk JP, Maddox JF and Ganey PE (2003) Inflammation and drug idiosyncrasy - Is there a connection? Journal of Pharmacology and Experimental Therapeutics 307:1-8.

Roujeau JC (2006) Immune mechanisms in drug allergy. Allergology International 55:27-33.

Roychowdhury S and Svensson CK (2005) Mechanisms of drug-induced delayed-type hypersensitivity reactions in the skin. AAPS Journal 7.

Roychowdhury S, Cram AE, Aly A and Svensson CK (2007a) Detection of haptenated proteins in organotypic human skin explant cultures exposed to dapsone. Drug Metabolism and Disposition 35:1463-1465.

Roychowdhury S, Vyas PM and Svensson CK (2007b) Formation and uptake of arylhydroxylamine-haptenated proteins in human dendritic cells. Drug Metabolism and Disposition 35:676-681.

Roychowdhury S, Vyas PM, Reilly TP, Gaspari AA and Svensson CK (2005) Characterization of the formation and localization of sulfamethoxazole and dapsone-associated drug-protein adducts in human epidermal keratinocytes. Journal of Pharmacology and Experimental Therapeutics 314:43-52.

Rozieres A, Hennino A, Rodet K, Gutowski MC, Gunera-Saad N, Berard F, Cozon G, Bienvenu J and Nicolas JF (2009a) Detection and quantification of drug-specific T cells in penicillin allergy. Allergy: European Journal of Allergy and Clinical Immunology 64:534-542.

Rozieres A, Vocanson M, Said BB, Nosbaum A and Nicolas JF (2009b) Role of T cells in non immediate allergic drug reactions. Current Opinion in Allergy and Clinical Immunology 9:305-310.

Rudolph MG, Stanfield RL and Wilson IA (2006) How TCRs bind MHCs, peptides, and coreceptors, in Annual Review of Immunology pp 419-466.

Rys-Sikora KE, Konger RL, Schoggins JW, Malaviya R and Pentland AP (2000) Coordinate expression of secretory phospholipase A2 and cyclooxygenase-2 in activated human keratinocytes. American Journal of Physiology - Cell Physiology 278.

Sacco JC and Trepanier LA (2010) Cytochrome b5 and NADH cytochrome b5 reductase: Genotype-phenotype correlations for hydroxylamine reduction. Pharmacogenetics and Genomics 20:26-37.

Sachs B, Erdmann S, Al-Masaoudi T and Merk HF (2001) In vitro drug allergy detection system incorporating human liver microsomes in chlorazepate-induced skin rash: Drug-specific proliferation associated with interleukin-5 secretion. British Journal of Dermatology 144:316-320.

Saeki M, Saito Y, Nagano M, Teshima R, Ozawa S and Sawada J (2002) mRNA expression of multiple cytochrome p450 isozymes in four types of cultured skin cells. Int Arch Allergy Immunol 127:333-336.

Saito T and Yamasaki S (2003) Negative feedback of T cell activation through inhibitory adapters and costimulatory receptors. Immunological Reviews 192:143-160.

Sakaguchi S (2004) Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses, in Annual Review of Immunology pp 531-562.

Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, Shimizu J, Takahashi T and Nomura T(2006) $Foxp3^+$ $CD25^+$ $CD4^+$ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunological Reviews 212:8-27.

Sallusto F and Lanzavecchia A (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. Journal of Experimental Medicine 179:1109-1118.

Sallusto F, Cella M, Danieli C and Lanzavecchia A (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: Downregulation by cytokines and bacterial products. Journal of Experimental Medicine 182:389-400.

Sallusto F, Schaerli P, Loetscher P, Schaniel C, Lenig D, Mackay CR, Qin S and Lanzavecchia A (1998) Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. European Journal of Immunology 28:2760-2769.

Salomon B and Bluestone JA (2001) Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation, in Annual Review of Immunology pp 225-252.

Sanderson JP, Naisbitt DJ and Park BK (2006) Role of bioactivation in drug-induced hypersensitivity reactions. AAPS Journal 8.

Sanderson JP, Naisbitt DJ, Farrell J, Ashby CA, Tucker MJ, Rieder MJ, Pirmohamed M, Clarke SE and Park BK (2007) Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling. Journal of Immunology 178:5533-5542.

Saurin AT, Neubert H, Brennan JP and Eaton P (2004) Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. Proceedings of the National Academy of Sciences of the United States of America 101:17982-17987.

Scaffidi P, Misteli T and Bianchi ME (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418:191-195.

Schaerli P and Moser B (2005) Chemokines: Control of primary and memory T-cell traffic. Immunologic Research 31:57-74.

Schirrmaker V (2003) Improvements of survival in nine phase II clinical studies with different types of cancer upon anti-tumor vaccination with an autologous tumor cell vaccine modified by virus infection to introduce danger signals, in Advances in Experimental Medicine and Biology pp 175-193.

Schmid DA, Depta JPH and Pichler WJ (2006) T cell-mediated hypersensitivity to quinolones: Mechanisms and cross-reactivity. Clinical and Experimental Allergy 36:59-69.

Schnyder B, Burkhart C, Schnyder-Frutig K, Von Greyerz S, Naisbitt DJ, Pirmohamed M, Park BK and Pichler WJ (2000) Recognition of sulfamethoxazole and its reactive metabolites by drug- specific CD4+ T cells from allergic individuals. Journal of Immunology 164:6647-6654.

Schnyder B, Frutig K, Mauri-Hellweg D, Limat A, Yawalkar N and Pichler WJ (1998) T-cell-mediated cytotoxicity against keratinocytes in sulfamethoxazol- induced skin reaction. Clinical and Experimental Allergy 28:1412-1417.

Schnyder B, Mauri-Hellweg D, Zanni M, Bettens F and Pichler WJ (1997) Direct, MHC-dependent presentation of the drug sulfamethoxazole to human αβ T cell clones. Journal of Clinical Investigation 100:136-141.

Schodin BA, Tsomides TJ and Kranz DM (1996) Correlation between the number of T cell receptors required for T cell activation and TCR-ligand affinity. Immunity 5:137-146.

Senst BL, Achusim LE, Genest RP, Cosentino LA, Ford CC, Little JA, Raybon SJ and Bates DW (2001) Practical approach to determining costs and frequency of adverse drug events in a health care network. American Journal of Health-System Pharmacy 58:1126-1132.

Seong SY and Matzinger P (2004) Hydrophobicity: An ancient damage-associated molecular pattern that initiates innate immune responses. Nature Reviews Immunology 4:469-478.

Sercarz EE and Maverakis E (2003) MHC-guided processing: Binding of large antigen fragments. Nature Reviews Immunology 3:621-629.

Shear NH and Spielberg SP (1985) An in vitro lymphocytotoxicity assay for studying adverse reactions to sulphonamides. British Journal of Dermatology 113:112-113.

Shear NH, Spielberg SP and Grant DM (1986) Differences in metabolism of sulfonamides predisposing to idiosyncratic toxicity. Annals of Internal Medicine 105:179-184.

Shi Y, Evans JE and Rock KL (2003) Molecular identification of a danger signal that alerts the immune system to dying cells. Nature 425:516-521.

Shi Y, Galusha SA and Rock KL (2006) Cutting edge: Elimination of an endogenous adjuvant reduces the activation of CD8 T lymphocytes to transplanted cells and in an autoimmune diabetes model. Journal of Immunology 176:3905-3908.

Shimada T, Yamazaki H, Mimura M, Inui Y and Guengerich FP (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: Studies with liver microsomes of 30 Japanese and 30 Caucasians. Journal of Pharmacology and Experimental Therapeutics 270:414-423.

Shimonkevitz R, Kappler J, Marrack P and Grey H (1983) Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. Journal of Experimental Medicine 158:303-316.

Shiohara T, Iijima M, Ikezawa Z and Hashimoto K (2007) The diagnosis of a DRESS syndrome has been sufficiently established on the basis of typical clinical features and viral reactivations [21]. British Journal of Dermatology 156:1083-1084.

Shortman K and Liu YJ (2002) Mouse and human dendritic cell subtypes. Nature Reviews Immunology 2:151-161.

Sidoroff A, Dunant A, Viboud C, Halevy S, Bavinck JNB, Naldi L, Mockenhaupt M, Fagot JP and Roujeau JC (2007) Risk factors for acute generalized exanthematous pustulosis (AGEP) - Results of a multinational case-control study (EuroSCAR). British Journal of Dermatology 157:989-996.

Sieben S, Baron JM, Blömeke B and Merk HF (1999) Multiple cytochrome P450-isoenzymes mRNA are expressed in dendritic cells. International Archives of Allergy and Immunology 118:358-361.

Sieben S, Kawakubo Y, Masaoudi TA, Merk HF and Blömeke B (2002) Delayed-type hypersensitivity reaction to paraphenylenediamine is mediated by 2 different pathways of antigen recognition by specific αβ+ human t-cell clones. Journal of Allergy and Clinical Immunology 109:1005-1011.

Siest G, Jeannesson E, Marteau JB, Samara A, Marie B, Pfister M and Visvikis-Siest S (2008) Transcription factor and drug-metabolizing enzyme gene expression in lymphocytes from healthy human subjects. Drug Metabolism and Disposition 36:182-189.

Singer A and Bosselut R (2004) CD4/CD8 coreceptors in thymocyte development, selection, and lineage commitment: Analysis of the CD4/CD8 lineage decision, in Advances in Immunology pp 91-131.

Skapenko A, Leipe J, Niesner U, Devriendt K, Beetz R, Radbruch A, Kalden JR, Lipsky PE and Schulze-Koops H (2004) GATA-3 in Human T Cell Helper Type 2 Development. Journal of Experimental Medicine 199:423-428.

Smith-Garvin JE, Koretzky GA and Jordan MS (2009) T cell activation, in Annual Review of Immunology pp 591-619.

Song J, Lei FT, Xiong X and Haque R (2008) Intracellular signals of T cell costimulation. Cellular & molecular immunology 5:239-247.

Spencer CT, Gilchuk P, Dragovic SM and Joyce S (2010) Minor histocompatibility antigens: Presentation principles, recognition logic and the potential for a healing hand. Current Opinion in Organ Transplantation 15:512-525.

Spencer DL, Masten SA, Lanier KM, Yang X, Grassman JA, Miller CR, Sutter TR, Lucier GW and Walker NJ (1999) Quantitative analysis of constitutive and 2,3,7,8-tetrachlorodibenzo-p- dioxin-induced cytochrome P450 1B1 expression in human lymphocytes. Cancer Epidemiology Biomarkers and Prevention 8:139-146.

Spielberg SP, Gordon GB and Blake DA (1981) Predisposition to phenytoin hepatotoxicity assessed in vitro. New England Journal of Medicine 305:722-727.

Starkel P, Sempoux C, Van den Berge V, Stevens M, De Saeger C, Desager JP and Horsmans Y (1999) CYP 3A proteins are expressed in human neutrophils and lymphocytes but are not induced by rifampicin. Life Sciences 64:643-653.

Steinman L (2007) A brief history of TH17, the first major revision in the T H1/TH2 hypothesis of T cell-mediated tissue damage. Nature Medicine 13:139-145.

Steinman RM (1991) The dendritic cell system and its role in immunogenicity. Annual Review of Immunology 9:271-296.

Stern LJ, Potolicchio I and Santambrogio L (2006) MHC class II compartment subtypes: Structure and function. Current Opinion in Immunology 18:64-69.

Stockinger B and Veldhoen M (2007) Differentiation and function of Th17 T cells. Current Opinion in Immunology 19:281-286.

Strobl H, Bello-Fernandez C, Riedl E, Pickl WF, Majdic O, Lyman SD and Knapp W (1997) flt3 ligand in cooperation with transforming growth factor-beta1 potentiates in vitro development of Langerhans-type dendritic cells and allows single-cell dendritic cell cluster formation under serum-free conditions. Blood 90:1425-1434.

Subudhi SK, Alegre ML and Fu YX (2005) The balance of immune responses: Costimulation verse coinhibition. Journal of Molecular Medicine 83:193-202.

Summan M and Cribb AE (2002) Novel non-labile covalent binding of sulfamethoxazole reactive metabolites to cultured human lymphoid cells. Chemico-Biological Interactions 142:155-173.

Sundrud MS, Grill SM, Ni D, Nagata K, Alkan SS, Subramaniam A and Unutmaz D (2003) Genetic reprogramming of primary human T cells reveals functional plasticity in Th cell differentiation. Journal of Immunology 171:3542-3549.

Svensson CK, Cowen EW and Gaspari AA (2001) Cutaneous drug reactions. Pharmacological Reviews 53:357-379.

Swanson HI (2004) Cytochrome P450 expression in human keratinocytes: An aryl hydrocarbon receptor perspective. Chemico-Biological Interactions 149:69-79.

Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG and Glimcher LH (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100:655-669.

Szabo SJ, Sullivan BM, Peng SL and Glimcher LH (2003) Molecular mechanisms regulating Th1 immune responses, in Annual Review of Immunology pp 713-758.

Tafazoli S, Spehar DD and O'Brien PJ (2005) Oxidative stress mediated idiosyncratic drug toxicity. Drug Metabolism Reviews 37:311-325.

Tahvanainen J, Pykäläinen, M, Kallonen T, Lähteenmäki, H, Rasool O and Lahesmaa R (2006) Enrichment of nucleofected primary human CD4+ T cells: A novel and efficient method for studying gene function and role in human primary T helper cell differentiation. Journal of Immunological Methods 310:30-39.

Takeda H, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura SI, Nakanishi K, Yoshida N, Kishimoto T and Akira S (1996) Essential role of Stat6 in IL-4 signalling. Nature 380:627-630.

Tanaka K (1994) Role of proteasomes modified by interferon-γ in antigen processing. Journal of Leukocyte Biology 56:571-575.

Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, Miyake K, Freudenberg M, Galanos C and Simon JC (2002) Oligosaccharides of hyaluronan activate dendritic cells via Toll-like receptor 4. Journal of Experimental Medicine 195:99-111.

Thierfelder WE, Van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, Sangster MY, Vignali DAA, Doherty PC, Grosveld GC and Ihle JN (1996) Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature 382:171-174.

Thomson RG, Rawlins MD, James OFW, Wood P and Williams FM (1988) The acute and subchronic effects of ketoconazole on hepatic microsomal monooxygenases in the rat. Biochemical Pharmacology 37:3975-3980.

Toebak MJ, Moed H, Von Blomberg MBE, Bruynzeel DP, Gibbs S, Scheper RJ and Rustemeyer T (2006) Intrinsic characteristics of contact and respiratory allergens influence production of polarizing cytokines by dendritic cells. Contact Dermatitis 55:238-245.

Toldbod HE, Agger R, Bolund L and Hokland M (2003) Potent influence of bovine serum proteins in experimental dendritic cell-based vaccination protocols. Scandinavian Journal of Immunology 58:43-50.

Treluyer JM, Bowers G, Cazali N, Sonnier M, Rey E, Pons G and Cresteil T (2003) Oxidative metabolism of amprenavir in the human liver. Effect of the CYP3A maturation. Drug Metabolism and Disposition 31:275-281.

Trepanier LA and Miller JL (2000) NADH-dependent reduction of sulphamethoxazole hydroxylamine in dog and human liver microsomes. Xenobiotica 30:1111-1121.

Trepanier LA, Yoder AR, Bajad S, Beckwith MD, Bellehumeur JL and Graziano FM (2004) Plasma ascorbate deficiency is associated with impaired reduction of sulfamethoxazole-nitroso in HIV infection. Journal of Acquired Immune Deficiency Syndromes 36:1041-1050.

Tsuge I, Okumura A, Kondo Y, Itomi S, Kakami M, Kawamura M, Nakajima Y, Komatsubara R and Urisu A (2007) Allergen-specific T-cell response in

patients with phenytoin hypersensitivity; simultaneous analysis of proliferation and cytokine production by Carboxyfluorescein Succinimidyl Ester (CFSE) dilution assay. Allergology International 56:149-155.

Uetrecht J (2002) N-oxidation of drugs associated with idiosyncratic drug reactions. Drug Metabolism Reviews 34:651-665.

Uetrecht J (2006) Evaluation of which reactive metabolite, if any, is responsible for a specific idiosyncratic reaction. Drug Metabolism Reviews 38:745-753.

Uetrecht J (2007) Idiosyncratic drug reactions: Current understanding, in Annual Review of Pharmacology and Toxicology pp 513-539.

Uetrecht J (2008) Idiosyncratic drug reactions: Past, present, and future. Chemical Research in Toxicology 21:84-92.

Uetrecht JP (1995) Myeloperoxidase as a generator of drug free radicals. Biochemical Society symposium 61:163-170.

Uetrecht JP (1999) New concepts in immunology relevant to idiosyncratic drug reactions: The 'danger hypothesis' and innate immune system. Chemical Research in Toxicology 12:387-395.

Uetrecht JP, Shear NH and Zahid N (1993) N-chlorination of sulfamethoxazole and dapsone by the myeloperoxidase system. Drug Metabolism and Disposition 21:830-834.

Usui T, Nishikomori R, Kitani A and Strober W (2003) GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rβ2 chain or T-bet. Immunity 18:415-428.

Valentine CR, Valentine JL, Seng J, Leakey J and Casciano D (1996) The use of transgenic cell lines for evaluating toxic metabolites of carbamazepine. Cell Biology and Toxicology 12:155-165.

Vallabhapurapu S and Karin M (2009) Regulation and function of NF-κB transcription factors in the immune system, in Annual Review of Immunology pp 693-733.

Van Berkel MEAT, Schrijver EHR, Hofhuis FMA, Sharpe AH, Coyle AJ, Broeren CP, Tesselaar K and Oosterwegel MA (2005) ICOS contributes to

T cell expansion in CTLA-4 deficient mice. Journal of Immunology 175:182-188.

Van Der Hooft CS, Sturkenboom MCJM, Van Grootheest K, Kingma HJ and Stricker BHC (2006) Adverse drug reaction-related hospitalisations: A nationwide study in The Netherlands. Drug Safety 29:161-168.

Van Der Merwe PA and Davis SJ (2003) Molecular interactions mediating T cell antigen recognition, in Annual Review of Immunology pp 659-684.

Van Gool SW, Vandenberghe P, De Boer M and Ceuppens JL (1996) CD80, CD86 and CD40 provide accessory signals in a multiple-step T-Cell activation model. Immunological Reviews:47-83.

Van Kooten G and Banchereau J (2000) CD40-CD40 ligand. Journal of Leukocyte Biology 67:2-17.

Vandeputte C, Guizon I, Genestie-Denis I, Vannier B and Lorenzon G (1994) A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: Performance study of a new miniaturized protocol. Cell Biology and Toxicology 10:415-421.

Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM and Stockinger B (2006) TGF β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 24:179-189.

Venkatakrishnan K, Von Moltke LL and Greenblatt DJ (2001) Human drug metabolism and the cytochromes P450: Application and relevance of in vitro models. Journal of Clinical Pharmacology 41:1149-1179.

Venning GR (1983) Identification of adverse reactions to new drugs. III: Alerting processes and early warning systems. British Medical Journal 286:458-460.

Vermes I, Haanen C, Steffens-Nakken H and Reutelingsperger C (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. Journal of Immunological Methods 184:39-51.

Viard I, Wehrli P, Bullani R, Schneider P, Holler N, Salomon D, Hunziker T, Saurat JH, Tschopp J and French LE (1998) Inhibition of toxic epidermal

necrolysis by blockade of CD95 with human intravenous immunoglobulin. Science 282:490-493.

Vogel C (2000) Prostaglandin H Synthases and their Importance in Chemical Toxicity. Current Drug Metabolism 1:391-404.

Vyas PM, Roychowdhury S and Svensson CK (2006b) Role of human cyclooxygenase-2 in the bioactivation of dapsone and sulfamethoxazole. Drug Metabolism and Disposition 34:16-18.

Vyas PM, Roychowdhury S, Koukouritaki SB, Hines RN, Krueger SK, Williams DE, Nauseef WM and Svensson CK (2006a) Enzyme-mediated protein haptenation of dapsone and sulfamethoxazole in human keratinocytes: II. Expression and role of flavin-containing monooxygenases and peroxidases. Journal of Pharmacology and Experimental Therapeutics 319:497-505.

Vyas PM, Roychowdhury S, Woster PM and Svensson CK (2005) Reactive oxygen species generation and its role in the differential cytotoxicity of the arylhydroxylamine metabolites of sulfamethoxazole and dapsone in normal human epidermal keratinocytes. Biochemical Pharmacology 70:275-286.

Wade O and Beeley L (1976) Adverse reactions to drugs. 2nd ed. London: William Heinemann Medical Books Ltd, Chapter II.

Wagner Jr DH, Vaitaitis G, Sanderson R, Poulin M, Dobbs C and Haskins K (2002) Expression of CD40 identifies a unique pathogenic T cell population in type 1 diabetes. Proceedings of the National Academy of Sciences of the United States of America 99:3782-3787.

Wallin RPA, Lundqvist A, Moré SH, Von Bonin A, Kiessling R and Ljunggren HG (2002) Heat-shock proteins as activators of the innate immune system. Trends in Immunology 23:130-135.

Walmsley SL, Khorasheh S, Singer J and Djurdjev O (1998) A randomized trial of N-acetylcysteine for prevention of trimethoprim- sulfamethoxazole hypersensitivity reactions in pneumocystis carinii Pneumonia prophylaxis (CTN 057). Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology 19:498-505.

Walmsley SL, Winn LM, Harrison ML, Uetrecht JP and Wells PG (1997) Oxidative stress and thiol depletion in plasma and peripheral blood lymphocytes from HIV-infected patients: Toxicological and pathological implications. AIDS 11:1689-1697.

Walunas TL, Bakker CY and Bluestone JA (1996) CTLA-4 ligation blocks CD28-dependent T cell activation. Journal of Experimental Medicine 183:2541-2550.

Warncke M, Dodero A, Dierbach H, Follo M and Veelken H (2006) Murine dendritic cells generated under serum-free conditions have a mature phenotype and efficiently induce primary immune responses. Journal of Immunological Methods 310:1-11.

Waszkowycz B, Clark DE, Frenkel D, Li J, Murray CW, Robson B and Westhead DR (1994) PRO_LIGAND: an approach to de novo molecular design. 2. Design of novel molecules from molecular field analysis (MFA) models and pharmacophores. J Med Chem 37:3994-4002.

Watanabe S, Ogawa S, Hara Y, Tanabe K, Toma H and Abe R (2006) Expression level of costimulatory receptor ICOS is critical for determining the polarization of helper T cell function. Transplant Immunology 15:255-263.

Watts C (2004) The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. Nature Immunology 5:685-692.

Wayne E (1985) Problems of toxicity in clinical medicine. In: Walpole AL, Spinks A, editors. The evaluation of drug toxicity. London: J & A Churchill Ltd, 1-11.

Wei X, Tromberg BJ and Cahalan MD (1999) Mapping the sensitivity of T cells with an optical trap: Polarity and minimal number of receptors for $Ca2^{+}$ signaling. Proceedings of the National Academy of Sciences of the United States of America 96:8471-8476.

Wells JW, Darling D, Farzaneh F and Galea-Lauri J (2005) Influence of interleukin-4 on the phenotype and function of bone marrow-derived murine dendritic cells generated under serum-free conditions. Scandinavian Journal of Immunology 61:251-259.

Wester K, Jonsson AK, Spigset O, Druid H and Hagg S (2008) Incidence of fatal adverse drug reactions: A population based study. British Journal of Clinical Pharmacology 65:573-579.

Wetmore DR, Joseloff E, Pilewski J, Lee DP, Lawton KA, Mitchell MW, Milburn MV, Ryals JA and Guo L (2010) Metabolomic profiling revealed biochemical pahtways and biomarkers associated with pathogensis in cystic fibrosis cells. J Biol Chem.

WHO (1966) International Drug Monitoring: The Role of the Hospital. A WHO report.

WHO (1972) International drug monitoring: the role of national centres. Technical report series No 498. Geneva.

WHO (1975) Requirements for adverse reaction reporting. Geneva, Switzerland.

WHO (1992) International monitoring of adverse reactions to drugs: adverse reaction terminology. Uppsala: WHO Collaborating Centre for International Drug Monitoring.

WHO (2002) Safety of medicines – a guide to detecting and reporting adverse drug reactions. A noxious and unintended response to a drug which occurs at doses normally used in man.

Wijsman JA, Dekaban GA and Rieder MJ (2005) Differential toxicity of reactive metabolites of clindamycin and sulfonamides in HIV-infected cells: Influence of HIV infection on clindamycin toxicity in vitro. Journal of Clinical Pharmacology 45:346-351.

Williams DP, Kitteringham NR, Naisbitt DJ, Pirmohamed M, Smith DA and Park BK (2002) Are chemically reactive metabolites responsible for adverse reactions to drugs? Current Drug Metabolism 3:351-366.

Williams FM (1987) Serum enzymes of drug metabolism. Pharmacology and Therapeutics 34:99-109.

Williams FM (2008) Potential for metabolism locally in the skin of dermally absorbed compounds. Human and Experimental Toxicology 27:277-280.

Wolkenstein P, Charue D, Laurent P, Revuz J, Roujeau JC and Bagot M (1995) Metabolic predisposition to cutaneous adverse drug reactions: Role in

toxic epidermal necrolysis caused by sulfonamides and anticonvulsants. Archives of Dermatology 131:544-551.

Wolkenstein PE, Roujeau JC and Revuz J (1998) Drug-induced toxic epidermal necrolysis. Clinics in Dermatology 16:399-409.

Worrall S and Dickinson RG (1995) Rat serum albumin modified by diflunisal acyl glucuronide is immunogenic in rats. Life Sciences 56:1921-1930.

Wu Y, Farrell J, Pirmohamed M, Park BK and Naisbitt DJ (2007) Generation and characterization of antigen-specific CD4+, CD8+, and CD4+CD8+ T-cell clones from patients with carbamazepine hypersensitivity. Journal of Allergy and Clinical Immunology 119:973-981.

Wu Y, Sanderson JP, Farrell J, Drummond NS, Hanson A, Bowkett E, Berry N, Stachulski AV, Clarke SE, Pichler WJ, Pirmohamed M, Park BK and Naisbitt DJ (2006) Activation of T cells by carbamazepine and carbamazepine metabolites. Journal of Allergy and Clinical Immunology 118:233-241.

Yawalkar N, Egli F, Hari Y, Nievergelt H, Braathen LR and Pichler WJ (2000) Infiltration of cytotoxic T cells in drug-induced cutaneous eruptions. Clinical and Experimental Allergy 30:847-855.

Yewdell JW and Haeryfar SMM (2005) Understanding presentation of viral antigens to CD8+ T cells in vivo: The key to rational vaccine design, in Annual Review of Immunology pp 651-682.

Yoshimura S, Bondeson J, Foxwell BMJ, Brennan FM and Feldmann M (2001) Effective antigen presentation by dendritic cells is NF-κB dependent: Coordinate regulation of MHC, co-stimulatory molecules and cytokines. International Immunology 13:675-683.

Zanni MP, Mauri-Hellweg D, Brander C, Wendland T, Schnyder B, Frei E, Von Greyerz S, Bircher A and Pichler WJ (1997) Characterization of Lidocaine-Specific T Cells. Journal of Immunology 158:1139-1148.

Zanni MP, Von Greyerz S, Hari Y, Schnyder B and Pichler WJ (1999) Recognition of local anesthetics by $\alpha\beta^+$ T cells. Journal of Investigative Dermatology 112:197-204.

Zanni MP, Von Greyerz S, Schnyder B, Brander KA, Frutig K, Hari Y, Valitutti S and Pichler WJ (1998) HLA-restricted, processing- and metabolism-independent pathway of drug recognition by human alpha beta T lymphocytes. Journal of Clinical Investigation 102:1591-1598.

Zhang W, Sloan-Lancaster J, Kitchen J, Trible RP and Samelson LE (1998) LAT: The ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell 92:83-92.

Zhang W, Sommers CL, Burshtyn DN, Stebbins CC, DeJarnette JB, Trible RP, Grinberg A, Tsay HC, Jacobs HM, Kessler CM, Long EO, Love PE and Samelson LE (1999) Essential role of LAT in T cell development. Immunity 10:323-332.

Zhao Chao L and Uetrecht JP (1995) Clozapine is oxidized by activated human neutrophils to a reactive nitrenium ion that irreversibly binds to the cells. Journal of Pharmacology and Experimental Therapeutics 275:1476-1483.

Zhou S, Chan E, Duan W, Huang M and Chen YZ (2005) Drug bioactivation, covalent binding to target proteins and toxicity relevance. Drug Metabolism Reviews 37:41-213.