The responsive and suppressive activities of CD4$^+$ T cells to neoantigens generated in procainamide drug-induced Lupus

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To my family

Quidquid agas, prudenter agas, et respice finem –

Whatever you do, do cautiously, and look to the end.

GESTA ROMANORUM (CAP.103)

ZUSAMMENFASSUNG

Das Arzneimittel Procainamid (PA, p-Aminobenzosäure-β-diäthylaminoäthylamid) kann beim Menschen einen systemischen Lupus erythematosus, eine Autoimmunkrankheit, auslösen. Es wird daher in der Maus als Modellsubstanz zur Untersuchung der Mechanismen arzneimittelinduzierter unerwünschter Immunreaktionen verwendet, auch hier führt die Behandlung mit PA T-Zell-abhängig zur Bildung von antinukleären Autoantikörpern (ANA). PA wird im Körper metabolisiert, wobei proteinreaktive Zwischenprodukte entstehen. T-Zellen reagieren normalerweise nicht auf körpereigene Proteine, deren immunrelevante Peptide auf der Oberfläche der antigenpräsentierenden Zellen (APZ) verankert sind. Veränderungen dieser Peptide, sei es durch Addukte mit proteinreaktiven Chemikalien („Neoantigene"), sei es durch Präsentation normalerweise nicht-präsentierter, sogenannter kryptischer Peptide, können zu unerwünschter T-Zell-Aktivität führen. ANA werden sowohl in Patienten als auch in Mäusestämmen, die genetisch bedingt einen langsamen Acetyliererphänotyp besitzen, schneller und in größerer Menge gebildet. In Individuen mit langsamem Acetyliererphänotyp wird der größte Teil des PA im *N*-Oxidations-Pfad in Zellen des Immunsystems, wie neutrophilen Granulozyten, metabolisiert. Die Produkte dieses Abbauweges, der intermediäre Metabolit *N*-Hydroxylamin-PA (HAPA) und besonders das instabile, reaktive Nitroso-PA, die ineinander umgewandelt werden, können an Proteine binden und somit Neoantigene induzieren, die wiederum T-Zellen stimulieren können. Wie bei den meisten chemikalieninduzierten Immunreaktionen sind auch für PA die exakten Neoantigene noch nicht identifiziert.

Im ersten Teil dieser Arbeit wurden T-Zell-Reaktionen auf PA-induzierte Neoantigene der Maus untersucht. Diese Experimente zeigen, dass die Metabolisierung von PA durch Makrophagen zur Bildung von PA-induzierten Proteinkonjugaten führte. Diese PA-induzierten Neoantigene lösten sowohl *in vivo* als auch *in vitro* spezifische T-Zell-Antworten aus. Passend hierzu wiesen A/J-Mäuse, die zu den langsamen Acetylierern gehören, nach achtmonatiger Gabe von PA im Trinkwasser erhöhte IgG1 ANA Titer auf. Zudem besaß ein signifikanter Anteil der Seren dieser Mäuse spezifische Antikörper für PA und/ oder HAPA. Weiterhin wiesen einige der PA-behandelten Mäuse Antikörper auf, die mit einem 35 kDa Protein aus Zelllysaten einer humanen Epithelzelllinie reagierten. Auch konnte ein Addukt, bestehend aus einem 35 kDa Protein und PA oder HAPA, mittels PA-spezifischer Antikörper in Zytoplasmalysaten von Mausknochenmarkszellen nachgewiesen werden, die mit PA-vorinkubiert worden waren. Dies legt die Schlussfolgerung nahe, dass eins der PA-induzierten Neoantigene ein Addukt aus PA oder seinen Metaboliten und dem obengenannten 35 kDa Protein ist.

$CD4^+$ T-Zellen, nicht jedoch $CD8^+$ T-Zellen von PA-behandelten A/J-Mäusen reagierten spezifisch gegen PA-gepulste Makrophagen oder auf die direkte Zugabe von HAPA in einem *In-vitro*-System. Zur Identifizierung der $CD4^+$ T-Zell-Subpopulation, die für die Induktion der ANA in PA-behandelten Mäusen verantwortlich war, wurden isolierte $CD4^+CD25^-$ und $CD4^+CD25^+$ T-Zell-Subpopulationen in adoptiven Transfers verwendet. Die $CD4^+CD25^-$ T-Zellen von PA-behandelten Spendermäusen waren überraschenderweise dazu in der Lage, ANA in nicht mit PA behandelten Empfängern zu induzieren. Offenbar waren diese $CD4^+CD25^-$ T-Zellen in der Lage, autoreaktive (ANA-produzierende) B-Zellen, denen normalerweise die erforderliche T-Zell-Hilfe fehlt, zu aktivieren. Die Tatsache, dass die B-Zellen in diesem Fall reaktiv wurden und ANA produzieten, legt die Schlussfolgerung nahe, dass es im Laufe der PA-Behandlung der T-Zell-Spender zu „epitope spreading" hin zu unveränderten Selbstproteinen und somit zu einer Aktivierung autoreaktiver T-Helfer-Zellen kam. Der Adoptive Transfer von $CD4^+CD25^+$ T-Zellen (T-Suppressor-Zellen) von PA-behandelten Spendermäusen hingegen unterdrückte die Bildung von ANA in den Empfängermäusen, und zwar unabhängig davon, ob die Empfänger mit PA oder aber mit anderen ANA-induzierenden Substanzen behandelt wurden. In dieser Arbeit konnte somit zum ersten Mal gezeigt werden, dass $CD4^+CD25^+$ T-Zellen einen suppressiven Effekt auf Arzneimittel-induzierte Autoimmunkrankheiten besitzen. Weiterhin wurde gezeigt, dass sich während einer PA-Behandlung die T-Zell-Reaktivität von PA-induzierten Neoantigenen auf Peptide von unveränderten Nukleoproteinen ausweitet, und somit auch auf der T-Zell-Ebene zu einer echten Autoimmunreaktion.

THIS THESIS IS BASED ON THE FOLLOWING ORIGINAL PUBLICATIONS

L. Layland, M. Wulferink, E. Gleichmann. 1999. Production of $CD4^+$ T cell hybridomas against procainamide, a drug-inducing Lupus. *Immunobiology*. **C13:** 348-349.

L. Layland, M. Wulferink, E. Gleichmann. 2001. Long term procainamide treatment in the drinking water of A/J mice produces a positive T cell reaction to an unidentified neo-antigen in the lymphocyte transformation test. *Immunobiology*. **H7:** 120.

L. Layland, M. Wulferink, E. Gleichmann. 2002. Prevention of drug-induced antinuclear autoantibody formation by the adoptive transfer of previously exposed $CD4^+CD25^+$ regulatory T cells. *Immunobiology*. **K8:** 150.

Oral Presentation "Prevention of drug-induced antinuclear autoantibody formation by the adoptive transfer of previously exposed $CD4^+CD25^+$ regulatory T cells" at 33rd Annual Meeting of the German Society of Immunology, September 25th-28th, 2002 in Marburg Germany.

Oral Presentation "Drug-induced autoimmunity in mice: Analysis of T-cell cross-reactivity: Demonstration of T-Suppressor cells", at the ENDA Meeting, October 19th-20th, 2002, in Aachen Germany.

L. Layland, M. Wulferink, E. Gleichmann. 2003. Prevention of drug-induced antinuclear autoantibody formation by the adoptive transfer of previously exposed $CD4^+CD25^+$ regulatory T cells. *Archives in Dermatological Research.* **PO70:** 479.

L. Layland, M. Wulferink, S. Dierkes and E. Gleichmann. 2003. Drug-induced autoantibody formation in mice: triggering by primed $CD4^+CD25^-$ T cells, prevention by primed $CD4^+CD25^+$ T cells. *Eur. J. Immunol*. In submission.

ABBREVIATIONS

ACT	ammoniumchloride-tris
AID	autoimmune disease
ANA	antinuclear antibodies
ANOVA	analysis of variance
APC	antigen presenting cell
APS	ammonium persulphate
ATCC	American cell culture
BSS	balanced salt solution
Con A	concanvalin A
CO_2	carbon dioxide
cpm	counts per minute
°C	degrees centigrade
DDW	double distilled water
DIL	drug-induced Lupus
DMSO	dimethyl sulphoxide
DNFB	2,4-dinitro-fluorobenzene (Sangers Reagent)
DNBS	2,4-dinitrobenzenesulphonic acid
DNA	deoxyribnucleic acid
DTT	dithiothretiol
ECL	enhanced chemiluminescence
EDTA	ethylene diaminetetraacetic acid
ELISA	enzyme-linked immunsorbent assay
FACScan	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
HAT	hypoxanthin, aminopterin, thymidine
HT	hypoxanthin, thymidine
HAPA	*N*-hydroxylamino-procainamide
hr	hour
HRP	horse radish peroxidase
H_2SO_4	sulphuric acid
IFA	Incomplete Freund's adjuvant
IFN-γ	interferon-gamma
IIF	indirect immunofluorescence
IEF	isoelectric focusing
IL	Interleukin
ip	intraperitoneal injection
kBq	kiloBequeral
KCl	potassium chloride
KD	kilodalton
LTT	lymphocyte transformation test
mAb	monoclonal antibody
MACS	magnetic activated cell sorter
MHC	major histocompatibility complex
min	minute
ml	millilitre
MW	molecular weight
NaN_3	sodium azide
ng	nanogram
%	percentage
PA	procainamide
PBS	phosphate buffered saline
PE	phycoerythrine
PEG 1500	Polyethyldiamineglycol

PLN	popliteal lymph node assay
PMA	phorbol myristate acid
PMφ	macrophage
PMSF	phenylmethylsulphonylfluoride
POSSEL	AutoMACS positive selection programme
RBC	red blood cell
rpm	rotations per minute
RNA	ribonucleic acid
RT	room temperature
sc	subcutaneous injection
SC	standard cocktail
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SI	stimulation index
SLE	Systemic Lupus Erythematosus
SNSD	self-non-self discrimination
Tc	cytotoxic T cell
TC	tumour cocktail
Th	helper T cell
TCR	T cell receptor
Thd	thymidine
TEMED	N,N,N,N-tetramethylethylenediamine
T_{reg}	T regulatory cell
Tris	tris(hydroxymethyl)aminomethane
µl	microlitre
µM	micromolar
WBMC	white bone marrow cell

1 Introduction

A wise man will make more opportunities than he finds –
FRANCIS BACON

1.1. THE ESSENCE OF IMMUNOLOGY

Immunology is the study of all aspects regarding host defence against infection and the adverse consequences of immune responses. The immune potential of mammalian species arises from a highly complex system involving numerous interacting cells which together, are intricately regulated by a variety of non-specific accessory cells and associated factors. The maturity of this intricate system begins in early foetal life and ultimately develops the ability to ignore self-components whilst permitting the recognition and destruction of copious pathogens.

Our immune system is often associated with battle or army terminology, we are after all, fighting enemies of pathogens and viruses and do so in a highly orchestrated manner. Upon entry into the mammalian system, foreign pathogens are confronted by the foot soldiers and cavalry such as mucous membranes and phagocytic cells, who provide abundant non-specific defence mechanisms. When pathogens manage to slip through these primary defence shields they are confronted with the special forces, derived from the primary lymphoid organs, namely T and B cells. The activities of these specific cells can be further ameliorated by certain task forces such as, adhesion molecules, co-stimulatory molecules, growth hormones and soluble factors such as cytokines and chemokines. These non-specific factors and signals can have either a direct influence upon the activation of antigen specific cells or a more remote regulatory one.

1.2. INNATE AND ADAPTIVE IMMUNITY

In AD 39-65, the roman poet Marcus Annaeus Lacunas used the word "*immunes"* to describe in his epic poem "Pharsalia", the resistance to snakebite by the Psylli tribe of North Africa. However, it was only in the 19th century and the ground breaking smallpox vaccination of Edward Jenners that the term immunity (*L. immunis*) and indeed immunology as a whole became a scientific term [1].

In 1896, Lord Lister addressed the British Medical Association and suggested "that if any chapters in the history of pathology were romantic it was definitely those concerned with the theories of immunity". Although also referring to the basic nature of the inflammatory reaction, his words were mainly directed to the battle over whether innate and acquired immunity to infection could be explained by cellular or humoral mechanisms [1].

Cellularists at that time were lead by Elie Metchnikoff at the Pasteur Institute in Paris. They argued that within the body, the chief line of defence against infection resided in the phagocytic and digestive powers of the macrophage cells. These cells are instantaneously available to combat a wide range of pathogens without requiring prior exposure and act in the same manner in all healthy individuals. Therefore, this line of defence was coined INNATE IMMUNITY. Controversially, the humoralists claimed that only the soluble substances of the blood and lymph could immobilise and destroy invading pathogens. The latter group was centred around Robert Koch at the Koch Institute in Berlin, Germany. The continual production of successful vaccines to cholera and rabies eventually lead to the discovery in 1890, by Emil von Behring and Shibasaburi Kitasato, that the sera from vaccinated individuals contained antibodies that could specifically bind to the relevant pathogens [2]. This outstanding evidence for a protective mechanism provided the necessary triumph for the humoralists and in 1908 the cellular immunology field slept for the next 60 years.

Nowadays, these two divisions of immunity are termed INNATE and ADAPTIVE and are both equally important in the understanding of the immune system. As mentioned, the adaptive immune response is responsible for the production of antibodies to an invading pathogen. Since this phenomenon occurs during the lifetime of an individual it is hence an "adaptation" to infection. Adaptive immunity has an extra feature and that is the ability to provide specific defence and memorise the previous strategy, so that upon re-infection the pathogen is immediately eliminated [3].

1.3. LYMPHOCYTE DEVELOPMENT AND DIFFERENTIATION

The cells involved in both innate and adaptive immunity arise from pluripotent hematopoietic stem cells in the bone marrow. The majority of the cells also mature within that environment before being released to survey and regulate the body. Collectively these cells are termed lymphocytes and upon activation have distant roles in their defence mechanisms against pathogens. However, although they may act separately these cells also have a close association and are often dependent upon one another for their activation and protective abilities.

The name for B lymphocytes is derived from their site of maturation, the bursa of Fabricus in birds [4]. Their distinguishing features are the well defined surface immunoglobulin (Ig) receptors or B cell receptors (BCR), composed of heavy and light chains. These are membrane bound forms of the antibody that are secreted upon activation. Mature B cells possess functional surface proteins including the major histocompatibility proteins (MHC) and Igs (IgM, IgD, IgA, IgE and upon activation IgG). Each B cell contains surface Ig receptors of a single specificity and therefore upon activation, each cell and its progeny produce antibody molecules of a single specific affinity for its epitope [3, 5]. When B cells are activated they differentiate into effector plasma cells whose primary

function is the production and secretion of antibodies. However, they also have important accessory properties such as antigen presentation in the activation of T cells. The diversity of Ig are further enhanced by the mechanism of class switching and somatic mutations. Class switching is alterations in the constant (C) region of the Ig heavy chain which leads to the acquisition of distinct biological functions. Thus, a B cell continues to express the same variable Ig region of its epitope but change the expression of its Ig class; this provides a more diversified approach for handling antigenic attacks. This phenomenon occurs since the IgH chain "Fc" dictates the function and the tissue specificity of the Ig molecule.

The development of T cells is depicted in figure 1.3. Mature T cells constitute a number of different T cell types and subsets possessing a variety of activities in order to allow an effective confrontation during a pathogenic encounter [3]. Helper T cells, which possess the CD4 protein, provide aid to both B cell antibody responses and cytotoxic T cells. The cytotoxic T cells destroy both viral infected and tumour cells and are designated by the expression of the CD8 protein. Fully competent T cells are generated by the extra developmental stages that occur in the thymus. As with B cells, T cells are borne in the bone marrow and undergo gene rearrangement in a specialised microenvironment to produce a unique antigen T cell receptor (TCR) on each cell. As with Ig, during gene rearrangement, the arising different combinations of TCR gene segments establish a diversity that allows a broad range of antigens (peptides) to be recognised [6]. The TCR is connected with a group of transmembrane proteins referred to as the CD3 complex which is composed of γ, δ, and ε chains [3, 7]. The complex is further associated with either a dimer of two ζ chains or a heterodimer of ζ and η chains.

The thymus is organised into three physically distinct areas: the outer subcapsular zone, the cortex and the inner medulla [3]. Upon entry into the thymus, pre T cells ($CD3^-CD4^-CD8^-$) enter the subcapsular zone and proliferate at an exceedingly fast rate producing $CD4^+$ and $CD8^+$ blasts. These cells then migrate deeper into the thymus and enter the cortex as immature $CD3^+TCR^{lo2+}$ cells. Upon cytoplasmic expression of the $CD3^+$ molecule, cells undergo a rearrangement of the TCR α, β or γδ genes. Cells that rearrange the TCRγδ chain acquire and then lose both $CD4^+$ and $CD8^+$ proteins and enter the periphery as TCRγδ T cells [8]. The function of these γδ T cells is still unclear, although the largest circulating population, designated by the expression Vγ9Vδ2, are thought to play a sentinel role [9]. Others however, expressing Vδ1 are known to recognise non-classical antigens such as lipids expressed on CD1 molecules [10].

Alternatively, cells which rearrange the TCR α and β chain genes become double positive T cell thymocytes ($CD4^+CD8^+$) and these can interact with the major histocompatibility complexes (MHC^+) on thymic epithelial cells. These epithelial cells are abundant in the cortex and express high levels of class I and class II MHC-encoded molecules. These MHC molecules present antigens composed of self-components to the double positive thymocytes. The specificity and affinity of the TCR on immature thymocytes for a self-peptide-MHC complex exposed on the epithelial cells will determine the fate of the thymocytes [11]. If the immature thymocytes do not recognise the self-MHC

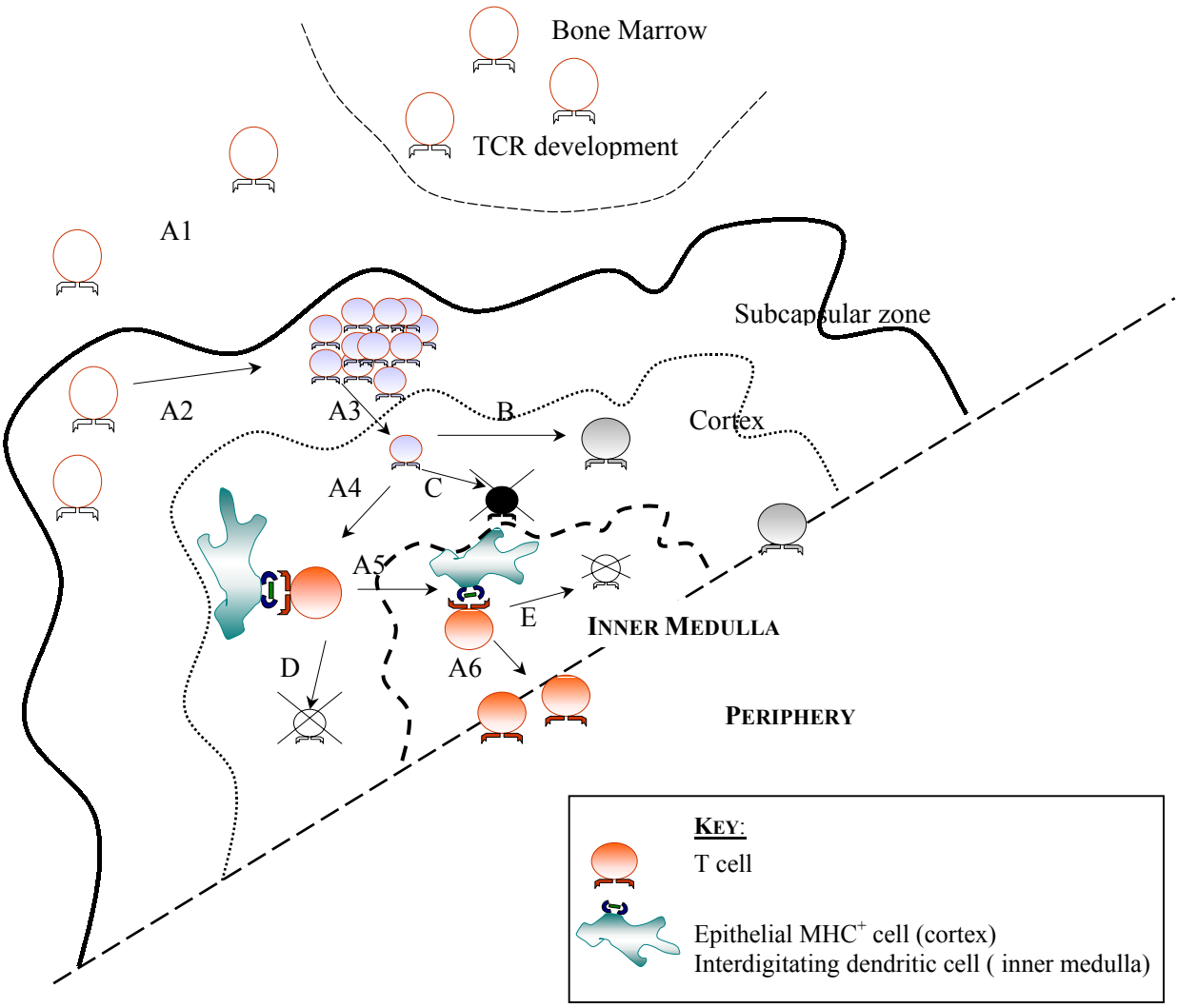


Figure 1.3. T cell development
T cells are borne in the bone marrow and develop individual TCR structures (A1). These pre T cells ($CD3^-CD4^-CD8^-$) enter the SUBCAPSULAR ZONE of the thymus (A2) and rapidly proliferate to produce $CD4^+$ and $CD8^+$ blasts (A3). The blasts then pass into the CORTEX as immature $CD3^+TCR^{lo2+}$ where the TCR α, β, and γδ genes are rearranged. Cells that rearrange the TCRγδ chain, loose both $CD4^+$ and $CD8^+$ proteins and leave the thymus as γδ T cells (B). Alternatively, the remaining $CD4^+CD8^+$ thymocytes either undergo POSITIVE SELECTION with EPITHELIAL MHC^+ cells (A4) or they die (C). Cells that respond with moderate avidity to presented proteins differentiate into mature $CD4^+$ or $CD8^+$ T cells (A5) and travel towards the PERIPHERY whereas others undergo programmed cell death (D). Before entering the periphery, mature T cells also undergo NEGATIVE SELECTION with INTERDIGITATING DENDRITIC CELLS in the INNER MEDULLA. T cells that display a very strong avidity for the presented proteins undergo apoptosis (E) whereas moderately responding T cells enter the periphery.

peptide complexes within 3-4 days, they immediately undergo programmed cell death. In contrast, when double positive thymocytes react with the MHC-peptide-complexes with a low but measurable avidity the cells will be POSITIVELY SELECTED, that is, allowed to differentiate into mature $CD4^+$ or $CD8^+$ T cells and migrate to the secondary lymphoid organs [12, 13]. Before entering the periphery, the positively selected T cells must first undergo another selection procedure since this population also contains T cells that have a too strong avidity for self-peptide-MHC complexes and are therefore potentially autoreactive. This process occurs via physical deletion involving apoptosis [14]. Depending on the availability of the antigen, this NEGATIVE SELECTION procedure or clonal deletion, may occur in the cortex with epithelial cells [15] or in the corticomedullary junction with

interdigitating dendritic cells [16]. T cells which survive both processes rapidly migrate to the periphery and enter the spleen or lymph nodes.

1.4. ANTIGEN RECOGNITION AND PRESENTATION

The pathways by which B and T cells recognise antigens is somewhat different. As described in 1.3, activated B cells secrete antibodies which bind pathogens or their toxic products in the extracellular spaces of the body. This binding leads to the neutralization of the pathogens and marks pathogens for destruction by phagocytes and complement. In contrast, classical αβT cells do not recognise intact whole protein molecules but linear antigens (peptides) that are presented on the MHC molecules of circulating APC [17].

The peptide MHC-class II complex is recognised by the complementary TCR on $CD4^+$ T cells [18] whereas MHC-class I complexes are recognised by $CD8^+$ T cells [19], figure 1.4. Class I molecules are found on virtually all cells in the body and generally present antigenic peptides from cytoplasmic sources. These peptides include those that arise from pathogens which have developed ways of entering the cells and replicating therein. MHC-class II molecules receive their antigenic peptides from extracellular sources by endocytosis and are present on cells only with the potential for antigen presentation such as macrophages, dendritic cells and B cells. In the mouse the class II molecules are encoded by genes within the "I region" of the MHC and referred to as I region associated antigens. These molecules exist as I-A and I-E subsets and the response to peptides may be either I-A or I-E restricted. Humans express the class II molecules HLA-DR, HLA-DP and HLA-DQ [3]. In both situations the presentation is genetically restricted in that only APC from members within the species which share certain MHC features are capable of presenting antigen to T cells of any given member of the species [3]. As a point of interest, it is important to remember that although class I and II molecules are defined as the endogenous and exogenous pathways of antigen presentation respectively, this distinction is by no ways absolute. Investigations into cross-presentation are rapidly demonstrating that this phenomenon does occur and is an intricate series of steps [20].

1.5. T CELL ACTIVATION

Activation, growth and differentiation of lymphocytes is under the control of intracellular signals. The lymphocyte activation pathway differs from other cells in that a) there are distinct receptors with precise specificity and diversity and b) the manner in which the signal is transduced from the surface of the cell into the cytoplasmic biochemical pathway. The activation process begins upon the engagement of the T cell TCR-CD3 complex to an MHC-expressed peptide which provokes the proliferation and differentiation of naïve T cells. In terms of classical self-non-self discrimination (SNSD) models this provides what has been designated as signal 1 [21]. Once recognition has been clarified, there is an activation of protein tyrosine kinases and protein tyrosine phosphatases which induce a multitude of phosphorylating and dephosphorylating events. Finally, activation of multiple

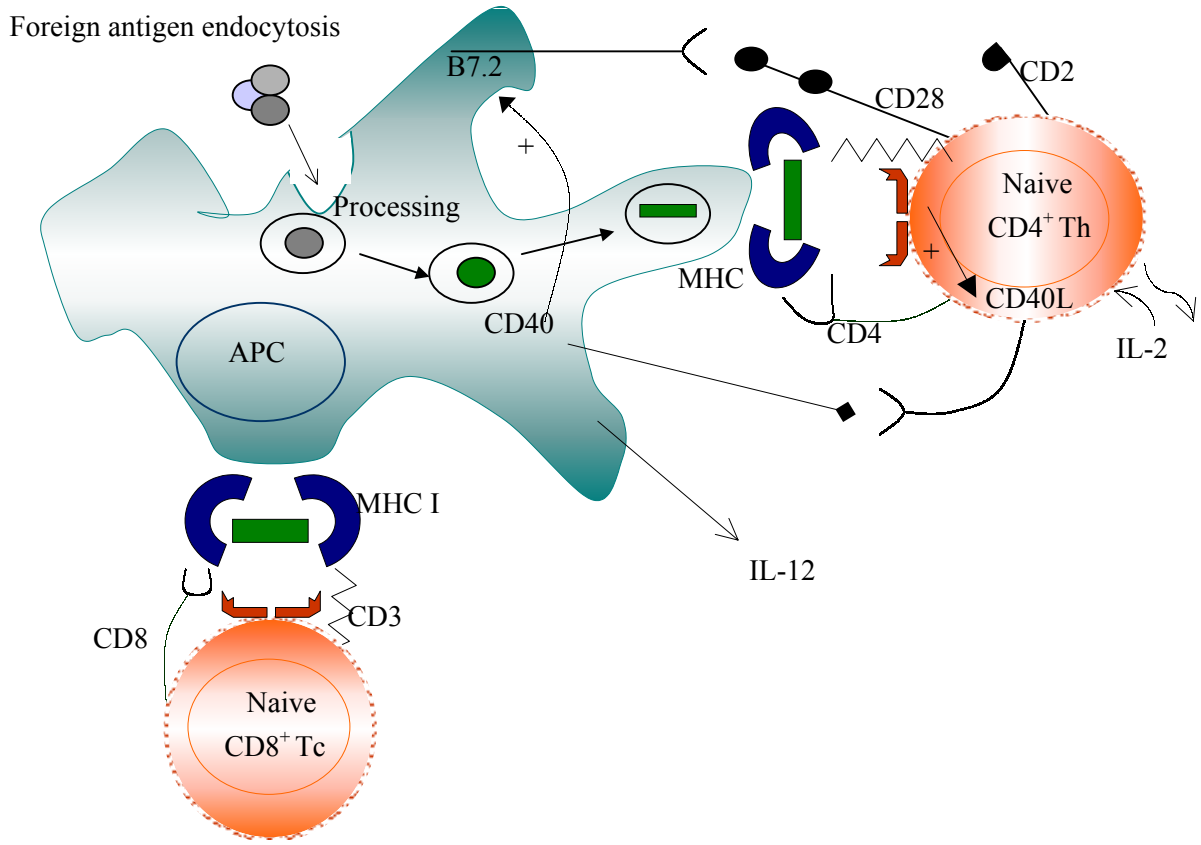


Figure 1.4 Antigen Processing and Presentation
Peptides that are derived from the endocytotic pathways within the APC are presented on the MHC-class II molecules. TCR molecules on $CD4^+$ T cells that are specific for the presented antigens engage the MHC-class II molecule leading to proliferation and differentiation of the naïve T cell into an effector cell. Costimulation is provided by the engagement of the CD28 membrane bound protein to its ligand B7-2 on the APC. CD8 T cells are activated by the presentation of antigens on the MHC-class I molecules, these peptides are usually derived from proteins in the cytoplasm.

transcription factors results in intracellular signalling [3]. In order to produce full lymphocyte activation, secondary signals are also required. In humans antigen-specific T cell events require costimulation signals, which are provided by the connection of the membrane bound protein CD28 and its ligands B7-1 and B7-2 (CD80 and CD86) on the APC surface [22]. CD28 delivers this secondary signal to $CD4^+$ T cells producing IL-2 and IFN-γ. Stimulation of T cells through the ligation of the CD28 molecule results in the augmentation of T cell gene expression and a multitude of cytokines. Cytokines play a fundamental role in the immune system and with the aid of monoclonal antibodies their expression patterns produced upon T cell activation, have been useful characterisation markers for isolating T lymphocyte subsets [23]. After activation, T cells can provide B cells with signal 2 and thereby stimulate the production of antibodies [3].

1.6. IMMUNOLOGICAL TOLERANCE

In 1953, Peter Medawar demonstrated that if exposed to foreign tissues during embryonic development, animals became immunologically tolerant to those tissues and thus failed to mount an immune response [24]. Consequently, MacFarlene Burnet hypothesised that developing lymphocytes, which are potentially self-reactive, are eliminated before they can mature and that unresponsiveness to

foreign antigens could be induced in animals if they were exposed to it in early life [25]. Since then, it has been demonstrated in a variety of animal species that immunological tolerance can be induced by numerous antigens [26]. Research into the cellular mechanisms of acquired tolerance to foreign antigens have provided considerable insight into the cellular events involved in self-tolerance. However, the relationship between experimentally induced tolerance to foreign antigens and naturally occurring tolerance to self has both practical and theoretical implications. For instance, the ability to start an immune response to foreign substances but not respond to one's own body constituents, indicates that the immune mechanisms must possess the ability to discriminate between self and foreign antigens.

In the majority of circumstances, tolerance to self-antigens (autoantigens) is initiated in the thymus through the positive and negative selection mechanisms described in detail in 1.3. To reiterate, during negative selection, T cells possessing a TCR with high avidity for self-antigen-MHCI/II complexes are deleted establishing CENTRAL TOLERANCE [27]. The remaining fraction which interacts with an intermediate avidity to self-antigen-MHC I/II complexes join the peripheral T cell repertoire. Even though some antigens located in the periphery have also been described in the thymus [28], the majority of self-antigens circulating in the periphery are absent in the thymus or only present in limited amounts. This is especially true for self-antigens such as tissue specific antigens. Therefore, this leaves a window of opportunity for some self-reactive T cells to enter the periphery and escape the negative selection regulations. In fact, a population of these T cells exists in both diseased and healthy individuals [29, 30]. However, despite the presence of autoreactive T cells, autoimmune illnesses within the general population are not a common occurrence, indicating that mechanisms must exist within the periphery to dampen the activity of these T cells.

Within these possible PERIPHERAL TOLERANCE mechanisms are clonal deletion, ignorance or anergy [31, 32]. Tolerance by ignorance is reserved for self-antigens which are sequestered and never come into contact with the immune system. Clonal deletion implies that the cells are eliminated and are no longer present within the system, whereas anergy refers to the situation in which the cells are functionally dormant but actively regulating the system. Anergy was first described for B cell tolerance [33] but has been subsequently demonstrated in both antigen specific T and B cell transgenic murine models [34]. The state of anergy occurs when the TCR of a T cell binds to a presented MHC-peptide but fails to receive the second costimulation signal. At this point the T cell becomes refractory to further activation by the specific antigen, even when presented by an APC.

There are currently four known criteria which can determine whether a self-reactive T cell becomes ignorant, anergic or is deleted: 1) the amount of MHC-class I/II complexes bearing the self-component (ligand density), 2) the localisation of the self-antigen, 3) the affinity of the TCR to the MHC-peptide complex and finally 4) the presence of costimulation. During the investigations into the above criteria it became clear that there were actually several active levels of peripheral tolerance [35, 36] which can act in unison or alone [37, 38]. In conclusion, all experiments confirmed the earlier studies by Bretscher and Cohn [39] which stated that an effective T cell response is only generated when signal 1 from the TCR is accompanied by signal 2 from the APC-derived costimulatory signals. Although the classical SNSD models have been continually adapted and altered they still fail to cover

all relevant questions and points about how immunological tolerance occurs. In 1989, Janeway Jr. found a way to meld costimulation and SNSD by insinuating that the innate immune system could discriminate between infectious and non-infectious self [40]. In this Pattern Recognition Receptor (PRR) model, he proposed that APC express distinct PRR which can recognise conserved patterns of molecules found only on evolutionarily distant organisms like bacteria. The binding of the bacteria onto the PRR induces the activation of the APC which then internalise the bacteria and re-express the bacterial antigens as peptides on their MHC-class II molecules. In turn, this upregulates the costimulation molecules, activates the neighbouring T cells and finally initiates the adaptive immune system [40]. Unfortunately, this mechanism still fell short of explaining how autoimmune diseases or immune responses to tumours could arise.

As a result, Matzinger and colleagues [41, 42] have extended this second signal theory by adding a third "DANGER" signal. Akin to the PRR model, the basis for the "Danger Model" was to try and incorporate costimulation into a functioning model of immunity. However, they discarded the old concept that the immune system is primarily concerned about non-self, and focused upon a more primitive mechanism for controlling immunity. This model is based on the idea that the ultimate controlling agents are not exogenous but endogenous and are actually the ALARM signals that emanate from stressed or injured tissues. Within this model a stressed cell alerts and activates a neighbouring APC by sending out signal 0 (the alarm signal). This communication subsequently activates the costimulation signals within the APC and activates the local T cells. Thus, in the context of this model the foreignness of the entity does not play a role unless the pathogen causes injury or stress. Likewise, an abnormality within self-physiology could cause an internal stress that could lead to an immune response to self. These danger signals can arise from products released on the destruction of a pathogen or by the overproduction of cytokines or oxygen radicals [41]. In the next section the growing topic of regulatory cells will be discussed and their ever increasing involvement in peripheral tolerance mechanisms.

1.7. IMMUNOREGULATORY T CELL POPULATIONS

A section of immunology which has gained increasing importance over the last few years is immunoregulatory T cells and their contribution, alongside anergy and clonal deletion, to the induction and maintenance of peripheral tolerance. Originally, suppressor T cells were conceived as dedicated populations that were $CD8^+$ and induced by $CD4^+$ T cells. These suppressor T cells were thought to recognise "whole" protein antigen in the context of the product of a putative I-J region purported to be located between the I-A and I-E MHC-region [43]. Their effects were supposedly mediated by secreted antigen-specific soluble "suppressor factors" [44], however, these factors could never be fully elucidated or cloned [45, 46] and this resulted in the downfall of this research area.

After several years and the development of precise sorting techniques with specific antibodies, researchers have been able to confirm the existence of these T cell sub-populations and have begun to unravel their abilities within various models of cell mediated immunity, such as autoimmunity, transplantation and tumour immunology [reviewed in 47, 48]. The first active contribution of these peripheral based cells was demonstrated by the transfer of $CD4^+$ T cells from normal donors into

lymphopenic animals and the subsequent prevention of different organ-specific autoimmune diseases in the recipients [49]. Nowadays, the general consensus is that the majority of these cell cohorts are $CD4^+$ in origin and resemble "normal" T cells both phenotypically and behaviourally. That is, they possess the typical αβTCR that can potentially recognise known MHC-restricted antigenic peptides in the classical manner. These subsets are thought to develop during normal T cell development and peripheral T cell responses. The latter are presumed to be influenced by both the nature of antigen stimulus and the cytokine and costimulation milieus. These regulatory cells can both enhance and suppress the immune response.

1.7.1. $CD4^+CD25^+$ T SUPPRESSOR CELLS

At the moment the $CD4^+CD25^+$ T cell subset is regarded as a suppressive subset since the majority of the literature demonstrates their ability to downregulate immune responses. The story surrounding the elucidation of $CD4^+CD25^+$ T cells spans almost three decades and their isolation was actually the turning point which revived the interest in T-cell-mediated suppression. The phenomenon began when it was reported that if mice were thymectomised on the third day of life, they developed autoimmune disease, the severity of which depended on the mouse strain. Three decades later Sakaguchi and associates isolated a minor $CD4^+$ T cell population (~ 10%), which constitutively expressed the α-chain of the interleukin-2 receptor (CD25) [48]. On re-performing the earlier experiments, investigators found that the development of autoimmune disease in three day old thymectomised mice, could be prevented by the injection of these $CD4^+CD25^+$ T cells [48]. Subsequent investigations have further revealed that this population is both hyporesponsive and suppressive [50, 51]. Although originally isolated in mice, it was soon discovered that the same population also existed in humans and possessed identical phenotypic and functional properties [47, 48, 52]. Alongside the "naturally" occurring $CD4^+CD25^+$ population, *in vivo* and *in vitro* experiments have also shown that several treatments (such as anergy or oral tolerance) can induce a broad array of suppressor cells. However, the relationship between these induced suppressor T cell populations (Ts) and the natural population remains a puzzle.

$CD4^+CD25^+$ T cells are anergic and absolutely dependent on exogenous IL-2 for their generation and survival *in vitro* and *in vivo* [53]. Their dependency on exogenous IL-2 has been underscored by the observations that mice genetically deficient for either IL-2, CD25, [53], IL-2β [54] CD28 or B7 [55] all lacked these Ts cells and succumbed to a variety of spontaneous autoimmune diseases. The first studies to define the suppressor function of $CD4^+CD25^+$ T cells *in vitro* was demonstrated by their ability to prevent CD3 activated $CD25^-$ T cells at a ratio of one $CD25^+$ to four $CD25^-$ T cells [50, 52]. Suppression only occurred when the $CD25^+$ T cells were activated through their TCR [55]. Therefore, the suppressive mechanism appeared to be via the inhibition of IL-2 transcription in the responding population, since the suppression could be overcome by both exogenous IL-2 and enhancing endogenous IL-2 production in the responders by additional anti-CD28 antibody [52]. The latter virtually enhances the aforementioned signal 2 (see section 1.5). $CD25^+$ T cells have been demonstrated to directly suppress $CD8^+$ T cells by a T cell-T cell interaction using specific peptide-MHC tetramers [57], indicating that APC are not required for the delivery of the suppressive signal to the responding cells. However, although direct suppressive effects on the APC

have not been observed [55] this does not exclude the possibility that $CD25^+$ T cells are able to exert effects on the APC or simply use them as platforms for attaching to the responder cells. In fact, very recently, $CD4^+CD25^+$ Ts cells have been shown to directly suppress activated B cells [58] and this point will be discusses later in section 4.7.2. Both types of indirect and direct suppression are depicted in figure 1.7.1.

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) and TGF-β have also been proposed as candidates for the suppressive mechanism. CTLA-4 is constitutively expressed on human $CD25^+$ T cells but whether this expression is just consistent with the activated/memory phenotype of these cells or it has a functional role is still not clarified. Studies have demonstrated that the addition of anti-CTLA-4 or its Fab fragment to an *in vitro* $CD25^+$ and $CD25^-$ culture could reverse suppression [51] and INFLAMMATORY BOWEL DISEASE could be suppressed by the simultaneous injection of these antibodies with $CD4^+CD45RB^{low}$ and $CD4^+CD45RB^{hi}$ T cells into susceptible recipients [59]. However, to date, no direct evidence with human cells has been established [52]. Nevertheless, despite these interspecies differences, research does indicate that the signals induced upon the engagement of CTLA-4 by its ligands CD80 or CD86 are necessary for the induction of suppression. Moreover, it remains a possibility that CTLA-4 is expressed on the $CD25^-$ T cells and the observed effects using these antibodies are actually directed towards that population.

In vitro the majority of studies to date have failed to produce definite evidence for a soluble suppressor cytokine. *Il4*$^{-/-}$ and *Il10*$^{-/-}$ knockout mice develop normal suppressor cells and the addition of specific neutralizing antibodies to these cytokines or transforming growth factor β (TGF-β) does not effect suppression. Even so, these findings do not rule out the involvement of cytokines especially ones that may act over short distances or are cell bound. For example, Nakamura *et al*., have hypothesised that latent TGF-β, bound to the surface of activated $CD25^+$ T cells, by an as yet unknown surface molecule, could be delivered directly to the responder $CD25^-$ T cells by a cell-contact-dependent mechanism. This in turn could switch the latent TGF-β into its active suppressive form [60]. Consequently, this theory could clarify why a high concentration of TGF-β has been shown to reverse suppression since it must first penetrate the interface between the two cells [61].

Although some agreement exists about the lack of involvement of suppressor cytokines *in vitro*, the *in vivo* mechanisms of $CD25^+$ T cells are more complex and several suppressor cytokines have been implicated as having crucial roles. The majority of these experiments have been performed using $CD25^+$ T cells from cytokine deficient animals or by treating reconstituted animals with neutralizing anti-cytokine antibodies [62-64]. In the latter animal models, it continues to remain a possibility that suppressor cytokines are produced by the host cells as a result of suppressor interaction. For example, in INFLAMMATORY BOWEL DISEASE, IL-10 has been shown to be produced by $CD25^-$ T cells [64] but the simultaneous source of TGF-β could be the $CD25^+$ T cells, other T cell populations or even non-lymphoid cells such as epithelium which is required in the healing process [65]. Nevertheless, IL-10, IL-4 and TGF-β remain three possible suppressor cytokine candidates although not necessarily altogether or in the same concentration. One possible explanation for this could be that there are distinct subsets of $CD4^+CD25^+$ T cells which can inhibit by either cell-contact-dependent mechanisms or by the secretion of different suppressor cytokines.

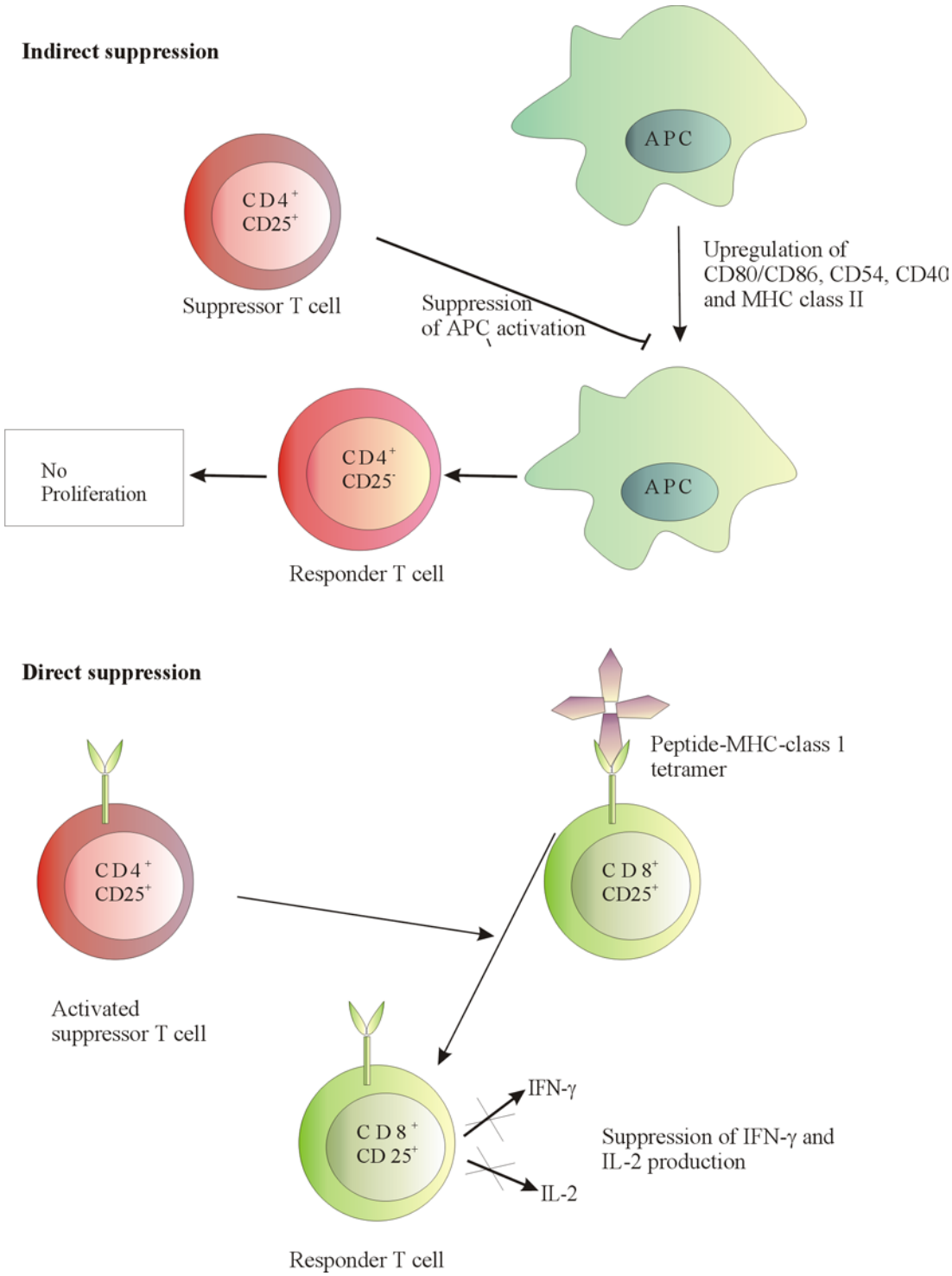


Figure 1.7.1. Indirect or direct suppression?
Two possible suppressive mechanisms of $CD4^+CD25^+$ T cells. A) Indirect suppression on $CD4^+CD25^-$ T cells via APC. $CD4^+CD25^+$ T cells act upon the APC to inhibit the upregulation of costimulation molecules required for the activation of $CD4^+CD25^-$ T cells and therefore indirectly inhibit the IL-2 production and hence the proliferation of $CD25^-$ responder T cells. B) Alternatively, direct T cell-T cell suppression by $CD4^+CD25^+$ T cells has been demonstrated through their inhibition of the IFN-γ production by $CD8^+$ responder T cells after *in vitro* activation using peptide-MHC-class I tetramers. This effect was demonstrated in the absence of APC.

1.7.2. OTHER IMMUNOREGULATORY SUBSETS

Although the most highly recognized, $CD4^+CD25^+$ T cells are not the only known regulatory population. Other types of $CD4^+$ regulatory T cells do exist, for instance, investigations have demonstrated that TGF-β and IL-10 can mediate the protective effects of the T cell population described as $CD45RB^{low}CD4^+$ [62, 66]. This population has been shown to prevent the development of "WASTING DISEASE" and "COLITIS" in the BALB/c murine strain and a subpopulation of these $CD45RB^{low}CD4^+$ T cells have been described to protect against "DIABETES" in NOD mice [67]. These $CD45RB^{low}CD38^+CD4^+$ T cells have been found to preferentially expand in mice protected from diabetes development by mycobacterial infection, indicating that these T cells contribute to the protection provided by TGF-β, CTLA-4 and Fas mechanisms [68]. In addition, this subpopulation can also downregulate the proliferation of $CD45RC^{low}CD38^-CD4^+$ T cells *in vitro* and this suppression was found to be mediated not by cytokines but by a mechanism related to those described for anergic cells [69].

$CD8^+$ regulatory T cells are also considered to have a regulatory role in oral tolerance since suppression can be adoptively transferred by either $CD4^+$ and $CD8^+$ subsets [70, 71]. Investigations have indicated that these $CD8^+$ T cells are required for local intestine suppression [72] but not for systemic hyporesponsiveness [70, 73]. Other experiments have shown that systemic $CD8^+$ Ts cells are induced after *in vivo* administration of autoreactive $CD4^+$ T cells. These $CD8^+$ T cells then suppressed the activities of the autoreactive $CD4^+$ T cells via both antigen-specific and non-specific mechanisms [74]. Recently allo- and xeno-specific $CD8^+CD28^-$ T regulatory cell lines, that may induce transplantation tolerance, have been isolated *in vitro* after multiple stimulations [75]. Thereafter, these cells could suppress alloreactive $CD4^+$ T helper cells by inhibiting the ability of APC to induce T helper cell activation. These APC have an impairment in their CD40 signalling pathway and thus fail to upregulate B7 molecules [76].

As mentioned above, the suppressive activities of several regulatory T cell subsets are mediated in a cytokine dependent fashion. However, double negative (DN) $\{CD8^-CD4^-TCR\alpha\beta^+\}$ T regulatory cells isolated from (C57BL/6 x BALB/c)F1 mice, have recently been shown to mediate tolerance in a different manner. These DN T cells are reactive with classical MHC-class I molecules, are present in pretransplant donor transfusions and mediate donor-specific skin allograft alloantigen–specific tolerance. The suppressive effects of these cells on the proliferation and cytotoxic activity of $CD8^+$ T cells, with the same antigen specificity, has been attributed to the Fas-mediated apoptosis of the alloreactive T cells [77].

Accompanying the classical αβ Treg cells are the non-classical γδ T cells and NKT cells (natural killer) which can recognise lipid antigens presented on the CD1 molecules of circulating APC. $CD8\alpha\alpha$ γδ T cells have been demonstrated to have an immunosuppressive, antidiabetogenic effect that is mediated by IL-10 [78] but their role in oral tolerance to OVA is still controversial [79, 80]. Evidence for an immunoregulatory role by NKT cells stems from the studies in NOD mice, in which depletion of NKT cells resulted in the accelerated onset of diabetes [81]. Conclusive evidence that NKT cells were required for systemic tolerance has arisen from the investigations using the ACAID

(anterior-chamber-associated immune deviation) model [82]. CD1 deficient mice, which specifically lack NKT cells could not be tolerised to OVA in this ACAID model unless they were reconstituted with NKT cells and $CD1^+$APC. However, NKT cells could not transfer the tolerance suggesting that they play a role in the induction phase of tolerance and are responsible for the differentiation of another regulatory subset, which in this case appears to be $CD8^+$ T cells [71].

As this field of interest continues to develop so does the intricate details and to fully comprehend the entire picture requires an understanding of the different factors and dimensions. In the case of $CD4^+CD25^+$ T cells, the role of IL-2 receptor expression on the cell surface has, to all current understanding, no actual role in the regulatory properties of the subpopulation. Therefore, other surface markers or combinations thereof, may discriminate between regulatory and non-regulatory cells. One such example is the recently described $\alpha_E\beta_7$ (CD103) on both $CD25^+$ and $CD25^-$ T cells. All combinations of these $CD4^+$ T cell subsets were demonstrated to have regulatory abilities with the $CD4^+CD25^+\alpha\varepsilon\beta7^+$ subset exhibiting the greatest influence [83].

1.8. XENOBIOTIC AND AUTOIMMUNE DISEASE

Immunotoxicology is the study of adverse effects on the immune system resulting from occupational, inadvertent or therapeutic exposure to drugs such as gold(I) [84] and procainamide [85], chemicals [86], environmental agents and in some instances, biological materials such as 3-pentadecyl-catechol. The latter is a representative catechol derivative in urushiol, the sensitizing component of poison ivy [87]. Generally, the immunotoxicology field can be divided into two research branches; one that deals with the unwanted immune reactions towards the xenobiotic and the other which studies the unwanted effects of the xenobiotic on the immune system. Within the first area, the immune system acts as a passive target (non-specific) for the chemicals, normally resulting in an increased incidence or severity of infectious diseases or neoplasia since there is an inadequate ability to respond to the invading agent.

The second area and the one in which the research in this thesis tends towards, delves into the study of allergic and autoimmune reactions that arise after xenobiotic exposure and the principles are summarised in figure 1.8. In this latter case, evidence has shown that chemicals and potential drugs can only be recognised by T cells if they bind to self-proteins, a process now termed HAPTENIZATION [88]. The arising haptenated self-peptides are then presented on the MHC-molecules of circulating APC [89]. Alternatively, chemical binding may change the physiological processing and presentation of self-proteins which may cause activation of self-specific $CD4^+$ T cells [84, 90]. The second mechanism allows for the presentation of cryptic self-peptides, peptides that are not normally presented. T cells are not tolerant towards these peptides and therefore react upon encounter [91]. The cytokines released by activated hapten- or self-specific $CD4^+$ T cells, determines the more prominent effector mechanisms of the immune response. The chosen effector mechanism, the strength and duration of the response and the site(s) of exposure to the eliciting antigen all determine the ultimate immunopathological picture [92].

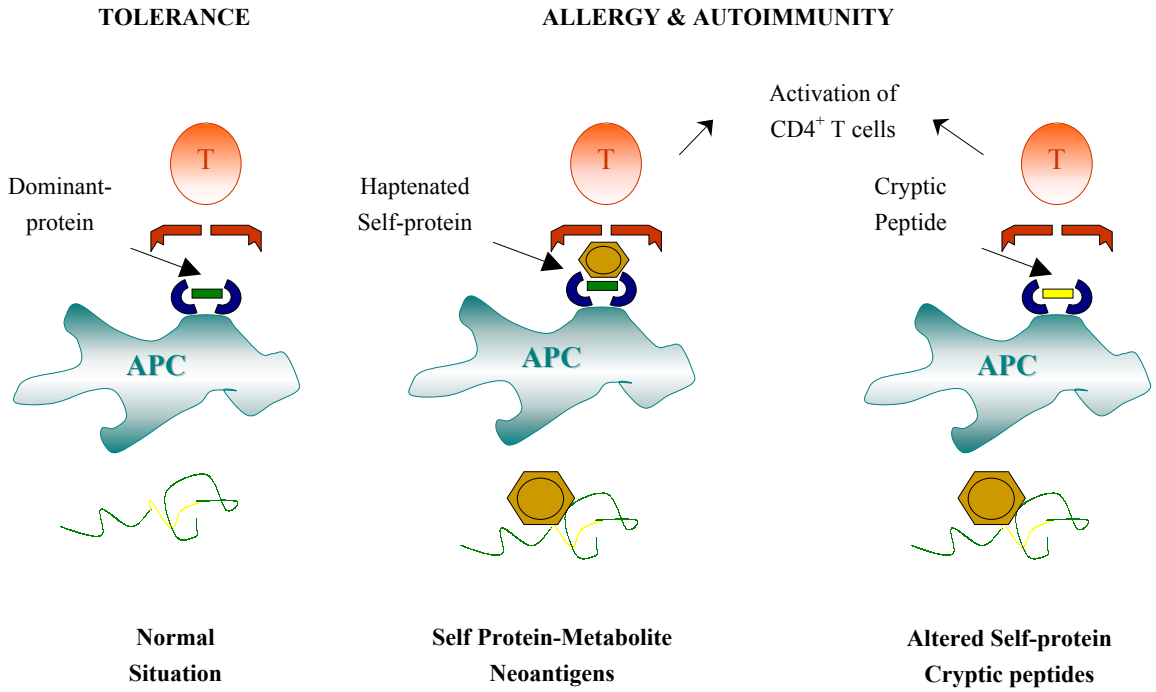


Figure 1.8 The initial steps involved in specific immune reactions to chemicals.
In the normal situation, dominant self-peptides are presented on the MHC-molecules of APC and ignored by circulating T cells resulting in tolerance to self. In the presence of chemicals, self-proteins can be altered in two ways. The chemicals can bind to the proteins and cause either the presentation of a hapten-self-peptide-complex or alter the protein so that a peptide is presented that is not normally seen. The latter are termed cryptic peptides. Both alterations initiate the activation of $CD4^+$ T cells and may eventually lead to adverse reactions.

Autoimmune diseases (AID) arise when an individual's own immune system mounts an attack upon self-tissues or organs, resulting in functional impairment, inflammation and sometimes permanent tissue damage. The pathogenesis of AID is a multifactorial and complex process, involving a large number of predisposing factors. These factors include the genetic specificities of the HLA and MHC-haplotype, viral exposure and hormonal, environmental or emotional influences. The manifestations of autoimmune disease include the production of autoantibodies, destructive inflammatory cell infiltrates into various target organs and the deposition of immune complexes at vascular sites. The well documented examples of xenobiotic-induced autoimmune syndromes have shown that through interference with the normal immunoregulation, xenobiotic exposure contributes to disease incidence [92].

As a number of factors participate in the development of xenobiotic-induced autoimmune disease and it initiates both diverse clinic symptomology and organ specificity, appropriate animals models have been difficult to develop. Consequently, predicative test models for the autoimmunogenic potential of xenobiotics have focused upon the detection of initiating responses rather than on symptomatic AID [93]. One method that has received attention is the Popliteal Lymph Node Assay (PLNA) which is based on the ability of chemicals, with the potential to induce adverse reactions, to cause non-specific changes in the draining lymph nodes [94]. This assay detects the influence of low molecular weight compounds with immunoregulatory potential, but fails to

discriminate between those that produce autoimmunity and those that may provide false positives results, as often seen with irritating agents [95]. Another test is the Lymphocyte Transformation Test (LTT) which demonstrates secondary responses of helper T cells to certain compounds using an *in vitro* based proliferation assay. Both of these assays have been incorporated into the research of this thesis and are shown in sections 3.1.2 and 3.2.4-6 respectively. A number of animal models displaying autoimmune diseases have been described but these representatives have rarely been used as screening models. They fall into three categories including, (i) those that develop spontaneous autoimmunity, such as the nonobese diabetic (NOD) mouse and the MLR lupus mouse, (ii) those which employ experimental autoimmunization, for instance immunisation with myelin basic protein which leads to experimental allergic encephalomyelitis (EAE) and (iii) those animal species or strains that are uniquely susceptible to developing autoimmune diseases following certain chemical exposure. An example of this latter model is mercury-induced glomerulonephritis detected in Brown Norway rats [96] and H-2^s mice such as the A.SW strain [97].

1.9. CHEMICALS AS HAPTENS

As mentioned above in section 1.8, two mechanisms of chemical-specific T cell activation are currently hypothesised. The first theory focuses upon the "*THE HAPTEN THEORY*" in which the xenobiotic must first chemically bind (haptenate) to a portion of a macromolecular carrier such as a protein [88, 92]. These chemical-protein complexes have been termed neoantigens. The second mechanism proposes that the chemical may induce the presentation of an altered set of self-peptides, including cryptic peptides, due to an alteration of the respective self-protein by either covalent binding or complex formation. This second mechanism has been described for metal ions such as mercury chloride and gold [98, 99].

1.9.1. HAPTEN-PROTEIN CONJUGATION

One of the most problematic aspects in studying T cell reactions to sensitizing chemicals is the fact that in most cases the ultimate neoantigens recognised by "xenobiotic-specific" T cells are unknown. Only a few specific neoantigens have been fully elucidated, examples of these are the classical hapten TNP and 3-pentadecyl-catechol [87, 89]. Following these findings, other xenobiotics are also thought to produce hapten-carrier conjugates or protein adducts. The term "hapten" denotes substances that are not immunogenic per se but become immunogenic when conjugated to a carrier protein. Majoritively, xenobiotic drugs are less than 1000 daltons in size and are not stable in a prohapten form. Nevertheless, these drugs can be metabolised and produce reactive species (haptens) that possess the ability to covalently bind to proteins which are then recognised as immunogens.

The molecular weight of an organic molecule is a major factor in determining whether a particular molecule has the potential to act as an immunogen [100]. Indeed the higher the molecular weight the more pronounced the immunological reaction [101]. The following two reasons highlight why the size of the molecule is an important criteria for the immunogenic potential of the prohapten, 1) the antigen must persist within the body long enough to be engulfed by antigen presenting cells and

activate the relevant clones of lymphocytes and thus induce an effective immune response. 2) a large molecule may be a more effective antigen because it displays multiple epitopes, which are required to stimulate a substantial portion of the T and/or B cell repertoire.

1.9.2. PROHAPTEN METABOLISM

Reactive chemicals such as quinones and aldehydes are capable of directly binding to self-proteins and can elicit irreversible alterations in the protein structure which can provide the aforementioned signal 1 (see section 1.5), necessary for T cell activation. However, the majority of xenobiotic compounds, including drugs, require metabolic bioactivation in order to produce chemically reactive intermediates [101]. Although having a special role in drug metabolism, the liver is considered to play a minor role in the initiation of drug-induced allergy and autoimmunity since T cells that encounter their antigen in this organ are either deleted or anergised rather than activated. The liver readily detoxifies harmful compounds, such as reactive metabolites, by initiating the coupling between them and molecules like glutathion and acetyl [102]. These metabolic activities however, are often under the influence of the cytochrome P450 enzymes which are responsible for the transformation of some chemicals into reactive metabolites. Research has demonstrated that some of these formed metabolites are able to bind to the cytochrome P450 enzyme and initiate drug-induced hepatitis. Examples of this phenomenon are tienilic acid and dihydralzine whose reactive metabolites directly bind to the metabolising P450 isoenzymes 2C9 and 1A2, producing IgG antibodies to these P450 isoenzymes [103].

In recent years, the notion that drug metabolism can occur in polymorphonuclear neutrophils and mononuclear phagocytes has become more established. This ability is largely due to the abundance of reactive oxygen species, such as hypochlorous acid (HOCl), residing within phagocytes which can generate reactive intermediate metabolites from a great variety of drugs and other chemicals [104]. In comparison to liver metabolism, this extra-hepatic drug metabolism may not be important in quantitative terms. However, it does appear to be a critical factor in the development of adverse immune reactions to drugs and other chemicals for four reasons. First, phagocytes indiscriminately engulf self-proteins as well as foreign substances. Second, phagocytes react to foreign substances in the same manner as to phagocytosed bacteria. For example, upon contact, the hypochlorous acid formed in the respiratory burst of phagocytes can oxidise a great variety of compounds [104], resulting in the formation of reactive metabolites that are immunogenic for T cells. This has been formally demonstrated with Au(I) [99, 105] and procainamide [106] which are rapidly transformed into their reactive and immunogenic forms Au(III) and *N*-hydroxyprocainamide (HAPA), respectively. Third, these activated mononuclear phagocytes cannot only oxidise the prohaptens into their hapten forms and present the resulting neoantigen to T cells, but can furthermore provide the necessary costimulation, that is, signal 2 [42]. Lastly, phagocytes are present in almost all tissues of the body, therefore allowing for the production of reactive metabolites and thus neoantigens at the anatomical site in which the adverse reaction occurs. This situation is more likely than the alternative transportation of the neoantigen from the liver.

1.10. PROCAINAMIDE-INDUCED IMMUNE REACTIONS

The main aim of this thesis work was to study T cell reactions that arise after the administration of procainamide (PA) both *in vitro* and *in vivo*, therefore this section covers the current knowledge surrounding PA. As mentioned earlier, upon metabolism, PA can form the reactive metabolites HAPA and nitroso-PA which can subsequently bind to proteins and form neoantigens. Therefore, the elucidation of these neoantigens was also a focal research point.

1.10.1. PROCAINAMIDE, A GENERAL INTRODUCTION

Over the past five decades it has become more apparent that during long term treatment, certain medications have the propensity to produce autoantibodies and occasionally lupus type syndromes. Since then, Drug Induced Lupus (DIL) has developed into the prototype for studying systemic autoimmune diseases caused by known environmental agents. Over forty six different drugs have been shown to induce DIL and thirty eight of these remain on the market. Nevertheless, DIL is not classed as a severe clinical problem since the normal healthy state of the treated individual usually returns after discontinuing the use of the drug. The type IA anti-arrhythmic drug procainamide belongs to the family of compounds termed arylamines, which are widely used in industry and medicine and notorious for their capacity to induce allergy and/or autoimmunity [92, 106, 107]. Alongside hydralazine and isoniazid, treatment with PA poses the greatest risk for developing perplexing idiosyncratic adverse drug reactions and generally occurs after several months or years of PA-treatment.

DIL occurs in approximately 20% of the PA-treated patients and almost all develop autoantibodies regardless as to whether full lupus-like symptoms appear. The onset of the disease is usually insidious with symptoms becoming more intense 1-2 months before diagnosis. Joint pain is a common complaint and is accompanied by fever, anaemia, lung problems, weight loss and a characteristic butterfly skin marking on the face [107]. In addition many patients treated with lupus-inducing drugs develop serologic abnormalities without disease symptoms. Antinuclear antibodies (ANA) in these symptomatic patients are clearly drug-induced and as with the more severe complaints subside after treatment is stopped. Different ANA have been reported for different DIL cases. Most patients undergoing prolonged PA therapy develop anti-denatured DNA (dDNA) [108] and anti-histone antibodies [109], whereas IgG antibodies, to the (H2A-H2B)-DNA subunit of chromatin, are a serologic marker in patients who develop symptomatic PA-induced lupus [110]. However, the pathogenesis of these DIL diseases are far from elucidated [85, 104, 107, 111] but one theory assumes the process to follow a graft-versus-host like pathway in which, T lymphocytes react to self-molecules on lymphoid cells that are then rendered “foreign” by the drug [112].

1.10.2. THE METABOLISM OF PROCAINAMIDE

DIL is a type B adverse drug reaction [113] which means that it cannot be predicted from the drug’s classified pharmacology. A possible pathway in which idiosyncratic drug reactions can occur is

through the *in vivo* generation of drug metabolites which possess different properties to the parent compound as discussed previously in section 1.8. Arylamines are considered to be prohaptens, which can undergo metabolism to produce chemically reactive metabolites that can bind to proteins and consequently form neoantigens. Figure 1.10.2 depicts the major metabolic pathways of PA which include, 1) the acetylation of the arylamine group producing *N*-acetylprocainamide (NAPA), or 2) the *N*-dealkylation of the aliphatic amine into desethylprocainamide followed by the sequential acetylation of the arylamine and *N*-dealkylation of the aliphatic amine to form *N*-acetyl-desethylprocainamide. Alternatively, the third pathway 3), involves the hydrolysis of the amide link producing *p*-aminobenzoic acid and its *N*-acetylated derivative. The final pathway 4), is the *N*-oxidation of the arylamine into the intermediate reactive metabolites *N*-hydroxyprocainamide (HAPA), which can be further converted into the unstable reactive nitrosoprocainamide and nitroprocainamide [114, 115].

The above described metabolic pathways can actually be metabolised via two different enzymatic pathways, the acetylation of PA (pathway 1) is mediated by the polymorphic *N*-acetyltransferase (NAT2) whereas cytochrome P450 isozymes are involved in the formation of other major metabolites especially those in the fourth oxidation pathway [116]. *N*-acetyltransferase activity occurs in the liver and is the pathway which predominates in individuals who are termed fast acetylators. In both mouse and man the gene for the fast acetylator phenotype is predominant. Individuals which are homozygous recessive for this particular gene enzyme usually have a two fold concentration of PA in their blood system. These individuals are therefore classed as slow acetylators and actually process PA by the oxidation pathway which consists of *N*-hydroxylation of the amino group by myeloperoxidase-generated hypochlorous acid. The products of this second pathway include *N*-hydroxyprocainamide-PA (HAPA), which is capable of covalently binding to proteins, in contrast to PA and *N*-acetyl-PA which cannot [117].

1.10.3. THE ROLE OF METABOLISM IN PROCAINAMIDE-INDUCED LUPUS

When treated with PA, slow acetylator phenotype humans and mice develop ANA faster and more frequently than fast acetylator controls [118]. In addition, DIL was not observed when PA treatment was substituted for treatment with the metabolite NAPA, suggesting that the predominant *N*-acetylation pathway actually plays a protective role [119]. Consequently, the reactive metabolite HAPA and its unstable nitroso derivative have been considered the responsible elements for the adverse immune effects that develop during PA treatment [115, 119, 120]. The formation of HAPA has been experimentally demonstrated after PA was incubated with either human or rat hepatic microsomes [114, 115] and this conversion was shown to be mediated by the cytochrome P450 isozyme, CYP2D6 [116]. The expression of CYP2D6 is bimodally distributed within the human population. Those with an active form of the isozymes are classed as extensive metabolisers whereas those with a poor or absent CYP2D6 are termed poor metabolisers [116].

HAPA has also been detected after the perfusion of rat liver with PA in a blood free environment [121]. Additionally, HAPA can also be engulfed by erythrocytes and within these cells the oxyhaemoglobin can apparently enhance the reactivity of this metabolite [120] and convert it into nitroso-PA [85, 121]. However, although research has shown that PA-treated rats have increased liver

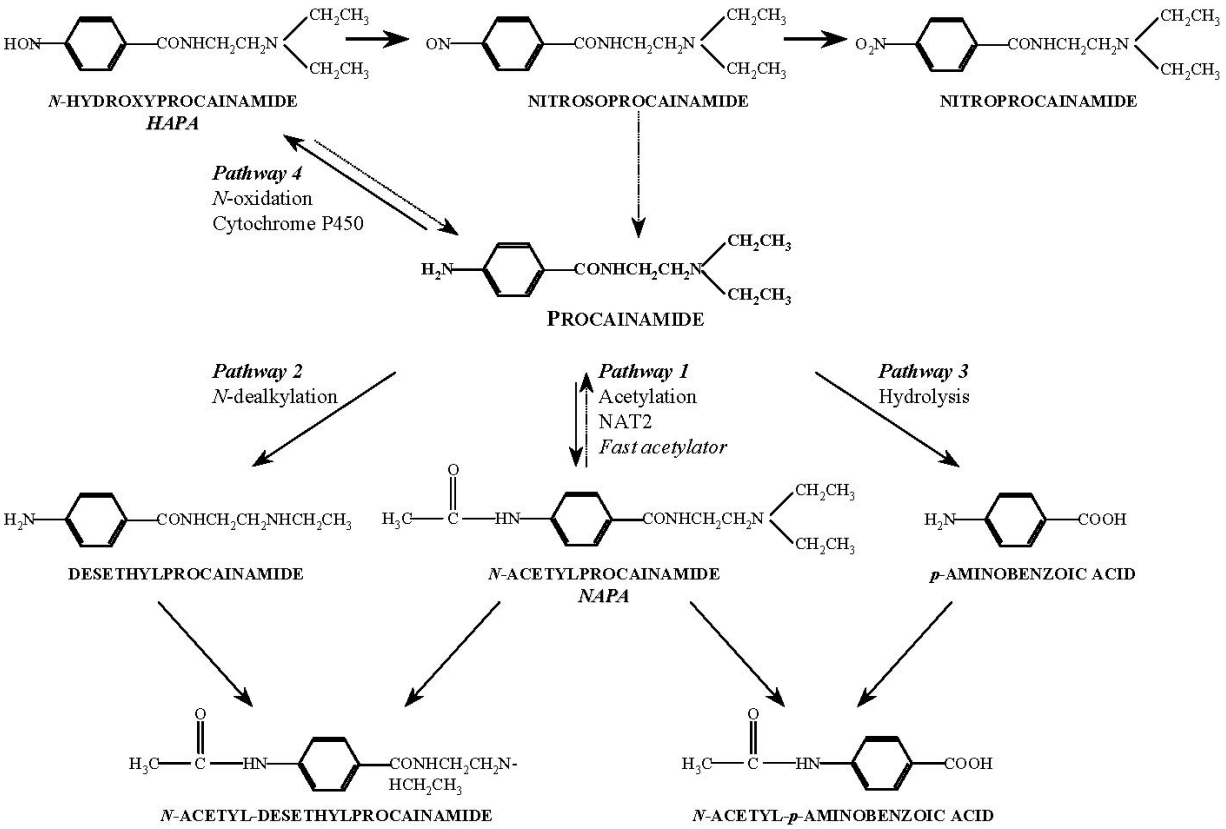


Figure 1.10.2. The major metabolic pathways of procainamide.
The four main pathways for the metabolism of PA are depicted in the above diagram. The main fast acetylator pathway 1, involves the acetylation of PA by the NAT2 enzymes leading to *N*-acetylprocainamide (NAPA). In contrast the slow acetylator pathway 4, is the *N*-oxidation of PA into the most reactive metabolite *N*-hydroxyprocainamide, HAPA.

lipid peroxide levels and the antioxidant activity within the liver could potentially metabolise the drug and produce metabolites [122], it is unlikely that liver mediated drug transformation plays an important role in the etiology of DIL. The explanations for this reasoning have been mentioned above in section 1.8. Alternatively, a more probable clarification is the involvement of an extrahepatic process, in which the reactive metabolites are produced within the immune compartment. Whilst there, they can directly act on the cellular elements of the immune system and ultimately prevent or break immune tolerance [115]. Phagocytic white blood cells and neutrophils are a potential source for oxidative metabolic processes. When the Fc or complement receptors of neutrophils are activated by opsonised particles, a respiratory burst is initiated. Thereafter, molecular O_2 is reduced by the ectoenzyme NADPH oxidase to produce the superoxide anion O_2^- which rapidly accumulates in the extracellular environment and forms hydrogen peroxide, H_2O_2. Accompanying the respiratory burst is neutrophil degranulation in which a portion of the neutrophil contents, containing specific and azurophil granules, are released into the extracellular environment. The dominant enzyme in granules granules, myeloperoxidase (MPO), is then released and can enzymatically oxidise locally present drugs using H_2O_2 that is produced upon neutrophil activation [123]. Rubin *et al*., were the first group to demonstrate that activated peripheral blood neutrophils have the ability to metabolise PA into HAPA and later described the role of the respiratory burst and degranulation events in detail [124].

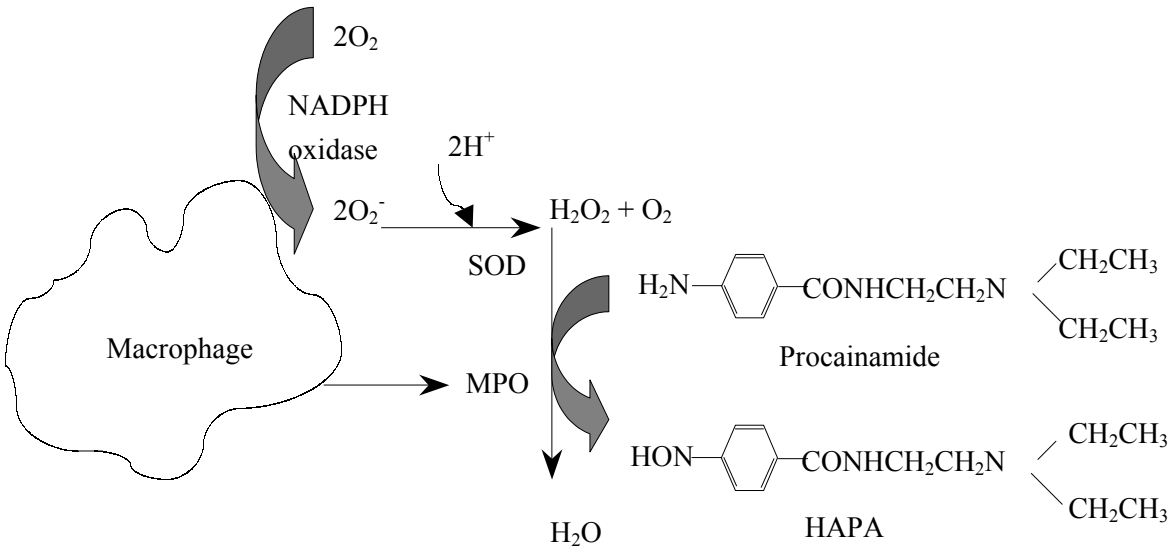


Figure 1.10.3. Neutrophil metabolism of procainamide**.**
The above depiction is the hypothesised mechanism by Rubin and co-workers for the metabolism of procainamide by activated neutrophils and macrophages. The H_2O_2 produced by the dismutation of O_2- by superoxide dismutase (SOD) serves as the primary substrate for myeloperoxidase (MPO) mediated co-oxidation of PA to it's reactive metabolite HAPA.

Their proposed mechanism for the bio-transformation of PA is depicted in figure 1.10.3. All major pharmacological classes of lupus-inducing drugs have the capacity to undergo transformation to reactive products after exposure to activated neutrophils [123]. However, the chemical analogues of these drugs are actually resistant to this metabolic pathway and this has been demonstrated with the analogue of procainamide, *N*-acetyl-PA. Furthermore, the ability of neutrophils to metabolise PA directly within the lymphoid tissues allows for the generation of reactive metabolites at the proposed sites of the autoimmune disease. Consequently, this would minimise: 1) the dilution of the reactive species, 2) the time dependent hydrolysis, 3) the circulatory distribution of reactive metabolites and 4) provide the necessary signal 2 via oxidative stress. In short it maximizes their ability to be recognised by lymphocytes.

1.10.4. T CELL REACTIVITY IN PROCAINAMIDE INVESTIGATIONS

The hallmark of DIL is the appearance of autoantibodies. However, as with protective immunity, development of such IgG antibodies to self requires the participation of cognate T-cell help. In order to investigate this hypothesis, the sensitising abilities of PA and its reactive metabolites have been extensively studied using the PLNA [106, 125-127]. The basis of this *in vivo* assay is described above in section 1.8. Within these studies a mixture of both the mother compound and the suspected primary reactive metabolite HAPA were used. Here, the aqueous solution of the PA derivatives consisted of both HAPA and nitroso-PA because HAPA is readily oxidised to the extremely reactive nitroso-PA, a compound that cannot be isolated due to its high instability [128]. From these investigations it was observed that PA and *N*-acetyl-PA (the chemical analogue) failed to produce a PLNA response. In contrast, vigorous PLNA responses did occur when murine strains were injected with either a mixture of PA metabolites including HAPA or homogenates of liver microsomes that had been previously incubated with PA. These experiments provided further evidence that only certain metabolites of PA

can elicit an immune reaction. Such exclusive immunogenicity to HAPA confirmed the earlier findings that HAPA but not PA can covalently bind to proteins [129].

In further studies, which incorporated the adoptive transfer PLNA, enriched T cells from mice that had received injections of the reactive metabolite mixture were shown to be specifically sensitised to the metabolite(s), but not to PA or *N*-acetyl-PA [106]. Using this system, an anamnestic T cell response could also be elicited when the challenging antigen consisted of homogenised peritoneal cells that had been extracted from four month long PA-treated mice. This result indicated that peritoneal cells, known to be rich in macrophages and neutrophils, from PA-treated animals contained the reactive metabolite(s) of procainamide. Consequently, this response was tested in two different murine strains that have been previously described as either slow or fast acetylators [130]. With the slow acetylator A/J murine strain, it was demonstrated that T cells from these mice, that had been immunised against HAPA, were able to mount a secondary immune response to this reactive metabolite. Furthermore, a secondary response could also be elicited to peritoneal macrophages (PMφ) obtained from syngenic donors, which were previously treated *in vivo* for four months with PA [106]. Indicating that this murine strain were able to generate sufficient quantities of HAPA *in vivo*. This observation provided further credence to the hypothesis that immunogenic metabolites are generated by extrahepatic phagocytic cells.

In contrast, the fast acetylator C57BL/6 mouse strain, required additional stimulation by the mitogen PMA in order to increase the oxidative metabolism of the phagocytes and thus production of the reactive metabolites [106]. This experimental result may be analogous to what clinicians have noted in patients since these adverse immune reactions to drugs often develop after the treated individual suffers from an infection, such as a common cold. Conceivably, this stimulates the oxidative burst in the patients phagocytes and thus enhances the production of reactive metabolites from the co-present drug. In this context, the enhanced production of certain cytokines such as TNF-α and IFN-γ during infections may be of relevance, since these cytokines may suppress drug metabolism in the liver and therefore lead to higher concentrations of the parent compound for extrahepatic metabolism by phagocytes.

1.11. HYPOTHESIS SURROUNDING PROCAINAMIDE DRUG INDUCED LUPUS

Since the initial observations that certain drugs possessed the ability to elicit adverse drug reactions, researchers have sought to find a common mechanism for their actions. Typical drug hypersensitivity reactions are characteristically drug dose independent and recur immediately after rechallenge with the inciting agent [131]. In contrast, DIL reactions stand apart from the hypersensitivity reactions due to: 1) their slow kinetics, which can be a delay of months to years from the onset of drug therapy to the development of autoantibodies and clinical symptoms, 2) correlations with the actual drug dose and 3) the inconsistent recurrence of symptoms upon rechallenge with the inciting drug [131]. To compound the problem, lupus-inducing drugs are heterogeneous with no common structural properties, or capacity to enhance or inhibit autoantibody binding. Nevertheless, investigations surrounding PA

drug-induced lupus have firmly established that autoantibodies are produced with varying specificities [132, 133] and a definite T cell response occurs [106, 127]. The current hypothesis for the complete pathogenesis of DIL is as yet unresolved but it is likely that the underlying immune perturbations responsible for the lupus like syndrome induced by all drugs are similar even though this mechanism remains an enigma.

The general consensus surrounding the first pre-immunological step involves the conversion of PA into its reactive metabolites HAPA and nitroso-PA [104, 106, 134, 135] but the subsequent steps remain a matter of debate. Our group proposes that due to the ability of the metabolites to bind to proteins [117], hapten-specific reactions could develop into an autoreactive T cell reaction [106]. In hapten-protein complexes, the hapten as well as the protein, can be recognised by T and B cells. These haptenated peptides are subsequently presented on the MHC-molecules of professional APC and thus activate T cells. In turn, this activation provides help to both hapten-specific and protein-specific B cells which are presenting the same MHC-bound haptenated peptides. Consequently, both hapten-specific antibodies and self-antigen-specific autoantibodies are produced. This mechanism of chemical induced autoantibody production has been well described for many chemicals [136]. The identification of these neoantigens has been a large focal point in our previous research and throughout this thesis further attempts have been made to elucidate these neoantigens.

Other theories have been voiced by the groups of Richardson and Rubin. In the former, this group has focused upon the mechanism by which PA can render T cells pathogenic. Amongst other DNA hypomethylating agents, PA has the ability to inhibit T cell DNA methylation and generate autoreactive $CD4^+$ T cells [134, 137]. Furthermore, following an adoptive transfer into non-irradiated recipients, these $CD4^+$ T cells could elicit an autoimmune reaction. PA also increases the expression of lymphocyte function-associated antigen 1 (LFA-1) and their research has demonstrated that T cell LFA-1 overexpression is sufficient to induce autoimmunity. Using a cloned murine Th2 cell line, LFA-1 overexpressing cells were adoptively transferred into syngenic recipients which subsequently developed a lupus-like disease state including anti-DNA antibodies, immune complex glomerulonephritis and pulmonary alveolitis [137]. Since T cells from patients with active lupus have hypomethylated DNA and overexpressed LFA-1, this mechanism is an alternative pathway for the development of human autoimmunity.

The concept of Rubin and co-workers tends more, like ours, to focus upon the activities of the metabolic products of PA. However, in recent publications the subsequent steps have moved towards a new theory which involves the possibility that it is the failure of central tolerance and not the loss of peripheral tolerance that might initiate DIL. Several years ago they developed a murine model exhibiting the hallmark features of DIL [138]. In that mouse model, two intra-thymic injections of HAPA resulted in high levels of ANA and chromatin specific T cells in the spleen. These observations suggested that the action of HAPA in the thymus resulted in the export of autoreactive T cells into the periphery where they provided T-helper-cell function to B cells with the potential to produce autoantibodies. This phenomenon did not occur if HAPA was injected directly into the periphery and therefore implied that disruption of central T cell tolerance maybe the reason [135]. Following these results, the group then sought to determine how tolerance to self in the thymus could

be disrupted by the presence of HAPA. Employing transgenic mice on different MHC backgrounds they were able to investigate whether the drug affected high-affinity or low affinity interactions of the TCR on immature T cells. They demonstrated that when the thymocytes undergo positive selection in the presence of HAPA, the resulting T cells have a lower threshold of activation than T cells derived from an untreated thymus, therefore permitting them to respond to low-affinity ligands. In view of that finding the group proposed that HAPA somehow prevents the establishment of anergy during T cell selection in the thymus. Their interpretation is that HAPA interferes in the accumulation of the putative negative regulators of activation that are normally responsible for setting a higher activation threshold during positive selection. Consequently, the resulting thymocytes can be activated by the selecting self-antigen rather than developing tolerance to it. When this occurs to an abundant self-protein such as chromatin, the T cells which enter the periphery maybe rapidly activated to the expressed chromatin and thus initiate a systemic autoimmune reaction [135, 139].

1.12. AIMS OF THIS THESIS

The essence of the working model for PA-induced adverse immune reactions postulates that due to the conversion of PA into the reactive metabolites HAPA and nitroso-PA, neoantigens are generated and presented by APC which are subsequently recognised by specific T cells and thus activates them. These T cells would then secrete cytokines and activate other cells including autoreactive B cells. The aims of this thesis work were to decipher several points in the current model including:

1) Direct evidence of PA-induced neoantigens after the metabolism of PA by WBMC.
2) The role of T cells in mediating specific immune responses to PA-neoantigens.
3) The establishment of PA-specific $CD4^+$ T cell hybridomas to investigate T cell responses to PA-neoantigens.
4) To determine specific secondary T cell responses to PA-neoantigens after long term oral PA treatment in slow acetylator A/J mice.
5) To identify, *ex vivo*, the responding T cell subset to PA-neoantigens from long term PA-treated A/J mice.
6) To investigate the possible requirement for APC during the specific secondary T cell responses to PA-neoantigens.
7) The induction and characterisation of ANA in A/J mice after long term oral PA treatment.
8) To investigate whether the sera from long term PA-treated A/J mice contained antibodies towards a common protein.
9) To determine the $CD4^+$ T cell subpopulation responsible for inducing ANA in long term PA-treated A/J mice
10) The role of PA-primed $CD4^+CD25^+$ donor T cells in the prevention of ANA in syngenic xenobiotic-treated recipients.

2 Materials and Methods

No amount of experimentation can ever prove me right;
a single experiment can prove me wrong
ALBERT EINSTEIN (1897-1955)

To describe the materials and methods used throughout this work this chapter is divided into eight sections. In section 2.1, there is a general summary of the necessary materials. This section is followed by detailed accounts regarding cell preparation methods 2.2, immunological assays 2.3, the long term murine treatment protocols of procainamide and pristane including the LTT assay and adoptive transfer assays 2.4. The latter part of the chapter focuses upon flow cytometry techniques 2.5 and the methods used to identify and characterise autoantibodies including cell fluorescence techniques 2.6 and ELISA assays 2.7. The protocols for the various buffers and solutions referred to in this chapter are described in appendices A-E.

2.1. MATERIALS

This section covers the variety of materials that were used throughout the study, beginning with the different mouse strains and cell lines and continuing with accounts of the plastic ware and the required antibodies. Finally, the purchase and preparations of PA and the metabolite HAPA are described.

2.1.1. MOUSE STRAINS

Specific pathogen free female mice were approximately six weeks of age at the beginning of each experiment. Strains were purchased from either (a) Janvier, France or (b) The Jackson Laboratories, USA. All mice were kept under pathogen free conditions in the animal facilities of the Institute of Environmental Medical Research, Düsseldorf in compliance with the German laws regulating animal husbandry. The mice were fed a standard diet (Ssniff Spezialdiäten GmbH Söest, Germany) and had access to water *ad libitum.*

STRAIN	MHC-HAPLOTYPE	SPECIALITY
BALB/c (a)	H-2^d I-A^d and I-E^d	Renowned for Th2 responses
A/J (b)	H-2^a I-A^a	Slow acetylators

2.1.2. CELL LINES

The following cell lines were used in the various assays described in the following sections. All cell lines were regularly tested for Mycoplasma infections with DAPI staining.

NAME	TYPE	SOURCE/REFERENCE
HEp2	Human epithelial carcinoma	ATCC
P338	DBA/2 macrophage line	ATCC
EXC-5	Culture supernatant rich in IL-2, IL-3 IL-4 and IL-5	Kindly provided by Dr. Weisner (Köln, Germany)
BW 5147 ($TCR\alpha^{-}\beta^{-}$)	Murine thymoma line. Fusion partner for T cell hybridomas	Kindly provided by H.G. Burgert (Freiburg Germany)

2.1.3. PLASTIC AND GLASS WARE

All plastic and glassware equipment was supplied from the by one of the following firms unless otherwise stated: Eppendorf, Hamburg, Germany; Flacon, Becton Dickinson, Heidelberg, Germany, Greiner, Frickenhausen, Germany or Schott, Düsseldorf, Germany.

2.1.4. ANTIBODIES AND MICROBEADS

Anti-CD19, anti-CD8α, anti-CD11c, anti-MHC class II, anti-CD4 and anti-PE microbeads were supplied by Miltenyi Biotech GmbH, Bergische Gladbach, Germany. Monoclonal CD16/32 and FITC, PE, PerCp, biotinylated or APC coupled antibodies, specific for a variety of murine cell surface markers and cytokines were purchased from Pharmingen, Hamburg, Germany. Monoclonal antibodies and their isotype controls specific for murine MHC-class II, $I\text{-}A^{d}$ and $I\text{-}E^{d}$ were also supplied by Pharmingen. FITC coupled goat-anti-mouse IgG type antibodies for immunofluorescent were purchased from Dianova, Hamburg, Germany. For the ELISA assays (see section 2.7), the monoclonal and biotinylated antibodies specific for mouse IgG1, IgG2a and IgM were also purchased from Dianova. Ku, smRNP and snRNP Protein A monoclonal antibodies were supplied from Dunn Labors, Germany.

2.1.5. PROCAINAMIDE AND HAPA

Procainamide was purchased from Sigma Chemicals (Taufkirchen, Germany). For *in vitro* research a sterile filtered stock solution prepared in naked RPMI 1640 medium (PAA, Austria) and then frozen at –20°C. For the long term oral treatment, PA water (6g/l) was prepared on a weekly basis. A sterile stock solution of the reactive metabolite HAPA, hydroxylamino-PA, was also prepared in the same naked medium and stored at –20°C. When HAPA is stored at room temperature in an aqueous solution it can very quickly undergo spontaneous oxidation.

2.2. CELL IMMUNOLOGY PROCEDURES

Following the cell counting and main centrifugation protocols, the general maintenance of cell lines and the preparation of lymphocytes from different mouse organs are described. For all cell preparations and cultures, RPMI 1640 medium was used. Depending on the cell type or assay, the additional supplements required can vary and these are described in Appendix D.

2.2.1. CELL COUNTING

Unless otherwise stated, cell suspensions were diluted 1:10 in tryptan blue solution (Appendix E) and the cell concentration counted using a Neubauser-Kammer haemocytometer under light microscope magnification, (DIAPLAN, Lietz). Dead cells could be identified by the blue staining caused by the tryptan blue. Cell suspensions with more than 20% dead cells after preparation were either discarded or removed using a Ficoll gradient (section 2.3.2.3).

2.2.2. CELL CENTRIFUGATION

Unless otherwise stated, all cell suspensions were centrifuged at 1200rpm for 10 minutes at 4°C using a MEGAFUGE, from Heraeus Instruments, Germany.

2.2.3. CELL CULTURES

All cell lines and primary cell cultures were incubated at 37°C with 6% CO_2 in a water saturated atmosphere. Cells were fed as required, this was essentially every two days and involved a change in the RPMI 1640 medium, which contained supplements specific for the individual cell types (Appendix D). For the adherent HEp-2 cells, cultures were first briefly rinsed with PBS and then incubated with Trypsin/EDTA (Sigma) for 5 minutes. After washing the cells were centrifuged and split in a ratio of 1:10 into new culture medium.

2.2.4. CELL FREEZING AND THAWING

For freezing, 1-5 $x10^6$ cells were centrifuged in sterile cryotubes and resuspended in 1 ml of ice-cold freezing medium (Appendix E). After standing on ice for 30 minutes cells were frozen at a rate of 1 degree per minute to –80°C. To thaw, the aliquots of cells were quickly heated in a beaker of warm water (25°C), tipped into a 50 ml tube and filled up with the appropriate fresh medium. Cells were then centrifuged and washed once again before being placed into culture flasks at 37°C. The cells were left for several days with at least one change of medium before use.

2.2.5. CELL PREPARATIONS

The mice were sacrificed by lethal anaesthetisation using a carbon dioxide filled atmosphere. Thereafter, areas of incision were sprayed with 70% ethanol and the mice were either pinned to an operation board or handled freely depending on the desired cells or organs required.

2.2.5.1. Spleen Cells

Whole mouse spleens were aseptically removed, placed in sterile BSS (see Appendix E) and placed to sterile conditions. In a small petri dish the spleens were homogenised in PBS until only the empty organ shells remained. Thereafter, the cell suspension was thoroughly mixed using a 10 ml pipette and then passed through a gauze filter into a 50 ml tube and centrifuged. The cell pellet was re-suspended by gentle tapping and 5 ml of ACT solution (Appendix E) added for 5 minutes at room temperature in order to lyse the erythrocytes. After that the suspension was passed through a second filter into a fresh 50 ml tube, filled up with PBS and centrifuged. The resultant supernatant was decanted and the cells resuspended in either 10 ml of appropriate medium or PBS for counting and further experimentation.

2.2.5.2. White Bone Marrow Cells

The complete hind legs from mice were aseptically removed and placed in sterile BSS solution. Under sterile conditions the excess fat and muscle were then removed. The femur and tibia bones were then separated at the knee joint to allow access to hollow stretches of bone. A 5 ml syringe was then filled with BSS and this solution pushed through the bone cavity using a Microlance 25 gauge needle. The emerging bone marrow was collected in a 50 ml tube. Once all the bone marrow was removed it was thoroughly mixed using a glass pipette in order to obtain a single cell suspension. Additional BSS was added and the cells centrifuged. Lysis of the RBC was performed as described above and after that the cells were resuspended in appropriate medium or PBS for counting and further use.

2.2.5.3. Peritoneal Macrophage Lavage

A 5 ml syringe with a Microlance 25 gauge needle was filled with BSS and injected into the peritoneum cavity of a sacrificed mouse. This area was gently massaged. The mouse was then pinned and a subcutaneous incision made from the lower abdomen to the neck. The skin was pinned to the left and right respectively displaying the intact peritoneal sheath. Thereafter, the needle was inserted into the exposed peritoneum cavity and the fluid drawn into the syringe. Once full, the needle was removed and the contents of the syringe expelled into a sterile tube. Another 5 ml of BSS was further injected into the cavity and the above procedure repeated. The cells were then recentrifuged at 4°C at 1800rpm for 10 minutes. The pellet was resuspended into either 10 ml PBS or appropriate medium and the cells counted. Due to the insignificant number of RBC, an ACT lysis step is not normally required.

2.3. IMMUNOLOGICAL CELL ASSAYS

Within this section, apart from the lymphocyte transformation test, detailed accounts regarding the different immunological assays are described. In summary, the PLN assay is explained first followed by the generation and specific testing of $CD4^+$ T cell hybridomas using the IL-2 bioassay. Variations on the IL-2 bioassay were employed in order to gain a deeper knowledge about the specificity and activity of these $CD4^+$ T cell hybridomas, these are also described. Finally the HPLC method used to identify the amount of procainamide metabolised by cells is recounted.

2.3.1. PRIMARY RESPONSE PLN ASSAY

This assay system is suitable for detecting the immunostimulatory capacity of low molecular weight substances and was performed as previously described [94]. For immunisation, WBMC were pulsed overnight with or without 500 µM procainamide. After which the cells were washed, counted and resuspended in PBS, pH7.2. Five naïve BALB/c mice per group were then injected into the ipsilateral hindfoot pad with 50 µl of either $5x10^6$, $2x10^6$ or $5x10^5$ sonicated (Labsonic V, B. Braun Melsungen AG, Melsungen, Germany) PA-pulsed or non-pulsed cells. For a definite primary response the popliteal lymph nodes were removed after six days. Mice were sacrificed and the areas for incision sprayed with ethanol. Individual ipsilateral and contralateral popliteal lymph nodes were aseptically removed and placed separately in 1 ml of BSS solution.

Under sterile conditions, the isolated lymph nodes were thoroughly homogenised using sterilised tweezers within the 1 ml of BSS medium and then mixed using a pipette to obtain an even cell suspension. 10 µl of the cell suspensions were then aliquoted into CASY mini-tubes with 5 ml of CASY buffer (Appendix E) immediately prior to cell count determination by the CASY 1 TT automatic counter, (Schärfe Systems GmbH, Reutlingen, Germany). As a parameter for the PLN response, the PLN cell count index can be calculated be dividing the ipsilateral cell counts by their corresponding contralateral values. A positive response is scientifically acknowledged if the index is greater than two.

2.3.2. GENERATION OF $CD4^+$ T CELL HYBRIDOMAS

2.3.2.1. Immunisation

For the immunization procedure, WBMC from naïve BALB/c or A/J mice were pulsed overnight with 500 µM PA at 37°C. Cells were then washed, counted, re-suspended in saline solution to the desired concentration and sonicated. An equal volume of Incomplete Freund's Adjuvant (Sigma) was then added and after thorough mixing, 50 µl of $5x10^5$ cells were injected into both hindfoot pads of naïve BALB/c mice.

2.3.2.2. Restimulation

Ten days after immunisation, PLNs were removed and single cell suspensions prepared by manual homogenisation. In a concentration of $2x10^6$/ ml, the cells were then re-stimulated for two days in TC medium (Appendix D) containing the appropriate antigen. In this instance, the antigen source was WBMC that had been previously pulsed with 500 µM PA for 24 hours.

2.3.2.3. Ficoll and Expansion

Following re-stimulation, remaining dead cells were extracted from the live cells using a Ficoll Gradient. In brief, the washed stimulated cell suspension was re-suspended in 3 ml of naked medium and slowly added by pipette on top of 5 ml of Ficoll solution (Ficoll-Plaque, Pharmacia, Freiburg, Germany). The gradient mixture was then centrifuged at 2200rpm for 20 minutes at 4°C and stopped without using the brake. The living T cells which remain ontop of the Ficoll solution, were then carefully removed with a glass pipette and placed in a fresh tube. After thorough washing with PBS the cells were propagated for a further two days using EXC-5 supernatant. Following this, T cells were fused with BW5147 $\alpha^-\beta^-$ TCR thymoma cells using polyethylene glycol PEG 1500, (Boehrigner, Mannheim, Germany).

2.3.2.4. Fusion

For fusion, the expanded T cells were first centrifuged with the specific murine fusion partner, BW5147 $\alpha^-\beta^-$ TCR cells, in a ratio of 1:2. The supernatant was then decanted and the unsuspended pellet held within a warm beaker of water. Over the course of 1 minute, 1 ml of pre-warmed PEG 1500 was slowly but vigorously pipetted into the pellet followed by a further minute of re-suspension. In the same manner, 1 ml of warm naked RPMI 1640 medium was also added. Then, over the course of 2 minutes another 3 ml of medium was added followed by a further 10 ml over another 2 minutes. After this, cells were allowed to rest for 5 minutes at 37°C before centrifugation. Cells were resuspended in fresh warm HAT medium (Appendix D) and 150 µl was pipetted into 4 x 96 round-bottom well plates and incubated at 37°C for 1-2 weeks during which a further 50 µl of warm HAT medium was added. The resultant hybridomas were then transferred into 24 well plates and cultured with increasing amounts of HT medium (Appendix D) so that after 2 weeks hybridomas were cultured solely in HT medium. HT medium was then gradually exchanged for TC medium. During the growth period, approximately $1x10^5$ cells were removed and incubated with anti-CD4 PE and anti-TCRβ FITC antibodies for 10 minutes in the dark. After washing the cells underwent flow cytometry analysis and individual hybridomas were checked for their expression of these two surface markers. Both characteristics are only present after a successful fusion and are a necessary requirement for a functional $CD4^+$ T cell hybridoma.

2.3.3. SUBCLONING

Following the specificity tests (section 2.3.5), those hybridomas producing a positive response were further subcloned in order to prevent the loss of their specificity. Using a limiting dilution technique, 9, 3, 1 or 0.3 cells from each hybridoma were plated onto 96-well-plates in 200 µl of TC medium. After a few days in culture, hybridomas that had grown to a sufficient capacity were transferred into 24 well plates and retested for their specificity.

2.3.4. PREPARATION OF CON A BLASTS

Mouse spleen cells were prepared as described in the subsection 2.2.5, and then cultured for 24 hours in TC medium with 1.25 µg/ ml Concanavalin A (Sigma). The cells were then washed and cultured for a further 24 hours in TC medium alone. After washing, these cells were used in the IL-2 bioassay.

2.3.5. T CELL HYBRIDOMA STIMULATION ASSAY (IL-2 BIOASSAY)

In TC or HT medium, 50 µl of the generated $CD4^+$ T cell hybridomas ($1x10^5$) were co-cultured in 96 round bottomed well plates at 37°C, with 100 µl of freshly prepared syngenic spleen cells acting as APC, $3\text{-}5x10^5$/well. The irradiation of the APC fraction was deemed unnecessary. Also co-cultured was 50 µl of the appropriate antigen or their controls, for example WBMC that had or had not been previously pulsed with 500 µM PA or not. After 24 hours, 50 µl of the culture supernatants were removed, transferred to new 96 well plates and frozen at –80°C for more than 2 hours. After thawing, the presence of IL-2 was tested by adding IL-2 dependent Con A blasts, $2x10^4$/ 50 µl. For a positive control, Con A blasts were incubated in TC medium with 10% EXC-5 supernatant. After 18 hrs at 37°C, 18.5 kBq [^{3}H] thymidine was added. Six hours later, the cells were harvested (PHD Cell Harvester Model 200A/290 Cambridge technology, Cambridge, MA USA) onto Ready Filters with Xtalscint (Beckmann Instruments, Fullerton, CA, USA). The filters were washed several times with water and left to dry for 30 minutes at 60°C. The radioactive thymidine incorporation was then measured in a β-counter (Model LS6000 IC Beckmann Instruments, München, Germany) and the counts per minute (cpm) obtained. Experimental wells were performed in triplicate and the entire assays were performed at least twice to ensure reproducibility.

2.3.6. MHC-RESTRICTION ANALYSIS

$1x10^5$ $CD4^+$ T cell hybridomas in 100 µl of HT or TC medium were incubated in a 96 round bottomed well plate with 100 µl of $5x10^5$ APC. Before adding 50 µl of antigen ($1x10^5$ PA-pulsed WBMC or controls) one of the following antibodies was added at a concentration of 40 µg/ ml: *Anti-I-A^d mAb; isotype control mAb; Anti-I-A^d/E^d mAb; isotype control mAb*. All antibodies were obtained from Pharmingen (Hamburg). All antibodies were dissolved in a 0.1% NaN_3 solution and therefore as an additional control an appropriate NaN_3 concentration was also added to the cultures. Each experimental combination was plated in triplicate and the IL-2 bioassay performed as described in 2.3.5 above. Assays were performed twice to ensure reproducibility.

2.3.7. APC FIXATION BY PARAFORMALDEHYDE

In order to determine whether or not the antigen presenting cells required a certain amount of time before they could present the PA-neoantigens formed from PA-pulsed WBMC, the following APC fixation experiments were performed. PA-pulsed WBMC or controls were incubated for 24 hours prior to the test. $1x10^5$/50 µl of these antigens were then added to prepared syngenic spleen APC and manipulated in one of the following manners.

1. 100 µl of $5x10^5$ APC and 50 µl of antigen were plated in a round bottomed 96 well plates and then immediately fixed with 4% paraformaldehyde for 5 minutes at 4°C. After washing twice, the cells were resuspended in 100 µl of fresh TC medium. 100 µl of $1x10^5$ hybridomas were then added and the IL-2 bioassay performed.
2. 100 µl of $5x10^5$ APC were incubated with 50 µl of antigen for 4 hours at 37°C. After this time the cells were fixed with 4% paraformaldehyde for 5 minutes at 4°C. After two washes the cells were resuspended in 100 µl of fresh medium and incubated with $1x10^5$ hybridomas at 37°C. The IL-2 bioassay was then continued as described above in 2.3.5.
3. As a control, hybridomas, APC and antigens were added altogether in the normal test manner and the IL-2 bioassay continued as described above in section 2.3.5.

2.3.8. TIME ASSAY

In order to determine the length of time required for the macrophages to produce sufficient quantities of the PA-neoantigen a time assay was devised. WBMC were incubated with or without 500 µM PA for 30 minutes, 1, 2, 4 and 8 hours at 37°C in TC medium. After these incubations, the cells were washed and co-cultured with $1x10^5$ T cell hybridomas and $5x10^5$ APC for a further 24 hours. The IL-2 bioassay was then continued as described in section 2.3.5.

2.3.9. INDIRECT DETERMINATION OF WBMC METABOLISM OF PA BY HPLC

In order to calculate the amount of PA which WBMC could metabolise over a period of time *in vitro*, a method was developed using HPLC (LaChrom, Merck, Germany). For this, WBMC were incubated overnight with 500 µM PA. After which, 1 ml of medium was taken from the samples and placed within glass HPLC vials. The control sample, medium and PA alone was ran on the HPLC and the peak of PA measured. The HPLC column was a Licospher® WP300 RP-18 (5 µM) column and the mobile phase a mixture of water, acetonitrile and trifluoric acid, starting at 90:10.0.05 and continuously changing to 10:90:0.05 over 40 minutes at a flow rate of 1 ml/min. The area of the peak was then used as the standard. WBMC and PA samples were then also measured and the area under the different peaks compared.

2.3.10. NITROCELLULOSE STRIP IL-2 BIOASSAY

Previous studies have detected a 35kDa PA-protein band in PA-pulsed peritoneal macrophages using a PA-specific antibody [127]. Other research [140] has shown the propensity of macrophages to digest nitrocellulose membrane *in vitro*. Thus, using this methodology the following protocol was devised to investigate whether the $CD4^+$ T cell hybridomas reacted towards a 35kDa protein generated by PA-pulsed WBMC. WBMC were incubated with or without 500 µM PA overnight in TC medium. After which the cells were centrifuged and resuspended in 200 µl of PBS. With an equal volume of x2 gel loading buffer (Appendix E) these lysates were sonicated and heated to 90°C. The samples were then run on a 12% polyacrylamide gel at 100V as described in section 2.8.2. After which, the gels were soaked in protein transfer buffer (Appendix E) and transferred onto nitrocellulose paper using a semi-dry blot membrane system (Biorad, Germany), as described in section 2.8.3. Using a sterile scalpel, these membranes were sliced into fragments that corresponded to different molecular weights and placed in 96 well plates. $5x10^5$ APC were then added for 1 hour at 37°C. $1x10^5$ $CD4^+$ T cell hybridomas in 100 µl of TC medium were then added and the IL-2 bioassay performed as described in section 2.3.5.

2.3.11. HYBRIDOMA RESPONSIVENESS TO CYTOPLASMIC OR NUCLEAR FRACTIONS

To determine whether the PA-neoantigen, which the $CD4^+$ T cell hybridomas were reacting towards was from a nuclear or cytoplasmic compartment the following assay was performed. WBMC or spleen cells were incubated overnight with or without 500 µM PA. After washing, the cells were resuspended in 1 ml PBS and recentrifuged on a bench top centrifuge for 10 minutes at 4000 rpm. The resultant pellets were resuspended on ice with 200 µl of 0.1% Triton X solution in water for 3 minutes. After which the cells underwent further centrifugation for 3 minutes at 4000 rpm. The cytoplasmic (supernatant) fraction was collected into a fresh tube and the nuclear (pellet) fraction was resuspended in saline. These antigen sources were then incubated with $5x10^5$ APC and $1x10^5$ $CD4^+$ T cell hybridomas in a total of 200 µl medium in a 96 well round bottomed plate. The IL-2 bioassay was then performed as described above in section 2.3.5.

2.4. LONG TERM PROCAINAMIDE AND PRISTANE TREATMENT

Within this section the long term effects on T cells after PA treatment in the drinking water of A/J mice was investigated. The following subsections describe the treatment regimes for both the PA and the positive controls, pristane- and DNFB-treated mice. The methodology for the lymphocyte transformation test (LTT) then follows with explanations on how T cells were isolated and purified. Finally, an adoptive transfer assay is described which was used in order to detect the ability of the $CD4^+CD25^+$ T cell subset in preventing the development of PA autoimmune disease in naïve recipients.

2.4.1. PROCAINAMIDE TREATMENT

A small trial experiment was performed in order to observe whether the A/J mouse strain could tolerate PA in their drinking water unless the water was sweetened. Therefore, four groups of 5 mice each received the following treatment: PA (6g/l) alone; PA(6g/l) with sweetener (1 teaspoon/l); water alone or sweetened water (1 teaspoon/l). This treatment continued over 4-6 months and every week the amount of water consumed and the weight of each mouse was noted. From this trial experiment it was observed that there was no significant difference in the amount of PA water consumed whether sweetener was added or not. Therefore, for the larger long term investigation, water or just PA water (6g/l), was provided *ad libitum* to the A/J groups (200 ml/cage). Each week the remaining water was measured and the concentration of PA calculated.

2.4.2. PRISTANE TREATMENT

Twelve, 6-8 week old naïve A/J female mice were given a single intra-peritoneal injection of 500 µl pure pristane (Sigma). Control mice were given a 500 µl intra-peritoneal injection of pyrogen free PBS. These mice were then housed in the same conditions as the PA-treated A/J mice and received no further treatment.

2.4.3. DNFB TREATMENT

For the positive control in the lymphocyte transformation test described below (2.4.4), mice treated with DNFB (Sigma) were used. 10 days prior to the LTT assay, naïve A/J mice were painted on their hindfoot pads with 50 µl/foot, 0.5% DNFB dissolved in an olive oil and acetone (1:4) solution. Popliteal lymph nodes were then aseptically removed and homogenised directly in SC medium (Appendix D). The cells were centrifuged and counted. $1x10^5$ DNFB-treated cells were then plated alongside irradiated APC and all the test antigens including 100 µM DNBS (Sigma) the antigen which they are specific for.

2.4.4. LYMPHOCYTE TRANSFORMATION TEST

This assay allows the detection of secondary T cell responses to antigens *in vitro*. In brief, responder T cells extracted and enriched from the treated animals are plated *in vitro* with APC and antigens and incubated for 96 hours. During the last 18 hours, [^{3}H] thymidine is added and the radioactive incorporation measured. The stimulation indices can be calculated by dividing the treated cpm over the control cpm and a stimulation index over two indicates a significant response.

2.4.4.1. Antigen Preparation

1. PA-pulsed cells: On the day prior to the LTT assay, spleen, WBMC and PM from naiveA/J mice were removed and pulsed overnight in SC medium with or without PA (500 µM) or

HAPA (20 μM). The following day the cells were centrifuged, re-suspended in fresh medium and counted. $1x10^5$ of these pulsed or non-pulsed cells were then plated in 50 μl of SC medium.

2. Another antigen used was one that contained spleen cells that had been pulsed with HAPA at 4°C. For this, on the day of the assay spleen cells from naïve mice were prepared and pre-cooled on ice for one hour. The cells were or were not then exposed to 20 μM HAPA for a further 30 minutes also on ice. After this time the cells were centrifuged and plated at a concentration of $1x10^5$/50 μl/well.
3. HAPA and PA were also added as single antigens on the day of the assay at a concentration of 500 μM and 20 μM each.
4. Heat shock protein 60, hsp60(Sigma) was added at a concentration of 5 ng/ml.
5. dsDNA (Sigma), Ku (Dunn Labors) and histone (Sigma) protein antigens were added at 50 μl/well at a concentration of 5 μg/ ml, 0.5 μg/ ml and 2.5 μg/ ml respectively.
6. Chromatin was prepared following the aqueous procedure: In summary naïve A/J spleen cells were seeded at $2x10^5$/ ml overnight at 37°C in SC medium with 0.2 μg/ ml Colchicine (Sigma). After which, the cells were centrifuged at 1000rpm for 10 minutes at 4°C and then re-suspended in 10 ml of medium. After cooling for 30 minutes on ice the cells were recentrifuged. The resultant pellet was then re-suspended in 75mM KCl solution and incubated for 30 minutes on ice. These hypotonic swollen cells were then centrifuged again followed by gentle resuspension in aqueous disruption buffer for 5 minutes on ice (Appendix E) In order to completely disrupt the cells, the suspension was passed through a 25 gauge needle at least ten times with care taken to avoid frothing. Cells were then centrifuged for 3 minutes at 400g, 4°C and the nuclei absent supernatant transferred into a clean tube. The supernatant was then centrifuged at 3000g for 15 minutes at 4°C to concentrate the chromosomes which were subsequently dissolved in saline. The concentration of chromosomes was determined by the Bradford Assay and a toxicity test performed with naïve A/J spleen cells in order to determine an acceptable non-toxic concentration. To conclude, chromatin was then added as antigen at a concentration of 5 μg/ ml in 50 μl of assay medium.
7. DNBS was diluted in RPMI 1640 naked medium and sterile filtered. The solution was then plated at a concentration of 100 μM/50 μl.

Before plating all antigens were irradiated with 20 Gy using a Gammacell 2000, Finland.

2.4.4.2. T cell Enrichment via Nylon Wool Columns

Unless otherwise stated, three PA-treated mice and three naivemice were used for each LTT assay. Only two pristane-treated mice were used. Spleen cells were prepared as described in section 2.2.5. For the initial LTT assays, T cells were enriched via nylon wool columns. These sterilised, cotton wool filled columns were clamped onto stands and sterile valves attached. Pre-warmed SC medium was then pipetted onto the columns and allowed to migrate slowly through the tubes so that all air bubbles were extinguished. After two or three passages of medium the valves were tightened, the columns filled up with medium, placed upright in glass beakers and incubated at 37°C for one hour.

The columns were then rinsed twice with pre-warmed SC medium taking care not to allow the columns to dry out. After the valves were secured, 1.5-2 x 10^8 prepared spleen cells in a volume of 1.5 ml of SC medium were pipetted carefully on top of the nylon wool. The valve was then opened and the cells allowed to run slowly into the column matrix until a small layer of cell suspension was left at the top of the column but without letting the cells pass completely through. The valves were then secured again and the columns placed at 37°C for 45-60 minutes. After this time the B cell population remains attached to the columns and therefore the enriched T cells could be collected very slowly into a sterile tubes at a rate of 1 ml per minute. The columns were constantly filled since it is very important that they do not run dry. Collection of the cells was stopped after a total volume of 15 ml had been reached. An aliquot was then removed to check for the purity of the cell separation by flow cytometry and the remaining cells centrifuged, resuspended in fresh medium and counted.

2.4.4.3. $CD4^+$ T cell Separation

In order to purify the nylon wool enriched T cells or to separate the cells further, either AutoMACS or MACS (Miltenyi) equipment was used. The nylon wool cell fractions were first washed in degassed AutoMACS running buffer (Appendix 1). 10 µl of anti-CD19 microbeads and 90 µl of cold running buffer were added for every $1x10^7$ cells to a final concentration of $1x10^8$ cells and left to incubate for 15 minutes at 4°C. Five times the volume of the cell mixture was then added and the cells centrifuged. Thereafter, the cells were resuspended in 500 µl of buffer and passed through either the positive selection "POSSEL" programme of the AutoMACS or through a prepared Mini-MACS column.

For the experiments in which both $CD4^+$ and $CD8^+$ T cell fractions were required, the whole cell population underwent the same procedure as above by additional separation using anti-CD11c and anti-MHC-class II microbeads together and then also with anti-CD8α microbeads. Aliquots of each cell fraction were then incubated with appropriate antibodies specific for B and T cells and the purity of the fractions verified by flow cytometry. When the fractions were over 95% pure, the cells were plated to a final concentration of $1x10^5$ cells per 50 µl.

2.4.4.4. APC isolation

For the majority of LTT assays, whole spleen lymphocytes were used as APC. Once the spleens were prepared the desired quantity of APC were removed and diluted to a concentration of 2-3x10^5 cells / 100 µl. These APC were then irradiated with 20 Gy and plated. For the assays in which pure B cell populations were required, spleen cells did not undergo nylon wool enrichment but were immediately incubated with anti-CD19 microbeads and passed through the AutoMACS using the POSSEL programme. An aliquot was then removed and the purity of the fraction tested using flow cytometry. When the purity was above 95%, these B cells were irradiated and plated at a final concentration of 3-5x10^5/well.

2.4.4.5. Proliferation assay

To conclude, 100 µl of $5x10^5$ irradiated APC, 50 µl of antigens and 50 µl of enriched $1x10^5$ T cells were plated together in sterile 96 round bottomed well plates and incubated at 37°C for 96 hours. For the last 18 hours, [^{3}H] thymidine was added and the proliferation measured after the cells were first harvested (Inotech, Switzerland) and then counted with a TRILUX β counter (1450 Microbeta Wallac, Oy, Finland). The stimulation indices were determined by the following formula:

$$\frac{\text{Treated T cells} + \text{APC} + \text{Antigen (cpm)}}{\text{Control T cells} + \text{APC} + \text{No Antigen (cpm)}}$$

2.4.5. ADOPTIVE TRANSFER

These investigations spanned a total of either 8 (section 3.2.12) or 14 weeks (3.2.15) and began with obtaining a preserum sample from all A/J recipients. In the initial transfer assay, 3.2.12, recipient mice were then divided into two groups, those receiving oral PA treatment and those that did not and this treatment continued for the entire investigation. One week later, recipient mice then received an intravenous T cell transfer consisting of either $CD4^+CD25^+$ or $CD4CD25^-$ T cells from either PA-treated or control donors. In the cross-transfer assay, recipient mice first received the T cell transfer of $CD4^+$ T cell subsets from PA-treated or untreated donors and then administered with either PA drinking water, gold sodium thiomalate (GST), or sodium thiomalate (ST). Again, treatment lasted the entire length of the experiment. In both assays, at different timepoints during the weeks after transfer, serum samples from all recipient mice were collected and tested for developing autoantibodies. Specific details regarding the treatment and separation of the T cells is provided in the following subsections and a scheme for the whole investigation is depicted in section 3.2.12.

2.4.5.1. Procainamide Treatment

Groups of recipient A/J mice requiring PA in their drinking water were treated in the same manner as with the long term experiment described in section 2.4.1. The concentration of procainamide was 6g/l. Control groups received untreated drinking water.

2.4.5.2. GST and ST Treatment

Groups of recipient A/J mice received weekly intramuscularly with either 50 µl of 0.45 mg gold sodium thiomalate (GST) or equimolar sodium thiomalate, both solutions were prepared in 0.9% saline. GST or Taureno® was obtained from Byk , Constance, Germany whereas sodium thiomalate was purchased from Sigma.

2.4.5.3. Serum Collections

Section 2.6.1 describes the method in how the sera were obtained and the processing procedure. For the recipients of the adoptive transfer assay, pre-serum samples from all recipient groups were

obtained one week before the intravenous injection of cells. After the injection, individual recipient serum was obtained at different timepoints by orbital puncture and at week 6 by heart puncture.

2.4.5.4. Cell preparation

Eight spleens and inguinal lymph nodes (ILN) from either long term PA-treated or age-related control, were aseptically removed and placed in pyrogen-free PBS solution (Sigma). Spleen cells were prepared and passed through nylon wool columns with a maximum cell concentration of $2x10^8$ cells per column. After collection the cells were centrifuged, resuspended in 5 ml of AutoMACS running buffer (Appendix E) and the cell counts determined. Lymph nodes were homogenised directly into PBS and passed through a gauze filter to remove lymph node shells. The ILN cell number was then determined and added to the remaining enriched T cells obtained from the spleens.

These enriched T cells were then re-centrifuged and incubated with the recommended volume of anti-CD19 microbeads for 15 minutes on ice. Before centrifugation three times the amount of incubation solution was added. The cells were then re-suspended in 500 µl of AutoMACS running buffer and passed through the AutoMACS "POSSEL" programme. The negative fraction, containing unlabelled cells, was then counted and recentrifuged. In order to remove the remaining APC, the cell fractions were incubated with anti-CD11c and anti-MHC-class II microbeads together. Finally, cells were incubated with anti-CD8 microbeads so that only $CD4^+$ T cells were left in the negative unlabelled fraction.

In order to fractionate the $CD4^+CD25^+$ T cell subsets, the $CD4^+$ T cell fraction was incubated with anti-CD25 PE antibody for 20 minutes at 4°C, in the dark. This fraction was then washed with running buffer and further incubated with anti-PE microbeads for 15 minutes on ice, followed by centrifugation. The cells were then passed through the "POSSELS" programme on the AutoMACS. The negative fraction, which now contained the $CD4^+CD25^-$ T cells was passed through the same programme a second time in order to ensure all $CD4^+CD25^+$ T cells were extracted. The cells were then counted and an aliquot removed for flow cytometry analysis see section 2.5. After three washes, the cells were resuspended in pyrogen-free PBS solution and 100 µl of $5x10^5$ T cells injected into the tail veins of A/J recipients. This procedure was performed twice on two consecutive days and the mice marked by shaving and tail labels in order to identify which mice were from which transfer.

2.4.6. PROTEINURIA CONCENTRATION

10-20 µl of mouse urine was pipetted onto Albustix strips (Bayer, Levekusen, Germany). The colour change on the strip was then compared to the provided chart and the concentration of the individual urine determined by three independent people. With the A/J murine strain, obtaining urine extracts was somewhat problematic, therefore, urine concentration measurements were only taken at the time of sacrifice.

2.5. FACSCALIBUR ANALYSIS

2.5.1. ANTIBODY CONCENTRATION

From previous investigations in the laboratory, the majority of antibody concentrations had already been optimised for signal to noise ratios. For this determination, 10^6 spleen cells per aliquot had been stained with increasing concentrations (1 ng-1 µg) of antibodies in 20 µl of PBS/0.01%NaN_3 for 10 minutes at 4°C in the dark. NaN_3 blocks the respiration chain and prevents the capping of antibodies on the cell surface [142]. After washing, the cells were then aquisited by using the FACSCalibur (Becton Dickinson, Heidelberg, Germany). After analysis a suitable concentration of antibody was chosen for further use. This value was chosen when the positive and negative subpopulations of cells were clearly distinguishable and no unspecific staining of negative cells could be detected. With the appropriate antibody concentrations A/J spleen cells were stained with various antibodies and aquisited with the FACSCalibur. Whilst acquisiting, adjustments were made in order to clearly distinguish the cell populations, once satisfied this status was saved for use.

2.5.2. SURFACE MARKER STAINING

For the LTT assays and hybridoma work, only surface marker stainings were required. $1x10^5$ of individual cell fractions were a concentration of incubated for 10 minutes at 4°C in the dark with combinations of different antibodies in PBS/BSA at dilutions of 1:100. Table 2.5.2 depicts the typical antibody staining combinations:

FITC LABELLED	PE LABELLED	PERCP LABELLED	APC LABELLED
CD19 FITC			CD3 APC
		CD4PerCp	CD3 APC
	CD8 PE		CD3 APC
CD19 FITC		CD4 PerCp	
CD11b FITC	CD3 PE		CD11c APC
TCRb FITC			CD3 APC
TCRb FITC	CD8 PE	CD4 PerCp	
CD8 FITC	CD25 PE	CD4 PerCp	
	CD25 PE		CD3 APC

Table 2.5.2 Typical Surface Marker Staining Combinations for FACSCalibur analysis on treated and untreated cells. Antibodies were diluted 1:100 in PBS/BSA and incubated with $1x10^5$ cells for 10 minutes at 4°C in the dark. All antibodies were purchased from Pharmingen, Germany

2.5.3. INTRACELLULAR CYTOKINE STAINING

For the detection of cytokines, cells must first be activated and then fixed with 4% paraformaldehyde. Treated and non-treated A/J spleen cells were plated in SC medium at a concentration of $5x10^5$/ 200 µl and stimulated for 4 hours at 37°C with the mitogens PMA (150ng/ ml), Ionomycin (1.5 µg/ ml) and Brefeldine A (30 µg/ ml). Thereafter, the cells were centrifuged at 1000rpm for 5 minutes and resuspended in 4% paraformaldehyde (Appendix E) at 4°C for 15 minutes for fixation. Cells were

then recentrifuged and 150 µl/well of opening buffer (Appendix E) added for 10 minutes so that the cells became permeable. Thereafter, cells were again centrifuged followed by incubation with 50 µl of anti-CD16/CD32 antibody for 10 minutes in the dark. After a further centrifugation the cells were incubated with 50 µl of cytokine antibody cocktail prepared in opening buffer, for 40 minutes at 4°C in the dark. Table 2.5.3 describes the typical antibody staining combinations. After centrifugation, cells were incubated with the 150 µl closing buffer (Appendix E) for 10 minutes at 4°C in the dark in order to close the cell membranes. Cells were then resuspended in 100 µl of PBS and kept on ice and in darkness whilst undergoing acquisition.

STAINING 1	STAINING 2	STAINING 3
CD19 FITC	IL-2, IL-4, IL-6, IL-10, IL-12, IL-18 or IFN-γ PE	CD3 APC
CD8 FITC	As above	CD4 PerCp
TGF-β FITC	CD8b PE	CD4 PerCp
TGF-β FITC	CD19 PE	CD3 APC

Table 2.5.3 Cytokine Combinations for flow cytometry analysis on treated and untreated cells. Antibodies were diluted 1:100 in opening buffer and incubated with cells for 40 mins at 4°C in the dark. Thereafter the cells were washed and acquisited. All antibodies were purchased from Pharmingen, Germany.

2.6. AUTOANTIBODY DETECTION

Within this section the removal of blood from all experimental mice is described followed by the description of autoantibody detection through HEp2 cell slides and immuno-fluorescence.

2.6.1. SERUM PREPARATION

To obtain serum from mice the following procedure was used. Mice were first anaesthetised in a chamber containing diethyl ether for approximately 20 seconds or until the mouse was asleep. Individual mice were then removed and placed on a clean cloth whilst blood was taken by retro-orbital puncture with a sterile glass pipette and collected into sterile 1.5 ml tubes. After one hour at 37 °C and 2 hours at 4°C, the coagulated blood was centrifuged at 1200rpm for 8 minutes. The supernatant was then transferred into fresh 1.5 ml tubes and re-centrifuged for 6 minutes at 1200rpm. The resultant clear serum was then transferred into 600 µl tubes, which were labelled and frozen at –20°C until required. For multiple use of the same serum, the samples were subdivided into 10 µl aliquots and then frozen to avoid destruction of proteins. For final serum collections, blood was obtained by heart puncture. For this procedure, the sacrificed mouse was pinned and an incision made so that the chest cavity was exposed. The rib cage was then removed and a 25 Gauge needle (Microlance) attached to a 2.5 ml syringe inserted into the exposed heart. Slowly, blood was drawn into the syringe until a loss of pressure prevented further withdrawal. The sera were then processed as described above.

2.6.2. INDIRECT IMMUNOFLUORESCENCE DETECTION OF ANTINUCLEAR AUTOANTIBODIES

HEp-2 pre-coated slides, (The Binding Site, Birmingham, UK) were incubated with 33 µl of treated or untreated mouse sera in PBS for 1 hour in a wet, dark chamber. Slides were underwent three 10 minute washes. Each slide also contained a positive and negative (PBS) control. The slides were then briefly dried around the well and incubated with 33 µl of the secondary rat anti-mouse IgG or IgM FITC coupled antibody (Dianova,) in PBS for 45 minutes, also in a wet dark chamber. Slides were then washed in darkness and excess solution removed using a cotton bud with care not to touch the surface of the well. One drop of VECTASHIELD™ (Vecta , USA) was placed onto the individual slide wells and then covered with a cover slip, (10 mm x 35 mm). Air bubbles and excess fluid was then removed and the slides sealed around the edges with nail varnish. The slides were stored at 4°C until read using a fluorescence microscope (Leiza, Germany).

For characterisation and photographic record of the detected ANA, self-made HEp-2 slides were prepared. For this, cultured HEp-2 cells were seeded on sterile glass coverslips at a density of $5x10^4$ cells/ ml in complete SC medium and grown for two days in a 6 well plate. The coverslips were then removed from the wells and rinsed once with PBS in small coplin jars. Thereafter cells were fixed with ice-cold methanol for 5 minutes at –20°C followed directly by a further 3 minutes incubation at –20°C with ice-cold acetone. The coverslips were then washed three times at room temperature in PBS for 5, 8 and 12 minutes respectively. These fixed cells were then incubated for one hour at room temperature with 50 µl of the desired serum (1:100) or PBS in a dark moist chamber. Following this, cells were washed in PBS a further three times spanning 10 minutes each. The secondary rat-anti mouse IgG, IgG1 and IgG2a FITC-labelled antibodies were then added at a concentration of 1:100 for 45 minutes followed by washing. Whilst washing, the slides were kept covered with aluminium foil to prevent early bleaching of the FITC flurochrome. Coverslips were then carefully inverted onto labelled glass slides after the addition of a drop of VECTASHIELD™ mounting medium on each slide. The coverslips were finally sealed with nail polish, air dried in the dark and stored at 4°C in the dark. Images of the autoantibodies were obtained using an Olympus Microscope and Photoshop software.

2.7. AUTOANTIBODY CHARACTERISATION BY ELISA

Here describes the different ELISA assays used to determine the specificity of the serum obtained from the treated an non-treated mice.

2.7.1. IGM, IGG1 AND IGG2A ANTIBODY CONCENTRATIONS

96-well-microtitre ELISA plates (Dynex technologies, Denkendorf, Germany) were coated overnight at 4°C with either 50 µl of IgM, IgG1 and IgG2a monoclonal antibodies (Dianova) at a concentration of 1 µg/ ml dissolved in binding buffer (Appendix E). The following day, the plates were blocked at room temperature with 200 µl of blocking buffer (Appendix E) for 2 hours. The plates were then

washed three times with PBS/Tween followed by once with PBS alone. Still at room temperature, excess fluid was removed and 100 µl of serum, diluted in PBS to a concentration of 1:500, was added to triplicate wells for three hours. After four washes with PBS/Tween the secondary IgM, IgG1 and IgG2a biotin labelled antibodies (Dianova) were added at a concentration of 1 µg/ ml in 100 µl of blocking buffer/Tween (Appendix E) and left for 1 hour at room temperature. Plates were washed four times with PBS/Tween followed by the addition of 100 µl of 1:2000 Strep-avidin HRP (Sigma) in blocking buffer/Tween for 30 minutes. After several washes the plates were incubated with 100 µl of TMB substrate which was left in the dark at room temperature for 30-40 minutes. 100 µl of 0.2 M sulphuric acid was then added for a further 10 minutes also in the dark before measuring using a 96-well-plate ELISA reader (Dynex Technologies)) at dual wavelengths 450 nm and 620 nm. This protocol was adapted from [143].

2.7.2. PROCAINAMIDE AND HAPA ELISA

96-well-Microtitre well plates were coated overnight at 4°C with 50 µl of procainamide and HAPA in binding buffer at a concentration of 10mg/ ml and 10 µg/ ml respectively. Plates were then blocked for 2 hours with 200 µl/well of blocking buffer at room temperature. After three washes with PBS/Tween, the plates were washed once more in PBS alone before 100 µl/well of the treated or untreated serum, (1:100 in PBS) was added in triplicate for 3 hours at room temperature. After four washes, (PBS/Tween) the secondary antibody, total Ig HRP (Sigma) in blocking buffer /Tween was added at 100 µl/well at a concentration of 1:4000 for 90 minutes at room temperature. Plates were again washed with PBS/Tween followed by the addition of 100 µl of TMB substrate for 30-40 minutes at room temperature in the dark. 100 µl of 0.2M sulphuric acid was then added for a further 10 minutes also in the dark before measuring with a 96-well-plate ELISA reader (Dynex) at dual wavelengths 450 nm and 620 nm.

2.7.3. DNA ELISA

For the DNA specific ELISA study, 96-well-microtitre plates were coated overnight at 4°C with 50 µl/well of 5 µg/ ml of DNA (Sigma) diluted in binding buffer. The plates were blocked with 200 µl/well of blocking buffer at room temperature for 2 hours followed by three washes with PBS/Tween and one with PBS alone. Sera were then plated in triplicate in 100 µl PBS dilutions of either 1:500 or 1:1000 and left for 3 hours at room temperature. After thorough washing, the secondary antibody, total conjugated Ig HRP, was added at a dilution of 1:4000 in blocking buffer/Tween for 90 minutes at room temperature. Plates were again washed as before and developed with 100 µl TMB substrate for 30-40 minutes in the dark at 4°C. 0.2M sulphuric acid was then added and the plates read after a further 10 minute incubation in the dark with a 96-well-plate ELISA reader (Dynex) at dual wavelengths 450nm and 630nm.

2.7.4. HISTONE ELISA

50 µl of 2.5 µg/ ml total calf histone proteins (Sigma) in binding buffer was used to coat 96-well-microtitre plates overnight at 4°C. After two hours blocking with 200 µl blocking buffer at room temperature plates were washed three times with PBS/Tween and once with PBS alone. Sera were then diluted 1:200 in PBS and plated for 3 hours in triplicate at 100 µl/well. Plates were then thoroughly washed with PBS/Tween and the secondary, total Ig HRP conjugate antibody (100 µl/well) added for 90 minutes at a dilution of 1:4000 in blocking buffer/Tween. After four washings with PBS/Tween, 100 µl/well of TMB substrate was pipetted for 30-40 minutes in the dark at room temperature. 0.2M sulphuric acid was then added and the plates read after a further 10 minute incubation in the dark with a 96-well-plate ELISA reader (Dynex) at dual wavelengths 450nm and 630nm.

2.7.5. KU, SMRNP, SNRNP PROTEIN A ELISA

In binding buffer, 50 µl of 0.1 µg/ ml, 0.5 µg/ ml and 0.2 µg/ ml of Ku, smRNP and snRNP Protein A (Dunn Labors) proteins respectively were used to coat 96-well-Microtitre plates overnight at 4°C. After two hours blocking with 200 µl blocking buffer at room temperature, plates were washed three times with PBS/Tween and once with PBS alone. Sera were then diluted 1:500 in PBS and plated at room temperature for 3 hours in triplicate at 100 µl /well. Plates were then thoroughly washed with PBS/Tween and the secondary, total Ig HRP conjugate antibody (100 µl/well) added for 90 mins at a dilution of 1:4000 in blocking buffer/Tween. After four washings with PBS/Tween, 100 µl/well of TMB substrate was added for 30-40 minutes in the dark at room temperature. After that time, 100 µl of 0.2M sulphuric acid was added and the plates read after a further 10 minute incubation period in the dark with a 96-well-plate ELISA reader (Dynex) at dual wavelengths 450nm and 630nm.

2.8. MOLECULAR BIOLOGICAL TECHNIQUES

2.8.1. SAMPLE PREPARATION

500 µM of PA, 20 µM of HAPA or medium alone were added to either WBMC, spleen, peritoneal macrophages or P338 cells overnight at 37°C. P338 experiments were performed using P338 medium (Appendix D), whereas all others were incubated in SC medium (Appendix D). In some of the experiments, the cells were subjected to PMA for 15 minutes at the end of the incubation period. After washing the cells were resuspended in 1 ml of PBS. 200 µl of the total cell lysate sample was removed into a fresh ependorf tube. The remaining cells were re-centrifuged on a bench top centrifuge at 300rpm for 5 minutes. 200 µl of pre-cooled Triton X 100 solution was then added and the pellet re-suspended, this was left for 3 minutes on ice. After centrifugation for 4 minutes at 300rpm, the cytoplasmic supernatant fraction was removed into a fresh tube and the nuclear pellet fraction re-suspended in 200 µl PBS. The samples were then placed on ice for immediate use or frozen at –20°C. For immediate use 10 µl of each sample was added to an equal volume of x2 gel loading buffer (Appendix E). In order to disrupt the DNA, the samples were either briefly sonicated

or passed through a needle several times. Following this, the samples were heated to 95°C for 5 minutes and then briefly centrifuged. The prepared samples were then loaded into the wells of the prepared gel (Appendix E).

For the detection of antibodies in the treated sera, HEp-2 cells were employed. Confluent HEp-2 cells were first washed with PBS and centrifuged. Whole cell lysates or Triton X 100 separated fractions were then prepared as described above except that these cells were loaded into large comb wells which expanded the entire gel length. To identify the size of the protein bands, Rainbow protein markers (Amersham, Germany) from 14.3kD to 200kD were ran alongside the samples.

2.8.2. SDS-PAGE GEL ELECTROPHORESIS

SDS-PAGE gel electrophoresis was performed in accordance with Lamelli *et al*, [144]. Clean glass plates were coupled together with 1.0 mm spacers. A 12% resolving gel solution (Appendix E) was then prepared and poured into the apparatus. Whilst the gel was setting a layer of 10% *sec*-2-butanol was added. After 40 minutes, the *sec*-2-butanol was discarded and the top of the gel carefully dried using blotting paper before the stacking gel was loaded (Appendix E). Combs of appropriate size and number were inserted into the stacking gel layer and left to set for 30 minutes. After the combs were removed the gels were assembled into a running tank. Running buffer (Appendix E) was then added and the samples loaded. The level of the running buffer solution was maintained to ensure good connection between the gels and electrodes. The protein samples were then separated by vertical electrophoresis at 100V for approximately 90 minutes.

2.8.3. PROTEIN TRANSFER AND WESTERN BLOT

After separation, the gels were soaked in a protein transfer buffer (Appendix E) alongside nitrocellulose membrane (Hybond™-C-super, Amersham) and blotting paper. The separated proteins were then transferred onto the nitrocellulose paper using the Trans-Blot™ SD semi-dry electrophoretic transfer cell (Biorad, Germany). The nitrocellulose strips were then blocked in blocking buffer (Appendix E) for 2 hours followed by two short 0.5% PBS/Tween 20 rinses. The strips were placed in a wet dark chamber and primary antibody added at the required concentration for 1 hour. For the detection of PA-specific protein bands from PA-pulsed macrophages, the primary antibody was a specific rabbit-anti-mouse PA-antibody (1:3000), obtained from J. Utrecht in Canada. For detection of common antibodies in the sera of treated mice, the primary antibody was the serum itself and was used in a dilution of 1:200. After three 10 minute washes with 0.5% PBS/Tween 20, strips were placed in the chamber again and the appropriate species specific HRP-labelled secondary antibodies were diluted to 1:10,000 and added for 45 minutes. After further washings with 0.5% PBS/Tween 20, the strips were placed in the correct orientation inside plastic coverings and ECL reagent added. After different periods of exposure time to Hyperfilm™ECL™ film (Amersham), the films were developed the autoradiography machine, Curix 60 (AGFA).

2.8.4. 2D GEL ELECTROPHORESIS

2.8.4.1. First Dimension:

Rehydration of the strips: Rehydration buffer (Appendix E) and samples were slowly thawed on ice prior to use. The appropriate amounts of IPG buffer (Amersham), DTT (Sigma) and sample (up to 1 mg) were added to 200 µl of rehydration buffer. This mixture was then pipetted onto a clean dry lane of the Immobiline Drystrip Reswelling Tray (Amersham), avoiding any air bubbles. The IEF strips (pH3-11 cm) were then removed from their protective covers and using tweezers gently lowered onto the sample solution with the pointed end of the strip going towards the sloped end of the tray. In order to aid equilibrium they were gently rocked back and forth. A layer of IPG cover fluid was then placed over the strips, the lid placed in position and the strips left to rehydrate overnight.

Isoelectric Focusing: It is essential that all equipment must be clean and dry before use. Firstly, the cooling plate was positioned on an levelled Multiphor II unit. 10 ml of IPG cover fluid was pipetted onto the plate and the Immobiline Drystrip Tray placed on top with the anodic electrode connection near the cooling tubes. Any large air bubbles were removed. A further 15 ml of IPG fluid was poured into the tray and the Drystrip aligner (Amersham) placed so the side with 13 grooves was face up. Two 11 cm long IEF electrode strips were cut and briefly soaked in distilled water. Rehydrated IEF strips were then removed and briefly rinsed with DDW. After gently blotting to remove excess water the strips were placed on the Drystrip aligner with the pointed acidic end at the anodic end. Once all the strips were aligned, the damp electrode strips were placed over the top and bottom ends of the strips with partial contact over the gel surface. The electrodes were then placed on top of the strips and secured into position. The chamber was then filled with IPG cover fluid and the appropriate running programme started. For these particular samples the optimal conditions were found to be 1 hour at 300v, 1 hour at 600v and 4 hours at 2700v.

2.8.4.2. Second Dimension

Equilibration: Frozen prepared SDS equilibration buffer (Appendix 4) was thawed and the appropriate amount of DTT added (100mg/10 ml). After the first dimension was complete, the strips were removed and briefly rinsed with DDW. These strips were then placed in 15 ml tubes that contained 11 cm of equilibration buffer. The backs of the strips were positioned to the tube surface. The strips were gently rocked for 15 minutes.

SDS-PAGE Gel: 12.5% polyacrylamide gels (Appendix E) were prepared and allowed to set at least two hours before use in Hoeffer vertical chamber equipment using 1.0 mm combs. A *sec*-2-butanol layer was added to ensure an even surface in the setting. Once set, the *sec*-2-butanol layer was removed, the gels rinsed with DDW and the excess solution removed with filter paper. After the equilibration period the strips were rinsed briefly with DDW and dipped into running buffer (Appendix E). The strips were then placed along the length of the set gel ensuring that the gel surface was not touching the glass plate. 3 µl of 800 MW Rainbow markers (Amersham) were pipetted onto

IEF sample application pieces (Amersham) and positioned slightly within the gel surface. The position of the strips was secured by sealing with 0.5% agarose (Appendix E). The gel apparatus was then assembled, running buffer added and ran for 5 hours at 100V.

2.8.5. SILVER STAINING PROTOCOL

Heukeshoven Protocol: Several staining techniques can be employed for developing 2D images. For this system, the silver staining protocol described by Heukeshoven [145] was found to be the most sensitive and accurate. After the 2D SDS-PAGE run, gels were carefully removed and placed in ethanol cleaned containers with fixation solution A (Appendix E) for 30 minutes, fixation solution B (Appendix E) for 1 hour and then fixation solution C (Appendix E) for 1.5 hours or overnight. The amount of each solution used depended on the chamber size. After fixation the gels were washed with DDW for 1-2 hours with changes every 15 minutes. Silver staining solution (Appendix E) was then added for 30 minutes followed by two brief rinses (20 seconds) with DDW. Developing solution (Appendix E) was then added for 1-5 minutes followed directly by Stopping solution (Appendix E) for a further 5-10 minutes. Glycerin solution (Appendix E) was then added for a further 30 minutes. After which the gels were scanned and dried.

3 Results

Studies serve for delight, for ornament, and for ability
FRANCIS BACON, ("OF STUDIES")

3.1. T CELL REACTIVITY TOWARDS PROCAINAMIDE

Within this results section, three different aspects regarding the activities of procainamide and one of its major reactive metabolites, *N*-hydroxyprocainamide (HAPA), will be described. The first section 3.1 deals with the initial evidence that procainamide can elicit a T cell response both *in vivo* and *in vitro*. Through the popliteal lymph node assay (PLNA), a definite primary PLN T cell response was observed after injecting homogenates of WBMC that had been pulsed with PA *in vitro* beforehand. Following this observation, $CD4^+$ T cell hybridomas were then generated to the potential reactive neoantigens formed when PA is metabolised by WBMC *in vitro* and their specificity further elucidated.

3.1.1. *IN VITRO* CYTOTOXICITY OF PROCAINAMIDE AND HAPA

In order to perform experimental investigations and procedures with PA such as immunisation, T cell restimulation and macrophage metabolism, it was necessary to establish the concentration of PA and HAPA that was tolerable to cells. Therefore, *in vitro* toxicity tests were performed to determine the influence of the aforementioned chemicals on the proliferation of naivespleen lymphocytes and WBMC derived from the BALB/c mouse strain. These cells were then further incubated with or without the T cell mitogen concavalin A (Con A). With increasing concentrations of PA and HAPA, a decrease in proliferation was observed with both cell types, regardless of whether Con A was present or not. A 20-30 % decrease in proliferation was seen for both cell types incubated with the highest concentration of PA (500 μM), as shown in figures 3.1A and 3.1B, respectively.

In contrast, the proliferation of both cell types was dramatically reduced to nearly 50 % upon addition of only 125 μM HAPA. This effect was mirrored with the additional presence of Con A figures 3.1C and 3.1D (△ spleen and ◇ WBMC symbols respectively). This observation confirms the earlier findings which showed that HAPA was more cytotoxic than the prohapten PA [104, 128]. The

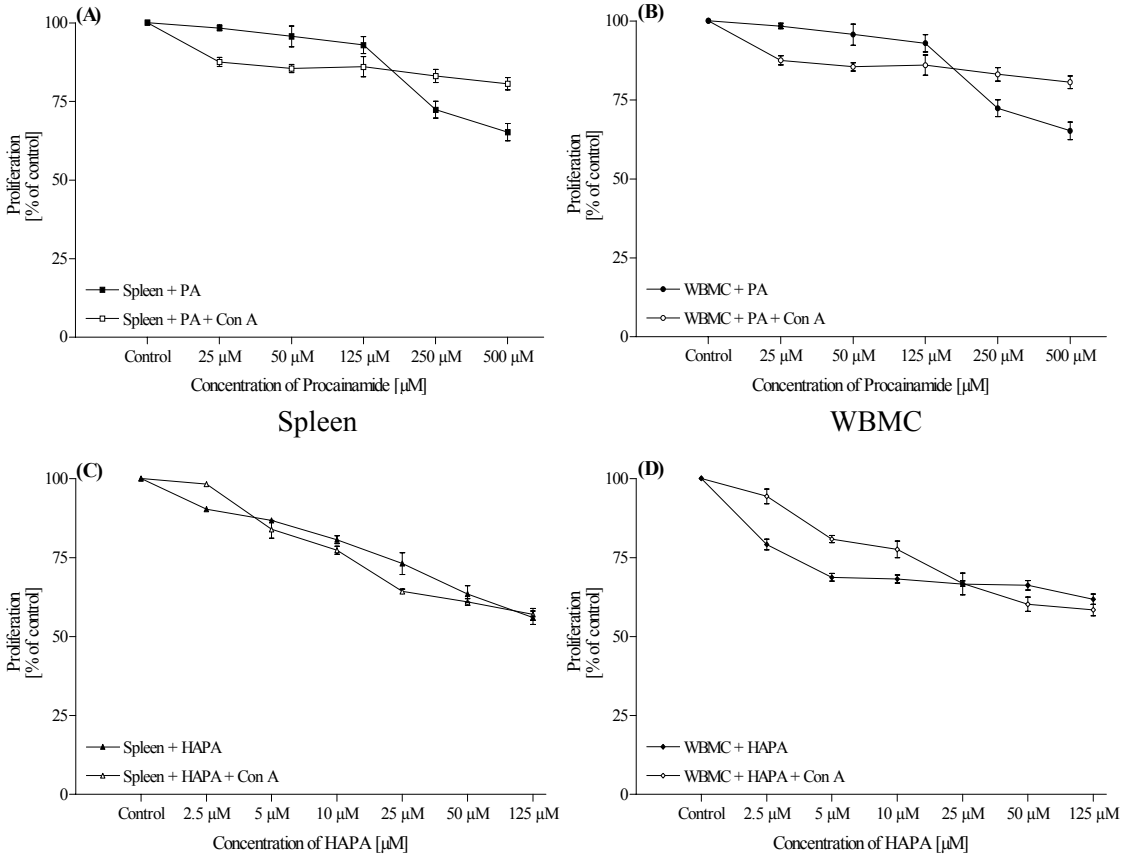


Figure 3.1.1.A-D. Cytotoxicity of procainamide and HAPA on WBMC and spleen cells.
WBMC or spleen cells ($2x10^5$) were incubated with or without increasing concentrations of PA (upper panel) or HAPA (lower panel) and in the presence or absence of Con A (2 µg/ml). After 78 hours in culture, [^{3}H] thymidine was added and the radioactive incorporation measured after a further 18 hours. Graphs represent the percentage decrease from the control and symbols represent the mean + SD of triplicate wells at the tested concentration.

above toxicity tests were performed over both 24 (data not shown) and 96 hours since the planned *in vitro* assays would span a minimum of 24 hours and a maximum of four days. Therefore it was necessary to study the effects of these compounds over those time periods. From these results the concentrations of 500 µM PA or 20 µM HAPA were always used unless otherwise stated. Toxicity tests were also performed with both cell types derived from the A/J mouse strain and the effects of HAPA and PA on those cell types did not significantly differ to those seen above for the BALB/c derived cells. Therefore in experiments using A/J derived cells the same concentrations were used.

3.1.2. THE ABILITY OF PA-PULSED WBMC TO ELICIT A PRIMARY PLN RESPONSE

An initial objective of this research was to elucidate the neoantigens thought to be created by the metabolism of PA by macrophages. Previous studies have established that a single injection of PA is unable to induce a primary PLN response in either fast acetylator (C57BL/6) or slow acetylator (A/J) mice. However, homogenates of peritoneal macrophages (PMφ) that had been previously exposed to PA *in vitro* for 48 hours, were able to induce a significant primary PLN response in comparison to the control homogenates. This finding was identical in both the fast and slow acetylator mouse strains

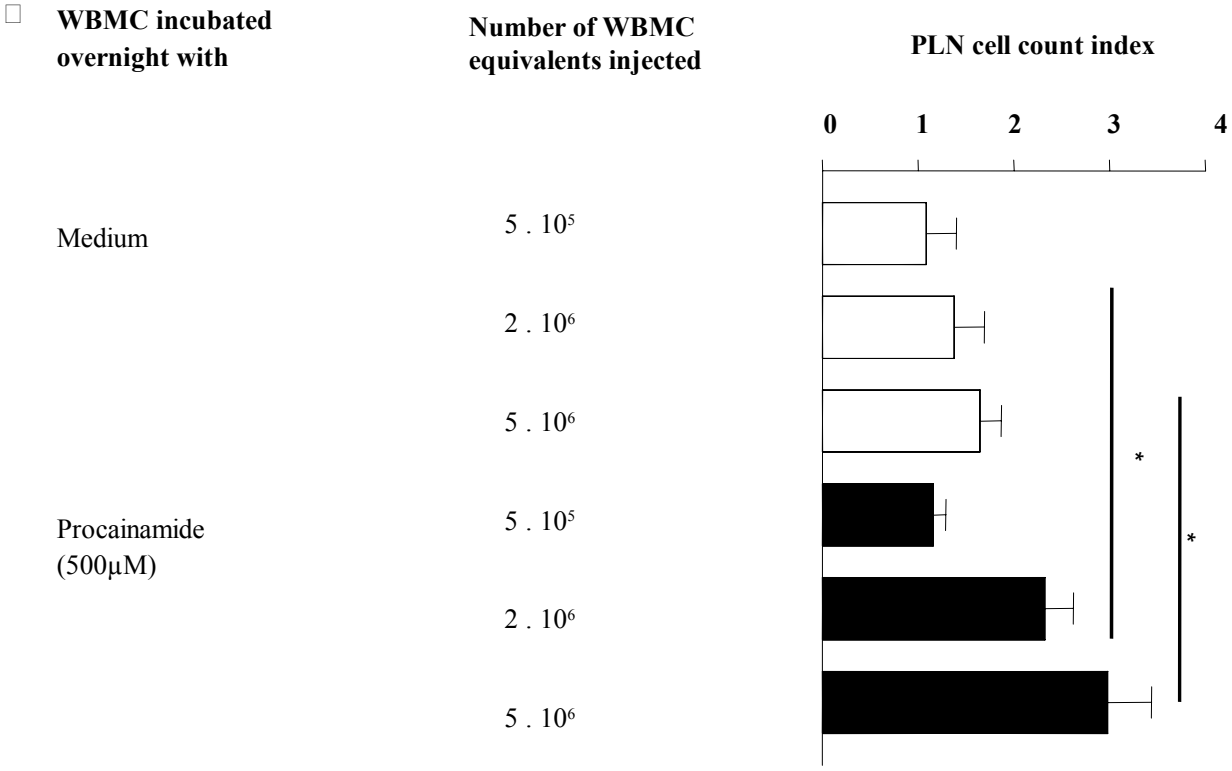


Figure 3.1.2. Primary PLN response against procainamide-pulsed WBMC.
WBMC were incubated overnight in medium alone or with 500 µM PA. WBMC were then washed, re-suspended in PBS and homogenised. On day 0, groups of BALB/c mice received a subcutaneous injection of 50 µl PBS containing the indicated cell equivalents of WBMC. PLN cell count indices were determined on day 6. Bars represent arithmetic means + SD from five mice per group. Asterisks indicate a significant difference (**P*<0.05) between the groups which received the same amount of PA-pulsed cells compared to the respective control group.

[127], indicating that the observed primary immune response was towards the reactive metabolites of PA generated *in vitro* by PMφ. Therefore, the following experiments were to determine whether other macrophage sources, such as WBMC, could also generate reactive metabolites and in turn neoantigens that could induce a primary immune response. Consequently, WBMC from BALB/c mice were pulsed with procainamide for 24 hours *in vitro* and thereafter stringently washed in sterile pyrogen-free PBS. Directly before injection, individual homogenates were sonicated and then subcutaneously administered (50 µl) into the ipsilateral hindfoot pads of naïve BALB/c mice. Six days later, the draining PLN were removed and the cell count indices determined in accordance to [94], that is, by dividing the total cell count of the treated side (ipsilateral) by that of the untreated side (contralateral).

As shown in figure 3.1.2, the homogenates of control WBMC (white bars) failed to induce a PLN response and this reaction was independent on the amount of cells injected. In contrast, the homogenates containing varying concentrations of PA-pulsed WBMC (black bars) were able to elicit a PLN response and this strengthened as the concentration of injected cells was increased, **P*<0.05. An injection of $5x10^5$ PA-pulsed WBMC was unable to produce a PLN response since the calculated index was comparable to the injection of $5x10^5$ unpulsed cells. Nevertheless, the significant response after the injection of $2x10^6$ PA-pulsed WBMC indicated that PA-pulsed WBMC could induce a primary T cell response, and furthermore implied that WBMC possessed the same metabolising capacity as previously found with PMφ [127]. Moreover, this result provided yet further evidence to

confirm our hypothesis that when PA is metabolised, reactive species are formed that can generate neoantigens which can subsequently induce immune responses.

3.1.3. GENERATION OF CD4$^+$ T CELL HYBRIDOMAS SPECIFIC FOR A NEOANTIGEN PRODUCED BY PA-PULSED WBMC

Since the primary PLN response, described in section 3.1.2 produced a positive result, the next objective was to further identify the possible PA-neoantigen(s) produced by macrophages. For this task T cell hybridoma development has proven a valuable tool since they provide an immortal *in vitro* test system with specificity to the antigen in question. Here, CD4$^+$ T cell hybridomas were generated after the immunization of naïve BALB/c mice with sonicated PA-pulsed WBMC homogenates and IFA. The resulting T cells were then fused with an immortal murine BW1548 TCR$\alpha^-\beta^-$ cell line.

Individual hybridomas were stained with both PE-conjugated anti-CD4 antibody and FITC-conjugated anti-αβTCR antibody in order to detect whether the fusion had functioned correctly. Within the hybridoma, the T cells provides the CD4 protein and the TCR whilst the BW 1548 cell provides the immortality. From two separate fusions 613 clones were produced and 427 of these hybridomas had a strong expression for both the CD4$^+$ and TCR$^+$ markers after analysis with specific antibodies and flow cytometry. Hybridomas that do not posses a TCR and/or CD4 protein after fusion can be explained by a poor fusion product or chromosome loss. Once these positive immortalised cells were in a confluent state, their specificity was tested through the IL-2 bioassay, as described in section 2.3.5.

In brief, WBMC from naivesyngenic mice were pulsed *in vitro* with or without PA, 24 hours prior to the test. 1x10^5 T cell hybridomas, bearing both the CD4$^+$ and TCR$^+$ surface markers, were then plated in round bottomed plates with 5x10^5 syngenic spleen APC. 1x10^5 PA-pulsed WBMC or untreated WBMC were then added and incubated for 24 hours at 37°C. The supernatants were then removed into fresh 96 well plates and frozen for a minimum of two hours. After thawing, the supernatants were further incubated for 18 hours with freshly prepared syngenic Con A blasts, (2x10^4 cells/50µl). [^{3}H] thymidine (1 µCi) was included for the last 6 hours and the radioactive incorporation measured.

In the IL-2 bioassay, 75 hybridomas demonstrated a clear specific response to PA-pulsed WBMC but not to WBMC alone. This response was ascertained when the resulting radioactive thymidine incorporation exceeded at least a factor of two over the control. To guarantee reproducible results these experiments were repeated at least twice. Figure 3.1.3A depicts a representative result obtained from four subcloned PA-specific CD4$^+$ T cell hybridomas generated from the BALB/c mouse strain. For handling purposes, twelve of the strongest responding hybridomas were subcloned. From these depicted subcloned hybridomas one can observe that indeed a significant response to neoantigen(s) produced from PA-pulsed WBMC can occur (*P<0.05, ***P<0.001). These findings not only confirm that WBMC possess the capacity to metabolise PA but can also produce reactive species that can stimulate T cells.

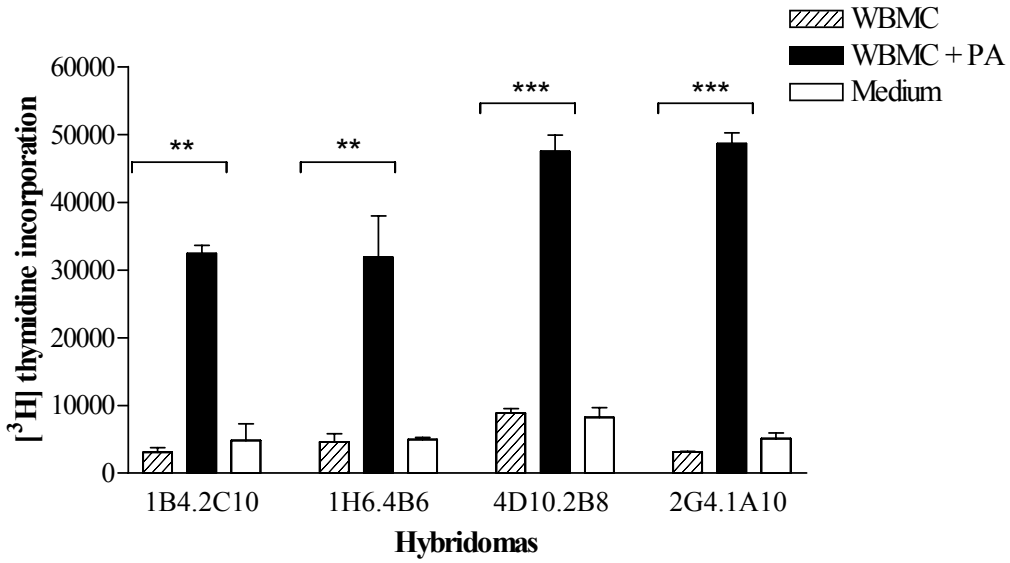


Figure 3.1.3.A. Reactivity of PA-specific $CD4^+$ T cell hybridomas.
$CD4^+$ T cells hybridomas, generated from the BALB/c mouse strain, were incubated with either previously PA-pulsed or untreated WBMC for 24 hours *in vitro*. The supernatants were then removed and analysed for IL-2 content through the proliferation of IL-2 dependent Con A blasts. Bars represent the arithmetic mean + SD of three reaction wells. Asterisks indicate significant differences [$^{**}P<0.01$, $^{***}P<0.001$] between the groups compared by the brackets.

For the long term *in vivo* PA investigations, (section 3.2) it was desirable to have $CD4^+$ T cells hybridomas that were generated from the A/J mouse strain, so that *in vivo* derived PA-neoantigens could be analysed. The generation of these hybridomas was initially successful and figure 3.1.3B shows a representative of $CD4^+$ T cell A/J hybridomas responding to WBMC pulsed with PA. In these experiments we also tried to boost the macrophage metabolism of PA by adding the mitogen PMA. The addition of PMA did appear to influence the response of these hybridomas to PA-antigen (black bars) but this was insignificant when compared to the normal PA-pulsed WBMC antigen source (grey bars).

Unfortunately, after several experiments these hybridomas lost their specificity to PA-neoantigens and on rechecking their expression of CD4 and TCR surface markers it was found that either the CD4 protein or the TCR were no longer expressed. Several more attempts at establishing A/J hybridomas were performed but the same pattern reoccurred. Possible explanations for this is that these hybridomas were specific for different antigens and those hybridomas specific for the PA-neoantigens were overgrown by a non-specific hybridoma before the sub-cloning could occur or the hybridomas expelled vital chromosomes. Although several investigations could be performed with the BALB/c $CD4^+$ T cell hybridomas, these too eventually lost their specificity and the frozen aliquots did not react after thawing. Further attempts to generate hybridomas specific to PA-pulsed PMφ, WBMC, spleen cells and HAPA-Globin proved unsuccessful in that either the fused cells did not grow, the hybridomas did not express the surface markers or specificity declined.

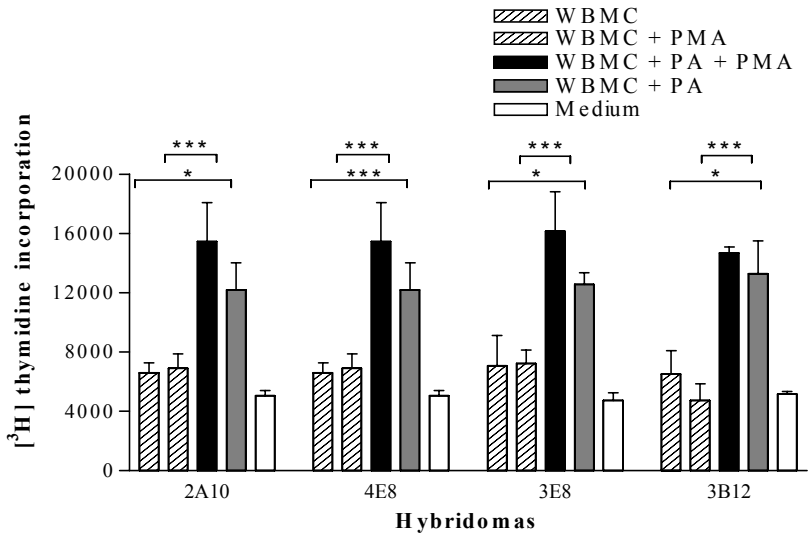


Figure 3.1.3.B. Reactivity of $CD4^+$ T cell hybridomas derived from the A/J murine strain.
A/J mice were immunised with sonicated PA-pulsed WBMC and IFA. After fusion with BW5147 cells the generated hybridomas were tested for their specificity towards neoantigens produced by WBMC that had been pulsed for 24hr with PA. To enhance metabolism, reaction wells were also briefly stimulated with PMA. After a 24hr incubation of hybridomas, APC and antigens, the supernatant was analysed for IL-2 content through the proliferation of IL-2 dependent Con A blasts. Bars represent mean + SD of triplicate wells. Asterisks indicate significant differences [$*P<0.05$, $***P<0.001$] between the groups compared by the brackets.

3.1.4. MHC-RESTRICTION ANALYSIS OF THE PA-SPECIFIC $CD4^+$ T CELL HYBRIDOMAS

To determine whether the recognition of the PA-induced neoantigens was MHC-class II dependent, restriction assays were performed. For this, the specific IL-2 bioassay was performed again but this time with prior blocking of the MHC molecules on the APC with monoclonal antibodies against $I\text{-}A^d$ or $I\text{-}A^d/I\text{-}E^d$ (see section 2.3.6). Hybridomas were classified as $I\text{-}A^d$ restricted when the hybridoma response towards PA-pulsed WBMC could be prevented with either of the mAb used. $I\text{-}E^d$ restricted hybridomas were ascertained when the reaction was blocked by only the $I\text{-}A^d/I\text{-}E^d$ mAb. Figure 3.1.4 depicts the findings from two subcloned PA-specific $CD4^+$ T cell hybridomas, whereas 2E10.0.3E4 is $I\text{-}A^d$ restricted (i) 4D10.0.32B8 is $I\text{-}E^d$ restricted (ii). The decrease in proliferation observed after specific blockage ($P<0.001$) was statistically significant in comparison to the PA-pulsed WBMC antigen. Since mAb are stored in azide solution, control wells containing appropriate concentrations of azide solution were also incubated. The reactive ability of the hybridomas was not affected by these controls, (grey bars).

To control for the specificity of the mAb, the corresponding isotype control antibodies were added to the hybridoma reaction cultures and these results are displayed in the striped columns directly after the mAb columns. As can be observed, these control incubations did not affect the ability of either hybridoma and indicated that the mAb blockage was specific. The above experiment was performed at twice to ensure reproducibility and provides evidence that these hybridomas respond to PA-derived neoantigens situated on the class II molecules presented by APC.

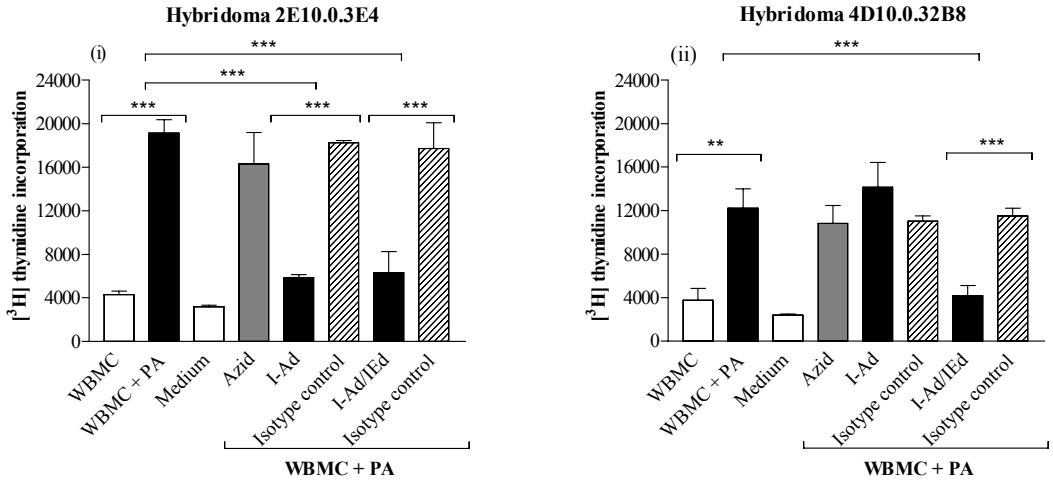


Figure 3.1.4. MHC-restriction of PA-specific BALB/c $CD4^+$ T cell hybridomas.
Clone 2E10.0.3E4 is 1-A^d restricted (i) whereas clone 4D10.0.32B8 is I-E^d restricted (ii). Hybridomas were incubated for 24 hr with PA-pulsed WBMC and APC, in the presence of restriction antibodies or their controls. Specificity was measured after the reaction supernatant was further incubated with IL-2 dependent Con A blasts and the IL-2 bioassay performed. Bars represent the arithmetic mean + SD of triplicate cultures and asterisks indicate significant differences between the groups compared by the brackets (**P<0.01 and ***P< 0.001).

3.1.5. PA-RELATED NEOANTIGEN FORMATION REQUIRES A PERIOD OF TIME

Previous studies have shown that in order for PA to elicit an immune response, this prohapten must first undergo metabolism by neutrophils or macrophages [106, 127, 128]. In addition to those findings, the previous sections of this chapter have shown that WBMC have the ability to metabolise PA and that the homogenates of those pulsed cells can elicit an immune response. Therefore, an assay was devised in order to determine whether or not a certain length of time was required for the WBMC to metabolise PA and produce sufficient quantities of the neoantigens for the $CD4^+$ T cell hybridomas to respond to and produce IL-2.

Section 2.3.8 describes the procedure in full but to summarise, WBMC that had been pulsed with PA for either 30 minutes, 1, 2, 4 or 8 hours were added to syngenic APC and BALB/c PA-specific $CD4^+$ T cell hybridomas. As shown in figure 3.1.5, neither hybridoma responded if PA was added to WBMC for only 30 minutes or 1 hour. However, from these experiments, it is apparent that enough neoantigen concentration is available for a hybridoma response after 2-4 hours. The different response times may depend on either the reactivity of the $CD4^+$ T cell hybridoma itself or the uptake, processing and presentation of the neoantigen by the APC. After 4 and 8 hour incubations the response from both hybridomas was strong enough to produce significant differences (***P<0.001) and these values did not significantly vary from those obtained with PA-pulsed WBMC after 24 hours. Consequently, it appears that WBMC require at least 4 hours to metabolise enough PA to generate significant quantities of the neoantigen for hybridomas activation.

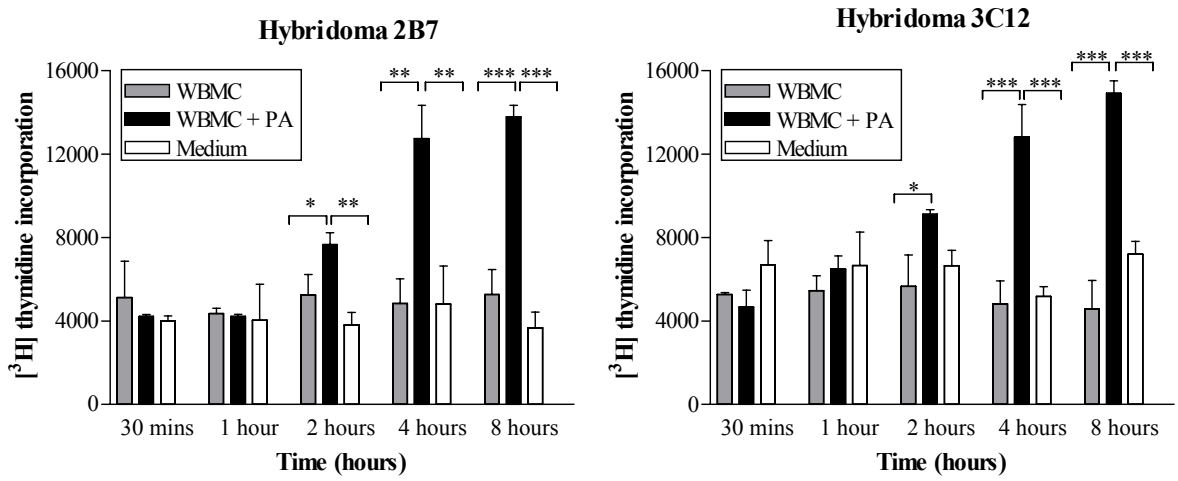


Figure 3.1.5. Time Assay.
PA-pulsed WBMC and APC were incubated together over different time periods followed by the addition of PA-specific $CD4^+$ T cell hybridomas. The IL-2 bioassay was then performed. Bars indicate the arithmetic mean + SD of duplicate wells. Asterisks indicate significant differences (*P<0.05, **P<0.01 and ***P<0.001) between the groups compared by the brackets.

3.1.6. PROCAINAMIDE-NEOANTIGEN RECOGNITION BY $CD4^+$ T CELL HYBRIDOMAS REQUIRES A LAG PHASE

In 1981, Ziegler and Unanue [146] described a lag period between the binding of an antigen to macrophages and the detection of the antigen by the T cells. They showed that T cells can recognise antigens arising from pulsed macrophages which have been rendered metabolically inactive by fixation with paraformaldehyde. Therefore, T cells actually recognise antigen at the surface of the APC rather than non-specifically binding to the APC and subsequently triggering the APC to produce an immunogenic moiety from a cytoplasmic compartment. In order to detect whether this was occurring in this system, APC were incubated with PA-pulsed or control WBMC and then subjected to either immediate fixation, fixation after 4 hours of culture or no fixation at all as a positive control, for details see section 2.3.7. PA-specific $CD4^+$ T cell hybridomas were then added and the IL-2 bioassay performed.

In figure 3.1.6, each graph depicts the proliferation results of one individual subcloned hybridoma after the different fixation treatments. As found previously (section 3.1.3), each hybridoma responded to PA-pulsed but not control WBMC (control data sets). Although slightly depreciated with some of the hybridomas, for example 4D10.0.32B8, this positive response to PA-neoantigens did not alter when the APC were fixed after a 4 hour incubation with the antigen source, (4 hours). In contrast, upon immediate fixation, (immediate), only background levels of IL-2 production were detectable, indicating that the APC required a certain amount of time to take up the antigen and process it to their surface MHC molecules so that the $CD4^+$ T cell hybridomas could recognise it and respond. These experiments were performed twice to ensure reproducibility.

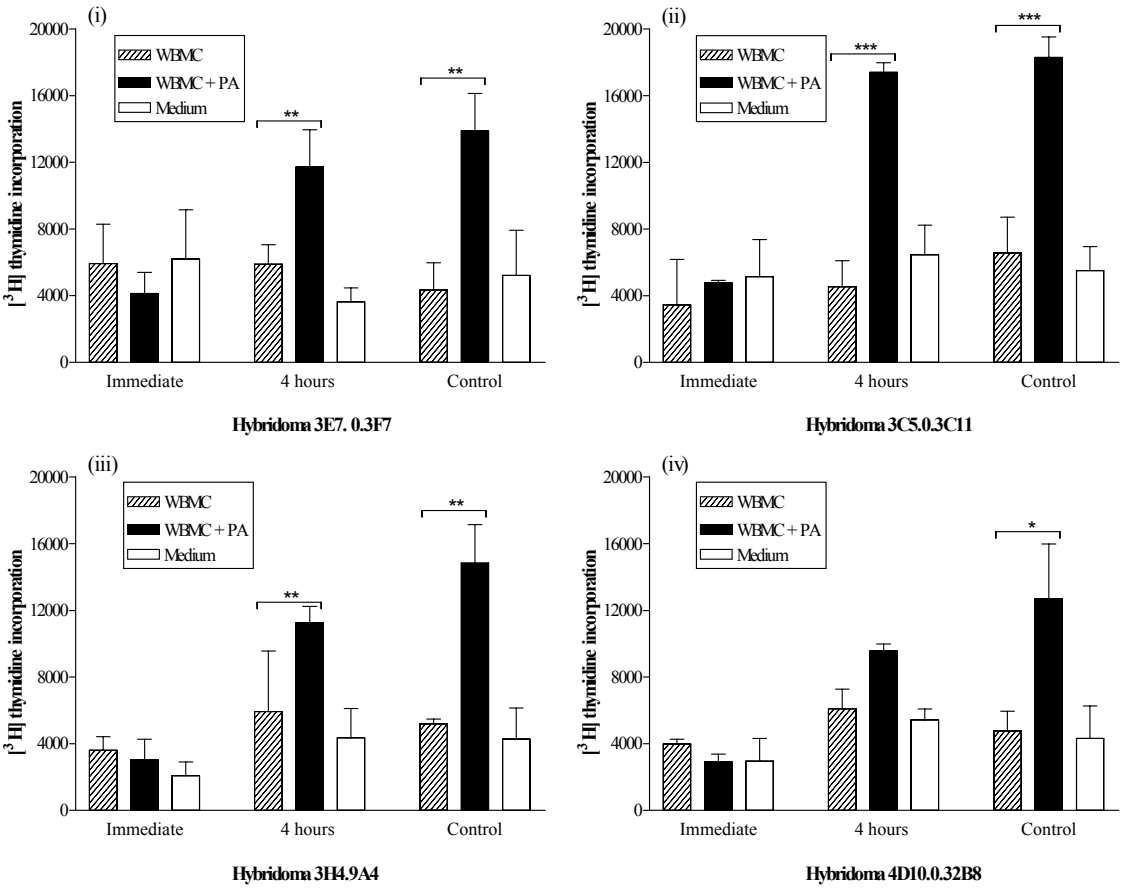


Figure 3.1.6. APC Fixation Assay.
PA-pulsed WBMC or control WBMC ($1x10^5$) were incubated together with syngenic APC ($5x10^5$) and then either directly fixed (immediate), fixed after four hours (4 hours) of culture or not fixed at all (control). Subcloned BALB/c derived PA-specific $CD4^+$ T cell hybridomas were then added for a further 24 hours. Thereafter the supernatant from each reaction well was analysed for IL-2 content through the proliferation of IL-2 dependent syngenic Con A blasts. Each of the above graphs depicts the proliferation results obtained from individual hybridomas after the different fixation treatments. Bars represent the arithmetic mean + SD of triplicate wells. Asterisks indicate significant difference (**P*<0.05, ***P*<0.01 and ****P*<0.001) between the groups compared by the brackets.

3.1.7. DETERMINATION OF PA METABOLISM BY HPLC

In order to try and determine how much PA was metabolised by WBMC *in vitro*, an indirect method using High Performance Liquid Chromatography was derived, (described in section 2.3.9). However, although several sets of data were produced this method proved insensitive and the results were collectively inconclusive. These findings are not shown here.

3.1.8. INVESTIGATIONS TO ELUCIDATE THE SPECIFICITY OF THE PA-NEOANTIGENS

From the evidence collected in sections 3.1.3, 3.1.4, 3.1.5 and 3.1.6 it was apparent that the generated $CD4^+$ T cell hybridomas reacted towards PA-neoantigen(s) produced from PA-pulsed WBMC *in vitro.* However, the nature of these neoantigens remained unknown. In numerous experiments, the reactivity of the PA-specific $CD4^+$ T cell hybridomas was tested to alternative antigen sources including histone,

chromatin, dsDNA, ssDNA, HAPA and HAPA-globin. Unfortunately, none of these antigens produced significant T cell hybridoma stimulation (data not shown). In retrospect, perhaps haptenisation of these self-proteins with HAPA could have provided the correct antigen.

Previous studies have shown that the cytoplasmic fraction of PA-pulsed PMφ, produced a 35kDa protein band after Western Blot analysis using a specific PA antibody [127]. To determine whether this protein region could contain the specific neoantigen, or one of the possible neoantigens, another variation on the IL-2 bioassay was performed that was in accordance to a similar assay performed by Kubicka-Muranyi *et al*., [140]. In brief, PA-pulsed or control WBMC nuclei or cytoplasmic homogenates were separated by SDS-PAGE gel electrophoresis. These separated proteins were then transferred onto nitrocellulose paper and various regions including the suspected 35kDa region were extracted. Proliferation responses to these nitrocellulose pieces by PA-specific $CD4^+$ T cell hybridomas were then tested in the IL-2 bioassay performed, for details see section 2.3.10. Similar IL-2 bioassays were also performed with separated cytoplasmic and nuclei fractions from PA-pulsed WBMC, see section 2.3.11 Although performed at least twice these investigations were inconclusive and therefore the data is not shown. Further investigations to elucidate PA-specific protein bands are described in section 3.3.1.

3.2. LONG TERM PA TREATMENT INDUCES T CELL REACTIVITY AND SUPPRESSION

The pathogenesis of procainamide drug-induced lupus in humans occurs only after several months of treatment. Therefore, when trying to mimic this phenomenon in an *in vivo* murine model, it is essential to bear these facets of the disease in mind. Within this section, the onset of procainamide drug-induced lupus is monitored in orally treated slow acetylator A/J mice mainly through the formation of autoantibodies (ANA). Furthermore, the *ex vivo* activity of PA-treated $CD4^+$ T cells and the suppressive effects of primed regulatory $CD4^+CD25^+$ T cells are described. In brief, using the lymphocyte transformation test assay (LTT), $CD4^+$ T cells from long term PA-treated A/J mice but not their age related controls were able to respond *ex vivo,* to PA-pulsed cells or the reactive metabolite HAPA. This response was further shown to be APC dependent and that the B cell population could efficiently act in this manner. These PA-treated A/J mice also developed significant titres of ANA which were IgG1 specific and reacted towards several different proteins and PA or HAPA.

Finally, the question arose as to whether the $CD4^+CD25^+$ T cell subset from PA-treated mice could prevent the formation of ANA in naïve mice receiving PA treatment. Thus, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets from PA-treated or control mice were used as donor cells and transferred into naïve recipients. After six weeks, PA-treated recipients that had received PA-treated $CD4^+CD25^-$ cells began to develop ANA whereas those which received $CD4^+CD25^+$ did not. The $CD4^+$ T cell subsets from the untreated donors had no affect on the ANA formation in the recipients. The results obtained from the transfer of all T cell subsets into PA-treated recipients were mirrored in the untreated recipients, indicating that the PA-treated $CD4^+CD25^-$ donor T cells were initiating the development of ANA to self-proteins.

3.2.1. DAILY WATER AND PROCAINAMIDE INTAKE BY A/J MICE

Previous *in vivo* experiments have shown that the A/J mouse strain cannot tolerate repeated injections of HAPA but can withstand repeated PA injections or PA drinking water [127, 133]. To determine whether the A/J strain could tolerate the concentration of PA in the drinking water that was required here, a trial experiment was performed over several months with four groups of A/J mice receiving either water, sweetened water, PA or PA and sweetener. PA was administered at a concentration of 6 g/L.

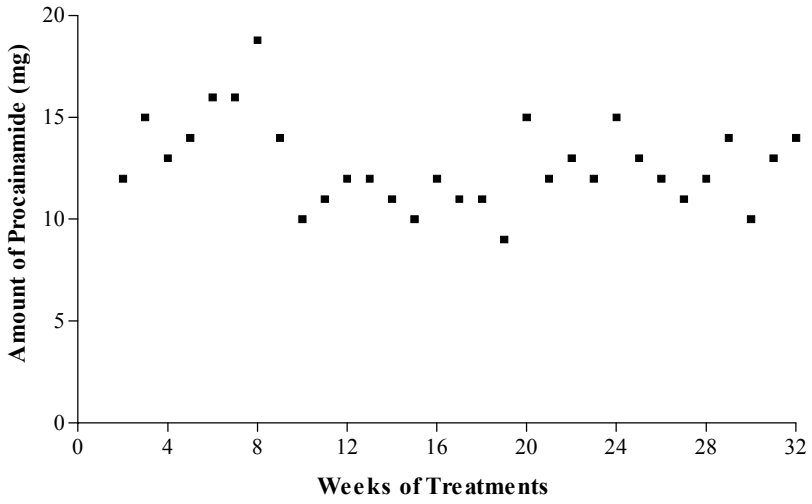


Figure 3.2.1. Consumption of procainamide.
The average amount of PA absorbed by individual mice through the drinking water was calculated by the following formula: Volume of water (L) x 6g/L PA / number of days / number of mice. Symbols represent the weekly average amount of PA (mg) consumed by each mouse per day (n=60). Overall the mean value over 8 months was 12.62 ± 2.12 mg of PA/ per mouse/ per day.

Each week the mice were weighed and the water consumption recorded so that the uptake of PA could be calculated. From this investigation it became apparent that there was no difference in the intake of PA between the mice that received PA sweetened water or PA water. On average each mouse drank approximately 22 ml of water over a four day period and thus each mouse was consuming on average between 10–15 mg of PA per day. This observation is much lower than that previously recorded which stated that oral PA-treated A/J mice treated consumed 24 mg per day [133].

Consequently, in the large investigation A/J groups received only water or PA water and the weekly measurements were recorded throughout the whole investigation. Figure 3.2.1 depicts the average weekly amount of PA (mg) that individual mice absorbed each day over the eight month period. As with the trial experiment the amount of PA water consumed by the groups was approximately 3.428 ml/per mouse/per day, resulting in the average consumption of 12.62 mg ± 2.12 mg PA/per mouse/ per day. This is in comparison to the water intake by the control group which was 3.287 ml per day. Therefore, the plasma concentration of the treated mice should have been approximately 7 μg/ml.

3.2.2. PROTEIN CONCENTRATION IN THE URINE OF A/J TREATED MICE

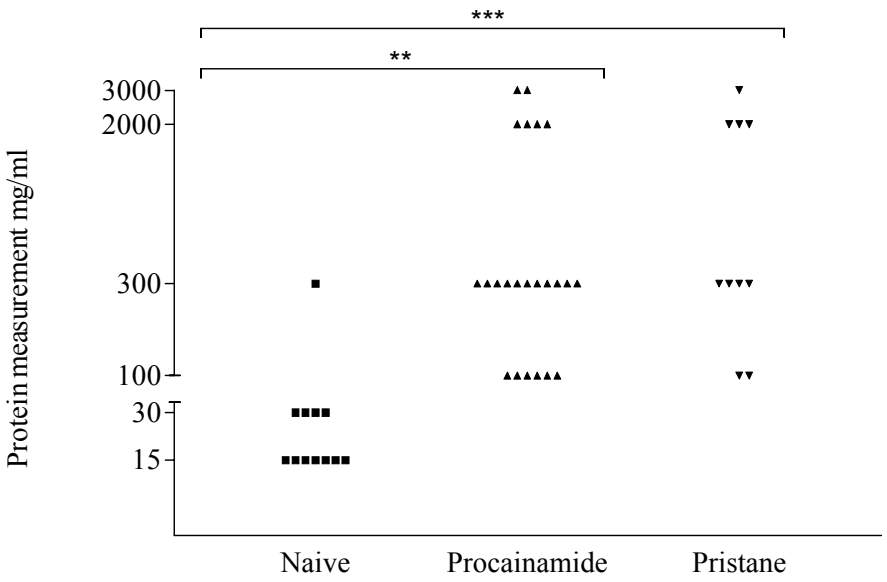


Figure 3.2.2. Increases in protein concentration in the urine of PA- and pristane-treated A/J mice
Individual measurements of protein concentration in the urine of 30 procainamide (▲), 12 pristane (▼) and 30 age related controls (■) were performed using Albustix® strips. Protein concentrations were determined in a blind fashion with three independent evaluations. Asterisks indicate significant differences (**P<0.01; *** P<0.001) between the groups compared by the brackets. .

With some autoimmune diseases an associated disorder termed glomerulonephritis can be initially detected by increased protein concentration in the urine. To assess the level of protein concentration in the urine of PA- and pristane-treated mice, the urine from all groups was collected upon death and determined with Albustix® sticks, (Bayer). Thereafter, three independent measurements were employed to calculate the average urine protein concentration in accordance with the provided grading chart. Figure 3.2.2 displays the differences in the average protein concentration of PA- and pristane-treated mice compared to age related controls, with significances of **P<0.01 and ***P<0.001 respectively. Several reports have demonstrated high protein concentrations in the urine of certain mouse strains after treatment with pristane [147, 148] and further assessment on the organs of these treated mice identified kidney damage and glomerulonephritis, thus indicating an autoimmune state.

3.2.3. AN INCREASE IN SPLEEN WEIGHTS IN PROCAINAMIDE AND PRISTANE-TREATED MICE IS ACCOMPANIED BY AN INCREASE IN THE B CELL POPULATION

A further finding in murine models that either spontaneously develop or have induced autoimmune disease is an increase in splenic weight and cell mass. A reason for this may lie in the increased B cell population which is producing the autoantibodies. This phenomenon has been reported with the treatment of pristane in several strains of inbred mice [147, 148].

To determine whether this observation also occurred for these pristane-treated A/J mice and for the PA-treated mice, individual spleen weights were measured at the time of death. Figure 3.2.3Ai depicts the differences in spleen mass between the groups with clear statistical significances between

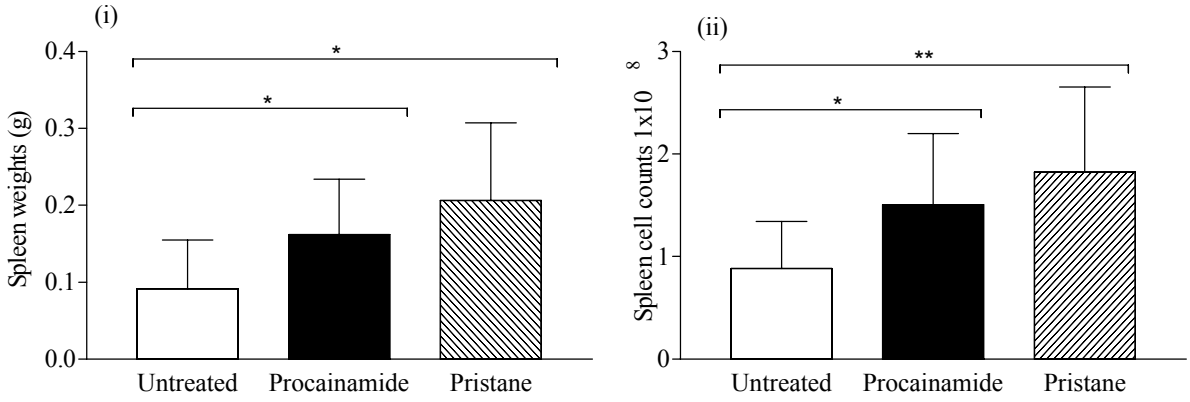


Figure 3.2.3.A. Spleen weights and cell counts.
From procainamide, pristane and untreated mice individual spleen mass values (i) and cell counts (ii) were recorded. Bars indicate the arithmetic mean + SD of pooled groups. Asterisks indicate a significant difference (**P*<0.05 and ***P*<0.01) between the groups compared by the brackets.

the treated groups and age related controls, **P*<0.05. The average cell number of each individual mouse spleen was also calculated (figure 3.2.3Aii) and the significant differences between the untreated group and either the PA-treated (**P*<0.05) or the pristane-treated (***P*<0.01) groups are clearly identifiable.

As mentioned above, the enlarged spleen sizes and cell mass could correlate with an enlarged B cell population. In order to evaluate this question, spleen lymphocytes from individual mice were incubated with a variety of antibodies towards specific cell surface markers and the population sizes determined via flow cytometry, see section 2.5. The total T cell population, as detected via an anti-CD3 antibody and the major T cell subsets, (anti-$CD4^+$ and anti-$CD8^+$ antibodies), showed no significant variations between the different groups, apart from a lower CD8 T cell population in pristane-treated mice, figure 3.2.3Bi. However, when the B cell populations from each group were compared using an anti-CD19 antibody, a significant increase in this population could be detected in the two treated groups but not in the age related controls, ****P*<0.001 figure 3.2.3Bii. Further FACS analysis was also performed using other surface markers specific for DC cells (CD11c and CD11b) and co-stimulation markers (CD80 and CD86). Further markers were also tested, for example, the L-selectin ligand (CD62L) which is reported to be an autoantibody in MLR mice which can spontaneously develop lupus [149]. With such markers no differences could be observed and therefore the data is not shown.

Cytokines and indeed chemokines play a vivid role in all immune responses and their involvement is steadily being unravelled. There is currently no literature regarding cytokine activities in long term PA-treated mice. Therefore, upon sacrifice, lymphocyte spleen cells from treated and untreated mice were activated with mitogenic stimuli (PMA, Ionomycin and Brefeldin A), and analysed for cytokine variations by intracellular staining and flow cytometry, (section 2.5.3). Although in some PA-treated A/J mice an up-regulation of some cytokines could be detected, for example IL-2 and IL-10 (data not shown), a general pattern could not be observed. A major fact to remember regarding cytokines is that their role and influences are usually very early on in an immune

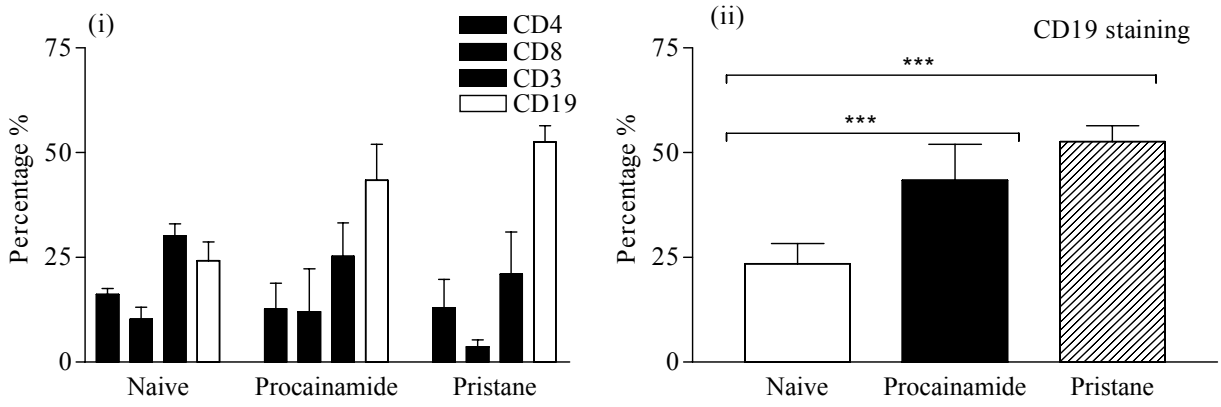


Figure 3.2.3.B. *Ex vivo* T and B cell population analysis.
$1x10^5$ lymphocyte spleen cells from individual PA, pristane or age related control mice were stained with anti-CD3, anti-CD4, anti-CD8 or anti-CD19 antibodies and then analysed for cell population differences using the FACSCalibur (i). B cell populations are depicted again in (ii). Bars indicate the arithmetic means + SD and asterisks indicate a significant difference (***P<0.001) between the groups that are compared by the brackets.

response. Here the long term affects were being analysed and consequently the actions of cytokines have already occurred and regressed to the normal state again.

3.2.4. ARE T CELLS FROM LONG TERM PA-TREATED A/J MICE REACTIVE EX VIVO TO PROCAINAMIDE PULSED MACROPHAGES?

Research surrounding adverse xenobiotic reactions have focused upon the involvement and ability of T cells, especially $CD4^+$ T cells, in eliciting the observed immune responses. The PLN assay and hybridoma work described in section 3.1, clearly indicated that T cells indeed play a role in the immune response to PA. Alongside this, previous studies have shown that in an adoptive transfer PLN assay, using donor PMφ from four month PA-treated mice, the recipients responded to HAPA but not to PA alone [142]. The long term administration of PA drinking water here, provided not only a more physiological situation than repeated injections but also ample opportunity for excess PA to be metabolised by circulating macrophages and the T cells to respond to the possible generated neoantigens. Therefore, the question arose as to whether the T cells from these PA-treated mice could respond to PA-related neoantigens or other possible candidate antigens in an *in vitro* assay. For these experiments, the lymphocyte transformation test (LTT) was employed and is described in full in section 2.4.4. The results of this assay are considered significant if the responding T cells produce a stimulation index that is more than a factor of two over the control cell proliferation.

For the bulk T cell assays, nylon wool enriched T cells from three treated or untreated mice were cultured with irradiated APC (in order to prevent non-specific proliferation) and various antigens for 96 hours. [^{3}H] thymidine (1 μCi) was added for the last 18 hours. After harvesting the radioactive incorporation was measured with a TRILUX β-counter (1450 Microbeta™ WALLAC Oy, Turku, Finland). The stimulation indices were calculated using the following formula and statistics were determined by applying the one-way analysis of variance (ANOVA) with the PRISM 3 programme (GraphPad Software, Inc., USA).

$$\frac{\text{Treated T cell + APC + Antigen}}{\text{Treated T cell + APC + Only.}}$$

To avoid false-positive results, these assays were discarded if the denominator value was below 1000 cpm. In the initial experiments, all cell types from the different treatment groups were criss-crossed to ensure T cell proliferation was towards the plated antigen(s) and not towards residual antigen sources presented on the APC from treated mice or from the antigens alone. Table 3.2.4 depicts a typical criss-cross assay.

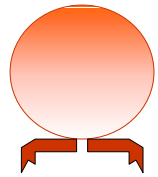
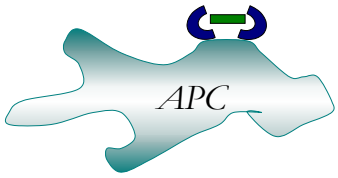
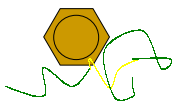
RESPONDER T CELL 	APC SOURCE 	ANTIGEN 
PA-treated T cells	Derived from PA-treated A/J mice	All
PA-treated T cells	Derived from naïve A/J mice	All
PA-treated T cells	None	All
PA-treated T cells	None	None
Naïve T cells	Derived from PA-treated A/J mice	All
Naïve T cells	Derived from naïve A/J mice	All
Naïve T cells	None	All
Naïve T cells	None	None
No T cells	Derived from PA-treated A/J mice	All
No T cells	Derived from naïve A/J mice	All
No T cells	Derived from PA-treated A/J mice	None
No T cells	Derived from naïve A/J mice	None
No T cells	None	None

Table 3.2.4. Typical criss-cross scheme for the lymphocyte transformation test.
This table depicts the combinations for only the PA-treated and naïve T cells. In the actual assays pristane- and DNFB-treated T cells were also included in the same fashion.

To determine whether the T cells from any of the treated groups had the ability to respond in the assay, Con A (2 μg/ml) controls were also included. The radioactive counts for this control were always between 16,000–25,000 cpm showing a clear ability for all tested T cells to proliferate. As a specificity control and to test the functional capacity of the assay, DNFB-treated mice were employed. For this, ten days prior to the assay, a solution of DNFB was painted onto the hindfoot pads of naïve A/J mice. On the day of the assay, the PLN were removed and the cells plated alongside irradiated APC and the antigens to be tested in the LTT assay. As expected, these bulk PLN cells only reacted to the specific antigen DNBS as depicted in figure 3.2.4iv (*** $P<0.001$). The T cells from naïve mice failed to react to any antigens and this was irrespective of the APC source used, figure 3.2.4i.

Also irrespective of the APC origin was the reaction observed by the T cells from PA-treated mice, figure 3.2.4ii. Here, bulk T cells proliferated to PMφ, WBMC and spleen cells but only when these macrophage sources had been previously pulsed with PA or HAPA (***$P<0.001$). PA-treated T cells also reacted towards HAPA (20 μM) alone or HAPA that had been incubated on ice with pre-cooled spleen cells for 30 minutes. Although these PA-related T cells elicited a response to allogenic chromatin (**$P<0.01$) and hsp60 (**$P<0.01$), they did not react to histone, dsDNA, Ku or smRNP antigens or the positive DNBS control.

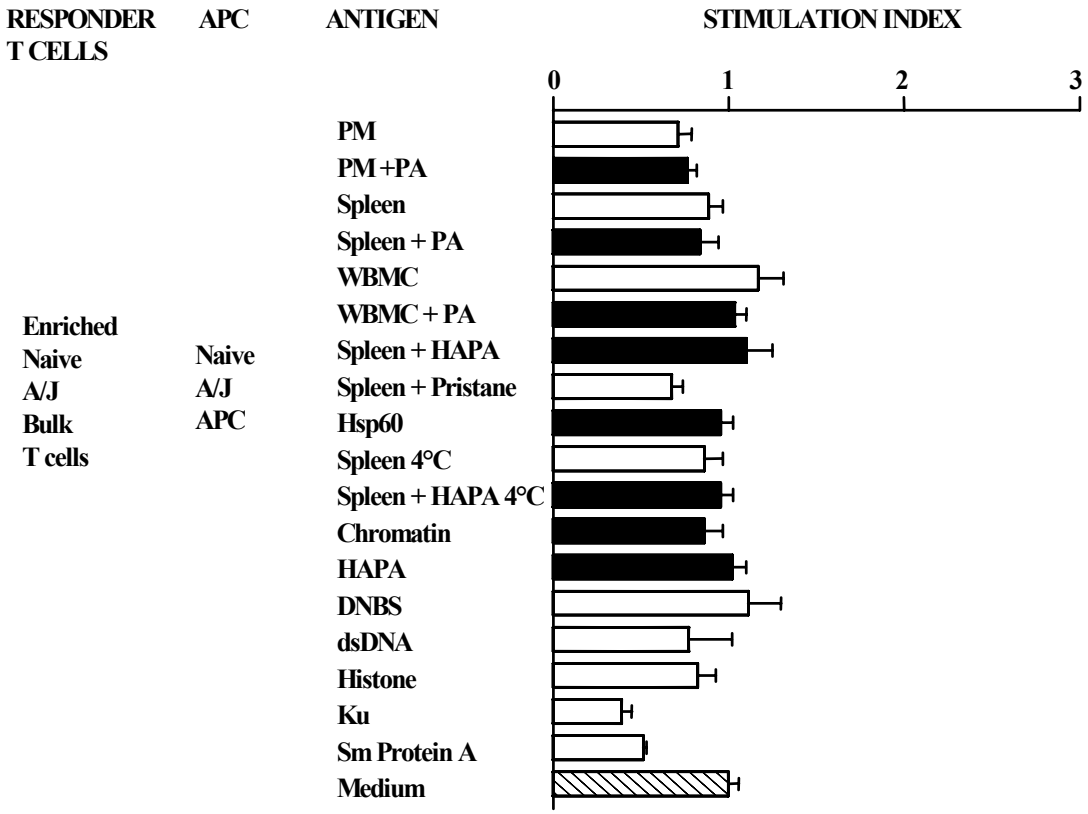


Figure 3.2.4.i. A/J derived naïve bulk T cells fail to respond to PA-related antigens
Enriched T cells from naïve donors were incubated with irradiated APC and the displayed antigens for 96 hours. [^{3}H] thymidine was added for the last 18 hours and the incorporated radioactivity measured. Bars indicate the SI + SD of 6 replicate wells which were calculated by the formula: *T cell + APC + Ag / T cell + APC + medium*. Control criss-cross experiments using APC from PA-, DNFB- and pristane-treated mice were also tested but no differences to the obtained stimulation indices were observed.

Bulk T cells from age related A/J mice that had been given a one time injection of pristane at six weeks of age, were also used in these LTT assays. The T cells from these mice were then used to determine whether or not the responses found from the T cells of PA-treated mice towards PA-induced neoantigens were specific. Previous studies have shown that certain mouse strains that are treated with a single injection of pristane can develop an autoimmune state [147, 148]. Furthermore, these responses have been described as T cell mediated [147]. Indeed, after four months of treatment these pristane-treated A/J mice developed significant IgG1 autoantibody titres (see section 3.2.7). Consequently, these mice provided an essential control since 1) they developed signs of an autoimmune disease like state section 3.2.3, 2) they were age-related and maintained in the same husbandry conditions and 3) they were treated with an entirely different xenobiotic and had no exposure to PA. These pristane-treated bulk T cells did not react to any of the antigens containing PA but slightly reacted (SI of 2) to pristane-pulsed spleen cells and hsp60, figure 3.2.4iii. Unfortunately, the specific antigen(s) for these reactive T cells are also unknown and pristane itself cannot be used since this oil based substance is difficult to dissolve and hence work with *in vitro* for long periods.

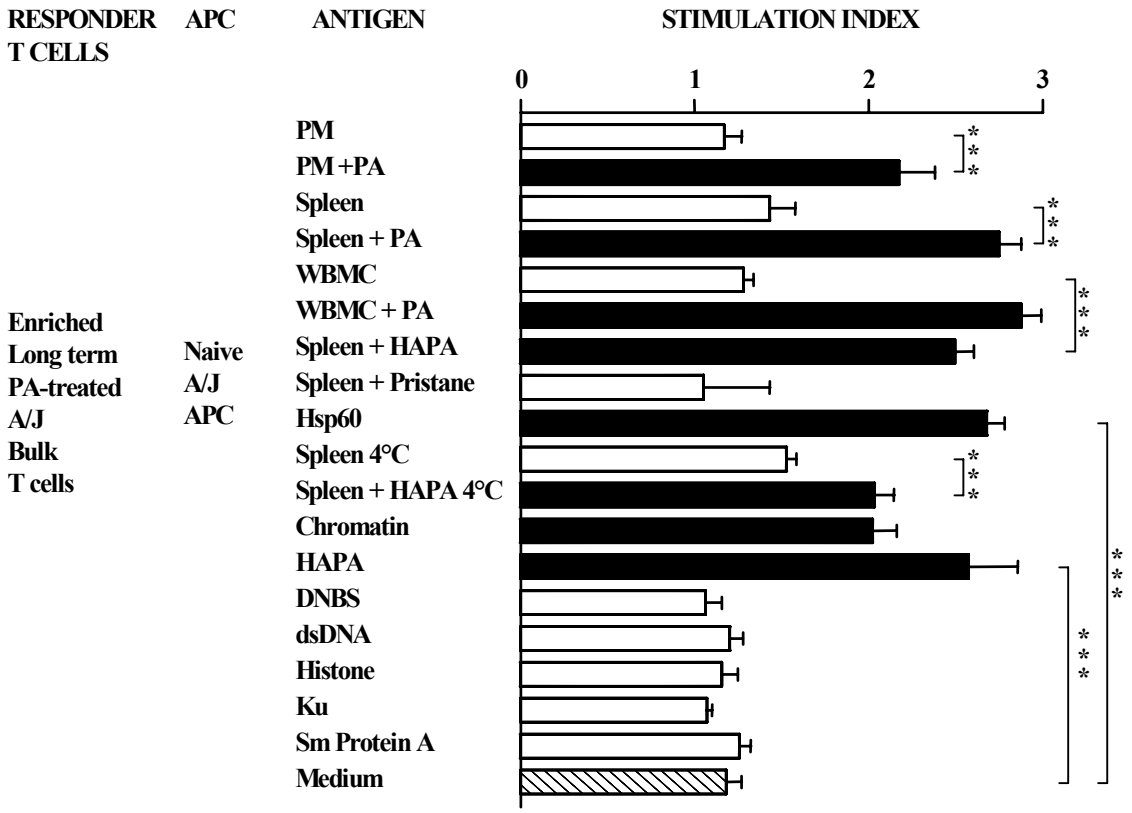


Figure 3.2.4.ii. *In vitro* LTT assay with *ex vivo* responding T cells from PA-treated A/J mice.
Enriched long term PA-treated donor T cells were incubated with APC and the depicted antigens for 96 hours. For the last 18 hours, [^{3}H] thymidine was added and the incorporated radioactivity measured. Bars indicate the SI + SD of 6 replicates which were calculated by the formula : *T cell + APC + Ag / T cell + APC + medium.* For control criss-cross experiments using APC from naïve, DNFB- and pristane-treated mice were also used but no differences to the depicted SI were observed. All black bars indicate a positive response and asterisks indicate significant differences between the groups compared by the brackets, ** P<0.05, **P< 0.01.

To summarise, T cells from long term PA-treated but not from naïve, pristane- or DNFB-treated mice, could be stimulated *ex vivo* with PA-pulsed macrophage sources or free HAPA. These bulk T cell assays were performed three times to ensure reproducibility. After enrichment, the T cell purity was examined using flow cytometry and was > 95 %. On one occasion, inguinal lymph nodes (ILN) from the PA-treated or control groups were removed and tested in the LTT assay for a response to PA-pulsed antigen sources or HAPA. The naïve ILN cells did not produce a response to any of the antigens tested and all the stimulation indices remained around one which is considered insignificant, data not shown.

However, although the ILN cells from PA-treated mice responded to PA-pulsed macrophage antigens the stimulation indices remained below two and were therefore also considered insignificant. Two possible explanations could perhaps explain this reduced response: 1) there is a smaller number of PA-neoantigen specific T cells in the ILN than the spleen or 2) since these ILN cells were not separated the amount of actual plated T cells that could respond was greatly reduced, see discussion section 4.2.

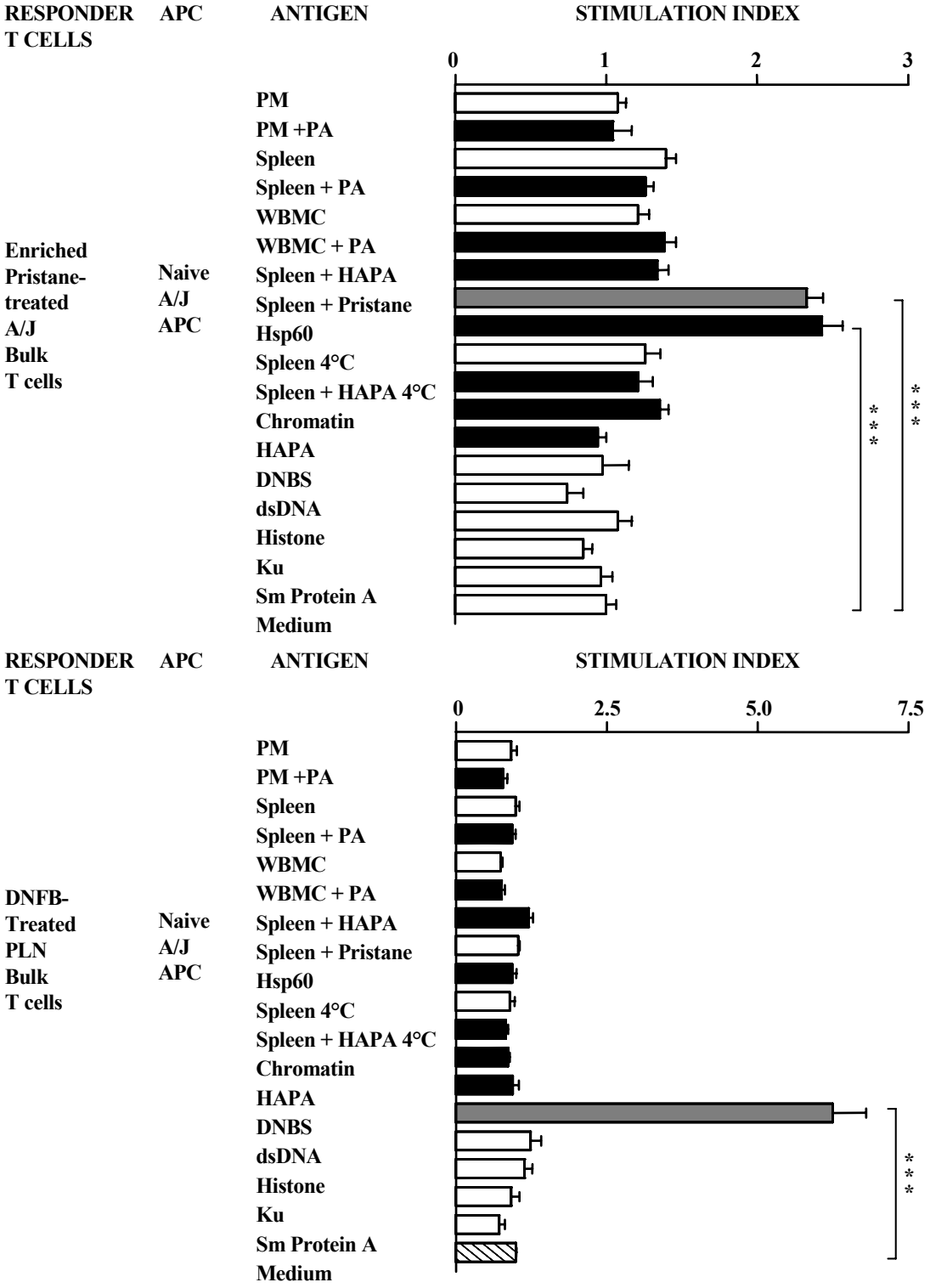


Figure 3.2.4.iii and iv. *In vitro* LTT assay with *ex vivo* pristane- or DNFB-treated T cells.
Irradiated APC, Ag and either pristane-treated (upper graph) or DNFB-treated PLN cells (lower graph) were plated together for 96 hours. For the last 18 hours, [^{3}H] thymidine was added and the incorporated radioactivity measured. Bars indicate the mean SI + SD of 6 replicates which were calculated by the formula: *T cell + APC + Ag / T cell + APC + medium*. Asterisks indicate a significant difference between the groups compared by the brackets.

3.2.5. CD4$^+$ T CELLS FROM PA-TREATED A/J MICE ARE THE RESPONSIVE T CELL SUBSET

From section 3.2.4 it became apparent that the T cells from long term PA-treated A/J mice could respond *ex vivo* to PA-induced neoantigens. Therefore, the next interesting and logical question was whether the CD4$^+$, the CD8$^+$ or both major T cell subsets were responsible for the observed proliferation. To evaluate this question, further LTT assays were performed. These assays contained T cells that had not only been enriched via nylon wool columns but also separated into distinct T cell subsets via the AutoMACS using specific microbeads (see sections 2.4.4.2 and 2.4.4.3). The purity of these T cell populations was then determined by flow cytometry using specific antibodies and the fractions were only used when the purity was over 95 %. Since the previous LTT assays had shown that PA-treated T cells responded to antigenic stimuli regardless of which APC source was employed, all following experiments contained only naïve APC fractions. Con A was used once again as a control for T cell responsiveness and this time also as an indication of how pure the CD4$^+$ T cells, were since this mitogen is MHC-class II specific. In all of these experiments the CD4$^+$ T cell fractions only proliferated to Con A in the presence of APC, data are not shown.

Figure 3.2.5i shows a comparison between the responses of separated CD4$^+$ T cells from either PA-treated mice or the age related controls to the different antigenic sources. As mentioned above, these assays were carried out in the presence of naïve syngenic APC. As observed in the previous bulk T cells assays, see section 3.2.4, no proliferation was detected if the APC or antigens were absent. Naïve CD4$^+$ T cells failed to respond to any of the antigens. In contrast, CD4$^+$ T cells from PA-treated mice did respond to purified HAPA (20 μM) or macrophage sources (WBMC, PMφ, spleen) that had been previously incubated with PA (black bars) but not to their controls (corresponding white bars) or DNBS. These T cell responses produced stimulation indices between 2 and 4 and were significantly different to the controls (**P<0.01 and ***P<0.001).

PA-treated CD4$^+$ T cells also responded to allogenic chromatin and hsp60 (grey bars) albeit weaker than the PA-related antigens. All antigens and APC were irradiated and therefore any non-specific proliferation could be considered negligible. The response to PA-related antigens or their controls by separated CD8$^+$ T cells from either the PA-treated or untreated mice can be observed in figure 3.2.5ii. At a glance one can clearly observe that neither of these T cell subsets responded to any of these antigens since all the SI remained approximately equal to the control. Although from these experiments a role of CD8$^+$ T cells cannot be ruled out, a definite CD4$^+$ T cell reponse was observed. Under these conditions the proliferation of CD8$^+$ T cells would probably require additional IL-2 from conditioned medium. Therefore, the lack of CD8 proliferation does not signify the absence of HAPA-specific or autoimmune CD8 T cells which could be activated *in vivo* by PA treatment.

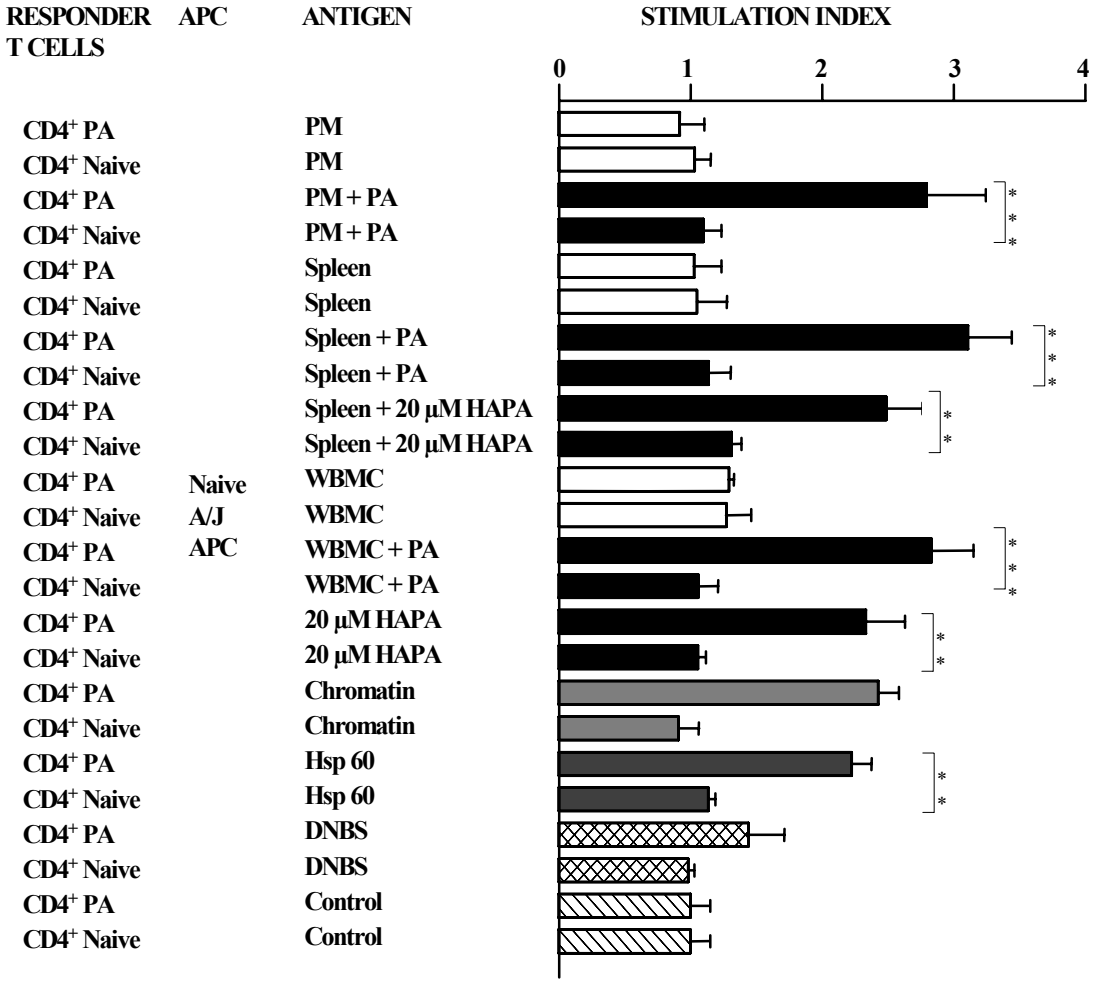


Figure 3.2.5.i. $CD4^+$ T cells are the responding T cell subset to PA-related neoantigens.
Enriched $CD4^+$ T cells from long term PA-treated or control mice were incubated together with irradiated APC and the indicated antigens in the LTT assay. [^{3}H] thymidine was added for the last 18 hours and the radioactive incorporation measured. Bars represent the mean SI indices + SD of 6 replicate wells which were calculated by the formula mentioned earlier. Asterisks indicate significant differences between the groups compared by the brackets, ** $P<0.05$, ** $P< 0.01$.

Since it was unknown which T cell subset was the responsible cell type for the positive reaction to PA-neoantigens and whether or not the responding T cell subset required the presence of the opposite subset, the LTT assays described in this section were also performed in a criss-cross fashion. For example, $CD4^+$ T cells derived from long term PA-treated mice were incubated with APC and antigens either 1) alone, 2) in the presence of $CD8^+$ T cells from the PA-treated group or 3) $CD8^+$ T cells from the age related controls. These different combinations were also performed with control $CD4^+$ T cells. Figure 3.2.5i, displays the stimulation indices resulting from the incubation of $CD4^+$ T cells from either PA-treated or untreated mice with PA-related neoantigens. These SI were unaffected by the presence or absence of $CD8^+$ T cells and the origin of these cells.

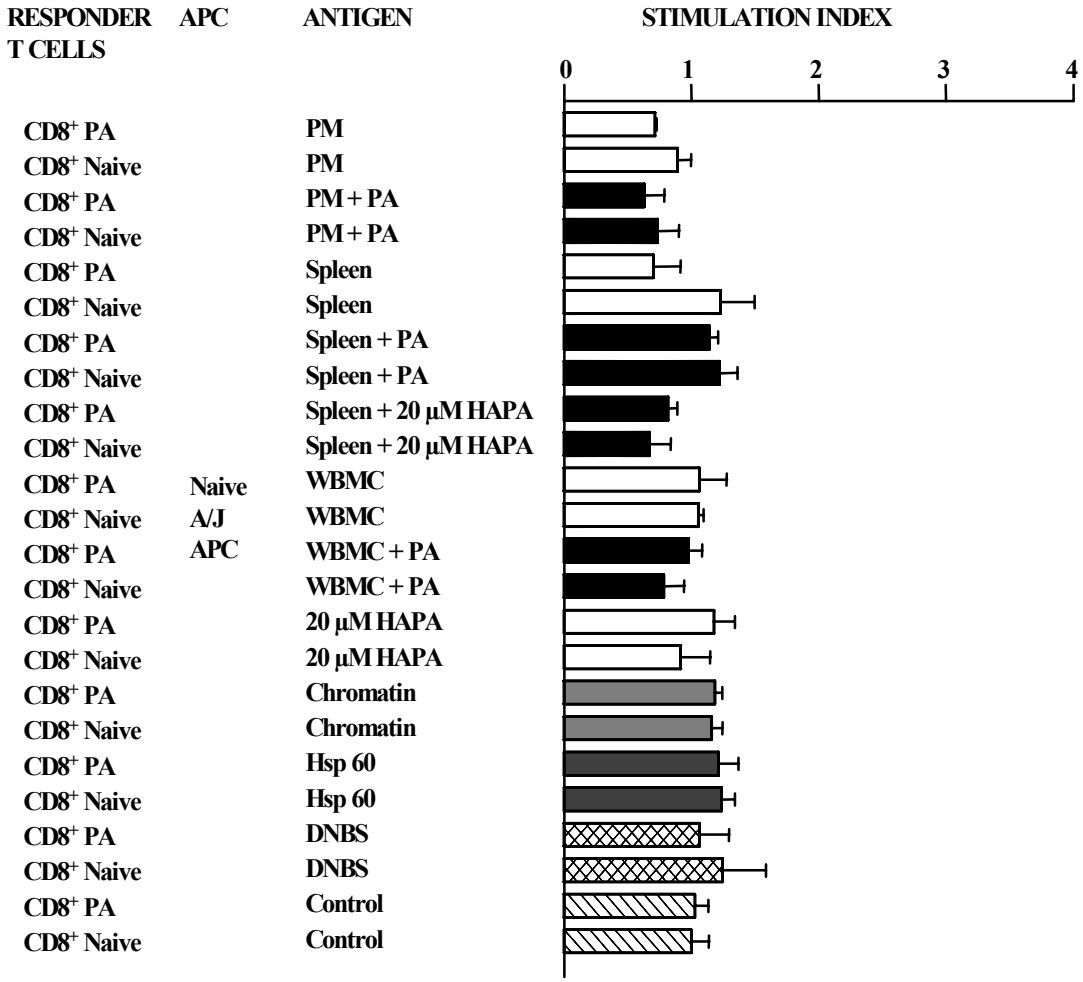


Figure 3.2.5.ii. CD8$^+$ T cells do not respond to PA-related neoantigens.
Enriched CD8$^+$ T cells from long term PA-treated or control mice were incubated together with irradiated APC and the indicated antigens in the LTT assay. [^{3}H] thymidine was added for the last 18 hours and the radioactive incorporation measured. Bars represent the mean SI + SD from 6 replicate wells which were calculated by the formula mentioned earlier.

3.2.6. CAN B CELLS ACT AS THE APC SOURCE IN THE LTT ASSAY?

The experiments performed in sections 3.2.4 and 3.2.5 indicated that the PA-rpimed CD4$^+$ T cells only reacted to the PA containing antigens when APC were present. Thus, to ascertain whether B cells could present the PA-neoantigens in a sufficient manner these cells were also purified. In the previous assays, the APC fraction had consisted of total spleen lymphocytes whereas here the spleen cells from naïve mice were enriched to a > 98 % pure B cell fraction. The separation protocol is described in detail in section 2.4.4.4 but in brief, the B cell fraction was separated in a cascade manner by incubations with anti-CD4$^+$ microbeads followed by anti-CD8$^+\alpha$ microbeads and finally anti-CD11c$^+$ and anti-CD11b$^+$ microbeads together. After each incubation the selected cell populations were separated using the AutoMACS so that the remaining fraction contained only unlabelled B cells. An aliquot of this fraction was then stained with FITC-conjugated anti-CD19 antibody and the purity determined using flow cytometry.

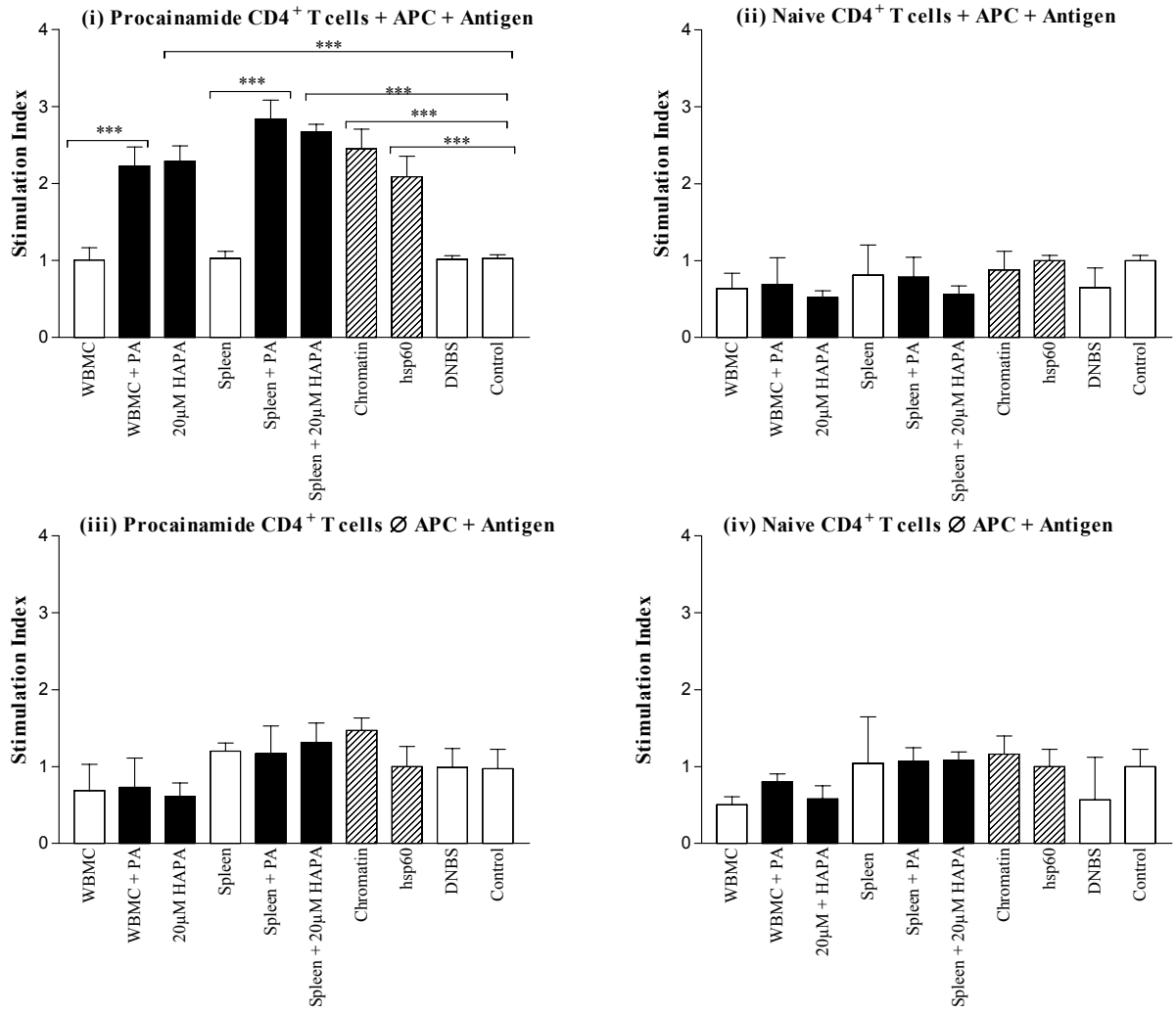


Figure 3.2.6.i-iv. B cells can act as APC for PA-related neoantigens
Enriched $CD4^+$ T cells from PA-treated (i and iii) or age related controls (ii and iv) were plated with the indicated antigens and in the presence or absence of purified B cells in the LTT assay. For the last 18 hours, [^{3}H] thymidine was added and the radioactive thymidine incorporation measured. Stimulation indices were calculated with the formula as described previously. Bars indicate the mean SI + SD of 6 replicate wells. Asterisks indicate significant differences between the groups compared by the brackets, ***P<0.001.

Purified and irradiated B cells were then plated with or without PA-treated or untreated $CD4^+$ T cells and in the presence or absence of irradiated antigens. Figures 3.2.6(i) and (ii) depict a comparison between the responses produced by $CD4^+$ T cells from PA-treated or untreated mice when incubated with the purified B cell fraction and antigens. As found in section 3.2.5, only the $CD4^+$ T cells from PA-treated mice responded to PA-pulsed antigens.

However, when the APC or in this case the purified B cells were absent, the ability of these $CD4^+$ T cells to respond was reduced to insignificant levels, figure 3.2.6(iii). This reduced activation also occurred with syngenic chromatin and free hsp60 antigens indicating that both of these antigens also required the presentation through the APC. Although not depicted here, further controls were also performed, these included the absence of either the antigens or T cells from the reaction wells. As before, no T cell responses occurred when antigens were absent and no proliferation was detected when only purified B cells and antigens were incubated together, indicating that the PA-treated $CD4^+$

T cell response was specific to PA-neoantigens and that it was necessary for these neoantigens to be presented by APC.

As mentioned earlier, (section 3.2.4), T cells from pristane-treated mice were also incorporated into the LTT assay in order to demonstrate that only PA-treated T cells responded to PA-pulsed neoantigens. Therefore as above, purified B cells were plated alongside $CD4^+$ T cells enriched from the pristane-treated mice. Similar results were obtained as described earlier in section 3.2.4, that is, these $CD4^+$ T cells from pristane-treated mice only responded to the antigen hsp60 (SI = 2.3) and not at all to the PA-related antigens or DNBS. When the B cell fraction was removed, the reaction by the T cells towards hsp60 also vanished indicating that B cells were also necessary for this response, data not displayed. As a control for the whole experiment, DNFB-treated mice were once again employed. As displayed in the earlier LTT assays (see figure 3.2.5iii), these treated PLN cells only responded to the specific antigen DNBS.

3.2.7. ASSESSMENT OF ANA PRODUCTION IN LONG TERM PA-TREATED A/J MICE

The production of antinuclear antibodies (ANA) is one of the defining features of SLE and indeed most drug-induced reactions. In both humans and mice that have been administered with PA, the development of ANA is a well established observation with a positive ANA reported in up to 90% of treated human patients. Previous research has shown that mouse strains treated with PA drinking water developed ANA over time [133]. This finding was further extended by demonstrating that slow acetylator mice such as the A/J strain, were more susceptible to ANA development than a fast acetylator phenotype mouse strain [130]. This result correlated to the findings found within human acetylator phenotype studies [118]. However, within these murine studies, the classification of these ANA was never elucidated.

In idiopathic SLE, the autoantibodies produced are varied whereas in drug-induced lupus they have been shown to be more distinct and often histone associated. Humans treated with PA tend to develop H2A-2B dimer antibodies whereas anti-histone antibodies from hydralzine patients recognise a boarder array of autoantigenic epitopes including individual histones [107]. Research has shown similar trends in mouse strains treated with these drugs [110, 132]. Furthermore, after two intra-thymic injections of HAPA into the (C57BL/6J xDBA/2)F1 mouse strain, the development of chromatin antibodies was detected in the periphery [138]. Therefore, the sera from both PA-treated and untreated A/J mice were tested for autoantibody production as an indication that an autoimmune reaction had occurred and also to try and classify the type of autoantibodies formed.

In order to screen for ANA production, all mouse sera collected over the eight months of treatment were examined on pre-fixed HEp2 cell slides and their endpoint titres determined, see section 2.4. For each slide a positive and negative control were used to grade the intensity of detected fluorescence generated by the tested sera. The secondary antibody used was a FITC-conjugated rat-anti-mouse IgG1, thus all results in figure 3.2.7 i-vi are the highest endpoint titres observed for each individual mouse at the indicated time points. Preserum titres (figure 3.2.7i) were low in both groups with titre ranges between 1:50 – 1:100 for both the PA-treated (▲) and age related controls (△). After

two months of treatment, the autoantibody titres had already begun to increase in the PA-treated mice with the majority of mice (14/60) producing an endpoint titre of 1:250. In comparison the control group remained low (figure 3.2.7ii).

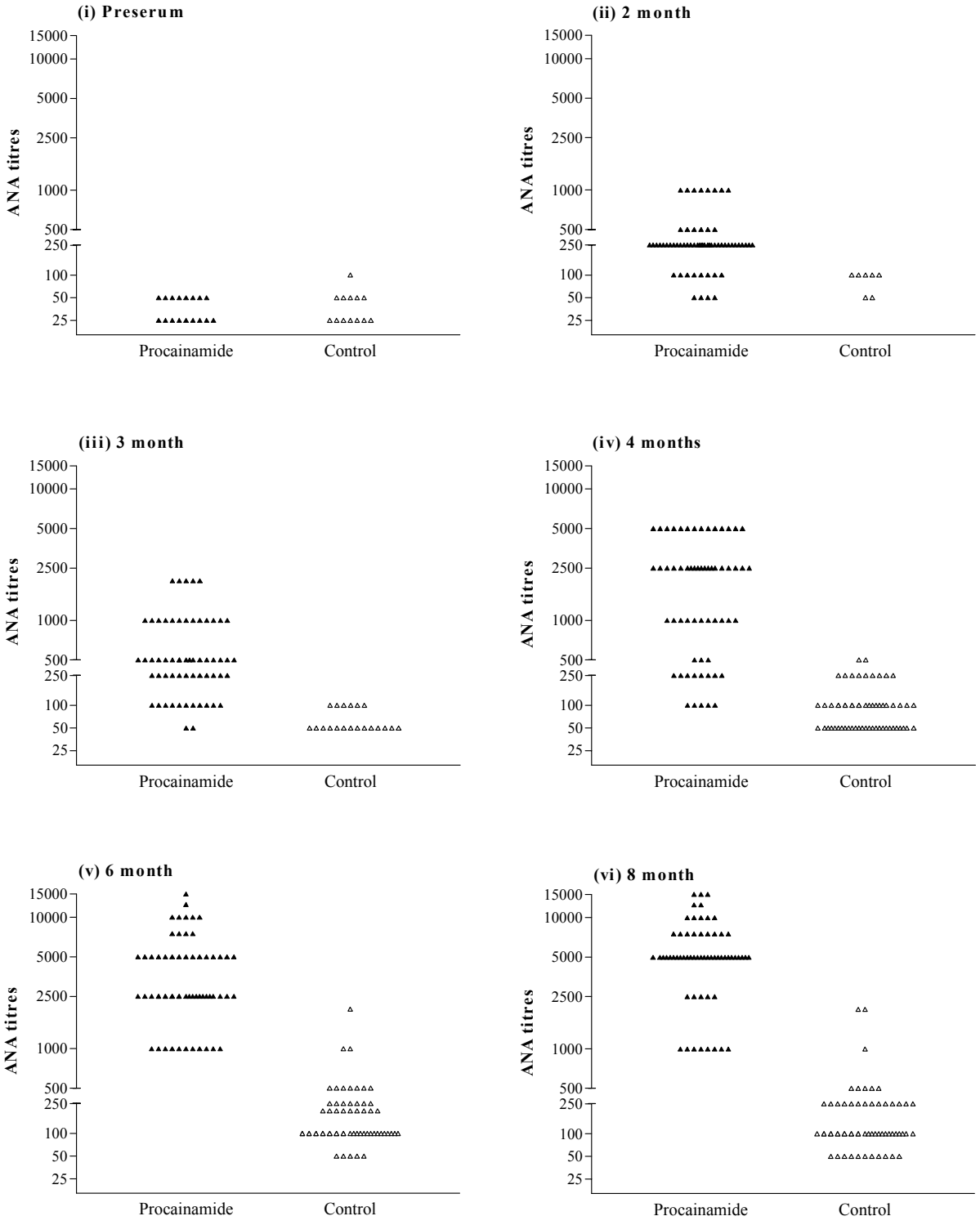


Figure 3.2.7. Autoantibody titres in procainamide treated A/J mice.
Sera collected over the 8 months of PA-treatment were examined for autoantibody development using pre-fixed HEp-2 cell slide and a FITC-conjugated rat-anti-mouse IgG1 secondary antibody. Graphs depict the endpoint titres determined for each individual PA-treated (▲) or control mouse (△). Slides were prepared and read in a blind fashion.

The ANA development in the PA-treated mice continued to rise through the third and fourth months of treatment with the median endpoint titre reaching 1:500 and 1:2500 figures 3.2.7iii and iv respectively. As one can also observe from these figures, the ANA values from the untreated mice remained mainly insignificant. After six months the titres in the PA-treated mice rose drastically with endpoint titres reaching 1:15,000, an approximately 17 fold increase over the age related controls. At eight months, some PA-treated mice reached titres of 1:15,000 with the group median titre equally 1:5,000. Even without treatment the titres of the untreated group rose, this result is not entirely unexpected since an increase in age is known to produce a certain amount of autoantibody development [133]. Although the data is not shown here, ANA titres were also analysed for the pristane-treated A/J mice. From the second to fourth month of treatment, ANA titres steadily rose with several mice reaching endpoint titres of 1:17, 500 at eight months. Therefore, these treated mice were an appropriate control for the PA experiments since under the same SPF conditions, but using a different xenobiotic, these A/J mice also developed autoantibodies. This is the first time that A/J mice have been reported to develop significant ANA titres with pristane treatment.

3.2.8. SERUM IMMUNOGLOBULIN CONCENTRATIONS ASSESSED BY ELISA

To obtain an overview regarding the progressive immunoglobulin (Ig) concentrations during the PA treatment and in consequence the tendency towards either a Th1 or Th2 response, analysis of the IgM, IgG1 and IgG2a levels were performed as described in section 2.5.1 Briefly, microtitre plates were coated with rat-anti-mouse IgG1, IgG2a or IgM mAb for 24 hours. After blocking, sera from treated or untreated mice were plated in triplicate for three hours at room temperature in a dilution of 1:500 or 1:1000 in PBS. Levels of bound IgG1, IgG2a and IgM were then detected using biotinylated rat-anti-mouse IgG1, IgG2a or IgM antibodies respectively and TMB substrate. ELISA plates were read at dual wavelengths of 490 and 630nm using the Dynex 96 well plate ELISA reader. The average PBS control values were then subtracted from the average value obtained from each individual serum.

Figures 3.2.8i-iii display the results obtained from each serum at each indicated month with the average mean value of each treated group depicted by the bars. As expected, the IgM concentrations in the preserum of each group were negative, figure 3.2.8i. However, after two months the IgM levels in the PA-treated sera (av:0.530) were already elevated in comparison to the untreated (av:0.275) and pristane-treated sera (av:0.3172) and these levels remained elevated throughout treatment until the sixth month. At eight months, the IgM levels in the PA-treated mice had receded so that there were comparable to the untreated group (av:0.228 and 0:294 respectively). In contrast, the IgM concentrations generated from pristane-treated mice only began to accelerate after the fourth month (av:0.693) and these levels remained constant until the end of the investigation period (av:0.794).

On analysis of the IgG2a data, no significant increases were detected in either the untreated or the PA-treated group. The latter did display a slight fluctuation after four months but the values were insignificant in comparison with the untreated group, figure 3.2.8ii. The pristane-treated mice also demonstrated no increase in this IgG2a concentration. The IgG1 results were in stark contrast to the IgG2a values, (figure 3.2.8iii). After four months of treatment, the PA-treated mice had significantly increased IgG1 concentrations (***P<0.001) in comparison to the untreated group. These values

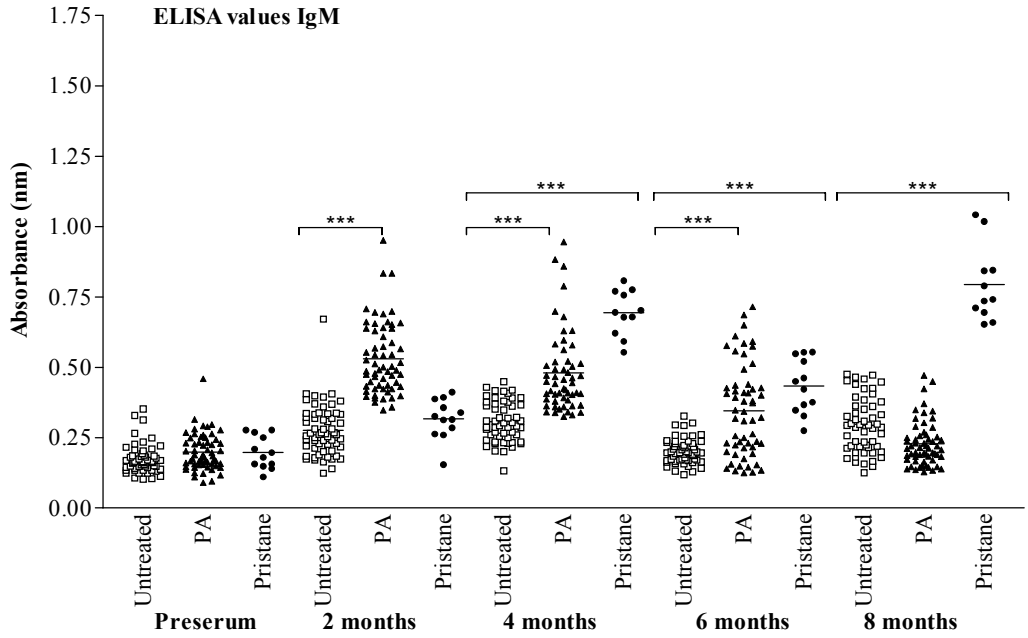


Figure 3.2.8.i. Levels of IgM concentrations during 8 months of either PA or pristane treatment.
With specific antibodies to mouse IgM, ELISA assays were performed and the increases in serum IgG2a levels observed in untreated (□), PA- (▲) or pristane-treated (●) sera. Symbols represent the average value obtained from each individual mouse from of triplicate wells. Group mean values are indicated by the lines. Sera were diluted 1:500.

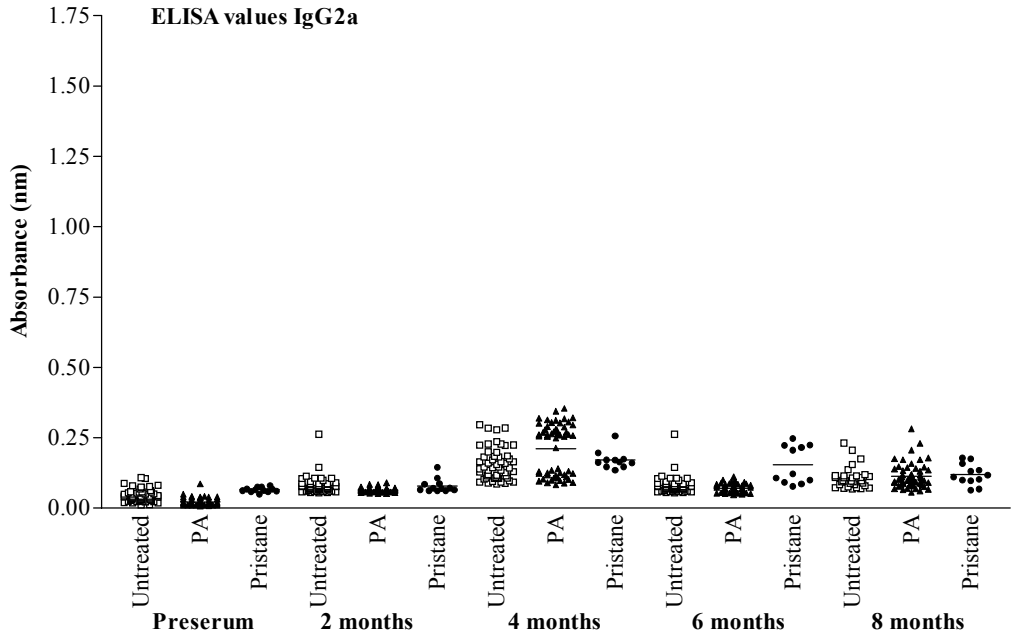


Figure 3.2.8.ii. Levels of IgG2a concentrations during 8 months of either PA or pristane treatment.
With specific antibodies to mouse IgG2a, ELISA assays were performed and the increases in serum IgG2a levels observed in untreated (□), PA- (▲) or pristane-treated (●) sera. Symbols represent the average value obtained from each individual mouse from of triplicate wells. Group mean values are indicated by the lines. Sera were diluted 1:500.

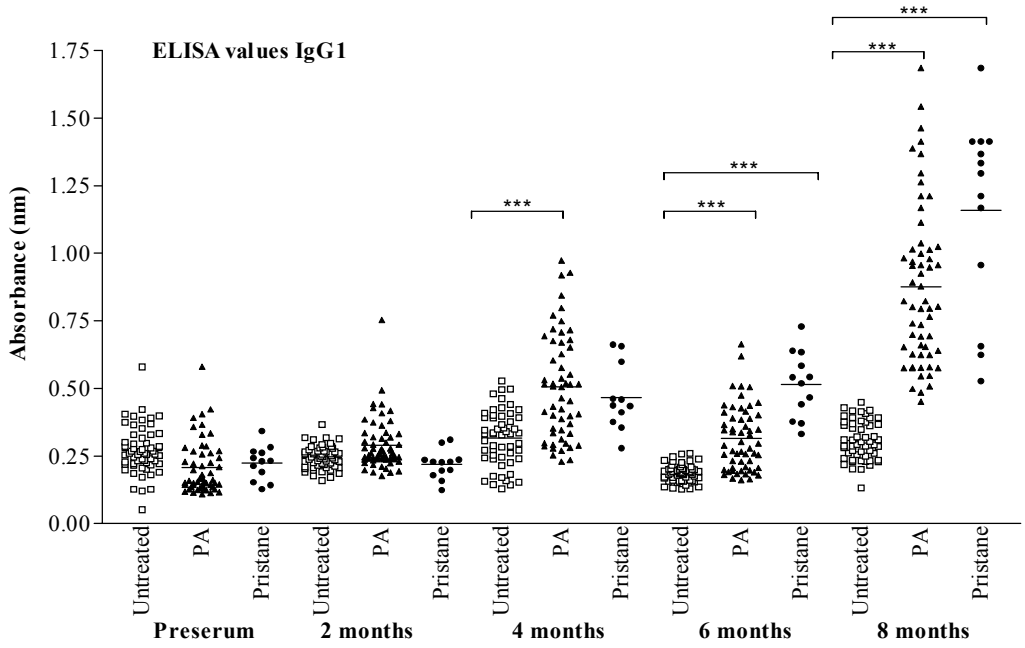


Figure 3.2.8.iii. IgG1 fluctuations in A/J mice after 8 months of PA or pristane treatment.
With specific antibodies to mouse IgG1, ELISA assays were performed and the increases in IgG1 levels observed in the untreated (□), PA-(▲) or pristane-treated (●) sera. Symbols represent the average value obtained from each individual mouse from triplicate wells. Group mean values are indicated by the lines. Asterisks indicate significant differences in the values ***P<0.001 between the groups compared by the brackets. Sera were diluted 1:500 (preserum – 4 months) or 1:1000 (6-8 months).

remained significantly elevated in comparison to the untreated group throughout the investigation period producing exceedingly high absorbance levels after 8 months in which the mean average was 0.876. The pristane-treated IgG1 levels were exceedingly elevated after 8 months of treatment with a group mean average of 1.160. Both the PA and pristane group values were determined in dilutions of 1:1000 and the differences were statistically significant in comparison to the untreated group, ***P<0.001. These findings indicate that both treatments were producing a Type 1 immune response and were therefore T cell mediated.

3.2.9. CLASSIFICATION OF ANA

The HEp-2 cell line is often used for the assessment of antinuclear autoantibodies (ANA). Historically rodent tissues were important but their use has been superseded by prefixed HEp-2 cell slides because of the latter's diagnostic and technical superiority. Quality control surveys have demonstrated major errors in the reported ANA using rodent tissues. HEp-2 cells are a valuable tool for distinguishing the patterns found in any treated serum since over thirty different patterns can be identified. In order to classify the ANA found in the PA- and pristane-treated sera, self-prepared HEp-2 cell slides were produced since these have been found to reduce the background and non-specific staining often observed with commercial pre-fixed HEp-2 cell slides [150]. In brief, HEp-2 cells were cultured *in vitro* onto coverslips for 24 hours and then fixed. These slides were then used in the normal staining

procedure with all PA-treated and control sera as described in section 2.6.2. The patterns produced by the treated sera were then observed and classified with a fluorescent microscope in a dilution of 1:200.

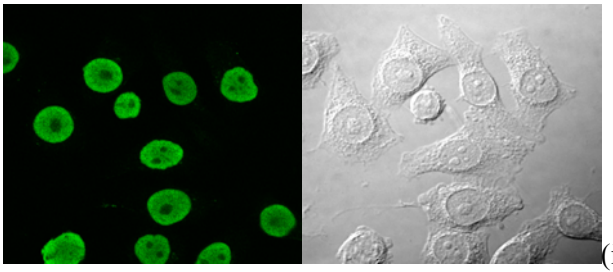
NUMBER OF MICE	TREATED GROUPS	STAINING PARAMETERS											
		NUCLEUS				NUCLEOLAR				CYTOPLASMIC			
		+/-	+	++	+++	+/-	+	++	+++	+/-	+	++	+++
60	Naïve	15	6	-	-	12	3	-	-	16	8	-	-
60	PA	-	14	31	15	-	18	23	-	-	12	4	2
15	Pristane	-	-	6	9	-	5	11	-	-	13	2	-

Table 3.2.9. Classification of autoantibodies after 8 months of treatment.
Individual sera from treated or untreated mice were diluted 1:100 and incubated onto self-prepared HEp-2 cell slides. After counterstaining with an FITC-conjugated rat-anti-mouse IgG1 antibody, individual patterns were identified using a fluorescent microscope. Intensity gradings were given to the above three parameters in a scale of minor staining (+/-) to extremely positive (+++).

On the assessment of two individuals, Table 3.2.9 summarises the findings obtained after classifying the ANA patterns from the sera of PA- and pristane-treated mice. Three criteria were taken into account and a grading for cytoplasmic, nucleus and nucleoli were given with +/-, +, ++ and +++ indicating the strength of the staining. This classification has not been performed before in long term PA-treated A/J mice but as stated earlier it is the dogma that when treated with PA, humans and mice develop a histone type pattern which appears as a homogenous staining.

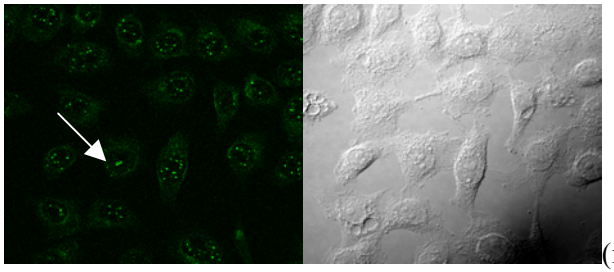
The pictures in figure 3.2.9 display the typical ANA patterns that developed in the treated A/J mice. Figure 3.2.9viii shows a typical untreated serum staining. In summary, of the 60 mice treated with PA, eight mice showed the typical staining expected with histone ANA development, figure 3.2.9i. This number of positive sera correlates to the amount of sera which were specific for histone proteins as detected using an ELISA assay in section 3.2.11. Only 16% of the sera showed histone specificity. The most frequent staining patterns in PA-treated sera were those depicted in figure 3.2.9v and vii which, when compared to an ANA pattern atlas, correlated to the pattern specific for centromere bodies. All ANA analysis using HEp-2 cells were performed with a secondary antibody to IgG1. Several treated sera were re-tested using IgG2a specific labelled antibodies. In correlation with the ELISA assays, the IgG2a antibody produced insignificant staining. It is the general consensus that testing ANA development using an IgM antibody is futile since the levels of these types of antibodies circulating in the healthy individual can be already elevated. Pristane-treated sera produced patterns depicted in figure 3.2.9 (i), (ii) and (vii) also indicating a various specificity.

The photographs in figure 3.2.9 are representatives of common patterns observed from both the PA- and pristane-treated sera. However, it must be acknowledged that the treated sera displayed numerous combinations of different patterns on the HEp-2 cell slides. Therefore, no single pattern could be defined for the entire treatment group. In fact, only a few sera showed a complete uniform pattern with the majority of sera displaying more than one pattern indicating that each sera contained several different autoantibody specificities. For example, many of the tested sera produced staining within the HEp-2 cells which corresponded to a nucleolar pattern (depicted by the white arrow) but



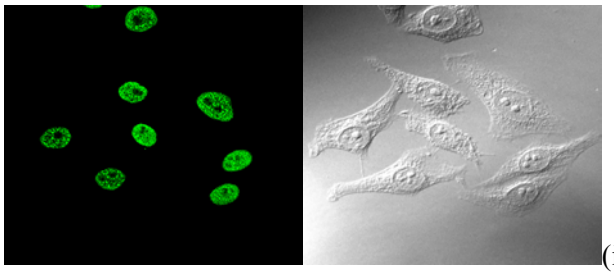
(i)

HOMOGENOUS AND NUCLEAR MEMBRANE



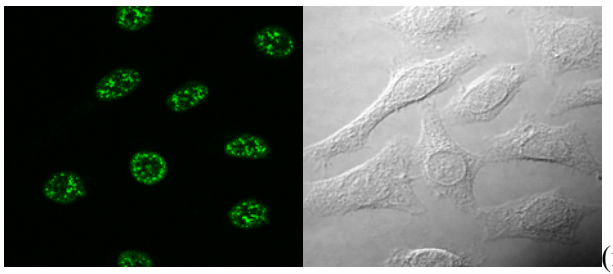
(ii)

NUCLEAR DOTS AND NUCLEAR HOMOGENOUS

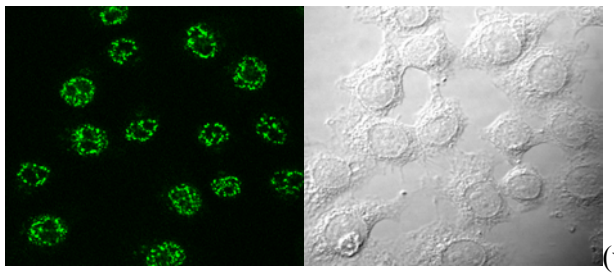


(iii)

HOMOGENOUS NUCLEAR MATRIX

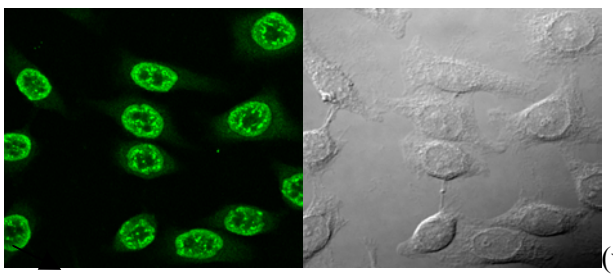


(iv)



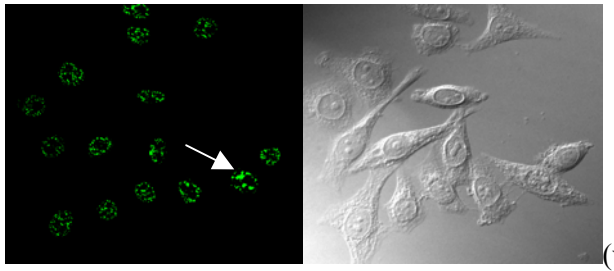
(v)

CENTROMERE BODIES



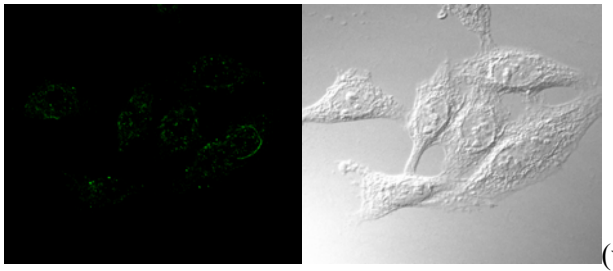
(vi)

NUCLEAR MATRIX



(vii)

CENTROMERE BODIES



(viii)

Figure 3.2.9.i-viii. Common ANA patterns found in PA and pristane treated serum.
Self-made HEp-2 slides were incubated with individual treated sera in a 1:100 dilution and then counterstained with an FITC rat-anti-mouse IgG secondary antibody. Pictures were processed with Adobe Photoshop.

these cells were distributed through a field of cells stained with an homogenous pattern such as those seen in figure 3.2.9i. Consequently, this varied patterning made the task of assigning one actual pattern to these treated mice extremely difficult. In correlation, as described later in section 3.2.11, these sera also demonstrated varied reactivity to several common self-proteins in specific ELISA tests, once again indicating that, as found with pristane-treated murine strains [147, 148], PA treatment can also induce several different autoantibodies.

3.2.10. PROCAINAMIDE SPECIFIC ANTIBODY PRODUCTION IN PA-TREATED MICE

A perplexing observation in the ANA analysis of the PA-treated sera was the low frequency of histone patterns. Therefore, the question arose as to whether or not these sera could be specific for other proteins or even PA or HAPA. Thus, the ELISA method was adapted for these purposes and is explained in detail in section 2.7.2. In summary, microtitre plates were coated overnight with either 10 mg/ml PA or 10 µg/ml HAPA. After blocking, PA-treated, pristane-treated or age related control sera were plated in triplicate at a dilution of 1:200 in PBS for 3 hours. HRP conjugated anti-mouse IgG was then added and the amount absorbance, after stringent washing, determined using TMB substrate and an ELISA reader (Dynex Technologies, Germany).

Figure 3.2.10 displays the results obtained from the 60 PA-treated sera. Of these, 27 PA-treated sera (43%) demonstrated a specific response to PA (Figure 3.2.10A) and 15 PA-treated sera reacted to HAPA (Figure 3.2.10B). 10 sera cross-reacted to both compounds. Individual sera were classed as positive when their mean absorbance value was greater than a factor of 2 over the group mean absorbance value of the age-related controls. Control values were comparable to the PBS negative controls incorporated onto each plate. For the positive control, a specific PA antibody was used, for more information see section 2.2.2. Several of these PA and HAPA positive sera responded with indices over 5 and three responded with a factor of 10 over the control, a stark contrast to the naïve or pristane sera. As expected, these latter control groups showed no specificity to either antigen.

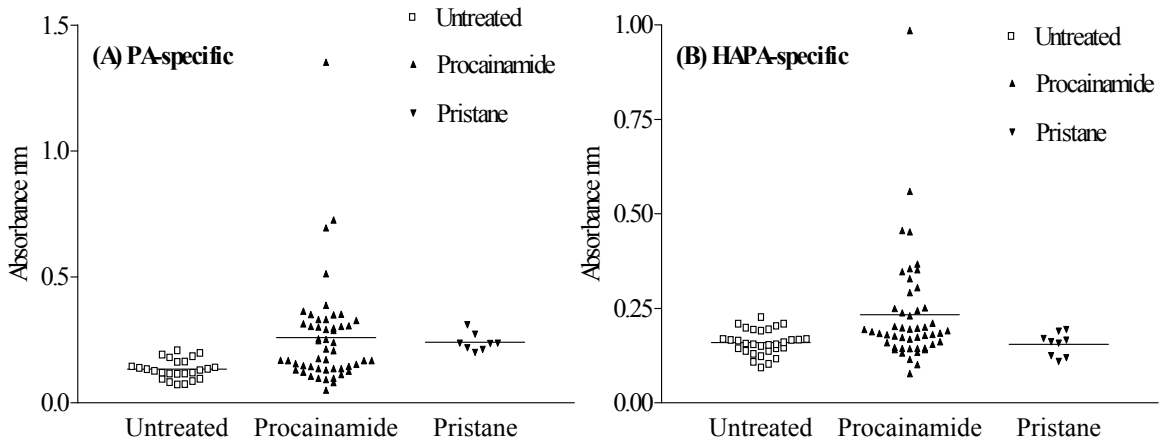


Figure 3.2.10: Long term procainamide-treated sera contain antibodies specific for PA or HAPA.
The presence of PA or HAPA antibodies in the A/J PA-treated sera was determined through specific PA (A) or HAPA (B) ELISA assays. PA and HAPA antibodies were detected in the sera of individual PA-treated A/J mice (▲) but not in the pristane-treated (▼) or age related controls (□). Symbols represent the average mean from triplicate results for each individual mouse. Group means are indicated by the bars.

Using the characterisation charts from the HEp-2 slide analysis (see section 3.2.9), it could be determined that each of these PA or HAPA positive sera displayed similar but not identical patterns. The strongest PA or HAPA responding sera displayed the pattern types depicted in figures 3.2.9v and vii, that is nuclear homogenates or centromere bodies. This observation has not been previously reported in long term investigations with PA-treated A/J mice and demonstrates first, that indeed PA was incorporated through the drinking water and that second, sufficient PA was available *in vivo* to bind to carrier proteins and elicit a reaction. Furthermore, it proves that HAPA is not the only PA metabolite to produce an immune response and that these two compounds can perhaps bind to different proteins.

3.2.11. CLASSIFICATION OF ANA TYPES IN PA- AND PRISTANE-TREATED SERA

Even though 50% of the PA-treated sera were specific for PA and HAPA and a number of these sera showed a staining pattern that could indicate histone specificity, a significant portion of the observed autoantibodies remained unidentified. From previous studies it has been demonstrated that pristane treatment in mouse strains can induce the development of a number of different autoantibodies and these differ in each treated strain [147, 148]. Therefore, to determine whether this observations also applied to these PA- and pristane-treated A/J mice, individual sera were analysed for their specific responses to several proteins including smRNP, snRNP protein A, Ku, histone, chromatin, dsDNA and ssDNA in ELISA assays as described in section 2.7.

Table 3.2.11 summarises the percentages of the different treated sera which were specific for a certain protein. When tested with a total histone protein, only 16% of the PA-treated sera produced a response and this value approximately correlates to the observations found using HEp-2 cell slide analysis see section 3.2.9. Only one PA-treated sera responded to the Ku antigen whereas 91 % of the

	TREATMENT GROUPS					
	PROCAINAMIDE		PRISTANE		UNTREATED	
	%age	*Index*	*%age*	*Index*	*%age*	*Index*
ssDNA	53 %	Av: 3.20	55 %	Av: 1.80	15.0 %	
dsDNA	36 %	Av: 1.80	44 %	Av: 1.85	3.0 %	---
Ku	1.6 %	Av:1.90	91 %	Av: 2.41	0.0 %	---
snRNP Protein A	40 %	Av: 2.15	100 %	Av: 3.04	0.0 %	---
smRNP	17 %	Av: 1.52	100 %	Av: 3.20	0.0 %	---
Histone	16 %	Av: 1.70	42 %	Av: 3.25	0.0 %	---
Chromatin	22 %	Av: 1.50	12 %	Av: 1.48	0.0 %	---
HAPA	25 %	Av: 2.12	0.0 %	---	0.0%	---
Procainamide	43 %	Av: 2.80	0.0 %	---	0.0%	---

Table 3.2.11. Summary of autoantibody specificity produced in PA- and pristane-treated A/J mice.
With specific ELISA assays, sera from PA-treated, pristane-treated and age-related control A/J mice were tested for their reactivity towards the antigens listed in the table above. The percentage of responsive sera are shown in the percentage column. From triplicate absorbance values, individual means were calculated for each responding sera using the PBS negative control as a reference. The group average index value for these positive sera is depicted in the "index" column.

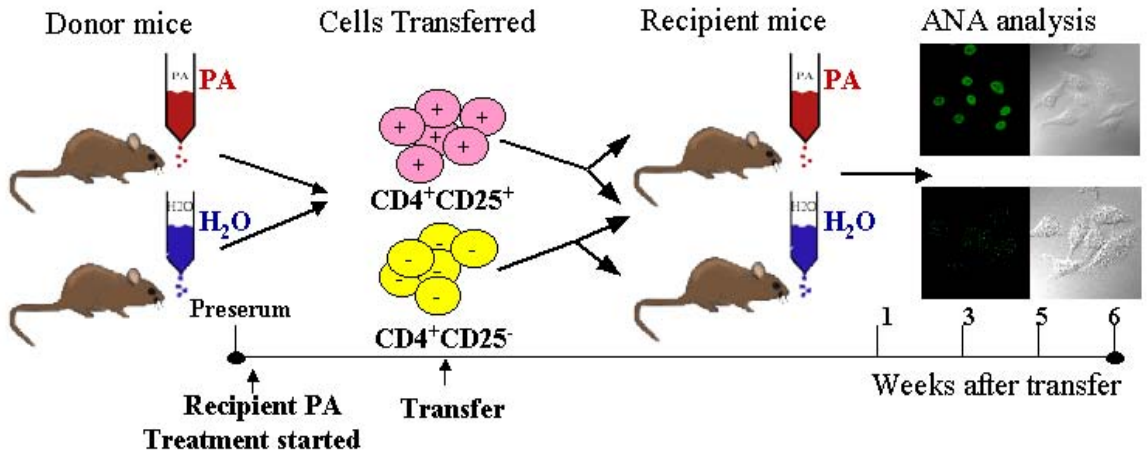
pristane-treated mice reacted positively. 100 % of the pristane-treated sera produced very strong responses to snRNP protein A and smRNP. This was in contrast to the PA-treated sera which responded less dramatically to these antigens with only 17 % or 40 % of the sera showing specificity for the snRNP protein A and smRNP protein respectively. 22% of the PA-treated sera were responsive to syngenic chromatin. Only 12 % of the pristane-treated sera displayed specificity to chromatin. PA-treated sera responded strongly to ssDNA (53 %), but not to dsDNA (36 %). This was a surprising result since more dsDNA antibodies were expected [150]. Similar percentage values were obtained with the pristane-treated sera. Several naïve sera responded to DNA proteins but these findings were expected since these aged mice have shown some ANA formation as described in section 3.2.7, and ANA formation in older mice is a well established observation [133].

In conclusion, the findings that several different self-proteins elicited a positive reaction in the ELISA assays from the PA- and pristane-treated sera confirmed the earlier observations found from the HEp2 cell slide analysis as observed in section 3.2.9 and the PA/HAPA specific ELISA assays (section 3.2.10). That is, each treated mouse sera contains several ANA specificities. Perhaps the different ANA arise from epitope spreading due to the alterations made by the presence of PA and HAPA *in vivo*. Research into Lupus related illnesses and indeed other xenobiotic-induced diseases, continually report the findings of more unusual and defined antibodies, such as L-selectin [149], thus verifying that the classification of these PA-induced ANA cannot be confined to one definite protein.

3.2.12. ADOPTIVE TRANSFER OF $CD4^+CD25^-$ T CELLS FROM PA-TREATED MICE BREAKS SELF-TOLERANCE AND INDUCES AUTOANTIBODY PRODUCTION IN NAÏVE RECIPIENTS

The revival of suppressor T cell research, especially $CD4^+CD25^+$ T cells, is largely impart to the continual advances in technology that have allowed more efficient separation and accurate labelling of small cell subpopulations. At the present time however, the search for an absolute regulatory marker

still continues with much emphasis focusing upon GITR, $\alpha_E\beta_7$ and Fox*p*3 [47, 83, 151]. Meanwhile, the general consensus dictates that the appearance of the IL-2 receptor (CD25) on $CD4^+$ T cells indicates a regulatory subset and therefore using this knowledge techniques have been developed so that these T cells can be enriched. The $CD4^+CD25^+$ T cells have been recognised since the mid-90's for their involvement in anergy and autoimmune suppression, see section 1.7. Increasing evidence shows that those cells are involved in regulating a variety of autoimmune diseases including diabetes and inflammatory bowel disease [reviewed in 47, 52]. The earliest studies demonstrated the ability of these cells to suppress autoimmune development in three day old thymectomised mice [48]. Investigations into the activity of suppressor T cells in xenobiotic-induced autoimmune diseases remains rather sparse. As with many aspects regarding this research field, experiments have been probably hindered due to the fact that the antigens involved remain largely unknown. Therefore, the question arose as to whether an adoptive transfer of the $CD4^+CD25^+$ T cell subset from the long term PA-treated mice but not control A/J mice could prevent the formation of ANA in PA-treated syngenic recipients.



Scheme 3.1. Adoptive transfer model for determining the role of $CD4^+CD25^+$ T cells from PA-treated mice $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets from long term PA-treated or untreated A/J donors were enriched and adoptively transferred into syngenic recipients that were or were not receiving PA drinking water (6 g/L). PA treatment began one week before transfer just after preserum samples were obtained. After the cell transfer, the sera from all recipients were periodically taken over six weeks and tested for ANA changes through HEp-2 cell slide analysis.

The experimental procedure for the assay is depicted in scheme 3.1 and is described in full in section 2.4.5. To summarize, $CD4^+$ T cells from the spleens and inguinal lymph nodes of PA-treated A/J mice or age related controls were enriched using the AutoMACS so that the purity of the fraction was more than 98%. Thereafter, the $CD4^+CD25^+$ T cell subpopulation was further extracted with the AutoMACS using an anti-CD25 PE antibody and anti-PE microbeads. The purity of both populations was then verified by flow cytometry and subsequently intravenously injected into the tail veins of recipient A/J mice. These recipients had or had not been placed under PA drinking water treatment (6g/L) one week prior to transfer, after the initial preserum was obtained. Treatment continued throughout the whole period of investigation. During the course of six weeks after the transfer, sera

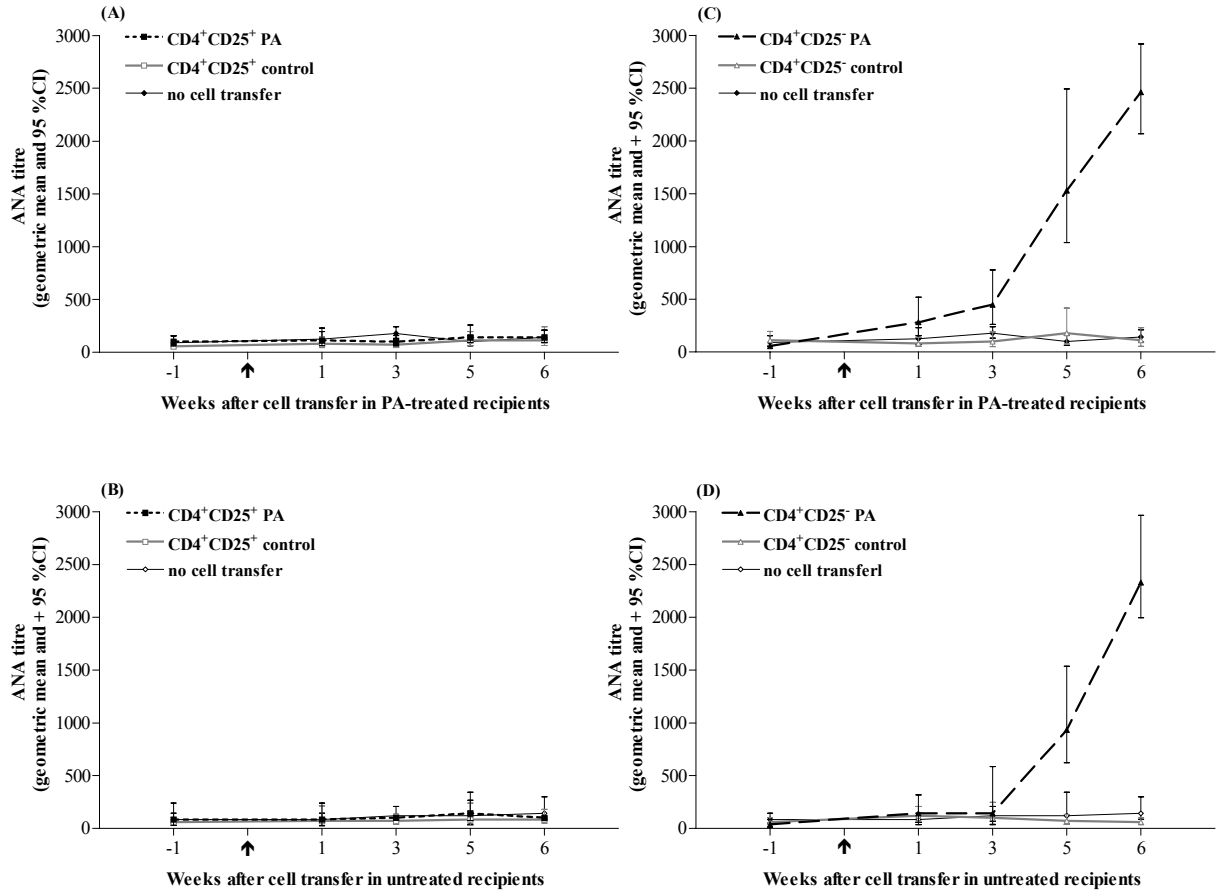


Figure 3.2.12.A-D. $CD4^+CD25^+$ T cells from PA-treated donors but not control donors can suppress ANA formation in PA-treated recipients.
$CD4^+CD25^+$ or $CD4^+CD25^-$ T cell subpopulations from either PA-treated or untreated (control) were adoptively transferred into naïve A/J recipients at the time point indicated by ↑. Recipient groups were divided into two, those receiving PA in their drinking water (upper panel) and those not (lower panel). Recipient treatment began directly after the preserum samples were obtained (-1) and continued throughout the investigation period. At the indicated timepoints after cell transfer, sera was taken from each recipient and tested for ANA formation. Symbols show the group geometric mean ANA titre with 95% CI. Dotted lines represent the recipient groups which received $CD4^+CD25^+$ T cells from PA-treated donors (■) whereas dashed lines depict the ANA titres in recipient groups after the transfer of $CD4^+CD25^-$ T cells from the same donors (□). The grey lines within each graph depict the ANA titres obtained after the transfer of the corresponding control T cell subset. Black lines indicate the recipients which received no cell transfer but were treated with PA drinking (◆) or not (◇).

from the recipient mice were taken and analysed with diagnostic HEp-2 cell slides for autoantibody formation using a FITC-conjugated anti-IgG1 antibody. Slides were prepared and read in a blind fashion.

Figure 3.2.12 A-D represents the combined results from two independent adoptive transfer experiments performed in parallel with one another. Recipients groups which received PA drinking water are depicted in the upper panel whereas the untreated recipients are displayed in the bottom panel. Each graph displays the obtained recipient group mean ANA titre and 95% confidence intervals (CI) at different time points indicated after the cell transfer. All ANA titre values were determined using the prefixed HEp-2 cell slides and a FITC-conjugated IgG1 antibody. After the transfer of $CD4^+CD25^+$ T cells from either PA-treated (dotted lines) or control donors, no increases in

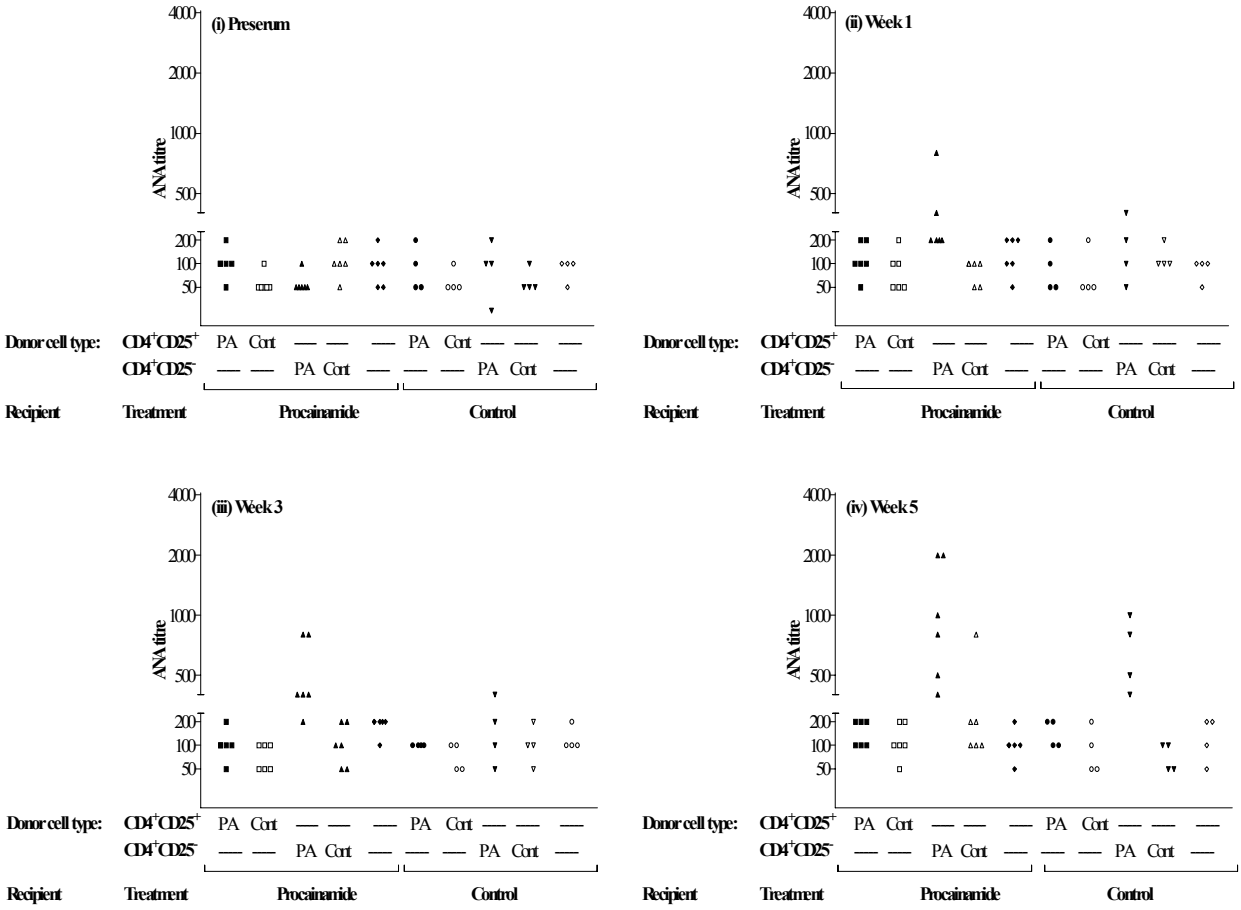


Figure 3.2.12.i-v. Graphs ii-iv represent the individual endpoint titres found in A/J recipients over the six weeks after an adoptive transfer of either $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells from long term PA-treated (PA) or control (Cont). On each graph, the first five recipient groups were treated with PA drinking water (6g/L) whereas the latter five were not. This treatment began directly after the preserum samples were obtained. Symbols represent the recipient groups injected with the following T cell subsets or PBS: 1) (■) PA-treated $CD4^+CD25^+$, 2) (□) Cont. $CD4^+CD25^+$, 3) (▲) PA-treated $CD4^+CD25^-$, 4) (△) Cont. $CD4^+CD25^-$, 5) (◆) PBS, 6) (●) PA.-treated $CD4^+CD25^+$, 7) (○) Cont. $CD4^+CD25^+$, 8) (▼) PA-treated $CD4^+CD25^-$, 9) (▽) Cont. $CD4^+CD25^-$ and 10) (◇) PBS. ANA Analysis was performed in a blind manner.

ANA formation could be detected and this was irrespective to the recipient PA treatment (Figure 3.2.12 A and B). In fact these recipient ANA titres did not exceed the preserum values.

In contrast, the adoptive transfer of $CD4^+CD25^-$ T cells from PA-treated donors (dashed lines) induced ANA formation (Figure 3.2.12 C and D) and this result was also irrespective of whether the recipients received PA treatment or not. This ANA formation in naïve recipients was not detected

after the transfer of $CD4^+CD25^-$ T cells from the control donors, corresponding open symbols (figure 3.2.12 C and D). These recipient groups which received the $CD4^+CD25^-$ T cells from PA-treated donors developed ANA titres three weeks after transfer. ANA formation was also not detected in recipient groups which did not receive a cell transfer but were treated with PA drinking water (upper panel) or not (lower panel). The lack of ANA formation in PA-treated recipients that did not receive a cell transfer is consistent with the earlier findings which demonstrated that ANA titres only began to increase after two months of treatment with PA drinking water, see section 3.2.7.

Figure 3.2.12i-v shows the actual individual endpoint titres determined for each recipient over the weeks of investigation. In the preserum values, the ANA titres in all recipient groups were low (Figure 3.2.12i) and apart from one mouse in the PA-treated recipient group which received $CD4^+CD25^-$ T cells from PA-treated donors, this was also the case one week after transfer (Figure 3.2.12ii). Thereafter, increases in ANA titres only occurred in the recipient groups which received $CD4^+CD25^-$ T cells from PA-treated donors. All of the ANA titres in these recipient mice were significantly positive after 6 weeks (***$P<0.001$) and this ANA formation was independent of PA treatment (figure 3.2.12v).

3.2.13. IG FLUCTUATIONS IN THE SERA FROM THE ADOPTIVE TRANSFER A/J RECIPIENTS

In order to assess whether there were increased levels of Ig in the sera of the above mentioned adoptive transfer recipients (section 3.2.12), specific ELISA assays were performed and are explained in detail in section 2.7.1. In the similar assays performed to determine the Ig concentrations within the long term PA-treated donors (see section 3.2.8), the negative PBS control value was subtracted from the average value of each individual serum obtained from triplicate results. Figures 3.2.13 A-D and E-H respectively depict the IgM and IgG1 variations between each recipient group over the investigation period. Recipient groups which received additional PA drinking water are shown in A, B, E and F. Only the recipient group which received $CD4^+CD25^-$ T cells from long term PA-treated mice (PA), produced an increase in the IgM levels after two weeks (dashed lines in figure 3.2.13B). These IgM levels steadily increased over the course of the investigation period until at week 6 a slight decline was observed. This increase was not as apparent in the corresponding recipient group which was not treated with PA drinking water (figure 3.2.13D). No IgM increases could be detected in the recipients which received either the PA-treated or control $CD4^+CD25^+$ donor T cells (figure 3.2.13A and C).

As observed with the original long term PA-treated donors (see figure 3.2.8ii), none of the adoptive transfer recipient groups showed increases in the concentration levels of IgG2a and therefore this data is not shown. In contrast, an increase in the levels of IgG1 was apparent and are depicted in Figure 3.2.13 E-H. Similar to the IgM concentrations, these IgG1 variations were only detected within the recipient groups which received $CD4^+CD25^-$ T cells from long term PA-treated donors (dashed lines in figure 3.2.13 F and H) and this finding was once again independent of additional PA treatment. However, unlike the IgM levels the increase in IgG1 only significantly appeared in the fifth week following the adoptive transfer. This result of increased IgG1 levels after 5 weeks correlates to the autoantibody development stage observed within the same recipient group with the HEp-2 slides, see section 3.2.12.

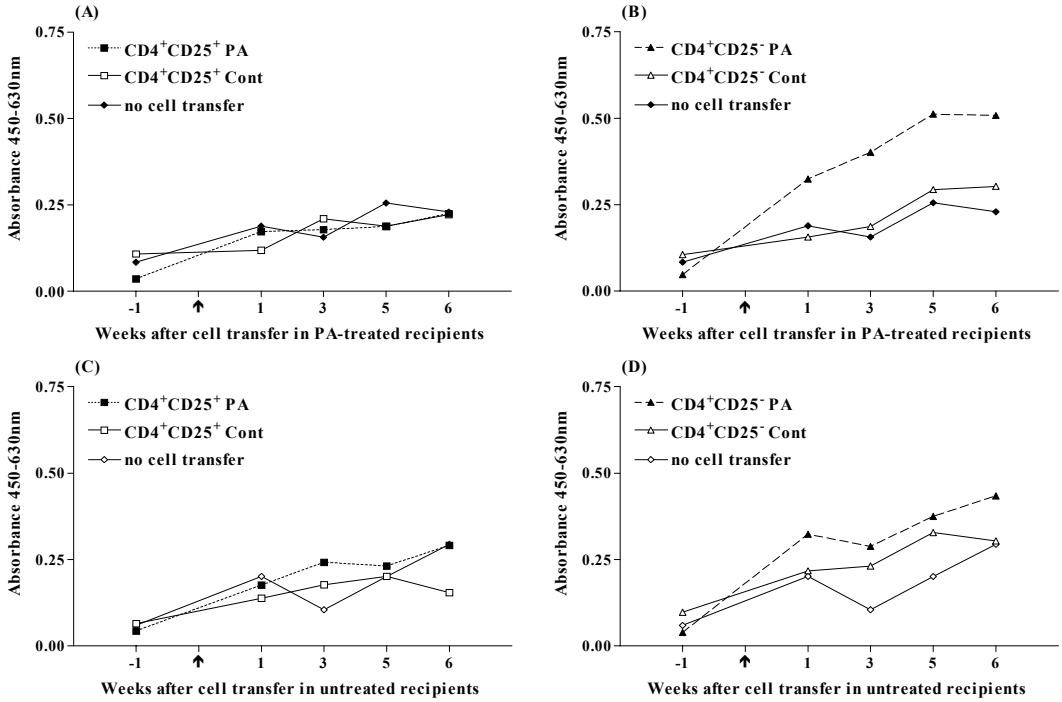


Figure 3.2.13.A-D. Combined summary of IgM concentrations in recipient A/J mice.
Serum from each recipient was tested using a specific ELISA for IgM concentrations during the six weeks after the adoptive transfer (↑) of either $CD4^+CD25^-$ or $CD4^+CD25^+$ T cells from either long term PA-treated (PA) or control donors (Cont). The above figures depict the combined results from two separate experiments. A and B depict additionally PA-treated recipients whereas the recipients in C and D remained untreated. Symbols represent the mean absorbance value from each recipient group measured at the indicated time points.

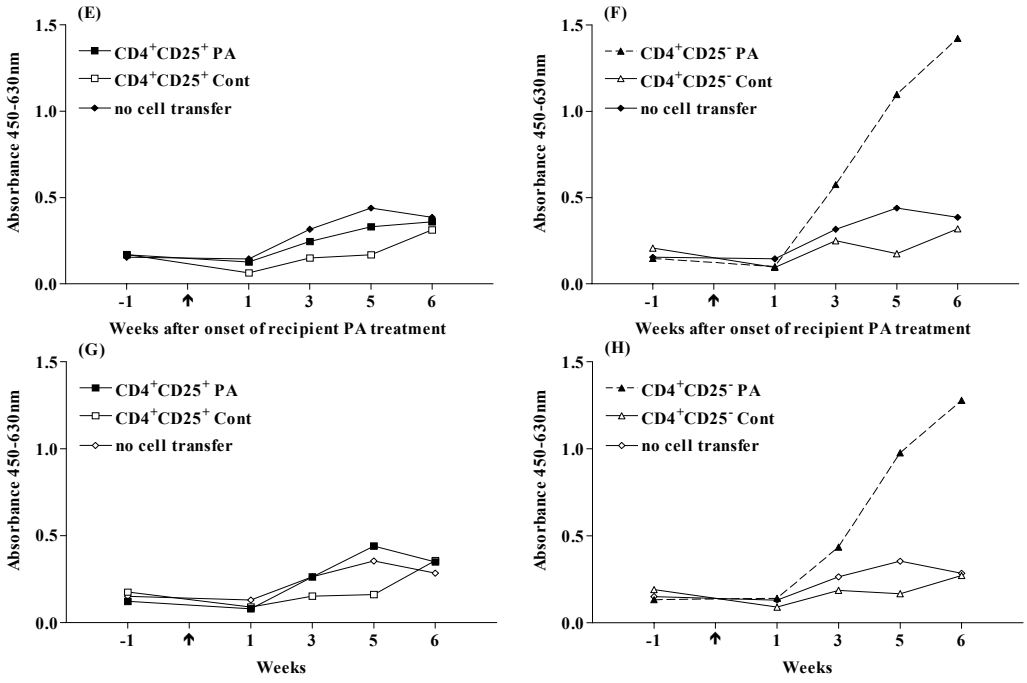


Figure 3.2.13.E-H. Combined summary of IgG1 concentrations in recipient A/J mice.
Serum from each recipient was tested using a specific ELISA for IgG1 concentrations during the six weeks after the adoptive transfer (↑) of either $CD4^+CD25^-$ or $CD4^+CD25^+$ T cells from either long term PA-treated (PA) or control donors (Cont). The above figures depict the combined results from two separate experiments. E and F depict additionally PA-treated recipients whereas the recipients in G and H remained untreated. Symbols represent the mean absorbance value from each recipient group measured at the indicated time points.

3.2.14. CLASSIFICATION OF THE AUTOANTIBODY PATTERN PRODUCED IN RECIPIENT MICE AFTER THE ADOPTIVE TRANSFER OF $CD4^+CD25^-$ T CELLS PA-TREATED DONORS.

In order to classify the autoantibody type which developed in the recipients after the adoptive transfer of long term PA-treated $CD4^+CD25^-$ T cells, sera from these groups were incubated onto self-prepared HEp-2 cell slides and the patterns viewed with fluorescent microscopy. As displayed in figure 3.2.14, a nucleolar type staining pattern developed in these recipients which was not present in the other groups. This staining is directed against the nucleoli of the cells. As described earlier (section 3.2.9), the long term, PA-treated donors produced very varied ANA patterns. Therefore, it is feasible that this detected ANA pattern in the recipients may have arose from the injected donor cells.

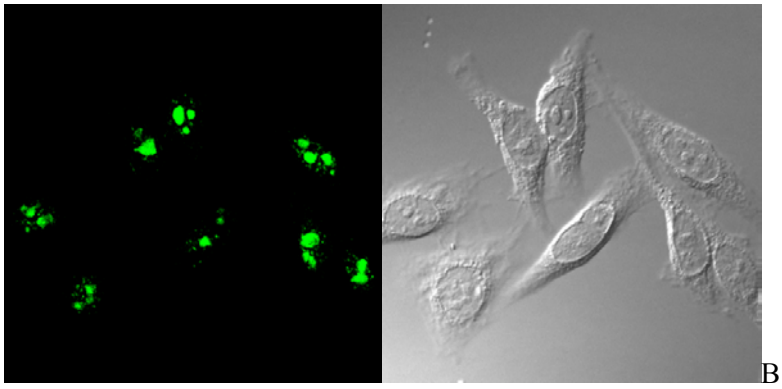
A  B

Figure 3.2.14. Autoantibody pattern in recipient A/J mice.
Typical pattern found in the recipient groups which received an adoptive transfer of $CD4^+CD25^-$ T cells from long term PA-treated A/J mice. Sera were diluted 1:100 and incubated on self-prepared HEp-2 cell slides. After secondary staining with a FITC labelled anti-IgG1 antibody, the staining patterns were detected using a fluorescent microscope. Picture A depicts the typical ANA pattern found within the above mentioned recipient group and the location of the staining can be mapped using the ghost image (B). Slides were prepared and read in a blind fashion.

3.2.15. $CD4^+CD25^+$ T CELLS FROM PA-TREATED DONORS CAN PARTIALLY SUPPRESS THE ANA FORMATION INDUCED BY GOLD SODIUM THIOMALATE

To evaluate the specificity of PA-primed suppressive $CD4^+CD25^+$ T cells, a cross transfer experiment was performed. This term denotes the adoptive transfer of T cell subsets derived from donors treated with one particular ANA-inducing xenobiotic into syngenic recipients that were being treated with a different xenobiotic. Gold I salts {Au(I)}, are used as antirheumatic drugs and frequently cause adverse immune reactions including glomerulonephritis, increased levels of serum Ig, ANA and rarely, aplastic anemia [99]. In the case of Au(I) drugs, the identification of the ultimate neoantigens has been as difficult as for PA. However, it has been shown that Au(I) can be oxidised into the reactive, intermediate element Au(III), which, in contrast to Au(I), can irreversibly denature proteins [84, 92, 105]. In comparison to PA, Au(I)-pulsed macrophages have been shown to generate gold-neoantigens that could be specifically recognised by "Au(III)-specific" T cells. Furthermore, Au(III) has been demonstrated to induce the presentation of cryptic self-peptides [84] and the production of ANA in susceptible mouse strains [99]. Moderately susceptible strains have also been shown to develop ANA after gold sodium thiomalate (GST) exposure but not the control substance sodium thiomalate (ST) [99, 152]. These strains include the C57Bl/6 ($H\text{-}2^b$) and the A/J strain ($H\text{-}2^a$) and both strains develop ANA after twelve to fourteen weeks of treatment [99, 152].

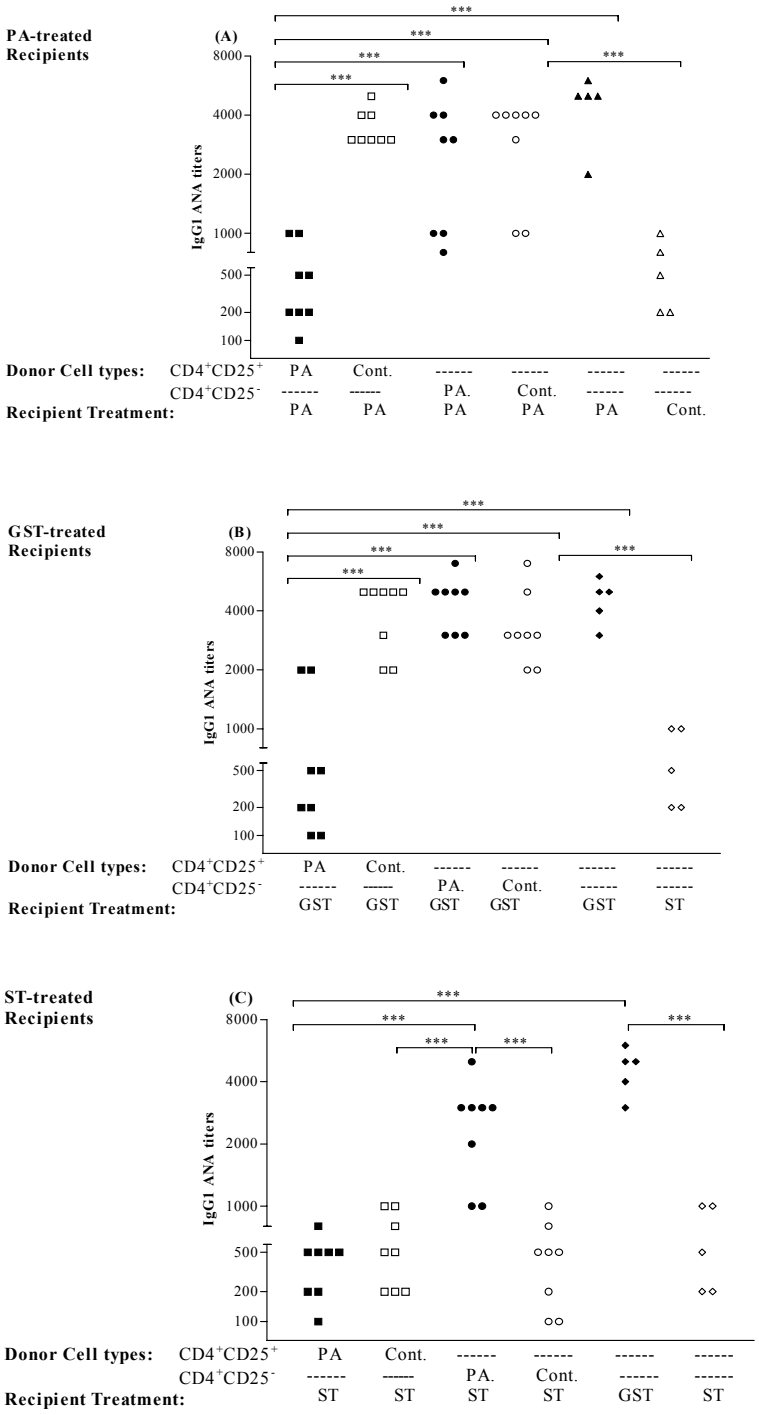


Figure 3.2.15. A-C: ANA formation in GST-treated A/J recipients can be partially prevented by $CD4^+CD25^+$ T cells from PA-treated syngenic donors. $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells (1×10^5) were adoptively transferred into syngenic recipients from donor A/J mice that had or had not been treated for 6-8 months with 6 g/L PA drinking water (PA and Cont., respectively). Recipient groups were subsequently treated with PA drinking water (A), or weekly i.m. injections of either GST (B) or ST (C). Recipient treatment began directly after the transfer of cells and continued throughout the 12 week observation period. Each graph depicts the endpoint titers of individual sera obtained at week 12. Symbols represent the recipient groups which received an adoptive transfer of the following donor cell types: PA-treated $CD4^+CD25^+$ (■), PA-treated $CD4^+CD25^-$ (●), control $CD4^+CD25^+$ (□), and control $CD4^+CD25^-$ T cells (○). Recipient groups which were treated with the different xenobiotics but did not receive a cell transfer are shown as (▲) PA, (△) control, (◆) GST, and (◇) ST. The depicted results are from two pooled independent experiments, each containing 4 mice per group. Asterisks indicate a significant difference [*** $P<0.001$] between the bars compared by the brackets.

The principal of this transfer experiment was the same as that performed in section 3.2.12. In short, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from the PA-treated or untreated A/J donors were enriched and intravenously injected into syngenic recipients. Directly after the transfer, the recipient groups were divided into three, those that were continuously treated with PA drinking water (A) and those that received weekly intramuscular injections of either GST, gold sodium thiomalate, (B) or ST, sodium thiomalate, (C). At several timepoints during the weeks after transfer, the sera from each recipient was obtained and evaluated for ANA formation using the pre-fixed HEp-2 cell slides.

Figure 3.2.15 depicts the individual endpoint ANA titres obtained from each recipient 12 weeks after transfer. In the recipient groups that did not receive a cell transfer, ANA titres only developed in the groups that were treated with either PA drinking water (A) or GST (B), indicating that without the influence of donor cells, these xenobiotics could induce ANA formation. Once again these ANA were IgG1 positive demonstrating a T cell response. As previously found (section 3.2.12), only the enriched $CD4^+CD25^+$ T cells from PA-treated donors were able to prevent ANA formation in PA-treated recipients and this was statistically significant in comparison to the corresponding transfer of untreated donor T cells (***P<0.001). Figure 3.2.15C depicts the ANA titres observed in the ST-treated recipients. As one can observe, no ANA formation was apparent in the recipient group that received no cell transfer but were treated with ST. In contrast, the ST-treated recipient group which received $CD4^+CD25^-$ T cells from PA-treated donors (● in figure 3.2.15), steadily developed ANA titres reaching endpoint titres of 1:6000 at week 12 (***P>0.001), this is also consistent with the findings described in section 3.2.12. ANA titres remained low in the ST-treated recipient groups that received either of the $CD4^+$ T cells subsets from untreated donor mice or the $CD4^+CD25^+$ T cell subset from PA-treated donors. This ANA formation only after the transfer of $CD4^+CD25^-$ T cells from PA-treated donors suggests that indeed these T cells can induce an ANA to a self-protein.

In the GST-treated recipients (B), the clear induction of ANA can be seen after the transfer of $CD4^+CD25^-$ T cells from both PA-treated (●) or untreated (○) donors an endpoint titre range of 1:3000 to 1:7000 and 1:2000 to 1:7000 respectively. ANA formation was also detected after the transfer of $CD4^+CD25^+$ T cells from untreated (□) donors indicating that these cells cannot prevent the induction of ANA during GST treatment. Here a comparable endpoint titre range was observed with the highest ANA titre reaching 1:6000. These titres were comparable to the recipient group which received no cell transfer but were treated with GST. Finally, PA-treated $CD4^+CD25^+$ donor T cells (■) that were transferred into GST-treated recipients demonstrated a partial prevention of the induced ANA. Six of the eight recipients in this group did not develop significant ANA titres over the 12 weeks. The group endpoint titres ranged from 1:100 to 1:2000. As a group, these titre values were significantly different (***P>0.001) to the other GST-treated recipient groups.

3.3. ELUCIDATION OF PA-INDUCED NEOANTIGENS

Within this section, the focus of the research was more molecular orientated. As discussed throughout this entire thesis, the identification of neoantigens which are involved in immune reactions to xenobiotics is the most challenging and difficult aspect of this field. Without the full elucidation of these neoantigens, further experimental work becomes more problematic and any possible therapeutic treatments almost impossible. Research involving procainamide has one advantage over other sensitising chemicals such as metals, in that this xenobiotic is metabolised and produces known metabolites that can bind to proteins [117]. This phenomenon was first described with neutrophils [124] and in the first section of this chapter, WBMC were shown to act in a similar manner (3.1.2). Furthermore, previous research within our laboratory has shown that *in vivo* casein-stimulated peritoneal macrophages enriched from C57Bl/6 mice, have the ability to metabolise PA *ex vivo* and form a 35kDa protein complex which was detected by Western blot analysis using a specific PA antibody [127]. Therefore, described in the following sections are additional attempts to identify the

specificity of the neoantigens produced during both the long term PA treatment and *in vitro* pulsation experiments.

Within the first part of this section *in vitro* experiments were performed to compare any protein bands produced by PA-pulsed WBMC with those protein bands previously found with PA-pulsed PMφ [127]. These experiments also included the pulsation of WBMC with 20 μM HAPA. This direct incubation of WBMC with HAPA resulted in several detectable (HA)PA-protein complexes as identified through the Western Blot technique and are described in section 3.3.1. Similar experiments were also performed using the specific mouse macrophage cell line termed P338 and here, PA-specific protein bands were also observed, providing direct evidence that macrophages are capable of producing PA-specific neoantigens (section 3.3.2). Attempts were then performed to separate these PA-protein bands using 2D gel electrophoresis (see section 3.3.3). In the closing sections of this chapter, *in vitro* cultured HEp-2 cell lysates were used to identify common protein bands in the sera obtained from the long term PA-treated A/J mice which were described in section 3.2.7. Several protein bands were identified within the PA-treated sera which were absent in the untreated sera. However, almost 40% of these long term PA-treated sera contained a common protein band at the 35kDa level indicating that indeed PA is able to induce specific neoantigens.

3.3.1. IDENTIFICATION OF ARISING NEOANTIGENS IN PA-PULSED WBMC

As previously mentioned, earlier investigations have shown that *in vivo* casein-stimulated PMφ, from fast acetylator C57BL/6 mice, could metabolise PA *ex vivo* and form a specific 35kDa protein band as detected through the Western blot technique [127]. Following this result and the evidence that both PA-pulsed WBMC and PMφ could elicit a primary PLN response [section 3.1.2 and 106], the question arose as to whether PA-pulsed WBMC could also produce this 35kDa band or other PA-induced protein bands when incubated *in vitro* with either PA or HAPA. Consequently, WBMC, spleen cells and PMφ were prepared from the A/J mouse strain as described in section 2.2.3 and incubated *in vitro* with or without 500 μM PA or 20 μM HAPA for 24 hours. After washing, cells were either kept as whole cell lysates or separated into cytoplasmic or nuclear fractions using Triton X100. Samples were then mixed with sample buffer, briefly sonicated and heated to 95 °C before being loaded onto 12% SDS-PAGE gels. Gel electrophoresis was then performed at 100V until protein separation was complete. After transfer onto nitrocellulose paper and a blocking step, the separated proteins were identified through Western Blot analysis. Nitrocellulose blots were first incubated with a specific PA antibody (1:3000) and thereafter with a secondary rat-anti-rabbit HRP conjugate. Protein bands were then detected using ECL reagents and films were developed, for details see section 2.2.10.

In the aforementioned *ex vivo* re-stimulation assays (see section 3.2.5), enriched $CD4^+$ T cells from long term PA-treated A/J mice responded to antigens consisting of HAPA or PA-pulsed WBMC, spleen or PMφ cells. Therefore, to correlate any possible neoantigens identified via molecular techniques, the cell lysates in the following investigations were prepared from naïve A/J mice that were not previously injected with casein. After several experiments using whole cell lysates or

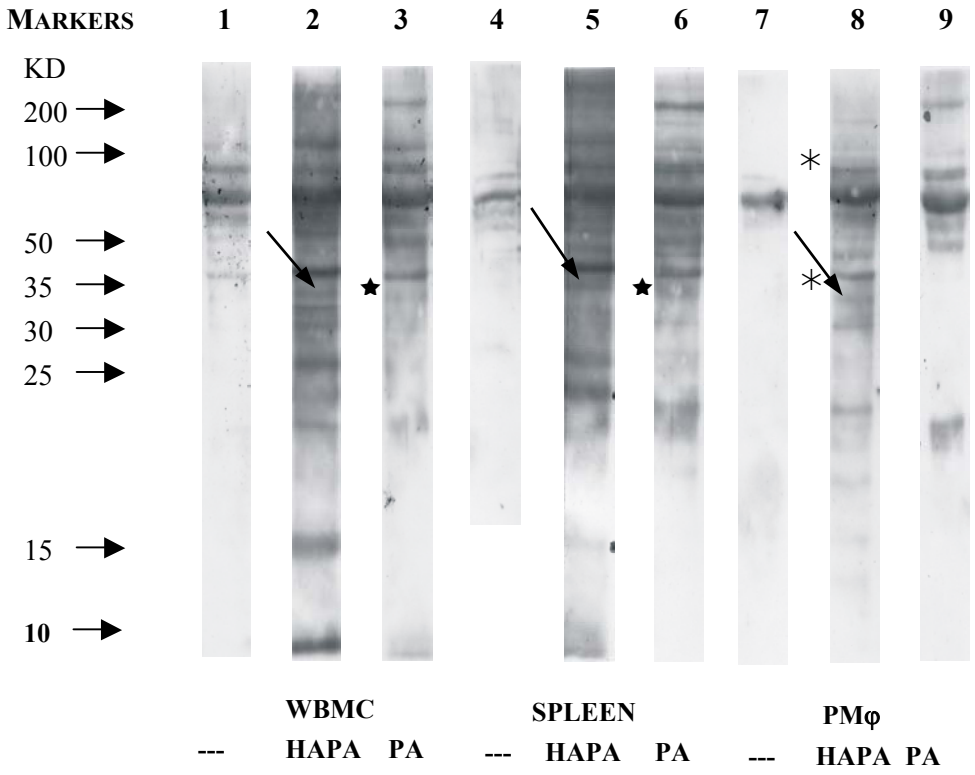


Figure 3.3.1. Identification of PA-specific protein bands from PA- or HAPA-pulsed cells.
WBMC, spleen cells and PMφ, derived from the A/J mouse strain, were pulsed overnight with or without 500 μM PA or 20 μM HAPA for 24 hours. Thereafter the cytoplasmic fractions from these treated or untreated cells were isolated by Triton X100 solution, added to an equal volume of sample buffer, briefly sonicated and heated at 95°C for 5 minutes. After separation on a 12% SDS-PAGE gel, proteins were transferred onto nitrocellulose paper and specific PA-protein bands detected through Western blot analysis using a specific PA antibody. Marker sizes are shown on the left hand side. Lanes 1-9 contain the following samples: 1) WBMC alone, 2) WBMC and HAPA, 3) WBMC and PA, 4) spleen alone, 5) spleen and HAPA, 6) spleen and PA, 7) PMφ alone, 8) PMφ and HAPA and 9) PMφ and PA.

separated nuclear and cytoplasmic fractions, it became apparent that the PA-related protein bands were contained within the cytoplasmic fraction. This correlates to the earlier findings observed with the *ex vivo* PA-pulsed PMφ [127]. Consequently, figure 3.3.1 displays a representative result of the different PA-protein bands observed in the cytoplasmic fractions from isolated PA-pulsed cell types. The results from PA- or HAPA-pulsed WBMC, spleen cells and PMφ are located within lanes 1-3, 4-6 and 7-9 respectively. The WBMC fraction that had been pulsed with 20 μM HAPA (lane 2) produced multiple PA-protein bands. In accordance with the molecular weight markers (left hand side), the strongest responding bands corresponded to sizes of approximately 10kDa, 15kDa, 20kDa, 35kDa, 50kDa and 100kDa. These bands could not be identified in the control WBMC fraction (lane 1). Although much weaker in intensity, HAPA-pulsed spleen cells also produced similar bands to those found in the WBMC fraction (lane 5). PMφ that had been pulsed with HAPA (lane 8) produced only three bands which correlated with those identified in lanes 2 and 5 (indicated by ∗). These bands were absent in the PMφ control lane (lane7). All the HAPA-pulsed cell types produced a protein band corresponding to a molecular weight of 35kDa band and is depicted by the arrows in lanes 2, 5 and 8.

In contrast to the previous findings [127], no detectable 35kDa band could be identified within the PA-pulsed PMφ (lane 9) but was faintly visible in lanes containing the PA-pulsed WBMC and spleen fractions (lanes 3 and 6 respectively) and is indicated by the ★ symbols. This 35kDa protein band could not be induced in the PA-pulsed A/J PMφ fractions even after metabolic enhancement by additional PMA (data not shown). This difference in the production of protein bands may well be influenced by the metabolic capacities of the different mouse strains. In summary, when different cell types from A/J mice are pulsed with either PA or HAPA, multiple bands are obtained indicating that indeed both PA and HAPA have the ability to bind to several proteins and hence form several possible neoantigens.

3.3.2. PA-PULSED MACROPHAGES ALSO PRODUCE PA-SPECIFIC NEOANTIGENS

For additional verification that macrophages were capable of producing PA-specific neoantigens, identical experiments to those described in section 3.3.1 were performed but this time employing the macrophage cell line, P338. Figure 3.3.2 displays the results obtained in both the nuclear and cytoplasmic fractions from PA- or HAPA-pulsed P338 cells. Once again, protein bands were detected with the specific PA antibody (1:3000). Multiple protein bands corresponding to sizes of 10kDa, 18kDa, 20kDa, 35kDa, 50kDa and 100kDa were visible within the cytoplasmic fraction of HAPA-pulsed P338 cells as indicated by ⋆ in lane 2. These bands were completely absent in the untreated cytoplasmic fraction (lane 1). In this experiment, the 35kDa band in the HAPA-pulsed fraction was very prominent (arrow in lane 2). In contrast to the previous experiments with A/J derived PA-pulsed cell types (lanes 3, 6 and 9 in figure 3.3.1), the 35kDa band was this time also detectable in the cytoplasmic fraction containing PA-pulsed P338 (arrow in lane 3).

These P338 experiments were then even further distinguished from those performed in section 3.3.1, by the fact that the 200kDa band found in section 3.3.1 within the PA-pulsed cells (lanes 3, 6 and 9 in figure 3.3.1) was absent from the equivalent P338 samples. A possible explanation for this difference is that within a mixed cell population, the formed PA-neoantigens maybe more varied than in the P338 macrophage cultures due to an abundance of different proteins from other cells. Also in lane 3 is another band at approximately 25kDa which was not present, at least not as vivid, in the PA- or HAPA-pulsed A/J cell types (figure 3.3.1) indicating that different cells types may produce different PA-protein complexes.

As mentioned previously, the cytoplasmic fractions of PA-pulsed or HAPA-pulsed A/J macrophage containing cells produced the more distinguished PA-specific protein bands. Whether this was also the case for this macrophage cell line was unknown. Therefore, the nuclear fractions from the P338 pulsed cells were also tested for PA-related protein bands and are included in figure 3.3.2, lanes 4 –6 respectively. As one can observe, specific PA-protein bands correlating to those found in the cytoplasmic fractions cannot be detected. Furthermore, no unusual bands were produced either, indicating that after a 24 hour incubation, the PA-neoantigens are only contained within the cytoplasmic fraction.

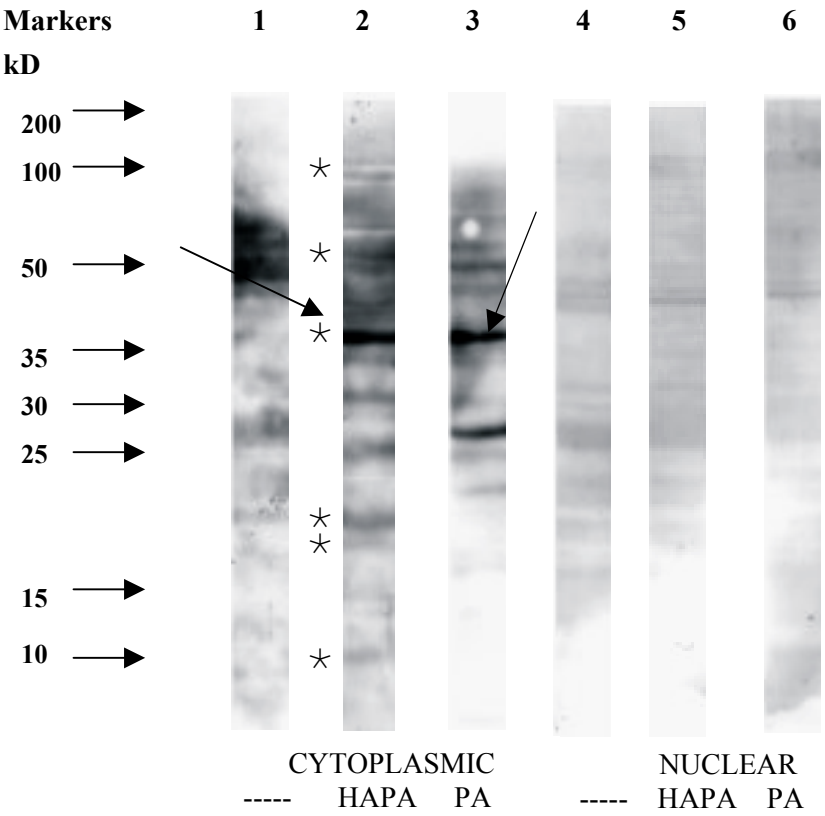


Figure 3.3.2. Identification of PA-related protein bands in PA- and HAPA-pulsed P338 macrophages. P338 cells were pulsed overnight with or without 20 µM HAPA or 500 µM PA. After washing, cells were separated into nuclear and cytoplasmic fractions using Triton X100. These fractions were then added to an equal volume of sample buffer, briefly sonicated and heated to 95°C before loading onto a 12% SDS-PAGE gel. Separated proteins were then transferred onto nitrocellulose paper and Western Blot analysis performed using a specific PA antibody. Protein bands were detected using the ECL reagent. The contents of each lane is as follows: 1) cytoplasmic fraction of P338 cells alone, 2) P338 cytoplasmic fraction and 20 µM HAPA, 3) P338 cytoplasmic fraction and 500 µM PA 4) nuclear fraction of P338 cells alone 5) nuclear fraction of P338 cells and 20 µM HAPA, and 6) nuclear fraction of P338 cells and 500 µM PA.

3.3.3. 2D SEPARATION OF PROCAINAMIDE-NEOANTIGENS

Although PA-related protein bands could be easily identified with one dimensional SDS-PAGE gel electrophoresis, no further evaluation could be determined since this separation technique cannot distinguish multiple proteins situated within one position. Consequently, in attempts to further elucidate information from these PA-related protein bands found with pulsed WBMC or macrophages, high resolution 2D gel electrophoresis techniques were established. These 2D electrophoretic methods are described in detail in section 2.3.3 and were performed in accordance to Klose and Görg [153, 154]. This method incorporates two phases, the first isoelectric focusing phase is carried out in the presence of near saturation point urea (8-9 molar) and a non-ionic detergent such as Triton X-100 or Nonidet NP-40. The second dimension is either a vertical or horizontal SDS-PAGE electrophoresis. The separation parameter of the first dimension run, the pI, is independent of the molecular weight which is the separation parameter of the second dimensional.

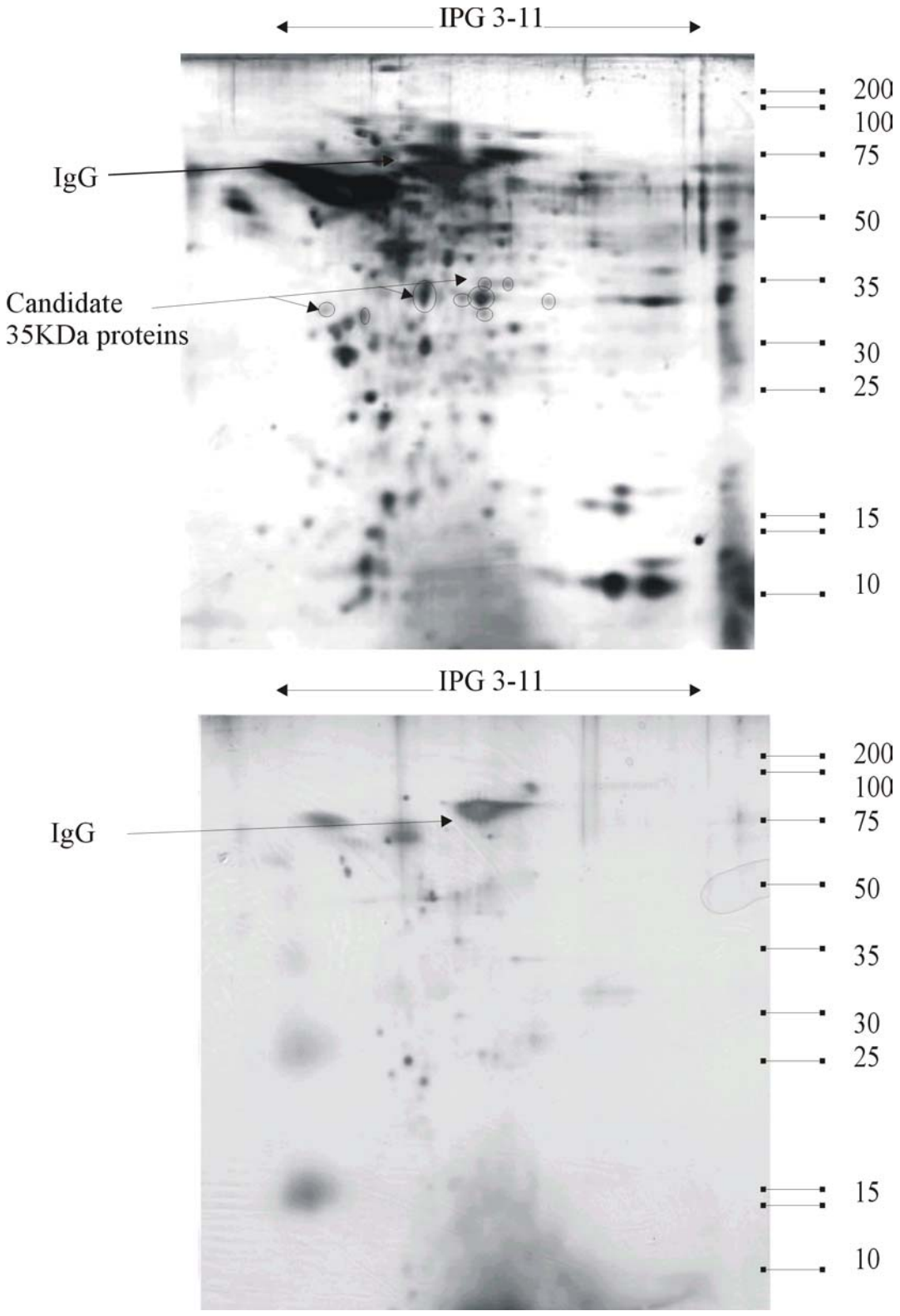


Figure 3.3.3.a and b. High resolution 2D electrophoresis of cytoplasmic proteins from PA-pulsed or control P338 macrophage cells.
Separated cytoplasmic fractions from PA-pulsed (upper gel) or control (lower gel) P338 cells were subjected to 2D electrophoresis using immobilized pH gradients (pH3-11) for the first dimension and vertical SDS-PAGE gel electrophoresis for the second. Proteins were then detected by silver staining. Protein sizes can be determined by the molecular weight markers shown on the right hand side. Possible PA-related 35kDa protein candidates are individually circled in the upper gel (A).

The results of a separation is a pattern of spots. According to the Cartesian coordinate system, the following standard of representation has gained acceptance: from left to right – increasing pI, from bottom to top – increasing molecular weight (see axis labelling on figure 3.3.3). At the moment, these two dimensional protein maps afford the highest resolution of protein separation. By lengthening the distance of separation, thinner gels and the development of more sensitive detection methods such as autoradiography of labelled proteins and silver staining, many research laboratories are able to increase the number of visible proteins. High reproducibility of the spot positions is important for interpretation and further sequencing steps. Immobilised pH gradients (IPG) have been shown to considerably enhance the reproducibility of the separated protein patterns [154]. Although several choices of IPG strips can be obtained, the strips used in the separations performed here covered the pH gradients between 3-11. Vertical SDS-PAGE was employed for the second stage using a 10% polyacrylamide gel. In the initial 2D separation investigations, the Western blot technique was used to visualise the specific PA-protein complexes. As in the one dimensional SDS-PAGE experiments (see section 3.3.1 and 3.3.2), the PA–specific antibody was used. Although some protein spots could be detected, even in the 35kDa range, this method proved to be far too insensitive and thus not shown. Consequently, silver staining techniques were employed. Two different silver staining techniques can be used depending on whether the experiment is to obtain protein patterns or elucidate the protein for sequencing [145].

Figure 3.3.3 depicts the protein separation patterns found in the cytoplasmic fractions of PA-pulsed (A) and untreated P338 cells (B) after the second dimension run. As mentioned above, protein separation in this technique is dependent on two parameters: pH and molecular weight. These parameters are depicted on the x and y axis respectively. From left to right the pI of the proteins increases whereas from top to bottom the molecular weight decreases. After protein detection by silver staining, the protein patterns found in the PA-pulsed fraction and the control were compared. From the first glance one can see that the control fraction contains less proteins than the PA-pulsed fraction. However, although difficult to determine from these scanned representatives many of the proteins in the PA-pulsed fraction were present in the control but were significantly fainter. In all of the experiments performed, a large protein mass was visible in the 70kDa region and probably depicts the immunoglobulins. In section 3.3.2, one dimensional SDS-PAGE gel electrophoresis detected several PA-specific protein bands. The sizes of these proteins were approximated at 18kDa, 20kDa, 35kDa, 50kDa and 100kDa. In this previous experiment, the protein band at 35kDa was very prominent and therefore this protein became the focal point for the 2D electrophoresis. As one can see, this region contains an abundance of proteins which can obviously not be individually detected in the one dimensional SDS-PAGE electrophoresis. Possible 35kDa candidate proteins are individually marked in figure 3.3.3A. These proteins can only be fully determined after enough protein material is obtained for protein sequencing, this is currently being performed.

3.3.4. HEP-2 CELL IDENTIFICATION

Another usage of HEp-2 cells is their ability to help identify serum antibodies via the Western blot technique. Within this technique, cultured cells are harvested and the resulting lysates prepared in a manner that can be loaded onto one dimensional SDS-PAGE gels. For these investigations, whole cell

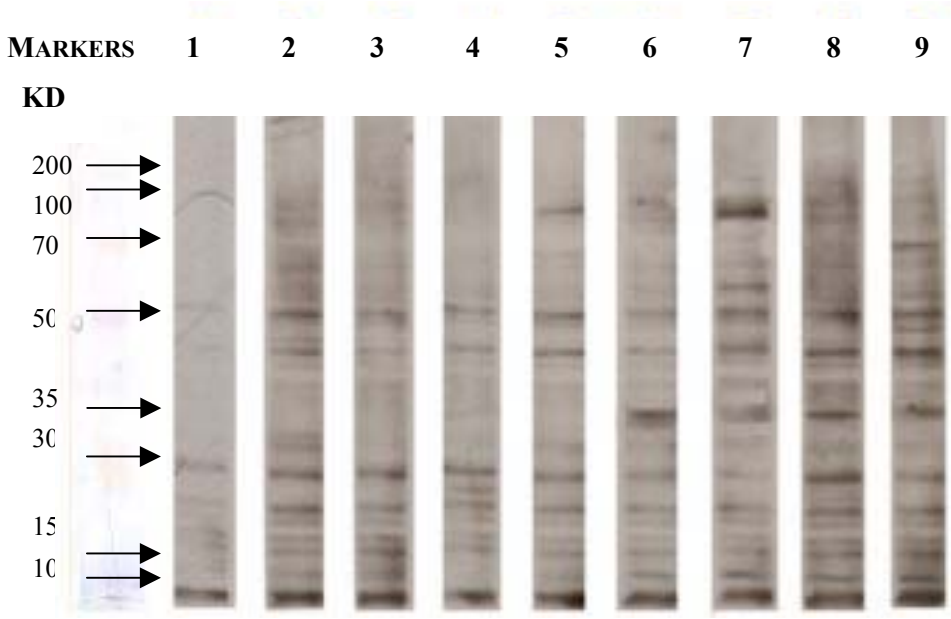


Figure 3.4.4. Protein bands detected in PA-treated sera using HEp-2 cell lysates.
Cultured HEp-2 cells were harvested and the total cell lysates were prepared for loading onto 12% SDS-PAGE gels. After transfer onto nitrocellulose sheets, even strips were cut and incubated for one hour with either PA-treated or untreated A/J sera. After a further incubation with a secondary goat-anti-mouse HRP antibody, protein bands were detected using ECL reagents. On the left hand side are the control markers depicting the size of the protein bands from 10-270kDa. Lanes two to five were incubated with untreated sera whereas lanes 6-9 were incubated with PA-treated sera. Lane one contained no sera.

lysates were ran on 12% SDS-PAGE gels as described in section 2.2.12. After transfer onto nitrocellulose paper the sheets were cut into approximately 4 mm strips and placed in blocking buffer to remove background staining. Thereafter, the individual strips were incubated with either long term PA-treated or control sera from A/J mice for one hour at room temperature. Antibodies present in the sera were then detected using a goat-anti-mouse HRP secondary antibody and ECL reagents. Films were developed after 30 seconds, 1, 5 and 10 minutes depending on the intensity of the bands. With the aid of rainbow markers the sizes of the visible bands could be approximated.

Figure 3.3.4 depicts a representative of the antibody patterns found within the PA-treated and untreated sera. Lanes 2-5 show the results obtained when HEp-2 loaded nitrocellulose strips were incubated with untreated A/J mouse sera. As one can observe, all mice produced the same bands albeit in varying intensities. Lanes 6-9 display the bands produced when the HEp-2 cell containing strips were incubated with individual PA-treated sera. Within these sera, a protein band, not seen in the control sera, can be observed just below the 35kDa marker. In all the of sera tested from both the initial trial study and later in the long term treatment investigation none of the control A/J mice developed this protein band using this technique. However, 29 of the 60 long term PA-treated and 50% of the trial PA-treated A/J mice displayed this specific protein band. Upon comparison with the HEp-2 cell classification analysis (section 3.2.9) and the specific ELISA assays that were performed (section 3.2.10), it was not possible to associate this prominent serum protein band with any one pattern or self-protein. Once again however, the centromere and nuclear clusters patterns were the most prominent, (figure 3.2.v and vii). Similarly although 32% of the PA-treated sera were positive

towards PA and/or HAPA in the specific ELISA assays (3.2.10) not all of these sera produced this band either. As one can also see in lanes 7 and 8, the 35kDa protein band was not the only unusual protein band that was seen. These two particular sera also produced other protein bands that were unique to that serum. Whereas both sera showed a protein band at 55kDa the serum in lane 9 also developed bands at 48kDa and 65kDa.

The various antibodies produced within these PA-treated sera correlate to the findings found with the pristane-sera also tested in this manner. These treated mouse sera produced numerous and very intense protein bands that were not detectable in the control sera. Moreover, no common protein band could be identified within these sera either (data not shown). This finding correlates to the results obtained with these sera in the specific ELISA assays indicating that there is not one specific antigen but maybe multiple specific ANA for one disease. Only the 35kDa protein band appears to be a consistent marker both in the PA-treated sera and the *in vitro* PA- and HAPA-pulsed cells (section 3.3.1, 3.3.2).

4 Discussion

Science is organised knowledge. Wisdom is organised life.
IMMANUEL KANT 1724-1804

There are nearly 100 medical disorders involving autoimmunity, a phenomenon in which the body's immune system attacks its own tissues [155]. However, despite the numerous reports on how these diseases affect the body, the specific etiological and initial pathogenic steps remain unknown. A large number of drugs and environmental agents can result in the appearance of various autoantibodies and in many instances also develop a wide range of autoimmune clinical syndromes [155]. Consequently, a vast amount of experimental autoimmune models arising from the relevant etiological agents have been established. One of the major diseases in which there have been numerous studies with environmental factors is lupus [155]. Drug-induced lupus is an unwanted side effect after the ingestion of various medications but its etiology, pathogenesis and underlying mechanisms remain a mystery. In fact, the three xenobiotics used within this study, PA, pristane and gold salts, have all been associated with lupus-like syndromes [155]. The *in vivo* metabolic transformation of lupus-inducing drugs, such as PA and gold salts, into reactive products provides an explanation of how a heterogeneous set of drugs could mediate the same syndrome. HAPA, the reactive metabolite of PA, displays diverse biological properties [106, 128, 156] and throughout this thesis the immunoreactive potential of this hapten have been studied using a slow acetylator mouse model. Significant pieces of evidence for the reactive properties of HAPA were observed in the $CD4^+$ T cell reactions to HAPA and PA-related neoantigens both *in vivo* and *in vitro*. These $CD4^+$ T cells were further shown to be effector cells since B cell presentation of neoantigens could initiate their activation. In this chapter, the findings described in the results section will be related to the important topics of drug-induced adverse reactions including the hapten theory (4.2), autoantigens and autoantibodies (4.5 and 4.6) and T suppressor cells (4.7 and 4.8).

4.1. IDIOSYNCRATIC DRUG REACTIONS FOLLOW A SIMILAR PATHOGENIC PATHWAY AS GRAFT-VERSUS HOST REACTIONS

Type B drug reactions can be divided into immune-mediated (hypersensitivity reactions) and non-immune mediated (also referred to as metabolic idiosyncrasy). These drug reactions are curious since they do not occur in every individual, can arise at any dose and unlike Type A drug reactions cannot

be predicted from the known pharmacological properties of the drug [157]. Although they are classed as rare, the frequency of these type B reactions are quite common and their serious nature and unpredictability makes them not only a significant clinical problem but a hindrance to drug development [113]. Therefore, in order to effectively deal with adverse immune reactions to drugs, a fundamental aspect is to try and understand their underlying mechanisms.

The clinical characteristics that arise during an adverse immune reaction can be considerably different between drugs and even for the same drug in different individuals. Although a number of these reactions are organ specific such as skin, liver or bone marrow, systemic reactions also occur and involve fever, skin rash and lymphadenopathy. The adverse immune reactions to PA and gold(I) salts are classical examples of generalised immunopathological effects and moreover, these clinical symptoms are strikingly similar to those seen during systemic graft-versus-host reactions (GVH). Consequently, the same mechanisms underlying a GVH reaction are also thought to trigger the pathogenesis of drug reactions [158, 159, 160]. In a GVH situation the pivotal reactions are carried out by the grafted T lymphocytes that can respond to the incomplete tissue antigens of the host. Therefore, the varying immunopathological pictures of the GVH reactions depend upon the distal effector mechanisms activated by the predominating donor T cell subsets and their cytokine production [159, 160]. The concept of GVH-like reactions to drugs and chemicals postulates that the involved etiological agent alters either the MHC-molecules or MHC-embedded self-peptides. In either case autologous T cells would react to the MHC-peptide complexes in a GVH-like fashion. This concept was proposed over twenty years ago [158] but confirmation has been restricted by the difficulty to measure T cell reactions to drugs *in vitro* and two intrinsic properties of the drugs. These issues which centre around the hapten theory will be described in the following sections.

4.2. FURTHER SUPPORT FOR THE HAPTEN THEORY

To date, the most prevalent hypothesis for the mechanism of idiosyncratic drug reactions remains the hapten hypothesis [92, 161]. This hypothesis proposes that drugs (for example penicillin) or more commonly their reactive metabolites can act as haptens and irreversibly bind to proteins or other macromolecules (figure 4.2A and B). These hapten-protein complexes are consequently "perceived" as foreign and may provide two important signals: (i) to stimulate T cell proliferation and/or (ii) to direct the effector arm of the immune response to the targeted cells (tissues) [156]. There exists a considerable amount of evidence that supports the hypothesis that reactive metabolites are involved in idiosyncratic drug reactions, especially since drugs that are not metabolised rarely cause hypersensitivity reactions [157, 161, 164]. Procainamide for example, is definitely associated with drug-induced lupus-like syndromes [155] and can be metabolised by cells of the immune system, such as macrophages, into HAPA and nitroso-PA which are the proposed main reactive metabolites [123, 128]. In section 3.2.10, slow acetylator A/J mice that were chronically treated with PA drinking water developed antibodies to HAPA and/or PA. Comparable findings have been previously observed with halothane, since patients with idiosyncratic halothane-induced hepatotoxicity developed antibodies to the reactive metabolite of halothane, trifluoroacetyl chloride [165, 166].

Another aspect to the theory is that the site of reactive metabolite formation usually correlates to the site of toxicity, for example, the oxidation of the halothane C-H bond is essentially limited to the cytochrome P450 enzyme and therefore corresponds to the liver damage observed during treatment [167]. In the case of PA, the individual acetylator phenotype, in both humans and mice, has been shown to influence the arising dominant metabolite [116, 118]. As described in section 1.10, there are a number of different PA metabolites that arise through either the slow or fast acetylator pathways. The slow acetylator phenotype leads to the majority of PA metabolism in the macrophages of the extrahepatic tissues. There, the *N*-hydroxylation of the arylamine group leads to a higher concentration of reactive metabolites of which the most stable is HAPA. These metabolites possess reactive abilities *in vivo* [106, 156] and can readily bind to cells and soluble proteins in a covalent manner [124, 129]. The majority of the latter *in vivo* activities were demonstrated using the PLNA and adoptive PLNA test systems. Several groups have shown that a direct injection of PA into the hindfoot pads of C57BL/6 mice failed to induce PLN responses, whereas an injection of HAPA produced vigorous PLN reactions which occurred in a dose dependent manner [106, 127]. Using the adoptive transfer PLNA, T cells from C57BL/6 mice that had received three injections of the HAPA metabolite based mixture were also shown to be sensitised to those metabolites [106]. Furthermore, splenic T cells from chronically PA/PMA-treated C57BL/6 mice, responded to HAPA metabolites but ignored PA in the adoptive PLNA. The same results were obtained with the A/J mouse strain but there, PMA injections were not necessary. In the C57BL/6 mice, PMA was used to enhance the oxidation of the tiny residual PA which escapes metabolism in the liver. To confirm these observations of generated PA-neoantigens with macrophages, another variation on the PLN assay was performed. This experiment demonstrated that homogenised WBMC were able to elicit a positive PLN response if they had been previously cultured *in vitro* with PA (section 3.1.2). The strength of this response intensified as the number of injected cells was also increased and was significantly different to the groups receiving injections of control WBMC. All of these findings are analogous to those obtained with gold(I) salts [99] and, more importantly, they provide direct experimental evidence to support the hypothesis that the haptens derived from *N*-hydroxylation of PA are generated *in vivo* and can sensitise T lymphocytes [107, 125].

The fundamental concept that protein-conjugation is an obligatory step in the process of immune recognition of drugs has been challenged by the group of Pichler. This group have demonstrated that T cell clones generated from idiosyncratic drug-treated patients were able to proliferate *in vitro* towards the mother compound without additional metabolic steps [168, 169] figure 4.2C. Consequently, it was proposed that a drug may act in a similar way as a superantigen, that is, it interacts with the MHC-molecules in a non-covalent manner and thereby generates a more general immune response [169]. However, although *in vitro* evidence for this pathway exists, it is important to note that: (a) in a polyclonal immune response to a drug antigen, it is possible that there will be some T cell clones that can respond to the parent drug alone [169], and (b) since the level of drug metabolism during *in vitro* investigations is low and the relevant APC are absent, it is plausible that the relevant antigen may not be presented [157]. Nevertheless, if such T cells as Pichler describes are actually involved in idiosyncratic reactions, this would provide a second pathway by which drugs could induce an immune response, pathway C in figure 4.4 [113]. However, in contrast to these findings, evidence provided in this thesis for T cell responses towards PA-induced neoantigens are

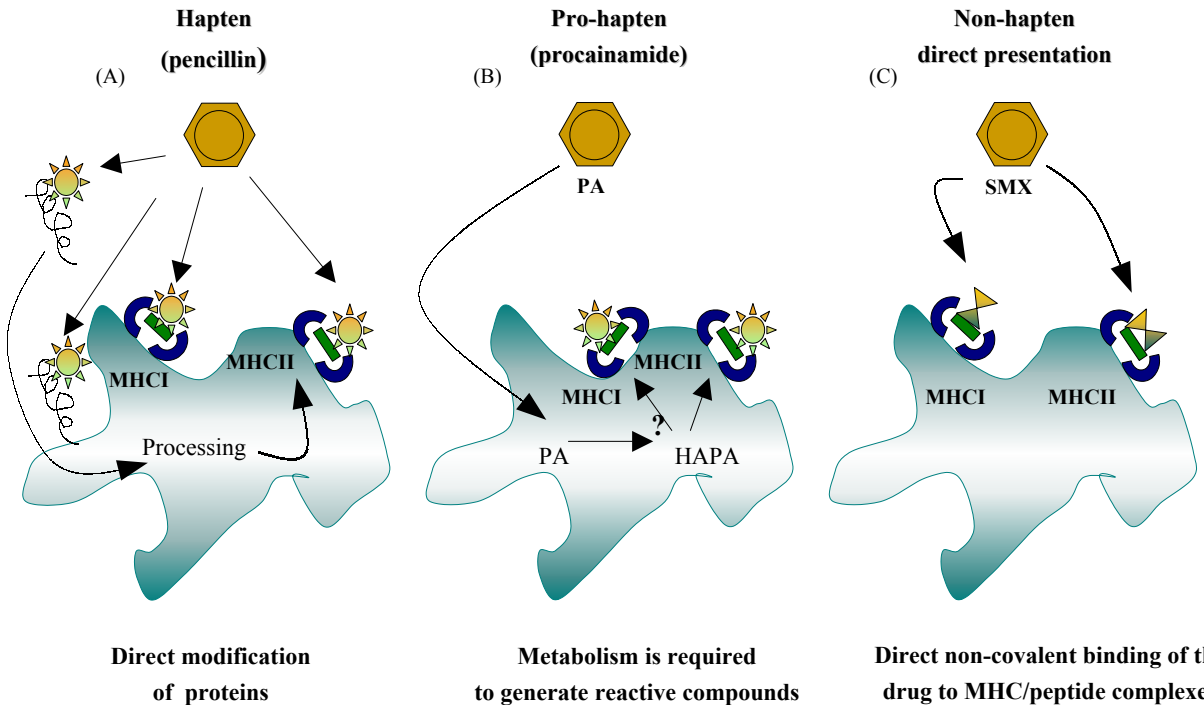


Figure 4.2. Alternative drug presentation pathways to T cells
(A) Some drugs, such as penicillin, are natural haptens and can directly modify soluble and cell bound proteins through covalent binding. (B) According to the HAPTEN THEORY, a prohapten drug, e.g. PA, must be first metabolised into its reactive metabolites. This metabolism occurs within macrophages or neutrophils and the presence of the metabolite, e.g HAPA, can lead to the modification of cell bound or soluble proteins. (C) The final possible pathway relies on the direct non-covalent binding of the parent drug to MHC-peptide complexes. This has been found *in vitro* with SMX. This figure is adapted from [178] with kind permission from W. Pichler.

depicted in the hybridoma work (section 3.1.3), the LTT assays (section 3.2.4-3.2.6) and the adoptive transfer assays (section 3.2.12, 3.2.15) indicating that both theories are possible.

Currently, the second part of the hapten theory has insinuated that the resulting damage is mediated by a classical antibody or cytotoxic T cell response towards the hapten. Despite numerous reports of antibodies specific for the reactive metabolites, as described for halothane and PA [127, 165, 170 and section 3.2.10], evidence to substantiate this hypothesis has proven more difficult to obtain. For either of these responses, helper T cells should be involved and this is the principle basis of the LTT assay. This *in vitro* test system allows for the detection of T cell responses in drug-hypersensitive individuals [36, 171]. The details of this assay are described in sections 2.4.4 and 3.2.4. In the studies performed here, bulk T cells or enriched $CD4^+$ T cells from chronically PA-treated A/J mice were able to respond to either 20 µM HAPA or previously PA-pulsed macrophage sources. These responses were significant when compared to those obtained from age-related controls (see section 3.2.4-3.2.6). Furthermore, unlike the positive control DNFB-primed PLN cells, the T cells from PA-treated mice failed to react to DNBS indicating that the responses were directed towards PA-induced neoantigens. Likewise, DNFB-treated PLN cells did not respond to PA-induced neoantigens (figure 3.2.4iv).

Numerous adverse drug reactions, such as penicillin and ciprofloxacin, have been detected using the LTT assay but with some drugs false-positive or false-negative results have been obtained.

A possible explanation for these latter misleading results maybe attributed to the incorrect antigen present in the culture. For example, the responding T cells may recognise metabolite-protein conjugates and unless metabolism occurs during the assay these hapten-complexes would be absent. This study ensured that PA metabolism had occurred since the macrophage sources were cultured with PA prior to the assay. The generation of PA-induced neoantigens during that incubation period were clarified in both the positive PLN assay using PA-pulsed WBMC (section 3.1.2) and the molecular experiments performed in section 3.3. The latter demonstrated PA-bound proteins in the cytoplasmic fractions of both PA-pulsed WBMC and P338 cells after a 24 hour incubation period (sections 3.3.1 and 3.3.2 respectively), indicating that the *in vitro* incubation period of WBMC and PA was sufficient to induce PA-neoantigens.

A further discrepancy is that although enriched $CD4^+$ T cells from PA-treated mice were able to respond to HAPA in these LTT assays (section 3.2.4-3.2.6), Kalish et al., have demonstrated that adding the reactive metabolite to the LTT cultures does not decrease the amount of false-negative results [172]. A possible explanation for their negative result is that there was a limited number of specific T cells for that drug making it difficult to detect specific proliferation of those reactive cells in the midst of others [172]. Although this does not apply to the enriched splenic $CD4^+$ T cells from PA-treated mice, this could explain why the positive responses to PA-pulsed macrophages and HAPA were significantly lower when bulk ILN cells from PA-treated mice were tested (section 3.2.4). In support of the original hapten hypothesis, the research performed within this thesis shows the generation of PA-reactive species both *in vitro* (section 3.3) and *in vivo* (section 3.1.2), the response of $CD4^+$ T cells to PA-neoantigens using both generated PA-specific hybridomas (section 3.1.3-3.1.4) and T cells from PA-treated mice in the LTT assays (section 3.2.4-3.2.6).

4.3. MODELS OF PA-INDUCED AUTOIMMUNITY

As discussed earlier (section 1.11), there are little doubts that the initial part of PA-induced autoimmunity is caused by the reactive metabolites produced during drug metabolism and their consequential binding to proteins [128]. Nevertheless, how these reactive metabolites can elicit secondary immune responses remains unsolved. Figure 4.3 depicts the initial metabolic and immunogenic steps considered to be involved in the pathogenesis of PA-induced lupus. This working model provides the basis for the research performed in this thesis and shows that the propensity for HAPA or the non protein-reactive metabolite *N*-acetyl-PA largely depends on the acetylator phenotype. As mentioned before, in contrast to fast acetylator phenotypes, the expression of a defective NAT-2 in slow acetylator A/J mice increases their hepatic elimination of PA and therefore allows macrophages or neutrophils to generate HAPA via the enzymes PGHS-2, MPO and CYP450 [127]. The former enzyme is abundant in macrophages and can be induced by PA [127]. The subsequent *N*-oxidation of PA into HAPA and nitroso-PA is then thought to lead to the MHC presentation of PA-related neoantigens and subsequent T cell sensitisation. These activated T cells would then produce cytokines and initiate a cascade reaction that would activate other cells, including autoreactive B cells. Accordingly, this process would be analogous to the chronic GVH reactions observed in mice [158]. Various points of this hypothetical scheme have been demonstrated in previous research [92, 106, 127, 137] and are described in detail in sections 4.1 and 1.11.

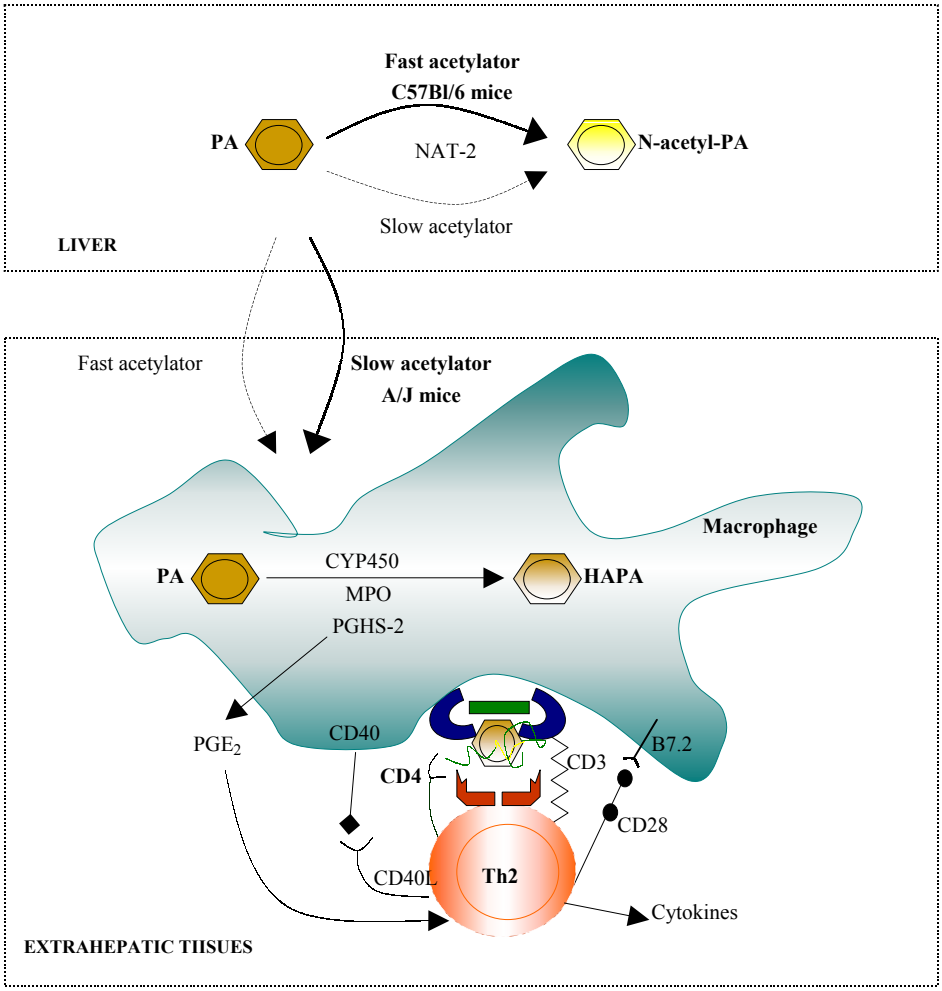


Figure 4.3. Working model for the initial metabolic and immunogenic steps thought to be involved in the pathogenesis of PA-induced lupus.
Depending on acetylator phenotype, PA metabolism can occur in two locations, the liver (A) or extrahepatic tissue (B). In the liver, the conversion of PA via *N*-acetylation into the harmless *N*-acetyl-PA metabolite occurs mainly in the fast acetylator phenotypes. This effective metabolism allows little HAPA to pass through to the extrahepatic tissues. In contrast, due to expression of a defective NAT-2, the macrophages in the extrahepatic tissue are the predominant metabolising agents of PA in the slow acetylator phenotypes. These macrophages contain the enzymes CYP450, MPO and PGHS-2 which can cause the *N*-oxidation of PA into the reactive metabolite HAPA. Consequently, this can lead to the presentation of HAPA-related neoantigens and subsequent T cells sensitization. These T cells would then secrete cytokines and activate other cells including autoreactive B cells in a fashion analogous to GVH reaction. Adapted from [127] with kind permission from M. Wulferink.

In conformation with this hypothesis, $CD4^+$ T cells from long term PA-treated A/J mice were able to demonstrate a positive response to PA-pulsed macrophages or the reactive metabolite HAPA indicating that within these treated mice the formation and presentation of PA-related neoantigens is sufficient enough to induce neoantigen specific T cells (section 3.2.4-3.2.6). These A/J mice actually developed further signs of autoimmune disease including enlarged spleens with increased B cell populations (section 3.2.3) and increases in the urine protein concentration (section 3.2.2). Alongside these observations, the points described in the following sections (4.3.1-4.3.4) confirm our working hypothesis. Similar symptoms were also found in the pristane-treated mice confirming the development of lupus autoimmunity in mice after pristane oil treatment [147].

4.3.1. PROTEIN REACTIVE PROCAINAMIDE METABOLITES ARE PRODUCED UPON INCUBATION WITH MACROPHAGES

Evidence for extrahepatic oxidation of PA was first shown indirectly by the ability of PA-pulsed WBMC to induce a positive PLN response in BALB/c mice, section 3.1.2. Further and more direct evidence arose from the numerous PA-bound proteins identified in the cytoplasmic fractions of PA-pulsed WBMC using SDS-PAGE techniques section 3.3.1. Indeed PA-bound proteins were not only visible in PA-pulsed WBMC derived from A/J mice but also in the cytoplasmic fractions of PA-pulsed P338 cells, a macrophage cell line (section 3.3.2). Similar PA-protein bands were also observed in the cytoplasmic fractions of WBMC incubated with HAPA indicating that this metabolite could form hapten-protein complexes as previously described [128]. Experiments employing PA-specific $CD4^+$ T cell hybridomas also determined that a certain amount of time was required for WBMC to produce PA-neoantigens, section 3.1.5. In short, these T cell hybridomas only responded to PA-pulsed WBMC after the macrophage sources had been pulsed for more than 4 hours. Furthermore increasing the incubation times amplified the response of the hybridomas suggesting progressively mounting PA-neoantigen concentrations.

4.3.2. APC ARE REQUIRED FOR PA-ANTIGEN PROCESSING AND PRESENTATION

Verification regarding the processing and presentation of these neoantigens by APC was first indicated in the hybridoma assays (section 3.1.3-6). Both BALB/c and A/J procainamide-specific $CD4^+$ T cell hybridomas only reacted to PA-pulsed WBMC when APC were present (section 3.1.3). Moreover, the generated neoantigens were presented within the MHC-class II molecules since blockage of the MHC-II molecules with specific antibodies prevented the hybridomas from responding to the antigen source (section 3.1.4). APC were also necessary in the LTT assays as described in sections 3.2.4-6. In the final LTT assay, B cells were shown to be capable of presenting the PA-neoantigens that subsequently activated $CD4^+$ T cells from PA-treated mice. Simultaneously, this also demonstrated that these responding PA-primed $CD4^+$ T cells were effector cells since they could be stimulated by the presentation of antigen on B cells.

4.3.3. INDUCTION OF AUTOREACTIVE B CELLS BY PA-PRIMED $CD4^+$ T CELLS

The concluding step of the model, that is, the induction of autoreactive B cells by activated T cells, was demonstrated by the production of ANA in untreated recipients after the adoptive transfer of $CD4^+CD25^-$ T cells from PA-treated mice (see section 3.2.12). This step was also indirectly observed by the production of ANA specific for several self-proteins in long term PA-treated A/J mice (section 3.2.11). Both of these points will be discussed further in the following sections 4.5.1 and 4.7.

4.3.4. OTHER MODELS OF PA-INDUCED AUTOIMMUNITY

Conceptually, autoimmunity is the converse of immune self-tolerance, therefore lupus inducing drugs could either act by breaking the tolerance of peripheral T cells to self-antigens or preventing

acquisition of self-tolerance during T cell development in the thymus [156]. The former theory of breaking peripheral tolerance has been demonstrated after the adoptive transfer of Th2 cells which had been modified *in vitro* with DNA-methylation inhibitors. This resulted in an interesting lupus-like pathology of autoantibodies and glomerulonephritis in the recipients [173]. These results, obtained by the group of Richarson *et al*. have been associated with the autoimmune side effects accompanying the GVH reactions upon adoptive transfer of semi-alloreactive T cells [159]. Another model of PA-induced autoimmunity has focused upon the second possibility, failure of central T cell tolerance. In several publications, Rubin *et al*. have shown that intra-thymic injections of HAPA into normal adult mice interfered with the activation threshold of positively selected thymocytes and eventually lead to the export of chromatin-reactive T cells [138-139, 156]. These T cells were then shown to break B cell tolerance in the periphery by assisting in the production of autoantibodies against nuclear compartments. This interesting model of drug-induced lupus incorporates some of the serological features of spontaneous systemic lupus erythematosus, which is characterised by the production of autoantibodies against DNA and other nuclear antigens [156]. Moreover, this model contradicts the normal perception that autoimmune diseases arise through defects in thymic negative selection.

Even though this model leads to the provocative idea that autoimmunity could arise from impaired positive selection, there is doubt that it can be a general model for SLE diseases since it fails to cover the following points raised by Datta [174]. 1) If HAPA can lower the activation threshold of all positively selected thymocytes, why is the autoreactive population skewed towards chromatin? Rubin claims that chromatin is the most abundant autoantigen in the thymus but peptide elution from class II molecules of thymic APC have indicated no predominance of chromatin-derived peptides [139]. Actually, Rubin *et al*. had to add exogenous chromatin to expand and detect chromatin reactive T cell populations from the HAPA-injected mice [138-139]. 2) The autoantibodies produced by the autoreactive B cells in this model do not incorporate the highly pathogenic varieties seen in SLE due to the lack of intrinsic B cell hypersensitivity that occurs in humans with SLE diseases [149]. The involvement of B cells is probably crucial since they may be responsible for sustaining and amplifying autoimmune T helper cells [149, 175] and recruiting T cells into lesions [176]. Further questions include, 3) why are the thymocytes of the HAPA-injected animals not deleted during subsequent transit through the medulla, despite their lower threshold? and 4) in a physiological context, how does HAPA enter the thymus? Regardless to those questions both the model described in this thesis and the model from Rubin indicate the involvement of autoreactive T cells induced by HAPA *in vivo* and the subsequent stimulation of autoreactive B cells.

4.4. THE ROLE OF THE INNATE IMMUNE SYSTEM IN DRUG-INDUCED LUPUS

From the literature it appears that immunotoxicologists have mainly focused upon the adaptive immune system in xenobiotic-induced adverse reactions [136]. Essentially in this response, antigen is processed and presented in the context of MHC-class II to helper T cells by APC. In turn, these cells stimulate B cells to differentiate and proliferate into antibody producing plasma cells and/or stimulate cytotoxic T cells that recognise the same antigen although in the form of peptides presented by MHC-

class I instead of MHC-class II. In many respects, the research performed in this thesis has followed this line of thought. However, research has now realised that both the adaptive and innate immune systems work hand in hand. Gene rearrangement allows adaptive responses to occur but this ability is absent in cells of the innate system. Thus, these latter cells can only respond to stimuli that have been encoded in an organism's DNA [177]. The types of constant structures which stimulate these cells are lipopolysaccharides that are present in the cell walls of many bacteria, viral hypomethylated DNA and several non-peptidic phosphoantigens that are derived from various infectious agents [177, 178]. These alternative antigens can also stimulate the γδT cells in a non-MHC-dependent manner through the CD1 molecules and evidence for γδT cells in drug reactions have been observed by γδT cell clones derived from idiosyncratic drug-treated patients [179].

In mammals, it is proposed that the innate immune system may determine whether the adaptive immune system will respond to a stimulus with tolerance or an active immune response [177]. In fact, the innate system may deliver the danger signal described in section 1.6, especially when one considers that in most experimental situations an antigen produces little response unless it is co-immunised with lipopolysaccharide based adjuvant [3]. This may even account for the ability of pristane to induce a lupus-like syndrome [147, section 3.2]. Consequently, one has to consider the involvement of the innate immune system in idiosyncratic drug reactions. For example, since there are no memory cells in the innate system there is no rapid anamnestic response and therefore this could explain the perplexing time course of clozapine-induced agranulocytosis upon re-exposure [178]. Based on the current research in chemical and cellular immunological concepts, three possible (or complementary) pathways of immune activation can be conceived to be involved in the pathogenesis of drug hypersensitivity reactions and these are depicted in figure 4.4. In pathway A, the reactive metabolite provides the antigenic stimulus but the danger develops from the oxidative stress or damage that arises whilst the binding process occurs [157]. Alternatively, Uetrecht has proposed a model in which the reactive metabolite could provide the antigenic stimulus whilst danger is derived from an exogenous source such as surgery, pathway B [113]. Actually, open heart surgery, which should clearly produce a danger signal, appears to increase the risk of procainamide-induced agranulocytosis by a factor of 10 [180, 181]. Finally, as mentioned before in section 4.2, the parent form of the drug can provide the antigenic stimulus through direct non-covalent binding and the danger in this situation could arise from either of the other two pathways [179]. The more prominent pathway will be essentially dependent on both the drug (it's structure and whether it undergoes bioactivation) and the patient (genetic disposition and concomitant diseases).

Therefore, if one was to substitute procainamide into this danger concept model, it would imply that if a small amount of HAPA is produced then no response would occur, this would be the situation in fast acetylator phenotypes [127]. However, if an abundance of HAPA is formed (slow acetylator phenotype), the metabolising macrophage or neutrophil would undergo apoptosis either because of senescence or because the reactive metabolite leads to apoptosis. The PA-neoantigens would then be processed and presented as haptenised peptides to T cells in the absence of signal 2. Presentation in the absence of signal 2 is expected to lead to immune tolerance to the drug or more accurately, tolerance to the reactive metabolite of the drug acting as hapten (bottom APC in figure 4.4).

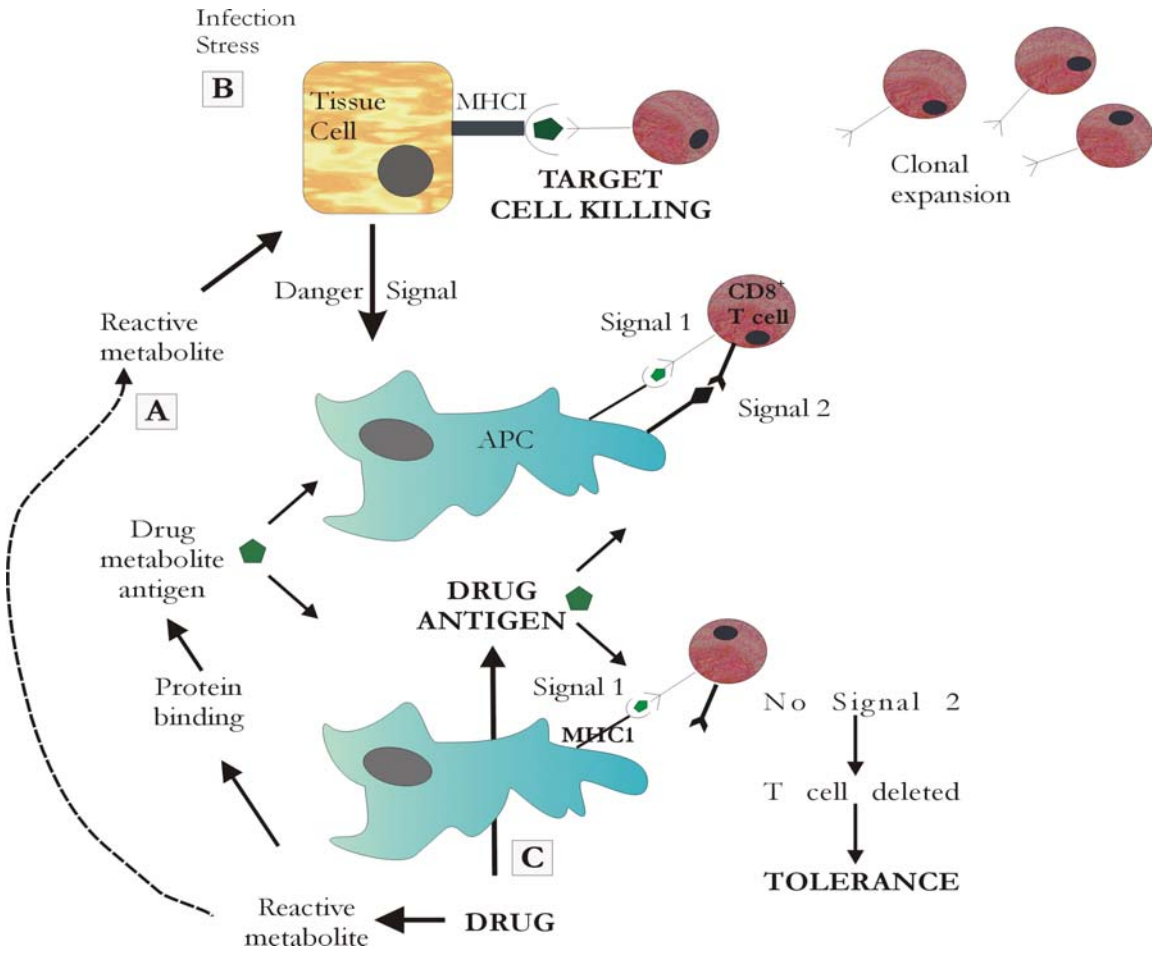


Figure 4.4 Three possible pathways in which drug metabolites may initiate adverse reactions.
A) A reactive metabolite may provide the antigenic stimulus through covalent binding, while the danger signal is provided by the oxidative stress and /or cellular damage resulting from binding of the reactive metabolite [157] B) A reactive metabolite may provide the antigenic stimulus, while danger is derived from an exogenous stimulus such as infection or surgical trauma [113]. C) the parent drug may provide the antigenic stimulus through a direct non-covalent interaction with MHC and the danger signal may derive from a chemically reactive metabolite, infection or surgical trauma [179]. Adapted from [157] with kind permission from M. Pirmhamed.

If the reactive metabolite is more cytotoxic, either because of the amount formed or simply its reactive nature like HAPA or nitroso-PA, it may lead to cell stress or necrosis and consequently a danger signal and upregulation of costimulation (signal 2) on APC (central APC on figure 4.4). In the danger model, the danger signals are endogenous and are derived from cells either stressed or undergoing necrotic cell death [41]. In dispute of this theory, recent *in vivo* experiments have shown that cells dying from either apoptosis and necrosis release endogenous signals that stimulate cytotoxic T cells responses [182]. Theoretically, apoptotic cells may also produce dual functioned endogenous signals such as the cytokine TNF-α, which may act as a danger signal [183] and/or a mediator of apoptosis [184]. Although various molecules have been proposed, the absolute nature of the danger signal(s) have not been defined and with respect to drugs much more research is required [157].

The danger theory may also explain why drug hypersensitivity reactions are more common in patients with certain concomitant viral infections. The higher frequency of allergic drug reactions

during viral infections such as Epstein-Barr virus and HIV have been well established [185]. These viral infections may result in elevated levels of cytokines and cell surface markers that increase the secondary signals and thereby act in concert with the drug antigen resulting in amplification of the drug's potential to cause an immune reaction. Alternatively cells of the innate immune system may detect cell stress and produce cytokines or other factors that could up regulate costimulation (signal 2), or the innate system may provide signal 2 by directly stimulating helper T cells. A final, and as yet unproved theory, is that cells of the innate immune system could detect a danger signal on cells that have been modified by reactive metabolites. Consequently, these cells would then become target cells, either because they formed a large amount of reactive metabolite or because they were more sensitive to the toxic effects of the reactive metabolite. The cells of the innate immune system could then directly induce apoptosis in the affected target cells.

4.5. FIGHTING OURSELVES – AUTOANTIGENS AND AUTOREACTIVE T CELLS

Immunology is based on the notion that an immune response is initiated when cells of the immune system clonally expand and differentiate upon antigen recognition [3]. This paradigm, when applied to autoimmune disease has focused attention on the search for the antigen or epitope that initiates and/or perpetuates the autoimmune response. Indeed, the literature has described a plethora of candidate autoantigens found in both experimental animal models and in humans with autoimmune diseases [186]. The search for these autoantigens as well as those involved in drug-induced autoimmune reactions is extremely important, especially when focusing upon possible clinical therapies that would target the autoantigen-specific T cells. Such therapies have been hindered by the unknown autoantigens and this directly applies to the research encompassing procainamide.

There are two possible explanations on how autoimmunity may arise, the first involves viral or microbial infections that can trigger the initial processes by initiating a response that is cross-reactive with a host antigen [187]. This is termed molecular mimicry. Alternatively, anti-self reactive B and T cells that are normally silent under the control of regulatory mechanisms are activated upon the onset of autoimmune disease. This second possibility may account for the results described in sections 3.2.12, since $CD4^+CD25^-$ T cells from chronically PA-treated donors were capable of inducing ANA directed towards self-antigens in untreated syngenic recipients. The constant presence of auto- or neoantigens is thought to be necessary for the continuation of autoimmune diseases. This notion would correlate to the regression of PA-induced adverse immune reactions after the removal of drug therapy [156]. This is actually the case with most idiosyncratic drug reactions [136]. Autoantibodies from patients or animal models have been used to screen appropriate expression libraries that are derived from tissues stained by those autoantibodies leading to the identification of specific autoantigens [188, 189]. Some of these candidate autoantigens have then been shown to transfer the disease pathology into naïve recipients. A possibility to identify PA-neoantigens is perhaps by mapping the autoantigenic epitopes identified by the $CD4^+CD25^-$ T cells from long term PA-treated donors. Similar research has prevailed in this manner after elucidating the autoantigens present in T cell mediated autoimmune diseases analogous to multiple sclerosis and type 1 diabetes [190, 191]. Recently, more evidence implicating the pathogenic role for autoantigens has been demonstrated after the initiation of autoimmune encephalitis by the transfer of antigen-specific human autoreactive T cells

into HLA-transgenic RAG-2$^{-/-}$ mice (devoid of T and B cells). In a fashion, this correlates to the induction of ANA in the untreated recipients after the adoptive transfer of $CD4^{+}CD25^{-}$ T cells from xenobiotic-treated donors (section 3.2.12).

4.5.1. EPITOPE SPREADING IN IMMUNE-MEDIATED DISEASES

Other major questions that arise are whether drug-induced autoimmune diseases are initiated by a primary antigen, whether other neoantigen epitopes are involved in the perpetuation of the disease and if possible treatment requires the identification of one or all of the neoantigens. Although none of these questions have been addressed within this thesis, models of induced autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), have provided direct access to the role and relationship of individual autoantigenic epitopes. A phenomenon that is generically termed "EPITOPE SPREADING" has been observed in EAE and is thought to be initiated as a result of tissue damage and in figure 4.5 epitope spreading is demonstrated in the context of hapten-protein complexes. Epitope spreading is the diversification of T and B cell responses to a particular antigen or group of antigens over time, where the specificity of determinants that are recognised increase from simple and restricted to complex and more diverse. Epitope spreading may occur either within a single antigen, within different antigens from a single macromolecular complex or within different antigens that are not physically linked but colocalise to a particular anatomical site. Despite the difficulties in studying this phenomenon in humans, numerous observations have shown epitope spreading in human autoimmune diseases [192]. Another issue is whether the determinant spreading involves T cells activated in the peripheral organs or whether diversification occurs after infiltration of the initiating T cells into the target organs. In EAE, the issue of epitope spreading is even more interesting since this is a relapsing disease and each relapse may reflect autoaggression to new epitopes that are exposed by the determinant spreading [193, 194].

The fact that autoreactive T cells are part of the normal peripheral repertoire raises important questions including the extent that determinant spreading depends on tissue damage and more importantly what breaks their tolerance. As mentioned above, the induction of ANA in recipients after the adoptive transfer of PA-primed $CD4^{+}CD25^{-}$ T cells occurred in the absence of procainamide and therefore the initiating antigen. In accordance to such studies as those performed by Mamula et al., [195], this heavily insinuates a role for epitope spreading. This earlier study described a mechanism for breaking T cell tolerance to a dominant autoantigen of SLE namely the U1 small nuclear ribonucleoprotein particle (snRNP). They demonstrated that T cells specific for self-snRNP were a component of the T cell repertoire but were neither thymically deleted nor irreversibly anergic. Additionally, it was demonstrated that when a B cell was elicited with a foreign cross-reactive antigen, it also presented self-snRNPs and consequently autoreactive T cells were triggered. Therefore, autoreactive B cells were considered to play a pivotal role in systemic autoimmune diseases since they did not only produce ANA to lupus autoantigens but at the same time recruited autoreactive T_H cells. Theoretically, by acting as APC, autoreactive B cells may diversify T cell responses and allow for determinant spreading.

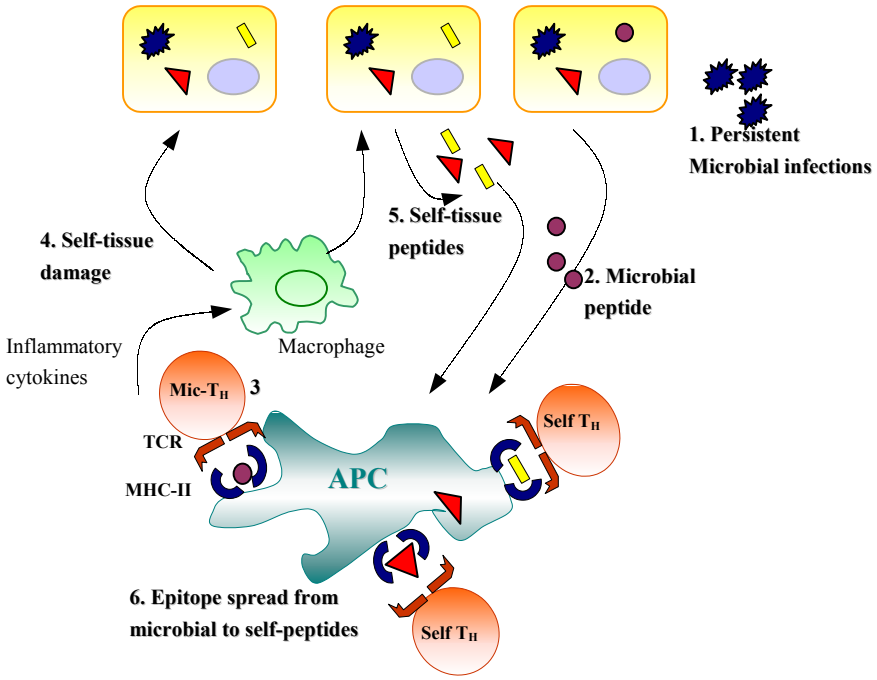


Figure 4.5. Epitope spreading inducement by hapten-self-protein complexes.
Persistent hapten-complexes (1) could initiate the activation of hapten-specific T cells (2,3) which would mediate possible tissue damage (4). This might lead to the release of self-peptides (5) which would be engulfed by APC and presented to self-reactive T_H cells (6). Continual damage and release of self-peptides would result in the spread of the self-reactive immune response to multiple self-epitopes.

In the context of xenobiotic-induced adverse reactions, T cell dependent polyclonal B cell activation has also been detected in both rat and mouse models of metal ion-induced autoimmunity [196, 197]. In the mercury model, "Hg-specific" T cell reactions have been shown to provide B cell help in the presence of Hg(II) [198]. Furthermore, one week after $HgCl_2$ administration, treated bulk T cells responded in an anamnestic fashion to the nuclear self-protein fibrillarin complexed with Hg(II), whereas after eight weeks of treatment there was a predominant autoreactive T cell response to untreated fibrillarin [198]. Collectively these results suggest that with time there was a determinant spreading of T cell reactivity from Hg(II)-modified fibrillarin to the native nuclear self-protein. Consistent with that hypothesis, when individual $CD4^+$ T cell hybridomas generated from $HgCl_2$-treated autoimmune mice were tested for their specificity, some responded to Hg-complexed fibrillarin while others responded to the native protein [140].

4.5.2. CRYPTIC PEPTIDES

Whereas the findings with the mercury mouse model suggest that mercury can induce T cell responses to self-proteins via determinant spreading, studies with gold(I) salts have indicated that the presentation of such proteins occurs immediately [84, 92, 198]. In either case, cryptic self-peptides are thought to be targets of "metal-specific" T cells. As described in section 1.8, cryptic peptides may arise when the binding of the hapten to a self-protein influences the processing of this carrier [88, 89]. These "foreign" cryptic self-peptides are not normally presented [91], therefore T cells are not tolerant towards them and would react upon encounter. T cells specific for cryptic peptides have not been

identified in PA-induced autoimmunity but have been demonstrated using $CD4^+$ T cell hybridomas which were raised solely against Au(III)-pretreated ribonuclease A. These hybridomas recognised two cryptic peptides of ribonuclease A and displayed cross-reactivity when incubated with ribonuclease A that had been treated with palladium (II), palladium (IV), nickel (II), or platinum (IV) ions [84]. Employing physico-chemical methods, an identical conformational change was elucidated in the ribonuclease A molecule upon treatment with the "cross-reacting" metals suggesting that this molecular denaturation gave rise to altered processing and the presentation of an identical cryptic peptide [84].

4.5.3. PROCAINAMIDE-NEOANTIGEN IDENTIFICATION

A major part of this thesis work was the elucidation of PA-neoantigens. As mentioned in section 1.4, a major difficulty in studying T cell reactions to xenobiotics is that, apart from a few exceptions [87, 89] most neoantigens are unknown. From the generated $CD4^+$ T cell hybridomas discussed in section 3.1.3-5, it was established that neoantigen(s) created by PA-pulsed WBMC could elicit a response from these hybridomas. Since further elucidation using these hybridomas was not possible, identification of these neoantigens was continued with molecular biology techniques. Using a PA-specific antibody, several PA-bound proteins of different molecular weights were identified in both PA-pulsed WBMC and P338 cells, sections 3.3.1 and 3.3.2 respectively. Similar and even more PA-specific bands were identified in A/J derived WBMC, spleen cells and PMφ that had been pulsed with HAPA, section 3.3.1. One of these HAPA-induced bands was approximately 35kDa in weight and correlated to the previous studies that showed a 35kDa PA-protein band using PA-pulsed PMφ from casein-treated C57BL/6 mice [127]. This 35kDa band was also detected when the macrophage cell line P338 was pulsed with either PA or HAPA (section 3.3.2).

There are numerous cytoplasmic proteins that reside around the 35kDa molecular weight such as fibrillarin. One dimensional SDS-PAGE cannot distinguish between proteins of the same molecular weight, therefore to identify these PA-specific proteins further, 2D gel electrophoresis was employed and these results are described in detail in section 3.3.3. Using the cytoplasmic fractions of the PA-pulsed P338 cells an enormous array of proteins around the 35KDa range could be seen which will be characterised in the future.

4.6. MANY NEEDLES IN ONE HAYSTACK – ANA DETERMINATION IN PA-INDUCED AUTOIMMUNITY

In autoimmune diseases, autoantibodies can be the actual pathogenic agents of the disease, as in autoimmune haemolytic anaemia; they may arise as a consequence of another process, for example antibodies which may react with heart muscle or tissue damage or they maybe harmless footprints of an etiologic agent while they themselves do not cause damage. In both the PA- and pristane-treated A/J mice, antinuclear autoantibody (ANA) development was initially determined via HEp-2 cell slide analysis, section 3.2.9. Attempts to clarify the specificity of these ANA was then performed using HEp-2 cell slides and specific ELISA assays, section 3.2.9 and 3.2.11. Although analysis of the PA-

treated mice was the focal point of this study, the pristane-treated mice provided the opportunity to study the ANA development in the same mouse strain treated with another ANA-inducing xenobiotic under the same conditions.

Pristane is an oil based substance that induces a non-specific activation of the immune system several months after injection [155]. The group of Reeves has provided the majority of the interesting data regarding *in vivo* pristane treatment. Research from this group has shown that different mouse strains develop different ANA specificities after four months of treatment [147, 148]. These ANA include anti-snRNP, anti-Sm, anti-Su, anti-ribosomal P and anti-DNA/chromatin. Furthermore, the developing range of ANA depends on the mouse strain and subsequent work from this group has proposed that the different subsets of ANA that arise during pristane treatment are regulated by cytokine stimulation and/or Fas signalling [148]. In this study, the A/J mouse strain was shown for the first time to produce ANA during pristane treatment (see section 3.2.7). As observed in the research of Reeves, these mice developed ANA in high titres and the sera were specific towards a broad variety of self-proteins. In fact nearly 100% of the treated mice showed specificity towards the proteins snRNP Protein A, smRNP and Ku (see section 3.2.11). These sera did not react to PA and/or HAPA in the ELISA assays described in section 3.2.14., indicating that the PA-treated mice, which had equally induced ANA titres, developed specific antibodies to those compounds. On a different point, bulk T cells or isolated $CD4^+$ T cell from pristane-treated mice did not respond to any of the PA-related neoantigens in the LTT assay, nor did they react to DNBS (section 3.2.4). Minimal responses were observed with hsp60 or spleen cells that had been previously incubated with pristane. However, the barely significant LTT stimulation indices indicate that these T cells would be perhaps more reactive to other antigens. An interesting point is that although the sera from these treated mice responded to Ku, histone and smRNP in the ELISA assays, T cells from these mice did not react to these antigens in the LTT, indicating that the T cells may react to altered self-proteins or, as mentioned above (section 4.5) to cryptic epitopes of these proteins.

Although ANA development has already been demonstrated in PA-treated A/J mice [133], the characterisation of these ANA was left unresolved. Using HEp-2 cells, a single staining pattern could not be defined for the all the PA-treated sera but the most frequent patterns were those corresponding to centromere bodies (see section 3.2.9). This was a rather unexpected result since the majority of studies have shown that both patients and animals that were treated with PA develop histone antibodies. The low number of nuclear homogenous stainings patterns (characteristic of histones) was confirmed by the small amount of sera (16%) responding to histones in the ELISA assays (see section 3.2.11). Nevertheless, this anomaly could be in accordance to the epitope spreading phenomenon, which proposes that as time progresses the dominant epitopes also change. Histone specificity was not tested in the sera obtained from earlier months and therefore, a possibility remains that these ANA would be prominent at a different time point. Similar observations have been shown in lupus mice whose spontaneous autoantibody specificity changes from nucleosomes to histone as they age. *In vitro*, $CD4^+$ T cells but not $CD8^+$ T cells from these mice also induced different autoantibody production when incubated with APC and nucleosomes [199] indicating that the nucleosomes were engulfed by the APC and presented self-peptides of different specificities.

In comparison to the pristane treatment, the sera from PA-treated mice also displayed a broad range of self-protein specificity in the ELISA assays with the largest portion corresponding to snRNP protein A, see section 3.2.11. Furthermore, using the HEp-2 cells as antigenic material, antibodies were identified in the sera of these PA-treated mice but not in the untreated controls, section 3.3.4. Although, several different antibodies could be detected within the individual sera, a portion of these sera displayed a common antibody specific for a protein located at the 35kDa range (section 3.3.4). Therefore, one could assume that the 35kDa protein detected by this antibody is the same antigen that was observed using a PA-specific antibody in the HAPA-pulsed macrophages (section 3.3.1, 3.3.2).

4.7. THE ROLE OF REGULATORY T CELLS IN XENOBIOTIC-INDUCED AUTOIMMUNE DISEASES

Regulatory T cells have become a hot topic over the last decade with multiple findings producing an ever increasing complex picture. In accordance to the current literature, the role of regulatory T cells in PA-induced autoimmunity has not been investigated. Actually, only a few research teams have investigated the potential role of these cells in xenobiotic models [200, 201]. From the adoptive transfer assay performed in section 3.2.12, the question arose as to why the PA-primed $CD4^+CD25^-$ T cells failed to raise the titres of induced ANA in the treated recipients. Explanations for this maybe that the xenobiotic treatment *per se* induced sufficient T cell help for the recipients' autoreactive B cells or that the PA-primed $CD4^+CD25^-$ T cells may have induced optimal help so that extra treatment was irrelevant. Alternatively one might consider the possible regulatory activities of $CD8^+$ T cells in the recipient mice. $CD8^+$ T cells are currently thought to mediate antigen-specific immunosuppression by either killing $CD4^+$ T helper cells [74, 202] or APC [203], or by non-cytolytic pathways that have not yet been defined. Subsets of $CD8^+$ T_{reg} cells have been demonstrated to act upon $CD4^+$ T cells in both antigen-specific and non-specific mechanisms [204]. $CD8^+CD28^-$ T_{reg} cells have recently become a focal point of transplantation immunology [75, 76] and appear to interfere with the maturation of immature DC by preventing their expression of the costimulatory molecules CD80 and CD86 [205]. These regulatory cells further induced the expression of the inhibitory ILT3 and ILT4 (immunoglobulin-like transcript 3 and 4) molecules on DC [205]. Both these ILT molecules contain an inhibitory ITIM motif in their cytoplasmic domains and mediate inhibition of cell activation via recruitment of the modulatory tyrosine phosphatase SHP-1. The resulting $CD8^+CD28^-$ T_{reg}-induced DC, with the phenotype $CD80^-CD86^-$ $ILT3^+ILT4^+$, (of donor origin) were then shown to anergize, in a HLA-restricted manner, alloreactive $CD4^+$ T helper cells (of recipient origin) in tissue culture [205]. Therefore, these allogenic DC are proposed to act, in a non-specific manner, as bridges for cross-talk between the T cell subsets.

Another group of $CD8^+$ T_{reg} cells were shown to be induced by *in vivo* vaccination with autoreactive $CD4^+$ T cells. These $CD8^+$ T_{reg} cells then specifically deleted autologous $CD4^+$ T cells expressing V beta 8 TCR, implying a role for the Qa-1 molecule [74]. On consideration of the latter $CD8^+$ T_{reg} activity, one could envisage that these cells were responsible for the moderate ANA production induced after the transfer of into the untreated recipients see section 3.2.12. Although there is no evidence for this here, the recipient CD8 T_{reg} cells could have dampened the transferred ANA-

inducing PA-primed $CD4^+CD25^-$ T cells since the former are known to counter-regulate Th2 responses [211].

4.7.1. A DAY OFF FOR CD8 T_{REG} CELLS

The PA adoptive transfer assays, (section 3.2.17 and 19) were the first to show that ANA in PA-treated recipients could be prevented by PA-treated $CD4^+CD25^+$ T cells. Although not contained in the main body of this thesis, identical adoptive transfer assays were performed using the xenobiotics gold sodium thiomalate (GST) and mercuric chloride ($HgCl_2$). These experiments are part of the submitted paper "Drug-induced autoantibody formation in mice: triggering by primed $CD4^+CD25^-$ T cells and prevention by $CD4^+CD25^+$ T cells" [208]. Similar to the PA adoptive transfers, $CD4^+CD25^+$ T cells from these treated donors showed suppression of ANA in treated recipients whilst primed $CD4^+CD25^-$ T cells initiated ANA in untreated recipients. Within these assays, isolated $CD8^+$ T cells from the treated donors were also transferred but curiously, these cells failed to initiate or prevent ANA production in either untreated or treated recipients. This observation is somewhat contradictory to earlier data regarding CD8 T_{reg} cells in the rat $HgCl_2$ model [200, 201] and to a great variety of different autoimmune mouse models in which $CD8^+$ T suppressor cells have been demonstrated [74].

Interestingly, a recent observation has shown that $CD8^+$ T cells are involved in neonatal tolerance to mercuric salt-induced Th2 autoimmune disease. This tolerance was further demonstrated to be transferable into naivesyngeneic recipients [201]. These $CD8^+$ T cells also expressed CD25 and are thought to inhibit the proliferation of activated $CD134^+$MHC-class II^+ T cells [201]. However, when trying to reason why $CD8^+$ T_{reg} cells do not play a role in the ANA suppression here, one must consider where the action of the $CD8^+$ T_{reg} cells has been reported. One particular subset has been demonstrated, as mentioned above, in neonatal circumstances [201]. Another subset of $CD8^+$ T_{reg} cells have been reported to play a role in oral tolerance [70, 71] but are considered to be required only for local intestinal suppression [72] and not for systemic hyporesponsiveness [70, 73]. A third subset of $CD8^+$ T_{reg} cells have been associated not only with the suppression of $CD8^+$ T cells during an influenza infection but also with long term acceptance of allogenic kidney transplants [208]. These IL-10 producing $CD8^+$ T_{reg} cells can be generated after one round of stimulation by dendritic cells and require antigen specific restimulation to deliver the immunosuppression through the production of IL-10. Currently, this subset is considered to be the most related to $CD4^+CD25^+$ T cells since they are anergic, their generation depends on IL-10 and they suppress primary T cell responses by IL-10 but not TGF-β [208].

4.7.2. RECENT UPDATES ON THE MECHANISMS OF $CD4^+CD25^+$ T CELLS

A current hypothesis regarding the regulatory mechanism of $CD4^+CD25^+$ T cells is that they require cell-cell contact and express the costimulatory molecule CTLA-4 [52, 59, 60]. In the LTT assay, described in section 3.2.4-6, not only did we show that the sole responding T cells were of $CD4^+$ origin but that APC (and finally B cells) were an absolute requirement. Research has shown that the cell-cell contact mechanism by $CD4^+CD25^+$ T cells may use APC as platforms [52, 56] and if one is to

assume that within the system described here epitope spreading is the actual mechanism of ANA induction, then the presentation of self and altered self-peptides on the MHC-molecules of APC combines both of these factors. $CD4^+CD25^+$ T cells that constitutively express CTLA-4 can regulate autoimmune responses by TGF-β production [60, 61]. Recently, Wang *et al*. demonstrated that blockage of CTLA-4 *in vivo* enhanced the ability of autoreactive T cells to provide help to B cells. Within their experimental autoimmune myasthenia gravis (EMAG) murine model they conclude that the enhanced B cell function may induce autoantibody production and APC might contribute to disease development and enhance T cell determinant spreading [209].

One important clue to how ANA could be downregulated in the recipients after the transfer of PA-primed $CD4^+CD25^+$ T cells is the postulation by Bystry *et al*. that B cells recruit $CD4^+CD25^+$ T_{reg} cells by secreting the chemokine CCL4. In fact the receptor for this chemokines, CCR5, is predominantly located on $CD4^+CD25^+$ T cells and not $CD4^+CD25^-$ T cells. Within their investigation they showed that $CD4^+CD25^+$ T cells recruited by activated CCL4 secreting B cells were able to directly suppress the B cells and in turn the IgG1 specific autoantibody production [58]. Interestingly, this suppression of autoantibodies was abolished by either the removal of $CD4^+CD25^+$ T cells or anti-CCL4 treatment indicating a direct relationship between activated B cells and regulatory cells. In confirmation to this theory, a very recent study using the inflammatory bowel disease (IBD) mouse model has shown a direct contact between T_{reg} cells ($CD4^+CD25^+$), T effector cells ($CD4^+CD45RB^{high}$) and $CD11c^+$ cells, not only in the lymph nodes but also at the infected tissue site [210]. In a different transgenic system, anti-DNA antibodies have also been shown to be inhibited by $CD4^+CD25^+$ T cells [211] indicating that these regulatory T cells can regulate antibody responses against self and non-self antigens and may exert their effects by a direct inhibitory effect on B lymphocytes [58] or via inhibition of T_H cell differentiation.

As a final point, earlier studies have claimed that there is a fine balance between autoreactive T cells and the T_{reg} that control them [49, 63]. This general explanation may not only account of the ANA development in untreated recipients after the transfer of PA-primed $CD4^+CD25^-$ T cells but also the suppression of ANA formation in treated recipients by $CD4^+CD25^+$ T cells. This latter point also covers the partial suppression observed in the cross-transfer experiment (section 3.2.15). For this explanation, the donor and recipient treatment will be referred to as xenobiotic(I) and xenobiotic(II) respectively. Hypothetically, within the xenobiotic(I)-treated donors, $CD4^+CD25^+$ T_{reg} arise not only with specificity to xenobiotic(I)-neoantigens but also self-antigens. Upon transfer into xenobiotic(II)-treated recipients, these primed xenobiotic(I)-$CD4^+CD25^+$ T cells not only swing the balance of autoreactive T cells versus T_{reg} cells towards the latter (since $CD4^+CD25^+T_{reg}$ can respond to peripheral neoantigens [63]) but also suppress developing xenobiotic(II)-induced neoantigens. The second mechanism may occur via the recruitment of these injected T_{reg} cells by the above described CCL4 secreting B cells [58].

4.8. CD25 OR NOT CD25 – THAT IS THE QUESTION

In 1995, Sakaguchi and colleagues discovered that the expression of the IL-2 receptor in $CD4^+$ T cells conferred the ability of these cells to provide autoimmune disease protection. Since then abundant studies have shown that these cells can suppress a variety of autoimmune diseases [reviewed in 47, 48,52,]. As a more detailed analysis of these $CD4^+CD25^+$ T cells arises, it becomes more and more apparent that although these cells are able to confer a substantial amount of suppressive abilities, the expression of CD25 might not hold the key to a regulatory phenotype. These recent doubts stem from research performed by a number of groups including Sakaguchi. They find that although CD25 is a common marker, these cells must also express the glycoprotein GITR, the $\alpha_E\beta_7$ intergrin, and CTLA-4. In fact, these markers fail to distinguish between regulatory cells, effector cells or memory cells.

Very recently, Sakaguchi and colleagues have re-examined the expression of $CD4^+$ T_{reg} cells and found a new marker [151]. Fox*p3* has become the new hot star in the world of immunoregulatory markers. This gene is expressed in the thymus and periphery and contained only in $CD4^+CD25^+$ T cells. A minute amount was found in the $CD4^+CD25^-$ T cell fraction but was further isolated to the $CD4^+CD45RB^{low}$ fractions which have also been shown to have regulatory abilities in the IBD model [59, 66]. Sakaguchi's group have now found that a disregulation in the Fox*p3* gene initiates autoimmune diseases and have proposed that this gene is the regulatory marker [151]. This exciting research also showed that the transduced expression of this gene into naïve $CD25^-$ T cells could swing these cells into a regulatory phenotype which have the same capabilities to prevent autoimmune induction *in vivo* as $CD4^+CD25^+$ T cells. The functional capacity of $CD4^+CD25^+$ T cells has also been shown to be dependent on the surface expression of certain markers. For example, the simultaneous expression of GITR and $\alpha_E\beta_7$ intergrin swings them into IL-10 producing cells whereas when GITR is removed, these cells secrete TGF-β [212]. This latter group further showed that the removal of both of these markers reduced these cells back into a non-regulatory phenotype indicating that CD25 did not play a role at all. Another group have speculated that $CD25^+$ T cells can induce $CD25^-$ T cells to produce IL-10 and that this cytokine suppresses the naïve $CD4^+$ T cells [214].

Further conflicting data over the role of CD25 expression has stemmed from a number of reports that $CD4^+CD25^-$ T cells can act in exactly the same way as $CD4^+CD25^+$ T cells [214]. Much research surrounding autoimmune gastritis and EAE have shown that both of these diseases can be prevented *in vivo* with either $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells. Apostolsol *et al*. found that $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells can have suppressive effects to the same antigen depending on where the antigen is presented. They suggest that suppressive $CD25^+$ T cells mature in the thymus whereas $CD25^-$ T cells mature in the periphery [214]. However, these groups have not deciphered the exact expression of the surface markers on these cells, therefore the presentation of GITR, integrins or other molecules may indeed influence which subset is involved. In the adoptive transfer assays performed within this study, no further analysis of the expression of certain surface markers on the transferred cells was performed. Nevertheless, we could clearly identify the different suppressive or inductive abilities of the $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets, indicating that here, the expression of CD25 indeed played a role in suppressing xenobiotic-induced ANA formation.

4.9. CONCLUDING WORDS

In the research field of immunotoxicology, it becomes more apparent that abundant xenobiotics can initiate autoimmune diseases in multiple ways. In the case of PA and gold(I) salts there appears to be some overlap in their inductive mechanisms since the reactive metabolites have been shown to play a crucial role. In the case of pristane however, reactive metabolites have not been considered so far. These examples show that xenobiotics may produce similar immune responses but via different mechanisms. There are currently six possible mechanisms proposed for xenobiotic-induced autoimmune diseases: 1) the xenobiotic could elicit an antigen-specific immune response that may result in a polyclonal B cell activation and the production of autoantibodies; 2) through direct toxic effects on immune or other cells, the xenobiotics could cause impairment of the immune response and/or toxicity, which would result in the release of intracellular constituents and the induction of autoantibodies; 3) molecular mimicry could, due to cross-reactivity between similar structures of the xenobiotics and the cellular constituents, give rise to autoreactive immune cells; alternatively, both the xenobiotic and the host may share molecular epitopes; 4) the xenobiotics may directly interact with the regulatory factors that modify gene activity; 5) the xenobiotic might bind to self-proteins leading to processing alteration and subsequent presentation of cryptic peptides; 6) and finally, the xenobiotics might stimulate the generation of free radicals initiating an inflammatory response which may initiate autoimmunity. Besides the proposed mechanisms above, other factors such as genetic predisposition would also play a deciding role.

To conclude, this thesis aimed towards elucidating the possible mechanisms in which procainamide-induced adverse immune reactions arise. This was achieved by focusing upon the formation of PA-neoantigens (4.5.3, 4.3.1), the processing and presentation of these induced neoantigens (4.3.2), the recognition of the neoantigens by T cells (4.3, 4.5) and the formation of ANA induced *in vivo* after exposure of these neoantigens (4.6). Finally, the role of PA-primed $CD4^+CD25^+$ T cells in preventing ANA formation *in vivo* was studied (4.7), and their abilities were further substantiated by the discovery that these primed T_{reg} cells could prevent ANA formation in alternatively treated recipients see section 3.2.15. This is the first time that $CD4^+CD25^+$ T_{reg} cells have been reported to play a role in xenobiotic-induced autoimmune diseases.

5 Appendix A: Reagents and Chemicals

Acetic acid, glacial 100%	J. T. Baker, Germany
Acetone	Merck, Germany
AgNO3	Merck, Germany
Ammonium persulphate	Merck, Germany
Ammonium sulphate	Sigma-Aldrich, Taufkirchen, Germany
Brefeldin A	Sigma-Aldrich, Taufkirchen, Germany
Bromophenol Blue	Plus One Amersham Biotech
Concavalin A	Sigma-Aldrich, Taufkirchen, Germany
Coomassie Brilliant blue	Sigma-Aldrich, Taufkirchen, Germany
DNBS	Fluka Chemicals, Taufkirchen, Germany
DNFB	Fluka Chemicals, Taufkirchen, Germany
DMSO	Sigma-Aldrich, Taufkirchen, Germany
DTT	Sigma-Aldrich, Taufkirchen, Germany
EGTA	Merck, Germany
Ethanol absolute	J. T. Baker, Germany
Ficoll Plaque	Pharmacia, Freiburg, Germany
Formaldehyde	Sigma-Aldrich, Taufkirchen, Germany
Glycerol	Sigma-Aldrich, Taufkirchen, Germany
Glycine	Merck, Germany
Hydrochloric acid	Merck, Germany
HAPA	A gift from Jack Uetrecht, Toronto, Canada
Freuds Adjuvant (IFA)	Sigma-Aldrich, Taufkirchen, Germany
Ionomycin	Sigma-Aldrich, Taufkirchen, Germany
KCl	Merck, Germany
KH2PO4	Merck, Germany
Methanol	J. T. Baker Germany
NaCl	Merck, Germany
Ha2HPO4.2H20	Merck, Germany
NaOH	Merck, Germany
NaN_3	Sigma-Aldrich, Taufkirchen, Germany
PEG 1500	Boehringer, Germany
PMA	Sigma-Aldrich, Taufkirchen, Germany
Procainamide HCl	Sigma-Aldrich, Taufkirchen, Germany
Pristane	Sigma-Aldrich, Taufkirchen, Germany
Ponceau S	Carl Rith, (Karlsruhe, Germany)
Acrylamide stock solution	AppliChem, Germany
Saccharin	Sigma-Aldrich, Taufkirchen, Germany
Sec-2-butanol	Sigma-Aldrich, Taufkirchen, Germany
Laurly sulphate SDS	Sigma-Aldrich, Taufkirchen, Germany
Sulphuric acid	J. T. Baker Germany
TEMED	Sigma-Aldrich, Taufkirchen, Germany

thymidine	ICN, Eschwege, Germany
Tris (ultra pure)	Merck, Germany
Triton X-100	Sigma-Aldrich, Taufkirchen, Germany
Trizma base	Sigma-Aldrich, Taufkirchen, Germany
Tryptan blue	Flow laboratories, USA
Tween 20	Merck, Germany
VECTASHIELDTM	Vector Laboratories Inc, Burlingame, CA, USA

6 Appendix B: Materials and Special Solutions

Albustix strips	Bayer Diagnostics GmbH, Munchen, Germany
Blotting Paper	Amersham, Pharmicia Biotech Germany
dsDNA	Sigma-Aldrich, Taufkirchen, Germany
Foetal Calf serum	Gibco, USA
Ku monoclonal antibody	Dunn Labors, Germany
HAT	PAA Austria
Histone calf protein	Sigma-Aldrich, Taufkirchen, Germany
HEp-2 coated ANA slides	The Binding Site, UK
Horse serum	Gibco, USA
Hsp-60	Sigma-Aldrich, USA
HT	PAA Austria
Hybond™-C-Super nitrocellulose paper	Amersham Pharmicia Biotech, Germany
Hyperfilm™ ECL™	Amersham Pharmicia Biotech, Germany
IPG buffer	Amersham Pharmicia Biotech, Germany
IPG fluid	Amersham Pharmicia Biotech, Germany
IPG 11cm strips, pH3-11	Amersham Pharmicia Biotech, Germany
Microlance needles, variety of gauges	Microlance, Gemany
Microscope slides	
ONCE syringes	Germany
Penicllin/Streptomycin	Germany
Rainbow markers 800	Amersham Pharmicia Biotech, Germany
RPMI 1640 Medium	PAA, Austria
0.9% Saline	Fresenius, Germany
smRNP antibody	Dunn Labors Germany
snRNP Protein A antibody	Dunn Labors, Germany
TMB	Kectcoh, Denmark
Trypsin/EDTA	Sigma-Aldrich, Taufkirchen, Germany

7 Appendix C: Equipment

Neubauser-Kammer heamocytometer	Germany
DIAPLAN, Light microscope magnification.	Lietz, Germany
MEGAFUGE, centrifuge	Heraeus Instruments, Germany
CASY 1 TT automatic counter	Schärfe Systems GmbH, Germany
Labsonic sonicator.	Braun, Germany
PHD Cell Harvester Model 200A/290	Cambridge technology, MA, USA
96 well plate Harvester	Inotech, Switzerland
Ready Filters with Xtalscint	Beckmann Instruments, CA, USA
Printed filtermat A	Wallac Oy, Finland
Meltilex™ AA, Scintillator sheets	PerkinElmer life sciences, Finland
β-counter: Model LS6000 IC	Beckmann Instruments, Germany
TRILUX,1450 Microbeta counter	Wallac Oy
La Chrom HPLC	Merck, Germany
Gammacell 2000 cell irradiator	Molsgaard, Denmark
Cytoperm Incubators	Heraeus, Germany
96 well plate ELISA reader	Dynex Technologies, Germany
MACS, magnetic cell sorter	Miltenyi, GmbH, Germany
AutoMACS, magnctic cell sorter	Miltenyi, GmbH, Germany
FACScalibur	Becton Dickinson, Germany
-80°C freezers	Germany
3000v Powerpack	Biorad, Germany
pH meter	Germany
Heat block	Germany
CURIY 60 Autoradiography	AGFA, Germany
MiniProtean® II cell	Biorad, Germany
Vertical Slab Gel Unit	Hoefer Scientific Instruments, USA
Transblot™ semi-dry transfer cell	Biorad, Germany
2D gel electrophoresis	Amersham Biotech, Germany

8 Appendix D: Supplements

8.1. FOETAL CALF AND HORSE SERUM

One litre of FCS or Horse Serum (Sigma) were heated to 60°C and centrifuged at 4°C, 1200rpm for 10 mins. Serum was then aliquotted into 25ml or 50 ml portions and stored at –20°C until required.

8.2. PENICILLIN AND STREPTOMYCIN

100ml bottles were aliquotted into 5ml portions and frozen at –20°C until required. One 5ml aliquot was then added to 500ml of naked medium to provide 50IU/ml penicillin and 50µg/ml streptomycin.

8.3. MEDIUMS

8.3.1. SC MEDIUM.

100ml 0.2M Glutamine (x100)
10ml Pen/Strep (10000 U/ml)
100ml 0.1M Sodium pyruvate
100ml non essential amino-acids MEM (x100)

Sterile filtered and controlled for 2 days at 37°C. Frozen in 21ml aliquots at –20°C until required. One aliquot was then added to 500ml RPMI 1640 medium (PAA, Austria) along with 5% FCS and 5ml of (5000U/ml) of Pen/Strep.

8.3.2. TC MEDIUM

59.5ml essential amino acids (x50)
29.8ml non-essential amino acids (x100)
79.4ml sodium pyruvate
6.7g NaHCO3
7.9ml gentamycinsulphate
7.9ml DDW
27µl β-Mercapto-ethanol

After sterile filtration, the above solution was pipetted into 48ml aliquots and frozen at –20°C until further required. One aliquot then provided supplement for one 500ml bottle of RPMI 1640 medium (PAA, Austria), along with 5% FCS and 5ml of (5000U/ml) Pen/Strep.

8.3.3. NAKED RPMI 1640 MEDIUM

For the fusion of hybridomas, the preparation of antigens and the labelling of thymidine, naked medium was employed. Therefore, medium without supplements was used.

8.3.4. HAT MEDIUM

500ml of TC medium was supplemented with sterile aliquots of HAT (x50) so that the end concentration was 100µM hypoxanthine, 0.4µM aminopterin and 16µM thymidine

8.3.5. HT MEDIUM

500ml of TC medium was supplemented with sterile aliquots of HAT (x50) so that the end concentration was 100µM hypoxanthine and 16µM thymidine

8.3.6. P388 MEDIUM

For the culture of P338 cells, 500ml of SC medium was supplemented further with 10% prepared horse serum.

9 Appendix E: Buffers and Solutions:

9.1. CELL IMMUNOLOGY PROCEDURES

9.1.1. PBS (10X)

80g $NaCl_2$ (132mM)
2g KCL (2.7mM)
11.5g $Na_2HPO_4.2H_20$ (4.3mM)
2g KH_2PO_4 (1.4mM)
Add approximately 900ml DDW. Mix to dissolve. Adjust pH to 7.3 with NaOH. Make up to a final volume of 1000ml. Filter under sterile conditions. Store at 4°C

9.1.2. PMA

1mg/ml stock solution dissolved in DMSO and stored at 4°C

9.1.3. ACT

2.06g Tris (17mM)
8.55g NH_4Cl (0.16M)
Add approximately 600ml ddw, adjust the pH to 7.2 and make to 1 litre stock, sterile filter.

9.1.4. FREEZING MEDIUM

10%DMSO, 20%FCS 25% RPMI 1640 naked medium

9.1.5. CASY BUFFER (X5)

39.65g/l NaCl
1.9g/l EDTA
2g/l KCl
0.95g/l NaH_2PO_4
9.75g/l Na_2HPO_4 x$2H_2O$
1.5g/l NaF (sodium fluoride)
After diluting to a x1 stock, buffer was sterile filtered and stored at 4°C. For use, buffer was warmed to room temperature.

9.1.6. BSS (BALANCED SALT SOLUTION)

BSSI: BSS II :

10g	Glucose	1.86g	$CaCl_2.2H2O$
0.6g	KH_2PO_4	4.0g	KCl
2.38g	$NaHPO_4.2H_2O$	80.0g	NaCl
0.1g	Phenol	2.0	$MgCl_2.6H_20$
		2.0g	$MgSO_4.7H_20$

BSS 100ml BSS I plus 100ml BSS II and 800ml DDW

9.1.7. TRYPTAN BLUE SOLUTION.

Stock solutions were diluted with PBS and sodium azide before being sterile filtered.
50ml: 31,25ml Tryptan blue
18.75ml PBS
250µl NaN_3.

9.1.8. AQUEOUS BUFFER FOR CHROMATIN PREPARATION.

10mM	Tris-HCl, pH 7.4
120mM	KCl
20mM	NaCl
0.1%	Triton X-100
0.1mM	PMSF containing 2mM $CaCl_2$

9.2. SDS-PAGE GEL ELECTROPHORESIS AND WESTERN BLOT

9.2.1. 2XSDS GEL-LOADING BUFFER

100mM Tris-	HCl
200mM	DTT
4%	Bromophenol blue
20%	Glycerol

Store at –20°C

9.2.2. RESOLVING GEL BUFFER

90.8g	Trizma base
2.0g	SDS

Add approximately 900 ml distilled water. Mix to dissolve. Adjust pH 8.8 with hydrochloric acid. Make up to a final volume of 1000ml Store at 4°C

9.2.3. RESOLVING GEL SOLUTION

9ml	Resolving gel buffer
7.5ml	Acrylamide stock solution (30%(w/v))
2ml	DDW

mix thoroughly and degas is required.

180µl	10% APS
18µl	TEMED

The above amount is sufficient for 2 mini-gels, Biorad.

9.2.4. STACKING GEL BUFFER

30.0g Trizma base
2.0g SDS
Add approximately 900 ml DDW. Mix to dissolve. Adjust pH to 6.8 with HCl. Make up to a final volume of 1000ml. Store at 4°C

9.2.5. STACKING GEL SOLUTION

5ml Stacking gel buffer
1.8ml 30% Acrylamide stock solution (30%(w/v))
4ml DDW
100µl 10% APS
10µl TEMED

9.2.6. RUNNING BUFFER

30.g Trizma base
14.4 g Glycine
1.0g SDS

Add approximately 900 ml DDW. Mix to dissolve, make up to a final volume of 1000ml. Store at 4°C

9.2.7. PROTEIN TRANSFER BUFFER

3.0g Trizma Base
14.4g Glycine
200 ml Ethanol
Add approximately 900 ml DDW. Mix to dissolve. Make up to a final volume of 1000ml. Store at 4°C

9.2.8. BLOCKING BUFFER FOR WESTERN BLOT

4%(w/v) nonfat dried milk
200µl Tween 20
Add PBS up to a final volume of 200ml. Mix to dissolve for at least 1 hour before application.

9.3. PROTEIN DETECTION TECHNIQUES

9.3.1. PONCEAU-S STOCK SOLUTION

2.5g Ponseau-S
5ml Glacial acetic acid

Add distilled water to produce a final volume of 500ml

9.3.2. COOMASSIE BRILLIANT BLUE STAINING SOLUTION

0.25g Coomassie brilliant blue
10ml Glacial acetic acid
45ml ethanol absolute

Add 45ml DDW to reach a final volume of 100ml. Mix to dissolve. Store at RT

9.3.3. SDS-PADE DESTAIN SOLUTION

50ml Glacial acetic acid
225ml Ethanol

Add 225ml DDW to a final volume of 500ml. Mix to dissolve. Store at RT.

9.3.4. SILVER STAINING – HEUKESHOVEN PROTOCOL

9.3.4.1. Fixation Solution A

40% Ethanol
10% Acetic acid
In every litre of DDW. Store at room temperature.

9.3.4.2. Fixation Solution B

30% Ethanol
78g Sodium Acetate (NaAc)
2g Sodium thiosulphate ($Na_2S_2O_3$)
20ml Glutaraldehyde (25%)

Dissolve in one litre of water and use fresh each time

9.3.4.3. Fixation Solution C

30% Ethanol
5% Acetic acid
In every litre of DDW. Store at room temperature.

9.3.4.4. Silver Solution

1g/litre of $AgNO_3$
500µl Formaldehyde (37%)
Dissolve in one litre of DDW and use fresh each time

9.3.4.5. Developing Solution

25g Sodium carbonate ($NaCO_3$)
250µl Formaldehyde (37%)
Dissolve in one litre of DDW and use fresh each time
Stopping Solution

10% Glycin in 1 litre of DDW. Store at room temperature.

9.4. 2D GEL ELECTROPHORESIS

9.4.1. REHYDRATION STOCK SOLUTION.

12g (8M) Urea
0.5g (2%w/v) CHAPS
Bromophenol Blue few grains

DDW 25ml

Mix to dissolve, store in 2.5ml aliquots at –20°C. DTT and IPG buffer are added just prior to use. Add 7 mg per 2.5ml aliquot of rehydration stock solution and the recommended amount of IPG buffer.

9.4.2. SDS EQUILIBRATION BUFFER

6.7ml	1.5mM Tris-HCL, pH 8.8
72.02g	Urea (6M)
69ml (30v/v)	Glycerol (87%v/v)
4.0g	SDS 2%(w/v)
few grains	Bromophenol Blue
200ml	DDW

Store in 40ml aliquots at –20°C. Prior to use add 100mg of DTT per 10ml solution.

9.4.3. X4 RESOLVING GEL BUFFER

181.5g	1.5M Tris Base
750ml	DDW

Adjust pH to 8.8 with HCl and make up the final volume to 1 litre. Filter through 0.45µm filter and store at 4°C.

9.4.4. 12.5% RESOLVING GEL SOLUTION

41.7ml	Acrylamide stock solution (30%(w/v))
25ml	x4 Resolving gel buffer
31.8ml	DDW
500µl	10% SDS
Swirl thoroughly and degas is necessary	
500µl	10% APS
33µl	TEMED

The above 100ml solution is enough for two Hoeffer vertical gels. To ensure a smooth surface, a layer of *sec*-2-butanol was added.

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ERKLÄRUNG

Hiermit erkläre ich an Eidesstatt, dass ich die vorliegende Arbeit mit dem Titel „The responsive and suppressive activities of $CD4^+$ T cells to neoantigens generated in procainamide drug-induced Lupus“ selbständig angefertigt und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe.

Ich habe die vorgelegte Dissertation weder in dieser noch in ähnlicher Form bei einer anderen Fakultät eingereicht.

Laura E. Layland

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