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Development of a Novel Oral Mouse Model to Predict Drug-Induced Immune-Mediated Hypersensitivity Reactions

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Abstract

Idiosyncratic drug reactions (IDRs), among the most serious adverse drug reactions, are a major clinical problem accounting for significant morbidity and mortality.

It is generally assumed that various mechanisms are responsible for triggering IDRs, and the research in the IDR field is headed in the direction of trying to understand the mechanisms of these reactions.

The unpredictable nature and low incidence of IDRs limit human studies and necessitate the use of animal models. Animal models, however, are limited and they have to be scientifically justified in each case. The exact mechanisms of idiosyncratic drug reactions are unknown; however, the drug reactive metabolite formation and the stimulation of patient's immune system have been suggested to play a role in their onset. The two drugs known to induce IDRs in patients that were applied in this doctoral work to further investigate IDR mechanism of action are nevirapine and D-Penicillamine.

In this thesis an already established local popliteal lymph node assay (PLNA) and reporter antigen popliteal lymph node assays (RA-PLNA) were used together with a newly established oral-mouse model to further investigate the individual steps involved in the onset of IDRs with both compounds. An increase in PLN weight, cell count, the total number of T & B cells, cell surface activation markers and also TNP-specific immunoglobulins were observed with D-Penicillamine in the local assay. These effects were also observed with nevirapine in RA-PLNA with TNP-OVA but not TNP-Ficoll. The results from the novel 10-days-oral mouse model showed increase in spleen and draining lymph nodes weight, activation of T and B cell markers, induction of various cytokines and most importantly, isotype switching from TNP-specific IgM to IgG in the spleen upon treatment with either nevirapine or D-Penicillamine. This latter effect was specifically significant in Nevirapine case. These data confirmed that an adaptation of the well-established local RA-PLNA to an oral mouse model was successful with respect to enabling identification of the immunostimulatory potential of nevirapine in mice using a reporter antigen. Hence, this model is a good starting point for further validation and testing other compounds.

Abstract

Idiosynkratische Reaktionen (IDRs) gehören zu den schwerwiegendsten Nebenwirkungen von Medikamenten. Sie sind ein grosses klinisches Problem, dass zu erheblicher Morbidität und Mortalität führt. Es wird angenommen, dass unterschiedliche Mechanismen für die Auslösung der IDRs verantwortlich sind und in der Forschung werden grosse Anstrengungen unternommen, um diese zu verstehen Aufgrund der Unvorhersehbarkeit und geringen Inzidenzrate von IDRs sind Studien im Menschen begrenzt und erfordern daher die Verwendung von Tiermodellen. Tiermodelle sind jedoch ebenso Beschränkungen unterworfen und müssen für jeden Einzelfall wissenschaftlich gerechtfertigt sein. Die genauen Mechanismen von IDRs sind unbekannt, aber es gibt Hinweise, dass die Bildung reaktiver Metabolite und die Stimulierung des Immunsystems bei der Entstehung von IDRs eine Rolle spielen können. Zur Untersuchung des IDRs-Wirkmechanismus wurden in dieser Doktorarbeit zwei bereits als IDR auslösende bekannte Substanzen Nevirapin und D-Penicillamin untersucht.

Im Rahmen dieser Doktorarbeit wurde ein bereits bestehender lokaler Poplitealen Lymphknoten-Assay (PLNA) und ein Reporter Antigen-Poplitealen Lymphknoten Assay (RA-PLNA) zusammen mit einem neuartigen oralen Mausmodell getestet, um die einzelnen Schritte bei der Entstehung der IDRs zu untersuchen.

Eine Erhöhung in PLN-Gewicht, Zellzahl, Gesamtzahl der B- und T-Zellen, Zelloberflächen-Aktivierungsmarker und auch der TNP-spezifischen Immunglobuline wurden im lokalen Assay mit D-Penicillamin beobachtet. Diese Effekte wurden auch mit Nevirapin im RA-PLNA mit TNP-OVA, aber nicht TNP-Ficoll beobachtet. Die Ergebnisse aus dem oralen 10-Tage-Mausmodell zeigten einen Anstieg in Milz- und Lymphknotengewicht, Aktivierung von T- und B- Zellmarkern, Induktion verschiedener Zytokine und vor allem Isotyp-Wechsel von TNP-spezifischem IgM zu IgG in der Milz, sowohl nach Nevirapine- als auch D-Penicillamine-Behandlung. Der letztgenannte Effekt war nach der Nevirapin-Behandlung besonders deutlich erkennbar. Diese Daten bestätigen die erfolgreiche Anpassung des etablierten lokalen RA-PLNA auf ein orales Mausmodell zur Identifizierung des immunstimulatorischen Potenzial von Nevirapin bei Mäusen mit einem Reporter-Antigen. Daher ist dieses Modell ein guter Ausgangspunkt für die weitere Validierung und Prüfung anderer Substanzen.

List of Abbreviations

ADR	Adverse drug reaction
AIDS	Autoimmune Deficiency Syndrome
ANA	Antinuclear antibodies
APC	Antigen presenting cell
BN	Brown Norway
BSA	Bovine serum albumin
CD4+	CD4 antigen expressing (helper) T lymphocytes
CD8+	CD8 antigen expressing (cytotoxic) T lymphocytes
CM	Costimulatory Molecule
CNS	Central nervous system
CYP450	Cytochrome P45O
DMSO	Dimethyl sulfoxide
DPH	Diphenylhydantoin
D-Pen	D-Penicillamine
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzym-linked immunospot Assay
FDA	Food and Drug Administration of U.S.A.
FITC	Fluorescein isothiocyanate
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
ICAM	Intracellular adhesion molecule
IDR	Idiosyncratic Drug Reaction
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NNRTI	Non-nucleoside Reverse Transcriptase Inhibitor

NSAID	Nonsteroidal anti-inflammatory drug
NVP	Nevirapine
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PE	Phycoerythrin
P-I	Pharmacological interaction with Immune receptors
PRR	Pattern recognition receptor
PLN	Popliteal lymph node
PLNA	Popliteal lymph node assay
PA	Procainamid
RA	Reporter Antigen
RM	Reactive Metabolite
SD	Sprague-Dawley
SJS	Stevens-Johnson syndrome
SLE	Systemic lupus erythematosus
SNS	Self-non-self
STZ	Streptozotocin
TCR	T cell receptor
TEN	Toxic epidermal necrolysis
Th	Helper T cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha

1 Introduction

1.1 An Overview of Thesis Research

IDRs are adverse drug reactions (ADRs) that occur in a small number of patients but can be fatal, are unpredictable and can not be explained by the pharmacological effects of drugs [1, 2]. They are estimated to account for 6 to 10% of all ADRs [3]. An attempt to prevent IDRs could potentially be successful if their mechanisms were understood. Although there is no clear mechanistic understanding of these reactions, evidence acquired to date suggests that drug reactive metabolites might play a key role in triggering their onset, Furthermore, additional research points in the direction of immune-mediated mechanisms playing a role in the onset of IDRs [4].

Specifically, in the case of the two drugs that will be further discussed in this thesis, D-Penicillamine (D-Pen) and nevirapine (NVP), immune-mediated mechanisms of action has been associated with the onset of IDRs observed in a patient population with both of these drugs.

NVP is a non-nucleoside reverse transcriptase inhibitor (NNRTI), used in the treatment of human immunodeficiency virus (HIV) infections. In the 1990s Uetrecht's lab began studying the mechanism of nevirapine-induced IDRs: liver toxicity and skin rash, by utilizing rats as an animal model. Upon administration of NVP (150 mg/kg/day) to female Sprague-Dawley rats they observed that 2 out of 4 rats developed erythema between weeks 4 and 6 from treatment onset. Also, they reported excessive scratching of rats around the nose/mouth area and the loss of body weight. Further work with NVP was performed in Brown Norway rats, and females of this strain displayed skin rashes in 100% of cases when treated with the same dose. Skin rash characteristic detected in the Brown Norway (BN) female rats resembled the ones previously reported in humans. This finding was captured in Shenton et al. publication describing a newly-discovered animal model of NVP-induced skin rash in rats. Further characterization of the new animal model led to more detailed analyses of the exact mechanism of action leading to the rash development in rats [5][6].

The model of D-Pen-induced autoimmunity in the BN rat is another animal model of IDRs. Brown Norway rats administered high doses of D-Penicillamine (>20 mg/day) develop an autoimmune disease resembling idiosyncratic drug-induced lupus observed in a small

percentage of patients on D-Pen therapy [7]. By studying the mechanism of NVP and D-Pen in mice the goal of this thesis was to gain additional insight into the possible mechanism(s) of drug-induced IDRs in humans.

This thesis is focused on further analyzing of the steps amounting to D-Pen and NVP-induced immune response in more than one animal species, primarily in mice by studying these two compounds in local as well as oral exposure mouse models.

One of the strategies presently utilized (although still in a standardization/validation phase) is a first tier/fast screening process assessing the risk of drug sensitization ability utilizing the PLNA and also the modified version of the PLNA assay, RA-PLNA [8]. Both assays focus on evaluating antibody response to a specific antigen in the draining lymph nodes of mice or rats, subcutaneously (s.c.) injected with the test compound either alone (PLNA) or in combination with a reporter antigen (trinitrophenyl (TNP)-Ovalbumin, or TNP-Ficoll) directly into the animal's foot pads. As mentioned previously PLNA is in the process of being validated, a necessary step prior to its utilization as an official screening tool. The first part of this thesis focuses on further validation of the PLNA/RA-PLNA assay; the second part focuses on the oral PLNA initially tested in R. Pieters' laboratory [9].

Although the PLNA/RA-PLNA is a fast and a simple preclinical assay, it has a disadvantage of chemicals being injected s.c. into animal's foot pads instead of being administered orally which is the usual route of drug administration. Subcutaneous injection of drugs cause also other problems such as lack of metabolic components and therefore missing the immune response in case of drugs that the reactive metabolite is responsible for induction of immune response. The other problem that one can encounter by injecting the drug s.c. is having unspecific immune response due to the crystal formation when the drug of interest is not water soluble. These different aspects/issues will be discussed further in the next chapter. Therefore the second part of this thesis focuses on investigating whether drugs are able to induce systemic immune changes via oral route of exposure which is the common route of drug administration.

The antigen-specificity of drug-induced immune response can vary from adjuvant like effect to T cell-specific event. Using RAs; TNP-OVA; T-dependent RA or TNP-Ficoll; T-independent RA as a read-out allowed differentiation between these two effects that can be caused by drugs. Efficient immune responses to TNP-OVA require cognate T-B cell interactions and costimulatory adjuvant signals [10]. Therefore, any drug that induces

inflammatory mediators, including nonsensitizing irritants or adjuvants, will induce the formation of TNP-specific ASC. On the other hand, TNP-Ficoll alone does not elicit TNP-specific ASC with Ig isotypes other than IgM. Therefore, a TNP-specific IgG response can only occur in the presence of T cell help. Importantly, naive T cells are incapable of specifically responding to TNP-Ficoll. Hence, the formation of TNP-specific IgG ASC in response that is induced upon coinjection of TNP-Ficoll together with a drug indicates soluble help from hapten- or neo-antigen-specific T cells. Thus, the use of these two RAs provides evidence for chemical-induced sensitization to either the compound itself (allergy) or neo-epitopes (autoimmune hypersensitivity) and differentiates sensitizing drugs from inflammatory (but nonsensitizing) compounds and innocent (non-inflammatory, non-sensitizing) drugs.

1.2 Adverse Drug Reactions

Adverse drug reactions are undesired effects of a drug, which occur at doses used in humans for prophylaxis, diagnosis or therapy. Adverse drug reactions have been classified according to various criteria, and they represent a major clinical problem, accounting for 5% of hospital admissions [11, 12]. It is essential that a better mechanistic understanding of ADRs is obtained in order to prevent their onset in patients undergoing the treatment with a drug.

Park et al. [13] classify the reactions based on their clinical, pharmacological, and chemical characteristics.

- **Type A: Augmented Reactions -** These reactions represent an exaggeration of the pharmacological effects of the drug. They are the most common type, accounting for 80% of all ADRs and they can be predicted based on the known pharmacological properties of the drug. They can be eliminated by drug dose reduction (e.g. hemorrhage development with anticoagulants).

- **Type B: Bizarre, Idiosyncratic Reactions -** Type B reactions, accounting for between 6 to 10% of ADRs, represent the minority of drug reactions. As the mechanisms of type B reactions are poorly understood, they have also been termed ‘idiosyncratic’ or ‘bizarre’ drug reactions. The term ‘idiosyncratic’ highlights the complexity of these reactions and will therefore be employed throughout this thesis. Unlike type A reactions, IDRs can not be predicted based on the known pharmacology of the drug. The majority of these reactions have characteristics that suggest immune pathogenesis; however, the mechanism(s) by which small

molecule drugs elicit pathogenic immune responses are unclear. For the most part, type B reactions are host-dependant and they lack a simple dose-response relationship; most patients will not develop an IDR at any dose. Although they account for a smaller percentage of ADRs, the unpredictable and serious nature of IDRs makes them a significant clinical problem.

- **Type C: Chemical Reactions –** Biological effects of these reactions can be predicted based on the chemical structure of a drug, or its reactive metabolite (e.g. acetaminophen-induced liver toxicity)
- **Type D: Delayed Reactions –** These reactions occur months, or years after the treatment has been completed (e.g. fetal hydantoin syndrome with phenytoin)
- **Type E: End of the Treatment Reactions** – These are the adverse reactions that occur upon sudden drug withdrawal (e.g. withdrawal seizures after stopping phenytoin treatment)

The focus of this thesis is on the type B reactions, also known as IDRs. Idiosyncratic drug reactions are rare events, but can be fatal to patient's health, they usually missed during the preclinical testing and clinical drug trials, and are often detected only once the drug is marketed because of the limited number of animals and the restrictions by complexity regarding different genetic background, environmental factors and etc [14]. Drug-induced IDRs in animals are as rare as in humans, so it is very difficult to detect idiosyncratic effects of a drug in preclinical animal testing. The number of animals and humans tested with the drug before it is marketed is limited, so only once the drug reaches the market and hundreds of thousands of patients are exposed to it, is the incidence of IDRs more apparent [13].

Lasser et al. reported in 2002 that approximately 10% of drugs approved in the last three decades had to be withdrawn from the market or were given a Black Box warning because of ADRs that they caused, which were not predicted during the drug development process [15].

Drugs known to cause IDRs have diverse chemical structures and functions, complicating the problem even further. Moreover, a drug can cause different reactions in different people. For example, NVP causes liver toxicity and skin rash in some patients, but can also cause just one, or the other [16]. Skin rash and liver toxicity are just some of the clinical manifestations of IDRs, others including autoimmunity, anaphylaxis, and bone marrow toxicity among others [17].

Mechanistic studies of IDRs are challenging: options available to study them include clinical studies, animal studies and in vitro studies. Clinical trials in humans would be the best option; however, prospective clinical studies are generally not feasible due to the low absolute incidence of IDRs. Retrospective clinical studies may be of use for the study of genetic risk factors for these reactions, but the nature of these reactions suggests that environmental risk factors may also be important in their onset; there is evidence that surgery [18] and underlying infection [19] are risk factors for the development of IDRs. Moreover, some studies that can be carried out retrospectively, such as rechallenge studies in patients initially challenged with the same drug, are generally unethical as re-exposure to the drug could result in a response more severe than on the first exposure. Conclusions drawn only from in vitro studies should be viewed carefully and critically, since test tube experiments are not set to successfully reproduce and address the complex pathways involved in pathogenesis of an IDR. As in vivo studies in humans are generally unethical and the relevance of in vitro studies is uncertain, the best option is to rely on animal models.

Animal models are important tools for mechanistic studies; however, IDRs are just as rare in animals as they are in humans so most animal models have been found by luck [20]. Still, there is a big need for the development of new animal models in order to be able to predict IDRs [21], hence finding a new animal model of IDRs has been a major objective in the ADR research.

1.3 Mechanisms of Idiosyncratic Drug Reactions

Evidence to date suggests a role for both the immune system and metabolism in the pathogenesis of IDRs. Immune system involvement was initially suspected due to a delay between starting the drug and the onset of the reaction, and a decreased delay on re-exposure. Additionally, in certain cases, anti-drug antibodies have been detected in patients who suffered from IDRs [22]. Often IDRs are also referred to hypersensitivity reactions [23].

In the late 1960s, Coombs and Gell proposed a model that categorized hypersensitivity reactions into four different types:

Type I - Immediate Hypersensitivity Reactions

These reactions are mediated by IgE antibodies, which are directed against soluble antigens. Once antigen binds to the IgE on the surface of mast cells or basophils, it leads to cross-linking of IgE antibodies causing the release of histamines, prostaglandins, and leukotrines, resulting in urticaria, asthma and anaphylaxis.

Type II – Immunoglobulin-Mediated Cytotoxicity Reactions

Circulating IgG antibodies react with cell surface antigens, leading to complement-mediated cytotoxic reactions resulting in blood cell dyscrasias.

Type III – Immune Complex Reactions

When IgG antibodies are directed against soluble antigens, immune complexes can get formed that lead to initiation of effecter mechanisms such as complement activation, monocyte and granulocyte recruitment that lead to tissue damage such as vasculitis.

Type IV – Delayed Type Hypersensitivity (DTH)

DTH is antigen specific and causes erythema at the site of antigen injection in immunized animals. Systemic injection of antigen results in fever, synthesis of acute phase proteins. The nature of this antigen can be varied. Mycobacteria, protein, hapten, and even grafted tissue are all capable of inducing delayed type hypersensitivity reactions [24]. The histology of DTH can be different for different species, but the general characteristics are an infiltration of immune cells at the site of injection, either macrophages and basophils in humans and mice or neutrophils in guinea pigs[25].

In addition to Coombs & Gell differentiation of hypersensitivity reaction types, evidence to date has lead to the development of three main hypotheses that outline mechanistically the steps that result in IDR reactions: Hapten hypothesis, Danger hypothesis and Pharmacological Interaction with Immune receptors (P-I) hypothesis [26, 27].

1.3.1 Hapten Hypothesis

A hapten is a small molecular weight compound, almost always less than 1,000 Dalton in size that can not induce an immune response by itself but can act as either an immunogen or antigen when conjugated to a macromolecular carrier [28]. In 1935 Landsteiner showed that chemicals only become potent sensitizing agents when they bind to a protein of molecular

weight larger than 50,000 Da, and form a hapten-protein complex. The hapten hypothesis proposes that either a drug, or its reactive metabolite, must covalently bind to host tissue proteins and form hapten-protein complex in order to be able to induce host immune response [29]. For a drug to be a hapten, its electrophilic group has to form a stable bond with a nucleophilic group on tissue proteins. This bond must not be rapidly hydrolyzed in aqueous conditions around pH 7.4, because, in that case, the hapten-protein complex would not survive antigen processing [28].

Most drugs are not sufficiently reactive to directly modify proteins; they first need to be metabolized to reactive metabolites. The major enzymes involved in drug metabolism are Phase I metabolizing enzymes [30].

1.3.2 Danger Hypothesis

The classic view of immune responsiveness is that the immune system discriminates between self and non-self i.e., the self-non-self (SNS) model. Thus, an immune response to a haptenated protein may occur because adduction of the protein by drug has rendered the protein as non-self or foreign; it is the immune response to alter self that is believed to manifest clinically as an idiosyncratic drug reaction. However, many drugs form metabolites that covalently bind to protein, but rarely cause idiosyncratic drug reactions. Therefore, it appears that the immune system is not recognizing drug-protein adducts as foreign in many cases and suggests that a reactive metabolite must do something in addition to covalently binding in order to cause an idiosyncratic reaction. Thus, when Matzinger [31] proposed an alternative mechanism for the regulation of immune responsiveness, researchers in the field of idiosyncratic drug reactions were quick to apply her tenets to the development of idiosyncratic toxicity [32, 33]. Matzinger theorizes that it is not SNS, but rather the presence or absence of a ‘danger signal’ that guides immune responsiveness i.e., only if a cell dies by necrosis, or become stressed or damaged, is an immune response initiated [34]. This theory is called the ‘Danger Hypothesis’. Both the SNS model and the Danger Hypothesis rely on the same molecular on/off switch for activation or tolerance of the cells of the immune system, i.e., co-stimulation. It is well known that the induction of an immune response is regulated by two signals: signal 1 and signal 2. Signal 1 represents the interaction of peptide/major histocompatibility complex (MHC) on an antigen-presenting cell (APC) with a cognate T cell receptor (TCR). Signal 2 is delivered by cytokines and co-stimulatory molecule-receptor

interactions (e.g., B7 molecules on APC bind to CD28 on the T cell). If a T cell receives signal 1 in the absence of signal 2 then tolerance ensues; whereas, activation occurs if the T cell receives both signals. Co-stimulatory molecules are expressed at very low levels prior to activation of APCs. In the SNS model it is believed that detection of non-self by APCs – pattern recognition receptors expressed on the surface of APCs recognize pathogen-associated molecular patterns results in up-regulation of co-stimulatory molecules; whereas, in the danger model it is believed that cell stress or cell death up regulate co-stimulatory factors [34]. Importantly, binding of a pathogen-associated molecular pattern, such as bacterial cell wall lipopolysaccharide (LPS), to a pattern recognition receptor (the Toll-like receptor (TLR)-4) on an APC could also result in cell stress as signaling through TLR-4 induces the production of reactive oxygen species [35]. Thus, the fundamental difference between the two theories is that the trigger in the SNS model is exogenous; whereas, the trigger in the danger model is endogenous. It is not clear how covalent binding of a drug to protein would up-regulate signal 2 as described in the SNS model. However, it follows, applying Matzinger's Danger Hypothesis, that a reactive metabolite must not just bind to endogenous protein (subsequent processing and presentation of these adducts by APCs can provide signal 1), but also cause some type of cell damage or cell stress in order to up-regulate signal 2, which could induce an immune response and result in an idiosyncratic drug reaction. It is also possible that danger is not caused by the drug itself, but rather by some environmental factor. For instance, HIV-positive patients have a higher risk of developing idiosyncratic drug reactions than the general population and virus-infected cells are certainly stressed cells. On the other hand, most patients do not have idiosyncratic reactions to drugs associated with such reactions even in the context of surgery or other factors that are likely to constitute a danger signal [36].

1.3.3 The Pharmacological-Interaction (P-I) Hypothesis

This hypothesis suggests that drugs can bind directly to MHC peptides on APCs and cause an immune response without prior need for antigen processing and presentation. The T cell can have a high affinity for a drug bound to MHC peptide, triggering an immune response [26]. Although it is still unknown how and where the binding between the drug and MHC-peptide complex occurs, T cell clones from patients with an IDR specific for sulfamethoxazole and carbamazepine have been generated [37, 38]. Metals can also bind directly to MHC proteins.

In the case of nickel, binding occurs through coordinate bonds to the MHC protein on APCs, inducing contact hypersensitivity, which elicits T cell responses [39].

Overall, it may be that hapten presentation on the surface of the MHC molecules in the presence of a danger signal leads to hypersensitivity reactions.

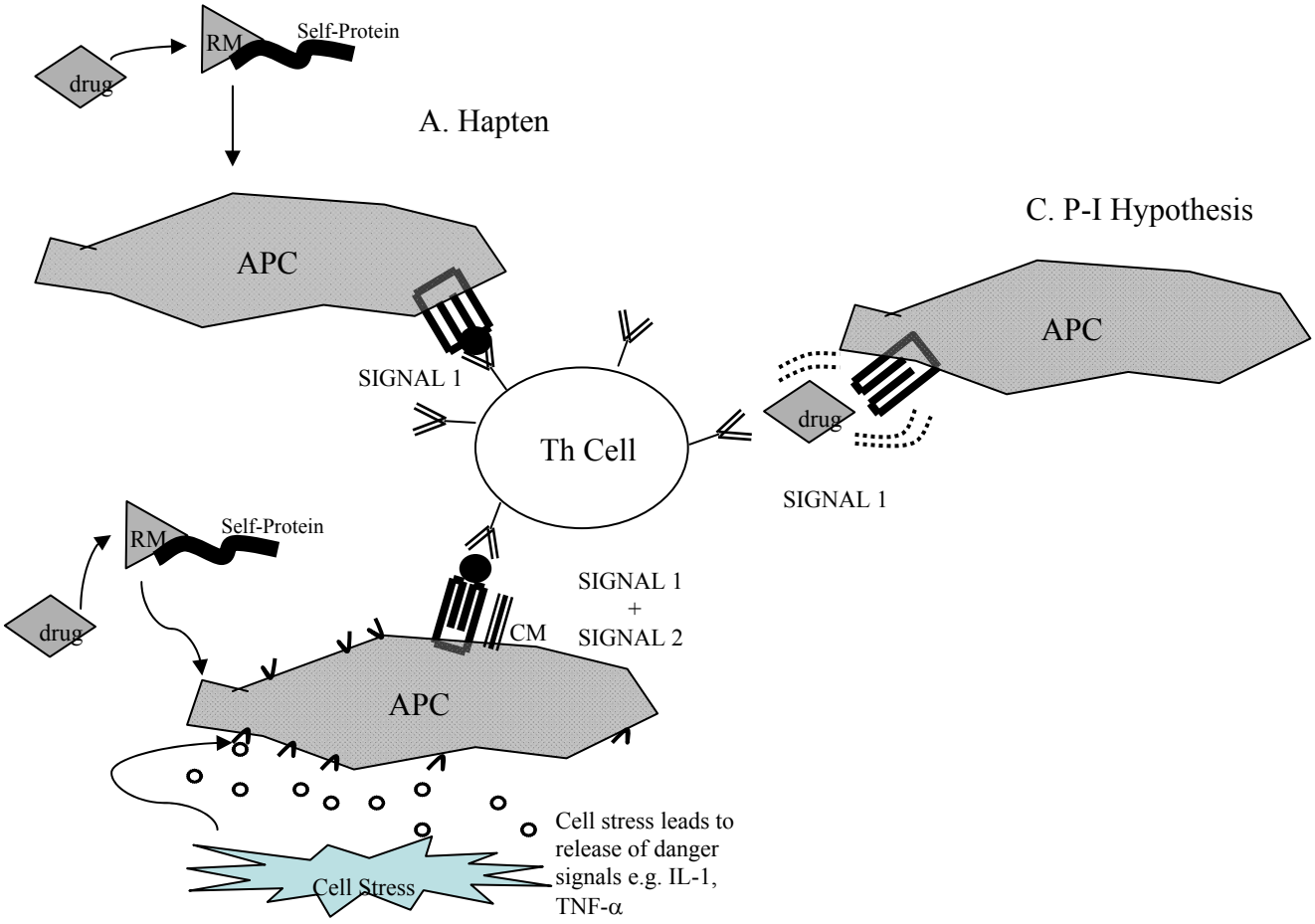


Figure 1. Proposed mechanisms of IDRs. A. The hapten hypothesis proposes that a parent drug or its reactive metabolite (RM) binds to an endogenous protein. The drug-protein complex is processed and presented by antigen-presenting cells (APCs) as signal 1. This model does not address signal 2. B. The danger hypothesis is based on the observation that both signal 1 and signal 2 are necessary to generate an immune response. Signal 1 is generated by covalently bound protein as shown in A. Signal 2 is generated from release of danger signals, e.g. IL-1 and TNF-α, by stressed or damaged cells. C. The P-I hypothesis proposes that drugs non-covalently associate with MHCII, independently of RM formation and antigen processing. Specific T helper (Th) cells are then activated by the drug-MHCII complex. This model does not address the issue of signal 2.

1.4 Immune Response Induction

In the context of immune response induction "self-non-self" dogma still holds a central place. This dogma proposes that the immune system is able to differentiate between "self" antigens and foreign "non-self" antigens, and responds to "self" by inducing tolerance, and to "non-self" by initiating an immune response. This dogma was useful for understanding most of immunological principles; however, some phenomena such as induction of immune tolerance in presence of antigens that are expressed for the first time during puberty or pregnancy, could not be explained [40]. Danger signal hypothesis was a good explanation for this latter phenomenon.

Over the years the dogma got modified and it evolved to a point that scientists concluding that two signals are necessary for an immune response to occur. The first signal is binding of a T cell receptor to a major histocompatibility complex (MHC) on antigen presenting cell (APC). The second signal is a co-stimulatory signal. Signal one in the absence of signal two results in tolerance. As it is almost impossible for APCs to distinguish between self and foreign antigens and specifically up regulate co-stimulatory surface proteins in the presence of the latter.

Janeway suggested that it is the role of innate immune system (e.g. macrophages), to send signals to APCs to up regulate co-stimulatory molecules [41, 42]. Innate immune cells have pattern recognition receptors (PRR) on their surface that recognize patterns associated with pathogens. These receptors are called Toll-like receptors (TLRs), and they are specific for bacterial molecules such as lipopolysaccharide, unmethylated DNA and viruses with double-stranded RNA [43]. Once TLRs recognize pathogens, they initiate a cell signal transduction cascade, resulting in release of cytokines that can activate APCs [44].

Applying immunological concepts to understanding IDRs leads us to hypothesize that once a hapten-protein complex is formed, it can be taken up, processed and presented on the surface of MHC proteins on APCs to the T cell receptors (TCRs) on T cells [45]. This represents signal 1 of immune stimulation. Once a co-stimulatory signal occurs, hapten-specific naïve T cell activation and clonal expansion take place followed by initiation of an immune response against the hapten-protein complex [46].

Activation of naïve CD8+ T and CD4+ T cells is associated with recognition of antigens in the context of MHC I and MHC II molecules, respectively. In general, peptides formed from intracellular antigens are presented on the MHC I complex and interact with CD8+ T cells

and peptides derived from internalized extra cellular antigens are presented by MHC II proteins to CD4+ T cells [47]. All nucleated cells express MHC I, but only some cell types express MHC II, or can be induced to express it. In addition, only professional APCs, such as dendritic cells (DCs), constitutively express MHC II on their surface and have the ability to initiate immune response in naïve T cells. Dendritic cells can capture and process antigens and then retain them for long periods in an immunogenic form [48]. Once dendritic cells uptake foreign antigens and present them on the surface of their MHC II proteins, they can activate antigen-specific helper CD4+ T cells which produce cytokines, and/or directly interact with B cells and CD8+ cytotoxic T cells and induce them to proliferate and differentiate into effecter cells [49].

1.4.1 Immune Tolerance Induction

The ability of the immune system to mount an immune response is very important as pathological immune responses to 'self' can be devastating. Therefore, the concept of immune tolerance is central to the study of immunology. Immune tolerance that occurs in the thymus, before T cells mature and start circulating, is referred to as 'central tolerance'. Immune tolerance that occurs in the periphery, after T cell maturation, is referred to as 'peripheral tolerance'.

1.4.1.1 Central Tolerance

Early on in development, before T cells enter the periphery, they are subjected to test in the thymus to eliminate potentially self-reactive cells. T cells that do not recognize antigen in the context of self-MHCII die in the thymus, a process referred to as 'positive selection'. T cells that recognize self-antigen is deleted, a process referred to as 'negative selection'. After passing positive and negative selection tests, mature T cells leave the thymus ready to respond to antigen. However, there must be additional mechanisms to induce tolerance in the periphery as the thymus does not contain all the self-antigens T cells encounter in the body [50].

1.4.1.2 Peripheral Tolerance

Peripheral tolerance is specific for a given antigen, it has memory, and in some cases, it can be adoptively transferred to naïve animals. Once peripheral tolerance has been established, the immune system can no longer mount an immune response to that antigen. The three

mechanisms of peripheral tolerance are: 1. Anergy, 2. Activation-induced cell death and 3. Suppression.

1.4.1.2.1 Anergy

To generate an adaptive immune response, T cells must receive both signal 1 and signal 2 from activated, competent APCs. In this instance, signal 1 refers to antigen in the context of MHCII and signal 2 refers to costimulation. T cells receiving both signals 1 and 2 undergo activation and start to differentiate and proliferate in response to the specific antigen. In the case that T cells encounter antigen presented by resting APCs lacking costimulatory molecules, they will not receive signal 2. Instead of undergoing activation, these T cells will enter a state of unresponsiveness that has been termed 'anergy' [51].

In vitro experiments provide evidence that signaling through the TCR in the absence of signal 2 leads to the induction of anergy. When incubated with fixed APCs lacking costimulatory molecules, murine T helper (Th) cells enter a long-term anergic state [52]. The mechanism of anergy is not well understood on a biochemical basis. It appears that in the absence of costimulation-derived IL-2 production, signaling through the TCR leads to a rise in Ca^{2+} and the induction of anergy [53].

As of yet, the fate of anergic T cells in the body is unknown. In vitro, the proliferative response of anergic T cells can be restored by exogenous IL-2 or IL-12 [54, 55]. The ability of exogenous cytokines to break peripheral tolerance has led to a hypothesis regarding the mechanism of autoimmunity. This hypothesis proposes that the induction of peripheral tolerance to self-antigens results in a population of circulating anergic autoreactive T cells. If a 'danger' signal (e.g. viral infection) were to induce IL-12 secretion by activated APCs, the normal immunoregulatory status could be disrupted leading to bystander activation of autoreactive T cells [54]. Consistent with this hypothesis is the observation that multiple sclerosis patients are more likely to relapse during an infectious illness [56].

1.4.1.2.2 Activation-Induced Cell Death

Activation-induced cell death is thought to be evolved as a mechanism to prevent uncontrolled T cell activation during an immune response. When T cells are repeatedly stimulated, they become susceptible to Fas-mediated apoptosis. In fact, more than 90% of responding T cells die during an immune response [57]. In a mixed lymphocyte reaction, the

percentage of responding T cells is fixed at 2 to 5% of the total $CD4^+$ T cell population. Therefore most of the cells must undergo apoptosis [58]. This homeostatic regulation is necessary to prevent on-going T cell activation in the absence of antigen, and also to regulate the total number of T cells as there is a limited amount of space available for lymphocytes in the body.

1.4.1.2.3 Suppression

In the 1990s, the demonstration of a T cell subset that prevents autoimmune disease in mice led to a interest in the suppressor cell [59].

Adoptive transfer experiments are generally considered to be the classic method of detecting suppressor or regulatory T cell involvement in vivo. In these experiments, T cells from tolerant donors are transferred to naïve recipients. The ability of the transferred T cells to actively suppress the recipients native immune response demonstrates the infectious nature of regulatory T cells. Although the exact mechanism of regulatory T cell differentiation is not known, they are usually generated by orally ingesting antigen, or by injecting antigen in the absence of adjuvant. The suppressor cells are thought to suppress naïve T cell proliferation by secreting large amounts of IL-5, IL-10 and TGF-β [60].

In the mouse, it is the subset of T cells co-expressing CD25 (IL-2R α-chain) and CD4 antigens that have been identified as regulatory T cells. $CD25^+CD4^+$ T cells account for about 10% of the total $CD4^+$ T cell population [61]. The first experiments to demonstrate the regulatory function of $CD25^+CD4^+$ T cells were performed by Sakaguchi et al [61]. Syngeneic *nu/nu* recipients injected with $CD25^-CD4^+$ T cells from BALB/c mice developed an autoimmune syndrome characterized by a wasting disease resembling GVHD. Injecting $CD25^+CD4^+$ T cells within 10 days of the transfer prevents the autoimmune syndrome. Since then, evidence supporting the regulatory function of $CD25^+CD4^+$ T cells has been found in many autoimmune animal models [62-64].

The inhibitory effects of regulatory T cells are mediated by TGF-β and IL-10 cytokines. The regulatory actions of TGF-β and IL-10 have been demonstrated in a number of autoimmune models [65, 66]. IL-10 inhibits T cell responses through its inhibitory effects on macrophage activation and cytokine secretion [67]. IL-10 knockout mice develop severe inflammatory bowel disease that can be prevented by exogenous IL-10 [68]. In addition, TGF-β also

inhibits the antigen presenting and effecter functions of macrophages [69]. Two weeks after birth, TGF-β knockout mice develop a severe wasting disease resembling GVHD [70].

1.5 Clinical Features of IDRs

Although IDRs do not occur in most patients and they are not predictable, some risk factors associated with their onset have already been identified: sex, age, genetic background, stress, and presence of viral or bacterial infections in the suffering patients. The risk of developing an IDR is greater among females than males [71]. Specific example is nevirapine-induced skin rash [72]. The incidence of IDRs is also higher among the elderly, although the increased risk may be due to multi-drug therapy and various diseases in these patients. Stress and viral or bacterial infections also seem to increase the risk of IDRs. Open-heart surgery, a major stress inducer, appears to increase the risk of procainamide-induced agranulocytosis by a factor of 10 [73]. Among HIV patients, the incidence of hypersensitivity reactions is increased during drug treatment with cotrimoxazole, carbamazepine and penicillins [74, 75].

The average time to IDR onset varies from weeks, to months, to years. For some IDRs, the time to onset is shorter upon secondary exposure, suggesting an adaptive immune response [76]. Idiosyncratic drug reactions can affect different target organs; the clinical features of most commonly affected target organs are described below.

1.5.1 Skin Reactions

One of the most common organ targets of IDRs is skin [77]. These reactions range from very mild, to more severe such as Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). In general, skin rashes occur soon after starting drug therapy, with most severe rashes appearing within four weeks of treatment [78]. Carbamazepine, sulfamethoxazole, penicillins, and phenytoin are some of drugs commonly associated with skin reactions [79].

Widely distributed blisters, particularly on the face, characterize SJS, while TEN is distinguished by loss of epidermis. Epidermal infiltration of $CD8^{+}$T cells and macrophages suggests immune involvement [80].

1.5.2 Hepatic Reactions

Liver is one of the other common targets of IDRs. Over 1,000 drugs have been reported to induce hepatotoxicity [81]. The symptoms range from very mild, non-symptomatic to severe hepatic failure. It has been shown that more than 50% of all hepatic failure cases are a result

of drug toxicity [82]. Evidence suggests that the liver damage induced by certain drugs is immune-mediated, as the incidence of hepatotoxicity increases upon secondary exposure. Drug-specific antibodies against phenytoin, carbamazepine, halothane, and diclofenac are an evidence that these effects are immune-mediated [83].

1.5.3 Blood Dyscrasias

Drugs can affect red blood cells, neutrophils, or the elements of the bone marrow, resulting in dramatic decreases of these blood cell types; the consequences can be fatal.

Aplastic anemia is the most serious blood dyscrasia. Interference with pluripotent stem cells results in the elimination of all bone marrow elements [84]. Recovery is slow and the mortality rate is 50% [85]. The most common drug-induced blood dyscrasia is agranulocytosis. This disorder is characterized by neutrophil depletion that could be due to either decreased production of neutrophil precursors in the bone marrow or destruction of mature neutrophils in the periphery. Aminopyrine, mianserin, propylthiouracil, captopril, and trimethoprim are drugs that are associated with a high incidence of agranulocytosis [79]. There is evidence of immune involvement for most drugs associated with agranulocytosis. In the case of aminopyrine, re-challenge with drug results in the onset of agranulocytosis; neutrophil levels drop dramatically within hours to days. Drug-specific antibodies that could be involved to the pathogenesis of agranulocytosis are seen in aminopyrine, amodiaquine, and mianserin patients [86-88].

Hemolytic anemia is another drug-induced blood dyscrasia that affects red blood cells. Hemolytic anemia is most commonly caused by high dose penicillin, but other drugs such as diclofenac is also associated with this reaction. Drug-induced hemolytic anemia is thought to be immune-mediated. Drug-specific IgG and IgM antibodies are found in penicillin patients [79].

1.5.4 Anaphylaxis

Drug-induced anaphylaxis is one of the IDRs that is immune-mediated. The onset of anaphylaxis is occurring upon secondary exposure to the drug. In the case of β-lactam antibiotics, 1/10000 patients experience anaphylactic drug reactions [89]. Drugs such as penicillin induce drug-specific IgE antibodies that bind to Fc receptors on mast cells. Upon secondary exposure the drugs bind IgE stimulating the release of histamine and other

inflammatory mediators that lead to vasodilation, causing a sudden drop in blood pressure, and airway constriction [90].

1.5.5 Drug-Induced Lupus

Drug-Induced lupus is a systemic autoimmune response characterized by the presence of anti-nuclear antibodies (ANA). Due to its similarity to idiopathic systemic lupus erythematosus (SLE), it is difficult to diagnose as drug-induced. The most common symptoms are skin rashes, myalgia and fever [91]. Diffuse myalgia is seen in 40% of patients with procainamide-induced lupus [92].

procainamide, methyldopa, isoniazid and phenytoin [93]. For hydralazine and procainamide, the incidence of drug-induced lupus is high, 5-10% [94] and 10-20% [23] of patients, respectively. Although the drugs listed above are older and no longer used frequently, drug-induced lupus is still a problem as some new drugs have recently been found to cause idiosyncratic drug-induced lupus. Among these newer drugs are minocycline, used to treat acne [95].

There are several characteristics of drug-induced lupus that differentiate it from the idiopathic lupus. Males and females are equally susceptible to drug-induced lupus, unlike the idiopathic form [96]. Anti-histone anti-nuclear antibodies are found in more than 95% of drug-induced lupus patients, whereas the anti-double stranded DNA antibodies commonly associated with idiopathic SLE are present in only 5% of drug-induced patients [93]. Most of the time, there is a time lag of months to years before patients develop the signs of disease and the symptoms of disease generally go away quickly upon drug withdrawal [91].

1.5.5.1 Proposed Mechanisms of Drug-Induced Lupus

Several mechanisms have been proposed to explain drug-induced lupus:

a) Inhibition of Complement: Deficiencies of C1, C2, and C4 components have shown to increase the risk of idiopathic SLE [97]. Several drugs associated with drug-induced lupus, e.g. hydralazine, isoniazid, D-penicillamine, and the hydroxylamine of procainamide inhibit the covalent binding of C4 by binding directly to the active site and thus lead to impaired clearance of immune complexes [76]. However, except in vitro studies, no other evidence exists to support this hypothesis.

b) Covalent Binding to Nuclear Antigens: It has also been proposed that by binding to histones or other nucleoproteins, drugs could act as haptens leading to the formation of Over 80 drugs have been associated with drug-induced lupus including hydralazine, anti-nuclear antibodies [98].

c) Activation by Mononuclear Phagocytes: A number of drugs associated with drug-induced lupus are oxidized to reactive metabolites within monocytes and macrophages. It has been proposed that these reactive metabolites could covalently bind to MHC II of macrophages leading to a graft versus host (GVHD)-type response [99].

d) Inhibition of DNA Methylation: As another mechanism of drug-induced lupus, inhibition of DNA methylation and subsequent alteration of gene expression has been proposed. Hydralazine and procainamide have been shown to induce autoreactive T cells by inhibiting DNA methylation. In an experimental model, T cells incubated with agents that inhibit DNA methylation adoptively transferred a lupus-like disease to naïve recipients [100].

1.6 Animal Models of Idiosyncratic Drug Reactions

As previously discussed, animal models are the best tool to understand the drug-induced IDRs. The greatest asset of an animal model is for the study of mechanisms of drug-induced IDRs, but it is an imperative that adverse reactions, which are induced in animals, closely resemble those observed in patients.

Animals most often used in study of the IDRs are rodents. The advantage of using small animals like mice in research is that they are widely commercially available. In addition, most biological and chemical reagents for scientific use have been designed for use in small animals. Furthermore, in the last decade, mice genome has been sequenced, and functions of many genes identified, making research with mice more viable.

Using inbred animals enables us to repeat observed drug-induced toxicity within the same strain of animals while studying IDRs, when all or most of the animals respond to the drug in a similar way.

Up to now, only several models of drug-induced idiosyncratic reactions have been found.

These include D-Penicillamine-induced autoimmunity in the male Brown Norway rat, propylthiouracil-associated autoimmunity in cats, sulphonamide-induced hypersensitivity in dogs, and nevirapine-triggered skin rash in the female Brown Norway rat. As none of these animal models of idiosyncratic reactions is able to predict the likelihood that a drug will cause idiosyncratic reaction in a patient, it is important for us to keep trying to develop more animal models.

1.6.1 Animal Model of D-Penicillamine-Induced Autoimmunity in the BN Rat

1.6.1.1 Chemical Properties of D-Penicillamine

D-Penicillamine (D-Pen) is a sulphydryl compound that is a structural analog of cysteine. Penicillamine can exist as either the D or the L stereoisomer due to the asymmetric carbon. The D form is the naturally occurring isomer and is used clinically. Due to the presence of the free sulphydryl group, D-Pen does not require enzyme-mediated metabolism to covalently bind to endogenous proteins. It is readily oxidized to form mixed disulphides or dimers. D-Pen is also able to form stable thiazolidine rings by reacting with aldehyde or ketone groups on proteins.

1.6.1.2 Clinical Uses of D-Penicillamine

In clinical practice the use of D-Pen is limited as it causes drug induced autoimuune diseases in 30 to 40% of long-term patients [101]. However, D-Pen is used in the treatment of some rare diseases such as Wilson's disease for which alternative therapies do not exist.

Wilson's disease is an inherited disorder whereby excessive copper leads to degenerative changes in the brain and liver cirrhosis. D-Pen promotes copper excretion and prevents further absorption.

D-Pen is an effective therapeutic drug in the treatment of rheumatoid arthritis. Rheumatoid arthritis is a chronic systemic autoimmune inflammatory disease that affects between 1 and 2% of the population [102].

1.6.1.3 Adverse Drug Reactions Associated with D-Penicillamine Use

Blood dyscrasias are the most important D-Pen-associated toxicities as they account for the majority of the mortality cases associated with D-Pen use. The most common blood dyscrasia is thrombocytopenia [103].

Skin rashes are the most common ADRs that are associated with D-Pen use [103]. Early rash, characterized by urticarial eruptions, occurs within days to weeks of drug treatment and is responsible for the majority of D-Pen-induced skin rashes. UV radiation is thought to increase the incidence of skin rash and as a result, D-Pen patients are advised to avoid strong sunlight [103]. In severe cases, a short course of corticosteroid therapy is necessary to alleviate symptoms [103]. D-Pen treatment is also associated with a number of autoimmune diseases including drug-induced lupus [104].

Adverse Drug Reactions Associated with D-Pen Use:

ADR	Incidence	Time to Onset
Early Rash [103]	25-50%	Days to weeks
Late Rash (pemphigus) [103]	1%	7 months to years
Blood Dyscrasias [103]	1%	3 to 18 months
Proteineuria [105]	13%	7 to 12 months
Drug-Induced Lupus [106]	2%	6 months to several years

1.6.1.4 D-Penicillamine-Induced Autoimmunity in the Brown Norway Rat

D-Pen induces autoimmunity in several animal models. In monkeys [107] and Wistar rats [108], D-Pen treatment is associated with nephropathy. D-Pen also induces ANAs in susceptible H-2^s mice [109]. Although D-Pen induces an immune response in these models, the animals do not get sick. It is very important to use models where the animal develops clinical symptoms of disease in response to drug treatment as ANAs are present in many patients that never develop IDRs. BN rats treated with D-Pen develop an autoimmune disease that can become very severe if drug is not withdrawn.

1.6.1.4.1 History of the D-Penicillamine Model

Donker et al. was the first one who described the BN rat model of D-Pen-induced autoimmunity. Seventy three percent of BN rats treated with 20 or 50 mg/day D-Pen developed a systemic autoimmune response characterized by severe dermatitis (ears and snout) and weight loss within 4 to 8 weeks. The mortality rate was near 100% in rats that were continued on drug treatment. Presence of ANAs and immune complex deposition along the glomerular capillary walls led to the comparison of this syndrome with D-Pen-induced

lupus in humans. Necrotic lesions were also seen in the spleen, liver, lungs, and skin of diseased rats. Both Lewis and Sprague Dawley rats are resistant to disease, so the disease is strain-specific. Donker et al. speculated that the toxicity might result from D-penicillamine binding to MHCII, leading to a GVHD response [7].

Druet et al. also studied the D-Pen model. He discovered that the immune responses to D-Pen and mercuric chloride are very similar in BN rats. Specifically, both compounds induce autoreactive T cells, anti-glomerular basement membrane (GBM) antibodies, and a rapid rise in IgE titers [110].

The two models are very similar but mercuric chloride is able to induce a stronger immune response than D-Pen. Because of the similarities between the two models, findings from the mercuric chloride model are often used to reference new findings from the D-Pen model.

1.6.1.4.2 Use of the D-Penicillamine Model in Investigating Mechanisms of IDR

The clinical symptoms of D-Pen-induced autoimmunity are similar in rats and humans: ANAs, skin rash, IgG deposits along the GBM and weight loss. The model also has other characteristic features of IDRs. For example, all BN rats respond to D-Pen treatment (ANAs and a rapid increase in IgE titers), but only a percentage of treated rats will develop clinical symptoms of disease and within the treatment group it is not possible to predict which animal will develop autoimmunity. There also appears to be a genetic component as only BN rats are susceptible while Lewis and Sprague Dawley rats are resistant.

1.6.2 Nevirapine-Induced Skin Rash and Hepatotoxicity

Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) used for the treatment of HIV type 1 [111]. NVP acts by binding to the tyrosine components of the reverse transcriptase enzyme located near the catalytic site [112]. The effect of NVP is termination of viral RNA transcription into viral DNA, thereby blocking its integration into human genome. NVP absorption is greater than 90%. It widely distributes throughout the body, including the nervous system [113]. NVP is often used in combination with another anti-HIV drug, reducing the chance of viral resistance and increasing the efficacy in patient treatment. It is often used as a post-exposure prophylaxis treatment, or to prevent transmission of HIV from mother to child [114, 115].

NVP therapy consists of bi-daily dosing, 400 mg in total [116]. Adverse events associated with treatment have been observed during clinical tests. Rash occurred at a 16% incidence within the first six weeks of therapy and hepatotoxicity with a 1% incidence [113]. Analyses of NVP-induced skin rash and hepatotoxicity incidences by Boehringer-Ingelheim indicate that NVP-induced skin rash occurs in 8.6% of patients and liver toxicity in about 3% of the patients. One-fifth of the reported rashes were severe or life-threatening. SJS and TEN syndromes account for 8% and 0.3-1%, respectively of the detected rashes [117, 118]. In patients given a lower dose (200 mg/day) dose in the first two weeks of treatment, decrease in the frequency of NVP -associated rash was observed [119]. The first 12 weeks of NVP treatment are a critical period during which intensive clinical and laboratory monitoring, including liver function tests, should be performed [120]. Fever is a predictor for the development of rash in patients receiving NVP [121]. Bersoff-Matcha shows that the incidence of severe rash was higher in women than in men [72]. NVP -induced skin rash and hepatotoxicity seem to be CD4+T cell dependent [122]. A higher incidence of skin rash [115] and severe hepatotoxicity have been reported in non-HIV-infected individuals [16]. The incidence of rash and liver toxicity are lower with low CD4+T cell counts; HIV-infected patients often have decreased CD4+ T cells counts, as these cells become infected by the virus and destroyed by the host immune system, decreasing the susceptibility of patients to develop NVP -mediated rash [123].

NVP is also used prophylactically. A single dose of NVP can be administered for prevention of perinatal HIV transmission without significant risk of serious adverse reactions [111]. However, by 2000 FDA had received reports of 22 serious adverse events related to NVP taken for post-exposure prophylaxis, including hepatotoxicity and skin reaction [124].

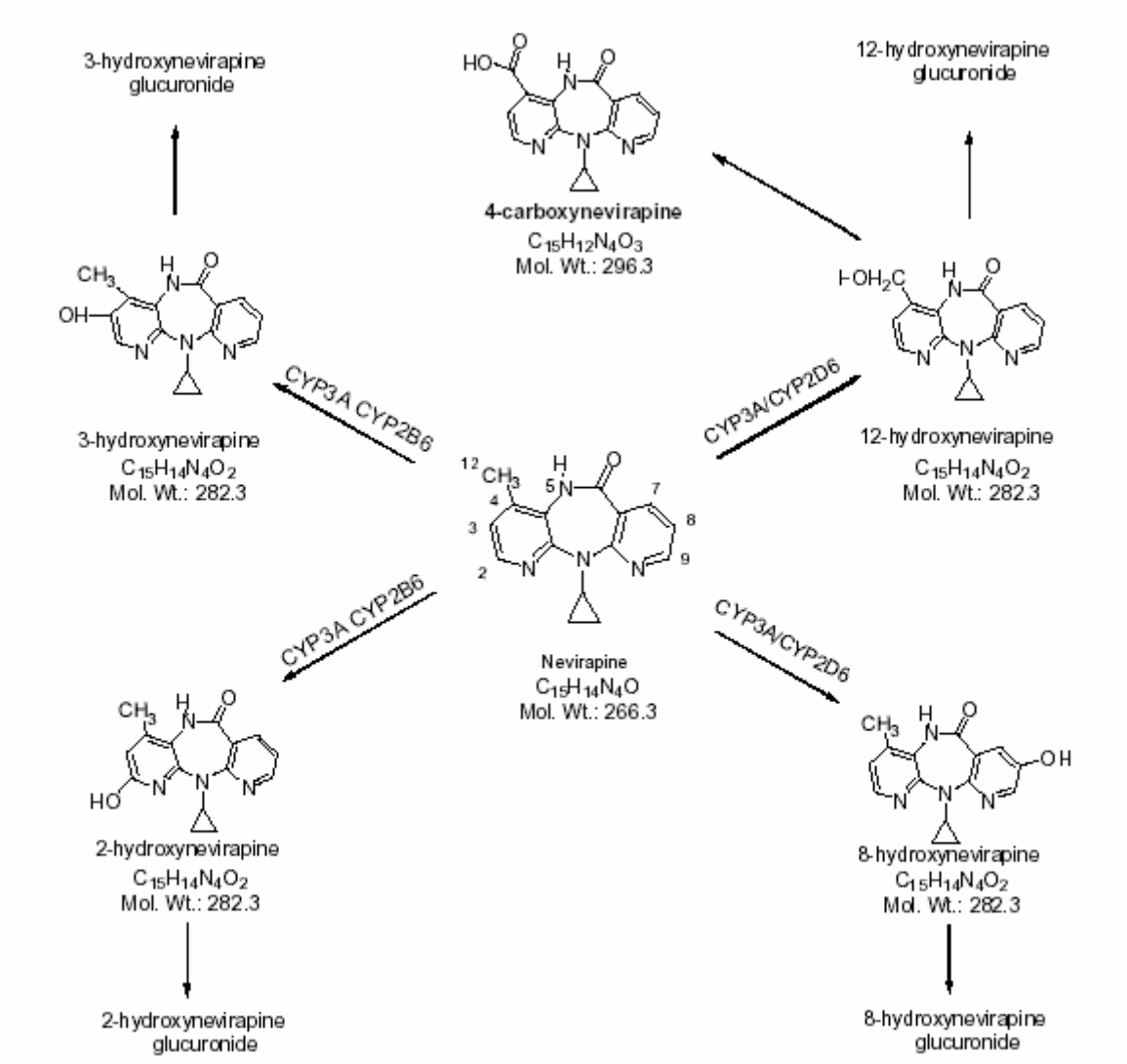
It is still not clear whether it is NVP or one of its metabolites that triggers immune reaction in patients. One of the hypothesis is that 12-hydroxynevirapine can act as a precursor to a reactive quninone methide formation. Once 12-hydroxynevirapine is formed in the liver, it can travel to various peripheral sites, including the skin. Sulfotransferases in the skin may sulfate 12-hydroxynevirapine which, would form a reactive quinone methide that can haptenate skin proteins and lead to an immune response [125].

The metabolic scheme of NVP is displayed in Figure 2A, and proposed scheme of quinone methide formation is displayed in Figure 2B.

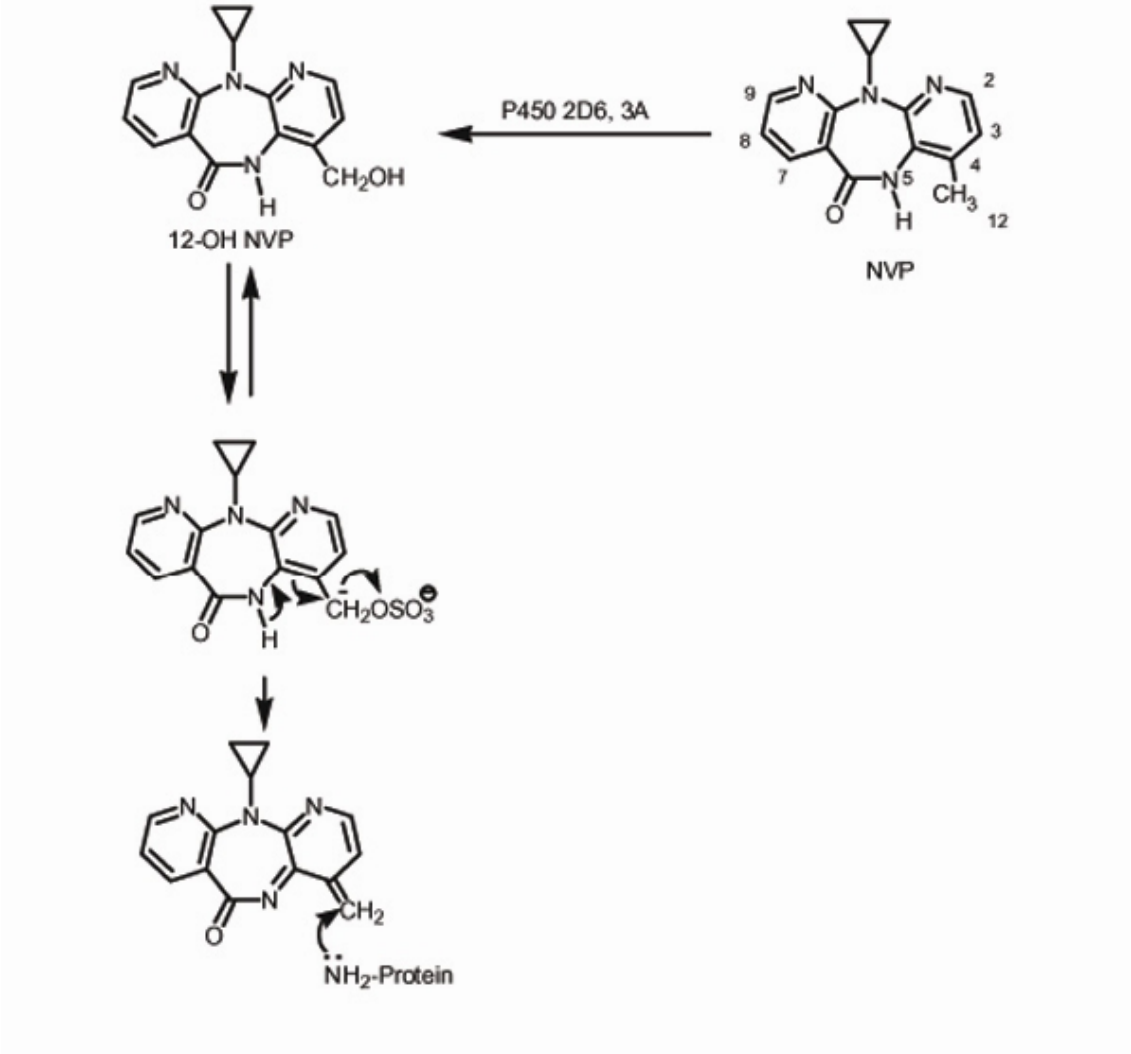
Figure 2. A) Nevirapine Metabolic Pathways, modified from [126]

B) Proposed Scheme for Quinone Methide Formation, modified from [126]

A)



B)



1.6.2.1 Animal Model of Nevirapine-Induced Skin Rash

Studies on NVP began about 10 years ago in Uetrecht's laboratory, where they treated four female Sprague Dawley rats with a 150 mg/kg/day dose of NVP for 6 weeks. They observed that during this time, 2 out of 4 NVP -treated rats developed erythema. In addition to treating female Sprague Dawley rats, Shenton et al. also treated female and male BN rats, female Lewis rats, and female SJL mice with NVP. After a month, they observed that rash developed in 20% of female Sprague Dawley and 100% of female BN rats. As all female BN rats responded to the treatment by developing skin rash, the work was continued with this sex and strain of rats. Initially, characterization studies took place [5] with one of the first questions being addressed was whether the BN model of NVP-induced skin rash is truly idiosyncratic.

As previously mentioned, IDRs do not occur in all patients nor animals treated with the drug; however, skin rash develops in all BN rats treated with NVP. Even though the incidence of skin rash is 100% in NVP-treated BN rats, the reaction is still considered idiosyncratic.

Idiosyncratic drug reactions do not occur in most patients, mainly because people have a wide array of genetic profiles, with differing predisposition for development of hypersensitivity reactions to drugs. As BN rats are highly inbred rats, there is a higher chance that if one BN rat develops skin rash during NVP treatment, all of them will. Increased predisposition to development of drug-induced hypersensitivity reaction has also been observed in monozygotic twins [120, 121].

The incidence of rash in the outbred Sprague Dawley rat strain was 20%, approximating the observed incidence of rash in humans [111]. The BN rat has also been used in the studies of gold-, mercury- and D-Pen-induced autoimmunity [122, 123], whereas Lewis rats are resistant to autoimmunity by these compounds [127]. SJL mice have also been found susceptible to mercury-induced autoimmunity; but, they were not susceptible to NVP -induced skin rash [128].

Comparison of the rash characteristics in rats and humans led to conclusion that there are a number of parallels between the two species:

a) Time to Onset – Patients are at the highest risk of developing skin rash within the first six weeks of therapy; most display the rash symptoms between week one and three [111]. In rats NVP treatment results in red ear onset by day 7, and skin rash onset between weeks two and six. All female BN rats develop skin rash within first three weeks of the treatment, while 20% of female Sprague Dawley rats develop rash at a later time point, between weeks four and six [5].

b) Sex – Females (both in patients and rats) are at a higher risk of developing the rash than males [72].

c) Characteristics of the Rash – In patients a range of rashes varying from mild erythematous to blistering skin eruptions, such as erythema multiforme, SJS and TEN were detected [111]. In female rats the rash starts with mild erythematous lesions, which over time progresses to a more severe phenotype. No blistering skin reactions are detected in rats because of their thin epidermis [5].

d) Histology – In skin lesions of patients, dermis is populated with a perivascular lymphocytic infiltrate associated with the endothelial cell swelling [113]. Most of the

mononuclear cells are monocytes, $CD8^+$T cells are predominantly located in the epidermis, and $CD4^+$T cells in the dermis of the skin. In rats mononuclear infiltrate is observed in the dermis. The keratinocyte death is present in the epidermis and in the dermal-epidermal junction [5].

e) Dose Response – When 400 mg NVP is administered once a day, 32 to 48% of patients develop skin rash, versus 9% when 200 mg is given [129]. All female BN rats dosed with 150 mg/kg/day NVP developed skin rash, half developed it when administered 100 mg/kg/day, and none developed the rash when given 50 mg/kg/day dose [5].

f) Tolerance Induction – To decrease the incidence of skin rash in patients 200 mg of NVP is administered daily for the first two weeks of the treatment, followed by the full 200 mg twice daily dosing. This regime successfully decreases the rash incidence by about 50%. In rats daily treatment with 40 or 75 mg/kg nevirapine for the first two weeks followed by the full 150 mg/kg daily dosing completely prevents the onset of skin rash [130].

g) Rechallenge – In previously sensitized patients, NVP rechallenge results in a faster and a more severe rash than on the initial exposure. In sensitized rats, the same is observed, primary challenge results in onset of red ears at day 7 and skin rash at day 14 of the treatment, while on rechallenge red ears and skin rash occur within 24 hours and 9 days, respectively [5].

h) T cells – Both in the skin lesions of NVP -treated patients and rats, the T cell infiltration has been documented. Female patients with $CD4^+$T cell counts higher than 250 cells/mm^3, and males with counts higher than 400 cells/mm^3 have a predisposition for developing the skin rash during NVP treatment. In rats with skin rash T cell infiltrates are present in the dermis, and the depletion of these cells is partially protective of the rash onset [6].

1.6.2.1.1 Immune Component of the Skin Rash in Rats

Popovic et al. published on the specific steps involved in immune-mediated mechanism of NVP -induced skin rash. They outlined that when auricular lymph nodes, ear and neck skin sections of the NVP -treated BN rats were analyzed an increase in the macrophage and eosinophil infiltration into the dermis by treatment day 7 (time point at which rats present

with red ears), with one third of the cells expressing ICAM-1 or MHC II cell surface activation markers was observed [130]. This precedes the lymphocyte infiltration into the skin, which takes place between treatment days 14 and 21, when skin rash starts. In rats with skin lesions skin MHC I and MHC II receptor expression was significantly increased, additionally, elevated IFN-γ, IL-1, IL-6, IL-10 and TNF-α cytokine levels were detected in the sera of the sick rats; IFN-γ plays a crucial role in the MHC up regulation [128, 129], while IL-1, IL-6 and TNF-α are proinflammatory cytokines that act as propagators of the immune response onset [131]. Shenton et al. has published that upon termination of a daily NVP dosing, it takes a month for skin rash to resolve. If rats are then rechallenged with at least 5 mg/kg NVP, red ears develop within 24h and skin lesions accompanied by malaise by day 4-7 of treatment. Additionally, the rat findings tightly correlate with the clinical picture; patients rechallenged with NVP develop faster and more severe skin rashes than during the primary exposure. In rats, rechallenge results in the loss of weight, hair, overall malaise and systemic sickness, which prompt for their euthanasia by day 9 of treatment. The main proponents of the skin rash onset in both primary and rechallenge exposures appeared to be host T cells. Shenton et al. was able to transfer the sensitivity from the NVP rechallenged rats into naïve recipients with the intravenous injection of the total splenocyte T cell population [130]. Recipients of $CD4^+$ T cells developed skin rash nine days later, while $CD8^+$T cell recipients behaved as NVP naïve rats, and only developed red ears by day 7 and skin rash by day 21 of the treatment [6]. $CD4^+$ T cell role was confirmed when partial depletion of these cells delayed the onset of the skin rash in rats, while a complete $CD8^+$T cell depletion led to a more severe rash [6]. In one patient with NVP -induced hepatitis, T cells specific for a parent drug have been found [132]. Nevertheless, for a number of idiosyncratic drug reactions, circumstantial evidence points out the importance of reactive drug metabolites in triggering adverse events, rather than parent compounds themselves. In case of NVP, its reactive metabolite quinone methide is suspected to trigger development of adverse skin reactions [130]. Once formed in the liver, oxidized NVP metabolite, 12-hydroxy NVP travels to the skin, where by action of sulfotransferase enzyme it may be converted into a 12-sulfa-nevirapine, which can spontaneously lose a sulfate moiety to form the reactive quinone methide.

1.6.2.1.2 Metabolic Component of the Skin Rash in Rats

Female BN, female Lewis and female Sprague Dawley rats treated with NVP at a daily 150 mg/kg dose showed 100%, 50% and 20% incidence of skin rash, respectively. Male BN, and male Lewis rats showed 25% and 0% incidence, respectively. When analyzed for the NVP blood levels, female BNs displayed the highest concentration, ranging from 30 to 40μg/mL, while male BN rats had the lowest, less than 10μg/ml. Male BN rats do not usually develop skin rash at a 150mg/kg NVP dose, and their blood levels are less than 10% of the levels detected in the females, however, when the males are co-treated with NVP and aminobenzotriazole (inhibitor of cytochrome P450), all rats develop skin rash. This cotreatment produces NVP blood levels similar to the ones observed in females at a 150mg/kg dose. When NVP blood concentrations were compared to the incidence of skin rash in rats, conclusion was made that the incidence was directly related to the NVP blood concentration. Although BN and Lewis rats have different predispositions for mounting immune responses against foreign antigens, in both of them skin rash occurs as long as the threshold NVP blood concentration is reached, indicating that one of the key components in initiating the skin rash in rats is host metabolism. Therefore, it is no surprise that the highest incidence of skin rash was observed in female BN rats, who also displayed the highest NVP blood concentration levels [126].

1.7 Predictive Mouse Models

1.7.1 Popliteal Lymph Node Assay/Reporter-Antigen Popliteal Lymph Node Assay (PLNA/RA-PLNA)

As discussed before IDRs do not occur in most patients at any dose. They can not be explained based on pharmacological properties of the drugs and are unpredictable [133]. Ultimate goal is to be able to treat, prevent and predict these reactions. One of the most promising strategies to predict the risk of sensitization by the drug is a PLNA [134]. The PLNA is an assay that utilizes popliteal lymph node hyperplasia as a way to measure the stimulating potential of a low molecular weight compounds (LMWC). In the primary PLNA, the LMWC is injected subcutaneously (s.c.) into the hind footpad of a mouse, or a rat. Seven days later the weight and cellularity of the LMWC treated lymph node is determined. Over 130 compounds have been tested in the PLNA and the results correlate with human data.

However PLNA needs further validation before it can be used as a screening tool, as there are a few false negatives including procainamide. This might be due to not looking at the sufficiently sensitive parameters in this assay. Furthermore, the involvement of T cells can not be assessed in the primary PLNA, as none of the parameters can discriminate irritant from sensitizing compounds. Parameters looked at can only assess for the presence of lymph node proliferation, effect that can also happen in the presence of an adjuvant alone [135]. For this purpose, detection of T cell-specific responses to reporter antigens in the modified PLNA is essential. In the modified PLNA the reporter antigen TNP-ovalbumin (TNP-OVA, T cell-dependent antigen) or TNP-Ficoll (T cell-independent antigen) is used to investigate the immunostimulatory potential of LMWC [135].

In this method, the LMWC is injected together with a sub-sensitizing dose of one of the reporter antigens and the formation of the TNP-specific antibody isotypes is followed seven days later. The isotype switching from IgM to IgG, due to the presence of TNP-OVA indicates an efficient interaction between T and B cells and non-specific inflammatory action of LMWC that leads to increase the production of this antibody response. An IgG response to TNP-Ficoll strictly depends on T cell help. But as T cells are unable to recognize TNP-Ficoll, such an IgG response implies the activation of LMWC- or neoantigen-specific T cells. Assessment of the IgG response to TNP-OVA and TNP-Ficoll offers the possibility to distinguish between immunosensitizing, and non-sensitizing (positive IgG response, or not to TNP-Ficoll, respectively), and also between irritant and innocent chemicals (no IgG response to TNP-Ficoll yet a positive IgG response, or no response at all to either TNP-OVA or TNP-Ficoll, respectively). The RA-PLNA also allows mechanistic studies, for instance, by detecting different antibody isotypes to the RA, Th1 (IgG2a) and Th2 (IgG1, IgE) responses can be discriminated [135].

Like all other assays the PLNA/RA-PLNA has its disadvantages. The main issue in this assay is using the irrelevant route of exposure, as most often the drugs are not administered s.c. but orally. Another aspect is the ethical problem that one can have with animal welfare committee for injecting potentially inflammatory compounds into a mouse footpad. An alternative way was recently addressed in a study that tested lymph node proliferation induced by selected drugs following s.c. injection in between mice ears. In another study that attempted to find an alternative assay to the traditional PLNA, injection of a range of pharmaceuticals together

with TNP-Ficoll into the ear resulted in the findings in the draining lymph node of the ear similar to those obtained with the RA-PLNA [136].

PLNA is a fast screening tool that allows testing a series of compounds and deciding which compound deserves further investigation without having a high chance of developing IDRs. However, for further hazard assessment a second way of testing, preferably oral, is required.

1.7.2 Oral Mouse Models of Drug-Induced Sensitization

Establishing an oral PLNA model includes an additional component of the immune system, mucosal. The mucosal immune system is very complicated because it has to tolerate many non-self antigens in an environment with potentially dangerous microbes [137]. The mucosal immune system accomplishes this by using a wide and complex range of regulatory mechanisms that eventually determine whether a substance is tolerated, or whether an immune response is mounted. Only a few attempts for inducing an immune response via oral route of administration of different drugs have been successful. In case of oral exposure to D-penicillamine, an increase in autoantibody levels in A.SW/Sn (H2s) mice [138] after 7–8 months was observed. Procainamide is able to induce an increase in antinuclear antibodies (ANA) in A/J mice after 8 months of exposure via the drinking water [139]. Diphenylhydantoin was administered for 6 months in genetically predisposed mice, C57BL/6-lpr/lpr. But in this case it appeared that after 6 months exposure, diphenylhydantoin depressed the levels of ANA [140]. In another study 16 different MHC-defined mouse strains were compared for induction of ANA by HgCl2; upon subcutaneous treatment they were detected after 0.5–2 months, for gold salts upon intramuscular exposure, they were detected after 1–5 months and upon oral D-penicillamine exposure, they were detected after 4.5–5 months.

The RA approach has recently been tested in combination with systemic (oral or intraperitoneal, i.p.) exposures. Results of these studies are hopeful. For instance, it was found that oral exposure to D-penicillamine, or diclofenac stimulated the RA-specific antibody and delayed type hypersensitivity (DTH) responses to i.p. co-administered TNP-OVA [9]. nevirapine stimulated DTH to TNP-OVA after oral and i.p. administration of the drug, but antibody responses were only activated after i.p. administration of the drug, suggesting potential for oral tolerance onset to nevirapine-induced antibody responses [9].

Single doses of either D-penicillamine or diclofenac [9] were also capable of stimulating drug-specific responses. These responses were detected by assessing TNP-specific antibody

formation in the PLN after injecting TNP-Ficoll together with a non-stimulating dose of the drug. These recent results suggest that the RA approach may be useful in assessing sensitizing potential following oral drug exposures. However, much more research, including examination of the role of mucosal immunoregulation, is required before an oral exposure RA-models can be used as a predictive immunotoxicological tool.

1.8 Research Hypotheses

The main research attempts of this thesis were:

- Validate PLNA/RA-PLNA assays and address the problem of false negative finding in case of Procainamide, while at the same time testing unknown compound (nevirapine) for their outcome in the same assay, by utilizing more sensitive molecular and immunological markers

- In the case of D-Penicillamine, based on the assumption that the parent drug is responsible for drug-induced lupus, a sequence of immune steps starting with immune cell activation leads to development of an oral mouse model of D-Penicillamine-induced immune response in female C3H/HeOuJ mice.

In case of nevirapine, based on the animal model of nevirapine-induced skin rash in the female BN rats and previous characterization of this already developed animal model implicated the immune system in skin rash development in rats [5] we tested the following hypotheses using a mouse animal model:

- Nevirapine initiate an immune response in RA-PLNA
- A sequence of immune steps including immune cell activation leads to development of an oral mouse model of nevirapine-induced immune response in female C3H/HeOuJ mice.

2 Materials and Methods

2.1 Chemicals, Reagents and Equipments

Acetic Acid	Sigma-Aldrich, Steinheim, Germany
Albumin fraction V (Bovine; BSA)	Sigma-Aldrich, Steinheim, Germany
5-Bromo-4-Chloro-3-Indolylphosphate	Sigma-Aldrich, Steinheim, Germany
Citric acid	Sigma-Aldrich, Steinheim, Germany
Coating Solution	KPL, Gaithersburg-MD, USA
Concanavalin A, Canavalia ensiformis	Merck KGaA, Darmstadt, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, Germany
D-Penicillamine hydrochloride	MP Biomedicals, Inc, Illkirch, France
Diphenylhydantoin	Sigma-Aldrich, Steinheim, Germany
Ethanol (absolute)	Merck, Darmstadt, Germany
Foetal bovine serum (FBS)	Gibco,Invitrogen, Karlsruhe,Germany
HEPES (N-2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid)	Gibco Life Technologies, Eggenstein, Germany
Lipopolysaccharides (LPS) (*Escherichia coli* 0111:B4)	Sigma-Aldrich, Steinheim, Germany
Magnesium chloride	Sigma-Aldrich, Steinheim, Germany
NEAA	Gibco, Scotland, UK
2-Mercaptoethanol	Gibco, Scotland, UK
Methanol	Merck, Darmstadt, Germany
Methyl Cellulose	Sigma-Aldrich, Steinheim, Germany
N,N-Dimethylformamid	Merck KGaA, Darmstadt, Germany
Nevirapine	Toronto Research, North York, ON, Canada

Nitro Blue Tetrazolium	Sigma-Aldrich, Steinheim, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
Penicillin-Streptomycin-Glutamine	Gibco, Scotland, UK
Phosphate buffered saline (PBS)	Sigma-Aldrich, Steinheim, Germany
Potassium chloride	Sigma-Aldrich, Steinheim, Germany
Potassium phosphate monobasic	Sigma-Aldrich, Steinheim, Germany
Procainamide	Sigma-Aldrich, Steinheim, Germany
Red blood cell lysing buffer	Sigma-Aldrich, Steinheim, Germany
RPMI 1640+GlutaMAX™-l	Gibco, Scotland, UK
Sodium acetate	Sigma chemical co.MO, USA
Sodium chloride	Sigma-Aldrich, Steinheim, Germany
Sodium citrate	Sigma-Aldrich, Steinheim, Germany
Sodium phosphate	Sigma-Aldrich, Steinheim, Germany
Sodium phosphate dibasicheptahydrate	Sigma-Aldrich, Steinheim, Germany
Sodium pyruvate	Gibco, Scotland, UK
Streptozocin, minimum 98% HPLC	Sigma-Aldrich, Steinheim, Germany
TNP (19)-BSA	Biosearch Technologies, INC
TNP (12)-OVAL	Biosearch Technologies, INC
TNP (90)-AECM-Ficoll	Biosearch Technologies, INC
Trizma® base, minimum 99.9% titration	Sigma-Aldrich, Seelze, Germany
Tween® 20 (polysorbate)	Sigma-Aldrich, Steinheim, Germany

2.1.1 Antibodies, Enzymes, and Kits

Antibodies:

List of mouse Antibodies	Fluorochrome	clone	Dilution
Anti-Mouse CD3e	Biotinylated	145-2C11	1/100
Anti-Mouse CD4	FITC	H129.19	1/200
Anti-Mouse CD8a	PE	53-6.7	1/50
Anti-Mouse CD25	PE	PC61	1/30
Anti-Mouse CD69	FITC	H1.2F3	1/30
Anti-Mouse CD69	PE	H1.2F3	1/30
Anti-Mouse CD19	PE/FITC	1D3/1D3	1/100/ / 1/200
Anti-Mouse MHC II	Biotinylated/PE	2G9	1/100
Anti-Mouse CD80	Biotinylated	16-10A1	1/50
Anti-Mouse CD86	Biotinylated	GL1	1/100
Anti-Mouse CD16/32	Mouse Fc Block	2.4G2	1/100
Anti-Mouse IgM (μ chain specific)	AP/Biotin		1/2000
Anti-Mouse IgG1 (γ_1 chain specific)	AP/Biotin		1/2000
Anti-Mouse IgM (γ_{2a} chain specific)	AP/Biotin		1/2000

Enzymes:

Streptavidin-APC — BD Pharmingen™

Kits:

BD Cytophix/Cytoperm™ (Fixation/Permeabilization Kit) — BD Biosciences, San Diego, CA, USA

Bio-Plex™, Mouse cytokine 10-Plex Assay (IL-1α, IL-2, IL-4, IL-6, IL-10, IL-12 (p40), IL-17, IFN-γ, TNF-α and RANTES) — Bio-Rad Laboratories, Inc. Hercules, CA, USA

Bio-Plex™ Calibration Kit — Bio-Rad, Hercules, CA, USA

Coating solution concentrate	KPL, MD, USA

Water was generally of Millipore-purified or distilled quality, produced by Milli-Q advantage A10 equipment

2.1.2 Equipment

Balances	Mettler, Toledo, Switzerland
(AT261 Delta Range and Mettler PM3000)	
Bio-Plex (Luminex) system	Bio Rad
Centrifuge (Heraeus instruments)	Thermo Fischer Scientific, Zuerich, Switzerland
CASY® cell counter + Analyzer system	Schaerfe system, Reutlingen, Germany
Digital pH meter	Thermo Orion, USA
FACS Calibur	Becton Dickinson, N.Y., USA
HI03 microscope	Carl Zeiss, Zuerich, Switzerland
shaker platform	Janke & Kunkel GmbH & CoKG
Vortex-Genie2$_{TM}$	Scientific industries, INC, N.Y., USA
VSE-2000-120 Sterile Bank	Skan, Switzerland
Water bath	VWR International, USA

2.1.3 Materials

Acrodisc® Syringe filters	Pall Corporation, MI, USA
BD plastipak™, syringe	Becton Dickinson, Madrid, Spain
Cell strainer (40 µm)	BD Biosciences, MA, USA
Centrifuge tubes (15, 50 ml)	Falcon, Heidelberg, Germany
Costar® Pipettes	Corning, N.Y., USA
Cover slips (various sizes)	Fisher Scientific, Pittsburgh, PA
Cryo vials	Nunc, Wiesbaden, Germany
Hypodermic injection needle	Rose GmbH, Trier, Germany
Microtest tubes (0.5, 1.5, 2 ml)	Eppendorf, Hamburg, Germany
MultiScreen$_{HTS}$® Assay system, 96-well filtration plate	Millipore, USA
Multi-well™ cell culture plates (6, 24, 96)	Beckton Dickinson, NJ, USA
Parafilm®	Laboratory film
Polypropylene conical tube (Falcon Tube)	Becton Dickinson, NJ., USA
Polystyrene round bottom tube (FACS Tube)	BD Biosciences, MA., USA
Steritop, presterilized receiver flask/	Millipore, Massachusetts, USA

2.2 Principle of Methods

This chapter includes the principles underlying the experiments performed in this study.

2.2.1 Organ Cell Count

Total counts of the cellularity of all organs are done by CASY cell counter after mechanical tissue disaggregating using a steel mesh.

2.2.2 Enzyme-Linked Immuno Spot Assay (ELISPOT)

ELISPOT assay is a tool for detecting individual cells that secrete a particular biomarker, in this study the ELISPOT assay was used to detect Antibody secreting cells.

In the ELISPOT procedure either a monoclonal or polyclonal antibody specific for the chosen analyte is pre-coated onto a PVDF 96-well plate. Cells are pipetted into the wells and the 96-well plate is placed into a humidified 37°C CO2 incubator for a specified period of time. During this incubation period, the immobilized antibodies bind to the secreted analyte. After washing away unspecific cells, an alkaline-phosphatase conjugated antibody specific for the chosen analyte is added to the wells. Following a wash step a substrate solution (BCIP/NBT) is added. Finally, spots will appear as blue-colored particles. Each individual spot represents an individual secreting cell. The spots can be counted with an automated ELISPOT reader system.

2.2.3 Mouse Cytokine Bio-Plex Cytokine Assay (Luminex)

The Luminex laser assay employs microspheres. Each bead can be covered with a reagent specific to a particular bioassay, allowing the detection of specific analytes from a sample. Within the Luminex analyzer, lasers provoke the internal dyes that detect each microsphere particle. Many readings are made on each bead set. In this way, xMAP technology allows multiplexing of up to 100 unique assays within a single sample, rapidly and precisely. Luminex technology has ideal speed and sensitivity for cytokine measurements. Its sensitivity is comparable with traditional ELISA-based systems, but with additional advantages including an expanded dynamic range and smaller sample size.

2.2.4 Flow Cytometry

The term "flow cytometry" derives from the measurement (meter) of single cells (cyto) as they flow past a series of detectors. The fundamental concept is the light scattering, light excitation, and emission of fluorochrome molecules to generate specific data from particles and cells in the size range of 0.5um to 40um diameter. Cells are hydro-dynamically focused in a sheath of PBS before intercepting an optimally focused light source (lasers). The signals which are given out will be picked up by detectors. These signals are then converted for computer storage and data analysis, and can provide information about various cellular properties. In order to make the measurement of biological/biochemical properties of interest easier, the cells are usually stained with fluorescent dyes which bind specifically to cellular constituents. The dyes are excited by the laser beam, and emit light at longer wavelengths. This emitted light is picked up by detectors, and these analogue signals are converted to digital so that they for later analysis.

2.2.5 Statistical Analysis

All data were analyzed using Sigma Stat version 3.1. Statistical comparisons between groups were performed using Anova one way test. Statistical significance was taken as $P < 0.05$.

2.3 Experimental Part

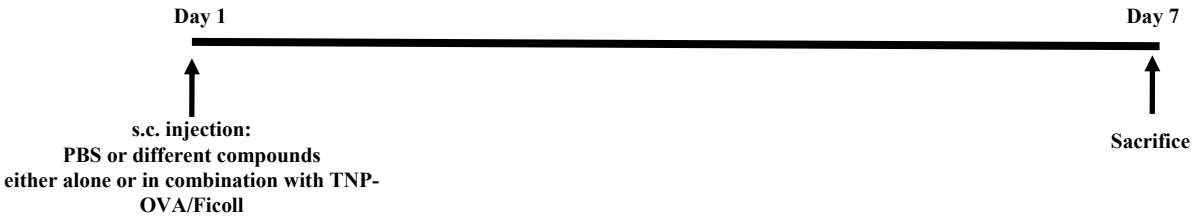
This chapter consists of the methods utilized in the studies.

2.3.1 Animal Care

Female pathogen-free C3H/HeOuJ mice (7-9 weeks old, 20-30 g) were obtained from Charles River Laboratories France, and were randomly assigned to specific treatment. Mice were allowed to acclimatize for a week while kept in Macrolon cages at a mean temperature of 23 ± 2°C, 50-55% relative humidity and a 12h light/dark cycle. Drinking water and standard laboratory food pellets were provided. The studies were performed in conformity with the Swiss Animal Welfare Law and Cantonal Veterinary Office, Basel land.

2.3.2 Study Design

In the local model mice were treated subcutaneously at 7-9 weeks (20-30 g) with either PBS or D-Penicillamine/Streptozotocin (1 mg/mouse) or Diphenylhydantoin (2 mg/mouse) or Nevirapine and Procainamide (1 and 3 mg/mouse) either alone or in combination with TNP-OVA/TNP-Ficoll. After 7 days animal were sacrificed by exsanguination under CO_2 and PLN was taken out from the right footpad to measure different immunological end points.



Although the PLNA/RA-PLNA are fast and simple preclinical assays, they have a disadvantage that the chemicals are injected subcutaneously (s.c.) instead of being orally administered. This is not a usual route of exposure for most drugs and chemicals. Therefore it is important to investigate whether drugs are able to induce systemic immunological changes via oral route of exposure which is a normal route of drug administration.

The antigen-specificity of drug induced immune response can vary from adjuvant like effect to T cell-specific. Using RAs as a read-out would enable us to differentiate between these two effects that can be caused by drugs. As drug induced hypersensitivity reactions are also very rare in animals only a limited number of animal models have been successfully established to

focus on this effect. In this thesis the focus for establishing an oral mouse model will be on Nevirapine and D-Penicillamine as reference compounds that have been investigated by different groups specifically in BN rats.

It was previously reported that female Brown Norway rats treated orally daily with 150 mg/kg nevirapine developed red ears between days 7 and 10, and skin rash between days 10 and 21 of the treatment [5].

To further investigate the mechanism(s) of Nevirapine-induced immune response in mice, C3H/HeOuJ mice were gavaged for 3 days with 200, 350 and 600 mg/kg NVP. These concentrations were chosen based on Shenton's data explained above [5]. They were able to induce skin rash in BN rats upon treatment with 150 mg/kg NVP. They were also able to induce low dose oral tolerance by treating BN rats with 40 or 75 mg/kg. To cover a broad range of different doses of NVP, the decision was to go ahead with the 200, 350 and 600 mg/kg daily dosing in mice, which corresponds to doses in the rats on a mg/m2 basis.

On day 4, in addition to the oral NVP treatment mice were i.p. injected with a suboptimal dose of reporter antigen (RA), TNP-OVA (200 μg/mouse, the concentration of TNP-OVA is based on a titration that was done in a pilot study to find a suboptimal dose). oral NVP treatment was continued for another 7 days. Finally, mice were sacrificed on day 10 and their organs (liver, ear, blood, spleen, auricular and inguinal lymph nodes) were taken and analyzed for the signs of an immune response onset.

Shenton's studies on BN rats show, necrotic lesions in the liver, infiltration of immune cells in the skin and ears and also increase in spleen weight in rats treated with 150 mg/kg NVP, hence these organs were also analyzed in our oral exposure mouse model with NVP.

Inguinal lymph node was selected as organ of interest based on the preliminary study that have been performed for finding the optimal concentration of TNP-OVA that should be i.p. injected. The data from this study showed upon systemic injection of RA, TNP-OVA the most pronounced immune response was detected in Inguinal lymph nodes.

In case of D-Pen, Donker et al. described for the first time the BN rat model of D-Pen-induced autoimmunity in 1984. Within 4 to 8 weeks, 73% of BN rats treated with 20 or 50 mg/day D-Pen developed a systemic autoimmune response characterized by severe dermatitis, especially observed on ears and snout of rats as well as overall weight loss. The mortality rate was near 100% in diseased rats that were continued on drug treatment. Anti-nuclear antibodies (ANAs) and immune complex deposition along the glomerular capillary walls led to the comparison of

this syndrome with D-Pen-induced lupus in humans. As well, necrotic lesions were found in the spleen, liver and skin of diseased animals. The disease is strain-specific as both Lewis and Sprague Dawley rats are resistant to disease. Within 4 to 8 weeks, 73% of BN rats treated with 20 or 50 mg/day D-Pen developed a systemic autoimmune response characterized by severe dermatitis, especially on ears and snout and weight loss. The mortality rate was near 100% in diseased rats that were continued on drug treatment. ANAs and immune complex deposition along the glomerular capillary walls led to the comparison of this syndrome with D-Pen-induced lupus in humans. As well, necrotic lesions were found in the spleen, liver and skin of diseased animals. The disease is strain-specific as both Lewis and Sprague Dawley rats are resistant to disease [7].

The BN rat model is a good model of autoimmune IDRs. The symptoms of disease are reproducible and the incidence is high within the BN strain. As well, the clinical symptoms of D-Pen-induced autoimmunity are similar in rats and humans: ANAs, skin rash and weight loss. The model also possesses other characteristic features of IDRs. While all BN rats initially respond to D-Pen treatment (ANAs and a rapid increase in IgE titers), only a percentage of those rats will develop clinical symptoms of disease. within a group, it is not possible to predict which animals will develop autoimmunity. There also appears to be a genetic component as only BN rats are susceptible; Lewis and Sprague Dawley are resistant [141].

In this case the same protocol is followed like in NVP, C3H/HeOuJ mice are gavaged 3 days with 50, 150 and 600 mg/kg D-Pen. These concentrations were chosen based on Donker's data and also Masson's study that was explained above. They were able to induce autoimmune response in BN rats upon treatment with 20 and 50 mg/rat D-Pen. Masson et al. was also able to induce low dose oral tolerance by treating BN rats with 5 mg/rat. To cover a broad range of different doses of D-Pen, the decision was to go ahead with the 50, 150 and 600 mg/kg daily dosing in mice, which corresponds to doses in the rats on a mg/m2 basis [141].

On day 4, in addition to the oral treatment of D-Pen mice are injected i.p. with the suboptimal dose of reporter antigen, TNP-OVA (200 μg/mouse) followed by the oral treatment with different concentrations of D-Pen being continued for another 7 days. Finally, mice were sacrificed on day 10 and their organs (caecum, skin, blood, spleen, mesenteric and inguinal lymph nodes) are taken and analyzed for the signs of an immune response onset.

The organs are chosen based on previous studies that have been performed by other groups in BN rats. Qasim et al. shows macroscopic lesions in caecum by day 15 and also histological studies that have been done by this group shows caecal vasculitis by day 3.
Masson et al. also shows infiltration of macrophages and costimulatory molecules in to the skin (ear) and caecum. Higher expression of macrophges and also costimulatory molecules was shown by Masson et al. in the spleen. Both groups show that IgE levels peak in serum after 2 weeks of treatment with D-Pen [141]. Necrotic lesions were seen in the spleen [7]. Mesenteric and Inguinal lymph nodes were studied based on titration study that has been performed for TNP-OVA i.p. titration. In this study after systemic injection of reporter antigen, TNP-OVA the most pronounced immune response was detected in Inguinal and mesenteric lymph nodes with the two highest concentrations of TNP-OVA (300 & 1000 μg/mouse).

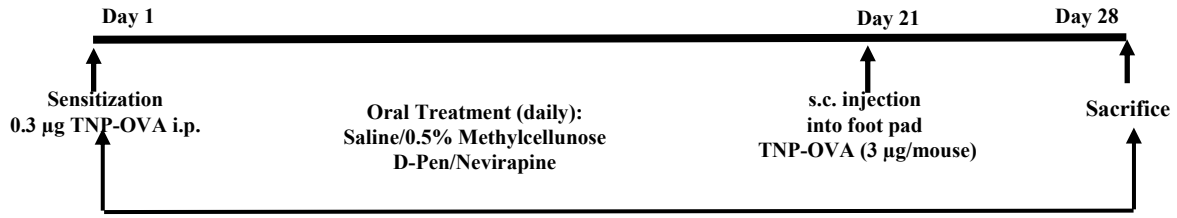
This protocol was designed based on RA-PLNA. In this assay the drug in combination with RA is s.c. injected to the foot pad. As we are looking at a systemic immune response the routs of administration for both drugs and RA are systemic, drugs are gavaged, RA is i.p. injected and the duration of the study was similar to RA-PLNA. The reason for giving the drugs by gavage three days in advance is just to reach enough amount of the drug in the circulation by the time that RA is i.p. injected.

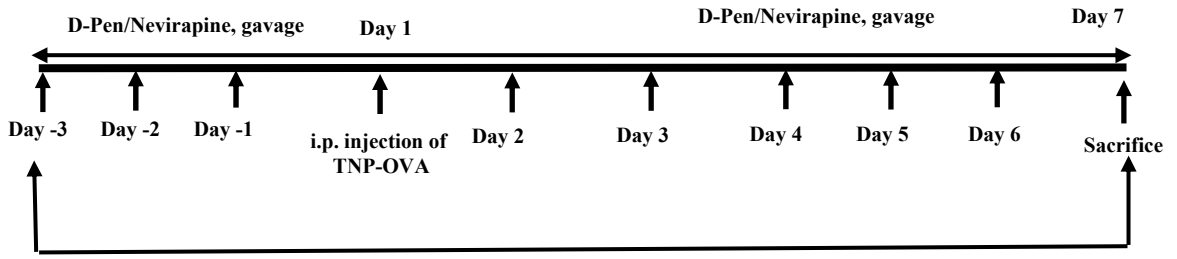
The second protocol for oral treatment was designed based on the Nierkens et al. attempt to establish an oral mouse model of drug induced IDR [9]. Additionaly, preliminary studies in two different strains of mice have been performed in our lab: C3H/HeOuJ and C57BL/6 in which mice were i.p. injected with TNP-OVA (10 μg) on the first day and then gavaged once daily with either 150 mg/kg D-Pen or 200, 350 and 500 mg/kg Nevirapine for 28 days. After three weeks mice were s.c. rechallenged in their right foot pads with a suboptimal dose of reporter antigen, TNP-OVA (10 μg/mouse). A week later different organs (e.g. PLN, liver, spleen) were sampled from these mice, and prepared for the signs of an immune response onset. The results of this preliminary experiment showed signs of an immune response onset: in C3H/HeOuJ mice orally dosed with 200 mg/kg Nevirapine, cytokine levels and IgG1 production by PLN cells was elevated. Elevation of IL-1 and IFN-γ was also seen with 150 mg/kg D-Pen, but the increases in both cases (NVP & D-Pen) were not significant and also not supported by increase in other immune markers. One of the explanations was that the doses used for presensitizing and resensitizing animals with TNP-OVA i.p. and s.c.,

respectively were too high, so the effect of the drug could be masked by the response which is caused by reporter antigen. Therefore we decided to titer the TNP-OVA in both cases. The results of the titration studies show that the suboptimal concentrations of TNP-OVA that should be used in the main study are 0.3 µg/mouse TNP-OVA i.p. on day 1 for presensitizing and 3 µg/mouse TNP-OVA s.c. on day 21 for resensitizing in C3H/HeOuJ mice.

To repeat this study under optimal conditions to investigate the mechanism(s) of NVP and D-Pen-induced immune response in mice, C3H/HeOuJ mice were i.p. injected with TNP-OVA (0.3 µg/mouse) on the first day and then gavaged once daily with either 150, 600 mg/kg D-Pen or 200, 600 mg/kg Nevirapine for 28 days. After three weeks mice were s.c. rechallenged in their right foot pads with a suboptimal dose of reporter antigen, TNP-OVA (3 µg/mouse). The compound's concentrations are chosen based on previous studies in BN rats that was explained above. Finally, mice were sacrificed on day 28 and their organs (liver, ear, blood, spleen, auricular and inguinal lymph nodes) were taken and analyzed for the signs of an immune response onset.

The two protocols that are designed for oral treatment are illustrated below. Animals were sacrificed by exsanguination under CO_2. Organs were taken and either stored on ice for further investigations, or frozen at -20°C, or snap frozen and stored at -80°C respectively, until used for one of the following techniques:





2.3.3 Quantification of Anti-TNP IgM, IgG1, and IgG2a Producing Cells (ELISPOT)

Immobilon PVDF membranes (Millipore) (as 96-well-plate) were pre-wet with 20 µl 50% ethanol, washed with PBS, and coated overnight with 100 µl 20 µg/ml TNP-BSA (in PBS) at 4°C. Following washing with PBS, membranes were blocked with 200 µl 20% FCS (in PBS) at room temperature for two hours. 500,000 tissue cells or 0.10, 100 and 1000 mouse myeloma cells (ATCC-TIB-191) which produce IgG1 against 2,4,6-trinitrophenyl which were used as positive control (in 100 µl medium containing 10% FCS) were added to each well and incubated at 37°C in a 5% CO_2 and humidified incubator for 20 hours. Following aspiration of cell suspensions and washing of wells with first deionized water (2x), then PBS (2x), and then PBS/0.05% Tween-20 (3x), 100 µl alkaline phosphatase (AP)-conjugated detection antibodies in PBS/BSA 0.5% were added to the wells for 2 hours at room temperature: *1.* AP-conjugated goat-anti-mouse IgM (Southern Biotech; 1/5000); *2.* AP-conjugated goat-anti-mouse IgG1 (Southern Biotech; 1/5000); *3.* AP-conjugated goat-anti-mouse IgG2a (Southern Biotech; 1/5000). After discarding detection antibody solution and 4x washing with 200 µl/well PBS/0.05% Tween-20 (allow wells to soak for 2 minutes at each wash step) and further 2x washing with 200 µl/well PBS, 100 µl/well final substrate solution (NBT/BCIP reagent) were added. Spot development was monitored after 10-20 min incubation. It was ensured that spots do not overdevelop in order to control the background. The substrate reaction was stopped by washing the wells with DI water. The plates were air-dried at room temperature overnight until they were completely dry. Plates are stored in a sealed plastic bag in the dark, until they are analyzed. Spots were enumerated manually by inspection under a dissecting microscope and automatically using an ELISPOT plate reader.

2.3.4 Cell Culture and Cytokine Measurement

Cell suspensions ($2x10^5$cells) in complete RPMI 1640 with Glutamax-I supplemented with 10% FBS and 2% penicillin-streptomycin (Invitrogen) were incubated with medium, LPS (2 µg/ml), or ConA (5 µg/ml) in 96-well plates (Highbond 3590; Costar, Cambridge, MA) overnight at 37°C and 5% CO2. Supernatant was collected and stored at –20°C until analysis. Cytokine levels were determined by Luminex analysis.

2.3.5 Determination of Cytokines by the Luminex™ Microbead Assay

The plasma levels of cytokines (IL-1α, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, TNF-α , IFN-γ and RANTES) were determined by using the Luminex™ microbead assay according to the manufacturer's instruction (BioSource Europe S.A. Belgium).

2.3.6 Immunophenotyping

The organs used for flow cytometric analysis were kept in ice-cold PBS/0.5% BSA on ice. Organ suspensions were prepared by mechanical tissue disruption using a stainless steel mesh. From each organ cell suspension, 10 µl was individually mixed with 10 ml CASY®ton. Individual cell counts were determined in a conductometer (CASY®TTC, Schärfe System, Reutlingen, Germany).

The organ cell suspensions used for flow cytometric analysis was kept on ice. About 1 x 10^6 cells from each organ cell suspension was washed with PBS/FCS 2% , after centrifugation , following mouse FC blocking (clone 2.4G2) cells were washed once more and then incubated in predetermined dilutions of FITC-, PE-, PB- and APC-conjugated monoclonal antibodies in FACS tubes for 30 min at 4°C in the dark. Samples incubated with biotin conjugated monoclonal antibodies were once again centrifuged and incubated with streptavidin-APC in the same way. Cells were washed with PBS and resuspended and stored in PBS. Samples were analyzed by flow cytometry using the LSR II flow cytometer. At least 100,000 cells in the appropriate live cell gate (FSC/SSC plot) were acquired for each staining.

2.3.7 Analysis of Cytokine mRNA

2.3.7.1 RNA Extraction and Purification

Total RNA was obtained by acid guanidinium thiocyanate-phenol-chloroform extraction (Trizol, Invitrogen Life Technologies) from each frozen tissue section and the total RNA was then purified on an affinity resin (Rneasy, Qiagen) according to the manufacturer's instructions and quantified. Total RNA was quantified by the absorbance at $\lambda = 260$ nm (A_{260nm}), and the purity was estimated by the ratio A_{260nm}/A_{280nm}. Integrity of the RNA molecules was confirmed by non-denaturing agarose gel electrophoresis. RNA was stored at approximately -80°C until analysis.

2.3.7.2 Gene Chip Experiment

The LIMS database is connected to a UNIX Sun Solaris server through a network filing system that allows for the average of all Gene Chip experiments were conducted as recommended by the manufacturer of the Gene Chip system (Affymetrix, Santa Clara, CA). Double stranded cDNA was synthesized with a starting amount of approximately 5 µg full-length total RNA using the Superscript Choice System (Invitrogen Life Technologies) in the presence of a T7-(dT)24 DNA oligonucleotide primer. Following synthesis, the cDNA was purified by phenol/chloroform/isoamylalkohol extraction and ethanol precipitation. The purified cDNA was then transcribed in vitro using the BioArray® High Yield RNA Transcript Labeling Kit (ENZO) in the presence of biotinylated ribonucleotides form biotin labeled cRNA. The labeled cRNA was then purified on an affinity resin (Rneasy, Qiagen), quantified and fragmented. An amount of approximately 10 µg labeled cRNA was hybridized for approximately 16 hours at 45ºC to an expression probe array. The array was then washed and stained twice with streptavidin-phycoerythrin (Molecular Probes) using the Gene Chip Fluidics Workstation (Affymetrix). The array was then scanned twice using a confocal laser scanner (Gene Array Scanner, Agilent) resulting in one scanned image. This resulting ".dat-file" was processed using the MAS5 program (Affymetrix) into a ".cel-file" which was captured and loaded into the Affymetrix Gene Chip Laboratory Information Management System (LIMS). nsities for all probes cells (CEL file) to be downloaded into an Oracle database (Genomics Data Layer; GDL). Raw data was converted to expression levels using a "target intensity" of 150. The Gene Chip® array used was the Mouse Genome MOE430 2.0 expression probe array (Affymetrix, Inc., San Diego, CA, USA), which allows for analysis of more than 39,000 transcripts.

2.3.7.3 Data Analysis

The data were checked for quality, exported from our internal Genomics Data Layer database (GDL database) and loaded in COMPARE 3.2.10 (internal software analytical tool) for analysis. COMPARE 3.2.10 was used exclusively for the analysis of this particular study.

The decision to consider a specific gene relevant for a signature is based on a conjunction of numerical changes identified by exploratory filtering and the relationship to other modulated genes that point to a common biological theme. Similarly modulated genes were identified by

using a Pearson correlation against the entire chip. The weight of that relationship is assessed by the analyst through a review of the relevant scientific literature. Therefore, additional genes, based on their biological relationship to statistically significant genes were also included in the analysis. Established gene signatures were scored using the in-house developed software Compare 3.2.10. The score is calculated as the geometric mean of the fold changes of each gene present in the signature.

The information content of these data sets is a conjunction of numerical changes and biological information. The decision to consider a specific gene relevant was based on a interpretation of numerical changes identified by comparative and statistical algorithms and the relationship to other modulated genes that point to a common biological theme. The value of that relationship was assessed by the analyst through a review of the relevant scientific literature.

3 Results

3.1 Local Assays (PLNA/RA-PLNA)

3.1.1 Popliteal Lymph Node Assay (PLNA)

Based on previous works which were done in humans and other species with PA and NVP, these two drugs were chosen as test compounds for local assays in this thesis.

In this part D-Penicillamine (D-Pen), Diphenylhydantoin (DPH) and Streptozotocin (STZ) were used as reference compounds, known to trigger an immune response in PLNA. D-Pen and DPH induce increases in IL-4 secretion [11]. STZ induces increases in macrophages, IFN-γ and TNF-α production and CD8+ T cells proliferation in the PLNA [142].
To perform the PLNA, 50 µl of freshly prepared drugs are injected s.c. into the right footpad of female C3H/HeOuJ mice. Drugs are injected in quantities that were determined to be immunostimulatory in the previous PLNA studies [143]: 1 mg D-Pen, 2 mg DPH, 1 mg STZ , 1 or 3 mg PA, 1 or 3 mg nevirapine (NVP) dissolved/suspended in PBS. Control group is treated with PBS. Seven days after drug injection, mice are sacrificed.

3.1.1.1 Popliteal Lymph Node Weight and Cell Count Determination

Figure 1A shows the PLN weight and figure 1B shows PLN cell count after s.c. injection of the compound of interest. Both weight and the total cell numbers of PLN were increased in mice upon treatment with D-Pen, DPH and STZ, whereas the weight and cellularity were similar to control in case of PA and NVP.

Exposure to D-Pen, DPH and STZ was associated with the induction of inflammation in footpad of mice, whereas paws of mice that were treated with different concentrations of PA or NVP were not different from control mice.

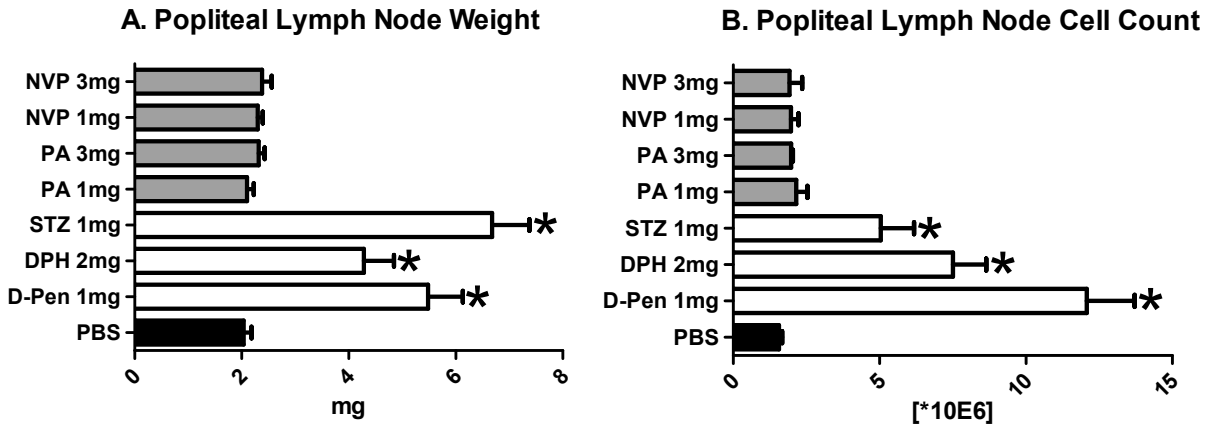


Figure 1. PLN weight and cell number. Mice were injected s.c. with either PBS or the indicated compounds in the footpad. After 7 days, PLN was isolated, weight and cellularity were measured. Bars represent the mean value ± STD of 5 mice. *, shows significant differences ($p<0.05$) referred to the control group.

3.1.1.2 The Effect of Different Drugs on Cytokine Levels in PLN Supernatant

A panel of 10 cytokines/chemokine (IL-1α, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IFN-γ, TNF-α and RANTES) was measured in PLN supernatant of animals that were treated with either PBS or different compounds (Figure 2A-D). D-Pen and DPH were tested as reference of type 2 immune response and STZ as a reference of type 1 immune response. The difference between type 1 and type 2 responses is based on differentiation of T helper cells in to either Th1 or Th2 cells that are characterized by their pattern of cytokine secretion [144]. In mice, IFN-γ is generally used as type 1 prototypes, whereas IL-4 is an indicator of type 2 response.

After 7 days PLNs were isolated and restimulated with either ConA or LPS for 24 h. As STZ and NVP are known as macrophage activating chemicals in addition to IL-4 and IFN-γ, IL-1α, IL-6, IL-12 and TNF-α were measured as proinflammatory cytokines. IL-2 was measured as an indicator of T cell activation.

IL-10 was studied as an anti-inflammatory cytokine, mainly expressed by monocytes, Th2, mast cells, CD4+CD25+Foxp3+ regulatory T cells, and also in a certain subset of activated T cells and B cells. It down-regulates the expression of Th1 cytokines, MHC class II antigens, and costimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production.

IL-17 was measured as a subset of helper T cells that produce IL-17, it has an important role in the induction of autoimmune tissue injury. Th17 cells are distinct from Th1 or Th2 cells since they do not produce classical Th1 or Th2 cytokines such as IFN-γ or IL-4.

RANTES was investigated as a chemokine which plays an active role in recruiting leukocytes into inflammatory sites.

As can be taken from the information in Figure 2, the levels of IL-2, IL-6, IL-12 and RANTES were significantly increased in STZ treated mice. No changes were observed in D-Pen and DPH treated mice.

An increase was observed in the levels of RANTES upon treatment with PA & NVP, specifically with 3 mg, however this increase was not supported by other measured immunological parameters.

The levels of other investigated cytokines (IL-1α, IL-4, IL-10, IL-17, IFN-γ and TNF-α) were under detection limit.

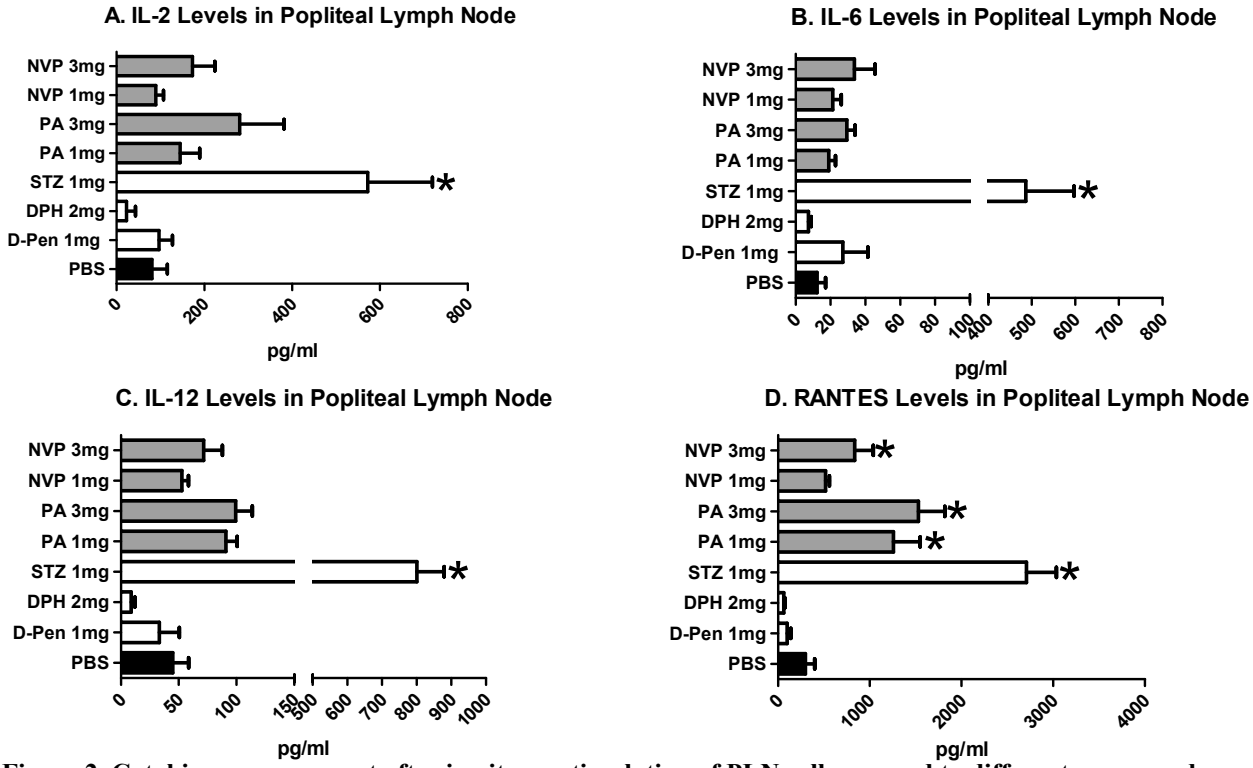
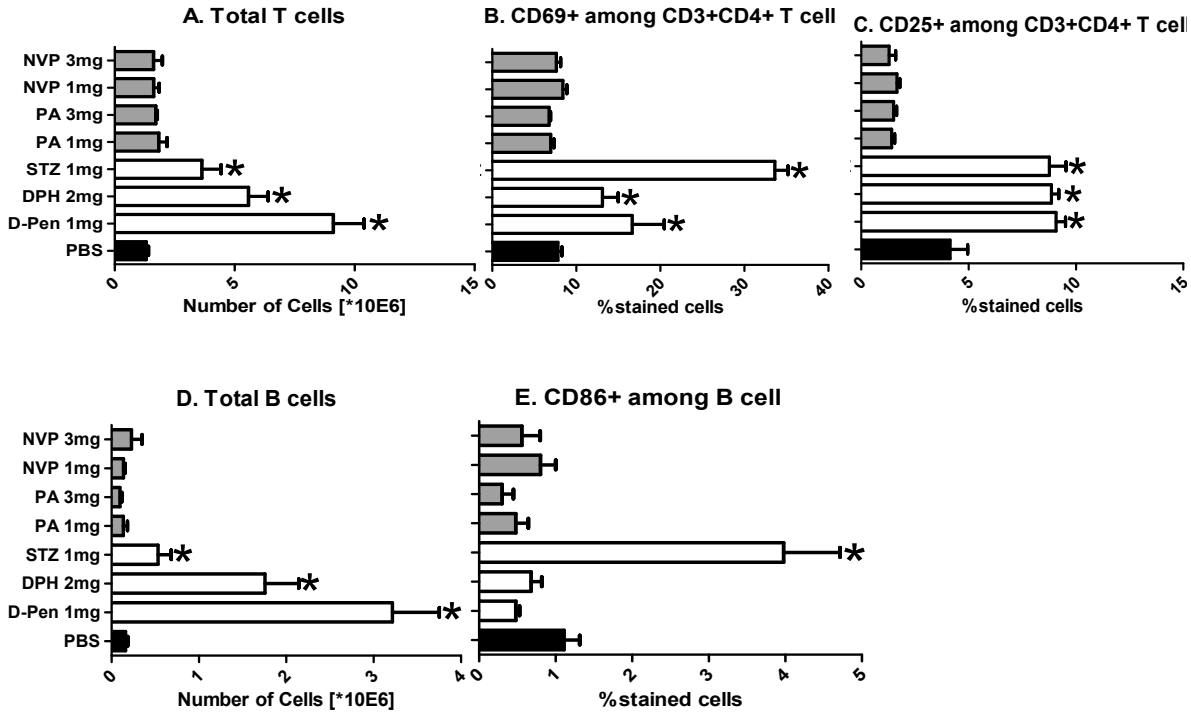
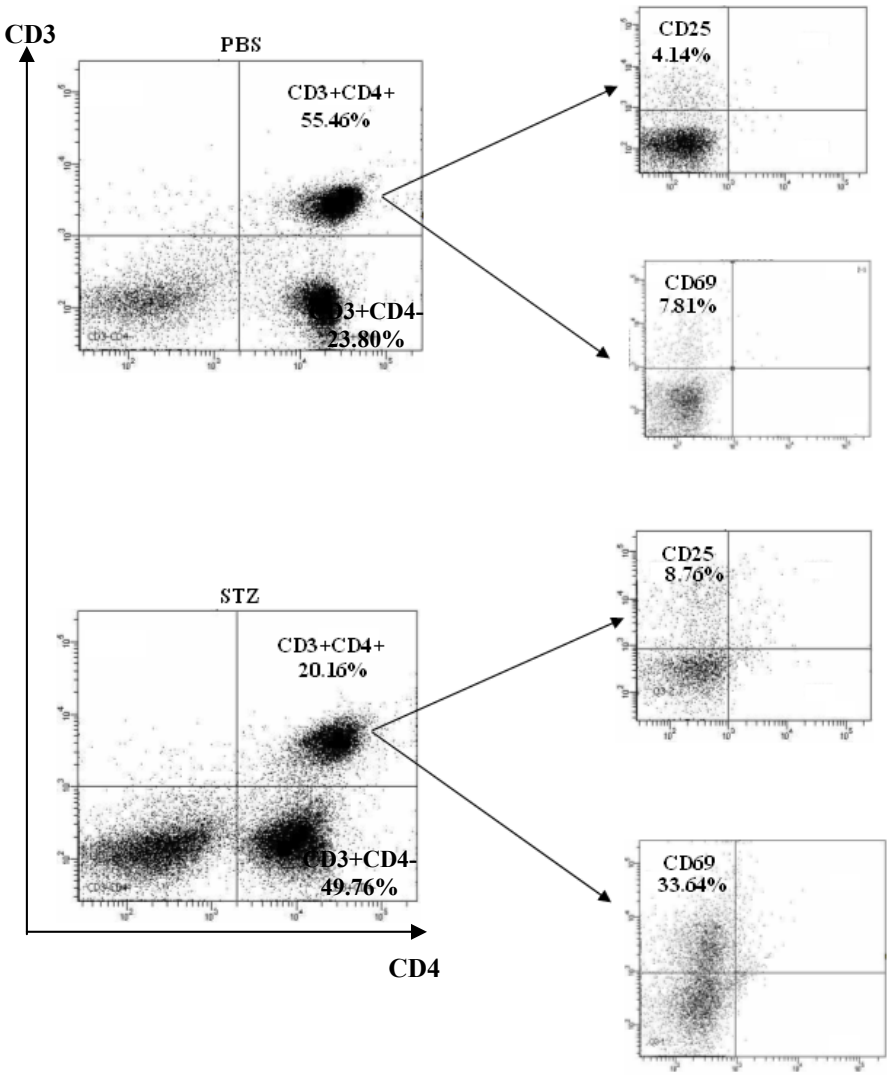


Figure 2. Cytokine measurement after in vitro restimulation of PLN cells exposed to different compounds. Mice were treated s.c. with either PBS or the indicated compounds in the footpad. After 7 days, PLN was isolated and restimmulated with either ConA or LPS for 24h. Bars represent the mean value of cytokine levels ± STD of maximum 5 mice. *, shows significant differences ($p<0.05$) referred to the control group.

3.1.1.3 Immunological Phenotype, the Effect of Various Compounds on Proportion of Different Cell Populations in PLN

The changes in percentages of T cells, B cells and their activation markers were evaluated in response to different chemicals. The results of this experiment (Figure 3A-E) showed a decrease in percentage of T cells after D-Pen, DPH and STZ treatment. This decrease was mainly seen in the percent of CD4+ T cells. An increase in percent of CD8+ T cells was seen in STZ treated mice. An increase in percent of B cells was also seen in D-Pen and DPH treated mice. The total number of both T and B cells were increased in D-Pen, DPH and STZ compounds. There was also an increase in T cell activation markers observed after D-Pen, DPH and STZ treated mice. An increase was observed in B cell activation markers (CD86) in STZ treated mice. No changes were observed either in T and B cells or in their activation markers in the mice that were treated with test compounds (PA and NVP).




CD3
PBS
CD3+CD4+
55.46%
CD3+CD4-
23.80%
CD25
4.14%
CD69
7.81%
STZ
CD3+CD4+
20.16%
CD3+CD4-
49.76%
CD25
8.76%
CD69
33.64%
CD4

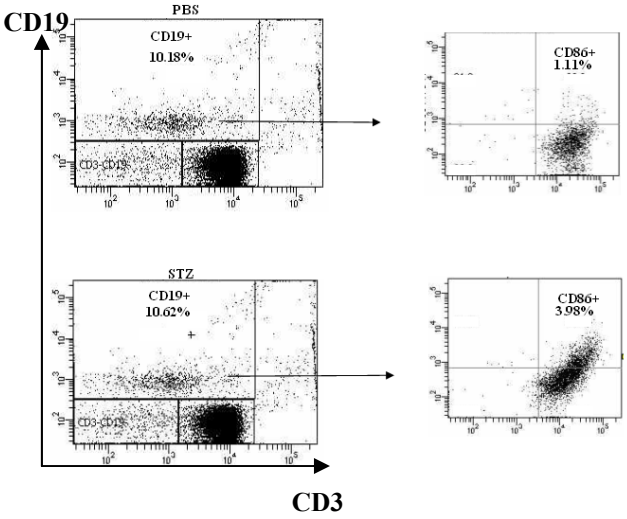


Figure 3. The effect of various compounds on proportion of different cell populations in PLN Mice were treated s.c. with either PBS or the indicated compounds in the footpad. After 7 days, PLN was isolated and changes in percentages of T cells, B cells and their activation markers were evaluated in response to different chemicals. Bars represent the mean value of cytokine levels ± STD of maximum 5 mice. *, shows significant differences ($p<0.05$) referred to the control group. CD3 was used to identify T cells and CD19 was used to identify B cells.

3.1.1.4 Effects of Different Compounds on Cytokine/Chemokine mRNA Expression

Semi-quantitative RT-PCR was performed on lymphocytes of treated mice to analyze changes in cytokine or chemokine expression (Figure 4A-H).

In lymphocytes, STZ up regulates mRNA expression of all cytokines also cytotoxic T cells. In mice treated with D-Pen, DPH or test compounds PA or NVP no significant changes in mRNA levels of cytokines or chemokine were observed. The values of mRNA expression for IL-4, IL-10 and IL-17 were to close to the background, so they were considered undetectable.

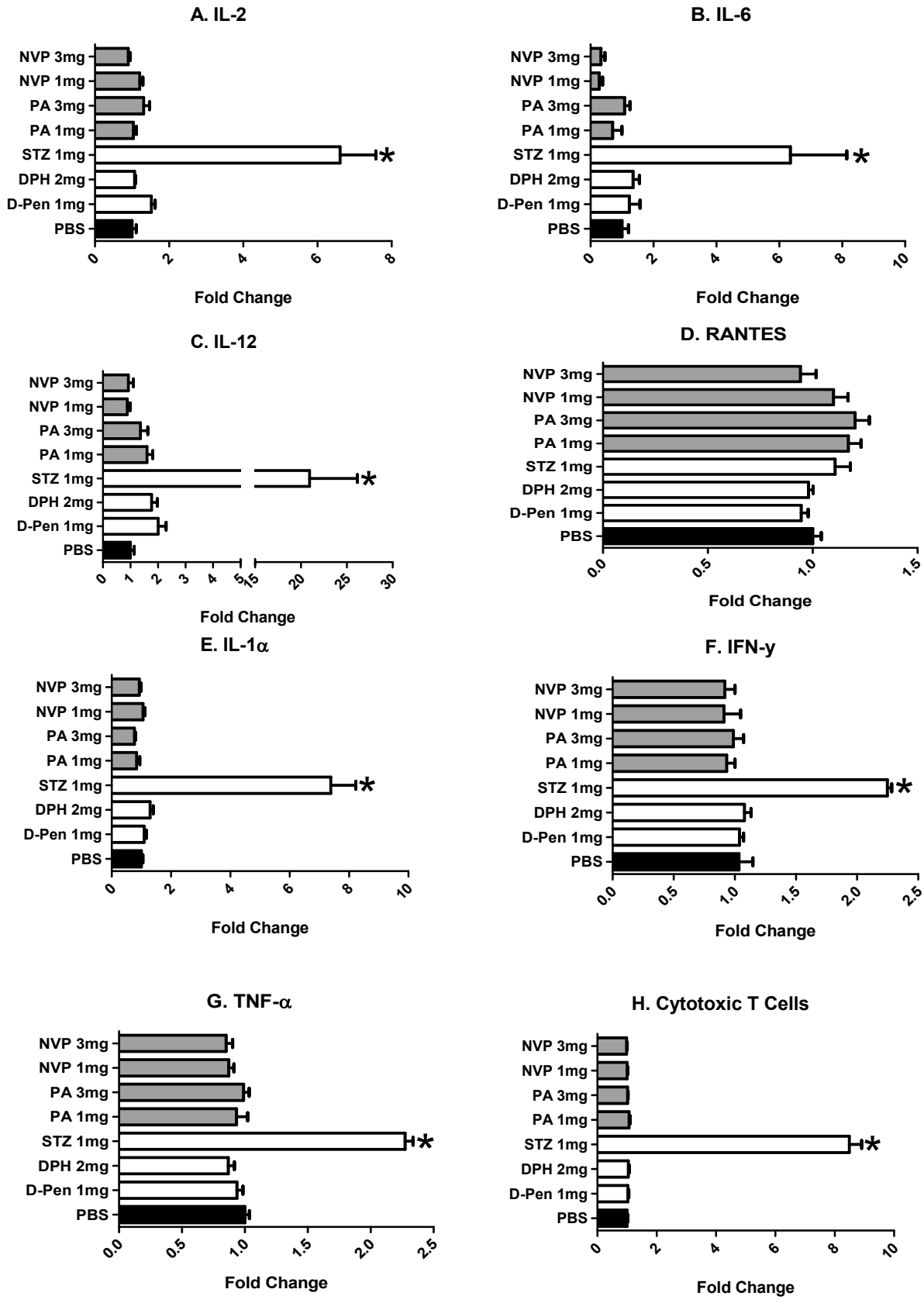

A. IL-2
NVP 3mg
NVP 1mg
PA 3mg
PA 1mg
STZ 1mg
DPH 2mg
D-Pen 1mg
PBS
0
2
4
6
8
*
Fold Change
B. IL-6
NVP 3mg
NVP 1mg
PA 3mg
PA 1mg
STZ 1mg
DPH 2mg
D-Pen 1mg
PBS
0
2
4
6
8
10
*
Fold Change
C. IL-12
NVP 3mg
NVP 1mg
PA 3mg
PA 1mg
STZ 1mg
DPH 2mg
D-Pen 1mg
PBS
0
1
2
3
4
5
15
20
25
30
*
Fold Change
D. RANTES
NVP 3mg
NVP 1mg
PA 3mg
PA 1mg
STZ 1mg
DPH 2mg
D-Pen 1mg
PBS
0.0
0.5
1.0
1.5
Fold Change
E. IL-1α
NVP 3mg
NVP 1mg
PA 3mg
PA 1mg
STZ 1mg
DPH 2mg
D-Pen 1mg
PBS
0
2
4
6
8
10
*
Fold Change
F. IFN-y
NVP 3mg
NVP 1mg
PA 3mg
PA 1mg
STZ 1mg
DPH 2mg
D-Pen 1mg
PBS
0.0
0.5
1.0
1.5
2.0
2.5
*
Fold Change
G. TNF-α
NVP 3mg
NVP 1mg
PA 3mg
PA 1mg
STZ 1mg
DPH 2mg
D-Pen 1mg
PBS
0.0
0.5
1.0
1.5
2.0
2.5
*
Fold Change
H. Cytotoxic T Cells
NVP 3mg
NVP 1mg
PA 3mg
PA 1mg
STZ 1mg
DPH 2mg
D-Pen 1mg
PBS
0
2
4
6
8
10
*
Fold Change

Figure 4. cytokine/chemokine mRNA expression after treatment of mice with different compounds. Mice were treated with either PBS or the indicated compounds in the footpad. After 7 days, PLN was isolated and processed for measurement of cytokine/chemokine mRNA expression in individual mice. Bars represent the mean value of the mRNA expression change compared to the control group ± STD of maximum 5-8 mice. *, shows significant differences ($p<0.05$) referred to the control group.

3.1.1.5 Summery of Primary PLNA Results

There was an increase in expression of T& B cells and T cell activation markers; CD25 & CD69 upon D-Pen and DPH. These findings were new and confirmed the immunostimulatory potential of both drugs. Increase in cytokine levels of IL-6, IL-12 and RANTES upon STZ and also increase in IL-1α, IL-2, IL-6, IL-12, TNF-α, IFN-γ and cytotoxic T cells on mRNA level upon STZ treatment were new findings that strengthen the idea of STZ acting as a potent Th1-prone drug. No significant changes in any immune parameter were detected after PA & NVP treatment in primary PLNA. In order to trigger the immune response to detect drug induced immune response, PA & NVP were combined with reporter antigens (TNP-OVA & Ficoll) in the next steps.

3.1.2 Reporter Antigen-Popliteal Lymph Node Assay, TNP-OVA (RA-PLNA)

RA-PLNA focuses on the initiation of lymphocyte proliferation in the popliteal lymph nodes of mice upon footpad injection of the test compound in combination with a suboptimal dose of a reporter antigen, trinitrophenyl-ovalbumine (TNP-OVA). The use of a suboptimal dose of a reporter antigen enables detection of specific antibody isotypes against the RA when reaction to the chemical induces an inflammatory immune response.

In this study D-Pen was used as reference of type 2 immune response and STZ as a reference of type 1 immune response. The difference between type 1 and type 2 responses is based on differentiation of Th cells in either Th1 and Th2 cells that are characterized by their pattern of cytokine secretion and also specific immunoglobulin isotype [144]. In mice, IFN-γ and IgG2a are generally known as type 1 prototypes, whereas IL-4 and IgG1 are indicators of type 2 response.

To perform the RA-PLNA, 50 µl of freshly prepared drugs in combination with 10 µg/mouse TNP-OVA were injected s.c. in to the right footpad of female C3H/HeOuJ mice. The TNP-OVA concentration was determined by performing a pilot study to find a suboptimal dose of TNP-OVA that can be combined with the compound of interest. The drugs were injected in

quantities that are determined to be immunostimulatory in the previous RA-PLNA studies [143]: 1 mg D-Pen or STZ, 3 mg PA, 3 mg NVP dissolved/suspended in PBS. Control groups were treated with PBS or PBS+TNP-OVA. Seven days after drug treatment, mice were sacrificed. PLNs were taken out and processed in order to measure different immunological parameters.

3.1.2.1 Popliteal Lymph Node Weight and Cell Count Determination

Figure 5A shows the PLN weights and PLN cell counts (B) after s.c. injection of the compound of interest in combination with TNP-OVA. There was a tendency to increased PLN weight and cellularity, which was however impaired by a relatively high individual variance of animals treated with reference compounds, so no significant increase was seen with D-Pen and STZ, as positive controls compared to previous work done with these compounds. PLN weight is increased in NVP treated groups. The total cell numbers of PLN were also increased in mice upon treatment with NVP as the test compound. The increase in PLN weight and cellularity after NVP treatment was not observed in previous study that NVP was injected alone and in the absence of RA, TNP-OVA. PLN weight and cellularity were not changed upon PA treatment.

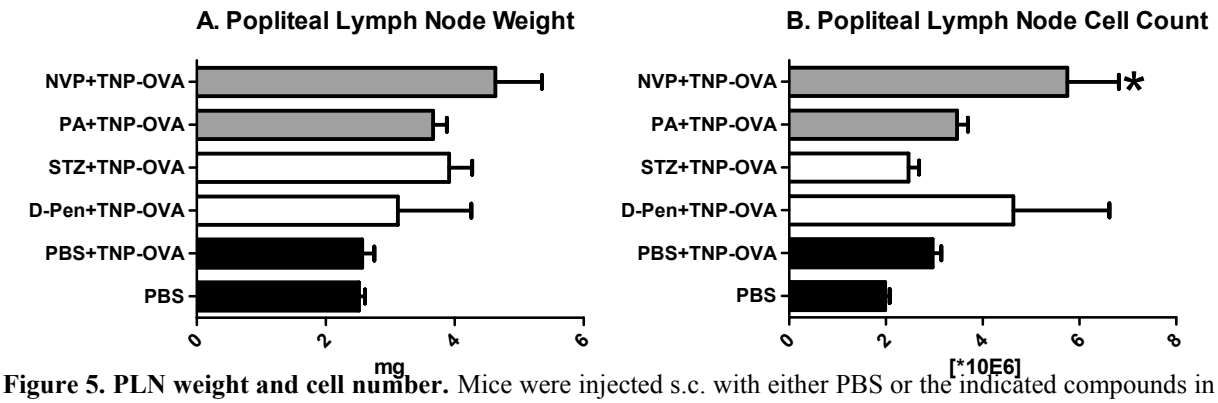


Figure 5. PLN weight and cell number. Mice were injected s.c. with either PBS or the indicated compounds in combination with TNP-OVA in the footpad. After 7 days, PLN was isolated, weight and cellularity were measured. Bars represent the mean value ± STD of 6 mice. *, shows significant differences ($p<0.05$) compared to the control group.

3.1.2.2 The Effect of Different Drugs on Cytokine Levels in PLN Supernatant

The same panel of 10 cytokines that was measured in direct PLNA was studied in the PLN supernatant of animals that were treated with either PBS or different compounds in combination with TNP-OVA (Figure 6A-D).

The levels of IL-6, IL-12 and RANTES were increased in STZ treated mice.

Animals that were exposed to NVP showed increase in IL-2 levels.

PA and D-Pen treatment did not lead to any change in cytokines/chemokine levels.

The levels of other investigated cytokines (IL-1α, IL-10, IL-17, IFN-γ and TNF-α) were below detection limit and the data are not shown here.

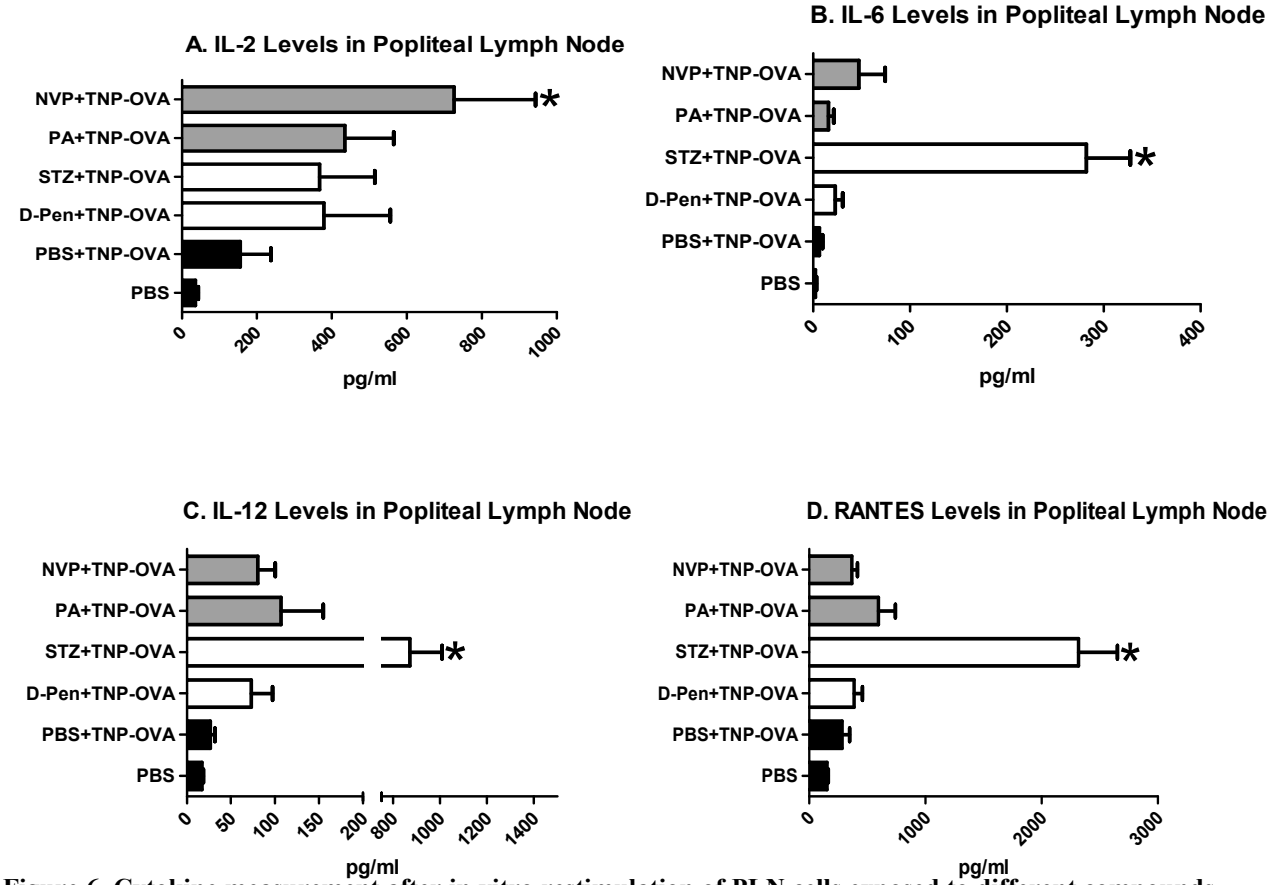
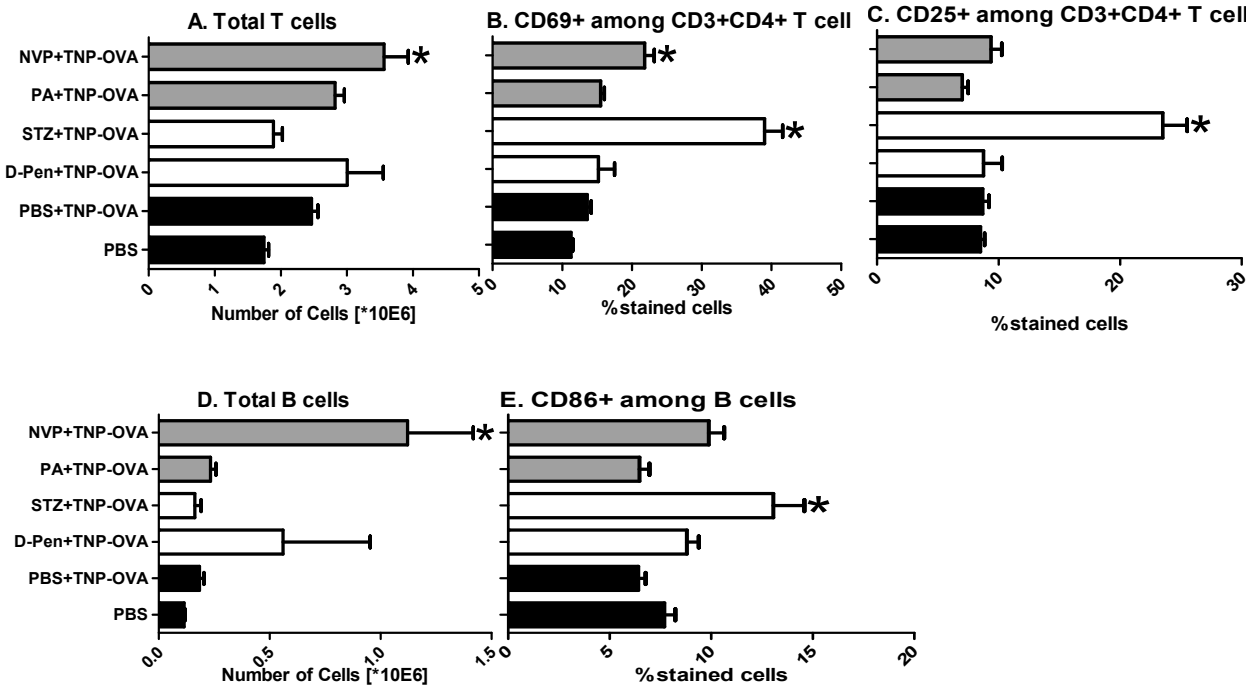


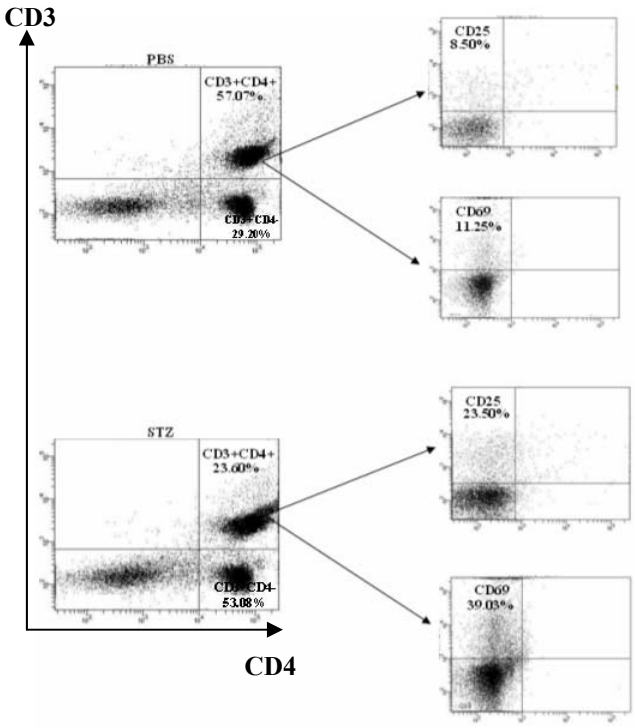
Figure 6. Cytokine measurement after in vitro restimulation of PLN cells exposed to different compounds in combination with TNP-OVA. Mice were treated s.c. with either PBS or the indicated compounds in combination with TNP-OVA in the footpad. After 7 days, PLN was isolated and restimmulated with ConA or LPS for 24h. Bars represent the mean value of cytokine levels ± STD of 6 mice. *, shows significant differences ($p<0.05$) compared to the control group.

3.1.2.3 Immunological Phenotype, the Effect of Various Compounds in Combination with TNP-OVA on Proportion of Different Cell Populations in PLN

The changes in percentages of T cells, B cells and their activation markers were evaluated in response to different chemicals + TNP-OVA (Figure 7A-E). The results of this study showed a decrease in percent of T cells after STZ and NVP treatment. This decrease in the percent level was significant in CD4+ T cells upon STZ and NVP treatments. There was an increase in the percent level of CD8+ T cells in STZ-treated mice and an increase on the percent level of B cells in NVP-treated mice. The total number of T and B cells was significantly increased upon NVP treatment.

There was an increase in CD69+ in STZ- and NVP-treated animals, an increase in CD25+ cells (among CD3+CD4+ T cell population) and CD86+ cells (among B cell population) in STZ-treated mice was also detected. No changes were observed in PA and D-Pen-treated mice.


A. Total T cells
B. CD69+ among CD3+CD4+ T cell
C. CD25+ among CD3+CD4+ T cell
NVP+TNP-OVA
PA+TNP-OVA
STZ+TNP-OVA
D-Pen+TNP-OVA
PBS+TNP-OVA
PBS
Number of Cells [*10E6]
%stained cells
%stained cells
D. Total B cells
E. CD86+ among B cells
NVP+TNP-OVA
PA+TNP-OVA
STZ+TNP-OVA
D-Pen+TNP-OVA
PBS+TNP-OVA
PBS
Number of Cells [*10E6]
%stained cells


CD3
PBS
CD3+CD4+
57.07%
CD25
8.50%
CD69
11.25%
STZ
CD3+CD4+
23.60%
CD25
23.50%
CD69
39.03%
CD4

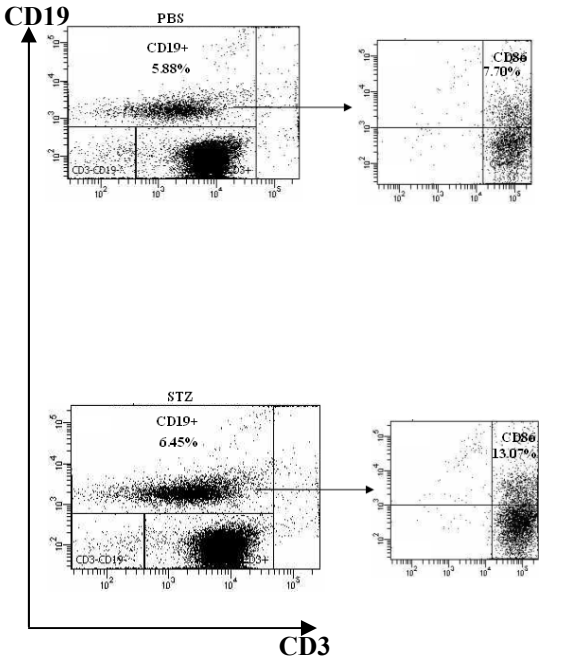


Figure 7. the effect of various compounds on proportion of different cell populations in PLN Mice were treated s.c. with either PBS or the indicated compounds in combination with TNP-OVA in to the footpad. After 7 days, PLN was isolated and changes in percentages of T cells, B cells and their activation markers were evaluated in response to different chemicals. Bars represent the mean value of cytokine levels ± STD of maximum 5 mice. *, shows significant differences ($p<0.05$) referred to the control group. CD3 was used to identify T cells and CD19 was used to identify B cells.

3.1.2.4 Determination of TNP-Specific Antibody Secreting Cell Numbers

Adjuvant activity of indicated compounds was assessed by detecting T-cell dependent responses to the reporter antigen, TNP-OVA (Figure 8A-C). Mice were treated with either PBS or different compounds in combination with TNP-OVA, after 7 days PLN was isolated and TNP-specific IgM, IgG1 and IgG2a were measured by performing ELISPOT. Co-injection of D-Pen and NVP with TNP-OVA lead to significant increase in the number of B cells producing isotypes IgM and IgG1 and also insignificant increase in the number of B cells producing IgG2a isotype. Mice that were treated with STZ+TNP-OVA showed a significant increase in the number of B cells producing IgG2a isotype. PA did not induce any significant changes in Igs response.

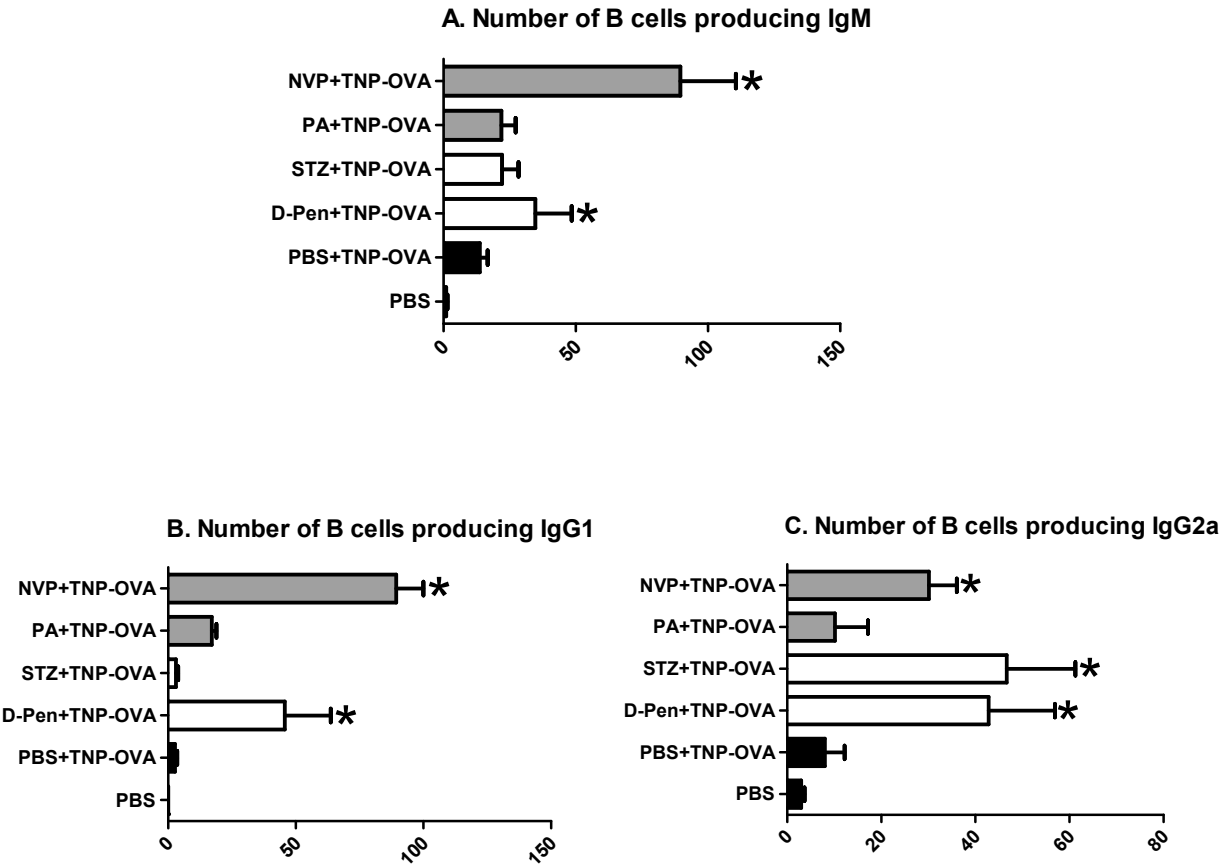


Figure 8. TNP-specific IgM (A), IgG1 (B) and IgG2a (C) Antibody Secreting Cells (ASCs) in the PLN. Mice were injected s.c. with PBS or indicated compounds in combination with TNP-OVA in the right footpad. After 7 days PLN was isolated and the number of B cells producing TNP-specific IgM, IgG1 and IgG2a were measured. Bars represent the mean value of TNP-specific ASCs ± STD of 6 mice. *, shows significant differences ($p<0.05$) compared to the control group.

3.1.2.5 Summery of RA-PLNA (TNP-OVA)

Based on immunoglobulin isotype switching and also expression of different cell markers, NVP shows an immune triggering effect. This effect was not detected in the absence of TNP-OVA. In case of PA utilizing new immunological end-points were not helpful to detect any immunostimulatory effect. In order to find out if the immunostimulatory effect of nevirapine is a T cell specific response, this was further investigated by combining NVP with TNP-Ficoll, as a T cell independent reporter antigen.

3.1.3 Reporter Antigen-Popliteal Lymph Node Assay, TNP-Ficoll (RA-PLNA)

The use of a subsensitizing dose of the reporter antigen, TNP-Ficoll enables detection of specific antibody isotypes when the reaction induced by the chemical involves a specific T cell reaction, which helps developing IgG response to the T cell independent antigen TNP-Ficoll.

Any effect of tested drugs in combination with TNP-OVA is a proof for an immunostimulatory potential and combining the drug with TNP-Ficoll in the next step as a T cell independent antigen, allowed detecting any drug-specific effect.
In this study D-Pen and STZ were used as reference of type 2 and type 1 immune response, respectively. The difference between type 1 and type 2 responses is based on differentiation of Th cells in either Th1 and Th2 cells that are characterized by their pattern of cytokine secretion and also specific immunoglobulin isotype [10]. In mice, IFN-γ and IgG2a are generally known as type 1 prototypes, whereas IL-4 and IgG1 are indicators of type 2 response.

To perform the RA-PLNA, 50 µl of freshly prepared drugs in combination with 30 µg/mouse TNP-Ficoll were injected s.c. into the right footpad of C3H/HeOuJ mice. The TNP-Ficoll concentration was determined by performing a pilot study to find a suboptimal dose of TNP-Ficoll that can be combined with the compound of interest. The drugs were injected in quantities that are determined to be immunostimulatory in the previous RA-PLNA studies [143]: 1 mg D-Pen or STZ, 3 mg PA, 3 mg NVP dissolved/suspended in PBS. Control groups were treated with PBS or PBS+TNP-Ficoll. Seven days after drug injection, mice were sacrificed.

3.1.3.1 Popliteal Lymph Node Weight and Cell Count Determination

Figure 9A indicates the PLN weights and PLN cell counts (B) after s.c. injection of the compound of interest in combination with TNP-Ficoll. PLN weight was significantly increased in D-Pen and STZ treated groups. An increase was also seen in PLN weight of mice that were treated with NVP. The total cell numbers of PLN were significantly increased in mice upon treatment with D-Pen as positive control and NVP as tested compound. There was

also an increase in PLN cellularity after STZ treatment. No changes were observed in PLN weight and cellularity of mice that were treated with PA.

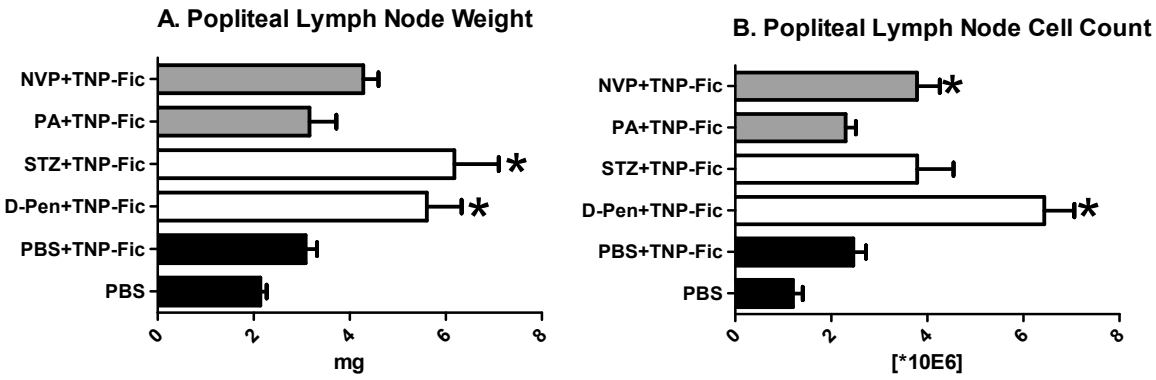


Figure 9. PLN weight and cell number. Mice were injected with either PBS or the indicated compounds in combination with TNP-Ficoll in the footpad. After 7 days, PLN was isolated and weight/cellularity were measured. Bars represent the mean value ± STD of 5 mice. *, shows significant differences ($p<0.05$) compared to the control group.

3.1.3.2 The Effect of Different Drugs on Cytokine Levels in PLN Supernatant

The same panel of 10 cytokines that was measured in direct PLNA was determined in PLN supernatant of animals that were treated with either PBS or different compounds in combination with TNP-Ficoll (Figure 10A-D).

The levels of IL-2, IL-6, IL-12 and RANTES were increased in D-Pen-treated mice. The levels of IL-6, IL-12 and RANTES were also increased upon STZ treatment. No increase in cytokine levels was observed in mice that were treated with the test compounds (PA & NVP, 3mg). The levels of other investigated cytokines (IL-1α, IL-4, IL-10, IL-17, IFN-γ and TNF-α) were below detection limit.

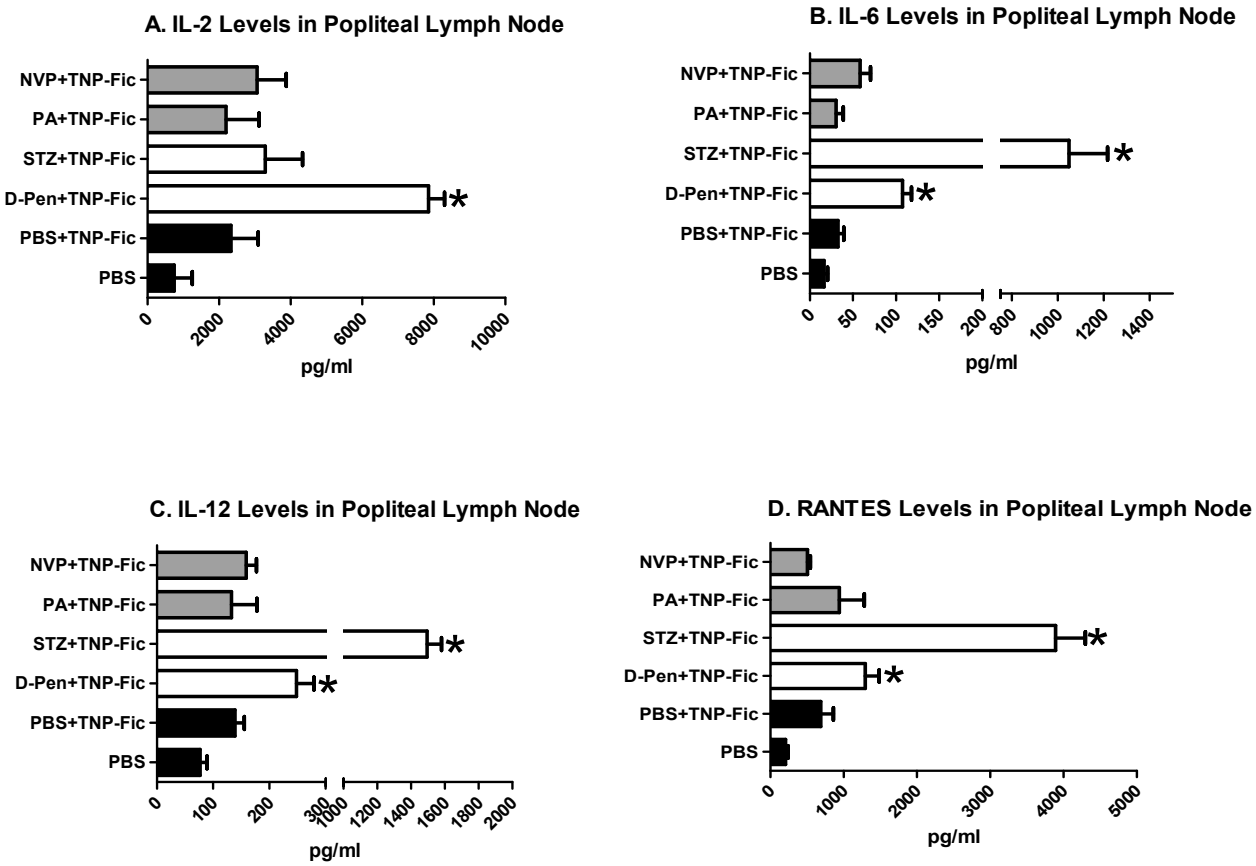
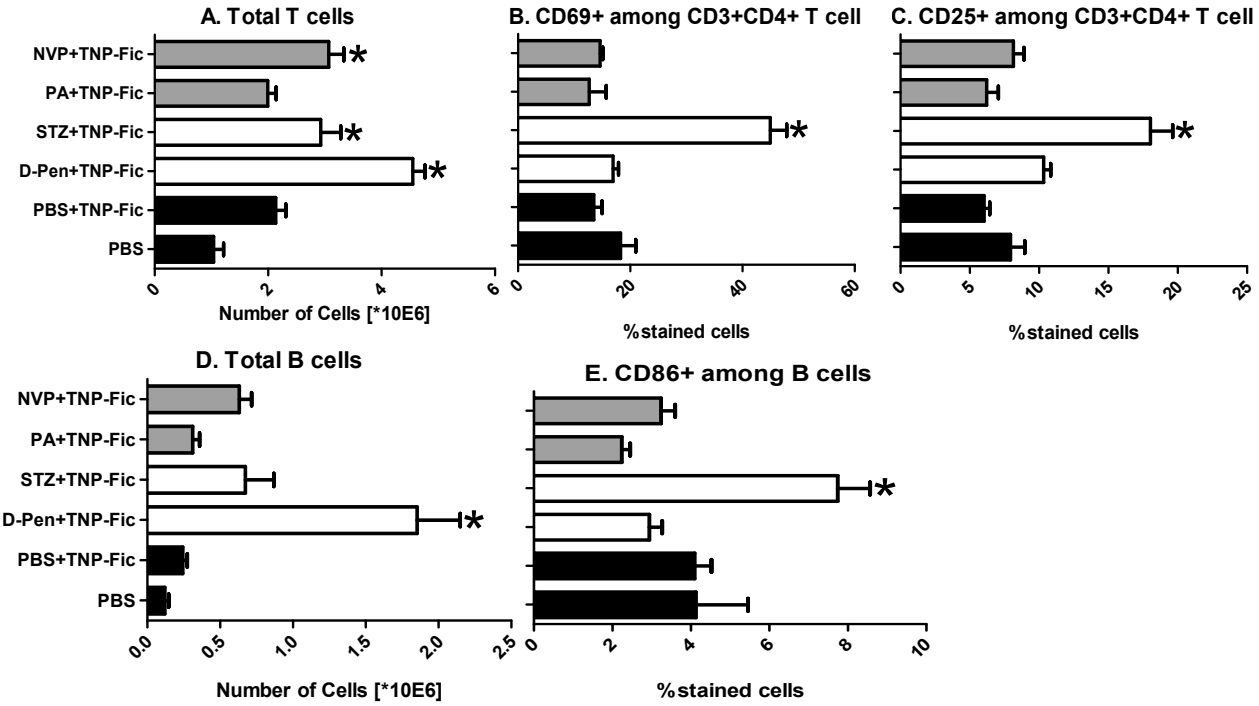


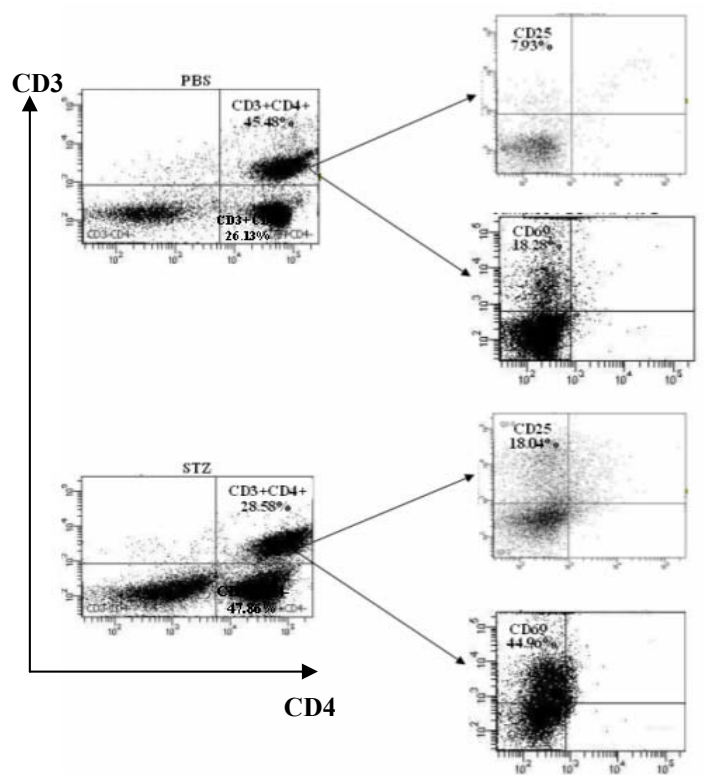
Figure 10. Cytokine measurement after in vitro restimulation of PLN cells exposed to different compounds in combination with TNP-Ficoll. Mice were treated with either PBS or the indicated compounds in combination with TNP-Ficoll in the footpad. After 7 days, PLN was isolated and restimmulated with ConA or LPS for 24h. Bars represent the mean value of cytokine levels ± STD of 5 mice. *, shows significant differences ($p<0.05$) compared to the control group.

3.1.3.3 Immunological Phenotype, the Effect of Various Compounds in Combination with TNP-Ficoll on Proportion of Different Cell Populations in PLN

The changes in percent of T cells, B cells and their activation markers were evaluated in response to different chemicals + TNP-Ficoll (Figure 11A-E). The results of this study showed a decrease in percent of T cells after D-Pen and STZ treatment. In D-Pen treated animals an increase in percent of B cells was observed and in STZ treated animals a relative increase in percent of CD8+ T cells was observed. The total number of T and B cells was increased upon treatment with D-Pen, STZ and NVP. The increase in total number of T and B cells upon NVP treatment was due to the increase in PLN cell count, on the percent level no changes was detected in T and B cell expression after NVP treatment.

There was an increase in % CD69+ and %CD25+ cells (among CD3+CD4+ T cell population) and increase in CD86+ cells (among B cell population) in STZ treated mice. No changes were observed on percent level of T and B cells activation markers in D-Pen treated mice. No changes were observed on percent level of T or B cells and their activation markers level in PA and NVP treated mice.





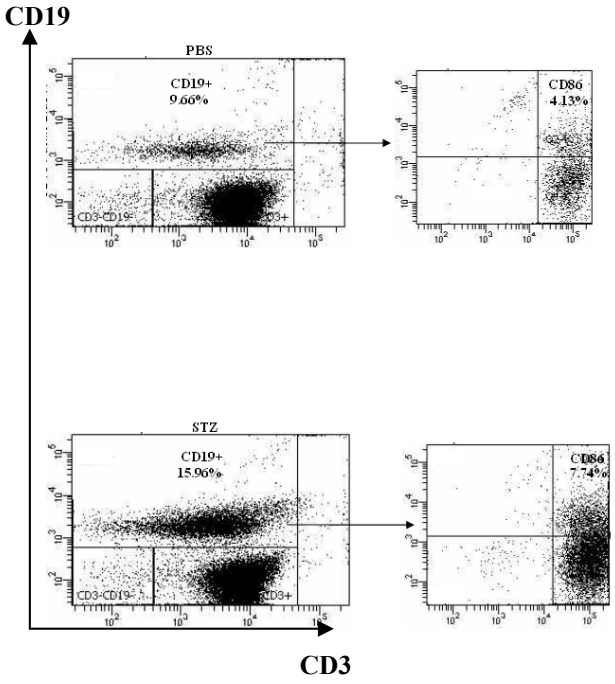


Figure 11. the effect of various compounds on proportion of different cell populations in PLN Mice were treated s.c. with either PBS or the indicated compounds in combination with TNP-Fic in to the footpad. After 7 days, PLN was isolated and changes in percentages of T cells, B cells and their activation markers were evaluated in response to different chemicals. Bars represent the mean value of cytokine levels ± STD of maximum 5 mice. *, shows significant differences ($p<0.05$) referred to the control group. CD3 was used to identify T cells and CD19 was used to identify B cells.

3.1.3.4 Determination of TNP-Specific Antibody Secreting Cell Numbers

T-cell specific activity of indicated compounds was assessed by detecting T-cell independent responses to the reporter antigen, TNP-Ficoll (Figure 12A-C). Mice were treated with either PBS or different compounds in combination with TNP-Ficoll, after 7 days PLN was isolated and TNP-specific IgM, IgG1 and IgG2a were measured by performing ELISPOT. Co injection of D-Pen and STZ with TNP-Ficoll leads to increase in the number of B cells producing isotypes IgM, IgG1 and IgG2a. No changes were seen in the number of B cells producing immunoglobulin in mice that were treated with PA or NVP+TNP-Ficoll.

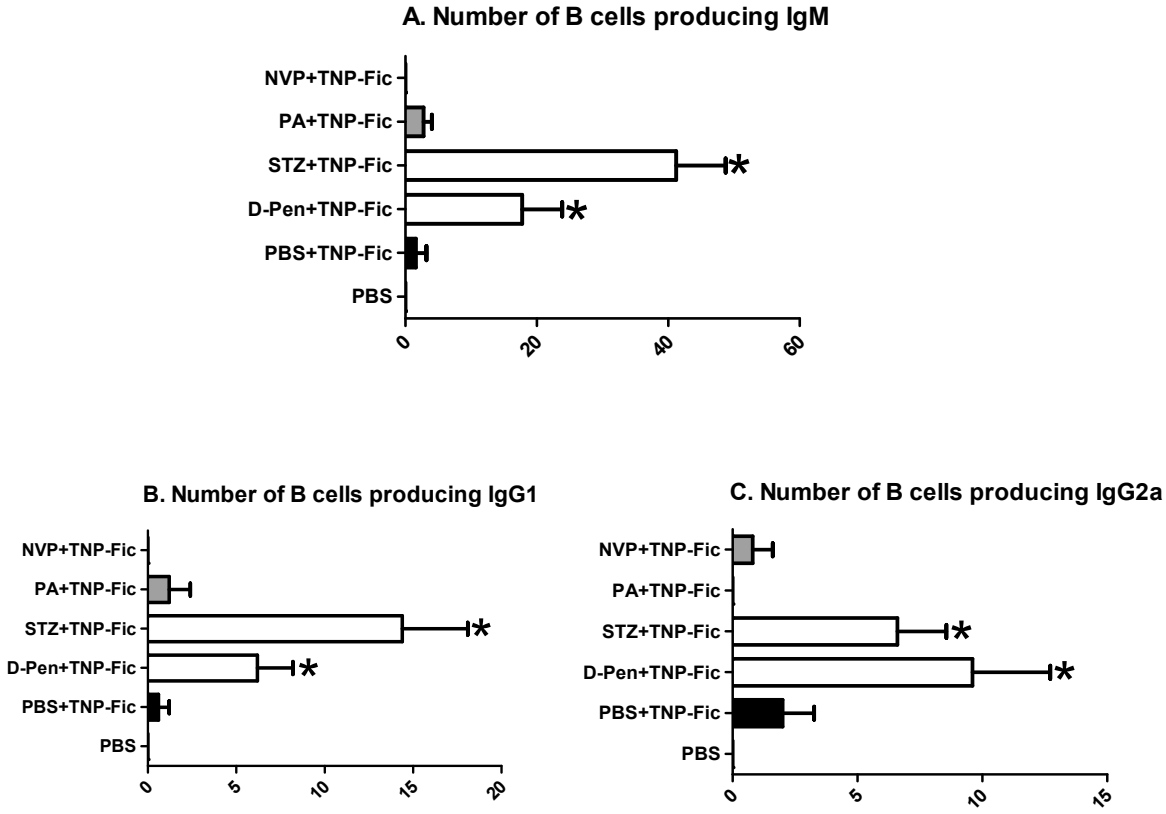


Figure 12. TNP-specific IgM (A), IgG1 (B) and IgG2a (C) Antibody Secreting Cells (ASCs) in the PLN. Mice were injected with PBS or indicated compounds in combination with TNP-Ficoll in the right footpad. After 7 days PLN was isolated and the number of B cells producing TNP-specific IgM, IgG1 and IgG2a were measured. Bars represent the mean value of TNP-specific ASCs ± STD of 5 mice. *, shows significant differences ($p<0.05$) compared to the control group.

3.1.3.5 Summery of RA-PLNA (TNP-Ficoll)

Cytokine/chemokine levels, immunoglobulin and also expression of different cell markers indicate that PA and NVP are not able to induce T cell specific immune response in local assays (PLNA/RA-PLNA). These data confirmed the existing hypothesis that in both cases, the reactive metabolite is responsible for induction of immune response. To overcome the lack of metabolism in the local assay, investigating in establishment of oral mouse models was essential.

3.2 Oral Assays

The focus of the first part of this thesis was validation of additional endpoints in PLNA and RA-PLNA to assess the risk of sensitization by the drug [8]. Although the RA-PLNA is a fast and simple preclinical assay, it has a disadvantage that the chemicals are injected subcutaneously (s.c.) instead of being administered orally. This is not a usual route of exposure for most drugs and chemicals. Therefore it is important to investigate whether drugs are able to induce systemic immunological changes via oral route of exposure which is the usual route of drug administration.

Based on the clinical investigations and findings with D-Pen and NVP and also already existing BN rat models in both cases, these two drugs were selected as reference compounds for establishment of the oral models in mice in this thesis.

3.2.1 Assessment of Immunstimulatory Effect of D-Penicillamine and Nevirapine Using the 28-Days Oral Exposure Mouse Model

Preliminary studies in two different strains of mice have already been performed. In these studies C3H/HeOuJ and C57BL/6 were i.p. injected with TNP-OVA (10 µg) on the first day and then gavaged once daily with 150 mg/kg D-Pen or 200, 350 and 500 mg/kg nevirapine for 28 days. After three weeks mice were s.c. rechallenged in their right foot pads with a suboptimal dose of reporter antigen, TNP-OVA (10 µg/mouse). A week later different organs (e.g. PLN, liver, spleen) were sampled from these mice, and prepared for the signs of an immune response onset. The results of this preliminary experiment showed signs of an immune response onset: in C3H/HeOuJ mice orally dosed with 200 mg/kg nevirapine,

cytokine levels and IgG1 production by PLN cells was elevated. Elevation of IL-1 and IFN-γ was also seen with 150 mg/kg D-Pen, but the increase in both cases (NVP & D-Pen) were not significant and also not supported by increase in other immune markers (data are not shown here). One of the explanations for this unclear and insignificant response was that the doses, which are used for pre-sensitizing and re-sensitizing the animals with TNP-OVA i.p. and s.c., respectively, are too high. Thus, the effect of the drug could be masked by the response caused by reporter antigen. Therefore the TNP-OVA were titrated in both cases. The results of the titration studies showed that the suboptimal concentrations of TNP-OVA that should be used in the main study is 0.3 μg/mouse TNP-OVA i.p. on day 1 for pre-sensitizing and 3 μg/mouse TNP-OVA s.c. on day 21 for re-sesitizing in C3H/HeOuJ mice.

To repeat this study under the optimal conditions found after titration studies and to investigate the mechanism(s) of NVP and D-Pen-induced immune response in mice, C3H/HeOuJ mice were i.p. injected with TNP-OVA (0.3 μg/mouse) on the first day and then gavaged once daily with either 150, 600 mg/kg D-Pen or 200, 600 mg/kg NVP for 28 days. After three weeks mice were s.c. rechallenged in their right footpads with a suboptimal dose of reporter antigen, TNP-OVA (3 μg/mouse), Finally, mice were sacrificed on day 28 and their organs (liver, ear, blood, spleen, auricular, popliteal and inguinal lymph nodes) were taken and analyzed for the signs of an immune response onset.

The compound's concentrations and also organs that should be investigated were chosen based on previous studies in BN rats.

In case of D-Pen, Qasim et al. were able to detect macroscopic lesions in caecum that appear on day 15 and also histological studies that have been done by this group shows caecal vacuities appears by day 3 [145].

Masson et al. also demonstrated infiltration of macrophages and costimulatory molecules in to the skin (ear) and caecum [141]. Both groups published that IgE levels peak in serum is after 2 weeks of treatment with D-Pen. Necrotic lesions were seen in the spleen [7]. Higher expression of macrophges and also costimulatory molecules was shown by Masson et al in the spleen [141].

In case of NVP, Shenton studies on BN rats described, necrotic lesions in the liver, infiltration of immune cells in to the skin and ears and also increasing in spleen weight in rats treated

with 150 mg/kg NVP. Therefore these organs were also analyzed in the oral exposure mouse model with NVP.

Auricular and Inguinal lymph nodes were studied based on titration studies that have been performed for TNP-OVA i.p. injection. In these studies after systemic injection of reporter antigen, TNP-OVA the most pronounced immune response was detected in Inguinal and auricular lymph nodes with the two highest concentrations of TNP-OVA (300 & 1000 µg/mouse).

3.2.1.1 28 Days-Oral Treatment with D-Penicillamine

3.2.1.1.1 Organ Weight and Cellularity

Figure 13A shows the spleen weights and spleen cell counts (B) in D-Penicillamine treated mice. In both cases, a significant increase was seen after 28 days oral treatment in the last two groups (600 mg/kg D-Pen + PBS i.p. or TNP-OVA i.p. + TNP-OVA s.c.). No changes were observed either in weight or in cellularity of investigated lymph nodes (auricular, inguinal and popliteal). All groups were treated with 0.3 µg/mouse TNP-OVA s.c.

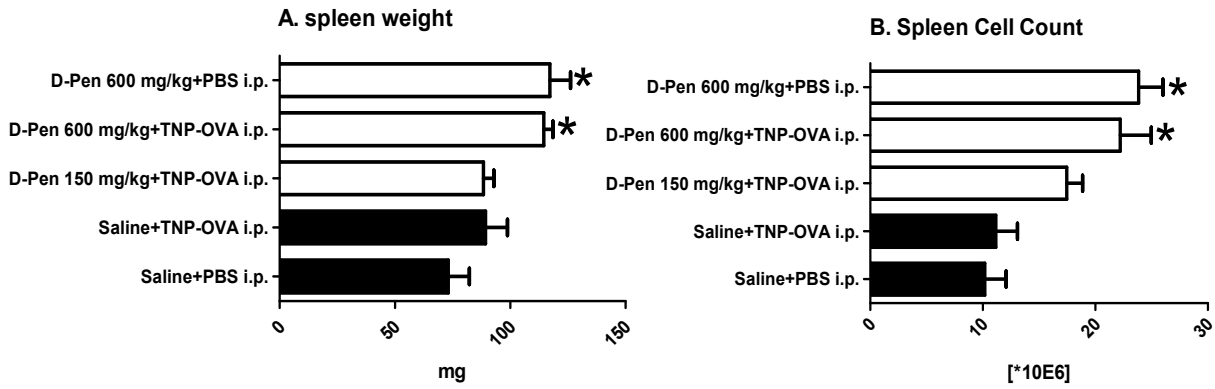
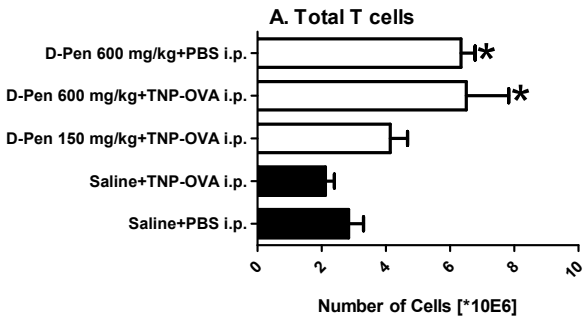


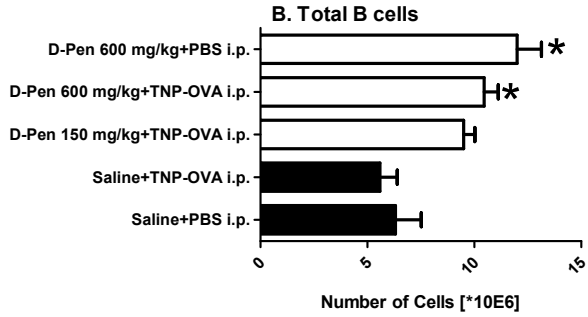
Figure 13. Spleen weight and cellularity. Mice were gavaged with either saline or the indicated concentrations of D-Pen in combination with TNP-OVA/PBS i.p. on day 1 and also TNP-OVA s.c. injection in to the footpad on day 21. After 28 days, spleen was isolated, weight and cellularity were measured. Bars represent the mean value ± STD of 5 mice. *, shows significant differences ($p<0.05$) compared to the control group.

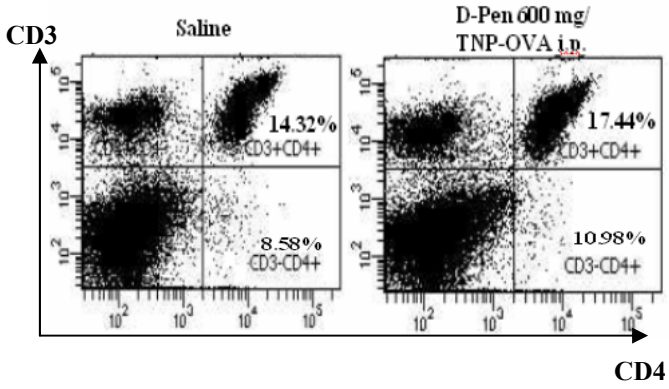
3.2.1.1.2 Immunological Phenotype, the Effect of Oral Treatment of D-Penicillamine on Proportion of Different cell Populations in Spleen and Inguinal lymph node

The changes in percent and total number of T cells, B cells and the percent of their activation markers, CD69 and CD25 (among T cell population) and CD80 and CD86 (among B cell

population) were evaluated in response to different concentrations of D-Pen (gavage, daily) + PBS/TNP-OVA (i.p., on day 1)+TNP-OVA (s.c., on day 21) (Figure 14A-B). The results of this study showed no changes in % of T cells after D-Pen (gavage, daily) treatment in the presence or absence of TNP-OVA (i.p., on day 1) in spleen. No changes were seen in the percent level of any activation markers in spleen either. There was a significant increase in the total number of T and B cells with the highest dose of D-Pen in the presence or absence of TNP-OVA i.p. in the two last groups, while no changes were observed in the percent level of either T or B cells or in their activation markers in auricular, inguinal and popliteal lymph nodes.







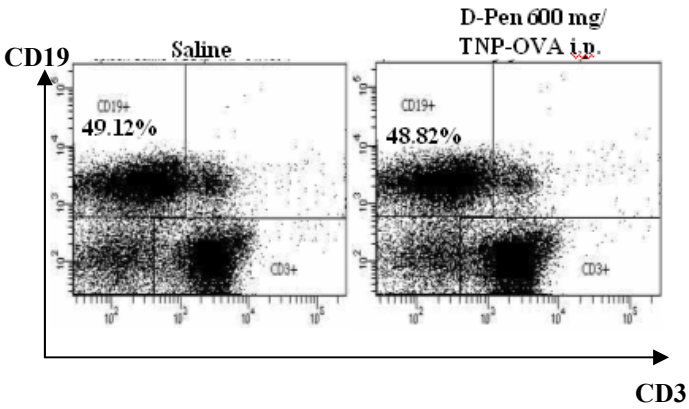


Figure 14. the effect of D-Pen on proportion of different cell populations in spleen Mice were gavaged with either saline or the indicated concentrations of D-Pen in combination with TNP-OVA/PBS i.p. on day 1 and also TNP-OVA s.c. injection in to the footpad on day 21. After 28 days, spleen was isolated and changes in percentages of T cells, B cells and their activation markers were evaluated in response to different chemicals. Bars represent the mean value of cytokine levels ± STD of maximum 5 mice. *, shows significant differences ($p<0.05$) referred to the control group. CD3 was used to identify T cells and CD19 was used to identify B cells.

3.2.1.1.3 Determination of TNP-Specific Antibody Secreting Cell Numbers

T-cell specific activity of indicated compounds was assessed by detecting T-cell responses to the reporter antigen, TNP-OVA. The animals were orally treated with either saline or different concentrations of D-Pen for 28 days. These mice got in addition to daily oral treatment an i.p. injection of TNP-OVA or PBS on day 1 of the treatment and s.c. TNP-OVA injection in the footpad on day 21.

TNP-specific IgM, IgG1 and IgG2a were measured by performing ELISPOT in spleen inguinal and auricular lymph nodes. No changes were seen in the number of B cells producing immunoglobulins in any organs.

3.2.1.2 28 Days-Oral treatment with Nevirapine

3.2.1.2.1 Organ Weight and Cellularity

Figure 15A shows the spleen weights and spleen cell counts (B) in Nevirapine treated mice. In both cases, an increase was seen after 28 days oral treatment with 600 mg/kg NVP+PBS i.p. + TNP-OVA s.c. which was not statistically significant. No changes were observed either in weight or in cellularity of investigated lymph nodes (auricular, inguinal and popliteal). All groups were treated with 0.3 µg/mouse TNP-OVA s.c.

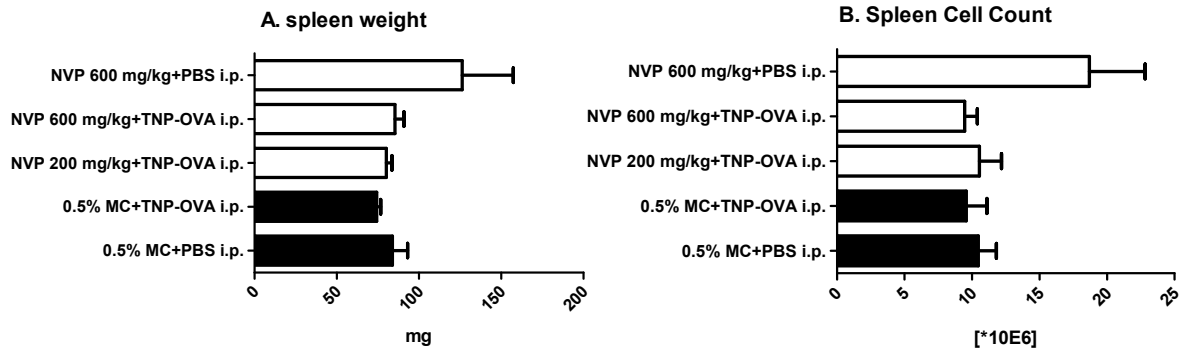
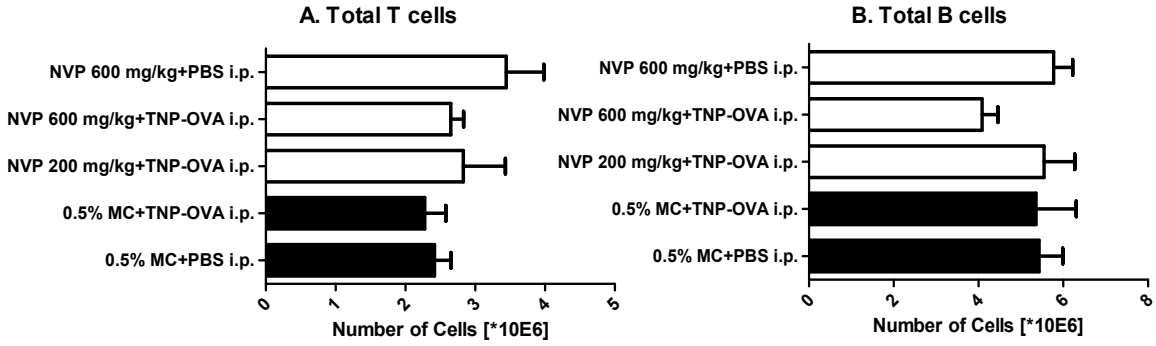


Figure 15. Spleen weight and cellularity. Mice were gavaged with either 0.5% Methylcellulose or the indicated concentrations of NVP in combination with TNP-OVA/PBS i.p. on day 1and also TNP-OVA s.c. injection in to the footpad on day 21. After 28 days, spleen was isolated, weight and cellularity were measured. Bars represent the mean value ± STD of 5 mice. *, shows significant differences ($p<0.05$) compared to the control group.

3.2.1.2.2 Immunological Phenotype, the Effect of Oral Treatment of Nevirapine on Proportion of Different Cell Populations in Spleen and Inguinal Lymph Node

The changes in percent/total number of T cells, B cells and % of their activation markers were evaluated in response to different concentrations of NVP (gavage, daily) + PBS/TNP-OVA (i.p., on day 1)+TNP-OVA (s.c., on day 21) (Figure 16A-B). The results of this study showed no changes in % or total number of T cells, B cells and % of their activation markers CD69 and CD25 (among T cell population) or CD80 and CD86 (among B cell population) in neither spleen nor auricular, inguinal and popliteal lymph nodes.



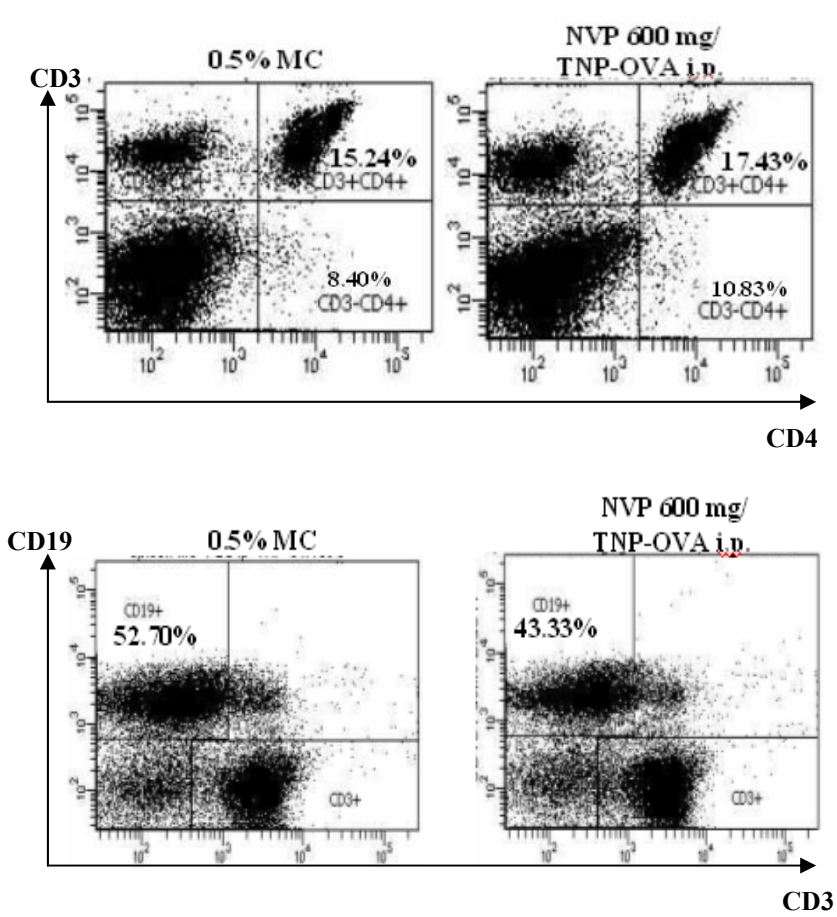


Figure 16. The effect of NVP on proportion of different cell populations in spleen Mice were gavaged with either 0.5% Methylcellulose or the indicated concentrations of NVP in combination with TNP-OVA/PBS i.p. on day 1 and also TNP-OVA s.c. injection in to the footpad on day 21. After 28 days, spleen was isolated and changes in percentages of T cells, B cells and their activation markers were evaluated in response to different chemicals. Bars represent the mean value of cytokine levels ± STD of maximum 5 mice. *, shows significant differences ($p<0.05$) referred to the control group. CD3 was used to identify T cells and CD19 was used to identify B cells.

3.2.1.2.3 Determination of TNP-specific Antibody Secreting Cell Numbers

T-cell specific activity of indicated compounds was assessed by detecting T-cell responses to the reporter antigen, TNP-OVA. The animals were orally treated with either 0.5% methylcellulose or different concentrations of NVP for 28 days. These mice got in addition to daily oral treatment an i.p. injection of TNP-OVA or PBS on day 1 of the treatment and s.c. TNP-OVA injection in the footpad on day 21. TNP-specific IgM, IgG1 and IgG2a were measured by performing ELISPOT in spleen inguinal and auricular lymph nodes. No changes were seen in the number of B cells producing immunoglobulins in any organs.

3.2.1.3 Summery of Oral Study (28-days)

There was an increase in spleen weight and cellularity with 600 mg/kg D-Pen in the presence or absence of TNP-OVA i.p. but no significant changes was observed in spleen weight and cellularity upon NVP treatment.

No change was observed in % of T & B cell markers, or their activation markers (CD25, CD69, CD80 or CD86) in spleen either with D-Pen or with NVP.

The results from ELISPOT analysis showed no isotype switching from TNP-specific IgM to TNP-specific IgG1 and IgG2a with any of the compounds of interest. As no significant sign of immune response specifically TNP-specific immune response was observed either with D-Pen or NVP, next step was to redesign the oral mouse model and try to investigate in a new oral mouse model.

3.2.2 Assessment of Immunstimulatory Effect of D-Penicillamine and Nevirapine Using a New Oral Exposure Mouse Model

3.2.2.1 10 Days-Oral Treatment with D-Penicillamine

To attempt for establishing a new oral exposure mouse model of drug-induced immune-mediated hypersensitivity, C3H/HeOuJ mice were gavaged 3 days with 50, 150 or 600 mg/kg D-Pen, These concentrations were chosen based on Donker's data and also Masson's study that was explained in the 28 days oral mouse model. They were able to induce autoimmune response in BN rats upon treatment with 20 and 50 mg/rat D-Pen. Masson et al. was also able to induce low dose oral tolerance by treating BN rats with 5 mg/rat [7, 141]. To cover a broad range of different doses of D-Pen, the mice were treated with the 50, 150 and 600 mg/kg daily, which corresponds to doses in the rats on a mg/m2 basis.

On day 4, in addition to the oral treatment of D-Pen mice were injected i.p. with a suboptimal dose of reporter antigen, TNP-OVA (200 µg/mouse, the concentration of TNP-OVA is based on a titration that was done to find a suboptimal dose of this RA), then the oral treatment of different concentrations of D-Pen were continued for another 7 days. Finally, mice were sacrificed on day 10 and their organs (caecum, skin, blood, spleen, mesenteric and inguinal lymph nodes) were taken and analyzed for the signs of an immune response onset.

This protocol was designed based on RA-PLNA. In this assay the reference compounds are injected in combination with RA s.c. to the foot pad. As the purpose of performing of the oral studies was to investigate a systemic immune response the routs of administration for both drug and RA were systemically, drug were gavaged and RA was i.p. injected but the duration of the study was similar to RA-PLNA. The reason for giving the drug by gavage three days in advance was to reach the enough amount of the drug in the circulation by the time that RA is i.p. injected.

3.2.2.1.1 Organ Weight

Figure 17A shows the spleen weights and Inguinal lymph node weights (B). In both organs, weight is significantly increased after 10 days oral treatment with 600 mg/kg D-Pen. This increase was seen in presence or absence of TNP-OVA (i.p.).

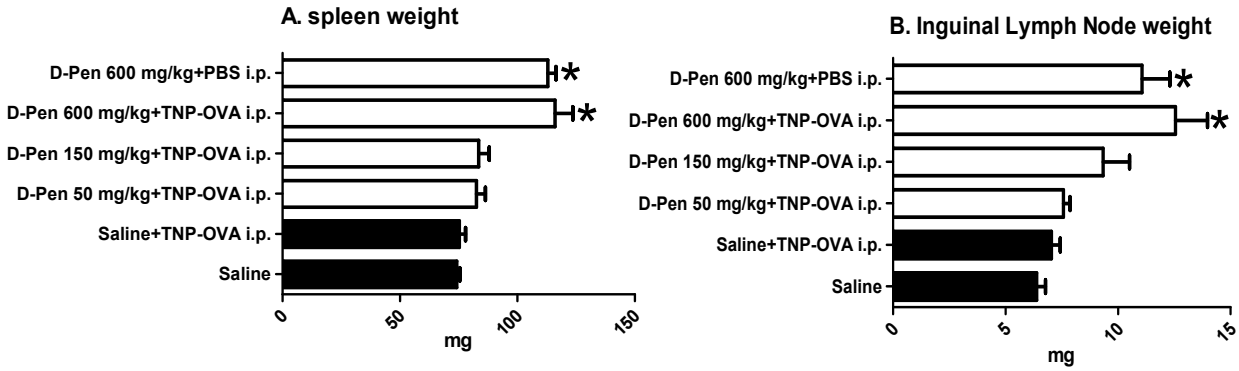


Figure 17. Organ weight. Mice were gavaged with either saline or the indicated concentrations of D-Pen in combination with TNP-OVA or PBS i.p. on day 4. After 10 days, organs were isolated and weight was measured. Bars represent the mean value ± STD of 6-10 mice. *, shows significant differences ($p<0.05$) compared to the control group.

3.2.2.1.2 The Effect of Oral treatment with D-Penicillamine on Cytokine Levels in Spleen and Inguinal Lymph Node Supernatant

The panel of 10 cytokines/chemokine (IL-1α, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IFN-γ, TNF-α and RANTES) was determined in inguinal lymph nodes supernatants of animals that were orally treated with either saline or different concentrations of D-Pen for 10 days. These mice got in addition to oral treatment an i.p. injection of TNP-OVA or PBS on day 4 of the treatment (Figure 18A-C).

In inguinal lymph node, the levels of IL-6, IL-12 and RANTES were increased in the group of animals which were treated with 600 mg/kg D-Pen+TNP-OVA i.p.. The levels of other investigated cytokines (IL-1α, IL-4, IL-10, TNF-α and IFN-γ) were below detection limit. The

levels of IL-2 and IL-17 were also measured but the data from these two cytokines due to the technical issues remain inconclusive.

The 10 above mentioned cytokines were also measured in the spleen supernatants but the results were not reliable due to the technical issues that we faced in spleen samples.

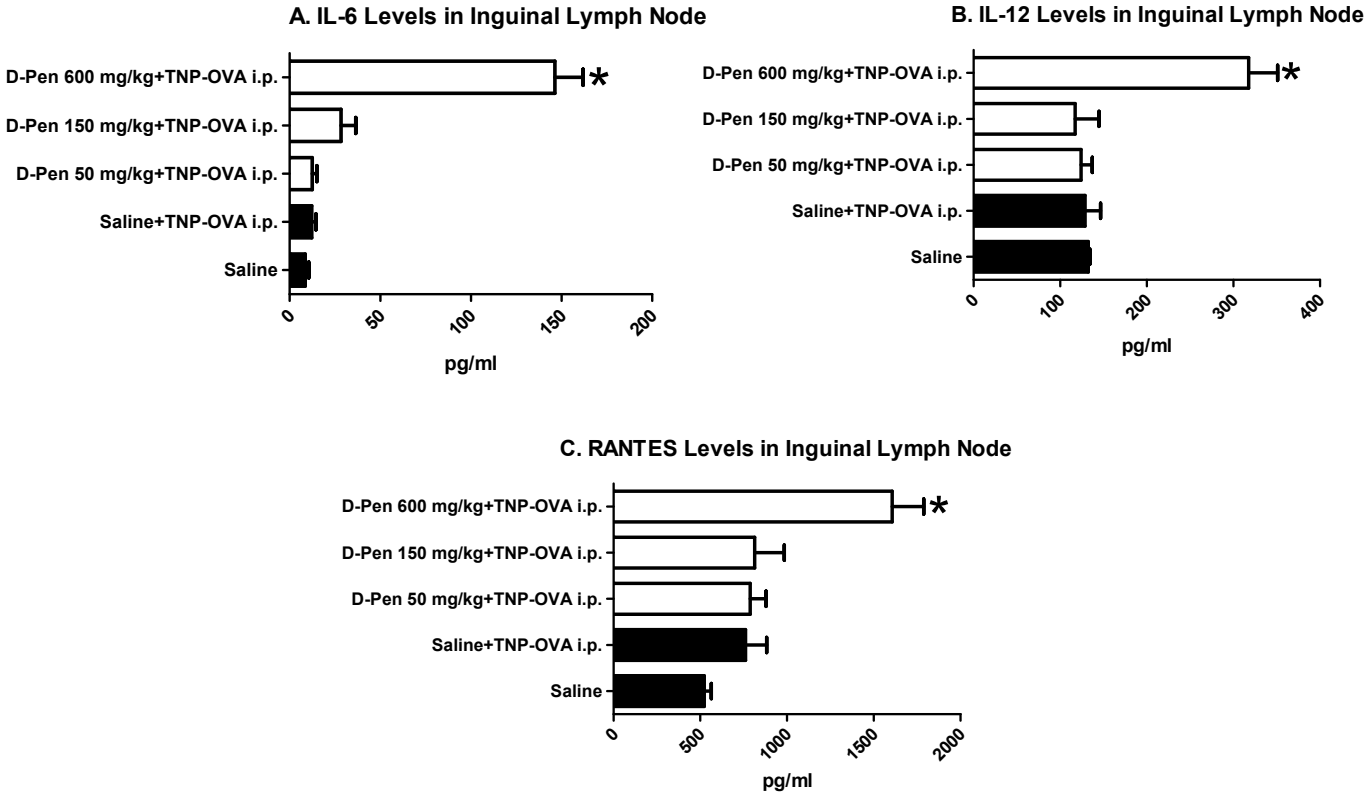


Figure 18. Cytokine measurement after in vitro restimulation of inguinal cells exposed to different concentrations of D-Pen orally in combination with TNP-OVA i.p.. Mice were gavaged with either saline or the indicated concentrations of D-Pen daily; they also were injected i.p. with TNP-OVA once on day 4. After 10 days, inguinal lymph node was isolated and restimmulated with ConA or LPS for 24h. Bars represent the mean value of cytokine levels ± STD of 6 mice. *, shows significant differences ($p<0.05$) compared to the control group.

3.2.2.1.3 Determination of TNP-specific Antibody Secreting Cell Numbers

T-cell specific activity of indicated compounds was assessed by detecting T-cell responses to the reporter antigen, TNP-OVA (Figure 19A-B). Mice were daily orally treated with either saline or different concentrations of D-Pen for 10 days. The mice (excepting the last group) got in addition to oral treatment an i.p. injection of TNP-OVA or PBS on day 4 of the treatment.

TNP-specific IgG1 and IgG2a were measured by performing ELISPOT in spleen and inguinal lymph nodes. No significant changes were seen in the number of B cells producing immunoglobulins (IgG1 and IgG2a) in either spleen or inguinal lymph nodes.

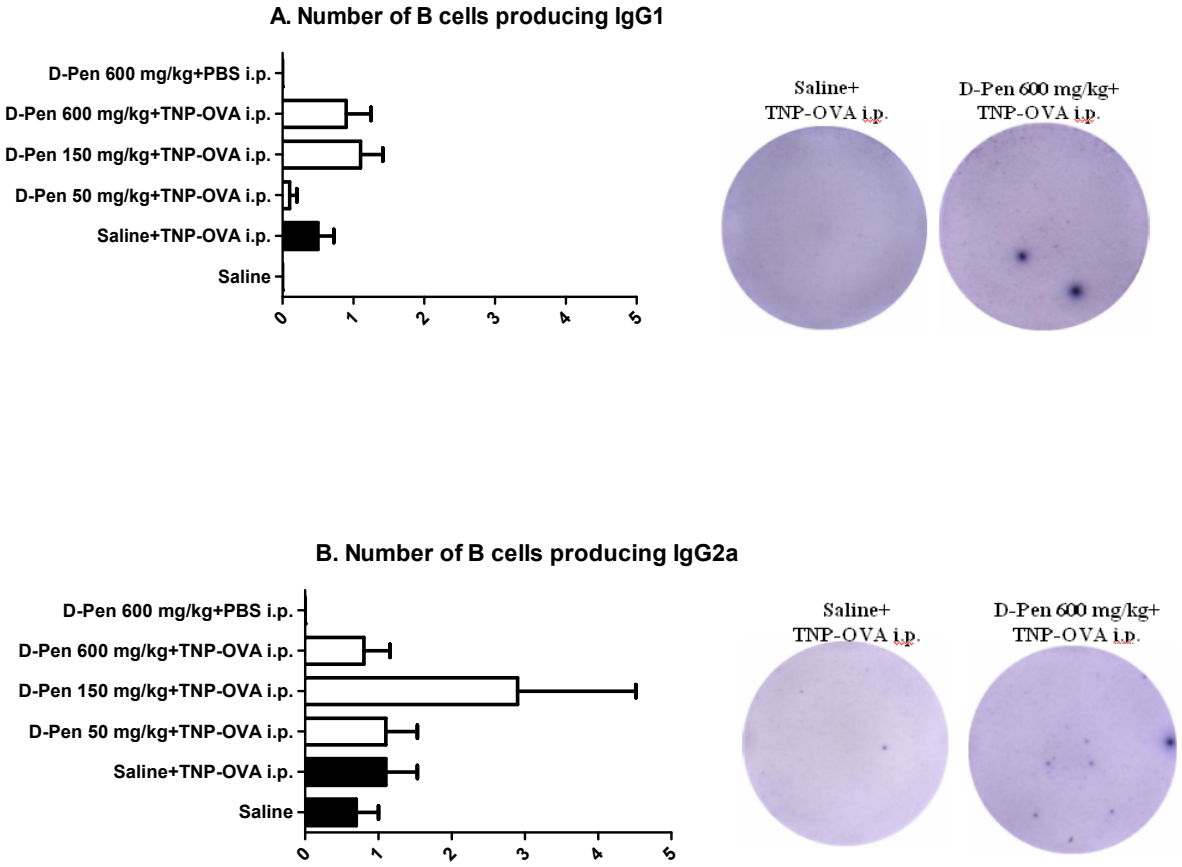
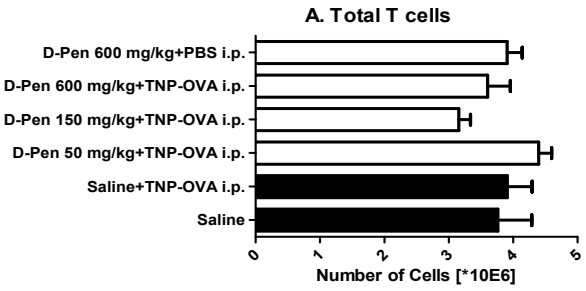


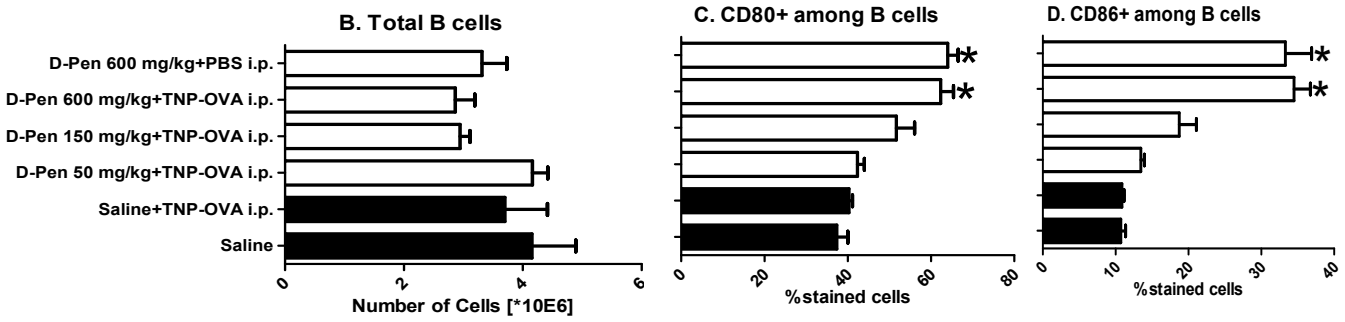
Figure 19. TNP-specific IgG1 (A) and IgG2a (B) Antibody Secreting Cells (ASCs) in the spleen. Mice were gavaged with either saline or the indicated concentrations of D-Pen daily; they also were injected i.p. with TNP-OVA once on day 4. After 10 days, spleen was isolated and the number of B cells producing TNP-specific IgG1 and IgG2a were measured. Bars represent the mean value of TNP-specific ASCs ± STD of 10 mice. *, shows significant differences ($p<0.05$) compared to the control group.

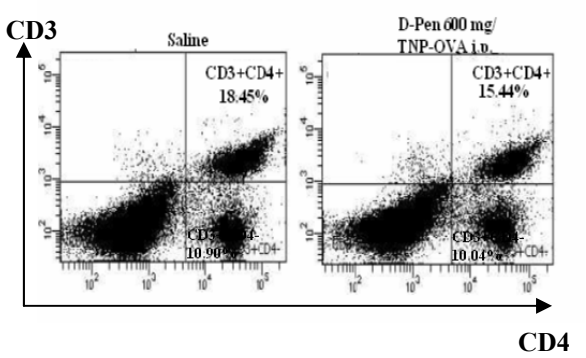
3.2.2.1.4 Immunological Phenotype, the Effect of Oral Treatment of D-Pen in Combination with i.p. Injection of TNP-OVA on Proportion of Different Cell Populations in Spleen and Inguinal Lymph Node

The changes in percent of T cells, B cells and their activation markers, CD69 or CD25 (among T cells population) and CD80 or CD86 (among B cell population) were evaluated in response to different concentrations of D-Pen (gavage, daily) + TNP-OVA (i.p., on day 4) (Figure 20A-D). The results of this study showed a decrease in % and total number of B cells

and an increase in CD80+ and CD86+ cells (among B cell population) cells after D-Pen 600 mg/kg (gavage, daily) in the presence or absence of TNP-OVA (i.p., on day 4) in spleen. No changes were observed neither in the % level of T cells nor in total number of T cells and its activation markers (CD69 and CD25) in spleen.







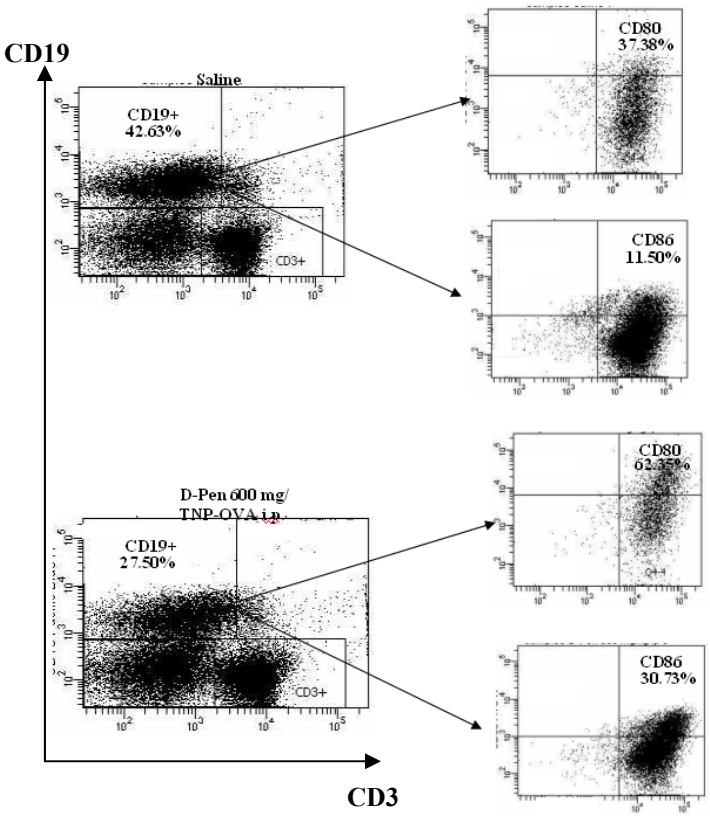
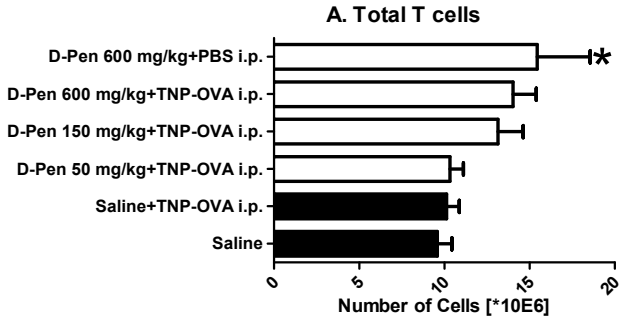
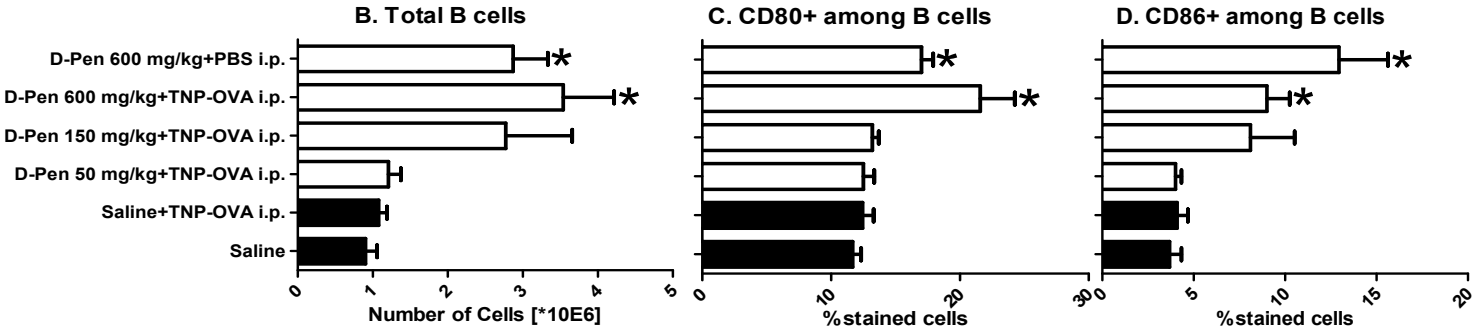
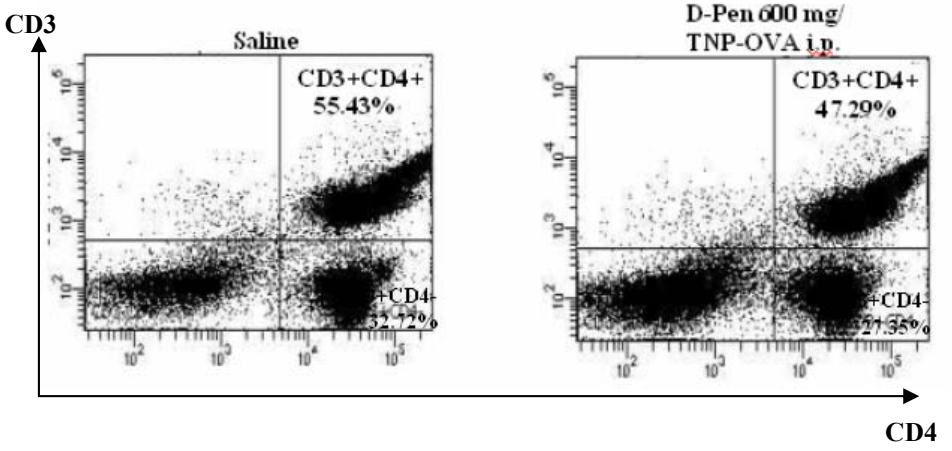


Figure 20. The effect of D-Pen on proportion of different cell populations in Spleen Mice were gavaged with either saline or the indicated concentrations of D-Pen daily, they also were injected i.p. with TNP-OVA once on day 4. After 10 days, spleen was isolated and changes in percentages of T cells, B cells and their activation markers were evaluated in response to different chemicals. Bars represent the mean value of cytokine levels ± STD of maximum 4-10 mice. *, shows significant differences ($p<0.05$) referred to the control group. CD3 was used to identify T cells and CD19 was used to identify B cells.

In inguinal lymph node (Figure 21A-D), an increase in total number of T and B cells in the two last treated groups of mice was observed. A decrease in % of T cells (CD3+, CD4+, CD8+) and an increase in % of B cells and also in % of CD80+ and CD86+ cells (among B cell population) after D-Pen 600 mg/kg (gavage, daily) in the presence or absence of TNP-OVA (i.p., on day 4). No changes were observed in the % level of T cell activation markers (CD69 and CD25).


A. Total T cells
D-Pen 600 mg/kg+PBS i.p.
D-Pen 600 mg/kg+TNP-OVA i.p.
D-Pen 150 mg/kg+TNP-OVA i.p.
D-Pen 50 mg/kg+TNP-OVA i.p.
Saline+TNP-OVA i.p.
Saline
0
5
10
15
20
Number of Cells [*10E6]


B. Total B cells
C. CD80+ among B cells
D. CD86+ among B cells
D-Pen 600 mg/kg+PBS i.p.
D-Pen 600 mg/kg+TNP-OVA i.p.
D-Pen 150 mg/kg+TNP-OVA i.p.
D-Pen 50 mg/kg+TNP-OVA i.p.
Saline+TNP-OVA i.p.
Saline
0
1
2
3
4
5
Number of Cells [*10E6]
0
10
20
30
%stained cells
0
5
10
15
20
%stained cells


CD3
Saline
CD3+CD4+
55.43%
D-Pen 600 mg/
TNP-OVA i.p.
CD3+CD4+
47.29%
CD4

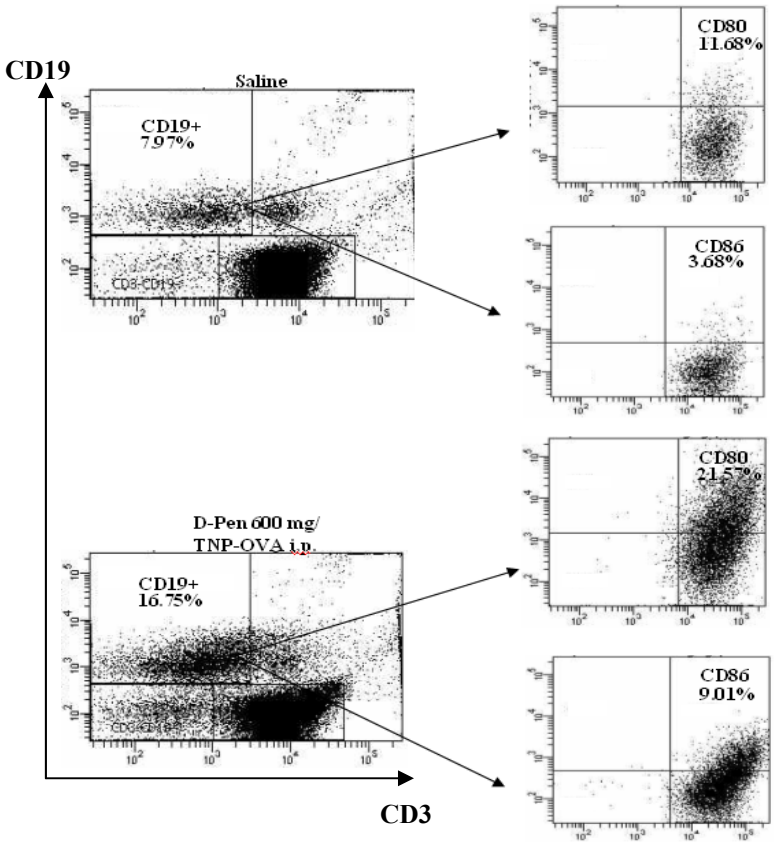


Figure 21. The effect of D-Pen on proportion of different cell populations in Inguinal lymph node Mice were gavaged with either saline or the indicated concentrations of D-Pen daily, they also were injected i.p. with TNP-OVA once on day 4. After 10 days, inguinal lymph node was isolated and changes in percentages of T cells, B cells and their activation markers were evaluated in response to different chemicals. Bars represent the mean value of cytokine levels ± STD of maximum 4-10 mice. *, shows significant differences ($p<0.05$) referred to the control group. CD3 was used to identify T cells and CD19 was used to identify B cells.

3.2.2.2 10 Days-Oral Treatment with Nevirapine

To further investigate the mechanism(s) of NVP-induced immune response in mice, C3H/HeOuJ mice were gavaged 3 days with 200, 350 and 600 mg/kg NVP; these concentrations were chosen based on Shenton's data. They were able to induce skin rash in BN rats upon treatment with 150 mg/kg NVP. They were also able to induce low dose oral tolerance by treating BN rats with 40 or 75 mg/kg [5]. To cover a broad range of different doses of NVP, the animals were treated with the 200, 350 or 600 mg/kg daily dosing, which corresponds to doses in the rats on a mg/m^2 basis.

The design of the protocol was the same like D-Pen; On day 4, in addition to the oral treatment of NVP mice were injected i.p. with a suboptimal dose of reporter antigen, TNP-OVA (200 μg/mouse), then the oral treatment of different concentrations of NVP were continued for another 7 days. Finally, mice were sacrificed on day 10 and their organs (liver, ear, blood, spleen, auricular and inguinal lymph nodes) were taken and analyzed for the signs of an immune response onset. Shenton's studies on BN rats described necrotic lesions in the liver, infiltration of immune cells in to the skin and ears and also increasing in spleen weight in rats treated with 150 mg/kg NVP. Therefore these organs were also analyzed in this oral exposure mouse model with NVP.

Auricular and inguinal lymph nodes were studied based on titration study that have been performed for TNP-OVA i.p. injection. In this study after systemic injection of reporter antigen, TNP-OVA the most pronounced immune response was detected in Inguinal lymph nodes with the two highest concentrations of TNP-OVA (300 & 1000 μg/mouse). Based on these data the above mentioned organs were examined in the oral exposure study of NVP.

3.2.2.2.1 Organ Weight

Figure 22A shows the spleen weight and auricular lymph node weight (B). In both organs, weight is increased after 10 days oral treatment with 600 mg/kg NVP. This increase was seen in presence or absence of TNP-OVA (i.p.), however the increase was more pronounced in the presence of TNP-OVA (i.p.) in auricular lymph node.

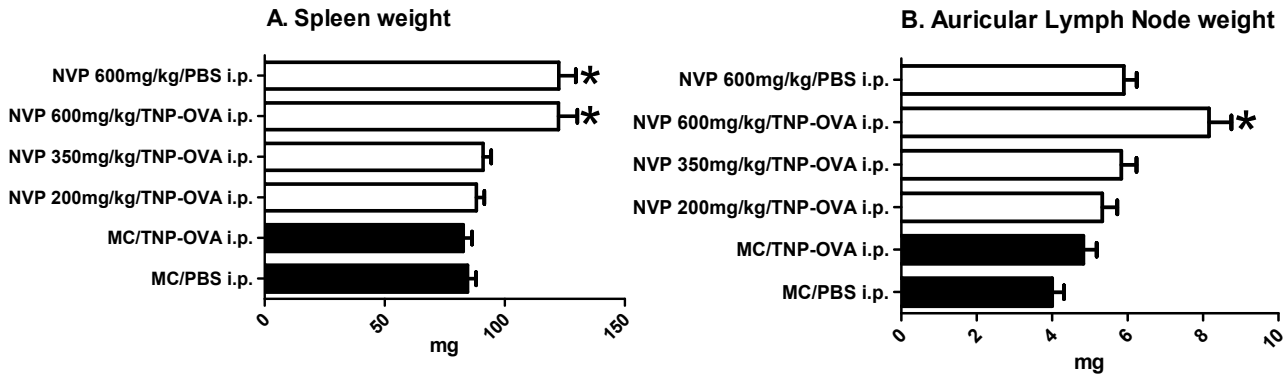


Figure 22. Organ weight. Mice were gavaged with either 0.5% methylcellulose or the indicated concentrations of NVP in combination with TNP-OVA/PBS i.p. on day 4. After 10 days, organs were isolated and weight was measured. Bars represent the mean value ± STD of 10 mice. *, shows significant differences ($p<0.05$) compared to the control group.

3.2.2.2.2 The Effect of Oral Treatment with Nevirapine on Cytokine Levels in Spleen and Auricular Lymph Node Supernatant

The panel of 10 cytokines/chemokine (IL-1α, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IFN-γ, TNF-α and RANTES) was determined in spleen (Figure 23A-C) and auricular lymph node (Figure 23A-C) supernatant of animals that were daily orally treated with either 0.5% methylcellulose or different concentrations of NVP for 10 days. These mice got in addition to oral treatment an i.p. injection of TNP-OVA or PBS on day 4 of the treatment.

In spleen, the levels of IL-6, IL-12 and RANTES were increased in the groups of animals which were treated with either 350 or 600 mg/kg NVP+TNP-OVA i.p. or in the presence of compound alone (600 mg/kg NVP) without having any reporter antigen on board.

The levels of IL-2, 10, 17 and IFN-γ were also measured but the data from these cytokines due to the technical issues remain inconclusive. The levels of IL-1α, IL-4 and TNF-α were below detection limit.

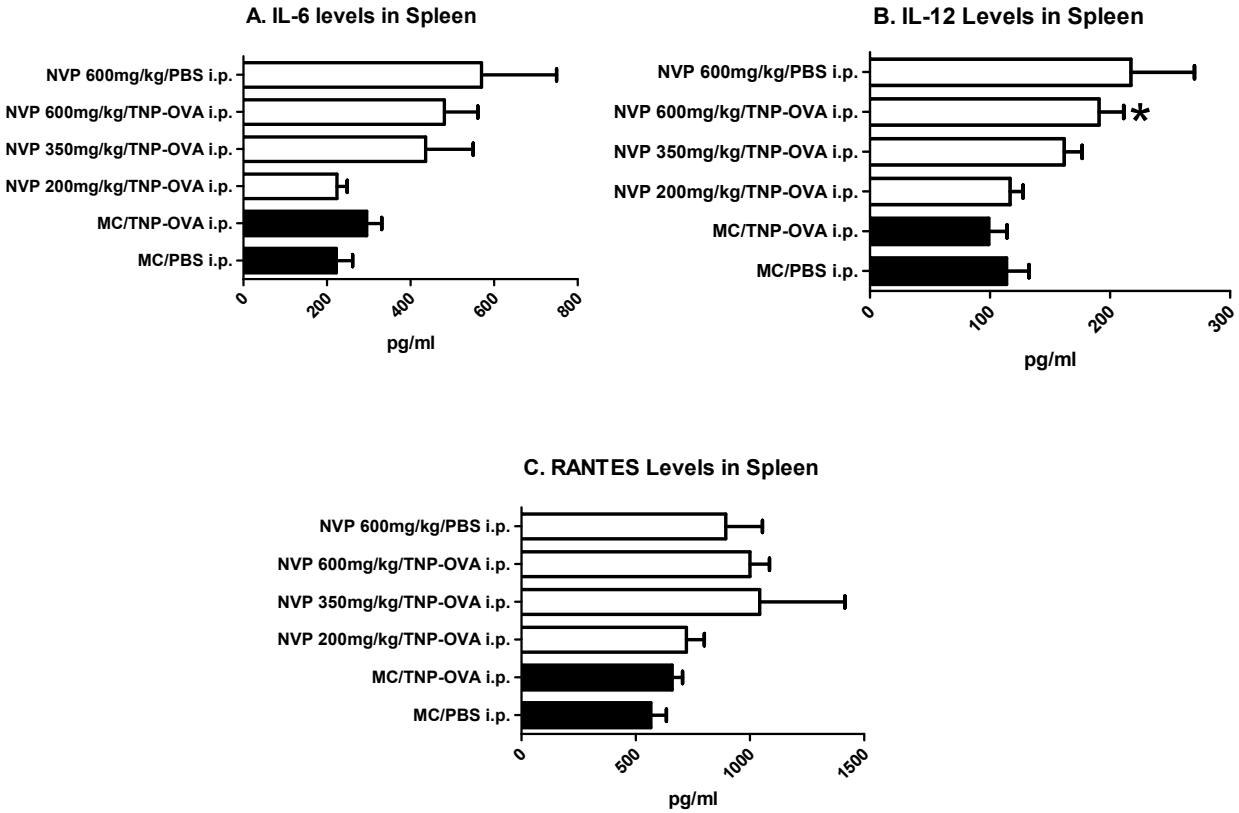


Figure 23. Cytokine measurement after in vitro restimulation of spleen cells exposed to different concentrations of NVP orally in combination with TNP-OVA i.p.. Mice were gavaged with either 0.5% methylcellulose or the indicated concentrations of NVP daily, they also were injected i.p. with TNP-OVA or PBS once on day 4 (excepting the last group). After 10 days, spleen was isolated and restimmulated with ConA or LPS for 24h. Bars represent the mean value of cytokine levels ± STD of 5 mice. *, shows significant differences ($p<0.05$) compared to the control group.

In auricular lymph node (Figure 24A-C), the levels of IL-12 and RANTES were increased in the groups of animals which were treated with either 350 or 600 mg/kg NVP+TNP-OVA i.p. or in the presence of compound alone (600 mg/kg NVP) without having any reporter antigen on board.

The levels of IL-1α, IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α were under detection limit.

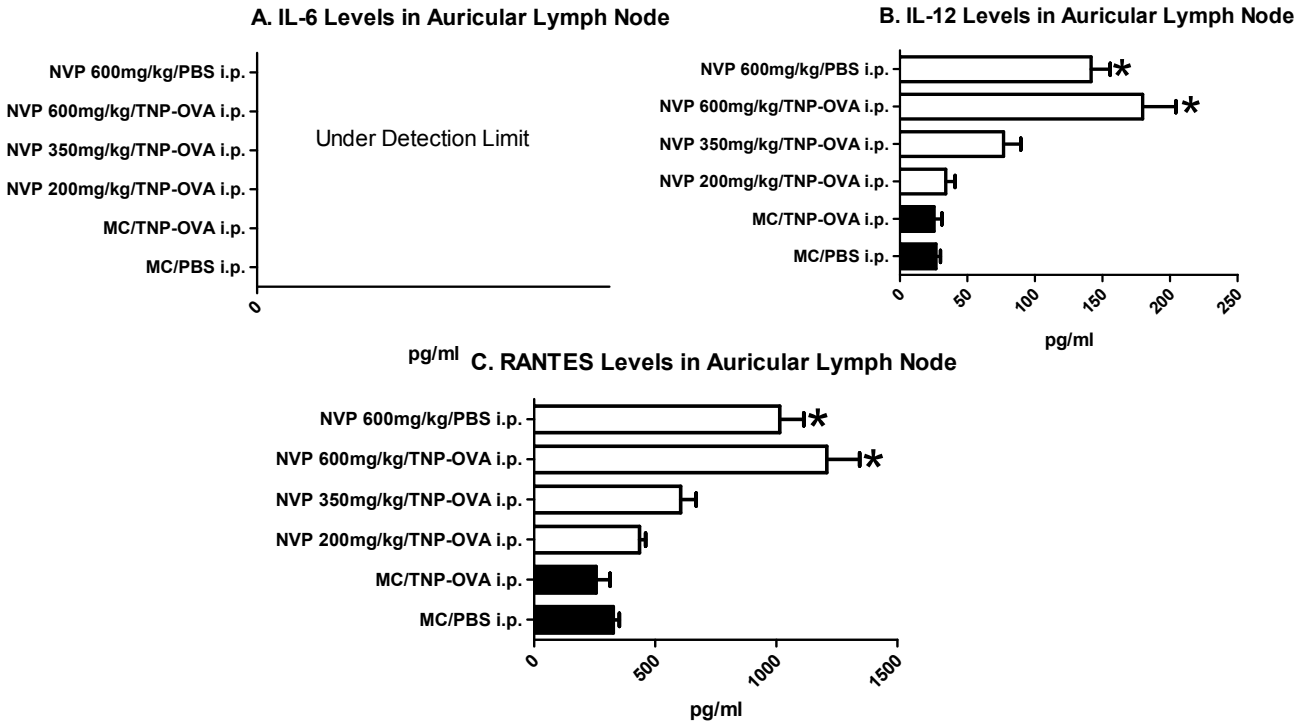


Figure 24. Cytokine measurement after in vitro restimulation of auricular lymph node cells exposed to different concentrations of NVP orally in combination with TNP-OVA i.p.. Mice were gavaged with either 0.5% methylcellulose or the indicated concentrations of NVP daily, they also were injected i.p. with TNP-OVA or PBS once on day 4 (excepting the last group). After 10 days, auricular lymph node was isolated and restimmulated with ConA or LPS for 24h. Bars represent the mean value of cytokine levels ± STD of 5 mice. *, shows significant differences ($p<0.05$) compared to the control group.

3.2.2.2.3 Determination of TNP-specific Antibody Secreting Cell Numbers

T-cell specific activity of indicated compounds was assessed by detecting T-cell responses to the reporter antigen, TNP-OVA (Figure 25A-B). Mice were daily orally treated with either 0.5% methylcellulose or different concentrations of NVP for 10 days. The mice (excepting the last group) got in addition to oral treatment an i.p. injection of TNP-OVA or PBS on day 4 of the treatment.

TNP-specific IgG1 and IgG2a were measured by performing ELISPOT in spleen inguinal and auricular lymph nodes. Daily treatment 600 mg/kg NVP orally + TNP-OVA i.p. on day 4 leads to increase in the number of B cells producing isotypes IgG1 and IgG2a in spleen. No changes were seen in the number of B cells producing immunoglobulins in lymph nodes.

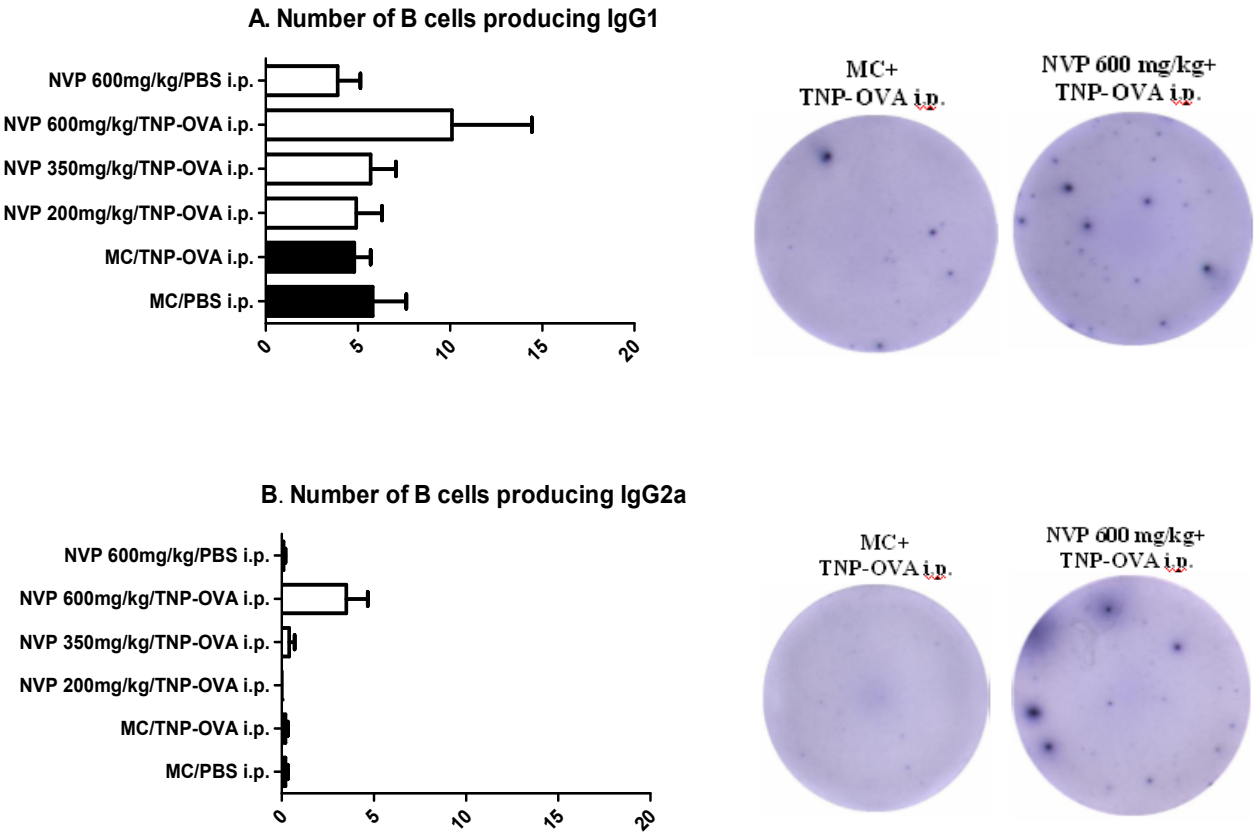
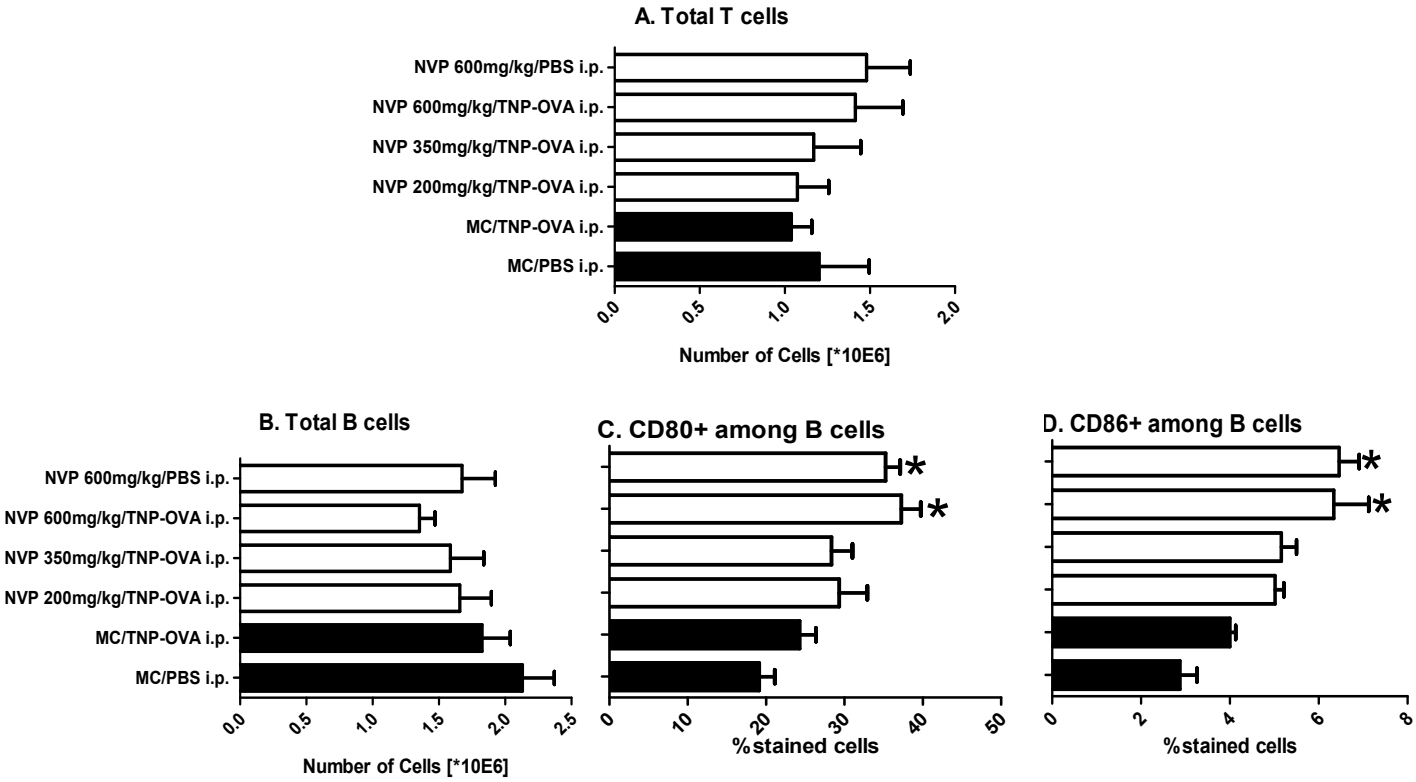
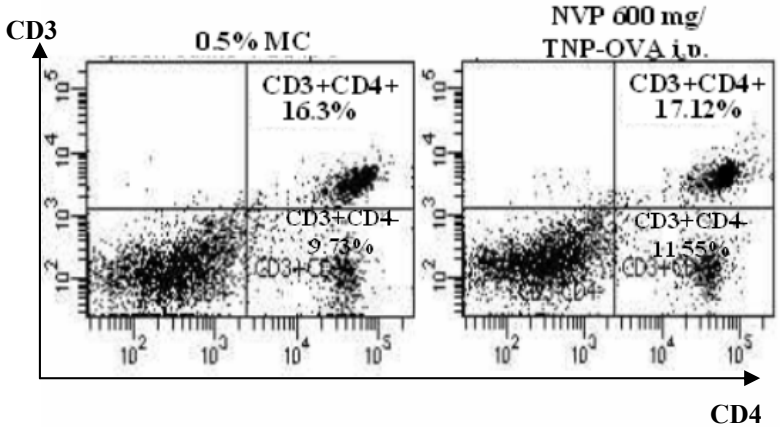


Figure 25. TNP-specific IgG1 (A) and IgG2a (B) Antibody Secreting Cells (ASCs) in spleen. Mice were daily orally treated with either 0.5% methylcellulose or different concentrations of NVP for 10 days. The mice (excepting the last group) got in addition to oral treatment an i.p. injection of TNP-OVA on day 4 of the treatment. After 10 days spleen was isolated and the number of B cells producing TNP-specific IgG1 and IgG2a was measured. Bars represent the mean value of TNP-specific ASCs ± STD of 10 mice.

3.2.2.2.4 Immunological Phenotype, the Effect of Oral Treatment of NVP in Combination with i.p. Injection of TNP-OVA on Proportion of Different Cell Populations in Spleen and Inguinal Lymph Node

The changes in percent of T cells, B cells and their activation markers CD69 and CD25 (among T cells population) and CD80 and CD86 (among B cell population) were evaluated in response to different concentrations of NVP (gavage, daily) + TNP-OVA or PBS (i.p., on day 4) (Figure 26A-D). The results of this study showed no changes in the total number of T and B cells, no changes in % of T cells but a decrease in % of B cells and an increase in CD80+ and CD86+ cells (among B cell population) after NVP 600 mg/kg (gavage, daily) in the presence or absence of TNP-OVA (i.p., on day 4) in spleen. No changes were observed in the % of T cell activation markers CD69 and CD25.


A. Total T cells
NVP 600mg/kg/PBS i.p.
NVP 600mg/kg/TNP-OVA i.p.
NVP 350mg/kg/TNP-OVA i.p.
NVP 200mg/kg/TNP-OVA i.p.
MC/TNP-OVA i.p.
MC/PBS i.p.
0.0
0.5
1.0
1.5
2.0
Number of Cells [*10E6]
B. Total B cells
NVP 600mg/kg/PBS i.p.
NVP 600mg/kg/TNP-OVA i.p.
NVP 350mg/kg/TNP-OVA i.p.
NVP 200mg/kg/TNP-OVA i.p.
MC/TNP-OVA i.p.
MC/PBS i.p.
0.0
0.5
1.0
1.5
2.0
2.5
Number of Cells [*10E6]
C. CD80+ among B cells
*
*
0
10
20
30
40
50
%stained cells
D. CD86+ among B cells
*
*
0
2
4
6
8
%stained cells


CD3
0.5% MC
CD3+CD4+
16.3%
CD3+CD4-
9.73%
NVP 600 mg/
TNP-OVA i.p.
CD3+CD4+
17.12%
CD3+CD4-
11.55%
CD4

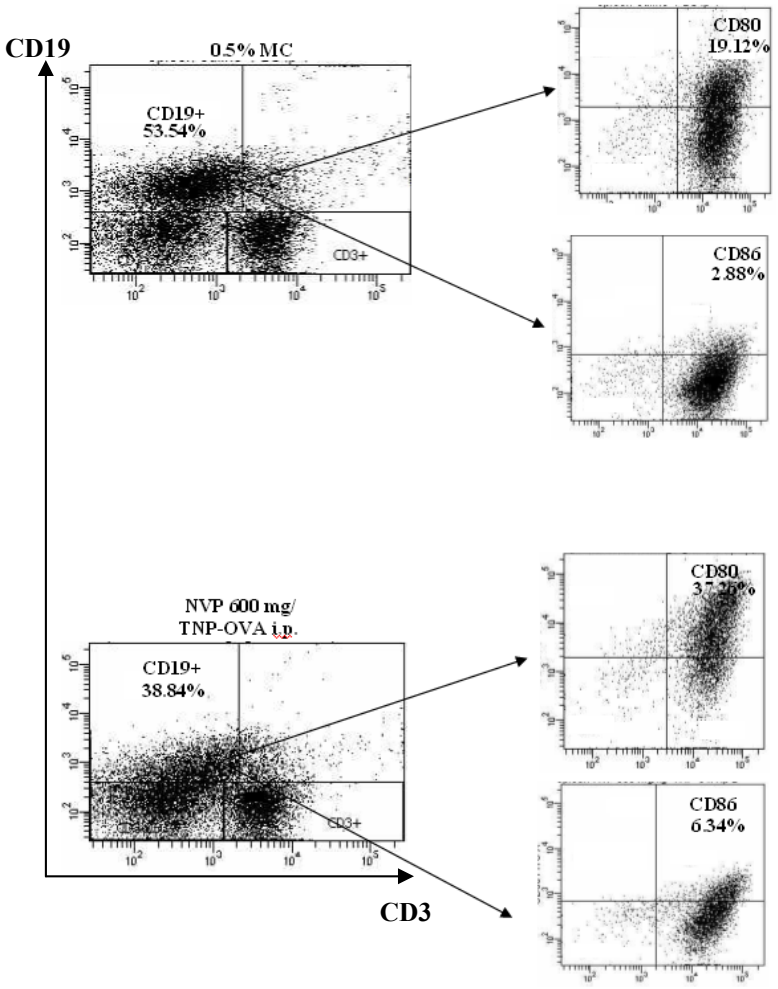
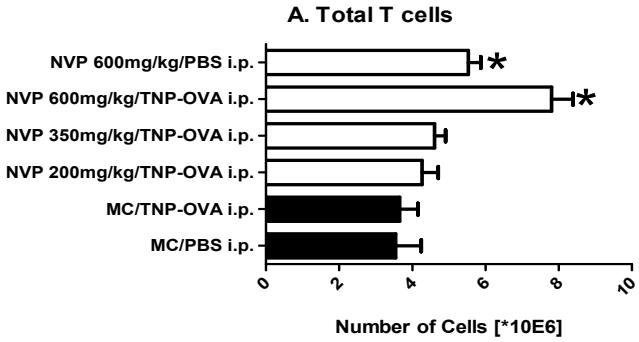
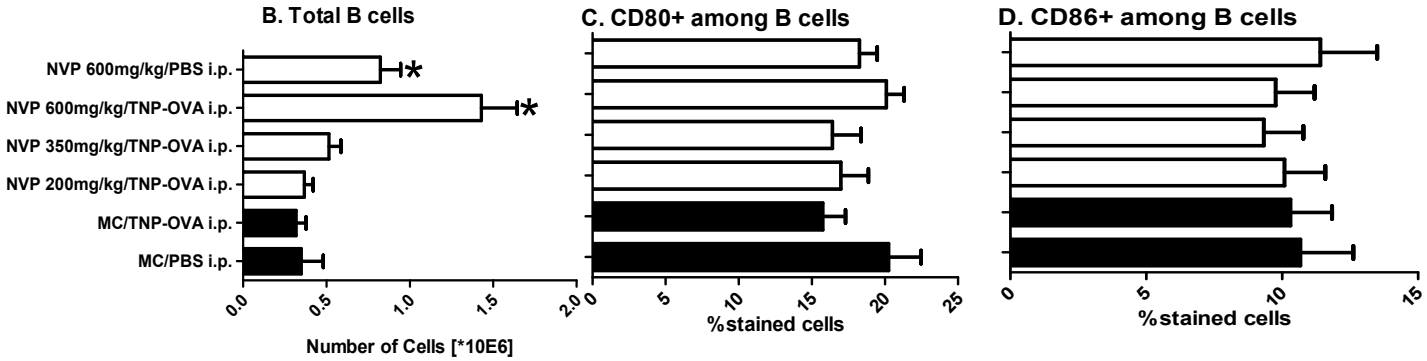
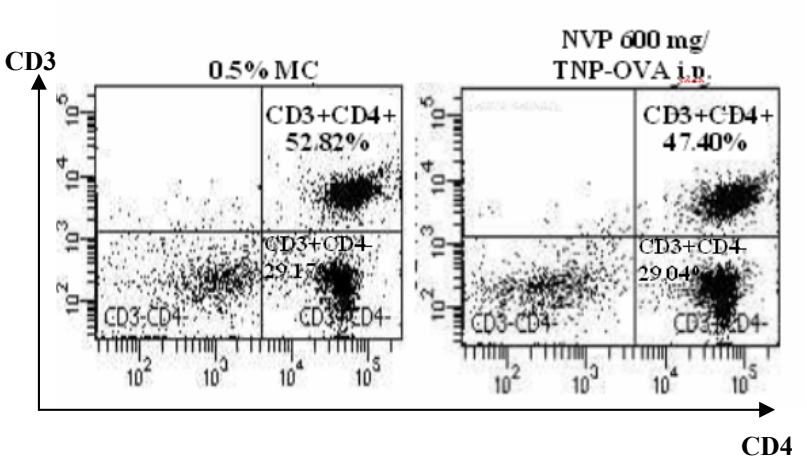


Figure 26. The effect of NVP on proportion of different cell populations in spleen Mice were daily orally treated with either 0.5% methylcellulose or different concentrations of NVP for 10 days. The mice (excepting the last group) got in addition to oral treatment an i.p. injection of TNP-OVA on day 4 of the treatment. After 10 days spleen was isolated and changes in percentages of T cells, B cells and their activation markers were evaluated in response to different chemicals. Bars represent the mean value of cytokine levels ± STD of maximum 4-10 mice. *, shows significant differences ($p<0.05$) referred to the control group. CD3 was used to identify T cells and CD19 was used to identify B cells.

In auricular lymph node (Figure 27A-D), an increase in total number of both T and B cells, a decrease in % of T cells (CD3+, CD4+) and an increase in % of B cells after NVP 600 mg/kg (gavage, daily) in the presence or absence of TNP-OVA (i.p., on day 4), while no changes was observed in either % of T cell activation markers CD69 and CD25 or B cell activation markers CD80 and CD86.


A. Total T cells
NVP 600mg/kg/PBS i.p.
NVP 600mg/kg/TNP-OVA i.p.
NVP 350mg/kg/TNP-OVA i.p.
NVP 200mg/kg/TNP-OVA i.p.
MC/TNP-OVA i.p.
MC/PBS i.p.
0
2
4
6
8
10
Number of Cells [*10E6]


B. Total B cells
NVP 600mg/kg/PBS i.p.
NVP 600mg/kg/TNP-OVA i.p.
NVP 350mg/kg/TNP-OVA i.p.
NVP 200mg/kg/TNP-OVA i.p.
MC/TNP-OVA i.p.
MC/PBS i.p.
0.0
0.5
1.0
1.5
2.0
Number of Cells [*10E6]
C. CD80+ among B cells
0
5
10
15
20
25
%stained cells
D. CD86+ among B cells
0
5
10
15
%stained cells


CD3
0.5% MC
CD3+CD4+
52.82%
CD3+CD4-
29.17%
CD3-CD4-
CD3-CD4-
NVP 600 mg/
TNP-OVA i.p.
CD3+CD4+
47.40%
CD3+CD4-
29.04%
CD3-CD4-
CD3-CD4-
CD4

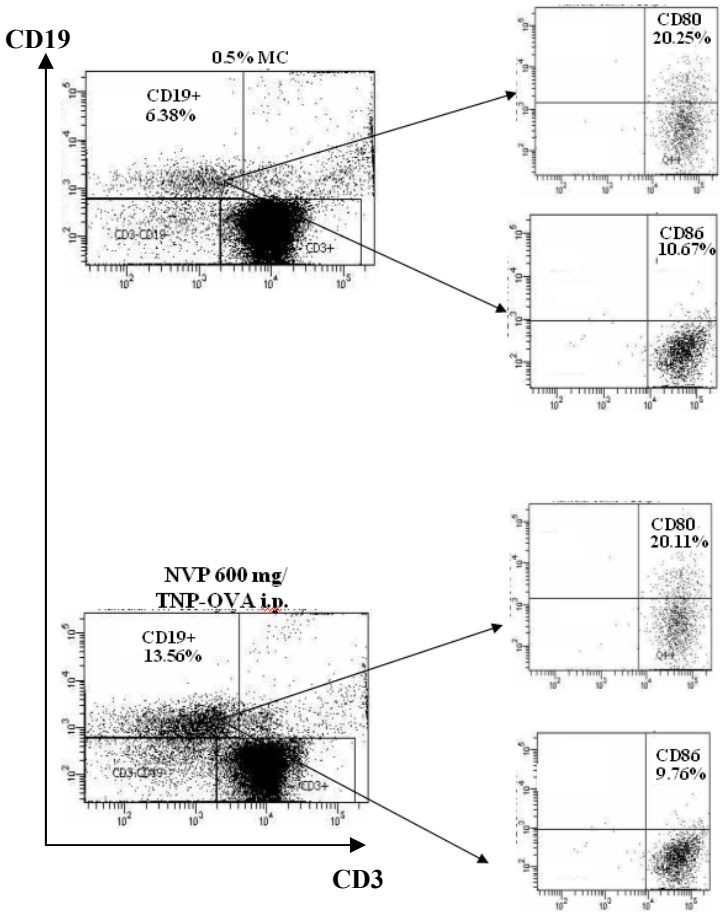


Figure 27. the effect of NVP on proportion of different cell populations in Auricular lymph node Mice were daily orally treated with either 0.5% methylcellulose or different concentrations of NVP for 10 days. The mice (excepting the last group) got in addition to oral treatment an i.p. injection of TNP-OVA on day 4 of the treatment. After 10 days Auricular lymph node was isolated and changes in percentages of T cells, B cells and their activation markers were evaluated in response to different chemicals. Bars represent the mean value of cytokine levels ± STD of maximum 4-10 mice. *, shows significant differences ($p<0.05$) referred to the control group. CD3 was used to identify T cells and CD19 was used to identify B cells.

3.2.2.3 Summery of Oral Study (10-Days)

There was an increase in spleen weight in 600 mg/kg NVP & D-Pen-treated mice in the presence or absence of TNP-OVA. Increase in expression of B cell activation markers (CD80 & CD86) with the highest dose of NVP & D-Pen with or without TNP-OVA was also observed. Isotype switching from TNP-specific IgM to TNP-specific IgG1 and IgG2a with NVP (600 mg/kg). There was also a tendency for Isotype switching from TNP-specific IgM to TNP-specific IgG1 and IgG2a in D-Pen (600 mg/kg), however the data from D-Pen isotype switching were not as convincing as in NVP case. These data reveal that an adoption of the

well-established local RA-PLNA to an oral mouse model was successful. In addition, using RA, TNP-OVA allows detecting systemic TNP-specific immune response.

4 Discussion

There is a long history of failed attempts to study idiosyncratic reactions using animals, because these reactions are just as idiosyncratic in animals as in humans, but attempts to create new and valuable screening animal models is constantly ongoing.

Nevertheless, there are still possibilities to increase the probability that an animal would develop an idiosyncratic reaction to a drug. Specifically, if reactive metabolites are responsible, elevation of the reactive metabolite's production or inhibition of their detoxification might lead to an animal model.

Up to date, the popliteal lymph node assay (PLNA) or the modified version of PLNA, reporter antigen popliteal lymph node assay (RA-PLNA) has been one of the main assays used in screening of immunogenic potential of candidate drugs during the preclinical phase of the drug development process, and helping distinguish innocent from immunogenic compounds [146].

There are some important advantages that lead to use the both PLNA as well as RA-PLNA as a promising tool to study drug induced IDRs; this local assays are very simple and fast profiling model. There are a number of compounds that have been already tested in these assays and the findings from them correlate with what have been found clinically with this compounds [147].

Besides above mentioned advantages of PLNA/RA-PLNA there are some disadvantages of these assays. One of the biggest issues in this regard is the irrelevant route of exposure (subcutaneous injection). Oral administration is the relevant route of drug exposure. Subcutaneous injection of drugs causes additional problems such as lack of metabolic components, so for drugs, like PA that the reactive metabolite is supposed to be the responsible one to cause drug induced IDRs, the induction of immune response in a local assay like PLNA/RA-PLNA could be easily missed and therefore there are some drugs including PA which are known as false negative in PLNA/RA-PLNA.

The first focus of this thesis was to establish and refine the PLNA/RA-PLNA. For this purpose, in addition to PLN weight and cellularity that are usually looked for in the PLNA/RA-PLNA as standard immunological parameters, additional and more sensitive

markers like cytokine/chemokine production and also additional cell surface activation markers were measured to detect signs of immune response with drugs, which have been tested falsely negative in these assays.

The goal was by measurement of additional and more sensitive markers in the PLNA/RA-PLNA to be able to overcome the problem of having false negative compound in this assay.

Another issue that one can encounter in PLNA/RA-PLNA is the solubility problem. Some compounds like NVP are not water soluble. In such a case, there are two possibilities; one is using another vehicle, which in most of the cases this vehicle could be the cause of immune response by itself even if it is used in a little amount. The other possibility would be injecting the suspension. This could be again an issue, as injecting a suspension can lead to induction of a non-specific immune response which is not drug-driven and it is caused due to the crystal formation and by non-specific activation of phagocytes releasing inflammatory cyto- and chemokines.

To circumvent the above mentioned issues in the local assay, establishing an oral mouse model was the second focus of this thesis. The goal was by using a systemic route of exposure to be able to overcome the issue of requiring reactive metabolite on board to induce immune response and also to solve the problem of inducing an unspecific immune response by not water soluble compounds.

In this thesis the mechanisms of D-Penicillamine (D-Pen), nevirapine (NVP), streptozocin (STZ), Procainamide (PA) and Diphenylhydantoin (DPH)-induced IDRs in the local assays and also establishing an oral exposure mouse model by using two different reference compounds, D-Pen and NVP, were investigated.

These drugs will be outlined and discussed one by one in this chapter.

4.1 D-Penicillamine (D-Pen)

4.1.1 Proposed Mechanism of D-Penicillamine Induced Immune Response

D-Pen treatment decreases the number of circulating immune complexes and overall IgG and IgM titers in rheumatoid arthritis patients [148]. Though the mechanism by which D-Pen down regulates the immune response is largely unknown, evidence suggests that D-Pen affects macrophage function. In vitro, D-Pen preferentially binds to the membrane of rat monocytes [149]. This preferential binding of D-Pen to peripheral blood monocytes is also

observed in vivo in humans [150]. By activating macrophages, D-Pen is thought to increase the clearance of immune complexes and IgG in rheumatoid arthritis patients. In vitro, D-Pen enhances the uptake of C^{14} glucosamine and I^{125} IgG by macrophages [151]. There is also an increase in the clearance of carbon particles by macrophages in the New Zealand white rabbit in vivo [152]. It is hypothesized that the clearance of IgG and immune complexes leads to the alleviation of rheumatoid arthritis symptoms.

Interestingly, it has been proposed that the mechanism of D-Pen toxicity is also related to the activation of macrophages. The presence of D-Pen abolishes the ability of macrophages to suppress concavalin A-mediated activation of T cells [153]. Studies using mouse splenocytes have demonstrated that primed T cells proliferate in response to D-Pen bound to MHCII in an antigen processing-independent manner [154]. By being recognized as foreign, it is possible that D-Pen bound to MHCII could result in Graft-versus-Host-Reactions (GVHD)-type response.

In Table (4-1-1) the new findings were compared with what has been done up to now in primary PLNA with D-Pen

Table 4-1-1

	D-Pen	
	Literature	**New findings**
PLN weight	↑[155]	↑
PLN cell count	↑[156]	↑
Cytokine secretion	Has not been done to date	No changes in IL-2, 6, 12 & RANTES
Flow cytometry	Has not been done to date	Total Nr of T & B cells↑, CD25+&CD69+ T cells↑
Gene expression profiling	Has not been done to date	No changes in any parameters

In Table (4-1-2) the new findings were compared with what has been done up to now in RA-PLNA (TNP-OVA) with D-Pen

Table 4-1-2

	D-Pen	
	Literature	**New findings**
PLN weight	↑	No changes compared to control group
PLN cell count	↑[143]	No changes compared to control group
Cytokine secretion	IL-4, TNF-α↑, no changes in IFN-γ levels [10]	No change in IL-2, IL-6, IL-12 & RANTES levels. The levels of IL-4, IFN-γ and TNF-α was below detection limit
Immunoglobulines	TNP-OVA specific IgM, IgG1, IgE↑, no changes in IgG2a [10, 143]	TNP-OVA specific IgM & IgG1↑, IgG2a ↑ but to a lesser extent compared to IgM and IgG1
Flow cytometry	Total Nr of T cells, %CD25+ and CD69+ T cells↑. Total Nr of B cells and % CD86+B cells↑, no changes in %CD80+Bcells. % CD11c, F4/80↑. No changes in CD80+B cells & CTL-4 [143, 157]	No changes in any cell markers

In Table (4-1-3) the new findings were compared with what has been done up to now in RA-PLNA (TNP-Ficoll) with D-Pen.

Table 4-1-3

	D-Pen	
	Literature	**New findings**
PLN weight	↑	↑
PLN cell count	↑ [136]	↑
Cytokine secretion	IL-4, IFN-γ↑, no changes in IL-1 and TNF-α levels [136]	IL-2, 6, 12 and RANTES↑, IL-1 and TNF-α levels were below detection limit
Immunoglobulines	TNP-Ficoll specific IgM, IgG1↑ [136]	TNP-Ficoll specific IgM, IgG1& IG2a↑
Flow cytometry	Total Nr of T & B cells↑ [136]	Total Nr of T & B cells↑

D-Pen is used as a reference or positive control for type-2 immune responses in different RA-PLNA studies, as it leads to increase in mainly typical classical Type-2 related immune parameters like, IL-4 cytokine or IgG1 immunoglobulin. These findings in primary PLNA reinforced what has been done up to now and based on the increase in T cell activation

markers as additional, new markers it was possible to show that there is a sign for a T cell specific immune response that have to be confirmed in RA-PLNA. In RA-PLNA (TNP-OVA), it was not possible to reproduce exactly what had been shown before on PLN weight and cellularity basis. There was no increase in either PLN weight and cellularity or cytokine release or activation of any cell markers. The only immunological marker that showed an increase which was correlating with previous studies was immunoglobulines. The only explanation for not being able to reproduce what has been done with D-Pen in RA-PLNA (TNP-OVA) is due to the technical issues, as the compound that is used in literature is D-Pen hydrochloride [10], but this batch is not produced any more so D-Pen (PH=2) had been used in these sets of studies. In primary PLNA, it has already been shown that D-Pen (PH=2) can lead to increase in PLN weight [155]. However, any little changes in PH could influence the results. Although the PH was measured after preparing D-Pen solution but the PH issue could be the only explanation for not being able to reproduce the positive expected response with D-Pen in RA-PLNA (TNP-OVA).

In RA-PLNA (TNP-Ficoll) what had been shown by other groups up to now with D-Pen on PLN weight, cellularity, immunoglubulines and also activation of T and B cell markers was reproduced. On cytokine levels, the levels of IL-4 and IFN-γ were below detection limit and the increase that has been shown with D-Pen by other groups was not detectable [172]. The reason for not being able to detect any changes in this cytokines could be using Luminex assay to measure cytokines in this thesis in stead of ELISA. The Luminex allowed measuring a broad range of cytokines in a small amount of samples but this assay is not sensitive enough to detect cytokines that are expressed in very low amount. However, an increase in cytokine levels for IL-2, IL-6, IL-12 and RANTES with D-Pen + TNP-Ficoll was observed.

IL-2 is the first T cell growth factor to be identified and molecularly cloned. Since its discovery, it has been shown to promote T cell proliferation and survival in vitro. Helper cells produce IL-2 upon activation by APCs, being the major source of this cytokine in vivo. In vitro studies have shown that helper cells, cultured with APCs and anti-CD3, produce large amounts of IL-2 [158]. IL-2 production occurs as a burst associated with the transition of helper cells, from the resting to the activated state. Helper cells proliferate and survive, upon TCR ligation with APCs and signaling received from IL-2, or other homeostatic cytokines: In vitro experiments have shown that IL-2 is a growth/survival factor for activated T cells [159], it enhances T cell immunity in vivo in cases of viral infection [160] and vaccination [161]. In

addition to IL-2, IL-12 levels were also increased in PLN supernatant derived from mice treated with D-Pen + TNP-Ficoll. IL-12 can induce IFN-γ by promoting differentiation of naive T cells directly towards Th1 effectors that enable them to produce IFN-γ [162, 163], or by acting as a co-stimulator for differentiated Th1 cells to enhance IFN-γ production. It can also induce switching of IFN-γ producing memory Th1 cells to their effecter stages and enable them to secret IFN-γ. IL-12 activates NK cells to produce IFN-γ and promotes NK cells and CTL differentiation and enhances their cytotoxicity. Although IL-12 might directly affect B-cell proliferation, differentiation and IFN-γ production, the effect of IL-12 on B-cell activation and immunoglobulin isotype switching is mediated by IFN-γ. IL-12 also synergizes with IL-18 to induce IFN-γ in Th1 cells and IgG2a switching in B cells.

In addition, it serves as a direct chemotactic factor for NK cell infiltration, further increasing their cytotoxicity [164]. One of these chemokines is RANTES which is known for acting as a chemo attractant for monocytes, T cells [165], eosinophils, NK cells [166], and dendritic cells [167]. It also promotes T cell adherence to activated endothelial cells and stimulates T cell proliferation [168]. In vivo analysis of RANTES-deficient mice reveals that RANTES plays a role in the delayed-type hypersensitivity (DTH) response, T cell proliferation, and production of IL-2 [169].

Although the RA-PLNA is a promising preclinical assay, it has a disadvantage that the chemicals are injected subcutaneously (s.c.) instead of being administered orally, the usual route of exposure for most drugs and chemicals. Therefore, it was important to investigate whether D-Pen is able to induce systemic immunological changes via oral route of exposure which is a normal route of drug administration.

As discussed previously, the antigen-specificity of drug induced immune response can vary from adjuvant like effect to T cell-specific. Using reporter antigens as a read-out allows differentiating between these two effects that can be caused by drugs.

D-Pen was the first candidate that were selected for attempts to establish an oral model in this thesis, based on previous experience with these compounds in humans as well as in BN rats. The first attempt to establish an oral mouse model was done by Nierkens et al. In this study the reporter antigen was combined with oral exposure of the drug to investigate whether this approach can be used to identify systemic immunosensitizing potential of the drug. The results of this study showed increased antibody levels specific for the co-injected bystander antigen, TNP-OVA and induce DTH responses upon challenge with TNP-OVA [9].

Preliminary studies in two different strains of mice have also been performed, based on the suggested oral model by Nierkens in this work. In these studies C3H/HeJ and C57BL/6 were i.p. injected with TNP-OVA (10 μg) on the first day and then gavaged once daily with 150 mg/kg D-Pen for 28 days. After three weeks mice were s.c. rechallenged in their right footpads with a suboptimal dose of reporter antigen, TNP-OVA (10 μg/mouse). A week later different organs (e.g. PLN, liver, spleen) were sampled from these mice, and prepared for the immune onset analysis. The results of this preliminary experiment showed signs of an immune response onset which were not significant and also not supported by increase in other immune markers. One of the explanations for this not clear and significant response was that the doses which are used for presensitizing and resensitizing animals with TNP-OVA i.p. and s.c., respectively, are too high, so the effect of the drug could be masked by the response which is caused by reporter antigen. In addition, the use of higher doses or repeated dosing of TNP-OVA is also able to induce specific T-cell memory. Hence, the TNP-specific response can be elevated by each extra dose of antigen. Despite all attempts to establish an oral exposure mouse model Nierkens' group was not able to detect any systemic immune response and this model could not be used as a profiling model.

Therefore the first step was to titrate TNP-OVA in both cases (i.p. and s.c. injection). The results of the titration studies showed that the suboptimal concentrations of TNP-OVA that should be used in the main study are 0.3 μg/mouse TNP-OVA i.p. on day 1 for presensitizing and 3 μg/mouse TNP-OVA s.c. on day 21 for resensitizing in C3H/HeJ mice. In addition, dose and treatment regimens need to be optimized to allow the induction of sensitization rather than the induction of tolerance. Nierkens studies showed that continuous exposure to D-Pen compared to seven-day exposure caused lower serum IgG1 and IgE (indicator of humoral Th2 responses), but higher DTH responses (indicator of Th1 responses).

To repeat this study under the optimal conditions that were found after titration studies and to investigate the mechanism(s) of D-Pen-induced immune response in mice, C3H/HeOuJ mice were i.p. injected with TNP-OVA (0.3 μg/mouse) on the first day and then gavaged once daily with either 150 or 600 mg/kg D-Pen for 28 days. After three weeks mice were s.c. rechallenged in their right foot pads with a suboptimal dose of reporter antigen, TNP-OVA (3 μg/mouse). Finally, mice were sacrificed on day 28 and their organs (liver, ear, blood, spleen, auricular, popliteal and inguinal lymph nodes) taken and analyzed for signs of an immune response onset.

The results of 28 days-oral treatment with 600 mg/kg/day D-Pen indicated a significant increase in spleen weight and cellularity and also an increase in total number of T and B cells in the spleen but there was no TNP-specific systemic response to be seen by measurement of IgM, IgG1 and IgG2a. Therefore the oral treatment study was redesigned.

Attempts for redesigning the oral exposure RA-mouse model lead to have a novel mouse model which is based on RA-PLNA. As the purpose of performing oral RA studies was to investigate a systemic immune response, the administration routes chosen for both the drug and the RA were systemic, with the drug being gavaged and RA i.p. injected, but the duration of the study itself was similar to the traditional local RA-PLNA study. The drug of interest was given by gavage for 3 days, on day 4 additionally mice were i.p. injected with a suboptimal dose of RA, TNP-OVA, upon which oral treatment was continued for another 7 days. Finally, mice were sacrificed on day 10 of treatment and their organs taken and analyzed for the signs of an immune response onset. The drug of interest was gavaged three days in advance in order to reach enough drug concentration in animal's systemic circulation by the time that RA was i.p. injected.

Data from the oral treatment of D-Pen in the newly designed oral mouse model showed a significant increase in spleen and inguinal lymph node weight and cellularity with the highest concentration of D-Pen either in the presence or in the absence of the RA, however presence of TNP-OVA lead to increased response.

A significant decrease of T cells (on percent basis) was observed after oral treatment with 600 mg/kg D-Pen. This decrease of T cells was counteracted by increase in percent of B cells, which additionally had activation markers CD80 and CD86 up regulated in both organs spleen and inguinal lymph node. The B cell activation in spleen was confirmed by Isotype switching data from IgM to IgG1 and IgG2a.

Increases in inguinal lymph node levels of IL-6 and IL-12 cytokines or RANTES chemokine, which are all prototypic type 1 cytokines/chemokine were also observed.
An important aspect of the cytokine profile is the Th1/Th2 balance. Notably, D-Pen induces IL-4 mRNA expression in the spleen and caecum in BN rats [170], which in turn drives IgE production. IL-4, the prototypic type 2 cytokine, can be taken to represent changes in type 2 cytokines. IFN-γ, the prototypic type 1 cytokine, can be taken to represent changes in type 1 cytokines [171].

High dose D-Pen induced an increase in type 1 related cytokines and chemokine in inguinal lymph node. This increase in type 1 cytokines is not surprising when compared to evidence obtained from the mercuric chloride model of autoimmunity in the Brown Norway rat. In the mercuric chloride model, the initial predominant Th2 response is followed by a period of immunosuppression, mediated by the production of IFN-γ and other Type 1 cytokines [172]. Similarly in the D-Pen BN rat model, IL-4 is decreased, in contrast, type 1 related cytokines and chemokines peak at the highest dose. It is possible that the peak in type 1 related cytokines and chemokines expression represents the onset of such immunoregulation.

The ELISPOT data showed Isotype switching from TNP-specific IgM to TNP-specific IgG1 and IgG2a in spleen but the data were not significantly high compared to another compound (Nevirapine) tested, which will be discussed later. The use of higher concentrations of D-Pen or increase in the concentration of RA (TNP-OVA) may consider as further improvement.

It could be possible that in this case a higher dose of TNP-OVA is required to boost the immune response and induce TNP-specific immunoglobulines systemically in case of D-Pen.

In Table (4-1-4) the new findings in oral mouse model with D-Pen in the 10-days oral assay model were compared with what has been done up to now in BN rats.

Table 4-1-4

	Rats	Mice (10 day-oral model)
Strain Susceptibility	Brown Norways are susceptible; Lewis and Sprague Dawley rats are resistant [7]	C3H/HeOuJ mice are susceptible
Time to Onset	Symptoms of disease appear between week 3 and week 7 [7]	No clinical signs
Incidence	Disease occurs in 60 to 80% of rats [7]	-
Weight Loss	Dramatic weight loss only occurs after disease onset [7]	No weight loss
Dermatitis	Dermatitis appears between weeks 3 and 7 [141]	No Dermititis
Caecum, skin and spleen	Macroscopic lesions in caecum appear by day 15 Necrotic lesions that are granulomatous in nature [7]	No finding
Splenomegaly	Only occurs in diseased rats [173]	Occur in the highest dose (600 mg/kg)
Cytokine secretion	IL-4 levels increase in spleen and caecum by day 4[170]	Due to the high induction levels of all cytokines in control groups, the data from cytokine measurement are inconclusive
Expression of cell markers	Has not been done to date	Decrease in % B cells, increase in %CD80 & CD86+ B cells in spleen. Increase in total T and B cells and also %CD80 & CD86+ B cells in inguinal lymph node
Immunoglobulines	Total IgE levels peak at week 2 [170]	TNP-OVA specific IgG1 increase with 150 & 600 mg/kg in spleen
Disease Regulation	Incidence of disease is 100% upon rechallenge [174] Low dose pretreatment prevents autoimmunity [7]	No sign of disease

4.2 Nevirapine (NVP)

4.2.1 Proposed Mechanism of Nevirapine Induced Skin Rash

NVP is a NNRTI used for the treatment of HIV type 1 [111]. NVP acts by binding to the tyrosine components of the reverse transcriptase enzyme located near the catalytic site [112]. The effect of NVP is termination of viral RNA transcription into viral DNA, thereby blocking its integration into human genome [113]. Due to the intrinsic reactivity of a bioactivated drug, it is generally observed that a reactive metabolite binds at or near the site to which it was formed. Thus, it follows that for a coetaneous drug reaction like in case of NVP the implicated drug may be bioactivated in the skin. The skin most certainly has metabolic capabilities and it seems that an immune response against reactive metabolites formed, and subsequently covalently bound in the skin, may pose a reasonable scenario for the development of coetaneous idiosyncratic drug toxicity. There is some evidence for the metabolism of systemically administered drugs in the skin [175]. This metabolism may be due to skin-resident cells or, alternatively, to circulating cells that infiltrate the skin, such as monocytes/macrophages [176].

NVP is delivered systemically and is known to result in induction of the liver P450 enzymes responsible for its metabolism i.e., autoinduction. The primary enzymes responsible for metabolizing NVP in the liver of humans are CYP 3A and CYP 2B6. Both of these enzymes have also been detected in human keratinocytes, as have CYP 1A1 and 2E1 [177]. It is hypothesized that metabolism of NVP in skin or in liver is important for the development of NVP -induced skin rash. It is also possible that hydroxylated- NVP metabolites formed in the liver circulate to the skin and then undergo bioactivation within the skin. its reactive metabolite quinone methide has been suspected to act as a trigger in the development of adverse liver and skin reactions [130]. Once formed in the liver, oxidized NVP metabolite, 12-hydroxy NVP, travels to the skin whereby sulfotransferase enzyme converts it into a 12-sulfa- NVP, which spontaneously loses a sulfate moiety to form the reactive quinone methide. It is thought that the reactive quinone methide is the responsible one to induce skin rash.

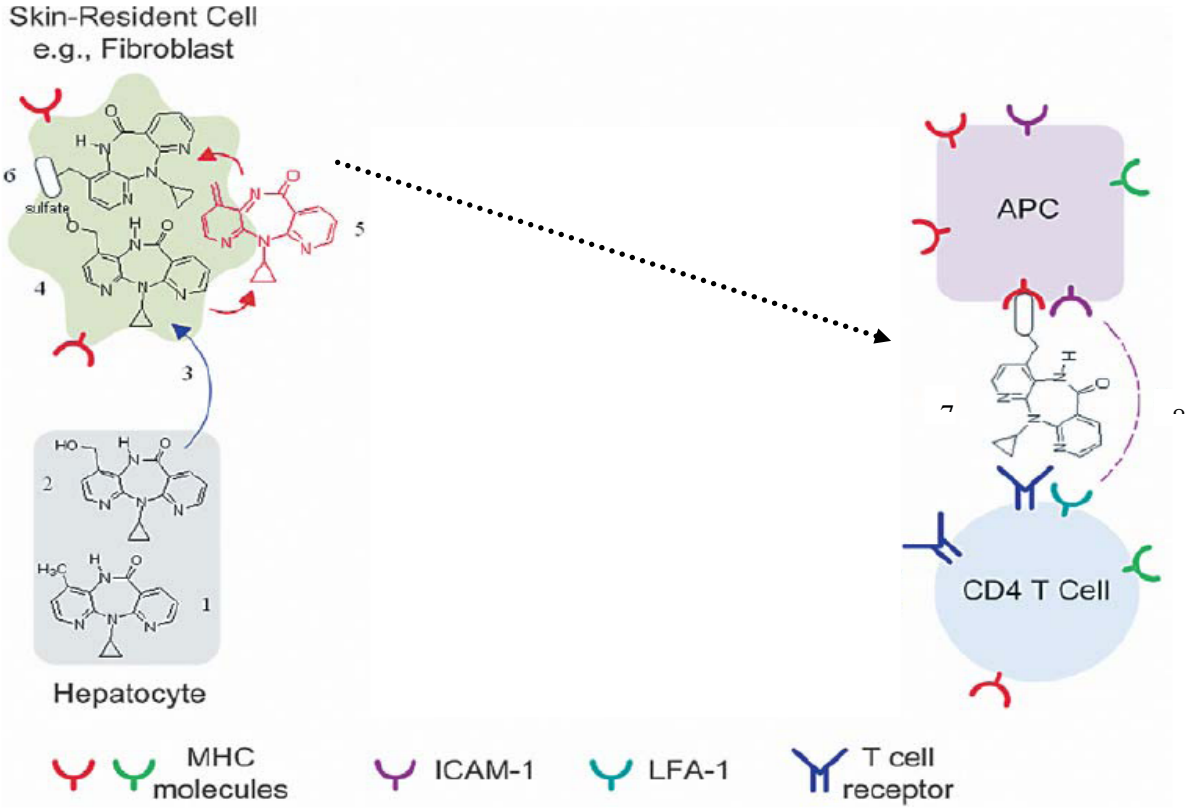


Figure 4.1. Potential pathway leading to NVP-induced rash

In Table (4-2-1) the new findings were compared with what has been done up to now in primary PLNA with NVP

Table 4-2-1

	NVP	
	Literature	**New findings**
PLN weight	Has not been done to date	No changes compared to control group
PLN cell count	Has not been done to date	No changes compared to control group
Cytokine secretion	Has not been done to date	RANTES↑ (insignificant)
Flow cytometry	Has not been done to date	No changes in any cell marker
Gene expression profiling	Has not been done to date	No changes in any parameters

In Table (4-2-2) the new findings were compared with what has been done up to now in RA-PLNA (TNP-OVA) with NVP

Table 4-2-2

	NVP	
	Literature	**New findings**
PLN weight	Has not been done to date	↑
PLN cell count	Has not been done to date	↑
Cytokine Secretion	Has not been done to date	IL-2 ↑, no change in IL-6, IL-12 & RANTES
Immunoglobulines	Has not been done to date	TNP-OVA specific IgM, IgG1 & IgG2a↑
Flow Cytometry	Has not been done to date	Total Nr of T cells, %CD69+ T cells↑. Total Nr of B cells↑

In Table (4-2-3) the new findings were compared with what has been done up to now in RA-PLNA (TNP-Ficoll) with NVP.

Table 4-2-3

	NVP	
	Literature	**New findings**
PLN weight	Has not been done to date	↑
PLN cell count	Has not been done to date	↑
Cytokine Secretion	Has not been done to date	No change in IL-2, IL-6, IL-12 & RANTES
Immunoglobulines	Has not been done to date	No changes in any TNP-Ficoll specific Igs
Flow Cytometry	Has not been done to date	Total Nr of T & B cells↑

NVP was investigated in PLNA/RA-PLNA for the first time. It was chosen based on already mentioned investigations and findings in humans as well as in BN rats. NVP is one of the very few compounds that have a well-established existing animal model in BN rats associated with its adverse potential. It was previously reported that female BN rats daily orally treated with 150 mg/kg NVP develop red ears between days 7 and 10, and skin rash between days 10 and 21 of the treatment [5].

NVP was tested in this thesis in the local, as well as in the mouse oral model to gain more mechanistic information about NVP-induced immune response.

In primary PLNA, no changes in any immunological parameter were observed after NVP treatment neither on RNA level nor on protein level.

In the next step NVP was combined with RA (TNP-OVA) to test if RA can trigger the immune response and make borderline effects visible.

So, NVP, in the next step, was combined with reporter antigens. In the RA-PLNA, RA-specific antibodies are analyzed after simultaneous injection of NVP and TNP-OVA; T-dependent RA or TNP-Ficoll; T-independent RA. The RAs are injected in a small amount (10/30 μg) such that no TNP-specific, antibody-secreting cells (ASC) are measured. Efficient immune responses to TNP-OVA require cognate T-B cell interactions and costimulatory adjuvant signals [10]. Therefore, any reactive drug that induces inflammatory mediators, including non sensitizing irritants, or adjuvants, will induce the formation of TNP-specific ASC. On the other hand, TNP-Ficoll alone does not elicit TNP-specific ASC with Ig isotypes other than IgM. Therefore, a TNP-specific IgG response can only occur in the presence of T cell help which are activated by the drug. Importantly, naive T cells are incapable of specifically responding to TNP-Ficoll, as Ficoll does not represent a T cell epitope. Therefore, the formation of TNP-specific IgG ASC in response that is induced upon co injection of TNP-Ficoll together with a drug indicates help from hapten- or neo-antigen-specific T cells. Thus, the use of these two RAs in the PLNA provides evidence for chemical-induced sensitization to either the compound itself (allergy) or neo-epitopes (autoimmune hypersensitivity) and differentiates sensitizing drugs from inflammatory (but nonsensitizing) compounds and innocent (non-inflammatory, non-sensitizing) drugs [143]. In this approach, the response to well-defined bystander antigens enables the use of a single, generalized and specific, T cell–dependent read-out parameter to assess the immunosensitizing potential of a wide variety of drugs.

From the RA-PLNA (TNP-OVA) experiments it was deduced that both CD4+ and CD8+ T cells seem to play a role in inducing immunostimulatory response in mice treated with NVP in combination with TNP-OVA. Investigating different cell markers and their activation levels indicated a more pronounced CD4+ T cell response. On the other hand the same group of mice showed an increase in levels of IL-2 cytokine. As discussed previously IL-2 is mainly involved in Th1 immune response. The results from immunoglobulines measurement

confirmed our findings from cytokine and cell markers response. There was an increase in IgM and also signs of isotype switching from IgM to IgG1 and IgG2a in NVP + TNP-OVA treated mice.

These data showed that adding the suboptimal dose of RA would be helpful to trigger the immune response and by looking at more sensitive immunological parameters, it would be possible to detect subtle immune-mediated changes that are caused by drugs like NVP and also to gain more mechanistic clues about drugs like NVP.

The question then remains whether this response was evoked by NVP activated T cells and their mediators or imply by general inflammatory mechanism which acts as an adjuvant with regard to TNP-OVA.

For this purpose NVP was combined in the third step with TNP-Ficoll. TNP-Ficoll allowed testing whether NVP is able to induce a T cell-specific immune response on its own.

With NVP, there was an increase in PLN weight and cellularity. That was an indicator of cell proliferation in PLN but no changes were observed in any other immunological parameters that were measured.

RA-PLNA might be less convenient for the injection of relatively insoluble compounds like NVP. Moreover, the injection volume used in the RA-PLNA may not be large enough to enable higher exposure amounts and less difficulty with relatively insoluble compounds. Using organic solvents, such as dimethyl sulphoxide (DMSO), might solve the lack of aqueous solubility. However, 10 µl DMSO was shown to cause moderate PLN enlargement upon injection into the footpad [178]. Therefore, NVP was suspended in PBS and to prepare homogenous suspensions an ultrasound bath was used.

The data from NVP+TNP-Ficoll study showed that injection of the not readily soluble NVP resulted in negative responses in RA-PLNA with TNP-Ficoll. So, limited aqueous solubility and the particle size may be responsible for induction of inflammation.

As a future step, immunogenicity of NVP reactive metabolites should also be tested in the RA-PLNA system, allowing unique opportunity to directly compare the results obtained from an existing animal model with the findings from the widely used screening tool PLNA/RA-PLNA.

The solubility issue of NVP and not being able to inject high enough amount of drug to induce immune response locally lead to test NVP in both already discussed oral mouse models (28 and 10 days).

Findings with NVP from Nierkens et al. attempts to establish an oral mouse model indicates that oral administration increased DTH responsiveness but failed to increase TNP-specific Ig serum levels, whereas i.p. dosing clearly induced IgG1 and IgE isotype production as well [9]. So, the preferential immunological response to NVP via the oral route could be via Th1 cells, which would correlate with the development of rash and CD8+ T cells and macrophages in skin infiltrates in BN rats [5].

Preliminary studies in two different strains of mice have also been performed, based on the suggested oral model by Nierkens. This approach was described in D-Pen chapter. In this attempt NVP was orally administered in two different concentrations, 350 and 500 mg/kg for 28 days. The results of this preliminary experiment showed signs of an immune response onset which were not significant and also not supported by increase in other immune markers. As previously discussed, one of the explanations for this not clear and non-significant response was that the doses which are used for TNP-OVA i.p. and s.c., respectively were too high, so the effect of the drug could be masked by the response which is caused by reporter antigen. Therefore the TNP-OVA was titrated in both cases. Then, C3H/HeJ mice were orally administered with 200 or 600 mg/kg NVP for 28 days. Dose and treatment regimens need to be optimized to allow the induction of sensitization rather than the induction of tolerance. For instance, administration of low doses; 40 or 75 mg/kg/day of NVP were shown to induce tolerance in rats, while these rats developed skin rash after exposure to 100 mg/kg/day [5]. This phenomena may be attributed to induction of low-dose oral tolerance [179] and indicate that the development of high or low dose tolerance vs. sensitization occurs within a small dose range.

Data from optimized 28 days oral mouse model did not show any significant change in any immunological parameters that was measured. Neither organ weight and cellularity nor expression of different T and B cell markers nor induction of cytokines in spleen and draining lymph nodes indicated any sort of immune response. In addition no TNP-specific immunoglubulines either in splenocates or in lymphcytes was detected.

Therefore, NVP was tested in the newly established 10 days oral mouse model that has already been described in D-Pen chapter.

NVP was given by gavage for 3 days, on day 4 additionally mice were i.p. injected with a suboptimal dose of RA, TNP-OVA, upon which oral treatment was continued for another 7 days.

Data from the oral treatment of NVP in the newly designed oral mouse model showed a significant increase in spleen and auricular lymph node weight with the highest concentration of NVP either in the presence or in the absence of the RA, however presence of TNP-OVA lead to increased response in the draining lymph node.

A significant increase in total T and B cells was observed in draining lymph node. A significant decrease of B cells (on percent basis) was observed after oral treatment with 600 mg/kg NVP in spleen. This decrease of % B cells, was most likely caused by the increase in non T non B cells as the percent level of T cells was unchanged, but no direct staining was done for non B non T cells. The activation of B cells was shown by up regulation of activation markers CD80 and CD86 in spleen. The B cell activation in spleen was confirmed by Isotype switching data from IgM to IgG1 and IgG2a in spleen. The significant increase in IgG1 and IgG2a after 600 mg/kg NVP was a novel finding in terms of observing a TNP-specific systemically immune response. This latter finding reinforced the need for having a RA on board in an oral/systemic model to gain more clue regarding mechanistic pathways that could be involved in drug induced hypersensitivity reactions.

Increase in the levels of IL-12 and RANTES in auricular lymph node and also increase in IL-6 and IL-12 cytokines and RANTES chemokine which are all prototypic type 1 cytokine/chemokine was observed with specifically highest dose of NVP in spleen.
In Table (4-2-4) new findings in oral mouse model with NVP were compared with what has been done up to now in rats.
Analyses with NVP in this thesis confirmed previously reported observations by Popovic *et al*. that CD4+ T cells appear to be essential for onset of NVP-induced skin rash; in contrast, it appears that CD8+ T cells may have more of a protective role [130].
These findings reinforce the hypothesis that the reaction in this model is similar to that in humans because the incidence of idiosyncratic reactions to NVP in humans appears to be lower in patients with low CD4+ T cell counts [180].
It has been shown that skin cells involved in mounting immune response to a foreign antigen increase production levels of both MIG (CXCL9) and IP-10 (CXCL10) chemokines, which

help recruit CD183 (CXCR3+ effectors T cells) from systemic circulation to the site of inflammatory challenge [181]. For resident immature Langerhans cells to traffic from the epidermis to draining lymph nodes, decreased skin cell E-cadherin expression and up regulated expression of the chemokine receptor CD197 (CCR7) on the surface of Langerhans cells need to take place. Chemokine receptor CD197 (CCR7) up regulation allows cell migration into lymphoid organs in response to the signaling of a chemokine SLC (CCL21), which is expressed by endothelial cells [182]. Additionally, increased APC production of MIP 1α (CCL3), MCP-3 (CCL7) and RANTES (CCL5) chemokines has been observed in patients with erythema multiforme lesions. These cytokines recruit mononuclear cells from the systemic circulation into periphery during inflammation in the skin [183]. The changes in the cell surface marker expression and chemokine production in mice may further elucidate the mechanism of NVP-induced skin rash in humans.

These recent results suggest that the RA approach can be useful in assessing sensitizing potential following oral drug exposure. However, much more research is required before an oral exposure RA-models can be used as a predictive model.

In Table (4-2-4) the new findings in oral mouse model with NVP were compared with what has been done up to now in rats.

Table 4-2-4

	Rats	Mice (10 day-oral model)
Strain Susceptibility	Brown Norways are susceptible [5]	C3H/HeOuJ mice are susceptible
Time to Onset	Symptoms of disease appear between week 2 and week 3 [5]	No clinical signs
Incidence	Disease occurs in 60 to 80% of rats [5]	-
Weight Loss	Dramatic weight loss only occurs after disease onset [5]	No weight loss
Dermatitis	Dermatitis appears between weeks 2 and 3 [5]	No Dermititis
Skin, liver and spleen	CD4+, CD8+ and macrophages infiltration in to the skin [5]	No finding
Splenomegaly	Only occurs in diseased rats [5]	Occur in the highest dose (600 mg/kg)
Cytokine secretion	Has not been done to date	Increase in IL-6, IL-12 and RANTES levels with the highest dose in spleen supernatant, and increase in IL-12 and RANTES levels with the highest dose in auricular lymph node
Expression of cell markers	Increase in expression of either ICAM-1, or MHC II cell surface activation markers, in auricular lymph nodes [130]	B cell activation, increase in B cell activation markers in spleen. Increase in total number of T & B cells in auricular lymph node
Immunoglobulines	Total IgE levels peak at week 2 [6]	TNP-OVA specific IgG1 & IgG2a increase in spleen in (600 mg/kg)

4.3 Procainamide (PA)

4.3.1 Proposed Mechanism of Procainamide Induced Lupus

The antiarrhythmic, PA, has been associated with drug-induced lupus [91]. Despite all attempts to induce lupus with these drugs in experimental animals, from mice to pigs, this attempts have been inconsistent or met with failure [184].

Rommel et al. have reported an animal model of PA-induced lupus [185]. A metabolite of PA, PA-hydroxylamine (PAHA), is thought to be responsible for inducing autoimmunity [186]. Also, Rommel et al. believed that PA may be inducing lupus by interfering with the production of central T cell tolerance in the thymus; thus, two injections of PAHA directly into the thymus of (C57BL/6 x DBA/2) F_1 mice were carried out 2 weeks apart [185]. PAHA injection resulted in a delayed and fairly long-lasting loss of IgG tolerance to chromatin and a more immediate short-lived IgM response to denatured DNA-findings consistent with that of PA-treated humans. Specifically, asymptomatic patients usually present with IgM antibodies against denatured DNA; whereas, patients with symptoms of lupus have significantly elevated IgG antibodies against chromatin, but fewer of the anti-denatured DNA antibodies [187]. Interestingly, antibodies against chromatin in both patients and mice were against the same subunit of chromatin [185]. However, the animals do not develop any clinical signs of autoimmunity. Another limitation of this model is that other drugs associated with lupus have not been tested, and it is quite possible that any toxic or even corrosive agent, injected directly into the thymus may interfere with tolerance leading to autoantibodies. Certainly, the concentration of the reactive metabolite that was injected is likely to be directly cytotoxic but would not occur in vivo.

An alternative theory on the pathogenesis of autoimmunity has been proposed by Richardson et al. [188], He reasons that inhibition of DNA methylation in mature T cells can result in autoimmunity. Hypomethylated DNA sequences have been shown to correlate with gene expression [189], and it is thought that autoreactivity is a result of altered gene expression. PA has been shown to inhibit T cell DNA methylation and induce autoreactivity in cloned human CD4 T cells [190]. Mouse CD4 T cells also become autoreactive after treatment with PA, and when these cells were adoptively transferred into naïve syngeneic recipients a lupus-like disease ensued including immune complex nephritis and IgG antibodies against DNA and histones [191]. Hypomethylation of T cell DNA has been found to result in the over

expression of the adhesion molecule LFA-1, and this over expression has been found to be sufficient to cause autoimmunity [192]. Since there are several characteristics in the DNA hypomethylation model that reflect the characteristics of idiopathic lupus in humans [193], this model may play an important role in our understanding of drug-induced autoimmune reactions.

In Table (4-3-1) the new findings were compared with what has been done up to now in primary PLNA with PA

Table 4-3-1

	PA	
	Literature	**New findings**
PLN weight	No changes compare to control group [194, 195]	No changes compared to control group
PLN cell count	Has not been done to date	No changes compared to control group
Cytokine secretion	Has not been done to date	RANTES↑ (insignificant)
Flow cytometry	Has not been done to date	No changes in any cell marker
Gene expression profiling	Has not been done to date	No changes in any parameters

In Table (4-3-2) the new findings were compared with what has been done up to now in RA-PLNA (TNP-OVA) with PA

Table 4-3-2

	PA	
	Literature	**New findings**
PLN weight	Has not been done to date	↑
PLN cell count	↑ [143]	No changes compared to control group
Cytokine Secretion	Has not been done to date	No change in IL-2, IL-6, IL-12 & RANTES
Immunoglobulines	TNP-OVA specific IgM↑ [143]	No changes in any TNP-OVA specific Igs
Flow Cytometry	Has not been done to date	No changes in any cell marker

In Table (4-3-3) the new findings were compared with what has been done up to now in RA-PLNA (TNP-Ficoll) with PA.

Table 4-3-3

	PA	
	Literature	**New findings**
PLN weight	Has not been done to date	No changes compared to control group
PLN cell count	No changes compared to control group [143]	No changes compared to control group
Cytokine secretion	Has not been done to date	No change in IL-2, IL-6, IL-12 & RANTES
Immunoglobulines	TNP-Ficoll specific IgM↑ [143]	No changes in any TNP-Ficoll specific Igs
Flow cytometry	Has not been done to date	No changes in any cell marker

Previous works with PA showed that activated peripheral blood neutrophils have capacity to metabolize PA to PA-hydroxylamine (PAHA). Drug metabolism by neutrophils occurs in the extracellular environment, in which released myloperoxidase, acting with hydrogen peroxide produced upon neutrophil activation, enzymatically oxidizes present drugs [196].

All drugs that are suspected to induce lupus have the capacity to undergo transformation to reactive products by exposure to activated neutrophils. There is a general correlation between susceptibility to neutrophil-mediated transformation and propensity to induce lupus [196]. Chemical analogs of each drug such as the non-lupus inducing analog of PA were resistant to metabolic transformation by this mechanism [196].

Metabolism of drugs by activated neutrophils provides a mechanism for generating highly reactive metabolites directly within lymphoid tissue, where autoimmunity presumably develops. Because neutrophils are present in high concentration in the circulation, local production of reactive metabolites from certain compounds like PA can potentially occur in any lymphoid compartment where neutrophils have access. This process would minimize dilution, time-dependent hydrolysis, and circulatory distribution of reactive metabolites, thereby maximizing their biologic effect on the tolerance status of lymphocytes.

The question then remains why PA is not able to induce immune response in a local assay like PLNA/RA-PLNA. One possibility to overcome this problem was to look for a broader range of immune parameters in PLNA and RA-PLNA.

In primary PLNA, no changes in any immunological parameter that was measured after PA treatment either on RNA level or on protein level was detected.
In the next step PA was combined with RAs (TNP-OVA or TNP-Ficoll) to test if RA can trigger the immune response and make small, borderline effects visible.
None of the RAs could be helpful to detect PA-induced immune response in RA-PLNA.
Looking at a broad range and more sensitive immunological parameters, like different cell markers, various cytokines, TNP-specific immunoglobulins or gene expression profiling was not helpful to detect any PA-induced immune response in none of our local assays (PLNA/RA-PLNA).
Lack of metabolism and not having enough metabolic components (HAPA) in case of PA is a drawback for the local assay, PLNA/RA-PLNA. To overcome the metabolism issue Katsutani et al. tried to inject an ultrafiltered mixture of 10 mg PA (5 parts) and S-9 (1 part) in to the foot pad of mice. The results of this attempt showed enlargement of PLN. The enlargement of PLN was observed as follows in the three different mouse strains, C3H/He>C57BL76>BALB/c [195]. The interpretation of this finding is compromised due to the likely presence of potentially immunogenic peptides derived from the ultrafiltrate S-9 mixture.
To overcome the problem of lack of metabolism in the local assays and investigate further in PA-induced Ides, PA should be given systemically namely orally to the animals. Therefore, PA could be one of the prior compounds that should be tested in the newly established oral mouse model (10-days model) to test if we could detect any systemic drug induced immune response by oral administration of PA.

4.4 Streptozotocin (STZ)

4.4.1 The Mechanism of Streptozotocin Action

STZ is used to induce diabetes mellitus in rats [197]. STZ is taken up by pancreatic B cells via glucose transporter GLUT2. A reduced expression of GLUT2 has been found to prevent the diabetogenic action of STZ [198]. It was also observed that STZ itself restricts GLUT2 expression in vivo and in vitro when administered in multiple doses. Intracellular action of STZ results in changes of DNA in pancreatic B cells comprising its fragmentation [199]. Recent experiments have proved that the main reason for the STZ-induced B cell death is

alkylation of DNA [200]. The alkylating activity of STZ is related to its nitrosourea moiety, especially at the O_6 position of guanine. After STZ injection to rats, different methylated purines were found in tissues of these animals [201]. Since STZ is a nitric oxide (NO) donor and NO was found to bring about the destruction of pancreatic islet cells, it was proposed that this molecule contributes to STZ-induced DNA damage [202]. The participation of NO in the cytotoxic effect of STZ was confirmed in several experiments [202].

However, the results of other additional experiments provide the evidence that NO is not the only molecule responsible for the cytotoxic effect of STZ. STZ was found to generate reactive oxygen species, which also contribute to DNA fragmentation and evoke other deleterious changes in the cells [203].

These effects strongly limit mitochondrial ATP production and cause depletion of this nucleotide in B cells [204]. It can be stated that potent alkylating properties of STZ are the main reason of its toxicity. However, the synergistic action of both NO and reactive oxygen species may also contribute to DNA fragmentation and other deleterious changes caused by STZ. STZ-induced DNA damage activates poly ADP-ribosylation [205]. This process leads to depletion of cellular NAD+, further reduction of the ATP content [206] and subsequent inhibition of insulin synthesis and secretion [207]. The concept of unfavorable consequences of augmented poly ADP-ribosylation as a result of STZ action was confirmed by experiments revealing that the inhibition of this process prevents the toxicity of this diabetogenic agent. It was found that 3-aminobenzamide, a strong inhibitor of poly(ADP-ribose) syntheses, protected against the action of STZ in rats [208]. recent investigations in poly (ADP-ribose) polymerase deficient mice demonstrated that the inhibition of poly ADP-ribosylation itself prevents STZ-induced B cell damage and hyperglycemia [209]. Thus, it can be concluded that the activation of poly ADP-ribosylation is of greater importance for the diabetogenicity of STZ than generation of free radicals and DNA damage.

In Table (4-4-1) the new findings were compared with what has been done up to now in primary PLNA with STZ

Table 4-4-1

	STZ	
	Literature	**New findings**
PLN weight	↑↑[210]	↑↑
PLN cell count	↑↑[211]	↑↑
Cytokine secretion	Has not been done to date	IL-2, 6, 12 & RANTES↑↑
Flow cytometry	APC, T & B cells, CD25+&CD69+ IFN-γ, IL-2↑[211]	Total Nr of T & B cells↑, CD25+&CD69+ Tcells↑, CD86+ Bcells↑
Gene expression profiling	IFN-γ mRNA↑, no changes in IL-4 mRNA[212]	IL-1α↑, IL-2↑, IL-6↑, IL-12↑, IFN-γ↑, TNF-α↑, cytotoxic T cells & cell division mRNA ↑

In Table (4-4-2) the new findings were compared with what has been done up to now in RA-PLNA (TNP-OVA) with STZ

Table 4-4-2

	STZ	
	Literature	**New findings**
PLN weight	Has not been done to date	↑
PLN cell count	↑ [143]	No changes compare to control group
Cytokine Secretion	TNF-α, IFN-γ levels↑ [10]	IL-6, IL-12, RANTES↑, no changes in IL-2
Immunoglobulines	IgM, IgG2a↑ [143]	IgG2a↑
Flow Cytometry	Total Nr of T cells, %CD25+ and CD69+ T cells↑. Total Nr of B cells and % CD80+ & CD86+B cells↑. % CD11c, F4/80 & CTL-4↑ [10]	Total Nr of CD8+T cells, %CD25+ and CD69+ T cells↑. % CD86+B cells↑.

In Table (4-4-3) the new findings were compared with what has been done up to now in RA-PLNA (TNP-Ficoll) with STZ.

Table 4-4-3

	STZ

	Literature	New findings
PLN weight	Has not been done to date	↑
PLN cell count	↑ [136]	↑
Cytokine secretion	IL-1, IL-4, IFN-γ, TNF-α↑ [136]	IL-6, IL-12, RANTES↑. No change in IL-2
Immunoglobulines	IgM, IgG1↑ [136]	IgM, IgG1 & IgG2a↑
Flow cytometry	Total Nr of T , B and % F4/80 cells↑ [136]	Total Nr of T cells and %CD25+ or CD69+Tcells↑. %CD86+B cells↑

In agreement with other studies the results from this work showed that PLN responses in primary and RA-PLNA induced by STZ were characterized by a decrease in CD4+ T cell percentages and an increase in the frequencies of CD8+ T cells and macrophages. In addition, significantly higher amounts of IL-6, IL-12 and RANTES were produced by cells from STZ-treated mice when compared to PLN cells from mice exposed to PBS. Both cytokines may be produced by macrophages and APC during the innate phase of the response, whereas RANTES may also be produced by NK (T) cells, or activated CD8+ T cells. Increased production of IL-12 during the first days after exposure may be an important marker for type 1-modulating properties of chemicals because this cytokine stimulates T cells to differentiate into Th1 cells [213].

In STZ-induced responses, there is an increase in B cells expression of activation markers CD80 and CD86. Increased levels of IgG2a ASC were found on day 7, which is indicative of type 1 responses. In summary, the production of IL-12 and RANTES and the frequency of CD8+ T-cells and macrophages may be used to differentiate the STZ induced Th1 responses from the Th2 response that was induced by D-Pen.

STZ is a strong alkylating compound [214] and gives rise to oxidative products [215]. STZ, or its metabolites, might directly, or indirectly by induction of PLN tissue damage, activate macrophages. Alternatively, STZ treatment might directly stimulate Th1 cytokines secretion by TCTL cells, which in turn attract and activate macrophages. Both TCTL lymphocytes and macrophages seem to be important cells for the immunogenic effect of STZ in the PLNA.

In line with its diabetogenic effect, STZ lead to increase in characteristic type 1 cytokine mRNA expression of IL-1α, IL-2, IL-6, IL-12, IFN-γ, TNF-α and also cytotoxic T cells in the primary PLNA in this thesis. In the primary PLNA study, the significant enhancement of IL-6 mRNAs production after STZ treatment was perhaps the reflection of inflammation related to the increase in PLN weight and cellularity, as was the increased production of IL-1α,
and TNF- α. The other cytokines of the acute phase have also been detected at significant mRNAs levels at day 7 after STZ treatment. TNF- α was present in large amounts. Recently this cytokine was evidenced to be crucial for the development of autoimmune diabetes. Indeed, diabetic mice lacking the TNF- α receptor on pancreatic islets were protected from the occurrence of diabetes [216]. Cytokines with inflammatory properties: e.g. IL-1 α, IL-6 and TNF- α may lead to activation of antigen-presenting cells [217] and thus efficient co-stimulation of T cell favoring the immune response against the xenobiotic or modified self antigens. Using mRNA extraction and microarray analyses in primary PLNA as an additional marker was helpful to elucidate the role of various immune cells in mediating idiosyncratic reactions. Microarray analyses provided us with much needed understanding of immune roles of drug-induced IDRs. Although potentially difficult, isolation of cells and analyses of their gene expression during the early stages of drug treatment allowed studying the initiating steps involved in propagation of immune response. Nevertheless, the mRNA extraction itself can induce gene expression changes in the proteins that can furthermore complicating our analyses and introducing errors in the conclusions that one can draw from these studies.

4.5 Diphenylhydantoin (DPH)

4.5.1 The Mechanism of Diphenylhydantoin Action

Various adverse reactions, e.g. lymphoadenopathy, toxic epidermal necrolysis and systemic lupus erythematosus (SLE) can develop in patients upon administration of the anticonvulsant drug DPH [218].

The DPH-induced immunopathological symptoms are strikingly similar to those which can develop graft-versus host reactions (GVHR) in animal models and man [218]. Based on these findings it has been proposed that a GVH-like abnormal T-B cell cooperation might be triggered in autologous systems by drugs such as DPH. It has been postulated that binding of

DPH, or its metabolites, to membrane of B lymphocytes and/or antigen-presenting cells might result in stimulating autologous T cells. The stimulated Th cells, in turn, would deliver proliferative and activating signals to B cells, including the normally existing autoreactive B cells. Initial results obtained from PLNA in mice were consistent with this concept [219].

In Table (4-5-1) new findings were compared with what has been done up to now in primary PLNA with DPH

Table 4-5-1

	DPH	
	Literature	**New findings**
PLN weight	↑[220]	↑
PLN cell count	↑[220]	↑
Cytokine secretion	Has not been done to date	No changes in IL-2, 6, 12 & RANTES
Flow cytometry	Has not been done to date	Total Nr of T & B cells↑, CD25+&CD69+ Tcells↑
Gene expression profiling	Has not been done to date	No changes in mRNA expression of any cytokines

Gleichmann et al. reported that s.c. injection of DPH into the foot pads of mice led to a T cell-dependent enlargement of the draining PLN [221]. The data presented in this thesis from primary PLNA further strengthens this concept.

Gleichmann et al. proposed that there are two mechanisms contributing to the DPH-induced PLN enlargement, lymphocyte proliferation and trapping of lymphocytes from the circulation. The major portion of the PLN lymphadenopathy was due to proliferation, whereas trapping appears to be a secondary event resulting from a longer recirculation pathway of lymphocytes in the enlarged PLN [222]. These observations and conclusions are identical with those made with the PLN enlargement caused by the genuine GVHR [223].

Although T lymphocytes were required for the DPH-induced lymphadenopathy, B cells comprise the major population proliferating and differentiating in these nodes [221]. DPH induced a tremendous increase of the total number of IgM- and especially IgG1-secreting cells in the draining PLN [10]. The DPH-induced functional activation of B cells required the presence of T lymphocytes. Presumably, this functional activation of B cells was caused by T

cells reacting to DPH, or its metabolites, bound to B cells or antigen-presenting cells in the draining PLN [221].

There are two pharmacologic properties that might enable DPH to induce the T cell dependent lymphadenopathy. DPH may bind to lymphoid cells either directly, in unchanged form, or through its metabolites. Because DPH belongs to the class of lipid soluble drugs [224], it is expected to be incorporated into the lipid bilayer of cell membranes and creates new membrane determinants. Alternatively arene oxide metabolites of DPH might be involved in the DPH-induced lymphadenopathy. In studies on the metabolism of DPH it has been found that the biotransformation of DPH by cytochrome P450, a microsomal enzyme, to its metabolites involves the formation of highly reactive arene oxide intermediates. It is conceivable that DPH, after insertion into the cell membrane of lymphocytes, may be oxidized by these cells because lymphocytes have also the cytochrome P450 system. Covalent binding of the reactive metabolite to membrane constituents of lymphoid cells may provide stable structures able to stimulate autologous T cells comparable to the effect of allogeneic structures. The finding that injection of hydantoin only, i.e. a compound lacking the two phenyl groups, failed to induce any PLN reactivity, supports the assumption that the PLN reactions are caused by the phenyl groups or the highly reactive intermediates of DPH.

Interestingly, injection of phenobarbital failed to induce PLN reactions. Phenobarbital was chosen for comparison because it is chemically and pharmacologically related to DPH, but is not known to cause significant immunopathological symptoms. The assumption is that phenobarbital, which is water-soluble, can not bind to lymphoid cells in the same manner as the hydrophobic compound DPH and thus fails to cause non-self structures capable of stimulating autologous T cells.

4.6 General Discussion

Discovering novel animal models of drug-induced idiosyncratic reactions allows determining the mechanisms of idiosyncratic reactions.

The PLNA provides a straightforward and simple tool to assess chemical induced immunostimulation effects. In addition, the measurement of new immunological parameters like cell markers, cytokines and gene expression profiling provides better understanding of the initiating mechanism of IDRs in the reference compounds; D-Pen, DPH and STZ. The RA-approach is particularly suitable to test drugs that lead to induce subtle

immunostimmulatory response e.g. NVP. In this case although the differences in affected immunological parameters induced by compounds like NVP are relatively small, having an additional antigen like TNP-OVA allowed detecting borderline effects of the drug that can not be detected by primary PLNA.

The RA-PLNA with TNP-OVA was used to assess the adjuvant activity of NVP. As the response was positive the model with TNP-Ficoll was used to assess whether the drug elicits the activation of neoepitope specific T cells.

The drawback of RA-PLNA was the detection of negative responses (like in PA case) when testing compounds that possibly require metabolism to become reactive. Therefore it was essential to develop oral models to assess the actual hazard of drug induced systemic sensitization.

Findings with NVP from Nierkens et al. attempts to establish an oral mouse model indicates that oral administration increased DTH responsiveness but failed to increase TNP-specific immunoglobulin in serum [9].

Preliminary studies in two different strains of mice have also been performed with NVP, based on the suggested oral model by Nierkens in this thesis. The results of this preliminary experiment showed signs of an immune response onset which were not significant and also not supported by increase in other immune markers. One of the explanations for this unclear response was that the doses, which are used for immunizing and challenging animals with TNP-OVA i.p. and s.c., respectively are too high, so the effect of the drug could be masked by the response which is caused by reporter antigen. In addition, the use of higher doses of TNP-OVA is also able to induce specific T-cell memory. Therefore, dose and treatment regimens were optimized to allow the induction of sensitization rather than the induction of tolerance. Data from optimized 28 days oral mouse model did not show any significant change in any immunological parameters that was measured. Neither organ weight and cellularity nor expression of different T and B cell markers nor induction of cytokines in spleen and draining lymph nodes indicated any sort of immune response. In addition no TNP-specific immunoglobulin either in splenocytes or in lymphocytes with D-Pen and NVP was detected. Therefore, the next attempt was to test D-Pen and NVP in the newly established 10 days oral mouse model. Data from the oral treatment of NVP in the newly designed oral mouse model showed a significant increase in spleen and auricular lymph node weight with the highest

concentration of NVP either in the presence or in the absence of the RA, however presence of TNP-OVA lead to increased response in the draining lymph node.

A significant increase in different cell markers and also cytokines was observed with 600 mg/kg NVP in the spleen. The B cell activation in spleen was confirmed by Isotype switching data from IgM to IgG1 and IgG2a. The significant increase in IgG1 and IgG2a after 600 mg/kg NVP was a novel finding in terms of observing a TNP-specific systemically immune response. This latter finding reinforced the need for having a RA on board in an oral/systemic model to gain more clue regarding mechanistic pathways that could be involved in drug induced hypersensitivity reactions.

These recent results suggest that the RA approach is useful in assessing sensitizing potential following oral drug exposure. However, much more research is required before an oral exposure RA-models can be used as a predictive model.

In case of D-Pen the systemic TNP-specific immune response was not as pronounced as in case of NVP. Tolerance may be the most common response in this case. Once the mechanisms of tolerance induction will be better understood, it would be possible to inhibit tolerance and potentially develop more animal models of drug-induced idiosyncratic reactions. Inhibition of enzyme induction in case of metabolic tolerance or activity of suppressor cells (i.e. CD4+CD25+) in immune tolerance may enable us to turn the tolerance switch off in resistant animal strains thus producing animals more susceptible to develop idiosyncratic drug reactions. Nevertheless, most of these animal models are far from perfect, and none has the ability to predict patient susceptibility to develop idiosyncratic reaction to a drug. Although it is likely impossible that an animal model will ever developed, which will be able to predict all types of drug-induced organ toxicities, one should strive to develop new valid animal models, which would allow performing additional mechanistic investigations in the field of idiosyncratic drug reactions. The research in the field of adverse reactions is around a half century old, it is still facing problems of understanding how and why idiosyncratic reactions occur in patients. The goal is with the development of new experimental approaches, to be able to ask more probing questions that will bring additional mechanistic understanding of idiosyncratic drug reactions. Likewise, discovery and use of additional animal models are required for further studies of the mechanisms of idiosyncratic drug reactions. Understanding the mechanism of one drug-induced idiosyncratic reaction does not mean that the mechanisms of all idiosyncratic drug reactions are understood, because there appear to be many different

mechanisms of these reactions. However, there may be common mechanistic features that will allow the production of better and safer medicines for patients.

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