INVESTIGATION OF THE MECHANISM OF
IDIOSYNCRATIC DRUG-INDUCED LIVER INJURY

by

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A thesis submitted in conformity with the requirements for the degree of

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ABSTRACT

Idiosyncratic drug reactions represent a special problem because of their unpredictable nature, and idiosyncratic drug-induced liver injury (IDILI) is a major reason for drug withdrawal. Our objective was to investigate the mechanism of IDILI caused by isoniazid and amodiaquine. Isoniazid-induced liver injury was believed to be due to bioactivation of N-acetylhydrazine, a metabolite of isoniazid; however, that conclusion was based on a rat model with characteristics very different from isoniazid-induced IDILI in patients. I found that isoniazid is directly bioactivated and covalently binds to hepatic proteins; furthermore, mice are a better model for human isoniazid metabolism than rats. We found that mild isoniazid-induced liver injury in patients is associated with an increase in Th17 cells and IL-10-producing T cells. I also found anti-isoniazid antibodies in the serum of patients with isoniazid-induced liver failure. These results suggest that isoniazid-induced IDILI is immune-mediated rather than metabolic idiosyncrasy as previously believed. Treatment of mice with isoniazid failed to lead to significant liver injury. We postulated that this was because of immune tolerance; however, attempts to develop an animal model using mice with impaired immune tolerance were unsuccessful. Treatment of mice with amodiaquine resulted in mild liver injury that resolved despite continued treatment similar to the more common type of IDILI observed in humans. In
this model, liver injury is immune-mediated with the initial injury mediated by natural killer cells, while the resolution of liver injury appears to involve the adaptive immune system because resolution was slower in Rag-/- mice. In summary, my studies have resulted a change in what is considered to be the mechanism of isoniazid-induced IDILI. We have developed an animal model of mild immune-mediated amodiaquine-induced liver injury, but we failed to develop a valid animal model of severe IDILI, most likely because the dominant immune response in the liver is tolerance.
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SUMMARY OF ABBREVIATIONS

- AcINH – acetylisoniazid
- AcHz – acetylhydrazine
- ADR – adverse drug reactions
- ALFSG – acute liver failure study group
- ALT – alanine aminotransferase
- AMA – anti-mitochondrial antibodies
- ANA – antinuclear antibodies
- APAP – acetaminophen
- APC – antigen presenting cell
- AQ – amodiaquine
- AQQI – amodiaquine quinine imine
- ASMA – anti-smooth muscle antibodies
- AST – aspartate aminotransferase
- BCG – bacillus Calmette-Guerin
- BN – brown Norway
- BSA – bovine serum albumin
- CARD – caspase recruitment domain
- Cbl-b-/– casitas B cell lymphoma-b knockout
- CFA – complete Freund’s adjuvant
- CNS – central nervous system
- CYP – cytochrome P450
- CYP2E1-INH – CYP2E1 chemically modified to mimic covalent binding by the reactive metabolite of INH
- DAMP – damage associated molecular pattern
- DEAQ – N-desethylamodiaquine
- DILI – drug induced liver injury
- EAH – experimental autoimmune hepatitis
- ELISA – enzyme-linked immunosorbent assay
- ESI-MS – Electron spray ionization mass spectrometry
- FACS – flow cytometry
- FICZ – 6 formylindolo(3,2-b)carbazole
- GLDH – glutamate dehydrogenase
- H&E – hematoxylin and eosin
- HLA – human leukocyte antigen
- HLM – human liver microsomes
- HBV – hepatitis B virus
- Hz – hydrazine
- IDILI – idiosyncratic drug-induced liver injury
- IDR – Idiosyncratic drug reaction
- IFA – incomplete freund’s adjuvant
- IFN – interferon
- IgG – Immunoglobulin G
- IHC – immunohistochemistry
• IL-10 – Interleukin 10
• INA – isonicotinic acid
• INA-BSA – isonicotinic acid coupled to bovine serum albumin
• INA-INH – isonicotinic acid coupled to isoniazid
• INA-NAL – isonicotinic acid coupled to N-α-acetyl-l-lysine
• INA-NHS – reactive N-hydroxysuccinimide ester of isonicotinic acid
• INH – isoniazid
• INR – international normalized ratio
• IRF – interferon regulatory factor
• ITAM – immunoreceptor tyrosine-based activation motif
• ITIM – immunoreceptor tyrosine-based inhibition motif
• KIR – killer cell immunoglobulin-like receptor
• KLR – killer lectin-like receptor
• L – lysozyme
• LC-MS – liquid chromatography coupled to mass spectrometry
• L-INH – lysozyme chemically modified to mimic binding by the reactive metabolite of INH
• LKM – anti-liver/kidney microsome antibody
• LKR – leukocyte receptor complex
• LTT – lymphocyte transformation test
• LPS – lipopolysaccharide
• LRC – leukocyte receptor complex
• MAL – MyD88 adaptor like
• MAPK – mitogen-associated protein kinases
• MHC – major histocompatibility complex
• MLM – mouse liver microsomes
• MyD88 – myeloid differentiatiation factor 88
• NAL – N-α-acetyl-l-lysine
• NALP – Nod-like receptors with a purine domain
• NAT1/2−− – N-acetyltransferase 1 and 2 knockout
• NFκB – nuclear factor kappa B
• NK cells – natural killer cells
• NKT cells – natural killer T cells
• NK1.1 – Natural killer 1.1 surface marker
• NLR – Nod-like receptor
• NOD – nucleotide-binding oligomerization domain
• NHS – N-hydroxysuccinimide
• PAMP – pathogen associated molecular pattern
• PBMC – peripheral blood mononuclear cell
• PI hypothesis – pharmacological interaction hypothesis
• RLM – rat liver microsomes
• SDH – sorbitol dehydrogenase
• S9 – supernatant of liver proteins after 9,000g centrifugation
• S9-INH – supernatant of liver proteins after 9,000g centrifugation and after reaction with an activated ester of isonicotinic acid
• S100 – supernatant of a liver homogenate after 100,000g centrifugation
• S100-INA – supernatant of a liver homogenate after 100,000g centrifugation and after reaction with an activated ester of isonicotinic acid
• TB – tuberculosis
• TBST – tris-buffered saline with Tween
• TCR – T cell receptor
• Th-17 cells – T helper 17 cells
• TIR – Toll-IL-1 receptor
• TLR – toll like receptor
• TRAM – TRIF-related adaptor molecule
• TRIF – TIR domain-containing adaptor-including IFN-β
• TMB – 3,3′,5,5′-tetramethylbenzidine
• T_{reg} cells – regulatory T cells
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CHAPTER 1: INTRODUCTION

STATEMENT OF RESEARCH PROBLEM

The two most important reasons for a drug candidate failure are lack of efficacy and adverse reactions. In particular, idiosyncratic drug reactions (IDRs) are very difficult to deal with because they are rare and unpredictable. In addition, IDRs represent a significant cause of patient morbidity and mortality and add to the cost of drug development. The pharmaceutical industry has tried to develop in vitro assays to screen for drugs that might cause an adverse event; however, this has not been successful, and given the complexity of the mechanism, this is not surprising. To date, there is no clear understanding of how and why these reactions occur. However, most of the evidence suggests that in most cases, reactive metabolites are responsible, and the IDRs are immune-mediated. Two hypothesis used to explain how reactive metabolites can lead to an immune response that results in an IDR are the hapten hypothesis and the danger hypothesis. However, the exact steps of how this immune reaction occurs and why it only affects some individuals is currently unknown. In addition not everyone accepts the hypothesis that IDRs are immune-mediated.

The three most common targets of IDRs are the skin, liver, and blood cells. For drugs such as isoniazid that can cause idiosyncratic drug-induced liver injury, it is still believed that liver injury is not immune-mediated, rather it has been called “metabolic idiosyncrasy”. In addition, without a clear understanding of the mechanistic steps involved in such reactions, it is unlikely that much progress will be made in this field.

Animal models represent an invaluable tool in which controlled experiments can be used to rigorously test mechanistic hypotheses. Such experiments would be impossible to perform in humans. However, in order to be able to translate such findings to humans, it is
absolutely crucial that the animal model have characteristics similar to the idiosyncratic reaction that occurs in humans and also have a high incidence that would make studies practical. However, such models have been extremely difficult to develop, especially models of liver injury, and a main reason is that these reactions are just as idiosyncratic in animals as they are in humans. Therefore, given the difficulty of studying such mechanisms in humans, it is unlikely that much progress will be made in this field without valid animal models.
PURPOSE OF THE STUDY AND OBJECTIVES

The purpose of this study was to understand the mechanism of idiosyncratic drug-induced liver injury (IDILI). The specific objective was to study the immune response to isoniazid (INH) in humans, and with this information to then develop an animal model of IDILI with similar characteristics to the IDILI that occurs in humans; this animal model would provide an essential tool to test mechanistic hypothesis.

STATEMENT OF RESEARCH HYPOTHESES AND RATIONALE FOR HYPOTHESES

1) INH itself is oxidised directly to a reactive metabolite, and this binds to liver macromolecules and is responsible for initiating an immune response and liver injury.

**Rationale:** INH-induced IDILI was believed to be due to bioactivation of acetylhydrazine (AcHz), a metabolite of INH. However, INH also has a hydrazine group; therefore, the chemistry of INH and the AcHz metabolite should be similar.

2) Idiosyncratic INH-induced liver injury is immune-mediated, and mild cases of liver injury with adaptation (IDILI which resolves despite continued INH treatment) represent immune tolerance.

**Rationale:** There are examples in the clinic where patients that were re-challenged with INH have fever, rash, and an eosinophilic infiltrate in the liver. In addition, positive lymphocyte transformation tests have been reported in patients receiving INH. This implies an immune response against INH.

3) The major barrier to the development of an animal model of IDILI is immune tolerance.

**Rationale:** The easiest way to explain the idiosyncratic nature of IDR is through the variability in the immune response. If the injury is immune-mediated but most cases of IDILI resolve despite continued treatment, this resolution must involve immune tolerance.
1.0: ADVERSE DRUG REACTIONS

Adverse Drug Reactions (ADRs) have been reported to represent the 4th leading cause of death in North America. In general, an ADR refers to a harmful or unpleasant reaction resulting from the normal use of a medication. ADRs have been classified into six types: 1) Augmented (dose-related); 2) Bizarre (incorrectly termed dose-independent); 3) Chronic (dose-related and time related); 4) Delayed effects; 5) End of use (withdrawal) and 6) Failure (failure of therapy).

1) The augmented type of ADRs (also called type A) are the easiest to deal with. These reactions are quite common, and they are usually related to the therapeutic effect of the drug. Type A reactions will occur in most patients at a high dose of a drug, but the dose required to cause such reactions varies from individual to individual. It is often - but not always - possible to individualize the dose so that the desired therapeutic effect can be achieved without serious toxicity. An example is digoxin, which can result in a variety of reactions such as dizziness, insomnia, agitation, depression, and a variety of arrhythmias, which can be fatal.

2) The bizarre reactions (also called type B) are the most difficult to deal with. These reactions are also called idiosyncratic, and this is the type of reaction that this thesis will focus on. Type B reactions are uncommon, not related to the pharmacological effect of the drug, unpredictable, and associated with high mortality. They are often referred to as dose-independent; however, everything is dose-dependent. What is true is that most patients will not have a type B reaction at any dose, but it is also true that a dose can always be found below which no one will have a type B reaction. In fact there is a correlation, although certainly not perfect, between the daily dose of a drug and the risk that it will cause type B reactions, and it is rare for a drug given at a dose of less
than 10 mg/day to cause type B reactions as discussed later. Examples include: penicillin hypersensitivity, clozapine-induced agranulocytosis, and isoniazid/amodiaquine-induced liver injury. The best way to deal with these reactions is to withhold the drug and avoid future rechallenge, but in some cases the reaction progresses after the drug is stopped.

3) The chronic type of ADRs (also known as type C) are also uncommon and usually manifest themselves after prolonged treatment with the drug, which results in a cumulative effect. An example is adrenal suppression caused by corticosteroids. In general, these types of reactions can be avoided by reducing the dose and preventing accumulation of drug or its effects.

4) Time-related ADRs (type D) occur sometime after the introduction of the drug. They are uncommon and usually dose-related. Drugs used are usually in the class of teratogens or carcinogens such as vaginal adenocarcinoma with diethylstilbestrol.

5) The withdrawal type of ADRs (type E) occur after the drug has been withdrawn. An example is myocardial ischemia seen after the withdrawal of β-blockers. To avoid these reactions, often the drug is reintroduced or withdrawn slowly.

6) The therapeutic failure type of ADRs (type F) are quite common, related to the dose of the drug and often are caused by drug interactions. An example is pregnancy that results from an interaction between oral contraceptives and agents that induce metabolic enzymes in the liver.
1.1: IDIOSYNCRATIC DRUG REACTIONS

As mentioned above, the term idiosyncratic drug reaction refers to an adverse drug reaction that does not occur in most patients and does not involve the therapeutic effects of the drug. These reactions are specific to an individual and do not occur in most people within the range of therapeutic doses used clinically. IDRs (type B reactions) are also commonly referred to as hypersensitivity or allergic reactions. Often, IDRs can manifest themselves as liver injury, blood dyscrasias, or severe skin rashes.

Recently, a study conducted in United Kingdom found that adverse drug reactions are responsible for more than 6% of hospital admissions, but only around 5% of these adverse reactions were idiosyncratic. Other studies have found a higher incidence of idiosyncratic adverse reactions. Even though IDRs are responsible for a smaller percentage of hospitalization admissions, they are a major issue for drug development because they are unpredictable and can be quite serious, sometimes fatal. Due to the low incidence of an IDR and the insufficient number of subjects in clinical trials, IDRs usually go undetected until the drug is marketed. This means that a drug that could potentially cause an IDR could be approved, and only when the drug is launched onto the market will there be a sufficient number of patients exposed to detect unusual reactions to the drug. If one in one thousand patients do indeed develop a serious IDR, the drug is likely to be withdrawn; this would cost billions of dollars to the pharmaceutical company involved. From 1975 to 2000, about 10% of the approved drugs had to be withdrawn or achieved a “black box” warning due to an adverse drug reaction that was not detected during clinical trials. Therefore, there is great interest in trying to understand why these IDRs occur. In the future it would be beneficial to pharmaceutical companies and society if we could predict the risk of such reactions and deal with possible “signals” of IDRs during clinical trials and post marketing surveillance. To date not much
progress has been made in this field, and the inability to predict IDRs continues to result in significant patient morbidity as well as increased costs and uncertainty of drug development.

1.1.1: CLINICAL CHARACTERISTICS OF IDRs

An important characteristic of IDRs is that there is almost always a delay in the onset of the reaction;\textsuperscript{5} and this varies with the type of IDR and the drug used. For example, skin rashes tend to occur within a few weeks, liver injury occurs between 1 – 6 months, and autoimmune reactions can take more than a year. When the drug is stopped the IDR usually resolves, but there are exceptions where IDRs occur even after stopping the drug. If the drug is reintroduced there is usually a faster onset of the IDR compared to the first time the drug was administered,\textsuperscript{6} but there are exceptions to that as well. For example, the time to onset of clozapine-induced agranulocytosis is often as long upon rechallenge as it was with the initial exposure,\textsuperscript{7} and sometimes the IDR does not occur on rechallenge.\textsuperscript{8,9} On the other hand, there are cases, as with some cases of aplastic anemia and liver injury, where even after stopping the drug the patient progresses to liver failure or death. One of the most intriguing characteristics of IDRs is that the IDR will often resolve despite continued treatment with the drug. Most clinicians refer to this resolution as adaptation; however, there is evidence that the immune system is involved; therefore, if the reaction is immune-mediated the resolution must involve immune tolerance. Drug-induced skin rashes are often associated with an infiltration of lymphocytes and there are also usually other features of an immune response; therefore, there is not much scepticism that they are likely immune-mediated.\textsuperscript{10} However, because the liver is commonly involved in bioactivation of xenobiotics, there is controversy about whether liver injury is immune mediated.\textsuperscript{11} An alternative hypothesis is that cumulative binding of reactive metabolites could
be what induces liver injury; this has given the rise to the term “metabolic idiosyncrasy” and will be investigated throughout this thesis.

1.1.2: GENETIC ASSOCIATIONS AND IDR}s

There have been efforts to find genetic variants that can explain the idiosyncratic nature of IDR}s. In the case of isoniazid (INH)-induced liver injury, slow acetylators have been shown to be at increased risk relative to rapid acetylators, but the increase in risk is just above two fold;\textsuperscript{12} in addition, half of the population are slow acetylators, but yet most patients who are treated with INH do not develop significant liver injury. Therefore, acetylator phenotype does not explain the idiosyncratic nature of INH-induced liver injury. The strongest genetic associations with IDR}s are specific human leukocyte antigen (HLA) associations, and this supports an immune mechanism. A strong association between carbamazepine-induced Stevens-Johnson syndrome and HLA-B*1502 has been found, but only in Han Chinese.\textsuperscript{13} Similarly, strong associations have also been found with other drugs such as: severe hypersensitivity to abacavir and the presence of HLA-B*5701,\textsuperscript{14} severe reactions to allopurinol and the presence of HLA-B*5801,\textsuperscript{15} and flucloxacillin hepatotoxicity and the presence of HLA-B*5701.\textsuperscript{16} These associations provide evidence that these IDR}s are immune-mediated; presumably, patients with the appropriate HLA can more effectively present drug-modified peptides to T cells, and this leads to an immune response. However, most patients with the required HLA who are treated with the drug will not develop an IDR to that drug. It appears that in order to develop the immune response, the correct TCR is also required. It has been shown that for carbamazepine-induced Stevens-Johnson Syndrome, in addition to carrying the HLA-B*1502 genotype, another risk factor is a specific peptide sequence in the T cell receptor.\textsuperscript{17} These data strongly support an immune mechanism for these IDR}s, and that
recognition of antigens produced by reactive metabolites and presented by antigen presenting cells (APCs) also require a specific TCR in order to mount a full immune response.

1.2 : THE ROLE OF INNATE IMMUNE SYSTEM IN IDRs

Although most of the evidence suggests that IDRs are mediated by the adaptive immune system, the innate immune system also plays a very important role in immune responses and presumably this also applies to IDRs. In fact, to a large degree it is the innate immune system that determines whether there will be a strong adaptive immune response and what the character of that response will be.\(^\text{18}\) Amongst other organs, the liver, which will be the focus of this thesis, contains a large number of innate immune cells such as Kupffer cells (which are resident macrophages), natural killer (NK) and NKT cells. In fact, a number of drugs such as penicillamine, isoniazid, and hydralazine have been shown to activate macrophages by binding irreversibly with aldehyde groups on macrophages.\(^\text{19}\) In addition, macrophages have been implicated to be a major cell type involved in severe cases of IDILI which resulted in liver failure.\(^\text{20}\) Also, there is overwhelming evidence that NK cells play an important role in the pathogenesis of liver injury and inflammation.\(^\text{21}\) Another main type of cells of the innate immune system are the neutrophils. Neutrophils have been implicated in the pathogenesis of liver injury in an acute model of liver injury that involves co-treatment of mice with lipopolysaccharide (LPS) and ranitidine.\(^\text{22}\) However, this is an acute model of liver toxicity and is unlikely to represent the type of IDILI that occurs in humans. In humans, neutrophils are not commonly seen in liver biopsies of patients that have liver failure due to IDILI; in contrast, many cases of IDILI do involve eosinophils, both in the liver biopsy and in the peripheral circulation.\(^\text{20}\) Another important question is whether the innate immune system is directly involved in pathogenesis of IDR or whether it is only involved through activation of
an adaptive immune response. Again, this is a difficult question to answer without valid animal models.

1.2.1: THE INNATE IMMUNE SYSTEM

The innate immune system and the adaptive immune system both have the ability to distinguish self from nonself. The innate immune system has only a limited number of receptors that can recognize pathogens while the adaptive immune system can generate an enormous repertoire of antigen receptors, which allows for more precise specificity. Although the innate immune response is less specific than the adaptive immune response, it provides a rapid defence because it recognizes most pathogens. Pathogens are recognized through common structures on their surface, which are referred to as pathogen associated molecular patterns (PAMPs). Similarly, damage associated molecular patterns (DAMPs) can also activate innate immune cells. Cells of the innate immune system have special receptors that bind PAMPs and initiate an innate immune response in order to clear the threat. These receptors are referred to as pattern recognition receptors which are classified into four types: 1) free receptors in the serum, 2) membrane-bound phagocytic receptors, 3) membrane-bound signalling receptors, and 4) cytoplasmic signalling receptors. Phagocytic receptors stimulate ingestion of pathogens while signalling receptors guide cells to the site of infection and stimulate production of effector molecules.

1.2.2: RECOGNITION OF PATHOGEN

A pathogen that crosses physical barriers such as the skin, in most cases, is immediately recognised by phagocytic cells of the innate immune system. There are three main classes of phagocytes: 1) macrophages/monocytes, 2) granulocytes and 3) dendritic cells. Monocytes are
immature macrophages and continuously migrate throughout various tissues. Once monocytes enter tissue they mature into macrophages. The granulocytes include neutrophils, eosinophils, and basophils with neutrophils having the largest phagocytic activity and the most immediate involvement in innate immune responses. Macrophages and granulocytes can ingest and destroy pathogens without the aid of an adaptive immune response. The last class of phagocytes includes the dendritic cells which can arise from both myeloid and lymphoid progenitors from the bone marrow. Although dendritic cells have phagocytic activity, their primary role is to serve as a bridge between the innate and adaptive immunity by ingesting pathogens and generating peptides and producing cytokines that can activate T cells and initiate an immune response. The most common process of pathogen internalization is that of phagocytosis. This is achieved by phagocytic cells first surrounding the pathogen and then internalizing the pathogen to form a phagosome. To kill the pathogen, the phagosome fuses with lysozymes which acidify the environment and kill the pathogen. An alternative process which can kill pathogens is through receptor mediated endocytosis, which makes use of surface receptors on phagocytes; this also leads to the formation of an endosome and is not restricted to phagocytosis.

Some common receptors found on surface of macrophages and other granulocytes such as neutrophils that facilitate phagocytosis and trigger cytokine production include:

- **dectin-1 receptor:** which is highly expressed on macrophages, dendritic cells, and neutrophils; it recognizes polymers of glucose that are expressed on fungal cell walls.
- **mannose receptor:** which is expressed by macrophages and dendritic cells and recognizes mannosylated ligands (present on fungi, bacteria, and viruses).
- **scavenger receptor:** which recognizes anionic polymers, lipids, and acetylated low-density lipoproteins.
• complement receptor; which is a complex of 30 plasma proteins and contributes through to pathogen clearance through one of the following four processes: 1) opsonisation, 2) leukocyte recruitment, 3) histamine secretion, and 4) membrane attack complex formation which opens a pore in virus infected cells. Complement can be activated through three distinct pathways: the classical pathway, which is facilitated by antibodies that are bound to pathogens, the lectin pathway, and the alternative pathway.

In addition to phagocytic receptors there also exists membrane-bound receptors that stimulate production of various cytokines. One type of these receptors, which are also the best characterised, are the toll like receptors (TLRs) and the NOD-like receptors (NLRs). Through various signalling pathways by these receptors, one of the most common transcription factors that is activated is NFκB. Normally, NFκB is found in its inactive form in the cytoplasm bound to IkBα. Stimulation through TNF receptors and IKKα/IKKβ dependent kinases leads to phosphorylation of IkBα. Phosphorylation of IkBα releases free NFκB so that it can translocate into the nucleus and regulate transcription of various cytokines, growth factors, adhesion molecules, and apoptosis. Another transcription factor that is activated through TLRs and NLRs by signalling through mitogen-activated protein kinases (MAPKs) is the interferon regulatory factor (IRF).

1.2.3: TOLL-LIKE RECEPTORS

Toll like receptors are expressed in a variety of cells, which include macrophages, dendritic cells, NK cells, B cells, and epithelial cells. There are a total of 10 expressed TLR genes in humans and 13 in mice with TLR-11 only expressed by mice and not humans. Each of these receptors recognises a distinct molecular pattern (i.e. PAMPS) that is not present in
normal endogenous cells. Some TLRs (such as TLR-1, 2, 4, 5, and 6) are expressed on the surface of the plasma membrane while others (TLR-3, 7, and 9) are located on the membrane of endosomes. TLRs recognize a variety of pathogens; for example, TLR-3 is expressed by macrophages, dendritic cells, and NK cells, and it recognizes double-stranded RNA. TLR-4 recognizes LPS which upon binding results in production of TNF-α. A table illustrating the various TLRs and their ligands is shown in Table 1.

<table>
<thead>
<tr>
<th>Toll-like receptor</th>
<th>Ligand</th>
<th>Cellular distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-1 and TLR-2</td>
<td>Lipomannas (mycobacteria) Lipoproteins Gram+ bacteria Fungi (zymosan) Cell wall of bacteria and fungi</td>
<td>Monocytes, dendritic cells, mast cells, eosinophils, basophils</td>
</tr>
<tr>
<td>TLR-3</td>
<td>Double-stranded RNA (ex: poly:IC)</td>
<td>NK cells</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Gram- bacteria (ex: LPS)</td>
<td>Macrophages, dendritic cells, mast cells, eosinophils</td>
</tr>
<tr>
<td>TLR-5</td>
<td>Bacteria (flagellin)</td>
<td>Epithelium, macrophages, dendritic cells</td>
</tr>
<tr>
<td>TLR-6</td>
<td>Same as TLR-1</td>
<td>Same as TLR-1</td>
</tr>
<tr>
<td>TLR-7</td>
<td>Viruses (single stranded RNA) imiquimod</td>
<td>Dendritic cells, NK cells, eosinophils, B cells</td>
</tr>
<tr>
<td>TLR-8</td>
<td>Viruses (single stranded RNA)</td>
<td>NK cells</td>
</tr>
<tr>
<td>TLR-9</td>
<td>Bacteria and herpes viruses</td>
<td>Dendritic cells, NK cells, B cells, eosinophils</td>
</tr>
<tr>
<td>TLR-10</td>
<td>Unknown</td>
<td>Dendritic cells, eosinophils, B cells and basophils</td>
</tr>
<tr>
<td>TLR-11</td>
<td>Bacteria (ex: profiling)</td>
<td>Macrophages, dendritic cells, liver and kidney</td>
</tr>
</tbody>
</table>

Adapted from Janeway’s Immunobiology. 8th ed. 2011

TLR signalling is activated by dimerization of two TLR domains, which brings their Toll-IL-1 receptor (TIR) cytoplasmic domains into close proximity and allows them to interact with TIR domains of cytoplasmic adaptor molecules. These adaptor molecules initiate intracellular signalling. There are four adaptor molecules that have been identified: 1) MyD88
(myeloid differentiation factor 88), 2) MAL (MyD88 adaptor like), 3) TRIF (TIR domain-containing adaptor-inducing IFN-β), and 4) TRAM (TRIF-related adaptor molecule). Different TLRs interact with various cytoplasmic adaptors such as TLR-5, 7, and 9 interact with MyD88 while TLR-3 interacts with TRIF. Other TLRs use a combination of MyD88 and MAL or TRIF and TRAM. Whether it is signalling through MyD88 or TRIF, the end result of activation of these signal transduction cascades that involve kinase proteins like serine-threonine kinases is the activation of IRFs and NFκB, which leads to the production of interferons and proinflammatory cytokines such as IFN-α and β.

1.2.3.1: TLR Activation Through MyD88:

Two domains of MyD88 are crucial for its function: the TIR domain and the death domain. The TIR domain of MyD88 interacts with the TIR domain of the TLR while the death domain of MyD88 activates two serine-threonine protein kinases (IRAK1 and IRAK4). The IRAK complex recruits an E3 ubiquitin ligase (TRAF-6) which together with a serine threonine kinase TAK1 and IKK phosphorylate IκBα which releases NFκB so that it can translocate into the nucleus and start transcription of pro inflammatory genes such as TNF-α, IL-1β, and IL-6.
1.2.4: NOD-LIKE RECEPTORS

Another type of receptor involved in innate immune defences are NOD-like receptors. While TLRs are located on cell surface or in the membrane of intracellular vesicles and sense extracellular pathogens, NLRs are located in the cytoplasm. NLRs also activate NFκB to initiate similar inflammatory responses as TLRs.23 NLRs contain a nucleotide-binding oligomerization domain (NOD) which has an amino terminus referred to as the caspase recruitment domain (CARD), which is used to recognise various ligands. Like the TIR domain of MyD88, CARD can dimerize with CARD domains on other proteins. When the ligand is recognized through the NOD domain it recruits the CARD-containing serine-threonine kinase RIPK2, which activates TAK1 and leads to the activation of NFκB through IKK.

Another subfamily of NLRs are the NALPs which have a purine domain that interacts with other purine domains. There are 14 NLRPs in humans with NALP3 (also called NLRP3) being the best characterized. A variety of signals can activate NALP; some of these signals include PAMPs on pathogens, bacterial toxins, various danger signals, reactive oxygen species, and K+ efflux from the cell.29 Any of these signals would result in NLRP3 being assembled with an adaptor protein known as ASC and caspase 1 to form a complex called the inflammasome.29 The role of caspase 1 is to cleave proinflammatory cytokine precursors, which can then be secreted in their active form. An example is proteolytic cleavage of pro-IL-1β and pro-IL-18 into IL-1β and IL-18, respectively, by caspase 1. The role of NALP3 inflammasome in liver injury is unclear because studies with acetaminophen and NALP3 deficient mice have shown no impact on acetaminophen hepatotoxicity.30 However, acetaminophen hepatotoxicity is not idiosyncratic, and it is possible that inflammasome may play a more important role in immune-mediated liver disease.
1.2.5: NATURAL KILLER CELLS

NK cells develop in the bone marrow, circulate into the blood, and they are a key lymphocyte that functions at the interface of the innate and adaptive immune systems. NK cells have cytoplasmic granules that contain cytotoxic proteins. NK cells protect against viruses and pathogens by inducing cytotoxicity through degranulation, cytokine secretion, and regulating antigen presenting cells.\(^{31}\) Killing activity of NK cells is significantly increased when they are exposed to interferons (IFN-\(\alpha\) and \(\beta\)).\(^{23}\) Also, it has been shown that IL-1 (which is secreted by inflammasomes) and IL-12 (released by activated macrophages) can play a role in activation of NK cells.\(^{32,33}\) Furthermore, IL-12 and IL-18 can activate macrophages and stimulate NK cells to produce IFN-\(\gamma\).\(^{23}\)

1.2.5.1: ACTIVATION OF NK CELLS

NK cells have evolved so that they can distinguish between normal and abnormal cells. NK cells are prevented from killing normal cells by a set of activating and inhibitory receptors expressed on their surface. Activating receptors trigger NK cells to kill the target cell and induce the release of cytokines such as IFN-\(\gamma\).\(^{34}\) In addition, NK cells also have receptors that recognise the Fc constant region of immunoglobulins, and binding of antibody to the Fc receptors activates NK cells to release perforin.\(^{23}\) The inhibitory receptors prevent NK cells from killing normal cells. Inhibitory receptors are particularly specific for MHC I, which explains why NK cells don’t usually to kill normal cells with high expression of MHC I. In particular, interferons help to up-regulate MHC I expression in healthy cells.\(^{34}\)

As mentioned, one key molecule that NK cells recognize is MHC I which is expressed by all nucleated cells, while MHC II is only expressed on specialized APCs. A common feature of pathogen-infected cells is that they have low expression of MHC I, in this way preventing
antigen presentation to T cells. In order for NK cells to induce cell death, two signals have to occur, one is the binding of the activating receptor on NK cells to its ligand and the other is the absence of an inhibitory signal, which is normally binding of the inhibitory receptor to MHC I. Therefore, since inhibitory receptors recognize MHC I molecules; NK cells tend to kill those cells that have low expression of MHC I (also known as the “missing self” hypothesis).

1.2.5.2: ACTIVATING/INHIBITORY RECEPTORS ON NK CELLS

There are two major families of receptors that activate NK cells. One family of receptors on the surface of NK cells is the killer cell immunoglobulin-like receptors (KIRs). KIR genes code for various numbers of immunoglobulin domains such as two or three (KIR-2D and KIR-3D) immunoglobulin domains, and they form a large cluster of receptors known as leukocyte receptor complex (LRC). The second family of NK-cell receptors are the killer lectin-like receptors (KLRs), and the genes for these receptors are found on a gene cluster called the NK receptor complex.

The KIR family of receptors consists both of activating and inhibitory receptors, and they mainly serve to sense MHC I protein levels. The activating KIR receptors have short cytoplasmic tails while the inhibitory receptors have long cytoplasmic tails and contain immunoreceptor tyrosine-based inhibition motif (ITIM). Activating KIR-2DS or KIR-3DS receptors have a charged residue that associates with a signalling protein called DAP12. DAP12 contains an immunoreceptor tyrosine-based activation motif (ITAM), which upon ligand binding to KIR receptor, becomes phosphorylated and turns on intracellular signalling pathways that activates NK cells.
The KLR family of NK-cell receptors also has both activating and inhibitory receptors. A common KLR receptor is the heterodimer between CD94 and NKG2, which recognizes MHC I molecules.\textsuperscript{23} In humans, there are five NKG2 families (NKG2A, C, D, E, and F) with NKG2A being inhibitory (since it contains an ITIM) while NKG2C, which associates with DAP12, is activating.\textsuperscript{23,36,37} Currently more NK receptors are being discovered and their roles defined. The NK-cell regulation is complex, and whether a NK-cell will be activated or not depends on the balance of activating and inhibitory receptors. Another added level of difficulty in determining whether NK-cells will be activated or not is the fact that the \textit{KIR} gene is polymorphic, and for the same \textit{KIR} gene there can be two alleles that would code for an activating or inhibitory receptor.

\textbf{1.2.5.3: NKG2D ACTIVATING RECEPTOR ON NK CELLS}

In addition to the KIR and KLR receptors, NK cells also have other surface receptors that serve to sense the presence of infections or perturbations in a cell. These include the activating receptors NKp30, NKp44, NKp46, and NKG2D. While other members of the NKG2 family form heterodimers with CD94 and bind MHC I; NKG2D does not, and it does not normally recognize MHC I. The ligands for NKG2D are the MHC I-like molecules, MIC-A and MIC-B, and RAET1 protein family.\textsuperscript{23} Mice do not have the equivalent of MIC molecules, and the ligands for mouse NKG2D are similar in structure to RAET1. These ligands for NKG2D are expressed in response to cellular or metabolic stress. Therefore, recognition by NKG2D acts as a generalized danger signal. The signalling pathway through NKG2D receptor is different from other pathways of NK-cell activation. NKG2D does not bind ITAMs, but rather binds a different adaptor protein known as DAP10, which does not contain the ITAM sequence and activates the intracellular lipid kinase phosphatidylinositol-3-kinase (PI 3-
kinase), which enhances cell survival. Activation of NK cells through these receptors induces the release IFN-γ and degranulation, which induces cell killing.

1.2.6: MACROPHAGES

Macrophages are found in lymphoid organs. They originate in the bone marrow from the common myeloid progenitor, and once in blood, they can differentiate into monocytes and when they enter tissue they mature into macrophages that have greater phagocytic activity. Resident macrophages do not normally express high levels of MHC II or the co-stimulatory molecule B7, which activates T cells by binding to their CD28 surface marker. The expression of MHC II and B7 on macrophages is induced upon ingestion of pathogens. Macrophages have a variety of receptors that recognise pathogens such as TLRs, NLRs, mannose receptor, scavenger receptor, and complement receptor. All of these receptors are involved in ingestion of pathogens and cytokine signalling pathways that activate other leukocytes.

1.2.6.1: MACROPHAGES ACTIVATE CELLS THROUGH CYTOKINE PRODUCTION

Cytokines are small proteins that are released by cells in response to an activating stimulus, and they induce immune responses by binding to specific receptors. A number of cytokines that are secreted by activated macrophages include IL-1β, IL-6, IL-12, IL-8, and TNF-α. These cytokines bind to their respective receptors; a number of important receptors include the IL-1 family, IL-6 receptor, receptors for IFN, TNF family receptors, and other receptor for chemokines.

The main cytokines that are active in innate immunity involve the IL-1 family, TNF family, and type I interferons. The IL-1 family contains 11 members, but the most common are IL-1α, IL-1β, and IL-18. As previously mentioned, these cytokines are produced as inactive
proteins and require proteolytic cleavage by the inflammasome so that they can be released into the circulation. The IL-1 family of receptors have TIR domains and signal through the NFκB pathway.\textsuperscript{23} The interferon receptor is a heterodimer that recognizes type I interferons and IL-4; these receptors signal through JAK-STAT pathway to activate transcription of various genes important in adaptive immunity. The TNF family is important in adaptive and innate immunity. Besides behaving as cytokines, many members of the TNF family are also transmembrane proteins found as homodimers. TNF-\(\alpha\) is usually membrane bound, but it can be released from the membrane and bind to its TNF receptor I (TNFRI) or TNFRII which leads to activation of NFκB.\textsuperscript{38}

The release of cytokines by activated macrophages is not only important for an innate immune response but also for the activation of an adaptive immune response. For example, IL-6 activates lymphocytes and increases antibody production and IL-12, which activates NK cells and induces differentiation of naïve T cells into Th1 cells.

\textbf{1.2.6.2: CELL RECRUITMENT BY MACROPHAGES}

In addition to the release of cytokines there are also chemokines that are released during the early phases of pathogen invasion. Chemokines are chemoattractant cytokines which involve small proteins that induce chemotaxis in nearby cells; this recruits the cells to where the chemokines are located.\textsuperscript{18} The receptors of chemokines are G-protein-coupled receptors, and activation leads to changes in cell adhesion, which results in cell migration. Chemokines serve as chemoattractants for a variety of leukocytes and drive these cells into the site of infection.

There are two distinct classes of chemokines: the CC and CXC chemokines. The CC chemokines have two adjacent cysteine residues near the amino terminus while in the CXC
chemokines the cysteine residues are separated by one amino acid. One important chemokine produced by macrophages and monocytes is CCL2, which upon binding to its CCR2B receptor attracts monocytes, NK and T cells, basophils, and dendritic cells to the site of infection. Upon induced expression, chemokines act on leukocytes by converting their rolling action into stable binding. This is achieved through the help of integrins and selectins that enable cells to bind strongly to their ligands on endothelial cells. This allows the leukocyte to cross blood vessels and endothelial cells and extravasate into tissue where the pathogen might be located. The recruitment of leukocytes by chemokines is facilitated by vasoactive mediators such as TNF-α that induce the necessary adhesion molecules on endothelial cells.

1.2.6.3: TYPES OF MACROPHAGES

Macrophages can be classified into two major types: The pro-inflammatory M1 macrophages and the anti-inflammatory M2 macrophages. The M1 macrophages, which are also called classically activated macrophages, are activated primarily by IFN-γ, TNF-α, GM-CSF, danger signals such as HMGB1, and LPS. Upon activation, M1 macrophages release a number of proinflammatory cytokines such as IL-1, IL-6, IL-12, TNF-α, induce expression of MHC II, present antigens to T cells, and induce cytotoxicity. All of these processes eventually lead to tissue injury. In contrast, M2 macrophages have been classified as anti-inflammatory and are more involved in tissue repair. M2 macrophages serve as antagonists of the M1 type of macrophages. The M2 macrophages are subdivided into three different types. M2a macrophages are activated by IL-4 and IL-13. This type of macrophage produces IL-10 and is involved in phagocytosis, stimulates cell proliferation, and promotes Th2 responses. M2b macrophages are activated by IL-1β and TLR agonists such as LPS; they are also involved in phagocytosis and promote Th2 responses. M2c macrophages are activated by IL-
10, TGF-β, and glucocorticoids. M2c produce IL-10 and are considered to be immuno-
suppressve, anti-inflammatory and promote wound repair, tissue remodelling and
angiogenesis.24 Through production of IL-12, M1 macrophages promote a strong Th1 immu-
ne response and also cytotoxic activities due to the release of their reactive oxygen and reactive
nitrogen species. In contrast, M2 macrophages promote a Th2 immune response and produce
IL-10 which leads to immunosuppressive effects and inhibits T cell proliferation.24

Macrophages have been heavily implicated in IDR s, and in particular IDILI. Studies
performed in animals have indicated that treatment with hepatotoxic doses of acetaminophen,
carbon tetrachloride, and phenobarbital increased the number of macrophages in the liver.24,41
Furthermore, the role of M1 as pathogenic macrophages has been elucidated in studies where
depletion of M1 macrophages by gadolinium chloride protected from acetaminophen liver
injury while treatment with clodronate-filled liposomes, which target M2 macrophages,
exacerbated liver injury.24 However, acetaminophen-induced liver injury is not an example of
an idiosyncratic drug reaction, and the role of macrophages remains to be tested in an animal
model in which the IDR resembles that in humans.
1.3: THE ROLE OF ADAPTIVE IMMUNE SYSTEM IN IDRs

1.3.1: ADAPTIVE IMMUNE RESPONSES

The major focus in understanding the mechanism of IDRs has been on the adaptive immune system. This is because the most common characteristics of a delayed onset, faster onset upon rechallenge, and the presence of anti-drug antibodies argue that the adaptive immune system is involved in the mechanism of IDRs. Currently, there is no clear understanding of IDRs, and it is thought that a combination of the innate immune system and the adaptive immune system may be what really triggers such reactions to occur. However, a major concern is whether mild reactions and severe reactions are mediated by the same type of immune response.

If an innate immune response fails to clear the infection, than a stronger adaptive immune response is induced. The adaptive immune response is composed of lymphocytes. The two major types of lymphocytes are B and T lymphocytes. Development of T cells occurs in the thymus and then they enter the bloodstream from which they reach peripheral organs. A mature T cell that has not encountered its antigen is known as naive T cell. An activated T cell that has acquired abilities to fight the pathogen is known as effector T cell. Unlike the innate immune system, which has a limited number of receptors for pathogen recognition, the adaptive immune system has a huge number of antigenic receptors that are determined by random genetic events; this generates a very large number of lymphocytes that help initiate an adaptive immune response. The adaptive immune system is more specific and involves specific cell types that recognize specific pathogens. It is diverse because it can recognize a wide variety of pathogens, and it has immunological memory, which means that it can respond faster to pathogens that it has already encountered. The main difference between B and T
lymphocytes is that the B cell receptor is structurally similar to an antibody which is used to recognize the pathogen, while T cells have the T cell receptor which recognises the antigen.

1.3.2: ENTRY OF T CELLS INTO PERIPHERAL LYMPHOID ORGANS

In order for a T cell-mediated immune response to occur, the naive T cell must meet an APC in a peripheral lymphoid organ or tissue in order to initiate an immune response. Naive T cells, in general, circulate from the blood to peripheral lymphoid tissues and then back into the blood. During this time, the T cell is always on the lookout for APCs that have encountered pathogens, and if the T cell encounters an APC that presents a peptide recognized by the T cell receptor than it starts to differentiate and proliferate through a process called clonal expansion. The entering of lymphocytes into lymphoid tissues depends on the ability of the T cell to bind to the endothelium through cell-cell interactions through the use of adhesion molecules such as selectins and integrins. The entering of lymphocytes into tissue involves several steps which starts by rolling of the lymphocyte along the endothelium, activation of integrins/selectins, firm adhesion, and transmigration into the tissue.23 One family of molecules that guides leukocytes into tissues is the selectins. L-selectin (CD62L) guides migration of T cells from blood into tissues by initiating the attachment of the T cell to the endothelium which results in rolling.42 However, for the T cell to be able to cross the epithelial layer, it is required that integrins and chemokines also help facilitate this process. Signalling through chemokines activates integrins to bind tightly to their ligands. Integrins consist of an alpha chain and one smaller beta chain. All T cells express LFA-1, a common integrin which is also present on macrophages.43 Other integrins expressed by T cells include members of CD2 and β1 integrins, the expression of which increases during T cell activation.23 For chemokines, three members are important for T cell activation: ICAMs (ICAM1-3) which serve as adhesion molecules and bind to T cell
integrin LFA-1; CD2, which binds LFA-3; and CD58, which binds LFA-2. T cells are recruited into the tissue by cytokines secreted by cells in that tissue. A key chemokine that enhances extravasation of naive T cells is CCL21 which is expressed on endothelial cells and binds to CCR7 on naive T cells. The general steps for a T cell entering a tissue involve circulating lymphocytes, which enter the endothelium and binds to GlyCAM-1, or CD34 ligands that bind to the endothelium through L-selectin. LFA-1 is activated by chemokines on the extracellular matrix and binds to ICAM-1 on the endothelium. This helps the lymphocyte migrate into the tissue and extravasate through the endothelium. This process completes the migration of the T lymphocyte from the blood into the tissue where it will encounter an APC and proliferate to induce an immune response; after which the lymphocyte will return back into the blood.

1.3.3: T CELLS AND IMMUNE RESPONSES

Upon activation, the naive T cell can generate effector T cells and memory T cells. Three signals are required for successful activation and survival of T cells: 1) the T cell binds to MHC/peptide through the TCR (this leads to activation of naive T cells), 2) CD28 surface chain on the T cell binds to B7 on an APC and this promotes the survival of T cell, 3) the T cell receives another signal from an APC in the form of a cytokine that allows it to differentiate. More on the mechanism of hypothesis of activation of T cells during IDR will be discussed in the following sections. T lymphocytes are divided into cytotoxic (express CD8 surface marker) and helper (express CD4 surface marker) lymphocytes. CD4 T cells recognise MHC-II molecules while CD8 T cells recognise MHC-I. The proliferation and differentiation of naive T cells is driven by IL-2 which is produced by activated T cells. IL-2 serves as a survival factor and without IL-2, activated T cells will die. Activation of T cells induces the synthesis of
transcription factors such as NFAT, AP-1, and NFκB which leads to transcription of the IL-2 gene. The co-stimulatory molecule CD28 helps in the production of IL-2 by activating PI3-kinases, which increase production of AP-1 and NFκB.

In addition to CD28, another co-stimulatory molecule on T cells that binds to B7 is ICOS. ICOS does not induce IL-2 but rather it is important for driving T helper cell differentiation and for B cells to perform isotype switching. ICOS regulates expression of IL-4 and IFN-γ.

Aside from co-stimulatory molecules that help with T cell activation, there are also co-stimulatory molecules that prevent activation. One example is CTLA4, which also binds B7 twenty times more avidly than CD28 and inhibits T cells. CTLA4 competes with CD28 for B7 binding and activated T cells express higher levels of CTLA-4 than naive T cells; therefore, it restricts IL-2 production. This property of CTLA-4 has been taken advantage of therapeutically for cancer treatment because tumours promote immune tolerance that prevents the immune system from killing the tumour. Therefore, treatment with anti-CTLA4 antibody is being exploited as a therapy to prevent tumours from inducing negative regulation of immune responses. In addition, there are also drugs that are used to prevent T cell proliferation; for example, cyclosporine inhibits IL-2 production by inhibiting signalling through the T cell receptor.

Cytotoxic T cells kill by the same mechanism as NK cells. Because CD8 T cells have a destructive function, they require more co-stimulatory activity in order to become fully activated than do CD4 helper T cells. This is provided by activated dendritic cells that produce enough IL-2 for proliferation and differentiation of CD8 T cells without the help of CD4 T cells. However, in other scenarios, activation of CD8 T cells is aided by CD4 T cells, which produce IL-2 that serves as growth factor to promote CD8 T cell differentiation.
T helper cells are subdivided into various categories as shown in Table 2. The division of Th cells in these classes is based on the cytokines they produce. The presence of IL-12 can promote differentiation into Th1 cells that produce IFN-γ while IL-4 drives Th2 cell differentiation. More recently, a novel subset of T cell has been discovered that can produce IL-17, which recruits neutrophils to the site of infection (defined as Th17 cell). There are also anti-inflammatory cell types such as T-regulatory cells, which express CTLA4 and produce anti-inflammatory cytokines such as IL-10 and TGF-β; these cytokines result in immune suppression.

Table 2: Differentiation of various T helper cells from naive CD4 T cells

<table>
<thead>
<tr>
<th>Cytokines required to induce differentiation</th>
<th>Type of T helper cell (transcription factor)</th>
<th>Cytokines Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12, IFN-γ</td>
<td>Th1 (STAT4, T-bet)</td>
<td>IFN-γ, TNF-α</td>
</tr>
<tr>
<td>IL-4</td>
<td>Th2 (STAT6, GATA3)</td>
<td>IL-4, IL-5, IL-13</td>
</tr>
<tr>
<td>IL-6, IL-21, TGF-β</td>
<td>Th17 (STAT3, ROR-γt)</td>
<td>IL-17, IL-21, IL-22, TNF-α</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Treg (STAT5, FoxP3)</td>
<td>TGF-β, IL-10</td>
</tr>
</tbody>
</table>

1.3.4: B CELLS AND IMMUNE RESPONSES

The extracellular spaces are protected by the humoral immune response which uses B cells and plasma cells to produce antibodies for destruction of pathogens. Activation of naive B cells usually requires helper T cells; after activation, B cells proliferate and differentiate into antibody-secreting plasma cells and memory cells. Antibodies protect in three main ways: 1) neutralization where antibodies bind to pathogens and prevent them from binding to cells or tissue, 2) opsonisation where antibodies coat the surface of pathogens and enhance phagocytosis and 3) complement activation.

B cells can act as lymphocytes and also as APCs. Once the antigen is recognized by the immunoglobulin on the surface of the B cell, it is then internalized, processed into peptides and
presented by MHC II to activate effector helper T cells. This process is facilitated by the co-stimulatory molecules CD40 on B cell and CD40L on T cells. This results in the production of cytokines such as IL-4, IL-5, and IL-6 from the helper T cell, which induce B cell proliferation and differentiation into memory cells and plasma cells that secrete antibodies. Since this process uses help from T cells in order to produce antibodies, these antigens are referred to as thymus-dependent antigens. Another possibility is that the pathogen can induce antibody production in the absence of helper T cells; these antigens are known as thymus-independent antigens. In this case, the second signal required to produce antibodies would be provided by the pathogen or by cross linking of B cell receptor, which would occur from binding repeating epitopes on the pathogen.

There are five major classes of antibodies found in humans and they include IgM, IgD, IgG, IgE, and IgA. In order for B cells to give rise to all these various isotypes of antibodies, B cells undergo class switching. This process begins after the B cell becomes activated by helper T cells; class switching is driven by cytokines released by effector helper T cells, and it also involves CD40L ligation. All naive B cells express IgM and IgD cell surface immunoglobulins, with IgM being the first type of antibody produced by activated B cells, although it make up less than 10% of the immunoglobulins found in plasma. IgG is the most abundant form found in plasma while IgD is present in very low amounts. Therefore the initial response is mediated by IgM and then later by IgG. IgA and IgE are less abundant and serve specific functions. Th2 cells result in the production of cytokines that favor humoral immunity and production of IgM, IgA, and IgE. It has been established by experiments performed in mice that IL-4 induced switching to IgG1 and IgE. TGF-β induces switching to IgG2b and IgA. IL-5 induces switching to IgA and IFN-γ induces switching to IgG2a and IgG3.
1.3.5: ANTI-DRUG ANTIBODIES AND THEIR IMPLICATION FOR AN IMMUNE MEDIATED REACTION

The presence of anti-drug antibodies has been used as strong evidence to argue that an IDR is immune mediated; however, their absence is not conclusive evidence to suggest that an IDR is not immune mediated.\(^5^2\) In particular, studies have identified antibodies against sulfomethoxazole or trimethoprim,\(^5^3\) amodiaquine,\(^5^4\) halothane,\(^5^5\) tienilic acid\(^5^6\) and now isoniazid.\(^5^7\) For drugs such as halothane it has been shown directly that the sera of patients that developed halothane-induced hepatotoxicity contains antibodies that react with microsomal proteins that have been covalently modified by the reactive metabolite of halothane; furthermore, these antibodies also reacted to microsomal proteins that were not modified by the reactive metabolite of halothane.\(^5^8\) Since these antibodies were not detected in the serum from normal individuals, from patients exposed to halothane without evidence of liver damage, from patients with viral hepatitis, or liver injury due to acetaminophen, and the fact that these antibodies could mediate lymphocyte killing of hepatocytes \textit{in vitro},\(^5^9\) it was suggested that halothane hepatitis is caused in susceptible individuals by humoral immune response against neoantigens;\(^6^0\) however, not all patients with halothane-induced hepatitis had autoantibodies.\(^5^9\) Autoantibodies were also detected for tienilic acid- and dihydralazine-induced hepatotoxicity. Tienilic acid is metabolised by cytochrome 2C9 (Cyp2C9), and it has been shown that sera from patients suffering from tienilic acid-induced hepatitis recognised Cyp2C9 while serum from control patients did not.\(^6^1\) Similarly, dihydralazine can bind to Cyp1A2 and generates antibodies against Cyp1A2.\(^6^2\) With drugs such as tienilic acid and dihydralazine, the reactive metabolite is short lived and binds the enzyme that generated the reactive metabolite. This gives rise to a neoantigen and this was clearly shown to induce an immune response mediated by antibodies that reacted with this neoantigens; furthermore, these antibodies could
also react with the native enzyme itself. This provides strong evidence for an immune mechanism and has been used to argue that these drugs induce an immune response via hapten mechanism which spreads to the native protein itself. This is similar to what happens in drug-induced autoimmune reactions where the immune system recognizes native proteins as foreign and initiates an immune response.

1.4: MECHANISTIC HYPOTHESIS OF IDR

There exist several hypothesis which try to explain the mechanism of IDR: 1) The hapten hypothesis, 2) the danger hypothesis, 3) the pharmacological interaction (PI) hypothesis and 4) non-immune hypotheses. A breakdown of these hypotheses is given in the subsequent section. However, these hypotheses are hard to test, and very little definitive evidence exists which supports any specific hypothesis.

1.4.1: HAPTEN HYPOTHESIS

The hapten hypothesis states that either the drug itself or a reactive metabolite of the drug binds to proteins, and this drug-protein complex is recognized by the immune system and leads to an immune response. The basis for the hapten hypothesis goes back to classical experiments done by Karl Landsteiner in 1936 in which small molecules did not elicit an immune response when injected in animals; however, injection of xenobiotic-modified proteins did. The liver has a high capacity to bioactivate xenobiotics, and covalent binding of most drugs in the liver is very common. In principle, the drug-protein adduct could induce an immune response against the drug or drug-modified protein, and this response could even spread to the native protein itself. This is what happens during a drug-induced autoimmune response. However, biological systems are very “intelligent”, and they have learned to adapt
and survive. In particular, the immune system has a way of determining how to destroy pathogens and not the host. This is known as “self-nonself discrimination”. For the hapten hypothesis to apply, the drug/reactive metabolite must bind to proteins, this drug/protein complex is then up-taken by APCs, the drug-modified peptides are processed by APCs into peptides, and these peptides are presented to T cells in the cleft of the major histocompatibility complex (MHC; Figure 1). The binding of the T-cell receptor to the drug-peptide fragments on MHC is referred as signal 1, and this is what initiates an immune response.¹

![Immune Response Diagram](image)

**Figure 1:** Hapten hypothesis. APC, antigen presenting cell; Ag, antigen (in this case protein modified by drug/reactive metabolite); TCR, T-cell receptor.

A caveat with the hapten hypothesis is the fact that almost all drugs and xenobiotics can, to some extent, be bioactivated and bind to hepatic macromolecules. However, not all
these drugs lead to a significant incidence of liver injury or IDRs. Therefore, signal 1 may be an important requirement in the pathogenesis of IDRs, but it is not universal to all drugs. An example of a drug consistent with hapten hypothesis is the reactivity of β-lactam antibiotics with lysine residues on proteins; this is a necessary step for the induction of the immune response that leads to allergic reactions to these antibiotics.67,68

1.4.2: DANGER HYPOTHESIS

An extension of the hapten hypothesis and the “self and non-self” hypothesis has been proposed by Polly Matzinger who proposed that the immune system does not try to distinguish self from non-self but rather it determines what may pose a danger.69 The driving force for the immune system is to recognize danger and to respond to it. In addition to signal 1, there is a second signal (signal 2) that is required for activation of a T cells (Figure 2). This second signal is provided by APCs, which have the unique ability to co-stimulate. Matzinger suggested that damaged cells can release danger signals, and this leads to up regulation of co-stimulatory molecules that are required for induction of signal 2 and the development of an immune response. In the absence of this second signal the result is tolerance. On the contrary, if the drug or its reactive metabolite can covalently bind to cells but does not induce the release of danger signals than no immune response will be initiated.

This danger hypothesis is attractive because it is an extension of the hapten hypothesis. According to this hypothesis, a drug or its reactive metabolite would have to cause significant cell damage in order to lead to the release of danger signals, which can be anything from endogenous danger signals (damage associated molecular patterns) or exogenous pathogen-associated molecular patterns. In fact, many drugs that cause IDRs in patients have been implicated to cause mitochondria injury,70,71 and damage to mitochondria can release damage-
associated molecular patterns\textsuperscript{72} into the extracellular matrix and activate innate immunity.\textsuperscript{73} Similarly, endoplasmic reticulum stress can be associated with the release of danger signals.\textsuperscript{74}

\textbf{Figure 2:} Danger hypothesis. APC, antigen presenting cell; Ag, antigen; TCR, T-cell receptor.
1.4.3: PHARMACOLOGICAL INTERACTION HYPOTHESIS

Another hypothesis that has been used to explain IDRIs is the pharmacological interaction (PI) hypothesis.\textsuperscript{75} This hypothesis states that, in addition to proteins that are modified by the reactive metabolite of the drug, the drug itself can induce an immune response by binding in a labile way to the MHC/peptide/TCR complex (Figure 3).

Many studies that led to this hypothesis were done with the aromatic amine sulfamethoxazole. Sulfamethoxazole can be bioactivated by P450 and lead to the formation of a hydroxylamine, which is not very reactive. A second oxidation leads to the formation of nitrososulfamethoxazole, which is reactive and can bind to proteins.\textsuperscript{45} Blood and skin T cells derived from hypersensitive patients could be stimulated by sulfamethoxazole and nitrososulphamethoxazole.\textsuperscript{76} In addition, Pichler found hydrogen bonds between sulfamethoxazole and the TCR, and he proposed that two types of PI mechanisms might occur. 1) The drug binds first to MHC, and this is recognized by TCRs, 2) Drugs might be associate with TCRs first, and this could induce some sort of initial signalling, which upon association with MHC would activate the T cells.\textsuperscript{77}

Regardless of the exact steps, presumably the PI hypothesis explains the allergic response of some drugs that do not form a reactive metabolite but still cause an allergic reaction; however, it does not address the issue of signal 2; therefore, it is consistent with the danger hypothesis. The fact that sulfamethoxazole and its nitroso metabolite induced the proliferation of the T cell clones does not distinguish whether it is the parent drug or its metabolite that can initiate the immune response. It is possible that, if the nitroso metabolite had initiated an immune response in these patients, the immune response could spread to the parent drug through a process known as “epitope spreading”. In addition, the antigenicity of nitrososulfamethoxazole compared to its hydroxyl metabolite has been demonstrated \textit{in vivo};
specifically, nitrososulfamethoxazole, but not sulfamethoxazole or hydroxsulfamethoxazole, resulted in antigen formation and immune response of lymphocytes, splenocytes, and epidermal keratinocytes.\textsuperscript{78}

\textbf{Figure 3:} The PI hypothesis. APC, antigen presenting cell; TCR, T-cell receptor.
1.4.4: OTHER HYPOTHESES

There is evidence that damage to mitochondria may be associated with IDRs, in particular liver injury.\textsuperscript{79} This may be true because there are several drugs that can cause steatosis, which implies inhibition of mitochondrial $\beta$-oxidation enzymes.\textsuperscript{79} However, steatosis is not a typical characteristic that is observed in liver biopsies from patients with idiosyncratic drug-induced liver injury (IDILI). While mitochondrial toxicity may be the basis for some types of DILI such as valproic acid-induced liver failure,\textsuperscript{80} it is unlikely that mitochondria damage is the basis for most cases of IDILI. It is possible that microvesicular steatosis and lipid peroxidation can progress to cell death/necrosis,\textsuperscript{81} and there are cases in which salicylic acid or valproic acid cause focal necrosis in addition to microvesicular steatosis.\textsuperscript{82} As mentioned above, mitochondrial injury could produce a danger signal that might lead to immune-mediated IDR; however, whether this association is true for most IDRs including IDILI remains to be established. In addition, it is possible that mitochondrial damage and steatosis could occur early, and in those patients who had an immune response and developed liver failure leading to a liver biopsy, the steatosis would no longer be evident on biopsy.

Another hypothesis used to explain IDRs is the inflammagen hypothesis proposed by Dr. Roth. This hypothesis is based on the observation that normal doses of drugs in animals do not result in hepatotoxicity, but coadministration of lipopolysaccharide (LPS) augments the hepatotoxic effects of certain drugs and chemicals.\textsuperscript{22} Briefly, the Roth hypothesis states that IDRs occur in humans when there is an episode of inflammation while being treated with a drug. This inflammatory response, just like in the animal studies, reduces the threshold of toxicity and results in an IDR that would otherwise not occur.\textsuperscript{22} The major drug used to study the inflammagen hypothesis has been ranitidine, where co-treatment of rats with ranitidine and LPS lead to acute toxicity associated with infiltration of neutrophils.\textsuperscript{83} This model is an acute
model of IDILI and does not have a delay in the onset like most cases of IDILI that occur clinically. In addition, the histology involves an infiltration of neutrophils and not lymphocytes or eosinophils. In clinical cases of IDILI, lymphocytes and sometimes eosinophils are the dominant cell in the inflammatory infiltrate, but never neutrophils. The presence of neutrophils is typical in LPS-induced DILI, and it appears that the drug is potentiating LPS-induced injury. This is supported by a recent study in a model of halothane-induced liver injury in which depletion of eosinophils prevented DILI, but depletion of neutrophils did nothing.\textsuperscript{84} Overall, the inflammagen hypothesis does not appear to involve features of liver injury that are seen in humans, and studies with other drugs, rather than ranitidine, should be performed to clarify the mechanism of liver injury. In fact, our laboratory has tried to develop animal models by treating animals with agents such as LPS to stimulate the immune system along with drugs such as nevirapine and amodiaquine, but it did not lead to significant liver injury, and certainly nothing similar to the IDILI that can occur in humans.

In addition to the hypotheses mentioned above, a new hypothesis has emerged in 2012 from studies conducted with the nucleoside reverse transcriptase inhibitor abacavir. The presence of a specific human leukocyte antigen (HLA-B*57:01) is a strong predictor of who will develop the hypersensitivity reaction to abacavir, which seems to be mediated by CD8\textsuperscript{+} cells.\textsuperscript{85} In several recent papers, it was shown that abacavir can bind within the F pocket of the peptide-binding groove of HLA-B*57:01.\textsuperscript{86-88} This alters the repertoire of endogenous peptides that bind to the MHC and can lead to an immune response analogous to the graft vs. host reaction. This is similar to the PI hypothesis but distinct and opens up new possibilities for the study of IDR.
1.5: TYPES OF IDIOSYNCRATIC DRUG REACTIONS

1.5.1: IDIOSYNCRATIC DRUG-INDUCED SKIN RASHES

Skin rashes are one of the most common types of IDRs and tend to occur at about 1–2 weeks after initiation of drug treatment.\(^8\) The high incidence may also reflect the fact that skin rashes are visible; therefore, even mild rashes are readily detected; in contrast mild liver injury is asymptomatic and only detected if an ALT measurement is performed during the time that it is elevated. Even with drugs such as INH in which ALT measurements are routine, they are only performed once a month and mild injury could easily be missed. In some countries, including the United States, routine ALT measurements are not even considered standard of care in patients treated with INH and so mild injury would never be detected. The severity of skin rashes can range anywhere from mild skin rashes (such as maculopapular rashes) to more severe skin rashes such as Stevens-Johnson syndrome and toxic epidermal necrolysis, which can lead to death.\(^9\) The skin, in general, is an immunologically active organ, and such reactions are believed to be immune-mediated. An example is nevirapine-induced hypersensitivity where patients with a low CD4 T cell count have a lower incidence of hypersensitivity,\(^9\) and cases of toxic epidermal necrolysis, which are believed to be CD8 T cell-mediated.\(^9\)

1.5.2: IDIOSYNCRATIC DRUG-INDUCED BLOOD DYSCRASIAS

Some examples of blood dyscrasias caused by drugs include: aplastic anemia, thrombocytopenia, and agranulocytosis. These reactions can take anywhere from 1–3 months; however, aplastic anemia is more commonly idiopathic and not drug-induced;\(^8\) and there are
cases where chloramphenicol-induced aplastic anemia occurred months after the drug was stopped,\cite{93} which makes it very difficult to associate the reaction to the drug.

Drug-induced thrombocytopenia appears to be immune mediated.\cite{94} A common example is heparin-induced thrombocytopenia, which is caused by antibodies formed against heparin-platelet factor 4 complex.\cite{95,96} This is a nice example of an immune-mediated IDR, yet it does not have immune memory because after the disappearance of pathogenic antibodies, there is no faster onset of thrombocytopenia upon rechallenge with heparin.

Agranulocytosis, is characterised by a drop in neutrophil count. Like most IDRs, the mechanism of agranulocytosis is currently not well understood; however, it is thought to be caused by damage of neutrophils, either in the periphery, in the bone marrow, or both.\cite{89} A major enzyme implicated in the induction of agranulocytosis is myeloperoxidase, which is located in neutrophils. Although the oxidation potential of myeloperoxidase is not as high as cytochromes P450, it still has the ability to oxidize many drugs such as clozapine to reactive metabolites. It is likely that these reactive metabolites are responsible for the cases of agranulocytosis that are associated with several such drugs.\cite{97,98}

1.5.3: IDIOSYNCRATIC DRUG-INDUCED LIVER INJURY

After cardiovascular toxicity, IDILI is the second most common reason for drug withdrawal,\cite{99} and it also contributes significantly to patient morbidity/mortality.\cite{3} The rest of this thesis will focus on IDILI, and in particular, with the hepatocellular type of liver injury which is the most common type of liver injury that is responsible for liver failure. A brief description of the different types of liver injury is presented below.
1.5.3.1: TYPES OF LIVER INJURY

There are several types of DILI, the most common ones include hepatocellular, cholestatic, autoimmune, and hepatic fibrosis.

_**Hepatocellular:**_ This is the most common type of DILI that leads to liver failure. It is characterized by hepatic necrosis with histology indistinguishable from viral hepatitis. This type of liver injury is also characterised by a large increase in ALT, aspartate aminotransferase (AST), bilirubin, and an increased international normalised ratio (INR), i.e. a decrease in clotting factors produced by the liver. The histopathology often includes an infiltration of eosinophils in the liver, which implies an immune-mediated reaction. Because the histology is virtually indistinguishable from viral hepatitis, the only way to tell that hepatic injury was drug-induced is to stop the drug and see if it resolves. In some cases there are mixed features of hepatocellular and cholestatic injury as in the case of amoxicillin/clavulanate-induced liver injury, and sometimes steatosis is observed.

_**Cholestatic:**_ Cholestatic DILI refers to injury of the biliary tract, and when it occurs, the bile cannot flow from liver to the duodenum. This shows up as yellow pigment on liver H&E-stained slides. Biochemical markers for this type of injury are an increase in alkaline phosphatase, and in particular an ALT/alkaline phosphatase ration of < 2; if the ratio is >5 then the IDILI is considered hepatocellular, while if the ration is in between 2 and 5 then the DILI is considered mixed. This type of liver injury does not usually cause liver failure, and it also can resolve despite continued treatment with the offending drug, although if anything, the recovery from cholestatic IDILI takes longer than from hepatocellular IDILI.
**Autoimmune:** By definition, this type of liver injury is immune-mediated. The characteristics of idiopathic autoimmune hepatitis\(^{103}\) and drug-induced autoimmune hepatitis (for example that caused by minocycline) are very similar.\(^{104}\) Autoimmune hepatitis is generally characterized by autoantibodies, e.g. antinuclear (ANA) and anti-smooth muscle antibodies, and histology consisting mainly of interface necrosis and plasma cells. Drug-induced autoimmune hepatitis is indistinguishable from idiopathic autoimmune hepatitis except that if the drug is stopped, the autoimmune reaction usually resolves.

**Fibrosis:** Hepatic fibrosis is the result of chronic treatment with certain drugs (but also acute in some cases) in conjunction with accumulation of extracellular matrix proteins.\(^{105}\) If left untreated, this can result in cirrhosis, which is the endstage of progressive fibrosis.

The rest of this thesis will focus on the hepatocellular type of IDILI because it is the most common reason for drug-induced liver failure.

### 1.5.3.2: CLINICAL CHARACTERISTICS OF IDILI

Although the characteristics of IDILI vary with the drug and the patient, as with most IDR s, the most common characteristic is a delay in the onset of liver injury (Figure 4). Most people treated with drugs that cause IDILI never develop any signs of liver damage, and most of those who do develop mild IDILI seem to adapt despite continued treatment with the drug, and the liver function returns to normal (Figure 4). Whether this adaptation is mediated by the immune system, and the particular role of the immune system remains to be determined; however, there is a large amount of evidence to suggest that with most drugs, when there is liver injury, the immune system is involved, and this is usually associated with inflammation.\(^{6,106,107}\) We think that this adaptation is most likely explained by a response of the immune system that prevents further liver injury, which would represent immune tolerance. In
addition, we propose that it is when this immune tolerance fails that more severe liver injury occurs; which only happens in a small fraction of the population (Figure 4).

Figure 4: Adaptation is common in IDILI. Most patients treated with drugs that cause IDILI never develop any signs of liver injury (green); up to 20% of patients develop mild signs of liver injury (orange), which are illustrated by a small increase in alanine aminotransferase (ALT); this resolves despite continued treatment with the drug; only a smaller fraction of patients develop overt liver injury or liver failure (red).

If a patient develops an increase in ALT when treated with a drug, and if the drug is stopped, the ALT usually returns to normal. Upon rechallenge with the drug, there usually is a earlier onset in the increase of ALT than what was seen with initial exposure to the drug. This has been considered a classic feature of an immune-mediated reaction, which suggests the development of immune memory and the involvement of an adaptive immune response.¹ However, there are cases of IDR, which are clearly immune-mediated but are not associated with a quicker onset of an increase in ALT upon rechallenge with the drug (this is common with INH-induced liver injury and will be discussed later). Features such as fever, rash, and an
eosinophilic infiltrate in the liver are also classic features of an allergic reaction/hypersensitivity. In general, the presence of anti-drug antibodies has also been used as circumstantial evidence to distinguish whether the mechanism of liver injury is immune-mediated or not. The presence of anti-drug antibodies is also consistent with the hypothesis that there is an adaptive immune response against the drug, and in several cases of IDILI, antibodies against the drug have been identified.\textsuperscript{52,63} With many cases of IDILI, ANA can be detected, and some drugs, especially hydrazines such as isoniazid and hydralazine, also cause an autoimmune reaction similar to lupus.\textsuperscript{108} This autoimmune response with drugs such as minocycline\textsuperscript{109} can take over a year to become clinically manifest. All of this evidence clearly suggests that there is an immune response against these drugs; however, not all cases of liver injury caused by drugs display the immunological features discussed above. In particular, mild cases of liver injury, which are more common than severe liver injury, are not usually associated with fever, rash, eosinophilic infiltrate in the liver, or anti-drug antibodies. This has been interpreted as indicating that these reactions are not immune-mediated but rather are caused by accumulation of covalent adducts formed by bioactivation of drugs in the liver.

1.5.3.3: PROGRESS IN MECHANISTIC UNDERSTANDING OF IDILI

There is currently no clear mechanistic understanding of hepatocellular IDILI. A major reason for this is that there are no good animal models that could be used to test mechanistic hypothesis. The low incidence and the unpredictable nature of IDILI in humans\textsuperscript{63} also makes controlled studies in humans very difficult. In addition, it is not possible to perform controlled mechanistic studies in humans because of the invasive nature of experiments.

\textit{In vitro} studies represent an interesting opportunity for maintenance and manipulation of experiments under controlled conditions. The use of freshly prepared primary hepatocytes
from rodents or humans has been used for many years to assess *in vitro* drug toxicity.\textsuperscript{110} However, the major question is whether the findings *in vitro* translate to *in vivo*. It is often true that depending on the dose used, *in vitro* experiments will lead to a response (effect); however, when a person or animal is exposed *in vivo*, the results could be completely different. Some challenges with *in vitro* experiments using primary hepatocytes are that the architecture of hepatocytes may not be similar to that *in vivo*.\textsuperscript{110} Although more recently 3D hepatocyte culture systems have been developed, there still remains the fact that in the liver there are many other cell types in addition to hepatocytes such as: liver endothelial cells, hepatic stellate cells, Kupffer cells, and natural killer cells.\textsuperscript{110} In addition, new technologies such as human hepatocytes derived from induced pluripotent stem cell don’t have cytochrome P450 expression levels comparable to those *in vivo*; this hampers drug bioactivation and reactive metabolite formation. In addition, *in vitro* studies also eliminate other lymphoid organs such as spleen and lymph nodes, which are involved in the initiation and propagation of an immune response. If IDILI is immune-mediated then this is a major limitation. Overall, the *in vitro* hepatocyte culture system represents an interesting tool to investigate the mechanism of drug toxicity; however, at this time, this has major limitations when extrapolating results to *in vivo*, and therefore valid animal models are the preferable tool for mechanistic studies.
1.6: RELATIONSHIP BETWEEN REACTIVE METABOLITES OR DRUG-INDUCED IMMUNE RESPONSES AND IDILI

1.6.1: REACTIVE METABOLITE AND IDILI

There is evidence to suggest that most IDILI is caused by a reactive metabolite rather than the parent drug; however, exceptions appear to exist.\textsuperscript{63} Decades ago, classic studies done at the National Institute of Health established the link between reactive metabolite formation and liver injury.\textsuperscript{100,111-113} Experiments involved a number of drugs such as acetaminophen, furosemide, carbon tetrachloride, halothane, isoniazid, and iproniazid. With these drugs it was found that bioactivation was required for covalent binding to occur in the liver. In addition, the amount of covalent binding was measured using \textsuperscript{14}C radiolabeled drug/metabolite, and it was found that the drug dose correlated with covalent binding, and this correlated with liver injury. This later led to the term “metabolic idiosyncrasy”, which suggested that differences in drug bioactivation and covalent binding led to the idiosyncratic nature of IDILI. If this were true, there should exist examples of polymorphisms in drug metabolising enzymes that are strong risk factors for IDILI; however, to date there is no such example. In addition, although earlier studies called halothane-induced liver injury “metabolic idiosyncrasy”, more recent studies have provided strong evidence that the mechanism of IDILI with halothane is not metabolic idiosyncrasy but rather immune-mediated.\textsuperscript{55,114} In addition, in studies of INH, we have determined that even with INH there is an immune component to the liver injury, and this will be discussed in later chapters. Although, it is well accepted that acetaminophen-induced liver injury is not immune-mediated and hepatotoxicity correlates with covalent binding, this is not the case for other drugs such as halothane and isoniazid for which there is now substantive evidence that the IDILI is immune-mediated.\textsuperscript{18,107} One important characteristic of the older studies that led to the term “metabolic idiosyncrasy”\textsuperscript{115} is that they were done in rats, and
animals were treated with very high doses of drugs which produced acute liver injury. For drugs that cause idiosyncratic toxicity such as halothane and isoniazid, the characteristics of these acute models of toxicity are very different from the clinical characteristics of IDILI, which involves a delayed onset; therefore, the mechanism of liver injury is likely to be completely different.

### 1.6.2: DRUG-INDUCED IMMUNE RESPONSES AND DILI

Evidence suggests that IDILI is immune-mediated; however, there is no definitive evidence for this hypothesis, and not everyone accepts the immune-mediated hypothesis. More importantly, the real question is whether the immune response is the cause of liver injury or simply a response to the injury. To date, there is no definitive evidence to argue either for or against the hypothesis that the immune system may be what is causing the injury.

The strongest piece of evidence that suggests that a drug involves an immune-mediated mechanism is an HLA association as in the case of flucloxacillin and presence of anti-drug antibodies as in the case of halothane, amodiaquine, and now INH. Also, a positive lymphocyte transformation test is considered strong evidence for an immune-mediated reaction as in the case of INH-induced liver injury. However, anti-drug antibodies are not present in all of the patients, and there are cases of liver injury that are not associated with the classical hallmarks of an immune-mediated reaction such as fever, rash, and eosinophilia. In addition, even when treating animals with drugs acutely, there is an increase in the number of mononuclear cells in the liver as well as an increase in serum cytokines. This does not distinguish whether the immune system causes the liver injury or is a response to the liver damage. A more convincing piece of evidence that argues that the immune system is responsible for induction of liver injury has recently been reported for the drug halothane,
where depletion of eosinophils prevented the liver injury that was caused by administration of the drug.\textsuperscript{84} However, even this is an acute animal model of IDILI and does not have the classical characteristic of a delayed onset that is seen in humans.-Whether IDILI is immune-mediated or not, in order to better understand its cause and to treat patients, a clear mechanistic understanding is required.

1.7: USE OF ANIMAL MODELS TO STUDY IDILI

Given the rare incidence of IDR\'s and how difficult it is to study them in humans, animal models represent an essential tool to study mechanistic hypothesis.\textsuperscript{6} However, it is important that the animal models have characteristics similar to the IDR that occurs in humans. As mentioned before, the most common characteristic of IDILI is that there is a delay in the onset. However, attempts to develop these models have been unsuccessful. Most models of IDILI involve acute studies in animals where high doses of the drugs are used.\textsuperscript{100,118} The pharmaceutical industry has tried to develop \textit{in vitro} assays to screen potential drug candidates that might cause IDILI. As mentioned above, this approach allows for greater manipulation of conditions to study mechanistic hypotheses relevant to hepatocyte damage \textit{in vitro}; however, the systems are so distant from what appears to be involved in the mechanism of IDILI \textit{in vivo} that it is unlikely that such methods will be reliable predictors of IDILI risk.
1.8: ISONIAZID-INDUCED LIVER INJURY

Reprinted from Clinical Pharmacology and Therapeutics. I G Metushi et al., “A Fresh Look at the Mechanism of Isoniazid-Induced Hepatotoxicity”.

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1.8.1. Characteristics of Clinical INH-Induced Hepatotoxicity

Isoniazid remains a mainstay for the treatment of tuberculosis (TB); however, its use is associated with serious hepatotoxicity and potentially fatal liver injury.\textsuperscript{119} Hepatotoxicity is often associated with nausea and right upper quadrant abdominal pain; however, it can be asymptomatic and diagnosis depends on measuring serum levels of bilirubin and the liver enzyme ALT.\textsuperscript{119,120} INH use is associated with elevated serum levels of ALT in up to 20\% of patients, while overt hepatotoxicity can occur in up to 1\% of patients.\textsuperscript{106,119,120} There is virtually always a delay between starting the drug and the onset of liver injury that is anywhere from 1 week to 6 months, but on occasion, more than 6 months.\textsuperscript{106,120} In most cases the increase in ALT will return to normal despite continued treatment; this is referred to as adaptation.\textsuperscript{121} Upon interruption of the treatment, liver enzymes can quickly return to normal, and it is only when INH is not stopped or when the injury continues despite discontinuation of the drug that liver failure results.\textsuperscript{106,120} If a patient is rechallenged with INH the response is quite variable.\textsuperscript{106,120} In some cases there is a prompt onset of symptoms, while in other cases it may require a month or more of treatment before the ALT rises again, and in many cases, especially when the toxicity was mild and the initial rechallenge dose is low, there is no increase in ALT.\textsuperscript{106,120}

Although the pathology can vary, in most cases liver biopsies from patients with severe hepatotoxicity are indistinguishable from those of patients with viral hepatitis including necrosis, inflammation, and in some cases, an infiltration of eosinophils.\textsuperscript{106,120,122} After a long course of treatment, INH is often associated with the induction of antinuclear antibodies, and in a few cases, a clinical syndrome of drug-induced autoimmunity similar to lupus.\textsuperscript{123}
1.8.2 Risk Factors Associated With INH Hepatotoxicity

The risk of INH-induced hepatotoxicity increases with age, alcohol abuse, and is higher when combined with other drugs such as rifampicin that induce cytochromes P450.124 There are also genetic risk factors that increase the risk of INH-induced hepatotoxicity. It was recently reported that the risk of hepatotoxicity with anti-TB drug treatment is higher in people with: slow acetylation phenotype (OR 3.7)12, CYP2E1 c1/c1 genotype (OR 2.5)125, homozygous null mutations in GSTM1126 or GSTT1127 (OR 2.13 and 2.6, respectively), the absence of HLA-DQA1*0102 (OR 4.0), and the presence of HLA-DQB1*0201 alleles (OR 1.9).128

Early studies suggested that acetylhydrazine was responsible for INH-induced hepatotoxicity;129 therefore, it was expected that patients with the rapid acetylator phenotype would be at increased risk (Figure 5).100 The first study appeared to support this hypothesis130; however, the consensus is now that slow acetylators are at higher risk of developing INH-induced hepatotoxicity. Even though the rate at which slow acetylators form acetylhydrazine is slower, the rate at which this is further acetylated to the nontoxic diacetylhydrazine is also slower, and the total area under the curve of acetylhydrazine is greater in slow acetylators than in rapid acetylators.131 In addition, patients who are slow acetylators and carried CYP2E1 c1/c1 genotype had higher risk of developing hepatotoxicity (OR 7.4).125 This is consistent with a mechanism that involves bioactivation of acetylhydrazine to toxic intermediates; however, the difference in the concentration of the parent drug, INH, between rapid and slow acetylators is greater than that for acetylhydrazine.131 Therefore, the increase in risk in slow acetylators is also consistent with toxicity involving the parent drug.
1.8.3: Proposed Mechanism of INH-Induced Hepatotoxicity

INH is a hydrazine derivative, and hydrazines are, in general, hepatotoxic, carcinogenic, and mutagenic.\textsuperscript{132} The toxicity of hydrazines is presumably because they are readily oxidized to chemically reactive species. Two metabolites of INH proposed to be responsible for toxicity are acetylhydrazine and hydrazine.

1.8.3.1: Acetylhydrazine

Acetylhydrazine is widely considered to be responsible for INH hepatotoxicity.\textsuperscript{100,129,131,133} This is based on early studies in rats that found treatment with isoniazid caused little or no hepatotoxicity.\textsuperscript{100} However, acetylisoniazid plus phenobarbital
caused acute hepatotoxicity, and the toxicity induced by acetylhydrazine plus phenobarbital was even greater.\textsuperscript{100} In addition, inhibition of P450s prevented acetylhydrazine–induced hepatotoxicity. In rats very little INH is hydrolyzed, but >30% of acetylisoniazid is hydrolyzed to isonicotinic acid and acetylhydrazine, and because INH itself did not cause hepatotoxicity, it was postulated that most toxicity resulted from INH being acetylated to acetylisoniazid and then hydrolysed to acetylhydrazine (Figure 5). This hypothesis was strengthened by the fact that co-treatment with bis-p-nitrophenyl phosphate to block the hydrolysis of acetylisoniazid prevented the hepatotoxicity of acetylisoniazid but not that of acetylhydrazine.\textsuperscript{100} Furthermore, covalent binding was observed when \textsuperscript{14}C-acetyl-labelled acetylisoniazid was administered to rats but not when the label was in the pyridine ring of INH.\textsuperscript{100,133}

\textbf{1.8.3.2: Hydrazine}

Although previous research focused on acetylhydrazine; more recently, hydrazine has also been proposed to be the hepatotoxic metabolite of INH.\textsuperscript{134-136} Hydrazine is known to be toxic, it is a INH metabolite, and slow acetylators have significantly higher plasma concentrations of hydrazine than fast acetylators.\textsuperscript{134} One patient who developed INH-induced hepatotoxicity had high levels of hydrazine.\textsuperscript{135} A rabbit model of INH-induced hepatotoxicity was developed in which toxicity correlated with hydrazine levels,\textsuperscript{136} but the rabbit model also involved acute toxicity requiring high doses of INH and is not consistent with a delayed onset observed in humans.

\textbf{1.8.3.3: Isoniazid}

The studies described above address the chemical species responsible for toxicity but not the mechanism by which it induces toxicity, and if the mechanism of the idiosyncratic reaction in humans is different from that of the acute toxicity in animal models, the chemical species responsible could also be different. Given that hydrazines are readily oxidized to
reactive metabolites, it seems likely that isoniazid would also be oxidized to a reactive metabolite. In previous studies we demonstrated that INH is oxidized by activated neutrophils or simply HOCl to isonicotinic acid\textsuperscript{137} which is likely to involve a reactive intermediate analogous to the reactive metabolite of acetylhydrazine (Figure 5). Another study also found covalent binding of \textsuperscript{14}C-INH in rats in vivo and in human and rat liver microsomes.\textsuperscript{138} Furthermore, incubation of INH with human microsomes leads to irreversible inhibition of P450, which implies the formation of a reactive metabolite.\textsuperscript{139} More recently, using an anti-INH antibody, we found in vivo covalent binding of INH in the liver of mice (Figure 6). We were able to trap a reactive metabolite of INH with N-\alpha-acetyl-l-lysine generated by human liver microsomes (unpublished data). This suggests nucleophilic attack on the diazene, or more likely the diazohydroxide intermediate because a radical or carbocation would likely be too reactive to trap in this way; however, the possibility of radical/carbocation formation is not excluded.\textsuperscript{140} All these observations indicate direct oxidation of the parent drug to a reactive intermediate.

![Figure 6](image.png)

**Figure 6:** Covalent binding of isoniazid in the liver of mice by Western blotting. C - control mice (n = 2). INH - INH treated mice (n = 2).
In addition, we found that INH is capable of directly activating macrophages, apparently by a mechanism involving nucleophilic attack of INH on a carbonyl-containing signalling molecule. The activation of macrophages by INH, either involving a reactive metabolite or the parent drug could lead to an immune response, which in turn could lead to immune-mediated liver toxicity or autoimmunity.

1.8.4: Mitochondrial Damage

As mentioned above, it has been suggested that most drug-induced hepatotoxicity is caused by mitochondrial damage. This appears to be the case for some acute toxicity such as acetaminophen-induced hepatotoxicity and delayed onset hepatotoxicity such as that caused by fialuridine and valproic acid that is associated with steatosis and/or lactic acidosis. In addition, mitochondrial injury could induce an immune response; however, it is unlikely that the mechanism of most idiosyncratic drug-induced hepatotoxicity is direct mitochondrial damage.

1.8.5: Evidence for the Involvement of the Immune System in INH-induced Hepatotoxicity

INH-induced IDILI has been classified as an example of metabolic idiosyncrasy implying that it involves direct cytotoxicity and is not an immune-mediated drug reaction. This is based on the fact that it is not usually associated with a fever, rash, or eosinophilia. However, this is not substantive evidence for the lack of an immune mechanism; most immune-mediated responses are not associated with fever, rash, and eosinophilia. In fact there are some cases in which INH-induced hepatotoxicity is associated with these features - 10% of isoniazid-treated patients develop eosinophilia - and there are also cases in which
rechallenge of a patient resulted in fever and liver injury within hours of rechallenge\textsuperscript{106} (Table 3). Also, the HLA association suggests an adaptive immune mechanism for INH-induced hepatotoxicity. These characteristics provide strong evidence for an immune-mediated reaction. It is possible that some cases of INH-induced hepatotoxicity involve an immune mechanism and others do not; however, this heterogeneity is common to idiosyncratic drug reactions, and the characteristics of INH-induced hepatotoxicity are similar to many other types of idiosyncratic drug reactions for which there is good evidence for an immune mechanism.\textsuperscript{63}

In addition, the relative risks related to the genetic polymorphisms in metabolic enzymes are too small to explain the idiosyncratic nature of INH-induced hepatotoxicity. It also does not explain the delay between starting the drug and onset of injury.

**Table 3:** Features of INH-induced liver injury that suggest that at least some are immune-mediated

<table>
<thead>
<tr>
<th>Feature</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A delay of a week or more between starting the drug and the onset of liver injury</td>
<td>\textsuperscript{106,120}</td>
</tr>
<tr>
<td>Cases in which rechallenge led to fever and liver injury within hours</td>
<td>\textsuperscript{106}</td>
</tr>
<tr>
<td>Cases in which the liver histology includes significant numbers of eosinophils</td>
<td>\textsuperscript{106,120,122}</td>
</tr>
<tr>
<td>The fact that INH can induce other immune responses, in particular, autoimmunity similar to lupus; and the presence of antinuclear antibodies</td>
<td>\textsuperscript{123}</td>
</tr>
<tr>
<td>Cases of INH-induced hepatotoxicity with a very long time to onset which is typical of drug-induced autoimmunity</td>
<td>\textsuperscript{106,120}</td>
</tr>
<tr>
<td>The fact that INH-induced hepatotoxicity has features very similar to other idiosyncratic drug reactions that are clearly immune-mediated</td>
<td>\textsuperscript{63}</td>
</tr>
<tr>
<td>The finding of a specific HLA genotype that is associated with an increased risk of INH-induced hepatotoxicity</td>
<td>\textsuperscript{128}</td>
</tr>
<tr>
<td>The fact that INH can activate macrophages</td>
<td>\textsuperscript{19}</td>
</tr>
<tr>
<td>The fact that INH-induced hepatotoxicity with features similar to those observed in humans cannot be reproduced in animals with high doses of the drug</td>
<td></td>
</tr>
<tr>
<td>A positive lymphocyte transformation test to INH and INH modified proteins</td>
<td>\textsuperscript{117,142}</td>
</tr>
<tr>
<td>Liver histology dominated by CD8\textsuperscript{7} cells in cases of INH-induced liver failure</td>
<td>\textsuperscript{20}</td>
</tr>
</tbody>
</table>

The fact that INH can induce a lupus-like syndrome makes it clear that it can induce an immune response. It is of note that many, if not most, drugs associated with hepatotoxicity also cause autoimmune syndromes as reviewed elsewhere.\textsuperscript{63} In some cases these drugs also cause hepatotoxicity with classic features of autoimmune hepatitis, but as in the case of INH, this is
not always true. Autoimmune hepatitis is a quite heterogeneous syndrome, but it usually occurs after a very long delay, and the cases of INH-induced hepatotoxicity that have a long time to onset may have an autoimmune component. In addition, the common occurrence of “adaptation” which occurs with the same time to onset as more severe hepatotoxicity may, in fact, represent immune tolerance, and this may be the basis for the lack of recurrence in many cases of rechallenge.
1.9: AMODIAQUINE-INDUCED LIVER INJURY

Amodiaquine (AQ), a 4 aminoquinoline, was introduced onto the market because it was more effective against malaria than chloroquine. However, AQ was withdrawn from the market due to severe IDR s which included hepatotoxicity,\textsuperscript{143,144} agranulocytosis,\textsuperscript{145} and aplastic anemia.\textsuperscript{146} The mechanism of AQ-induced adverse reactions is currently not well understood, but AQ and its N-desmethyl metabolite (DEAQ) formed by CYP2C8,\textsuperscript{147} are oxidised to a reactive quinoneimine (AQQI) that reacts with proteins and forms covalent adducts (Figure 7).\textsuperscript{148,149}

![Metabolism of AQ to the reactive intermediate, AQQI.](image)

**Figure 7:** Metabolism of AQ to the reactive intermediate, AQQI.
As with most drugs that cause idiosyncratic drug-induced liver injury (IDILI), the clinical characteristics of AQ-induced hepatotoxicity involve a delayed onset, and in most patients, the IDILI resolves despite continued treatment.\textsuperscript{144} There is a prompt onset in IDILI upon rechallenge, which is consistent with immune memory.\textsuperscript{144} Histopathology shows necrosis and inflammatory cells including Kupffer cells.\textsuperscript{143,144} It has also been reported that there are circulating anti-AQ IgG antibodies in humans\textsuperscript{54} and even in rats.\textsuperscript{150} This evidence suggests that AQ-induced IDILI is an immune-mediated reaction.

Despite the clinical characteristics of AQ-induced hepatotoxicity and other drugs that are associated with IDILI, the lack of a valid animal model has hampered progress in understanding the molecular mechanism and factors that lead to the initiation of liver injury, and in most extreme cases, liver failure. Recently, a model of AQ-induced IDILI has been reported.\textsuperscript{118} However, like with most other drugs, this is an acute model of hepatotoxicity where mice were treated with buthionine sulfoximine (BSO) and AQ for up to 24 h, and this resulted in an increase in alanine transaminase (ALT) levels. A similar liver injury was reported in Swiss mice.\textsuperscript{151} It is likely that the mechanism of AQ-induced IDLI in patients is very different from these acute models of hepatotoxicity because in patients there is a delay in the onset of the IDILI. We have used AQ to treat mice and develop an animal model with characteristics of liver injury similar to humans. Our animal model of liver injury has a delayed onset that resolves despite continued treatment which is a very common characteristics of most patients with mild IDILI in which the liver injury resolves despite continued treatment with the drug.
CHAPTER 2: MATERIALS AND METHODS

Trapping of the INH Reactive Metabolite:

INH (Sigma; Oakville, ON) and N-α-acetyl-l-lysine (NAL) or glutathione (both from Fisher Scientific; Ottawa, ON) were dissolved in water and incubated with 1 mg/mL of human liver microsomes (HLM, pooled from 50 donors, BD Biosciences; Mississauga, ON) in phosphate buffer (50 mM, pH 7.4) at concentrations of 500 µM for INH and 1 mM for NAL and glutathione. The reaction was initiated by addition of a NADPH-regenerating system (Solutions A and B; BD Biosciences) and incubated at 37 °C for 30 min. To quench the reaction, 3 volumes of ice-cold methanol were added and the mixture was allowed to sit at -20 °C for 30 min. After centrifugation (11,000g for 10 min), supernatants were dried under a stream of N₂. The samples were reconstituted with the initial mobile phase and 10 µL samples were analyzed using a 150×3mm Luna 3µ C18(2) 100A column (Phenomenex; Torrance, CA) with a methanol/10 mM aqueous ammonium acetate (pH 4.0) gradient at a flow of 0.2 mL/min. Initially, methanol was 10% for 2 min with a linear gradient to 95% methanol over 8 min.

INA Activated Ester, INH Dimer, and Isonicotinic Acid-N-α-acetyl-l-lysine (INA-NAL) Adduct:

The INH dimer (INA-INH) was synthesized as previously described; similarly the activated ester of isonicotinic acid (INA) was synthesized by reaction of isonicotinoyl chloride hydrochloride (Fisher Scientific) with N-hydroxysuccinimide (NHS, Sigma) to form INA-NHS as previously shown. For synthesis of INA-NAL; to a stirred solution of NAL (0.2 g; 0.001 mol) in anhydrous methanol (50 mL) was added INA-NHS (0.22 g; 0.001 mol). The mixture was
refluxed for 2 h and concentrated in vacuo. The residue was taken up in methanol and purified by column chromatography (silica gel, 230 x 33 mm, mesh 230-400, Sigma) using a gradient starting with 4% methanol in CHCl₃ and increasing up to 10% methanol. The structure was confirmed by ¹H-NMR, ¹³C-NMR, and mass spectrometry. Yield: 0.21 g (30%); ¹H-NMR (400 MHz; d-MeOH) δ: 8.7 (d; 2H); 7.8 (d; 2H); 4.4 (m; 1H); 3.4 (m; 2H); 1.95 (s; 3H); 1.9 (m; 2H); 1.6 (m; 2H); 1.5 (m; 2H). ¹³C-NMR (400 MHz; d-MeOH) δ: 175, 172, 166, 149, 143, 122, 53, 39, 32, 29, 23, 21. MS (ESI): m/z: 294 [M+H]⁺.

**Conjugation of INH to BSA/Blue Carrier Protein and Antibody Production:**

INH was coupled to bovine serum albumin (BSA, Sigma) by adding a 10 fold molar excess (based on 58 lysine residues/BSA) of the activated ester (INA-NHS, 0.2 g) to a stirred solution of BSA (0.1 g) in buffer (see Table 4). INH was coupled to Blue Carrier Protein (Fisher Scientific) in a similar manner with a 10 fold molar excess (based on a maximum of 699 lysine residues/Protein Blue protein) of INA-NHS (3.3 mg) to 20 mg of protein. The mixtures of INA-NHS with protein were stirred for 1-2 h at room temperature and low mass products removed with a 10,000 MW cut off filter. INA coupling to BSA was confirmed by ESI-MS (Supplemental figure 1 = Figure S1) while a trinitrobenzene sulfonic acid assay (Sigma) was used to measure free amino groups and calculate coupling to Blue Carrier Protein because of its high mass. All protein concentrations were measured using the bicinchoninic acid (BCA) kit (Fisher Scientific). Production of polyclonal antibodies in rabbits against INA was carried out by the Division of Comparative Medicine at the University of Toronto using INA coupled to Blue Carrier Protein coupled as the antigen. The antibody production schedule involved a primary immunization of rabbits with 500 µg of antigen dissolved in phosphate-buffered saline (pH 7.4) followed by two subsequent immunizations of 250 and 100 µg of
antigen. For primary immunizations, Freund’s complete adjuvant was used to induce an immune response and subsequent immunizations involved Freund’s incomplete adjuvant.

**Table 4:** Percentage of BSA and Blue Carrier protein lysine residues modified by INA-NHS

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH</th>
<th>Buffer</th>
<th>% modification of Lysine residues</th>
<th># of Lysine residues coupled</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>7.0</td>
<td>H₂O</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>BSA</td>
<td>4.7</td>
<td>0.9% NaCl</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>BSA</td>
<td>8.6</td>
<td>0.1M NaHCO₃</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>BSA</td>
<td>8.6</td>
<td>0.1 M Na₂HPO₄  + 0.15 M NaCl</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>Protein Blue</td>
<td>8.6</td>
<td>0.1 M Na₂HPO₄  + 0.15 M NaCl</td>
<td>25</td>
<td>75 - 150</td>
</tr>
</tbody>
</table>

BSA or Blue Carrier protein was reacted with INA-NHS in the presence of different buffers to evaluate the optimal conditions for INA coupling to protein. BSA was used as the model protein and the optimal conditions were also used for Blue Carrier protein. The % of modified Lysine proteins were calculated by monitoring absorbance at 335 nm and using the formula: \[ \text{Modification} \% = [1 - (\text{OD INA-BSA/OD BSA}) \times 100] \]. Calculations were based on a total number of 58 lysine residues for BSA and 300 – 600 lysine residues for Blue Carrier protein.
**INH, AcHz, and Hz Blood Level Measurements:**

The procedure for quantitation of serum INH and its metabolites was adapted from Sarich et al.\textsuperscript{136} To 25 µL of serum was added 375 µL of ice cold methanol containing 4-dimethylaminoantipyrene (Sigma) as the internal standard, the mixture was placed at -20 °C for 30 min, and then it was centrifuged at 11,000 g for 5 min. To 200 µL of supernatant was added 200 µL of 1 M formic acid in water. To this mixture was added 100 µL of derivatizing agent (3-methoxybenzaldehyde, Sigma) made up 1:10 in 50% 2-propanol in methanol and incubated at room temperature in the dark with rocking for 2 h. After two h the samples were diluted and the metabolite levels were analyzed using an LC-MS system with a 30×2 mm Gemini 5µ C18 100A column (Phenomenex) and a mobile phase consisting of methanol/10 mM aqueous ammonium acetate (pH 4.0) gradient at a flow of 0.2 mL/min. Initial % of methanol was 0 for 2 min with a linear gradient to 95% methanol over 5 min. Optimizations for the multi reaction monitoring were performed using the synthetic standards (Sigma) for N'-{(3-methoxybenzyldiene)isonicotinohydrazide} (Q1/Q3: 255.95/121.2), 3-methoxybenzaldehyde[(3-methoxyphenyl)methylene]hydrazone (Q1/Q3: 268.81/136.0) and N'-{(3-methoxybenzyldiene)acetohydrazide} (Q1/Q3: 192.82/151.2).

**Alternative Method for Blood Level Measurements of INH Only:**

To 5 µL of plasma was added 80 µL of methanol containing 4-dimethylaminoantipyrene (internal standard, Sigma) to precipitate the protein. The mixture was vortexed and placed at -20 °C for 30 min, and after centrifugation (11,000g for 10 min) the supernatant was dried under a stream of N\(_2\). The samples were reconstituted with the initial mobile phase, and 20 µL of sample was injected onto a 150×2mm Gemini 5µ C18 110 Å column (Phenomenex, Torrance, CA) with an isocratic mobile phase containing 10% methanol.
in water-ammonium acetate (10 mM, pH = 4) at a flow of 0.2 mL/min. Total run time was 5 min. The outlet from the HPLC was connected to a Sciex API3000 mass spectrometer. Metabolites were detected using an LC-MS system and optimizations for multireaction monitoring were performed using synthetic standards (Sigma); for INH (Q1/Q3: 138/79) and for 4-dimethylaminoantipyrine (Q1/Q3: 232.12/56.1).

**Blood Level Measurements of AQ:**

Methanol (80 µL) including 0.1 internal standard µM (4-dimethylaminoantipyrine from Sigma, Oakville, ON) was added to 10 µL of serum samples to precipitate the protein. The mixture was vortexed, allowed to sit at -20 °C for 30 min, then centrifuged at 13,200×g for 10 min. The supernatant was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 100 µL of the mobile phase and 20 µL of the aliquot was injected into the LC–MS/MS system. The LC-MS/MS system consisted of a Shimadzu LC10 HPLC and a API3000 mass spectrometer. Separation was done on a 2.1mm×50mm Luna® C18 3µm analytical column, and the flow-rate was 0.2 mL/min. The mobile phases used for chromatography were 0.1% formic acid (solvent A) and methanol (solvent B). The mobile phase was delivered using a linear gradient elution program: 5% solvent B at 0 min increased to 90% over a period of 2.5 min. Optimization for multiple reaction monitoring transitions were performed using synthetic standards (Sigma) for AQ (Q1/Q3: 356.1/283.2) and internal standard (232.1/56.1). Data were collected and processed using Analyst 1.4.2 software (Applied Biosystems, Burlington, ON).
Animals:

*Mice:* All mice used were between 6-8 weeks of age. Inbred mice (Balb/c AnNCrl, C57BL/6NCrl or C3H/HeNCrl) were purchased from Charles River Laboratories (Montreal, QC) and were allowed to acclimatize for one week before treatment. Cbl-b⁻/⁻ mice are on a C57BL/6 background and were bred in house with the permission of the developer, Dr. J. Penninger at the Institute of Molecular Biotechnology of the Austrian Academy of Science, Vienna. PD1⁻/⁻ mice are also on a C57BL/6 background and were bred in house with the permission of the developer, Dr. H. Honjo at the Kyoto University Graduate School of Medicine. NAT1/2⁻/⁻ mice are on a C57BL/6 background and were bred in house with the permission of the developer, Dr. D. Grant, at the University of Toronto, Department of Pharmacology and Toxicology. T and B cell immunodeficient Rag⁻⁻ (Rag1<sup>tm1Mom/J</sup>) mice were purchased from Jackson Laboratories (Bar Harbor, Maine).

*Rats:* Male rats of Wistar strain (Crl:WI), 100 – 150 g or 6-8 weeks of age and male Brown Norway rats, both 6-8 weeks of age, were purchased from Charles River Laboratories (Montreal, QC) and were allowed to acclimatize for one week before treatment.

Treatment of Animals:

*Mice:* INH (Sigma) was ground to fine powder, thoroughly mixed with food, and given to rodents at a dose of 0.1%, 0.15%, 0.2% or 0.4% of INH by weight (w/w) in food. Food was provided to the animals in small jars ad libitum, and the amount consumed was measured. This resulted in an INH dose of 150 – 450 mg/kg/day depending on the % dose of INH and the amount of daily food consumption by mice. This INH dose is similar to what we have reported previously and leads to INH blood levels in the therapeutic range.¹⁵⁴ Pyridoxine hydrochloride (MP Biomedicals, Solon, OH) was also mixed in food, and given at 0.05% by weight in food
which resulted in a dose of about 50 – 90 mg/kg/day. RMP (Sigma) was suspended in saline and given by gavage to female Cbl-b−/− mice at 50 mg/kg/day; saline was given as vehicle to control group. Alternatively, INH was dissolved in saline and given by oral gavage to female NAT1/2−/− mice at a dose of 100 mg/kg/day; saline was administered as the vehicle control. AQ was thoroughly mixed with food and given to rodents at a dose of 0.2% w/w in food. Food was provided in small jars ad libitum, and the amount consumed was measured; this resulted in an AQ dose of about 250 – 350 mg/kg/day.

*Rats*: INH and rifampicin (RMP) were dissolved in saline and given to Wistar rats by gavage (75 mg/kg/day and 50 mg/kg/day, respectively) for 4 weeks. In addition, 6 – 8 week old Wistar rats were gavaged with 150 mg/kg/day of INH only for 4 weeks. BN rats were treated with 150, 200, or 400 mg/kg/day of INH by gavage for up to 5 weeks as previously described.154 In all cases, saline alone was given to the control group.

**Depletion of T-regulatory (T_{reg}) cells:**

Depletion was carried out as previously described.155,156 Briefly, Cbl-b−/− mice were injected i.p. with 1 µg/mouse of 6 formylindolo(3,2-b)carbazole (FICZ; Enzo Life Sciences, Brockville, ON) or with 0.25 mg/mouse anti-CD25 antibody (BioXcell, West Lebanon, NH). INH was started two days from the first injection of FICZ or anti-CD25 antibody, and one additional subsequent injection with FICZ or anti-CD25 antibody were repeated one week after the first.

**Depletion of NK cells:**

Depletion was carried out by injecting 200 µg of anti-NK1.1 antibody/mouse at day -3 and -1 of treatment, AQ treatment was started on day 0, and injections of 200 µg of anti-NK1.1
antibody were repeated every week to maintain depletion of NK cells; IgG1 isotype control was used as vehicle.

**Western Blotting:**

Mouse liver microsomes (MLM) and rat liver microsomes (RLM) were prepared from male C57BL/6 mice or male BN rats. Briefly, liver was homogenised in phosphate buffered saline (pH 7.4) and centrifuged at 9,000g for 10 min at 4°C, the supernatant from this centrifugation (S9) was recovered and centrifuged again at 100,000g for 50 min at 4°C. The pellet from this last centrifugation contained the microsomes and was resuspended in 20% glycerol, 0.4% KCl in phosphate-buffered saline (pH 7.4). In vitro incubations of microsomes with INH utilized a microsome concentration of 0.5 mg/mL, and 10 µg of protein/lane was loaded on the gel for western blotting. For *in vivo* studies, the S9 fraction (supernatant after 9,000g centrifugation) was prepared in the presence of protease inhibitors (Sigma) and 20 µg of protein/lane was loaded on the gel. Other subcellular fractions involved differential centrifugation as previously described. Briefly, cell debris was separated by 2,500 g centrifugation for 10 min, the supernatant was centrifuged at 20,000 g for 10 min to collect the mitochondria, and centrifugation at 110,000 g for 1 h precipitated the microsomes. This last supernatant was collected as the cytosolic fraction. The protein was separated by electrophoresis (8% SDS-PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad, Mississauga, ON). Each western blot was repeated at least twice and each time the concentration of protein loaded was measured using the BCA kit. Rabbit anti-INH or rabbit anti-AQ antibody was used as the primary antibody and goat anti-rabbit IgG-peroxidase (Sigma) was used as the secondary antibody. Bound peroxidase was detected using Supersignal West Pico Chemiluminescent Substrate (Fisher Scientific). Mouse monoclonal anti-GAPDH
(Sigma) was used as the loading control and detected by goat anti-mouse IgG-peroxidase (Jackson ImmunoResearch; West Grove, PA). Super signal enhanced molecular weight markers were used (Fisher Scientific).

**Histopathology:**

At the endpoint, animals were sacrificed, livers perfused, and together with the spleen, were extracted and placed in 10% neutral-buffered formalin solution (Sigma; Oakville, ON) overnight. For preparation of frozen sections, liver tissue was placed in OCT medium (VWR International; Radnor, PA) and immediately frozen using liquid nitrogen. Slides were prepared and H&E staining was performed by the Department of Pathology at the Hospital for Sick Children (University of Toronto). Oil Red O staining was performed on frozen slides by the Department of Pathology at the Hospital for Sick Children (University of Toronto).

**Immunohistochemistry:**

Rat monoclonal primary antibodies against mouse CD11b (clone M1/70), F4/80 (clone Cl:A3-1), and CD45R (clone RA3-6B2) were purchased from Abcam (Cambridge, MA). Rabbit anti-INH antibody was produced as previously described. Rabbit anti-AQ was produced in our laboratory similarly to the anti-INH antibody; however, the work was carried out by another graduate student (Alexandra Lobach). Rabbit polyclonal antibody against mouse KI67 was also purchased from Abcam. Monoclonal antibodies against mouse CD4 (clone GK 1.5) and CD8 (clone YTS169) were donated by Pamela Ohashi`s laboratory, Princess Margaret Hospital, University of Toronto. The proliferating cell nuclear antigen (PCNA) kit was purchased from Invitrogen (Camarillo, CA), and mouse monoclonal antibody against rat CD68 (clone ED1) was purchased from Abcam. Polyclonal rabbit secondary antibody anti-rat IgG-
biotinylated, anti-mouse IgG-biotinylated and streptavidin-peroxidase were purchased from Dako (Burlington, ON). Goat anti-rabbit IgG-peroxidase was purchased from Sigma. Each experiment was repeated at least twice and the signal was developed using 3,3′-diaminobenzidine for paraffin-embedded slides or NovaRed for frozen slides (Vector; Burlington, ON) with Mayer’s hematoxylin (Sigma) as the counter stain. Paraffin-embedded slides were stained with antibodies against F4/80, CD45R, and KI-67. Antibodies against CD11b, CD4, and CD8 were used to stain frozen sections. Immunohistochemical grading was done by counting the number of cells per field of view under a microscope; at least two slices of tissue (3 – 6 mm²) were mounted on glass slides and 5 areas from each slice were counted under the microscope.

**Biochemical Measures of Liver Injury in Animals:**

Liver enzyme activities were measure by collecting blood from the saphenous vein in mice and from the tail vein in rats. As biomarkers of liver injury, the activity of alanine aminotransferase (ALT, Thermo Scientific, Middletown, VA) and sorbitol dehydrogenase (SDH, Catachem, Oxford, CT) were measured as described by the manufacturer. The method for glutamate dehydrogenase (GLDH, Randox, Crumlin, UK) was slightly modified where 200 µL of reagent 1 was premixed with 8 µL of reagent 2, 200 µL of this mixture was loaded into wells of a 96 well plate, and to this was added either 10 or 20 µL of serum. All the reagents were reconstituted as per manufacturer’s specification, and the absorbance was monitored for at least 5 min at 25 °C as described in the kit.
Correlation of GLDH/SDH Assay with ALT:

Because the ALT assay is inhibited by INH, and given the limited amount of blood that can be drawn from mice, the GLDH and SDH assays were optimized to decrease the amount of serum required for assessing liver injury. Treatment of Cbl-b^{-/-} mice with amodiaquine (AQ) produced liver injury which resulted in higher activities for ALT, SDH, and GLDH compared to controls (Figure 8A-C); this was also consistent with H&E staining, which showed infiltration of lymphocytes (Figure 8D, E). By using 10 µL of serum, SDH correlated well with ALT, and by modifying the GLDH assay, a good correlation with ALT was also observed ($R^2 = 0.74$ and 0.82, respectively; Figure 8F, G). Correlation of SDH with GLDH gave an $R^2 = 0.71$. As expected, the modified GLDH assay and SDH also correlated well with ALT when 20 µL of serum were used (Figure 9). Treatment of mice with acetaminophen also resulted in high values for the modified GLDH assay (Figure 10). In addition, we compared the fold difference in enzyme activities between control and mice treated with amodiaquine and found that the highest fold difference was for ALT (5.6 fold higher than control) followed by GLDH (2.1 fold higher than control) and SDH (1.7 fold higher than control).
Figure 8: Treatment of Cbl-b−/− mice with AQ produces mild liver injury. Amodiaquine hydrochloride was provided from IPCA Laboratories (Mumbai, India) and was also used as a positive control of liver injury and given to female Cbl-b−/− mice (18 – 20 g of weight) at 0.15% or 0.2% w/w in food. This resulted in an amodiaquine dose of about 225 – 350 mg/kg/day. A-C) Activities of ALT, SDH, and GLDH were compared between control female Cbl-b−/− mice (n = 4) or mice treated with either 0.15% or 0.2% AQ w/w in food for 22 days. D-E) H&E staining of the liver in control or AQ treated female Cbl-b−/− mice. The red arrow shows lymphocyte infiltration; the green arrow shows foamy hepatocytes. Magnification 40X. F-H) Correlation analysis between ALT, SDH, and GLDH in female Cbl-b−/− mice. Serum from the same mice as in parts A-C were used for analysis. Analysed for statistical significance by One-Way ANOVA with Dunns post-test to compare all pairs of columns. Statistical significant from control (*p < 0.05, **p < 0.01).
Figure 9: Activities and correlation analysis between ALT, SDH, and GLDH in female Cbl-b−/− mice. Control female Cbl-b−/− mice (n = 4) or treated with either 0.15% or 0.2% AQ w/w in food for 22 days.
**Figure 10:** ALT, SDH, and GLDH activities in mice treated with acetaminophen. Male Balb/c mice (n = 2) were treated with Acetaminophen (APAP). APAP (Sigma, Oakville, ON) was dissolved in saline and given to male Balb/c mice at a dose of 300 mg/kg by i.p injection. Mice were fasted 15 h prior to treatment and sacrificed 2 hours after acetaminophen administration.

**Human Subjects:**

Upon research ethics board approval, a total of 29 patients undergoing prophylaxis with INH were recruited by the Toronto Western hospital (Toronto, ON) between June 2010 and June 2013. After obtaining informed consent, blood was drawn into heparinised tubes from these 29 patients before the initiation of INH therapy to be used as baseline measurement, and patients were followed every month until they finished INH therapy. None of the 29 patients developed severe hepatotoxicity; five patients out of twenty nine developed a mild increase in alanine aminotransferase (ALT, 47 – 144 U/L). Patients with an abnormal ALT baseline were excluded from the study. Serum samples were also obtained from 19 patients enrolled in the Acute Liver Failure Study Group (ALFSG) registry and who were presumed to have INH-induced toxicity leading to encephalopathy and coagulopathy as stipulated by entry criteria for the study. Each patient’s clinical history was reviewed by the site principal investigator and by the study center principal investigator (WML) and were adjudicated as at least probable or higher (>50% likelihood) due to INH. Results of serum antinuclear antibodies (ANA),
antibodies against liver/kidney microsomes (LKM), anti-mitochondrial antibodies (AMA),
anti-smooth muscle antibodies (ASMA) and ALT were also available for some patients from
the ALFSG database as measured by the respective hospitals. One paraffin embedded block
from a patient who had severe hepatotoxicity due to INH was obtained from the Acute Liver
Failure Study Group (ALFSG) registry; this patient was determined to have INH-induced
toxicity leading to encephalopathy and coagulopathy as stipulated by entry criteria for the
study. In addition, as control slide: a patient with hepatitis B virus (HBV) was obtained by
ALFSG.

**Method for Detection of Anti-INH antibodies in Human Serum:**

Lysozyme (L; Sigma, Oakville, ON) was modified with an N-hydroxysuccinimide
activated ester of isonicotinic acid (INA-NHS) using a previously described procedure to give
lysozyme coupled to INH (L-INH)\textsuperscript{154}. The activated ester of isonicotinic acid and the reactive
metabolite of INH both react with amino groups on proteins and form the same product;
therefore, this method should mimic the covalent binding to proteins that occurs \textit{in vivo}\textsuperscript{154}. Briefly lysozyme (2.5 mL at 2 mg/mL solution in PBS pH 7.4) was incubated with 10 mg of
INA-NHS and reacted for 1 h at room temperature. The mixture was dialysed using a 1,000
MW cut-off filter. Protein concentration was measured by a bicinchoninic acid kit (Fisher
Scientific, Ottawa, ON) and 10 µg of protein/well was loaded on the gel. The protein was
separated by electrophoresis (8% SDS-PAGE) and transferred onto a nitrocellulose membrane
(Bio-Rad, Mississauga, ON). Human serum was used as the primary antibody diluted in tris-
buffered saline with Tween (TBST) pH = 7.4 containing 4% milk. The secondary antibody was
anti-human Ig-peroxidase (AbD Serotec, Raleigh, NC). As a positive control, rabbit anti-INH
antibody was used to detect INH bound to lysozyme, detected by goat anti-rabbit IgG-
peroxidase (Sigma) as described before. Bound peroxidase was detected using Supersignal West Pico Chemiluminescent Substrate (Fisher Scientific).

**Detection of Anti-CYP Antibodies in Human Serum:**

A pool of human liver microsomes (HLM) from 50 donors, CYP2E1, CYP3A4, and CYP2C9 were purchased from (BD Biosciences, Mississauga, ON). CYP2E1 was modified by the reactive metabolite of INH in two ways: 1) by reacting CYP2E1 with INA-NHS, which we refer to as CYP2E1-INH, in a similar manner to the modification of lysozyme and 2) by incubation of CYP2E1 (0.5 mg/mL) with INH (500 µM) and a NADPH-generating system (Solutions A and B; BD Biosciences) for 1 h at 37 ºC.

*Enzyme-linked immunosorbent assay (ELISA):* 96-well microtiter plates (Bethyl Laboratories, Montgomery, TX) were coated with 400 ng of protein overnight. Proteins included HLM, CYP2E1, CYP2E1-INH, CYP3A4, CYP2C9, lysozyme or L-INH. Human serum diluted 1:1000 in TBST containing 4% milk was used as primary antibody and allowed to sit overnight with slight agitation. As secondary antibody, polyclonal goat anti-human IgG-peroxidase (AbD Serotec) was used. Color was developed using 3,3’,5,5’-tetramethylbenzidine (TMB) solution and absorbance was monitored at 450 and 540 nm.

**Covalent Binding of INH to Human CYP Isozymes:**

INH (100 µM) was incubated with a total protein concentration of 0.5 mg/mL for CYP2E1, CYP2C9, or CYP3A4 for 30 min at 37°C in the presence or absence of an NADPH-generating system. After 30 min, the reaction was stopped by placing the reaction mixture on ice. Protein concentration was measured by BCA kit and 5 µg of protein/lane was loaded a gel. Covalent binding of INH to each CYP isozyme was determined by western blotting using the
anti-INH antibody that we have previously used to demonstrate INH binding to rodent liver and human liver microsomes.\textsuperscript{154}

**Flow Cytometry:**

**Cell Isolation from Various Organs**

**Cell Isolation from the Liver of Animals:**

Liver (from rat or mice) were perfused with phosphate-buffered saline (PBS) pH 7.4 and extracted. Single cell suspension was prepared by passing the liver through a 100µM cell strainer with a syringe plunger. The homogenate was centrifuged at 400g for 5 min, the pellet was resuspended in 35% Percoll (Mississauga, ON) made up in PBS. The lymphocytes were collected at the bottom after centrifugation of the 35% Percoll solution at 1000g for 15 min.

**Cell Isolation from the Spleen and Cervical Lymph Nodes of Animals:**

Half of the spleen and five cervical lymph nodes were collected from each rat or mouse. Single cell suspensions were prepared by passing the lymph nodes and spleen through a 100 µm cell strainer with a syringe plunger. The cell homogenate was washed twice with 10% fetal calf serum in PBS buffer.

**PBMC Isolation from Humans:**

Blood (10 mL) was collected from patients into a heparinised tube and mixed with 10 mL of phosphate buffered saline (PBS) pH = 7.4. The mixture was slowly overlaid on top of 15 mL of Ficoll (GE Healthcare, Cooksville, ON) and spun at 600xg for 30 min by setting the deceleration of the centrifuge to its lowest. The peripheral blood mononuclear cells (PBMCs) were pelleted and washed twice with 5 mL of cold PBS.
Cell Counting

Followed lymphocyte isolation, red cell lysis was performed followed by a second filtration through a 40 µm cell strainer, and then the cells were resuspended in 10% fetal calf serum in PBS buffer for cell counting. The number of lymphocytes was counted using the Countless Automated Cell Counter by Invitrogen (Life Technologies, Grand Island, NY), and 1 million cells were stained for surface markers with antibodies as shown below.

FACS Antibody Staining

Rat: CD3-PB (AbSerotec, Raleigh, NC), CD4-APCCY7 (Biolegend, San Diego, CA), CD8-PECY7 (eBiosciences, San Diego, CA), CD25-APC (eBioscience), CTLA4-PE (eBioscience) using the manufacturer’s protocols. For intracellular staining, one million cells/mL were suspended for 5 hr in culture medium which contained: 10% fetal bovine serum in RPMI medium, 50 ng phorbol myristate acetate/mL, 750 ng/mL ionomycin and 5 µL of golgi stop (BD Biosciences, Mississauga, ON) and stained with anti-rat IL-10-PE (BD Biosciences, Mississauga, ON) and anti-mouse IL-17-APC (eBioscience) in addition to CD3, CD4, and CD8 surface staining. As a viability marker E506 was used (eBioscience). The anti-mouse IL-17-APC antibody was previously tested in our laboratory to cross-reacts with rat IL-17.

Mouse: Anti-mouse CD11b-AF700 (Biolegend, San Diego, CA), anti-F4/80-PE (Life Technologies, Burlington, ON), CD4-PECY7 (eBioscience, San Diego, CA), CD8-APCCY7 (BD Biosciences, Mississauga, ON), NK1.1-FITC (Sunnybrook, Toronto, ON), CD62L-PE (Sunnybrook), CD44-Biotin (Sunnybrook), CD279-APC (BD Biosciences), CD152-Biotin (Sunnybrook), Streptavidin-PECY5 (eBioscience), and DAPI (Molecular Probes, Burlington, ON) as a marker of live cells at 4º C for 30 min. For macrophage phenotyping, cells were
washed three times with cold FACS buffer, fixed, and permeabilized with cold Perm/Fix buffer (eBioscience) for 30 min. Cells were washed again and incubated for 1 h at 4º C with anti-IL-10-PB (Cedarlane, Burlington, ON), anti-TNF-α-FITC (eBioscience), or anti-CCL2-APC (Cedarlane) in combinations to allow the correct measurement of each cell marker.

**Humans:** Th17 cell staining: One million cells/mL were suspended for 5 hr in culture medium which contained 10% fetal bovine serum in RPMI 1640 medium (Life Technologies, Burlington, ON), 50 ng phorbol myristate acetate/mL (Sigma, Oakville, ON), 750 ng/mL ionomycin (Sigma), and 5 µL of golgi stop (BD Biosciences, Mississauga, ON). After stimulation, cells were collected and nonspecific binding was blocked by incubating the cells for 20 min with 20 µL/one million cells of human Fc binding receptor inhibitor (eBioscience, San Diego, CA). Surface staining with antibodies against human CD3-APC/CY7 (Biolegend, San Diego, CA), CD4-eFluor@450 (eBioscience) and CD8-APC (eBiosciences) was performed first. Following surface staining, cells were washed, fixed, and resuspended in flow cytometry staining buffer (eBioscience) overnight. The next day intracellular staining was performed by spinning the cells down, pre-incubating them for 30 min with 1X permeabilization buffer (eBioscience) and reincubating cells with anti-human IL-17-FITC (eBioscience), IL-10-PE, and human Fc binding receptor inhibitor for 1 h in 1X permeabilization buffer. Cells were washed twice with 1X permeabilization buffer and resuspended in flow cytometry staining buffer for analysis. This procedure is similar to the one previously used for mice. For the analysis of IL-10, in patient 4 the cell percentages at revisit after 77 days was used as baseline because baseline measurements were not available for CD3⁺IL-10⁺ cells

**For Treg Cells:** After PBMC isolation. Cells were stained for CD3, CD4, CD8 (as described above) and CD25-Alex Fluor 488 and fixed by using the fixation/permeabilization
buffer (eBioscience) for 30 min. Cells were washed and stained for Foxp3-PE by using the 1X permeabilization buffer for 30 min. A total of 100,000 events were collected for analysis, the initial gate was drawn on the lymphocyte population, cell aggregates were gated out; the data was analysed using FlowJo software (Tree Star, Ashland, OR).

**Analysis of Flow Cytometry**

Analysis was performed by first gating on the lymphocyte population, doublets were gated out, and live cells were selected for analysis by gating on DAPI or E506 negative cells. Cells were analyzed in a flow cytometer (LSR II) recording 50,000 events per gate. Data was analyzed using the FlowJo software. Results are reported as percentages and total cell numbers, which are derived by multiplying the percentage of cells with the total number of lymphocytes from each organ.

**Serum Cytokine Measurements:**

Mouse serum cytokines in 25 µL of serum were measured using Bio-Rad’s Bio-Plex Pro Mouse Cytokine 23 Plex following the manufacturer’s instructions. Rat serum cytokines were measured using Millipore’s rat 23 cytokine/chemokine Milliplex map. Human serum cytokines were analysed using Bio-Rad’s Bio-Plex Pro Human Cytokine 27 plex. The cytokines measured are listed below.

**Mouse Cytokine 23 plex:** IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, eotaxin, GCSF, GMCSF, IFNγ, KC, MCP-1 (MCAF), MIP-1α, MIP-1β, RANTES, and TNF-α.
Rat Cytokine 23 map: eotaxin, GCSF, GMCSF, CXCL1 (GRO/KC), IFNγ, IL-10, IL-12p70, IL-13, IL-17, IL-18, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IP10, leptin, MCP-1, MIP1α, RANTES, TNF-α, and VEGF.

Human Cytokine 27 plex: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF. Five samples from patients who received INH but did not have an increase in ALT, five samples from patients who received INH and had a mild increase in ALT, and five patients who had INH-induced liver failure and anti-INH antibodies were analyzed.

Preparation of Haptenated S100-INA or S9-INA:

Modification of the S100 and S9 fractions were accomplished by adding 35 mg of the hydroxysuccinimide ester of isonicotinic acid, which mimics the covalent binding of the reactive metabolite of INH to lysine amino groups, to 25 mg of protein in PBS and stirring for 1 h at room temperature. The synthesis of the hydroxysuccinimide ester and procedure for reaction with protein was reported previously. The modification of S100 with the reactive ester of isonicotinic acid was confirmed by western blotting (Figure 11). These products are abbreviated S100-INA and S9-INA, respectively.
Figure 11: Western blot of S100 hepatic proteins and S100 proteins after reaction with an activated ester of isonicotinic acid (S100-INA) and visualized by an anti-INH antibody.

**Induction of Autoimmune Hepatitis:**

Induction of EAH was initiated by intraperitoneal immunization of mice with S100 or S100-INA at a concentration of 2.5 mg/mouse (4 mg/mouse was used for S9 or S9-INA) in 150 µL of PBS and emulsified with 150 µL of Freund’s adjuvant. Immunization was performed weekly for up to three times. The first immunization always involved complete Freund’s adjuvant (CFA) while subsequent immunizations involved incomplete Freund’s adjuvant (IFA). In contrast to Lohse et al., we used IFA for subsequent immunizations in order to reduce the discomfort to animals. The schedule of immunization and drug treatment are shown in Figure 12; the experiment was performed three times.
Figure 12: Schedule for immunization of mice with hepatic protein (S100 or S9) or INH-modified hepatic protein to mimic covalent binding of INH in the liver (S100-INA or S9-INA). Scheme 1) Immunization of mice with S100. C57BL/6 mice or Cbl-b⁻/⁻ mice were immunized with S100 or S100-INA in CFA once a week for three weeks. A) INH was given orally to C57BL/6 mice immediately after the first immunization; INH was stopped after 6 weeks of treatment, mice were allowed to be off drug for 4 weeks to allow CYP450 synthesis to recover due to CFA inhibition, and then mice were put back on the drug for another 6 weeks. In C57BL/6 mice, at the end of the first 6 weeks, one mouse was sacrificed from each group to look for evidence of autoimmune hepatitis. B) INH was given orally to Cbl-b⁻/⁻ mice immediately after the first immunization for two weeks; INH was stopped after the second week and mice were kept off drug for 2 weeks, then mice were put back on INH for another 4 weeks. Scheme 2) Mice were immunized 2X with S9 or S9-INA in CFA and 1 time with S9 or S9-INA in IFA. After immunization, mice were not treated with anything to allow CYP450 synthesis to recover from inhibitory effects of CFA. The drug was started after 4 weeks from the last immunization and mice were kept on INH for 3 weeks. Scheme 3) Mice were immunized one time with S100 or S100-INA in CFA and 2 times with S100 or S100-INA in IFA. Then mice were not treated with anything for 4 weeks after the last immunization to allow CYP450 synthesis to recover due to the inhibitory effects of CFA, after the 4 week period mice were treated with INH in food for 5 weeks.
Statistical Analysis:

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data were analysed using two-way ANOVA, one-way ANOVA (Kruskal-Wallis test), two-tailed non-parametric t-test or Mann-Whitney U test. A p value < 0.05 was considered significant (*p < 0.05; **p < 0.01; ***p < 0.001).
CHAPTER 3: RESULTS

3.1: Direct Oxidation and Covalent Binding of Isoniazid to Rodent Liver and Human Hepatic Microsomes: Humans Are More Like Mice than Rats

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All experiments and experimental design, manuscript writing, and data analysis were performed by Imir G. Metushi. The manuscript was edited by Dr. Jack Uetrecht. Dr. Nakagawa performed in vitro trapping studies of INH with lysine and glutathione to identify the reactive metabolite formed.

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Results

The reactive metabolite of INH was trapped with NAL in an incubation of HLM with a NADPH-generating system. Two products were observed (m/z 292 and 243), which corresponded to INA-NAL with a retention time of 9 min and an INH dimer (INA-INH) with a retention time of 9.9 min (Table 5 and Figure 13). The NAL adduct and INH dimer were identical on LC-MS/MS to the synthetic products. The fragmentation pattern of the protonated molecular ion of INA-INH (m/z 243) in the positive ion mode was: m/z 243 (0%), 137.0 (11%), 124.4 (31%), 121.3 (57%), 107 (13%), 105.1 (23%), 93.1 (47%), 79.1 (100%), 66.2 (27%) and that for the dimer, INA-NAL, in the negative ion mode were: m/z 292 (0%), 121.1 (11%), 77.9 (100%), 57.9 (7%). No glutathione adduct was detected when the NAL was replaced by glutathione. Given the structure of the product, it is likely that this reactive metabolite is a diazohydroxide that reacts with hard nucleophiles such as primary amines to form an amide.

<table>
<thead>
<tr>
<th>HLM</th>
<th>NADPH</th>
<th>INH</th>
<th>NAL</th>
<th>Adducts Observed</th>
<th>t_r (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No Peak</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>No Peak</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>INA-INH</td>
<td>9.9</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>INA-NAL</td>
<td>9.0</td>
</tr>
</tbody>
</table>

INA-INH = the INH dimer and INA-NAL = the N-α-acetyl-l-lysine adduct
An antibody was produced by immunization of rabbits with a protein modified by reaction with an activated ester of INA that should mimic the covalent binding of this diazohydroxide reactive metabolite of INH. This antibody was tested for specificity against INH and cross reactivity with binding of AcHz by both ELISA and western blotting (Figure 14). The antibody detected INH binding to many hepatic proteins and the binding was specific; i.e., no binding was observed to hepatic proteins from untreated controls, and binding was blocked by preincubation of the serum with INH.
Figure 14: Specificity of the anti-INH antibody. A) Antibody specificity was tested by ELISA. The plate was either coated with BSA modified with INA (BSA-INA) or BSA alone. Pre-immune serum (S_{Pre}) or serum after immunization with Blue Carrier Protein modified with INA (S_{Aft}) diluted at 1:100,000 was used as the primary antibody. In the 3rd and 5th column, the primary antibody was preincubated with INH or NAL, respectively at a concentration of 200 µM for 30 min at room temperature. B) Antibody was tested for cross reactivity with binding due to AcHz. Female C57BL/6 mice were treated with either INH or AcHz (Fisher Scientific) by gavage for 7 days at 50 mg/kg/day (n = 2 for each group). There was no binding to hepatic proteins from untreated control and AcHz-treated mice, whereas hepatic proteins from INH-treated mice showed a large number of bands modified with INH. C) Antiserum was tested for specificity on western blots by pre-incubation with INH at 200 µM or 2 mM for 1 hr at 4 ºC which prevented binding to the INH-modified hepatic proteins. D) Binding of the anti-INH serum to INH-modified liver proteins on western blots was compared to that of the pre-immune serum from the same animal.

We found that after chronic treatment of mice with INH, a large amount of INH was bound to the livers of male C57BL/6 mice (Figure 15A). This was also true for female C57BL/6 and Balb/c mice (Figure S2). No major differences were observed between the degree of covalent binding in male vs. female C57BL/6 mice (Figure 15B). There may be slightly more INH binding in female C57BL/6 than female Balb/c mice, but the duration of treatment was also longer (Figure 15C), and there was a similar difference in binding between male and female Balb/c mice (Figure S2). The amount of covalent binding increased with each dose of INH with day 7 having much greater covalent binding than days 1 and 3, and the
amount of binding was greatest when the drug was given in food for three weeks (Figure 15D). Presumably this reflects the half-lives of the proteins modified. Immunohistochemical analysis showed that the binding was mostly centrilobular (Figure 15E,F).

Figure 15: Covalent binding of INH to hepatic proteins in mice. A) Male C57BL/6 (n = 4) untreated controls or treated with INH (0.2% of INH by weight in food) for 5 weeks. B) Male vs. female C57BL/6 (n = 4) treated with INH (0.2% of INH by weight in food) for 5 weeks. C) Female C57BL/6 mice (n = 3) treated for 5 weeks vs. Balb/c mice (n = 3) treated for 3 weeks, both with 0.2% INH by weight in food. D) Female Balb/c mice treated with INH; either by gavage at 100 mg/kg/day (n = 2) for a period of 1, 3 or 7 days or with 0.2% INH by weight in food for 3 weeks. E, F) Immunohistochemical staining in the livers of female C57BL/6 mice untreated control vs. treated with 0.2% INH by weight in food for 5 weeks. 5X magnification.
Covalent binding also occurred in rats, both BN and Wistar rats (Figure 16A,B), with Wistar rats having a slightly greater amount of covalent binding than BN rats even though Wistar rats were treated for four weeks instead of five (Figure 16C). The densest bands were observed at about 37 and 50 kDa. In BN rats, there was not much difference in the amount of covalent binding when the drug was given by gavage rather than in food (Figure 16A). In comparison to mice, the covalent binding of INH to hepatic proteins in rats was less (Figure 16D). Because of this, the sensitivity had to be increased, which led to noticeable artefact bands in proteins from untreated control rats; however, the difference between treated and control animals was clear (Figure 16A,B).

**Figure 16:** Covalent binding of INH to hepatic proteins in rats. A) BN rats (n = 3) untreated controls or treated with INH either by gavage at a dose of 150 mg/kg/day or with 0.2% INH by weight in food for 5 weeks. B) Wistar rats (n = 4) untreated controls or treated with INH by gavage at 150 mg/kg/day for 4 weeks. C) Comparison of covalent binding between male Wistar rats and male BN rats (n = 4) treated with INH by gavage at a dose of 150 mg/kg/day for 4 and 5 weeks respectively. D) Comparison of covalent binding between male C57BL/6 mice (n =4) treated with 0.2% INH by weight in food for 5 weeks vs. male Wistar rats (n = 4) treated with INH by gavage at a dose of 150 mg/kg/day for 4 weeks.
*In vitro* Binding of INH to Human, Mouse, and Rat Liver Microsomes

To relate our *in vivo* animal studies to humans, we investigated the oxidation of INH by HLM to a reactive metabolite. INH binding to HLM was greater at 100 µM INH than 10 µM (Figure 17A). There was a small amount of INH binding to HLM in the absence of a NADPH-generating system (Figure 17A). This could be due to a small amount of endogenous peroxidase activity or to nucleophilic attack on proteins by the hydrazine group of INH. Binding of INH to MLM was also apparent and is consistent with the *in vivo* studies (Figure 17B). The higher concentrations are above therapeutic serum concentrations, but such concentrations may occur in the liver clinically during first pass through the liver. Covalent binding of INH to HLM was less than to MLM and it was time dependent, and concentration dependent (Figure 17C-F). The difference between HLM and MLM was more apparent with the higher concentrations of INH. A comparison between rat, mouse, and human liver microsomes revealed that the amount of covalent binding is greatest in mouse followed by rat and human liver microsomes (Figure 18).
Figure 17: *In vitro* covalent binding of INH to hepatic microsomes. A) To HLM with and without an NADPH-generating system. B) To MLM with and without a NADPH-generating system. C) To HLM as a function of time and INH concentration. D) To MLM as a function of time and INH concentration. E-F) direct comparison of binding between HLM and MLM at INH concentrations of 10 and 100 µM, respectively.
Treatment of Wistar and BN Rats with INH

In other studies we have found BN rats to be more sensitive to immune-mediated reactions than other strains;\textsuperscript{162} therefore, we treated male BN rats with INH using a variety of protocols to try to develop an animal model. A dose of 200 mg/kg/day caused rats to appear ill after 7 days, but again histology only showed signs of steatosis. Lower doses such as 150 mg/kg/day and 50 mg/kg twice daily or INH given in food for up to five weeks also did not lead to an increase in SDH with the exception of when INH was given by gavage, and then after the first week the SDH returned to normal despite continued treatment (Figure 19A).
Figure 19: Serum SDH activities and body weights in INH-treated BN rats. A) SDH activity in BN rats treated with INH by gavage at 150 mg/kg/day, 50 mg/kg twice daily or given at 0.2% INH by weight in food. B) Body weight of BN rats given INH as in part A. Values represent the mean ± S.E. Analysed for statistical significance by two-way ANOVA. Significantly different from control group (*p < 0.05; **p < 0.01; ***p < 0.001).
An experiment in which INH was administered by gavage twice daily had to be discontinued at the end of the first week because the animals lost significant weight mainly due to reduced food intake (Table 6).

**Table 6: Food consumption in mice/rats and daily INH dose**

<table>
<thead>
<tr>
<th>BN Rats</th>
<th>Food Consumption (g/day)</th>
<th>Avg Animal Weight Range (g)</th>
<th>Avg INH Intake in mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.6 ± 1.1</td>
<td>240 - 300</td>
<td>-</td>
</tr>
<tr>
<td>Gavaged at 150 mg/kg/day</td>
<td>4.7 ± 1.0 ***</td>
<td>220 - 240</td>
<td>-</td>
</tr>
<tr>
<td>Gavaged at 50 mg/kg twice daily</td>
<td>3.5 ± 2.0 ***</td>
<td>180 - 240</td>
<td>-</td>
</tr>
<tr>
<td>INH given in food</td>
<td>9.1 ± 0.6 ***</td>
<td>210 - 240</td>
<td>79 – 87</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C57BL/6 Mice</th>
<th>Male</th>
<th></th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.0 ± 0.3</td>
<td>22 - 26</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>INH</td>
<td>4.6 ± 0.3</td>
<td>22 - 24</td>
<td>4.2 ± 0.3 **</td>
</tr>
<tr>
<td>Male Balb/c</td>
<td>4.6 ± 0.3</td>
<td>22 - 23</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>INH</td>
<td>2.9 ± 0.2 ***</td>
<td>18 - 20</td>
<td>3.1 ± 0.3 ***</td>
</tr>
</tbody>
</table>

INH was given to BN rats (n = 4) by gavage as specified or at 0.2% INH by weight in food for 5 weeks maximum. C57BL/6 (n = 4) mice were treated with INH at 0.2% by weight in food for 5 weeks and Balb/c (n = 4) were treated at the same dose for 3 weeks. Animal body weight range was estimated from Figures 5 & 6 and the amount of food consumed was calculated based on weight range and by assuming homogenous mixing of drug with food. Values represent as mean ± Standard Error of the Mean (S.E.) with 4 animals per group. Analysed for statistical significance by Mann-Whitney U test. Significantly different from control group (*p < 0.05; **p < 0.01; ***p < 0.001).

The group that was gavaged at an INH dose of 150 mg/kg/day also lost weight after one week; however, at the end of the first week, powdered food was mixed with water to make the food more palatable, and this resulted in the rats eating food again and they could be treated for up to 5 weeks (Figure 19B). At the endpoint, ALT and GLDH were also measured in addition to SDH in all groups, but no change in SDH or GLDH was observed but rather a decrease in
ALT was seen when the drug was given in food and by gavage (Table S1). The decrease in ALT levels in treated animals is consistent with the fact that the ALT assay is inhibited by drugs such as INH that can react with pyridoxal-5’-phosphate. This is because INH forms a Schiff base with pyridoxal-5’-phosphate, a cofactor for the ALT assay. We recently demonstrated the inhibition of the ALT assay where BN rats were dosed with INH (400 mg/kg/day) for up to 7 days, which produced systemic toxicity. As predicted, ALT levels were lower in treated rats, but the SDH levels were elevated. The H&E slides from BN rats dosed with 400 mg/kg/day showed signs of steatosis but no other evidence of liver injury (Figure S3). Treatment of Wistar rats for up to 4 weeks with 150 mg/kg/day of INH by gavage produced no obvious signs of liver injury and no steatosis as seen by H and E staining.

In none of the groups were we able to find evidence of liver injury that represented an animal model with hepatotoxicity similar to that which occurs in humans. The biochemical findings were consistent with the H&E staining that showed no necrosis or inflammatory cell infiltrate, although some steatosis was observed when BN rats were gavaged with INH at a dose of 200 or 400 mg/kg/day for 7 days (Figure S3), but it resolved despite continued treatment when INH was given for up to 5 weeks either by gavage or in food.

**Treatment of C57BL/6 and Balb/c Mice with INH**

Because INH failed to cause significant liver injury in rats and we found greater covalent binding of INH in mice, we also investigated INH-induced liver injury in mice. Treatment of C57BL/6 mice with INH for up to 5 weeks did not result in an increase in GLDH (Figure 20A,B). In contrast, GLDH levels were elevated in Balb/c mice, with male Balb/c mice having a greater increase at week 1 (Figure 20E) and female Balb/c mice at week 3 (Figure 20F).
Figure 20: GLDH activities and body weights in mice. A-D) Male and female C57BL/6 mice were treated at 0.2% INH by weight in food for 5 weeks. E-H) Male and female Balb/c mice were treated at 0.2% INH by weight in food for 3 weeks. Values represent the mean ± S.E. from 4 animals per group. Analysed for statistical significance by two-way ANOVA. Significantly different from control group (*p < 0.05; **p < 0.01; ***p < 0.001).
At the 3 week endpoint, SDH was only elevated in female Balb/c mice, while ALT was generally decreased in all the treated groups (Table S2). Liver histology was normal in Balb/c mice and C57BL/6 (Figure S3). Food intake was decreased in Balb/c mice (Table 6) resulting in a significant decrease in body weight (Figure 20G,H). In general C57BL/6 mice ate more food that was mixed with drug than Balb/c mice and this resulted in a higher body weight, higher INH dose, and C57BL/6 mice could be treated for longer. We gave the drug in food because INH has a short half-life and this provided a more consistent blood level than once-a-day oral gavage. Given the amount of food that mice and rats consume, and based on their average body weight, the mice received a greater dose of INH (about 400 mg/kg/day) than rats (about 90 mg/kg/day, Table 6). This method of drug administration produced blood levels in mice that were comparable to the \( C_{\text{max}} \) in humans.\(^{164}\) To compare INH and its metabolite blood levels in mice vs. rats we treated male C57BL/6 and male BN rats with INH in food for one week. Mice had INH blood levels of \( 4.5 \pm 0.9 \mu g/mL \), which is comparable to the \( C_{\text{max}} \) in humans,\(^{164}\) but rats had about three fold lower INH concentrations (\( 1.5 \pm 0.1 \mu g/mL \); Figure 21A). In contrast, rats had more than double the concentration of AcHz compared to mice (Rat/Mouse AcHz ratio of 2.5) while the concentration of Hz was only slightly higher in rats compared to mice (Rat/Mouse Hz ratio of 1.5). This resulted in a higher INH to AcHz ratio in mice (Figure 21B) indicating that the relative exposure of mice and rats to INH and AcHz is quite different.
Figure 21: Serum concentrations of INH and INH/AcHz ratio in mice and rats. INH was given at a dose of 0.2% by weight in food for up to one week. Values represent the mean ± S.E; for mice (n = 5) and for rats (n = 4). Analysed for statistical significance by Mann-Whitney U test. Significantly different groups (*p < 0.05).
**Discussion**

Based on acute toxicity studies in rats from several decades ago, it is generally accepted that the hepatotoxic effects of INH are due to bioactivation of AcHz.\textsuperscript{100,129,133,165} However, the present study demonstrates that INH itself can be oxidized to a reactive metabolite that binds to hepatic proteins. The ability to trap this reactive metabolite with NAL and the structure of the adduct suggest that the metabolite responsible for the binding is a diazohydroxide (Figure 22).

![Proposed bioactivation pathway of INH.](image)

Figure 22: Proposed bioactivation pathway of INH.

The alternative carbocation formed by the loss of nitrogen would have an extraordinarily short half-life\textsuperscript{166} making it difficult to trap with a nucleophile and unlikely to bind \textit{in vivo} to any proteins other than the enzyme that formed it; however, it is conceivable that the diazene is susceptible to nucleophilic attack. This is also not the chemistry that would be expected of a free radical intermediate. The chemistry of INH and AcHz oxidation is similar; therefore, their relative contribution to covalent binding would be determined by the relative rates of acetylation, hydrolysis, the affinities of P450 isozymes for the two hydrazides, and the relative contributions of other clearance pathways such as pyruvate conjugation. These
parameters are likely to be species dependent, and although we do not have quantitative data for all of these parameters, the relative concentration of AcHz was greater in rats than mice while the covalent binding and blood levels of INH were greater in mice (Figure 16D and Figure 21A). The contribution of INH bioactivation relative to that of AcHz to covalent binding is presumably greater in mice than rats; therefore, human slow acetylators, who are at increased risk of hepatotoxicity, are more comparable to mice. This makes conclusions based on studies in rats suspect.

With this information about the bioactivation of INH we tried to develop an animal model of INH-induced hepatotoxicity with characteristics similar to those of INH-induced liver injury in humans. The typical features of INH-induced hepatotoxicity in humans include a delay in onset, and in more severe cases of liver injury the histopathology is associated with centrilobular necrosis with a mild lymphocytic infiltrate, often with eosinophils. We found that ALT was not a reliable assay to measure INH liver injury because INH interferes with the assay as previously reported. Even though this has been reported it is important to note because this problem is not generally known, and ALT is commonly used in studies of INH-induced liver injury, both in animals and humans. This may be less of an issue in humans because it is commonly recognized that INH depletes pyridoxal phosphate leading to peripheral neuropathies and so patients are usually, but not always, given vitamin B6 to prevent this problem. In subsequent studies we used SDH or GLDH rather than to try to modify the ALT assay.

Another difficulty in developing an animal model of INH-induced hepatotoxicity is the route of administration. Because of its short half life, it was found that smaller, more frequent doses of INH lead to greater hepatotoxicity than one large dose. Treatment of Wistar rats did not display any signs of hepatotoxicity as seen by H & E staining. We found that in BN rats,
high doses of INH (200 and 400 mg/kg/day) led to signs of central nervous system (CNS) toxicity. A smaller dose of 150 mg/kg/day decreased food intake and weight gain (Figure 19F) so animals could be maintained for longer, but even 50 mg/kg given twice daily lead to signs of CNS toxicity. When INH was administered in food it produced INH blood levels in mice that were comparable to therapeutic levels in humans, but the blood levels in rats at the same dose were less than half those in mice (Figure 21A). Thus administration of INH to rats in food led to fewer signs of CNS toxicity, and animals could be maintained for five weeks. Because of the short half-life of INH, this method of administration has the advantage of providing more constant blood levels. However, despite trying different species and strains of animals and experimentation with dose and mode of administration, we were not able to develop an animal model of INH-induced liver injury similar to what occurs in humans. We did see evidence of steatosis that resolved despite continued treatment, which suggests that INH can cause mitochondrial injury (Figure S3). This is consistent with other published studies where co-treatment of INH with rifampicin caused steatosis and mitochondria oxidative stress in mice and rats. The amount of covalent binding was not the only predictor of hepatotoxicity because INH binding was similar to both C57BL/6 and Balb/c (Figure 15C), but Balb/c mice had a greater increase in GLDH and SDH levels (Figure 20 and Table S2). Although there was liver injury with some treatments, in no case did it appear to be a good model of the idiosyncratic liver injury observed in humans. Unfortunately, this inability to reproduce idiosyncratic drug reactions in animals is typical of such studies.

How do these data relate to INH-induced liver injury in humans? Most studies indicate that slow acetylators are at increased risk of INH-induced liver injury. The fact that slow acetylators appear to be at increased risk has been explained on the basis that they are exposed to more AcHz. This is because, although slow acetylators form less AcHz, clearance of AcHz
by a second acetylation is also slower, and the net result is a small increase in AcHz exposure.\textsuperscript{131,169} However, the difference in blood levels of INH between fast and slow acetylators is even greater than that of AcHz\textsuperscript{131,164}, therefore, the increased risk associated with the slow acetylator phenotype in humans is more easily explained if a reactive metabolite of INH is responsible for the liver injury.

Although INH-induced liver injury has been classed as metabolic idiosyncrasy, implying that it is not immune-mediated, there are cases which have clear evidence of an immune mechanism with immediate fever and increase in ALT on re-challenge.\textsuperscript{107} An immune mechanism is also the easiest way to explain the idiosyncratic nature of INH-induced liver injury, and the absence of features of a hypersensitivity reaction is not good evidence against an immune mechanism. Furthermore, we have found that patients treated with INH who have an increase in ALT also have an increase in Th17 cells which provides additional evidence for involvement of the immune system (unpublished observations). In addition, there is direct evidence for an immune mechanism involving covalent binding of INH. Specifically, Warrington et al. found that lymphocytes from patients with mild INH-induced liver injury proliferated when incubated with INH- or INA-modified protein but not to INH itself.\textsuperscript{117,142} If the injury was more severe, the lymphocytes also proliferated when incubated with INH itself. This positive lymphocyte transformation test provides strong evidence that INH-induced liver injury is mediated by the adaptive immune system, and furthermore, the recognition of INH-modified proteins suggests that the immune response is against INH-modified proteins, which fits nicely with our observation that INH binds to human hepatic proteins. In more severe cases the immune response spreads so that the parent drug is also recognized. In addition, although it has yet to be replicated, the finding of an increased risk of INH-induced liver injury in patients who have the HLA-DQB1 gene *0201 provides additional evidence for an immune
mechanism.\textsuperscript{128} It also seems that modification of protein by INH would be more likely to induce an immune response than would acetylation (the result of AcHz reactive metabolite binding) because its structure is more “foreign”. However, cell damage caused by bioactivation of AcHz could also contribute to the induction of an immune response; therefore, both reactive metabolites may play a role in INH-induced liver injury. In addition, INH and AcHz could also be oxidized to free radicals that cause cell injury that would not be detected by covalent binding assays but could contribute to cell damage and the induction of an immune response.

In conclusion, in contrast to previous studies in rats, we found that INH is oxidized to a reactive metabolite that covalently binds to mouse and human hepatic proteins. This is consistent with previous studies that found that lymphocytes from patients with INH-induced liver injury responded to INH or INH-modified proteins. Also, in contrast to previous assertions that INH-induced liver injury is not immune-mediated, the lymphocyte transformation test data along with other data such as clinical cases that have features of an immune response and an association with a specific HLA genotype provide evidence that INH-induced liver injury is immune-mediated.
Acknowledgments

Imir G Metushi is current trainee of the “Drug Safety and Effectiveness Cross-Disciplinary Training” program which is funded by CIHR. Jack Uetrecht holds the Canada’s Research Chair in Adverse Drug Reactions.
Significance

After over 30 years of considering INH-induced hepatotoxicity to be due to a reactive metabolite of acetylhydrazine, I was able to successfully synthesize an antibody that recognizes INH bound to proteins and demonstrate that a reactive metabolite of the parent drug binds to hepatic proteins. The likely reactive metabolite responsible for this binding involves a 4 electron oxidation to form a diazohydroxide because we could trap the reactive metabolite with lysine; the alternative carbocation or free radical metabolites would not be trapped in this way. In addition, mice seemed to be the better model for humans than rats, which had been use for the previous studies, because there was much more binding to mouse and human hepatic proteins than to rat hepatic proteins. We found that the ALT assay is inhibited by INH so when working with INH, the ALT assay should be avoided.

This study, changes the way that hepatologists look at INH-induced hepatotoxicity because our finding that the parent drug can form covalent adducts with proteins is in agreement with previous studies from Warrington that found that it is INH, not N-acetylhydrazine that lymphocytes from patients with INH-induced IDILI recognize. Together these studies provide strong evidence that INH-induced IDILI is immune-mediated and it is INH-modified proteins that the immune response is directed against.
3.2: Detection of Anti-Isoniazid and Anti-CYP Antibodies in Patients with Isoniazid-Induced Liver Failure

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2. Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada
3. Division of Digestive and Liver Disease, Department of Internal Medicine, UT Southwestern Medical Center, Dallas, Texas

All experiments and experimental design, manuscript writing, and data analysis were performed by Imir G. Metushi. Corron Sanders helped in co-ordinating and sending samples from patients with INH-induced liver failure. Dr. William M. Lee helped provide the serum samples from patients with INH-induced liver failure and edit the manuscript. Dr. Jack Uetrecht helped design the study and edited the manuscript.

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Results

Lysozyme was modified by reacting it with an activated ester of isonicotinic acid to give L-INH (Figure 23); this mimics the *in vivo* bioactivation and covalent binding of INH to protein and is similar to what we previously used to make an anti-INH antibody that was used for these western blots\(^{154}\).

![Western blot image](image)

**Figure 23:** Western blot of lysozyme (L) or lysozyme modified by INA-NHS (L-INH), which mimics the covalent binding of the reactive metabolite of INH. Detection was with an anti-INH antibody as previously described\(^{154}\).

We also modified other proteins such as human serum albumin and Blue Carrier Immunogenic Protein (Fisher Scientific) with INA-NHS analogous to lysozyme or used a liver homogenate from untreated or INH-treated mice instead of L-INH in order to detect anti-INH antibodies; however, when control sera (sera obtained from patients before the start of INH prophylaxis as described in the Methods) were used as the primary antibody for the western blots, we observed extensive background binding that precluded the use of these proteins for detection of anti-INH antibodies (Figure S4). This was in contrast to lysozyme and L-INH in which there was no binding of control sera, and therefore L-INH was used in subsequent experiments. Preliminary experiments using western blots showed the presence of anti-INH
antibodies in the serum of two INH-induced liver failure patients, but not in a patient who had been treated with INH and developed mild liver injury (ALT = 144 U/L; Figure 24).

Figure 24: Detection of anti-INH antibodies by western blotting. L = lysozyme and L-INH = INH-modified lysozyme. Serum was diluted 1:400; #1-0 = control serum from a patient at baseline, #1-2 = serum from the same patient 98 days after initiation of therapy (ALT = 144 U/L). ALF-19 and ALF-3 are sera from two patients with INH-induced liver failure.

However, not all of the patients who had liver failure had anti-INH antibodies. We used ELISA to screen for the presence of anti-INH antibodies in all of the available serum samples. In addition to patients with liver failure, 20 patients undergoing prophylactic INH treatment without severe liver injury were tested; only 5/20 of these patients had a mild increase in ALT (between 47 and 144 U/L which was associated with an increase in peripheral Th17 cells); however, none of these 20 patients had detectable anti-INH antibodies (Figure 25A). Given the positive preliminary results that detected anti-INH antibodies by western blotting, we used ELISA to screen all of the sera that were available from patients who had been diagnosed as having INH-induced liver failure for anti-INH antibodies. Of these 19 serum samples, 8 tested positive for the presence of anti-INH antibodies (Figure 25A). Preincubation of the serum from those 8 patients who had anti-INH antibodies with INH prevented binding, which indicated that binding was specific for INH-modified proteins (Figure 25B). In addition, all sera that were positive for anti-INH antibodies by ELISA were also analyzed for anti-INH antibodies by
western blotting which confirmed the ELISA results. Specifically, in all cases incubation of the western blots with sera from patients who tested positive for anti-INH antibodies by ELISA showed a strong band that appeared only on the lanes loaded with INH-modified lysozyme, but not with lysozyme itself, and preincubation of the human serum with INH almost completely eliminated binding indicating specificity of antiserum for INH (Figure 25C).
Figure 25: Detection of anti-INH antibodies by ELISA and western blotting. A) Baseline sera before initiation of INH therapy (Baseline), sera from patients who were treated with INH but did not develop significant hepatotoxicity (Prophylaxis), sera from patients who developed INH-induced liver failure but did not test positive for anti-INH antibodies (Liver Failure -) and sera from patients who had liver failure and tested positive for anti-INH antibodies (Liver Failure +) were diluted 1:1000. The ELISA plate was coated either with lysozyme (L) or INH-modified lysozyme (L-INH); the ratio of OD absorbance from L-INH/L was used to determine the presence of anti-INH antibodies. B) To determine if the antibodies in the sera from the 8 patients that tested positive for anti-INH antibodies were, in fact, specific for INH, the sera were preincubated with 200 µM INH for 1 h at 4 ºC (+INH) and this blocked the binding. C) The presence of anti-INH antibodies in the remaining 6 serum samples that tested positive for anti-INH antibodies by ELISA were confirmed by western blotting. Either lysozyme (L) or INH-modified lysozyme (L-INH) was loaded on a gel and transferred into a nitrocellulose membrane as described in the Methods section. Poncau S staining is shown as the loading control. Serum was diluted 1:400; + INH = serum was preincubated with 1 mM INH at 4 ºC for 1 h. Values represent Mean ± S.E. Statistically significant from control **p < 0.01.
Four serum samples from patients on INH prophylaxis and 4 from patients with liver failure that did not have anti-INH antibodies as determined by ELISA were analyzed by western blotting and there were no bands corresponding to anti-INH antibodies thus confirming the ELISA results (Figure S5). Demographic data from patients who were on INH prophylaxis only and patients with acute liver failure who had taken INH are shown in Table 7 and Table 8. No correlation between the presence of anti-INH antibodies and other patient characteristics were found.

**Table 7: Demographic data in patients with mild or no liver injury due to INH prophylaxis only.**

<table>
<thead>
<tr>
<th>#</th>
<th>Gender</th>
<th>Country of Origin</th>
<th>Age</th>
<th>Days on INH</th>
<th>Peak ALT (U/L)/AST (U/L)/Bilirubin (µmol/L)</th>
<th>Year Enrolled</th>
<th>Concomitant Disease</th>
<th>Other Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>Philippines</td>
<td>56</td>
<td>98</td>
<td>144/63/11</td>
<td>2010</td>
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</tr>
<tr>
<td>2</td>
<td>F</td>
<td>Vietnam</td>
<td>47</td>
<td>231</td>
<td>73/52/10</td>
<td>2010</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>Iraq</td>
<td>43</td>
<td>205</td>
<td>59/41/9</td>
<td>2010</td>
<td>Hypertension</td>
<td>Lisinopril</td>
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<tr>
<td>4</td>
<td>M</td>
<td>Canada</td>
<td>20</td>
<td>77</td>
<td>47/36/12</td>
<td>2011</td>
<td>Topical acne product – name unknown</td>
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</tr>
<tr>
<td>5</td>
<td>M</td>
<td>Canada</td>
<td>19</td>
<td>64</td>
<td>52/28/8</td>
<td>2011</td>
<td></td>
<td>Vitamin C, D</td>
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<tr>
<td>6</td>
<td>F</td>
<td>Philippines</td>
<td>38</td>
<td>56</td>
<td></td>
<td>2010</td>
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<td>43</td>
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<td>8</td>
<td>F</td>
<td>South Korea</td>
<td>25</td>
<td>196</td>
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<td>Multivitamin</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>China</td>
<td>24</td>
<td>221</td>
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<tr>
<td>10</td>
<td>M</td>
<td>Ghana</td>
<td>54</td>
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<td>Hypertension, High Cholesterol</td>
<td>Alfase, Lipitor, Lipidil, ASA</td>
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<td>11</td>
<td>F</td>
<td>Moldova</td>
<td>28</td>
<td>238</td>
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<td>F</td>
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<td>M</td>
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<td>28</td>
<td></td>
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<td>Advil</td>
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<td>17</td>
<td>F</td>
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<td>170</td>
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<td>2011</td>
<td>Asthma</td>
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<td>18</td>
<td>M</td>
<td>Pakistan</td>
<td>41</td>
<td>75</td>
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<td>F</td>
<td>Philippines</td>
<td>48</td>
<td>28</td>
<td></td>
<td>2012</td>
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<tr>
<td>20</td>
<td>F</td>
<td>Brazil</td>
<td>24</td>
<td>35</td>
<td></td>
<td>2012</td>
<td>Bulimia X 8 years</td>
<td>Vitamin B12</td>
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The serum assayed for the patients who had mild liver injury due to INH (n = 5) was when their ALT levels were highest. All the other patients had normal liver function tests before (baseline) and after treatment with INH for the duration of days specified above. M = male; F = female
Table 8: Demographic data in patients with liver failure.

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<th>Patient ID</th>
<th>Gender</th>
<th>Race</th>
<th>Age</th>
<th>Year Enrolled</th>
<th>Medications Taken</th>
<th>Total Dose</th>
<th>Duration</th>
<th>Date Last Taken</th>
<th>Concomitant Disease</th>
</tr>
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<td>ALF-1</td>
<td>F</td>
<td>African American</td>
<td>65</td>
<td>2007</td>
<td>INH 300 mg/day</td>
<td>8 mo</td>
<td>-5</td>
<td>-5</td>
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</tr>
<tr>
<td>ALF-2</td>
<td>F</td>
<td>African American</td>
<td>63</td>
<td>2001</td>
<td>INH 300 mg/day</td>
<td>1 mo</td>
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<tr>
<td>ALF-3</td>
<td>F</td>
<td>African American</td>
<td>21</td>
<td>1998</td>
<td>INH 300 mg/day</td>
<td>5 mo</td>
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<td>-16</td>
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</tr>
<tr>
<td>ALF-4</td>
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<td>White</td>
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<td>2001</td>
<td>INH 300 mg/day</td>
<td>30 days</td>
<td>-8</td>
<td>-8</td>
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</tr>
<tr>
<td>ALF-5</td>
<td>F</td>
<td>White</td>
<td>59</td>
<td>2002</td>
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<td>2 mo</td>
<td>-19</td>
<td>-19</td>
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<tr>
<td>ALF-6*</td>
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<td>Other</td>
<td>41</td>
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<td>6 mo</td>
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<td>-25</td>
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<td>28</td>
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<td>Patient ID</td>
<td>Gender</td>
<td>Race</td>
<td>Age</td>
<td>Year Enrolled In Study</td>
<td>Medications Taken</td>
<td>Total Dose</td>
<td>Duration</td>
<td>Date Last taken</td>
<td>Date Last taken expressed as number of days from enrolment</td>
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<td>diabetes, high cholesterol, hypertension</td>
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<td>Amoxicillin 500 mg/day</td>
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<td>3 mo</td>
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<td>RMP 300 mg/day</td>
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</tbody>
</table>

Bold indicates patients who tested positive for the presence of anti-INH antibodies. ALF = Acute liver failure. M = male; F = female; INH = isoniazid; RMP = rifampicin; PZA = pyrazinamide; mo = months. Concomitant disease were anything from collagen/vascular disease, chronic liver disease, endocrine/diabetes, psychiatric disease, neurological, hypertension, heart disease, renal, pulmonary disease, substance abuse, GI disease, HIV/AIDS, intravenous drug use at any time in past, other, or none.
We looked for antibodies against human liver microsomes (HLM) because in a previous study we found that they bioactivate INH; however, as with mouse liver homogenate high background binding was observed with control sera. We tested CYP2E1 as an antigen to detect INH-induced antibodies because patients who carry the high activity CYP2E1 c1/c1 genotype appear to have a higher risk of developing liver injury. Eleven patients who had liver failure also had anti-CYP2E1 antibodies, but no anti-CYP2E1 antibodies were detected in patients who underwent INH prophylaxis (Figure 26A). Fourteen patients had antibodies against CYP2E1-INH (Figure 26), and the concentration of these antibodies appeared to be greater than antibodies against INH or CYP2E1 because the average difference in the signal compared to control was significantly greater. This suggests that the protein is part of the epitope recognized by many of the antibodies. We also tested the ability of CYP2E1 that had been incubated with INH and NADPH to detect antibodies not detected by L-INH or CYP2E1 chemically modified by INH. However, there was no difference between CYP2E1 modified in this way and unmodified CYP2E1. This is presumably because, although we can detect covalent binding of INH to CYP2E1, under these conditions, the turnover of substrate and the amount of covalent binding would be much less than with chemical modification of CYP2E1, and it insufficient to allow detection of a difference in binding when compared with native CYP2E1. INH has also been shown to irreversibly inhibit other CYPs such as CYP3A4 and CYP2C9, which suggests that INH covalently binds to these CYPs.
Figure 26: Detection of anti-CYP antibodies in the serum from patients treated with INH. Control sera were from patients before starting INH (Baseline), sera from patients treated with INH but without significant liver injury (Prophylaxis), sera from patients with INH-induced liver failure but without anti-CYP antibodies (Liver Failure (-)), sera from patients with INH-induced liver failure that do have anti-CYP antibodies (Liver Failure (+)) or sera from all of the patients who had INH-induced liver failure (All Liver Failure). A) autoantibodies against CYP2E1. B) antibodies against INH-modified CYP2E1, C) autoantibodies against CYP3A4. D) autoantibodies against CYP2C9. Values represent Mean ± S.E. Statistically significant from control **p < 0.01, ***p < 0.001.
Incubation of INH with CYP2E1, CYP2C9, or CYP3A4 confirmed that INH does bind to these isozymes, and the binding requires the presence of NADPH (Figure 27) indicating that it is a metabolite that binds.

\[
\begin{array}{cccccccccc}
\text{CYP450} & + & + & + & + & + & + & + & + & + \\
\text{INH} & + & + & + & + & + & + & + & + & + \\
\text{NADPH} & + & + & + & + & + & + & + & + & + \\
\end{array}
\]

**Figure 27:** In vitro covalent binding of INH to CYP2E1, CYP2C9, or CYP3A4. Each CYP isozyme was incubated with INH (100 µM) in the presence or absence of an NADPH regenerating system. Rabbit, anti-INH antibody was used to detect covalent binding of INH to CYP isozyme as previously described.\(^{154}\)

Such binding could lead to antibodies against these proteins and this was also tested and confirmed. Specifically, 14 patients had anti-CYP3A4 antibodies and 10 patients had anti-CYP2C9 antibodies (Figure 26C, D). Patients either had antibodies against one of the tested proteins or they did not; therefore, in Figure 25, these two groups were separated. However, even if the results of the positive and negative patients are combined (indicated as “All Liver Failure” in the figure), there is a clear difference between patients with liver failure and either untreated controls or INH-treated patients without significant liver injury.

As part of their clinical workup, some of the patients with INH-induced liver failure were tested for the presence of autoantibodies. A low titre of antinuclear antibodies (ANA) was found in 4 out of 11, anti-smooth muscle antibodies (ASMA) in 1 out of 8, anti-mitochondrial antibodies (AMA) in 1 out of 6, and anti-liver/kidney microsome antibody (KLM) in none of the 2 patients tested. The antibody profile for each patient is presented in Table 9. In addition,
we looked to see if there was a correlation between patient ALT, bilirubin, or INR and the presence of anti-INH antibodies, but no correlation was observed. Data showing ALT, bilirubin, and INR levels for patients with liver failure are shown in Figure S6.

**Table 9:** Pattern of antibodies in the sera from 20 patients with INH-induced liver failure.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Anti-INH</th>
<th>Anti-CYP2E1</th>
<th>Anti-CYP2E1-INH</th>
<th>Anti-CYP3A4</th>
<th>Anti-CYP2C9</th>
<th>ASMA ratio</th>
<th>ANA ratio</th>
<th>AMA ratio</th>
<th>KLM ratio</th>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>640</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
</tbody>
</table>

Blank or – means that the antibody was not detected, ND means not done. ALF = Acute liver failure. AMA, anti-mitochondrial antibodies; ANA, anti-nuclear antibodies; ASMA, anti-smooth muscle antibodies; LKM, anti-liver/kidney microsome.
The mechanism of INH-induced hepatotoxicity remains poorly understood. However, it is generally not considered to be immune-mediated and has been referred to as metabolic idiosyncrasy. One reason for this belief is that, in contrast to the liver injury caused by several other drugs such as halothane, it was reported not to be associated with anti-INH antibodies.\textsuperscript{11,120} To the best of our knowledge, this study is the first to find anti-INH and anti-CYP autoantibodies in the serum of patients with INH-induced liver failure. Anti-INH antibodies were found in the serum of 8/19 patients who had INH-induced liver failure (Figure 24), but not in patients treated with INH who had only very mild liver injury or no increase in ALT at all. In the case of other drugs such as halothane that induce idiosyncratic liver injury and are associated with antibodies, a range of antibodies have been observed.\textsuperscript{55,56,114} This includes antibodies against drug-modified proteins, anti-CYP antibodies, and other autoantibodies. We demonstrated that INH is bioactivated by and covalently binds to CYP2E1, in the presence of a NADPH regenerating system (Figure 27), which is consistent with the observation that the high activity variant \textit{CYP2E1 c1/c1} genotype is associated with more severe liver injury.\textsuperscript{125} INH has been shown to be an inhibitor of 2C9, 2E1, and 3A4, which suggests that INH is bioactivated by several CYPs, and therefore these modified proteins might also induce antibody formation.\textsuperscript{139,170} In addition to CYP2E1, we showed that INH can form covalent adducts to CYP2C9 and CYP3A4 (Figure 27). We also found that 11 patients who had INH-induced liver failure had anti-CYP2E1 antibodies, 14 patients had antibodies against CYP2E1 modified by INH, 14 patients had anti-CYP3A4 antibodies, and 10 patients had anti-CYP2C9 antibodies (Figure 26). Out of 19 patients who had liver failure, 15 (79\%) had antibodies against INH, CYP3A4, 2E1, or 2C9, and most patients had antibodies to several
native or INH-modified proteins (Table 9). Given the large number of proteins that are modified by the reactive metabolite of INH,\textsuperscript{154} it is quite possible that if we tested for other antibodies, all of the patients would have one or more antibody against drug-modified or native proteins. INH induces a different spectrum of antibodies in different patients (Table 9), and such heterogeneity is common in idiosyncratic drug reactions.\textsuperscript{6}

The previous two reports that did not detect anti-INH antibodies only studied patients with mild liver injury;\textsuperscript{117,171} we also did not detect such antibodies in patients with mild liver injury (n = 5). In addition, some of the antigens that we tried gave unacceptable background binding so finding the best antigen is important. Our data clearly indicate that most cases of severe INH-induced liver injury associated with liver failure have antibodies that were induced by INH, and the absence of such antibodies in patients without injury or mild injury suggests that INH-induced liver failure is immune-mediated. The presence of anti-INH antibodies also suggests that the reactive metabolite responsible for this immune response came from bioactivation of INH itself and not acetylhydrazine as previously believed. Although the presence of anti-INH antibodies only in patients with INH-induced liver failure suggests an immune-mediated mechanism, we cannot rule out the possibility that the liver failure was instrumental in the induction of these antibodies. In addition, some of those patients who had anti-INH antibodies were also being treated with other drugs that could contribute to the presence of autoantibodies. However, in most cases the timing made INH or INH/pyrazinamide more likely, and there were two clean cases of INH-induced liver failure (patient ID ALF-10 and 14), which were associated with the presence of anti-INH antibodies and autoantibodies similar to most of the other samples (Table 8,9).

There are other data that strongly suggest that INH-induced liver injury is immune-mediated, and further that it is INH rather than acetylhydrazine that is recognized by the
immune cells involved in INH-induced liver injury. Specifically, Warrington found a positive lymphocyte transformation test (LTT) when lymphocytes from cases of mild INH-induced liver injury were incubated with INH-modified protein but not with INH itself; however, more severe cases of DILI also had a positive LTT to INH.\textsuperscript{117,142} Maria and Victorino also reported a positive LTT test from a patient with INH-induced hepatotoxicity.\textsuperscript{172} The liver injury caused by other drugs such as halothane and tienilic acid can be associated with a variety of antibodies.\textsuperscript{55,56,114} Some of these antibodies are against drug-modified proteins and some are autoantibodies against native proteins, especially the CYP that formed the reactive metabolite, and the pattern varies from patient to patient. INH also commonly induces the production of autoantibodies, sometimes resulting in a lupus-like syndrome.\textsuperscript{123} This panel of anti-drug and anti-CYP antibodies appear to have sufficient sensitivity and specificity that they could be useful in causality assessment where INH is one of possible causes of severe liver injury. It suggests that anti-CYP antibodies may be present in many other cases of idiosyncratic drug-induced liver injury, although this is less likely for drugs in which the degree of covalent binding to CYPs is insufficient to lead to suicide inhibition of the enzymes.

Although the presence of anti-INH and anti-CYP autoantibodies only in patients with INH-induced liver failure suggests that INH-induced liver injury is immune-mediated, there is no evidence that these antibodies are actually responsible for the liver injury. They could be a result of an immune response to the liver injury or even an attempt to resolve the immune response; many immune cells have Fc antibody receptors, and intravenous immunoglobulin is used to treat immune-mediated idiosyncratic drug reactions such as toxic epidermal necrolysis. However, these antibodies do indicate that severe INH-induced liver injury involves the immune system. The positive LTT indicates the presence of sensitized lymphocytes, which may represent the major mechanism of liver injury. Combining our data with the Warrington
data suggests that mild cases of INH-induced liver injury resolve with immune tolerance, and it is only when this immune tolerance fails and the immune response spreads that more severe liver injury occurs.
Acknowledgments

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SIGNIFICANCE

Building logically from our last publication, in this paper we show that INH-induced liver injury is associated with the presence of anti-INH antibodies and autoantibodies. These antibodies were present only in cases of INH-induced liver failure and not in patients who had no liver injury or mild liver injury when taking INH. This indicates that at least some cases of severe INH-induced liver injury are associated with an immune response. However, whether this immune response is pathogenic or not is unclear. Previously we showed that INH itself, and not just its acetylhydrazine metabolite, can form a covalent adduct with hepatic macromolecules, and this could be responsible for liver injury. The presence of these antibodies provide additional evidence that, in contrast to what was previously believed, INH-induced liver injury is immune-mediated, and the immune response is directed against the parent drug and drug-modified proteins, not against acetylhydrazine. In addition, the fact that a greater number of patients had anti-CYP450 antibodies indicates that the immune response to INH may spread to hepatic proteins and induce autoimmunity.
3.3: Mild Isoniazid-Induced Liver Injury is associated with an Increase in Th17 cells and Intracellular IL-10

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1. Department of Pharmacology and Toxicology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada
2. Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada

Xu Zhu helped with initial study design and collection of data from the summer of 2010 – April 2012. Imir G. Metushi assisted Xu Zhu with data collection until April 2012 and continued the study until fall 2013. Imir G Metushi also designed additional experiments necessary for the study and analysed the data (with initial help from Xu Zhu) and wrote the manuscript. Dr. William M. Lee helped to provide the liver biopsies from one patient with INH-induced liver failure and another patient with hepatitis B virus. Dr. Jack P. Uetrecht helped design the study and edit the manuscript.

Manuscript in Preparation. I G Metushi et al., “Mild Isoniazid-Induced Liver Injury is associated with an Increase in Th17 cells and intracellular IL-10”
Results

The histopathological characteristic of INH-induced hepatotoxicity can range from hepatocellular necrosis to centrilobular necrosis and massive multilobular necrosis, which makes it indistinguishable from viral hepatitis.\textsuperscript{106,120} These features are similar to one patient who received INH and had liver failure (Figure 28A); however, another patient with liver failure due to INH also had the less common feature of cholestasis and steatosis, which is not typically seen with INH (Figure 28B). It is well known that the acetylhydrazine metabolite of INH can bind to hepatic proteins in rats;\textsuperscript{100} Recently, we also demonstrated that INH itself is bioactivated and binds to mouse and rat liver proteins \textit{in vivo}, and to human liver microsomes \textit{in vitro}. We determined whether INH covalently binds in the liver of human subjects that were treated with INH. In the patient treated with INH there was covalent binding of INH in the liver and this was not observed in a liver slice from the patient with viral hepatitis (Figure 28C, D). The binding of INH in the liver could be prevented by preincubation of the primary anti-INH antibody with INH (Figure 28E), and no binding was observed when the primary antibody was omitted, which indicates that the binding is specific for INH. The binding is very heterogeneous, which is presumably caused by the fact that most of the normal hepatocytes that would be able to metabolize the drug are gone. In addition, the liver biopsy was obtained 24 days after the INH was stopped and much of the covalently bound drug was presumably cleared.
Figure 28: H&E and immunohistochemical staining for INH in patients with INH-induced liver failure. A, B) H&E of two patients who had liver failure caused to INH. C) IHC using anti-INH antibody to detect binding of INH in a patient who had HVB virus or; D) in patient who had INH-induced liver failure. This patient had taken INH for 5 months, liver biopsy was taken 24 days after stopping INH. E) IHC using anti-INH antibody to detect binding of INH in the same patient as in part D, but the anti-INH antibody was pre incubated with 500 µM INH.

We phenotyped the lymphocytes from patients treated with INH by flow cytometry. Out of a total of 29 patients who enrolled in the study we were only able to collect sufficient data on 13 patients (Table 10). Five patients developed a small increase in ALT during INH treatment, and the time to the increase in ALT varied from 56 – 160 days (Table 11); individual data from all the patients can be found at the end of this manuscript after the discussion.
Table 10: Number of patients enrolled in the cellular phenotyping study

<p>| | |</p>
<table>
<thead>
<tr>
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<tr>
<td>Patients enrolled in study</td>
<td>29</td>
</tr>
<tr>
<td>Patients with high ALT baseline</td>
<td>3</td>
</tr>
<tr>
<td>Patients who withdrew from study</td>
<td>13</td>
</tr>
<tr>
<td>Patients for which data was collected</td>
<td>13</td>
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Table 11: Time to onset and peak ALT elevation in the five patients who had mild INH-induced liver injury

<table>
<thead>
<tr>
<th>Patient</th>
<th>Days to onset of hepatotoxicity</th>
<th>Peak ALT (U/L)</th>
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<td>1</td>
<td>56</td>
<td>144</td>
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<tr>
<td>2</td>
<td>140</td>
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<td>20</td>
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<td>52</td>
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</table>
We looked for evidence of a Th1 or Th2 immune response using CD3, CD4, CD8, IL-4, and INFγ, but we did not find anything significant with respect to this classification of immune response. However, we did find that patients who had an small increase in ALT also had an increase in peripheral Th17 cells (defined as CD4+IL-17+) (Figure 29A). In addition, the increase in Th17 cells was associated with an increase in cells that stained for intracellular IL-10 (Figure 29B) (defined as CD3+IL-10+).

**Figure 29:** Changes in the percentage of specific cell types in the blood coincident with the increase in ALT. A) Change in % Th17 cells. B) Change in % CD3+IL-10+ cells. C) Change in % Tregs (CD4+/IL-10+/FoxP3+) cell percentages were plotted at baseline and when there was the highest ALT. D) Ratio of Th17/Treg cells were plotted at baseline and when there was the highest ALT.
Illustration for these changes is shown in Figure 30 where patient #17 who had a small increase in ALT, also had an increase in Th17 cells (Figure 30A) and CD3^+IL-10^+ cells (Figure 30B); most of the cells that produced IL-10 were CD4^+ (Figure 30C). However, the source of IL-17 and IL-10 was from different CD4^+ cells (Figure 30D).

**Figure 30:** Lymphocyte phenotyping in patient 17. A) Th17 cells at baseline (ALT = 15) and then again when there was a small increase in ALT from 15 to 47, which was the lowest ALT of the group. B) Change in IL-10^+ cells in the same patient. C) Percentage of CD4 vs. CD8 positive cells from the CD3^+IL-10^+ panel when the ALT = 47. D) Distribution of IL-10^+ and IL-17^+ cells when ALT = 47 by first gating on CD4^+ cells.
Most of the IL-10+ cells were also CD4+ (Figure 30C), but they were not FoxP3+ so they were not the classic T regulatory cells (Tregs). A better predictor was the ratio of Th17/T_{reg} cells which also seemed to increase between baseline and when there was an increase in ALT (Figure 29D). There is an weak correlation between the % Th17 cells and the ALT (Figure 31A-E). We also looked at the correlation when the ALT was the highest in the five patients and the % of Th17 cells but poor correlation was observed; this is not surprising since each patient had slightly different baseline of Th17 cell levels. Patient #27 had an increase in Th17 cells but no increase in ALT after one month, however this patient had a skin rash and discontinued treatment (for individual flow data on all patients, see page 135). In addition, serum IL-17 and IL-10 did not show much of a change between patients who had mild liver injury and those that took INH but did not have an increase in ALT (Table 12), suggesting that flow cytometry staining for IL-17 and IL-10 may be a more sensitive assay to look for an immune response to drugs that may initiate a Th17 immune response.
Figure 31: Correlation between ALT and the % of Th17 cells. F) Correlation between the highest ALT and the % of Th17 cells in all five patients.
Table 12: Serum cytokines in patients taking INH

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<th>Patient #</th>
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<th>IL-10 (pg/mL)</th>
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<td>5-2</td>
<td>50.97</td>
<td>31.11</td>
</tr>
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<td>6-3</td>
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<td>24.00</td>
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</table>

The first five patients were patients who received INH but did not have an increase in ALT. Bold indicates patients who had mild INH-induced liver injury. The serum assayed was from when the ALT was highest as shown in Table 11.
Discussion

The mechanism of INH-induced hepatotoxicity remains poorly understood. However, it is generally not considered to be immune-mediated and has been referred to as metabolic idiosyncrasy. The main reason for this has been the fact that INH-induced hepatotoxicity takes time to occur, it often does not recur rapidly upon rechallenge, is not associated with fever or rash, and there are no anti-INH antibodies. However, there are cases in which INH-induced hepatotoxicity is associated with fever, rash, eosinophilic infiltrate in the liver, and a faster onset of liver injury upon rechallenge with the drug. More recently we have re-evaluated the issue of anti-INH antibodies and found anti-INH and anti-P450 antibodies in cases of INH-induced liver failure. In addition, INH-induced hepatotoxicity has been considered to be due to bioactivation and covalent binding of the acetylhydrazine metabolite. However, we have shown that INH itself can be bioactivated and bind covalently to rodent livers in vivo and to human liver microsomes in vitro. In the present study, we demonstrated that INH covalently binds to human hepatic liver proteins using a liver of biopsy of a patient with INH-induced liver failure (Figure 28D). In addition, we demonstrated that in patients undergoing INH treatment because of positive tuberculosis skin test, when there was a small increase in ALT, there was also an increase in Th17 cells and T cells that produce IL-10 (Figure 29). This data suggests that Th17 cells are involved in the liver injury and upregulation of IL-10-producing cells limit the liver injury. The % in Th17 cells did not always seem to correlate with the increase in ALT, therefore it is difficult to say which came first, the increase in ALT or Th17/IL-10 response (Figure 31). Also, given the difficulty in performing these clinical studies and the inability to control when the patients come in for their visit, it is unlikely that this question can be answered without a valid animal model. Treg cells did not
seem to change when there was an increase in ALT (Figure 29C). One patient who had an increase in Th17 cells but no increase in ALT had a skin rash, which is an indication of an immune-mediated reaction (Patient 27, data located at the end of manuscript); therefore, liver injury and rash due to INH may share a similar mechanism. In addition, in our study the incidence of INH-induced liver injury was high (5/29 patients or 17%) compared to some other studies where they reported an incidence of up to 0.15% and suggested that INH is not as hepatotoxic as it has been considered.174

Overall, this study adds to the data that suggests that INH-induced liver injury is mediated by the adaptive immune system. The increase in Th17 cells is interesting because this cell type has been implicated in liver disease.49 Th17 cells have been shown to produce key cytokines such as IL-17, IL6, TNFα, and IL22, which are important to promote an immune response against extracellular pathogens.175 However, acute acetaminophen-induced liver injury in mice, which is clearly not mediated by the adaptive immune system, is associated with an increase in Th17 cells just 2 h after treatment.161 This raises the question as to whether the role of Th17 cells in liver disease is pathogenic or is just a response to the injury. Without a valid animal model of idiosyncratic drug-induced liver injury (IDILI) it is unlikely that this question will be answered, to a large degree because it is hard to control the timing of blood sampling in humans. On the other hand, an animal model must be related to the IDILI in humans to be useful.

There are other data that strongly suggest that INH-induced liver injury is immune-mediated, and it is INH rather than acetylhydrazine that is recognized by the immune cells involved in INH-induced liver injury. Specifically, Warrington found a positive lymphocyte transformation test (LTT) when lymphocytes from cases of mild INH-induced liver injury were incubated with INH-modified protein, but not with INH itself; however, more severe cases of
DILI also had a positive LTT to INH.\textsuperscript{117,142} Maria and Victorino also reported a positive LTT test from a patient with INH-induced hepatotoxicity.\textsuperscript{172} INH also commonly induces the production of autoantibodies, sometimes resulting in a lupus-like syndrome.\textsuperscript{123} In conclusion, these data, together with our previous investigations suggest that INH-induced hepatotoxicity is immune-mediated, not metabolic idiosyncrasy, and furthermore, it is caused by direct bioactivation of INH.
**INDIVIDUAL PATIENT DATA SHOWING THE TH17 OR IL-10 RESPONSE**

*Patients marked in red indicate those who had an increase in ALT.*

### Patient #1

<table>
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<tr>
<th>Days to revisit</th>
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<th>% Treg</th>
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After day 42, patient discontinued INH treatment due to a high increase in ALT.

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Patient had skin rash and discontinued INH treatment after day 28.
Acknowledgments

This work is supported by grants from the Canadian Institutes of Health Research. Jack Uetrecht holds the Canadian Research chair in Adverse Drug Reactions. Imir G Metushi is a trainee of the Drug Safety and Effectiveness Cross Disciplinary Training Program, which is funded by CIHR. We would like to thank Dr. Michael Gardam and the nurses: Judith Lang, Peggy Howard and Andrea Moore for recruiting patients at the Toronto Western Hospital for this study.
SIGNIFICANCE OF CHAPTER

In our recent studies we demonstrated the presence of anti-drug antibodies and autoantibodies in the serum of patients with severe INH-induced liver injury, but not in patients with mild liver injury. In this work we show that mild cases of liver injury are also associated with an adaptive immune response. In particular, when INH caused a small increase in ALT, there was a coincident increase in Th17 cells and IL-10+ T cells. In mild cases of INH-induced liver injury the proinflammatory effects of Th17 cells appear to be balanced by the anti-inflammatory effects of IL-10; this is an important finding because it may explain why most people do not progress to liver failure. In addition we demonstrated that INH can directly covalently bind in the liver of humans who are treated with the drug, providing a direct link between INH bioactivation and an immune response against INH-modified proteins. These data suggests that INH-induced liver injury is immune-mediated and the immune response involves INH-modified proteins. These mechanistic clues may help in the quest for an animal model of INH-induced liver injury that could be used to study the details of INH-induced liver injury.
3.4: Attempts to Develop an Animal Model of Isoniazid-Induced Hepatitis Reveal Immunosuppressive Effects of the Drug

Imir G Metushi¹ and Jack P Uetrecht¹,²

1. Department of Pharmacology and Toxicology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada
2. Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada

Imir G Metushi has been responsible for designed the study, conducting experiments, collecting and analysing the data as well as writing the manuscript. Dr. Jack Uetrecht helped design the study and edited the manuscript.

Manuscript in Preparation. I G Metushi et al., “Attempts to Develop an Animal Model of Isoniazid-Induced Hepatitis Reveal Immunosuppressive Effects of the Drug”
Results

Treatment of rats with INH/RMP

We tried to replicate the published animal model in which treatment of Wistar rats with INH and RMP produced an increase in ALT that was associated with liver necrosis; however, we saw no increase in ALT (Figure 32A). Because ALT activity is inhibited by INH, we monitored GLDH and SDH activities as alternative biomarkers of liver injury, but no change in enzyme activity was observed (Figure 32B,C). The body weight of the treated group was slightly less than control, but animals continued to gain weight throughout the treatment (Figure 32D); however, the liver and spleen weights in the INH + RMP cotreatment group were less than those from controls: (21.1 g ± 0.6 for control vs. 17.5 ± 0.7 for INH + RMP cotreated group, mean ± S.E.; *p < 0.05) and (1.2 g ± 0.06 for control vs. 1.0 g ± 0.1 for INH + RMP cotreated group, mean ± S.E.; p > 0.05), respectively.
Figure 32: ALT, GLDH, SDH, and body weight in Wistar rats treated with INH/RMP. A-D) Wistar rats (n = 4) were treated with 75 mg/kg/day INH and 50 mg/kg/day RMP by gavage daily for 4 weeks. E-H) Wistar rats (n = 4) were treated with 150 mg/kg/day INH by gavage daily for 4 weeks.
Another group reported that treatment of Wistar rats with 50 mg/kg/day of INH resulted in a mild increase in ALT by weeks 3-4, which continued to increase for up to 90 days. We treated Wistar rats with up to three times higher doses of INH (150 mg/kg/day) for 4 weeks, but no increase in ALT, SDH, or GLDH was observed (Figure 32E-G). In fact, the ALT assay showed decreased activity, which is consistent with our previous results indicating that the assay is inhibited by INH. No liver injury/necrosis was observed by H&E in any of the INH-treated groups.

INH has been shown to activate macrophages in vitro. We examined macrophages in the liver/spleen of Wistar or Brown Norway rats treated with INH (150 mg/kg/day) for 4 or 5 weeks, respectively, by staining for the CD68 surface marker. No change in the number of cells staining positive for CD68 in the liver (Figure 33) or the spleen (Figure S7) was observed. Even when BN rats were gavaged with high doses of INH (200 or 400 mg/kg/day) for one week, which produced steatosis, there was no change in the number of cells staining positive for CD68 in the liver of control vs. INH treated rats (data not shown).

![Image A: Wistar Rat Control](image1.png) ![Image B: Wistar Rat + INH](image2.png)

**Figure 33:** Immunohistochemical staining for CD68 (macrophages/Kupffer cells) in the liver of control Wistar rats (A) or treated with INH (B). Rats were treated with INH at 150 mg/kg/day by gavage for 4 weeks; (20X Magnification).
Several serum cytokines were decreased both in Wistar rats and BN rats after 1 week of treatment with INH. Specifically, in Wistar rats CXCL1 (GRO/KC) and MCP-1 were significantly decreased (Figure 34A); a similar decrease was observed in BN rats; and in addition, RANTES (CCL5) and leptin were also down regulated (Figure 34B).
Figure 34: Serum cytokines in male Wistar rats and Brown Norway rats. A) Wistar rats (n = 4) or B) Brown Norway rats were treated with INH (150 mg/kg/day) for 7 days. Analysed for significance by Mann Whitney-U test; * = p < 0.05.
We examined the expression of CD25 and CTLA4 as potential markers of immune tolerance as well as CD4⁺/IL-17⁺ and CD3⁺/IL-10⁺ cells because an increase in IL-17- and IL-10-producing cells was observed in patients treated with INH who had a small increase in ALT.¹⁰⁷ No differences in the percentages of CD3⁺, CD4⁺, or CD8⁺ cells were observed between control or in INH + RMP-treated rats. There was an increase in cells staining positive for CD4⁺/IL-17⁺ cells and CTLA4⁺ cells (Figure S8), but the difference was only statistically significant in lymph nodes (Figure 35). No change in CD3⁺IL-10⁺ cells was observed.

**Figure 35:** Phenotype of leukocytes in the liver and cervical lymph nodes determined by flow cytometry in Wistar rats treated with INH + RMP. Wistar rats (n = 4) were treated with INH + RMP for 4 weeks. Statistical significance was determined by the Mann-Whitney U test with * = p< 0.05.
Treatment of Balb/c mice with INH

Recently, we reported that treatment of Balb/c mice with 0.2% of INH w/w in food resulted in mild increases in GLDH levels; however, mice could not be treated for longer than three weeks because of body weight loss. To develop an animal model of chronic liver injury similar to that observed in humans, Balb/c mice were treated with lower doses of INH. Treatment of male Balb/c mice with 0.1% or 0.15% of INH w/w in food for up to 5 weeks produced mild increases in GLDH without severe weight loss (Figure 36A,C). Covalent binding appeared to be a little greater in male Balb/c mice that received the higher dose (0.15% of INH; Table 13; Figure 36E).

Table 13: Food Consumption in mice

<table>
<thead>
<tr>
<th></th>
<th>food consumption (g/day)</th>
<th>animal weight range (g)</th>
<th>avg INH/Pyr intake in mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male Balb/c Mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH (0.1%)</td>
<td>3.1 ± 0.2</td>
<td>18 – 21</td>
<td>147 – 172</td>
</tr>
<tr>
<td>INH (0.15%)</td>
<td>3.6 ± 0.4</td>
<td>18 – 21</td>
<td>257 – 300</td>
</tr>
<tr>
<td>Female Balb/c Mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH (0.1%)</td>
<td>3.5 ± 0.5</td>
<td>17 – 20</td>
<td>175 – 206</td>
</tr>
<tr>
<td>INH (0.15%)</td>
<td>2.7 ± 0.3</td>
<td>15 – 17</td>
<td>238 – 270</td>
</tr>
<tr>
<td>INH (0.2% + Pyr)</td>
<td>1.9 ± 0.4</td>
<td>13 – 17</td>
<td>224 – 292/56 – 73</td>
</tr>
<tr>
<td>Male Cbl-b^− Mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.0 ± 0.5</td>
<td>23 – 28</td>
<td></td>
</tr>
<tr>
<td>INH</td>
<td>3.8 ± 0.5</td>
<td>21 – 23</td>
<td>330 – 362</td>
</tr>
<tr>
<td>INH + Pyr</td>
<td>3.8 ± 0.4</td>
<td>21 – 23</td>
<td>330 – 362/83 – 90</td>
</tr>
<tr>
<td>Female Cbl-b^− Mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.5 ± 0.4</td>
<td>18 – 21</td>
<td></td>
</tr>
<tr>
<td>INH</td>
<td>3.8 ± 0.3*</td>
<td>16 – 18</td>
<td>422 – 475</td>
</tr>
<tr>
<td>Female PD1^−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.1 ± 0.4</td>
<td>15 – 25</td>
<td></td>
</tr>
<tr>
<td>INH (0.1 - 0.2%)</td>
<td>1.1 – 2.4*</td>
<td>14 – 20</td>
<td>110 - 170</td>
</tr>
<tr>
<td>Female C3H + INH (0.2%)</td>
<td>4.5 ± 0.4</td>
<td>20 – 21</td>
<td>428 – 450</td>
</tr>
<tr>
<td>Female Rag^− + INH (0.2%)</td>
<td>2.9 ± 0.4</td>
<td>15 – 18</td>
<td>280 - 430</td>
</tr>
</tbody>
</table>

INH was given to mice at 0.1%, 0.15% or 0.2% w/w in food. Pyr = pyridoxine hydrochloride given to mice at 0.05% w/w in food. Values represented as mean ± S.E of 4 mice per group except female C3H and female Rag^− mice (n = 3). The data were analysed for significance using the Mann-Whitney U test. Significantly different from control group *p < 0.05.
Figure 36: GLDH activities, body weight, and INH covalent binding in INH-treated Balb/c mice. A) GLDH in male Balb/c mice treated at 0.1 or 0.15% INH w/w in food for 5 weeks. B) GLDH in female Balb/c mice treated at 0.1% or 0.15% of INH w/w in food for 3 weeks or treated with 0.2% of INH and 0.05% of pyridoxine hydrochloride (Pyr) w/w in food for 2 weeks. C-D) Body weight in male and female Balb/c mice. E-F) Covalent binding of INH in the livers of male and female Balb/c mice. Values represent the mean ± S.E. from 4 animals per group. Analysed for statistical significance by Mann-Whitney U test with * = p < 0.05.
However, the H&E staining in the livers and spleen of male Balb/c mice at the end of 5 weeks was normal. Treatment of female Balb/c mice with 0.1% and 0.15% of INH by weight in food also resulted in slight elevations in GLDH levels (Figure 36B). Pyridoxine hydrochloride was added to the group of mice treated with the highest dose of INH (0.2% INH w/w in food) as a supplement to protect from the depletion of vitamin B6 and to prevent weight loss; this approach is similar to what has been used to prevent extensive weight loss in rabbits\textsuperscript{178} and is consistent with the B6 supplement which is given to patients undergoing INH therapy. However, despite administration of pyridoxine, the group of mice treated with 0.2% INH lost significant body weight and could not be maintained for more than 2 weeks (Figure 36D). Covalent binding of INH in female Balb/c mice revealed no difference between the groups (Figure 36F), presumably because, although there was an increase in concentration of INH in the food, these mice also ate less food making the total INH intake similar between the groups (Table 13). The histology (H&E) of the liver and spleen from female Balb/c mice was normal.

**Treatment of Cbl-b\textsuperscript{−/−}, PD1\textsuperscript{−/−} and Rag\textsuperscript{−/−} mice with INH**

Treatment of C57BL/6 mice with INH did not cause liver injury.\textsuperscript{154} If the lack of injury is due to immune tolerance it is possible that treatment of Cbl-b\textsuperscript{−/−} mice, which lack an E3 ubiquitin ligase, a molecule important for regulating immune tolerance, would be more susceptible to injury.\textsuperscript{179} Male Cbl-b\textsuperscript{−/−} mice treated with 0.2% of INH by weight in food had mild elevations in GLDH, which returned to normal despite continued treatment (Figure 37A). We tried to prevent the increase in GLDH by also adding pyridoxine hydrochloride, but no significant difference from the INH-only group was observed. Male Cbl\textsuperscript{−/−} mice did not eat as much food as the control animals (Table 13), but their body weight was stable for 5 weeks.
(Figure 37C). Female Cbl-b<sup>−/−</sup> mice also had a mild increase in GLDH activity and could be maintained for up to 5 weeks when treated with 0.2% of INH w/w in food (Figure 37B).

Figure 37: GLDH activities and body weights in INH-treated Cbl-b<sup>−/−</sup> mice. A) GLDH in male Cbl-b<sup>−/−</sup> mice treated with 0.2% INH w/w in food or 0.2% INH and 0.05% pyridoxine hydrochloride (INH + Pyr) w/w in food for 5 weeks. B) GLDH in female Cbl-b<sup>−/−</sup> mice treated with INH at 0.2% w/w in food for 5 weeks. C-D) Body weight of male and female INH-treated Cbl-b<sup>−/−</sup> mice. Values represent the mean ± S.E. from 4 animals per group. Analysed for statistical significance by two-way ANOVA with * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
The blood level of INH appeared slightly higher in female mice than males, but the difference was not statistically significant (Figure S9C), and it is similar to the peak therapeutic concentration of INH in humans. Food consumption in female mice was also less than in the control animals, but it was sufficient to maintain stable body weight (Table 6). The liver histology of one female Cbl-b−/− mouse had an infiltration of lymphocytes, focal necrosis, steatosis, and cholestasis as shown by H&E staining in Figure 38. This mouse also had an increase in cells staining positive for F4/80, CD45R, and PCNA, which implies an immune response and cell regeneration in the liver. An increase in cells staining positive for CD45R and F4/80 was also observed in the spleen of the mouse that had abnormal liver histology (Figure S10), but not in the other three mice that were treated with INH.
Figure 38: H&E and immunohistochemical staining for anti-CD45R, F4/80, and PCNA in the livers of Cbl-b−/− mice treated with INH (0.2% w/w in food) for 5 weeks. Control = untreated mice (n = 4); INH = mice treated with INH that did not develop abnormal liver histology (n = 3); INH steatosis = one mouse treated with INH that developed significant abnormal liver histology (n = 1). Red arrow = lymphocyte infiltration, yellow arrow = microvesicular steatosis, green arrow = macrovesicular steatosis, blue arrow = focal necrosis, orange arrow = cholestasis. 40X magnification for H&E and 20X magnification for CD45R, F4/80, and PCNA.
Staining for F4/80 in the livers and spleens of C57BL/6 mice showed no changes compared to controls (Figure S11). A decrease in serum IL-12 (p70) and IL-1α was observed in female Cbl-b−/− mice treated with INH at the end of the 5 weeks (Figure 39); the rest of cytokines are shown in Figure S12.

![Figure 39: Serum IL-1α and IL-12 (p70) in Cbl-b−/− mice (n = 4) treated with 0.2% INH w/w in food for 5 weeks.](image)

Treatment of C3H female mice with INH also produced a mild increase in GLDH which returned to normal (Figure S13); however, no histological changes were observed. Comparison of INH covalent binding revealed no difference between INH- and INH + pyridoxine (INH + Pyr)-treated male Cbl-b−/− mice (Figure 40A). There was no difference in covalent binding between male and female Cbl-b−/− mice treated with INH (Figure 40B); this is similar to our previous results in C57BL/6 mice. Also, no difference in covalent binding was observed between female Cbl-b−/− mice and female C57BL/6 or C3H mice (Figure 40C,D), suggesting again that covalent binding is not the only determinant for hepatotoxicity.
Figure 40: Comparison of the amount of INH covalent binding in the liver of different mice. A) Male Cbl-b<sup>−/−</sup> mice treated with INH (0.2% or 0.2% + 0.05% pyridoxine hydrochloride w/w food, n = 3). B) Female (n = 4) and male (n = 3) Cbl-b<sup>−/−</sup> mice treated with 0.2% INH w/w in food. C) Female C57BL/6 and Cbl-b<sup>−/−</sup> mice treated with 0.2% INH w/w in food (n = 4). D) Female Cbl-b<sup>−/−</sup> control (n = 2), Cbl-b<sup>−/−</sup> (n=4) or C3H mice (n=3) treated with 0.2% INH w/w in food.
Treatment of female PD1−/− mice with INH also produced mild increases in GLDH at week 5 (Figure 41A). However, these mice seemed to lose much weight after one week of treatment, and the % dose of INH had to be reduced for two weeks (Figure S14). Upon immunohistochemical grading, no infiltration of cells staining positive for KI67, F4/80, CD11b, CD4, CD8, CD45R was observed in the liver by the end of 5 weeks (Figure S15). Only in the spleen was there a decrease in cell proliferation as determined by KI67 staining (Figure S16). Treatment of Rag−/− mice, which are B and T cell deficient, for up to 12 weeks resulted in consistently higher GLDH levels, but mice did not progress to liver failure (Figure 41B).
Figure 41: GLDH in female PD1⁻/⁻ and Rag⁻/⁻ mice treated with INH. A) INH was given at 0.2% w/w in food until week 2, then the dose was decreased to 0.1% w/w in food for one week (until week 3) because mice lost weigh; mice were put back on 0.2% INH w/w from week 3 – 5. B) Female C57BL/6 control mice (n = 4) or female Rag⁻/⁻ mice (n = 3) were treated with INH at 0.2% w/w for up to 12 weeks. Analysed for statistical significance by two- way ANOVA. A p value < 0.05 was considered significant (*p < 0.05; **p < 0.01; ***p < 0.001).
Attempts to break immune tolerance: treatment of Cbl-b⁻/⁻ mice with INH/RMP and depletion of T₉ₑᵣg cells

Female Cbl-b⁻/⁻ mice appeared to be the most promising strain in which to induce liver injury; therefore, we tried to cotreat animals with RMP, which is an inducer of P450 in humans, and INH in order to increase covalent binding of INH in the liver. Treatment of female Cbl-b⁻/⁻ mice with INH/RMP for up to two weeks resulted in slight elevations in GLDH and SDH, which seemed to be higher in the INH + RMP group, but the difference was not significant (Figure S17 A,B). Covalent binding was greater in the INH + RMP group (Figure S17C) suggesting induction of drug metabolism, but the mice that were co-treated with INH + RMP, on average, also ate more INH-containing food (2.9 ± 0.32 for INH group vs. 3.8 ± 0.41 for INH + RMP group; values represented as mean ± S.E). However, despite the increased covalent binding, the H&E slides of the liver indicated no evidence of damage in either.

Another strategy to break immune tolerance was to deplete Treg cells by treating Cbl-b⁻/⁻ mice with anti-CD25 antibody or treatment with FICZ. These studies were done before we switched to GLDH/SDH as biomarkers of liver injury, and ALT was used as biomarker of hepatotoxicity, but no changes in histopathology were seen in the liver between controls and the INH group cotreated with FICZ or anti-CD25 antibodies (Figure S9 A,B). We also tried to give a higher INH dose to Cbl-b⁻/⁻ mice (0.4% of INH w/w in food), but the drug had to be discontinued in 2/4 mice at the end of the second week because of extensive weight loss; the other two animals also lost weight but could be treated for 6 weeks. In addition, the blood levels of INH were not much higher in the high dose group (0.4% INH w/w in food) because of decreased food consumption (Figure S9 C).
Discussion

The mechanism of idiosyncratic INH-induced hepatotoxicity in humans remains unknown, although there is increasing evidence that it is immune-mediated. Specifically, although mild INH-induced liver injury is often not associated with immune memory, with more severe injury, there are reports of a large increase in ALT and fever within hours of rechallenge of patients. In addition, more severe injury is associated with a positive lymphocyte transformation test, antibodies against INH-modified proteins or cytochromes P450, and the histology is dominated by CD8+ T cells, sometimes also with eosinophils. In order to determine exactly how INH induces an immune response leading to liver injury and what risk factors might predict who is at increased risk, it would be very helpful to have a valid animal model. However, there are no suitable animal models of INH-induced liver injury, or any other idiosyncratic drug-induced liver injury, that could be used to test hypotheses. To be a good model of the liver injury caused by INH in humans it should have the following characteristics: a delay in onset of more than a week, the principal type of injury should be hepatic necrosis, the lymphocyte transformation test should be positive, and the dominant inflammatory cell in the liver should be a CD8+ T cell.

Although the previous report of liver injury in rats with the combination of INH and rifampicin was mild and did not meet the criteria listed above, it was interesting in that it was delayed in onset. However, when we tried to replicate this study we found no evidence of liver injury (Figure 32). In rats, no change in macrophage numbers were observed in the liver or spleen, but there was a decrease in serum GRO/KC and MCP-1 (Figure 34) and an increase in CTLA4 expression in cervical lymph nodes (Figure 35), which suggests immune tolerance. The decrease in MCP-1 is paradoxical because MCP-1 is induced by endoplasmic stress and
given the extensive binding of INH to hepatic proteins including cytochromes P450 an increase would be expected. A noticeable difference between our Wistar rats and those in the study that we tried to replicate is that our Wistar rats gained about 200 g of weight over 4 weeks while the animals from the previous study gained only about 30 g. It is possible that the difference in response and the much smaller weight gain in previous studies was due to an undetected viral infection, but that is speculative, and the model still would not mimic what happens in humans. In addition, rodents may not be a good model to study effects of RMP on INH toxicity because RMP appears to be a human specific pregnane X receptor (PXR) activator, which is required for induction of cytochrome P450s.181,182

The metabolite that was believed to be responsible for INH-induced hepatotoxicity was acetylhydrazine, which was implicated in an acute model in rats.100,129,133 However, we have shown that INH itself covalently binds in the livers of mice in vivo and in human liver microsomes in vitro.154 Furthermore, the specificity of the lymphocyte transformation test in patients with INH-induced liver injury argues that it is binding of INH that induces the immune response.107 We found that the amount of INH covalent binding in mice is much greater than in rats154 suggesting that mice would be the better species for the development of an animal model.

In our previous study, no evidence of liver injury in male or female C57BL/6 mice was observed; a small increase in GLDH was observed in male and female Balb/c mice, but at the dose utilized (300 – 350 mg/kg/d of INH given in food) the animals lost significant weight and the treatment could not be continued for long.154 In this study, male Balb/c were treated with a lower dose of INH, which allowed chronic treatment with INH, and there was still a significant increase in GLDH, which was greater in females even though there was no significant difference in the amount of covalent binding (Figure 36).
We have postulated that the reason why it is difficult to develop animal models of idiosyncratic drug reactions is that the usual response is immune tolerance. In contrast to skin where there is little bioactivation, the liver contains a large amount of P450 and covalent binding is routine but the general response of the liver is immune tolerance.\textsuperscript{6,183} There are many redundant systems involved in immune tolerance. One is the ubiquitin ligase pathway including Cbl-b. Although Cbl-b\textsuperscript{−/−} mice are generally healthy, they have been shown to have a hyperproliferative T cell response and are resistant to anergy induction compared to the wild type C57BL/6 mice from which they were developed.\textsuperscript{179} Treatment of Cbl-b\textsuperscript{−/−} mice with INH resulted in a small increase in GLDH activity which appeared to return towards normal despite continued treatment (Figure 37). One female Cbl-b\textsuperscript{−/−} mouse developed abnormal liver histology that featured lymphocyte infiltration, focal necrosis, cholestasis, and steatosis. This mouse also had an increase in the number of macrophages, B cells, and hepatocyte proliferation in the liver and spleen implying an immune response (Figure 38 and Figure S10). The idiosyncratic response of this mouse could represent a model of the idiosyncratic liver injury observed in humans, although unlike severe liver injury in humans, the necrosis was focal and the major lymphocytes at this early time point were B cells. The amount of INH covalent binding was similar between female Cbl-b\textsuperscript{−/−} mice and female C57BL/6 mice (Figure 40C). Serum cytokines in female Cbl-b\textsuperscript{−/−} mice were generally down regulated after 5 weeks of treatment with INH; specifically, IL-1\textalpha and IL-12 were significantly decreased (Figure 39). This could indicate immune suppression. This is in contrast to serum cytokines in INH-treated wild type C57BL/6 mice in which there was no increase in GLDH and no change in serum cytokines after 1 or 3 weeks of treatment. Overall, Cbl-b\textsuperscript{−/−} mice tolerated INH quite well despite the increase in GLDH, but there was still a lack of serious liver injury with the exception of one mouse. INH also led to an increase in GLDH in PD-1\textsuperscript{−/−} mice, but they did not
tolerate the treatment as well as Cbl-b-/- mice. Isolated animals in different treatment groups had more serious liver injury, but it is not clear what the basis for this difference was. As with the Cbl-b-/- mice, the PD-1-/- mice were developed from C57BL/6 mice in which we saw no evidence of INH-induced liver injury. The observation that Cbl-b-/- and PD-1-/- mice are more sensitive than the wild type is consistent with an immune mechanism, but the fact that Rag-/- mice, which lack competent T and B cells, also had an increase in GLDH, which appeared more persistent than in other strains suggests that the initial immune response may be mediated by the innate immune system, and that T and/or B cells can modulate this response. In addition, the finding of steatosis that resolved despite continued treatment suggests that mitochondrial injury could also play a role in mild injury. Although we utilized mouse strains that have somewhat impaired immune tolerance, there are several redundant mechanisms for immune tolerance and these animals are by no means totally unable to develop immune tolerance.

Attempts to increase the liver injury in Cbl-b-/- mice by depleting T regulatory cells were not successful. Specifically, the use of FICZ has been shown to interfere with T_reg cell development and to increase the severity of an experimental autoimmune model of encephalomyelitis in mice. Likewise, although T_reg are not the only cells that express CD25, administration of anti-CD25 mAb was reported to reduce the number of CD4+CD25+ cells in peripheral lymphoid tissues, and this was associated with regression of tumours that grew in syngeneic mice.

So what do these data mean and why is it so difficult to develop an animal model of idiosyncratic drug-induced liver injury? Given that severe liver injury is idiosyncratic in humans maybe it should not be surprising that it is difficult to reproduce this injury in animals. If it is immune-mediated as argued above, it is possible that there is a requirement for a specific MHC/T cell receptor combination in order to produce an immune response that leads
to severe liver injury. There is one report of an HLA association, but it does not appear to be a strong association. In addition, given that INH covalently binds to a very large number of hepatic proteins, which would lead to an even greater number of drug-modified peptides, it seems likely that at least one MHC/T cell receptor pair would have the necessary fit, although it is possible that the affinity would not be sufficient to produce a strong response. It is possible that some environmental exposure, such as an infection, might be required to stimulate a strong immune response; however, patients with active tuberculosis do not appear to be at significantly increased risk, and it does not appear that infections or other agents are required to precipitate INH-induced liver failure. This also appears to be true of other idiosyncratic drug-induced liver failure; specifically, preexisting liver disease does not appear to be a major risk factor. Therefore, immune tolerance/immunosuppression is still a reasonable hypothesis for the reason that most patients and animals do not develop severe INH-induced liver injury.

There are other observations that suggest that INH can lead to immune suppression, which include the fact that administration of INH together with intravesical bacillus Calmette-Guérin (BCG) therapy for superficial bladder cancer seemed to: reduce the induction of mononuclear cell infiltrate in the bladder wall, inhibit enlargement of regional lymph nodes, inhibit the increase in MHC II expression of lymph node cells, and diminish systemic immunity that was induced by BCG administration. Signs of immune impairment were seen even if INH was administered later on during the course of BCG therapy, or if the dose of BCG was increased. Similarly, in humans, the absolute number of granulocytes and the concentration of IgG antibodies after BCG instillation were significantly suppressed by INH administration. This immunosuppression may be part of a response that prevents an immune response to covalent binding of INH and protects the liver. However, the effects of INH on the
immune system are complex; it commonly induces autoantibody production and can also lead
to a lupus-like autoimmune reaction.\textsuperscript{123}

In short, we were unable to replicate a previous report of INH-induced liver injury in rats, and even that model did not represent a true model of INH-induced liver injury in humans. Given that INH-induced liver injury appears to be immune-mediated and the default response of the liver is immune tolerance, we used methods to attempt to overcome immune tolerance. Although we saw some liver injury, the response in mouse knockout models also do not represent a true model of INH-induced severe liver injury in humans. The two most likely explanations for this failure are that there are many redundant mechanisms of immune tolerance and/or that the mice simply did not have the requisite MHC/T cell receptor repertoire to develop a strong immune response.
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Jack Uetrecht holds the Canada Research Chair in Adverse Drug Reactions. Imir G Metushi is a trainee of the Drug Safety and Effectiveness Cross Disciplinary Training Program which is funded by the Canadian Institutes for Health Research. The operating funds for this research were also supplied by the Canadian Institutes for Health Research. We would like to thank Dr Pamela Ohashi for giving us breeding pairs of PD1−/− and Cbl−/− mice as well as for providing us with anti-CD4/CD8 antibody. We thank Dr Tony Hayes for interpretation of the liver H & E slides. We also thank Winnie Ng for helping with the optimization of the antibodies for flow cytometry and Kristin Wohanka for helping with the rat treatment.
SIGNIFICANCE

In this chapter we tried to develop an animal model of INH-induced hepatotoxicity, but were unsuccessful. Building logically from our previous studies in which we found that Balb/c may be the most susceptible strain to INH-induced liver injury and from the human studies that suggest that injury is immune-mediated; we tried a variety of strategies in order to induce liver injury in mice by treatment with INH. Independent of the dose, Balb/c mice did not appear to be the ideal strain for developing an animal model because they lost a large amount of weight when treated with INH. In contrast, knockout mice such as Cbl-b and PD1 knockout, which should not develop as much immune tolerance, had more INH-induced liver injury compared to their C57BL/6 wild type background. This suggests that, as we had previously speculated, immune tolerance may be the most important barrier to developing this type of animal model. Interestingly, treatment of Rag knockout mice also displayed a mild form of liver injury implying that the adaptive immune system may not be what initiates hepatotoxicity in mice. Overall, we observed a down regulation of proinflammatory cytokines, which suggests that INH may cause immunosuppression, and this may contribute to immune tolerance that prevents the induction of more severe liver injury and an animal model of significant INH-induced liver injury.
3.5: Autoimmune Hepatitis Induced in Mice by Immunization with Isoniazid-Modified Hepatic Proteins is Prevented by Oral Administration of Isoniazid

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Imir G Metushi was responsible for designing the study, conducting experiments, collecting and analysing the data as well as writing the manuscript. Dr Ping Cai helped design the original study involving S100 immunization and he also helped in conducting the study. Dr Libia Vega has designed and performed the experiment as well as analyse results for the phenotyping of macrophages. Dr. Jack Uetrecht helped design the study and edited the manuscript.

Manuscript in Preparation. I G Metushi et al., “Autoimmune Hepatitis Induced in Mice by Immunization with Isoniazid-Modified Hepatic Proteins is Prevented by Oral Administration of Isoniazid”
Results

Immunization of C57BL/6 and Cbl-b<sup>−/−</sup> Mice with S100

Our most recent attempts to develop an animal model of INH-induced hepatotoxicity have been unsuccessful, however, the results suggested that immune tolerance is the most likely barrier to developing this animal model. To break immune tolerance in the liver we immunized mice with INH-modified hepatic proteins (S100-INA) to sensitize the immune system to INH, which we know binds in the livers of mice in vivo, and then we treated the animals orally with INH in order to produce ongoing drug-protein conjugates and liver injury that could represent a model INH-induced liver injury in humans.

As previously described, immunization of mice with S100 resulted in induction of EAH (Figure 42A).
Figure 42: H&E slides from mice immunized with S100 or S100-INH or alternatively S9 or S9-INA and some were also treated with INH orally. (A-C) C57BL/6 mice were immunized with S100 or S100-INH in CFA every week for 3 weeks as described in Figure 2/Scheme1A. INH was given in food from the first week as described in the methods section. Animals which were sacrificed after 16 weeks from the first immunization. S100 are mice immunized with S100 only. S100 + INH are mice immunized with S100 while being treated with INH in food. S100-INA + INH are mice immunized with S100-INA while also being treated with INH orally. Figures are representative of 4 animals/group. Magnification is 10X. (D-F) Mice were immunized with S9 along with CFA/IFA and INH was given in food (for details see Figure 2/Scheme 2). S9 are mice immunized with S9 only. S9 + INH are mice immunized with S9 and treated orally with INH in food. S9-INA + INH are mice immunized with S9-INA while given INH orally. Animals were sacrificed after 3 weeks of being treated with INH orally. Figures are representative of 4 animals per group. Magnification 20X.

By histology, EAH was present at 6 and 16 weeks after initial S100 immunization with week 6 being more severe than week 16 (refer to Figure 12/Scheme 1A for Methods and Figure S18A for ALT activity). However, a marked reduction in the degree of autoimmune hepatitis was observed when immunization was followed by treatment with oral INH; this was consistent even when animals were immunized with S100-INA (Figure 42B,C). A confounding factor with the use of CFA is that it induces inflammation, which decreases cytochromes P450 (P450) synthesis, and therefore it can inhibit drug bioactivation.\textsuperscript{187,188} In the initial experiments we treated animals with INH during the immunization schedule; later we waited for 4 weeks before starting the INH to mitigate this effect as shown in Figure 12. We confirmed that there was a high degree of covalent binding of INH, and therefore this is not a significant issue when
we delayed the onset of INH treatment (Figure S19). In previous studies we found that Cbl-b−/− mice were more susceptible to INH-induced hepatotoxicity than C57BL/6 mice; this is in accordance with the fact that Cbl-b−/− mice have impaired immune tolerance relative to their C57BL/6 background. Cbl-b−/− mice were also susceptible to autoimmune hepatitis induced by immunization with S100, but the decrease in autoimmune hepatitis by oral administration of INH was also observed. In initial studies we used ALT as a biomarker of liver injury, but we now know that the ALT assay is inhibited by INH, and there was no change in ALT (Figure S18). Given the focal and chronic nature of the hepatic necrosis, histology provided a better indication of liver injury; however, no differences between C57BL/6 and Cbl-b−/− mice were observed in the extent of EAH or prevention of EAH by oral INH administration.
Immunization of C57BL/6 and Cbl-b−/− Mice with S9

S100 does not contain P450s, and we have shown that INH binds to P450 with the greatest covalent binding localised around the central vein where most P450’s are located. Therefore, we tried replacing S100 with S9, which does contain the P450s. Mice immunized with S9 could not be maintained for more than 7 weeks after the last immunization because they developed peritonitis (see Figure 12/Scheme 2 for the immunization schedule). Oral administration of INH to S9 or S9-INA-immunized mice prevented autoimmune hepatitis in a similar way as S100-treated mice (Figure 42 D-E). The SDH activity in S9/S9-INA-treated mice was increased, but there were no differences between S9 group and the co-treated with oral INH (Figure S20). As with S100 immunization, when mice were immunized with S9/S9-INA and INH was given orally, no changes in the severity or prevention of EAH were observed between C57BL/6 mice and Cbl-b−/− mice.

Immunization With S100-INH Induced More Severe EAH than S100 Alone

To determine if the modification of S100 with activated INH (S100-INA) or the oral administration of INH prevented autoimmune hepatitis, mice were immunized (schematic shown in Figure 12/Scheme 3) and sacrificed after 5 weeks of oral treatment with INH. We allowed CYP450 synthesis to recover for 4 weeks after the last immunization with IFA in order not to interfere with in vivo INH bioactivation. The body weight of animals exposed to INH was slightly reduced after one week of treatment, but no other differences were observed between groups (Figure S21A). The ALT activity was measured weekly for 4 weeks after the last immunization, but it did not change (Figure S21B). GLDH and SDH activity were significantly increased in the S100/S100-INA immunized groups with the S100-INA group having a more prominent increase (Figure 43A,B). Immunization with S100-INA produced a
higher grade of EAH when compared with the group receiving S100 alone, as shown by H&E staining (Figure 44), but paradoxically, when this was followed by oral INH administration, the severity of autoimmune hepatitis induced either by S100 or S100-INA was markedly reduced.

**Figure 43:** GLDH (A) and SDH activities (B) in female C57BL/6 mice immunized with S100 or S100-INA and/or treated with INH orally in food. Mice were treated as described in Figure 1/Scheme 3. INH was started in food at week 4 resulting in a 5 week treatment with INH until the end of experiment. Mean ± S.E. *p < 0.05, **p < 0.01, ***p < 0.001, Two-way ANOVA.
Figure 44: H&E and immunohistochemical staining for F4/80, CD11b, and CD8 in the liver of mice immunized with S100 or S100-INA. S100 = mice were immunized with S100 hepatic protein only; S100-INA = mice were immunized with S100 modified to mimic covalently bound INH; S100-INA + INH = mice were immunized with S100-INA and treated with 0.2% w/w of INH in food for 5 weeks. Figures are representative of 4 animals per group.
Along with the increase in severity of EAH in the S100-INA-treatment group there was an increase in cells that stained positive for F4/80, CD11b, CD8, CD4, CD45R, and KI-67 markers (Figure 44-46). Serum cytokine measurements by Luminex revealed small increases in eotaxin, IFN\(\gamma\), IL9, and IL13 in the S100-INA group; however, the large variation between samples made any possible differences not statistically significant (Figure 47).

Figure 45: H&E and immunohistochemical staining for CD4, CD45R, and KI-67 in the liver of mice immunized with S100 or S100-INA. S100 = mice were immunized with S100 hepatic protein only; S100-INA = mice were immunized with S100 modified to mimic covalently bound INH (S100-INA) only; S100-INA + INH = mice were immunized with S100-INA and treated with 0.2% w/w of INH in food for 5 weeks. Figures are representative of 4 animals per group.
Figure 46: Immunohistochemical grading of EAH in female C57BL/6 mice immunized with S100 or S100-INA with or without oral treatment with INH. Cells (clusters of lymphocytes, F4/80 macrophages, Cd11b macrophages, CD8⁺ T cells, CD4⁺ T cells, or CD45R B cells) were counted under 10X or 40X magnification. Mean ± S.E. *p < 0.05, **p < 0.01, ***p < 0.001, One-way ANOVA.
Figure 47: Serum cytokine measurements in female C57BL/6 mice. Control mice, mice treated with INH only for 5 weeks or mice immunized with S100 or S100-INA only and/or treated with INH in food for 5 weeks. Serum was taken at endpoint of Scheme 3 (Figure 2). Values represented as Mean ± S.E of 4 animals per group.
Chronic Treatment of NAT1/2-/- mice with INH

We have shown that mice treated with INH have higher blood levels of INH and more covalent binding than rats;\textsuperscript{154} therefore, mice appear to be a better model for human slow acetylators who are at increased risk of hepatotoxicity.\textsuperscript{12} We went one step farther and treated NAT1/2-/- mice, which should not eliminate INH by acetylation; this is presumed to result in greater covalent binding of INH in the liver and more hepatotoxicity. Treatment of NAT1/2-/- mice with INH resulted in mild elevations in GLDH and SDH only in female mice (Figure 48A-D). Treatment with INH reduced the body weight gain in both male and female NAT1/2-/- mice (Figure 48E,F), with female mice being more sensitive than male mice.
Figure 48: GLDH, SDH, and body weights in NAT1/2−/− male (A, C, and E) and female (B, D, and F) mice from control or treated for 5 weeks with 0.2% INH w/w (n=4). A-B) GLDH activity in NAT 1/2−/− mice. C-D) SDH activity in NAT 1/2−/− mice. E-F) Body weight in NAT 1/2−/− mice. Mean ± S.E. *p < 0.05, **p < 0.01, ***p < 0.001, Two-way ANOVA.
We expected to find more covalent binding of INH in NAT1/2−/− mice; however, female NAT1/2−/− mice had similar covalent binding as female C57BL/6 mice (Figure 49A). Interestingly, male NAT1/2−/− mice had higher covalent binding than females, although female NAT1/2−/− mice had higher GLDH elevations and more covalent binding than Balb/c mice (Figure 49B-D).

**Figure 49:** Western blotting comparing the amount of INH covalent binding in the liver of mice treated with 0.2% INH w/w in food for 5 weeks. (A) Female C57BL/6 or NAT1/2−/− mice (n = 4); (B) male or female NAT1/2−/− mice (n = 4); (C-D) male or female NAT1/2−/− (n = 3) and male or female Balb/c mice.
Acute Treatment of Female NAT1/2\(^{-/-}\) Mice with INH.

Only a small increase in GLDH, indicating mild liver injury, was observed when treating female NAT1/2\(^{-/-}\) mice with INH for up to 5 weeks; therefore we looked for signs of an immune response after acute treatment. We determined the amount of INH covalent binding as a function of time and found that INH covalent binding to liver proteins was higher in NAT1/2\(^{-/-}\) mice treated for 7 days than in mice treated for only 1 or 3 days (Figure S22); however, there was no significant increase in GLDH or SDH levels (Table S3). Hepatic steatosis was observed as early as day one of treatment, and this was sustained until day 7 (Figure 50). There was a considerable increase in lipid as shown by Oil Red O staining after 3 days of treatment and electron microscopy revealed depletion of glycogen, an increase in lipid vesicles, and abnormal mitochondria and endoplasmic reticulum shape (Figure 50). There were no changes in the proportion of F4/80 positive cells in liver (Figure 50).
Figure 50: Steatosis as an early hepatic response to treatment of NAT1/2⁻ mice with INH (100 mg/kg/day by gavage for 3 days). Stains were H&E, Oil Red O, transmission electron microscopy (TEM, n = 3) or anti-F4/80 staining in the liver (n = 4).
A comparison of INH covalent binding in different fractions of liver homogenate revealed that the highest covalent binding was to microsomes followed by mitochondria, and the lowest was to the cytosol; however, binding occurred to all fractions and the differences were small (Figure 51). This indicates that INH has the potential to cause mitochondrial damage; however, no inflammatory response leading to an increase in the number of macrophages was observed (Figure 50).

**Figure 51:** Covalent binding of INH to subcellular fractions of the liver. Female C57BL/6 mice (n = 2) were treated for 7 days with INH in food at 0.2% w/w.
Phenotyping of M1 vs. M2 Macrophages in Female NAT1/2<sup>−/−</sup> Mice treated with INH

Flow cytometry was used to phenotype macrophages in the cervical nodes of INH-treated NAT1/2<sup>−/−</sup> mice as shown in Figure 52. The markers utilized were adapted from the literature.<sup>24,189-191</sup> Monocytes were first gated by forward and side scatter, and then by the membrane expression of CD11b and/or F4/80 <i>versus</i> the intracellular expression of TNF-α and/or IL-10. In these plots we also evaluated the presence of intracellular CCL2<sup>+</sup>. With this combination of cell markers we determined the phenotype and the proportion of activated macrophages present in peripheral lymph nodes. Treatment with INH led to an increase in the percentage of macrophages after 1, 3, and 7 days of treatment, but this returned to baseline levels by day 35 despite continued treatment (Figure 52A). Further analysis of macrophage markers indicated that INH treatment increased the proportion of F4/80 only macrophages, which suggested recruitment of M1 polarized cells at days 1-7, while at day 35 of treatment, a dramatic decrease in F4/80 only macrophages was observed (Figure 52B). Although no statistical changes in the proportion of F4/80<sup>+/</sup>/CD11b<sup>+</sup> macrophages (which likely represents M2 polarization) were observed, a reduction on this particular phenotype was suggested, and the increase in the M1/M2 ratio compared with the control was unequivocal. Further analysis of the proportion of activated M1 and M2 cells by analyzing the intracellular cytokine production revealed that treatment with INH decreased the percentage of activated M1 macrophages from days 1 to 7, while there was an increase in the activation of M2a and M2b macrophages. All these changes returned to baseline levels by day 35 of treatment (Figure 52C).
Figure 52: Macrophage phenotyping by flow cytometry in the cervical lymph nodes of NAT1/2<sup>−/−</sup> mice. Day 0 is before INH treatment (n = 4) while for days 1, 3, and 7, INH was given by gavage at a dose of 100 mg/kg/day (n = 3); and for the 35 day data, INH was administered in food at 0.2% w/w (n = 4). (A) Percentage of total events from the monocyte gate after 1, 3, 7, or 35 days of INH treatment, (B) Phenotyping of macrophages into M1 (F4/80<sup>+</sup> only) and M2 (F4/80<sup>+</sup>/CD11b<sup>+</sup>). (C) Activation of M1 (TNFα<sup>+</sup>/IL-10<sup>−</sup>/CCL2<sup>+</sup>), M2a (CD11b<sup>+</sup>/IL-10<sup>+</sup>), M2b (IL-10<sup>+</sup>/TNFα<sup>−</sup>/CCL2<sup>−</sup>), and M2c (IL-10<sup>+</sup>/TNFα<sup>+</sup>/CCL2<sup>−</sup>). Mean ± S.D. *p<0.05, **p<0.01, ***p<0.001, Mann-Whitney’s U test.
**Discussion**

Due to its high efficacy, INH remains the drug of choice for the treatment of tuberculosis. However, in a small fraction of the patients, INH can lead to liver failure. Progress in this field has been hampered by the lack of a valid animal model of INH-induced liver injury. Recently, we determined that INH itself, and not just the acetylhydrazine metabolite, is responsible for covalent binding in the liver, and binding of the parent drug appears to be able to initiate an immune response as determined by lymphocyte transformation tests. Furthermore, we have identified anti-INH and anti-P450 antibodies in the serum of patients with INH-induced liver failure, and it has been showed that the major cell type in liver biopsies from cases of INH-induced hepatotoxicity are CD8+ cells. Together, these data provide strong evidence that INH-induced liver injury is immune-mediated, and in particular there is an immune response against INH rather than the acetylhydrazine metabolite.

Previously we showed that mice are a better species to develop an animal model than rats because ratio of INH/acetylhydrazine and covalent binding of INH in the livers of mice are more similar to that of humans than rats. Previous efforts to develop an animal model of INH-induced hepatotoxicity by treating mice orally with INH have been unsuccessful. More recently, we found a higher incidence of mild liver injury in various knockout mouse strains that have impaired immune tolerance relative to wild type C57BL/6 mice. This suggested that immune tolerance may be the major barrier to overcome in order to induce liver injury. However, attempts to convert these models of mild hepatotoxicity into models of INH-induced liver failure have been unsuccessful. In this study we utilised a previously reported model of EAH in mice to try to overcome immune tolerance. Specifically, we immunized mice with S100 or S9 hepatic proteins that had been modified to mimic the in vivo covalent binding of
INH, this was followed by treatment with INH which also leads to modification of hepatic proteins. Modification of these proteins with INH increased their ability to induce EAH relative to native proteins (Figures 42 and 44), but paradoxically, subsequent treatment with INH markedly decreased the liver injury caused by this immunization. The inhibition of EAH occurred independent of whether the hepatic proteins had been modified to mimic covalent binding of the reactive metabolite of INH. This implies that INH acts as an immunosuppressant. The prevention of autoimmune hepatitis by oral administration of INH was also associated with a decrease in immune cell infiltration of macrophages/dendritic cells and T/B lymphocytes (Figures 45-46), which suggests suppression of an immune response in the liver. The most dramatic change was when oral INH treatment prevented the marked infiltration of F4/80+ macrophages in the liver that was caused by immunization with INH-modified S100 hepatic proteins. Although, INH could prevent infiltration of leukocytes in the liver caused by S100/S100-INH, it did not completely prevent the increase in GLDH/SDH, which had been induced before initiation treatment with oral INH (Figure 43). One possible reason for this is the fact that due to extensive tissue injury from the CFA immunization, the GLDH/SDH could be leaking out from other organs such as kidney and intestine.

Because our attempts to induce an immune response by immunizing mice with INH-modified hepatic proteins while treating with oral INH failed, we tried to develop a model by treating NAT1/2−/− mice, which should not acetylate INH. The rationale for using acetylator knockout mice was the observation that patients who are slow acetylators are at increased risk of INH-induced hepatotoxicity. We expected greater covalent binding of INH in these animals and possibly an increase in liver injury. Interestingly, the amount of INH covalent binding between female C57BL/6 and NAT1/2−/− mice was similar. In fact male NAT1/2−/− mice had higher covalent binding of INH in the liver compared to female NAT1/2−/− mice, although,
however, treatment of female NAT1/2\(^{-/-}\) mice with INH produced a mild increase in serum SDH and GLDH while treatment of male mice did not (Figure 48). However, given the lack of abnormal histology this does not represent a model of significant INH-induced liver injury. Treatment of NAT1/2\(^{-/-}\) mice with INH for 1-7 days also produced steatosis and an increase in Oil Red O staining, which indicates lipid accumulation, and this was followed by a reduction of glycogen and abnormal mitochondrial morphology (Figure 50). There are other reports that indicate that INH can cause mitochondrial injury in mice and rats,\(^{167,168}\) and more recently, in a mouse diversity panel of 34 strains, microvesicular steatosis was observed when mice were treated with 100 mg/kg/day INH for 3 days.\(^{194}\) In addition, changes in the expression of several genes related to the mitochondria dysfunction were observed.\(^{194}\) This suggests that in animals, treatment with INH can cause mitochondrial damage at early time points. We also found that there is significant binding of the reactive metabolite of INH to mitochondrial proteins, suggesting that the reactive metabolite of INH can directly bind to mitochondrial protein, and this may be responsible for induction of steatosis. Steatosis has also been observed in a few clinical cases of INH-induced liver injury.\(^{195-197}\) However, these cases involve administration of INH together with other drugs such as rifampicin, ethambutol, and/or pyrazinamide, and these cases do not involve the common histological feature of hepatocellular necrosis, which is observed in most cases of INH-induced liver failure.\(^{106}\) In our studies, steatosis appeared to resolve despite continued treatment because we don’t see steatosis after 5 weeks of INH treatment. However, in the studies involving chronic treatment the drug was administered in food rather than by gavage. It is possible that mitochondrial injury is an early event that helps to initiate an immune response, but it is no longer evident at later times when liver biopsies are likely to be performed on patients with INH-induced liver failure.
In INH-treated NAT1/2−/− mice we observed a decrease in the percentage of activated M1 macrophages, which are thought to be pro-inflammatory, and an increase in the proportion of M2a and M2b macrophages, which are characterised as anti-inflammatory (Figure 52). This is also consistent with an immunosuppressive effect of INH, and this may prevent most patients from developing significant INH-induced liver injury. This is in contrast to the fact that INH treatment commonly induces antinuclear antibodies, and in some cases, a lupus-like autoimmune syndrome.

So what does this data mean? Just like with most humans who don’t develop liver injury due to drugs that can cause idiosyncratic drug-induced liver injury, the present studies highlight the complex immune response to drugs such as INH. In humans, INH-induced hepatotoxicity appears to be immune-mediated and caused by covalent binding of INH; therefore, we used interventions that should stimulate an immune response against INH-modified proteins. However, the observation that oral treatment with INH prevented EAH and increased activated M2 macrophages is consistent with our previous observations that INH can cause immune suppression. This provides a plausible explanation of why it is so difficult to develop an animal model of severe INH-induced hepatotoxicity similar to the idiosyncratic reaction in humans that can lead to liver failure.
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SIGNIFICANCE OF CHAPTER

This chapter builds on the previous chapters and emphasizes again the difficulty in trying to develop animal models of IDILI. In addition, we found that immunization of mice with S100 modified by INH produces greater liver injury than S100 itself, implying a greater immune response against drug modified protein; however, paradoxically, subsequent oral treatment with INH prevented liver injury and in addition prevented infiltration of immune cells in the liver. This suggests that INH may cause immune suppression, and this may partially explain why INH does not cause liver injury in most patients or animals. In addition, we show again that during chronic treatment with INH, covalent binding did not correlate with liver injury because male NAT1/2−/− mice had more covalent binding, but female NAT1/2−/− mice developed slightly higher GLDH levels implying mild injury. That does not mean that covalent binding is not important, rather covalent binding may be necessary but not sufficient to induce significant liver injury. The fact that oral INH treatment resulted in an upregulation of anti-inflammatory M2 macrophages after 1 day of treatment suggests again that INH may cause immune suppression. Overall, we were not able to develop an animal model of INH-induced hepatotoxicity, but we have shown again that oral treatment with the drug leads to an immune response which resolves with time rather than causing significant liver injury, and this resolution is most easily explained by immune tolerance.
3.6: Amodiaquine-Induced Liver Injury in Mice: A Novel Animal Model of Liver Injury with a Delayed Onset is Mediated by NK Cells

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Imir G Metushi has been responsible for designing the study, conducting experiments, collecting and analysing the data as well as writing the manuscript. Ping Cai helped in establishing the animal model in which treatment of female C57BL/6 mice produced mild liver injury. Dzana Dervovic helped to set up several flow cytometry experiments. Dr. Jack P. Uetrecht helped design the study and edit the manuscript.

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Results

Treatment of C57BL/6 Mice with AQ Results in Mild Liver Injury

Treatment of female C57BL/6 mice with AQ resulted in a mild increase in ALT which had a delayed onset of at least one week and resolved despite continued treatment (Figure 53A). There was no increase in ALT activity for male C57BL/6 mice (Figure 53B). This is likely because the blood levels of AQ and its major metabolite (desethyl AQ) were much higher in female than male C57BL/6 mice; female mice had AQ levels of 265.8 ng/mL ± 37.8; mean ± S.E. while male mice had AQ levels of 103.7 ng/mL ± 19.9; Mean ± S.E., *p < 0.05 analysed by Mann-Whitney U test. A similar trend was observed for the desethyl metabolite of AQ where female mice had levels of 1680.8 ng/mL ± 218.5; mean ± S.E. and male mice had levels of 454.5 ng/mL ± 62.4; mean ± S.E., *p < 0.05, analysed by Mann-Whitney U test. The blood levels for AQ as a function of time in female mice were similar between week 1, 3, and 7 (week 1 = 369.2 ± 75.5, week 3 = 242.6 ± 115.8, and week 7 = 259.6 ± 154.9; all concentrations in ng/mL reported as mean ± S.E.).

AQ covalent binds to macromolecules in the liver and spleen. Most of the binding in the liver was around the centrilobular region (Figure 53C,D), which is where most of the cytochrome P450 is located, and AQ was also shown to bind in the red pulp of the spleen, which contains antigen presenting cells (Figure 53E,F). A comparison of AQ covalent binding in the livers of female C57BL/6 mice as a function of time revealed that binding peaks after two days of treatment, and remains relatively constant after that time (Figure 54).
Figure 53. Liver injury and covalent binding of AQ in C57BL/6 mice. (A) female or (B) male mice were treated with AQ in food for up to 6 weeks. (C,D) Covalent binding of AQ in the liver of a female C57BL/6 mouse treated with AQ for 3 weeks. (E, F) covalent binding in the spleen of the same mouse. Figures are representative of 4 animals per group. The ALT levels were analysed for statistical significance by two-way ANOVA. A p value < 0.05 was considered significant (*p < 0.05; **p < 0.01; ***p < 0.001).
The Increase in ALT is Associated with Lymphocyte Infiltration in the Liver

The immune response to AQ treatment was analyzed by immunohistochemistry and flow cytometry in female C57BL/6 mice treated with AQ for 1, 3, or 7 weeks. These time points were chosen to determine the immunological changes before ALT increased, when ALT was highest, and after ALT returned back to normal. The ALT was significantly higher at week 3 compared to control mice (Figure 55A). The liver weights in control and treated mice were similar, but the total number of lymphocytes isolated from the liver was higher in AQ-treated mice at week 3, which indicates infiltration of immune cells (Figure 55B,D). The spleen weight was also increased at week 1 and 3, and this was associated by an increase in cell numbers as well (Figure 55C, E). In lymph nodes, there was a significant increase in the number of lymphocytes by week 1 (Figure 55F). Immunohistochemical analysis revealed an increase in immune cells staining positive for CD4, CD8, CD11b, F4/80, and CD45R in the liver of mice treated with AQ as well as increased cell proliferation as indicated by the number of cells stained by KI67 (Figure 56A); with the highest cell numbers observed around week 3 when ALT was the highest (Figure 56B).
Figure 55: ALT, liver and spleen weights, and lymphocyte numbers in the liver, spleen and lymph nodes of mice treated with AQ. (A) ALT activity after AQ treatment for 1, 3, or 7 weeks. (B-C) Liver and spleen weights of 4 mice from part A. (D-F) Total number of lymphocytes in 4 control or AQ-treated animals from part A. Panel A was analysed for significance by one-way ANOVA while the rest of the panels were analysed by Mann-Whitney U test. A p value < 0.05 was considered significant (*p < 0.05; **p < 0.01; ***p < 0.001).
Figure 56: Immunohistochemical staining of livers of AQ-treated mice. (A) Staining for CD4, CD8, CD11b, KI67, F4/80, or CD45R. (B) Grading of the number of cells in part A; the grading was determined from the results of statistical analysis (data not shown). Figures are representative of 4 animals per group. For illustration purposes, slides from mice treated with AQ for 3 weeks are shown because they showed the largest changes.
Flow cytometry analysis revealed a small increase in CD4⁺ cells in the spleen and cervical lymph nodes while a greater increase for CD8⁺ or NK1.1⁺ cells in the liver, spleen, and cervical lymph nodes was observed (Figure 57). The dominant immune cell infiltrating the livers of patients with drug-induced liver failure are CD8⁺ cells. We used CD62L, CD44, and CD69 markers to test for T cell activation. There was no indication of activation of CD4⁺ cells in the liver, but there was an increase in cells expressing CD44 in the spleen. (Figure 58). In contrast there was an increase in CD8⁺ T cells expressing CD44, CD69 as well as low CD62L expression in the liver but not in the spleen (Figure 58).
**Figure 57:** Effect of AQ on the numbers of CD4\(^+\), CD8\(^+\), and NK leukocytes in the liver, spleen, and lymph nodes. The cells from female C57BL/6 untreated control mice (n = 4) or AQ-treated mice (n = 4) were phenotyped at 1, 3, and 7 weeks. Total cell numbers were calculated by multiplying the percentage of each cell subtype by the total number of lymphocytes from each organ. Analyzed for statistical significance by Mann-Whitney U test. A p value < 0.05 was considered significant (*p < 0.05; **p < 0.01; ***p < 0.001).
Figure 58: Lymphocyte activation in AQ-treated mice. The lymphocytes from female C57BL/6 control (n = 4) or AQ-treated mice (n = 4) were stained for CD62L, CD44, and CD69. Total cell numbers were calculated by multiplying the percentage of each cell subtype by the total number of lymphocytes from each organ. Analysed by statistical significance by Mann-Whitney U test. A p value < 0.05 was considered significant (*p < 0.05; **p < 0.01; ***p < 0.001).
There was an increase in the number of CD4^+CD279^+ and CD8^+CD279^+ cells in the liver (Figure 59) which suggests an increase in PD-1 and induction of immune tolerance; however, there were no apparent changes in surface CTLA4 staining (Figure S23). Serum cytokines such as IL-1α, IL-17, IL-12 (p40), and INF-γ were upregulated either by week 1 or 3, and they returned to baseline or lower than baseline levels by week 7 when the ALT also returned to normal (Figure 60).

Figure 59: Expression of PD1 (CD279) on T cells from AQ-treated mice. Total cell numbers were calculated by multiplying the percentage of each cell subtype by the total number of lymphocytes from each organ. Analysed by statistical significance by Mann-Whitney U test. A p value < 0.05 was considered significant (*p < 0.05; **p < 0.01; ***p < 0.001).
Figure 60: Serum cytokine measurements in AQ-treated mice. Female C57BL/6 mice control (n = 4) or AQ-treated (n = 8) for 1, 3, or 7 weeks. Analysed by statistical significance by Mann-Whitney U test. A p value < 0.05 was considered significant (*p < 0.05; **p < 0.01; ***p < 0.001).
Treatment of PD1−/−, Cbl-b−/−, or Rag−/− mice with AQ Results in More Severe Liver Injury

An attempt was made to increase AQ-induced liver injury by using knockout animals that have impaired immune tolerance. Treatment of Cbl-b−/− mice and PD1−/− mice, in general, resulted in slightly higher ALT levels than what we usually observe in female C57BL/6 mice (Figure 61A,C). However, as with C57BL/6 mice, ALT resolved despite continued treatment suggesting that even in these knockout mice there is still potential for induction of immune tolerance. The amount of AQ covalent binding in the liver of Cbl-b−/− and PD1−/− mice was similar to that in C57BL/6 mice, which suggests again that covalent binding of AQ in the liver is not the only predictor of liver injury (Figure 61B,D). Attempts, to break immune tolerance by treating with AQ and FICZ or anti-CD25 antibody in an attempt to deplete Treg cells did not make the liver injury worse (Figure S24).
Figure 61: AQ-induced liver injury and covalent binding in Cbl-b−/− and PD1−/− mice. Mice were treated with 0.2% w/w AQ in food for up to 6 weeks. Covalent binding of AQ was determined for the liver at endpoint.
In PD1\(^{-/-}\) mice we consistently observe a larger number of necroinflammatory foci than we see in C57BL/6 mice. In particular, we see that when there are focal areas of necrosis, there is an infiltration of CD4, CD8, CD11b, F4/80, and CD45R positive cells (Figure 62). If AQ-induced liver injury in mice is immune-mediated, there should be no injury in Rag\(^{-/-}\) that do not have competent B and T cells.

Figure 62: Immunohistochemical staining of the liver of PD1\(^{-/-}\) mice treated with AQ for 5 weeks.
Contrary to expectations, treatment of female Rag\(^{-/-}\) mice with AQ also resulted in an increase in ALT and this injury did not resolve as effectively as in the wildtype mice (Figure 63A). Covalent binding at the end of the 5 week treatment was similar between Rag\(^{-/-}\) mice and C57BL/6 mice suggesting that covalent binding of AQ was not the basis for this difference in ALT at week 5 (Figure 63C). A repeat of this experiment for up to 11 weeks revealed that Rag\(^{-/-}\) mice consistently had higher ALT levels than C57BL/6 mice; however, they still did not progress to liver failure (Figure 63B); as in the earlier experiment, covalent binding of AQ in the livers of these mice was not the basis for this difference (Figure 63D).

**Figure 63:** Treatment of female C57BL/6 or Rag\(^{-/-}\) mice with AQ. A) ALT levels in female C57BL/6 mice vs. female Rag\(^{-/-}\) mice. * indicates a statistically significant difference compared to week 0; analysed by Mann-Whitney U test (* < 0.05).
The results from the Rag\(^{-/-}\) mouse experiment revealed that it is not the adaptive immune system that is responsible for AQ-induced liver injury in mice. In previous experiments we found an increase in cells expressing the NK1.1 surface marker. Although, some of these cells may be NKT cells, from the results of Rag\(^{-/-}\) experiment, we can conclude that NKT cells are not responsible for injury in this model. We depleted the NK cells by using an anti-NK1.1 antibody. Administration of this antibody lead to depletion of NK1.1 cells in the liver, spleen, blood, and cervical lymph nodes (Figure 64).

**Expression of NK1.1**

![Image](image.png)

**Figure 64:** Depletion of NK cells in female C57BL/6 mice. Control = female C57BL/6 mice not treated with anything. IgG1 = female C57BL/6 mice treated with AQ in food and i.p. injections of IgG1 isotype control. NK1.1 = female C57BL/6 mice treated with AQ in food and i.p. with anti-NK1.1 antibody.

Depletion of NK cells prevented the increase in ALT in female C57BL/6 mice treated with AQ (Figure 65A) suggesting an important role for the innate immune system in this animal model. Consistent with our previous results, covalent binding was not the basis for this difference (Figure 65B).
Figure 65: ALT levels and covalent binding in NK cell-depleted mice. A) Control = untreated female C57BL/6 mice. IgG1 = female C57BL/6 mice treated with AQ in food and i.p. injections of IgG1 isotype control. NK1.1 = female C57BL/6 mice treated with AQ in food and i.p. with anti-NK1.1 antibody. B) Western blotting from mice treated in part A showing that the amount of AQ covalent binding is did not change between groups.
Discussion

Drug-induced liver injury represents a significant cause of patient morbidity and mortality, and it also adds to the uncertainty of drug development. The mechanism of idiosyncratic drug-induced liver injury (IDILI) is currently unknown, and a major impediment to mechanistic studies is the lack of a good animal. To be a good animal model, the injury in animals should have essentially the same mechanism as the injury in humans and that means the characteristics should also be very similar. The most common characteristics of IDILI in humans is a delay in onset and infiltration of CD8 cells in the liver.

AQ was withdrawn from the market because of a significant incidence of severe hepatotoxicity and/or agranulocytosis. In human studies, the mechanism of AQ-induced hepatotoxicity is believed to be immune-mediated because hepatotoxicity has a delayed in onset, occurs more rapidly upon rechallenge, is associated with features such as fever and/or rash, and anti-AQ antibodies have been detected in patients’ sera.

In this study we found that treatment of female C57BL/6 mice with AQ results in a delayed onset of hepatotoxicity, which resolves despite continued treatment (Figure 53A). The liver injury was not apparent in male mice, and this may be explained by the fact that male mice had significantly lower blood levels of AQ and its major metabolite (desethyl AQ) than female mice. Also, the amount of AQ covalent binding was higher in female mice compared to males; however, AQ covalent binding was maximal after two days in females suggesting that it is not covalent binding per se that causes the injury (Figure 54). The pattern of covalent binding was centrilobular in the liver and around the red pulp in the spleen, which is where most of the antigen presenting cells are located (Figure 53). When there was an increase in ALT, there also was an increase in cells staining positive for CD4, CD8, CD11b, KI67, F4/80,
and CD45R in the liver, which implies an immune response (Figure 56). The infiltration of these cells correlated with the increase in ALT. The response was not limited to the liver, and the spleen of treated mice was enlarged at week three along with significant cell proliferation (Figure 56). There was also evidence of lymphocyte activation in the liver and spleen (Figure 58). In general, these effects returned to baseline levels by week 7, which suggests the induction of immune tolerance. Attempts to convert this mild model of liver injury into a model of more severe injury by treating Cbl-b−/− and PD1−/− mice, which have impaired immune tolerance, resulted in somewhat greater liver injury, but it also resolved despite continued AQ treatment (Figure 61). In PD1−/− mice we also observed an increase in the number of necroinflammatory foci infiltrate in the liver (Figure 62). These results were consistent with injury mediated by an adaptive immune response that resolved with induction of immune tolerance. The ultimate test of this hypothesis was performed by treating Rag−/− mice, which lack competent T and B cells. Paradoxically, AQ-treated Rag−/− mice sustained more liver injury than wildtype mice, and the resolution in liver injury was less complete (Figure 63). This implies that rather than being responsible for the liver injury, cells of the adaptive immune system are involved in the resolution of liver injury. Another cell type that was increased by AQ treatment expressed the NK1.1 surface marker and are presumed to be NK cells (Figure 57). Depletion of NK cells prevented the AQ-induced increase in ALT (Figure 64), which provides strong evidence that these are the cells that mediate the liver injury. Although NKT cells also express NK1.1, the experiment with Rag−/− mice indicates that it is not NKT cells that mediate the liver injury.

We were not able to reproduce AQ-induced liver failure in mice; however, we did observe liver injury that has characteristics very similar to the mild liver injury induced by AQ in patients that is more common than liver failure. One possible interpretation of these results
is that NK cells mediate mild liver injury in patients, and it is only when this innate immune response evolves into an adaptive immune response that severe liver injury results. More studies will be required to test this hypothesis.
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SIGNIFICANCE OF CHAPTER

Throughout this thesis I have emphasised how difficult it is to predict and prevent IDRs. The major reason being a lack of mechanistic understanding, which in turn is because it is almost impossible to perform prospective and controlled experiments in humans and the fact that there are no valid animal models. The final chapter of this thesis illustrates what may be the first valid animal model of mild immune-mediated drug-induced liver injury. In female C57BL/6 mice, AQ led to mild liver injury that had a delayed onset and resolved despite continued treatment. This type of liver injury is much more common than drug-induced liver failure. The liver injury was mediated by NK cells, and cells of the adaptive immune system appeared to be involved in the resolution of this injury. This suggests the hypothesis that severe idiosyncratic drug-induced liver injury starts with an innate immune response that resolves with the help of cells of the adaptive immune system. It is when the adaptive immune response fails to induce tolerance and rather causes liver injury that severe injury results. This model provides an important tool to test mechanistic hypothesis of IDILI and additional studies will be performed to test this hypothesis and to test whether these findings translate to humans.
Despite extensive research, IDRs remain a significant medical problem. In addition, the unpredictable nature of IDRs significantly adds to uncertainty and cost of drug development. IDILI is the most common type of IDR responsible for drug withdrawal. Although there have been many studies of the hepatotoxicity of drugs that cause IDILI, almost universally the experiments involve acute toxicity with high doses of the drugs, and the results have little relevance to the mechanism of IDILI. As I have mentioned throughout this thesis, the most common characteristic of IDILI, and other IDRs, is a delay in onset of at least one week. If we understood the mechanism of IDRs we might be able to predict and prevent such reactions; however, we don’t, and the major question is: how do we proceed in order to predict the unpredictable?

As mentioned in the introduction of this thesis, the fundamental principle that is used to explain such reactions lies within the “Hapten Hypothesis”. This hypothesis states that there is binding of a drug or its reactive metabolite to a protein and this makes the protein “foreign”, and this leads to an immune response that in some patients can cause injury to the organ involved. Extensions of this hypothesis are the danger hypothesis, which in addition to the drug-modified protein, require danger signals to be released in order to induce an immune response. Central to most hypotheses is an adaptive immune response, but the exact steps involved and what makes some individuals susceptible are unknown. Older studies made the connection between reactive metabolite production, covalent binding, and hepatic necrosis; however, as indicated above, most were acute studies where animals were given one large dose and the toxicity was studied a few hours later. Therefore, the claims of those papers that
covalent binding of the drug correlated with hepatic necrosis are unlikely to be relevant to IDILI. Now there is compelling evidence that IDILI involves an immune response; the question is: what kind of immune response, and how does it contribute to tissue injury? So far, this has been very difficult to answer, and it appears that different drugs can initiate different types of immune responses. In this thesis, we have studied two drugs: INH and AQ.

Both INH and AQ can cause liver injury in humans that has a delayed onset and features such as fever, rash, and an eosinophilic infiltrate in the liver. With INH, the belief until now has been that liver injury is not immune-mediated, but rather it has been called “metabolic idiosyncrasy”. Two important characteristics led people to classify INH-induced liver injury as “metabolic idiosyncrasy” compared to AQ-induced liver injury, which was more generally accepted to be immune-mediated. The first characteristic was that most cases of INH-induced liver injury do not have the classic features of an allergic immune response such as fever, rash, eosinophilic infiltrate in the liver, and also limited attempts to detect anti-INH antibodies were unsuccessful. However, the lack of allergic features is not significant evidence against an immune-mediated reaction. Most immune responses do not involve a fever or rash, and even the lack of immune memory can be observed in clearly immune-mediated reactions such as heparin-induced thrombocytopenia. Furthermore, the metabolite responsible for the acute liver injury in rats was found to be AcHz, and INH itself was not thought to be important in this toxicity. The reasons for this conclusion were: INH was known to be acetylated to acetyl-INH and then hydrolysed to AcHz; furthermore, in animal studies it was shown that AcHz covalent binding in the liver correlated with hepatotoxicity, but INH binding was not detected at all. However, these studies were performed in rats and involved acute toxicity. This model has very different characteristics from the toxicity in humans, and therefore the metabolites responsible for the liver injury could be different. We
have completely reopened the chapter of INH-induced hepatotoxicity and viewed this problem from a different perspective. First, we demonstrated that INH itself can be bioactivated and bind to macromolecules *in vivo* in rodents and covalent binding of INH was also observed in human hepatic microsomes and in human liver biopsies from a patient with INH-induced liver failure. In fact the metabolism of INH in rats is significantly different from that in humans, and mice are a better model for INH metabolism in humans. This finding opened a range of opportunities that could be investigated. I reinvestigated the possibility that patients with INH-induced IDILI have anti-INH antibodies. I found anti-INH antibodies in 42% of patients with INH-induced liver failure, and in addition, I also found autoantibodies against P450s that bioactivate INH. The patterns of anti-INH antibodies and autoantibodies were different in different patients, but almost all patients had one or more such antibodies. Given that INH binds to hundreds of hepatic proteins, if I looked for other autoantibodies it is likely that all patients would have one or more such antibodies. In contrast, no antibodies were detected in patients who had mild liver injury or in patients who took INH and did not have liver injury at all. This implied an immune response against the parent drug itself, but it does not exclude the possibility that this immune response could be in response to liver injury and not what caused liver failure. Other drugs that cause liver injury with evidence of an immune mechanism such as halothane also have a similar spectrum of anti-drug and autoantibodies.\(^\text{55,114}\) In addition, I found an increase in Th17 cells and cells that express IL-10 in patients who are treated with INH and have only mild liver injury. This suggests the hypothesis that the injury is mediated, at least in part, by Th-17 cells, and this injury resolves because of cells that produce the immunosuppressive cytokine IL-10. However, there is quite a bit of controversy about the role of Th17 cells in liver injury.\(^\text{200}\) More recently, we have shown that for acetaminophen, in which the liver injury is almost certainly not immune-mediated, there was an increase in Th17
cells two hours after administration of the acetaminophen. Even though Th17 cells are CD4+ T cells and classically part of the adaptive immune system, this very rapid response suggests that they can also be part of the innate immune response.\textsuperscript{161}

The detailed steps and interactions between different cells in the initiation of IDILI and the answer to why most cases resolve despite continued treatment with the drug are yet to be determined. There is additional evidence that INH-induced IDILI is immune-mediated. Specifically, Warrington found a positive lymphocyte transformation test (LTT) when lymphocytes from patients who had mild liver injury to INH were incubated with INH-modified proteins but not to INH itself.\textsuperscript{117,142} However, more severe cases of INH-induced liver injury had a positive LTT to INH-modified proteins and also to INH itself, implying an immune response against drug-modified proteins, and in the most severe cases this immune response spread to the parent drug itself. Another important piece of evidence that supports the hypothesis that INH-induced IDILI may be immune-mediated and not “metabolic idiosyncrasy” is the finding that there was an increased risk of liver injury in patients receiving anti-TB therapy who carry the gene HLA-DQB1*0201 and a decreased risk in those who carry the gene HLA-DQA1*0102; however, the odds ratio was only about 1.9 and 4.0, respectively.\textsuperscript{128} These associations are certainly not nearly as strong as the association of abacavir hypersensitivity syndrome and HLA-B*5701 (odds ration > 900). In addition, the histology of patients who have liver failure, including that caused by INH, is dominated by CD8 cells.\textsuperscript{20} Together, this data implies that the mechanism of INH-induced liver injury in humans is not “metabolic idiosyncrasy” as it has been considered for decades, but rather an immune-mediated reaction, and most likely against INH rather than any other metabolite.\textsuperscript{107}
With the data from our clinical studies, we set out to try to develop an animal model of INH-induced hepatotoxicity with characteristics similar to the liver injury that occurs in humans. We tried to replicate an animal model of INH-induced hepatotoxicity in Wistar rats, which was reported to have a delayed onset of ALT and histological characteristics of hepatic necrosis. However, we saw no evidence of liver injury. We tried various doses of INH in various strains of rats, but never saw any evidence of significant hepatic injury. In addition, we found that the amount of INH covalent binding was higher in mice than rats; similarly, the blood levels of INH were higher in mice than in rats. This suggests that mice might be a better species in which to develop an animal model because they are more like humans, especially slow acetylators, which are known to be at increased risk of hepatotoxicity. Despite more covalent binding, treatment of C57BL/6 mice did not result in significant liver injury. Treatment of Balb/c mice had slightly higher SDH levels, but they could not be treated for longer than 3 weeks because of extensive weight loss. High doses of INH simply cause general toxicity; they do not develop a specific delayed onset liver injury.

Working on the hypothesis that it is immune tolerance that prevents the development of idiosyncratic liver injury, we treated knockout mice such as Cbl-b<sup>-/-</sup> mice and PD1<sup>-/-</sup> mice that have impaired immune tolerance with INH. In both of these knockouts we observed higher GLDH levels compared to the C57BL/6 background strain. In addition, we noticed that INH treatment led to a decrease in several serum cytokines, which suggests the induction of immune suppression. We tried other strategies to overcome tolerance such as depleting T<sub>reg</sub> cells but these attempts were unsuccessful. It is likely that published effects of these agents are not as simple as stated in the publications. More surprising, attempts to break immune tolerance by immunizing mice with S100 hepatic protein that had been modified by INH followed by oral INH not only failed, but the treatment with oral INH prevented the autoimmune hepatitis that
was induced by immunization by INH-modified hepatic protein alone. These results indicate that INH causes some type of immune suppression. This observation was also confirmed by the fact that treatment of NAT1/2-/- mice with INH resulted in the down-regulation of activated proinflammatory M1 macrophages and up regulation of antiinflammatory M2 macrophages. These data are consistent with the hypothesis that the dominant response to INH is immune tolerance, and this is why it is extremely difficult to produce an animal model of INH-induced IDILI. The effects of INH on the liver are significantly different from those of AQ for which we do have an animal model of mild immune-mediated liver injury, which has a delayed onset and resolves despite continued treatment. As with INH, we think that covalent binding of AQ in the liver of mice is important to initiate an immune response, but that is insufficient to produce an immune response that results in liver failure. The mild increase in ALT induced by AQ is clearly associated with an increase in antigen presenting cells and T and B lymphocytes, which we do not see when mice are treated with INH. In addition, in the AQ model, serum cytokines are mainly down regulated when ALT returns to normal. This model provides a unique opportunity to study the mechanism of mild IDILI, and in the AQ model we have shown that the initial injury is not mediated by the adaptive immune system, but rather by NK cells, because treatment of Rag2/- mice, which are B and T cell deficient, did not prevent liver injury but depletion of NK cells did.

Overall, we have changed the prevailing view for some 40 years that INH-induced IDILI is caused by a reactive metabolite of acetylhydrazine and that it is not immune-mediated. We have also developed an animal model of mild AQ-induced liver injury that is mediated by NK cells and has characteristics similar to the mild liver injury that is much more common than liver failure.
4.2: CONCLUSION AND RECOMMENDATIONS

Ultimately, we think that virtually all IDRs are immune mediated. Even for INH, a drug that was not thought to have an immune component, we have shown that the immune system is involved, although it is difficult to make sure that the immune response causes the liver injury rather than being a result of the injury. In animals, just as in most humans, the dominant response in the liver appears to be immune tolerance. The liver is a very tolerogenic organ because it is exposed to many xenobiotics, and covalent binding in the liver happens frequently; therefore, the liver has developed mechanisms to prevent an immune response that would cause harm. In our most successful model of DILI, i.e. AQ-induced liver injury, we determined that it is the innate immune system, specifically NK cells, that mediate the injury. In this model, the adaptive immune system actually appears to play a role in the induction of tolerance. Overall, the AQ model provides an excellent opportunity to further study the mechanism of IDILI because, just like in humans, the liver injury has a delay in onset and resolves despite continued treatment. In this model it will be important to study the role that macrophages play because macrophages have been implicated in other acute animal models of IDILI with drugs such as halothane. Additional, strategies would include using MyD88⁻/⁻ mice which is critical for TLR signalling and γc⁻/⁻ mice which do not have NK cells or T/B cells. In addition, a key question to answer in the AQ model would be to identify what constitutes “immune tolerance”. If we could understand what prevents further injury then it would be easier to intervene in a way that would result in a more serious liver injury. And if we understood exactly what mechanism leads to immune tolerance, we might be able to predict which patients are likely to develop idiosyncratic drug-induced liver failure.
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LIST OF PUBLICATIONS AND ABSTRACTS

1  Metushi, I. G., Cai, P., Nakagawa, T., Dervovic, D and Uetrecht J. P. Hepatotoxicity in a Novel Model of Amodiaquine-Induced Liver Injury with a Delayed Onset is Mediated by NK Cells. To be submitted to Chemical Research in Toxicology.

Abstract

Amodiaquine was withdrawn from the market because of a relatively high incidence of severe liver injury and/or agranulocytosis. As with other drugs that cause idiosyncratic drug reactions, the mechanism of this type of liver injury is currently unknown. We report a novel animal model of amodiaquine-induced liver injury with a delay in onset which is one important characteristic of the liver injury that occurs in patients. The liver injury induced in female C57BL/6 mice resolves despite continued treatment; this is also what happens with most patients who develop mild idiosyncratic drug induced liver injury. Liver injury in these mice appeared to be immune-mediated because when there is an increase in ALT, there is also an infiltration of leukocytes in the liver as well as an increase in serum cytokines. Treatment of Cbl-b-/- or PD1-/- mice resulted in greater infiltration of lymphocytes in the liver and slightly higher ALT levels, but still the liver injury resolved despite continued treatment. However, Rag-/- mice developed slightly more severe injury than C57BL/6 mice and it did not resolve as quickly; this provided definitive evidence that the adaptive immune system is not responsible for the liver injury; instead it may be involved in the induction of tolerance. In contrast, depletion of NK cells prevented the liver injury, which suggests that the innate immune system may be responsible for the liver injury in this model. Although this does not represent a model
of the severe liver injury that can be induced by amodiaquine, it suggests that the initial step in
this liver injury may be an innate immune response, and only when this progresses to an
adaptive immune response does severe liver injury occur.
Metushi, I. G., Cai, P., Vega, L and Uetrecht, J.P. Autoimmune Hepatitis Induced in Mice by Immunization with Isoniazid-Modified Hepatic Proteins is Prevented by Oral Administration of Isoniazid. To be submitted to Chemical Research in Toxicology.

Abstract

Isoniazid (INH) treatment can cause hepatotoxicity and autoimmunity. We have shown that INH is directly oxidized to a reactive metabolite that binds to hepatic proteins. There is growing evidence that INH-induced hepatotoxicity in humans is immune-mediated, but this type of liver injury has not been reproduced in animals, possibly because immune tolerance is the dominant response of the liver. In this study we immunized mice with INH-modified proteins and Freund’s adjuvant leading to mild experimental autoimmune hepatitis (EAH) and an increase in cells in the liver staining for F4/80, CD11b, CD8, CD4, CD45R, and KI67. We expected that subsequent treatment of mice with oral INH would lead to more serious immune-mediated liver injury, but paradoxically it prevented the EAH caused by immunization with INH-modified hepatic proteins. Patients of the slow acetylator phenotype are at increased risk of INH-induced hepatotoxicity. Treatment of acetylator knockout (NAT1/2\(^{-/-}\)) mice with INH for up to 5 weeks produced mild increases in GLDH and SDH activities, but not severe liver injury. Female NAT1/2\(^{-/-}\) mice treated with INH for 1, 3, or 7 days developed steatosis, an increase in Oil Red O staining, and abnormal mitochondrial morphology in the liver. A decrease in M1 and an increase in M2a,b macrophages were observed in female NAT1/2\(^{-/-}\) mice treated with INH for 1, 3, or 7 days; these changes returned to baseline levels by day 35 of treatment. These data indicate that INH has immunosuppressive effects even though it is known to induce autoantibody production and a lupus-like syndrome in humans.
Abstract

Isoniazid (INH) can cause serious idiosyncratic hepatotoxicity. An animal model would greatly facilitate mechanistic studies, but it is essential that the mechanism in the model be similar to the hepatotoxicity in humans. We attempted to replicate a previous study in which Wistar rats treated with INH and rifampicin developed a delayed-onset hepatotoxicity; however, we observed no liver injury. An increase in CTLA4-positive cells as well as a decrease in serum CXCL1 and MCP-1 was observed, which suggests the induction of immune tolerance. Previously, we found that covalent binding of INH in the liver of mice was greater than in rats. Treatment of Balb/c mice with INH resulted in small increases in GLDH. Treatment of Cbl-b−/− and PD1−/− mice, which have impaired tolerance, resulted in greater elevations of GLDH, but not liver failure. The fact that these mouse strains sustained greater injury than the wild type mice suggests that the injury is mediated by the adaptive immune system; however, Rag−/− mice, which do not have competent T and B cells, also sustained more liver injury than the wild type mice. Serum cytokines such as IL-1α and IL-12 were down regulated, but attempts to break immune tolerance by depleting Treg cells failed to increase INH-induced liver injury. In short, although we saw evidence INH-induced liver injury in mice, we were unable to develop a model of INH-induced hepatotoxicity similar to that which occurs in humans. In fact, the cytokine and chemokine response suggested that INH is immunosuppressive.
Abstract

Isoniazid (INH) remains a mainstay for the treatment of tuberculosis despite the fact that is associated with liver failure. This form of liver injury is not believed to be immune-mediated because it is not usually associated with fever or rash, and often does not recur more rapidly on rechallenge. We have recently reported that severe cases of INH-induced hepatotoxicity are associated with the presence of anti-INH antibodies which argues strongly for an immune response against INH itself. In this paper we show by immunohistochemistry that INH itself can bind in the liver of patients who have been receiving INH. In addition we found that patients who had a small increase in alanine aminotransferase (ALT) also had an increase in Th17 cells and cells that stained for intracellular IL-10, which suggests stimulation of an adaptive immune response and this was not observed in any of the patients who did not have an increase in ALT. Th17 cells are considered inflammatory and could be involved in causing the liver injury. IL-10 is considered anti-inflammatory and could be the reason that more serious liver injury did not occur. Although Treg cells produce IL-10 and are an attractive candidate for the IL-10 producing cells, these cells were not positive for Foxp3 so they are not classic Tregs. Conclusion: These data provide strong evidence that the INH induces an immune response which may be responsible for INH-induced liver injury.
Abstract

Isoniazid (INH)-induced hepatotoxicity remains one of the most common causes of drug-induced idiosyncratic liver injury and liver failure. This form of liver injury is not believed to be immune-mediated because it is not usually associated with fever or rash, does not recur more rapidly on rechallenge, and previous studies have failed to identify anti-INH antibodies. In this paper we found antibodies present in the sera of 15/19 cases of INH-induced liver failure. Anti-INH antibodies were present in 8; 11 sera had anti-CYP2E1 antibodies, 14 sera had antibodies against CYP2E1 modified by INH, 14 sera had anti-CYP3A4 antibodies, and 10 sera had anti-CYP2C9 antibodies. INH was found to form covalent adducts with CYP2E1, CYP3A4, and CYP2C9. None of these antibodies was detected in sera from INH-treated controls without significant liver injury. The presence of a range of anti-drug and autoantibodies has been observed in other drug-induced liver injury that is presumed to be immune-mediated. Conclusion: These data provide strong evidence that INH induces an immune response that causes INH-induced liver injury.

Abstract

Isoniazid (INH) is associated with serious liver injury and autoimmunity. Classic studies in rats indicated that a reactive metabolite of acetylhydrazine is responsible for the covalent binding and toxicity of INH. Studies in rabbits suggested that hydrazine might be the toxic species. However, these models involved acute toxicity with high doses of INH, and INH-induced liver injury in humans has very different features than such animal models. In this study we demonstrated that a reactive metabolite of INH itself can covalently bind in the liver of mice, and also to human liver microsomes. Covalent binding also occurred in rats, but it was much less than in mice. We were able to trap the reactive metabolite of INH with N-α-acetyl-l-lysine in incubations with human liver microsomes. This suggests that the reactive intermediate of INH that leads to covalent binding is a diazohydroxide rather than a radical or carbocation because those reactive metabolites would be too reactive to trap in this way. Treatment of mice or rats with INH for up to 5 weeks did not produce severe liver injury. The alanine transaminase assay (ALT) is inhibited by INH, and other assays such as glutamate and sorbitol dehydrogenase (SDH) were better biomarkers of INH-induced liver injury. High doses of INH (200 and 400 mg/kg/day) for one week produced steatosis in rats and an increase in SDH, which suggests that it can cause mitochondrial injury. However, steatosis was not observed when INH was given at lower doses for longer periods of time to either mice or rats. We propose that covalent binding of the parent drug can contribute to INH-induced hepatotoxicity.
and autoimmunity. We also propose that these are immune-mediated reactions and there are clinical data to support these hypotheses.
Abstract

If we could predict and prevent idiosyncratic drug reactions (IDRs) it would have a profound effect on drug development and therapy. Given our present lack of mechanistic understanding, this goal remains elusive. Hypothesis testing requires valid animal models with characteristics similar to the idiosyncratic reactions that occur in patients. Although it has not been conclusively demonstrated, it appears that almost all IDRs are immune-mediated, and a dominant characteristic is a delay between starting the drug and the onset of the adverse reaction. In contrast, most animal models are acute and therefore involve a different mechanism than idiosyncratic reactions. There are, however, a few animal models such as the nevirapine-induced skin rash in rats that have characteristics very similar to the idiosyncratic reaction that occurs in humans and presumably have a very similar mechanism. These models have allowed testing hypotheses that would be impossible to test in any other way. In addition there are models in which there is a delayed onset of mild hepatic injury that resolves despite continued treatment similar to the “adaptation” reactions that are more common than severe idiosyncratic hepatotoxicity in humans. This probably represents the development of immune tolerance. However, most attempts to develop animal models by stimulating the immune system have been failures. A specific combination of MHC and T cell receptor may be
required, but it is likely more complex. Animal studies that determine the requirements for an immune response would provide vital clues about risk factors for IDRs in patients.
SUPPLEMENTAL TABLES

**Table S1:** GLDH and ALT activities after 5 weeks of treatment of BN rats with INH.

<table>
<thead>
<tr>
<th></th>
<th>GLDH</th>
<th>ALT</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.2 ± 0.1</td>
<td>23.8 ± 0.6</td>
</tr>
<tr>
<td>INH (gavage)</td>
<td>2.0 ± 0.2</td>
<td>7.4 ± 0.3 *</td>
</tr>
<tr>
<td>INH (food)</td>
<td>2.3 ± 0.2</td>
<td>1.1 ± 0.2 *</td>
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</table>

INH was given by gavage at 150 mg/kg/day or in food at 0.2% of INH by weight in food. Values represented as mean ± S.E. of 4 animals per group. Analysed for statistical significance by Mann-Whitney U test. Significantly different from control group (*p < 0.05).
**Table S2:** SDH and ALT activities in Balb/c and C57BL/6 mice.

<table>
<thead>
<tr>
<th></th>
<th>SDH (U/L)</th>
<th>ALT (U/L)</th>
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<tbody>
<tr>
<td></td>
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<td>Control INH</td>
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<tr>
<td></td>
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<td>INH</td>
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<tr>
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<td>Female Balb/c</td>
<td>Control INH</td>
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<tr>
<td></td>
<td></td>
<td>INH</td>
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<tr>
<td></td>
<td>Male C57BL/6</td>
<td>Control INH</td>
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<tr>
<td></td>
<td></td>
<td>INH</td>
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<tr>
<td></td>
<td>Female C57BL/6</td>
<td>Control INH</td>
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<td></td>
<td></td>
<td>INH</td>
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</table>

Balb/c mice were treated at 0.2% of INH by weight in food for 3 weeks and C57BL/6 mice were treated at 0.2% of INH by weight in food for 5 weeks. Values represented as mean ± S.E. of 4 animals per group. Analysed for statistical significance by Mann-Whitney U test. Significantly different from the control group (*p < 0.05).
Table S3. SDH and GLDH activities in NAT1/2\(^{-/-}\) mice

<table>
<thead>
<tr>
<th></th>
<th>Female NAT1/2(^{-/-})</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SDH</td>
</tr>
<tr>
<td>Control (5)(^a)</td>
<td>20.8 ± 0.6</td>
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<tr>
<td>Day 1 (3)</td>
<td>18.7 ± 1.1</td>
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<tr>
<td>Day 3 (5)</td>
<td>21.2 ± 1.8</td>
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<tr>
<td>Day 7 (3)</td>
<td>23.9 ± 1.1</td>
</tr>
</tbody>
</table>

\(^a\)Number of animals. Mean ± S.E. Analysed for statistical significance by Mann-Whitney’s U test, *p<0.05.
Figure S1: ESI-MS of INH coupled to BSA. Top; BSA. Bottom; INH coupled to BSA with a MW = 66430 + 105 x n Da, where n is molecules on INH. After coupling of INH to BSA we expected multiple fragments of 105 Da to be added. On average, by using the 8 most intense peaks, the mean mass difference is about 104 Da which is within 1 Da of the expected mass. For large molecules the accuracy of ESI-MS is about 0.01% which would give a mass range of about 6 Da from the targeted 105 Da. On average, by looking at the two most intense peaks 10 molecules of INH were coupled to one molecule of BSA: (67476-66430)/150 = 9.9
Figure S2: Covalent binding of INH to hepatic proteins in mice. A) Female C57BL/6 (n = 4) untreated controls or treated with INH (0.2% of INH by weight in food) for 5 weeks. B) Female or male Balb/c mice (n = 2) untreated controls or treated with INH (0.2% of INH by weight in food) for 3 weeks. C) Male vs. female Balb/c mice (n = 3) treated with INH (0.2% of INH by weight in food) for 3 weeks.
Figure S3: Liver histology of mice and rats treated with INH. A-B) Wistar rats, control or treated with INH at a dose of 150 mg/kg/day for up to 4 weeks. C-D) BN rats, control or treated with INH by gavage at 400 mg/kg/day for 7 days. D-F) Female C57BL/6 mice, control or treated at 0.2 % of INH by weight in food. H&E stain, 100X magnification.
Figure S4: Optimization of INH modified protein for antibody detection. A) HSA or HSA modified with INH. B) Lysozyme or Protein Blu unmodified. C) Control or 5 week INH treated liver from female C57BL/6 mouse. In all cases, serum from a patient at baseline was used as primary antibody to determine the background level of non specific binding. Lysozyme showed the least non specific binding.
**Figure S5:** Absence of anti-INH antibodies in samples from patients before starting INH treatment or after treatment with INH in the absence of significant liver injury compared with sera without anti-INH antibodies from patients with INH-induced liver failure. Lysozyme (L) or INH-modified lysozyme (L-INH) was loaded on a western blot, and serum from patients diluted 1:400 was used as primary antibody. #4-0 = serum from a patient at baseline (ALT = 23), #4-5 = serum from the same patient at revisit number 5 from initiation of INH treatment and a small increase in ALT (ALT = 49). Likewise, #10-0 was from a patient before treatment (ALT = 12) and #10-5 was the same patient at revisit number 5 (ALT = 15); #11-0 was before treatment (ALT = 11) and #11-5 was at revisit number 5 (ALT = 24); #27-0 was before treatment (ALT = 15) and #27-1 was at revisit number 1 (ALT = 16). ALF-6, ALF-11, ALF-5, and ALF-16 are samples from patients with INH-induced liver failure who tested negative for anti-INH antibodies by ELISA. In these blots, the contrast was adjusted to a maximum to show the background and illustrate that there were no bands that could be attributed to anti-INH antibodies.
Figure S6: ALT, bilirubin and international normalized ratio (INR) for patients who had liver failure due to INH as a function of days after initial hospitalization. In Y-axis, values are expressed as upper limit of normal (ULN); the upper limit of normal for each parameter was considered to be: 40 U/L for ALT, 1.2 mg/dL for bilirubin and 1.5 for INR. A) Patients who had anti-INH antibodies. B) Patients negative for anti-INH antibodies.
Figure S7: Immunohistochemical staining of CD68 in rats. Male Wistar rats (n = 4) or male Brown Norway rats (n = 4) were treated with INH (150 mg/kg/day by gavage for 4 and 5 weeks, respectively).
Figure S8: Representative figure showing flow cytometry analysis from the cervical lymph node of a rat.
Figure S9: Effect of FICZ and anti-CD25 on serum ALT in Cbl-b^-/- mice. A-B) male or female Cbl-b^-/- mice (n = 4) were treated with 0.2% INH w/w in food alone or also treated with FICZ or anti-CD25 as described in the methods section. C) Blood levels of INH in male and female Cbl-b^-/- from part A and B, another group of mice treated with 0.4% by weight of INH was added.
**Figure S10:** H&E and immunohistochemical staining for CD45R and F4/80 in the spleen of Cbl-b<sup>-/-</sup> mice. Control = untreated mice (n = 4); INH = mice treated with INH that did not develop abnormal liver histology (n = 3); INH steatosis = one mouse treated with INH that developed significant abnormal liver histology (n = 1); 20X magnification.
Figure S11: Immunohistochemical staining for F4/80 in mice. Male or female C57BL/6 mice (n = 4) were treated with 0.2% of INH by weight in food for 5 weeks.
Figure S12: Serum cytokines in Cbl-b^{-/-} mice treated with 0.2% INH w/w in food for 5 weeks.
**Figure S13:** GLDH activity and body weight in Female C3H mice. Mice were treated at 0.2% INH by weigh in food for 5 weeks. Values represent the mean ± S.E. from 3 animals per group. Analysed for statistical significance by Mann-Whitney U test, * = p < 0.05.
**Figure S14**: Body weight in female PD1−/− mice treated with INH. INH was given at 0.2% w/w in food until week 2, then the dose was decreased to 0.1% w/w in food for one week (until week 3) because mice lost much weight; mice were put back on 0.2% INH w/w from week 3 – 5.
Figure S15: H&E and immunohistochemical staining in the liver of PD1−/− mice. Female PD1−/− control or treated with INH in food for 5 weeks. Slides are representative of 4 animals per group. Immunohistochemistry was graded but no significant difference were observed between control or INH treated mice; 20X magnification.
Figure S16: H&E and immunohistochemical staining in the spleen of PD1−/− mice. Female PD1−/− control or treated with INH in food for 5 weeks. Slides are representative of 4 animals per group. No significant changes were observed between control or INH group upon immunohistochemical evaluation with the exception of KI-67, for which a decrease in the spleen was observed; 20X magnification.
Figure S17: GLDH, SDH, and covalent binding of INH in INH/RMP treated mice. Cbl-b−/− mice control (n = 2), treated with INH (n = 4) at 0.2% w/w in food or treated with INH at 0.2% w/w in food + RMP (50 mg/kg/day by gavage) (n = 4) for two weeks.
Figure S18: ALT activity in mice immunized with S100 or S100-INA. A) Immunization schedule is described in Figure 2, Scheme 1A. A total of 4 mice were treated with INH in food for 6 weeks, at week 6, mice were taken off INH for 4 weeks (weeks 7 – 11) and one mouse was sacrificed to look for evidence of EAH resulting in a total of 3 mice used until the end of experiment, then mice were put back on INH for another 6 weeks (weeks 11 – 16). B) Immunization schedule is described in Figure 2, scheme 1B. For female mice n = 3, for male mice n = 2. Mean ± S.E. Cbl-b<sup>-/-</sup> mice were treated with INH in food for 2 weeks (week 1 - 2), mice were allowed off INH for 2 weeks (week 3 – 4), then mice were put back on INH for another 4 weeks (week 5 – 8).
Figure S19: Immunization of C57BL/6 mice with S100-INA protein in CFA does not decrease hepatic binding in INH-treated animals after 5 weeks of treatment. Western blotting showing covalent binding of INH to S9 liver homogenate. INH = mice treated with 0.2% INH w/w in food for 5 weeks (n = 4). S100-INA + INH = mice immunized with S100-INA along with CFA, as shown in Scheme 3, while also being treated with 0.2% INH in food w/w (n = 4). Animals were sacrificed at the endpoint of Scheme 3.
Figure S20: SDH activity in S9-treated mice. SDH was measured for three weeks after starting INH treatment in food.
Figure S21: Body weight (A) and serum ALT (B) in S100- or S100-INA-immunized mice. Mice were treated as described in Scheme 3 and body weights were determined for 9 weeks after the last immunization. ALT activity was measured for 4 weeks after the last immunization; during these 4 weeks, no group of mice were receiving INH. Mean ± S.E. *p < 0.05, **p < 0.01, ***p < 0.001, Two-way ANOVA.
**Female NAT1/2−/− Mice + 100 mg/kg/day of INH by gavage**

<table>
<thead>
<tr>
<th>Control</th>
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**GAPDH**

**Figure S22:** Time course of covalent binding of INH in the liver of NAT1/2−/− mice (n = 3). Mice were treated with 100 mg/kg/d for up to 7 days. Saline was the vehicle control.
Figure S23: Expression of surface CTLA4 (CD152) on T cells from AQ-treated mice. Female C57BL/6 mice control (n = 4) or AQ-treated (n = 4) were treated for 1, 3, or 7 weeks. Analysed by statistical significance by Mann-Whitney U test. A p value < 0.05 was considered significant (*p < 0.05; **p < 0.01; ***p < 0.001).
Figure S24: Treatment of Cbl-b-/- mice with AQ and FICZ or anti-CD25 antibody.