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A MOUSE MODEL OF HALOTHANE HEPATITIS BASED ON HUMAN RISK FACTORS: A SEXUALLY DIMORPHIC IMMUNE-MEDIATED MECHANISM

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By

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ABSTRACT

A MOUSE MODEL OF HALOTHANE HEPATITIS BASED ON HUMAN RISK FACTORS: A SEXUALLY DIMORPHIC IMMUNE-MEDIATED MECHANISM

By

Christine Marie Dugan

Halothane (HAL) is an inhaled anesthetic that induces a severe, idiosyncratic liver injury also referred to as HAL hepatitis, in 1 out of 6,000-30,000 human patients. We used known human risk factors (female sex, adult age, genetics) as well as probable risk factors (fasting and inflammatory stress) to develop a murine model having the known characteristics of human HAL hepatitis. Female and male BALB/cJ mice treated with halothane developed dose-dependent liver injury within 24hrs; however, only females developed severe liver injury. Livers had extensive centrilobular necrosis, inflammatory cell infiltrate, and steatosis. Fasting rendered mice more sensitive to HAL hepatotoxicity, and 8 week-old female mice were the most sensitive compared to males of the same age and younger (4 week-old) females. HAL-treated female BALB/cJ mice had higher plasma concentrations of tumor necrosis factor-alpha than male HAL treated mice. Also, neutrophils were recruited to the liver more rapidly and to a greater extent in HAL-treated female BALB/cJ mice. AntiCD18 serum attenuated HAL-induced liver injury in the female mice, suggesting that neutrophil migration and/or activation are required for injury. Ovariectomized (OVX) BALB/cJ mice developed only mild liver injury at a HAL dose (15mmol/kg, ip) that produced severe liver injury in control mice. Plasma

interferon-gamma (IFN-γ) was elevated 10-fold in HAL-treated females compared to similarly-treated OVX and male mice. IFN-γ knockout mice were resistant to severe HAL-induced liver injury. The deactivation of NK cells with anti-asialo GM1 treatment attenuated liver injury and plasma IFN-γ compared to IgG-treated control mice. Mice with mutated perforin, a protein involved in granule-mediated cytotoxicity, were protected from HAL, whereas wild-type mice were not. Furthermore, HAL increased the activity of NK cells in vivo, as indicated by increased surface expression of CD69, an early activation marker. Hepatocyte surface proteins are altered in response to HAL treatment in vivo, as indicated by the decreased expression of self, MHC class I molecules, H-2D^{d}, and increased expression of stress ligand, Rae-1. In conclusion, this is an animal model of an idiosyncratic adverse drug reaction that is based on human risk factors and produces reproducible, severe hepatitis from HAL exposure with lesions characteristic of human halothane hepatitis. Furthermore, this dissertation provides evidence that IFN-γ, perforin, and NK cells have essential roles in the development of HAL hepatotoxicity and contribute to the sexual dimorphic response seen in HAL-treated mice.

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Christine M. Dugan

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LIST OF ABBREVIATIONS

ADRs	adverse drug reactions
CYP	cytochrome P450
NAPQI	N-acetyl-p- benzoquinoneimine
INH	isoniazid
HAL	halothane
LPS	lipopolysaccharide
PAMP	pathogen associated molecular pattern
TLR	toll-like receptor
DAMP	danger associated molecular pattern
HMGB-1	high molecular weight group box protein 1
MHC	major histocompatibility complex
DCs	dendritic cells
PMN	polymorphonuclear cell
APCs	antigen presenting cells
IFN-γ	interferon gamma
NVP	nevirapine
TFA	trifluoroaetyl
polyI:C	Polyinosinic:polycytidylic acid
MCP-1	Monocyte chemoattractant protein-1
MIP-2	Macrophage inflammatory protein 2
VCAMs	vascular cell adhesion molecules
PIBF	progesterone-induced blocking factor
Kc	Kupffer cells
Tc	cytotoxic T cells

Th	helper T cells
IADRs	Idiosyncratic adverse drug reactions
TNF-α	tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand
LBP	LPS-binding protein
KpOmpA	Klebsiella pneumoniae
NNRTI	non-nucleoside reverse transcriptase inhibitor
AIDS	autoimmune deficiency syndrome
ART	antiretroviral therapy
NRTI	nucleoside reverse transcription inhibitors
PI	protease inhibitors

Chapter 1

General Introduction

1. Introduction

1.1 Hepatic Adverse Drug Reactions:

Although they account for less than 5% of cases of jaundice in the hospital, hepatic adverse drug reactions are the leading cause of acute liver failure in the United States (1), and drug-induced hepatotoxicity is the most common cause of drug withdrawal of U.S. Food and Drug Administration-approved drugs from the pharmaceutical market (2). In 2002, the global incidence of hepatic adverse drug reactions (ADRs) was estimated to be 13.9 +/- 2.4 per 100,000 inhabitants (3). However, a prospective study of the French general population reported that symptomatic cases of hepatic ADRs were underreported in spontaneous post marketing surveillance and further suggested that the actual incidence may be sixteen times greater than that reported (3). Furthermore, the detection and identification of hepatic ADRs in clinics is hindered by the reliance on plasma transaminase activity as a biomarker for hepatic injury, which may not detect drugs for which hepatotoxicity progresses slowly. Therefore, it is likely that the plasma transaminase activity does not increase to levels that are clinically relevant until later- after the patient has left the hospital or clinic. This is the case for the selective serotonin reuptake inhibitor nefazodone which was removed from the market following reports of hepatotoxicity (4) that developed after several weeks of treatment (5). It is possible that the incidence and seriousness of hepatic ADRs in the general population is underestimated.

1.2. Liver as a Target Organ

The liver is the first major organ to be exposed to ingested toxicants through its portal blood supply and eliminates many toxicants on their first pass through the circulation. Unfortunately, it is also a common location for drug-induced toxicity because it is the major organ of xenobiotic metabolism and subsequent elimination. While metabolic transformation and elimination of xenobiotics is a normal function of the liver, the resulting metabolites can be more toxic than the parent compounds as in the case with acetaminophen-induced liver injury. Acetaminophen is metabolized by several cytochrome P450 (CYP) isoforms, with the major contribution coming from CYP2E1(6), and minor contributions from the CYP1A2, CYP3A4, and CYP2D6 isoforms (6, 7). The product is the reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI). When acetaminophen is given at non-toxic doses, NAPQI is detoxified by reduced glutathione forming an acetaminophen-glutathione conjugate (8). However, at toxic doses, the NAPQI depletes glutathione and it subsequently covalently binds to protein (8). The amount of covalent binding correlates with hepatotoxicity (9). Accordingly, the centrilobular distribution of CYP enzymes is consistent with the pattern of liver injury seen in patients with acetaminophen-induced hepatotoxicity.

1.3 Intrinsic versus Idiosyncratic Adverse Drug Reactions:

In 1978, drugs causing adverse reactions were categorized as "intrinsic" or "non-predictable" by Zimmerman (10), or Type A or Type B reactions, respectively by

Pirmohamed (11). Intrinsic drug reactions are dose-dependent reactions related to the pharmacological or toxicological activities of the drug and are generally predictable. In contrast, non-predictable reactions, also called idiosyncratic reactions, are not seemingly dose-dependent and occur in less than 5% of patients. Recent reports indicate that patients consuming a daily dose of 50mg of oral medications have a higher incidence of idiosyncratic reactions than patients consuming a smaller daily dose (12) indicating that idiosyncratic adverse drug reactions (IADRS) are not dose-independent. For the purpose of this dissertation, an idiosyncratic adverse drug reaction, or IADR, is the response that is not predicted by the pharmacology of the drug and occurs in a minority of patients.

Although IADRs occur in 1-20,000 to 1-100,000 patients, they account for up to 20% of cases of severe liver injury requiring hospitalization (13). This represents a unique problem for our healthcare system because drugs responsible for these serious reactions are often efficacious to a large majority of patients. When these drugs are withdrawn from the market, the resulting publicity contributes to the public's lack of confidence in the healthcare and pharmaceutical industries. It is likely that the mechanisms of IADRs are multifactorial, and their rarity and complex mechanisms of development are barriers to the prediction of which drugs will produce IADRs in preclinical testing. The lack preclinical testing leads to widespread drug exposure before the idiosyncratic liability is discovered. An animal model in which IADRs occur with much higher frequency than in the human patient population will facilitate future

studies aimed at studying the underlying mechanisms that produce IADRs. This would ultimately help us discover ways to predict, prevent, and develop therapeutic modalities to treat IADRs in susceptible patients.

1.4 Mechanisms of Idiosyncrasy:

Several hypotheses have been proposed for the mechanism of pathogenesis of IADRs and many of them fall into one of two categories: metabolism-mediated or immune-mediated. This categorization is based on the absence or presence clinical signs and symptoms of hypersensitivity (fever, rash, eosinophilia), and a more robust response after multiple exposures indicating an acquired-immune mediated response (14). However, neither immune nor metabolic pathogenesis has been strictly proven to be the sole mechanism of IADR for any drug.

1.4.1. Metabolic Idiosyncrasy

Variation in drug metabolism is one proposed mechanism of IADR pathogenesis. For example, a polymorphism in a metabolic enzyme necessary for drug metabolism may lead to the accumulation of harmful reactive metabolites. Such is the case for the anti-tuberculosis drug isoniazid (INH). INH can be acetylated to acetyl-isoniazid, or biotransformed by CYP2E1 into hydrazine, the major toxic reactive metabolite (15). Patients are categorized as rapid or slow acetylators based on the rate at which the INH can be acetylated. Slow acetylation leads to higher plasma INH and hydrazine concentrations with chronic administration of the drug, with subsequent increased risk

of toxicity. A meta analysis of clinical studies reported that genetic polymorphisms in metabolic enzymes N-acetyltransferase 2, CYP2E1, and glutathione S-transferase are associated with increased risk for INH-induced hepatotoxicity in human patients afflicted with tuberculosis (16). These polymorphisms are thought to contribute to the wide intraethnic and interethnic variability in INH-induced hepatotoxicity. Since differences in metabolism can be overcome by increasing drug dose, genetic polymorphisms are not likely the sole explanation for most idiosyncratic reactions. Interestingly, it has recently been illustrated that large plasma concentrations of INH inhibit the function of CYP2E1 (17) and the formation of hydrazine. Therefore, several pharmacokinetic factors may contribute to the unpredictable nature of INH-induced liver injury.

1.4.2 Failure to Adapt

Drugs that cause IADRs are generally identified because of their rare occurrence. However, one characteristic of drugs with idiosyncratic tendencies is the development of mild hepatic injury in 5-15% of patients that resolves without the necessity of drug withdrawal. For example, the proportion of patients treated with INH who develop asymptomatic transaminase elevations is approximately 10-20% (18), whereas symptomatic hepatotoxicity (fever, jaundice, abdominal pain) and fatal hepatitis from INH-treatment occur in 1 in 1,000 patients (19). It is thought that the patients whose livers "fail to adapt" and progress to severe liver injury represent a subset of those patients with initial minor plasma liver enzyme elevations. Support for this idea is given in the clinical trials of troglitazone, an hepatic IADR-producing anti-diabetic drug, in

which alanine aminotransferase (ALT) elevations mirror the timing and pattern of acute liver injury initially, but then resolve (20, 21) for most patients. However, a small number of patients have liver injury that becomes more severe. It is not known why some patients develop liver failure while the majority do not. It is possible that "failure to adapt" may result from the inhibition of hepatocellular regenerative processes or an accumulation of intracellular stresses.

1.4.3 Hypotheses Involving the Immune System

The immune response is thought to mediate the pathogenesis of IADRs. The clinical presentation of patients with signs of an autoimmune reaction (i.e. pruritis, skin rash, eosinophilia) has been used to classify IADRs as immune-mediated reactions, whereas the lack of these clinical signs has been used to classify IADRs as "metabolism-mediated reactions" (14). The "hapten hypothesis", the "danger hypothesis", and the "inflammagen hypothesis" postulate that the acquired and/or the innate immune system mediate the pathogenesis. In order to explain these proposed mechanisms, a brief description of the acquired and innate arms of the immune response will be presented below.

1.4.3.1 Innate and Acquired Immunity

The innate immune response is the first line of defense against microbial infection and relies on the recognition of pathogen-associated molecular pattern (PAMPs) or danger-associated molecular patterns (DAMPs) derived from microbes or

host-cell factors, respectively (22, 23). Innate immune cells such as Kupffer cells (Kcs), dendritic cells (DCs), natural killer (NK) cells, and polymorphonuclear leukocytes (PMNs) recognize these motifs through evolutionarily conserved pattern recognition receptors (PRRs) (24). Each innate immune cell can respond to many different stimuli depending on the repertoire of receptors. Following ligation of PRRs, such as toll-like receptors (TLRs), a signaling cascade follows, leading to effector responses. In contrast, T and B lymphocytes, effectors of the acquired immune response, require somatic rearrangement of their T- and B-cell receptor gene loci to achieve highly specific receptors to a single antigen, and then undergo clonal expansion to increase the numbers of antigen-specific cells. Upon subsequent exposure, the acquired arm mounts a quicker and more robust response to a familiar antigen due to immunological memory. Delayed initial onset, faster onset upon re-exposure, and immunological memory are all hallmarks of the acquired immune response; whereas, in the non-specific (innate) response there is similar time to onset and magnitude of response for each exposure (25). The arms of the immune system orchestrate a defense by initially utilizing the non-specific innate arm, followed by the progressively more sensitive and highly selective acquired arm to thwart the progress of invading pathogens.

1.4.3.2. Acquired Immunity:

The antibiotic, penicillin, elicits anaphalytic shock in those patients who develop an immune response to the parent drug. Classically, for immune-mediated drug-induced adverse reactions there is a close temporal relationship between the drug

exposure and the reaction. Also, the adverse reaction resolves upon removal of the offending drug. In contrast, many presumably immune-mediated IADRs demonstrate a delayed temporal relationship between the drug exposure and the adverse reaction, and the reaction does not resolve upon removal of the drug. These characteristics present an obstacle for the clinicians attempting to identify the causative agent. Regardless of the dissimilarity between allergic-type drug-induced reactions, many IADRs are thought to be immune-mediated.

Most drugs are too small to be recognized by the immune system (< 1000kDA). However, drugs or their metabolites can become covalently bound to endogenous carrier proteins to form immunogenic conjugates (26). Some drugs are relatively unstable and become reactive without the requirement of metabolizing enzymes. For example, penicillin spontaneously becomes penicillenic acid in solution which then can bind directly to endogenous macromolecules. Most drugs, however, are not intrinsically reactive and require biotransformation to form reactive metabolites that are capable of binding tissue macromolecules. Once a drug-carrier conjugate is formed, it may act as an immunogen and elicit a specific humoral (antibody) response, a specific cellular (cytotoxic T lymphocyte) response, or both.

A specific humoral response is orchestrated by B lymphocytes that have surface immunoglobulin molecules which serve as antigen receptors. When an immunogenic conjugate binds to the immunoglobulin with the appropriate specificity, the B cell is triggered to proliferate clonally and differentiate into plasma cells that release

antibodies with the same specificity as the parent B cell or become a memory cell. In order for a strong B cell response to occur, helper T lymphocytes (Th, CD4+,CD3+ lymphocytes) recognize the antigen through their T-cell receptors (TCRs) and assist in activating B cells. Parker et al. (27) prepared an excellent review of T-cell dependent B-cell activation. The receptors on the T cell recognize the immunogen complexed in a self glycoprotein that is presented on the surface of antigen presenting cells (APCs), such as macrophages and dendritic cells. These glycoproteins are the major histocompatibility complex (MHC) class II for humans and H-2 molecules for mice. T cells will recognize an antigen only when it is bound to a particular MHC molecule. Therefore, Th cells are considered "MHC restricted". Antibodies can induce cytotoxicity and tissue damage by initiating the complement cascade on the cell surface of the target cell and by binding Fc receptors to stimulate cytotoxic lymphocytes to release lytic granules to kill target cells. For example eosinophils use Fc receptors to recognize immunoglobulin class E (IgE)-coated Shistosoma parasites and kill them (28).

Tc cells (cytotoxic T cells, CD8+,CD3+ lymphocytes) and NK cells are responsible for cell-mediated immunity. NK cells are considered innate immune cells and will be discussed in section 1.4.3.4. Tc and Th cells have T cell receptors (TCRs) on their surface that bind to the immunogenic conjugate complexed with the self glycoprotein, or MHC class I molecule, located on the surface of all nucleated cells. Costimulatory molecules, such as B7-1 and B7-2, bind to CD28 on the surface of Tc cells and induce the expression of IL-2 receptor (29). These costimulatory molecules are required for this immune

response. Tc use two distinct cytolytic pathways: the exocytosis of preformed perforin granules and the Fas ligand/Fas pathway leading to caspase activation and cell death.

1.4.3.3. Innate versus Acquired Immune System: A Limited Paradigm

Some cells blur the distinction between innate and acquired immunity by having characteristics of both arms of the immune response. For example, NK cells do not require prior sensitization and respond quickly to many stimuli through PRR, a feature of the innate response; however, they also have a repertoire of specificities through the integration of signals from surface receptors, a feature of the acquired response. In addition to acting in a non-MHC-restricted manner to lyse tumor cells and virally contaminated cells, NK cells also participate in and promote the acquired immune response. For example, the rapid production of interferon gamma (IFN-γ) by Tc cells, NK cells, Natural Killer T (NKT) cells, and Th cells upregulates the expression of MHC class I and II molecules on nucleated cells and APCs, and thereby encourages an acquired immune response (30). Furthermore, IFN-γ promotes the differentiation of Th cells to become Th1 cells which assist in cell-mediated cytotoxicity. IFN-γ also induces immunoglobulin class switching in B cells, whereby a portion of heavy chain of the antibody is changed to produce an antibody with a different isotype class (IgE, IgG, IgG, IgM) while unaltering its antigenic specificity. Furthermore, NK cells can participate in the acquired immune response through antibody-dependent cellular cytotoxicity through ligation of its CD16 FcγRIIIA immunoglobulin Fc receptor (31). Therefore, instead of classifying cells into innate or adaptive immune cell categories, it is probably

more accurate to consider the stimulating ligand, the effector cell receptors, and the nature of the effector response when describing an immune response.

1.4.3.4. Hypotheses Involving the Acquired Immune Response

1.4.3.4.1 The Hapten Hypothesis:

The prevailing theory for the mechanism of immune-mediated IADRs is an extension of the "hapten hypothesis". According to this theory, small molecules bind irreversibly to self-proteins, becoming neoantigens, which elicit an acquired immune reaction upon a subsequent exposure. Extending this process to drug metabolism and adverse drug reactions, reactive drugs, or their reactive metabolites, are thought to act like haptens and bind irreversibly to macromolecules, eliciting an autoimmune response. Often the immune response targets the carrier protein instead of the hapten and this initiates an autoimmune response. Severe halothane (HAL) hepatitis is considered a classic example of acquired, immune-mediated IADR; however, the evidence for this hypothesis is largely circumstantial.

1.4.3.4.2. Danger Hypothesis

In 2002, Matzinger suggested that the driving force of the immune system is the recognition of "danger signals" from dying or damaged cells. This represents a shift from the previous paradigm in which recognition of "self or not self" was the principal driving force (32). Within this construct, tissue injury as well as foreign material can direct the immune system to mediate deleterious and/or beneficial events leading to progression

or resolution of injury. Several groups have attempted to apply this hypothesis to the pathogenesis of acquired immune-mediated IADRs (33, 34), in which cytokines, such as Kupffer cell-derived tumor necrosis factor-alpha (TNF-α), exacerbate T and B lymphocyte activity to develop a specific adaptive immune response.

1.4.3.5. Hypotheses Involving the Innate Immune Response

1.4.3.5.1 Inflammatory Stress Hypothesis

The innate immune response could also contribute to the pathogenesis of IADRs (35). The "inflammatory stress hypothesis" proposes that a modest inflammatory stress lowers an individual's threshold to toxicity from drugs with idiosyncratic potential (36). This hypothesis has been supported by the development of several rodent models in which cotreatment with a modestly inflammatory dose of lipopolysaccharide (LPS) and various drugs with idiosyncratic potential (e.g., sulindac, trovafloxacin, and HAL) results in liver injury (37-40). The lack of hepatic injury in other rodent models without additional inflammatory stress lends credence to this mechanism for hepatic IADRs.

LPS is a recognized PAMP that can bind to pattern recognition receptors of the innate immune system, such as TLR4. A point of convergence between the "danger signal" and the "inflammatory stress" hypotheses is the finding that endogenous DAMPs can also signal through TLRs. For example, high molecular weight group box 1 (HMGB-1) protein is a DAMP that is released from necrotic cells or immune cells. HMGB-1 is an endogenous ligand for TLR4 and has been shown to contribute to acetaminophen-

induced liver injury (41). It is not known whether HMGB-1 contributes to pathogenesis of hepatic IADRs. In addition, there are other signals, such as prolonged plasma TNF-α, that are a common feature of LPS- drug co-administration liver injury animal models that could play a role in pathogenesis (39, 42) of hepatic IADRs. It seems very likely that danger signals can also mitigate innate immune-mediated drug-induced pathology.

1.4.3.5.2 Immunosurveilance and Intracellular Stress

There are many stressors, such as heat shock, intracellular pathogens, aberrant molecular machinery and chemical toxicity, that induce a change in cellular homeostasis, or intracellular stress. All of the stressors mentioned above produce misfolded or abnormal proteins that are potentially damaging to the cell or the organism. Although the precise signaling mechanism is not known, abnormal proteins trigger an intracellular stress response that drives the removal of offending proteins (43). This is carried out by halting the synthesis of protein at transcriptional and translational levels (44), activating the ubiquitination machinery to increase the turnover of protein (45), and increasing expression of chaperone proteins, such as heat shock proteins, to assist in protein refolding (46). In general, any stimulus that triggers the upregulation of chaperone proteins is considered a stress stimulus. Hepatocellular lipid accumulation is a gross sign of this stress response which accompanies the inhibition of protein synthesis and is a histopathological feature of many some forms of toxicant-induced liver injury.

While beneficial for the cell to achieve a new homeostasis to survive, the stress response may also target the cell for destruction by immune cells. Damaged, infected, or malignant cells must be cleared to limit the potential for progressive inflammation, infection, or pathology. By changing the repertoire of MHC class I-like molecules on the surface of cells and/ or the protein mounted on the MHC class I molecule, individual cells can communicate a stressed status to immune cells (47). MHC class I-like proteins are included in the MHC class I protein superfamily and are thought to have arisen from a genome duplication event; however, these proteins are not involved in antigen presentation. In addition to other diverse functions, MHC class I-like molecules provide stress immune recognition. For example, the expression of MHC class I-like molecules, MICA and MICB proteins, in human gastroepithelial cells are under the regulation of heat-shock promoters and show an increased expression in stressed cells (48). MICA is also increased in hepatocellular carcinoma cells and can induce NK cell anti-tumor activity through NKG2D receptor (49). In contrast, classical and non-classical MHC class I molecules are expressed on nucleated cells and function by binding intracellular peptides and nonpeptides to present them on the cell surface for recognition by Tc and NK cells (50). In this way, stressed cells are targeted for destruction to minimize their adverse impact on the organism.

1.4.3.6 Natural Killer Cell Function.

As mentioned earlier, NK cells are innate immune cells. They are large granular lymphocytes in lymphoid and peripheral tissue that participate in tumor inhibition, viral

immunity, graft rejection, and the inhibition of intracellular microbial infections (51). The effector functions of NK cells are direct cytolysis of target cells and the production and secretion of cytokines and chemokines, such as IFN-γ as shown in response to polyinosinic:polycytidylic acid (polyI:C) (52, 53). NK cell-mediated cytotoxicity is accomplished by several mechanisms, including the release of preformed cytotoxic proteins perforin and granzyme B, signaling with TNF-related apoptosis-inducing ligand (TRAIL), FASL, and signaling through Fc receptors. The nature of the NK cell effector response (cell-mediated cytolysis, cytokine/chemokine release, or both) depends on the nature of the insult. In the liver, NK-cell mediated liver injury can be promoted by polyI:C-induced accumulation and activation of NK cells to kill hepatocytes by TRAIL signaling and/or granzyme B release (54, 55). NK cells can also be activated by cells that express up-regulated NK cell-activating ligands, such as Rae-1 and MULT, or decreased NK cell-inhibitory ligands. IFN-γ can also promote NK cell-mediated liver injury (56).

Hepatic NK cells, originally called pit cells, are located in the sinusoids loosely attached to endothelial cells through adhesion molecules. The immunophenotypical, morphological, and functional characteristics of hepatic NK cells are different from circulating or splenic NK cells (57). The higher protein expression of granzyme, perforin, and FasL, as well as their enhanced ability to kill metastatic tumor cells that evade other splenic NK cells, indicates that hepatic NK cells have a more active phenotype (53). Furthermore, there is emerging evidences to suggest that hepatic NK cells are similar to IL-2-activated splenic NK cells (53, 58, 59). The distribution of hepatic NK cells mirrors

the portal distribution of the Kcs, which are thought to be intimately involved in the differentiation and maturation of hepatic NK cells. In addition to interacting with Kcs physically, the pseudopodia of NK cells can penetrate the fenestrae of endothelial cells to contact hepatic parenchymal cells directly (57). This could provide a mechanism for hepatocyte surveillance by liver resident NK cells.

1.4.3.6.1 NK Cell Receptor Specificity

Karre et al. (60) demonstrated that NK cell-mediated cytotoxicity inversely correlates with the level of major MHC class I expression on target cells and proposed that NK cells perform immune surveillance by monitoring the loss of self molecules, or MHC class I molecules. This is known as the "missing self hypothesis." Inhibitory receptors were subsequently discovered on NK cells that can sequester phosphastases on the cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIM) (61). By occurring near the interface between the target cell and the NK cell, these phosphatases inhibit Ca^{2+} influx, cytokine production, and degranulation. There are two classes of inhibitor receptors found on NK cells: type 1 membrane proteins that belong to the Ig superfamily, such as killer immunoglobulin-like receptor (KIR) and leukocyte Ig-like receptors in humans and gp-49B1 in mice; and type II membrane proteins that have homology to C-type lectins, such as CD49/NKG2 receptors in humans and mice and Ly49 receptors in mice (62). KIR and Ly49 receptors bind directly to MHC class I molecules, such as HLA-E and Qa-1b in human and mouse, respectively.

The existence of multiple receptor strategies to inhibit NK cell activity led many scientists to speculate that NK cells are constitutively activated. However, stimulatory receptors on NK cells were also discovered. These receptors use cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) to signal activation. These stimulatory receptors recognize constitutively expressed self-motifs, stress motifs, and some infectious pathogen components. For example, NKG2D protein is an activating receptor, and is distinct from inhibitory NKG2 receptors that recognize stress-induced proteins. In humans, the NKG2D ligands are MICA, MICB, and ULBP/RAE1 molecules; in mice, they are H60, MULT, and Rae1 molecules (63). These ligands are expressed very selectively and at low levels by normal adult cells (63, 64). The NKG2D ligands MICA, MICB, and Rae-1 are upregulated in tumor cells and the overexpression of NKG2D ligands in murine tumors invokes an enhanced NK-cell cytotoxic response (63, 64). This represents a direct mechanism for cancer surveillance by NK cells.

Many cells, including NK cells, express TLRs (1-10), evolutionarily conserved pattern recognition receptors found in vertebrates that bind PAMPs and DAMPs to elicit innate immune responses. LPS, a component of the outer membrane of gram negative bacteria, binds LPS-binding protein (LBP), TLR4, and co-receptor CD14 on monocytes and macrophages to produce pro-inflammatory cytokines (65, 66). Until recently, PAMP-induced activation of NK cells was thought to be mediated indirectly. For example, the ligation of TLRs on the surface of DCs leads to the release of IL-12 and subsequent activation of NK cells. However, there is growing evidence that NK cells can be directly

activated by PAMPs. Exogenous administration of protein A from Klebsiella pneumoniae (KpOmpA), polyI:C, bacterial fibrial component (FimH), and flagellin directly bind TLR2, TLR3, TLR4, and TLR5 respectively to activate NK cells (67-69). Therefore, PAMP signaling is present and functional on NK cells. In conclusion, NK receptors are expressed in a variegated, overlapping fashion, such that each NK cell expresses several inhibitory and stimulatory receptors the integrated signals of which identifies healthy cells and targets unhealthy or infected cells (70).

1.5. Nevirapine as an Example of a Drug with Idiosyncratic Potential:

Since the nevirapine (NVP)-induced skin lesion rat model is widely considered one the first models of IADRs demonstrating an acquired immune-mediated mechanism, I will introduce NVP-induced adverse reactions in humans and the rat model. The introduction of both NVP-induced and HAL-induced adverse reactions in animal models is intended to illustrate that different mechanisms of pathogenesis could exist for different IADRs, and that location of the adverse reaction could also contribute to the mechanism.

1.5.1 Clinical Spectrum of NVP-associated ADRs

Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) used to treat patients with human immunodeficiency virus (HIV) infection or induced acquired immune deficiency syndromes (AIDS). NVP binds to the reverse transcriptase enzyme and inhibits the replication of HIV. Since virus mutation and resistance occurs

quickly in monotherapy, NVP is administered as a component of combination antiretroviral therapy (ART) with nucleoside reverse transcription inhibitors (NRTI) and protease inhibitors (PI). Like other NNRTI drugs, rash is a common adverse reaction with 32% to 48% of NVP-treated patients developing a rash within the first month of exposure (71, 72). Severe mucocutaneous or cutaneous reactions are more rare, with incidence of 1% and 8% of patients, respectively (73).

Asymptomatic liver toxicity, or liver enzyme elevations without concurrent symptoms of abdominal pain, nausea, vomiting, malaise, rash, or fever, has been noted in 5-10% of HIV-positive patients treated for more than 6 months with combination ART therapy (74). Symptomatic clinical hepatic events during ART therapy are associated with NVP use, as well as female sex, chronic viral hepatitis, higher baseline ALT and AST values, and recreational drug use (75, 75). NVP, in particular, has been associated with several cases of liver failure (76-79). Retrospective studies report symptomatic liver injury in 3 to 4.9% of patients administered NVP (Nevirapine product insert) (80, 80); whereas two prospective studies containing NVP cite symptomatic hepatotoxicity in 6-10% to 17% of patients within the first month of dosing (81, 82). Reports of acute hepatotoxicity due to NVP therapy led to a change in the NVP product label in Europe and the United States (83),in which a 200mg/kg/day lead-in dose is administered for two weeks prior to administering the full dose of 400mg/kg/day.

The adverse reactions to NVP therapy in humans can be either cutaneous, hepatic, or both, and usually occur within 14 and 21 days of treatment (74, 80). These

adverse reactions are also reported to be more severe upon NVP readministration (84). As mentioned previously, delayed onset and increased severity upon rechallenge are common characteristics of hypersensitivity immune reactions (85), and clinical hallmarks for hypersensitivity reactions are fever, rash, arthralgias, myalgias, and hypereosinophilia (77). The occasional concurrent occurrence, delayed onset of adverse reaction from the initial NVP exposure, and shared risk factors (female sex and plasma NVP concentration) support a common mechanism for hepatic and cutaneous NVP-reactions. It is generally assumed that the cutaneous and hepatic manifestations are derived from the same hypersensitivity mechanism; however, there is no clear evidence to support a common mechanism.

Since NVP-induced adverse reactions do not occur in some patients at any dose, they are considered idiosyncratic (84). The human risk factors for NVP-induced hepatotoxicity are: female sex (82), plasma NVP concentration (86, 87), length of dosing regimen (88), concurrent hepatitis C or B infection (89), and genetics (90, 90, 90). A higher CD4+ T cell count is reported to be a risk factor for NVP-induced hepatotoxicity (90, 91), however, other reports refute this claim (92, 93).

1.5.2 Metabolism of NVP:

The majority of drugs with idiosyncratic tendency are metabolized to reactive metabolites that are thought to be indirectly responsible for toxicity (94) by acting like neoantigens and producing an autoimmune response. It is possible that other

mechanisms are predominant in different organs or with IADRs induced by other drugs. This indirect, neoantigen-mediated cutaneous reaction is consistent with the scarcity of metabolizing enzymes in the skin. NVP is metabolized by CYPs, and skin reaction is a common manifestation. The administration of deuterated NVP attenuated the incidence of rash in rats (95) and exogenous administration of 12-hydroxy-nevirapine, a NVP metabolite, produced the rash. These studies confirm that metabolism is important for the development of rash in rats.

1.5.2 Rodent Model of NVP-induced IADR:

Animal models of adverse reactions are essential to study the mechanisms of toxicity that occur in human patients. It is likely that IADRs caused by different drugs vary in their mechanism of injury, and no single animal model will reproduce the toxicities seen in the human population. One of the first rodent models of an idiosyncratic adverse drug reaction that clearly indicates an adaptive immune mechanism is the NVP-induced rash rat model. In 2003, Shenton et al. (96) developed an animal model in which NVP (150mg/kg/day) induced rash in 100% of female, Brown-Norway rats and 21% of female, Sprague-Dawley rats, whereas no cutaneous adverse reactions were noted in male Brown-Norway rats, male Sprague-Dawley rats, or Lewis rats regardless of sex. This model is considered idiosyncratic because only some of the strains of rats respond in a dose-dependent manner, whereas other rats are insensitive at all doses (96).

The female Brown-Norway rats developed red ears on days 7-10 and skin lesions on days 10-21 after the initiation of the NVP regimen. Upon rechallenge, the rats had a quicker time to onset of rash, increased CD4+ and CD8+ cellular infiltrate in their skin lesions, and appeared generally less well than the primary challenged rats (96). The initial delay in toxicity, the quickened onset of toxicity upon re-challenge, and the increased T cells in the tissue specimens are suggestive of an acquired immune response(95). Adaptive transfer of splenocytes from re-challenged rats conferred a faster time of rash onset compared to the primary challenged rats upon NVP exposure, and T cell depletion decreased the incidence of NVP-induced rash in this rat model confirming the role of T cells in the pathogenesis (97).

Although this model does not produce the NVP-induced liver or severe skin manifestations that curtailed its use in human patients, it is thought there are some common mechanistic features to all of the NVP-induced adverse reactions (98). Evidence for this is needed. However, this is the first animal model that accurately depicts the idiosyncratic NVP-induced mild rash seen in some patients by sharing the characteristic delayed time of onset, increased severity on NVP rechallenge, and histological features seen in patients with NVP-associated adverse reactions. Whether the cutaneous effects of NVP are related mechanistically to its hepatic effects remain to be determined, in view of the fact that liver injury did not occur in this model.

1.6 Introduction to Halothane (HAL)

HAL is an anesthetic that was introduced to the United States in 1958. By 1963, the drug manufacturer issued a warning due to clinical reports of fatal hepatic necrosis following HAL administration. In 1966, a large retrospective study was unable to establish or refute a causal relationship between HAL and fulminant liver injury due to an incidence rate that was rarer than 1 in 10,000. The report also indicated that HAL had a good safety record compared to other anesthetic options available at the time (i.e. ether) (99). Although increasing numbers of reports of HAL-associated liver failure ultimately curtailed the use of HAL in the United States, it is still used in other countries and is included on the current "World Health Organization's Model List of Essential Medicine" released in March 2007. Careful assessment of the risk factors for HAL-associated liver failure, such as female sex, genetics, age (the young are resistant), and multiple HAL exposures (100, 101), are useful guidelines for physicians trying to avoid the HAL-associated injury in patients.

HAL is vaporized and administered as an inhaled gas in human patients. It is widely distributed in the body and achieves anesthetic effect quickly. HAL also has a low blood:gas partition coefficient which reflects a low affinity of blood for the anesthetic. This is a desirable property because it engenders precise control over the anesthetic state and a more rapid recovery from anesthesia. Other attractive characteristics of this anesthetic that led to its widespread use are the lack of bronchospasm and laryngospasm, as well as, reduced respiratory secretions. However,

there are also adverse pharmacologic effects of HAL, namely a decrease in arterial pressure, cardiac output and heart rate and increased peripheral circulation (102). These adverse effects are transient. A decrease in glomerular filtration rate has also been noted in patients treated with HAL. In dogs, diminished renal function was proportional to the degree of hypotension induced by HAL (103). Therefore, HAL did not directly affect the kidneys.

1.6.1 Clinical Spectrum of HAL Hepatotoxicity

Although initially thought not to impact hepatic function, HAL produces a non-icteric, mild liver injury in 1 in 5 patients (104, 105). A more severe idiosyncratic liver injury, or HAL hepatitis, was observed in 1 in 6,000-30,000 adult patients who received the drug (99, 106, 107). The clinical signs of HAL hepatitis are fever, jaundice, vomiting, and coma (108). The laboratory findings are characteristic of hepatocellular injury, with increases in plasma liver enzyme activity, hyperbilirubinemia, and decrease in prothrombin time (108). Fever is usually the first sign of HAL hepatitis, with onset between 8 and 14 days (107, 109); however, not all patients present with fevers. Retrospective studies indicate a shortened time until onset of fever presentation to 4 days following non-primary HAL administration (107). Fevers with temperatures reaching 104°F are seen in some cases of HAL hepatitis. Another prominent clinical sign is the onset of jaundice. Kline and colleagues (109) noted that patients became icteric 10-28 days following the primary exposure of HAL and 3-17 days following a non-primary exposure. The shortened latency of symptoms following a non-primary

exposure may indicate that the primary HAL exposure sensitized patients to the subsequent HAL exposures. In contrast, other groups reported that patients developed jaundice 8 and 7 days following primary and non-primary HAL exposures, respectively (107).

Transaminase activity and bilirubin concentration are elevated in HAL hepatitis patients and indicate damage to the liver parenchyma. Bottiger and colleagues (107) discovered that patients who had serum bilirubin concentrations that were greater than 10mg/dl had a 60% mortality rate, whereas there were no deaths in patients who had serum bilirubin concentrations less than 5mg/dl. Historically, HAL hepatitis was one of the clinical entities considered in the development of Hy's law for risk assessment of drug sthat cause hepatic injury. A useful guideline that developed from Hy's law is that elevated plasma transaminase activity (3X the upper limit of normal) and bilirubin concentrations (2X the upper limit of normal) are indicators of poor prognosis with an estimated 50% mortality or required liver transplant rate (2).

The most prominent histological feature seen in liver biopsies from patients who had HAL hepatitis is centrilobular necrosis (110). Other histopathological findings in postmortem liver specimens include fatty degeneration, vacuolation, and inflammatory infiltrate (108, 111). Eosinophilia was a minor finding and was a feature in only 10% of HAL hepatitis patients.

1.6.2. Two Pathologic Entities or One?

Whether the mild and severe forms of HAL hepatotoxicity are separate pathologic entities or different degrees of the same pathology is not known. Since enzyme induction exacerbates liver injury, the mild HAL hepatotoxicity is thought to be the result of direct hepatotoxicity of HAL metabolites (112). There is other evidence that supports the idea that severe and mild HAL-associated hepatotoxicity could have similar mechanisms of pathogenesis. The centrilobular necrosis seen in liver sections from patients with HAL hepatitis is similar to the pathology seen with other direct hepatotoxicants (110). Multiple HAL exposures produced mild HAL-associated hepatotoxicity in prospective studies (105, 113). Short intervals (less than 28 days) of multiple exposure are a risk factor for HAL hepatitis (106, 114-116) suggesting a cumulative dose effect from HAL exposure. Furthermore, nonvolatile metabolites are excreted for more than 2 weeks following HAL exposure in humans (117, 118). The HAL-associated pathology severity and/or quantity of covalent metabolite binding was increased by CYP enzyme induction in rats (119-123) and decreased by CYP enzyme inhibition in guinea pigs (124). The demonstration of both mild and severe forms of pathology within the same animal model suggests that the two forms may be different degrees of the same pathology.

Unlike the rare incidence of HAL hepatitis in humans, mild HAL hepatotoxicity is estimated to occur in 20% of patients treated with HAL (105). Pohl and colleagues (112) suggested that HAL hepatitis is a separate, immune-mediated reaction because of its

rare incidence in humans and the inability to reproduce similar lesions in animal models consistently. There is also clinical corroborative evidence supporting an acquired immune-mediated mechanism for the pathogenesis of HAL hepatitis. Several retrospective reports suggest that multiple HAL exposures is a risk factor for HAL hepatitis (99, 115, 125) with onset of clinical symptoms occurring faster upon subsequent exposure. Additionally, circulating antibodies reacting with cell membranes of hepatocytes from HAL-anesthetized rabbits were found in 9 of 11 patients with fulminant hepatic failure, whereas HAL-treated patients with no ill effects did not develop antibodies in their serum (126). Furthermore, antibodies from the sera of patients diagnosed with HAL hepatitis reacted specifically to a trifluoroacetate HAL metabolite (127) and the antibody production occurred at the time of hepatic injury (128). Also, the deposition antibody that recognize HAL metabolite adducted to proteins on the surface of hepatocytes suggested that antibodies specifically targeted hepatocytes (129). These findings along with the unpredictable nature of HAL hepatitis support an acquired immunological etiology, unlike the mild form of HAL hepatotoxicity. The findings that support either two separate clinical entities for HAL hepatotoxicity or one clinical entities with different degrees of severity is summarized in Table 1.1.

Table 1.1 Two Separate Clinical Entities of HAL Hepatotoxicity or One?

	Situations Producing Mild Pathology	Situations Producing Severe Pathology	Same or Different Pathogenesis
Prevalence in Humans	20% of patients taking HAL develop mild lesion (104, 105)	1 in 20,000 patients develop severe pathology (99, 106, 107)	Separate Etiology
Human - Clinical Hepatic Signs and Symptoms	Minor elevations of plasma ALT activity	fever, vomiting, abdominal pain, jaundice, coma (108).	Same Pathogenesis
Human -Liver Histopathological Analysis	Mild centrilobular necrosis	Extensive, centrilobular necrosis (110)	Same Pathogenesis
Human- Multiple Exposures	Multiple Exposures causes mild pathology following each exposure (105, 113)	Multiple exposures is a risk factor (106, 114-116)	Separate Etiology
Human - Clinical Signs of Immunological Involvement	No antibodies in sera from patients with mild HAL hepatotoxicity. (126).	Antibodies in the sera of patients with HAL hepatitis that recognize HAL-modified proteins in the liver of HAL exposed rabbits (126).	Separate Etiology
	Same time to onset of symptoms following non-primary exposure (107).	Faster time to onset of symptoms following a non-primary exposure (109)	Separate Etiology
Animal Models- Hepatic Involvement	Many models produce mild pathology (130-133)	None	Separate Etiology
Animal Models- Enzyme Induction, Glutathione Depletion, and/or Inflammatory Stress	Not necessary	Increased toxicity with P450 enzyme induction, glutathione pretreatments, or inflammatory stress (119-123)	Same Pathogenesis

1.6.3. Metabolism of HAL:

Approximately 20% of absorbed HAL is metabolized in the human body (118). Although initially thought to be chemically inert, experiments using whole animal autoradiography demonstrated that the liver was the primary site of metabolism and accumulation of ^{14}C-labelled HAL metabolites following intravenous administration of ^{14}C-HAL in mice (134, 135). Since HAL is widely dispersed in the body and the major site of the adverse reaction is the site of metabolism; it is likely that a reactive metabolite of HAL is involved in the initiation of pathogenesis of both clinical entities. It was subsequently demonstrated that pretreament of rats with phenobarbital, an inducer of some CYP enzymes, increased the covalent binding of HAL metabolites in the liver (136). CYP is responsible for both anaerobic and aerobic metabolism (137, 137) of HAL. Under aerobic conditions, the reductive pathway is inhibited. Therefore, the aerobic metabolism of HAL is dominant (137).

Gut et al. (138) provided a detailed description of the anerobic and aerobic metabolism of HAL, which is briefly described here (Figure 1.1). Under anerobic conditions, HAL, or $CF_3CHClBr$ (**1**), can be reduced to 1-chloro-2,2,2- trifluoroethyl radical (**6**) with the loss of bromine ion (138). The reactive radical (**6**) can react with double and single bonds to form adducts (**10**). The abstraction of an hydrogen leads to the production of 1-chloro-2,2,2-trifluoroethane (**7**) (139), whereas the abstraction of an electron (e-) forms 1-chloro-2,2,2-trifluoroethyl carbanion (**8**). The carbanion can

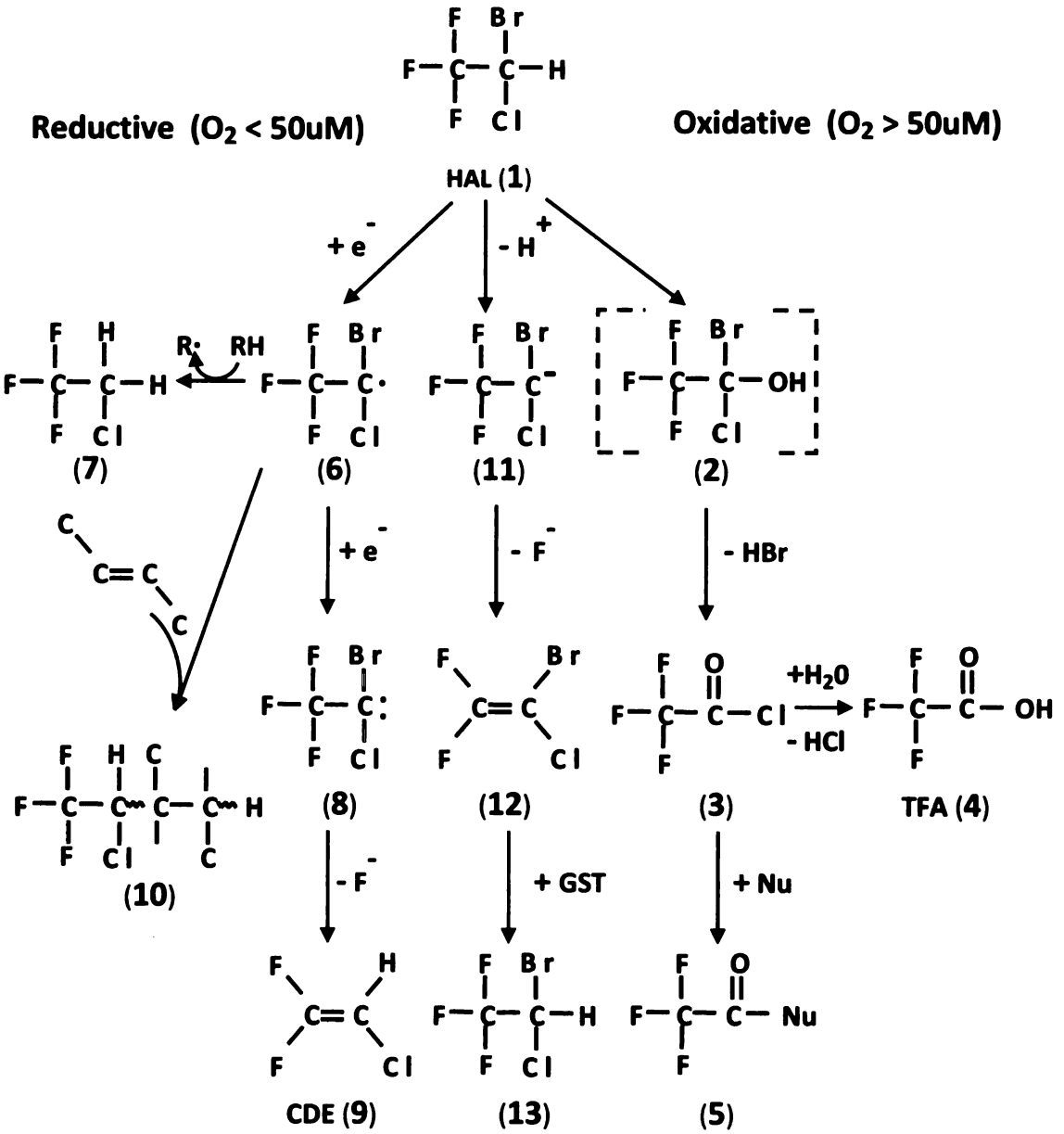


Figure 1.1. Reductive and Oxidative Metabolism of HAL
(Gut et al., 1997)

eliminate a fluoride ion to produce 1-chloro-2,2-difluoroethylene (CDE) (**9**) (139) or a chloride ion to form trifluoroethyl carbene (not shown). The carbene metabolites can react with liver proteins. Alternatively, CYP removes a proton (H+), followed by the loss of floride ion to yield the anion (**11**) and 2-bromo-2-chloro-1,1,-difluoroethylene (**12**) (140), which can then form metabolites that are released in the urine (117).

Under aerobic conditions, HAL is oxidized by CYP, mainly isoform 2E1 (141) to a halohydrin intermediate (**2**), which spontaneously loses hydrochloric acid or hydrobromic acid to form trifluoroacetyl (TFA-)chloride (**3**) (138). TFA-chloride interacts with nucleophiles (Nu) to form TFA-adducts (**5**) (142) or undergoes hydrolysis to form trifluoracetic acid (**4**). Intracellular pools of glutathione (GSH) can interact with TFA-chloride (**3**), preventing the formation of TFA-adducts (143). Trifluoracetic acid is a urinary end product found in HAL-exposed rabbits (144) and human patients (117, 118). The inhibition of CYP enzymes with disulfiram inhibits the formation of TFA-adducts in people (145) and animals (146). TFA-adducts have been shown to be a risk factor for HAL hepatotoxicity in guinea pigs (133).

Antibodies in the sera of HAL hepatitis patients have been shown to react with at least five polypeptides that were chemically altered by trifluoroacetyl (TFA) chloride and were approximately 100, 76, 59, 57, and 54 kDa (147, 148). These TFA-adducted proteins were predominately localized to the microsomal subcellular fraction of the livers (147). Reports indicate that these TFA-adducts can persist for at least 7d in rat hepatocyte cultures (127) and 21 days in guinea pig livers (149). Mice administered ^{14}C-

HAL weekly exhibited rapidly accumulating radioactivity in their livers (134), indicating either the induction of metabolizing enzymes or an accumulation of metabolites. Some of the adducted proteins were identified as the 94kDa glucose-regulated protein (GRP), GRP78, 72kDa endoplasmic reticulum protein (ERp72), calreticulin, a carboxylesterase, protein disulfide isomerase (PDI), and cytochrome P450 (150, 151). However, the contribution of these adducted proteins in the pathogenesis of HAL hepatotoxicity is not clear.

1.6.4 Animal Models of HAL Hepatotoxicity:

Animal models to study HAL hepatotoxicity have been developed, but most reproduce the mild type of HAL hepatotoxicity (130-133). Attempts to develop animal models of severe, HAL-induced hepatotoxicity have focused on using repeated HAL exposures (152, 153), drug metabolizing enzyme inducers, glutathione depletion techniques, and/or hypoxic conditions to elicit the hepatic injury (136, 154). Low incidence of responders and/or lack of severe hepatotoxicity limit the usefulness of these as models in studying the mechanism of pathogenesis. I will briefly discuss some of the single-exposure and multiple-exposure studies from the literature.

1.6.4.1 Single HAL Exposure Animal Models:

Since oxidative metabolism is energetically unfavorable for deuterated HAL, it is preferentially metabolized via reductive pathways. Guinea pigs treated with deuterated HAL develop less hepatotoxicity, compared to those treated with regular HAL. This

indicates that HAL-induced hepatotoxicity in guinea pigs is mediated through oxidative metabolism (155). The spectrum of liver injury in the single-exposure HAL treatment (1% HAL for 4 hours) closely resembles the mild HAL hepatotoxicity form seen in humans (156). Studies in which HAL-induced liver injury in guinea pigs was ameliorated by SKF-525A, a CYP inhibitor, and exacerbated by 4-methylpyrazole, a CYP2E1 inducer, underscore the importance of metabolism as prerequisite for hepatotoxicity (124, 157). Accordingly, the susceptibility of outbred Hartley guinea pigs to HAL-associated liver injury correlated with the development of TFA-adducts in the liver (133). These findings indicate that metabolism and the development of TFA-adducts is critical to the pathogenesis of HAL-induced liver injury. In other studies, HAL-treated, outbred, English short-haired guinea pigs developed similar amounts of TFA-adducts in their livers regardless of susceptibility to HAL-associated liver injury; however, inhibition of hepatic CYP2E1 attenuated liver injury in susceptible animals upon reexposure (157). Taken together, this indicates that metabolism and the development of TFA-adducts is necessary but not sufficient to cause liver injury in guinea pigs.

Reduced glutathione protects against accumulating damage from chemicals that produce reactive intermediates by reacting with the intermediates before they can bind covalently to subcellular macromolecules (158). Buthione sulfoximine (BSO), an agent that decreases intracellular reduced glutathione, induced hepatotoxicity in 4 out of 4 HAL-treated Hartley guinea pigs in response to a subanesthetic HAL dose (0.1% HAL, 4hrs) and increased the amount of bound organic fluorine. Furthermore, fatal

hepatitis occurred in 37-50% of BSO-pretreated guinea pigs administered an anesthetic dose of HAL (1%, 4hrs). This may indicate that the maintenance of subcellular protective mechanisms could prevent less susceptible patients from developing HAL-associated liver injury.

Following phenobarbital pretreatment (1mg/ml for 10 days), rats developed hepatotoxicity when administered HAL (1%, 4hr in 14% oxygen) in hypoxic conditions (136). This rat model, called the hypoxia-halothane model, represents direct hepatotoxic damage from the reductive metabolism of halothane (136). However, the usefulness of this model was called into question upon the discovery that hypoxia itself could produce mild liver injury (159, 160). Furthermore there has been no relationship between HAL-associated hepatotoxicity and the type or duration of surgical procedure, which challenges the claim that ischemic hypoxia may be involved in the pathogenesis of liver injury in humans (99, 109, 161).

Ju and colleagues (132) characterized a mouse model in which HAL (30mmol/kg in 2ml olive oil, i.p.) administration induced a mild, HAL hepatotoxicity that was sex- and strain- dependent. This study also indicated that HAL hepatotoxicity was dependent on PMN activity (132). Further studies demonstrated that PolyI:C exacerbated HAL-induced hepatotoxicity (162), indicating that inflammation can exacerbate HAL-induced hepatotoxicity in BALB/c mice. This PolyI:C exacerbation of HAL liver injury involved Kcs (162). More recently, NKT cells have also been shown to play a role in HAL hepatotoxicity in mice (163).

This mouse model of HAL hepatotoxicity can serve as a platform to develop a model of severe HAL hepatotoxicity (132). This is essential in order to due mechanistic studies on the pathogenesis of liver injury. Accordingly, one goal of my dissertation research was to test whether the simultaneously imposition of known and possible human risk factors could result in the expression of severe HAL hepatotoxicity would be occur in all mice exposed. There are several characteristics of the mouse model that closely parallel that which is found in humans with HAL hepatotoxicity. In brief, the histopathological analysis of the liver sections from mice treated with HAL reveal centrilobular necrosis, steatosis, and cellular infiltrate. These characteristics are also found in post-mortem specimens from patients who suffered with HAL hepatitis. The desired effects of anesthesia and sedation, as well as the undesired effects of respiratory depression and liver injury are evident in humans and BALB/cJ mice. A more detailed analysis of the similarities between the mouse mode of severe HAL-hepatotoxicity and the human clinical presentation of HAL hepatitis is covered in chapter 2.

There are also some differences between how mice are exposed to HAL and the clinical experience of humans. The route of exposure for the mouse model is intraperitoneal, whereas humans inhale volatilized HAL. The intraperitoneal administration of HAL in the animals was chosen to eliminate variation in dose due to differences in respiration rate between individual mice, as well as the pharmacologically-induced variation due to respiratory depression from HAL. Although

forced respiratory exposure is possible, this method could introduce additional injury by opposing both the mouse's natural respiratory rhythm at low doses and the pharmacologic effect of HAL to depress respiration at higher dose of HAL. Initial attempts to administer HAL (1% in medical oxygen for 2hrs) in an exposure chamber to female BALB and C57BL6 mice resulted in no liver injury (data not shown). Respiratory depression limited the HAL dose that was administered in these studies. It is possible that the high respiratory rate in mice (~200 breaths/sec), low HAL availability in the liver, or a lower rate of HAL metabolism could be reasons why mice appear to be less sensitive to HAL by inhalation route of exposure compared to some humans patients.

1.6.4.2. Multiple HAL Exposure Animal Models:

Some of the initial attempts to develop an animal model of HAL hepatitis used repeated exposures to HAL. Hughes et al. (164) demonstrated that inhaled HAL(4L/min for 1hour) administered in 100% oxygen at 2 week intervals induced hepatic changes in guinea pigs. These studies also indicated that liver injury could be dose-dependent, since animals exposed HAL more frequently within an hour had a higher incidence of liver injury. Unfortunately, no relationship with single HAL exposure was tested, so it is unknown whether HAL sensitized mice to subsequent HAL-induced liver injury. Another guinea pig model with HAL exposures(1% in 20% oxygen for 4hr) of 6-day- or 5-week-intervals demonstrated that susceptible guinea pigs responded similarly upon re-exposure regardless of number of exposures or interval of administration (165). Therefore, these studies indicate that HAL is not sensitizing guinea pigs to HAL-induced

liver injury following secondary HAL exposure. Several other attempts have been made to develop a repeat-exposure HAL hepatitis model by sensitizing animals to a TFA epitope prior to the HAL exposure either by using TFA-conjugated proteins or HAL preexposure (149, 152, 153). However, these models demonstrated enhanced humoral immune response to TFA or HAL without the associated enhanced liver pathology.

1.6.5. Clinical Incongruencies with the "Hapten Hypothesis"

Despite its popularity, there exists clinical evidence that is incongruent with a strictly acquired immune-mediated hypothesis. For example, there are reports of patients who developed severe HAL hepatotoxicity after a single exposure of HAL and of people with antibodies in their serum who did not develop liver injury (116, 166). A causal relationship between antibody presence and severe HAL hepatotoxicity has yet to be demonstrated (112). Furthermore, there are hepatotoxic drugs to which antibodies develop after exposure but are not involved in the pathogenesis of liver injury. For example, in the case of alcohol-induced liver injury, antibodies to acetaldehyde-modified protein epitopes and autoantibodies can be found in human alcoholics (167, 168), but these are not usually thought to be involved in the pathogenesis. Accordingly, it is appropriate to consider additional hypotheses for the pathogenesis of HAL hepatitis.

Considering the overwhelming support for the hapten hypothesis as the mechanism of HAL hepatitis, there is surprisingly little definitive evidence that HAL-induced neoantigens play a causal role in the development of HAL hepatotoxicity. Based on morphological changes seen in specimens of halothane hepatitis patients,

Blackburn et al. (108) suggested that HAL is a direct toxicant in the severe form of toxicity, similar to histopathological changes seen in carbon tetrachloride and chloroform toxicity. Peters et al. (110) suggested that the clinical presentation of post exposure fever followed by jaundice several days later suggests that the clinical syndrome is similar to infectious hepatitis. Although HAL is probably the most well studied drug with idiosyncratic potential, the mechanisms of pathology for HAL are still largely unknown.

1.7.1 Female Sex and Hepatic Adverse Drug Reactions

Female sex has been implicated as a risk factor in ADRs, such as in NVP-induced skin lesions and amiodarone-induced cardiac arrhythmia (169, 170). In a hospital-based prospective study, 18.7% of patients admitted to the internal medicine department developed an ADR. Female sex was a risk factor for developing ADRs, and women were more prone to develop a gastrointestinal-type ADR compared to men (171). Another prospective study confirmed the female-sex bias cases of gastrointestinal adverse reactions as well as allergic cutaneous reactions (172). In particular, women are more susceptible to hepatic adverse reactions associated with HAL, flucloxacillin, INH, erthromycin, nitrofurantoin, and alcohol, whereas, males are more susceptible to azathioprine-induced liver injury (173-176). Additionally, women are overrepresented in drug-induced liver injury cases leading to fulminant liver injury (177). There is also a sex bias in the pattern of liver pathology, with hepatocellular injury (ALT/alkaline phosphatase ratio >5) being more prominent in women, whereas men have more

cholestatic liver injury (ALT/alkaline phosphatase < 2) in response to drug-induced liver injury (177).

1.7.1 Pharmacokinetic and Pharmacodynamic Factors

The exact reasons for the sex-related vulnerability are not known. Pharmacokinetic factors may influence an individual's propensity to develop an ADR. It is reported that women achieve higher plasma drug concentrations for some drugs such as tricyclic antidepressants (178). This could be due to the fact that women have a smaller body mass, and therefore have more drug per kg at the same dose of drug. Alternatively, sex-differences in gastric emptying can alter drug absorption leading to sex-related differences in plasma drug concentrations. Reports indicate that women have slower gastric emptying than males, and that the rate of gastric emptying can vary with the menstrual cycle (179-181). Also, women tend to have a greater ratio of adipose to lean muscle tissue. This alters the bioavailability of lipophilic drugs which have an affinity for adipose tissue, leading to longer plasma drug half-life and elevated serum drug concentrations.

A sex-specific difference in drug metabolism, permitting toxic drugs or toxic metabolites to accumulate in females, could lead to sex differences in the sensitivity to ADRs. One example is that women have less activity of alcohol dehydrogenase leading to a greater plasma alcohol concentration (182) which may contribute to the increased alcohol-induced pathology seen in women compared to men (176). There are also

reports that genetic polymorphism such as that of glutathione-s-transferase associates with enhanced susceptibility to idiosyncratic hepatic ADRs and is predominately found in women (183). There are several reports that CYP enzymes responsible for phase I reactions are influenced by sex. For example, the quantity and activity of hepatic CYP enzymes and uridine diphosphate (UDP)-glucuronyltransferase enzymes were greater in male rats compared to females (184). This corresponds to a shorter sedation time in male rats administered hexobarbital, which requires bioactivation, as compared to the female counterparts (185). A literature review comparing benzodiazepines demonstrated sex-related differences in metabolism for the following 5 drugs: chlordiazepoxie, diazepam, desmethlydiazepam, oxazepam and temazepam. For these drugs the plasma half-life was consistently longer in women (186). However, the sex-related differences are minor compared to the influence of age and genetics and do not warrant dose modification according to sex (187). Therefore, sex disparity in drug metabolism is apparent in both human and rodents; whether the bias is similar across species for the same drugs is not clear.

Differences in pharmacodynamics, or the biochemical and physiological effects of drugs on the body, can lead to alterations in the desired and adverse effect profiles of a drug. Sex-related differences in pharmacodynamics could cause sex-related differences in drug sensitivity. In contrast to pharmacokinetics parameters, pharmacodynamic effects, such as the perception of pain, can be more challenging to assay. Accordingly, sex-related differences in pharmacodynamics are less well studied

compared to those associated with pharmacokinetics. One example of a sex-related difference in pharmacodynamics is the finding that women show greater improvement and have more severe adverse reactions to some psychotic agents (188). It is thought that estrogen can act like a dopamine antagonist and exacerbates the effect of antipsychotic drugs (188). Therefore, women require a lower dose to achieve the desired pharmacologic effect. Also, women have a longer corrected QT interval, time interval between ventricular depolarization and repolarization, and a greater response to drugs that block potassium ion pumps in cardiac muscle (170). In the presence of these drugs, the lengthened QT interval predisposes women to cardiac arrhythmias and torsade de pointes (189). It is thought that this is due to an intrinsic electrophysiolgical difference between women and men (170).

1.7.2 Sex Hormones in Adverse Reactions

Sex hormones can also influence the propensity to develop ADRs. For example, estrogen inhibits the repolarization of cardiac muscle by acting on potassium pumps in cardiac myocytes and thereby facilitates the higher incidence of drug-induced torsade de points and/or arrhythmias in women (170). Likewise, hepatic pathophysiology and drug metabolism are influenced by sex hormones, which are similarly metabolized in the liver. Increasing doses of estrogen exacerbate carbon tetrachloride (CCl4)-induced inhibition of hexobarbital metabolism and further increases the sedative effects of hexobarbital (190). Some drugs, such as methaqualone, also demonstrate menstrual

cycle-related differences in drug kinetics (186). Therefore, sex hormones and the menstrual cycle can predispose a person to develop ADRs.

In addition to playing a role in reproductive health, sex hormones can aggravate or lessen hepatic injury depending on the type of hepatic stress. For example, estrogen is beneficial in ischemia/reperfusion or hemorrhagic shock largely by upregulating endothelial nitric oxide synthase to produce vasodilatory, nitric oxide (191). Furthermore, estrogens inhibit the upregulation of integrin components CD11 and CD18 on leukocytes thereby blocking attachment to the endothelial cells and diapedesis out of the circulation. Estrogen also promotes liver regeneration in response to liver resection, as demonstrated by the observation that pharmacologic antagonism with tamoxifen attenuated liver weight gain following partial hepatectomy in rodents (192). The increased incidence of benign hepatocellular adenoma with oral contraceptives also highlights this point (193, 194). Counter-intuitively, hepatocellular carcinomas occur more often in men than women (2:1-4:1) (195), suggesting that additional mechanisms regulate tumorigenesis.

Interestingly, Naugler et al. (196) demonstrated that toxicant-induced hepatocellular carcinomas are mediated through MYD88-dependent IL-6 production, an innate immune cascade. Exogenous estrogen administration abrogated tumor formation in male mice. Therefore, sex hormones can modulate immune response to impact liver pathophysiology. The enhanced propensity for females to develop hepatic ADRs may also be due to an enhanced immune response compared to males. In rat models of

chronic alcohol-induced liver injury, as in people, female rats develop more severe liver injury than their male counterparts (176). In the sensitive females, serum endotoxin and hepatic CD14 protein levels were increased, suggesting an enhancement of the inflammatory pathway through TLR4 signaling (197). Additionally, alcohol-treated females have elevated inflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 2 (MIP-2), and TNF-α (198) Furthermore, inflammation-mediated liver injury induced by staphylococcal enterotoxin is more severe in female mice compared to males (199). Thus, enhanced inflammation in females is associated with a greater hepatotoxic response to alcohol and other agents..

1.8 Female Sex as a Predisposing Factor in Immune-mediated Pathology

Females display quicker, more robust cell-mediated and humoral immune responses to infectious and non-infectious insults in many species. For instance female deer have a lower parasite load compared to males (200). Women develop higher immunoglobulin levels in response to antigen stimulation and exhibit lower susceptibility to bacterial sepsis than their male counterparts (201). Response to traumatic injury and diseases of non-immune origin can also be influenced by gender-specific immune response. For example, epidemiological studies have shown than men develop immune suppression as a result of major blood loss leading to higher rates of bacteremia (202). Both immune suppression in males and enhanced response in females to injury have been reproduced in rodent models of burn and hemorrhagic shock (201).

Epidemiological reports indicate that sex is correlated with the development of immune-related pathology as well. There is a higher incidence of autoimmune disease such as rheumatoid arthritis, systemic lupus erythematosus, Graves disease, and Hashimoto's thyroiditis in females than males (203). Females more rapidly reject skin allografts compared to males (204-206) whereas men suffer higher rates of certain tumors, including colorectal (207), renal cell (208), and liver carcinomas (209). Interestingly, sex has also been associated with inflammation-mediated toxicant-induced liver cancer. In a recent study, elevated serum levels of IL-6 in male rodents contributed to the higher incidence of MYD88-dependent diethylnitrosamine-induced liver tumor formation in male rats (196).

1.8.1 Female Sex Hormones and Innate Immune Cells:

Immunological sexual dimorphism appears to be primarily regulated by gonadal hormones, and secondarily from the thymus and hypothalamus. This is reviewed by Grossman et al. (210). The distribution and number of leukocytes varies with menstrual cycle and the use of oral contraceptive in female cyclists (211). In particular, the number of NK cells is higher in the blood during the preovulation phase of the menstrual cycle (212). Estradiol is a female sex hormone that acts on many cells. One of the effects of estrogens is to inhibit the transmigration of circulating leukocytes (monocytes, neutrophils, NK cells) into the tissue parenchyma by decreasing integrins and vascular cell adhesion molecules (VCAMs) on leukocytes and endothelial cells, respectively, and by inhibiting NF-κB activity in vivo (213). Furthermore, gonadal hormones can influence immune cell activity. For example, NK cell activity is increased in the first trimester of

pregnancy and severely suppressed in the 2nd and third trimester due to the influence of sex hormones (214). The following brief discussion of some of the influences of estrogen and progesterone on innate immune cells.

Innate immune cells including macrophages, neutrophils, mast cells, and NK cells express estrogen receptors (ER-α and ER-β) and progesterone receptors(PR-A and PR-B). Signaling through these receptors can modulate immune cell activity (215). The literature indicates that the action of estrogen is both cell type- and tissue type- specific. For example, estrogen induces the production of nitric oxide in peritoneal macrophages (216), whereas it inhibits iNOS expression in alveolar macrophages (217). Estriol (a weak estrogen with high levels during pregnancy) enhances the sensitivity of Kcs to endotoxin by upregulating the CD14 receptor (218). In contrast, other reports indicate that estrogens decrease the LPS-induced upregulation of TLR4 mRNA and inhibit NFκB signaling and cytokine production in splenic macrophages and macrophage cell-lines (219-222). In ovariectomoized and sham-operated BALB/c mice on chronic estrogen therapy, estrogen decreases the surface expression of the activation marker of CD69 and increases intracellular IFN-γ of NK cells (223). This could indicate that estrogen increased capacity for NK cell IFN-γ production, while decreasing their ability to transmigrate and signal through receptors, such as CD69.

Progesterone is another female sex hormone that is also metabolized in the liver. Progesterone stimulates cytokine (TNF-α and IL-1β) and chemokine (MIP-2) release from peritoneal macrophages. Large concentrations of progesterone prolong

the survival of xenotransgenic and allogeneic grafts (224, 225) and inhibit fetal tissue resorption in healthy pregnancies (226), activities thought to be largely mediated by NK cells and CD8+ T cells. Interestingly, mifepristone, an progesterone receptor antagonist, increases fetal resorption rates in humans (227) and enhances the anti-tumor NK cell activity in mice (228). Since splenic NK cells do not have progesterone receptors, it is thought that progesterone's effect on NK cells is indirect through progesterone-induced blocking factor (PIBF). PIBF is synthesized by leukocytes in the presence of progesterone and inhibits arachadonic acid and prostaglandin release and the production of IL-12, a cytokine known to stimulate IFN-γ production in NK cells (229). Accordingly, some tumor cells have adapted fetal tissue mechanisms by expressing PIBF to inhibit NK cells (230). Whether these same indirect mechanisms are involved in controlling progesterone-induced activity of hepatic NK cells is unknown.

1.9. Multiple Determinants

It is clear that no one mechanism explains all situations of IADRs (98). This is underscored by seemingly different mechanisms involved in the pathogenesis of NVP-induced rash and the HAL-induced liver injury in rodents. It is possible that IADRs can occur from a confluence of susceptibility factors, as proposed by the multiple determinant hypothesis, in which an individual's chance of developing an IADR is a product of several separate probabilities of specific risk factors particular to the individual, the environment, and the drug (231). This product would be expected to be small, which would explain the rare frequency of IADRs in humans. If the major risk

factors could be incorporated into an animal model, it follows that liver injury would occur with high frequency. This is different for each drug. For example, it seems probable that metabolism and the innate immune response has a greater involvement in the hepatic ADRs, whereas the acquired immune response is likely more prominent in IADRs of the skin. Figure 1.2 illustrates that the innate and adaptive immune responses are both possible mechanisms of HAL pathogenesis. Also, it is possible that the acquired and innate immune mechanisms are not mutually exclusive. The determinants of susceptibility could predispose an individual to one arm of the immune response or the other with regard to HAL-associated pathogenesis.

Figure 1.2 illustrates how the determinants of susceptibility could exacerbate a seemingly mild injury to become much more severe. HAL is metabolized in the liver and forms a reactive metabolite, trifluoroacetyl chloride, which can bind to proteins and macromolecules, forming protein conjugates, such as TFA adducts. In the acquired immune-mediated hypothesis, these adducts become neoantigens that, upon second exposure, precipitate severe liver injury. In the innate immune hypothesis, hepatocytes are rendered sensitive by HAL exposure through the development of TFA-protein conjugates. The altered hepatocyte homeostasis and the unique risk factors (sex, genetics, fasted state) induce an innate immune response that precipitates HAL hepatitis.

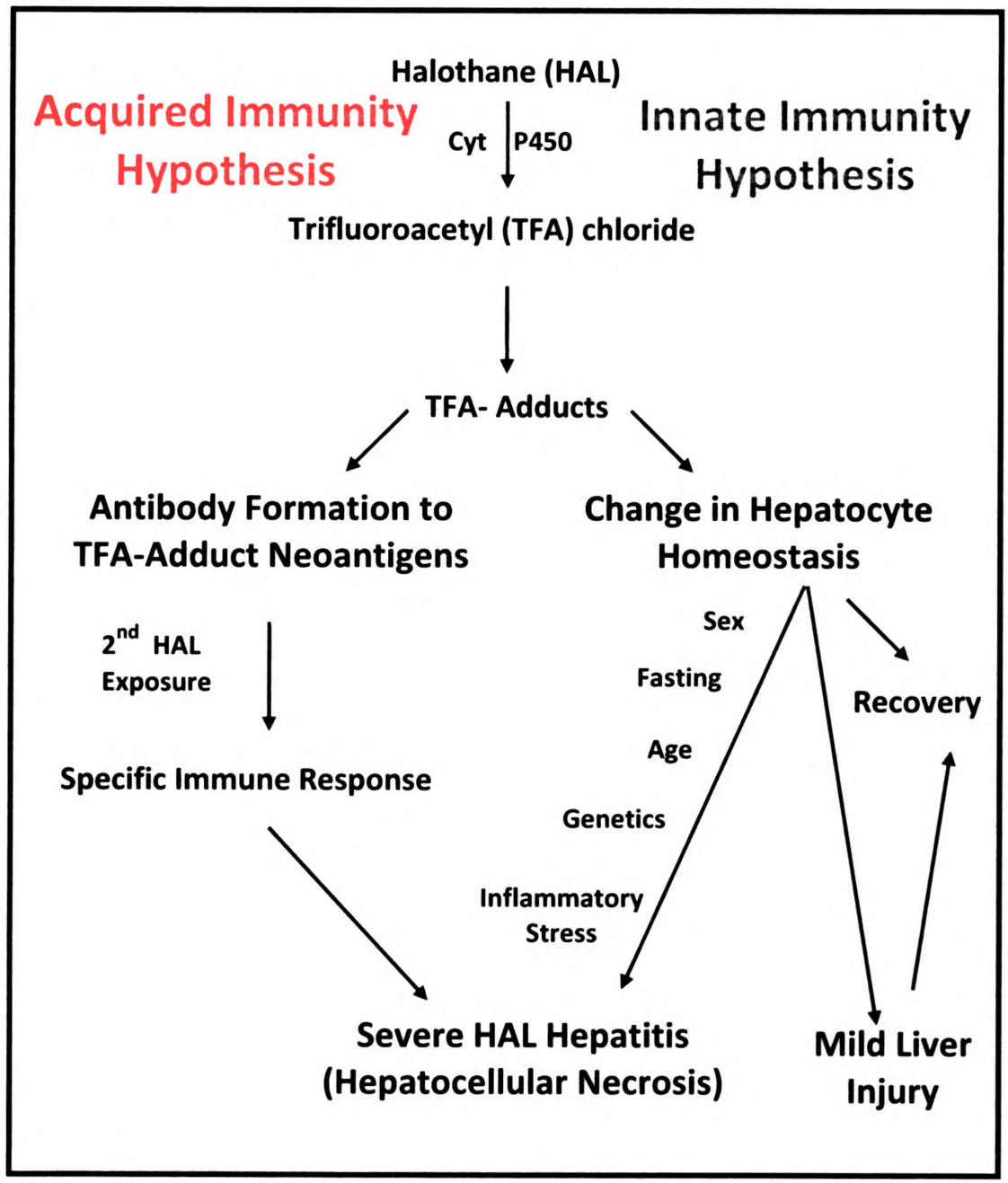


Figure 1.2. HAL Hepatotoxicity

1.10 Summary and Overview of Dissertation:

Rare adverse drug reactions, like the severe form of halothane-induced liver injury, are considered IADRs. IADRs represent a unique problem for public health because drug withdrawal from the market constitutes loss of a therapeutic modality that would benefit the majority of patients. They are also an economic loss to pharmaceutical companies. By utilizing animal models that have clinically determined sensitivity factors for IADRs, the mechanisms by which IADRs occur can be explored, and predictive, preclinical screens for IADRs might be developed.

HAL, a once widely used, inhaled anesthetic, produces a mild clinical hepatitis in 1 in 5 patients and severe hepatic injury in 1 in 6-20,000 patients. Although widely believed to occur by an antibody-driven immune mechanism, there are clinical reports of patients who developed HAL hepatitis without prior sensitization, a requirement for an acquired immune response. This suggests that acquired immunity may not be the sole mechanism for pathogenesis. Clinical and animal studies indicate that there exists a sex disparity with respect to many inflammatory processes. Also, there exists a sex-disparity in the response to HAL in humans. Therefore, the studies presented here test the hypothesis that **a sexually dimorphic, innate immune response is evident in the mouse model of HAL hepatitis and enhances the sensitivity of female mice to develop HAL hepatotoxicity.** In the first portion of this dissertation, an animal model of HAL hepatitis is characterized. This model imposes human risk factors (female sex, mature age, genetics) as well as potential situational risk factors (fasted state, inflammatory

stress) to permit the expression of severe liver pathology. Inflammatory mediators such as hepatic PMNs and plasma TNF-α were evaluated. In the second portion of my dissertation research, the role of the IFN-γ and NK cells in the pathogenesis of HAL hepatotoxicity were evaluated. Furthermore, their contribution to the sexual dimorphic response to HAL was addressed Lastly, an alternative to the hapten hypothesis is proposed as a possible mechanism for the development of HAL hepatitis, in which a sexually dimorphic innate immune response can precipitate HAL hepatitis.

CHAPTER 2

A Mouse Model of Severe HAL Hepatitis Based on Human Risk Factors

Dugan CM, MacDonald AE, Roth RA, Ganey PE.J Pharmacol Exp Ther. 2010 May;333 (2):364-72.

2.1. Abstract:

HAL (HAL)is an inhaled anesthetic that induces severe, idiosyncratic liver injury, ie, "HAL hepatitis," in approximately 1 in 20,000 human patients. We employed known human risk factors (female sex, adult age, and genetics) as well as probable risk factors (fasting and inflammatory stress) to develop a murine model with characteristics of human HAL hepatitis. Female and male BALB/cJ mice treated with HAL developed dose-dependent liver injury within 24hrs; however, the liver injury was severe only in females. Livers had extensive centrilobular necrosis, inflammatory cell infiltrate and steatosis. Fasting rendered mice more sensitive to HAL hepatotoxicity, and 8 week-old female mice were more sensitive than males of the same age or than younger (4 week-old) females. C57BL/6 mice were insensitive to HAL, suggesting a strong genetic predisposition. In HAL-treated females, plasma concentration of tumor necrosis factor-alpha was greater than in males, and neutrophils were recruited to liver more rapidly and to a greater extent. AntiCD18 serum attenuated HAL-induced liver injury in female mice, suggesting that neutrophil migration and/or activation are required for injury. Coexposure of HAL-treated male mice to lipopolysaccharide to induce modest inflammatory stress converted their mild hepatotoxic response to a pronounced, female-like response. This is the first animal model of an idiosyncratic adverse drug reaction that is based on human risk factors and produces reproducible, severe hepatitis from HAL exposure with lesions characteristic of human HAL hepatitis. Moreover, these results suggest that a

more robust innate immune response underlies the pre-disposition of female mice to develop HAL hepatitis.

2.2. Introduction:

Idiosyncratic adverse drug reactions (IADRs) occur in a minority of patients during drug therapy. They pose a unique public health problem because they cause severe illness and also often result in the withdrawal of otherwise useful drugs from the market. There are currently no predictive preclinical tests to identify drugs that have idiosyncratic potential, and the mechanisms of IADRs are poorly understood. Animal models that share the same sensitivity factors as humans and reproduce the liver lesions seen in people would be useful to study mechanisms of pathogenesis of IADRs and to develop strategies for therapy and prevention. The first step toward this effort must occur with drugs that are known to cause IADRs in people.

HAL is an inhaled anesthetic that produces a mild and reversible liver injury in 1 in 5 patients (104). A more severe IADR, or "HAL hepatitis," is observed in 1 in 6,000-22,000 patients who receive the drug (106). Although increasing numbers of reports of HAL-associated liver failure curtailed the use of HAL in the United States, it is still used in other countries, and cases of liver failure from HAL continue to be reported (232). The most prominent histologic feature seen in liver biopsies from patients who had HAL hepatitis is centrilobular necrosis (111). Other findings include fatty degeneration, vacuolation, and inflammatory infiltrate. Risk factors for severe HAL hepatotoxicity are female sex, genetics, age, and multiple HAL exposures (100, 101). Other susceptibility factors, such as fasting prior to anesthesia and exposure to inflammagens that accompanies surgery, are possible. For example, it has been reported that the plasma

concentration of lipopolysaccharide (LPS) increases in patients at the initiation of several types of surgery (233).

HAL is metabolized by cytochrome P450 2E1 in hepatocytes to form trifluoroacetyl-(TFA-) chloride, which binds covalently to proteins and lipids making TFA-adducts. Direct toxicity from TFA adducts is thought to cause the mild form of injury (133). The severe form of HAL hepatotoxicity is widely thought to result from an adaptive immune response to TFA-adducted or HAL-modified macromolecules. This is supported by the finding that repeated exposure is a risk factor and by the appearance of antibodies and immune complexes in the sera of some HAL-treated patients (128).

Despite its popularity, there exists clinical evidence that is incongruent with a strictly adaptive immune-mediated hypothesis. For example, a recent retrospective study demonstrated that 39% of HAL hepatitis patients had no previous history of HAL exposure (232). Additionally, some people with antibodies in their serum did not develop liver injury (116, 166). Attempts at developing animal models of liver damage using a HAL sensitization and challenge paradigm have resulted in a humoral immune response without associated liver pathology (152). Pohl and Gillette noted in 1982 that after years of intensive investigation the adaptive immunity theory for HAL hepatitis was unproven (112). This remains true today.

It seems possible that HAL hepatitis occurs from a confluence of susceptibility factors, such as proposed by the "multiple determinant hypothesis," in which an individual's chance of developing an IADR is a product of several discreet probabilities of specific risk factors particular to the individual and the drug (231). This product would

be expected to be small, which would explain the rare frequency of IADRs. If the major risk factors could be incorporated into an animal model, it follows that liver injury would occur with high frequency. Animal models of HAL hepatotoxicity have been developed, but most reproduce the mild type of injury (40, 132, 133). Attempts to develop animal models of severe, HAL-induced hepatotoxicity have focused on using repeated exposures (234), drug metabolizing enzyme inducers, glutathione depletion techniques, and/or hypoxic conditions (136, 235). However, low incidence of responders and/or lack of severe hepatotoxicity limit the usefulness of these models. High incidence of severe HAL-induced liver injury (n=4) was reported in studies using female strain 2 guinea pigs (236), but few studies have followed the original report. Here we report a mouse model developed using risk factors seen in humans. The result is the first animal model of HAL hepatitis in which severe liver injury occurs reproducibly without extensive chemical manipulations and which demonstrates histopathologic findings consistent with those observed in humans. The results raise the possibility that human risk factors might be useful in developing models of hepatotoxic IADRs and suggest that inflammation is a critical factor in the pathogenesis of HAL hepatitis.

2.3. Methods:

2.3.1. Materials: HAL (2-bromo-2-chloro-1,1,1-trifluoro-ethane), highly refined, low acidity olive oil, sodium citrate, oil red O, and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). Isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) was purchased from Abbott Laboratories (Chicago, IL). LPS from Escherichia coli O55:B5 (Lot 075K4038) with an activity of 3.3×10^6 endotoxin units

(EU/mg) was used in these studies. Rabbit anti-CD18 antiserum against amino acids 89-100 was purchased from New England Peptide (Gardnew, MA). Alanine aminotransferase (ALT) reagent was purchased from Thermo Electron Corp. (Louisville, CO). Anti-TFA-adduct rabbit serum was generously donated by Dr. Lance Pohl. Fluorescein isothiocyanate (FITC) goat anti-rabbit immunoglobulin G (IgG) and horse radish peroxidase (HRP) goat anti-rabbit IgG secondary antibodies were purchased from Invitrogen (Eugene, OR) and Santa Cruz (Santa Cruz, CA), respectively. RIPA buffer and HALT protease inhibitor were purchased from Thermo Scientific (Santa Cruz, MA).

2.3.2. Animals: C57BL/6 or BALB/cJ mice were purchased from Jackson Laboratory (Bar Harbor, ME) and used at an age of 4,8, or 10-12 weeks, with weights ranging from 10g to 27g. They were housed under conditions of controlled temperature and humidity and 12 hr light/dark cycle. They were given continuous access to bottled spring water and fed a standard chow (Rodent Chow/Tek 2018, Harlan Teklad, Madison, WI*) ad libitum*. The mice were allowed to acclimate for 1 week prior to use. Unless otherwise stated, mice were fasted for 15hrs and injected with HAL between 10 AM and noon. Food was returned 30 min after HAL administration. All procedures were carried out according to the humane guidelines of the American Association for Laboratory Animal Science and the University Laboratory Animal Research Unit at Michigan State University.

2.3.3 Experimental Protocol: Unless otherwise stated, experiments were performed using BALB/cJ mice. HAL was mixed with olive oil in a septum-covered glass

vial at concentrations of 0.047M, 0.063M, 0.094M, 0.187M, 0.375M, 0.562M to deliver the respective doses of 3.75, 5, 7.5, 15, 30, 45mmol/kg. Isoflurane mixtures were prepared similarly. Mice were fasted overnight to mimic the duration humans are typically fasted prior to surgery. Mice were given HAL or olive oil, i.p. For polymorphonuclear leukocyte (PMN) functional inhibition, mice were treated with 100 ul of antiCD18 serum (1:1 in saline), normal rabbit serum (1:1 in saline), or saline i.v. 6 hrs after HAL administration. They were anesthetized with isoflurane and euthanized at various times after the administration of HAL. Blood was drawn from the vena cava into a syringe containing sodium citrate (final concentration of 0.76%) for preparation of plasma. The left lateral lobe of the liver was fixed in 10% neutral buffered formalin (Fisher Scientific, NJ) and then blocked in paraffin. The median lobe was snap frozen, and the caudal lobe was fixed in formalin, immersed in 20% sucrose, snap frozen in Tissue-Tek OCT embedding media from VWR Labshop (Batavia, IL), and cryosectioned.

Male, BALB/cJ mice were treated with 5.0 X 10^6 EU/kg LPS (i.v.) 5.5hrs following HAL administration. The dose chosen for LPS was nonhepatotoxic but consistently produces increased plasma cytokine concentrations and hepatic PMN accumulation.

2.3.4. Histopathology: Paraffin-embedded, left lateral liver lobes were sectioned, stained with hematoxylin & eosin and examined by light microscopy. Snap frozen sections were sectioned and stained with Oil Red O (0.5% Oil Red O in isopropranol) according to a previously described method (237).

2.3.5. Immunohistochemistry and Microscopy: For PMN identification, formalin-fixed liver sections were probed with anti-mouse PMN antibodies using the protocol described by Yee et al. (238). Stained PMNs were counted in 4-5 random, 400X fields for each animal with a Nikon Eclipse E400 light microscope. For evaluation of TFA-adducted proteins, 8 um frozen liver sections from treated animals were probed with TFA-adduct antiserum. Using a fluorescent microscope, optical fields were captured as computer images. Periportal and centrilobular region are defined as seven hepatic cells from either the hepatic arteries or central vein, respectively. Five centrilobular and periportal regions were analyzed, and the percent of positive pixels in the centrilobular area was calculated and divided by the percent in the periportal region, in which fluorescent staining was minimal in all sections examined.

2.3.6. Western Analysis: Frozen liver samples were processed for whole cell protein isolation with RIPA buffer supplemented with HALT protease inhibitor according to the manufacturer's directions. Twenty ug of protein were loaded onto Invitrogen NuPAGE 12%-TRIS gels (Carlsbad, CA) and electrophoresed in Invitrogen NuPAGE MOPS SDS Running Buffer at 200V for 1 hr. Protein was transferred onto BioRad Immuno-blot PVDF membranes (Hercules, CA) using Invitrogen NuPAGE transfer buffer at 150 mAmp for 2hrs. The blot was blocked in 5% bovine serum albumin (BSA) for 1hr and hybridized with 1:10,000 TFA antiserum in 5% BSA overnight at 4°C. It was washed in TRIS-buffered saline with 0.01% Tween-20 (TBST) three times and hybridized with 1:5,000 goat anti-rabbit HRP in 5% BSA for 2hrs at room temperature. Following TBST wash, the proteins

were detected using Amersham ECL Western Blotting Analysis System and Amersham Hyperfilm MP from GE Health Care (Uppsala, Sweden).

2.3.7. Tumor necrosis factor-alpha (TNFα) Analysis: The plasma concentration of TNFα was measured using a BD OpEIA mouse TNF ELISA kit (Cat. No 558534) purchased from BD Biosciences (San Diego, CA).

2.3.8. Statistical Analysis: Results are presented as mean +/- standard error of the mean. A Student's t-test was performed on comparisons of two groups. For comparison of more than two groups, a 1-or 2- way ANOVA was used as appropriate after data normalization. The Student-Newman-Keuls test was performed to compare means in studies in which the ANOVA indicated statistical significance. The criterion for significance was $p<0.05$ for all studies.

2.4. Results:

Dose- and Sex-dependent Liver Injury in HAL-treated Mice. Male and female BALB/cJ mice were treated with HAL, and hepatocellular injury was assessed 24hrs later from the activity of ALT in plasma and liver histopathology. Doses up to 15mmol/kg HAL proved to be subanesthetic, with mild ataxia evident at the 15mmol/kg dose. The 30mmol/kg dose induced a transient anesthesia for less than 30min. Resulting liver injury was dose-dependent in males and females (Figure 2.1A). Male mice developed relatively mild liver damage at doses up to 45mmol/kg. The response in females was greater than that in males and corresponded to severe hepatocellular injury, as plasma ALT activities

approached 10,000U/L. Livers from female mice treated with 30mmol/kg HAL had severe lesions located primarily in centrilobular regions. Lesions were characterized by hepatocellular necrosis and inflammatory cell infiltrate (Figure 1C). Similarly treated males had milder liver lesions than females (Figure 2.1B).

Sensitivity to HAL-Induced Liver Injury in Fasted Mice. To test whether fasting increases sensitivity to HAL-induced liver injury, female BALB/cJ mice were fasted for 15hrs before HAL administration, and plasma ALT activity was evaluated 24hrs later. In mice fed *ad libitum*, there was little or no hepatic injury at doses up to 15mmol/kg. In contrast, in fasted mice treated with 7.5 or 15mmol/kg, plasma ALT activity reached 4000U/L. Fasting shifted the dose-response curve to the left so that doses of 7.5mmol/kg or greater were hepatotoxic (Figure 2.2).

Genetic Background as a Sensitivity Factor for HAL-induced Liver Injury. Responses to HAL were compared in two inbred mouse strains, BALB/cJ and C57BL/6. As demonstrated in Figure 2, HAL caused dose-dependent hepatotoxicity in the BALB/cJ mice. In contrast, there were no significant increases in plasma ALT activity at any HAL dose up to 30mmol/kg in the C57BL/6 mice (Figure 2.3A). The extent of TFA-adduct

Figure 2.1. HAL-induced Hepatotoxicity in Male and Female Mice. A. Plasma ALT activity evaluated 24 hrs after HAL treatment (ip) of mice fed *ad libitum* (n = 3-6 per group). *significantly different from males given the same dose. # significantly different from 7.5 mmol/kg-treated sex-matched animals. B and C. H&E-stained liver sections from 30 mmol/kg HAL-treated male and female mice, respectively. Liver section from the male mouse shows minimal necrosis, whereas the lesion is more severe in the female mouse. Labeled in picture are central vein (CV) and portal triad (PT).

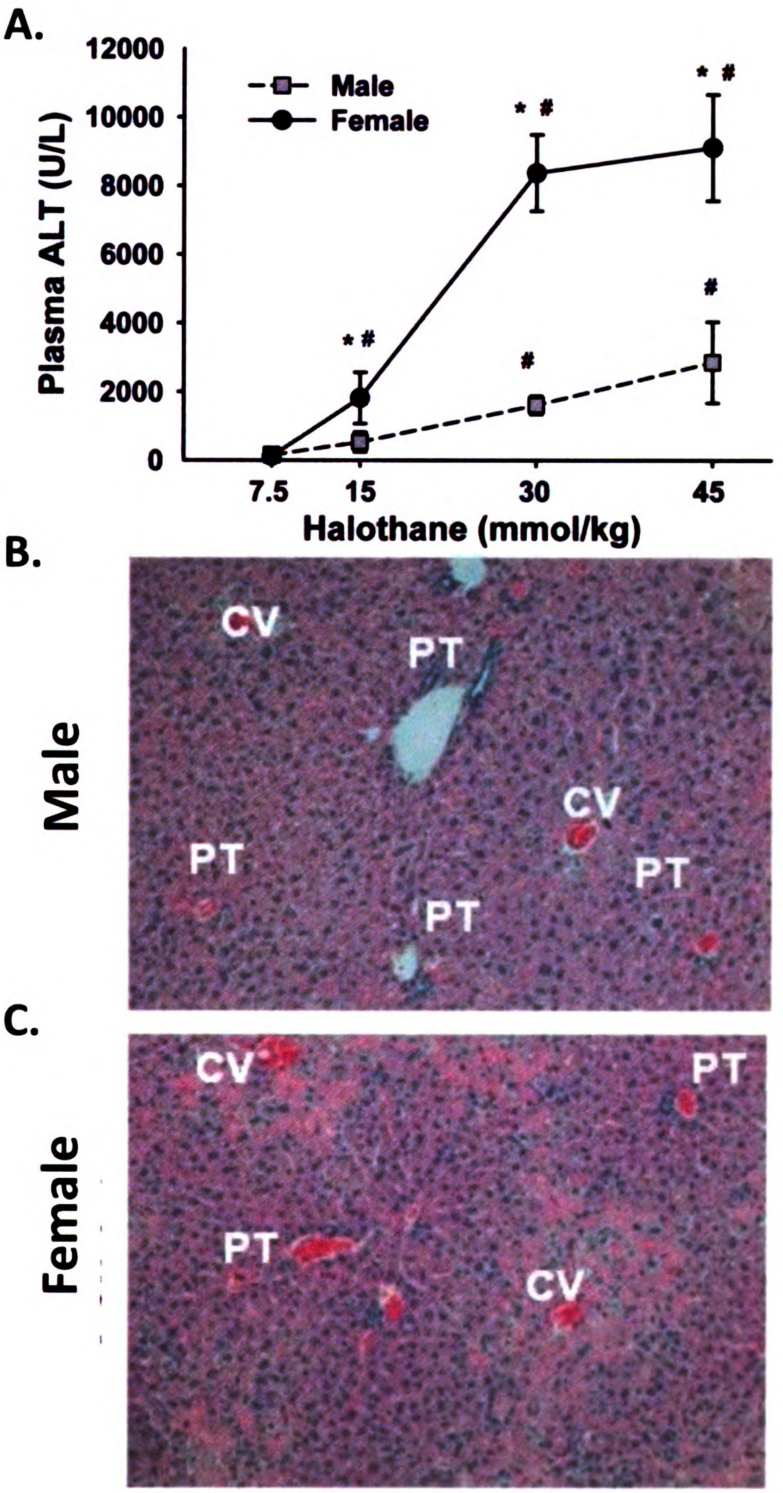

A.
12000
10000
8000
6000
4000
2000
0
Plasma ALT (U/L)
Male
Female
* #
* #
#
* #
#
7.5
15
30
45
Halothane (mmol/kg)
B.
Male
CV
PT
CV
PT
PT
PT
C.
Female
CV
PT
PT
CV

Figure 2.1

Figure 2.2. Fasting Enhances Sensitivity to HAL-induced Liver Injury. Female mice were either fasted overnight or not and then given HAL at the doses indicated (n = 3-5 per group). Blood was collected 24 hrs later for the determination serum ALT activity. *significantly different from fed mice given the same dose. #significantly different from 3.75 mmol/kg group.

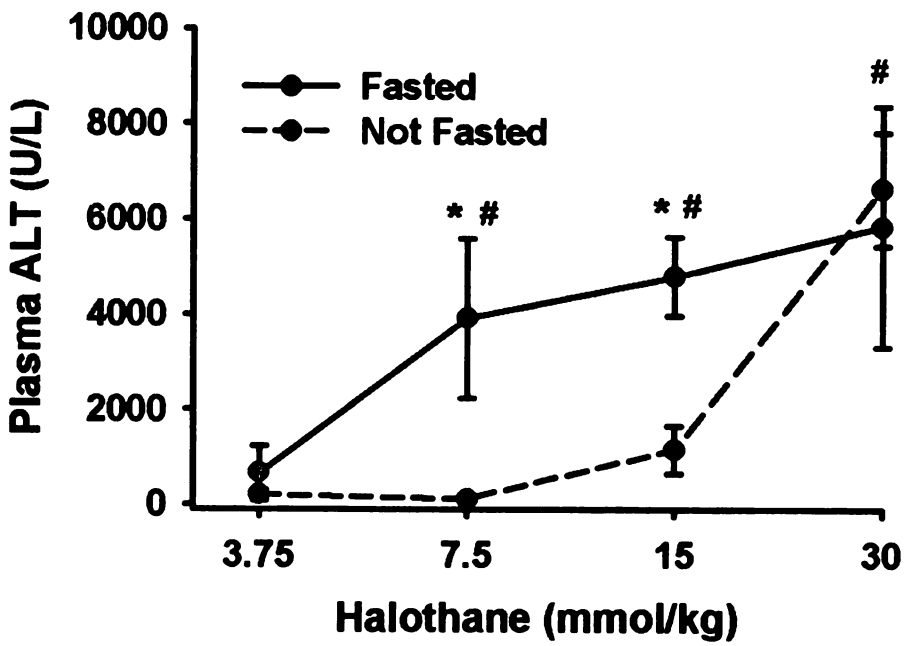
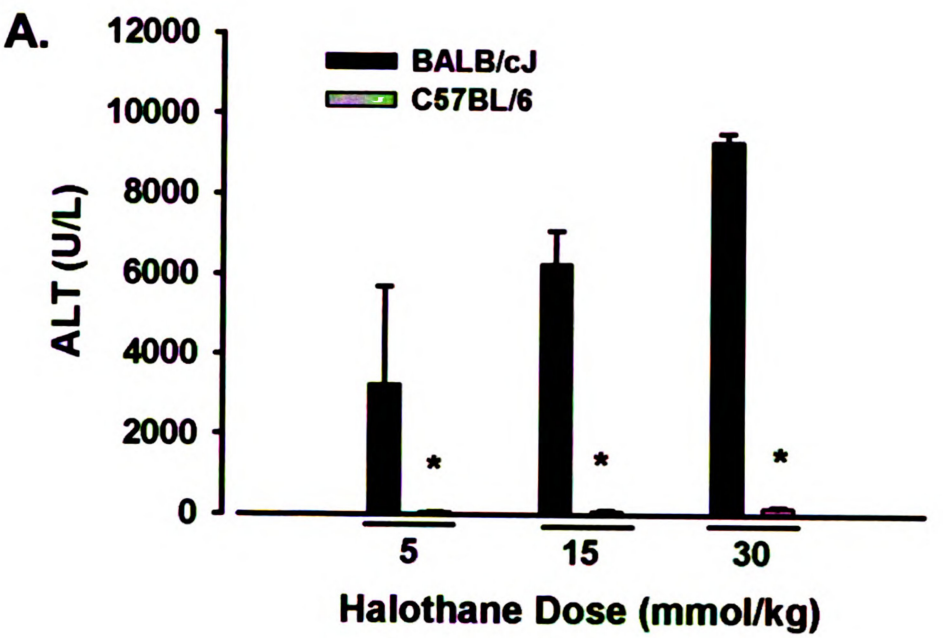

10000
8000
6000
4000
2000
0
Plasma ALT (U/L)
Fasted
Not Fasted
* #
* #
#
3.75
7.5
15
30
Halothane (mmol/kg)

Figure 2. 2

Figure 2.3. Genetic Background is a Sensitivity Factor for Severe HAL Hepatotoxicity in Mice. Fasted female C57BL/6 and BALB/cJ mice were given HAL at the doses indicated (n = 3-5 per group), and blood was collected 24 hrs later. A. ALT activity in plasma. B. Immunoblot detection of TFA-protein adducts in liver homogenates from mice treated with 15mmol/kg HAL. *significantly different from BALB/cJ mice.


A.
12000
10000
8000
6000
4000
2000
0
ALT (U/L)
BALB/cJ
C57BL/6
*
*
*
5
15
30
Halothane Dose (mmol/kg)

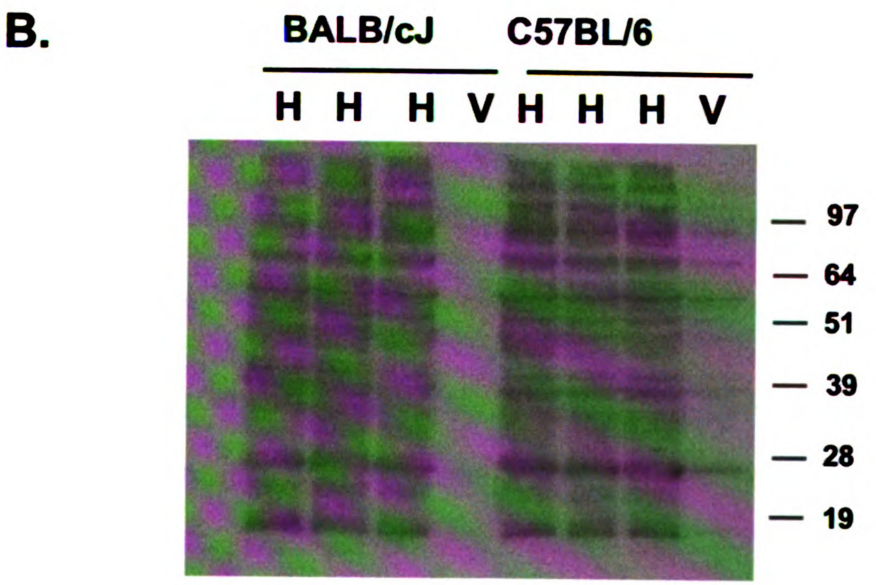

B.
BALB/cJ
C57BL/6
H H H V H H H V
97
64
51
39
28
19

Figure 2.3

formation in liver homogenates from HAL-treated C57BL/6 and BALB/cJ female mice was similar (Figure 2.3B).

Insensitivity to Isoflurane-induced Liver Injury. Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) is an inhaled anesthetic that is structurally similar to HAL but has less propensity than HAL to cause hepatotoxic IADRs in humans (239). In female BALB/cJ mice, HAL exposure caused dose-dependent hepatotoxicity, whereas ALT activity was not increased at doses of isoflurane up to 30mmol/kg (Figure 2.4).

Age as a Sensitivy Factor for HAL-Induced Liver Injury in BALB/cJ Mice. Responses to HAL were compared in 4- and 8-week old, male and female BALB/cJ mice. After 15mmol/kg HAL exposure, plasma ALT activity was <2,000U/L in 8-week old males and 4-week old mice of either sex (Figure 2.5). Eight-week old female mice had plasma ALT activity of ~8,000U/L.

Further characterization of Sex Related Differences in Sensitivity in HAL-induced Hepatotoxicity: Development of HAL-induced Liver Injury in Male and Female BALB/cJ Mice. Having evaluated the ability of sex, fasting, genetic background and age to modulate the response to HAL, we investigated further the factors involved in the sex-related difference in sensitivity. 10-12 week old male and female mice demonstrated signs of transient (<30min), mild coordination loss following administration of 15mmol/kg HAL. In male mice, HAL treatment caused an increase in plasma ALT activity

Figure 2.4. Lack of Isoflurane-induced Hepatotoxicity in Female Mice. Fasted, female BALB/cJ mice were given HAL or isoflurane at the doses indicated (n = 3-5 per group), and blood was collected at 12hrs for plasma ALT activity. All isoflurane-treated mice had plasma ALT activities < 50 U/L. *significantly different from the respective HAL-treated group.

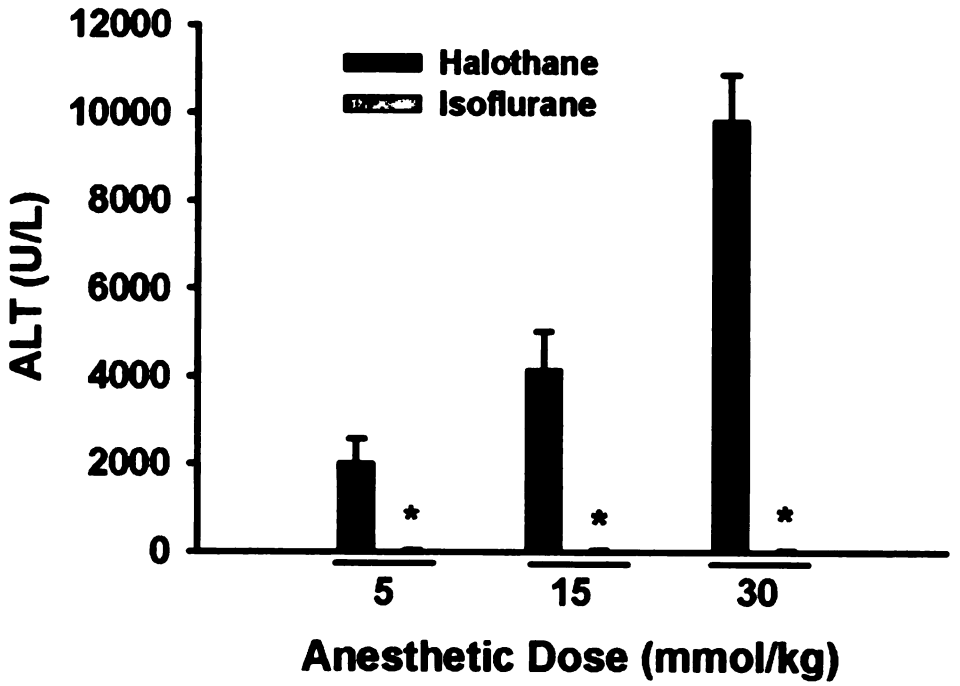

12000
10000
8000
6000
4000
2000
0
ALT (U/L)
Halothane
Isoflurane
*
*
*
5
15
30
Anesthetic Dose (mmol/kg)

Figure 2.4

Figure 2.5. Age is a Sensitivity Factor for Severe HAL Hepatotoxicity in BALB/cJ Mice. Fasted 4- and 8- week old, male and female BALBc/J mice (n = 5 per group) were given 15 mmol/kg HAL, i.p., and blood was collected 24 hrs later. *significantly different from age-matched males. #significantly different from 4-week old females.

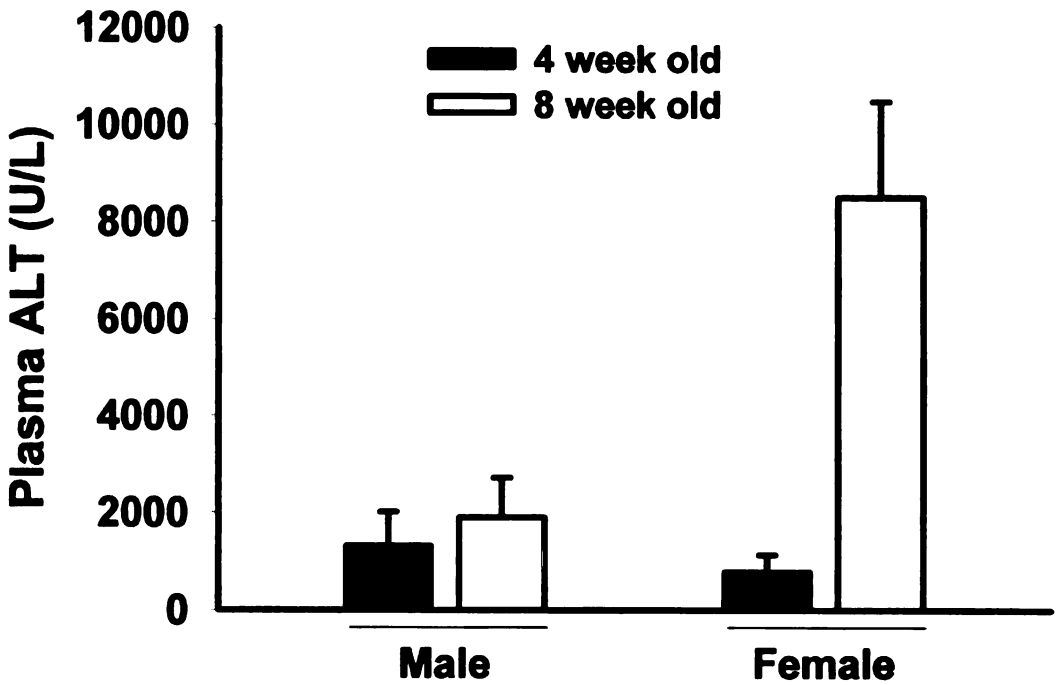

12000
10000
8000
6000
4000
2000
0
Plasma ALT (U/L)
4 week old
8 week old
Male
Female

Figure 2.5

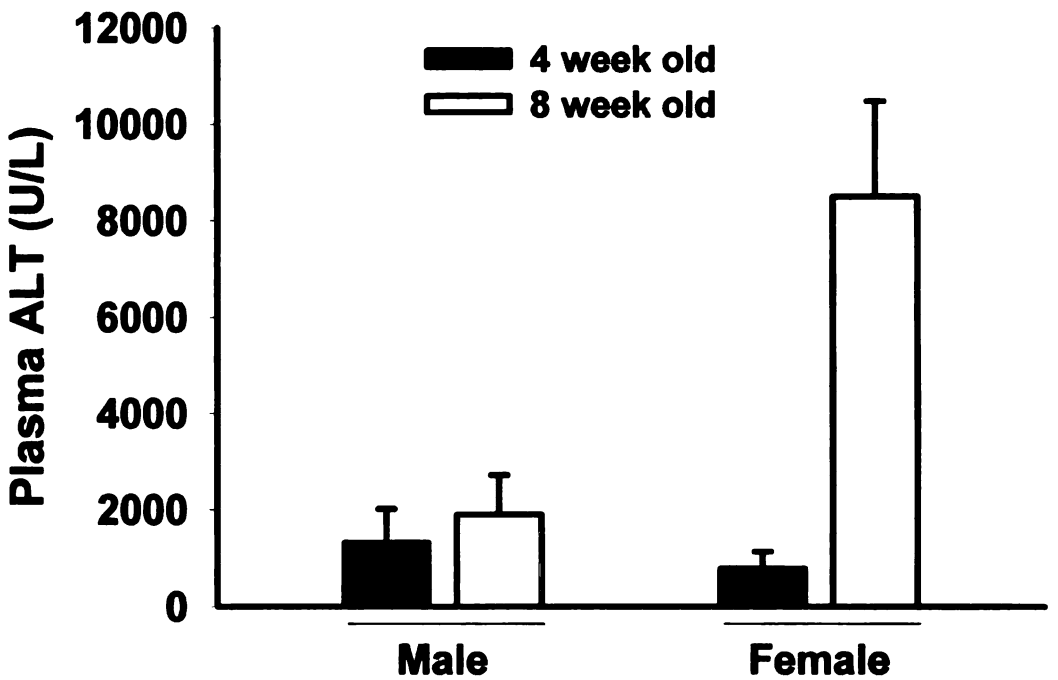

12000
10000
8000
6000
4000
2000
0
Plasma ALT (U/L)
4 week old
8 week old
Male
Female

Figure 2.5

Figure 2.6. Development of HAL-induced Liver Injury. Fasted female and male BALB/cJ mice were given vehicle or 15 mmol/kg HAL, i.p. (n = 4-6 per group). Blood was collected at various times for the determination of plasma ALT activity. There was no time- or sex-related difference in ALT activity in the vehicle-treated mice, so the results were combined and represented as zero-time. *significantly different from male mice at the same time. #significantly different from vehicle-treated animals (zero-time).

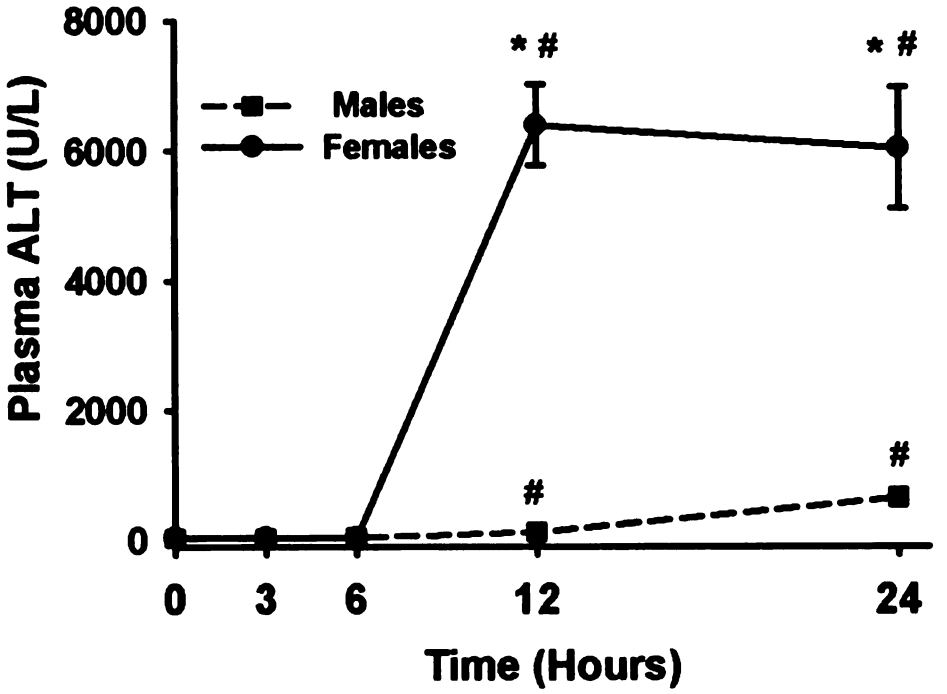

8000
6000
4000
2000
0
Plasma ALT (U/L)
Males
Females
* #
* #
#
#
0
3
6
12
24
Time (Hours)

Figure 2.6

at 12 and 24hrs after administration, at which times the ALT activity was 124 and 600U/L, respectively (Figure 2.6). The injury in female mice followed a similar time course but was much more severe, reaching an ALT activity of nearly 7000U/L at these times.

There were no significant lesions in untreated female, mice, whereas HAL-treated females developed pronounced centrilobular necrosis within 12hrs (Figure 2.7). Immunohistochemical staining for PMNs revealed a greater number in livers of HAL-treated mice compared to control mice. Livers from HAL-treated mice also had greater Oil Red O staining at 6hrs compared to livers from untreated mice at the same time (Figure 2.7), indicating that HAL administration caused steatosis.

HAL Bioactivation in Male and Female BALB/cJ Mice. A positive correlation has been reported between the severity of liver injury and the formation of TFA-adducts in livers of HAL-treated guinea pigs (133). As shown in Figure 2.8A, TFA-adducted proteins in the livers from HAL-treated male mice had a centrilobular distribution. A similar distribution was observed in HAL-treated female mice (not shown). There was a similar degree of TFA-adduct formation in livers from HAL-treated male and female mice given the same dose of HAL as determined by immunohistochemistry (Figure 2.8B) or by western analysis of liver homogenates (Figure 2.8C). There were no TFA-adducts in the livers of vehicle-treated animals of either sex (Figure 2.8C).

Figure 2.7. Histopathology of Livers from HAL-treated Mice. Female BALB/cJ mice were fasted overnight and then given vehicle or HAL (15 mmol/kg, i.p.). Representative liver sections from mice treated 12 hrs earlier were examined after hematoxylin and eosin (H&E) staining or after immunohistochemical staining for PMNs, in which PMNS appear as pink cells. Liver sections taken from mice 6hrs after HAL administration were stained with Oil Red O, in which lipid. Labeled in the picture are central vein (CV) and portal triad (PT). Photomicrograghs were taken at 200X magnification.

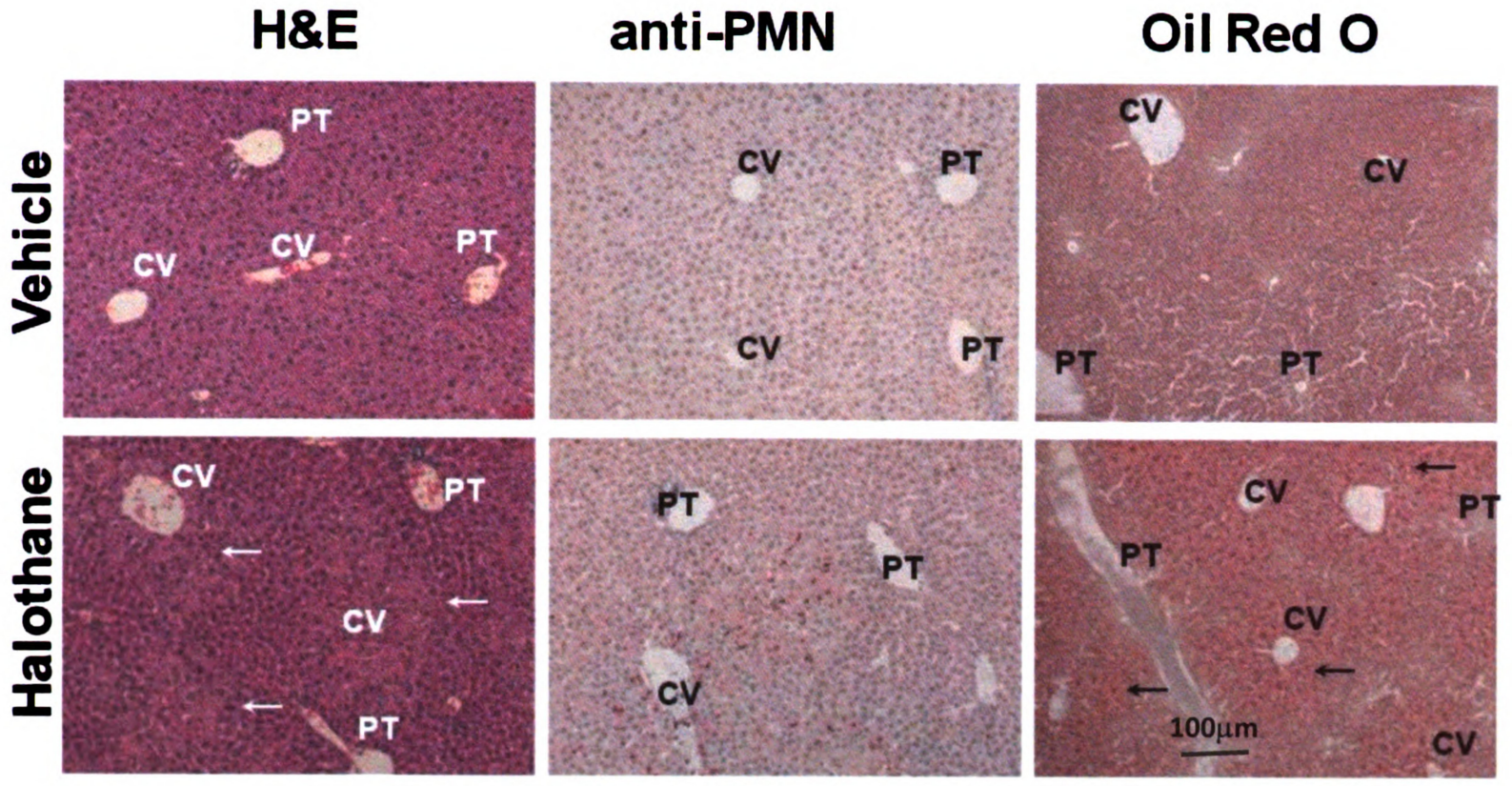


Figure 2.7

Figure 2.8. HAL Metabolism is Similar in Male and Female Mice. Fasted male and female BALB/c mice were treated with vehicle or 15 mmol/kg HAL, and liver samples were collected 12hrs later. A. Representative liver section from a HAL-treated, male mouse stained immunohistochemically for TFA-adducts and visualized with green color. DAPI nuclear stain appears blue. B. Ratio of positive pixels in the centrilobular and periportal regions (CV/PT)(n= 3 per group). C. Immunoblot detection of TFA-protein adducts in liver homogenates.

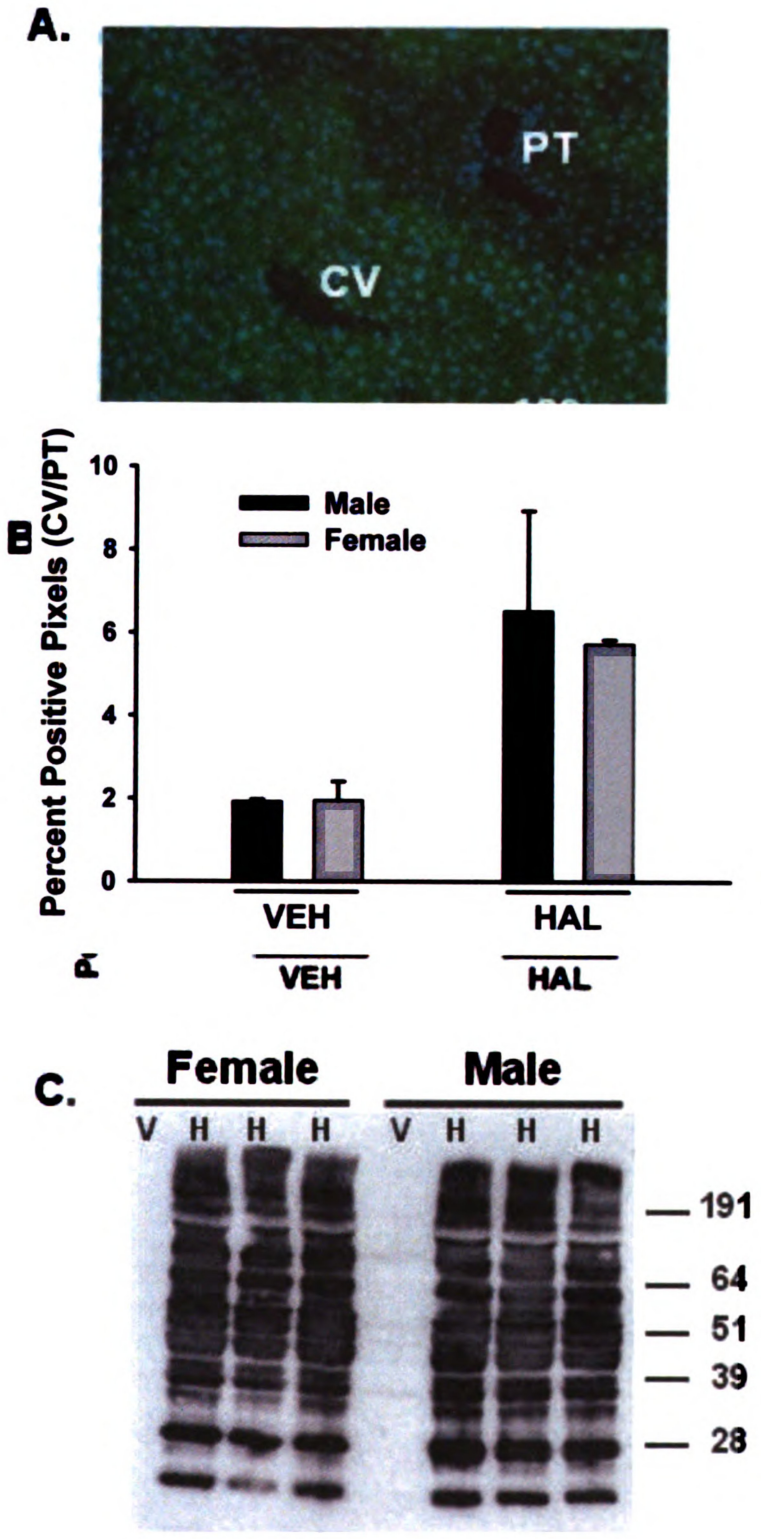

A.
PT
CV
B
Percent Positive Pixels (CV/PT)
10
8
6
4
2
0
Male
Female
VEH
HAL
Pt
VEH
HAL
C.
Female
Male
V H H H V H H H
191
64
51
39
28

Figure 2.8

Figure 2.9. Sex-specific Difference in Plasma TNF-α Concentration after HAL Exposure. Female and male BALB/cJ mice were given vehicle or 15 mmol/kg HAL (n = 4-6 per group), and blood was collected at various times. Plasma TNFα concentration was determined using a BD optEIA Mouse ELISA. The average plasma concentration of TNFα in vehicle-treated animals was less than 40pg/ml.*,significantly different from males at the same time. #, significantly different from vehicle controls (zero-time).

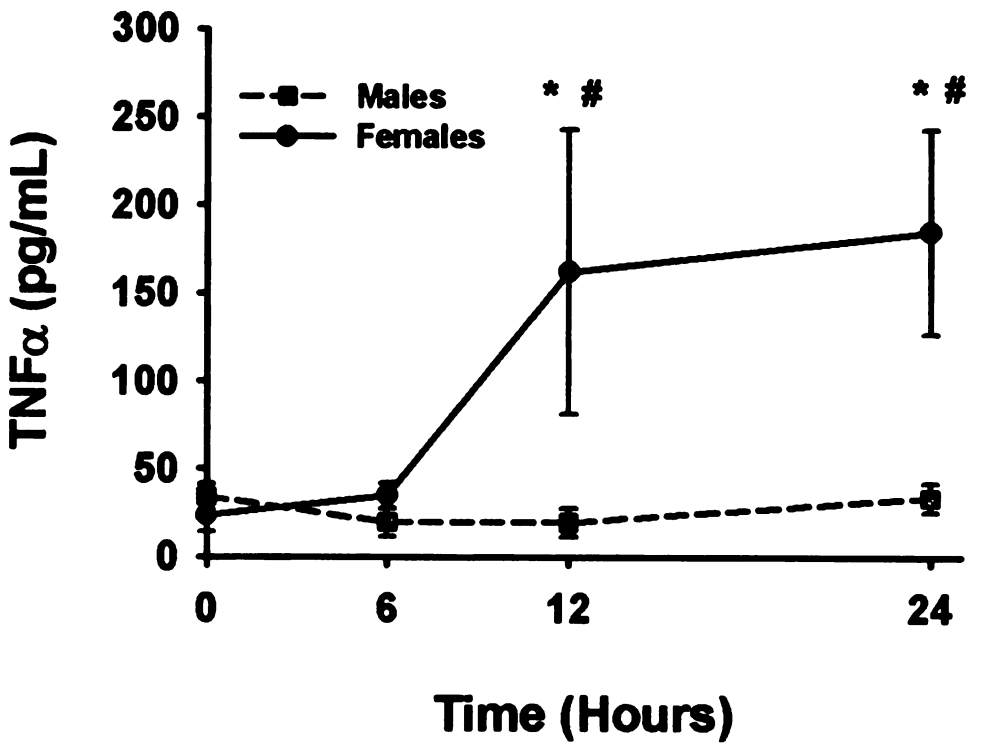

300
250
200
150
100
50
0
TNFα (pg/mL)
Males
Females
* #
* #
0
6
12
24
Time (Hours)

Figure 2.9

Plasma TNF-α Concentration in HAL-treated Mice. In vehicle-treated mice or in HAL-treated (15mmol/kg) males, there was no change in plasma TNF-α concentration at any time investigated. In contrast, plasma TNF-α concentration was significantly increased 12 and 24hrs after HAL exposure in female mice (Figure 2.9).

Hepatic PMN Recruitment in HAL-treated Mice. There was no change in the number of hepatic PMNs in vehicle-treated mice over time. PMN number was slightly elevated by 24hrs in livers of male mice given 15mmol/kg HAL. In contrast, there was a marked increase in hepatic PMNs in female mice at 12 and 24hrs after HAL administration. Hepatic PMN accumulation was significantly greater in female mice compared to male mice at 12 and 24hrs (Figure 2.10A). Interestingly, PMNs failed to accumulate in HAL-treated, C57BL/6 mice (Figure 10B), which were insensitive to HAL hepatotoxicity (see above).

HAL-induced Severe Liver Injury in Female Mice Given Anti-CD18 Serum. In a mouse model of mild HAL-induced hepatotoxicity, rabbit antiserum depleted PMNs and attenuated the increase in plasma ALT activity (132). CD18 is an integrin on leukocyte plasma membranes and is needed for adhesion to vascular endothelium and transmigration of PMNs into the liver parenchyma. Mice given CD18 antiserum in our model of severe HAL hepatitis had less hepatocellular injury compared to those treated with control serum or saline (Figure 2.11).

Enhanced Sensitivity of Male Mice to HAL upon LPS Coexposure. The results above suggest that the greater sensitivity of female mice might be due to a more robust

Figure 2.10. Hepatic PMN Recruitment is Sex- and Strain-Dependent. Female and male BALB/cJ mice were given vehicle or 15 mmol/kg HAL (n = 4-5 per group) and female BALB/cJ and C57BL/6 mice were given 30 mmol/kg HAL (n=4). The number of PMNs in liver sections was determined by counting PMNs in immunostained tissue. A. Hepatic PMNs in male and female BALB/cJ mice. The number of PMNs was < 8 for vehicle-treated mice and did not change with time, so those values were combined and represented as zero-time. *,significantly different from HAL-treated males. #,significantly different from vehicle controls (zero time). B. Hepatic PMNs in female, BALB/cJ and C57BL/6 mice. *,significantly different from HAL-treated BALB/cJ mice.

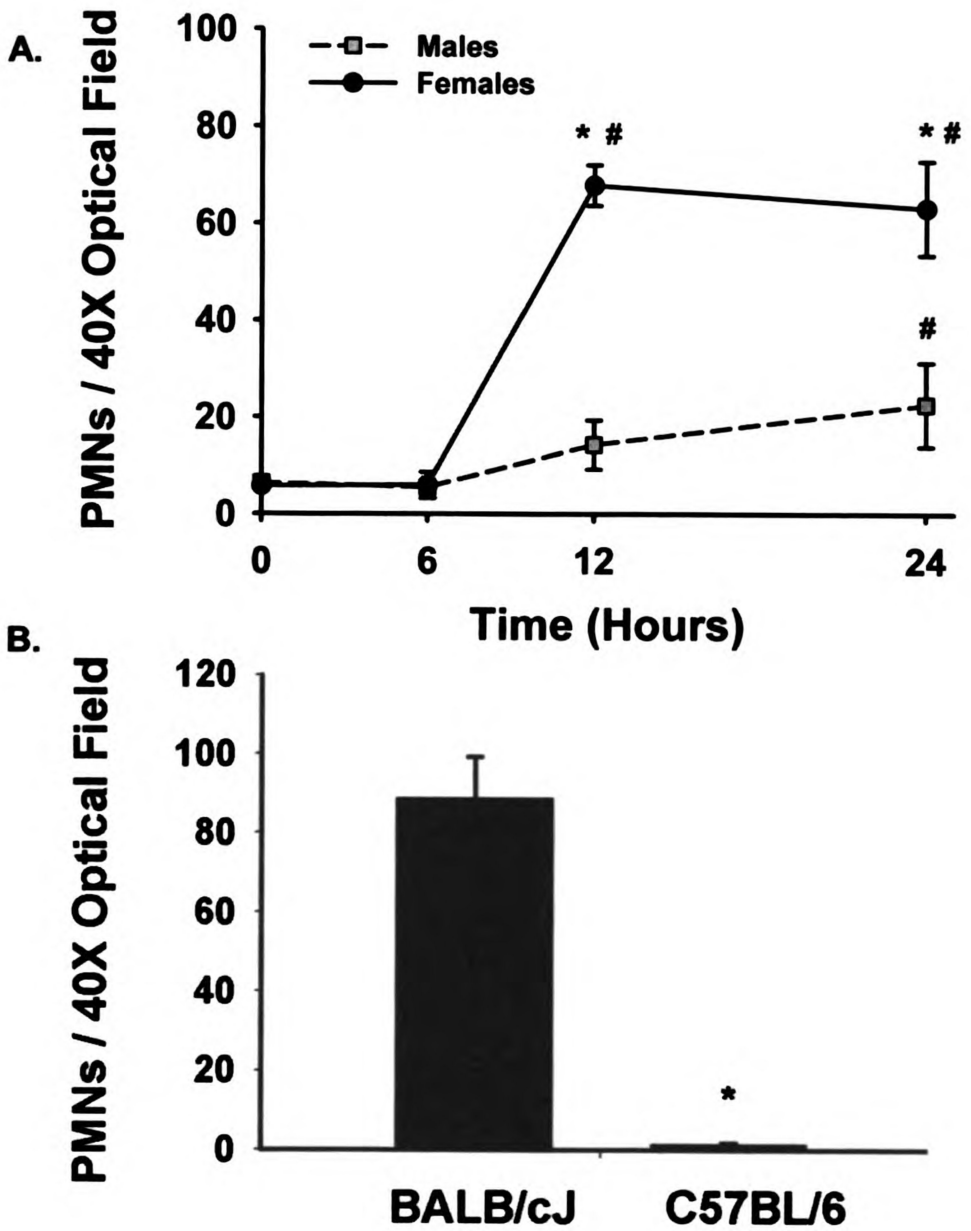

A.
PMNs / 40X Optical Field
100
80
60
40
20
0
Males
Females
* #
* #
#
0
6
12
24
Time (Hours)
B.
PMNs / 40X Optical Field
120
100
80
60
40
20
0
*
BALB/cJ
C57BL/6

Figure 2.10

Figure 2.11. CD18 Neutralization Attenuates Severe HAL Hepatotoxicity. Fasted, female BALB/cJ mice were treated with 15 mmol/kg HAL (i.p.) and either saline, control rabbit serum (NRS) or anti-CD18 rabbit serum (CD18 RS) as described in Methods (i.v.). Plasma was collected 12 hrs after HAL administration and evaluated for ALT activity (n=6 per group). *,significantly different from all other groups.

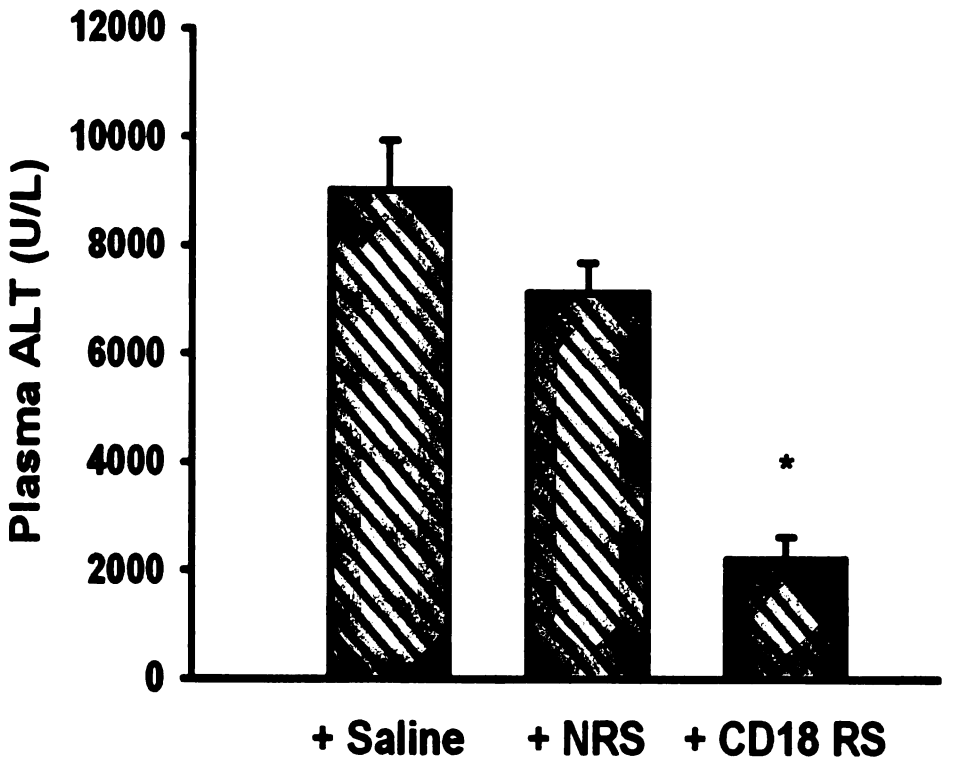

12000
10000
8000
6000
4000
2000
0
Plasma ALT (U/L)
*
+ Saline
+ NRS
+ CD18 RS

Figure 2.11

Figure 2.12. Inflammation Enhances Sensitivity to HAL Hepatotoxicity in Male BALB/cJ Mice. Mice were treated with vehicle or HAL and then either $5x10^6$ EU/kg LPS or saline vehicle 6 hrs later (n = 3-8 per group). Blood and liver samples were collected 24hrs after HAL administration. A. Plasma ALT activity. *significantly different from HAL-treated and LPS-treated animals. B. H&E section of liver from a representative HAL/LPS-cotreated mouse. C. Immunohistochemical staining for PMNs in a cotreated mouse; PMNs stain bright pink and nuclei stain blue. Both photomicrographs were taken at 200X magnification. Labeled in the pictures are central vein (CV) and portal triad (PT).

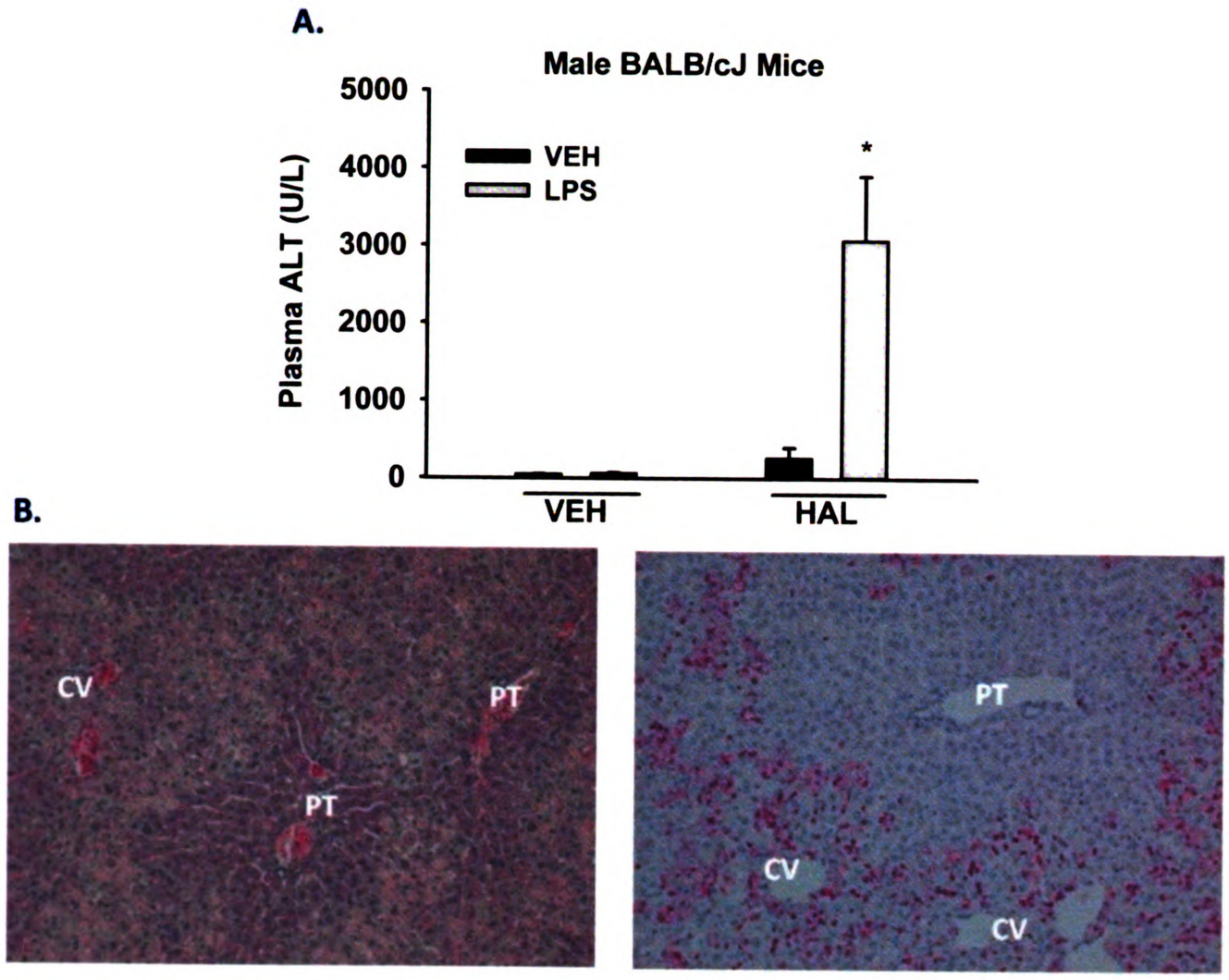

A.
Male BALB/cJ Mice
Plasma ALT (U/L)
5000
4000
3000
2000
1000
0
VEH
LPS
*
VEH
HAL
B.
CV
PT
PT
PT
CV
CV

Figure 2.12

inflammatory response accompanying HAL exposure. Accordingly, we determined whether LPS-induced inflammation could increase the sensitivity of male mice to HAL hepatotoxicity. Treatment with vehicle or LPS alone was not hepatotoxic (Figure 2.12A). The animals treated with HAL alone had a small increase in ALT activity, whereas the LPS-cotreated animals had a much larger increase (Figure 2.12A). Livers from mice treated with LPS and HAL demonstrated severe centrilobular hepatocellular necrosis with marked PMN accumulation (Figures 2.12B & 2.12C).

2.5. Discussion:

Few animal models reproduce severe liver injury caused by drugs with human idiosyncratic potential. Most of the models have involved administration of an inflammagen that prompts the appearance of liver injury from drugs that cause hepatotoxic IADRs in humans(240). In other attempts at developing animal models, liver injury has been either modest (132) or nonexistent (241). For example, nevirapine produces idiosyncratic dermal and liver toxicity in humans; a rat model reproduced the skin lesions but failed to reproduce the liver toxicity (96). Accordingly, it is noteworthy that the present model in mice based on human risk factors resulted in severe liver injury without the coadministration of other agents.

Although an adaptive immune response is commonly thought to be the mode of action of HAL hepatitis, not all human cases support a strictly adaptive-immune mechanism. For example, 4 out of 5 lymphocyte transformation tests and a leukocyte

migration inhibition test failed to demonstrate evidence of cellular hypersensitivity for HAL hepatitis patients (242). Antibodies to HAL-modified proteins form in humans (128); however, evidence linking antibodies to the pathogenesis of hepatitis is lacking (243) Several retrospective clinical studies indicate that HAL hepatitis can occur on the first exposure to HAL (244, 245), with the the most recent study reporting that 39% of affected patients had no previous exposure (246). Furthermore, multiple exposures was only a risk factor for HAL hepatitis when exposures occurred within 28 days (114, 115). This timeframe supports a paradigm in which the first exposure renders the liver more sensitive to a second exposure; however, an adaptive immune response is not the only explanation for the results. For example, TFA-adducted proteins persisted in guinea pigs for at least 21 days after HAL exposure (247). If the adducts formed in people have similar longevity, then repeated exposure at intervals that overwhelm the regenerative capacity of the liver could produce cumulative damage. Moreover, the observations that patients who developed clinical signs of liver failure upon first exposure to HAL but not upon subsequent exposure cast doubt on a strictly adaptive immune-mediated mode that explains all instances of idiosyncratic HAL hepatitis (244).

Data presented in Figure 2.1 demonstrate severe hepatotoxicity resulting from a single exposure of mice to HAL. All of HAL-treated mice developed liver injury at the largest dose administered. The lack of requirement for a priming exposure followed by reexposure indicates a mode of action other than adaptive immune-mediated pathogenesis in this model. Importantly, the histopathological findings in livers from

HAL-treated, female mice were consistent with the changes in postmortem specimens from patients who died from HAL-induced liver failure (111). Centrilobular necrosis, inflammatory cell infiltrate and steatosis, all hallmarks of human HAL hepatitis, were observed in livers of HAL-treated mice. Thus, this mouse model mimics to a substantial degree the histopathological features associated with idiosyncratic HAL hepatitis in human patients.

The present study extends the work of Ju and coworkers (248) by imposing several human risk factors to enable the expression of severe HAL hepatotoxicity. The magnitude of the response to HAL observed in this mouse model was much greater than the mild hepatotoxicity reported in a similar murine model. For example, the 6000-10,000U/L plasma ALT activity reported here for female, BALB/cJ given 30mmol/kg HAL is much greater than the 1200U/L reported earlier.

The rarity and heterogeneous clinical presentation of many IADRs and the unknown mechanisms of pathogenesis are barriers to the development of adequate animal models. We considered the influence of several risk factors known to be important in people when developing an animal model of severe HAL hepatotoxicity in mice. Female sex is a risk factor for HAL hepatotoxicity with a 2:1 preponderance in women (125). It was recently reported that females were the most at-risk group, representing 81.4% of patients who developed HAL hepatitis (246). Female mice developed severe hepatitis at doses of HAL that produced only a mild response in male mice (Figure 2.1). The disparity in toxicity between males and females was not due to

differences in bioactivation of HAL (Figure 2.8). The greater sensitivity of females has been reported previously for mice and guinea pigs (236, 248).

Children are thought to be less susceptible to HAL hepatotoxicity, with an estimated incidence of 1-80,000 to -200,000 (249, 250). Four-week old female mice had a milder response to HAL than 8-week old, female mice (Figure 2.5). These results indicate that the human risk factors of female sex and mature age can be recapitulated in a mouse model of severe HAL hepatitis

The occurrence of rare, HAL hepatitis among closely related family members and an increased frequency of the HLA-DR2 haplotype in patients with severe HAL hepatitis suggest a genetic predisposition (251, 252). The findings that guinea pigs sensitive to HAL produce offspring that are also sensitive to HAL (165) and that there are strain-dependent variations in HAL metabolism in rats (253) suggest genetic determinants of HAL sensitivity in animals as well. C57BL/6 mice were insensitive to severe HAL hepatitis at all doses tested (Figure 2.4A), consistent with reports in which sensitivity to more modest liver injury was strain-dependent (236, 248). The difference in sensitivity between C57BL/6 and BALB/cJ mice was not due to a difference in HAL bioactivation (Figure 2.4B). We are not aware of specific genetic differences between BALBc/J and C57BL/6 that may account for the difference in sensitivity; however, the results indicate that, as in humans, genetic differences are important in the hepatotoxic response to HAL in animals. BALB and C57BL/6 strains are vary in their immunologic response to pathogens. For example, a similar dose of the intracellular parasite Leishmania, is lethal

to BALB mice, whereas C57BL/6 mice mount an effective TH1 immune response to clear the pathogen (254). BALB/c mice are also more susceptible to Trypanosoma cruzi parasite infection compared to C57BL/6 mice. The susceptibility of the BALB/c mice due to an enhanced humoral immune response, whereas the C57BL/6 mice mount an effective cell-mediated immune response (255). Furthermore, it is well established that BALB mice have a greater propensity to develop allergic reactive airway disease, compare to C57Bl/6 mice (256). Whether the genetic determinants that render BALB/cJ mice sensitive are related to immune response and whether they are the same genetic determinants as in susceptible humans are not known.

Fasting has not been studied as a risk factor for human HAL hepatitis, yet patients are uniformly fasted prior to general anesthesia. Fasting mice before exposure to HAL increased their sensitivity to liver injury (Figure 2.2). Accordingly, consideration should be given to fasting as a potential contributor to risk for HAL hepatitis in humans. Fasting increases hepatic CYP2E1 expression and decreases hepatic glutathione stores in mice (257), and this may reduce protection against reactive metabolites generated during oxidative metabolism of HAL. Treatment of guinea pigs with an inhibitor of glutathione biosynthesis increased covalent binding of reactive HAL intermediates and enhanced liver injury (235).

HAL treatment resulted in elevated plasma TNFα concentration in female, but not male, mice (Figure 2.9). Moreover, in the HAL-treated, female mice hepatic PMN accumulation occurred to a greater extent than in males (Figure 2.10), and liver damage

was attenuated by neutralization of CD18, an adhesion molecule required for transmigration of PMNs into the hepatic parenchyma. These results support the hypothesis that PMNs contribute to the pathogenesis of severe HAL hepatitis in mice. The findings also point to a sexually dimorphic inflammatory response to HAL, inasmuch as male mice responded to HAL with only modest liver injury, minimal hepatic accumulation of PMNs, and no increase in serum TNFα. Sexually dimorphic inflammatory responses have been reported in both humans and experimental animals (201, 258). For example, female sex is a risk factor in alcohol-induced liver injury in people (176) as well as animals (198), and alcohol consumption produces greater concentration of inflammatory mediators in female compared to male rats.

Interestingly, although male, BALB/cJ mice responded to HAL with only modest liver injury and inflammation, cotreatment with a nontoxic dose of LPS led to pronounced hepatotoxicity. Similar results have been observed by others in a hypoxia-HAL model in rats (259). LPS effects biological responses through Toll-like receptor 4. A viral mimetic (PolyI:C) that stimulates Toll-like receptor 3 potentiated HAL hepatotoxicity in female mice (162). These results suggest that inflammation might contribute to HAL hepatotoxicity and that the contribution of inflammatory stimuli might be mediated through at least Toll-like receptors 3 or 4. It is tempting to speculate that the modest hepatotoxic response to HAL alone in male mice is tantamount to the mild liver injury seen in 20% of patients, whereas the pronounced response seen in combination with LPS relates to the rarer, severe HAL hepatitis seen in humans.

In summary, to our knowledge this is the first animal model of idiosyncratic hepatotoxicity based on human risk factors for an IADR. The animal model of severe HAL hepatitis shares the human risk factors of female sex, mature age, and genetic predisposition and a possible contribution of fasting, which is imposed on all individuals prior to general anesthesia. The model in female, BALB/cJ mice captured important aspects of the pathology of human HAL hepatitis, including severe centrilobular necrosis, inflammatory infiltrate and steatosis. Importantly, the results suggest that an adaptive immune response requiring sensitization and challenge exposures to HAL is not needed to precipitate severe HAL hepatitis, since injury was produced in mice upon a single exposure to the drug. The greater sensitivity of female mice was associated with a more robust inflammatory response than occurred in males, and the importance of inflammatory stress in the hepatotoxicity was suggested by the ameliorative effect of CD18 neutralization. The ability of a nontoxic dose of LPS to convert a modestly hepatotoxic response in males to a robust, female-like response further testifies to the importance of an inflammatory response in the expression of HAL hepatitis in this model. This animal model should inform our thinking about the mode(s) of action of human HAL hepatitis and may prove to be useful in the future study of mechanisms underlying IADRs from HAL and other drugs.

CHAPTER 3

Natural Killer Cells Mediate Severe Liver Injury in a Murine Model of Halothane Hepatitis

Dugan CM, Fullerton AM, Roth RA, Ganey PE. **in preparation**

3.1. Abstract:

Severe halothane (HAL)-induced hepatotoxicity occurs in 1 in 6,000-30,000 patients by an unknown mechanism. Female sex is a risk factor in humans and rodents. We tested the hypothesis that a sex difference in natural killer (NK) cell activity contributes to HAL-induced liver injury. HAL (15mmol/kg, i.p.) treatment resulted in severe liver injury at 12 hr in female, wild-type BALB/cJ mice, and the magnitude of liver injury varied with stage of the estrus cycle. Ovariectomized (OVX) mice developed only mild liver injury. Plasma interferon-gamma (IFN-γ) was elevated 10-fold in HAL-treated females compared to similarly-treated male mice and to OVX, female mice. IFN-γ knockout mice were resistant to severe HAL-induced liver injury. The deactivation of NK cells with anti-asialo GM1 treatment attenuated liver injury and the increase in plasma IFN-γ compared to IgG-treated control mice. Mice with a mutated form of perforin, a protein involved in granule-mediated cytotoxicity, were modestly protected from severe liver injury. Furthermore, HAL increased the activity of NK cells in vivo, as indicated by increased surface expression of CD69, an early activation marker. In response to HAL, NK cell receptor ligands on the surface of hepatocytes are expressed in a manner that can activate NK cells. These results suggest that IFN-γ and NK cells have essential roles in the development of severe, HAL hepatotoxicity and contribute to the sexual dimorphic response seen in HAL-treated mice. These results in a murine model of HAL hepatitis should inform our thinking about the mode(s) of action of human HAL hepatitis and other hepatic idiosyncratic adverse drug reactions.

3.2. Introduction:

Drug-induced liver injury is the leading cause of acute liver failure in the United States (1) and the leading cause of withdrawal of U.S. Food and Drug Administration-approved drugs. Some of these hepatotoxic responses are classified as idiosyncratic adverse drug reactions (IADRs) and account for up to 20% of cases of severe liver injury requiring hospitalization (13). IADRs typically occur in a small fraction of people undergoing drug therapy, and the reactions are unrelated to the pharmacology of the drug. It is likely that the mechanisms of IADRs are multifactorial, and their rarity and complex mechanisms of development are barriers to the ability to predict in preclinical testing which drugs will produce IADRs. Information gained from mechanistic studies using animal models that produce the injury seen in patients would be helpful to prevent and treat IADRs.

Halothane (HAL) is an inhaled anesthetic that produces a mild liver injury in 1 in 5 patients (104) and a more severe IADR, or "HAL hepatitis," in 1 in 6,000-30,000 patients who receive the drug (99, 106, 107). HAL is metabolized by cytochrome P450 2E1 in hepatocytes to form trifluoroacetyl- (TFA-) chloride, which binds covalently to proteins and lipids, forming TFA-adducts. Studies in which HAL hepatotoxicity in guinea pigs was ameliorated by SKF-525A, a P450 inhibitor, and exacerbated by 4-methylpyrazole, a CYP2E1 inducer, illustrate the requirement of metabolism in the development of hepatotoxicity (124, 260). Furthermore, susceptibility to HAL hepatotoxocity in a guinea pig model correlated with the formation of liver TFA-protein

adducts (133). This suggests that TFA-adducts are a prerequisite for pathogenesis; however, the mechanisms of pathogenesis for the mild and severe forms of HAL hepatotoxicity remain elusive. Hypotheses to explain the pathogenesis have been proposed; however, none have satisfactorily explained what is seen clinically in human patients..

Female sex is a risk factor for many idiosyncratic drug-induced liver injuries, including those from isoniazid, HAL, flucloxacillin, nitrofurantoin, and cloropromazine (261). It was recently reported that females were the most at-risk group, representing 81.4% of patients who developed severe HAL hepatitis (232). In mouse models of either mild or severe HAL hepatotoxicity, female mice developed liver injury at doses of HAL that produced no, or only mild injury in male mice, and this disparity was not due to differences in HAL bioactivation (132, 262). Previously, we demonstrated that HAL-treated female mice had a greater accumulation of polymorphonuclear leukocytes (PMNs) in the liver and a greater concentration of tumor necrosis factor-alpha (TNF-α) in plasma compared to similarly-treated male mice (262). The accumulation and activity of immune cells are influenced by sex hormones (263, 264).

The liver contains a preponderance of innate immune cells, including natural killer (NK) cells, natural killer T (NKT) cells, and Kupffer cells (Kcs) (265). NK and NKT cells participate in innate immune responses through the release of rapidly induced cytokines, such as interferon-gamma (IFN-γ) and through cell-mediated cytotoxicity via Fas-Fas ligand interactions, TNF-related apoptosis-inducing ligand (TRAIL) receptors,

and/or the exocytosis of cytotoxic granules. Their enrichment in the liver and the lack of requirement for prior sensitization to perform effector functions suggest that hepatic innate immune cells could also participate in the pathogenesis of drug-induced liver injury. Evidence exists for participation of NK and NKT cells in the hepatotoxicity of other drugs such as acetaminophen (266, 267). Polyinosinic:polycytidylic (PolyI:C), a double-stranded RNA viral mimetic, induced the accumulation and activation of NK and NKT cells and exacerbated HAL-induced liver injury in mice (162). However, the role of NK and NKT cells and IFN-γ in the initiation of HAL hepatotoxicity remains unclear. Accordingly, we investigated the possibility that innate immune cells contribute to the sex-disparity of HAL hepatotoxicity observed in mice.

3.3. Methods:

3.3.1. Materials: Halothane (HAL, 2-bromo-2-chloro-1,1,1,trifluoro-ethane), and highly refined, low acidity olive oil were purchased from Sigma-Aldrich (St. Louis, MO). Alanine aminotransferase (ALT) reagent was purchased from Thermo Electron Corp. (Louisville, CO). Gibco Liver Perfusion, WIlliams' E and Hepatocyte Wash Media, and fluorescein isothiocyanate (FITC) goat anti-rabbit immunoglobulin G (IgG) were purchased from Invitrogen (Carlsbad, CA). Dr. Lance Pohl generously donated anti-TFA-adduct rabbit serum (268). Horseradish peroxidase (HRP) goat anti-rabbit antibody was purchased from Santa Cruz (Santa Cruz, CA). RIPA buffer and HALT protease inhibitor were purchased from Thermo Scientific (Waltham, MA). Lympholyte Mammal density separation medium and anti-asialo GM1 Ig fraction were purchased from Cedarlane

Laboratories Limited (Hornby, Ontario, Canada). Rabbit IgG was purchased from EMD Chemicals (Gibbstown, NJ). Clodronate-containing and empty liposomes were purchased from Encapsula Nano Sciences (Nashville, TN). Allophycocyanin (APC)-anti mouse Rae-1 (pan specific) was purchased from R&D systems (Minneapolis, MN). APC rat IgG2a isotype, phycoertythrin (PE) anti-mouse H-2D^{d}, PE anti-mouse IgG2a isotype, APC-Cy7 anti-mouse CD3e, APC-Cy7 Armenian hamster IgG isotype, APC-anti mouse IFN-γ, APC-rat IgG1 isotype, RBC Lysis Buffer, and Fixation Buffer were purchased from Biolegends (San Diego, CA). Higgins India Ink was purchased from Utrecht (Canbury, NJ). Collegenase Type II was purchased from Worthington Biochemical Co (Lakewood, NJ). BD Cytofix/Cytoperm supplemented with GogiPlug, and 100um cell strainers were purchased from BD Biosciences (San Jose, CA). Phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} and Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} were purchased from Lonza Walkersville, Inc. (Walkersville, MD).

3.3.2. Animals: BALB/cJ, BALB/cByJ, BALB/cJ CD1d KO, BALB/cJ IFN-γ KO, BALB^{PRF1}, and Tlr4^{LPS-d}, RAG1^{NULL}, ovariectomized (OVX) BALB/cJ, and sham-operated (SHAM) BALB/cJ mice were purchased from Jackson Laboratory (Bar Harbor, ME). They were housed under conditions of controlled temperature and humidity and 12hr light/dark cycle. Mice were given continual access to spring water and fed a standard chow (Rodent Chow/Tek 2018, Harlan Teklad, Madison, WI) *ad libitum* and allowed to acclimate for a week prior to use. 8-12-week old mice were fasted for 15 hr prior to HAL administration, and food was returned following HAL administration. All procedures

were carried out according to the humane guidelines of the American Association for Laboratory Animal Science and the University Laboratory Animal Research Unit at Michigan State University.

3.3.3. Experimental Protocol: HAL solution was prepared as previously reported (262). Unless otherwise noted, mice fasted overnight were given 15mmol/kg HAL or olive oil, i.p. For appropriate studies, vaginal cytology was analyzed prior to HAL administration by methods described elsewhere (269). Mice were anesthetized with isoflurane and euthanized at various times after the administration of HAL, and blood was drawn from the vena cava into a syringe containing sodium citrate (final concentration of 0.76%) for preparation of plasma. The left lateral lobe of the liver was fixed in 10% neutral buffered formalin and embedded in paraffin. The median lobe was frozen in liquid nitrogen for liver protein analysis.

To reduce natural killer cell (NK) activity, mice were treated with 50ul anti-asialo GM1 (AsGM) or rabbit IgG, i.v., 48 and 24hr before HAL administration. To deplete Kupffer cells (Kcs), clodronate-containing or empty liposomes were administered 48hr before HAL administration. In some experiments, Higgins India ink (diluted 1:4 in saline) was injected i.v. 45 min before liver extraction to confirm Kc depletion.

3.3.4. Isolation of Hepatic Lymphocytes: The liver was isolated and gently pressed through a 100um mesh. The liver cell suspension was collected in PBS and centrifuged at 50g at 4°C for 3 min to remove hepatocytes and debris. Supernatant was centrifuged at 350g at 4°C for 5 min, and the pellet was resuspended in RBC Lysis Buffer

according to the manufacturer's protocol. After two washes in PBS, the cells from five animals were combined in PBS and underlayed with Lympholyte-Mammal density separation medium. Hepatic lymphocytes were obtained by centrifugation and washed in PBS.

3.3.5. Isolation of Primary Hepatocytes for Flow Cytometry: The protocol for primary mouse hepatocyte isolation was adapted from the protocol described by Renton (270). In brief, mice were anesthetized with pentobarbital (50mg/kg, i.p.), and their livers were retrograde-perfused with Gibco Liver Perfusion Medium through the inferior vena cava. Williams' Medium E supplemented with 0.4mg/ml collagenase Type II was then perfused to digest the liver. The liver was combed gently to disaggregate the hepatocytes into Gibco Hepatocyte Wash Medium. Cell debris was removed by straining the cell suspension through a 100um cell strainer. The hepatocytes were collected after centrifugation (50g, 5 min) and washed twice in Hepatocyte Wash Medium.

3.3.6. Immunophenotyping Liver Cells After 2 washes in staining buffer (HBSS supplemented with 1% bovine serum albumin (BSA), 0.09% sodium azide, pH 7.6), the lymphocytes were incubated at 4°C for 30min with cell surface antibodies, i.e., either PE -conjugated CD49b mAb (clone DX5), APC-Cy7 conjugated CD3e mAb (clone 145-2C11), APC-conjugated CD69 mAB (clone H1.2F3) or the appropriate isotype control. After two washes in staining buffer, the cells were fixed in Fixation Buffer for 20min at

4°C. Data were collected on a BD FACS Canto II flow cytometer and analyzed with FLO JO software (Tree Star, Inc).

3.3.7. Measurement of Plasma ALT Activity: Plasma samples were obtained at various times after HAL administration. Alanine aminotransferase (ALT) activity was evaluated with ALT reagent (Louisville, CO) used according to the manufacturer's protocol.

3.3.8. Histopathology: Paraffin-embedded, left-lateral liver lobes were sectioned, stained with hematoxylin & eosin (H&E) or eosin alone and examined by light microscopy.

3.3.9. Western Analysis: Frozen liver samples were processed for whole cell protein isolation with RIPA buffer supplemented with HALT protease inhibitor according to the manufacturer's directions. Twenty ug of protein were loaded onto Invitrogen NuPAGE 12%-TRIS gels (Carlsbad, CA) and electrophoresed in Invitrogen NuPAGE MOPS SDS Running Buffer at 200V for 1hr. Protein was transferred onto BioRad Immuno-blot PVDF membranes (Hercules, CA) using Invitrogen NuPAGE transfer buffer at 150 mAmp for 2hrs. The blot was blocked in 5% BSA for 1hr and hybridized with 1:10,000 TFA antiserum in 5% BSA overnight at 4°C. It was washed in TRIS-buffered saline with 0.01% Tween-20 (TBST) three times and hybridized with 1:5,000 goat anti-rabbit HRP in 5% BSA for 2hrs at room temperature. After TBST wash, the proteins were detected using Amersham ECL Western Blotting Analysis System and Amersham Hyperfilm MP from GE Health Care (Uppsala, Sweden).

3.3.10. Interferon-gamma (IFN-γ) Analysis: The plasma concentration of IFN-γ was measured using a BD OpEIA mouse IFN-γ ELISA kit purchased from BD Biosciences (San Diego, CA).

3.3.11. Statistical Analysis: Results are presented as mean +/- standard error of the mean. A Student's t-test was performed on comparisons of two groups. For comparison of more than two groups, a 1-or 2- way ANOVA was used as appropriate after data normalization. The Student-Newman-Keuls test was performed to compare means in studies in which the ANOVA indicated statistical significance. The criterion for significance was $p<0.05$ for all studies.

Probability binning was performed on flow cytometry data. In brief, this algorithm divides the control sample population into bins with the same number of events and then divides the test sample along the same boundaries and calculates the Chi Square value, X^2, of the two-binned data sets. The probability binning metric, or T(x), is derived from the X^2 value and provides a quantitative measurement of the probability that the populations are different (271, 272). For our studies, $T(x) > 3$, was used as the criterion for significance. Hepatic lymphocyte experiments involving pooled animals were replicated at least once, whereas hepatocyte experiments were repeated three times with similar results.

3.4. Results:

Estrous Cycle and Ovarian Hormones Influence HAL-induced Hepatotoxicity. Estrous cycle phase was determined before the administration of HAL (5mmol/kg, i.p.), and plasma ALT activities were evaluated 12 hr later. The plasma ALT activity of HAL-treated mice in diestrus or protestrus was approximately 1000U/L, whereas it was much greater (~5000U/L) in HAL-treated mice in estrus (Figure 3.1A).

OVX and SHAM BALB/cJ mice were administered HAL(15mmol/kg, i.p.) or olive oil. Vehicle-treated mice had plasma ALT activities <100U/L. HAL-treated SHAM mice developed severe liver injury as indicated by the 12 hr plasma ALT activity of >10,000 U/L, whereas similarly-treated OVX mice developed a mild injury with ALT activity of 600U/L (Figure 3.1B).

Cytokines in HAL-treated Mice: In our earlier publication (12), female mice developed more severe liver injury in response to HAL with a more robust inflammatory response compared to male mice. IFN-γ is a inflammatory cytokine that is released mainly by NK cells and NKT. High Mobility Group Box-1 (HMGB-1) is released from injured cells and can also signal inflammatory responses by binding to pattern recognition receptors, such as toll-like receptor 4 (TLR4) (273). Additionally, HMGB-1 mediates acetaminophen-induced liver injury (41). Here we evaluated the concentrations of interferon-gamma (IFN-γ) and high mobility group box-1 (HMGB-1), at 6, 12, and 24 hr in HAL-treated male and female mice. In VEH-treated mice, there was no elevation in plasma IFN-γ concentration at any time investigated. In male mice, there was a mild elevation in

plasma IFN-γ concentration (28pg/ml) 24 hr after HAL administration (Figure 3.2A). The plasma IFN-γ concentration in female mice increased to 250 and 680pg/ml at 12 and 24 hr following HAL administration, respectively (Figure 3.2A). HAL-treated SHAM and OVX mice had 12 hr plasma IFN-γ concentrations of 730 and 16pg/ml, respectively (Figure 2B).

There was a slight increased in the plasma HMGB-1 concentration in HAL-treated male mice at 12 and 24 hr (19 and 29 ng/ml), whereas the HMGB-1 modestly increased in HAL-treated females 12 hr after HAL administration (180 ng/ml). There were no significant increases in plasma HMGB-1 concentration in VEH-treated animals at any time point or HAL-treated animals in the 6 hr time group (Figure 2C).

IFN-γ KO mice are Protected from HAL-induced Liver Injury. To determine the contribution of IFN-γ in the pathogenesis of HAL-induced liver injury, wild-type BALB/cJ or IFN-γ KO mice were treated with HAL, and plasma and liver samples were collected at various times. HAL exposure caused significant liver injury at 8 and 12 hr in female WT mice as indicated by ALT activities of 2000U/L and 6000U/L, respectively (Figure 3.3A). IFN-γ KO mice were protected from severe, HAL-induced liver injury at both times. Histopathological examination of livers corroborated this result, inasmuch as lesions of hepatocellular necrosis were decreased in IFN-γ KO mice compared to wild-type mice (Figure 3.3C). All of the wild-type mice treated with HAL developed severe centrilobular necrosis in the livers collected 30 hr after HAL administration. In addition, livers of the

Figure 3.1. Sensitivity to HAL Hepatotoxicity is Dependent on Ovarian Hormones. A. For each mouse, the stage of estrous cycle was determined by vaginal cytology analysis before treatment with HAL(5mmol/kg, i.p.). Plasma ALT activity was measured 12 hr after HAL administration (n=3-5 per group). P, proestrus; E/M, estrus/metestrus; D, diestrus. *significantly different from other groups. B. Plasma ALT activity was evaluated 12 hr after vehicle (VEH) or HAL(15mmol/kg, i.p.) administration in ovariectomized (OVX) or sham-operated (SHAM) mice (n=3-5 per group). *,significantly different from respective VEH control; #,significantly different from HAL-treated SHAM mice.

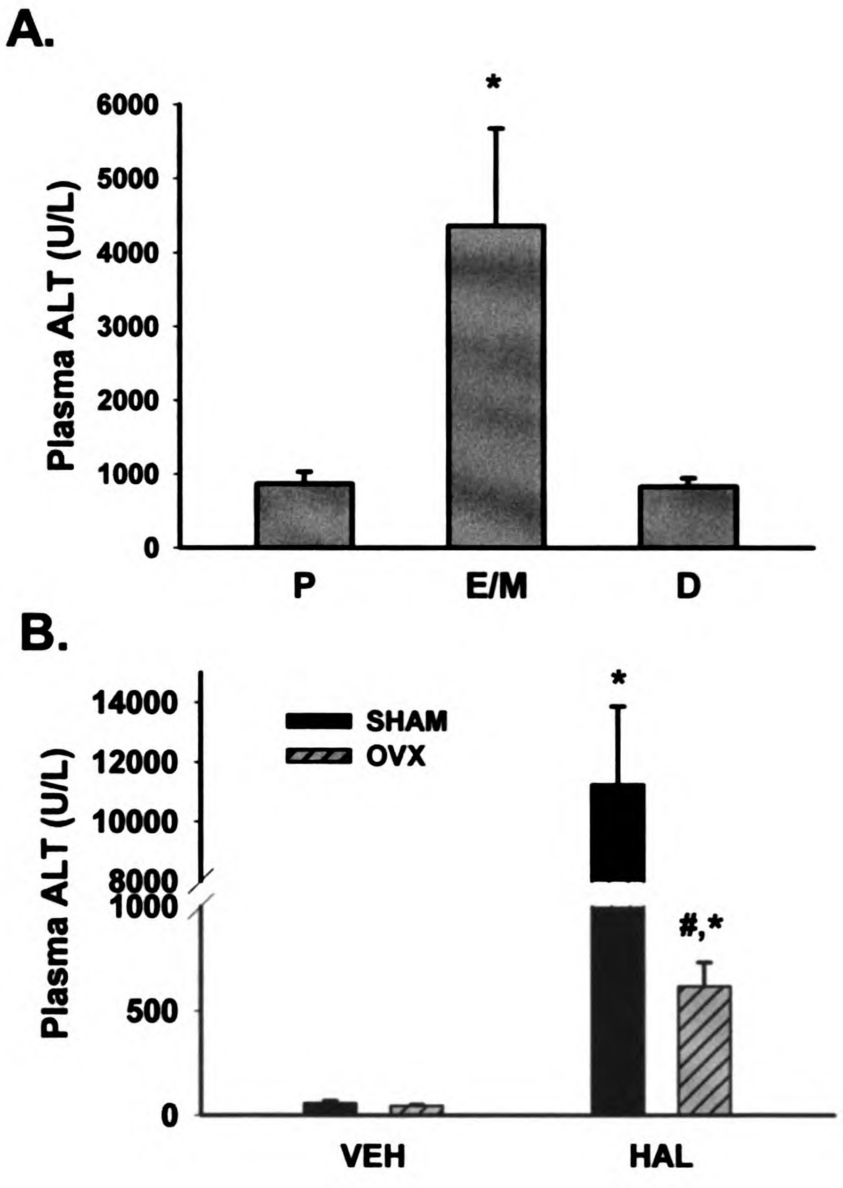

A.
Plasma ALT (U/L)
6000
5000
4000
3000
2000
1000
0
*
P
E/M
D
B.
Plasma ALT (U/L)
14000
12000
10000
8000
1000
500
0
SHAM
OVX
*
#,*
VEH
HAL

Figure 3.1

Figure 3.2. Plasma IFN-γ and HMGB-1 Concentrations in HAL-treated Mice. Naive female and male mice or OVX and SHAM mice were treated with HAL (15mmol/kg, i.p.), and plasma was collected at various times. A. Plasma IFN-γ concentration was evaluated in male and female mice (n=5-6 per group). *,significantly different from 6hr sex-matched animals and time-matched male group; #,significantly different from time-matched male group. B. IFN-γ concentration was evaluated 12 hr after HAL treatment in SHAM and OVX mice (n=4 per group). *,significantly different from SHAM group. C. Plasma HMGB-1 concentration was evaluated at various times after HAL treatment in male and female mice (n=6 per group). VEH-treated animals had plasma HMGB1 concentrations <5pg/ml. #, significantly different from sex-matched 6 hr time point. *,significantly different from time-matched male group.

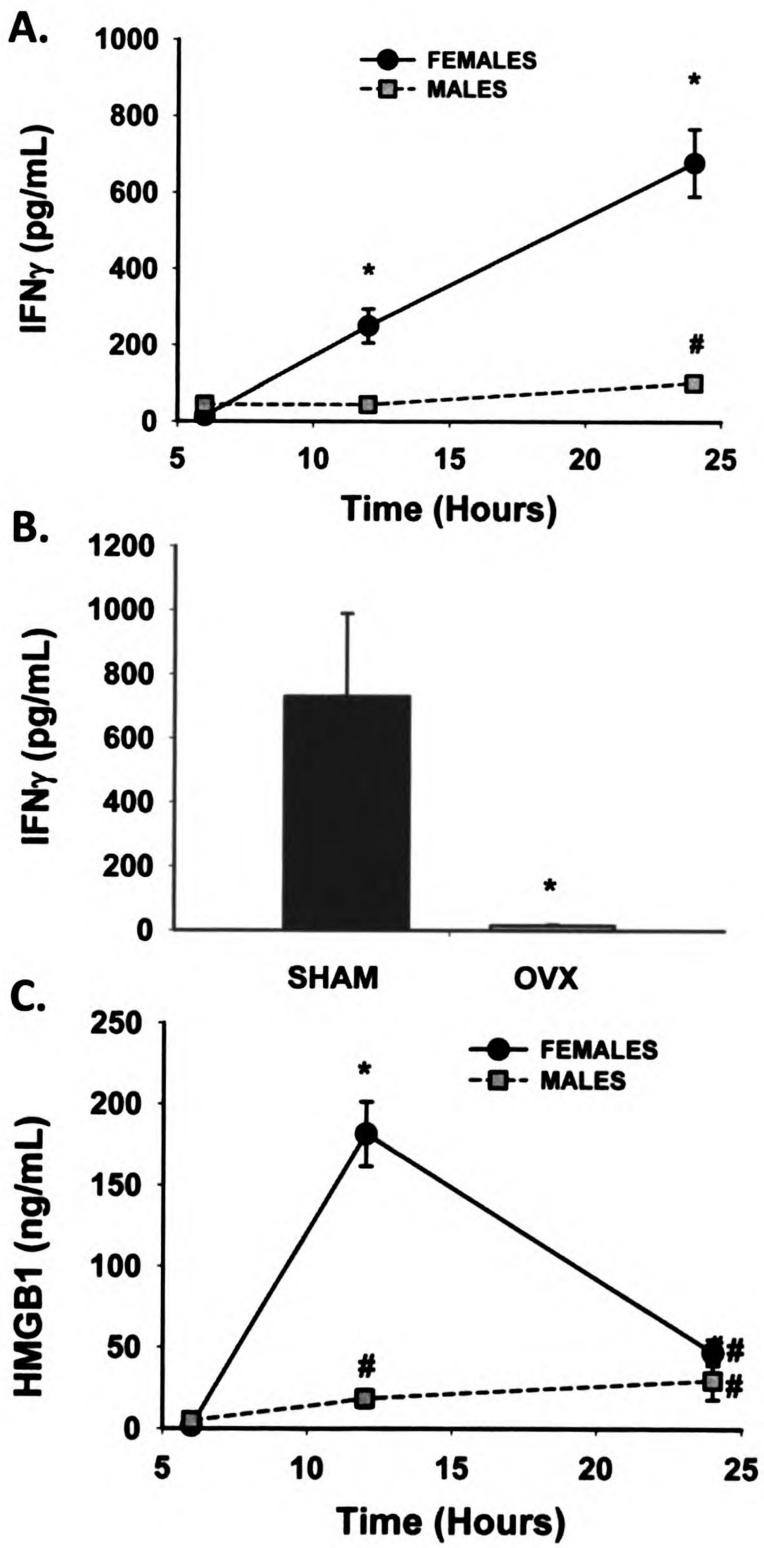

A.
1000
800
600
400
200
0
IFNγ (pg/mL)
FEMALES
MALES
*
*
#
5
10
15
20
25
Time (Hours)
B.
1200
1000
800
600
400
200
0
IFNγ (pg/mL)
*
SHAM
OVX
C.
250
200
150
100
50
0
HMGB1 (ng/mL)
FEMALES
MALES
*
#
#
#
5
10
15
20
25
Time (Hours)

Figure 3.2

Figure 3. 3 IFN-γ KO Mice are Protected from Developing Severe HAL Hepatotoxicity. Wild-type BALB/cJ (WT) and IFN-γ KO mice were treated with HAL(15mmol/kg, i.p.), and plasma and liver samples were collected at various times. A. Plasma ALT activity was evaluated 8 and 12 hr after HAL treatment (n=5-6 per group). *,significantly different from HAL-treated WT mice. B. Immunoblot detection of TFA-protein adducts in liver homogenates (n=3 per group). C.H&E liver sections from HAL-treated WT and IFN-γ KO mice 30 hr after treatment. Labeled in picture are central vein (CV) and portal triad (PT). Images were photographed at 200X magnification.

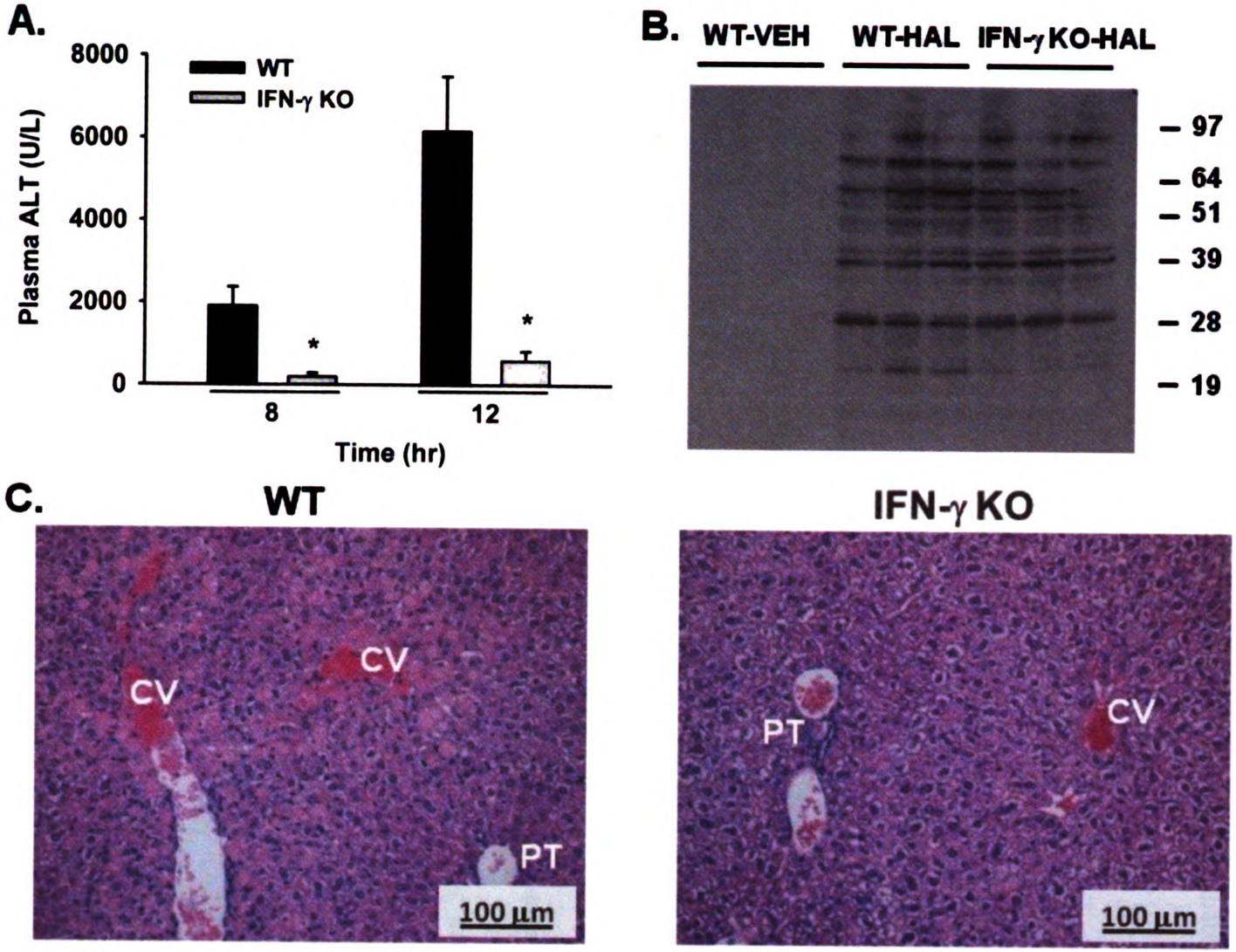

A.
8000
6000
4000
2000
0
Plasma ALT (U/L)
WT
IFN-γ KO
*
*
8
12
Time (hr)
B.
WT-VEH
WT-HAL
IFN-γ KO-HAL
97
64
51
39
28
19
C.
WT
CV
CV
PT
100 µm
IFN-γ KO
PT
CV
100 µm

Figure 3.3

protected IFN-γ KO mice showed reduced intracellular staining of hepatocytes, suggesting glycogen accumulation that was absent in the wild-type mice.

A positive correlation has been reported between the severity of liver injury and the formation of TFA-adducts in the livers of HAL-treated guinea pigs (133). There was a similar degree of TFA-adduct formation in livers from HAL-treated WT and HAL-treated IFN-γ KO mice given the same dose of HAL as determined by western blot analysis of liver homogenates (Figure 3.3B). There were no TFA-adducts in the livers of VEH-treated animals.

HAL Hepatotoxicity in Toll-like Receptor 4 Defective ($Tlr4^{LPS-d}$) Mice. Wild-type BALB/cByJ and $Tlr4^{LPS-d}$ mice were given HAL, and plasma and liver samples were collected 24 hr later. HAL-treated WT mice developed considerable liver injury, whereas similarly-treated $Tlr4^{LPS-d}$ mice had an attenuated response. Plasma IFN-γ concentration was reduced in HAL-treated $Tlr4^{LPS-d}$ mice compared to HAL-treated WT mice (Figure 3.4B). Histopathologically, there were fewer necrotic cells in the liver sections from $Tlr4^{LPS-d}$ mice compared to those from WT mice (Figure 3.4C).

HAL Hepatotoxicity in Kupffer Cell-depleted Mice. To deplete Kcs, clodronate-encapsulated liposomes were injected 48 and 24 hr into mice intravenously prior to HAL administration; empty liposomes were used as control. India ink-laden Kcs were visible in the hepatic sinusoids of liver sections from mice pretreated with control liposomes,

Figure 3.4. Attenuated HAL-induced Liver Injury in $Tlr4^{LPS-d}$ Mice. Wild-type BALB/cBYJ (WT) mice and $Tlr4^{LPS-d}$ mice were treated with HAL(15mmol/kg, i.p.). A&B. Plasma ALT activity and IFN-γ concentration were evaluated 24 hr after HAL treatment (n=4-5 per group). *,significantly different from WT controls. C. H&E liver sections taken 24hr after HAL treatment. Labeled in picture are central vein (CV) and portal triad (PT). Arrowheads indicate examples of necrotic cells. Images were photographed at 200X magnification.

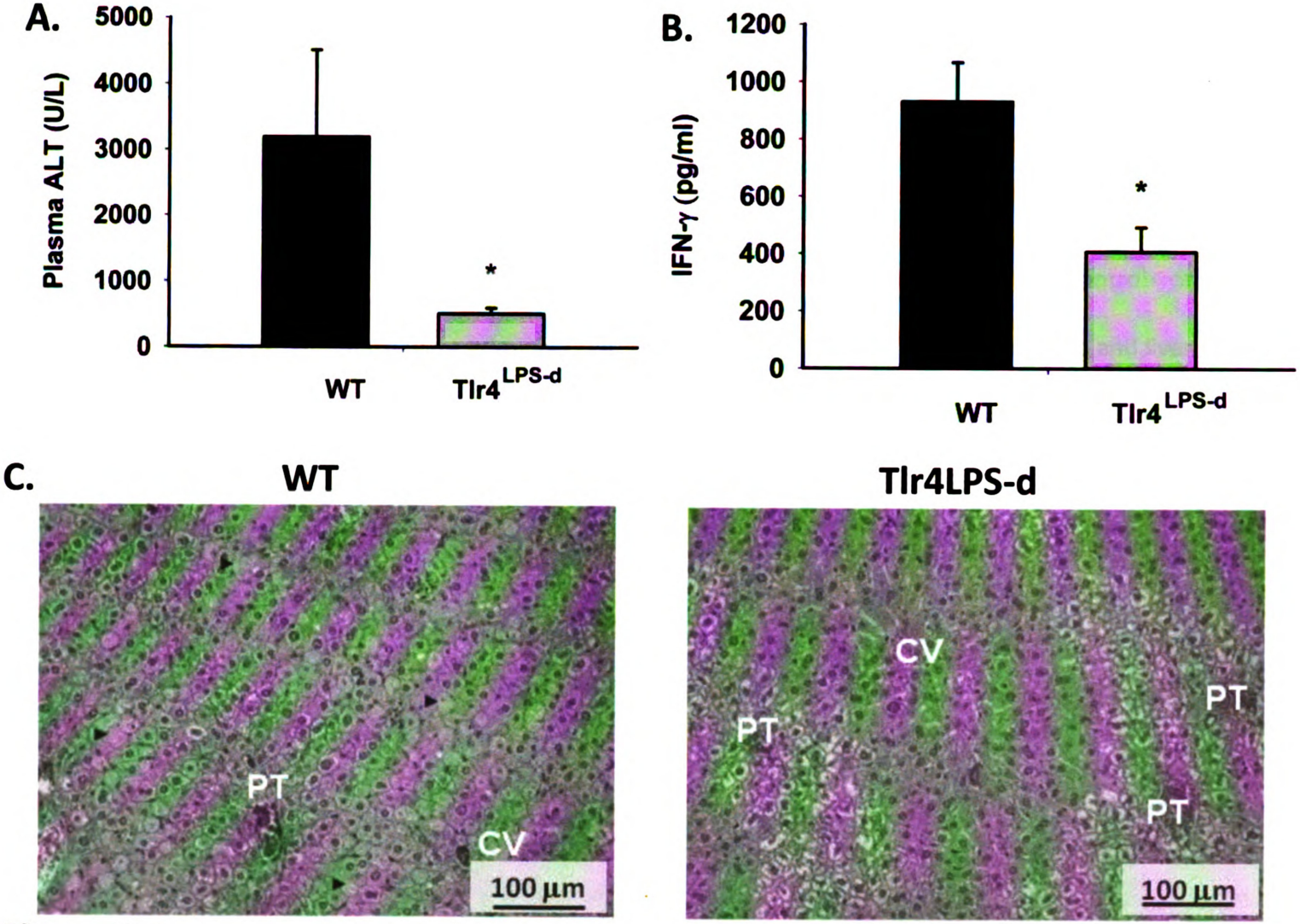

A.
Plasma ALT (U/L)
5000
4000
3000
2000
1000
0
*
WT
Tlr4LPS-d
B.
IFN-γ (pg/ml)
1200
1000
800
600
400
200
0
*
WT
Tlr4LPS-d
C.
WT
PT
CV
100 µm
Tlr4LPS-d
CV
PT
PT
PT
100 µm

Figure 3.4

Figure 3.5. Kupffer cell (KC)-depleted Mice are Sensitive to HAL-induced Liver Injury. Control- or clodronate-liposome pretreated mice were administered VEH or HAL (15mmol/kg, i.p.), and plasma and liver samples were collected 24 hr later. A&B. Plasma ALT activity and IFN-γ concentration were evaluated (n=4-6 per group). C. Representative eosin-stained liver sections from control- or clodronate-pretreated, VEH-treated mice after India ink administration. Arrowheads point to India-ink filled KCs. Arrows point to areas where India ink lines the sinusoids. Images were photographed at 200X magnification.

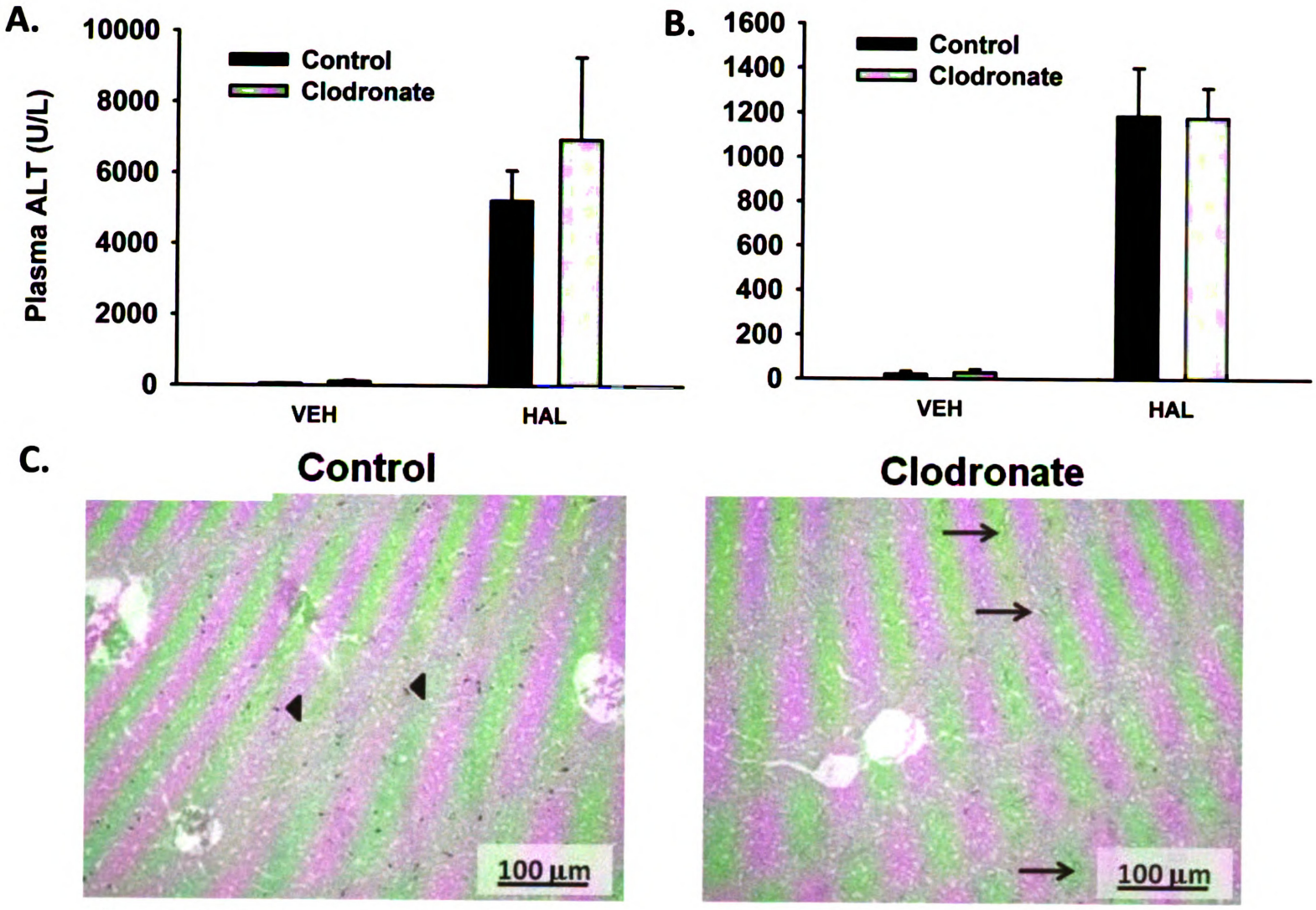

A.
Plasma ALT (U/L)
10000
8000
6000
4000
2000
0
Control
Clodronate
VEH
HAL
B.
1600
1400
1200
1000
800
600
400
200
0
Control
Clodronate
VEH
HAL
C.
Control
Clodronate
100 µm
100 µm

Figure 3.5

whereas they were not visible in clodronate-pretreated animals, confirming inhibition of Kc function (Figure 3.5C). There was no increase in plasma ALT activity in vehicle (VEH)-treated mice given control liposomes or the clodronate-encapsulated liposomes. Plasma ALT was elevated in HAL-treated mice, and the increase was similar irrespective of clodronate inclusion in the liposomes (Figure 3.5A). Similarly, plasma IFN-γ concentration was increased by HAL treatment similarly in clodronate-treated and control mice (Figure 5B).

HAL Hepatotoxicity in Mice with Reduced NK Cell Activity. anti-AsGM1 decreases NK cell activity in BALB/c mice (274). Compared to NK cells from IgG-treated mice, the NK cells taken from anti-AsGM1-treated mice had a decreased expression of CD69, an early activation marker, and an increased expression of CD107a, a marker of post-degranulation (Figure 3.6B-C).

IgG-pretreated, HAL-treated mice had plasma ALT activity >7000U/L at 12 hr, indicating severe HAL hepatotoxicity, whereas anti-AsGM1-pretreatment induced a milder HAL hepatotoxicity with ALT activity of ~1500U/L (Figure 3.7A). By 24 hr, the ALT activity was ~5000 and ~2500U/L for IgG- and ASGM1-pretreated, HAL-treated mice, respectively. The plasma IFN-γ concentration in HAL-treated mice was decreased markedly at 12 and 24 hr by AsGM1 pretreatment (Figure 3.7B).

NK cells are capable of granule-mediated cytotoxicity whereby preformed cytotoxic proteins, perforin and granzyme B, create pores in the cell membrane of target

Figure 3.6. Anti-AsGM1 Treatment Alters the Surface Protein Expression of Hepatic NK Cells. A. Hepatic lymphocytes from IgG- or anti-AsGM1-treated mice were pooled. NK cells were gated as DX5+,CD3- as shown in the dot plots. The percentage of NK cells in the population is labeled inside each graph. B&C. Surface expression of CD69 and CD107a on NK cells was analyzed by flow cytometry. The grey and black histograms depict the IgG and anti-AsGM1 treatment groups respectively. Gates are indicated by the horizontal bars, and percent positive cells is indicated in the legend.

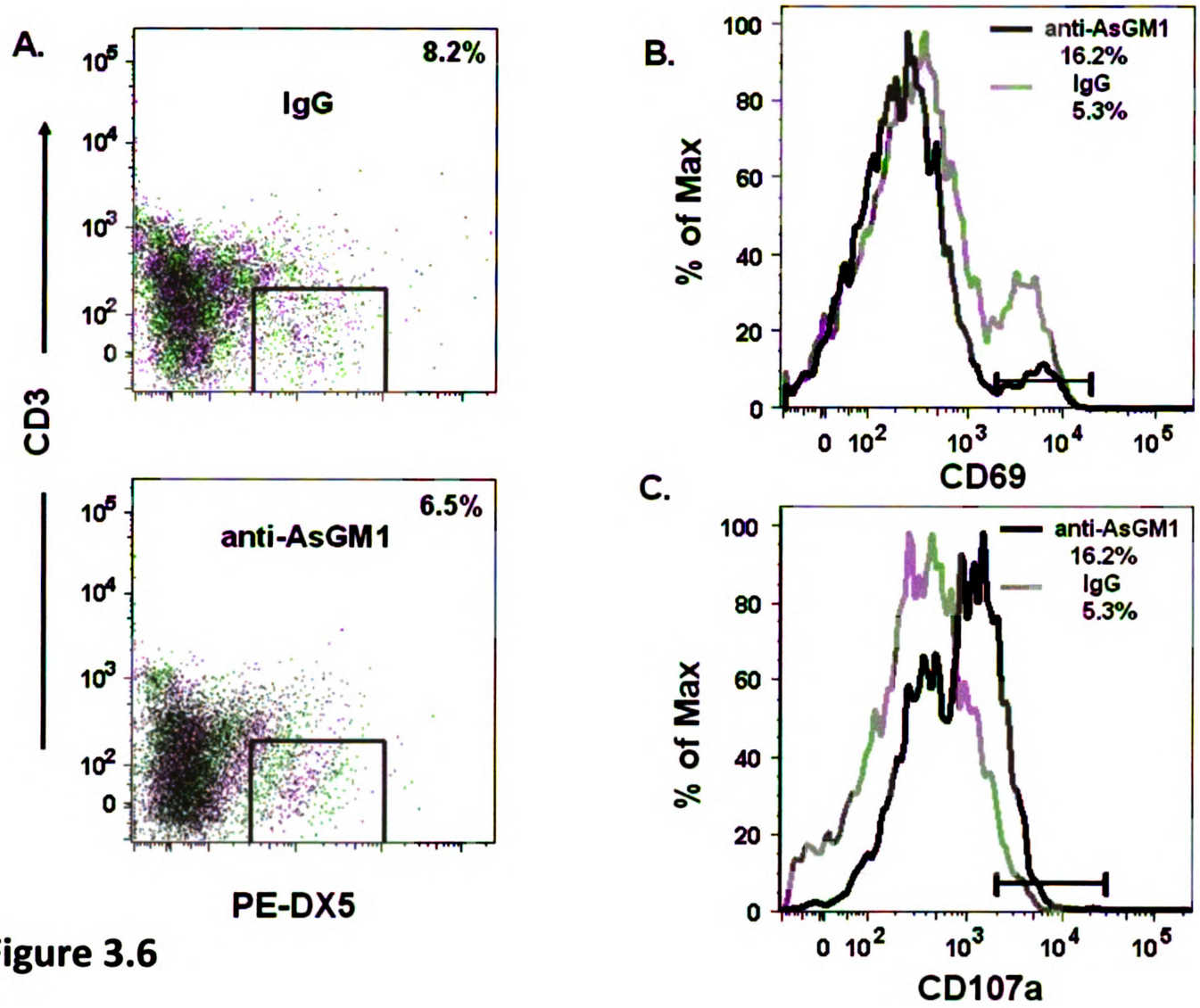

A.
8.2%
IgG
6.5%
anti-AsGM1
CD3
PE-DX5
B.
anti-AsGM1
16.2%
IgG
5.3%
% of Max
CD69
C.
anti-AsGM1
16.2%
IgG
5.3%
% of Max
CD107a

Figure 3.6

Figure 3.7. HAL-induced Hepatotoxicity Depends on NK Cell Activity. IgG- and anti-AsGM1-treated mice were given HAL (15mmol/kg, i.p.), and plasma samples were collected at 12 and 24 hr. A&B. Plasma ALT activity and IFN-γ concentration were evaluated (n=4-6 per group). *significantly different from time-matched controls. C&D. Plasma ALT activity and IFN-γ concentration were evaluated from HAL-treated wild-type BALB/cByJ (WT) and perforin-defective mice ($BALB^{PRF1}$) 12 hr after HAL administration (15mmol/kg, i.p.) (n=4 per group). *,significantly different

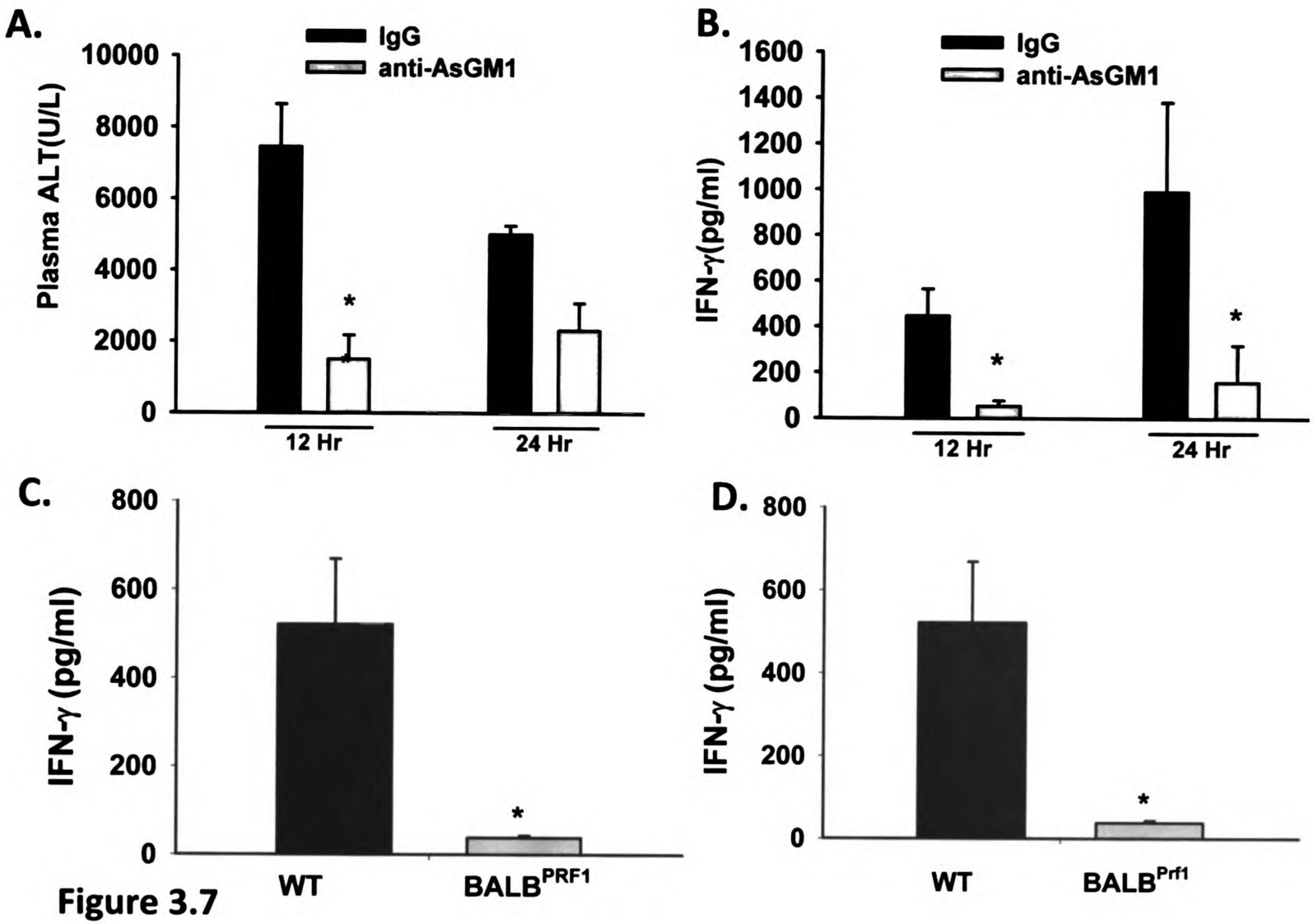

A.
IgG
anti-AsGM1
Plasma ALT(U/L)
10000
8000
6000
4000
2000
0
*
12 Hr
24 Hr
B.
IgG
anti-AsGM1
IFN-γ(pg/ml)
1600
1400
1200
1000
800
600
400
200
0
*
*
12 Hr
24 Hr
C.
IFN-γ (pg/ml)
800
600
400
200
0
*
WT
BALB^PRF1
D.
IFN-γ (pg/ml)
800
600
400
200
0
*
WT
BALB^Prf1

Figure 3.7

cells and mediate cell death (275). Mice with mutated perforin ($BALB^{PRF1}$) and wild-type mice were treated with HAL to determine if granule-mediated cytotoxicity participates in the development of HAL-induced liver injury. $BALB^{PRF1}$ mice treated with HAL had a much smaller increase in plasma ALT than WT mice (Figure 3.7C). Plasma IFN-γ concentration was also much smaller in HAL-treated $BALB^{PRF1}$ mice (~38pg/ml) compared to similarly-treated WT mice (~500pg/ml) (Figure 3.7D).

HAL Hepatotoxicity in Mice Deficient in NKT Cells. NKT cells have an invariant T cell receptor that is CD1d-restricted during development; therefore, CD1d knockout mice (CD1d KO) lack NKT cells, but otherwise have normal immune cells. HAL treatment caused similar, severe liver injury in wild-type and CD1d KO mice as marked by increases in plasma ALT activities that were not statistically different at either 12 or 24 hr (Figure 3.8A). Histopathological examination of livers revealed centrilobular necrosis in HAL-treated wild-type and CD1d KO mice (Figure 3.8B). Bridging necrosis was evident in many of the liver sections from CD1d KO mice at 24 hr (Figure 3.8B).

Activation of Hepatic NK Cells after HAL Administration In Vivo. The DX5+,CD3- NK cell population was significantly decreased 8 hr after HAL administration (18.6%) compared to vehicle-treated mice (22.4%) at the same time (Figure 3.9A). The number of DX5+,CD3-NK cells that were positive for CD69 increased significantly in HAL-treated mice (12.7%) compared to VEH-treated mice (9.6%) at 8 hr following HAL administration (Figure 3.9B).

Figure 3.8. CD1d KO Mice Develop Severe HAL-induced Liver Injury. Wild-type BALB/cJ (WT) and NKT deficient mice (CD1d KO) were treated with HAL(15mmol/kg, i.p.), and livers were collected at 24 hr. A. Plasma ALT activity was evaluated from HAL-treated wild-type BALB/cByJ (WT) and CD1d KO mice 12 and 24 hr after HAL administration (15mmol/kg, i.p.) (n=5 per group). B. H&E liver sections from HAL-treated WT and CD1d KO mice. Labeled in picture are central vein (CV) and portal triad (PT). Images were photographed at 200X magnification.

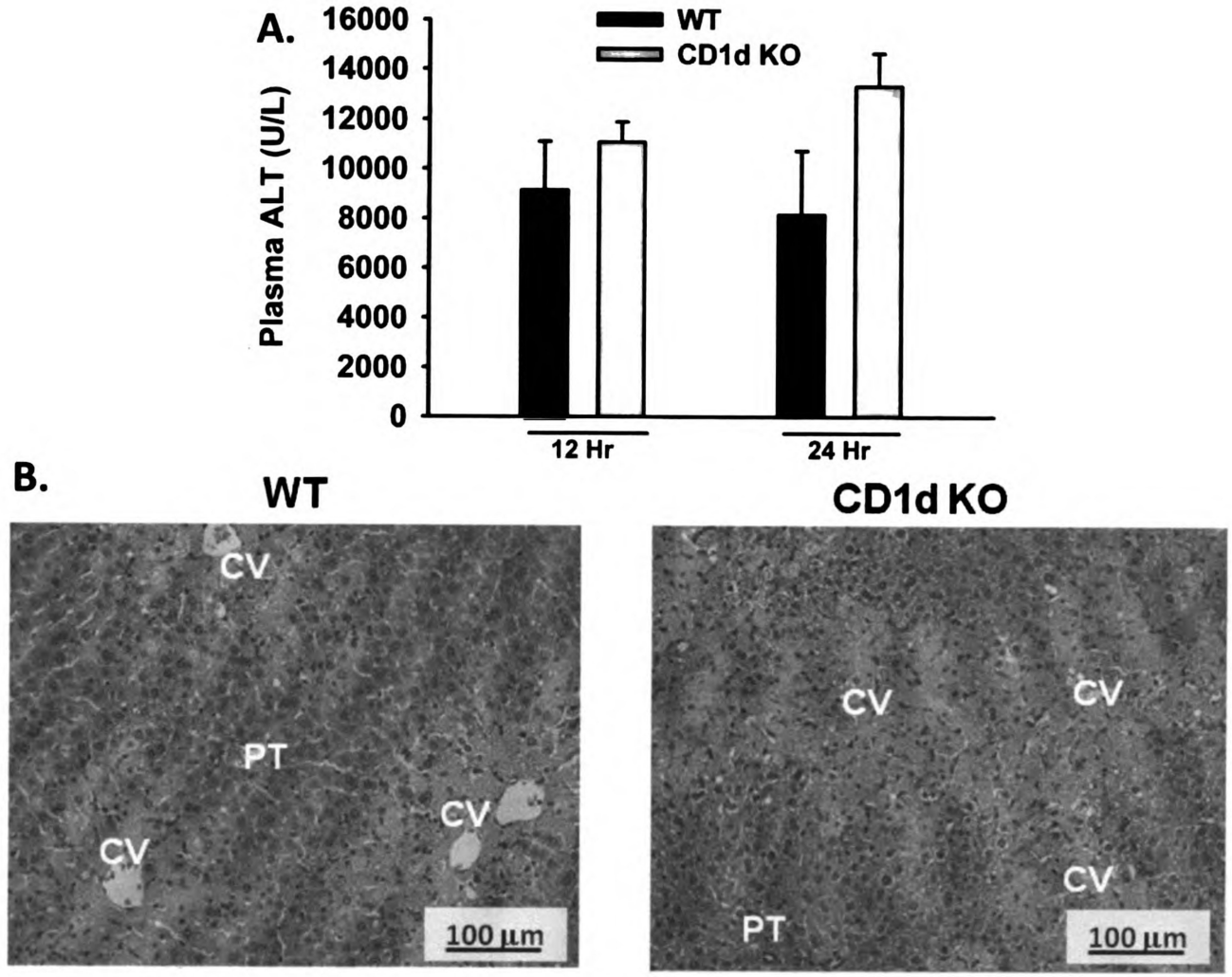

A.
Plasma ALT (U/L)
16000
14000
12000
10000
8000
6000
4000
2000
0
WT
CD1d KO
12 Hr
24 Hr
B.
WT
CD1d KO
CV
PT
CV
CV
100 µm
CV
CV
CV
PT
100 µm

Figure 3.8

Figure 3.9. HAL Treatment Alters the Phenotype of Hepatic NK Cells. A. Hepatic lymphocytes from VEH- or HAL- treated mice were pooled according to their respective groups. NK cells were gated as DX5+,CD3- as indicated in the dot plot graphs. Labeled in each graph is the percent of NK cells in the population. Similar results were obtained in 3 independent experiments of pooled lymphocytes. B. NK cell surface expression of CD69 analyzed by flow cytometry. The grey and black histograms depict the VEH and HAL treatment groups, respectively. Gates are indicated by the horizontal bars, and percent positive cells is indicated in the legend. Experimental replicate had similar

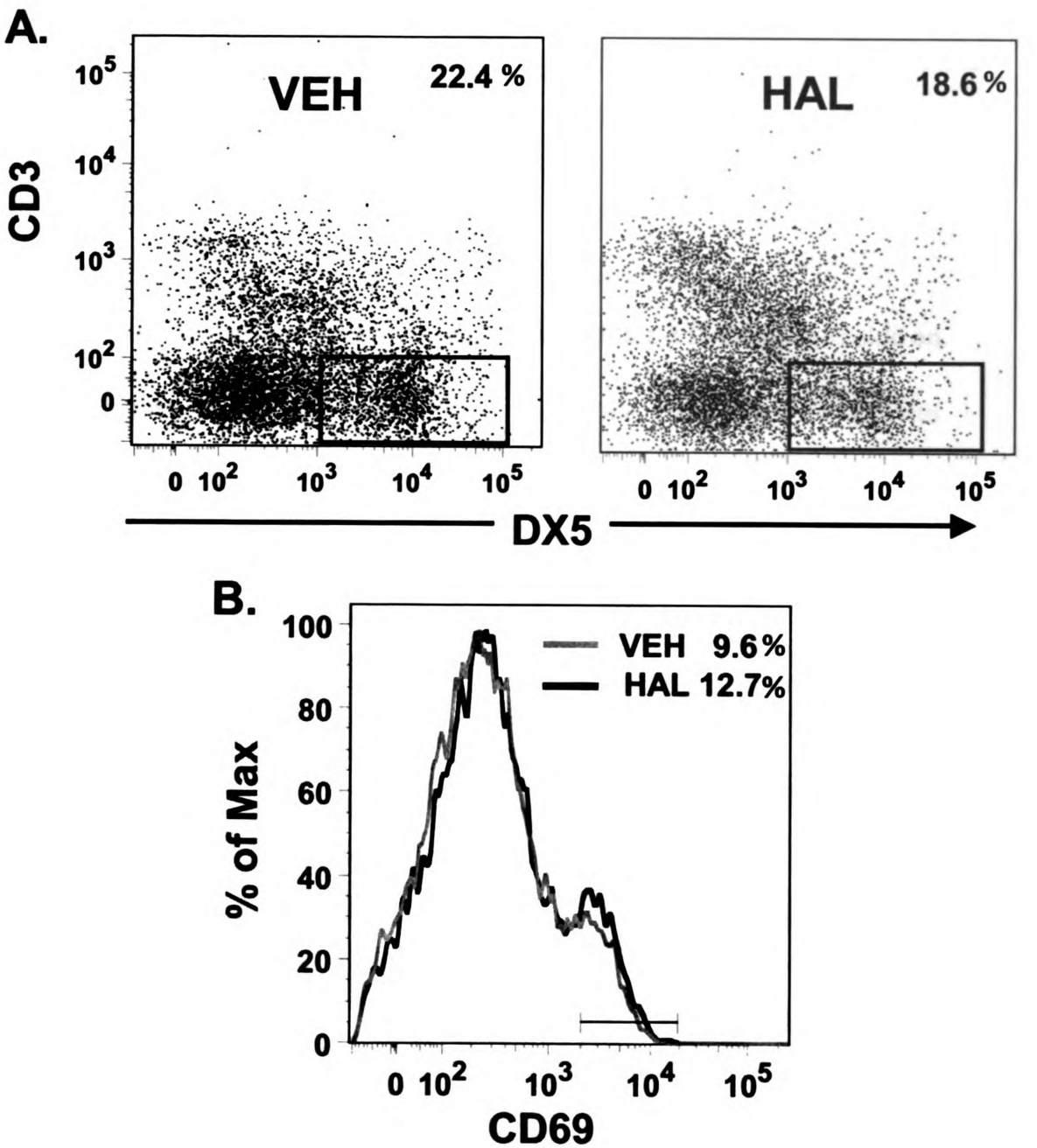


Figure 3.9

Figure 3.10. Decreased H-2D^d and Increased Rae-1 Surface Expression on Hepatocytes Isolated from HAL-treated Mice. A and B. Hepatocytes isolated from VEH- and HAL-treated mice were immunolabeled with anti-H2D^d and anti-Rae-1 antibodies and analyzed by flow cytometry. The grey and black histograms depict the VEH and HAL treatment, respectively. Gates are indicated by the horizontal bars, and percent positive cells is indicated in the legend. Similar results were obtained in 4 independent replicates.

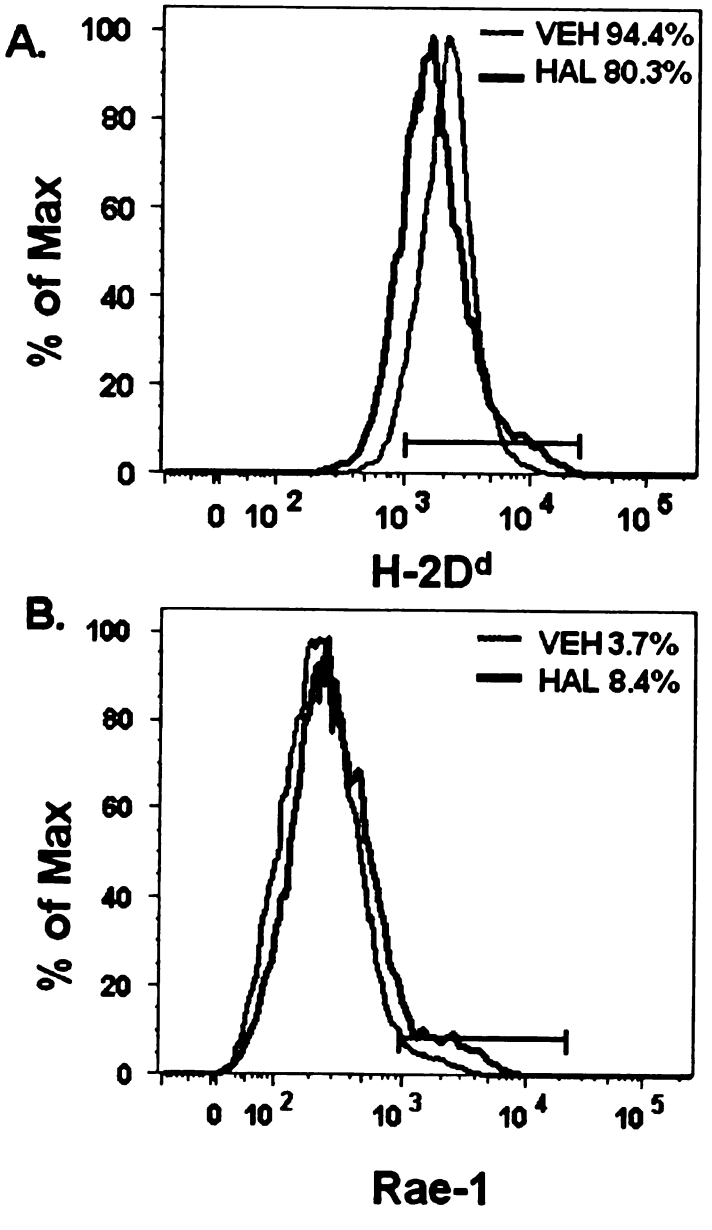

A.
VEH 94.4%
HAL 80.3%
% of Max
100
80
60
40
20
0
0 10 2
10 3
10 4
10 5
H-2Dd
B.
VEH 3.7%
HAL 8.4%
% of Max
100
80
60
40
20
0
0 10 2
10 3
10 4
10 5
Rae-1

Figure 3.10

Altered Surface Protein Expression on Hepatocytes after HAL Administration In Vivo. The expression of known ligands for NK receptors on hepatocytes was evaluated 8 hr after HAL or VEH treatment. There was a decrease in the percent of hepatocytes that express H-2D^{d} protein from HAL-treated mice (80.3%), as compared to hepatocytes from VEH-treated mice (95.1%) (Figure 3.10A). In addition, the surface expression of retinoic acid early inducible gene-1 (Rae-1), a stress ligand for stimulatory receptors on NK cells, increased with HAL treatment (8.38%) compared to hepatocytes isolated from VEH-treated mice (3.7%) (Figure 10B).

3.5. Discussion:

The exact mechanisms of pathogenesis of hepatic IADRs, and HAL hepatitis in particular, are not known. The hapten hypothesis, in which HAL's reactive metabolite, trifluoroacetyl chloride, modifies proteins and/or lipids to form neoantigens that trigger an adaptive immune response upon reexposure, has received wide support. Much of the support for this proposed mechanism stems from the discovery of antibodies to TFA in some patients with HAL hepatitis and that multiple exposures are a risk factor; however, these features do not apply to all cases of HAL hepatitis (100, 106, 116, 232, 276, 277). Accordingly, it remains possible that other processes could precipitate HAL hepatitis (278).

We recently characterized a model in which human risk factors (female sex, mature age, genetics and fasting) were imposed on mice to enable the expression of severe, HAL hepatotoxicity (262). Female sex is a risk factor for HAL hepatotoxicity in

humans as well as in the mild form of HAL-induced liver injury in mice (132, 232, 262). In the mouse model presented herein, HAL sensitivity in mice was ovarian hormone-dependent, as indicated by loss of HAL sensitivity in OVX mice (Figure 3.1). There was also an increased HAL sensitivity of mice in estrous phase in which plasma levels of progesterone are elevated compared to proestrous (high estrogen) and diestrous (low estrogen and progesterone) phases of the cycle (269). Interestingly, a recent report demonstrated that the glucocorticoid receptor antagonist, mifepristrone, attenuated HAL-induced liver injury in mice (279). However, mifepristrone, also known as RU486, is a well known progesterone receptor antagonist. Since, estrogen and progesterone receptors are found on many leukocytes and accessory cells (215), it is possible that sex hormones modulate leukocyte activity. For example, 17β-estradiol administration decreased NK cell activating receptors, such as CD69 and NKG2D, and increased intracellular IFN-γ pools in splenic NK cells stimulated with phorbol myristate acetate and ionomycin (280). Knowledge of the mechanisms of sex hormone-enhanced sensitivity to HAL-induced liver injury could provide useful insights into the pathogenesis of other IADRs for which female sex is a predisposing factor. If sensitivity to HAL hepatitis also varies with estrous cycle stage in humans, then risk might be decreased by scheduling general ansesthesia with HAL accordingly in women.

The sex disparity in sensitivity to HAL is unlikely to be due to hormonal influences on metabolic bioactivation of HAL, since male and female mice have similar hepatic TFA-adduct formation (262). Female mice that developed severe HAL hepatotoxicity have greater hepatic neutrophil accumulation and plasma TNF-alpha concentration (262),

suggesting that a more robust inflammatory response occurs in females than males. Sexually dimorphic inflammatory responses have been reported in humans and experimental animals. For example, females more rapidly reject skin allografts compared to males (204, 205) and suffer a smaller incidence of hepatocellular carcinoma (209), both of which involve immune surveillance mediated by NK cells. IFN-γ, which is released by NK cells, and HMGB-1 are elevated in HAL-treated, intact female mice compared to HAL-treated male mice or to HAL-treated OVX mice (Figure 3.2). This suggests that IFN-γ and HMGB-1 might be part of a sexually dimorphic innate immune response in the pathogenesis of HAL-induced liver injury.

Interferon-gamma (IFN-γ) is a pleiomorphic cytokine released mainly from cytotoxic T cells, NK cells and NKT cells (281). The main functions of IFN-γ are to activate macrophages and NK cells, upregulate major histocompatibility complex (MHC) class I and class II molecules, and participate in antiviral and antibacterial activities. IFN-γ also promotes liver injury in humans, as demonstrated by the finding that administration of IFN-γ to patients taking acetaminophen elevated liver enzyme activity in serum (282). Also, exogenous IFN-γ administration induced apoptosis in a hepatocyte cell line (HepG2.2.15) in vitro, and mice genetically engineered to overexpress IFN-γ spontaneously develop hepatitis (283, 284). IFN-γ has also been implicated in the pathogenesis of liver injury in concanavalin A and acetaminophen animal models (285, 286). In the present study, IFN-γ KO mice were protected from severe HAL-induced liver injury, as indicated by markedly smaller plasma ALT activity and the reduced necrosis in

liver sections compared to wild-type mice (Figure 3.3). Although IFN-γ has been reported to decrease the activity of some cytochrome P450 enzymes (287), the formation of TFA-adducts was similar in wild-type and IFN-γ KO mice (Figure 3.3), suggesting no difference in HAL bioactivation. There are several proposed mechanisms by which IFN-γ can enhance liver injury, such as through the production of inflammatory mediators, the recruitment of inflammatory cells, and the suppression of liver regeneration (288); however, the mechanism by which IFN-γ contributes to HAL-induced hepatotoxicity is not known.

Toll-like receptors (TLRs) are pattern recognition receptors that, upon ligation, activate the innate immune response (22). TLR4 binds to microbial ligands such as lipopolysaccharide (LPS), a bacterial component of Gram-negative bacteria, and to endogenous ligands, such as heat shock proteins and HMGB-1, to initiate inflammatory cascades. For example, TLR4 signaling can mediate aseptic tissue injury in animal models of acetaminophen and alcohol hepatotoxicity (289, 290). TLR4 signaling is also involved in HAL-induced liver injury, since $Tlr4^{LPS\text{-}d}$ mice have an attenuated response (Figure 3.4A). The cellular component responsible for this TLR4 signaling is not known; however, Kcs are not likely to be involved since Kc-depleted mice had a response to HAL that was similar to controls (Figure 3.5) (162). Our results indicate that female mice release HMGB-1 to a greater extent in response to HAL than male mice (Figure 3.2C). Since HMGB-1 is both an endogenous danger signal and a ligand for TLR4, it is possible that HMGB-1 provides a signal that enhances toxicity in the female mice.

NK cells participate in stress surveillance to destroy tumor cells, infected or aberrant host cells, foreign cells and pathogens. In humans, NK cells (CD56+,CD3-) constitute 30-50% of hepatic lymphocytes (265). Similarly, the present study demonstrates a large number of hepatic NK cells in BALB/cJ mice, with 22% of hepatic lymphocytes immunophenotyped as DX5+,CD3- NK cells (Figure 3.9A). Other reports indicate that 5-10% of the hepatic lymphocytes from female, C57BL/6 mice, a HAL insensitive strain (262), are NK cells (291). It is possible that different hepatic lymphocyte isolation techniques can introduce differences in NK cell yield, since these cells are only lightly anchored to the sinusoidal endothelium in liver. On the other hand, a greater number of resident NK cells in livers of BALB/c mice compared to C57/BL6 mice could indicate a disparity in immune response between the two strains, as has been reported for these strains in susceptibility to various pathogens (292), and this could underlie the difference in susceptibility to HAL hepatotoxicity. The observation that BALB/c mice and humans have similar proportions of hepatic NK cells could indicate that this strain is appropriate to model human NK cell-mediated liver pathology.

HAL administration decreases the numbers of DX5+,CD3- NK cells in the hepatic lymphocyte pool at 8 (Figure 3.9A) and 24hr (data not shown). It is possible that this decrease is the result of the increased sequestration of NK cells within the hepatic parenchyma that excludes them from the isolation procedure. The percentage of CD69+,DX5+,CD3- NK cells extracted from HAL-treated mice was increased compared to

NK cells isolated from vehicle-treated mice (Figure 3.9). This suggests that HAL treatment can enhance hepatic NK cell activity in vivo.

Anti-AsGM1 treatment and CD1d KO mice were used as pharmacologic and genetic approaches to distinguish the relative contributions of NK and NKT cells in the pathogenesis of HAL-induced liver injury. Anti-AsGM1 treatment diminished anti-tumor activity attributed to NK cells when administered every three days in mice (293) and decreased NK cell-mediated cytotoxicity in vitro (267, 294). In the present study, anti-AsGM1 treatment induced a phenotype shift in the expression of CD69 and CD107a, suggesting deactivation and degranulation of NK cells(Figure 3.6). Anti-AsGM1 also abrogated HAL-induced liver injury and plasma IFN-γ increases compared to IgG-treated control mice (Figure 3.7). Furthermore, mice with defective perforin were protected from HAL hepatotoxicity (Figure 3.7). Collectively, these data strongly support a role for NK cells in HAL-induced liver injury. This finding contrasts another report in which anti-AsGM1 did not affect HAL-hepatotoxicity (163). We suggest that anti-AsGM1 dosing regimen and experimental time points chosen to evaluate liver enzyme activity could have led to these disparate results.

Kcs are capable of promoting early IFN-γ release; however, it has been reported (162) and confirmed here (Figure 3.5) that Kcs are not necessary for the development of severe HAL hepatotoxicity in mice. Furthermore, Kcs do not contribute significantly to HAL-induced IFN-γ accumulation (Figure 3.5). Co-treatment with polyI:C, a double stranded RNA mimic and ligand for TLR3, exacerbated HAL hepatotoxicity in a Kc-

dependent manner (162). Therefore, Kcs are not essential for the initiation of HAL hepatotoxicity but can exacerbate liver injury. The role of NKT cells is less clear. CD1d KO mice deficient in NKT cells develop severe HAL-induced liver injury (Figure 3.8). This finding is in contrast to a report that CD1d KO mice were protected from HAL-induced liver injury (163). It is possible that differences in the two mouse models, such as the simultaneous imposition of fasting and a different method of HAL preparation, could have led to disparate results.

Since HAL hepatitis is widely thought to be mediated by an acquired immune mechanism, we evaluated the response to HAL in RAG^{NULL} mice deficient in mature T and B cells. Accordingly, RAG^{NULL} mice are deficient DX5-,CD3+ T cells (Figure S.3.2.C). RAG^{NULL} mice treated with 15mmol/kg HAL developed severe HAL hepatotoxicity (FigureS.3.2.A). Bioactivation is similar in HAL-treated WT and RAG^{NULL} mice (Figure S.3.2.B). Although several RAG^{NULL} mice had 12hr plasma ALT activity >8000 U/L, the mean 12hr plasma ALT for the RAG^{NULL} mice was ~4000 U/L, which is less than that of similarly-treated WT mice (data not shown). This suggests that cells expressing the RAG gene are not required for injury, but they might positively regulate the activity of cells that mediate HAL-induced liver injury. According, NK cells have Fc receptors that bind to immunoglobulin, indicating a mechanism whereby plasma antibodies levels could modulate NK cell activity. Similarly, NK cells in immunocompromised nude mice, lacking T and B cells, required preactivation with poly(I:C) to cause virus-induced liver injury,

whereas prior activation was not required in wild-type mice (295). This underscores the highly integrated nature of the two arms of the immune system, as well as suggest that immunoglobulin levels may be enhancing NK cells, as well as the antibody-mediated toxicity suggested by the hapten hypothesis.

Since the bioactivation of HAL occurs within hepatocytes, it is possible that the resulting TFA-adducts induce an intracellular stress response that targets affected cells for cytotoxic killing by surveillant immune cells. This is an attractive concept, since hepatic NK cells physically interact with hepatic parenchymal cells through endothelial fenestrae (57). One way that a cell can communicate its stressed status to adherent immune effector cells such as NK cells is by changing the expression of MHC class I and MHC class I-like molecules on its plasma membrane. (47). MHC class I and MHC class I-like molecules are very closely related genetically, however their functions have diverged. MHC class I molecules are involved in antigen presentation in cell-mediated immunity, whereas MHC class I-like molecules signal intracellular distress to surveillent immune cells. In addition to playing a role in presenting intracellular proteins to cytotoxic T cells, MHC class I molecules also downregulate NK cell activity (60), and the loss of MHC class I molecules activates NK cells (60). This is known as the "missing self hypothesis". After HAL treatment, there were fewer hepatocytes expressing H-2D^{d}, a murine MHC class I molecule (Figure 3.10). In mice, MHC class I-like molecules such as Rae-1 and Mult-2 bind NKG2D stimulatory receptors on NK cells (63) and induce NK cell activity (49). HAL-treated hepatocytes had increased Rae-1 expression compared to

hepatocytes from vehicle-treated mice (Figure 3.10). Therefore, the direct interaction and signaling of the HAL-stressed hepatocytes to the cytotoxic NK cell is one way in which the innate immune system may exacerbate HAL-induced hepatotoxicity in female mice.

The results of this study significantly enhance our understanding of which effector cells contribute to the sexually dimorphic HAL response in mice. Ovarian hormones predispose female mice to severe HAL hepatotoxicity through IFN-γ and possibly HMGB-1. NK cells contribute to the onset of pathogenesis in an IFN-γ- and perforin-dependent manner. IFN-γ and the phenotype of circulating NK cells might ultimately provide serum biomarkers and potential therapeutic targets for HAL hepatitis and possibly other drug-induced hepatotoxicities. We suggest, therefore, that the pathogenesis of IADRs in humans might include factors related to the innate immune response, such as activated NK cells, that enhance the sensitivity of females to develop drug-induced liver injury.

Figure S.3.1. Anti-Asialo GM1 and IgG Treatment Alter the Surface Protein Expression of Hepatic NK Cells. A. Hepatic lymphocytes from IgG-, anti-AsGM1-, or normal rabbit serum (NRS)-treated mice were pooled into respective groups. NK cells were gated at DX5+,CD3- as shown in the dot plots. Labeled in each graph is the percentage of NK cells in the population. B. Surface expression of CD107a was analyzed by flow cytometry. The grey and black histograms depict the IgG and AsGM1 treatment groups, respectively. The shaded histogram depicts the NRS treatment group. Gates are indicated by the horizontal bars, and percent positive cells is indicated in the legend.

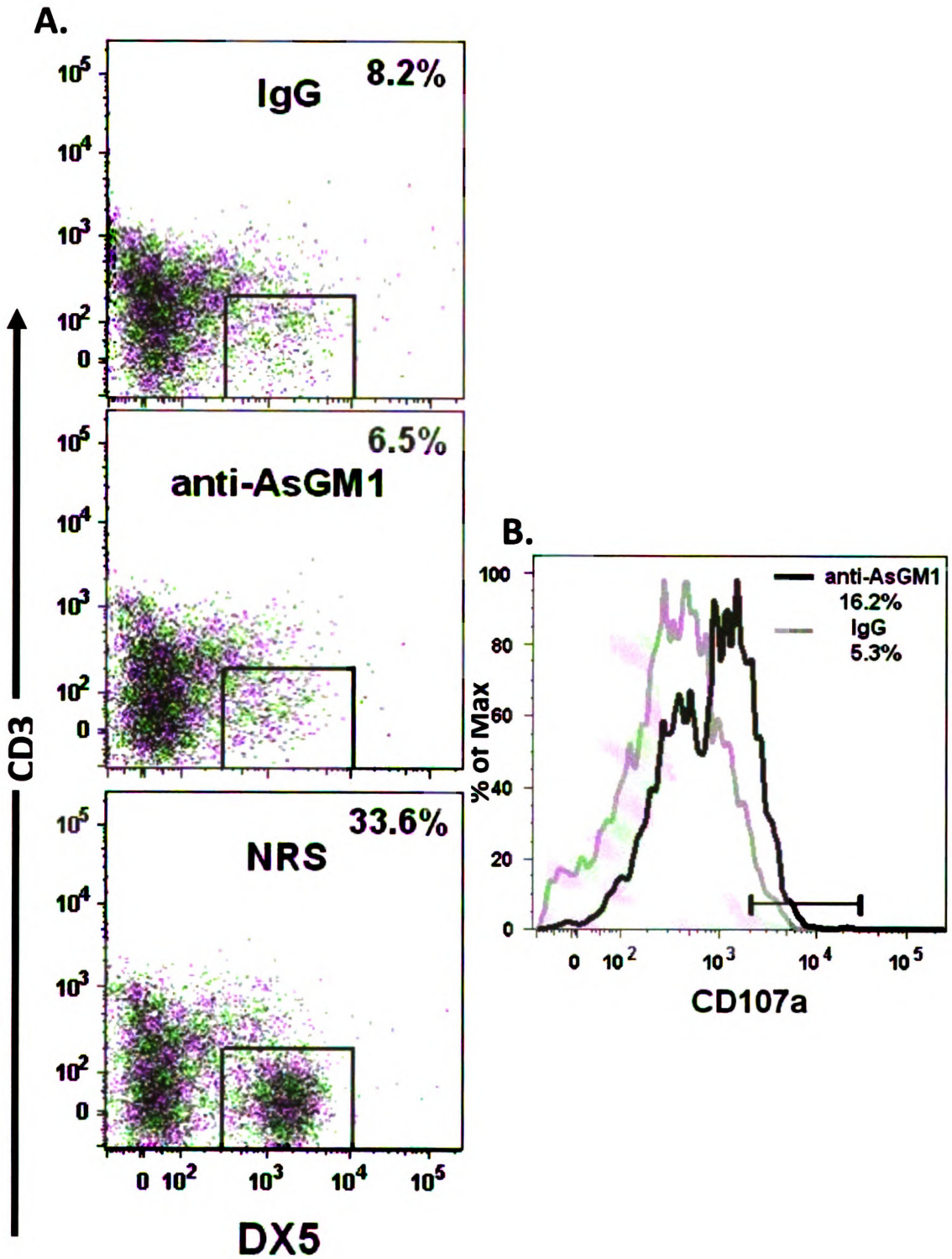

A.
IgG
8.2%
anti-AsGM1
6.5%
NRS
33.6%
CD3
DX5
B.
anti-AsGM1
16.2%
IgG
5.3%
% of Max
CD107a

Figure S.3.2. RAG-deficient Mice Develop HAL-induced Liver Injury. RAG^{NULL} mice deficient in mature T and B cells were treated with 15 or 30mmol/kg (i.p.). A. Plasma ALT activity was evaluated. B. H&E liver section of a 15mmol/kg HAL-treated RAG^{NULL} mouse. Labeled in picture are central vein (CV) and portal triad (PT). Arrowheads indicate necrotic areas. Images were photographed at 200X magnification. C. Hepatic lymphocytes from WT or RAG^{NULL} mice were pooled. T cells were gated as DX5-CD3- as shown in the dot plot, and the percentage of T cells is indicated accordingly. D. Immunoblot detection of TFA-adducts.

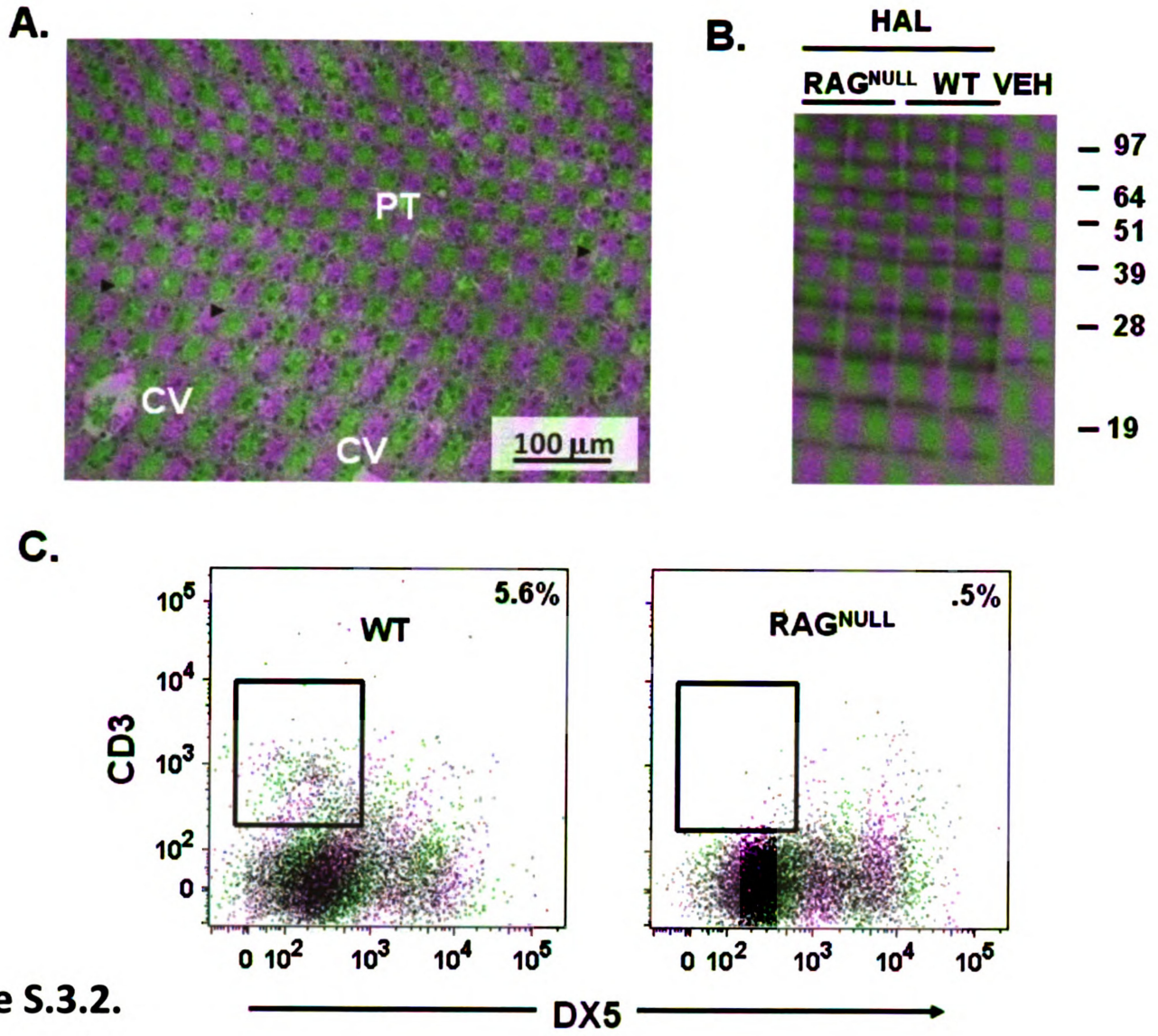

A.
PT
CV
CV
100 µm
B.
HAL
RAGNULL WT VEH
97
64
51
39
28
19
C.
WT
5.6%
RAGNULL
.5%
CD3
DX5
0 10^2 10^3 10^4 10^5

Figure S.3.2.

CHAPTER 4

Summary and Conclusions

4.1 Summary of Conducted Research:

In order to study the mechanism of rare hepatic ADRs, an animal model in which injury occurs at a high frequency is required. I tested the hypothesis that a mouse model of severe HAL hepatitis designed to have the risk factors associated with human clinical cases (female sex, genetics, fasted state, and mature age) would develop consistent, severe liver injury at a high enough frequency to be experimentally useful. Female, BALB/cJ mice developed severe hepatocellular toxicity in response to HAL administration, whereas males did not (Figure 2.1). Female, C57BL/6 mice were resistant to the development of severe HAL-induced liver injury at all doses tested (Figure 2.3). Overnight fasting shifted the dose response curve to the left, making previously non-toxic doses hepatotoxic in female BALB/cJ mice (Figure 2.2). Age was also determined to be an important risk factor. Eight week old female BALBc/J mice were more sensitive to HAL compared to four week old mice (Figure 2.5).

TFA-adduct formation was similar in extent in male and female mice (Figure 2.8). This suggests that HAL metabolism is not likely to be responsible for the observed sex disparity in HAL sensitivity. Anti-CD18 antiserum attenuated injury in female mice (Figure 2.11), indicating that PMNs are involved in the pathogenesis of injury. Inflammatory mediators such as TNF-α, IFN-γ, and hepatic neutrophil accumulation were increased in HAL-treated female mice compared to males (Figure 2.9, Figure 3.2, Figure 2.10). This suggests that an inflammatory response is responsible for the enhanced sensitivity observed in HAL-treated females. Accordingly, a non-hepatotoxic

dose of LPS resulted in increased recruitment of PMNs to the liver and potentiated HAL-induced liver injury in male mice (Figure 2.12).

I tested whether the state of the estrous cycle of the individual mice at the time of HAL treatment correlated with the severity of injury produced by HAL in order to determine whether female sex hormones sensitize female mice to HAL. Female mice in estrus were more sensitive to HAL, whereas the mice in proestrus and diestrus were less sensitive (Figure 3.1). In addition, ovariectomized mice were protected from the development of HAL-induced liver injury, as compared to sham-operated mice (Figure 3.1). These data indicate that female ovarian hormones influence the course of HAL hepatotoxicity.

IFN-γ is critical to the development of HAL toxicity and appears in a greater concentration in the plasma in sham-operated female mice compared to similarly-treated ovariectomized female mice and male mice (Figure 3.3, Figure 3.2). I tested the hypothesis that innate immune cells contribute to the IFN-γ-dependent pathogenesis of HAL-induced liver injury. Mice depleted of Kcs or NKT cells developed similar HAL-associated pathology and similar concentrations of plasma IFN-γ compared to wild-type mice (Figure 3.5, 3.8). Therefore, other cell type(s) must be responsible for the observed IFN-γ production (Figure 3.5, 3.8).

Taken together, my results indicate that NK cells mediate IFN-γ-dependent HAL hepatotoxicity. Anti-asialo GM1 treatment attenuated HAL hepatotoxicity and decreased HAL-induced increases of plasma IFN-γ in female mice (Figure 3.7). Mice with

defective perforin, a preformed protein involved in NK cell granule-mediated cytotoxicity, were also more resistant to HAL-induced liver injury. Additionally, HAL treatment activated hepatic NK cells in vivo, as indicated by increased percentage of CD69+ NK cells compared to vehicle-treated mice (Figure 3.9). Furthermore, HAL treatment in vivo increased the expression of an activating NK cell ligand, Rae-1, and decreased an inhibitory NK cell ligand, H-2D^{d}, on hepatocytes (Figure 3.10).

4.2 Proposed Mechanism of HAL Hepatitis

Figure 4.1 illustrates a proposed mechanism for HAL hepatitis that occurs in female mice in which the innate immune response mediates severe liver injury. This mechanism is based on the results discussed in this dissertation and ongoing research. It will likely evolve as we learn more about this model. HAL is metabolized in hepatocytes to TFA chloride, which binds macromolecules to form TFA-adducts. This induces a stress response in hepatocytes that alters the surface NK receptor ligands on affected hepatocytes and activates NK cells. NK cells release cytotoxic granules, such as perforin and granzyme B, as well as IFN-γ. Damaged hepatocytes release endogenous danger signals, such as HMGB-1. These endogenous danger signals are often ligands for TLR4 and some of these resultant signals are involved in a positive feedback loop that further activates NK cells, as well as recruits PMNs. Additionally, the resultant increase in IFN-γ induces the upregulation of MHC class I and MHC class II molecules, and

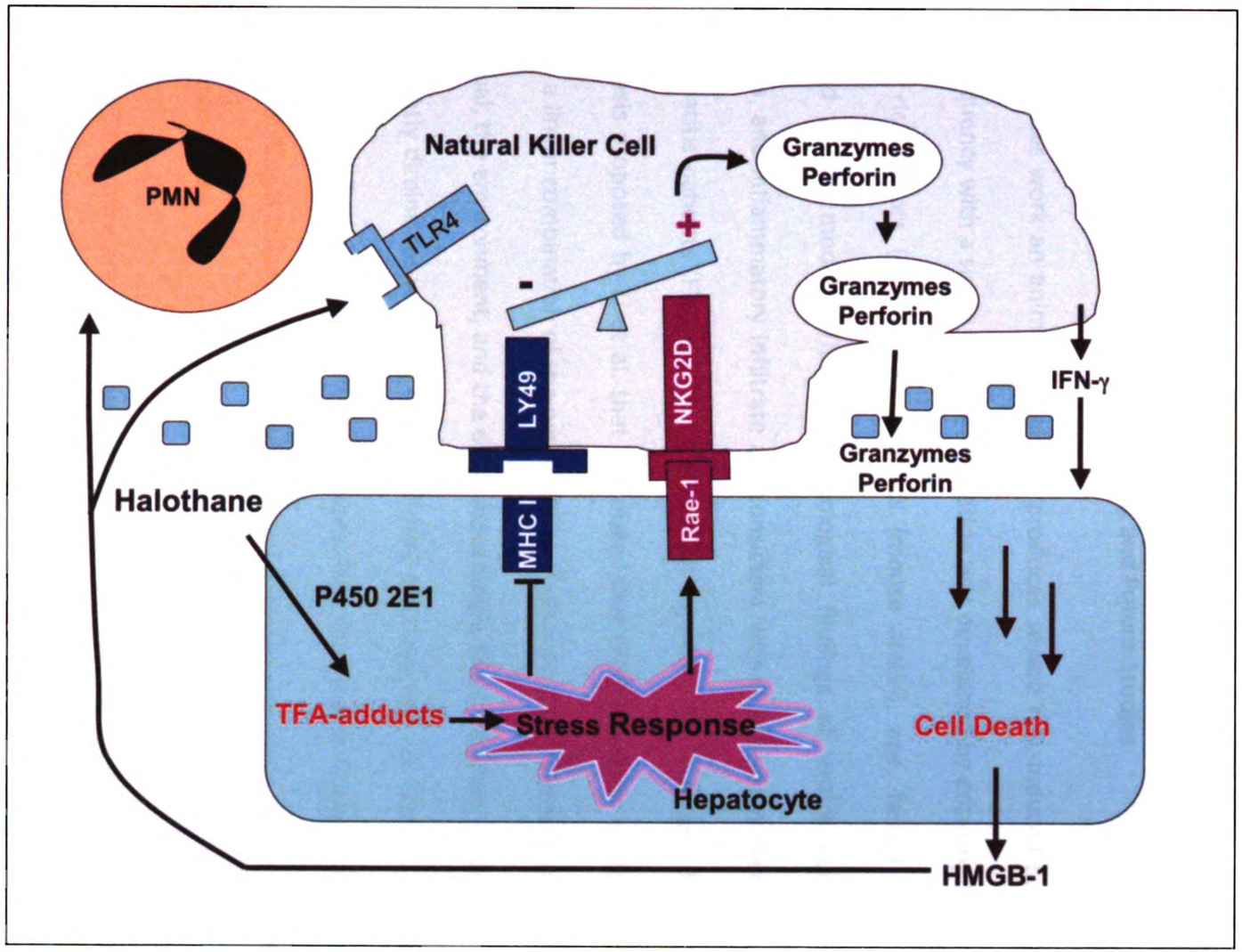

PMN
Natural Killer Cell
Granzymes
Perforin
TLR4
+
-
Granzymes
Perforin
LY49
NKG2D
IFN-γ
Granzymes
Perforin
Halothane
MHC I
Rae-1
P450 2E1
TFA-adducts
Stress Response
Cell Death
Hepatocyte
HMGB-1

Figure 4.1

thereby enhances the development of acquired, cell-mediated and humoral immune responses.

4.3 Significance of Research, Research Gaps, and Future Studies

In this work an animal model that produces severe HAL-induced liver injury at high frequency with a single HAL administration was developed by ensuring that several human risk factors (female sex, genetics (mouse strain), age, fasted state) were reflected in the model. The histopathological findings of centrilobular necrosis, steatosis, and inflammatory infiltrate are consistent with that seen in liver biopsies of HAL hepatitis patients (Figure 2.7). This model supports the "multiple determinant" hypothesis proposed by Li et al. that postulates that the probability of developing an IADR is a linear combination of the probabilities of discrete characteristics unique to the individual, the environment, and the drug. Accordingly, the probability of developing an IADR greatly diminishes when an individual does not have the risk factors. The dearth of animal models that develop hepatic lesions from drugs with idiosyncratic potential without additional inflammatory stress or chemical treatments underscores the value of this animal model.

C57BL/6 mice are insensitive to the toxic effects of HAL at all doses tested. This is consistent with other reports that there is a genetic component involved in the sensitivity to HAL hepatotoxicity for both animal models and humans. By using a panel of inbred strains intended to capture the genetic diversity seen in the human population, CD44 was identified as a sensitivity factor for acetaminophen-induced liver

injury in mice and human patients (296). Accordingly, the use of a panel of recombinant inbred mice from C57BL/6 mice, an insensitive strain, and BALB/c mice, a sensitive strain, could be a useful to gain further insight into the genetic basis of sensitivity to HAL.

In addition to being more sensitive to HAL hepatotoxicity, female mice had an enhanced inflammatory response, as indicated by more rapid accumulation and greater numbers of PMNs in the liver, and increased plasma TNF-α and IFN-γ compared to the male mice. The significance of the finding that female mice in estrus are most sensitive to HAL hepatotoxicity is that rodents in estrus have elevated plasma progesterone, whereas those in proestrus have high estrogen and in diestrus have low progesterone and estrogen. It is possible that progesterone could be acting to enhance the immune response to HAL. Interestingly, mifepristone attenuated HAL-induced hepatotoxicity (279). Mifepristone, also known as RU-486, is also a progesterone receptor antagonist. Therefore, the use of pharmacological progesterone receptor agonists, such as progesterone and medroxyprogesterone acetate, could be administered to ovariectomized mice to elucidate whether progesterone contributes to the sensitivity of female mice to HAL hepatotoxicity.

Another important finding from this research is the identification of some of the cellular mediators involved in the pathogenesis. Kcs are not necessary for the initiation of HAL-hepatitis in female mice. This conflicts with the mechanism proposed under the hapten hypothesis, whereby Kcs are antigen presenting cells of TFA neoantigens in

acquired immune-mediated injury (297). In our current research, NK cells contribute to the pathogenesis of HAL hepatitis as demonstrated by the attenuation of liver injury following the depletion of NK cell activity with anti-asialo GM1 treatment. We also determined that IFN-γ release and granule-mediated cytotoxicity, which are effector functions of NK cells, are critical mediators of HAL-associated liver injury in mice. A recent report demonstrates the constitutive expression of protease inhibitors in the liver that protect against granule-associated cytotoxicity (55). It would be of interest to determine whether this protective mechanism is compromised following HAL treatment and whether it contributes to the enhanced sensitivity of female mice to HAL. Furthermore, it is not known whether IFN-γ is acting directly on hepatocytes and/or NK cells or acting indirectly through other cells to mediate HAL hepatotoxicity. It is likely to be indirect, since hepatocytes have low levels of IFN-γ receptor expression (298). Co-culturing NK cells and HAL-treated hepatocytes could give some insight into whether other cells are necessary for the development of hepatocellular injury.

The finding that NK cells mediate liver injury is a valuable contribution to the field of idiosyncratic liver injury because it demonstrates another potential mechanism by which the innate immune system can mediate idiosyncratic drug-induced liver injury. Whether the immunological response to HAL hepatotoxicity is the result of accumulated stress injury from repeated HAL exposures is not known. To the best of my knowledge, this has yet to be demonstrated in any animal model; however, it has been demonstrated that HAL metabolites accumulate in the livers of mice exposed weekly to

HAL (134). It would be of interest to determine whether the accumulation of adducts correlates with enhanced severity of HAL hepatotoxicity, as well as determine whether the clearance of adducts impacts the sexually dimorphic HAL sensitivity seen in female BALB/cJ mice.

One of the challenges in the field of hepatic drug-induced liver injury is the identification of a biomarker that predicts which patients will progress to develop liver injury. The evaluation of hepatic enzyme activities, such as alanine aminotransferase, is useful in detecting hepatocellular injury in animal models; however, it is not a reliable prediction of patient outcome. Watkins and colleagues (299) analyzed urine and blood metabolites from acetaminophen-treated patients and correlated urine metabolites with the development of hepatic injury. The same group is advancing a method of transcriptome profiling of hepatocyte-specific circulating microRNA as potential biomarkers of hepatic injury in animals treated with hepatotoxins (300). It is not known whether plasma NK cell number or activity correlates with enhanced liver injury in humans or animal models. However, circulating NK cell number and phenotype fluctuates with menstrual cycle (301). Furthermore, endometrial NK cell number is proportional to the risk of spontaneous abortion in pregnant women (302)and mice(303).Using NK cell number or activation phenotype as a possible biomarker might provide valuable insight into possible susceptibility markers.

CHAPTER 5

Reference List

5.1 Reference List

1. Ostapowicz G, Fontana RJ, Schiodt FV, Larson A, Davern TJ, Han SH, et al. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. Ann Intern Med 2002 Dec 17;137(12):947-954.

2. Temple R. Hy's law: predicting serious hepatotoxicity. Pharmacoepidemiology and Drug Safety 2006;15(4):241-243.

3. Sgro C, Clinard F, Ouazir K, Chanay H, Allard C, Guilleminet C, et al. Incidence of drug-induced hepatic injuries: A French population-based study. Hepatology 2002;36(2):451-455.

4. Spigset O, Hägg S, Bate A. Hepatic injury and pancreatitis during treatment with serotonin reuptake inhibitors: Data from the World Health Organization (WHO) database of adverse drug reactions. International Clinical Psychopharmacology 2003;18(3):157-161.

5. Kostrubsky SE, Strom SC, Kalgutkar AS, Kulkarni S, Atherton J, Mireles R, et al. Inhibition of hepatobiliary transport as a predictive method for clinical hepatotoxicity of nefazodone. Toxicol Sci 2006;90(2):451-459.

6. Raucy JL, Lasker JM, Lieber CS, Black M. Acetaminophen activation by human liver cytochromes P450IIE1 and P450IA2. Arch Biochem Biophys 1989;271(2):270-283.

7. Dong H, Haining RL, Thummel KE, Rettie AE, Nelson SD. Involvement of human cytochrome P450 2D6 in the bioactivation of acetaminophen. Drug Metabolism and Disposition 2000;28(12):1397-1400.

8. Jollow DJ, Thorgeirsson SS, Potter WZ, Hashimoto M, Mitchell JR. Acetaminophen-induced hepatic necrosis. VI. Metabolic disposition of toxic and nontoxic doses of acetaminophen. Pharamacology 1974;12(4):251-271.

9. Jollow DJ, Mitchell JR, Potter WZ, Davis DC, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. II. Role of covalent binding in vivo. Journal of Pharmacology and Experimental Therapeutics 1973;187(1):195-202.

10. Zimmerman HJ. Drug-Induced Liver Disease. Drugs 1978;16(1):25-45.

11. Pirmohamed M, Breckenridge AM, Kitteringham NR, Park BK. Fortnightly review: Adverse drug reactions. BMJ 1998;316(7140):1295-1298.

12. Lammert C, Einarsson S, Saha C, Niklasson A, Bjornsson E, Chalasani N. Relationship between daily dose of oral medications and idiosyncratic drug-induced liver injury: Search for signals. Hepatology 2008;47(6):2003-2009.

13. Lee WM. Etiologies of acute liver failure. Semin Liver Dis 2008 May;28(2):142-152.

14. Zimmerman HJ. Various forms of chemically induced liver injury and their detection by diagnostic procedures. Environ Health Perspect 1976 Jun;15:3-12.

15. Preziosi P. Isoniazid: Metabolic aspects and toxicological correlates. Current Drug Metabolism 2007;8(8):839-851.

16. Sun F, Chen Y, Xiang Y, Zhan S. Drug-metabolising enzyme polymorphisms and predisposition to anti-tuberculosis drug-induced liver injury: a meta-analysis. Int J Tuberc Lung Dis 2008;12(9):994-1002.

17. O'Shea D, Kim RB, Wilkinson GR. Modulation of CYP2E1 activity by isoniazid in rapid and slow N-acetylators. Br J Clin Pharmacol 1997;43(1):99-103.

18. Forget EJ, Menzies D. Adverse reactions to first-line antituberculosis drugs. Expert Opinion on Drug Safety 2006;5(2):231-249.

19. Nolan CM, Goldberg SV, Buskin SE. Hepatotoxicity associated with isoniazid preventive therapy: a 7-year survey from a public health tuberculosis clinic. JAMA 1999;281(11):1014-1018.

20. Watkins PB. Idiosyncratic Liver Injury: Challenges and Approaches. Toxicologic Pathology 2005;33:1-5.

21. Watkins PB, Whitcomb RW. Hepatic dysfunction associated with troglitazone. N Engl J Med 1998;338(13):916-917.

22. Medzhitov R, Janeway C, Jr. Innate immune recognition: mechanisms and pathways. Immunol Rev 2000 Feb;173:89-97.

23. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 2007 Jan;81(1):1-5.

24. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010 May;11(5):373-384.

25. Francisco AB, Hans CO. Adaptive immunity. J Allergy Clin Immunol 2010 Feb 1;125(2):S33-S40.

26. GELL PG, HARINGTON CR, RIVERS RP. The antigenic function of simple chemical compounds; production of precipitins in rabbits. Br J Exp Pathol 1946 Oct;27(5):267-286.

27. Parker DC. T cell-dependent B cell activation. Annu Rev Immunol 1993;11:331-360.

28. Butterworth AE, Remold HG, Houba V, David JR, Franks D, David PH, et al. Antibody-dependent eosinophil-mediated damage to 51Cr-labeled schistosomula of Schistosoma mansoni: mediation by IgG, and inhibition by antigen-antibody complexes. J Immunol 1977 Jun;118(6):2230-2236.

29. Cerdan C, Martin Y, Courcoul M, Mawas C, Birg F, Olive D. CD28 costimulation regulates long-term expression of the three genes (alpha, beta, gamma) encoding the high-affinity IL2 receptor. Res Immunol 1995 Mar;146(3):164-168.

30. Hobart M, Ramassar V, Goes N, Urmson J, Halloran PF. IFN regulatory factor-1 plays a central role in the regulation of the expression of class I and II MHC genes in vivo. J Immunol 1997 May 1;158(9):4260-4269.

31. Lanier LL, Ruitenberg JJ, Phillips JH. Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes. J Immunol 1988 Nov 15;141(10):3478-3485.

32. Matzinger P. An innate sense of danger. Annals of the New York Academy of Sciences 2002;961:341-342.

33. Seuguin B, Uetrecht J. The danger hypothesis applied to idiosyncratic drug reactions. Current Opinion in Allergy and Clinical Immunology 2003;3(4):235-242.

34. Uetrecht JP. New concepts in immunology relevant to idiosyncratic drug reactions: The "danger hypothesis" and innate immune system. Chemical Research in Toxicology 1999;12(5):387-395.

35. Roth RA, Luyendyk JP, Maddox JF, Ganey PE. Inflammation and drug idiosyncrasy--is there a connection? J Pharmacol Exp Ther 2003;307(1):1-8.

36. Ganey PE, Luyendyk JP, Maddox JF, Roth RA. Adverse hepatic drug reactions: inflammatory episodes as consequence and contributor. Chemico-Biological Interactions 2004;150(1).

37. Shaw PJ, Hopfensperger MJ, Ganey PE, Roth RA. Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. Toxicol Sci 2007;100(1):259-266.

38. Luyendyk J, Maddox J, Cosma G, Ganey P, Cockerell G, Roth R. Ranitidine treatment during a modest inflammatory response precipitates idiosyncrasy-like liver injury in rats. J Pharmacol Exp Ther 2003;307(1):9-16.

39. Zou W, Devi SS, Sparkenbaugh E, Younis HS, Roth RA, Ganey PE. Hepatotoxic interaction of sulindac with lipopolysaccharide: role of the hemostatic system. Toxicol Sci 2009;108(1):184-193.

40. Lind R, Gandolfi A, Sipes G, B B. The Involvement of Endotoxin in the halothane-associated liver injury. Anesthesiology 1984;61:544-550.

41. Chen GY, Tang J, Zheng P, Liu Y. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. Science 2009;323(5922):1722-1725.

42. Shaw PJ, Beggs KM, Sparkenbaugh EM, Dugan CM, Ganey PE, Roth RA. Trovafloxacin enhances TNF-induced inflammatory stress and cell death signaling and reduces TNF clearance in a murine model of idiosyncratic hepatotoxicity. Toxicol Sci 2009;111(2):288-301.

43. Patil C, Walter P. Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Current Opinion in Cell Biology 2001 Jun 1;13(3):349-355.

44. Welihinda AA, Tirasophon W, Kaufman RJ. The cellular response to protein misfolding in the endoplasmic reticulum. Gene Expr 1999;7(4-6):293-300.

45. Wilkinson KD. Ubiquitin-dependent signaling: the role of ubiquitination in the response of cells to their environment. J Nutr 1999 Nov;129(11):1933-1936.

46. Bukau B, Horwich AL. The Hsp70 and Hsp60 Chaperone Machines. Cell 1998 Feb 6;92(3):351-366.

47. Gleimer M, Parham P. Stress management: MHC class I and class I-like molecules as reporters of cellular stress. Immunity 2003 Oct;19(4):469-477.

48. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci U S A 1996 Oct 29;93(22):12445-12450.

49. Jinushi M, Takehara T, Tatsumi T, Kanto T, Groh V, Spies T, et al. Expression and role of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acid. Int J Cancer 2003 Apr 10;104(3):354-361.

50. Viret C, Janeway CA, Jr. MHC and T cell development. Rev Immunogenet 1999;1(1):91-104.

51. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 2008 May;9(5):503-510.

52. Dong Z, Wei H, Sun R, Hu Z, Gao B, Tian Z. Involvement of natural killer cells in Polyl:C-induced liver injury. J Hepatol 2004;41(6).

53. Vermijlen D, Luo D, Froelich CJ, Medema JP, Kummer JA, Willems E, et al. Hepatic natural killer cells exclusively kill splenic/blood natural killer-resistant tumor cells by the perforin/granzyme pathway. J Leukoc Biol 2002 Oct;72(4):668-676.

54. Ochi M, Ohdan H, Mitsuta H, Onoe T, Tokita D, Hara H, et al. Liver NK cells expressing TRAIL are toxic against self hepatocytes in mice. Hepatology 2004 May;39(5):1321-1331.

55. Stout-Delgado HW, Getachew Y, Rogers TE, Miller BC, Thiele DL. The role of serpinb9/serine protease inhibitor 6 in preventing granzyme B-dependent hepatotoxicity. Hepatology 2007 Nov;46(5):1530-1540.

56. Chen Y, Sun R, Jiang W, Wei H, Tian Z. Liver-specific HBsAg transgenic mice are over-sensitive to Poly(I:C)-induced liver injury in NK cell- and IFN-gamma-dependent manner. J Hepatol 2007;47(2).

57. Wisse E, van't Noordende JM, van der Meulen J, Daems WT. The pit cell: description of a new type of cell occurring in rat liver sinusoids and peripheral blood. Cell Tissue Res 1976 Oct 19;173(4):423-435.

58. Ishiyama K, Ohdan H, Ohira M, Mitsuta H, Arihiro K, Asahara T. Difference in cytotoxicity against hepatocellular carcinoma between liver and periphery natural killer cells in humans. Hepatology 2006 Feb;43(2):362-372.

59. Vanderkerken K, Bouwens L, Wisse E. Characterization of a phenotypically and functionally distinct subset of large granular lymphocytes (pit cells) in rat liver sinusoids. Hepatology 1990 Jul;12(1):70-75.

60. Ljunggren HG, Karre K. Experimental strategies and interpretations in the analysis of changes in MHC gene expression during tumour progression. Opposing influences of T cell and natural killer mediated resistance? J Immunogenet 1986 Apr;13(2-3):141-151.

61. Vivier E, Daeron M. Immunoreceptor tyrosine-based inhibition motifs. Immunol Today 1997 Jun;18(6):286-291.

62. Raulet DH, Vance RE, McMahon CW. Regulation of the natural killer cell receptor repertoire. Annu Rev Immunol 2001;19:291-330.

63. Cerwenka A, Bakker AB, McClanahan T, Wagner J, Wu J, Phillips JH, et al. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. Immunity 2000 Jun;12(6):721-727.

64. Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat Immunol 2000 Aug;1(2):119-126.

65. Barton CC, Hill DA, Yee SB, Barton EX, Ganey PE, Roth RA. Bacterial lipopolysaccharide exposure augments aflatoxin B(1)-induced liver injury. Toxicol Sci 2000;55(2):444-452.

66. Su GL, Goyert SM, Fan MH, Aminlari A, Gong KQ, Klein RD, et al. Activation of human and mouse Kupffer cells by lipopolysaccharide is mediated by CD14. Am J Physiol Gastrointest Liver Physiol 2002 Sep;283(3):G640-G645.

67. Chalifour A, Jeannin P, Gauchat JF, Blaecke A, Malissard M, N'Guyen T, et al. Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers {alpha}-defensin production. Blood 2004 Sep 15;104(6):1778-1783.

68. Mian MF, Lauzon NM, Andrews DW, Lichty BD, Ashkar AA. FimH can directly activate human and murine natural killer cells via TLR4. Mol Ther 2010 May 4;1379-1388.

69. Wulff S, Pries R, Wollenberg B. Cytokine release of human NK cells solely triggered with Poly I:C. Cell Immunol 2010 Apr 4;263(2):135-137.

70. Chambers BJ, Salcedo M, Ljunggren HG. Triggering of natural killer cells by the costimulatory molecule CD80 (B7-1). Immunity 1996 Oct;5(4):311-317.

71. Carr A, Vella S, de Jong MD, Sorice F, Imrie A, Boucher CA, et al. A controlled trial of nevirapine plus zidovudine versus zidovudine alone in p24 antigenaemic HIV-infected patients. The Dutch-Italian-Australian Nevirapine Study Group. AIDS 1996;10:635-641.

72. Havlir D, Cheeseman SH, McLaughlin M, Murphy R, Erice A, Spector SA, et al. High-dose nevirapine: Safety, pharmacokinetics, and antiviral effect in patients with uuman immunodeficiency virus infection. The Journal of Infectious Diseases 1995;171(3):537-545.

73. Warren KJ, Boxwell DE, Kim NY, Drolet BA. Nevirapine-assoiciated Stevens-Johnson syndrome. Lancet 1998;351(9102):568.

74. Stern JO, Robinson PA, Love J, Lanes S, Imperiale MS, Mayers DL. A comprehensive hepatic safety analysis of nevirapine in different populations of HIV infected patients*. Journal of Acquired Immune Deficiency Syndromes 2003;34:S21-S33.

75. Wit F, Weverling G, Weel J, Jurriaans S, Lange J. Incidence of and risk factors for severe hepatotoxicity associated with antiretroviral combination therapy. The Journal of Infectious Diseases 2002;186(1):23-31.

76. Sha BE, Proia LA, Kessler HA. Adverse effects associated with use of nevirapine in HIV postexposure prophylaxis for 2 health care workers. JAMA 2000;284(21):2723.

77. Cattelan A, Erne E, Salatino A, Trevenzoli M, Carretta G, Meneghetti F, et al. Severe hepatic failure related to nevirapine treatment. Clinical Infectious Diseases 1999;29(2):455-456.

78. Maniar JK, Shah SR, Verma R, Kamath R, Gupta P, Maniar A. Nevirapine-induced fulminant hepatitis. J Assoc Physicians India 2006;54:957-958.

79. Piliero PJ, Purdy B. Nevirapine-induced hepatitis: a case series and review of the literature. AIDS Read 2001;11(7):379-282.

80. Dieterich D, Robinson P, Love J, Stern J. Drug-induced liver injury associated with the use of nonnucleoside reverse-transcriptase inhibitors. Clinical Infectious Diseases 2004;38(s2):S80-S89.

81. Sanne I. Severe liver toxicity in patients receiving two nucleoside analogues and a non-nucleoside reverse transcriptase inhibitor. AIDS 2000;14(suppl 4):S12.

82. Sanne I, Mommeja–Marin H, Hinkle J, Bartlett J, Lederman M, Maartens G, et al. Severe hepatotoxicity associated with nevirapine use in HIV+ infected subjects. The Journal of Infectious Diseases 2005;191(6):825-829.

83. no authors listed. Serious adverse events attributed to nevirapine regimens for post exposure prophylaxis after HIV exposures-worldwide, 1997-2000. MMWR Morb Wkly Rep 2001;49:1153-1156.

84. Uetrecht J. Is it possivle to more accurately predict which drug candidates will cause idiosyncratic drug reactions? Curr Drug Metab 2000;1(2):133-141.

85. Popovic M, Shenton JM, Chen J, Baban A, Tharmanathan T, Mannargudi B, et al. Nevirapine Hypersensitivity. Adverse Drug Reactions. 2010. 437-451.

86. González de Requena D, Núñez M, Jiménez-Nácher I, Soriano V. Liver toxicity caused by nevirapine. AIDS 2002;16(2):290-291.

87. Almond LM, Boffito M, Hoggard PG, Bonora S, Raiteri R, Reynolds HE, et al. The relationship between nevirapine plasma concentrations and abnormal liver function tests. AIDS Res Hum Retroviruses 2004;20(7):716-722.

88. McKoy JM, Bennett CL, Scheetz MH, Differding V, Chandler KL, Scarsi KK, et al. Hepatotoxicity Associated with Long-versus Short-Course HIV-Prophylactic Nevirapine Use A Systematic Review and Meta-Analysis from the Research on Adverse Drug events And Reports (RADAR) Project. Drug Saf 2009;32(2):147-158.

89. Mbougua JB, Laurent C, Kouanfack C, Bourgeois A, Ciaffi L, Calmy A, et al. Hepatotoxicity and effectiveness of a Nevirapine-based antiretroviral therapy in HIV-infected patients with or without viral hepatitis B or C infection in Cameroon. BMC Public Health 2010;1:105.

90. Martin AM, Nolan D, James I, Cameron P, Keller J, Moore C, et al. Predisposition to nevirapine hypersensitivity associated with HLA-DRB1*0101 and abrogated by low CD4 T-cell counts. AIDS 2005;19(1):97-99.

91. Taiwo BO. Nevirapine toxicity. Int J STD AIDS 2006;17(6):364-370.

92. Kesselring AM, Wit FW, Sabin CA, Lundgren JD, Gill MJ, Gatell JM, et al. Risk factors for treatment-limiting toxicities in patients starting nevirapine-containing antiretroviral therapy. AIDS 2009;23(13).

93. Antela A, Ocampo A, Gomez R, Lopez MJ, Marino A, Losada E, et al. Liver toxicity after switching or simplifying to nevirapine-based therapy is not related to CD4 cell counts: results of the TOSCANA study. HIV Clin Trials 2010 Jan;11(1):11-17.

94. Naisbitt DJ, Williams DP, Pirmohamed M, Kitteringham NR, Park BK. Reactive metabolites and their role in drug reactions. Curr Opin Allergy Clin Immunol 2001;1(4):317-325.

95. Chen J, Mannargudi MM, Xu L, Uetrecht J. Demonstration of the metaboic pathway responsible for neviropine-induced skin rash. Chem Res Toxicol 2008;21:1862-1870.

96. Shenton JM, Teranishi M, Abu-Asab MS, Yager JA, Uetrecht JP. Characterization of a potential animal model of an idiosyncratic drug reaction: Nevirapine-

induced skin rash in the rat. Chemical Research in Toxicology 2003;16(9):1078-1089.

97. Shenton JM, Popovic M, Chen J, Masson MJ, Uetrecht JP. Evidence of an immune-mediated mechanism for an idiosyncratic nevirapine-induced reaction in the female Brown Norway rat. Chemical Research in Toxicology 2005;18(12):1799-1813.

98. Shenton JM, Chen J, Uetrecht JP. Animal models of idiosyncratic drug reactions. Chemico-Biological Interactions 2004;150(1):53-70.

99. Bunker JP, Child CG, Davidson CS, Gall EA, Klatskin G, Laster L, et al. Summary of the National Halothane Study. JAMA 1966;197(10).

100. Inman W, Mushin W. Jaundice after repeated exposure to halothane: An analysis of reports to the committee on safety of medicines. Brit Med J 1974;1(5896).

101. Cousins M, Plummer J, Hall P. Toxicity of volatile anaesthetic agents. Clinics in Anaesthesiology 1985;2(3).

102. Black GW. A review of the pharmacology of halothane. Brit J Anaesth 1965;37:688.

103. Blackmore WP, Erwin KW, Wiegard OF, Lipsey R. Renal and cardiovascular effects of halothane. Anesthesiology 1960;21:489.

104. Trowell J, Peto R, Smith A. Controlled trial of repeated halothane anaesthetics in patients with carcionoma of the uterine cervix treated with radium. The Lancet 1975;305(7911).

105. Wright R, Eade OE, Chisholm M, Hawksley M, Lloyd B, Moles TM, et al. Controlled prospective study of the effect on liver function of multiple exposures to halothane. Lancet 1975 Apr 12;1(7911):817-820.

106. Mushin WW, Rosen M, Bowen DJ, Campbell H. Post-halothane jaundice in relation to previous administration of halothane. British Medical Journal 1971;3:18-22.

107. Bottiger LE, Dalen E, Hallen B. Halothane-induced liver damage: an analysis of the material reported to the Swedish Adverse Drug Reaction Committee, 1966-1973. Acta Anaesthesiol Scand 1976;20(1):40-46.

108. Blackburn WR, Ngai SH, Lindenbaum J. Morphologic changes in hepatic necrosis following halothane anesthesia in man. Anesthesiology 1964;25:270-283.

109. Klion FM, Schaffner F, Popper H. Hepatitis after exposure to halothane. Ann Intern Med 1969 Sep;71(3):467-477.

110. Peters RL, Edmondson HA, Reynolds TB, Meister JC, Curpey TJ. Hepatic necrosis associated with halothane anesthesia. Am J Med 1969 Nov;47(5):748-764.

111. Wills EJ, Walton B. A morphologic study of unexplained hepatitis following halothane anesthesia. Am J Pathol 1978;91(1):11-32.

112. Pohl LR, Gillette JR. A perspective on halothane-induced hepatotoxicity. Anesth Analg 1982;61(10):809-811.

113. Trowell J, Peto R, Smith AC. Controlled trial of repeated halothane anaesthetics in patients with carcionoma of the uterine cervix treated with radium. The Lancet 1975;305(7911):821-824.

114. Inman WH, Mushin WW. Jaundice after repeated exposure to halothane: An analysis of reports to the committee on safety of medicines. Brit Med J 1974;1(5896).

115. Inman WH, Mushin WW. Jaundice after repeated exposure to halothane: a further analysis of reports to the Committee on Safety of Medicines. Br Med J 1978;25(2).

116. Walton B, Hamblin A, Dumonde D, Simpson R. Absence of cellular hypersensitivity in patients with unexplained hepatitis following halothane. Anesthesiology 1976;44(5).

117. Cohen EN, Trudell JR, Edmunds HN, Watson E. Urinary metabolites of halothane in man. Anesthesiology 1975;43(4):392.

118. Rehder K. Biotransformation of halothane in humans. Anesthesiology 2003;99(5):1220-1221.

119. McLain G, Sipes I, Brown BJ. An animal model of halothane hepatotoxicity: roles of enzyme induction and hypoxia. Anesthesiology 1979;51(4):321-326.

120. Reynolds ES, Moslen MT. Liver injury following halothane anesthesia in phenobarbital-pretreated rats. Biochem Pharmacol 1974 Jan 15;23(2):189-195.

121. Reynolds ES, Moslen MT. Halothane hepatotoxicity: enhancement by polychlorinated biphenyl pretreatment. Anesthesiology 1977 Jul;47(1):19-27.

122. Rice SA, Maze M, Smith CM, Kosek JC, Mazze RI. Halothane hepatotoxicity in Fischer 344 rats pretreated with isoniazid. Toxicol Appl Pharmacol 1987 Mar 15;87(3):411-419.

123. Ross WT, Jr., Daggy BP, Cardell RR, Jr. Hepatic necrosis caused by halothane and hypoxia in phenobarbital-treated rats. Anesthesiology 1979 Oct;51(4):327-333.

124. Lunam CA, Cousins MJ, Hall PM. Guinea-pig model of halothane-associated hepatotoxicity in the absence of enzyme induction and hypoxia. J Pharmacol Exp Ther 1985 Mar;232(3):802-809.

125. Cousins MJ, Plummer JL, Hall PD. Toxicity of volatile anaesthetic agents. Clinics in Anaesthesiology 1985;2(3).

126. Kenna JG, Neuberger J, Williams R. Specific antibodies to halothane-induced liver antigens in halothane-associated hepatitis. Br J Anaesth 1987;59(10).

127. Kenna J, Satoh H, Christ D, Pohl L. Metabolic basis for a drug hypersensitivity: antibodies in sera from patients with halothane hepatitis recognize liver neoantigens that contain the trifluoroacetyl group derived from halothane. J Pharmacol Exp Ther 1988;245(3).

128. Bird G, Williams R. Detection of antibodies to a halothane metabolite hepaten in sera from patients with halothane-associated hepatitis. J Hepatol 1989;9.

129. Davis M, Eddleston A, Neuberger J, Vergani D, Mieli-Vergani G, Williams R. Halothane hepatitis. N Engl J Med 1980;303(19).

130. Furst S, Luedke D, Gaw H, Reich R, Gandolfi A. Demonstration of a cellular immune response in halothane-exposed guinea pigs. Toxicology and Applied Pharmacology 1997;143(2).

131. Lind R, Gandolf iA, Hall P. Covalent binding of oxidative biotransformation intermediates is associated with halothane hepatotoxicity in guinea pigs. Anesthesiology 1990;73(6).

132. You Q, Cheng L, Reilly T, Wegmann D, Ju C. Role of neutrophils in a mouse model of halothane-induced liver injury. Hepatology 2006;44(6).

133. Bourdi M, Amouzadeh H, Rushmore T, Martin J, Pohl L. Halothane-induced liver injury in outbred guinea pigs: role of trifluoracetylated protein adducts in animal susceptibility. Chemical Res Toxicol 2001;14.

134. Cohen EN. Metabolism of halothane-2 14C in the mouse. Anesthesiology 1969;31(6):560-565.

135. Cohen EN, Hood N. Application of low-temperature autoradiography to studies of the uptake and metabolism of volatile anesthetics in the mouse. Anesthesiology 1969;31(6):553-559.

136. McLain G, Sipes I, Brown BJr. An animal model of halothane hepatotoxicity: roles of enzyme induction and hypoxia. Anesthesiology 1979;51(4).

137. Fujii K, Miki N, Sugiyama T, Morio M, Yamano T, Miyake Y. Anaerobic dehalogenation of halothane by reconstituted liver microsomal cytochrome P-450 enzyme system. Biochemical and Biophysical Research Communications 1981;102(1):507-512.

138. Gut J, Christen U, Huwyler J. Mechanisms of halothane toxicity: novel insights. Pharmacol Ther 1993;58(2):133-155.

139. Ahr HJ, King LJ, Nastainczyk W, Ullrich V. The mechanism of reductive dehalogenation of halothane by liver cytochrome P450. Biochemical Pharmacology 1982;31(3):383-390.

140. Ullrich V, Schnabel KH. Formation and binding of carbanions by cytochrome P-450 of liver microsomes. Drug Metab Dispos 1973 Jan;1(1):176-183.

141. Gruenke LD, Konopka K, Koop DR, Waskell LA. Characterization of halothane oxidation by hepatic microsomes and purified cytochromes P-450 using a gas chromatographic mass spectrometric assay. J Pharmacol Exp Ther 1988 Aug;246(2):454-459.

142. Van Dyke RA, Gandolfi AJ. Studies on irreversible binding of radioactivity from (14C)halothane to rat hepatic microsomal lipids and protein. Drug Metab Dispos 1974;2(5):469-476.

143. Huwyler J, Aeschlimann D, Christen U, Gut J. The kidney as a novel target tissue for protein adduct formation associated with metabolism of halothane and the candidate chlorofluorocarbon replacement 2,2-dichloro-1,1,1-trifluoroethane. Eur J Biochem 1992 Jul 1;207(1):229-238.

144. Stier A. Trifluoroacetic acid as metabolite of halothane. Biochem Pharmacol 1964;13:1544.

145. Kharasch ED, Hankins D, Mautz D, Thummel KE. Identification of the enzyme responsible for oxidative halothane metabolism: implications for prevention of halothane hepatitis. Lancet 1996 May 18;347(9012):1367-1371.

146. Spracklin DK, Emery ME, Thummel KE, Kharasch ED. Concordance between trifluoroacetic acid and hepatic protein trifluoroacetylation after disulfiram

inhibition of halothane metabolism in rats. Acta Anaesthesiol Scand 2003 Jul;47(6):765-770.

147. Kenna JG, Neuberger J, Williams R. Identification by immunoblotting of three halothane-induced liver microsomal polypeptide antigens recognized by antibodies in sera from patients with halothane-associated hepatitis. J Pharmacol Exp Ther 1987 Aug;242(2):733-740.

148. Kenna JG, Satoh H, Christ DD, Pohl LR. Metabolic basis for a drug hypersensitivity: antibodies in sera from patients with halothane hepatitis recognize liver neoantigens that contain the trifluoroacetyl group derived from halothane. J Pharmacol Exp Ther 1988;245(3).

149. Chen M, Gandolfi A. Characterization of the humoral immune response and hepatotoxicity after multiple halothane exposures in guinea pigs. Drug Metab Rev 1997;29(1-2).

150. Amouzadeh HR, Pohl LR. Processing of endoplasmic reticulum luminal antigens associated with halothane hepatitis in rat hepatocytes. Hepatotology 1995;22(3):936-943.

151. Satoh H, Gillette JR, Davies HW, Schulick RD, Pohl LR. Immunochemical evidence of trifluoroacetylated cytochrome P-450 in the liver of halothane-treated rats. Mol Pharmacol 1985;28(5):468-474.

152. Hastings K, Thomas C, Brown A, Gandolfi A. Trifluoroacetylation potentiates the humoral immune response to halothane in the guinea pig. Immunopharmacology and Immunotoxicology 1995;17(1).

153. Mathieu A, DiPadua D, Kahan B, Galdabini J, Mills J. Correlation between specific immunity to a metabolite of halothane and hepatic lesions after multiple exposures. Anesth Analg 1975;54(3):332-339.

154. Lind R, Gandolfi A, Hall P. Glutathione depletion enhances subanesthetic halothane hepatotoxicity in guinea pigs. Anesthesiology 1992;77(4).

155. Lind R, Gandolfi A, Hall P. The role of oxdative biotransformation of halothane in guinea pig model of halothane-associated hepatotoxicity. Anesthesiology 1989;70(4).

156. Lunam CA, Hall PM, Cousins MJ. The pathology of halothane hepatotoxicity in a guinea-pig model: a comparison with human halothane hepatitis. Br J Exp Pathol 1989 Oct;70(5):533-541.

157. Farrell G, Prendergast D, Murray M. Halothane hepatitis. Detection of a constitutional susceptibility factor. N Engl J Med 1985;313(21).

158. Anders MW, Pohl LR. Halogenated alkanes, biotransformation of foreign compounds. Academic Press, 1985.

159. Shingu K, Eger EI, Johnson BH. Hypoxia may be more important than reductive metabolism in halothane-induced hepatic injury. Anesth Analg 1982 Oct;61(10):824-827.

160. Shingu K, Eger EI, Johnson BH. Hypoxia per se can produce hepatic damage without death in rats. Anesth Analg 1982 Oct;61(10):820-823.

161. Moult PA, Sherlock S. Halothane-related hepatitis: A clinical study of twenty-six cases. QJM 1975;44(1):99-114.

162. Cheng L, You Q, Yin H, Holt M, Franklin C, Ju C. Effect of polyI:C cotreatment on halothane-induced liver injury in mice. Hepatology 2009;49(1).

163. Cheng L, You Q, Yin H, Holt MP, Ju C. Involvement of natural killer T cells in halothane-induced liver injury in mice. Biochem Pharmacol 2010 Jul 15;80(2):255-261.

164. Hughes HC, Jr., Lang CM. Hepatic necrosis produced by repeated administration of halothane to guinea pigs. Anesthesiology 1972 May;36(5):466-471.

165. Lunam CA, Cousins MJ, de la Hall PM. Genetic predisposition to liver damage after halothane anesthesia in guinea pigs. Anesth Analg 1986;65(11):1143-1148.

166. Njoku D, Greenberg R, Bourdi M, Borkowf C, Dake E, Martin J, et al. Autoantibodies associated with volatile anesthetic hepatitis found in the sera of a large cohort of pediatric anesthesiologists. Anesth Analg 2002;94(2):243-249.

167. Laskin C, Vidins E, Blendis L, Soloninka C. Autoantibodies in alcoholic liver disease. The American journal of medicine 1990;89(2):129-133.

168. Niemela O, Klajner F, Orrego H, Vidins E, Blendis L, Israel Y. Antibodies against acetaldehyde-modified protein epitopes in human alcoholics. Hepatology 1987 Nov;7(6):1210-1214.

169. Zimmerman HJ. Drug-induced liver disease. Clin Liver Dis 2000 Feb;4(1):73-96.

170. Drici MD, Clement N. Is gender a risk factor for adverse drug reactions? The example of drug-induced long QT syndrome. Drug Saf 2001;24(8):575-585.

171. Klein U, Klein M, Sturm H, Rothenbuhler M, Huber R, Stucki P, et al. The frequency of adverse drug reactions as dependent upon age, sex and duration of hospitalization. Int J Clin Pharmacol Biopharm 1976 Apr;13(3):187-195.

172. Domecq C, Naranjo CA, Ruiz I, Busto U. Sex-related variations in the frequency and characteristics of adverse drug reactions. Int J Clin Pharmacol Ther Toxicol 1980;18(8):362-366.

173. Bell LN, Chalasani N. Epidemiology of idiosyncratic drug-induced liver injury. Semin Liver Dis 2009 Nov;29(4):337-347.

174. Larrey D. Epidemiology and individual susceptibility to adverse drug reactions affecting the liver. Semin Liver Dis 2002;22(2):145-155.

175. de Abajo FJ, Montero D, Madurga M, Garcia Rodriguez LA. Acute and clinically relevant drug-induced liver injury: a population based case-control study. Br J Clin Pharmacol 2004 Jul;58(1):71-80.

176. Becker PU, Deis A, Sorensen TI, Gronbaek MN, Borch-Johnsen K, Muller CF, et al. Alcohol intake and risk of liver disease--significance of gender. A population study. Ugeskr Laeger 1997;159(24).

177. Lucena MI, Andrade RJ, Kaplowitz N, Garcia-Cortes M, Fernandez MC, Romero-Gomez M, et al. Phenotypic characterization of idiosyncratic drug-induced liver injury: the influence of age and sex. Hepatology 2009 Jun;49(6):2001-2009.

178. Hildebrandt MG, Steyerberg EW, Stage KB, Passchier J, Kragh-Soerensen P. Are gender differences important for the clinical effects of antidepressants? Am J Psychiatry 2003 Sep;160(9):1643-1650.

179. Brennan IM, Feltrin KL, Nair NS, Hausken T, Little TJ, Gentilcore D, et al. Effects of the phases of the menstrual cycle on gastric emptying, glycemia, plasma GLP-1 and insulin, and energy intake in healthy lean women. Am J Physiol Gastrointest Liver Physiol 2009 Sep;297(3):G602-G610.

180. Caballero-Plasencia AM, Valenzuela-Barranco M, Martin-Ruiz JL, Herrerias-Gutierrez JM, Esteban-Carretero JM. Are there changes in gastric emptying during the menstrual cycle? Scand J Gastroenterol 1999 Aug;34(8):772-776.

181. Wald A, Van Thiel DH, Hoechstetter L, Gavaler JS, Egler KM, Verm R, et al. Gastrointestinal transit: the effect of the menstrual cycle. Gastroenterology 1981 Jun;80(6):1497-1500.

182. Frezza M, di PC, Pozzato G, Terpin M, Baraona E, Lieber CS. High blood alcohol levels in women. The role of decreased gastric alcohol dehydrogenase activity and first-pass metabolism. N Engl J Med 1990 Jan 11;322(2):95-99.

183. Lucena MI, Andrade RJ, Martinez C, Ulzurrun E, Garcia-Martin E, Borraz Y, et al. Glutathione S-transferase m1 and t1 null genotypes increase susceptibility to idiosyncratic drug-induced liver injury. Hepatology 2008 Aug;48(2):588-596.

184. Watanabe M, Tanaka M, Tateishi T, Nakura H, Kumai T, Kobayashi S. Effects of the estrous cycle and the gender difference on hepatic drug-meabolising enzyme activities. Pharmacological Research 1997;35(5):477-480.

185. Shapiro BH. Sexually dimorphic response of rat hepatic monooxygenases to low-dose phenobarbital. Biochem Pharmacol 1986 May 15;35(10):1766-1768.

186. Wilson K, Oram M, Horth CE, Burnett D. The influence of the menstrual cycle on the metabolism and clearance of methaqualone. Br J Clin Pharmacol 1982 Sep;14(3):333-339.

187. Giudicelli JF, Tillement JP. Influence of sex on drug kinetics in man. Clin Pharmacokinet 1977 May;2(3):157-166.

188. Yonkers KA, Kando JC, Cole JO, Blumenthal S. Gender differences in pharmacokinetics and pharmacodynamics of psychotropic medication. Am J Psychiatry 1992 May;149(5):587-595.

189. Anderson GD. Gender differences in pharmacological response. Int Rev Neurobiol 2008;83:1-10.

190. Kulscar-Gergely J, Kulcsar A, Gomba S. Estrogens as disposing factors in experimental liver injury. Exp Pathol (Jena) 1978;16(1-6):283-290.

191. Harada H, Bharwani S, Pavlick KP, Korach KS, Grisham MB. Estrogen receptor-alpha, sexual dimorphism and reduced-size liver ischemia and reperfusion injury in mice. Pediatr Res 2004 Mar;55(3):450-456.

192. Liddle C, Farrell GC. Role of the oestrogen receptor in liver regeneration in the male rat. J Gastroenterol Hepatol 1993 Nov;8(6):524-529.

193. Baum JK, Bookstein JJ, Holtz F, Klein EW. Possible association between benign hepatomas and oral contraceptives. Lancet 1973 Oct 27;2(7835):926-929.

194. Neuberger J, Portmann B, Nunnerley HB, Laws JW, Davis M, Williams R. Oral-contraceptive-associated liver tumours: occurrence of malignancy and difficulties in diagnosis. Lancet 1980 Feb 9;1(8163):273-276.

195. Montalto G, Cervello M, Giannitrapani L, Dantona F, Terranova A, Castagnetta LA. Epidemiology, risk factors, and natural history of hepatocellular carcinoma. Ann N Y Acad Sci 2002 Jun;963:13-20.

196. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 2007 Jul 6;317(5834):121-124.

197. Yin M, Ikejima K, Wheeler M, Bradford B, Seabra V, Forman DT, et al. Estrogen is involved in early alcohol-induced liver injury in a rat enteral feeding model. Hepatology 2000;31(1):117-123.

198. Nanji A, Jokelainen K, Fotouhinia M, Rahemtulla A, Thomas P, Tipoe GL, et al. Increased severity of alcoholic liver injury in female rats: role of oxidative stress, endotoxin, and chemokines. Am J Physiol Gastrointest Liver Physiol 2001;281(6):G1348-G1356.

199. Faulkner L, Altmann DM, Ellmerich S, Huhtaniemi I, Stamp G, Sriskandan S. Sexual Dimorphism in Superantigen Shock Involves Elevated TNF-{alpha} and TNF-{alpha} induced Hepatic Apoptosis. Am J Respir Crit Care Med 2007;176(5):473-482.

200. Zuk M, McKean KA. Sex differences in parasite infections: patterns and processes. Int J Parasitol 1996 Oct;26(10):1009-1023.

201. Wichmann MW, Zellweger R, DeMaso CM, Ayala A, Chaudry IH. Enhanced immune responses in females as opposed to decreased responses in males following hemorrhagic shock. Cytokine 1996;8(11):853-863.

202. McGowan JE, Jr., Barnes MW, Finland M. Bacteremia at Boston City Hospital: Occurrence and mortality during 12 selected years (1935-1972), with special reference to hospital-acquired cases. J Infect Dis 1975 Sep;132(3):316-335.

203. Olsen NJ, Kovacs WJ. Gonadal steroids and immunity. Endocr Rev 1996 Aug;17(4):369-384.

204. Hirasawa K, Enosawa S. Sex differences in the prolongation of allogeneic skin graft survival in rats treated with cyclosporin A: effects of orchiectomy and pregnancy. Transplant Proc 1989 Feb;21(1 Pt 1):881-884.

205. Graff RJ, Lappe MA, Snell GD. The influence of the gonads and adrenal glands on the immune response to skin grafts. Transplantation 1969 Feb;7(2):105-111.

206. Graff RJ, Hildemann WH, Snell GD. Histocompatibility genes of mice. VI. Allografts in mice congenic at various non-H-2 histocompatibility loci. Transplantation 1966 Jul;4(4):425-437.

207. McCashland TM, Brand R, Lyden E, de GP. Gender differences in colorectal polyps and tumors. Am J Gastroenterol 2001 Mar;96(3):882-886.

208. Woldrich JM, Mallin K, Ritchey J, Carroll PR, Kane CJ. Sex differences in renal cell cancer presentation and survival: an analysis of the National Cancer Database, 1993-2004. J Urol 2008 May;179(5):1709-1713.

209. Bosch FX, Ribes J, Cleries R, Diaz M. Epidemiology of hepatocellular carcinoma. Clin Liver Dis 2005 May;9(2):191-211, v.

210. Grossman C. Possible underlying mechanisms of sexual dimorphism in the immune response, fact and hypothesis. J Steroid Biochem 1989;34(1-6):241-251.

211. Timmons BW, Hamadeh MJ, Devries MC, Tarnopolsky MA. Influence of gender, menstrual phase, and oral contraceptive use on immunological changes in response to prolonged cycling. J Appl Physiol 2005 Sep;99(3):979-985.

212. Yovel G, Shakhar K, Ben-Eliyahu S. The effects of sex, menstrual cycle, and oral contraceptives on the number and activity of natural killer cells. Gynecol Oncol 2001 May;81(2):254-262.

213. Evans MJ, Eckert A, Lai K, Adelman SJ, Harnish DC. Reciprocal Antagonism Between Estrogen Receptor and NF-{kappa}B Activity In Vivo. Circ Res 2001;89(9):823-830.

214. Gabrilovac J, Zadjelovic J, Osmak M, Suchanek E, Zupanovic Z, Boranic M. NK cell activity and estrogen hormone levels during normal human pregnancy. Gynecol Obstet Invest 1988;25(3):165-172.

215. Gilliver SC. Sex steroids as inflammatory regulators. J Steroid Biochem Mol Biol 2010 May 31;120(2-3):105-115.

216. You HJ, Kim JY, Jeong HG. 17 beta-estradiol increases inducible nitric oxide synthase expression in macrophages. Biochem Biophys Res Commun 2003 Apr 18;303(4):1129-1134.

217. Robert R, Spitzer JA. Effects of female hormones (17beta-estradiol and progesterone) on nitric oxide production by alveolar macrophages in rats. Nitric Oxide 1997;1(6):453-462.

218. Enomoto N, Yamashina S, Schemmer P, Rivera CA, Bradford BU, Enomoto A, et al. Estriol sensitizes rat Kupffer cells via gut-derived endotoxin. Am J Physiol 1999 Sep;277(3 Pt 1):G671-G677.

219. Vegeto E, Ghisletti S, Meda C, Etteri S, Belcredito S, Maggi A. Regulation of the lipopolysaccharide signal transduction pathway by 17beta-estradiol in macrophage cells. J Steroid Biochem Mol Biol 2004 Jun;91(1-2):59-66.

220. Huang H, He J, Yuan Y, Aoyagi E, Takenaka H, Itagaki T, et al. Opposing effects of estradiol and progesterone on the oxidative stress-induced production of chemokine and proinflammatory cytokines in murine peritoneal macrophages. J Med Invest 2008 Feb;55(1-2):133-141.

221. Evans MJ, Eckert A, Lai K, Adelman SJ, Harnish DC. Reciprocal antagonism between estrogen receptor and NF-kappaB activity in vivo. Circ Res 2001 Oct 26;89(9):823-830.

222. Deshpande R, Khalili H, Pergolizzi RG, Michael SD, Chang MD. Estradiol down-regulates LPS-induced cytokine production and NFkB activation in murine macrophages. Am J Reprod Immunol 1997 Jul;38(1):46-54.

223. Hou X, Zhou R, Wei H, Sun R, Tian Z. NKG2D-retinoic acid early inducible-1 recognition between natural killer cells and Kupffer cells in a novel murine natural killer cell-dependent fulminant hepatitis. Hepatology 2009;49(3).

224. Moriyama I, Sugawa T. Progesterone facilitates implantation of xenogenic cultured cells in hamster uterus. Nat New Biol 1972 Apr 5;236(66):150-152.

225. Hansen PJ, Bazer FW, Segerson EC, Jr. Skin graft survival in the uterine lumen of ewes treated with progesterone. Am J Reprod Immunol Microbiol 1986 Oct;12(2):48-54.

226. Siiteri PK, Febres F, Clemens LE, Chang RJ, Gondos B, Stites D. Progesterone and maintenance of pregnancy: is progesterone nature's immunosuppressant? Ann N Y Acad Sci 1977 Mar 11;286:384-397.

227. Gomez O, Borras A, Rabanal A, Palacio M, Carceller A, Coll O, et al. Mifepristone-misoprostol midtrimester abortion: impact of gestational age on the induction-to-abortion interval. Contraception 2010 Feb;81(2):97-101.

228. Check JH, Sansoucie L, Chern J, Amadi N, Srivastava M, Larece K. Evidence that progesterone receptor antagonists may help in the treatment of a variety of cancers by locally suppressing natural killer cell activity. Clin Exp Obstet Gynecol 2007;34(4):207-211.

229. Szekeres-Bartho J, Faust Z, Varga P, Szereday L, Kelemen K. The immunological pregnancy protective effect of progesterone is manifested via controlling cytokine production. Am J Reprod Immunol 1996 Apr;35(4):348-351.

230. Srivastava MD, Thomas A, Srivastava BI, Check JH. Expression and modulation of progesterone induced blocking factor (PIBF) and innate immune factors in human leukemia cell lines by progesterone and mifepristone. Leuk Lymphoma 2007 Aug;48(8):1610-1617.

231. Li AP. A review of the common properties of drugs with idiosyncratic hepatotoxicity and the "multiple determinant hypothesis" for the manifestation of idiosyncratic drug toxicity. Chemico-Biological Interactions 2002;142(1-2).

232. Eghtesadi-Araghi P, Sohrabpour A, Vahedi H, Saberi-Firoozi M. Halothane hepatitis in Iran: A review of 59 cases. World J Gastroenterol 2008;14(34).

233. Berger D, Bölke E, Seidelmann M, Beger H. Time-scale of interleukin-6, myeloid related proteins (MRP), C reactive protein (CRP), and endotoxin plasma levels during the postoperative acute phase reaction. Shock 1997;7(6).

234. Hastings KL, Thomas C, Brown AP, Gandolfi AJ. Trifluoroacetylation potentiates the humoral immune response to halothane in the guinea pig. Immunopharmacology and Immunotoxicology 1995;17(1):201-213.

235. Lind RC, Gandolfi AJ, Hall P. Glutathione depletion enhances subanesthetic halothane hepatotoxicity in guinea pigs. Anesthesiology 1992;77(4).

236. Lind RC, Gandolfi AJ, Brown BR, Hall P. Halothane hepatotoxicity in guinea pigs. Anesth Analg 1987;66(3).

237. Lillie RD, Ashburn LL. Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degeneration not shown by Herxheimer's technique. Archs Path 1943;36.

238. Yee SB, Hanumegowda UM, Hotchkiss JA, Ganey PE, Roth RA. Role of neutrophils in the synergistic liver injury from monocrotaline and bacterial lipopolysaccharide exposure. Toxicol Sci 2003 Mar;72(1):43-56.

239. Hussey AJ, Aldridge LM, Paul D, Ray DC, Beckett GJ, Allan LG. Plasma glutathione S-transferase concentrations as a measure of hepatocellular integrity following a single general anaesthetic with halothane, enflurane or isoflurane. Br J Anaesth 1988;60(2).

240. Deng X, Luyendyk JP, Ganey PE, Roth RA. Inflammatory stress and idiosyncratic hepatotoxicity: hints from animal models. Pharmacol Rev 2009;61(3).

241. Ong MMK, Latchoumycandane C, Boelsterli U. Troglitazone-induced hepatic necrosis in an animal model of silent genetic mitochondrial abnormalities. Toxicol Sci 2007;97(1):205-213.

242. Walton B, Hamblin A, Dumonde DC, Simpson R. Absence of cellular hypersensitivity in patients with unexplained hepatitis following halothane. Anesthesiology 1976;44(5):391-397.

243. Pohl LR, Satoh H, Christ DD, Kenna JG. The immunologic and metabolic basis of drug hypersensitivities. Annual Review of Pharmacology and Toxicology 1988;28(1):367-387.

244. Walton B, Simpsn BR, Strunin L, Doniach D, Perrin J, Appleyard AJ. Unexplained hepatits following halothane. British Medical Journal 1976;1:1171-1176.

245. Weber P, Scheurlen M, Irkin I, Viebahn R, Schareck W, Becker W, et al. Liver transplantation in halothane-induced lliver necrosis. Zentralbl Chir 1994;119(5):305-308.

246. Eghtesadi-Araghi P, Sohrabpour A, Vahedi H, Saberi-Firoozi M. Halothane hepatitis in Iran: A review of 59 cases. World J Gastroenterol 2008;14(34).

247. Chen M, Gandolfi AJ. Characterization of the humoral immune response and hepatotoxicity after multiple halothane exposures in guinea pigs. Drug Metab Rev 1997;29(1-2).

248. You Q, Cheng L, Reilly T, Wegmann D, Ju C. Role of neutrophils in a mouse model of halothane-induced liver injury. Hepatology 2006;44(6):1421-1431.

249. Carney FMT, Van Dyke RA. Halothane hepatitis: a critical review. Anesth Analg 1972;51(1).

250. Warner LO, Beach TP, Garvin JP, Warner EJ. Halothane and children: The first quarter century. Anesth Analg 1984;63(9):838-840.

251. Hoft R, Bunker J, Goodman H, Gregory P. Halothane Hepatitis in 3 pairs of closely related women. N Enj J Med 1981;304(17):1023-1024.

252. Otsuka S, Yamamoto M, Kasuya S, Ohtomo H, Yamamoto Y, Yoshida T, et al. HLA antigens in patients with unexplained hepatitis following halothane anesthesia. Acta Anaesthesiol Scand 1985;29(5):497-501.

253. Gourlay GK, Adams JF, Cousins MJ, Hall P. Genetic differences in reductive metabolism and hepatotoxicity of halothane in three rat strains. Anesthesiology 1981;55(2).

254. Nasseri M, Modabber FZ. Generalized infection and lack of delayed hypersensitivity in BALB/c mice infected with Leishmania tropica major. Infect Immun 1979 Nov;26(2):611-614.

255. Bryan MA, Guyach SE, Norris KA. Specific humoral immunity versus polyclonal B cell activation in Trypanosoma cruzi infection of susceptible and resistant mice. PLoS Negl Trop Dis 2010;4(7):e733.

256. Gosselin M, Goulet S, Wu-Wong JR, Wessale JL, Opgenorth TJ, Boulet LP, et al. Effects of a selective ET(A)-receptor antagonist, atrasentan (ABT-627), in murine models of allergic asthma: demonstration of mouse strain specificity. Clin Sci (Lond) 2002 Aug;103 Suppl 48:367S-370S.

257. Walker RM, Massey TE, McElligott TF, Racz WJ. Acetaminophen toxicity in fed and fasted mice. Can J Physiol Pharmacol 1982 Mar;60(3):399-404.

258. Marriott I, Huet-Hudson Y. Sexual dimorphism in innate immune responses to infectious organisms. Immunologic Research 2006;34(3):177-192.

259. Lind RC, Gandolfi AJ, Sipes G, Brown B. The Involvement of endotoxin in the halothane-associated liver injury. Anesthesiology 1984;61.

260. Farrell GC, Frost L, Tapner M, Field J, Weltman M, Mahoney J. Halothane-induced liver injury in guinea-pigs: importance of cytochrome P450 enzyme activity and hepatic blood flow. J Gastroenterol Hepatol 1996 Jun;11(6):594-601.

261. Chalasani N, Bjornsson E. Risk factors for idiosyncratic drug-induced liver injury. Gastroenterology 2010 Jun;138(7):2246-2259.

262. Dugan CM, MacDonald AE, Roth RA, Ganey PE. A mouse model of severe halothane hepatitis based on human risk factors. J Pharmacol Exp Ther 2010 May;333(2):364-372.

263. Curran EM, Berghaus LJ, Vernetti NJ, Saporita AJ, Lubahn DB, Estes DM. Natural killer cells express estrogen receptor-alpha and estrogen receptor-beta and can respond to estrogen via a non-estrogen receptor-alpha-mediated pathway. Cell Immunol 2001 Nov 25;214(1):12-20.

264. Offner H, Adlard K, Zamora A, Vandenbark AA. Estrogen potentiates treatment with T-cell receptor protein of female mice with experimental encephalomyelitis. J Clin Invest 2000 May;105(10):1465-1472.

265. Nemeth E, Baird AW, O'Farrelly C. Microanatomy of the liver immune system. Semin Immunopathol 2009 Sep;31(3):333-343.

266. Liu ZX, Kaplowitz N. Role of innate immunity in acetaminophen-induced hepatotoxicity. Expert Opin Drug Metab Toxicol 2006 Aug;2(4):493-503.

267. Masson MJ, Carpenter LD, Graf ML, Pohl LR. Pathogenic role of natural killer T and natural killer cells in acetaminophen-induced liver injury in mice is dependent on the presence of dimethyl sulfoxide. Hepatology 2008 Sep;48(3):889-897.

268. Satoh H, Fukuda Y, Anderson DK, Ferrans VJ, Gillette JR, Pohl LR. Immunological studies on the mechanism of halothane-induced hepatotoxicity: immunohistochemical evidence of trifluoroacetylated hepatocytes. J Pharmacol Exp Ther 1985;233(3):857-862.

269. Goldman JM, Murr AS, Cooper RL. The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. Birth Defects Res B Dev Reprod Toxicol 2007 Apr;80(2):84-97.

270. Renton KW. Methyl red azo-reductase and its induction by 3-methylcholanthrene in the liver by different species. Xenobiotica 1980 Apr;10(4):243-246.

271. Roederer M, Hardy RR. Frequency difference gating: a multivariate method for identifying subsets that differ between samples. Cytometry 2001 Sep 1;45(1):56-64.

272. Roederer M, Moore W, Treister A, Hardy RR, Herzenberg LA. Probability binning comparison: a metric for quantitating multivariate distribution differences. Cytometry 2001 Sep 1;45(1):47-55.

273. van Zoelen MA, Yang H, Florquin S, Meijers JC, Akira S, Arnold B, et al. Role of toll-like receptors 2 and 4, and the receptor for advanced glycation end products in high-mobility group box 1-induced inflammation in vivo. Shock 2009;31(3):280-284.

274. Smyth MJ, Cretney E, Takeda K, Wiltrout RH, Sedger LM, Kayagaki N, et al. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. J Exp Med 2001 Mar 19;193(6):661-670.

275. Berke G. The CTL's kiss of death. Cell 1995 Apr 7;81(1):9-12.

276. Inman W, Mushin W. Jaundice after repeated exposure to halothane: a further analysis of reports to the Committee on Safety of Medicines. Br Med J 1978;25(2).

277. Moult P, Sherlock S. Halothane-Related Hepatitis: A Clinical Study of Twenty-Six Cases. QJM 1975;44(1).

278. Pohl L, Satoh H, Christ D, Kenna J. The Immunologic and Metabolic Basis of Drug Hypersensitivities. Annual Review of Pharmacology and Toxicology 1988;28(1).

279. Masson MJ, Collins LA, Carpenter LD, Graf ML, Ryan PM, Bourdi M, et al. Pathologic role of stressed-induced glucocorticoids in drug-induced liver injury in mice. Biochem Biophys Res Commun 2010 Jul 2;397(3):453-458.

280. Hao S, Zhao J, Zhou J, Zhao S, Hu Y, Hou Y. Modulation of 17beta-estradiol on the number and cytotoxicity of NK cells in vivo related to MCM and activating receptors. Int Immunopharmacol 2007 Dec 15;7(13):1765-1775.

281. Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol 1993;11:571-611.

282. Kellokumpu-Lehtinen P, Iisalo E, Nordman E. Hepatotoxicity of paracetamol in combination with interferon and vinblastine. Lancet 1989 May 20;1(8647):1143.

283. Shi H, Guan SH. Increased apoptosis in HepG2.2.15 cells with hepatitis B virus expression by synergistic induction of interferon-gamma and tumour necrosis factor-alpha. Liver Int 2009 Mar;29(3):349-355.

284. Toyonaga T, Hino O, Sugai S, Wakasugi S, Abe K, Shichiri M, et al. Chronic active hepatitis in transgenic mice expressing interferon-gamma in the liver. Proc Natl Acad Sci U S A 1994 Jan 18;91(2):614-618.

285. Ishida Y, Kondo T, Ohshima T, Fujiwara H, Iwakura Y, Mukaida N. A pivotal involvement of IFN-gamma in the pathogenesis of acetaminophen-induced acute liver injury. FASEB J 2002 Aug;16(10):1227-1236.

286. Tagawa Y, Sekikawa K, Iwakura Y. Suppression of concanavalin A-induced hepatitis in IFN-gamma(-/-) mice, but not in TNF-alpha(-/-) mice: role for IFN-gamma in activating apoptosis of hepatocytes. J Immunol 1997 Aug 1;159(3):1418-1428.

287. Renton KW, Deloria LB, Mannering GJ. Effects of polyribonoinosinic acid polyribocytidylic acid and a mouse interferon preparation on cytochrome P-450-dependent monooxygenase systems in cultures of primary mouse hepatocytes. Mol Pharmacol 1978 Jul;14(4):672-681.

288. Gao B. Cytokines, STATs and liver disease. Cell Mol Immunol 2005 Apr;2(2):92-100.

289. Uesugi T, Froh M, Arteel GE, Bradford BU, Thurman RG. Toll-like receptor 4 is involved in the mechanism of early alcohol-induced liver injury in mice. Hepatology 2001 Jul;34(1):101-108.

290. Yohe HC, O'Hara KA, Hunt JA, Kitzmiller TJ, Wood SG, Bement JL, et al. Involvement of Toll-like receptor 4 in acetaminophen hepatotoxicity. Am J Physiol Gastrointest Liver Physiol 2006 Jun;290(6):G1269-G1279.

291. Gregoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, et al. The trafficking of natural killer cells. Immunol Rev 2007 Dec;220:169-182.

292. Heinzel FP, Sadick MD, Holaday BJ, Coffman RL, Locksley RM. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. J Exp Med 1989 Jan 1;169(1):59-72.

293. Habu S, Fukui H, Shimamura K, Kasai M, Nagai Y, Okumura K, et al. In vivo effects of anti-asialo GM1. I. Reduction of NK activity and enhancement of transplanted tumor growth in nude mice. J Immunol 1981 Jul;127(1):34-38.

294. Kasai M, Iwamori M, Nagai Y, Okumura K, Tada T. A glycolipid on the surface of mouse natural killer cells. Eur J Immunol 1980 Mar;10(3):175-180.

295. Liu ZX, Govindarajan S, Okamoto S, Dennert G. NK cells cause liver injury and facilitate the induction of T cell-mediated immunity to a viral liver infection. J Immunol 2000 Jun 15;164(12):6480-6486.

296. Harrill AH, Watkins PB, Su S, Ross PK, Harbourt DE, Stylianou IM, et al. Mouse population-guided resequencing reveals that variants in CD44 contribute to acetaminophen-induced liver injury in humans. Genome Res 2009 Sep;19(9):1507-1515.

297. Furst SM, Luedke D, Gaw HH, Reich R, Gandolfi AJ. Demonstration of a cellular immune response in halothane-exposed guinea pigs. Toxicology and Applied Pharmacology 1997;143(2).

298. Valente G, Ozmen L, Novelli F, Geuna M, Palestro G, Forni G, et al. Distribution of interferon-gamma receptor in human tissues. Eur J Immunol 1992 Sep;22(9):2403-2412.

299. Winnike JH, Li Z, Wright FA, Macdonald JM, O'Connell TM, Watkins PB. Use of pharmaco-metabonomics for early prediction of acetaminophen-induced hepatotoxicity in humans. Clin Pharmacol Ther 2010 Jul;88(1):45-51.

300. Wetmore BA, Brees DJ, Singh R, Watkins PB, Andersen ME, Loy J, et al. Quantitative analyses and transcriptomic profiling of circulating messenger RNAs as biomarkers of rat liver injury. Hepatology 2010 Jun;51(6):2127-2139.

301. Lee S, Kim J, Jang B, Hur S, Jung U, Kil K, et al. Fluctuation of peripheral blood T, B, and NK cells during a menstrual cycle of normal healthy women. J Immunol 2010 Jul 1;185(1):756-762.

302. Vassiliadou N, Bulmer JN. Immunohistochemical evidence for increased numbers of 'classic' CD57+ natural killer cells in the endometrium of women suffering spontaneous early pregnancy loss. Hum Reprod 1996 Jul;11(7):1569-1574.

303. Gendron RL, Baines MG. Infiltrating decidual natural killer cells are associated with spontaneous abortion in mice. Cell Immunol 1988 May;113(2):261-267.