Insights into the Mechanism of Idiosyncratic Drug-Induced Agranulocytosis

by

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

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Abstract

Many drugs are known to cause idiosyncratic drug-induced agranulocytosis, although the mechanism is not well understood. This adverse event is suspected to be the result of the drug’s reactive metabolite binding to endogenous proteins and creating a foreign body capable of eliciting an immune response. In particular, clozapine has been shown to form reactive metabolites by neutrophil myeloperoxidase (MPO). These studies set out to further characterize MPO’s role in bioactivating drugs that cause idiosyncratic drug-induced agranulocytosis and to characterize the immune response seen due to clozapine. Overall this work further characterized the early immune response seen due to clozapine treatment using in vitro and in vivo methods. Through this work we have a better understanding of the immune response that may lead to clozapine-induced agranulocytosis. Understanding the clozapine-induced immune response may provide avenues for early detection of clozapine-induced agranulocytosis and ultimately limit the magnitude of this adverse event.
Acknowledgments

These past years have been some of my most rewarding, insightful, and challenging of my professional career. I would like to first thank my supervisor Dr. Jack Uetrecht, who throughout my time in his lab provided me with invaluable insight and guidance. Thank-you for always having your door open to your office, your home, and your cottage ready to discuss everything from your path to a life in academia to the documentary about the life of Amazing Randi. You have shown me what it means to be a passionate scientist, skeptic, and educator, and from that I have gained an immeasurable respect and wealth of knowledge in a multitude of facets of life that will no-doubt help me in the future. Thank-you very much for providing me with every opportunity to learn and grow in such a positive and rewarding experience.

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<th>Description</th>
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<tr>
<td>α-1-AGP</td>
<td>α-1-acid-glycoprotein</td>
</tr>
<tr>
<td>α-MEM</td>
<td>α-modified Eagle’s medium</td>
</tr>
<tr>
<td>ANC</td>
<td>absolute neutrophil count</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>AQ</td>
<td>amodiaquine</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type collection culture</td>
</tr>
<tr>
<td>BV 421</td>
<td>blue violet 421</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>CLZ</td>
<td>clozapine</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylfumarate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiolthreitol</td>
</tr>
<tr>
<td>EA</td>
<td>ethocrinic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IDR</td>
<td>idiosyncratic drug reaction</td>
</tr>
<tr>
<td>IDIAG</td>
<td>idiosyncratic drug-induced agranulocytosis</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IO</td>
<td>intraosseous</td>
</tr>
<tr>
<td>INF</td>
<td>interferon</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharides</td>
</tr>
<tr>
<td>MDSC</td>
<td>myeloid derived suppressor cell</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NK cell</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>P450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate-13-acetate</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptors</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SSC</td>
<td>side scatter</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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Chapter 1

Introduction
The estimated cost to a pharmaceutical company to bring a drug to market is over $2 billion. Each drug’s cost incorporates its own costs plus all other failed leads during its development. The cost that goes into a medication grows the further along a lead gets. Idiosyncratic drug reactions (IDRs) are rare adverse events for medications, but when they do occur, they are often considered the end for the development of a drug. IDRs are not that prevalent, and as such, are typically not caught in the normal phases of clinical trials where relatively few patients are tested for the risk of adverse events. Understanding the mechanism by which these reactions occur, and better yet, developing assays that determine the risk of developing these adverse events early in a drug development’s process can save a pharmaceutical company time and money; and more importantly, it can save patients from these often life-threatening adverse events.

1.1 Adverse drug reactions

Adverse drug reactions cause an estimated 100,000 deaths a year; in 1994 this made them the 4th-6th leading cause of death in the United States [Lazarou et al., 1998]. A more recent systematic review concluded that 5.3% of their included hospital admissions were related to adverse drug reactions [Kongkaew et al., 2008]. The exact impact of ADRs is not specifically known; however, they are unequivocally recognized as a very serious burden on the healthcare system, both as a financial burden, and more importantly, a healthcare burden as they are a danger to patients. There are several classifications of adverse drug reactions; however, types A and B are the most prevalent [Andrés et al., 2006]. Type A, or augmentation adverse reactions are caused by an exacerbated response to the intended pharmacological profile of a medication. As such, type A reactions are typically predictable and dose-dependent. Type A reactions can usually be prevented and are typically noted early in the drug development process. Drug-induced agranulocytosis caused by cytotoxic chemotherapeutic agents usually falls into the category of a type A adverse reaction. These agents are generally not intended to target the bone marrow;
however, due to their cytotoxic nature, they may do so leading to a type A drug-induced agranulocytosis.

In contrast, type B reactions, commonly referred to as bizarre or IDRs, are specific to an individual. Drug-induced agranulocytosis caused by variety of medications fall into the category of type B adverse reactions. Many agents that have a variety of different pharmacological efficacies may lead to type B drug-induced agranulocytosis, most likely by a range of mechanisms [Johnston & Uetrecht, 2015], which will be explained below.

Although IDRs are not as common as type A reactions, their unpredictable and potentially life-threatening nature makes them very difficult to deal with. Although the incidence of IDRs may be low, with a typical incidence between 1/100 to 1/100,000, given the large number of drugs that can cause IDRs and the number of patients who take these medications, IDRs actually affect many patients [Uetrecht, 2008].

1.2 Idiosyncratic drug reactions (IDRs)

The patient-specific response of IDRs is what makes them so rare and unpredictable making them extremely difficult to detect during clinical trials. Lasser et al. reported that over a 25 year period (1975 – 2000), 10.2% of newly approved drugs were labeled with black box warnings or completely withdrawn from the market due to adverse reactions that were not detected during clinical trials [Lasser et. Al., 2002]. The more we understand about the mechanism of these IDRs the higher the likelihood of finding good predictive measures of identifying IDRs early in the drug development process. However, as we learn more about IDRs, we recognize the increasing mechanistic complexity of these adverse events.
1.2.1 Clinical characteristics of IDRs

There are many different types of IDRs, and although there are certainly variations on the theme, many of their characteristics are quite similar. The onset of the reaction is usually delayed; however, this varies with the IDR and even with the drug. In some cases the onset occurs as much as a month after the drug has been stopped [Agnelli et al., 2009]. Usually there is a rapid onset on rechallenge, but there are many exceptions, especially for mild IDRs. Mild IDRs to a specific drug are always more common than severe IDRs of the same type, and they often resolve despite continued treatment with the drug [Uetrecht & Naisbitt, 2013]. Most patients will not have an IDR to a specific drug at any dose; however, IDRs are not dose independent. Drugs given at a high dose are much more likely to cause IDRs than drugs given at a low dose. Furthermore, a dose can always be found below which even individuals more prone to IDRs will not have an IDR, for instance, drugs given at a daily dose of 10 mg/day or less almost never cause IDRs [Uetrecht, 2008]. An important question is whether the mechanism of the mild IDR is the same as that of the serious IDR. There is increasing evidence that in many cases the mechanisms are the same; for example, mild lumiracoxib-induced liver injury is associated with the same human leukocyte antigen (HLA) as lumiracoxib-induced liver failure [Singer et al., 2010]. If the mechanisms are the same and they are immune mediated, resolution of the mild injury despite continued treatment must involve immune tolerance [Metushi et al., 2014a]. It is likely that many IDRs share similar mechanisms, and a good understanding of any one IDR may help suggest the mechanism of other IDRs.
1.2.2 Evidence that most IDRs are immune mediated

There are several hypotheses for the mechanisms of IDRs; however, because of their idiosyncratic nature, it is very difficult to perform mechanistic studies to test hypotheses. With a few notable exceptions, there is no definitive evidence for a specific mechanism, and the mechanism may be different, especially in detail, for different IDRs. Traditionally, the two main hypotheses for the mechanism of IDRs are an immune mechanism or a toxic mechanism. IDR clinical characteristics, genetic data, and other experimental findings as detailed below support an immune mechanism for most IDRs; however, cell damage may be essential for the induction of the immune response that can lead to IDRs [Séguin & Uetrecht, 2003].

1.2.2.1 General characteristics of IDRs

The idiosyncratic nature of IDRs is most easily explained by an immune mechanism. We readily accept that some individuals are allergic to some agents while most people are not. Delay in onset is characteristic of an immune mediated reaction, presumably because it takes time for the few T cells with the required specificity to proliferate, and for those with the highest affinity to become dominant [Uetrecht & Naisbitt, 2013]. As stated above, the onset of IDRs is typically delayed, usually between 1-6 months, although it varies somewhat with the drug and with the type of IDR [Andersohn et al., 2007]. In contrast, the time to onset on rechallenge is typically shorter. Such a rapid response on rechallenge is indicative of an immune mediated reaction and is the result of memory T cells. However, this shortened delay in onset is not universal, especially for mild IDRs [Warkentin & Kelton, 2001], and lack of an immediate reaction upon rechallenge should not be used as evidence against an immune mechanism. There are IDRs that are clearly immune mediated such as heparin-induced thrombocytopenia that lack immune
memory [Warkentin & Kelton, 2001]. Many IDRs are also associated with eosinophilia, which is characteristic of a drug hypersensitivity reaction.

### 1.2.2.2 Identification of drug associated antibodies

Many IDRs are associated with anti-drug and/or autoantibodies. In the case of penicillin- and other β-lactam-induced allergic reactions and some cases of aminopyrine-induced idiosyncratic drug-induced agranulocytosis (IDIAG), these antibodies mediate the IDR; therefore, the IDR is clearly immune mediated. In other cases such as idiosyncratic halothane- and isoniazid-induced liver injury, the antibodies may not contribute to the liver injury [Vergani et al., 1980; Metushi et al., 2014b]. Even though they may not contribute directly to the injury, the presence of such antibodies clearly indicates that the drug has induced an immune response, and the presence of anti-drug or autoantibodies is consistent with an immune mechanism. However, their presence does not definitively prove an immune mechanism because the antibodies could be a response to the injury rather than being a cause of the injury.

### 1.2.2.3 Positive lymphocyte transformation test

Many IDRs are associated with a positive lymphocyte transformation test [Nyfeler & Pichler, 1997]. This test is performed by incubation of peripheral blood mononuclear cells from a patient with a history of an IDR with the drug believed to have been responsible. If the T cells proliferate in response to the drug it is strong evidence that the IDR was immune mediated. The incidence of a positive test is highest with chemically reactive drugs such as β-lactams, presumably because no bioactivation is required for covalent binding of the drug to proteins. However, the incidence of a positive test with drugs that do require bioactivation is surprisingly high, about 50% [Maria & Victorino, 1997], and this appears to be because the immune response can spread from recognition of drug-modified proteins to recognition of the drug itself.
1.2.2.4 Genetic predisposition

Of course the puzzling aspect of IDRs is that so few patients are affected after exposure to a drug that can cause IDRs. This suggests that genetic factors play a role in predisposing individuals to IDRs. Several studies have found an association between single nucleotide polymorphisms and development of an IDR to a specific drug [Daly, 2012]. The fact that almost all of the strong associations that have been found are with specific HLA genes provides strong evidence that the IDRs involved are immune mediated.

Lieberman et al. found an increased incidence of the HLA haplotypes B38, DR4, and DQW3 in an Ashkenazic Jew population who developed clozapine-induced IDIAG [Lieberman et al., 1990]. Other genetic associations have also been found in Ashkenazic Jews who developed clozapine-induced agranulocytosis to non-HLA genes linked to particular IDRs including genes for the endogenous danger signals; heat shock proteins and TNF-α [Yunis et al., 1995; Ostrousky et al., 2003; Chowdhury et al., 2011]; this provides additional support for an immune mechanism of many IDRs. Genetic components for the occurrence of IDIAG in populations have also been suggested to be, in part, the result of single nucleotide polymorphisms in genes that control the metabolism of drugs [Wadelius et al., 2000; Daly, 2013].

1.2.3 Involvement of reactive metabolites

The mechanism of β-lactam-induced allergic reactions clearly involves the covalent binding of the drug to proteins with the production of IgE antibodies against drug-modified proteins that mediate the allergic reaction. There is a very large amount of circumstantial evidence to support the hypothesis that most, although not all, IDRs are caused by reactive metabolites of drugs rather than by the parent drug [Cho & Uetrecht, 2016]. However, without valid animal models it is very difficult to perform controlled experiments to demonstrate their involvement. One
exception is nevirapine-induced skin rash in which there is a valid animal model that made it possible to conclusively demonstrate that the rash is caused by a reactive benzylic sulfate formed in the skin [Sharma et al., 2013].

It has been demonstrated that the risk that a drug will cause idiosyncratic liver injury is related to the “body burden” of the reactive metabolite, which is determined by the total daily dose of the drug times the amount of covalent binding to hepatic microsomes at a fixed concentration of the drug [Obach et al., 2008; Nakayama et al., 2009]. The formation of these metabolites is often implicated in inducing toxicity, usually by means of protein interactions in the form of direct covalent modification [Uetrecht, 2003]. Protein modification can lead to immune activation and initiation of cellular apoptosis or necrosis. There are exceptions; for example, abacavir forms a reactive metabolite, but the current data suggests that this reactive metabolite is not involved in abacavir-induced hypersensitivity reactions [Norcross et al., 2012].

1.2.4 Proposed mechanisms of IDRs

There are currently two main mechanisms that have been used to explain immune mediated IDRs. These two hypotheses are the hapten hypothesis and the danger hypothesis and are depicted in Figure 1-1 [Uetrecht, 1999]. These hypotheses are not mutually exclusive, and both hypotheses are required to explain a strong immune response. These two hypotheses describe two signals, which are known to be required for an immune response; if only one signal is present tolerance is induced [Lafferty & Gazda, 1997; Miller & Basten, 1996].

1.2.4.1 Hapten hypothesis

The classic hypothesis for how a drug can induce an immune response is the hapten hypothesis. First reported in 1935, Landsteiner found that in order for a small molecule to induce an immune response it must first be bound to proteins [Landsteiner & Jacobs, 1935]. Since most molecules
are chemically inert, it is likely that they must first form an electrophilic reactive metabolite and covalently bind to an endogenous protein in order to produce a molecule of sufficient size to induce an immune reaction. Small molecules that modify proteins and lead to an immune response are known as haptens. According to the hapten hypothesis, hapten-modified proteins are taken up by antigen presenting cells (APCs), processed into peptide fragments, and presented via the major histocompatibility complex (MHC) to T cells. These hapten-modified peptides would not have been encountered by the immune system previously and would be considered “foreign” or “non-self”. In contrast, T cells that have a strong affinity for endogenous proteins are generally deleted during their development in the thymus. The interaction between the T cell receptor and the hapten-modified peptide presented by APCs constitutes signal 1; when combined with signal 2 (described below) this can lead to activation of T cells and produce an immune response.

The first IDR whose mechanism clearly fits the hapten hypothesis was that of penicillin-induced hemolytic anemia [Parker et al., 1962]. Penicillin, as stated previously, is a chemically reactive β-lactam that does not require metabolic activation in order to covalently bind to proteins. Penicillin-induced allergic reactions are clearly consistent with the hapten hypothesis because they are mediated by IgE antibodies that recognize penicillin-modified proteins [Parker, 1962].

1.2.4.2 Danger hypothesis

Although the hapten hypothesis is clearly involved in some IDRs, it is not without its limitations. In 1994 Matzinger proposed that the immune system’s main function was not to differentiate self from non-self, as implied by the hapten hypothesis, but rather to detect danger [Matzinger, 1994]. This is a fundamental difference in what induces an immune response.
In 2000 Anderson and Matzinger provided a detailed outline of the danger hypothesis and how it relates to the hapten hypothesis [Anderson & Matzinger, 2000]. The danger hypothesis describes signal 2, which is a co-stimulatory interaction between molecules such as, but not limited to, B7 on antigen presenting cells (APCs) and cluster of differentiation 28 (CD28) on T cells as depicted in Figure 1-1. Endogenous danger signals can be released by dying cells [Gallucci et al., 1999; Shi et al., 2000]; specifically, cells that have undergone necrosis [Matzinger, 1998]. It has been shown that endogenous danger signals can act as adjuvants to upregulate co-stimulatory molecules on APCs and therefore produce signal 2 resulting in strong T cell proliferation [Watts et al., 2003]. There are many examples of endogenous danger signals that are ligands for various immune cell pattern recognition receptors (PRRs), some of which are listed in Table 1-1 [Zhang & Mosser, 2008]. Although cell death releases danger signals, it is probably not required.

Table 1-1. Endogenous danger signals and their pattern recognition receptors (PRRs)

<table>
<thead>
<tr>
<th>Ligands</th>
<th>PRRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biglycan; gp60; Hsp60; Hsp70; HMGB1; hyaluronan fragments</td>
<td>TLR2</td>
</tr>
<tr>
<td>Endogenous mRNA</td>
<td>TLR3</td>
</tr>
<tr>
<td>αA crystalline; biglycan; fibrinogen; gp60; HMGB1; Hsp60; Hsp70; HSPB8; hyaluronan fragments; murine β-defensin 2; polysaccharide fragments of heparan sulphate; Apoptotic cells; oxidized LDL</td>
<td>TLR4</td>
</tr>
</tbody>
</table>

HSP: Heat Shock Protein; GP60: Glycoprotein 60; HMGB1: High-Mobility Group Protein B1; TLR: Toll-Like Receptor; LDL: Low Density Lipoprotein; CD36: Cluster of Differentiation 36 [Zhang, 2008]. Permission has been granted by the Journal of pathology to include this image in this thesis.
An important question to consider is; how does a reactive metabolite generate a danger signal? We have recently shown a correlation between the risk of an IDR and a drug’s ability to activate inflammasomes [Weston & Uetrecht, 2014]. Activation of inflammasomes leads to the production of the inflammatory cytokines IL-1β and IL-18. This is an attractive hypothesis for the mechanism by which a reactive metabolite can activate an immune response; this will be expanded upon below.

![Image](92x331 to 482x543)

**Figure 1-1. Hapten and danger hypothesis.**

A chemically reactive drug or reactive metabolite binds to proteins. These modified proteins are taken up by APCs, processed, and the peptides presented to T cells. This represents signal 1. Unless the APC is activated leading to upregulation of co-stimulatory molecules and production of signal 2, signal 1 alone leads to immune tolerance [Uetrecht, 2008]. Permission has been granted by the American Chemical Society to include this image in this thesis.

### 1.2.4.3 Pharmacological interaction (PI) and related hypotheses

An alternative hypothesis for how a drug can induce an immune response is the PI hypothesis. Recently proposed, it states that a drug can stimulate T cells through direct interaction with the MHC-T cell receptor complex [Medzhitov & Janeway, 1997]. The hypothesis is based on the observation that T cell clones obtained from sensitized patients respond to the parent drug in the absence of metabolism and in an MHC-dependent manner. However, the basis for the PI
hypothesis is false, i.e. what the T cells respond to is not necessarily what induced the immune response in the first place [Chen et al., 2009]. That does not make the PI hypothesis false, and it is an attractive hypothesis for drugs that induce IDRs but do not appear to form a reactive metabolite. The PI hypothesis has been implicated in various IDRs but not in IDIAG.

The mechanism by which abacavir appears to induce hypersensitivity is related to the PI hypothesis, but is distinct from it. Specifically, abacavir binds tightly, but reversibly, to HLA B5701 and changes the spectrum of endogenous peptides that are presented to T cells [Norcross et al., 2012]. This results in an immune response analogous to a graft vs. host reaction. At the present time this does not appear to be a common mechanism for IDRs.

1.2.4.4 Non-immune hypotheses

Although many IDRs, including most hematological IDRs, include an immune component, some have proposed other possible explanations. Recently, some drugs have been shown to produce epigenetic effects in which they directly affect a target cell’s genes via mechanisms such as methylation of DNA or histone deacetylation [Khan et al., 2008].

Others have suggested that certain IDRs may be the result of differences in metabolic enzymes resulting in more reactive metabolites capable of forming haptens and therefore increasing the risk of a possible IDR. For example, viral infections leading to increased incidence of IDRs may be mechanistically linked to a depletion of metabolic factors rather than immunological changes. Although different IDRs are likely to have different mechanisms, most IDRs are likely to be mainly mediated by immune mechanisms.

Still others have cited mitochondrial damage as a cause of IDRs. This hypothesis is based on the importance of mitochondrial function in cells. Additionally in the event of cellular dysfunction;
many damage-associated molecular patterns (DAMPs) have been shown to originate from the mitochondria and could lead to an immune response [Cho & Uetrecht, 2016]. Drug-induced mitochondrial damage has been linked to IDRs such as idiosyncratic drug-induced liver injury (IDILI) in which drugs could damage the mitochondria by inhibiting particular portions of the electron transport chain such as complex I and complex II or by inhibiting mitochondrial DNA synthesis [Cho & Uetrecht, 2016]. The DAMPs produced by mitochondrial dysfunction have also been explored as potential stimulants of the NLRP3 inflammasome, which may lead to an immune response [Lawlor & Vince, 2014]. This hypothesis is still in its early days and the majority of the studies have been completed in vitro. To validate these hypotheses, studies should be completed in vivo and ultimately linked to humans. For now this appears to have some characteristics that agree with the understanding of these reactions, but may be more relevant for non-IDR adverse events.

1.2.5 Models of idiosyncratic drug reactions

Predicting IDRs early on in drug development and limiting the effects of IDRs in humans remains the main reason to study these adverse events. In order to understand the mechanism of how these reactions occur, multiple models of IDRs have been developed, specifically from the Uetrecht lab [Ng et al., 2012]. The ultimate goal would be to develop animal models that perfectly reflect the clinical data seen in humans or to develop an in vitro assay that accurately and precisely predicts drugs that have the ability to cause IDRs. The Utrecht group has developed two animal models of IDRs, a rat model of idiosyncratic nevirapine-induced skin rash and a mouse model of idiosyncratic drug-induced liver injury. The Uetrecht group is also currently exploring the prospect of an in vitro model to distinguish between drugs that have the ability to cause IDRs and those that do not using a human monocytic cell line, THP-1, that appears promising [Weston & Uetrecht, 2014].
1.2.5.1 Idiosyncratic drug-induced liver injury animal model

The Uetrecht lab has recently explored utilizing C57BL/6 mice to develop an animal model of liver injury. Treatment of these mice with amodiaquine leads to mild liver injury with a delay in onset and resolution despite continued treatment [Metushi et al., 2014a]. This resolution was suspected to be due to immune tolerance. At this time novel immune checkpoint inhibitors were being explored for cancer treatment, and these immune checkpoint inhibitors were utilized in this mouse model to try to limit the immune tolerance observed. This led to the development of a novel mouse model of idiosyncratic drug-induced liver injury, which utilized PD1 -/- mice and a CTLA-4 antibody to limit the immune tolerance [Metushi et al., 2015]. This model has been further characterized using a variety of other drugs known to cause IDILI including isoniazid and nevirapine [Mak & Uetrecht, 2015]. This model shows the importance of immune tolerance in the suppression of IDILI, a concept that is exciting to explore in understanding the mechanism of other IDRs including IDIAG.

1.2.5.2 THP-1 cell IL-1β in vitro assay

The development of biomarkers to predict the risk of drugs to cause IDRs is an important investment to help identify problem drugs early in drug development. The Uetrecht lab has been exploring the use of THP-1 cells, a monocytic cell line, which responds to proinflammatory mediators with the production of IL-1β and IL-18 through the activation of inflammasomes. To date, this in vitro assay has shown the ability to distinguish between a few chemically similar drugs that are shown to lead to idiosyncratic drug-induced skin rash and drugs that are not able to do this. Specifically, the pairings dimethylfumarate/ethacrynic acid and telaprevir/boceprevir have been published as a proof of concept of how this assay can distinguish between drugs with a significant incidence of IDRs (dimethylfumarate and telaprevir) and drugs with a low incidence of IDRs (ethacrynic acid and boceprevir) [Weston & Uetrecht, 2014]. Since then, drug pairings
including clozapine/olanzapine, troglitazone/pioglitazone, and tolcapone/entacapone have been explored in this model with varying degrees of success [unpublished data].

1.2.5.3 Idiosyncratic nevirapine-induced skin rash animal model

Nevirapine is associated with an incidence of skin rash ranging from mild to serious, including Steven-Johnson’s syndrome and toxic epidermal necrolysis (TEN) [Fagot et al., 2001]. The Utrecht lab utilized female Brown Norway rats to understand the mechanism of idiosyncratic nevirapine-induced skin rash [Sharma et al., 2013]. In this model, female Brown Norway rats are administered 150 mg/kg/day nevirapine in their food for a maximum of 3 weeks; after such a time 100% develop a nevirapine-induced skin rash. This skin rash model was used to perform mechanistic studies, most notably this model was used to show that a reactive benzylic sulfate metabolite of nevirapine is formed in the skin and is responsible for the skin rash [Sharma, 2013]. It is studies like these that highlight the importance of the production of valid animal models in order to understand the mechanism of IDR.

1.3 Hematology overview

1.3.1 Structure and function of the bone marrow

The bone marrow resides as the spongy tissue inside the flat bones, short bones, and the heads of long bones in the body. The bone marrow is responsible for the production of blood cells through a process known as hematopoiesis. Hematopoiesis gives rise to all blood cells including erythrocytes, granulocytes (neutrophils, eosinophils, and basophils), monocytes, lymphocytes (T cells and B cells), and thrombocytes; the function of these cells are outlined in Table 1-2 [Chanarin, 1985]. Each stage of blood cell maturity is perpetuated by specific cytokines and growth factors; therefore the bone marrow is compartmentalized, providing specialized areas for a variety of different cell types at differing stages of maturity to have the microenvironment
necessary to proliferate. Hematopoiesis within the bone marrow is a continuous process in order to replenish the billions of short-lived mature blood cells in the body; however, it can be separated into distinct stages, which will be described below.

Table 1-2: The peripheral mature blood cells

<table>
<thead>
<tr>
<th>Type</th>
<th>Subtype</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes</td>
<td></td>
<td>Transports oxygen and carbon dioxide to and from tissues</td>
</tr>
<tr>
<td>Mononuclear</td>
<td>T Lymphocyte</td>
<td>An immune cell that matures in the thymus; involved in cell-mediated immunity</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>B Lymphocyte</td>
<td>An immune cell that matures in the bone marrow; involved in humoral immunity</td>
</tr>
<tr>
<td></td>
<td>NK T Cell</td>
<td>A cytotoxic T cell involved in innate immune responses</td>
</tr>
<tr>
<td>Polymorphonuclear Leukocytes</td>
<td>Neutrophil</td>
<td>An abundant, fast-acting innate immune cell, which contains granules used to digest microorganisms</td>
</tr>
<tr>
<td></td>
<td>Basophils</td>
<td>A phagocytic cell of the innate immune system which has granules containing heparin, histamine, and leukotrienes</td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td>An innate immune cell, which has granules to help fight parasites and particular infections</td>
</tr>
<tr>
<td>Thrombocyte</td>
<td></td>
<td>A small blood cell derived from megakaryocytes whose function is to clump together and clot to limit bleeding</td>
</tr>
</tbody>
</table>

1.3.2 Hematopoiesis

As mentioned above, hematopoiesis is a continuous process that results in the formation of a variety of different mature blood cells from a common hematopoietic stem cell. All blood cells begin their differentiation process as pluripotent stem cells, and as such, these cells are constantly being renewed. Two types of mature cell classes exist: lymphoid and myeloid cells, and hematopoietic stem cells differentiate into either lymphoid stem cells or multipotent myeloid
cells. Mature lymphoid cells include T lymphocytes and B lymphocytes, whereas mature myeloid cells include granulocytes, erythrocytes, monocytes, and megakaryocytes. The differentiation is decided as a result of local cytokines, which aid in differentiation, and are displayed in Figure 1-2.

1.3.3 Neutrophils
Neutrophils are the most abundant type of granulocytes in humans, comprising 40-70% of all leukocytes. They were the first type of innate immune cell known to effectively eliminate microorganisms, which was shown over a century ago [Amulic et al., 2012]. Neutrophils are typically the first responders to invading pathogens, where they have the ability to kill multiple pathogens through a variety of different mechanisms. These killing mechanisms are not very specific to a given pathogen and have also been known to cause harm to the host [Weiss, 1989]. Neutrophils are phagocytes, meaning they have the ability to internalize pathogens through engulfing and then killing them internally. For a neutrophil to internalize a pathogen, the pathogen must be coated in antibody through a process known as opsonization, which is explained in detail below. Once internalized, neutrophils are able to kill the pathogen via oxidation, using oxidants produced through a process known as the oxidative burst, also explained below. Neutrophils contain granules, which contain an assortment of antimicrobial proteins. The major mechanism neutrophils possess for killing is using these granules either internally or releasing them externally, through a process known as degranulation. Neutrophils contain three types of granules; the most abundant are the azurophilic granules or primary granules, which contain proteins such as myeloperoxidase (MPO), defensins and serine proteases. Specific granules or secondary granules contain NADPH oxidase, lysozyme, and phosphatases, and tertiary granules contain cathepsin and collagenase. Although phagocytosis and degranulation are thought to be the neutrophil’s primary method of killing, neutrophils
actually have a third mechanism of killing that is suicidal. Neutrophils have the ability to release their own DNA along with a variety of antimicrobial proteins found in their granules [Brinkmann & Zychlinsky, 2007]. This release of DNA has the ability to reach pathogens that are unable to be phagocytosed. These DNA fragments, which have the ability to wrap up pathogens to limit their movement and proliferation, have the fitting name of NETS (neutrophil extracellular traps). NETS usually contain concentrates of neutrophil antimicrobial proteins, such as MPO, allowing the NETS to be lethal to their target. Neutrophils also exhibit their antimicrobial ability though the use of chemokines. Upon interaction with a pathogen, traditionally neutrophils were only thought to kill pathogens through phagocytosis and oxidant production; however, a growing list of cytokines are being found that suggests neutrophils have the ability to recruit a wide array of immune cells to the site of inflammation [Lacy et al., 2015].

1.3.3.1 Neutrophil development

The average human produces neutrophils in the order of billions per day owing to a short lifespan and a rapid production rate [Furze & Rankin, 2008]. Although estimates vary, the typical lifespan of a neutrophil in the peripheral circulation is approximately 6-10 h and even shorter in infected patients, while the lifespan in tissues is about 1-5 days [Bainton et al., 1971; Fliedner et al., 1964; Athens et al., 1961; Summers et al., 2010]. A mature neutrophil is produced in the bone marrow from a pluripotent stem cell in only 4-6 days [Summers et al., 2010] and is typically destroyed by macrophages in the spleen, liver, or within the bone marrow itself [Savill et al., 1989]. After neutrophils are produced, many mature neutrophils remain retained within the bone marrow; these neutrophils are known as the neutrophil reserve. These mature neutrophils serve to replenish those terminated after their short lifespan, and they also serve in case increased numbers of neutrophils are required on short notice. Neutrophils from this reserve can increase the absolute neutrophil count (ANC) in the peripheral blood 10 fold within a matter of hours
[Furze & Rankin, 2008]. The neutrophil begins its life as any other blood cell, as a multipotential hematopoietic stem cell. During neutrophil development, the common myeloid progenitor is formed, followed by a myeloblast, which can become any granulocyte; most of which go on to form the numerous neutrophils (Figure 1-2). The various differential stages of hematopoiesis are regulated by a number of cytokines; G-CSF appears to play a major role in neutrophil production and mobilization under homeostatic conditions [Metcalf, 1989].

Figure 1-2. Bone marrow hematopoiesis.
(Adapted from: Mikael Häggström and A. Rad. Permission was granted to use this image under the terms of the GNU Free Documentation License.)

1.3.3.2 Neutrophil recruitment and activation

Mature neutrophils freely circulate in the peripheral blood in an inactivated state, waiting for recruitment in order to fight an infection. Pathogens usually enter the body through the skin,
respiratory system, gut, or the mucosa, and therefore resident macrophages on these barriers are likely the first to encounter a pathogen. When a macrophage encounters a pathogen it releases a variety of chemokines and cytokines, some of which serve as attractors and recruiters to neutrophils in the blood [Amulic et al., 2012]. The process of attracting neutrophils to the site of infection is known as chemotaxis and is depicted in Figure 1-3. Cell surface receptors on neutrophils respond to cytokines such as IL-8, INF-γ, and leukotriene B4 [Amulic et al., 2012]. At this stage the neutrophil is attracted to the site of infection, but it must be able to cross the blood vessel barrier. This process is known as extravasation, and it begins with the upregulation of a variety of cell adhesion molecules (CAM), most notably P-selectin glycoprotein ligand (PSGL) and L-selectin (CD62L) [Amulic et al., 2012]. This upregulation in CAMs on neutrophils is coupled with upregulation of binding partners on the epithelial cells closest to the site of infection. For example, PSGL binds to P-selectin on the epithelial cell membrane. These binding partners allow the neutrophil to slow down in the blood stream in order to prepare to cross the membrane, and as the neutrophil slows it begins to roll. The rolling neutrophil can then be further slowed by other adhesion molecules on the epithelial cell wall; this time ICAM-1 and ICAM-2 bind to β2 integrins (CD11b) on the surface of the neutrophil and bring the neutrophil to a halt [Amulic et al., 2012]. Neutrophils are then able to cross the vessel wall, most likely through epithelial cell junctions where they follow chemical gradients and pathogen signals towards the infection. Neutrophils begin their activation process upon the binding of their PSGL to the epithelial P-selectin. This activation continues, as it gets closer to the site of action where the local chemokine density increases. Upon the neutrophil’s arrival at its target, activation is at its highest. Cells are primed for all modes of destruction, i.e., production of proteins for oxidative burst peak, cellular processes necessary for the neutrophil to undergo degranulation, and NET release [Amulic et al., 2012].
1.3.3.3 The oxidative burst

The major oxidizing system in neutrophils is the NADPH oxidase system, which produces superoxide and is coupled with myeloperoxidase. This system utilizes the hydrogen peroxide produced from superoxide as an oxidant. The major substrate for the oxidized form of myeloperoxidase is chloride anion, which is converted to hypochlorous acid (Figure 1-4) [Segal, 2005]. Neutrophils use hypochlorous acid to kill pathogens, but it can also oxidize drugs [Uetrecht, 1992]. Myeloperoxidase is present in significant quantities in neutrophils, myeloid
progenitor cells starting at the promyelocyte stage, and also in monocytes and some macrophages
[Brown et al., 2001].

1.3.3.3.1 Reactive metabolite formation by neutrophils

Given their reactivity and short half-lives, most reactive metabolites are likely to be formed close
to where they covalently bind. Although oxidized myeloperoxidase and hypochlorite are strong
oxidants, their oxidation potential is limited to molecules that are readily oxidized. This is
consistent with the observation that most of the drugs that are associated with IDIAG are readily
oxidized to reactive metabolites by the myeloperoxidase system [Liu & Uetrecht, 1995; Lai et
al., 1999; Ju & Uetrecht, 1998]. Specifically, the functional groups that are readily oxidized by
the MPO system include aromatic amines, thiono sulfur compounds, and drugs that readily form
quinones or quinoneimines. Examples are shown in Table 1-3. Although the ability of a drug to
be oxidized to a reactive metabolite by MPO is a convenient explanation for the ability of drugs
to cause IDIAG, there are also other oxidizing systems in these cells that can form reactive
metabolites. Specifically, amodiaquine covalent binding to bone marrow cells was decreased in
MPO knockout mice, but binding was still significant [Lobach & Uetrecht, 2014]. Furthermore,
covalent binding of amodiaquine was not decreased in NADPH oxidase knockout mice. In
addition, patients with genetically low MPO activity are no less likely to develop clozapine-
induced IDIAG than patients with normal MPO activity [Mosyagin et al., 2004].

Agranulocytosis is characterized by a very low granulocyte count; however, it has been shown
that basophils may be spared [Besser et al., 2009]. This may be indicative of which enzyme is
involved because basophils lack the peroxidase activity that is present in the affected neutrophils
and eosinophils.

Table 1-3. Drugs that have been shown to be (or are expected to be) oxidized to reactive
metabolites by myeloperoxidase and are associated with a significant incidence of IDIAG.
<table>
<thead>
<tr>
<th>Primary aromatic amines</th>
<th>Thiono-sulfur drugs</th>
<th>Quinones/Quinoneimmines</th>
<th>Thiophenes</th>
<th>Miscellaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>procainamide</td>
<td>propylthiouracil</td>
<td>amodiaquine</td>
<td>clopidogrel</td>
<td>clozapine</td>
</tr>
<tr>
<td>dapsone</td>
<td>methimazole</td>
<td>benzene (not a drug)</td>
<td>ticlopidine</td>
<td>aminopyrine</td>
</tr>
<tr>
<td>aminogluthethimide</td>
<td>carbamazole</td>
<td>calcium dobesilate</td>
<td></td>
<td>mianserin</td>
</tr>
<tr>
<td>sulfamethoxazole</td>
<td></td>
<td>carbamazepine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sulfasalazine</td>
<td></td>
<td>diclofenac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aminosalicylic acid</td>
<td></td>
<td>carbamazepine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>metoclopramide</td>
<td></td>
<td>vesnarinone</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>phenothiazines</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>clomipramine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>trimethoprim</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>indomethacin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>acetaminophen</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Most drug metabolism and reactive metabolite formation occurs in the liver and is mediated by cytochromes P450 (P450). In the neutrophils and their precursors, however, the concentration of P450 is quite low. Given that myeloperoxidase is not as strong an oxidant as P450, if a drug can be oxidized to a reactive metabolite by myeloperoxidase it is likely that it can also be oxidized to a reactive metabolite by P450, although P450s display more substrate specificity than myeloperoxidase. Presumably that is why most drugs that can cause IDIAG can also cause other IDR such as liver injury or other types of hypersensitivity reactions. Specifically, if the drug covalently binds in multiple tissues it could induce an immune response against neutrophils in one patient and hepatocytes in another patient. This observation also supports the involvement of reactive metabolites in the mechanism of IDIAG.
Figure 1-4. Oxidative potential of neutrophils.

When neutrophils are activated, NADPH oxidase produces superoxide. The superoxide is converted to hydrogen peroxide, which in turn oxidizes MPO. Oxidized MPO oxidizes chloride anion to hypochlorous acid, which can oxidize particular drugs into reactive metabolites.

1.4 Immunology

The immune system is made up of a complex network of cells and signaling molecules that work together to protect the body by detecting, containing and eliminating foreign invaders. However, the immune system can create harmful situations for the body as well; for instance, in the case of autoimmune disorders or in the development of IDRs, which are likely mediated by the immune system. This shows the dichotomy that is the immune system’s power to both protect the body or to potentially create more harm than perhaps is necessary. To understand how the immune system possesses this power, and to understand how and why neutropenia and agranulocytosis present such life threatening symptoms, a brief description of a few immunology concepts are explored below.
1.4.1 Innate immunity
The primary barrier to the outside world is the skin; however, if a pathogen were to break this initial barrier the first responders are those of the innate immune system [MacLeod & Mansbridge, 2016]. The cells of the innate immune system are crude first responders. In general, they recognize non-specific non-self epitopes, engulf, process, and let out signaling molecules to warn about the possible invading pathogen. Macrophages are prime examples of the innate immune system at work. They are resident to almost all tissue types, most notably those facing the barriers to the outside world where they act as guardians; as such, they are often the first to encounter foreign attackers. Monocytes derive from a myeloid origin before maturing into macrophages in tissues [Gordon, 2003]. Macrophages respond to invading pathogens by recognizing, engulfing, and destroying them through a process known as phagocytosis. Upon phagocytosis, macrophages warn the rest of the host by emitting a variety of cytokines, which work as chemical messengers to attract other immune cells to the area [Gordon, 2003]. Although the progression of cellular response changes due to a variety of reasons, the next responders are typically peripheral neutrophils and monocytes, then eosinophils and basophils. These immune cells aid the macrophages in pathogen destruction and cellular recruitment through the release of their own unique set of cytokines [Rivera et al., 2016]. Another immune cell important to the innate immune system is the dendritic cell, another phagocyte that resides mainly in the skin as a first responder that holds the added responsibility of being antigen-presenting cells. After taking up presentable antigen, now mature dendritic cells travel to a lymph node where they are able to present that antigen to a T cell. By doing this, dendritic cells provide a link between the innate and adaptive immune systems [Merad et al., 2013]. Natural killer cells are the only cells of the innate immune system that are derived from lymphoid progenitor origin and are defined as large granular lymphocytes (LGL). NK cells are also unique in that they do not require activation in
order to function. One mechanism that allows this to be possible is that NK cells have the ability to detect a decrease in MHC molecules on host cells, a function that viral take-over of a cell can limit in hopes of hiding from T cells [Lanier, 2008]. NK cells induce apoptosis by releasing granzymes upon recognition of abnormal cells such as those infected by viruses or tumor cells [Cook et al., 2014]. Lastly, mast cells are innate immune cells that reside on the boundaries of the body, mainly the skin. They function similar to basophils by releasing granules full of heparin and histamine to cause local vasodilatation and anticoagulation in the infected area [Schroeder, 2009].

1.4.2 Adaptive immunity

The innate immune system is great at mounting an initial response to foreign invaders, but it is non-specific and lacks the memory required to provide long-lasting immunity to the host. The adaptive immune system is a subsystem that is highly specialized and unique to the host, and it has the ability to provide extensive and long-lasting protection. The cells that make up the adaptive immune system are lymphocytes: T lymphocytes and B lymphocytes. These cells begin their lives comparatively featureless, freely circulating in the blood. In the presence of an infection, these cells are recruited and differentiate into effector cells that participate in an effective adaptive immune response.

The adaptive immune response includes both the humoral immune response, which can be represented by the immune response mediated by antibodies, and the cell-mediated immune response, which is the adaptive immune response that does not involve antibodies. The cell-mediated adaptive immune response is mainly reliant on a variety of different subsets of T lymphocytes. T lymphocytes can be distinguished from other lymphocytes by the presence of a surface T cell receptor, which allows T cells to recognize antigens presented on MHC molecules.
The CD3+ T cells are subcategorized by transmembrane co-receptors, which aid the T cell in recognizing particular MHC molecules [Rangarajan & Mariuzza, 2014]. Cytotoxic T cells express the co-receptor CD8 along with the T cell receptor, and T helper cells express the co-receptor CD4 along with the T cell receptor [Koretzky, 2010]. Cytotoxic T cells are specific to virally infected cells to which internalized antigen is presented via MHC-I on the surface of the host cell. The cytotoxic T cell then directly destroys the infected cell and the pathogen within it. Cytotoxic T cells work by releasing perforin and granulysin to create holes in the infected cell membrane, and serine proteases, which enter into the cell and degrade the DNA of the host cell and the pathogen [Harty et al., 2000]. T helper cells are divided into many subtypes, which for simplicity sake are T_h1, T_h2, and T_h17. T helper cells circulate in the periphery until they meet antigen-presenting cells (macrophages or dendritic cells). This interaction typically occurs within a lymphoid organ where the interaction with MHC-II molecules is most likely. Depending on the required T helper cell, these cells will differentiate into one of the subsets [Zhu et al., 2010]. T_h1 cells are the most common subtype of helper T cells; they are the host immunity effector cells that function mainly against intracellular bacteria and protozoa. These cells maximize the killing efficacy of macrophages and the proliferation of CD8+ T cells by producing cytokines such as INF-γ, TNF-β, IL-2, and IL-10. The T_h2 subset has been shown to produce immunity against extracellular parasites such as helminthes. T_h2 cells mainly interact with the humoral immune system by stimulating B cell proliferation through the production of cytokines, e.g. IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 [Romagnani, 2000]. The T_h17 cells are developmentally distinct from the T_h1 and T_h2 subtypes and are characterized by their production of IL-17 [Zúñiga et al., 2013]. IL-17 is a proinflammatory cytokine, which has been shown to recruit monocytes and neutrophils. Lastly, T regulatory cells (Tregs) are a subset of T cells that promote an anti-inflammatory response and are currently characterized as CD4+, CD25+, FOXP3+ cells,
although these markers are still currently under debate [Corthay, 2009; Zheng et al., 2015]. These cells exhibit their anti-inflammatory response by down-regulating T cell responses. The mechanism of this remains unknown; however, recent evidence suggests that FOXP3, a transcription factor not-exclusively found in Tregs, has the potential to down-regulate T cell activation [Schubert et al., 2001].

1.4.3 Immune tolerance

Immune tolerance has been an exciting topic in immunology recently as it has been used to explore immune-related events including autoimmune diseases and treatments of tumor suppression [Pardoll, 2012]. Immune tolerance is a mechanism to regulate and resolve immune responses that are damaging to the host. As explained above, T regulatory cells are well known as cells of immune tolerance, although more immune tolerance cells are being discovered. Myeloid-derived suppressor cells (MDSC) are, as their name suggests, cells of myeloid lineage that participate in immune tolerance. MDSC have been defined as CD11b/c+, CD172a+, HIS48+ cells and further subdivided into granulocytic MDSC (Rp-1+) or mononuclear MDSC (RP-1-) in rats. These cells show their immunosuppressive functions in a variety of ways: deprivation of amino acids, production of nitric oxide, which disrupts T cell receptors, production of anti-inflammatory cytokines such as IL-10 and TGF-β1, and upregulation of immune checkpoints on T cells such as PD-1 receptors [Umansky et al., 2016].

Immune tolerance has been adopted as a treatment strategy in autoimmune reactions and cancers by utilizing the intrinsic functions of many anti-inflammatory cells. For instance the use of immune checkpoint inhibitors such as PD-1, PD-L1, and CTLA-4 inhibitors draw from the immunosuppressive abilities of MDSCs. These immune checkpoint inhibitors work by preventing a co-receptor interaction between APCs and T cells, which stop the T cell from
responding. As such, these checkpoint inhibitors are currently a hot topic in cancer treatment due to their ability to facilitate an APC-T cell interaction, leading to an immune response [Voutsadakis, 2016; Zhou et al. 2016; Facchinetti et al., 2016]. Our lab has recently explored these checkpoint inhibitors to limit immune tolerance in a mouse model of drug-induced liver injury, which led to a more valid model of human idiosyncratic drug-induced liver injury [Mak & Uetrecht, 2015; Metushi et al., 2015]. Presumably, using a similar method of limiting immune tolerance, other animal models of IDRs could be produced, including the potential for an animal model of IDIAG.

1.4.4 The NLRP3 inflammasome
Inflammasomes are multiprotein oligomers expressed within the cytoplasm of myeloid cells, mainly macrophages, and are a component of the innate immune system that allows the activation of inflammatory processes including the induction of pyroptosis [Guo et al., 2015]. Multiple types of inflammasomes exist, each being activated by varying arrays of stimulants, but the best characterized is the NLRP3 inflammasome, which has recently been implicated in a variety of inflammatory diseases [Abais et al., 2015]. The NLRP3 inflammasome is so named because of the receptor used to activate it, the nucleotide-binding oligomerization domain (NOD)-like receptor containing pyrin domain 3. Its main function is to convert pro-caspase-1 into caspase-1, which converts pro-IL-18 and pro-IL-1β into the proinflammatory cytokines IL-18 and IL-1β, respectively. The NLRP3 inflammasome is made up of the oligomerization of two inflammasome monomers. These monomers are the NLRP3 protein, the adaptor molecule apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain) (ASC), and the cysteine protease caspase-1, which together form this cytosolic multiprotein complex [Guo et al., 2015]. Upon cell surface pattern recognition receptor (PRR) stimulation of a wide range of signals, including DAMPS, pathogen-associated molecular patterns (PAMPs)
and other exogenous signals, these domains oligomerize to form an active inflammasome (Figure 1-5).

![NLRP3 inflammasome activation diagram](image)

**Figure 1-5. NLRP3 inflammasome activation**

The NLRP3 inflammasome is a dimerization of two inflammasome monomers. These monomers are made up of a NTPase domain (NACHT), a leucine rich-repeat domain (LRRs), and a pyrin domain, which collectively form the abbreviation NLRP. Dimerization of two NLRP3 molecules leads to activation of caspase-1, which converts pro-IL-1β into IL-1β ready for cell release. Permission has been granted by the Nature Publishing Group to include this image in this thesis. (Tschopp & Schroder, 2010).

### 1.5 Characteristics of idiosyncratic drug induced agranulocytosis

The typical neutrophil count in the peripheral blood, or ANC as it is often referred to, is about 4,000 cells/µL, and ranges from 2,000 to 8,000 cells/µL. The average ANC is different in different races with Blacks having a lower average ANC than Whites, but benign neutropenia also occurs in other ethnic groups [Bray, 2008]. In addition, unlike other blood cells, the ANC can change very rapidly. This is because many of the cells are sequestered or margined on the blood vessel walls and bone marrow, where they can be released with minor stimuli such as exercise or other stress.
If the ANC falls below 1,500 cells/µL it is referred to as neutropenia, although as mentioned above, this can be normal in Black individuals. The risk of infection is not high until the ANC falls below 500 cells/µL, defined as agranulocytosis. As mentioned previously, the typical time to onset of most IDIAG ranges from 1-6 months after starting the drug, although it is a little shorter for aminopyrine and longer for levamisole, and there are cases involving several different drugs where the offending drug was given for more than a year before the onset of IDIAG [Atkin et al., 1996]. The decrease in ANC can be gradual, but more commonly it is precipitous. There is often an increase in ANC before the onset of IDIAG, and more than 50% of patients given clozapine have a paradoxical increase in ANC early in the course of treatment. IDIAG is usually asymptomatic until an infection occurs, classically with a fever and sore throat.

When IDIAG occurs and the drug is stopped, the ANC usually recovers in 1-2 weeks [Andersohn et al., 2007]; a delay in recovery is associated with a higher mortality rate. In some cases the recovery appears to start while the patient is still taking the drug. In general, there is an overshoot in the ANC, and it sometimes peaks at >20,000 cells/µL. The major cause of mortality is infection, and if the patient recovers there are no permanent sequelae. If a patient is rechallenged there is usually a recurrence of IDIAG, often with a shortened time to onset, especially with aminopyrine, but in many cases the time to onset is similar as on initial exposure, especially with clozapine [Dunk et al., 2006].

1.5.1 Incidence and mortality

The majority of agranulocytosis cases appear to be drug-induced; it is estimated that two-thirds to three-quarters of agranulocytosis cases are drug-induced [Andrès et al., 2006; Kaufman et al., 1996]. Although studies report varying values, typically the reported incidence is between 2.4-15.4 cases per million [Andrès et al., 2006; Andrès et al., 2002]. Over the last 20 years the
incidence of IDIAG has remained fairly constant. The fairly constant incidence of drug-induced agranulocytosis persists even with increased awareness, strict monitoring of drugs associated with a high incidence, and elimination of several older drugs that were associated with a relatively high incidence of IDIAG. This is indicative of its idiosyncratic nature; IDIAG remains virtually impossible to predict. The strict monitoring of drugs associated with high incidence of agranulocytosis has however contributed to decreasing mortality rates associated with IDIAG.

1.5.2 Current treatment options

IDIAG is a rare condition, and there are currently no ways in which it can be predicted prior to the decrease in ANC. The most important aspect of treatment is early detection and stopping the responsible drug. Good supportive treatment with optimal treatment of IDIAG-related infections is also very important. In most cases stopping the offending drug leads to complete resolution.

If the rebound in ANC is delayed it is associated with an increased mortality; therefore, most cases of IDIAG are treated with granulocyte colony-stimulating factor (G-CSF) to stimulate neutrophil maturation and release. However, the typical overshoot of the ANC when the drug is stopped suggests that endogenous levels of G-CSF are already elevated. There is evidence that G-CSF decreases the duration of agranulocytosis [Andersohn et al., 2007]; however, reports of the success of this treatment in non-chemotherapy medications are conflicting [Andersohn et al., 2007, Fukata et al., 1999]. G-CSF has also been co-administered prophylactically with potential agranulocytosis-causing medications in an attempt to prevent IDIAG, although this is costly, and evidence for its ability to decrease agranulocytosis is conflicting [Andrès et al., 2010].

Close attention to patients’ white blood cell counts remains the first line of defense against DIAG. Many medications including clozapine, antithyroid drugs, and some other drugs require constant monitoring [Andersohn et al., 2007]; this monitoring has been shown to save lives
[Ibáñez et al., 2005; Imbarlina et al., 2004; Tajiri & Noguchi, 2004]. Because most cases occur in the first 6 months, modifications have been made to the frequency of blood monitoring, with individual countries having slightly different monitoring standards [Cohen et al., 2012].

1.6 Immune responses to drugs that cause IDIAG

The most extensive studies of IDIAG have been done with clozapine. It is known that most patients who are treated with clozapine have an increase in inflammatory cytokines including IL-6 and TNF-α during the first 3 months of treatment [Maes et al., 1997; Pollmacher et al., 1996; Pollmacher et al., 2000]. Many patients also have a paradoxical increase in ANC and fever. This indicates that clozapine induces an immune response in most patients; however, in most cases this resolves despite continued treatment with the drug. In contrast, olanzapine, which forms a similar reactive metabolite but rarely causes agranulocytosis, did not cause neutrophilia in a study utilizing a rat model [Ng & Uetrecht, 2014]. One possible reason for the difference in IDIAG risk between clozapine and olanzapine is dose. The therapeutic dose of clozapine is more than ten times that of olanzapine; however, in the rat study mentioned above the dose used was the same.

An important question is how reactive metabolites activate the immune system. Recent data suggest that an important mechanism by which a reactive species can induce an immune response is by activation of inflammasomes [Weston & Uetrecht, 2014]. We found that clozapine activated inflammasomes in THP-1 cells leading to the production of IL-1β (Figure 1-6) [unpublished observation]. Furthermore, despite its similar structure, olanzapine did not activate inflammasomes [unpublished observation]. We also found that amodiaquine, another drug associated with a relatively high incidence of agranulocytosis, also activated inflammasomes. These data suggest that even though clozapine and olanzapine have very similar
structures and both form a reactive metabolite, the important difference is that clozapine induces an immune response, and olanzapine does not. However, in most patients the clozapine immune response resolves, presumably with immune tolerance, and it is only when this fails that the patient develops IDIAG.

1-6. Clozapine stimulates IL-1ß release from THP-1 cells, while olanzapine does not.

Clozapine stimulates a dose-dependent response in IL-1ß release from THP-1 macrophages, while structurally similar olanzapine does not. This dose dependent IL-1ß response can be halted by the addition of a caspase inhibitor, ZVAD. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by one-way ANOVA, where *, p < 0.05 and **, p < 0.01 [unpublished data].

1.6.1 Cells affected in IDIAG

Agranulocytosis may be the result of interruption of the production of any of the cells in the hematopoietic differentiation pathway, or any of the specific cytokines. An important question is which cells are the targets of IDIAG? There is good evidence that at least some cases of aminopyrine-induced IDIAG are mediated by antibodies that destroy mature neutrophils. This was first demonstrated in 1952 when an investigator took aminopyrine and then injected himself with the serum from a patient with aminopyrine-induced agranulocytosis. This led to a very rapid onset of fever and profound neutropenia, suggesting that his mature neutrophils were rapidly destroyed by aminopyrine-dependent antineutrophil antibodies [Moeschlin & Wagner, 1952]. However, many patients with cases of aminopyrine-induced IDIAG lack neutrophil precursors in
their bone marrow, which indicates that neutrophil precursors are also often a target. Antineutrophil antibodies are not observed in most cases of IDIAG, although detection of antineutrophil antibodies is technically difficult [Tesfa et al., 2009]. The bone marrow of IDIAG typically reveals an absence of neutrophil precursors back to about the promyelocyte stage. It may be important that this is about the stage when the neutrophil precursors start to synthesize oxidative enzymes such as myeloperoxidase [Brederoo et al., 1986].

1.6.2 Characteristics of IDIAG caused by specific drugs

One systematic review listed 125 drugs definitely or probably related to agranulocytosis [Andersohn et al., 2007], and although there are many similarities in the IDIAG caused by different drugs, there are also differences, and these differences likely highlight differences in mechanism.

1.6.2.1 Clozapine

Clozapine (Clozaril) is an orally-dosed dibenzodiazepine derivative developed in 1958 and sold commercially in 1972 as an antipsychotic agent used to manage the symptoms of treatment-resistant schizophrenia. Clozapine was the first of the atypical or second-generation antipsychotics developed, which collectively have different neurotransmitter binding profiles to the typical, or first-generation antipsychotics. The first generation antipsychotics, therefore, have different side effect profiles; their binding profile infamously leads to an acute Parkinson’s-like side effect known as tardive dyskinesia. Clozapine exhibits its antipsychotic effects through its strong binding to a variety of dopamine and serotonin receptors [Iqbal et al., 2003]. Specifically, clozapine has a high binding affinity for D₁ and D₄ dopamine receptors and a strong antagonism for the 5-HT₂ serotonin receptor. In contrast to typical antipsychotics, clozapine has a low affinity to D₂ receptors [Iqbal et al., 2003]. This binding profile is believed to be the reason
clozapine was the first antipsychotic that showed reduced risk of patients developing tardive dyskinesia in its side effect profile. Clozapine, however, does have its own set of serious side effects. Side effects such as weight gain; hypersalivation, and gastrointestinal hypomotility are accompanied by more life-threatening side effects listed as 5 black box warnings. These warnings are seizures, myocarditis, other cardiovascular and respiratory effects, dementia-related psychosis, and agranulocytosis. The most serious of these black box warnings is the potential to develop life-threatening agranulocytosis, which is typically seen within the first 6 months of treatment in 0.5-1.0% of treated patients. The potential of clozapine to lead to agranulocytosis led to its withdrawal from the European and U.S. market in 1974, 2 years after its initial introduction on the market. However, due to its unique ability to treat the symptoms of treatment-resistant schizophrenia, the FDA reintroduced it into the market in 1990 with the aforementioned black box warnings. The FDA also implemented strict prescribing guidelines where a patient must fail on two antipsychotic medications before being considered for clozapine treatment. Upon treatment, patients must also have weekly preemptive monitoring of peripheral neutrophil counts, which has been shown to decrease deaths due to drug-induced agranulocytosis.

Clozapine-induced IDIAG exhibits characteristics that strongly suggest an immune mechanism such as a typical delay in onset. Genetic HLA associations such as DRB1*0402, DQB1*0301, and DQA1*0302 in Jewish patients, and in non-Jewish patients, HLA-DR*02, DQB1*0502, and DQA1*0102 [Yunis et al., 1995] also suggest an immune mechanism. However, rechallenge with clozapine does not usually lead to rapid onset of agranulocytosis and therefore there is a presumed lack of memory T cells [Dunk et al., 2006]. Although agranulocytosis is not immediately seen upon clozapine rechallenge, it does usually occur more rapidly than on initial exposure. This should not be used as strong evidence against an immune mechanism, and the
time to onset is usually shorter on rechallenge. As mentioned earlier, most patients treated with clozapine have an increase in inflammatory cytokines, a spike in temperature, a spike in monocytes, and a paradoxical increase in ANC early in the course of treatment [Pollmacher et al., 2000; Lee et al., 2015].

1.6.2.2 Amodiaquine

Amodiaquine (Camoquin) is an orally active 4-aminoquinoline derivative with antimalarial and anti-inflammatory properties in which the mechanism of therapeutic action is still not well understood [Piedade & Gil, 2011]. In the mid 1980’s amodiaquine had been reported to cause agranulocytosis and/or liver toxicity in humans using it prophylactically [Harrison et al., 1992]. Because of this, it is no longer recommended for prophylaxis, and it is now used only at a lower dose (10 mg/kg/day) and in combination with other agents. It is currently used in one of the most globally administered antimalarial combination therapies consisting of artesunate (4 mg/kg/day) and amodiaquine (10 mg/kg/day) [Olliaro & Mussano, 2003]. This combination therapy is particularly useful in the treatment of chloroquine-resistant Plasmodium falciparum malaria infections [Famin & Ginsburg, 2002]. The adverse reactions caused by amodiaquine are idiosyncratic with an incidence of 1:2,000 for agranulocytosis and 1:30,000 for liver injury [Taylor & White, 2004].

1.7 Research focus and rationale

As described in this introductory chapter, the mechanisms leading to IDR are not well understood. It is suspected that these adverse drug reactions are induced by the production of reactive metabolites, formation of drug-protein conjugates, and induction of an immune response [Uetrecht & Naisbitt, 2013]. IDIAG is suspected to follow this generalized mechanism of adverse event induction; however, little is known about the specifics of the mechanism.
Therefore, the objective of this body of work is to better understand the mechanism of the initial immune response seen as a result of initiating a drug known to cause agranulocytosis, and to characterize the immune response that may lead to the adverse event. To understand the mechanism of these reactions, production of a valid animal model is paramount to do mechanistic studies. This is not a trivial task. At this point it appears that treating female Sprague Dawley rats with clozapine leads to an early immune response similar to that in humans; however, clozapine-induced agranulocytosis is idiosyncratic in humans, and it is likely idiosyncratic in rats as well, so inducing agranulocytosis is difficult.

The animal model previously used to do mechanistic studies on the early immune response to clozapine previously utilized IP injection as the method of administration. This method has two key disadvantages: IP injection causes peritonitis after 10 days of treatment, and thus longer studies are impossible. In addition, the administration of drugs via IP injection can lead to its own irritation and immune response, which when doing immune studies, is a potential confounding factor. We thus set out to develop an animal model of the early immune response to clozapine that would allow the administration of clozapine in a less invasive manner and for a longer duration of time (Chapter 2). Previous to these studies, this animal model had been used to understand the early immune response in terms of cytokine changes and monitoring neutrophil levels and kinetics. To understand the immune response better, we monitored immune cell changes using flow cytometry in a variety of different organs (Chapter 3).

Myeloperoxidase bioactivation of clozapine had been implicated in producing reactive metabolites of clozapine that are capable of binding to neutrophils and neutrophil precursors. This finding had been validated in vitro and in vivo by using a myeloperoxidase knockout mouse model: however, clozapine does not cause an immune response in mice for an unknown reason.
Therefore, although bioactivation studies are possible in the mouse model, further mechanistic studies are not feasible. This led us to use the rat model of clozapine-induced immune response and to block myeloperoxidase using a chemical inhibitor. These studies allow for the identification of differences in clozapine bioactivation between mice and rats. Furthermore, it allowed us to identify clozapine bioactivation by myeloperoxidase and its role in causing downstream proinflammatory responses in the model (Chapter 4).

The combination of these studies has led to a better understanding of the early immune response to clozapine. Among other findings, these data show the importance of macrophages in the immune response to clozapine, both in vivo and in vitro, and add evidence for the importance of inflammasome-generated IL-1β in exacerbating the proinflammatory response to clozapine.
Chapter 2
Clozapine Administration in the Water of Female Sprague Dawley Rats and Female C57/BL6 Mice: A Dose-Finding Experiment.
2.1 Abstract

Clozapine, an atypical antipsychotic, is limited in its use due to the risk of idiosyncratic agranulocytosis. Although very few patients develop agranulocytosis, the majority of patients have an immune response highlighted by neutrophilia at about the second week of treatment. This response is generally paired with increases in proinflammatory cytokines. The mechanism of clozapine-induced agranulocytosis is currently unknown, and as such, an animal model would greatly aid in mechanistic studies. Early attempts at developing a rodent model of clozapine-induced agranulocytosis have mimicked particular human data; most notably the neutrophilia seen in 60% of patients has been seen in 100% of female Sprague Dawley rats treated with a 30 mg/kg/day IP dose of clozapine. A limitation in using IP administration is it can only be given for 10 days due to the risk of peritonitis. Administering clozapine via the drinking water is less invasive and can be utilized indefinitely. However, administering clozapine via the drinking water to mice does not result in a minimum effective concentration in the peripheral blood (0.18 µg/mL) and does not have a significant effect on mouse neutrophil kinetics. In contrast, administering clozapine via the drinking water to rats achieves therapeutic concentrations of clozapine in the peripheral blood (0.35 µg/mL–0.8 µg/mL) after 5 days of administration. In rats this method of clozapine administration also causes neutrophilia beginning around day 5 of treatment, peaking around day 7, and still significantly elevated around day 9, before returning to basal levels. These results suggest dosing rats with clozapine in the drinking water may provide a dosing regimen capable of eliciting an inflammatory response that more closely mirrors human data.
2.2 Introduction

Clozapine is a second-generation antipsychotic medication used for treatment-resistant schizophrenia. Clozapine is considered a second line medication prescribed to patients who have not responded to at least two other antipsychotic medications. Clozapine is a second line medication because it comes with a risk of developing agranulocytosis. Although less than 1% of patients taking clozapine develop agranulocytosis, those that do, are at risk of life-threatening infections due to extremely low neutrophil counts [Honigfeld et al., 1998]. The agranulocytosis seen as a result of clozapine administration is classified as an IDR. Like other IDRs, clozapine-induced agranulocytosis is thought to be immune mediated, although the mechanism is unclear at this point. Recent retrospective human data have shown that about 60% of humans taking clozapine develop transient paradoxical neutrophilia with accompanying increases in cytokines such as IL-6 at the beginning of treatment typically around week 2 [Lee et al., 2015]. This suggests that most patients develop an immune response to clozapine that resolves with time. We hypothesize that this immune response is counteracted by immune tolerance in most patients; however, in the rare cases that immune tolerance fails to resolve, agranulocytosis occurs. To understand the mechanism of immune responses to clozapine, and ultimately clozapine-induced agranulocytosis, we set out to develop an animal model. When developing an animal model, it is important that it mimics human clozapine blood levels, leukocyte responses, as well as cytokine responses. Neutrophilia at the beginning of treatment remains an important indicator in determining clozapine’s ability to cause an immune response that affects neutrophil kinetics. Treatment of female Sprague Dawley rats with 30 mg/kg/day clozapine IP produces an animal model that mimics some, but not all responses seen in human patients.
2.3 Materials and methods

2.3.1 Chemical materials

Clozapine was generously provided by Novartis Pharmaceuticals Inc. (Dorval, QC), DMP-406 was obtained from DuPont-Pharma (Wilmington, DW), and desmethylclozapine was purchased from Abcam Inc. (Cambridge, MA). HPLC-grade methanol was obtained from Caledon Laboratories Inc. (Georgetown, ON). Acetic acid was purchased from EMD Chemical Inc. (Gibbstown, NJ). Ammonium acetate, ammonium chloride, and EDTA were purchased from Sigma-Aldrich (St. Louis, MO). Sucrose (99.5% pure) and potassium bicarbonate were purchased from BioShop Canada Inc. (Burlington, ON).

2.3.2 Treatment of animals with clozapine

All rodents were acclimatized to a 12/12 h light/dark cycle at 22 °C for a minimum of 1 week and were given regular access to rodent meal prior to starting clozapine treatment (Harlan Teklad, Madison, WI). The University of Toronto Animal Care Committee preapproved these experimental protocols. Female Sprague-Dawley rats (200–250 g, Charles River), housed in triplets, were administered either 0.25 mg/mL, 0.5 mg/mL, or 1.0 mg/mL clozapine in their drinking water, which was sweetened with 0.25 g/mL, 0.5 g/mL, or 0.1 g/mL sucrose, and the pH was adjusted to 3.0 with 1 N HCl and 1N NaOH for up to 28 days. Female C57BL/6J mice (6 weeks, Jackson Laboratories), housed in triplets, were administered either 0.068 mg/mL, 0.135 mg/mL, or 0.2 mg/mL clozapine in their drinking water, which was sweetened with 0.1 g/mL sucrose, and the pH was adjusted to 3.0 with 1 N HCl and 1 N NaOH for up to 12 days. Control animals were treated with sweetened water devoid of clozapine.
2.3.3 Blood collection

Blood samples were collected from the tail vein and saphenous vein from rats and mice, respectively, at specified time points to measure leukocyte counts in whole blood and analyze clozapine concentrations in serum. Total leukocyte counts were obtained manually from whole blood samples mixed with Turk’s blood diluting fluid (Ricca Chemical Company, Arlington, TX). Leukocyte differentials were calculated from blood smears stained with Giemsa-Wright-like stain (CAMCO Stain Pak, Cambridge Diagnostic Products, Inc., Fort Lauderdale, FL) by manually classifying a minimum of 100 leukocytes per slide. Serum samples were obtained by collecting blood from the tail vein in a clot-activating microvette tube and allowing it to settle for 1 h. The serum was then isolated by centrifugation at 10,000g and frozen at -80 °C until the time of analysis. Some serum samples were analyzed for alpha-1-acid glycoprotein via an ELISA kit according to the protocol from Life Diagnostics Inc. (West Chester, PA).

2.3.4 Utilizing flow cytometry to analyze absolute neutrophil counts

Whole blood was collected in EDTA-anticoagulation microvette tubes via the mouse saphenous veins at days 0, 3, 6, 10, and 12 and immediately used to obtain total neutrophil counts. Briefly, 50 μL whole blood was added to the Trucount tubes (BD biosciences, Mississauga, ON) along with 10 μL of anti-mouse Ly6G antibody conjugated to APC-eFluor 710 and allowed to incubate in the dark for 15 min. Contaminating erythrocytes were removed by adding red cell lysis buffer (150 mM NH₄Cl, 11.3 mM KHCO₃, 1 mM EDTA) for 20-40 min and immediately analyzed on the flow cytometer. Trucount tubes contain R780+/B525+ positive beads, which appear as double positive populations, the Ly6G antibody appears as R780+/B525- population. Absolute neutrophil concentration is equal to [(cell events × beads/test) / (bead events × 500 μL)].
2.3.5 Clozapine and desmethylclozapine serum concentration analysis

Clozapine and desmethylclozapine standards were prepared in methanol (0.01–1.5 µg/mL), diluted two-fold with blank rat serum, and then combined with internal standard DMP-406 (0.1 µg/mL final concentration). Serum samples were collected from the rats at 2, 5, 9, 14, and 28 days post-clozapine administration. As was done for the standards, clozapine serum samples were diluted two-fold with blank rat serum followed by addition of DMP-406 (0.1 µg/mL). Serum proteins were allowed to precipitate in the methanol solvent (1:4, serum:methanol) for 30 min at -20 ºC. The supernatant was isolated by centrifugation at 16,000g, diluted two-fold with water, and analyzed using a PE Sciex API 3000 quadrupole mass spectrometer with an electrospray ionization source (Sciex, Concord, ON) interfaced with an HPLC system (Shimadzu, Columbia, MD). The mobile phase consisted of 50% methanol, 1% acetic acid, and 2 mM CH$_3$CO$_2$NH$_4$ and a Kinetex 2.6µ C18 100 A 50 x 4.6 mm column (Phenomenex, Torrance, CA) was employed.

2.3.6 Statistical analysis

GraphPad Prism (GraphPad, San Diego, CA) was employed for all statistical analyses using the paired Student’s t test or one-way ANOVA. Data sets were expressed as the mean ± SEM, and results were considered statistically significant if p < 0.05.

2.4 Results

2.4.1 A clozapine concentration of 0.135 mg/ml in drinking water gives the highest clozapine blood levels in mice

Mice drink an average of 5 mL per day (as per the University of Toronto’s mouse protocol), and an IP dose of 30 mg/kg/day achieved therapeutic concentrations in rodents; therefore, 3
concentrations of clozapine were chosen, that if drunk at normal levels, would achieve a relevant extrapolated dose assuming 100% bioavailability. Mice were treated with 0.068 mg/mL, 0.135 mg/mL, or 0.2 mg/ml clozapine in the drinking water supplemented with 0.5 g/mL sucrose and a pH of 3 for 17 days. To assess extrapolated doses, the volume of water consumed by each mouse was observed (Figure 2-1A). The average volume was 9.2 mL/day for the 0.068 mg/mL clozapine group, 6.95 mL/day for the 0.135 mg/mL clozapine group, and 3.78 mL/day for the 0.2 mg/ml clozapine group. All mice weights remained constant at 22 g throughout the treatment (data not shown). Neutrophil counts were monitored throughout treatment, and no significant changes were observed in any of the groups (Figure 2-2). In these mice, none of the treatment groups reached the average minimum effective concentrations in humans of 0.35 μg/mL (Figure 2-3).

**Figure 2-1. Amount of water consumed by three treatment groups over a 17 day treatment period.**

Mice were treated with clozapine in their drinking water at varying concentrations for 17 days. Results are shown as average water consumption by the mice in each treatment group from the previous time point to the indicated time point.
Figure 2-2. Manual absolute leukocyte and neutrophil counts in mice from three clozapine treatment groups.

Mice were treated with clozapine in their drinking water at varying concentrations. (A) Total leukocyte counts were determined manually and (B) absolute neutrophil counts were obtained from freshly prepared blood smears. Results are expressed as the mean ± SEM.

Figure 2-3. Clozapine serum levels in mice treated with three different clozapine concentrations in their drinking water.

Serum was collected from mice at 6:30 AM after 48 h access to clozapine in their drinking water at varying concentrations. Protein was removed by methanol precipitation, and the supernatant was analyzed by LC-MS/MS using a mobile phase of 50% methanol, 1% acetic acid, and 2 mM CH₃CO₂NH₄ and a Kinetex 2.6µ C18 100A 50 x 4.5 mm column. Results are shown from individual animals and expressed as the mean ± SEM. The segmented line represents the average minimum effective concentration reported in patients.
2.4.2 Dosing mice with clozapine in their drinking water did not lead to a proinflammatory response

No significant weight change was observed over a 12 day treatment period using the 0.135 mg/mL clozapine dose (Figure 2-4). Also, there were no significant differences in the neutrophil counts of mice as a result of clozapine treatment in the drinking water (Figure 2-5B). Alpha-1-acid glycoprotein is an acute phase protein released early in an immune response and was previously shown to increase significantly in rats treated with clozapine via IP injection, both acutely after a single dose, and chronically over a 10-day dosing regimen [unpublished data]. Both control and clozapine-treated mice had alpha-1-acid glycoprotein levels of about 0.6 µg/mL (Figure 2-6).

![Figure 2-4. Weights of mice administered 0.135 g/mL clozapine in drinking water.](image)

Mice were treated with clozapine in their drinking water for 12 days (0.135 mg/mL). Total body weights were determined by weighing the mice at specific times throughout the treatment. Results are expressed as the mean ± SEM.
Figure 2-5. Absolute neutrophil counts analyzed by flow cytometry from mice administered clozapine in drinking water.

Mice were treated with clozapine in their drinking water for 12 days (0.135 mg/mL). Whole blood was put into Trucount bead tubes along with an anti-RP1 antibody to tag neutrophils. Trucount beads are seen as B525+/R780+ events and neutrophils are seen as B525-/R780+ events. Gate placement was obtained using fluorescence minus one tagged whole blood. Absolute neutrophil counts were calculated using the following equation: \( \frac{\text{cell events} \times \text{beads/test}}{\text{bead events} \times 500 \mu\text{L}} \). Results are expressed as the mean ± SEM.

Figure 2-6. Alpha-1-acid glycoprotein serum levels in mice administered clozapine in drinking water.

Serum concentrations of alpha-1-acid glycoprotein were measured by ELISA over a period of 8 days of clozapine treatment by administering it in their drinking water (0.135 mg/mL). Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by two-way ANOVA.

2.4.3 Gavaging rats with clozapine led to therapeutic concentrations and caused neutrophilia

Another alternative, less invasive route of clozapine administration for rats is oral gavage, which can be used to administer drugs for up to 3 months if done correctly; therefore, clozapine administration via oral gavage was attempted. The clozapine blood levels following a single
100mg/kg/day dose appeared to have the highest peak blood levels of about 0.6 µg/mL; all doses revealed a clozapine half life of 2-5 h in these rats, and after 24 h, no drug was detectable via LC-MS/MS in any of the treatment groups (Figure 2-7). Absolute neutrophil counts analyzed via blood smears at the time of blood draw showed that a clozapine dose of 70 mg/kg/day by gavage resulted in neutrophilia between 1-5 h post dose, and chronically statistically significant neutrophilia was observed on day 4 of treatment (Figure 2-8).

Figure 2-7. Serum clozapine levels following varying doses of clozapine administered by oral gavage.

Serum was collected from rats at 0.5 h, 2 h, 3 h, 5 h, and 24 h post clozapine administration by oral gavage. Three different clozapine doses were administered. Protein was removed by methanol precipitation, and the supernatant was analyzed by LC-MS/MS using a mobile phase of 50% methanol, 1% acetic acid, and 2 mM CH₃CO₂NH₄ and a Kinetex 2.6µ C18 100Å 50 x 4.5 mm column. Results are shown from individual animals. The grey shaded area represents the average therapeutic range reported in patients.
Figure 2-8. Absolute neutrophil counts following a single PO dose of clozapine.

Rats were treated with clozapine administered by oral gavage (70 mg/kg) every 24 h. Absolute neutrophil counts were determined manually from freshly prepared blood smears after (A) a single dose and (B) over a 4 day treatment period. Results are expressed as the mean ± SEM, Total leucocyte counts were determined manually and used to calculate ANCs along with differential counts obtained from freshly prepared blood smears. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by two-way ANOVA, where ** *p < 0.01 and ****, *p < 0.0001.

2.4.4 A clozapine solution of 0.5 mg/mL clozapine, 0.1 g/mL sucrose, pH 3 solution in drinking water gives the highest clozapine blood levels in rats.

Rats drink an average of 25 mL per day (as per the University of Toronto’s rat protocol), and an IP dose of 30 mg/kg/day achieved therapeutic blood concentrations. We therefore chose 3 concentrations of clozapine, which if water consumption did not change, would achieve a relevant therapeutic concentration. A dose of 0.18 mg/mL was used to access when to take blood samples; 6:30 AM or 12:30 PM; the serum level was higher when blood samples were taken at 6:30 AM (Figure 2-9). Rats were treated with 0.5 mg/mL clozapine in the drinking water supplemented with 0.05 g/mL sucrose and a pH of 3, or 0.05 g/mL sucrose and a pH of 7, or 0.1 g/mL sucrose and a pH of 3 or a 0.1 g/mL sucrose and a pH of 7. All blood samples were taken at 6:30 AM, 2 days after beginning the drug treatment. None of the clozapine blood levels in these rats reached the average minimum effective dose seen in humans after 2 days of treatment. Blood levels were unaffected by the pH of the water, but an increase in drug level was seen with increased sucrose concentration (Figure 2-10).
Figure 2-9. Measurement of clozapine serum concentration by LC-MS/MS of varying blood draw times.

Serum was collected from rats at 6:30 AM or 12:30 PM after 48 h of access to clozapine in their drinking water (0.18 mg/mL). Protein was removed by methanol precipitation, and the supernatant was analyzed by LC-MS/MS using a mobile phase of 50% methanol, 1% acetic acid, and 2 mM CH$_3$CO$_2$NH$_4$ and a Kinetex 2.6µ C18 100 A 50 x 4.5 mm column. Results are shown from individual animals and expressed as the mean ± SEM. The segmented line represents the average minimum effective concentration reported in patients.

Figure 2-10. Clozapine serum concentrations in different treatment groups in which the clozapine was administered in the drinking water.

Serum was collected from rats at 6:30 AM after 48 h access to clozapine in their drinking water with varying sucrose concentrations and pH. Protein was removed by methanol precipitation, and the supernatant was analyzed by LC-MS/MS using a mobile phase of 50% methanol, 1% acetic acid, and 2 mM CH$_3$CO$_2$NH$_4$ and a Kinetex 2.6µ C18 100A 50 x 4.5 mm column. Results are shown from individual animals, and expressed as the mean ± SEM. The segmented line represents the average minimum effective concentration reported in patients.
2.4.5 Treatment of rats with clozapine in their drinking water resulted in therapeutic blood levels and neutrophilia.

Based on the preceding experiments, the solution of 0.5 mg/mL clozapine, 0.1 g/mL sucrose, pH 3 was chosen to achieve the highest clozapine blood levels in the rat model. The pH of 3 also helped to maintain clozapine solubility for an extended period of time. A 14 day study was completed to monitor clozapine blood levels for an extended period of time, as well as to monitor for clozapine-induced neutrophilia. Over a 14 day period, treated rats drank lower-than-normal amounts of drinking water solution over the first week of treatment (10 mL/day), but it rose to a normal level after 7 days (25 mL/day: Figure 2-11A). Over a 14 day period the extrapolated dose of clozapine in these rats was 40 mg/kg/day clozapine. Control rats drank on average 90 mL/day of the 0.5 g/mL sucrose, pH 3 water (Figure 2-11B). Treated rats lost a significant amount of weight (down from 250 g to 200 g) until drinking levels returned to normal, and their weight returned to 250 g (Figure 2-12). Clozapine levels were measured in blood taken at 6:30 AM and found to reach therapeutic levels of 0.6 µg/mL after day 5 that continues to day 27 (Figure 2-13A). Desmethylclozapine levels were found to be detectable in the blood at days 5 and 9 where levels reached about 0.2 µg/mL (Figure 2-13B). Leukocyte counts were obtained utilizing blood smears at the time of each blood draw (Figure 2-14A); lymphocyte counts (Figure 2-14B), neutrophil counts (Figure 2-14C), and monocyte counts (Figure 2-14D). Leukocyte counts showed no significant change due to treatment: lymphocytes were significantly decreased only at the day 14 time point where the treated group’s lymphocyte count was 5,000 cells/µL of blood compared to that of the control group’s 8,000 cells/µL of blood, neutrophilia was observed between days 5-9 and peaking at 4,000 cells/µL of blood compared to the control group’s 2,000 cells/µL; and there was no significant change in the number of monocytes. Clozapine-induced neutrophilia was observed, so serum alpha-1-acid
glycoprotein levels were assessed, but no significant changes were seen throughout 8 days of treatment (Figures 12-15).

Figure 2-11. Consumption of drinking water containing clozapine over a period of 2 weeks.

Rats were treated with clozapine in their drinking water (0.5 mg/mL) for 14 days. Results are shown as average water consumption of the rats from the previous time point to the indicated time point. Results show the amount of water consumed by (A) the treated group over a 14 day treatment time compared to normal rat water consumption as listed in the U of T rat module and depicted as the dotted line. (B) Water consumed by the treated group compared to water consumed by the control group over a 14 day treatment period.

Figure 2-12. Body weights over a 2 week period of clozapine treatment.

Rats were treated with clozapine for 14 days in their drinking water (0.5 mg/mL) and weighed periodically. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by two-way ANOVA, where *, p < 0.05.
Figure 2-13. Clozapine and desmethylclozapine blood levels over a 2 week period of clozapine treatment.

Serum was collected from rats at varying time points throughout a 27 day period of treatment with clozapine in their drinking water (0.5 mg/mL). (A) Clozapine blood levels were determined by LC-MS/MS and (B) desmethylclozapine blood levels were determined by LC-MS/MS. Protein was removed by methanol precipitation, and the supernatant was analyzed by LC-MS/MS using a mobile phase of 50% methanol, 1% acetic acid, and 2 mM CH₃CO₂NH₄ and a Kinetex 2.6μ C18 100 A 50 x 4.5 mm column. Results are shown from individual animals, and expressed as the mean ± SEM. The grey shaded area represents the average therapeutic range reported in patients.

Figure 2-14. Leukocyte counts over a 2 week period of clozapine treatment.
Rats were treated with clozapine in their drinking water (0.5 mg/mL) for 14 days. (A) Total leukocyte counts were determined manually and differential counts were obtained from freshly prepared blood smears to determine (B) absolute lymphocyte counts, (C) absolute neutrophil counts, and (D) absolute monocyte counts. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by two-way ANOVA, where *, p < 0.05 and **, p < 0.01.

![Graph of Alpha-1-acid glycoprotein levels over 8 days of clozapine treatment.](image)

**Figure 2-15. Alpha-1-acid glycoprotein levels over 8 days of clozapine treatment.**

Serum concentrations of alpha-1-acid glycoprotein were measured by ELISA over 8 days of clozapine treatment by administering it in their drinking water (0.5 mg/mL). Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by two-way ANOVA.

### 2.5 Discussion

Clozapine remains one of the most important treatment options for patients with schizophrenia, particularly those who are resistant to treatment with first and second line medications. Clozapine, however, is severely limited in its use due to its propensity to cause agranulocytosis in almost 1% of the patients treated. It remains a top priority to determine the mechanisms of idiosyncratic drug reactions such as clozapine-induced agranulocytosis. Various studies have been performed utilizing in vitro techniques, in vivo techniques, and through clinical studies. Developing a valid animal model of clozapine-induced agranulocytosis would allow for in depth mechanistic studies and would greatly facilitate the understanding of the mechanisms behind this idiosyncratic reaction. Previous attempts to develop an animal model of the early response to clozapine utilized an IP dosing strategy with female Sprague Daley rats [Lobach & Uetrecht,
This had positive results in that it caused neutrophilia at about day 5, but the studies were limited to 10 days. In this study, dosing rodents with clozapine in the drinking water was explored to achieve blood concentrations similar to therapeutic concentrations in humans over an extended period of time.

First, we attempted to dose mice with clozapine in drinking water. Previously mice were dosed with clozapine via IP injection, but resulted in severe lethargy or death [unpublished data]. Drinking water (pH 3) containing clozapine (0.135 mg/mL) and sucrose (0.1 g/mL) achieved the highest clozapine levels in the blood, as well as the highest extrapolated dose (52.13 mg/kg/day) of all clozapine solutions tested. This clozapine concentration did not significantly impact neutrophil kinetics (Figure 2-5). Although the extrapolated dose was higher than what is typically used in animal studies, the blood levels were low, and mouse neutrophil kinetics seemed to be unaffected by clozapine. In addition, mouse alpha-1-acid glycoprotein levels were not altered by clozapine treatment (Figure 2-6). This suggests that either clozapine levels must be higher and in the therapeutic range in humans, or mice simply do not have an immune response to clozapine. In either case the mouse is not a good model for the early immune response humans have to clozapine.

In contrast, we were able to achieve blood levels of clozapine in rats within the human therapeutic range by adding it to their drinking water at a concentration of 0.5 mg/mL clozapine with added sucrose (0.1 g/mL) and pH 3 to make to clozapine soluble. However, the concentration did not reach levels close to those seen after a single 30 mg/kg IP dose of clozapine (1.35 µg/mL). A lower Cmax is expected with constant dosing compared to a bolus dose, but levels are also affected by a very low oral bioavailability, shown to be around 5.32% in rats and 50% in humans [Sun & Lau, 2000]. Desmethylclozapine is the major metabolite of
clozapine, and it maintains the chemical structure capable of forming the nitrenium ion that is implicated in neutrophil covalent binding. Although clozapine is extensively metabolized [Centorrino et al., 1994], desmethylclozapine levels were only detectable via LC-MS/MS on days 5 and 9 and only reached levels of about 0.2 µg/mL. Desmethylclozapine could be contributing to what is covalently binding to neutrophils, but given the low blood levels, it is unlikely to make a significant contribution to the covalent binding and immune response. Neutrophilia was observed in the clozapine-treated rats between days 5 and 9 (Figure 2-14C), and lymphocyte numbers were significantly decreased on day 14 (Figure 2-14B), but monocytes appeared to remain unaffected by the clozapine treatment (Figure 2-14D). This lack of monocyte increase was unlike any data we have seen thus far in the female Sprague Dawley model. Typically, peripheral blood monocyte counts increase at day 9 of treatment; the lack of a monocyte increase in this case is likely due to the method of cell counting used during these studies. Creating and analyzing blood slides manually requires a skilled technician. Cells such as monocytes are not abundant and identifying their cell morphology among other leukocytes is not a trivial task. As such, hematology analyzers are becoming more popular as they count blood cells in a fully automated procedure based on known size ranges of each blood cell quickly and accurately. Unlike previous studies in which clozapine was administered via IP injection, alpha-1-acid glycoprotein was not significantly increased due to the clozapine treatment (Figure 2-15). These data indicate that clozapine delivered via the drinking water achieved therapeutic concentrations in female Sprague Dawley rats and caused neutrophilia, which is the common response in humans. This may represent a way to study the immune response induced by clozapine in an animal model; however, the fact that it did not result in an increase in alpha-1-acid glycoprotein suggests that the immune response at this dose is less in the rats than in humans who have higher sustained blood levels.
2.6 Acknowledgments

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Chapter 3

Clozapine’s Effect on Immune Cells in a Rat Model:
Implications for Idiosyncratic Drug-Induced
Agranulocytosis
3.1 Abstract

Clozapine is a very effective antipsychotic agent, but its use is limited by the fact it causes agranulocytosis. Most patients do not develop agranulocytosis, but they do develop a transient paradoxical neutrophilia and an increase in cytokines such as IL-6. Recently it was reported that 62% of patients treated with clozapine develop an increase in peripheral blood monocytes that precedes the increase in neutrophils. Interestingly, we found that treatment of THP-1 cells (a human monocyte cell line) with clozapine led to inflammasome activation with release of IL-1β. However, in most patients the immune response resolves without resulting in agranulocytosis, presumably via immune tolerance. If we could reproduce this immune response in animals it would allow us to study the mechanism by which clozapine induces an immune response, this could help to understand the mechanism of idiosyncratic agranulocytosis. Treatment of rats with clozapine (0.5 mg/mL + 0.1 g/mL sucrose in drinking water) resulted in clinically relevant serum concentrations of clozapine (0.7 µg/mL) and led to neutrophilia. In addition, there was an increase in the % peripheral monocytes (CD3-, CD11b/c+, and CD43+) from 3% to 9%. Given that clozapine can stimulate a proinflammatory response in THP-1 cells, we postulate that the proinflammatory response in vivo is due to activation of monocytes with the production of IL-1β; however, we were not able to detect an increase in serum IL-1β in rats.
3.2 Introduction

Clozapine is prescribed to about 5% of the schizophrenic population in the United States of America as a last resort for those who have treatment resistant schizophrenia, [Torrey et al., 2015]. About 20% to 30% of schizophrenic patients are thought to be treatment resistant, and 60% of this population respond to clozapine; therefore, it has been suggested that prescription rates should be closer to 12% to 18% [Bogers et al., 2016]. Physicians have a reluctance to prescribe clozapine because there is a risk of developing life-threatening agranulocytosis. Clozapine-induced agranulocytosis is an idiosyncratic drug reaction (IDR) and like other IDRs, it is likely immune mediated. Although only about 1% of patients taking clozapine develop agranulocytosis, the majority of patients do elicit an immune response. Currently in North America, patients taking clozapine must have their peripheral blood cell counts monitored weekly during the first 6 months of treatment, bi-weekly for the second 6 months and monthly for the remainder of the treatment plan. Data collected from these blood counts suggests that about 60% of human patients develop neutrophilia at the beginning of treatment at around week 2 and 62% of human patients develop monocytosis, typically around week 2 as well [Lee et al., 2015]. This is typically accompanied by an increase in inflammatory cytokines and a spike in body temperature [Pollmacher et al., 2000]. By assessing the ability of clozapine to affect immune cell changes in an animal model, we can achieve two things: first, we can verify the validity of the animal model by comparing peripheral blood immune cell changes to those reported in the literature, and second, we can gain a better understanding of clozapine’s ability to affect immune cell changes throughout the body in an animal, potentially providing insight as to what is occurring in humans.
3.3 Materials and methods

3.3.1 Chemical materials

Clozapine was generously provided by Novartis Pharmaceuticals Inc. (Dorval, QC). Lipopolysaccharide (LPS), phorbol-13-myristate-12-acetate (PMA), EDTA, KHCO₃, NH₄Cl, and Histopaque-1083 were purchased from Sigma-Aldrich (St. Louis, MO). Hanks’ balanced salt solution (HBSS); PBS (pH 7.4), fetal bovine serum (FBS), trypan blue, penicillin/streptomycin (Pen/Strep), and α-modified eagle’s medium (α-MEM) were purchased from Gibco (Life Technologies Inc., Burlington, ON). DMSO was purchased from BioShop Canada Inc. (Burlington, ON). Hydrogen peroxide was obtained from EMD Chemical Inc. (Gibbstown, NJ). Permeabilization buffer and fixation buffer were obtained from eBioscience (San Diego CA). Antibodies were obtained from the following companies: Fixable viability dye-eFluor506, CD11b/c-PE, CD4-FITC, CD8-PE/CY7, CD161-PerCPEFluor710, IL-17a-APC, CD25-PerCPEFluor710, FOXP3-APC, TNF-α-PE, CD11b-APC, MHC-II-PerCPEFluor710, HIS48-FITC, CD172a-APC, CD4-PerCPEFluor710, RPI-PE, and CD45-FITC were obtained from eBioscience (San Diego CA). CD32, CD62L-PE, IL-10-AlexaFluor647, CD3-BV421, CD45RC-PE, RP1-PE, INF-γ-PE, and CD71-PE were obtained from BD Biosciences (Mississauga, ON). CD45RA-PE, CD62L-FITC, IL-6-PE/CY7, and CD43-PE were obtained from Biolegend (San Diego, CA). CD163-AlexaFluor647 was obtained from BioRad (Mississauga, ON). VEGF-FITC was obtained from Abcam Inc. (Cambridge, MA).

3.3.2 Treatment of animals with clozapine

All rodents were acclimatized to a 12/12 h light/dark cycle at 22 °C for a minimum of 1 week prior to starting clozapine treatment and were given regular access to rodent meal (Harlan Teklad, Madison, WI). The University of Toronto Animal Care Committee approved the
experimental protocols. Female Sprague-Dawley rats (200–250 g, Charles River) housed in triplets were administered 0.5 mg/mL clozapine in drinking water, sweetened with 0.1 g/mL sucrose, and acidified to pH 3, for up to 28 days.

### 3.3.3 Blood and tissue collection

Blood samples were collected from the tail vein into EDTA-coated microvette tubes (Sarstedt, Montreal, QC, Canada) at specified time points to measure leukocyte counts in whole blood. Total blood cell counts were obtained from EDTA anti-coagulated whole blood samples using a Hemavet 950FS instrument (Drew Scientific, Dallas, TX, USA); each sample was analyzed in triplicate. At the one and two week time points, specific rat tissues were harvested following CO₂ euthanasia for flow cytometry analysis. Whole blood samples were collected via cardiac puncture into EDTA anti-coagulated Vacutainer tubes (BD Biosciences, San Jose, CA) to analyze the PBMCs by flow cytometry. PBMCs were isolated from anti-coagulated whole blood using Histopaque-1083. Briefly, whole blood was mixed in a 1:1 ratio with cold PBS and carefully layered over Histopaque-1083; this was centrifuged at 400g for 30 min with low acceleration/deceleration settings. The buffy coat was collected at the Histopaque-serum interface, and the contaminating red blood cells were removed using a red blood cell lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). The PBMCs were then washed twice with PBS and resuspended in FACS buffer for flow cytometry preparation. Cell viability was found to be > 95% using trypan blue exclusion for all PBMC isolations.

Splenocytes and cervical lymph node cells were also harvested at the time of necropsy for flow cytometric analysis. The tissues were mechanically minced with scissors, followed by pushing the cells, suspended in FACS buffer, through a 100 mm cell strainer with FACS buffer to create a single cell suspension. The cells were then washed twice with PBS and then re-suspended in
FACS buffer for flow cytometric preparation. Cell viability was found to be > 95% using trypan blue exclusion for all splenocyte and cervical lymph node isolations.

3.3.4 Measurement of immune cell changes

Clozapine was administered in the drinking water and PBMCs, splenocytes, and cervical lymph node cells were isolated as previously described at the end of weeks 1 and 2. Cells (1×10^6 cells/sample) were suspended in FACS buffer with a CD32 antibody for 15 min to block non-specific Fc binding. All incubations were carried out at 4 °C. Cells were washed with FACS buffer and stained with their respective extracellular antibodies for 30 min. Following three washes with PBS, cells were incubated for 30 min with viability dye. Cells were washed with FACS buffer then incubated for 30 min in 1× fixation buffer, washed again with FACS buffer, then incubated for 30 min with 1× permeabilization buffer, and then incubated with their respective intracellular antibodies in 1× permeabilization buffer for 60 min. Cells were washed two times with 1× permeabilization buffer, then resuspended in FACS buffer for flow cytometric analysis or kept overnight in 1:1 1× fixation: FACS buffer for analysis the next day. Cell populations were characterized using the following antibodies: macrophages were characterized as CD11b/c+/CD3-/CD68+ and further characterized as either M1 (CD11b/c+/CD68+/CD3-/CD163-) or M2 (CD11b/c+/CD68+/CD3-/CD163+). CD62L, MHC II, and IL-6 antibodies were used as macrophage activation markers. Alternatively macrophages were also classified according Dolen to et al. as follows: M1 (TNF-α+/MHC II+), M2b (MHC II+/IL-10+), M2a (VEGF-/IL-10+/MHC II+/TNF-α+) [Dolen et al., 2015]. Monocytes were characterized as CD11b+/CD3-/CD43+ and further characterized as either classical (CD11b+/CD43+/CD3-/CD4-/CD62L+) or non-classical (CD11b+/CD43+/CD3-/CD4+/CD62L-). NK cells were characterized as CD161a+/CD3-/CD8a+ cells, B cells were characterized as CD45RA+/CD3- cells, myeloid-derived suppressor cells (MDSC) were characterized as either monocytic MDSC
(HIS48+/CD172a+/RP-1+) or granulocytic MDSC (HIS48+/CD172a+/RP-1+). T cells were characterized as either helper T cells (CD3+/CD4+/CD8a-), cytotoxic T cells (CD3+/CD8a+/CD4-), TH17 cells (CD4+/CD3+/IL-17+), effector memory T cells (CD3+/INF-γ+, either CD4+ or CD8+), or T regulatory cells (CD3+/CD25+/FOXP3+). Flow cytometry analysis was carried out using a BD FACSCalibur configured with FlowJo software (Tree Star Inc., Ashland, OR), and compensation was carried out manually using fixed cells before sample analysis. For flow cytometric analysis, cells were initially gated on forward-scatter (FSC) and side-scatter (SSC) and then gated on live cells. A minimum of 50,000 live, correctly-gated cells/sample were acquired for each analysis.

3.3.5 Identification of progenitor populations in the bone marrow

The myeloid, lymphoid, and erythroid populations in the bone marrow were identified by modifications to the method of Saad et al. [Saad et al., 2000]. Bone marrow progenitor cells were obtained following CO2 euthanasia from the femurs and tibias of the Sprague Dawley rats. The bone marrow was flushed out with cold α-MEM and centrifuged at 750g for 5 min at 4 °C. Contaminating erythrocytes were removed with red cell lysis buffer, and the progenitor cells were washed and resuspended in FACS buffer. Trypan blue exclusion showed the viability to be > 95% for all cells used. Nucleated cells from the bone marrow (1 x 10^6 cells/sample) were resuspended in FACS buffer and blocked with CD32 antibody for 15 min. All incubations were carried out at 4 °C. Cells were washed with FACS buffer and stained with PE-anti-CD71 and FITC-anti-CD45 for 30 min. Following three washes, cells were incubated for 25 min with 1× fixation/permeabilization buffer (eBioscience), washed again, and then resuspended in FACS buffer. LDS-751 was added to all samples to identify the nucleated cells. Samples were analyzed
by flow cytometry using a BD FACSCanto II configured with FACSDiva software. Compensation was carried out before sample analysis using beads from BD Biosciences.

3.3.6 Measurement of IL-1β release in THP-1 cells stimulated with clozapine

THP-1 monocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in ATCC high glucose RPMI-1640 medium, supplemented with 10% FBS. Monocytes were cultured in 24-well plates at a concentration of \(4 \times 10^5\) cells/mL in a volume of 1 mL. Clozapine was dissolved in DMSO (0.25%) and added to the cells over a concentration range of 1–25 µg/mL. Cells were incubated at 37 °C, 5% CO₂ for 24 h, after which they were spun down, and the cell culture medium was then tested for IL-1β via ELISA (Life Technologies Inc., Burlington, ON).

Alternatively, monocytes were cultured in ATCC high glucose RPMI-1640 medium, supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol. Monocytes cultured in 24-well plates at a concentration of \(4 \times 10^5\) cells/mL in a volume of 1 mL and were differentiated to macrophages with PMA (25 ng/mL) over 3 days at 37 °C. Cells were washed once and resuspended in fresh medium without 2-mercaptoethanol. Clozapine was dissolved in DMSO (0.25%) and added to the cells over a concentration range of 1–10 µg/mL. Cells were incubated at 37 °C, 5% CO₂ for 24 h, at which point medium was collected for IL-1β analysis using an ELISA kit from R&D.

PBMCs were isolated from control or treated rats as previously described. Trypan blue exclusion showed the viability to be > 95% for all PBMC samples used. The PBMCs were cultured in 24-well plates at a concentration of \(1 \times 10^6\) cells/mL in a volume of 1 mL of ATCC high glucose RPMI-1640 medium supplemented with 10% FBS and 100 U Pen/Strep. Clozapine was
dissolved in DMSO (0.25%) and added to the cells over a concentration range of 2–25 µg/mL. Cells were incubated at 37 °C, 5% CO₂ for 24 h, at which point medium was collected for IL-1β analysis using an ELISA kit from R&D. After the supernatant was removed, more than 99% of the adherent cells were collected by rinsing the wells with cold HBSS and gentle mechanical scraping. The adherent cells were spun down at 600g and resuspended in FACS buffer for analysis by flow cytometry.

PBMCs were incubated at a concentration of 1×10⁶ cells/sample in FACS buffer with a CD32 antibody for 15 min to block non-specific Fc binding. All incubations were carried out at 4 °C. Cells were washed with FACS buffer and stained with their respective extracellular antibodies for 30 min. Following three washes with PBS, cells were incubated for 30 min with viability dye. Cells were washed with FACS buffer then resuspended in FACS buffer for flow cytometric analysis or kept overnight in 1:1 1× fixation:FACS buffer for analysis the next day. Cell populations were characterized using the following antibodies: monocytes were characterized as CD11b+/CD3-/CD43+ and further characterized as either classical (CD11b+/CD43+/CD3-/CD4-/CD62L+) or non-classical (CD11b+/CD43+/CD3-/CD4+/CD62L-).

3.3.7 Statistical analysis

GraphPad Prism (GraphPad, San Diego, CA) was employed for all statistical analyses by the paired Student’s t test, one-way ANOVA, or two-way ANOVA. Data sets were expressed as the mean ± SEM, and results were considered statistically significant if p < 0.05.
3.4 Results

3.4.1 Leukocyte counts in rats treated with clozapine in their drinking water

As previously described in Chapter 2, rats treated with drinking water containing 0.5 mg/mL clozapine, 0.1 g/mL sucrose, and pH 3 drank 10 mL/day of drinking water solution over the first week of treatment, which rose to 25 mL/day over the second week. The extrapolated dose of clozapine for the whole 14 day treatment period was 40 mg/kg/day. Control rats drank water containing 0.1 g/mL sucrose at pH 3, and they consumed on average 90 mL/day. Treated rats lost about 15% of their body weight during week 1 until drinking levels returned to normal, and their weights returned to normal shortly after. Blood was taken to determine total blood cell counts over a 17 day treatment period (Figure 3-1). Neutrophilia was observed between days 5 and 9, peaking around day 7 at 4,000 cells/µL (Figure 3-1B). An increase in monocytes was observed starting at day 8, peaking at day 12 at 1,000 cells/µL, still elevated at day 14, before returning to normal (Figure 3-1D). Leukocytes (Figure 3-1A), lymphocytes (Figure 3-1C), eosinophils (Figure 3-1E), basophils (Figure 3-1F), red blood cells (Figure 3-1G), and platelets (Figure 3-1H) all were not significantly altered compared to control throughout the 17 day treatment period.
Rats were treated with 0.5 mg/mL clozapine in their drinking water for 17 days. Blood samples were drawn from the tail vein at specified time periods throughout treatment into EDTA-anticoagulated microvette tubes. Each whole blood sample was analyzed on a Hemavet 950FS in triplicate. (A) Total leukocytes, (B) neutrophils, (C) lymphocytes, (D) monocytes, (E) eosinophils, (F) basophils, (G) red blood cells, and (H) platelets are shown. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by two-way ANOVA, where ***, p < 0.001.
3.4.2 Immune cell changes in various body organs

Only significant changes at either the 7 day or 14 day time point are shown; however, a multitude of different cells were analyzed that did not show any significant changes as a result of treatment.

In the peripheral blood, monocytes, marked by CD11b/c+/CD43+/CD3-, increased from 2.5% to 6% of viable cells between week 1 and week 2 due to clozapine treatment (Figure 3-2A,B). B cells, marked by CD45RA+/CD3-, decreased from 30% to 15% viable cells between week 1 and week 2 due to clozapine treatment (Figure 3-2C,D). IL-6-secreting monocytes were tested by looking at % viable CD11b/c+/CD43+/CD3-/IL-6+ cells, which were significantly elevated at week 2 (Figure 3-2E); however, the percentage of CD11b/c+/CD68+/CD3- cells that were IL-6+ was not significantly affected by clozapine administration (Figure 3-2F).

In the spleen of these animals, T regulatory cells, marked as CD3+/CD25+/FOXP3+ cells were increased over control at 2 weeks, from around 2% viable cells to 3% viable cells (Figure 3-3B). B cells, marked as CD45RA+/CD3- cells, residing in the spleen saw a significant decrease at the 2 week time point from around 60% viable cells to 40% viable cells between week 1 and week 2 due to clozapine treatment (Figure 3-3B).

In the cervical lymph nodes, B cells, marked as CD45RA+/CD3- cells, were significantly elevated at 2 weeks from around 30% at week 1 to around 50% at week 2 (Figure 3-4).

In the bone marrow of these animals the myeloid progenitor cells increased from week 1 (60%) to week 2 (70%) marked as LDS-751+/CD45+/CD71-/SSC_high and the lymphoid progenitor cells decreased from week 1 (8%) to week 2 (5%) marked as LDS751+/CD45+/CD71-/SSC_low (Figure 3-5).
Rats were treated with 0.5 mg/mL clozapine in their drinking water for either 7 days or 14 days. Flow cytometry staining of peripheral blood mononuclear cells are shown as the average for each group. Each graph is presented as % viable cells, gated for correct cell population based on FSC vs. SSC, single cells and viable cells. (A, B) Monocytes at 7 days and 14 days. (C, D) B cells at 7 days and 14 days. (E, F) Total IL-6+ monocytes and percentage of IL-6+ monocytes in control and treated groups at 14 days. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by student’s t test, where *, p < 0.05.
Figure 3-3. Immune cell changes in the splenocytes from clozapine-treated rats

Rats were treated with 0.5 mg/mL clozapine in their drinking water for either 7 days or 14 days. Flow cytometry staining of splenocytes are shown as the average for each group. Each graph is presented as % viable cells, gated for correct cell population based on FSC vs. SSC, single cells and viable cells. (A, B) T regulatory cells in the spleen at 7 days and 14 days. (C, D) B cells in the spleen at 7 days and 14 days. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by student's t test, where ***, p < 0.001

Figure 3-4. Immune cell changes in the cervical lymph nodes from clozapine-treated rats

Rats were treated with 0.5 mg/mL clozapine in their drinking water for either 7 days or 14 days. Flow cytometry staining of cervical lymph node cells are shown as the average for each group. Each graph is presented as % viable
cells, gated for correct cell population based on FSC vs. SSC, single cells and viable cells. (A, B) B cells in cervical lymph nodes at 7 days and 14 days. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by student’s t test, where *, p < 0.05.

Figure 3-5. Progenitor cell changes in the bone marrow from clozapine-treated rats

Rats were treated with 0.5 mg/mL clozapine in their drinking water for either 7 days or 14 days. Flow cytometry staining of bone marrow cells are shown as the average for each group. Each graph is presented as % viable cells, gated for correct cell population based on FSC vs. SSC, single cells and viable cells. (A, B) Myeloid progenitor cells in the bone marrow of rats at 7 days and 14 days of treatment. (C, D) Lymphoid progenitor cells in the bone marrow of rats at 7 days and 14 days of treatment. Results are expressed as the mean ± SEM, and statistical difference compared to control was determined by student’s t test, where *, p < 0.05, **, p<0.01.
3.4.3 Clozapine stimulation of THP-1 cells in vitro

![Bar chart showing IL-1β release from THP-1 cells stimulated with clozapine](image)

**Figure 3-6. IL-1β release from THP-1 cells stimulated with clozapine**

THP-1 cells were either cultured as monocytes or differentiated to macrophages for 48 h with PMA stimulation. THP-1 monocytes and macrophages were stimulated with varying concentrations of clozapine and the culture medium was used to determine IL-1β via ELISA. Results are expressed as the mean ± SEM, and statistical difference was determined by one-way ANOVA, where ****, \( p < 0.0001 \).

Both THP-1 monocytes and THP-1 macrophages were stimulated with varying concentrations of clozapine for 24 h, and the resulting supernatant was taken for ELISA analysis. THP-1 monocytes showed a significant IL-1β release after 25 µg/mL clozapine stimulation for 24 h (Figure 3-6). THP-1 macrophages showed a dose-dependent increase in IL-1β release with increases in clozapine concentration. Treated PBMCs stimulated with 2 µg/mL clozapine treatment resulted in an IL-1β release of 49 µg/mL; a 10 µg/mL clozapine stimulation resulted in 155 µg/mL IL-1β release.

3.4.3.1 Clozapine stimulation of PBMCs isolated from rats ex vivo

PBMCs were taken from rats treated with clozapine (treated) or control saline (saline). These PBMCs were cultured in 24 well plates at a contratration of 1×10⁶ cells/mL, of which the % monocytes seen in each culture were 0.4% (Figure 3-7A). Both control PBMCs and treated PBMCs were stimulated with varying concentrations of clozapine in vitro for 24 h, and the
resulting supernatant was taken for IL-1β analysis. Pre-treated PBMCs showed significantly greater IL-1β release than did control cells after 5, 10, and 25 µg/mL clozapine stimulation for 24 h (Figure 3-7B). Treated ex vivo PBMCs showed a dose-dependent increase in IL-1β release with increases in clozapine concentration. Treated PBMCs stimulated with 2 µg/mL clozapine treatment resulted in an IL-1β release of 92 µg/mL; a 25 µg/mL clozapine stimulation resulted in 195 µg/mL IL-1β release.

Figure 3-7. IL-1β release from isolated rat PBMCs stimulated with clozapine

Rats were treated with 0.5 mg/mL clozapine in their drinking water for 14 days. Blood samples were drawn from the tail vein at specified time periods throughout treatment into EDTA-anticoagulated microvette tubes. PBMCs were extracted using Histopaque 1083 and cultured in RPMI 1640 medium. PBMCs were stimulated with varying concentrations of clozapine and culture medium was used to determine IL-1β via ELISA. (A) Total monocytes (CD43+, CD11b+, CD3-) in the incubated PBMC population, (B) IL-1β release following stimulation of PBMCs with varying concentrations of clozapine for 24 h. Results are expressed as the mean ± SEM, and statistical difference was determined by one-way ANOVA, where *, p < 0.05.

3.5 Discussion

Clozapine is known to cause a proinflammatory response in the form of peripheral blood immune cell changes in the majority of patients, particularly during the first month of treatment. We set out to determine if the female Sprague Dawley rat model could elucidate the mechanism of the early immune response to clozapine. In using an animal model we were able to determine immune cell changes at various time points throughout treatment in tissues that are not practical
to assess in humans. From these findings we are able to assess the validity of this animal model in mirroring human response to clozapine as well as gather data not practical in humans on whole body cellular changes.

Despite the ability of clozapine to affect a variety of hematological indices in the majority of human patients [Lee et al., 2015], clozapine affected only neutrophil cell counts and monocyte cell counts in the female Sprague Dawley rats. Neutrophilia occurred between days 5 and 9, and an increase in monocytes following the neutrophilia between days 8 and 14. Neutrophil kinetics appeared to be affected by clozapine when administered in the drinking water, much the same as it is when administered as a single dose per day administration via IP injection [unpublished data]. However, the neutrophilia does not appear to be as pronounced as it is with the IP injection route of administration. This animal model reflects human data for its effects on neutrophils; however, the majority of humans also have increases in platelet counts, eosinophils, and red blood cells [Lee et al., 2015]. It is possible that neutrophils are more responsive to lower clozapine levels than the other leukocytes, and that is why there is no change in this model compared to what is seen in humans.

Clozapine’s ability to elicit a proinflammatory response can be best understood if we established a complete picture of how leukocytes are responding and the time course of the changes. To do this we utilized flow cytometry to identify a variety of different cell types in a variety of different organs at 7 days of treatment when the neutrophilia is at its peak, and at 14 days when the neutrophilia has subsided. Significant differences are seen at the two week time point when comparing rats in the treated group to those in the control group. Monocytes, as identified by size, granularity and the markers CD11b/c+/CD3-/CD68+ are significantly increased in the treated group at 14 days (Figure 3-2A, B). Also increased are the IL-6+ monocytes (Figure 3-
2E); however, the percentage of monocytes in the treated group is the same as the control group (Figure 3-2F). This suggests that an increased percentage of monocytes are being recruited into the peripheral blood, but more of them are not actively secreting IL-6, which is one of many monocyte activation markers. MHC-II and CD62L expression on monocytes was also monitored, both of which have increased expression when monocytes are activated, but neither of which were expressed in elevated amounts in the treated groups’ monocytes (data not shown). B cells, marked as CD45RA+/CD3-, were significantly decreased at week 2 in the peripheral blood, but not at week 1 (Figure 3-2D). Concurrently, B cells were elevated at 14 days in the cervical lymph nodes (Figure 3-4B). Although B cells increased in the cervical lymph nodes, the population was decreased in the spleen (Figure 3-3C,D), suggesting a possible relocation of the B cell population into the cervical lymph nodes at that time point. These changes at specific times give a glimpse into how B cell populations might respond during clozapine administration. Anti-inflammatory cell changes were also documented; of the MDSCs and Tregs, the only significant change in population was a significant increase in Tregs in the spleen at the 14 day time point (Figure 3-3B). Tregs are anti-inflammatory subsets of T cells, which generally act to down regulate the inflammatory action of T cells. Lastly the progenitor cells in the bone marrow of these animals responded in a similar fashion to what was seen as a result of clozapine administration via IP injection. The myeloid progenitor cell population increased after 14 days of treatment (Figure 3-5B); concurrently, the lymphoid progenitor cell population decreased at the 14 day time point (Figure 3-5D). Myeloid progenitors differentiate into monocytes and neutrophils among other myeloid cells, while lymphoid progenitor cells differentiate into NK cells, T cells, and B cells. The increase in myeloid progenitors may act to replenish those cells, which differentiated into neutrophils during the neutrophilia stage of treatment, or in response to the production of more monocytes, which is seen in the peripheral blood after 14 days of
treatment. A corresponding decrease in lymphoid progenitor cells reflects the decrease in peripheral blood and spleen B cells at the 2 week time point.

The increase in monocytes at 14 days as measured by flow cytometry is noteworthy because it mirrors the findings collected from human data. Monocytes and macrophages also appear to respond to drugs known to cause idiosyncratic drug reactions, while structurally similar medications known to not cause those reactions do not stimulate macrophage responses [Weston & Uetrecht, 2014]. Clozapine has the ability to stimulate THP-1 macrophages and monocytes in a dose-dependent fashion to release proinflammatory IL-1β (Figure 3-6). We hypothesize that macrophages and monocytes play a key role in eliciting a proinflammatory response to clozapine and other IDR-causing medications. Seeing increased monocytes in the peripheral blood fits in with this narrative, although these monocytes did not appear to be any more activated than control monocytes when looking at the expression of activation markers such as IL-6, MHC-II, and CD62L. Monocytes can be preactivated in the peripheral blood and have the ability to respond to stimuli faster than dormant monocytes. We explored this idea by isolating PBMCs from control and treated rats after 14 days of treatment and subjecting them to clozapine stimulation for 24 h. PBMCs from rats pretreated in vivo with clozapine responded with an increased IL-1β release when subjected to 5, 10, or 25 µg/mL clozapine compared to PBMCs isolated from control animals (Figure 3-7). This shows that these rat monocytes are not only increased in number, but are in a primed state, with the increased ability to respond to clozapine treatment.

Overall the rats in this model respond to clozapine with some similar characteristics to what is seen in humans, most notably a delayed, but early neutrophilia. All female Sprague Dawley rats responded to clozapine with neutrophilia as opposed to 60% of humans treated with clozapine.
The neutrophilia subsided in all of the rats, and none developed neutropenia or agranulocytosis, although it is possible that with a longer duration of treatment or greater number of animals, some may have developed an adverse reaction. The initial immune response, followed by a return to basal levels is indicative of immune tolerance mechanisms, and we suspect this is the overwhelmingly dominant response in humans as well. We hypothesize that immune tolerance stopped the immune response from leading to an autoimmune-like state where neutropenia and agranulocytosis occurs. From this rat model we can deduce the importance of monocytes and macrophages in creating a proinflammatory environment in response to clozapine treatment. Other cellular changes occurred in a variety of organ systems within the animal model in response to clozapine treatment that are difficult to access in human subjects, but interesting to monitor in an animal model.

3.6 Acknowledgments

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Chapter 4

In Vivo Myeloperoxidase Inhibition in a Sprague Dawley Rat Model of the Early Immune Response of Clozapine Administration
4.1 Abstract

Clozapine is a very effective antipsychotic agent, but its use is limited by the fact that it causes agranulocytosis in less than 1% of patients. Although most patients do not develop agranulocytosis, most do develop an immune response with an increase in serum IL-6, a transient paradoxical neutrophilia, and an increase in peripheral blood monocytes after 2 weeks of treatment. Two major hypotheses are used to explain how drugs are able to elicit an immune response leading to an idiosyncratic drug reaction (IDR): the hapten hypothesis and the danger hypothesis. The hapten hypothesis states that most drugs are first bioactivated into a reactive metabolite before covalently binding to native proteins, forming what is detected as a foreign hapten by the immune system. Previously our lab used myeloperoxidase (MPO) -/- mice to show that amodiaquine and clozapine are about 50% bioactivated by MPO in C57/BL6 mice. MPO is the main metabolic enzyme found in neutrophils and their precursors, and it appears important in generating reactive metabolites of drugs such as amodiaquine and clozapine. These reactive metabolites are capable of binding to neutrophils, which may be the hapten required to elicit an immune response. Mice are very sensitive to the neurologic effects of clozapine; the dose of clozapine that they tolerate is limited, and they do not respond with neutrophilia. In contrast, treatment of rats with clozapine (30 mg/kg IP) results in clinically relevant serum concentrations (1.4 µg/mL), and characteristic neutrophilia. In order to understand MPO’s role in bioactivating clozapine in rats, we used an irreversible inhibitor of MPO, PF-1355. PF-1355 is also able to prevent about 65% of clozapine covalent binding to neutrophil precursors in the rat model. Inhibiting MPO activity in the rat model also appears to significantly dampen the neutrophilia seen in the model. Various immune cell changes previously documented in this model appear to be affected by inhibiting MPO. Some notable findings include a decrease in T helper cells and
cytotoxic T cells due to clozapine treatment that appears to be attenuated by the MPO inhibition. An important immune cell change that appears not to be affected by MPO inhibition is a characteristic increase in monocytes, which follows the neutrophilia in this model. These studies show the role of MPO in bioactivating clozapine in a rat model of clozapine-induced immune activation. These findings have implications in understanding MPO’s involvement in the mechanism of idiosyncratic clozapine-induced agranulocytosis.

4.2 Introduction

Clozapine-induced agranulocytosis is an idiosyncratic drug reaction (IDR) that affects 0.1-1% of patients taking clozapine. It is a life-threatening reaction because it increases the risk of infections due to the drop in neutrophil count. The mechanism of idiosyncratic clozapine-induced agranulocytosis is not well understood, but like many IDRs, it is likely to involve an immune mechanism. Bioactivation of drugs that cause IDRs is likely key to the reaction. According to the hapten hypothesis, drugs or reactive metabolites of drugs, bind to endogenous proteins making a covalently bound hapten-protein complex that is seen as foreign to the immune system. In the case of idiosyncratic liver injury, the bioactivation occurs via enzymes in the liver. Because the liver is the site of the majority of the body’s metabolizing enzymes, many drugs that cause IDRs cause idiosyncratic drug-induced liver injury [Cho & Uetrecht, 2016]. In the case of idiosyncratic drug-induced agranulocytosis, it is likely that the drug is bioactivated into a reactive metabolite by the cell that is affected, in this case neutrophils and their precursors.

Neutrophil-mediated bioactivation of drugs such as clozapine has been well studied in vitro. Myeloperoxidase (MPO) is the main enzyme in neutrophils that is responsible for the neutrophils’ oxidizing power, and it is primarily used by the cell to kill bacteria and other
pathogens. MPO works by producing a very strong oxidant known as hypochlorous acid, which is able to oxidize easily oxidized drugs into reactive intermediates [Liu & Uetrecht, 1995; Tingle et al., 1995]. These in vitro studies showed, for the first time, that HOCl produced by the NADPH oxidase/MPO system within neutrophils results in a strong enough oxidizing agent to produce a reactive metabolite of drugs such as clozapine [Liu & Uetrecht, 1995] and amodiaquine [Tingle et al., 1995]. These papers also showed that the reactive metabolites of clozapine and amodiaquine are able to bind to neutrophil proteins. This binding could be limited if another competing nucleophile was introduced, such as glutathione (GSH). These studies support the hypothesis that clozapine metabolites have the ability to act as a hapten and bind to neutrophil proteins. These haptens could then stimulate an immune response leading to an immune mediated idiosyncratic reaction such as agranulocytosis. However, later data suggested that patients with under-functioning MPO due to a MPO allelic variant show no less susceptibility to developing clozapine-induced agranulocytosis [Mosyagin & Dettling, 2004]. This finding suggested that MPO might not be the sole enzyme responsible for the bioactivation of drugs by neutrophils. With no way of knowing how important MPO is in the bioactivation of clozapine in humans, the Uetrecht lab set out to understand the role of MPO in bioactivating clozapine and amodiaquine in vivo by utilizing a MPO knockout mouse. Mice do not tolerate clozapine well; they do not respond with increased inflammatory cytokines, and they do not develop the common paradoxical neutrophilia that is typically seen in humans [unpublished data]. Thus, the mouse model is limited to only bioactivation studies. To understand MPO’s role in this mouse model, neutrophils had to be spiked with clozapine ex-vivo. These studies indicated that MPO was responsible for creating 50% of the reactive metabolites that bind to neutrophil precursors in the bone marrow in mice [Lobach & Uetrecht, 2014]. The other 50% of bioactivation may come from other peroxidases expressed in neutrophils. Although there are
many possibilities, we tested lipoxygenase (LOX), partially due to the availability of a LOX knockout mouse. The results from the LOX knockout mouse showed that LOX does not appear to be important in the bioactivation of amodiaquine in the mouse model (data not shown).

Clozapine is an easily oxidized drug, and in vitro studies have shown that neutrophils can metabolize clozapine into reactive intermediates that can, in turn, covalently bind to neutrophil proteins [Liu & Uetrecht, 1995]. Moreover, binding of clozapine to neutrophils probably occurs in all patients who take the drug.

The goal of the present work is to understand MPO’s role in the immune response to clozapine in a rat model. This work utilized an MPO inhibitor, known as PF-1335, which is a mechanism-based MPO inhibitor that has been shown to inhibit MPO in an animal model and prevents immune complex vasculitis and anti–glomerular basement membrane glomerulonephritis [Zheng et al., 2015]. PF-1355 will be used to inhibit MPO in our rat model of early clozapine-induced immune responses in female Sprague-Dawley rats.

4.3 Materials and methods

4.3.1 Chemical materials

Clozapine was generously provided by Novartis Pharmaceuticals Inc. (Dorval, QC). PF-1355 was generously provided by Pfizer (Cambridge, MA). Ammonium chloride, anhydrous sodium sulfate, bovine serum albumin (BSA), Dextran-500, EDTA, horseradish peroxidase (HRP), manganese dioxide, sodium dodecyl sulfate (SDS), and Trizma base were purchased from Sigma-Aldrich (St. Louis, MO). Potassium bicarbonate, sodium chloride, tween-20, and 3,3’,5,5’-tetramethylbenzidine (TMB) were bought from BioShop Canada Inc. (Burlington, ON), and blue carrier protein (BCP), the lane marker reducing sample buffer, and SuperSignal West pico chemiluminescent substrate were purchased from Pierce Biotechnology (Rockford, IL).
Acrylamide/bis solution (30%), nitrocellulose membrane (0.2 µm), and nonfat blotting-grade milk powder were obtained from BioRad (Mississauga, ON). Hanks’ balanced salt solution (HBSS), PBS (pH 7.4), and α-modified Eagle’s medium (α-MEM) were purchased from Gibco (Life Technologies Inc., Burlington, ON). Hydrogen peroxide was obtained from EMD Chemical Inc. (Gibbstown, NJ). Antibodies were obtained from the following companies: HRP-conjugated goat-anti-rabbit IgG (H+L chains) was from Cedarlane (Burlington, ON), HRP-conjugated goat-anti-mouse IgG was from Jackson ImmunoResearch (West Grove, PA), and monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Sigma.

4.3.2 Blood and tissue collection

Blood samples were collected from the tail vein into EDTA-coated microvette tubes (Sarstedt, Montreal, QC, Canada) at specified time points to measure leukocyte counts in whole blood. Total blood cell counts were obtained from EDTA-anti-coagulated whole blood samples using a Hemavet 950 FS instrument (Drew Scientific, Dallas, TX, USA). At the one and two week time points, specific rat tissues were harvested for analysis by flow cytometry following CO₂ euthanasia. Whole blood samples were collected via cardiac puncture into EDTA-anti-coagulated Vacutainer tubes (BD Biosciences, San Jose, CA) to analyze the PBMCs by flow cytometry. PBMCs were isolated from anti-coagulated whole blood using Histopaque-1083. Briefly, whole blood was mixed in a 1:1 ratio with cold PBS and carefully layered over Histopaque-1083; this was centrifuged at 400g for 30 min with low acceleration/deceleration settings. The buffy coat was collected at the Histopaque-serum interface and the contaminating red blood cells were removed using a red blood cell lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). The PBMCs were then washed twice with PBS and resuspended in FACS buffer for flow cytometry preparation. Cell viability was found to be > 95% using trypan blue exclusion for all PBMC isolations.
Splenocytes and cervical lymph node cells were also harvested at the time of necropsy for analysis by flow cytometry. The tissues were mechanically minced with scissors, followed by pushing the cells through a 100 mm cell strainer with FACS buffer to create a single cell suspension. The cells were then washed twice with PBS and then re-suspended in FACS buffer for flow cytometric preparation. Cell viability was found to be > 95% using trypan blue exclusion for all splenocytes and cervical lymph node isolations.

### 4.3.3 Measurement of immune cell changes due to clozapine treatment

Clozapine was administered as a 30 mg/kg IP dose, and PBMCs, splenocytes, and cervical lymph node cells were isolated as previously described at the end of 9 days. Cells (1×10⁶ cells/sample) were suspended in FACS buffer with a CD32 antibody for 15 min to block non-specific Fc binding. All incubations were carried out at 4 °C. Cells were washed with FACS buffer and stained with their respective extracellular antibodies for 30 min. Following three washes with PBS, cells were incubated for 30 min with viability dye. Cells were washed with FACS buffer then incubated for 30 min in 1× fixation buffer, washed again with FACS buffer, then incubated for 30 min with 1× permeabilization buffer, then incubated with their respective intracellular antibodies in 1× permeabilization buffer for 60 min. Cells were washed two times with 1× permeabilization buffer, then resuspended in FACS buffer for flow cytometric analysis or kept overnight in 1:1 1× fixation:FACS buffer for analysis the next day. Cell populations were characterized using the following antibodies: macrophages were characterized as CD11b/c+/CD3-/CD68+ and further characterized as either M1 (CD11b/c+/CD68+/CD3-/CD163-) or M2 (CD11b/c+/CD68+/CD3-/CD163+), CD62L, MHC-II, and IL-6 antibodies were used as macrophage activation markers. Alternatively, macrophages were also classified according to Dolen to et. al. as follows: M1 (TNF-α+/MHC-II+, M2b MHC-II+/IL-10+), M2a (VEGF-/IL-10+/MHC-II+/TNF-α+) [Dolen et al., 2015]. Monocytes were characterized as
CD11b+/CD3-/CD43+ and further characterized as either classical (CD11b+/CD43+/CD3-/CD4-/CD62L+) or non-classical (CD11b+/CD43+/CD3-/CD4+/CD62L-). NK cells were characterized as CD161a+/CD3-/CD8a+ cells, B cells were characterized as CD45RA+/CD3- cells: myeloid derived suppressor cells (MDSC) were characterized as either monocytic MDSC (HIS48+/CD172a+/RP-1+) or granulocytic MDSC (HIS48+/CD172a+/RP-1-). T cells were characterized as either helper T cells (CD3+/CD4+/CD8a-), cytotoxic T cells (CD3+/CD8a+/CD4-), TH17 cells (CD4+/CD3+/IL-17+), effector memory T cells (CD3+/INF-γ+, either CD4+ or CD8+), or T regulatory cells (CD3+/CD25+/FOXP3+). Flow cytometry analysis was carried out using a BD FACSCalibur configured with FlowJo software (Tree Star Inc., Ashland, OR), and compensation was carried out manually using fixed cells before sample analysis. For flow cytometric analysis, cells were initially gated on forward-scatter (FSC) and side-scatter (SSC) and then gated on live cells. A minimum of 50,000 live, correctly-gated cells/sample were acquired for each analysis.

4.3.4 Neutrophil precursor isolation from rodents

Neutrophil precursors were isolated from the bone marrow of rats following CO2 euthanasia after 9 days on treatment. Femurs and tibias were obtained from rats, and the bone marrow was flushed out with cold α-MEM and centrifuged at 750g for 5 min at 4 °C. Bone marrow α-MEM suspensions were layered onto discontinuous Percoll (GE Healthcare) gradients of 80/65/55%. Samples were centrifuged at 750g for 30 min at 4 °C, and the neutrophil precursors were collected at the 80/65% interface. Contaminating erythrocytes were removed with red cell lysis buffer, and the neutrophil precursors were washed and resuspended in 1:1; 1× cell lysis buffer: protease inhibitor and stored at -80 °C until western analysis.
4.3.5 Treatment of animals with clozapine and PF-1355

Female Sprague-Dawley rats (200–250 g, Charles River) were housed in triplets and acclimatized to a 12/12 h light/dark cycle at 22 °C for a minimum of 1 week prior to starting treatment. All rodents were given regular access to rodent meal (Harlan Teklad, Madison, WI). The University of Toronto Animal Care Committee approved all experimental protocols. PF-1355 was suspended in 0.5% methylcellulose in PBS and administered by gavage 2 h prior to clozapine administration at a dose of 60 mg/kg for up to 9 days. Clozapine was dissolved in a small amount of 1 N HCl, followed by dilution in saline, and the pH was adjusted to 5 using 1 N NaOH. Clozapine was administered by IP injection at a dose of 30 mg/kg/day for up to 9 days.

4.3.6 SDS-PAGE and immunoblotting

Protein samples (20 – 60 µg) were combined with DTT reducing sample buffer and boiled for 5 min. Proteins were separated at 30 – 40 mA on 8% polyacrylamide gels using the Bio-Rad PROTEAN II mini-gel system followed by transfer to nitrocellulose at 250 mA in a mini Trans-Blot transfer cell. Clozapine blots were incubated in 3% hydrogen peroxide for 15 min. All membranes were blocked with 5% skim milk in wash buffer (50 mM Tris-HCl, 0.9% NaCl, 0.1% Tween-20, pH 7.5) for 2 h. After washing three times, membranes were incubated overnight at 4 °C with primary antibody (rabbit-anti-clozapine IgG) diluted in wash buffer, 2% milk. Membranes were washed five times and incubated for 1.5 h at room temperature with goat-anti-rabbit-IgG HRP diluted 1:20,000 in wash buffer, 2% milk. Following three washes, membranes were incubated with SuperSignal West pico chemiluminescent substrate and imaged using a FluoroChem Alpha Innotech imager with AlphaEase FC software, version 6.0.0 (ProteinSimple, Santa Clara, CA). Each immunoblot was repeated at least twice, and GAPDH was used as the loading control.
4.3.7 MPO activity after inhibition with PF-1355

Using a neutrophil activator and recruiter, zymosan, MPO activity was assessed. Zymosan was administered as a 2 mg dose in 500 µL of PBS via IP injection. Three hours post dose, blood samples were collected from the tail vein into clotting activator-coated microvette tubes (Sarstedt, Montreal, QC, Canada) and spun at 10,000g for 5 min at room temperature. The serum was collected and used right away to assess MPO activity using an amplex red hydrogen peroxide/peroxidase assay kit (Life Technologies Inc., Burlington, ON, Canada). Peritoneal exudate samples were collected 3 h post zymosan administration after euthanasia by flushing the peritoneal cavity with 10 mL of cold PBS, massaging PBS throughout the peritoneal space and collecting the exudate. The peritoneal exudate was used right away to assess MPO activity. A MPO activity assay was performed as per the manufacturer’s instructions. Briefly, plasma or peritoneal exudate samples were added to triplicate wells. Assay buffer (0.2225 M sodium phosphate buffer (1X reaction buffer), pH 7.4, 100 µM Amplex Red, and 2 mM H₂O₂) was added to the wells. The amplex red reagent reacts with H₂O₂ in the presence of peroxidase to generate resorufin, MPO activity was assessed based on the production of resorufin as a function of time with an excitation/emission wavelength of 530/580 nm on a fluorescence plate reader (Biorad, Hercules, CA). Horseradish peroxidase standards were prepared in 1X reaction buffer at concentrations ranging from 0.125 U to 2 U and used to calculate unknown peritoneal exudate and serum MPO concentrations.

4.4 Results

4.4.1 Clozapine at a dose of 30 mg/kg IP resulted in therapeutic blood levels

Following a 30 mg/kg IP injection of clozapine, serum levels of clozapine were found to peak at 1.4 µg/mL 30 min post injection. Clozapine blood levels fell to 0.35 µg/mL 6 h post dose and
were not detectable 24 h post dose. Clozapine dosed at 30 mg/kg IP in female Sprague Dawley rats resulted in blood levels similar to that seen in clozapine-treated human patients (0.35 µg/mL to 1.35 µg/mL). The apparent half-life as calculated using these data was approximately 3 h in female Sprague Dawley rats.

![Clozapine Levels Graph]

**Figure 4-1. Serum clozapine levels following a 30 mg/kg IP dose of clozapine**

Serum was collected from rats at 0 h, 0.5 h, 1 h, 3 h, 6 h, and 24 h post IP dose of clozapine. Protein was removed by methanol precipitation, and the supernatant was analyzed by LC-MS/MS using a mobile phase of 50% methanol, 1% acetic acid, and 2 mM CH₃CO₂NH and a Kinetex 2.6µ C18 100A 50 x 4.5 mm column. Results are shown from individual animals. The grey shaded area represents the average therapeutic range reported in patients (1.35 µg/mL - 0.35 µg/mL).

### 4.4.2 Zymosan is a neutrophil activator and recruiter

Zymosan has the ability to recruit neutrophils. Three hours after a 2 mg IP injection of zymosan neutrophils were elevated in the peripheral blood from 2,000 cells/µL to 6,000 cells/µL of blood (Figure 4-2D). Zymosan does not significantly affect peripheral blood leukocyte, lymphocyte, or monocyte blood counts (Figure 4-2A-C). Zymosan not only increased the number of circulating neutrophils, but also activates neutrophils at the site of injection. Neutrophils taken from the peritoneal cavity show an increased MPO activity level (Figure 4-3). These properties allow zymosan to be used for the demonstration of the ability of PF-1355 to inhibit MPO activity in neutrophils. A dose of 60 mg/kg via oral gavage 2 h pre-zymosan administration showed the
ability of PF-1355 to limit the MPO activity increase caused by zymosan administration (Figure 4-3). MPO activity in the serum was below the limit of detection (data not shown).

Figure 4-2. Zymosan is a neutrophil recruiter.

Rats were treated with a 2 mg dose of zymosan via the IP route. Blood samples were collected prior to dosing and 3 h post-dose into EDTA anticoagulant microvette tubes. Each whole blood sample was analyzed on a Hemavet 950FS in triplicate. (A) 0 h vs 3 h white blood cell counts, (B) 0 h vs 3 h lymphocyte counts, (C) 0 h vs 3 h monocyte counts, (D) 0 h vs 3 h neutrophil counts. Results are expressed as the mean ± SEM, and statistical difference was determined by one-way ANOVA, where ***, p < 0.0005.
Rats were treated with a 2 mg dose of zymosan via IP administration. Peritoneal lavage fluid was collected 3 h post-dose by flushing rat peritoneal space with 5 mL of cold PBS to collect MPO. An MPO activity assay was performed to determine MPO inhibition. Results are expressed as the mean ± SEM, and statistical difference was determined by one-way ANOVA, where *, p < 0.05.

4.4.3 PF-1355 limited the binding of clozapine to neutrophil precursors in the bone marrow.

MPO is expressed in mature neutrophils in the peripheral blood, but also in neutrophil precursors in the bone marrow going back to the promyelocyte stage of neutrophil development [Brown, 2001]. Clozapine covalently bound to neutrophil precursors in the bone marrow with increasing binding occurring during the first 3 days of treatment (Figure 4-4). This covalent binding was decreased by treatment with PF-1355 to inhibit MPO activity, and clozapine binding in the rats co-treated with clozapine and PF-1355 was decreased by about 70% (Figure 4-4). The western blot shows a solid non-specific dark blotch around 40 kDa, this may be due to a combination of non-specific binding of an old anti-clozapine antibody or the increased exposure needed to visualize binding using this specific antibody.
Figure 4-4. Clozapine binding to bone marrow derived neutrophils with and without PF-1355.

Rats were treated with 30 mg/kg/day IP doses of clozapine for 1-3 days with (PF+CLZ) or without PF-1355 administration (CLZ). Covalent binding of clozapine to bone marrow-derived neutrophils was determined on western blots with antibodies that recognize clozapine bound to proteins as described in the methods section. The same amount of protein was loaded in each lane so that the binding could be compared between different treatment durations. The density of clozapine binding was assessed using Image J, by taking the average pixel density at the 75 kDa band.

4.4.4 Decreasing covalent binding to neutrophil precursors limits clozapine-induced neutrophilia.

Acute neutrophilia is seen in female Sprague Dawley rats following a single dose of clozapine administered as a 30 mg/kg IP dose, where peripheral blood neutrophil counts reached 7,000 cells/µL 6 h post-dose compared to 2,000 cells/µL in control rats (Figure 4-5). This acute neutrophilia was attenuated when pretreatment with PF-1355 was administered via oral gavage 2 h prior to clozapine administration as a 20 mg/kg dose (4,000 cells/µL at 6 h) and as a 60 mg/kg dose (2,000 cells/µL), but not as a 100 mg/kg dose (7,000 cells/µL at 6 h) (Figure 4-5). Oral gavage of PF-1355 as a 100 mg/kg dose alone led to neutrophilia (6,000 cells/µL at 6 h) for an unknown reason (data not shown). When clozapine was administered as a 30 mg/kg/day dose via IP, neutrophilia was observed on days 6-9 (Figure 4-6D). This model did not significantly change total leukocytes (Figure 4-6A), lymphocytes (Figure 4-6B), or monocytes (Figure 4-6C) over this time period. The pre-administration of 60 mg/kg/day oral gavage PF-1355 significantly
limited the neutrophilia seen in this model on days 6 (4,000 cells/µL vs. 2,200 cells/µL) and 9 (4,000 cells/µL vs. 2,000 cells/µL) in clozapine treated vs. co-administration groups, respectively (Figure 4-6D).

![Bar chart showing neutrophil counts over time with different treatments]

**Figure 4-5.** PF-1355 attenuated the neutrophilia seen as a result of acute clozapine treatment

Rats were treated with one dose of 30 mg/kg clozapine IP± PF1355 via gavage. Blood samples were drawn from the tail vein at specified time periods throughout treatment into EDTA anticoagulated microvette tubes. Each whole blood sample was analyzed on a Hemavet 950FS in triplicate. Control, clozapine, clozapine + PF1355 20 mg/kg, clozapine + 60 mg/kg, clozapine 100 mg/kg neutrophil counts. Results are expressed as the mean ± SEM, and statistical difference was determined by two-way ANOVA, where ***, p < 0.001.
Rats were treated with 30 mg/kg/day clozapine IP ± PF1355 60 mg/kg via gavage for 9 days. Blood samples were drawn from the tail vein at specified time periods throughout treatment into EDTA-anticoagulant microvette tubes. Each whole blood sample was analyzed on a Hemavet 950FS in triplicate. (A) Leukocyte counts, (B) Lymphocyte counts. (C) Monocyte counts. (D) Neutrophil counts. Results are expressed as the mean ± SEM, and statistical difference compared was determined by two-way ANOVA, where *, p < 0.05.

4.4.5 Limiting clozapine binding to neutrophil precursors, limited the immune response to clozapine

Proinflammatory and anti-inflammatory blood cells were altered in a variety of organs within the rat model of early immune response to clozapine treatment. The PBMCs in this model were separated from whole blood using Histopaque-1077. The separation of PBMCs yielded a 39% recovery rate, which was not significantly affected by treatment group (Figure 4-7A, B). At the end of treatment (9 days), monocytes were increased, identified as CD11b/c+, CD43+, and CD3-cells from the control group (10% of viable cells and 2 million cells) to the clozapine treated group (23% of viable and 4.5 million cells). This increase was not attenuated by the inhibition of MPO in the co-treated group (20% of viable cells and 4 million total cells; Figure 4-7C, D). Peripheral blood Th cells, identified as CD3+, CD4+ cells were 25% of viable cells in the control group, and decreased in the clozapine treated group (11% of viable cells; Figure 4-7E). This
decrease was not significant when expressing cell counts as a percentage of total cells (Figure 4-7F). The T\textsubscript{h} cell change due to clozapine did appear to be attenuated by the co-treatment with PF-1355 (25% of viable cells; Figure 4-7E, F).

The spleen is a major organ of immune function; therefore, immune cell changes within the spleen are of special interest. A variety of different immune cell changes were assessed within the spleen using flow cytometry. A measure of a proinflammatory response is increased spleen size, and the average number of spleen cells in the clozapine-treated group was 250 million vs. 190 million in the control group (Figure 4-8A). These results were not significant, but the trend is interesting.

Immune cells within the cervical lymph node at the time of sacrifice were counted to compare between groups. T\textsubscript{h}17 cells, a proinflammatory helper T cell that produces IL-17, was found to be increased in the clozapine treated group: 0.25% of viable cells and 0.0025 million cells over control, 0.05% of viable cells and 0.001 million cells (Figure 4-9B, C). This increase in T\textsubscript{h}17 cells was also significant in the clozapine group over the co-treated group; 0.15% of viable cells and 0.001% of total cells (Figure 4-9B, C).
Figure 4-7. Immune cell changes in the peripheral blood of clozapine-treated rats ± PF-1355.

Rats were treated with 30 mg/kg/day clozapine via IP administration ± PF1355 60 mg/kg via gavage for 9 days. (A) Total PBMCs were measured before and after Histopaque separation measured using trypan blue stain. (B) % of PBMCs recovered per sample are listed, average PBMC recovery was 47%. Flow cytometry staining of peripheral blood mononuclear cells are shown as the average for each group. Graphs are either presented as % viable cells, gated for correct cell population based on FSC vs. SSC, single cells and viable cells or as total cells, % total cells multiplied by total PBMCs in the sample. (C, D) Monocytes. (E, F) T<sub>h</sub>17 cells are expressed as the mean ± SEM, and statistical difference compared to control was determined by one-way ANOVA, where *, p < 0.05.
Figure 4-8. Immune cell changes in the splenocytes from clozapine-treated rats ± PF-1355.

Rats were treated with either 30 mg/kg/day clozapine via IP injection, saline injection or both 60 mg/kg oral gavage PF-1355 followed by 30 mg/kg/day clozapine IP injection 2 h later for 9 days. Total leukocyte counts were measured in each spleen sample using trypan blue exclusion. Results are expressed as the mean ± SEM.

Figure 4-9. Immune cell changes in the cervical lymph nodes of clozapine-treated ± PF-1355.

Rats were treated with either 30 mg/kg/day clozapine via IP injection, saline injection or both 60 mg/kg oral gavage PF-1355 followed by 30 mg/kg/day clozapine IP injection 2 h later for 9 days. (A) Total leukocyte counts were measured in each cervical lymph node sample using trypan blue exclusion. Flow cytometry staining of cervical lymph node cells are shown as the average for each group. Each graph is presented as % viable cells, gated for correct cell population based on FSC vs. SSC, single cells and viable cells. (B, C) T17 cells in cervical lymph nodes. Results are expressed as the mean ± SEM, and statistical difference was determined by one-way ANOVA, where *, p < 0.05.

4.5 Discussion

There is strong evidence to suggest that IDIAG is mediated by the formation of a reactive metabolite of a drug leading to the formation of an immune response-inducing hapten. These
reactive metabolites are likely formed by the neutrophil, and MPO is likely a key contributor. There now exists evidence in vitro, in human data [Lee et al, 2015], in a mouse model [Lobach & Uetrecht, 2014], and now in a rat model that clozapine is bioactivated into reactive metabolites by neutrophils in the absence of MPO. The absence of MPO also does not halt all proinflammatory responses seen as a result of clozapine treatment.

In female Sprague Dawley rats, clozapine causes neutrophilia acutely, after a single dose, and chronically, after 6 to 9 days of treatment. This is similar to clinical data, although blood sampling is usually not done in patients at time points less than 1 week. A delayed neutrophilia occurs on average after 2 weeks of clozapine treatment [Lee et al., 2015]. Clozapine also appears to bind to neutrophils in 100% of human patients, similar to what is seen in this Sprague Dawley model. Unfortunately, there are no MPO knockout rats available on which to perform mechanistic studies. We set out to use a chemical inhibitor of MPO, PF-1355, to understand MPO’s role in bioactivating clozapine into a reactive metabolite in an in vivo rat model. This inhibitor provides a method to study the role of MPO in the immune response to clozapine

First, we demonstrated that PF-1355 does in fact have the ability to decrease the activity of MPO. To do this, we used zymosan to stimulate neutrophil release, recruit neutrophils to an easily accessible area, and also to increase MPO activity levels. Zymosan was able to recruit neutrophils to the site of injection (the peritoneal space) and activate MPO within; the MPO activity could be inhibited using PF-1355 (Figure 4-2, 4-3). Knowing that PF-1355 inhibits MPO activity, we set out to understand its role in bioactivating clozapine into a reactive metabolite in a rat model. Although MPO function could be effectively inhibited, clozapine was still able to bind to neutrophil precursors in the bone marrow, however, at a decreased amount. The binding seen in this model was about 30% of that in an uninhibited model (Figure 4-5). Compare that to the
50% seen in the mouse model, it appears as though MPO is more responsible for clozapine bioactivation by neutrophils in rats. However, it is important to note that there are still other mechanisms in play that can account for that remaining 30%. This model holds true for amodiaquine as well; inhibiting MPO using PF-1355 limited the binding of amodiaquine metabolites to neutrophil precursors in the bone marrow of rats by 50% (data not shown).

By using this animal model, we were able to assess the downstream effects of limiting the binding of clozapine to neutrophil proteins. The neutrophilia seen at the beginning of clozapine treatment is of interest, and therefore peripheral blood neutrophil counts were monitored throughout treatment. The neutrophilia seen after a single dose as well as after 9 days of chronic administration were both attenuated by the use of PF-1355 dosed at 60 mg/kg (Figure 4-5, 4-6D). These results suggest that binding of drug to neutrophil proteins leads to neutrophilia. This could be due to a variety of different reasons including, but not limited to, the release of neutrophil attracting cytokines, but further studies need to be performed to elucidate this mechanism.

Having access to an animal model of the early immune response to clozapine allows mechanistic studies that are not otherwise feasible in vitro or in humans. For instance, immune cell changes can be observed in organs such as the spleen and lymph nodes in response to clozapine treatment. In the peripheral blood of humans, an increase in monocytes has been reported in about 66% of patients at about the 2 week mark [Lee et al., 2015]; this increase in monocytes is also seen in the rat model. The increase in monocytes seen at the end of a clozapine treatment is not affected by limiting the binding to neutrophil proteins (Figure 4-7C, D). In the rat model there appears to be a decrease in peripheral blood T_h17 cells at day 9, which is attenuated by inhibiting MPO bioactivation. Samples taken from human blood are not generally characterized for T_h17 cells. This is due to the fact human samples are usually analyzed using a hematology
analyzer. This method of analyzing will tell you the total lymphocyte cell changes, but it is unable to detect specific subsets of lymphocytes such as T_h17 cells.

The size of spleens has traditionally been used as a measure of immune response. In the event of an immune reaction many cells meet and are developed in the spleen resulting in a larger size. In these rats, the spleen from clozapine treated animals contained the most cells, then the co-treated group, and finally the control group, which had the fewest number of cells in their spleens (Figure 4-8). Although these results were not significant, there is a trend that could outline something more significant if a larger group size was studied. No significant differences in immune cells were seen in the spleen at the end of treatment (data not shown).

Immune cells typically travel to the lymph nodes in response to inflammation; therefore, cervical lymph nodes were assessed. Of the cells assessed, the T_h17 were the only cells to show significant changes; these cells were increased in clozapine-treated animals. This increase seemed to be attenuated by MPO inhibition (Figure 4-9B, C). T_h17 cells produce the inflammatory cytokines IL-17, IL-17F, and IL-22. These cells have been associated with the pathogenesis of many experimental autoimmune diseases and inflammatory conditions, and they appear to aid in the clearance of pathogens not adequately resolved by T_h1 and T_h2 cells [Korn et al., 2009].

MPO has the ability to bioactivate clozapine, but it is also evident that rats, like mice and humans, have other mechanisms of oxidizing clozapine into a reactive metabolite. From these data we were able to demonstrate the downstream effect of a lack of MPO-generated clozapine reactive metabolites with some interesting results. It appears the neutrophilia seen is, at least in part, affected by the amount of clozapine covalent binding. It would be interesting to know to what degree MPO contributes to clozapine-induced neutrophilia in humans. In theory, one could
compare the cases of early neutrophilia between MPO normal patients and MPO deficient patients taking clozapine [Dettling et al., 2000]. To my knowledge no such study has been performed. It remains unknown what other mechanisms exist to produce clozapine reactive metabolites, but for now, MPO appears to be the major contributor.

4.6 Acknowledgements

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Chapter 5

Summary and conclusions
5.1 Summary of findings and conclusions

The overarching hypothesis for this work is that clozapine is metabolized into a reactive metabolite by neutrophils, which binds to neutrophil proteins to form haptens that are seen as foreign to the immune system. The immune system then mounts an immune response against these haptens, which results in a proinflammatory state in a patient. This proinflammatory state is highlighted by neutrophilia, an increase in monocytes, and an increase in a variety of proinflammatory cytokines. Monocytes and macrophages are increased, primed, and stimulated in vivo and in vitro. Using THP-1 cells, both monocytes and macrophages can be stimulated to release IL-1β in response to clozapine treatment in an inflammasome-dependent manner. The resulting proinflammatory state is suspected to be down regulated in the majority of patients via immune tolerance; however in a patient-specific manner, some patients have an exacerbated immune response leading to the destruction of the neutrophil-clozapine haptens as well as their unchanged neutrophils. This leads to a state of decreased peripheral blood neutrophils, or, idiosyncratic clozapine-induced agranulocytosis.

We hypothesize that IDRs are immune-mediated adverse reactions to particular medications. IDIAG is one such IDR that we believe to be immune mediated. In the case of aminopyrine-induced agranulocytosis the adverse drug reaction is definitively mediated by drug-dependent antibodies that lead to the destruction of neutrophils [Moeschlin & Wagner, 1952]. This mechanism was famously discovered when an investigator induced agranulocytosis in himself by taking aminopyrine and injecting himself with serum from a patient with aminopyrine induced agranulocytosis. This led to immediate and profound neutropenia. Other medications, such as β-lactam, have been implicated in positive T lymphocyte tests, indicating the involvement of T
cells [Nyfeler & Pichler, 1997]. Many drugs known to cause IDRs have been associated with a positive T lymphocyte test [Maria & Victorino, 1997]. Many drugs known to cause IDIAG are associated with genetic predispositions to the adverse reaction; of which almost all of these predispositions come from strong associations to the HLA genes. As stated above, clozapine-induced agranulocytosis is more common in patients of Jewish decent expressing particular haplotypes of specific HLA genes [Yunis et al., 1995]. Lastly, the majority of medications known to cause IDRs exhibit a characteristic delay in onset, typically between 1-6 months [Andersohn et al., 2007]. It is quite possible that this delay in onset is due to the time it takes for the immune system to find the antigen, proliferate, and mount a response. Many medications known to cause IDRs also have a quicker onset of the IDR on rechallenge of the drug, presumably due to memory T cells in the periphery of the patient. In order to perform controlled mechanistic studies, a valid animal model is required. IDRs are also idiosyncratic in animals, and so it is not surprising that administration of clozapine to female Sprague Dawley rats does not produce a model of idiosyncratic clozapine-induced agranulocytosis. However, it does show similarities to that of humans initially taking clozapine. The immune responses in the rat model known before we embarked upon the present studies ranged from peripheral leukocyte changes to cytokine changes in the peripheral blood. Most notably, a neutrophilia was known to occur after approximately 6 days of clozapine treatment in the rat model, which mimics a neutrophilia seen in 60% of human patients around week 2 [Lee et al., 2015]. In the rat model, an increase in proinflammatory cytokines G-CSF, alpha-1-acid glycoprotein, and CXCL1 mimic the increase in C reactive protein, G-CSF, and fever seen in the majority of patients who take clozapine [Lobach & Uetrecht, 2014]. From in vitro and in vivo data, it is known that MPO has the ability to metabolize clozapine into reactive metabolites within neutrophils [Liu & Uetrecht, 1995]. However, what has never been shown is how limiting drug binding can affect downstream
immunological changes from occurring. In this thesis, further characterization of the model allowed for insight into the early immune response to clozapine that was not seen before.

Administering clozapine via the drinking water allowed for longer studies, so the first task was to optimize a dose to generate the highest clozapine blood levels in the rats. A clozapine solution was found that gave therapeutic blood levels, but lower than the levels seen after a single clozapine administration via an IP route. However, administering clozapine in the drinking water did provide clozapine blood levels sufficient enough to elicit neutrophilia and other immune cell changes. This route of administration also presents the opportunity to extend studies, which will likely be necessary to produce a valid animal model of clozapine-induced agranulocytosis. Although this animal model does not entirely reflect human data, the similarities should still provide insight into the mechanism. Overall, the studies using clozapine administered in the drinking water provided further characterization of the use female Sprague Dawley rats for an animal model of the early immune response seen due to clozapine treatment. These studies showed the immunological changes occurring in the cervical lymph nodes, the spleen, the bone marrow, and the peripheral blood that were not known previously. These findings show the complexity of the early immune response in an in vivo model and provide novel pathways to follow for future studies. Although a clear picture of particular immune cells involved did not emerge as a result of these studies, both innate and adaptive immune cells were involved, and specific immune cell changes are interesting. Peripheral blood monocytes increased due to clozapine-treatment; this was seen with both IP administered and drinking water administered clozapine in rats at 9 days of treatment. Monocytes and macrophages appear to be an intriguing cell line to follow in future studies because they are early responders in an immune response and they appear important in the inflammatory response that results due to clozapine treatment.
Investigating MPO’s role in producing reactive metabolites of clozapine in vivo led to a number of novel findings. Prior to these present studies, MPO was suspected to be important in bioactivating clozapine; however, these studies were limited to in vitro studies and using mice neutrophils ex-vivo. The prospect of studying MPO’s role in producing reactive metabolites in a rat model was exciting not only because bioactivation studies could be completed in an in vivo setting, but also monitoring downstream immunological effects was possible using this model. Similar to past findings of MPO’s role in bioactivating drugs that are known to cause agranulocytosis [Lobach & Uetrecht, 2014], the result from this rat model suggests that MPO is involved in about 50% of the covalent binding to neutrophil proteins (Chapter 4). This suggests similarities between rat MPO and mouse MPO in their ability to bioactivate drugs. Humans lacking fully functioning MPO do not appear to be at a decreased risk of developing clozapine-induced agranulocytosis [Dettling et al., 2000]; this suggests that in humans, another pathway is capable of forming clozapine reactive metabolites in the neutrophils. In humans, MPO’s role is harder to quantify because we do not have access to bone marrow in order to perform binding assays. Perhaps most intriguing about these findings is that by blocking MPO, the neutrophilia seen acutely and chronically in this model is attenuated. Inhibiting MPO attenuated many other proinflammatroy cell changes seen by treating female Sprague Dawley rats with clozapine. Notably, peripheral T and B cell changes were attenuated. However, the monocyte increase at around day 9 was not affected by inhibiting MPO. It is possible that the degree of clozapine metabolite binding could affect different cell kinetics differently. Overall, these studies show that MPO is responsible for about 70% of the bioactivation of clozapine by neutrophil precursors in a rat model. Unfortunately, the anti-clozapine antibody used for the binding assay is poor, with heavy background binding. If these findings are to be validated, a better antibody against clozapine should be utilized. For the first time we were able to show the downstream effects of
MPO generated reactive metabolites binding to neutrophils in vivo. These results support the hypothesis that hapten formation can lead to an immune response; we hypothesize that this immune response has the potential to lead to IDIAG.

Understanding the mechanism of clozapine-induced agranulocytosis will be an impossible task without the availability of a valid animal model and supporting clinical data. All attempts we have made thus far to gather the patients necessary to validate the in vitro and in vivo findings have been unsuccessful to date. Therefore, developing an animal model remains an important first step toward understanding the mechanism of a clozapine-induced immune response and ultimately clozapine-induced agranulocytosis. Although the mechanism remains unknown, significant insight and future directions were gained through understanding clozapine’s reactive metabolite formation and immune activation in the preceding studies. Ultimately, by creating an animal model first, we would have a better idea of what to look for should the opportunity arise to collect human data.

5.2 Implications and future directions

Our next step to understanding clozapine-induced agranulocytosis is to develop an animal model that reflects the early immune response during clozapine treatment in humans. Clozapine is able to produce an immune response in female Sprague Dawley rats that partially mimics the human data; however, these experiments have only been studied for three weeks. If agranulocytosis could be achieved in this model, it is likely that it would occur after a longer duration of treatment because humans typically develop agranulocytosis after a month but within the first 6 months. We also hypothesize that the immune response seen in this model, i.e. the neutrophilia, the increases in proinflammatory cytokines, and the increases in a variety of proinflammatory
cells, all subside due to immune tolerance. A more valid animal model of idiosyncratic drug induced liver injury has recently been produced by the Uetrecht lab using immune-checkpoint inhibitors to impair immune tolerance [Metushi et al., 2015]. A PD1-/- rat has recently been produced by the Uetrecht lab. By utilizing this PD1-/- rat along with longer clozapine administration in the drinking water, it may be possible to develop a more valid model to reflect clozapine-induced agranulocytosis.

Designing studies to better determine how clozapine activates inflammasomes and how clozapine affects monocytes and macrophages in vivo remains a promising direction to investigate. Drugs known to cause IDRs have recently been shown to stimulate IL-1β release from THP-1 cells [Weston & Uetrecht, 2014]. Clozapine also affects monocyte kinetics, both in humans and in the rat model. The IL-1β released by THP-1 cells in response to clozapine is inflammasome-mediated. Attempts to limit inflammasome IL-1β generation in vivo in response to clozapine treatment produced conflicting results. Specifically, an IL-1 receptor antagonist, anakinra, showed successful results in limiting IL-1β downstream effects in the clozapine female Sprague Dawley rat model using one batch of anakinra, while a second batch showed conflicting results [unpublished data]. A successful in vivo IL-1β inhibitor is needed to link the in vitro results to in vivo findings. Understanding macrophages’ role in IDR progression is an interesting direction to explore. Monitoring monocyte and macrophage activation states throughout clozapine treatment using cellular markers: CD62L, MHC-II, CD206, and IL-6 have proven inconclusive; however, there are a plethora of other markers that could be explored.

MPO’s role in the bioactivation of drugs into reactive metabolites capable of binding to neutrophil proteins has been shown in vitro, in a mouse model, through human data, and now in a rat model. Each model indicates that neutrophils have other mechanisms to bioactivate drugs
known to cause IDIAG. We have tested a variety of other enzymes capable of forming these reactive metabolites without success. In the future, other metabolic enzymes should be tested for their ability to produce reactive metabolites of clozapine and other drugs capable of causing IDIAG. These enzymes, or oxidative pathways, are unknown at the moment; however, there appears to be other mechanisms in which oxidation of clozapine can occur within the neutrophils.

The ultimate goal of any pharmacology research is to make the findings relevant to humans. In order to validate any animal model, human patients will be needed to determine if the findings in controlled animal experiments extend to humans. For example, monitoring patient PBMC changes via flow cytometry or serum cytokines using an ELISA would give great insight into disease progression. Unfortunately, to date, any attempts at collecting a sufficient number of patients taking clozapine have been unsuccessful. This remains a vital task if we want to create valid models of clozapine-induced agranulocytosis or any IDR for that matter.

In conclusion, the finding that clozapine stimulates a variety of proinflammatory and anti-inflammatory immune cells in an animal model, and that some of these inflammatory cell changes can be attenuated by limiting covalent binding of clozapine, is novel. In vitro and in vivo data support the hypothesis that neutrophil hapten formation is important in clozapine stimulating an immune response. However, to test these hypotheses, it will be necessary to link immune activation to the induction of agranulocytosis, a task that will prove difficult in the absence of an animal model. A better understanding of the early immune response to clozapine has been achieved by monitoring a variety of immune cells within a variety of different tissues. This must be validated using human data; therefore, collecting human samples remains of utmost importance in understanding the mechanism of clozapine-induced agranulocytosis.
References


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Appendix I

Investigating the Role of Peripheral Blood Exosomes in Carrying Danger Signals Capable of Priming Macrophages to Respond to Stimulants
1.1 Abstract

An early model of idiosyncratic clozapine-induced agranulocytosis has shown that monocytes and macrophages appear to play a role in the proinflammatory response to clozapine. Recently exosomes have been explored for their ability to harbor and transfer damage associated proteins and RNA fragments. Exosomes have the ability to serve as a mode of communication between damaged cells and immune cells, specifically monocytes and macrophages. We explored the hypothesis that exosomes may carry damage-associated molecular patterns (DAMPs) with the ability to prime monocytes and macrophages in our animal model of early immune responses of clozapine. Exosomes taken from the peripheral blood of animals after a single IP dose of clozapine were incubated with THP-1 (a human monocytic cell line) monocytes or macrophages. When THP-1 monocytes and macrophages were incubated with exosomes from control and clozapine-treated animals, followed by stimulation with LPS, there was no significant difference in the release of IL-1β. This suggests that the exosomes taken at various time points from the serum of clozapine-treated rats do not carry any DAMPs capable of priming THP-1 monocytes and macrophages to future stimulants. It is possible that exosomes play a role in local cell-to-cell communication at some point in the immune response, but they do not appear important after a single dose of clozapine.

1.2 Introduction

Exosomes are small vesicles (50-150 nm) that are released from just about every cell type and have the ability to carry macromolecules, proteins, and non-coding RNAs [Théry et al., 2002].
Exosomes are gaining attention as detectable biomarkers for a variety of diseases, because they are released in response to cell damage and distress [Thakur et al., 2014]. Recent data suggest that exosomes play an important role in conveying information between various cell types and across different tissues through the transfer of the macromolecules they can harbor [Bakhshandeh, 2017; Raposo & Stoorvogel, 2013]. Recently, in an animal model of diabetes, exosomes have been shown to mediate the transfer of auto-antigens, and play a role in autoimmune trigger in diabetic mice [Silverman, 2010; Rahman et al., 2014].

Exosomes have shown the ability to induce monocytes and macrophages to become sensitized to other stimulants. Specifically exosomes, extracted from a liver injury model of ethanol exposure, harbored miRNAs. These miRNAs were capable of being transferred to monocytes where they sensitized monocytes to respond to inflammatory stimuli [Momen-Heravi et al., 2015]. In the liver injury model it was deduced to be a common miRNA, miRNA-122, that was responsible for the monocyte sensitization. The transferred miRNA increased the response of THP-1 macrophages to LPS.

Clozapine administration lead to elevated peripheral blood monocytes in our rat model. Clozapine also has the ability to stimulate monocytes and macrophages in vitro. Therefore, we hypothesized that peripheral blood exosomes in the clozapine-induced immune response model may have the ability to harbor macromolecules and sensitize macrophages.

1.3 Materials and methods

1.3.1 Chemical materials
Clozapine was generously provided by Novartis Pharmaceuticals Inc. (Dorval, QC). Dimethyl fumarate (DMF), ethacrynic acid (EA), lipopolysacharide (LPS), and phorbol 13-myristate 12-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO). Hanks’ balanced salt
solution (HBSS), PBS (pH 7.4), fetal bovine serum (FBS), and trypan blue were purchased from Gibco (Life Technologies Inc., Burlington, ON). DMSO was purchased from BioShop Canada Inc. (Burlington, ON).

1.3.2 Treatment of animals with clozapine

All rodents were acclimatized to a 12/12 h light/dark cycle at 22 °C for a minimum of 1 week prior to starting clozapine treatment and were given regular access to rodent meal (Harlan Teklad, Madison, WI) and tap water. The University of Toronto Animal Care Committee approved the experimental protocols. Female Sprague-Dawley rats (200–250 g, Charles River) were housed in triplets. Clozapine was dissolved in a small amount of 1 N HCl, followed by dilution in saline, and the pH was adjusted to approximately 5 using 1 N NaOH. Clozapine was administered by IP injection at a dose of 30 mg/kg.

1.3.3 Clozapine serum concentration analysis using liquid chromatography/mass spectrometry

Clozapine standards were prepared in methanol (0.01 – 1.5 µg/mL), diluted two-fold with blank rat serum, and then combined with internal standard DMP-406 (0.1 µg/mL final concentration). Serum samples were collected from the rats at 0.5 h, 1 h, 3 h, 6 h, and 24 h post-clozapine administration. As was done for the standards, clozapine serum samples were diluted two-fold with blank rat serum, followed by addition of DMP-406 (0.1 µg/mL). Protein was allowed to precipitate for 30 min at -20 °C. The supernatant was isolated by centrifugation at 16,000g, diluted two-fold with water, and analyzed using a PE Sciex API 3000 quadrupole mass spectrometer with an electrospray ionization source (Sciex, Concord, ON) interfaced with a HPLC system (Shimadzu, Columbia, MD). The mobile phase consisted of 50% methanol, 1% acetic acid, and 2 mM CH₃CO₂NH₄ and a Kinetex 2.6µ C18 100A 50 x 4.6 mm column (Phenomenex, Torrance, CA) was employed.
1.3.4 Isolation of exosomes and their incubation with THP-1 cells

Blood samples were collected from the tail vein of rats into clot-activating microvette tubes (Sarstedt, Montreal, QC, Canada) at 1 h, 3 h, 6 h, and 24 h post IP injection of clozapine or control saline and allowed to settle for 1 h. The plasma was then isolated by centrifugation at 10,000g for 5 min. Cell debris was removed by centrifuging the plasma at 3,000g for 15 min. Thrombin (500 U/mL in PBS) was added to 0.5 mL plasma and incubated at room temperature for 5 min. This mixture was centrifuged at 8,900g for 5 min to pellet the fibrin. The supernatant was mixed with Exoquick Exosome Precipitation Solution (System Biosciences, Mountain View, CA) at a ratio of 250 µL sample: 63 µL Exoquick solution. This was allowed to precipitate for 60 min at 4 °C. The exosomes were then pelleted via a centrifugation at 1,500g for 30 min and resuspended in H₂O. The concentration of protein in each sample was measured using a bicinchoninic acid (BCA) kit (Fisher Scientific, Ottawa, ON, Canada).

THP-1 monocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in ATCC high glucose RPMI-1640 medium, supplemented with 10% FBS. Monocytes were cultured in 96-well plates at a concentration of 1 × 10⁵ cells/mL in a volume of 0.1 mL.

Exosome preincubation followed by LPS stimulation was completed as follows; exosomes from control and treated animals were added to the cells at a concentration of 50, 75, or 100 µg/mL for 8 h, the cells were spun down via centrifugation at 890g, washed with PBS and resuspended in ATCC high glucose RPMI-1640 medium, supplemented with 10% FBS. After 10 h, 10nM LPS was added to the medium and incubated for an additional 8 h, at which point the cells were pelleted and the supernatant was harvested and stored at -80 °C until analysis. IL-1β analysis was completed using an ELISA kit from Life Technologies.
Exosome preincubation followed by clozapine stimulation was completed as follows. Exosomes from control and treated animals were added to the cells at a concentration of 50, 75, or 100 µg/mL for 8 h, the cells were spun down via centrifugation at 890g, washed with PBS and resuspended in ATCC high glucose RPMI-1640 medium, supplemented with 10% FBS. After 10 h, 10 µg/mL clozapine was added to the medium, and incubated for an additional 8 h, at which point the cells were pelleted, and the supernatant was harvested and stored at -80 °C until analysis. IL-1β analysis was completed using an ELISA kit from Life Technologies. Exosome stimulation alone was completed as the preincubation, but the cells were spun down for supernatant extraction before the addition of clozapine.

Alternatively, monocytes were cultured in ATCC high glucose RPMI-1640 medium, supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol. Monocytes were cultured in 24-well plates at a concentration of 4 × 10^5 cells/mL in a volume of 1 mL and were differentiated to macrophages with PMA (25 ng/mL) over 3 days at 37 °C. Cells were washed once and resuspended in fresh medium without 2-mercaptoethanol. Exosome pre-treatment experiments were done similar to experiments done with monocytes described above.

### 1.3.5 Treatment of THP-1 cells with drugs

THP-1 monocytes were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in ATCC high glucose RPMI-1640 medium, supplemented with 10% FBS. Monocytes were cultured in 24-well plates at a concentration of 4 × 10^5 cells/mL in a volume of 1 mL. Clozapine was dissolved in DMSO (0.25%) and added to the cells over a concentration range of 1-25 µg/mL. Dimethylfumarate (DMF) and ethacrynic acid (EA) acid were dissolved in DMSO (0.25%) and added to the cells over a concentration range of 5–30 µg/mL. Cells were
incubated at 37 °C, 5% CO₂ for 24 h, after which they were spun down, and the cell culture medium was then tested for IL-1β by ELISA (Life Technologies Inc., Burlington, ON).

Alternatively, monocytes were cultured in ATCC high glucose RPMI-1640 medium, supplemented with 10% FBS and 2-mercaptoethanol. Monocytes cultured in 24-well plates at a concentration of 4×10⁵ cells/mL in a volume of 1 mL, and were differentiated to macrophages with PMA (25 ng/mL) over 3 days at 37 °C. Cells were washed once and resuspended in fresh medium without 2-mercaptoethanol. Clozapine was dissolved in DMSO (0.25%) and added to the cells over a concentration range of 1–10 µg/mL. Clozapine was dissolved in DMSO (0.25%) and added to the cells over a concentration range of 1–25 µg/mL. DMF and EA acid were dissolved in DMSO (0.25%) and added to the cells over a concentration range of 5–30 µg/mL. Cells were incubated at 37 °C, 5% CO₂ for 24 h, at which point medium was collected for IL-1β analysis using an ELISA kit from Life Technologies.

1.3.6 Statistical analysis

GraphPad Prism (GraphPad, San Diego, CA) was employed for all statistical analyses by the paired Student’s t test or one-way ANOVA. Data sets were expressed as the mean ± SEM, and results were considered statistically significant if \( p < 0.05 \).

1.4 Results

1.4.1 IL-1β production via THP-1 cell stimulation shows a drug’s potential to elicit an immune response

It was previously shown that DMF and EA could be used as structurally similar positive and negative controls respectively for IL-1β stimulation in THP-1 cells [Weston, 2014]. THP-1
macrophages respond to DMF stimulation in a dose dependent manner with IL-1β release, where as structurally similar EA does not stimulate THP-1 macrophages (Figure A-1A). Clozapine, a drug thought to cause idiosyncratic drug-induced reactions (IDRs), also has the ability to stimulate THP-1 macrophages and monocytes in a dose dependent manner (Figure A-1B). In contrast, olanzapine, a structurally similar drug that does not cause IDRs did not stimulate THP-1 cells [unpublished data]. LPS is a common stimulant of macrophages and monocytes, eliciting a much more profound IL-1β release from macrophages and monocytes than clozapine, and a comparable amount to 30 µg/mL DMF (Figure A-1C, D).

Figure A-1. Production of IL-1β in monocytes or macrophages in response to clozapine and other agents known to activate inflammasomes.

(A) IL-1β release by THP-1 macrophages treated with ethacrynic acid (EA) or dimethylfumerate (DMF), known negative and positive THP-1 stimulants, respectively. (B) IL-1β release as a result of THP-1 monocytes and macrophages treated with varying concentrations of clozapine. (C) IL-1β release as a result of THP-1 macrophages treated with varying concentrations of clozapine and stimulated with LPS. (D) IL-1β release as a result of THP-1
monocytes treated with varying concentrations of clozapine and stimulated with LPS. Results are expressed as the mean ± SEM, and statistical difference was determined by two-way ANOVA, where *, p < 0.05, ***, p < 0.001, and ****, p< 0.0001.

1.4.2 Exosomes from clozapine treated rats do not stimulate THP-1 cells

Clozapine administered via a single IP dose of 30 mg/kg/day results in blood levels at the top of the human therapeutic range (1.35 µg/mL), which is quickly metabolized resulting in negligible concentrations after 24 h (Figure A-2A). This clozapine administration results in neutrophilia, peaking at 6 h and returning to normal levels after 24 h (Figure A-2B). This neutrophilia was previously shown to be the result of an increased release of immature neutrophils from the bone marrow [Lobach, 2015]. Exosomes extracted from the serum at various time points within 24 h post clozapine injection were taken and incubated with THP-1 monocytes and macrophages, and IL-1β release was used as a measure of stimulation. Neither THP-1 macrophages (Figure A-3) nor THP-1 monocytes (Figure A-4) responded with increased IL-1β release as a result of incubation with control or treated exosomes alone for 24 h.

![Figure A-2. Clozapine blood levels and ANC after a single IP dose](image)

Rats were treated with one dose of 30 mg/kg clozapine IP. Blood samples were drawn from the tail vein at specified time periods after treatment into clot-activating microvette tubes. Each serum sample was analyzed via LC-MS/MS for total parent clozapine levels. (A) Total clozapine blood levels at various time points post clozapine IP dose. The grey box signifies the human therapeutic range of clozapine. (B) Neutrophil counts analyzed via a Hemavet 950FS over 24 h post clozapine IP treatment. Results are expressed as the mean ± SEM, and statistical difference was determined by two-way ANOVA, where *, p < 0.05 and ****, p < 0.0001.
Rats were treated with one IP dose of clozapine (30 mg/kg). Blood samples were drawn from the tail vein at specified time periods throughout treatment into coagulant microvette tubes. Each whole blood sample was used to extract exosomes from the serum. (A) Treated rat exosomes were incubated with THP-1 macrophages for 24 h; supernatant was taken to measure IL-1β via ELISA. (B) Control rat exosomes were incubated with THP-1 macrophages for 24 h, and the supernatant was taken to measure IL-1β via ELISA. Results are expressed as the mean ± SEM.

**Figure A-4. IL-1β release from THP-1 monocytes in response to exosomes**

Rats were treated with a single IP dose of clozapine (30 mg/kg). Blood samples were drawn from the tail vein at specified time periods throughout treatment into coagulant microvette tubes. Each whole blood sample was used to extract exosomes from the serum. (A) Treated rat exosomes were incubated with THP-1 monocytes for 24 h; supernatant was taken to measure IL-1β via ELISA. (B) Control rat exosomes were incubated with THP-1 monocytes for 24 h; supernatant was taken to measure IL-1β via ELISA. Results are expressed as the mean ± SEM.
1.4.3 Pretreating THP-1 cells with exosomes did not affect their ability to respond to further stimulation with LPS

THP-1 macrophages were stimulated with treated or control exosomes (50, 75, and 100 µg/mL) taken 1 h, 3 h, 6 h, and 24 h post clozapine injection for 18 h followed by an 8 h LPS stimulation. LPS stimulated macrophages to release about 300 µg/mL IL-1β cytokine after an 8 h incubation (Figure A-5). There is no clear pattern in time after injection or dose dependent effect of exosomes on IL-1β release (Figure A-5). When comparing the effect of exosomes from treated animals (Figure A-5A) to control exosomes (Figure A-5B) there does not appear to be a significant difference.

THP-1 monocytes were treated with exosomes (50, 75, and 100 µg) from treated or control animals taken 1 h, 3 h, 6 h, or 24 h post clozapine injection at for 18 h followed by an 8 h LPS stimulation. LPS stimulates monocytes to release around 400 µg/mL after an 8 h incubation (Figure A-6). There is no clear pattern in the best time after injection or a dose dependent effect of exosomes on IL-1β release (Figure A-6). When comparing the effects on monocytes of exosomes from treated animals (Figure A-6A) to control animals (Figure A-6B) there does not appear to be a significant difference.
Figure A-5. IL-1β release from THP-1 macrophages in response to exosomes from clozapine-treated rats followed by LPS.

Rats were treated with one IP dose of clozapine (30 mg/kg). Blood samples were drawn from the tail vein at specified time periods throughout treatment into coagulant microvette tubes. Each whole blood sample was used to extract exosomes from the serum. (A) Treated rat exosomes were incubated with THP-1 macrophages for 18 h then stimulated with LPS for 8 h and the supernatant was taken to measure IL-1β via ELISA. (B) Control rat exosomes were incubated with THP-1 macrophages for 18 h then stimulated with LPS for 8 h and the supernatant was taken to measure IL-1β via ELISA. Results are expressed as the mean ± SEM.

Figure A-6. IL-1β release from THP-1 monocytes in response to exosomes from clozapine-treated rats followed by LPS.

Rats were treated with a single IP dose of clozapine (30 mg/kg). Blood samples were drawn from the tail vein at specified time periods throughout treatment into coagulant microvette tubes. Each whole blood sample was used to extract exosomes from the serum. (A) Treated rat exosomes were incubated with THP-1 monocytes for 18 h then stimulated with LPS for 8 h and the supernatant was taken to measure IL-1β via ELISA. (B) Control rat exosomes were incubated with THP-1 monocytes for 18 h then stimulated with LPS for 8 h and the supernatant was taken to measure IL-1β via ELISA. Results are expressed as the mean ± SEM.
1.5 Discussion

Clozapine is known to cause adverse side effects including life threatening idiosyncratic agranulocytosis. Recently, exosomes have been shown to be an important cell-to-cell communication mechanism in a variety of animal models of diseases. These exosomes have been shown to relay danger signals in the form of macromolecules, such as, protein and non-coding RNA sequences from damaged cells in the body that are capable of being horizontally transferred to monocytes and macrophages, thus priming them to respond to stimuli.

Recently we discovery that THP-1 cell monocytes and macrophages respond to drugs that can cause IDRs while not responding to structurally similar drugs that do not cause IDRs [Weston & Uetrecht, 2014]. We also recently discovered that clozapine administered to female Sprague Daley rats results in increased numbers of monocytes and macrophages [unpublished data]. Given these discoveries, we hypothesized that exosomes may play a role in carrying danger signals to monocytes and macrophages in our animal model that leads to sensitization and a proinflammatory response.

A single clozapine dose of 30 mg/kg IP achieves therapeutic blood levels (Figure A-2A) and is able to elicit an immune response in female Sprague Dawley rats in the form of neutrophilia (Figure A-2B), and a release in proinflammatroy cytokines, CXCL1, G-CSF, and α-1-AGP [Lobach & Uetrecht, 2014]. It is possible that exosomes taken within the first 24 h of the clozapine dose would harbor danger signals or cytokines capable of priming monocytes or macrophages.

To determine THP-1 cell stimulation we measured IL-1β release into the cell culture supernatant via ELISA. IL-1β is released from THP-1 cells through the activation of caspase-1 via the
inflammasome; caspase-1 converts pro-IL-1β into IL-1β, which is subsequently released by the cell [Guo et al., 2015]. IL-1β is also known as leukocyte pyrogen, and it has a variety of proinflammatory effects in the body including, as its name suggests, working as a fever-inducing substance. THP-1 monocytes and macrophages respond to common macrophage stimulants such as LPS with a large IL-1β release (Figure A-1C, D). They also respond to drugs that are known to cause IDRs. For example, EA and DMF are structurally similar medications; DMF causes IDRs, while EA does not [Weston & Uetrecht, 2014]. DMF causes a dose dependent stimulation of THP-1 macrophages, while EA does not cause any response (Figure A-1A). Clozapine is known to cause IDRs, and it has the potential to stimulate IL-1β production from THP-1 monocytes and macrophages (Figure A-1B); structurally similar olanzapine does not cause IDRs and does not have the capability to stimulate THP-1 cells [unpublished data].

We first set out to determine if exosomes taken from either clozapine treated or saline control animals would stimulate THP-1 monocytes or macrophages on their own. We incubated both THP-1 monocytes and THP-1 macrophages with varying concentrations of exosomes taken from at varying time points post clozapine injection. Neither the rat exosomes from treated nor control animals elicited an increase in cell culture IL-1β as a result of incubation alone (Figure A-3, A-4). This shows that these exosomes, and the proteins transported within them, do not have the capability to stimulate THP-1 cells themselves at these concentrations.

Next, we set out to find whether the exosomes were able to prime the THP-1 cells to have an increased response to future stimulants. One of the most common macrophage stimulants used is LPS. LPS is known to stimulate macrophages by binding to TLR4 and stimulating the production of NLRP3 inflammasomes leading to the production of IL-1β among stimulating other proinflammatory cytokines [Qiao et al., 2012]. Upregulation of a variety of proteins in the
pathway to IL-1β release can be achieved by various mechanisms and this is called priming or sensitizing the monocytes and macrophages to be better ready to respond to stimuli [Momen-Heravi et al., 2015]. However, preincubating THP-1 monocytes and macrophages with exosomes harvested from control and clozapine treated animals, prior to LPS stimulation did not appear to lead to an increased IL-1β response (Figure A-5, A-6). This suggests that these exosomes were not able to prime THP-1 cells to better respond to LPS stimulation. The time points of exosome extraction may not have been ideal for priming the THP-1 cells or LPS may be too strong of a stimulant to see differences in priming vs. not priming these cells. Alternatively, this animal model may not involve exosome release to carry signals to the periphery. Since these monocytes and macrophages did not show an increased sensitization as a result of pretreatment with exosomes, there was no need to further characterize the exosomes to identify the macromolecules harbored within.

1.6 Acknowledgements

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