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The Emerging Role of the Innate Immune Response in Idiosyncratic Drug Reactions[S]

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ABBREVIATIONS: AGEP, acute generalized exanthematous pustulosis; AIN, acute interstitial nephritis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; APC, antigen-presenting cell; BSEP, bile salt export pump; BSO, buthionine sulphoximine; CCL, chemokine (C-C motif) ligand; cDC, conventional DC; CXCL, C-X-C motif chemokine ligand; DAMP, damage-associated molecular pattern; DC, dendritic cell; DIAIN, drug-induced acute interstitial nephritis; DRESS, drug reaction with eosinophilia and systemic symptoms; ER, endoplasmic reticulum; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; HMGB1, high mobility group box 1; IDIAG, idiosyncratic drug-induced agranulocytosis; IDILI, idiosyncratic drug-induced liver injury; IDR, idiosyncratic drug reaction; IFN, interferon; IL, interleukin; ILC, innate lymphoid cell; MHC, major histocompatibility complex; NET, neutrophil extracellular trap; NF-κB, nuclear factor of the κ light chain enhancer of B cells; NK, natural killer; NLR, nucleotide-binding oligomerization domain-like receptor; NLRP3, NLR family pyrin domain containing 3; NSAID, nonsteroidal anti-inflammatory drug; PRR, pattern recognition receptor; Rel, v-rel avian reticuloendotheliosis viral oncogene homolog; ROS, reactive oxygen species; SJS, Stevens-Johnson syndrome; T_c cell, cytotoxic T cell; TCR, T-cell receptor; TEN, toxic epidermal necrolysis; T_h cell, helper T cell; TLR, Toll-like receptor; TNF, tumor necrosis factor; UPR, unfolded protein response.

Abstract——Idiosyncratic drug reactions (IDRs) range from relatively common, mild reactions to rarer, potentially life-threatening adverse effects that pose significant risks to both human health and successful drug discovery. Most frequently, IDRs target the liver, skin, and blood or bone marrow. Clinical data indicate that most IDRs are mediated by an adaptive immune response against drug-modified proteins, formed when chemically reactive species of a drug bind to self-proteins, making them appear foreign to the immune system. Although much emphasis has been placed on characterizing the clinical presentation of IDRs and noting implicated drugs, limited research has focused on the mechanisms preceding the manifestations of these severe responses. Therefore, we propose that to address the knowledge gap between drug administration and onset of a severe IDR, more research is required to understand IDR-initiating mechanisms; namely, the role of the innate immune response. In this review, we outline the immune processes involved from neoantigen formation to the result of the formation of the immunologic synapse and suggest that this framework be applied to IDR research. Using four drugs associated with severe IDRs as examples (amoxicillin, amodiaquine, clozapine, and nevirapine), we also summarize clinical and animal model data that are supportive of an early innate immune response. Finally, we discuss how understanding the early steps in innate immune activation in the development of an adaptive IDR will be fundamental in risk assessment during drug development.

Significance Statement——Although there is some understanding that certain adaptive immune mechanisms are involved in the development of idiosyncratic drug reactions, the early phase of these immune responses remains largely uncharacterized. The presented framework refocuses the investigation of IDR pathogenesis from severe clinical manifestations to the initiating innate immune mechanisms that, in contrast, may be quite mild or clinically silent. A comprehensive understanding of these early influences on IDR onset is crucial for accurate risk prediction, IDR prevention, and therapeutic intervention.

I. Introduction

Idiosyncratic drug reactions (IDRs) represent a spectrum of unpredictable adverse drug reactions, ranging from mild, more common reactions to potentially life-threatening, less common reactions. IDRs can affect any organ, but a common target of IDRs is the liver. This can lead to liver failure and liver transplantation or death. IDRs may affect the skin and can range in presentation from a mild rash to toxic epidermal necrolysis (TEN), which has a high mortality rate and leaves survivors with permanent scars and often blindness. The bone marrow is also a common target, presenting as agranulocytosis, which can lead to sepsis and death. IDRs are responsible for a substantial burden on patient morbidity, mortality, and health care expenses, and because we cannot predict which drugs may cause IDRs, it also represents a risk to drug development (Suh et al., 2000; Pirmohamed et al., 2004; Breckenridge, 2015).

Although their mechanisms are still poorly understood, there is considerable evidence to suggest that IDRs are immune-mediated. Clinical features such as antidrug or antinuclear antibody detection, human leukocyte antigen (HLA) associations, delayed reaction onset with rapid onset during rechallenge, and involvement of lymphocytes, particularly cytotoxic T cells (identified by histology and by their activation in response to drug exposure in vitro) are all highly suggestive that IDRs are the result of aberrant activation of the adaptive immune response. It is likely that specific attributes of the adaptive immune system are what make IDRs idiosyncratic. For example, HLA associations alone often do not accurately predict the risk of developing IDRs. It is possible that the correct combination of HLA and T-cell receptor (TCR), which are randomly generated in each individual, is required to initiate the adaptive response that leads to the IDR. However, the events that lead up to this, i.e., the innate immune response that precedes antigen presentation, may not be idiosyncratic.

The postulation that an innate immune response is a necessary initiating mechanism in the progression to a serious IDR has been proposed by a number of groups (Cho and Uetrecht, 2017; Sawalha, 2018; Holman et al., 2019; Ali et al., 2020; Hastings et al., 2020; Yokoi and Oda, 2021). However, IDR research to date has predominantly focused on the role of the adaptive immune response and the clinical manifestations of these reactions during the IDR itself, but the events leading up to the clinical manifestation of the IDR remain largely uncharacterized. Thus, this review aims to encourage prospective research on the mechanisms that are involved during the time between commencement of drug administration and the onset of an adaptive IDR by providing an overview of the innate immune system and supporting evidence that drugs that cause IDRs can also induce an innate response. First, we provide a brief overview of the major classes of IDRs with reference to general characteristics, treatment strategies, and drugs frequently associated with the reactions. We then introduce fundamental principles in innate immunology, as well as mechanisms of adaptive immune activation, that may play a mechanistic role in the subclinical phase preceding the development of an IDR. This includes the cells and soluble mediators of the innate immune system in addition to mechanisms of antigen formation, antigen uptake, antigen presentation, and adaptive immune activation. Subsequently, using four archetypal IDR-associated drugs (amodiaquine,

amoxicillin, clozapine, and nevirapine), we summarize the available clinical and animal model literature that is supportive of early immune involvement and activation. Patterns and differences among the data for these drugs will be discussed, and current knowledge gaps will also be emphasized. Lastly, we suggest the application of this research to relevant fields in toxicology.

II. Review of Types of Idiosyncratic Drug Reactions

IDRs have been extensively reviewed elsewhere (Uetrecht and Naisbitt, 2013; Böhm et al., 2018), and describing these reactions in considerable detail is not the purpose here. The main presentations of IDRs will be briefly described, with a focus on the clinical features and studies that illustrate the involvement of the immune system.

A. Idiosyncratic Drug-Induced Liver Injury

Between 1975 and 2007, of 47 drugs that were withdrawn from the market, 15 were withdrawn because of hepatotoxicity, highlighting the burden of this adverse event on patient safety and drug development (Stevens and Baker, 2009). The liver is likely such a common target of IDRs because of its role in drug metabolism. The LiverTox website (http://www.livertox.nih.gov/) identifies 12 different types of drug-induced liver injury based on clinical phenotype (Hoofnagle, 2013). Idiosyncratic drug-induced liver injury (IDILI) may occur unpredictably after drug administration. To broadly classify the type of IDILI, an R ratio is calculated using alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels, expressed as a multiple of the upper limit of normal: ALT/ALP ≤ 2 indicates cholestatic liver injury, ≥ 5 indicates hepatocellular liver injury, and intermediate values indicate a mixed phenotype. Particular HLA class II molecules may influence the pattern of liver injury (Andrade et al., 2004).

1. Hepatocellular Liver Injury. Hepatocellular liver injury is caused by hepatocyte death. The time to onset can vary widely, with 1–3 months being most common. The severity in presentation also varies, with mild and transient elevations in liver enzymes presenting more frequently than severe liver injury that may require liver transplantation or result in death (Uetrecht, 2019a). Symptoms can include allergic features such as fever or rash (Uetrecht and Naisbitt, 2013). Many drugs have been associated with causing hepatocellular IDILI, including various anti-infective agents (e.g., sulfonamides, minocycline, nitrofurantoin, rifampicin, isoniazid, nevirapine), troglitazone, lamotrigine, and diclofenac; immune checkpoint inhibitors are also emerging as a major cause of liver injury (Andrade et al., 2019; Uetrecht, 2019a; Shah et al., 2020).

Histologic examination has revealed the involvement of various cell types, although there is often a mononuclear infiltrate, and there may be eosinophils (Zimmerman, 1999). Eosinophilia in peripheral blood and liver biopsies was correlated with a better prognosis (Björnsson et al., 2007). Increases in CD8^{+} T cells [cytotoxic T cells (T_c cells)] and macrophages have been noted by immunohistochemical staining (Foureau et al., 2015). An immune response can be a response to injury rather than its cause; however, the major role of CD8^{+} T cells is to kill virus-infected cells and cancer cells, not to repair damage. In a mouse model, we showed that depletion of CD8^{+} T cells protected against amodiaquine-induced liver injury, suggesting that these cells do indeed mediate the injury (Mak and Uetrecht, 2015b). In patients treated with isoniazid who had a mild increase in liver enzymes, T_h17 cells secreting interleukin (IL)-10 were also increased in peripheral blood (Metushi et al., 2014).

Various antibodies have also been detected in patients with IDILI. For instance, a number of cases of anti–cytochrome P450 antibodies have been reported for different drugs (Kullak-Ublick et al., 2017), which suggests that drug bioactivation is important in producing the immune response. A recent study found that anti-mitochondrial antibodies correlated with the severity of liver injury better than did anti-nuclear antibodies (Weber et al., 2020).

Most genetic associations with the risk of IDILI development have been related to HLA polymorphisms (Kaliyaperumal et al., 2018). In some cases, other associations have been found, such as an association with an IL-10-low producing phenotype that correlated with an absence of peripheral eosinophilia and more severe liver injury (Pachkoria et al., 2008), an association between increased risk of IDILI with a genetic variant linked to differential expression of interferon regulatory factor-6 in the context of interferon (IFN)-β treatment in multiple sclerosis (Kowalec et al., 2018), and an association between increased risk of IDILI and a missense variant of the gene encoding protein tyrosine phosphatase, nonreceptor type 22 gene (Cirulli et al., 2019).

2. Autoimmune Liver Injury. Certain drugs cause a syndrome that closely resembles autoimmune hepatitis with hypergammaglobulinemia and detectable serum autoantibodies including anti-nuclear antibodies and smooth muscle antibodies (de Boer et al., 2017). The histology also tends to be consistent with that of autoimmune hepatitis, such as interface hepatitis and hepatic rosette formation (Hennes et al., 2008). The onset of autoimmune IDILI is typically later, often after over a year of drug administration. Nitrofurantoin and minocycline are two of the most commonly implicated drugs (Björnsson et al., 2010).

3. Cholestatic Liver Injury. Cholestatic liver injury arises from problems within the biliary system. In some

cases, cholestatic IDILI has been associated with a lower risk of death compared with hepatocellular IDILI (Andrade et al., 2005; Björnsson and Olsson, 2005), but in other cases, the mortality was found to be higher, although the cause of death was not often the liver injury itself (Chalasani et al., 2008). Such findings may depend upon the patient population, as cholestatic IDILI is more commonly observed in older patients (Lucena et al., 2009). In terms of the course of the liver injury, the recovery from cholestatic IDILI tends to be more prolonged than for hepatocellular IDILI, possibly because cholangiocytes regenerate more slowly than hepatocytes (Abboud and Kaplowitz, 2007). Cholestatic IDILI may also lead to ductal injury, such as vanishing bile duct syndrome (Hussaini and Farrington, 2007). Drugs associated with cholestatic drug–induced liver injury include various anti-infective agents (e.g., amoxicillin-clavulanate, flucloxacillin, penicillins) and oral contraceptives (Andrade et al., 2019).

Bile salt export pump (BSEP) inhibition has been identified as a possible mechanism that induces cholestatic IDILI. The rationale for this hypothesis is based upon the finding that genetic defects in BSEP activity cause liver failure with a cholestatic pattern (Jacquemin, 2012). Although correlations have been identified between in vitro BSEP inhibition and drugs that cause IDILI (Morgan et al., 2010), there has not been convincing evidence that this is the mechanism in vivo. Indeed, many of these drugs cause hepatocellular, rather than cholestatic, liver injury, so this is not consistent with the proposed mechanism. One group found that the in vitro results predict IDILI as well as the Biopharmaceutics Drug Disposition Classification System, but because this system is not based upon mechanistic hypotheses of liver injury, BSEP inhibition as a mechanism cannot be a reliable predictor of drug-induced liver injury (Chan and Benet, 2018). Additionally, although it is plausible that BSEP inhibition could lead to the accumulation of bile salts in the liver and induce cytotoxicity or cell stress, few clinical studies to examine bile salt levels in patient sera have been performed to further test this hypothesis.

Amoxicillin/clavulanic acid is most commonly associated with cholestatic IDILI, and multiple HLA associations have been identified in different ethnicities (Hautekeete et al., 1999; Lucena et al., 2011; Stephens et al., 2013). HLA associations have also been found for flucloxacillin (Daly et al., 2009; Nicoletti et al., 2019), and a polymorphism in BSEP 1331 has been found for cholestatic IDILI caused by estrogen (Meier et al., 2008).

B. Severe Cutaneous Adverse Reactions

Skin rash is a highly reported adverse effect likely because it is visible to the patient, even if it is not usually severe. Additionally, as a barrier between the host and the environment, the skin has high immune activity and contains a number of immune cells including macrophages, Langerhans cells, mast cells, and multiple lymphocytes (Sharma et al., 2019). Although the skin has very low cytochrome P450 activity relative to the liver (Rolsted et al., 2008), it contains other enzymes capable of xenobiotic metabolism, such as sulfotransferases and acetyltransferases, which can bioactivate drugs and generate covalently modified proteins (Baker et al., 1994; Dooley et al., 2000; Bhaiya et al., 2006; Luu-The et al., 2009). The focus of this section will be the severe cutaneous drug reactions, which can be life-threatening skin reactions with systemic involvement and fever.

1. Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis. Stevens-Johnson syndrome (SJS) and TEN are considered to be on the same spectrum of disease, wherein SJS is classified as involving ≤10% of total body surface area, TEN as ≥30% of body surface area, and SJS-TEN as intermediate involvement (Gerull et al., 2011). TEN is the most severe of the skin reactions and has a mortality rate of 30%. The onset usually ranges from about 1 to 3 weeks. Drugs with a high risk of causing SJS or TEN include antiepileptics (e.g., carbamazepine, lamotrigine, phenytoin, phenobarbital), antibiotics (e.g., trimethoprim-sulfamethoxazole, nevirapine), oxicam NSAIDs (e.g., meloxicam, piroxicam), allopurinol, and sulfasalazine (Mockenhaupt et al., 2008).

Full-thickness epidermal necrosis, keratinocyte apoptosis, and a mild mononuclear infiltrate characterize the histology (Uetrecht and Naisbitt, 2013). Involvement of various inflammatory mediators has been identified in the pathology of SJS/TEN, including tumor necrosis factor (TNF)-α (Paquet et al., 1994; Nassif et al., 2004b), soluble Fas ligand (Viard et al., 1998; Abe et al., 2003; Murata et al., 2008), granzyme B and perforin (Posadas et al., 2002; Nassif et al., 2004a), and granulysin (Chung et al., 2008). These mediators are highly suggestive of CD8^{+} T-cell involvement, and indeed these cells have been identified in patient blister fluid (Chung et al., 2008). In addition, CD8^{+} T cells from patients proliferate in response to culprit drugs in vitro (Nassif et al., 2004a; Hanafusa et al., 2012), although this is not always the case (Tang et al., 2012). Monocytes have also been identified in patient blister fluid (de Araujo et al., 2011; Tohyama and Hashimoto, 2012).

2. Drug Reaction with Eosinophilia and Systemic Symptoms. Drug reaction with eosinophilia and systemic symptoms (DRESS) was first identified as being caused by anticonvulsant medications and was originally referred to as anticonvulsant hypersensitivity syndrome (Shear and Spielberg, 1988), but this term is now seldom used (Bocquet et al., 1996; Uetrecht and Naisbitt, 2013). DRESS is characterized by rash, fever, and at least one additional symptom indicating organ involvement (lymph nodes, liver, kidney, lung, heart, thyroid, or blood) (Peyrière et al., 2006; Walsh and

Creamer, 2011). However, the presentation of the syndrome is highly heterogeneous, and diagnosis can be quite difficult; for example, a rash is not always present, and if it is, it can vary in its histopathology (Ortonne et al., 2015). The onset of DRESS is typically 2–6 weeks, and its associated mortality rate is about 10% (Cacoub et al., 2011). Moreover, multiple human herpesviruses and other viruses have been found to be reactivated in patients experiencing DRESS (Kano et al., 2006). Drugs that have been associated with DRESS include antiepileptics (e.g., carbamazepine, phenytoin, lamotrigine), antibiotics (e.g., trimethoprim-sulfamethoxazole, minocycline), allopurinol, and abacavir (Behera et al., 2018).

3. Acute Generalized Exanthematous Pustulosis. Acute generalized exanthematous pustulosis (AGEP) presents as a sterile pustular rash on the trunk, face, axillae, upper extremities, and groin. Neutrophilia and eosinophilia may be present, and systemic symptoms are less common than with the other skin reactions but may occur (~20% of cases) (Beylot et al., 1980; Sidoroff et al., 2001; Feldmeyer et al., 2016). The onset of AGEP is shorter than with other skin reactions and can be as short as less than a day or up to 23 days (Roujeau et al., 1991; Choi et al., 2010). Antibiotics (e.g., penicillins, sulfonamides, terbinafine) are the most common cause of AGEP, although other drugs have been implicated as well (e.g., hydroxychloroquine, diltiazem) (Sidoroff, 2012).

Both $CD4^+$ T cells (helper T cells, T_h cells) and $CD8^+$ T cells have been identified in the dermis and epidermis of patients with AGEP, and neutrophils are observed in the pustules (Britschgi et al., 2001). These T cells were found to be drug-reactive and secreted IL-8 [C-X-C motif chemokine ligand (CXCL) 8]. Both $CD4^+$ and $CD8^+$ T-cell subsets appear to be activated to a cytotoxic killer phenotype, and perforin, granzyme B, and Fas ligand are involved in the tissue damage (Schmid et al., 2002). Variants in interleukin 36 receptor antagonist (IL-36RN) have been associated with the development of AGEP (Navarini et al., 2013), as well as HLA-A*31:01 (McCormack et al., 2011).

C. Idiosyncratic Drug-Induced Blood Dyscrasias

Several IDRs affect blood cells, possibly by enhancing their destruction or impairing their production and maturation. These blood reactions include agranulocytosis, hemolytic anemia, and thrombocytopenia.

1. Idiosyncratic Drug-Induced Agranulocytosis. Agranulocytosis is a deficiency of granulocytes in the peripheral blood, which is classically defined as a neutrophil count below 500 cells per microliter of blood (Andrès and Maloisel, 2008; Andrès et al., 2011). Agranulocytosis can be the result of a sequestering of neutrophils in tissue reservoirs, decreased production of neutrophils in the bone marrow (where there is an absence of neutrophil precursors beginning at the promyelocyte stage), and/or increased destruction of neutrophils or their precursors (Schwartzberg, 2006). Like other IDRs targeting blood and bone marrow, the time to onset of idiosyncratic drug-induced agranulocytosis (IDIAG) is usually delayed, typically between 1 and 3 months (Andrès et al., 2017). It can present clinically as septicemia, septic shock, and/or severe infection; however, often patients may remain relatively asymptomatic, highlighting the need for routine monitoring of neutrophil counts for high-risk drugs (Palmblad et al., 2016; Andrès et al., 2019). Drugs frequently associated with this IDR include antibiotics (e.g., cotrimoxazole and amoxicillin ± clavulanic acid), antithyroid drugs (e.g., carbimazole), psychotropics (e.g., clozapine and carbamazepine), antiviral agents (e.g., valganciclovir), antiaggregants (e.g., ticlopidine), analgesics (e.g., metamizole), disease-modifying antirheumatic drugs (e.g., sulfasalazine), and immune checkpoint inhibitors (e.g., nivolumab and ipilimumab) (Andrès and Mourot-Cottet, 2017; Boegeholz et al., 2020). Some risk factors have been identified, such as the presence of certain HLA haplotypes. For instance, several HLA-B haplotypes and HLA-DQB1 are associated with an increased risk of agranulocytosis with clozapine (Legge and Walters, 2019).

Rescue of neutrophil counts to baseline levels can usually be achieved by halting treatment with the suspected drug, and recovery can be assisted with the administration of granulocyte colony stimulating factor or granulocyte-macrophage-colony stimulating factor, thereby reducing the likelihood of infections or other fatal complications (Andersohn et al., 2007; Andrès and Mourot-Cottet, 2017). Although this treatment is useful for patients who have already developed agranulocytosis, it does not prevent the onset of this IDR. Overall, the underlying mechanism of IDIAG is not well understood, but preclinical and clinical research suggests that the reaction likely involves an immune component linked with the formation of reactive metabolites of the drug by myeloperoxidase (Johnston and Uetrecht, 2015).

2. Idiosyncratic Drug-Induced Hemolytic Anemia. Hemolytic anemia is characterized by the premature destruction of erythrocytes that can occur intra- or extravascularly. Patients may be asymptomatic or present with a variety of symptoms, including dyspnea, fatigue, hematuria, tachycardia, and jaundice. Management simply involves discontinuation of the implicated agent (Phillips and Henderson, 2018). There is considerable overlap between drugs that cause agranulocytosis or thrombocytopenia and hemolytic anemia, with reports of patients experiencing more than one hematologic IDR from a single drug (Garratty, 2012). Frequently implicated drugs include the antiarrhythmics (e.g., quinidine, procainamide), antibiotics (e.g., piperacillin, minocycline), the antihypertensive α-methyldopa, and the diuretic hydrochlorothiazide (Al Qahtani, 2018). The suggested mechanisms of this IDR include either

drug-dependent or autoimmune antibodies (Gniadek et al., 2018), with some drug-dependent antibodies demonstrating potential selectivity for certain blood group antigens (Garratty, 2009).

3. *Idiosyncratic Drug-Induced Thrombocytopenia.* Thrombocytopenia is a deficiency in circulating platelets, typically characterized by a platelet count of less than 150,000 cells per microliter of blood, although patients may be asymptomatic until counts fall below 50,000 cells per microliter, at which point purpura may be observed (Gauer and Braun, 2012). With counts below 10,000 cells per microliter, spontaneous bleeding may occur; this constitutes a hematologic emergency (https://www.ncbi.nlm.nih.gov/books/NBK542208/). Typically, treatment involves discontinuation of the causative agent and allowing counts to recover without further intervention, although corticosteroids or platelet transfusions may be administered if the hemorrhage is life-threatening (Andrès et al., 2009). The most common drugs reported in association with immune thrombocytopenia include the anticoagulant heparin; the antimalarial quinine; the antiarrhythmic quinidine; the antibiotics rifampicin, cotrimoxazole, and penicillin; and several oral antidiabetic agents (Andrès et al., 2009). Depending on the offending drug, several mechanisms responsible for the decrease have been proposed, including myelosuppression or the expedited clearance of platelets caused by anti-platelet or anti-haptenated platelet antibodies or platelet-specific auto-antibodies (Narayanan et al., 2019). One recent example is a case of moxifloxacin-induced thrombocytopenia, in which IgM and IgG antiplatelet antibodies were detected in serologic testing and were found to be enhanced in the presence of moxifloxacin, but not with pantoprazole or esomeprazole (Moore et al., 2020).

D. Other Idiosyncratic Drug Reactions

Although reactions targeting the liver, skin, and blood cells are among the most common IDRs, several other classes exist, including autoimmune reactions and kidney injury.

1. *Idiosyncratic Drug-Induced Autoimmune Reactions.* A number of drugs may cause organ-specific autoimmune-type reactions, such as autoimmune hemolytic anemia or autoimmune hepatitis, as described above. Frequently, drugs may cause more than one type of autoimmune reaction, although the pattern of reactions observed may be unique for different drugs (Uetrecht and Naisbitt, 2013). Drug-induced vasculitis is another example of a delayed-type autoimmune reaction, whereby patients may develop antineutrophilic cytoplasmic antibodies against a variety of cytoplasmic neutrophil antigens, including myeloperoxidase, lactoferrin, or granule proteins (Guzman and Balagula, 2020). Notably, myeloperoxidase can oxidize many drugs that are associated with autoimmune reactions, and this likely represents a key mechanistic step in

the progression to IDRs (Hofstra and Uetrecht, 1993; Uetrecht, 2005). Drug-induced vasculitis may present with morbilliform eruptions but is also manifested by blood vessel wall inflammation and necrosis (Shavit et al., 2018). Medications from a variety of classes have been associated with rare cases of vasculitis, including TNF-α inhibitors such as etanercept (Shavit et al., 2018).

Conversely, the autoimmune reaction induced by hundreds of drugs and herbal medications presents with systemic lupus erythematosus-like clinical characteristics within the first few weeks to months of treatment (Solhjoo et al., 2020). Although the clinical manifestation of different drugs can vary considerably, a positive antinuclear antibody score usually is observed, with autoantibodies including anti-histone antibodies, anti-phospholipid antibodies, and anti-neutrophilic cytoplasmic antibodies. The necessity of both an innate and adaptive immune response in the onset of drug-induced autoimmunity has also been proposed (Sawalha, 2018). One of the earliest drugs reported to have a high incidence of drug-induced lupus during chronic treatment was procainamide, with the majority of patients presenting with anti-nuclear antibodies (Uetrecht and Woosley, 1981). Sulfasalazine, a disease-modifying antirheumatic drug, has also been associated with a significant number of autoimmune reactions (Atheymen et al., 2013), identified risk factors for which include HLA-DR4 and HLA-DR*03:01 (Gunnarsson et al., 2000). Resolution of drug-induced autoimmunity is commonly achieved by discontinuation of the implicated agent.

2. *Idiosyncratic Drug-Induced Nephropathy.* Drug-induced acute interstitial nephritis (DIAIN) is most prominent at the corticomedullary junction. Drug treatment accounts for between 70% and 90% of biopsy-confirmed acute interstitial nephritis (Nast, 2017), and it is the third most common reason for acute kidney injury in hospitalized patients (Raghavan and Shawar, 2017). Typically, symptoms of DIAIN are nonspecific (e.g., general fatigue, myalgia, and arthralgia) (Perazella, 2017), with approximately 50% of cases accompanied by cutaneous reactions (Raghavan and Eknoyan, 2014). The most accurate diagnosis of interstitial nephritis is achieved with a kidney biopsy, as blood tests are generally not useful and various imaging modalities (e.g., computed tomography scans, ultrasounds) and urinary tests (e.g., urine microscopy, eosinophiluria) do not provide highly sensitive and/or specific findings (Perazella, 2017). Key histopathological findings include focal to diffuse interstitial edema and an inflammatory infiltrate of T cells that is frequently accompanied by plasma cells and macrophages but infrequently may be accompanied by eosinophilia, depending upon the causative drug (Nast, 2017).

More than 250 drugs have been associated with the risk of DIAIN, including NSAIDs (e.g., diclofenac and naproxen), proton pump inhibitors (e.g., omeprazole

and esomeprazole), and antibiotics (e.g., penicillins and sulfonamides) (Eddy, 2020), each presenting with differing histology. On average, NSAIDs induce less severe injury and rarely have infiltrating interstitial eosinophils, whereas eosinophils are observed in more than 80% of proton pump inhibitor–induced acute interstitial nephritis (AIN), which also appears to be a more severe reaction and often takes more than 6 months to resolve (Valluri et al., 2015). DRESS may also involve the kidney in approximately 10%–30% of cases caused by antibiotics (Eddy, 2020).

The onset of AIN frequently occurs within the first few weeks of treatment with antibiotics, although cases of NSAID-induced AIN have been reported after 6–18 months of treatment (Eddy, 2020). With proton pump inhibitors, the range of onset is 1–18 months, and in many cases of drug-induced AIN, the initiating mechanisms are unclear (Nast, 2017). A possible initiating mechanism includes the covalent binding of the drug or its metabolites to proteins in the kidney, as may occur with β-lactam or sulfonamide antibiotics (Raghavan and Shawar, 2017). Resolution of injury often occurs after removal of the offending agent and may be aided with corticosteroid treatment; however, in the elderly population, return to baseline kidney function may not be achieved in up to 50% of patients (Valluri et al., 2015). Moreover, some acute cases of tubulointerstitial

nephritis may progress to chronic kidney disease with interstitial fibrosis and tubular atrophy (Perazella, 2017).

E. Rationale for Current Review

Throughout this section, it is clear that there is involvement of the adaptive immune system across IDRs affecting different organs. Delayed onset, multiple symptoms, and HLA-associated risk factors of severe IDRs are most consistent with an adaptive immune response. But cells of the adaptive immune system require activation from the innate immune system, and the following section outlines how drugs may cause activation of the innate immune system. Understanding this process is crucial in understanding the development of IDRs. Additionally, although the adaptive response appears to be idiosyncratic because of patient-specific factors, the innate response is unlikely to be idiosyncratic, as it is the body's first and nonspecific line of defense after the detection of pathogens and other harmful stimuli. Therefore, this may represent a means of identifying drug candidates that carry the risk of causing IDRs during drug development and will be discussed in more detail below.

Ultimately, the goal of this review is to highlight the need for research on the initiating factors of IDRs to delineate the events that occur between the commencement of drug
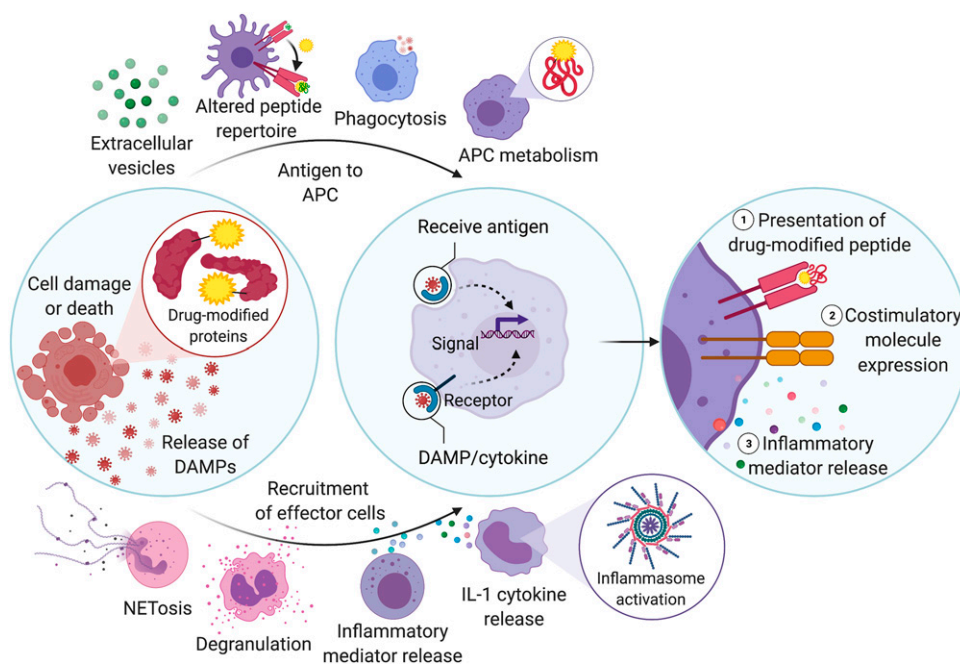
Fig. 1. A working hypothesis of the early immune mechanisms involved in idiosyncratic drug reactions. First, drugs may bind to MHC molecules and alter the repertoire of peptides presented by the MHC molecules, known as the altered peptide hypothesis. More commonly, drugs or their reactive metabolites (generated by various enzymes) covalently bind to cellular proteins, generating drug-modified, or haptenated, proteins. These haptenated proteins may be transported to APCs via extracellular vesicles or endocytosis mechanisms, or may be generated by the APC itself. Additionally, protein modification leads to cell stress, damage, or death, which prompts the release of proinflammatory molecules such as DAMPs. These mediators result in the recruitment of effector innate immune cells such as neutrophils or other granulocytes, which may degranulate or release NETs, monocytes or macrophages (which may result in cytokine release), and/or ILCs. Another response induced in these cells may include activation of the inflammasome and the subsequent release of IL-1 cytokines. Within APCs, the drug-modified proteins are processed and presented in the context of MHC molecules. The recognition of DAMPs and cytokines by APCs induces the upregulated expression of costimulatory molecules and also causes inflammatory cytokine release by the APCs themselves, ultimately resulting in the activation of T cells.

administration and the development of the IDR. To guide these investigations, we look to the fundamentals of immunology to describe how an immune response may develop in response to the administration of small-molecule drugs.

III. Innate Mechanisms Contributing to Adaptive Immune Activation

Overwhelmingly, the adaptive arm of the immune system has been the focus of IDR research, as this is the mechanism that is likely responsible for clinically significant IDRs. The adaptive immune response is likely also what makes IDRs idiosyncratic: individuals possess unique and dynamic TCR repertoires, formed through random somatic recombination events (Krangel, 2009), and without the major histocompatibility complex (MHC) presentation of drug-modified peptides to cognate TCRs, adaptive immune activation and subsequent IDR manifestation cannot occur (Usui and Naisbitt, 2017; Hwang et al., 2020).

However, a fundamental dogma of immunology is that an innate immune response is required to initiate an adaptive immune response, and although progression to a severe IDR may be uncommon, it is likely that a greater proportion of patients experience an innate immune response that resolves without intervention and without leading to a significant adaptive immune response. Therefore, a more comprehensive understanding of the subclinical early immune mechanisms preceding IDR onset is necessary to guide future strategies in disease management and prevention. Thus, from neoantigen formation to consequences of immunologic synapse formation, this section will provide a succinct overview of important principles in innate immunology as well as mechanisms of adaptive immune activation that are potentially involved preceding the development of an IDR. These concepts are summarized in Fig. 1. Admittedly, the innate immune response is much more complex and nuanced than can be adequately addressed here; however, these topics provide a basic framework to be considered when designing future mechanistic studies for drugs associated with the risk of IDRs. For those already familiar with the innate immune system, this section may be skimmed, skipped, and/or referred to when necessary in accompaniment with *Section IV. Support for Immune Activation Using Model Drugs*.

A. Cells of the Innate Immune System

Initiation of any inflammatory response is dependent upon the recruitment and activation of innate effector cells. When this immune response is triggered by the detection of endogenous danger signals without the detection of pathogens or pathogen-associated molecular patterns, it is described as sterile inflammation (Chen and Nuñez, 2010). Although the types of innate immune cells that may participate in this type of inflammation are generally similar, the function of the sterile response is not to clear an infection but, ultimately, to repair the damage caused by chemical or physical insult; thus, the role of effector cells may vary considerably. Responding leukocytes include granulocytes (neutrophils, eosinophils, basophils, and mast cells), professional antigen-presenting cells (APCs: monocytes, macrophages, and dendritic cells), and innate lymphoid cells (ILC groups 1–3). Other immune cells, including platelets, megakaryocytes, and erythrocytes, and nonimmune cells, such as mesenchymal stem cells, fibroblasts, and hepatocytes, may also function during the immune response and are introduced briefly. Ultimately, since the function of the innate immune system is to provide a first line of defense against foreign or potentially harmful stimuli, including potential damage caused by binding of drug-reactive metabolites, activation of innate immune cells represents a more universal, non–patient-specific mechanism to be studied in the context of IDRs.

1. Granulocytes.

a. Neutrophils. Neutrophils are essential for innate immunity, not only as phagocytes that engulf and destroy invading pathogens but also as rapid responders during sterile inflammation (McDonald et al., 2010; Lämmermann et al., 2013), and can even possess a reparative function (Wang et al., 2017). Moreover, in vitro, neutrophils have been demonstrated to function as APCs under inflammatory conditions, further highlighting the diverse roles of these cells (Mehrfeld et al., 2018).

Mature neutrophils, derived from common myeloid progenitors in the bone marrow, are the most abundant leukocyte present in the human circulation, although a large store of mature cells also exist in the bone marrow or transiently arrested within blood capillaries (Lawrence et al., 2018). After the detection of any of an extensive array of inflammatory stimuli [such as chemokines or damage-associated molecular patterns (DAMPs), discussed below], marginated neutrophils are released rapidly into the circulation and, through the process of chemotaxis, can migrate to the site of inflammation. Although once considered to be a single, short-lived population, significant neutrophil heterogeneity has been reported in the steady-state (Fine et al., 2019) as well as in the context of numerous inflammatory (Silvestre-Roig et al., 2016; Yang et al., 2017) and cancer models (Hellebrekers et al., 2018; Giese et al., 2019), with extended neutrophil life spans observed in the presence of inflammation (Filep and Ariel, 2020). Reparative and immunosuppressive phenotypes have also been described (Rosales, 2020).

Neutrophils contain several types of granules and secretory vesicles, the contents of which can be released in a stimulus-dependent manner, either intracellularly via fusion with a phagocytic vacuole or extracellularly

via degranulation or exocytosis (Giese et al., 2019). In addition to the many enzymes, receptors, and cytokines released in granules and secretory vesicles, neutrophils are also able to generate reactive oxygen species (Sheshachalam et al., 2014; Winterbourn et al., 2016). Together, these components can mediate pathogen destruction, induce recruitment of additional inflammatory cells, or contribute to tissue injury or repair (Silvestre-Roig et al., 2019).

Moreover, after stimulation, neutrophils can release web-like structures called neutrophil extracellular traps (NETs), composed of histone-linked DNA fragments, cathepsin G, elastase, and myeloperoxidase (Brinkmann et al., 2004). Interestingly, NET release can occur in a lytic or nonlytic manner, meaning neutrophil lysis and subsequent cell death may or may not occur during the process (Castanheira and Kubes, 2019). Of note, the enzyme myeloperoxidase, which is present in both neutrophil granules and NETs, has also been shown to bioactivate a variety of drugs to reactive metabolites in vitro (Hofstra and Uetrecht, 1993) and to contribute to the covalent binding of drugs observed in vivo (Lobach and Uetrecht, 2014b). Whereas IDIAG is the result of a delayed, adaptive immune response, paradoxical neutrophilia has been reported in the first few weeks of treatment with drugs associated with this IDR, namely clozapine (*Section IV. D. Clozapine*). Overall, neutrophils are integral for coordination and resolution of an inflammatory response and for tissue repair, and they can also play a role in the generation of neoantigens through myeloperoxidase-mediated reactive metabolite formation.

b. Eosinophils. Eosinophils are among the rarest leukocytes in circulation in a healthy state, but their numbers can increase up to 20-fold during certain pathologic conditions (Klion et al., 2020). They are fundamental effector cells in innate immunity against a wide variety of pathogens but also contribute to acute and chronic inflammatory conditions including asthma, eczema, and different types of autoimmunity and can mediate both tissue damage and repair (Ferrari et al., 2020; Nagata et al., 2020). In addition to granular proteins, eosinophils synthesize more than 40 proinflammatory mediators, such as TNF-α, IL-1 family cytokines, IL-4, IL-6, IL-8, granulocyte-macrophage-colony stimulating factor, leukotrienes, and reactive oxygen species (Spencer et al., 2014; Melo and Weller, 2018). These mediators can be released via classic exocytosis; through eosinophil cytolysis, whereby intact granules are liberated directly into target tissues; or through piecemeal degranulation, whereby cytokines are selectively mobilized to vesicles from the main granules and are then released (Spencer et al., 2014). Much like neutrophils, eosinophils can release extracellular traps of DNA and DAMPs, although these networks are more resistant to degradation compared with NETs (Ueki et al., 2016). Overall, eosinophils are

a hallmark of allergic inflammation, and as discussed in *Section IV. Support for Immune Activation Using Model Drugs*, eosinophilia is frequently reported during the initial weeks of clozapine therapy in patients, indicative of an innate immune response. More broadly speaking, eosinophilia is also seen in other IDRs, such as DRESS, AGEP, or liver injury.

c. Basophils. Although they are the rarest and weakest phagocytic granulocyte in circulation, basophils play a key role in tissue inflammation; namely, skin, lung, and gastrointestinal tract inflammatory responses that are commonly triggered by either an invading parasite or allergen (Schwartz et al., 2016). Basophils are activated by allergen-induced crosslinking of their IgE receptors (Knol, 2006). Indeed, the basophil activation test is used as a reliable diagnostic tool for identifying various allergens. In the context of drug allergy, however, the basophil activation test is not as sensitive as it is in identifying other types of allergens (Eberlein, 2020). Possibly, this could be because the covalent modification of proteins by drugs produces a range of antigens such that it is not accurately reproduced in vitro.

Basophils are a source of IL-13 and are known to constitutively express IL-4, which are cytokines necessary for B-cell stimulation and differentiation to plasma cells and also differentiation of naïve helper T cells to T_h2 cells (Liang et al., 2011), thus representing an important bridge between the innate and adaptive immune responses. Basophil-derived IL-4 has also been shown to have an important function in alternatively activated (M2) macrophages, which are involved not only in type 2 immunity but also in tissue repair and physiologic homeostasis (Yamanishi and Karasuyama, 2016). Basophils can quickly migrate to inflamed tissues and are among the first responding cells during skin injury (Chhiba et al., 2017). Activated basophils release a variety of mediators stored in cytoplasmic granules, including the bioactive lipids leukotrienes and prostaglandins, histamine, chemokines, and other cytokines (Chirumbolo et al., 2018), and also present with transcriptional heterogeneity, depending upon the stimuli (Chhiba et al., 2017). Additional innate effector cells such as eosinophils and ILC2 have also been demonstrated to be recruited by basophils to inflammatory sites (Schwartz et al., 2016). Although basophils were once considered a redundant counterpart of tissue-resident mast cells, they have more recently been acknowledged to play many unique roles during the inflammatory response that extend beyond allergy and hypersensitivity reactions.

d. Mast cells. Mast cells share functional and morphologic characteristics with basophils but are considered sentinels of the innate immune system, and although they are found in most tissues of the body, terminally differentiated mast cells are typically not detected in circulation. Although mast cells have diffuse

cytoplasmic granules comparable to basophils and other classic granulocytes, there has been considerable debate as to whether the progenitors of the mast cell lineage are more closely related to megakaryocyte/erythroid or granulocyte/macrophage progenitors. However, it appears that mast cells are derived independently from either group and only share the early common myeloid progenitor (Franco et al., 2010). Both positive and negative immunoregulatory roles have been ascribed to mast cells. They function as a first line of defense against pathogens, and they are particularly useful in degrading venoms and toxins (Dudeck et al., 2019). Additionally, they contribute to allergic inflammatory responses by recruiting additional innate cells to the site of inflammation and by activating adaptive immune cells, thus promoting chronic responses (Kubo, 2018; Olivera et al., 2018). Moreover, excessive and sustained activation of mast cells can cause anaphylaxis and tissue damage, respectively. These effector functions can be attributed to the release of mast cell secretory granules, which contain proteases, lysosomal enzymes, biogenic amines, and cytokines (TNF, IL-4, IL-5, IL-6, etc.), among numerous other constituents (Wernersson and Pejler, 2014). Mast cells are most abundant in areas exposed to high levels of antigen, including the skin, other connective tissues, and the gastrointestinal and respiratory tracts (Krystel-Whittemore et al., 2016), and their roles in innate immunity can vary depending on the local milieu of mediators.

2. Professional Antigen-Presenting Cells. Professional APCs are cells that possess constitutive or inducible expression of high levels of MHC II molecules, process antigen, and express costimulatory molecules to facilitate the development of adaptive immunity to specific antigens. Classically, dendritic cells, macrophages, and B cells are considered professional APCs. B cells will not be discussed here, aside from their antigen presentation function, which is briefly described later. Although it has been recognized that other cell types, referred to as atypical or nonprofessional APCs, can also express MHC II, there is little evidence that they can activate naïve T cells (Kambayashi and Laufer, 2014). APCs facilitate the surveying of antigen by $CD4^+$ T cells to efficiently expand the small subset of T cells expressing the cognate T-cell receptors to respond to antigenic challenge.

a. Dendritic cells. Dendritic cells (DCs) received their name from their many branched cellular processes (Steinman and Cohn, 1973). DCs can be categorized as conventional or plasmacytoid, both of which arise from a committed dendritic cell precursor in the bone marrow. These then diverge, as conventional dendritic cell precursors leave the bone marrow and seed other organs, whereas plasmacytoid dendritic cell precursors remain. Conventional DCs (cDCs) are the predominant cell type responsible for T-cell activation, and the far less abundant plasmacytoid DCs are specialized in sensing viral RNA and DNA and can produce large amounts of interferons to drive the antiviral response (Sichien et al., 2017; Musumeci et al., 2019). Historically, cDCs have been further categorized as migratory or lymphoid-resident; however, more recent studies have resulted in the classification of cDCs as cDC1 and cDC2 based upon surface marker expression and transcriptomic analyses (Ziegler-Heitbrock et al., 2010; Guilliams et al., 2014, 2016). Langerhans cells were originally presumed to be DCs based upon their function, but based upon their ontogeny, they are resident macrophages (Doebel et al., 2017), highlighting the complexity in classifying these types of cells. Langerhans cells likely play an important role in mediating skin IDRs.

DCs are usually found in a resting or immature state and survey their environment by sampling antigen. Because they have both low surface expression and rapid turnover of MHC II molecules, they are unable to activate naïve T cells (Drutman et al., 2012). In an inflammatory milieu, DAMPs and pathogen-associated molecular patterns are present and engage various pattern recognition receptors on the DC surface, causing the DC to mature. The cells are then able to express cytokine and chemokine receptors, facilitating migration to lymph nodes. MHC II turnover decreases while expression increases, allowing for presentation of the peptides found in the inflammatory context (Cella et al., 1997). Additionally, costimulatory molecule expression and cytokine secretion are induced, and the combination of these changes is sufficient to induce naïve T-cell activation (Curtsinger and Mescher, 2010). Depending upon the stimuli received by the DC, it will secrete different cytokines and influence the differentiation of cognate T cells into different subsets of effector T cells (Blanco et al., 2008).

DCs may also be tolerogenic in certain cases. Thymic DC populations appear to be important in maintaining central tolerance during T-cell development (Lopes et al., 2015). Peripherally, a small proportion of DCs undergo maturation under homeostatic conditions and upregulate MHC II expression, but this maturation results in tolerance rather than naïve T-cell activation (Lutz and Schuler, 2002). Indeed, antigen presentation without DC priming resulted in antigen-specific tolerance (Probst et al., 2003). The vast heterogeneity of DCs, as well as the varied outcomes of maturation based on environmental influences, results in numerous functions for DCs.

b. Monocytes. Monocytes are cells of the myeloid lineage that are derived from the bone marrow and are released into circulation. Monocytes are phagocytic and scavenge apoptotic cells and toxic macromolecules in circulation. They also function as important orchestrators of inflammatory responses by producing cytokines after the detection of tissue damage or pathogens.

Monocyte subsets exist across a spectrum of differentiation, initially taking on an "inflammatory" phenotype upon egress from the bone marrow and then taking on a "patrolling" phenotype over time because of transcriptional changes (Mildner et al., 2017). Under steady-state conditions, monocytes can enter tissue, return to circulation with minimal differentiation, and traffic to lymph nodes to present antigen to T cells (Jakubzick et al., 2013). Although monocytes may themselves function as APCs, upon entering inflamed tissue, they can also differentiate into macrophages or dendritic cells to propagate the inflammatory response (Jakubzick et al., 2017).

c. Macrophages. Macrophages are phagocytes that are usually found in tissues, and many have been named depending upon the organ in which they reside (e.g., Kupffer cells in the liver, microglia in the central nervous system, or osteoclasts in bones). The environment in which macrophages are found influences their phenotype and function; various tissue-resident macrophage populations express different surface proteins, and even within an organ may have different phenotypes (Hume et al., 2019). Macrophages survey the tissue in which they reside and phagocytose foreign molecules and debris.

Like DCs, macrophages express pattern recognition receptors, which can stimulate their activation. Macrophage activation states are highly complex and are often described as polarization to an "M1-like" or "M2-like" phenotype, correlating with T_h1 and T_h2 responses, respectively. As macrophage activation is a dynamic process, different macrophages in the same tissue likely express different mixtures of activation markers and perform different functions; this also evolves over time (Murray, 2017). For example, in response to IFN-γ, activated macrophages secrete proinflammatory cytokines (e.g., IL-1β, IL-6, IL-12). In response to IL-4, however, they can secrete insulin-like growth factor 1 and resistin-like molecule-α, which can stimulate fibroblast survival and promote extracellular matrix deposition, respectively (Mosser and Edwards, 2008).

Macrophages can participate in antigen presentation, but unlike DCs, they cannot activate naïve T cells. Their ability to present antigen can be influenced by their environment; for example, antigen presentation to T cells and CD40 expression were increased with the uptake of necrotic but not apoptotic cells (Barker et al., 2002). Crosspresentation of antigen by macrophages from dead tumor cells has been shown to be important in antitumor immunity (Asano et al., 2011). Macrophages have also been shown to present lipid antigens to invariant natural killer T cells (Barral et al., 2010).

Macrophages also have reparative functions and can secrete growth factors and anti-inflammatory mediators including IL-10 and TGF-β during tissue repair (Vannella and Wynn, 2017). Overall, macrophages play

varied and dynamic roles in the steady-state and throughout the inflammatory response.

3. Innate Lymphoid Cells. Over the past decade, ILCs have come to be recognized as fundamental effectors of the innate immune response (Moro et al., 2010; Neill et al., 2010; Price et al., 2010), both in health and in disease states ranging from type 2 inflammatory conditions (e.g., atopic dermatitis and asthma) to autoimmune diseases (e.g., psoriasis and inflammatory bowel disease) (Ebbo et al., 2017; Kobayashi et al., 2020). Aside from natural killer (NK) cells, which are localized in secondary lymphoid organs, ILCs are generally under-represented in lymphoid tissues but are predominantly found in the liver, skin, intestine, lungs, adipose tissue, and mesenteric lymph nodes and are most prominent at mucosal barriers (Klose and Artis, 2016). At the most rudimentary level, ILCs can be classified as group 1, group 2, and group 3, with each group sharing similarities in cytokine production and transcriptional regulation with a particular T-cell subset (although ILC antigenic receptors do not undergo the genetic rearrangement that adaptive lymphocytes undergo) (Spits et al., 2013). Group 1 ILCs include both ILC1s (T_h1-like) and natural killer cells (cytotoxic T cell-like) and are characterized by their production of IFN-γ and TNF (Spits et al., 2016), whereas group 2 ILCs are a single population (T_h2-like) that produce amphiregulin, IL-4, IL-5, and IL-13 (Klose and Artis, 2016). Group 3 ILCs comprise three populations (T_h17-like) —lymphoid tissue inducer cells, natural cytotoxicity receptor-negative cells, and natural cytotoxicity receptor-positive cells—that all secrete IL-22, but only the first two population also secrete IL-17A/IL-17F (Montaldo et al., 2015).

Additionally, some T-cell subsets are "preprogrammed" and behave like innate cells in that they can respond rapidly to a limited and conserved antigenic repertoire. These include invariant natural killer T cells, which differ most prominently from conventional T cells in that they recognize lipid-based antigens in the context of CD1d; mucosal-associated invariant T cells, subsets of $\gamma\delta$ T cells; and certain memory T-cell subsets (Vivier et al., 2018).

Like many cells, ILCs demonstrate significant plasticity, and their functionality is dependent on their local microenvironment. Even within specific ILC groups, significant heterogeneity has been reported. For example, among natural killer cell subsets, some possess more cytolytic activity and contain high concentrations of granzyme and perforin, whereas others are more reactive to activation by proinflammatory mediators, and surface receptor expression varies between hepatic, intraepithelial, and other natural killer cell populations (Spits et al., 2016). Natural killer cells, as discussed in the next section, have also been shown to mediate the inflammatory response induced by amodiaquine, and they are likely to play fundamental roles in the early

immune responses to other IDR-associated drugs, as well. Moreover, based on the emerging roles of various ILC subsets in inflammatory conditions, as well as their localization within organs most commonly associated with IDRs (e.g., the liver and skin), it is reasonable to question whether other members of the ILC family play a crucial part in the innate immune response to drugs associated with IDRs.

4. Other Innate Immune Cells.   Other immune cells, such as megakaryocytes, their platelet derivatives, and erythrocytes, may also be important contributors during an innate immune response. Although the immunologic role of megakaryocytes is not as well defined, their expression is dependent on the demand for platelets, which can be upregulated during inflammatory conditions or infection, vascular damage, and tissue repair (Noetzli et al., 2019). Beyond a role in hemostasis, platelets have significant immunomodulatory potential: they can release both proinflammatory [e.g., CXCL4, chemokine (C-C motif) ligand (CCL) 5, histamine, epinephrine, and high mobility group box 1 (HMGB1)] and proresolving mediators and can form complexes with a variety of immune cells, including neutrophils and monocytes (Margraf and Zarbock, 2019). Platelets can release these mediators through microvesicles and exosomes (Heijnen et al., 1999). Likewise, erythrocytes contribute to innate immunity and are more than just oxygen carriers. Interestingly, these cells can bind a variety of chemokines, in turn, either preventing recruitment of effector cells such as neutrophils or possibly extending the half-life of these mediators to prolong the inflammatory response, referred to as the sink hypothesis and reservoir hypothesis, respectively (Anderson et al., 2018).

5. Nonimmune Cells.   Although the focus of this section is to introduce the reader to cells of the innate immune system, it is also worth emphasizing that countless nonimmune cells can have inflammatory or immunoregulatory functions. Naturally, any damaged or dying cells can release DAMPs and proinflammatory mediators that can activate immune cells both locally and in circulation, resulting in recruitment to the location of injury and initiation of an innate immune response; this is not dependent on immune cell status. However, nonimmune cells can also secrete bioactive and chemotactic molecules in response to the detection of a stimulus, as well. Such cells include, but are not limited to, hepatocytes, mesenchymal stem cells, and fibroblasts.

a. Hepatocytes.   As the predominant parenchymal cell in the liver, hepatocytes are well known for their role in metabolism, xenobiotic detoxification, and protein synthesis, but they are also critical players in innate immunity (Mehrfeld et al., 2018). The liver is highly vascularized, receiving 25% of total cardiac output (Eipel et al., 2010), and is also responsible for the production of up to 50% of the lymph collected by the thoracic duct (Ohtani and Ohtani, 2008). While not in direct contact with the sinusoidal blood flow, hepatocytes can extend filopodia through fenestrations in the adjacent endothelium to enable interactions with circulating leukocytes (Warren et al., 2006). Under steady-state conditions, hepatocytes only express MHC I (Mehrfeld et al., 2018), but they may express MHC II under inflammatory conditions (Herkel et al., 2003). Thus, hepatocytes may function as APCs with the potential to interact with both helper and cytotoxic T cells; indeed, hepatocytes have been shown to activate CD8^{+} T cells, although they did not promote survival (Bertolino et al., 1998). Like most of the cells discussed thus far, hepatocytes not only share the ability to target pathogens for destruction but can also secrete a variety of proinflammatory mediators, such as soluble CD14, soluble myeloid differentiation 2, IL-6, CCL2, and CXCL1 (Zhou et al., 2016). Moreover, among the proteins synthesized and secreted into the blood by hepatocytes are acute phase proteins, such as C-reactive protein, serum amyloid A, and serum amyloid P. The concentrations of these mediators can dramatically increase after the detection of inflammation, thus acting to amplify the immune response (Schrödl et al., 2016). As most reactive metabolite formation occurs in the liver, and the liver is the target of a large proportion of IDRs, hepatocytes are likely fundamental in the initiation of the innate immune response to drugs that cause IDILI (Uetrecht, 2019b; Ali et al., 2020; Hastings et al., 2020; Mosedale and Watkins, 2020; Yokoi and Oda, 2021).

b. Mesenchymal stem cells.   Mesenchymal stem cells, also referred to as mesenchymal stromal cells, have been identified in various tissues and have the capacity to differentiate into chondrocytes, osteoblasts, and adipocytes (Dominici et al., 2006). Moreover, these multipotent stem cells help maintain the tissue microenvironment, under both normal and inflamed conditions, often promoting an immunosuppressive milieu via the release of growth factors and anti-inflammatory molecules (e.g., transforming growth factor-β, IL-1 receptor antagonist, IL-10, prostaglandin E2, etc.) after the recognition of proinflammatory stimuli (Wang et al., 2014). Exosomes have also been shown to contribute to the immunomodulatory capabilities of these cells, and even apoptotic mesenchymal stem cells maintain suppressive properties (Shi et al., 2018). Mesenchymal stem cells can also migrate to the site of tissue damage to participate in regeneration and can activate or suppress the activation of various innate cells, including neutrophils, macrophages, DCs, and mast cells (Le Blanc and Mougiakakos, 2012; Shi et al., 2018).

c. Fibroblasts.   Although a key function of fibroblasts is to maintain connective tissue structural integrity, these sentinel cells also have the capacity to respond to pathogens and endogenous danger signals, to secrete

inflammatory signals, and to initiate tissue repair (Hamada et al., 2019). For instance, intestinal immunity is shaped by the secretion of cytokines, chemokines, growth factors, and metalloproteinases by epithelial cells and myofibroblasts (Curciarello et al., 2019). These cells have also been shown to contribute to the persistence of inflammation, such as during rheumatoid arthritis, where synovial fibroblasts produce a variety of proinflammatory and matrix-degrading molecules (Frank-Bertoncelj et al., 2017).

B. Antigen Formation and Cell Damage

Multiple theories have been proposed to explain how drugs may cause IDRs; these are discussed in detail elsewhere (Cho and Uetrecht, 2017). We will present the hypotheses that are relevant to the discussion of innate immune system activation. It has long been understood that foreign peptides are recognized by the immune system. The hapten hypothesis and the altered peptide repertoire model describe distinct processes by which drug administration may ultimately result in the exposure of the immune system to novel peptides. These neoantigens may serve as targets for the immune response, potentially resulting in the development of an IDR.

More recently, it was also recognized that there needs to be a signal (e.g., a DAMP, discussed below) that damage is occurring. This is known as the danger hypothesis. This hypothesis most likely complements the hapten and altered peptide hypotheses. Similarly, in conjunction with the hapten hypothesis, it is plausible that sufficient covalent binding in the cell may result in cellular damage. The endoplasmic reticulum (ER) stress and unfolded protein response, as well as mitochondrial toxicity, may result from the generation of covalently modified proteins. These processes may also result in the release of DAMPs. Thus, these hypotheses likely work together to describe the initiating events in IDRs.

1. Hapten Hypothesis. Small-molecule drugs are too small to be detected by the immune system, which recognizes larger molecules, such as proteins (Erkes and Selvan, 2014). However, many drugs that cause IDRs have reactive metabolites. The hapten hypothesis posits that drugs are bioactivated to a reactive metabolite that then covalently binds to endogenous protein, thereby altering the protein and provoking an immune response (Landsteiner and Jacobs, 1935; Faulkner et al., 2014; Cho and Uetrecht, 2017). Although it is very difficult to prove that reactive metabolites cause IDRs, there are some cases in which they have been shown to be responsible. For example, penicillin hypersensitivity involves IgE, and IgE from hypersensitive patients has been shown to react to penicillin-modified protein (forming the basis of diagnostic skin tests) (Levine et al., 1967). There have also been studies of multiple drugs characterizing drug-protein adducts in

patient samples, although these have not necessarily been causally linked to IDR onset. Additionally, nevirapine is another case in which the reactive metabolite was identified. Female brown Norway rats develop a rash when chronically administered nevirapine. The findings that 12-hydroxynevirapine sulfate was covalently bound in the skin and that topical application of a sulfotransferase prevented both the covalent binding and the rash demonstrates that this reactive metabolite was indeed responsible for causing the skin rash (Sharma et al., 2013).

2. p-i Concept. The pharmacological interaction of drugs with immune receptors concept (p-i concept) attributes IDRs to the activation of immune receptors, MHC and the TCR specifically, by direct, noncovalent interaction of the culprit drug (Pichler, 2002). This is based on the observation that drugs can activate lymphocytes from patients who have experienced an IDR to that drug in the absence of metabolism. However, in the case of nevirapine-induced skin rash, it has been shown that the rash is caused by a reactive metabolite, and yet cells from rats or humans who have a history of nevirapine-induced skin rash are activated by the parent drug (Chen et al., 2009). Thus, although direct activation of immune cells by parent drug may occur in IDR patients, this mechanism may not play a role in the initiation of the IDR.

3. Altered Peptide Repertoire Model. A mechanism related to the p-i concept is the altered peptide repertoire model, which describes the noncovalent binding of drug to the HLA molecule itself, thereby altering its conformation and the peptide repertoire that it is able to present. This is illustrated by abacavir, which has been shown to reversibly bind to the F pocket of the peptide-binding groove of HLA-B*57:01 and alter the repertoire of peptides that it can present (Illing et al., 2012; Norcross et al., 2012; Ostrov et al., 2012).

4. Danger Hypothesis. Although foreign peptide is a requirement for activation of the immune response, it is not usually sufficient; indeed, the body is exposed to non–self-proteins constantly from food sources and gut microflora, for example. It would be detrimental if the immune system were constantly activated as a result of these sources. The danger hypothesis recognizes that it is not necessarily the detection of an entity that appears foreign but, in fact, an entity that causes damage that activates the immune system (Matzinger, 1994). Cell damage causes the release of DAMPs, which signal to the immune system that there is likely a pathogen that needs to be eliminated. Very broadly speaking, cell damage may manifest as cell death, in which intracellular contents may be passively released and serve as DAMPs, or the cell may continue to survive, in which case DAMPs may be actively secreted.

Additionally, the type of cell death influences the types of DAMPs that are released. In apoptosis, cellular contents are not necessarily released into the

extracellular milieu as membrane integrity is maintained; however, ATP is released in a controlled manner as a "find-me" signal (Elliott et al., 2009). In contrast, in cells dying by necrosis, cellular contents are released as cell death is uncontrolled. Necroptosis, a regulated form of necrosis mediated by death receptor activation, and pyroptosis, cell death regulated by inflammasome and caspase-1 activation, may similarly both result in the release of a number of DAMPs. Some DAMPs are released in the context of both types of programmed cell death (e.g., HMGB1, heat shock protein 90, ATP, IL-1α), whereas some have only been observed in necroptosis (e.g., S100A9, IL-33) or pyroptosis (e.g., ASC specks) to date (Frank and Vince, 2019).

5. Endoplasmic Reticulum Stress and the Unfolded Protein Response. The ER is the location of protein folding and post-translational modification in the cell. Disruption of this process can cause ER stress. A series of pathways, termed the unfolded protein response (UPR), maintain quality control of protein synthesis through sensing deficiencies in protein folding capacity. Proteins in their proper conformation proceed to the Golgi apparatus as the next step in the secretory pathway, whereas misfolded proteins are retained in the ER (Schröder and Kaufman, 2005). As cytochrome P450 enzymes tend to localize in the ER (Szczesna-Skorupa and Kemper, 1993), reactive metabolites can be formed in the ER and adduct to proteins, inducing the UPR.

Unfolded proteins take on a conformation of a higher free energy than that of their native conformations. A variety of chaperones can sense this increased free surface energy as hydrophobic residues are exposed to water. To maintain a balance with the folding capacity of the ER, the UPR employs two strategies: first, to increase folding capacity by inducing ER-resident molecule chaperones and foldases and by increasing the size of the ER, and second, to decrease the misfolded protein load by downregulating protein synthesis and by increasing clearance of misfolded protein by upregulating ER-associated degradation (Schröder and Kaufman, 2005).

Ultimately, if the unfolded protein burden remains too great, the UPR response can result in apoptosis. It has also been shown that chronic ER stress can lead to inflammation. For example, ER stress has been shown to induce nuclear factor of the κ light chain enhancer of B cells (NF-κB) activation (Deng et al., 2004), NLR family pyrin domain containing 3 (NLRP3) inflammasome activation (Menu et al., 2012), and DAMP secretion either freely (Andersohn et al., 2019) or packaged in extracellular vesicles (Collett et al., 2018).

Unfolded protein is not the only possible trigger of ER stress, although it is the most well studied. Aberrations in lipid homeostasis may also induce ER stress (Song and Malhi, 2019). Although, compared with proteins, changes to lipids have not been studied as extensively in the context of IDRs, this may be an interesting avenue to explore; for example, lipid-smooth ER inclusions were found in hepatocytes of brown Norway rats administered nevirapine (Sastry et al., 2018), which is also known to induce smooth ER hypertrophy (Sharma et al., 2012).

The absolute number of proteins modified by covalent binding of a drug is quite small (Evans et al., 2004), and compared with other sources of unfolded protein, drug modification of protein may not induce sufficient protein unfolding to trigger activation of the UPR. Additionally, a transcriptomic study of primary human hepatocytes predicted a suppression, rather than induction, of pathways related to the UPR (Terelius et al., 2016).

6. Mitochondrial Toxicity. Mitochondrial toxicity has been identified as an adverse effect of many medications. Its role in IDRs in particular, however, has been a matter of debate (Boelsterli and Lim, 2007; Cho and Uetrecht, 2017). The mechanisms underlying mitochondrial toxicity include inhibition of the electron transport chain, interference with mitochondrial transcription and translation, inhibition or uncoupling of ATP synthase, inhibition of enzymes in the citric acid cycle or mitochondrial transporters, and increased reactive oxygen species (ROS) production (Vuda and Kamath, 2016; Will et al., 2019). As these mechanisms have been extensively covered elsewhere, we will focus on how mitochondrial toxicity may result in inflammation.

Increased ROS production causes activation of redox-sensitive transcription factors such as NF-κB. It has also been shown that autophagy negatively regulates NLRP3 inflammasome activation, whereas increased ROS positively regulates NLRP3 inflammasome activation and inflammation, at least in part due to cytosolic localization of oxidized mitochondrial DNA (Nakahira et al., 2011; Zhou et al., 2011; Shimada et al., 2012). In addition to mitochondrial DNA, other mitochondrial-derived molecules can function as DAMPs, such as ATP, mitochondrial transcription factor A, N-formyl peptide, succinate, cardiolipin, and cytochrome-c (Nakahira et al., 2015; Grazioli and Pugin, 2018). Cytochrome-c release into the cytosol can induce apoptosis via inducing oligomerization of apoptosis-protease activating factor 1 and initiating caspase activation. Depending upon the context and the cleavage products of the caspases involved, this may result in apoptosis, but it may also result in cell differentiation and proliferation (Garrido et al., 2006). Thus, the causes and outcomes of mitochondrial toxicity are varied and complex.

In general, there is little direct evidence that drugs that cause IDRs do so by inducing mitochondrial damage. An exception is valproic acid–induced liver injury, however, which has been associated with variants in mitochondrial DNA polymerase γ (Stewart

et al., 2010) and which may present with steatosis and lactic acidosis (Chaudhry et al., 2013; Farinelli et al., 2015; Pham et al., 2015). Acetaminophen also causes mitochondrial damage, but it does not cause IDRs (Jaeschke et al., 2019).

C. Mediators of Inflammation

Depending on the location and severity of the tissue injury, a variety of factors may be involved in the initiation and propagation of a sterile inflammatory response, including DAMPs, cytokines, and chemoattractants. The transcriptional regulation of many of these proinflammatory molecules by NF-κB is also an important consideration. Moreover, other body systems such as the microbiome and the nervous system have the potential to contribution to inflammation.

1. Damage-Associated Molecular Patterns. As discussed above, the injury or death of a cell may result in the release of intracellular contents. Once outside of their normal subcellular location, these components are referred to as danger signals or DAMPs, at which point they can initiate an inflammatory response (Medzhitov, 2008). DAMPs can be classified based on their normal location in or on the cell and include stimuli such as nuclear and mitochondrial DNA, RNA, ATP, S100, heat shock proteins, HMGB1, and extracellular matrix fragments (Chen and Nuñez, 2010; Zindel and Kubes, 2020). The detection of DAMPs then leads to effector cell recruitment and propagation of the sterile inflammatory response.

In the context of efferocytosis, DAMPs such as ATP, UTP, lysophosphatidylcholine, and sphingosine-1-phosphate, in addition to adhesion molecules and receptors such as intracellular adhesion molecule 3 and CX3C chemokine receptor, can act as chemotactic find-me signals, and concurrent with surface expression of eat-me signals such as phosphatidylserine, contribute to the removal of apoptotic cells by phagocytes (Westman et al., 2020).

The concept of danger signals in the initiation of IDRs has been proposed several times (Pirmohamed et al., 2002; Li and Uetrecht, 2010; Hassan and Fontana, 2019; Uetrecht, 2019b). Although reactive metabolites of drugs associated with IDRs can covalently bind to cellular proteins that may, in turn, cause cell damage or cell death, the release of DAMPs can also occur in response to a wide array of insults, such as UV irradiation, hemorrhagic shock, starvation, and other forms of injury or trauma (Schaefer, 2014). Since DAMPs are simply a mechanism by which the immune system is alerted that there is tissue injury, they are not idiosyncratic in their release. Therefore, it is possible that a similar pattern of DAMPs may be released after treatment with a drug associated with IDRs. This pattern of DAMPs could function as potential biomarkers during preclinical development by indicating that a drug candidate may carry the risk of causing

IDRs; however, it is likely too nonspecific to be useful for clinical diagnosis of the early onset of an IDR. Thus, characterization of the specific DAMPs released after treatment with different drugs associated with IDRs is certainly an avenue for future research, as it may provide insight into the specific type and target of cell injury or death that is stimulating the observed innate immune response.

2. Cytokines, Chemokines, and Acute Phase Proteins. A wide range of classic soluble mediators have already been highlighted for various functions in innate immunity. To reiterate, cytokines such as TNF-α, IL-1β, IL-4, IL-6, and IL-18; chemokines such as CCL2, CCL5, CXCL1, CXCL2, and CXCL8; and acute phase proteins such as C-reactive protein, serum amyloid A, and serum amyloid P contribute to effector cell recruitment and activation and the propagation of the inflammatory response. These mediators are released in coordinated spatial and temporal responses, the patterns of which can influence the types and absolute numbers of innate leukocytes that are recruited to the site of injury. Not yet mentioned are the type I (i.e., α and β) and type II (γ) interferons, which act to potentiate proinflammatory signaling via enhanced cytokine production and antigen presentation, macrophage priming, and natural killer cell function, among numerous other effects (Kopitar-Jerala, 2017). Although additional proinflammatory molecules are elaborated on elsewhere (Turner et al., 2014; Akdis et al., 2016; Kapurniotu et al., 2019), the role of IL-1 family cytokines is worth emphasizing because of their fundamental importance in innate immunity.

a. Interleukin-1 cytokines and their activation. The IL-1 family of cytokines consists of 11 soluble mediators, including proinflammatory IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ, as well as several receptor antagonists and the anti-inflammatory cytokine IL-37 (Dinarello, 2018). IL-1 cytokines are first translated into inactive precursors (except for IL-1α), which then attain functional maturity after enzymatic cleavage in a process mediated predominantly by caspase-1 and the inflammasome (Mantovani et al., 2019). Fundamentally, the inflammasome is a multimeric protein complex that, when activated, leads to the maturation of caspase-1, the cleavage and release of mature IL-1 cytokines, and the induction of additional inflammatory effector mechanisms (Walsh et al., 2014). Several cytoplasmic pattern recognition receptors can assemble as independent inflammasomes, each responding to specific DAMPs or other stimuli. These pattern recognition receptors (PRRs) are expressed in multiple cells, including neutrophils, monocytes, macrophages, and dendritic cells, and play an important role in innate immunity (Sharma and Kanneganti, 2016).

The NLRP3 inflammasome may be the most relevant for the study of drug-induced immune activation, as it is activated by the widest array of stimulants,

although several other inflammasomes may be involved (Schroder and Tschopp, 2010; Latz et al., 2013). Several drugs associated with serious IDRs have also been demonstrated to activate inflammasomes and increase IL-1β release in vitro (Kato and Uetrecht, 2017; Mak et al., 2018; Kato et al., 2019, 2020). Additionally, it is known that animals deficient in components of the inflammasome are resistant to contact hypersensitivity (Watanabe et al., 2007), a reaction that may parallel events in the early immune response to drugs that cause IDRs. Whether inflammasome activation occurs in humans treated with these drugs has yet to be clearly demonstrated.

3. Bioactive Lipids. In addition to inflammatory protein mediators, several classes of bioactive lipids exist and play various roles in inflammation, immunoregulation, and maintenance of tissue homeostasis (Chiurchiù and Maccarrone, 2016). The main types of proinflammatory lipids include classic eicosanoids (Dennis and Norris, 2015), certain endocannabinoids (Chiurchiù et al., 2015), lysoglycerophospholipids, and sphingolipids (El Alwani et al., 2006), and specialized proresolving lipid mediators are a relatively new class of lipids that terminate acute inflammation and drive resolution and tissue repair (Serhan, 2014). These molecules are all generated from ω-6 or ω-3 essential polyunsaturated fatty acids precursors (e.g., arachidonic acid) but demonstrate significant heterogeneity in structure and function after maturation (Das, 2018).

Classic eicosanoids (e.g., certain prostaglandins, prostacyclins, thromboxanes, leukotrienes, hydroxyeicosatetraenoids, and lipoxins) are typically considered highly proinflammatory mediators that are usually produced by innate cells such as neutrophils and monocytes within the first several hours of an inflammatory stimulus. Specifically, leukotrienes can act as chemoattractants for neutrophils, macrophages, and eosinophils (De Caterina and Zampolli, 2004), and prostaglandins can function to enhance proinflammatory cytokine gene transcription and release and can also amplify the innate response to DAMPs (Hirata and Narumiya, 2012). However, some eicosanoids can be immunosuppressive and promote immune tolerance in certain contexts (Obermajer et al., 2012; Wang et al., 2014). The endocannabinoids, such as 2-arachidonoylglycerol, are ubiquitously expressed molecules that have diverse immunomodulatory effects on monocytes/macrophages, dendritic cells, and granulocytes, and unsurprisingly, perturbations in endocannabinoid homeostasis have been shown to contribute to neuroinflammatory and autoimmune diseases (Chiurchiù et al., 2018). Lysoglycerophospholipids (e.g., lysophosphatidylcholine and lysophosphatidylinositol) and sphingolipids (e.g., ceramide 1-phosphate and sphingosine 1-phosphate) are key signaling molecules controlling inflammatory cascades, trafficking and activation of immune cells, cell survival, and apoptosis (Sevastou et al., 2013; Gomez-Muñoz et al., 2016).

NSAIDs are one class of drugs for which the potential role of bioactive lipids in the innate immune response is particularly relevant. Although NSAIDs are the most frequently used medications for the management of pain and inflammation, they are also associated with some of the highest incidence rates of drug hypersensitivity reactions (Conaghan, 2012). Reported reactions include urticaria and other cutaneous reactions, acute interstitial nephritis, and hepatotoxicity (Nast, 2017; Yamashita et al., 2017; Wöhrl, 2018). Mechanistically, NSAIDs inhibit the enzymes cyclooxygenase-1 and -2, blocking the synthesis of inflammatory prostanoids such as prostaglandin E2. It has even been postulated that an innate immune response contributes to the onset of NSAID-mediated adaptive IDRs, potentially through the activation of eosinophil and mast cell degranulation or through the shunting of arachidonic acid precursors to the production of other proinflammatory lipid mediators such as leukotrienes (Doña et al., 2020). Based on the fundamental roles of bioactive lipids in the initiation and propagation of an inflammatory response, future research to delineate key mediators in the early immune response to drugs that are associated with IDRs is necessary.

4. Pattern Recognition Receptors. As part of the innate immune system, pattern recognition receptors have evolved to recognize conserved molecular patterns of danger or invading pathogens. Thus, PRRs represent a key aspect of the innate immune system that is not likely to be idiosyncratic, as they are conserved, germline-encoded receptors that are not antigen-specific and respond to a structurally diverse range of molecules (Gong et al., 2020), in contrast to an individual's randomly generated TCR repertoire. PRRs include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLR), retinoic acid–inducible gene-1–like receptors, C-type lectin receptors, receptor for advanced glycation end products, and scavenger receptors (Gordon, 2002; Xie et al., 2008; Palm and Medzhitov, 2009; Takeuchi and Akira, 2010). Notably, DAMPs are largely recognized by PRRs. For example, HMGB1 can signal through TLR4 or receptor for advanced glycation end products (Lu et al., 2013). TLR signaling can result in NF-κB signaling (discussed below) and ultimately the production of proinflammatory cytokines (Vidya et al., 2018). PRR signaling may also result in cell death (Amarante-Mendes et al., 2018). If signaling through PRRs was directly responsible for the onset of IDRs, however, it is likely that these severe reactions would be observed in most, if not all, patients given a particular drug because of the conserved nature of these receptors, but this is not what is observed clinically. Thus, although likely a necessary first step for the development of an IDR, pattern recognition is not itself sufficient to cause an IDR. Again, we

emphasize that this innate aspect of the immune response should occur in most patients taking drugs that are associated with IDRs if they cause cellular damage and is not idiosyncratic, but other downstream pathways contribute to the development of IDRs themselves.

5. Transcriptional Regulation of Inflammation. Several families of transcription factors are activated in response to inflammatory stimuli, such as signal transducers and activators of transcription, interferon regulatory factors, and most notably, NF-κB (Smale and Natoli, 2014; Irazoqui, 2020). The NF-κB family consists of several inducible transcription factors—NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel—that bind to κB enhancer DNA elements as dimers to modulate gene transcription (Liu et al., 2017). Activation can occur via the canonical pathway in response to ligand binding of various cytokine receptors, PRRs, and TNF receptors, or via the noncanonical pathway in response to ligand binding of a specific subset of TNF receptors (Cildir et al., 2016; Sun, 2017).

NF-κB signaling results in the upregulation of target genes related to cell adhesion, survival and proliferation, dendritic cell maturation, neutrophil recruitment, M1 macrophage polarization, and other inflammatory mediators that function to amplify the detected inflammatory response (Lambrou et al., 2020). Key proinflammatory cytokines and chemokines under NF-κB regulation include IL-6, IL-8, TNF-α, CCL2, CCL5, CXCL1, and CXCL2 (Liu et al., 2017). Moreover, activation of NF-κB is necessary for signal 1 (priming) in inflammasome activation, as transcription of inflammasome-related components such as pro-IL-1β, pro-IL-18, and NLRP3 is upregulated after NF-κB activation (Latz et al., 2013). If drugs associated with IDRs cause an inflammatory response that is characterized by elevated levels of NF-κB–regulated mediators, then this provides a starting point to determine which canonical or noncanonical receptors are activated after drug administration and may provide clues as to the types of cell damage or neoantigens formed (i.e., potential receptor ligands) with that drug.

6. Other Contributing Factors. In addition to the multifarious range of activation signals presented thus far, multiple junctures of interaction have been identified between the innate immune system and both the microbiome and the nervous system; however, these will only be introduced briefly.

a. Interaction with the microbiome. Although most commonly associated with the gut, the human microbiome refers to the collection of genes of all microorganisms (e.g., archaea, bacteria, fungi, protists, viruses) that reside on or in all bodily tissues and fluids, including the biliary tract, respiratory tract, and skin (Marchesi and Ravel, 2015). To maintain a commensal relationship and prevent the initiation of an inappropriate immune response, extensive crosstalk between the microbiota and immune cells, particularly ILCs (Thaiss et al., 2016; Negi et al., 2019), must occur. For instance, it has been shown that germ-free mice have a significantly altered innate immune system, with attenuated myeloid cell development in the bone marrow (Khosravi et al., 2014). Although this is an extreme example that would not be particularly relevant to humans, it does highlight the potentially profound impact of the microbiome on innate immunity.

Commensals are necessary to educate the immune system and often promote tolerance (Grice and Segre, 2011), but how these microorganism interactions may shape the metabolism of and subsequent inflammatory response to drugs that cause IDRs has yet to be adequately investigated (Marchesi and Ravel, 2015). One notable exception, however, is immune checkpoint inhibitor–induced colitis. A recent investigation demonstrated that ipilimumab altered microbiome composition and the subsequent risk of colitis (Dubin et al., 2016). Countless drugs can target components of the microbiome, the most obvious being the antibiotics; therefore, understanding the reverse of this relationship will likely provide novel insights into patient-specific risk factors for IDRs.

b. Communication with the nervous system. An important function of the nervous system is to interact with immune cells. Unsurprisingly, innate immune cells, including neutrophils, macrophages, and dendritic cells, express receptors for several neurotransmitters (e.g., α- and β-adrenergic and acetylcholinergic receptors), and neurons can express various pattern recognition and cytokine receptors, facilitating effective crosstalk between the systems (Chavan et al., 2017). Additionally, at peripheral sites of inflammation and tissue injury, both afferent and efferent neural circuits have been shown to have immunoregulatory functions (Pavlov and Tracey, 2015). As many drugs associated with IDRs have therapeutic effects in the CNS, including a multitude of anticonvulsant and antischizophrenic agents, it is necessary to consider how these psychotropics may influence the neuroimmune axis and the consequential immune activation.

D. Antigen Reception/Uptake by Antigen-Presenting Cells

There are multiple means by which APCs may obtain peptides or proteins and present them (Avalos and Ploegh, 2014; Roche and Furuta, 2015; Allen et al., 2016; Lindenbergh and Stoorvogel, 2018; Li and Hu, 2019). Antigen presented by APCs is most often thought to originate from within the cell itself or to be received via uptake from the extracellular environment by processes such as phagocytosis (Roche and Furuta, 2015; Kotsias et al., 2019). Based on the numerous HLA associations that have been identified as risk factors for different drugs and reactions (Usui and Naisbitt,

2017; Chen et al., 2018), this step is likely to be important in the pathogenesis of IDRs.

1. Presentation by Major Histocompatibility Complex I: Endogenous Protein, Crosspresentation. MHC I molecules are expressed by all nucleated cells, which allows for CD8^{+} T cells to survey host tissue for aberrations suggestive of intracellular pathogens or malignancy (Jongsma et al., 2019). Peptides originating from within the cell are usually presented in the context of MHC I (Neefjes et al., 2011). In what is considered the conventional processing route, proteins are digested by the proteasome to generate shorter peptide fragments, which are then translocated to the ER via the transporter associated with antigen processing for loading onto MHC I molecules after assembly of the peptide-loading complex (Vyas et al., 2008).

In some cases, proteins exogenous to the cell may be presented by MHC I, particularly in the case of DCs; this process is termed crosspresentation (Li and Hu, 2019). Peptide loading is described as following either the cytosolic pathway or the vacuolar pathway. The cytosolic pathway appears to require the proteasome for peptide processing, and peptide loading may occur in phagosomes or endosomes, whereas the vacuolar pathway is lysosome-dependent and both peptide processing and loading may occur in lysosomes (Embgenbroich and Burgdorf, 2018).

2. Presentation by Major Histocompatibility Complex II: Phagocytosis, Endocytosis, Macropinocytosis, Autophagy. Constitutive expression of MHC II is largely restricted to professional APCs, although myeloid cells, including eosinophils, neutrophils, and basophils, as well as ILCs, have been demonstrated to upregulate expression of MHC II in certain conditions (Kambayashi and Laufer, 2014). Peptides originating from exogenous sources are most often presented in the context of MHC II; however, endogenous peptides may also follow this pathway via autophagy (Duffy et al., 2017). Exogenous proteins may be acquired via different methods (Roche and Furuta, 2015). Phagocytosis is the internalization of pathogens or particulate antigens and is considered to be the most important mechanism of antigen uptake in vivo (Stuart and Ezekowitz, 2005). This process can be enhanced by opsonins, which are host proteins such as antibodies or complement that can coat foreign entities. Clathrin-mediated endocytosis is the internalizing of ligands complexed to surface receptors and soluble macromolecules (Mantegazza et al., 2013). Macropinocytosis is a nonspecific process during which uptake of extracellular fluid containing soluble antigens and macromolecules occurs (Liu and Roche, 2015). Proteins are then processed via the endocytic pathway to peptides in specialized late endosomes that are enriched with MHC II molecules for antigen presentation (Neefjes et al., 2011).

3. Crossdressing: Trogocytosis, Extracellular Vesicles, Nanotubes. In some cases, preformed MHC-peptide complexes may be transferred from the surface of a donor cell to a recipient cell; the process is referred to as crossdressing (Campana et al., 2015). Multiple mechanisms have been proposed to describe the transfer of these complexes. Trogocytosis refers to the phenomenon in which patches of the plasma membrane are rapidly transferred from one live cell to another upon cell-cell contact. In some cases, phagocytosis may not be possible if the target cell is too large; the phagocyte may instead ingest smaller pieces of the cell by "nibbling" at the membrane and potentially the cytoplasm (Dance, 2019).

Extracellular vesicles refer to either microvesicles, formed by plasma membrane budding, or exosomes, formed as intraluminal vesicles within endosomal multivesicular bodies and then released by fusion of the multivesicular body with the plasma membrane. Because these two types of vesicles are indistinguishable after their release, they are collectively termed extracellular vesicles (Groot Kormelink et al., 2018). In the context of IDRs, extracellular vesicles have been shown to be involved in the transport of drug-modified antigen to target cells, such as in the case of amoxicillin (Sánchez-Gómez et al., 2017; Ogese et al., 2019). Extracellular vesicles may also transfer proteins that can be processed by the recipient cell and presented by MHC molecules, or they may transfer the MHC-peptide complexes themselves. Additionally, extracellular vesicles derived from multiple cell types including B cells (Raposo et al., 1996) and DCs (Théry et al., 2002) have been shown to activate T cells themselves. Conversely, hepatocyte-derived exosomes have also been implicated in the promotion of immune tolerance in the liver, and dysregulation of this tolerogenic mechanism may be an important step in the onset of IDILI (Holman et al., 2019).

Tunneling nanotubes are intercellular structures that have been shown to mediate the exchange of MHC I molecules (Schiller et al., 2013). Such an exchange may be another means by which crossdressing can occur.

These varied methods of antigen acquisition, processing, and presentation describe different means by which antigen may be presented to the adaptive immune system. Understanding how antigen may reach both APCs and target T cells will aid in the understanding of the pathogenesis of IDRs.

E. Naïve Lymphocyte Activation by Antigen-Presenting Cells

Naïve T cells and B cells are activated by APCs once they receive sufficient activation signals (Mak et al., 2014). Classically, the three-signal model is used to describe this sequence of events: signal one refers to the binding involving the MHC molecule presenting the antigenic peptide of interest; signal two refers to costimulatory molecule engagement, which has been upregulated as a result of exposure to inflammatory

conditions; and signal three refers to the cytokine help that permits the lymphocyte to survive and proliferate. This model describes the activation process for helper T-cell, cytotoxic T-cell, and B-cell activation, although there are some differences between the cell types.

1. Helper T Cells. Only mature DCs can activate naïve T cells. For T$_h$ cell activation, the first signal between these cells is the binding of cognate TCRs to MHC II-peptide complexes on the DC; a strong interaction over several hours results in the signaling cascades that induce cell polarization and forms the immunologic synapse. T-cell receptor engagement induces NF-κB signaling (Liu et al., 2017). This also induces CD40L and CD28 expression on the T-cell surface; CD40 engagement on the DC surface by CD40L upregulates expression of B7 molecules by the DC.

In most cases, costimulatory molecule engagement is required for T-cell activation, although in some cases, the MHC-peptide complex may deliver a strong enough signal to bypass the need for signal two (Wang et al., 2000). The B7 molecules on the DC surface interact with CD28 on the T-cell surface. This permits upregulation of cytokine receptors and induces CD4$^+$ T-cell production of proinflammatory cytokines such as IL-2 and IFN-γ.

Signal three is delivered by APCs in the form of cytokine release. For CD4$^+$ T cells, these cytokines include IL-1, TNF-α, and IL-6 (Pape et al., 1997; Joseph et al., 1998; Curtsinger et al., 1999; Ben-Sasson et al., 2009). This results in activation, proliferation, and differentiation to T$_h$ effector cells as well as licensed DCs. Licensed DCs may then proceed to activate naïve T$_c$ cells.

2. Cytotoxic T Cells. Signal one is delivered to the T$_c$ cell by engagement of MHC I on a licensed DC, the product of T$_h$ cell activation, to the T-cell receptor (Joffre et al., 2009). Signal two, or costimulation of T$_c$ cells, is more dependent upon CD28 engagement, as B7 is already upregulated on the DC surface (Curtsinger et al., 2003). Finally, in signal three, the naïve T$_c$ receives cytokine help, such as IL-12, from activated T$_h$ cells and APCs, thus allowing for proliferation and differentiation to precytotoxic lymphocytes (Curtsinger et al., 2003; Curtsinger and Mescher, 2010). These precytotoxic lymphocytes may then leave the lymph node and migrate to the site of inflammation, where signals such as IL-12, IFN-γ, and IL-6 induce differentiation to armed cytotoxic lymphocytes (Mescher et al., 2007). Protein synthesis for the contents of the cytotoxic granules is induced. Finally, engagement of the T-cell receptor by antigen presented on MHC I within the tissue induces targeted cell destruction by the cytotoxic T$_c$ cell (Groscurth and Filgueira, 1998).

3. B Cells. Some antigens are considered to be T-independent in that the antigens themselves can stimulate the B cell to proliferate without T-cell help (Mond et al., 1995). Most antigens, however, are T-dependent and require the same three signals for B-cell activation (MacLennan et al., 1997).

Multiple antigens are required to bind to the B cell receptors on a single cell, termed the B-cell microcluster (Wan and Liu, 2012). This allows for the intracellular signaling cascades that prepare the B cell to receive T-cell help. An important distinction from the T-cell activation process is that the B cell can recognize whole antigen (Li et al., 2019).

Signal two is provided to B cells by activated T$_h$ cells: costimulatory signals are delivered by the T$_h$ cell, primarily by the interaction of CD40L, and the receptor, CD40, which is constitutively expressed on the B-cell surface (Banchereau et al., 1994). This induces the B cell to internalize the antigen engaged by its B-cell receptors, process the peptides, and present the peptides to the T cell. MHC II on the B cell is engaged by the T-cell receptor, which means that both the B and T$_h$ cells must recognize the same antigen, although not necessarily the same epitopes. This is known as linked recognition (Smith, 2012).

Finally, cytokines are also required as signal three for B-cell proliferation (Zubler and Kanagawa, 1982). The T$_h$ cell in contact with the B cell is usually the source of these cytokines. IL-4 is critical to induce the primed B cell to proliferate, while other cytokines support this process (Takatsu, 1997).

F. Fate of the Adaptive Immune Response

After the formation of the immunologic synapse, there are several potential outcomes with respect to the adaptive immune response that are dependent upon the strength of the signals received. At a fundamental level, the result of synapse formation may be 1) no adaptive immune activation (if signals are below the threshold of activation), 2) the promotion of tolerance via anergy or clonal deletion (if there is an engagement of coinhibitory molecules), or 3) the initiation of an adaptive immune response, resulting in T- and/or B-cell activation and effector cell maturation (after the successful formation of an immunologic synapse, complete with the engagement of the MHC-TCR complex and costimulatory receptors) (Finetti and Baldari, 2018). This spectrum of potential consequences likely explains why some individuals develop IDRs, whereas some develop mild reactions that resolve, and others may have no such adverse effects. Even if an individual has drug-modified proteins that have caused cell stress and have stimulated an innate immune response, it is unlikely that they will have the specific MHC molecule to present and/or the specific TCR clone to recognize the neoantigens in the correct conformation, or the interaction may not be strong enough to stimulate T-cell activation and expansion. There are likely other contributing factors to the idiosyncrasy of adaptive immune activation that
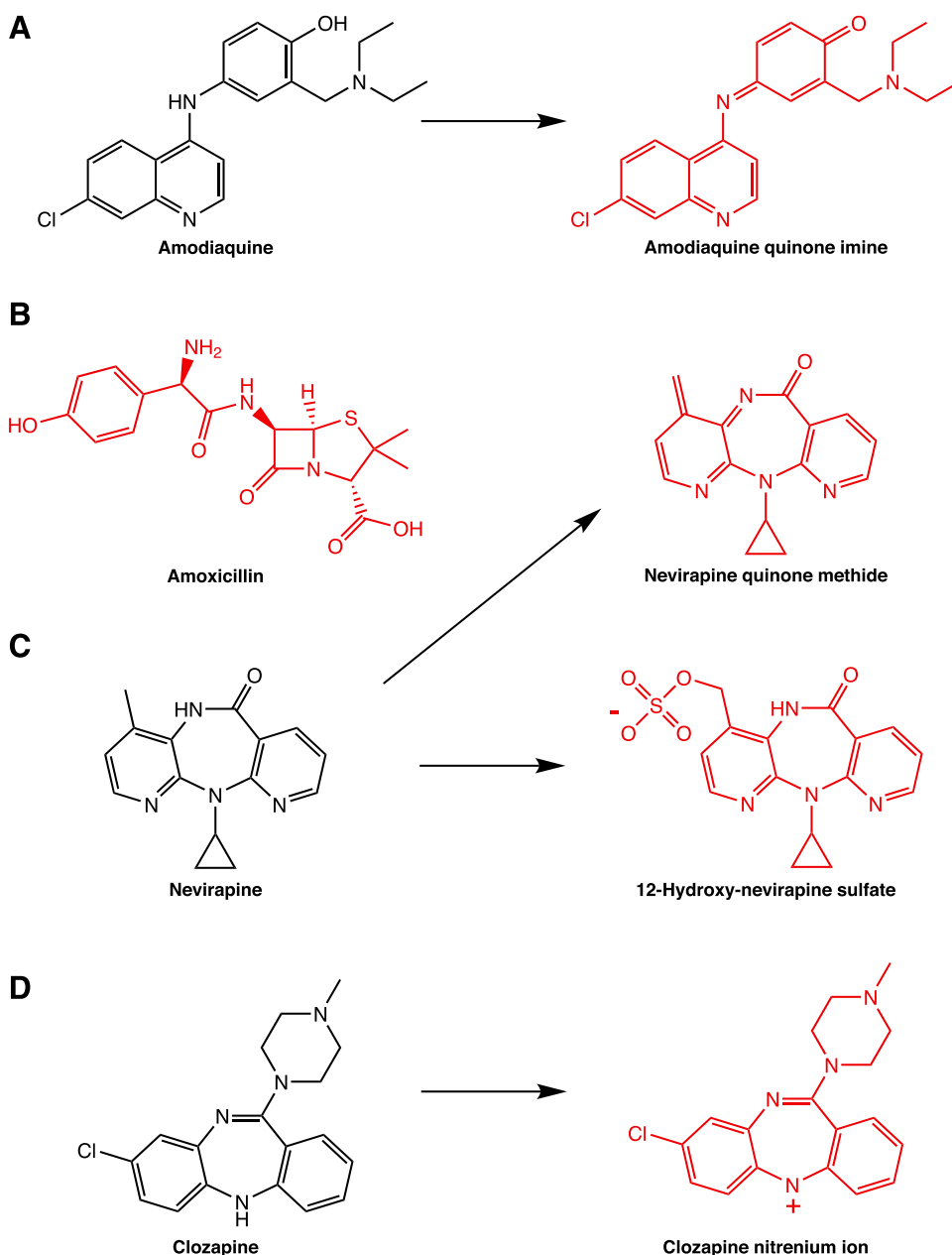
Fig. 2. Chemical structures of the drugs associated with idiosyncratic drug reactions that are discussed in this review: amodiaquine, amoxicillin, nevirapine, and clozapine. Reactive species of each drug that have been demonstrated or proposed to contribute to the onset of the associated idiosyncratic reactions are shown in red: (A) amodiaquine can form a quinone imine, (B) amoxicillin is itself a reactive β-lactam, (C) nevirapine can form both a 12-hydroxy species and a quinone methide, and (D) clozapine can form a nitrenium ion.

have yet to be characterized; thus, severe IDRs remain difficult to predict.

IV. Support for Immune Activation Using Model Drugs

Hundreds of drugs have been reported to cause various severe IDRs (Mockenhaupt et al., 2008; Andrès et al., 2009, 2019; Chalasani et al., 2014; Hussaini and Farrington, 2014; Björnsson, 2016; Al Qahtani, 2018; Behera et al., 2018; De et al., 2018; Eddy, 2020; Solhjoo et al., 2020). Although different drugs are associated with different IDRs, and many drugs can

cause more than one type of IDR, this section will summarize the available clinical and animal model literature demonstrating early immune involvement using four archetypal IDR-associated drugs: amodiaquine, amoxicillin, clozapine, and nevirapine (Fig. 2). Together, these IDR-associated drugs provide a representation of the majority of target organs, encompassing liver, skin, and blood reactions.

Using an extensive combination of keywords related to the innate immune response, many of which were presented in *Section III. Innate Mechanisms Contributing to Adaptive Immune Activation*, we searched the available literature for each drug of interest. Reviewed

studies were only included if the focus of the research was on early responses to drug treatment and not on the study of an IDR. In vitro studies were largely omitted to focus on the effect of drugs administered in vivo because of the complexity of the immune response, which is not adequately recapitulated using in vitro models. We also emphasize studies that focused on the healthy state, rather than disease or injury, to isolate the specific effects of the drug on the immune system.

Although the clinical manifestations of many IDRs have been well documented, research characterizing the mechanisms preceding these adaptive immune processes is limited, particularly for human data. Of course, such mechanistic studies are exceptionally difficult to undertake, as research in patients is usually limited to immune changes observed in blood samples; more detailed studies on organ effects cannot be performed. Additionally, the timing and duration of the innate immune response are likely to vary for different drugs, and the extensive patient monitoring required to capture such a response would be quite expensive, time-consuming, and generally impractical. Moreover, the characteristics of an early immune response can diverge greatly depending on the stimuli involved, and attempting to encapsulate all potential biomarkers of an innate response in clinical testing would be impossible. Therefore, in addition to data from patients, relevant studies investigating immune-related changes in experimental animal models are also discussed (refer to Supplemental Data for more detailed discussion of the individual studies). Although there are evident species differences and the immune response observed in animals may not be identical to that experienced by patients in the early weeks of drug treatment, such studies can provide important mechanistic insight into the general cells, pathways, and inflammatory mediators that may be involved in the immune response.

A. Amodiaquine

The 4-aminoquinolone amodiaquine was introduced as an alternative antimalarial medication to chloroquine. Although it is still in use in malaria-endemic areas, amodiaquine was withdrawn from most markets because of the occurrence of several serious IDRs, including agranulocytosis (Rouveix et al., 1989) and hepatotoxicity (Neftel et al., 1986).

Although the mechanisms of amodiaquine-induced IDRs are not completely understood, the bioactivation of amodiaquine in both the liver and immune cells has been extensively investigated, providing insights into the formation of neoantigens and potential immune activation. In the liver, amodiaquine is metabolized by CYP2C8 to N-desethylamodiaquine (Li et al., 2002). Both amodiaquine and N-desethylamodiaquine can be oxidized to a reactive quinone imine by cytochrome P450s in the liver and myeloperoxidase in neutrophils,

leading to significant levels of covalent binding (Maggs et al., 1987, 1988; Clarke et al., 1990; Tingle et al., 1995; Naisbitt et al., 1997, 1998; Lobach and Uetrecht, 2014b) (Fig. 2A). The sites of reactive metabolite formation, i.e., CYP450 enzymes in the liver and myeloperoxidase in neutrophils and their precursors, are consistent with the pattern of IDRs caused by amodiaquine, i.e., liver injury and agranulocytosis.

Moreover, amodiaquine has been found to activate inflammasomes in vitro in a human acute monocytic leukemia cell line (THP-1 cells), with or without prior bioactivation of the drug by human hepatocarcinoma functional liver cell-4 cells (Kato and Uetrecht, 2017). In an impaired immune tolerance model, treatment of female programmed cell death protein 1 knockout (PD-1$^{-/-}$) mice with anti–cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibodies and amodiaquine caused marked liver injury similar to IDILI in humans that was mediated by T_c cells (Mak et al., 2017).

These data support the role of early antigen formation in the progression to serious hepatotoxicity induced by amodiaquine, and further support of innate immune involvement is discussed below. Notably, no clinical studies reviewed reported any relevant data on early immune responses to amodiaquine, and thus, this section only highlights data obtained from rodent studies. This is likely reflective of discontinued amodiaquine use in many countries, although early clinical monitoring in areas actively using amodiaquine may reveal patterns of immune activation that could be leveraged to reduce progression to severe IDRs.

1. Data from Rodent and Human Studies. Several groups have investigated the impact of amodiaquine on hepatic structure and function. In general, studies characterizing the effects of amodiaquine monotherapy in the absence of a pre-existing disease have consistently demonstrated elevated ALT levels in the first few weeks of treatment, which then resolves (Clarke et al., 1990; Shimizu et al., 2009; Mak and Uetrecht, 2015a, 2019; Metushi et al., 2015; Liu et al., 2016).

Glutathione-depletion studies using buthionine sulphoximine (BSO) have been performed to evaluate the effect of detoxification of amodiaquine. However, the dosing paradigms used differ significantly. In one case, BSO (700 mg/kg intraperitoneally) was administered 1 hour before amodiaquine (180 mg/kg orally), and liver injury was greatly exacerbated in 6–48 hours compared with amodiaquine treatment alone (Shimizu et al., 2009). In contrast, BSO (4.4 g/l in drinking water), administered 1 week before amodiaquine (~200 mg/kg per day in rodent meal), in addition to diethyl maleate (4 mmol/kg, intraperitoneally), administered 1 day before amodiaquine, prevented liver injury (Liu et al., 2016). It is important to note that the liver injury occurred acutely in the former model, which was likely due to the higher exposure of the mice to amodiaquine by bolus

administration. Acute toxicity represents a different type of liver injury compared with what is observed clinically with patients with IDILI, as these drugs do not cause acute toxicity in humans at therapeutic doses. However, this does not preclude the fact that these drugs may cause a clinically silent immune response in patients.

Few studies have sought to characterize changes in inflammatory mediators with amodiaquine treatment. Amodiaquine monotherapy in female mice and male rats caused significant increases in numerous proinflammatory cytokines and chemokines beginning after 1 week of treatment (Metushi et al., 2015; Liu et al., 2016). Interestingly, the addition of amodiaquine was reported to attenuate increases in some inflammatory cytokines in models of acute tissue injury such as hepatitis or intracerebral hemorrhage (Yokoyama et al., 2007; Kinoshita et al., 2019). This could be due to the induction of tolerogenic mechanisms by amodiaquine, which prevents a pathogenic response to amodiaquine. This illustrates the complexity of immune responses.

Although the patterns observed are organ-specific with respect to timing and specific cell types, studies that investigated the effect of amodiaquine treatment on immune cells have consistently reported a decrease in leukocytes in the first several days to weeks of treatment, followed by an increase around a month of treatment (Clarke et al., 1990; Ajani et al., 2008; Mak and Uetrecht, 2015a, 2019; Metushi et al., 2015; Liu et al., 2016). In studies that undertook phenotyping of specific populations, NK cells were demonstrated to be the most important effector cell in response to amodiaquine treatment, with increased populations observed in the lymph node, spleen, and liver beginning after 1 week of treatment (Metushi et al., 2015; Liu et al., 2016; Mak and Uetrecht, 2019).

Only two studies, both using male rats, explored alterations in cell death pathways in response to amodiaquine treatment. Both reported an increase in apoptotic-related processes, either in the seminiferous tubules after 2 weeks (Niu et al., 2016) or in the liver after 5 weeks (Liu et al., 2016). These data suggest that amodiaquine-induced cell death may play a role in the activation of the immune response that ultimately results in severe IDRs. Covalent binding has been detected in several organs beyond the liver, including the kidney, spleen, and gut (Metushi et al., 2015), and thus, similar cell death effects may also occur elsewhere. Additional work is necessary to characterize the mechanisms preceding the onset of apoptosis and whether this occurs in other organs and, if so, at what time points.

Taken together, amodiaquine has been consistently shown to induce mild liver injury in rodent models that resolves spontaneously with continued treatment, and it has been shown that NK cells are important in mediating this injury. Whether the apoptosis that has been observed is induced by covalent binding of the drug itself or by the subsequent release of DAMPs and recruitment of NK cells or other immune cells remains to be determined. However, it is quite clear that amodiaquine induces an immune response that is not idiosyncratic.

B. Amoxicillin

Amoxicillin is a β-lactam antibiotic often used in the treatment of multiple bacterial infections. It is sometimes administered in combination with clavulanic acid, a β-lactamase inhibitor, to prevent the development of microbial resistance. Both of these agents are intrinsically reactive because of the β-lactam ring (Fig. 2B). Amoxicillin on its own is associated with different hypersensitivity reactions. Hypersensitivity reactions to β-lactam antibiotics can be classified as immediate or delayed. Immediate hypersensitivity reactions are IgE-mediated, involving basophil activation, and occur within 1 hour of drug administration, whereas delayed hypersensitivity, occurring over 1 hour after administration, tends to be T cell–mediated (Blanca et al., 2009).

The combination of amoxicillin and clavulanate is also associated with cholestatic IDILI (de Abajo et al., 2004). As amoxicillin alone is not associated with a high incidence of cholestatic IDILI (https://www.ncbi.nlm.nih.gov/books/NBK547854/), this suggests that clavulanate is the causative drug; however, as clavulanate is not used alone, there are no direct data to support this.

Covalent binding of amoxicillin to protein has been identified in in vitro studies, and some studies have also identified amoxicillin-modified proteins in exosomes, which may represent a means of transporting antigen to the immune system, as the exosomes were shown to activate naïve T cells in vitro in an HLA-A*02:01–dependent manner (Ogese et al., 2017, 2019; Sánchez-Gómez et al., 2017). Additionally, binding of amoxicillin and clavulanate to serum protein was identified in patients (Ariza et al., 2012; Meng et al., 2016).

1. Data from Rodent and Human Studies. There are few published studies on the immunomodulatory effects of amoxicillin in uninfected subjects. A study in rats administered amoxicillin/clavulanate for 30 mg/kg per day intraperitoneally (clavulanate dose not specified) for 14 days showed some signs of liver cell death, indicated by increased serum ALT and increased caspase expression in the liver. This appeared to have been caused by oxidative stress, as evidenced by increased malondialdehyde levels, cytochrome-c release, and increased ATPase activity (Oyebode et al., 2019). White blood cell counts were also elevated. However, another study in mice reported a decrease in white blood cell counts only at a higher dose of 500 mg/kg per day by oral gavage (amoxicillin only) for 28 days (Lebrec et al.,

TABLE 1
An overview of the most commonly observed findings from rodent and human studies that investigated the effects of amodiaquine, amoxicillin, nevirapine, or clozapine on various innate immune parameters, excluding models of injury or disease

Refer to Supplemental Data for a comprehensive list of rodent and human studies (including models of injury or disease), including information on the dose, route of administration, study duration, and primary outcomes.

Effect	Amodiaquine	Amoxicillin	Nevirapine	Clozapine
Organ weight	Multiple organs ↓/—[a]	Spleen —[a]	Liver —/↑[a]	Liver, heart —/↑[a]
Liver	ALT ↑[a] Covalent binding[a]	ALT ↑[a]	ALT ↑[a] Inflammatory lesions[a] Covalent binding[a]	ALT ↑[a] Inflammatory lesions[a] Covalent binding[a]
Other organs	Covalent binding: kidney, spleen, gut[a]	Covalent binding: serum albumin[b]	n.d.	Decreased splenic white pulp[a] Ovarian, kidney damage[a] Cardiac inflammation [a,b]
Cell death or proliferation	Apoptosis ↑[a]	Apoptosis ↑[a]	Apoptosis ↑[a] Apoptosis ↓[b]	Apoptosis ↑[a] Proliferation ↓[a] AIF translocation —[a,b]
Immune cells	Leukocytes ↓ then ↑[a] NK cells ↑[a]	Leukocytes ↓/↑[a] Leukocytes —[b]	Leukocytes ↓/↑[a] Leukocytes ↑[b]	Neutrophils ↑[a,b] Eosinophils, neutrophils, monocytes ↑[b]
Inflammatory mediators	Many cytokines ↑[a]	n.d.	TNF-α ↑[a] IFN-γ —[a] Mixed effect on cytokines ± HIV infection[b]	CXCL2, TNF-α ↑[a] Soluble TNFR, soluble CD8, soluble IL-2R, TNF-α ↑[b] Arachidonic acid signaling ↔[a] IL-6 ↑[a,b]
Signal transduction	n.d.	n.d.	n.d.	NF-κB ↑/—[a] AMPK-ULK1-Beclin1↑[a] PERK/eIF2α ER stress ↑[a]
Mitochondria and oxidative stress	n.d.	Cytochrome-c ↑[a] Malondialdehyde ↑[a]	Malondialdehyde ↑[a] Mitochondrial dysfunction ↑[a] Mitochondrial dysfunction ↑/—[b]	Malondialdehyde ↑[a] Mitochondrial dysfunction ↑[a]

↑, increase; —, no change; ↓, decrease; AIF, apoptosis-inducing factor; AMPK-ULK1-Beclin1, AMP-activated protein kinase-Unc-51-like kinase 1-Beclin1; IL-2R, IL-2 receptor; n.d., no data; PERK/eIF2α, protein kinase R–like ER kinase/eukaryotic translation initiation factor 2A; TNFR, TNF receptor.
[a]Rodent study
[b]Human study

1994). Decreased thymus cellularity was also noted but was only significant at a dose of 100 mg/kg.

As mentioned, in humans, amoxicillin (Ariza et al., 2012) and clavulanate (Meng et al., 2016) have been identified covalently bound to serum albumin. However, treatment with oral amoxicillin (1 g)/clavulanate potassium (125 mg) twice daily for 5 days resulted in no change in cell counts of multiple leukocytes, intracellular TNF-α concentrations in monocytes, and intracellular TNF-α and IFN-γ concentrations in NK cells or CD8$^+$ T cells stimulated ex vivo (Dufour et al., 2005).

Overall, these findings are perhaps not very surprising, as amoxicillin is used very frequently and is generally considered quite safe. The safety profile of amoxicillin illustrates the fact that covalent binding on its own is insufficient to cause IDRs. Liver toxicity was observed in the study of rats administered amoxicillin/clavulanate at 2 weeks, but such parameters were not measured in the clinical study. Although few innate parameters were measured in the clinical study, the general lack of changes reported suggests that there may not be a detectable systemic inflammatory response to amoxicillin and, possibly, that immune changes are localized to the liver.

C. Nevirapine

Nevirapine is a non-nucleoside reverse transcriptase inhibitor used in the treatment of HIV infections. Nevirapine is associated with skin reactions and IDILI (Popovic et al., 2010). It is noteworthy that, in some cases, such as perinatal transmission prophylaxis, it can be used as monotherapy; in others, it is administered in combination with other highly active antiretroviral therapy medications to avoid the development of resistance (Bardsley-Elliot and Perry, 2000). Thus, the effects of nevirapine treatment on immune parameters in clinical studies can be difficult to disentangle from the effect of other drugs when used in combination or from the background of HIV infection and subsequent effects of treatment efficacy (i.e., recovery of CD4$^+$ T-cell counts).

As already mentioned, 12-hydroxynevirpine sulfate was found to covalently bind in the skin of female brown Norway rats and was determined to be responsible for the observed skin rash because the application of a topical sulfotransferase inhibitor prevented the development of the rash (Sharma et al., 2013). However, although this metabolite is responsible for skin rash, a quinone methide formed by cytochrome P450 is the major metabolite responsible for covalent binding in the liver (Sharma et al., 2012) (Fig. 2C).

In patients taking nevirapine, protein-nevirapine adducts have been detected in blood samples. A 12-hydroxynevirapine sulfate-His146 adduct was detected on human serum albumin from patients taking nevirapine, which was replicated in vitro by treatment of human serum albumin with 12-hydroxynevirapine sulfate (Meng et al., 2013). Nevirapine-derived adducts to the N-terminal valine of hemoglobin were also detected in patient samples (Caixas et al., 2012).

1. Data from Rodent and Human Studies. Generally, nevirapine administration caused an increase in serum ALT levels in male rats and mice (Adaramoye et al., 2012; Sharma et al., 2012; Awodele et al., 2015), but not in female brown Norway rats (Bekker et al., 2012; Brown et al., 2016), although this observation is likely due to the shorter duration of the latter studies (7 or 14 days). In a clinical study, nevirapine exposure was associated with reduced fibrosis, although again it is difficult to speculate upon the mechanism, as this was in the context of HIV and hepatitis C coinfection (Berenguer et al., 2008).

Histologic findings indicative of hepatocyte cell death were sometimes found in rats and mice (Adaramoye et al., 2012; Bekker et al., 2012; Sharma et al., 2012). Gene expression changes in the skin of female brown Norway rats 6 hours after 12-hydroxynevirapine treatment also appeared to indicate apoptosis or altered mitochondrial function (Zhang et al., 2013). In rodent studies, nevirapine caused an increase in malondialdehyde, although changes in antioxidant enzymes were not observed (Adaramoye et al., 2012; Awodele et al., 2015). Altogether, the studies in rodents appear to suggest effects on cell death and mitochondrial function.

The effects of nevirapine on mitochondrial function are less clear in clinical studies. One study showed that nevirapine (coadministered with stavudine and lamivudine) increased mitochondrial depolarization and lymphocyte apoptosis (Karamchand et al., 2008). In contrast, another study showed that switching to nevirapine from nucleoside reverse transcriptase inhibitors improved mitochondrial parameters, but this may simply indicate that nevirapine has less of an effect on mitochondria than nucleoside reverse transcriptase inhibitors, which are known to cause mitochondrial toxicity (Negredo et al., 2009). Infants treated with nevirapine were not shown to have significant mitochondrial toxicity (Jao et al., 2017). In terms of oxidative stress, a study measured plasma F_2-isoprostane levels as a measure of lipid peroxidation and found that there was a trend toward decreased plasma F_2-isoprostane levels with nevirapine treatment (Redhage et al., 2009). Altogether, mitochondrial function may be impaired or unchanged with nevirapine exposure, but any observed effects do not appear to be as substantial as with other antiretrovirals.

In general, nevirapine did not have a clear impact on blood cell counts in rodents; depending upon the timing and the dosing, nevirapine was found to decrease leukocytes (compared with controls, 6 mg/kg per day orally, 60 days) (Awodele et al., 2015) or increase lymphocytes and platelets (compared with reference range, 200 mg/kg per day by oral gavage, 21 days) (Bekker et al., 2012) in rats. A low dose of nevirapine acutely increased leukocyte emigration in rats (Orden et al., 2014). In the female brown Norway rat model of skin rash, nevirapine treatment appeared to induce macrophage infiltration in auricular lymph nodes that preceded T-cell recruitment (Popovic et al., 2006). Infants exposed to prophylactic nevirapine treatment had elevated monocyte counts and percentages and basophil counts at birth (Schramm et al., 2010).

In rodent models, nevirapine caused some changes in cytokine levels, although in most cases, these changes occurred 3 weeks or longer after initiation of drug treatment. Serum TNF-α was increased at 24 hours in one study (Bekker et al., 2012), but no changes were seen with IFN-γ up to 3 weeks (Popovic et al., 2006; Bekker et al., 2012). In clinical studies of HIV infection, nevirapine exposure was associated with decreased serum or plasma cytokines such as CCL3 and IL-8 (Shalekoff et al., 2009), IL-6 (Borges et al., 2015), and potentially soluble CD14 (Allavena et al., 2013). The latter two studies compared the effects of multiple drugs, so these results may speak to a nevirapine-specific effect rather than a broad antiviral treatment effect. Although there are not many clear or consistent changes in specific inflammatory markers, nevirapine does seem to have modulatory effects on inflammatory markers in general, which may even contribute to its efficacy.

Overall, nevirapine has been shown to cause mild liver injury in otherwise healthy rodents. In some cases, histologic findings of cell death may complement the observation of liver injury. There are conflicting results regarding mitochondrial toxicity, leukocyte changes, and cytokine changes. There is no convincing evidence that nevirapine mediates mitochondrial injury; if anything, it appears less likely to do so than other antiretrovirals used in the treatment of HIV. Nevirapine does appear to have effects on peripheral blood cells, although the differences in study duration, doses, and models used are problematic. It would be informative to determine whether changes observed in female brown Norway rats, particularly the macrophage recruitment to lymph nodes, are reproduced in other rodent models that do not develop a skin rash.

D. Clozapine

Clozapine, an atypical antipsychotic, has unique efficacy in the treatment of schizophrenia. However, it is infrequently prescribed because of the risks of IDIAG and, more rarely, IDILI (Wu Chou et al., 2014; Li et al., 2020). As mentioned, several HLA haplotypes have been associated with an increased risk of clozapine-induced agranulocytosis (Legge and Walters, 2019).

The initiating mechanisms of clozapine-induced IDRs are poorly understood but are hypothesized to involve an aberrant adaptive immune response against clozapine-modified proteins. Clozapine can be bioactivated by cytochromes P450 in the liver and myeloperoxidase in neutrophils and monocytes to a reactive nitrenium ion that covalently binds to cellular proteins in vitro and in vivo (Liu and Uetrecht, 1995; Maggs

et al., 1995; Dragovic et al., 2013; Lobach and Uetrecht, 2014b) (Fig. 2D). If this neoantigen formation leads to significant cell damage or death, then the proinflammatory mediators and DAMPs released could stimulate an innate immune response that eventually leads to the onset of severe IDRs (Pirmohamed and Park, 1997; Johnston and Uetrecht, 2015).

Because of the rigorous hematologic monitoring required for patients starting clozapine, substantial evidence in support of an early innate immune response has been reported, as discussed below. Additionally, several animal models, predominantly albino rats, have been used to characterize immune changes throughout the first few weeks of clozapine treatment.

1. Data from Rodent and Human Studies. A review of the literature revealed more than 50 case reports and case series that noted the occurrence of an innate immune response with clozapine, most commonly evidenced by fever, eosinophilia, neutrophilia, leukocytosis, a left shift in blood cells, and increased C-reactive protein, ALT, ALP, and aspartate transaminase within the first 6 weeks of treatment (Lowe et al., 2007; Røge et al., 2012; Szota et al., 2013; Fonseka et al., 2016; Bellissima et al., 2018; Verdoux et al., 2019; de Leon et al., 2020). To avoid redundancy, those studies are not presented here.

Although short-term clozapine administration has been studied in close to 100 rodent studies, the focus of much of this work was to determine how clozapine alters disease and/or injury progression (e.g., in phencyclidine-induced schizophrenia) and not to characterize the effects of clozapine alone. Such models make it challenging to delineate a role for clozapine in the initiation of an innate immune response. Interestingly, many of these studies actually reported a protective effect of clozapine, often noting an attenuation of the disease model–induced inflammatory response. However, these disease models are physically or chemically induced, and such results may not reflect the true effects of clozapine monotherapy in patients.

Two male rat studies that evaluated clozapine effects in the liver demonstrated significant increases in injury (e.g., ALT increases, inflammatory cell infiltrates, increased liver weight) between 1 and 3 weeks of treatment (Jia et al., 2014; Zlatković et al., 2014). Significant covalent binding has also been demonstrated in the liver of clozapine-treated rats (Gardner et al., 2005; Ip and Uetrecht, 2008), and it is possible that the hepatic inflammation observed is in response to this haptenization.

The effects of clozapine on various other organs, including the brain, heart, and kidney, have been investigated using several rodent models. In studies characterizing the effects of clozapine in the absence of induced injury or disease, decreased splenic white pulp was observed in both female mice and male rats (Abdelrahman et al., 2014; Mohammed et al., 2020),

and both ovarian and kidney injury were reported in rats (Khalaf et al., 2019; Mohammed et al., 2020). Moreover, significant cardiac inflammation and morphologic aberrations were observed during the first few weeks of treatment (Wang et al., 2008; Abdel-Wahab and Metwally, 2014; Abdel-Wahab et al., 2014; Nikolić-Kokić et al., 2018; Mohammed et al., 2020). This parallels what is observed clinically because, in addition to severe IDRs, clozapine has been associated with an increased risk of myocarditis in patients, which can present with fever, eosinophilia, and increased troponin levels, often during weeks 2 and 3 of treatment (Kilian et al., 1999; Ronaldson et al., 2010; Curto et al., 2015). This is clearly an innate immune response due to the acute onset and effector cells and mediators observed.

The potential for clozapine to trigger cell death has been explored in several organs, including the liver, heart, blood, and brain. The majority of rodent studies demonstrated evidence of apoptosis (e.g., increased terminal deoxynucleotide transferase dUTP nick-end labeling staining or caspase-3 activation) between weeks 1 and 4 of treatment (Wasti et al., 2006; Jarskog et al., 2007; Huang et al., 2012; Abdel-Wahab and Metwally, 2014; Abdel-Wahab et al., 2014; Jia et al., 2014; Zlatković et al., 2014; Hsu and Fu, 2016; Khalaf et al., 2019) using doses that would approximate therapeutic concentrations in patients (Lobach and Uetrecht, 2014a). One study also noted that clozapine induced autophagy within hours of administration (Kim et al., 2018), and others noted decreased proliferation within the first few weeks of treatment (Huang et al., 2012; Hsu and Fu, 2016; Khalaf et al., 2019) as well. Translocation of apoptosis-inducing factor was not observed in the striatum of clozapine-treated patients or in rodents after 1 month of treatment (Skoblenick et al., 2006), suggesting against the involvement of caspase-independent cell death with clozapine.

In various models of acute injury and disease, clozapine was not consistently found to attenuate changes in immune cell populations. Only a small number of studies investigated the effects of clozapine in healthy animals, almost all of which demonstrated induction of an immune response by clozapine in the first 3 weeks of treatment. Most commonly, an increase in neutrophils was reported in clozapine-treated male and female rats (Wasti et al., 2006; Abdel-Wahab and Metwally, 2014; Lobach and Uetrecht, 2014a; Ng et al., 2014) or rabbits (Iverson et al., 2010). In the only two mouse studies, clozapine caused not only a decrease in several leukocyte populations (Abdelrahman et al., 2014; Jiang et al., 2016) but also an increase in monocytes, suggesting that the immunomodulatory effects of clozapine may differ across rodent species. In clinical studies, however, clozapine demonstrated strong evidence of innate immune cell activation during the first several weeks of treatment. Depending on the patient population, studies reported an increased incidence of eosinophilia,

neutrophilia, and/or leukocytosis that typically resolved with continued clozapine administration (Banov et al., 1993; Pollmächer et al., 1996; Chatterton, 1997; Tham and Dickson, 2002; Pui-yin Chung et al., 2008; Löffler et al., 2010). One small study also noted an increase in circulating CD34^{+} hemopoietic stem cells after 2 weeks of clozapine treatment (Löffler et al., 2010).

In rodent models in which immunomodulatory effects were investigated in the context of a disease or injury model, clozapine was frequently shown to attenuate the model-induced inflammation. Contrarily, few studies characterized the inflammatory mediator changes caused by clozapine alone. Of these studies, most demonstrated an increase in proinflammatory mediators in either rats or mice, including TNF-α, CXCL2, and heat shock protein 75 (Wang et al., 2008; Abdel-Wahab and Metwally, 2014; Abdel-Wahab et al., 2014; Lobach and Uetrecht, 2014a; Kedracka-Krok et al., 2016; Mohammed et al., 2020). Few studies have reported alterations in bioactive lipids in response to the drugs reviewed; however, dysregulated arachidonic acid signaling was also noted with clozapine treatment (Kim et al., 2012; Modi et al., 2013). Among the drugs investigated for this review, clozapine also provides the strongest support of innate immune activation in patients. All but one study reported an increased incidence of fever and/or increased serum levels of inflammatory mediators, such as TNF-α, soluble TNF receptor, soluble CD8, and soluble IL-2 receptor, most commonly occurring during the first month of treatment (Pollmächer et al., 1995, 1996, 1997; Maes et al., 1997, 2002; Hinze-Selch et al., 1998, 2000; Tham and Dickson, 2002; Pui-yin Chung et al., 2008; Kluge et al., 2009; Hung et al., 2017).

The research focused on the effects of amodiaquine, amoxicillin, or nevirapine on signal transduction pathways in rodent models is limited, although this is likely due to the preference for in vitro work in this area. Contrastingly, the effect of clozapine on a number of signaling pathways has been examined in rodents, although many of these observations have yet to be verified in subsequent studies. The reported effects of clozapine on the regulation of transcription vary greatly and depend on the timing of the studies, as well as the organs investigated. Clozapine-induced activation of hepatic and cardiac NF-κB was demonstrated in two rat models at 3 weeks (Abdel-Wahab and Metwally, 2014; Zlatković et al., 2014), but these changes were not observed in several brain regions in other models. Other studies have also characterized clozapine-induced activation of other signaling pathways, including the AMP-activated protein kinase (AMPK)-Unc-51–like kinase 1-Beclin1 pathway (Kim et al., 2018) and the protein kinase R–like ER kinase/eukaryotic translation initiation factor 2A ER stress axis (Weston-Green et al., 2018), although additional work is necessary to confirm the results of these reports. Notably,

AMPK signaling has been shown to play a role in many biochemical pathways, including autophagy, mitochondrial biogenesis, and lipid metabolism (Hardie et al., 2016); thus, further investigation of clozapine's impact on AMPK signaling and its potential role in inflammation should be undertaken.

Several rodent studies have also been conducted to evaluate changes in mitochondrial function due to clozapine. Clozapine often caused attenuated mitochondrial function or oxidative stress (e.g., increased malondialdehyde levels), which was noted most frequently in male rats after 3–4 weeks of treatment in various brain regions (Lara et al., 2001; La et al., 2006; Mehler-Wex et al., 2006; Streck et al., 2007; Bullock et al., 2008; Martins et al., 2008; Ji et al., 2009; Bishnoi et al., 2011; Zlatković et al., 2014; Cai et al., 2017), although cardiac-specific (Nikolić-Kokić et al., 2018) and ovarian-specific (Khalaf et al., 2019) aberrations were also reported. Additionally, another study reported increased markers of ER stress in the liver 1 hour after clozapine treatment (Lauressergues et al., 2012).

Although additional work is clearly needed to characterize the mechanisms underlying the findings discussed here, clozapine has frequently been shown to cause innate immune activation, both in patients and in various animal models. One avenue that should also be pursued moving forward is determining what initially triggers the immune response (e.g., triggers of myeloid cell recruitment) and, subsequently, whether inhibiting this immune response prevents progression to serious IDRs, effectively reducing the risks associated with clozapine use.

E. Summary

Overall, various early immune-related changes have been observed in animal models and human studies with the drugs presented here (Table 1). In rodents, the increased serum ALT observed with all drugs is indicative of liver damage. Additionally, the induction of apoptosis in many other organs was also observed with each of the drugs. A number of changes were described in various leukocyte populations, with some drugs causing increases in innate immune cells and, in fewer instances, some drugs causing decreases in leukocytes. In many cases, increases in proinflammatory cytokines were observed, and with clozapine, activated signal transduction pathways involved in proinflammatory signaling were also observed; this has not been studied in vivo for the other drugs examined. Markers of mitochondrial dysfunction were also reported after administration of amoxicillin, clozapine, and nevirapine.

The study of the inflammation caused by amodiaquine is limited to rodent models. However, there is a clear indication of NK cell–mediated liver injury, which spontaneously resolves with continued treatment, in addition to other immune cell infiltrates

detected in the liver, spleen, lymph node, and peripheral blood. The immune effects of amoxicillin ± clavulanic acid have not been studied in healthy subjects as extensively as some of the other drugs presented here. In general, the data do not suggest that amoxicillin causes an overt inflammatory response, but there are certainly changes that suggest some effects on mitochondria and leukocytes. Nevirapine treatment appears to cause liver damage but has variable effects on inflammatory mediators and immune cell counts. In rodents, clozapine shows the clearest pattern of a proinflammatory response of these drugs, which is not surprising, as it has been noted to induce fever, eosinophilia, neutrophilia, and increased proinflammatory cytokine release in patients.

Overall, the effects of each of these drugs are quite variable depending upon the models and doses used and the time points at which different parameters are evaluated. This highlights the complexity of the immune response and the potential differences that may be caused by different drugs, which likely depend upon the conditions in which their reactive metabolites are formed and which may also have a bearing on the types of IDRs that they cause. Studies that evaluate the time course of drug effects on inflammatory pathways are needed to better understand how drugs that cause IDRs induce innate immune responses.

V. Conclusions and Perspectives

Just as the drugs presented here are associated with different IDRs, they have also been demonstrated to cause a variety of immune-related effects during the first few weeks of treatment. These differences include the tissue localization of cellular dysfunction, injury, and death; the responding effector cells; the mechanisms contributing to inflammation; and the development of the immune response over time. These drug-specific observations emphasize the nuances and complexity underlying the activation and progression of an innate immune response. Based on the data presented here, it is clear that further research is necessary to expand our understanding of how drugs that are associated with severe IDRs can more frequently induce an early, transient immune response that typically resolves with continued treatment. Such research is fundamental to understanding the mechanisms of IDRs, and it is quite feasible to perform such research. Most drug metabolic pathways and immune responses share some similarities in animals and humans, and animal models are an important tool because they make it possible to perform controlled experiments and investigate organs such as the liver and spleen that could not be routinely looked at in patients. It is important to use doses in animals that would produce what would be

a therapeutic level in humans because high doses are more likely to cause overt toxicity that is not involved in the mechanism of IDRs. However, even though most features are likely to be similar in rodents and humans, there are clearly important differences between animals and humans; therefore, it is essential to follow up the animal studies with studies in humans to make sure that the results in animals correspond to the immune response to drugs in humans.

The innate immune response caused by these IDR-associated drugs is likely to be mild in comparison with the overt injury induced by disease models and would easily be overlooked in studies not designed to capture these relatively subtle changes. Moreover, additional consideration should be given to how these innate immune responses resolve with persistent treatment, as this resolution/tolerogenic response, or lack thereof, may provide clues as to why certain individuals eventually develop severe IDRs while the majority do not. Although certain risk factors for different IDRs have been identified, such as particular HLA haplotypes, these factors only account for a small proportion of risk; for most drugs, it remains difficult to predict which individuals will develop a severe IDR. An individual's T-cell receptor repertoire is likely to be a major factor, but it is much more difficult to study than HLA haplotypes. Many drugs, although highly efficacious in the treatment of their intended conditions, are therefore limited in their clinical use because of the risk of IDRs (including amodiaquine, clozapine, and nevirapine). Thus, a better understanding of the mechanisms contributing to the early immune response to these drugs may help predict and prevent or treat their associated IDRs, enabling the safer use of these agents. Although some work has been done in this area already, as reviewed here, the innate immune response has not been systematically studied across drugs that cause IDRs. More work is required to understand whether different drugs cause different responses, or whether there are certain commonalities in the immune changes caused by drugs that cause IDRs. Additionally, it will be important to test drugs that do not cause IDRs to ensure that they do not have the same effects. By identifying alterations in pathways that presage IDRs, these studies will identify potential biomarkers for drugs that can cause IDRs. These biomarkers could be used to develop a preclinical tool to screen drug candidates for the potential to cause serious IDRs. Such assays would facilitate the development of safer drugs and reduce the burden of IDRs on the drug discovery process. Additionally, understanding the specifics of the innate immune response to these drugs may reveal potential targets to inhibit to prevent the development of IDRs for drugs in clinical use. Altogether, although much work remains in this area, the study of the innate immune response is clearly important in improving drug safety.

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Authorship Contributions

Participated in research design: Sernoskie, Jee, Uetrecht.

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Pharmacological Reviews: The emerging role of the innate immune response in idiosyncratic drug reactions

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Supplemental Data

A. Effects on Organ Weights
    1. Rodent Studies
        a. Amodiaquine
Compared to untreated control rats, no effects on spleen, liver, or lung weights were observed when male Wistar rats were treated with amodiaquine (10 mg/kg/day, intraperitoneal (i.p.)) for up to 3 weeks (Elko and Cantrell, 1970). A second study also did not find any changes in liver weight during 7 weeks of amodiaquine (~200-300 mg/kg/day in rodent meal) treatment, although spleen weights were significantly increased from weeks 1 to 7, with peak weight at week 3 (Metushi *et al.*, 2015). In contrast, amodiaquine (10 mg/kg/day, per os (p.o.)) treatment of male Wistar rats for 4 days resulted in increased liver weights after 4 weeks compared to saline-treated rats, while heart, spleen, and kidney weights were unaffected (Ajani *et al.*, 2008).

Male Sprague Dawley rats administered amodiaquine (5, 10, or 15 mg/kg, p.o.) for 14 days, following which changes were evaluated up to 34 days (Niu *et al.*, 2016). The testis weights of amodiaquine-treated animals were found to be significantly lower than those of the control animals, although results from this study were presented as pooled groups for each drug dose and included data from 4 different endpoint times.

After 1 week of amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) treatment in male Brown Norway rats, another group noted significantly smaller livers and larger spleens, compared to control animals (Liu *et al.*, 2016).
        b. Amoxicillin
In female B6C3F1 mice dosed with 20, 100, or 500 mg/kg/day amoxicillin by oral gavage for 28 days, there were no changes in spleen weight or cellularity (Lebrec *et al.*, 1994).
        c. Nevirapine
In male rats administered 6 mg/kg nevirapine for 60 days, liver weights appeared slightly decreased compared to those of controls (Awodele *et al.*, 2015). This is unexpected as nevirapine induces liver enzymes, and an increase in liver weight is expected. In contrast, another study using male rats dosed nevirapine at 32 mg/kg for 4 weeks, an increase in liver weights was observed (Adaramoye *et al.*, 2012). The different results may be explained by the different doses used.
        d. Clozapine
To evaluate potential immunotoxicity, female BALB/c mice were treated with clozapine (1, 5, 10, or 20 mg/kg/day, i.p.) for 21 days (Abdelrahman *et al.*, 2014). Clozapine caused a dose-dependent reduction in spleen weight, reductions in both thymus and liver weight at the highest dose, and an overall decrease in body weight at 10 and 20 mg/kg, but no changes in kidney weight.

Conversely, following a week of clozapine treatment (25 mg/kg/day, p.o.) in male Sprague Dawley rats, liver weights were significantly increased compared to controls (Jia *et al.*,

2014). One study investigated the effects of clozapine (10, 15, or 25 mg/kg/day, i.p.) in male Wistar rats after 21 days of treatment (Abdel-Wahab and Metwally, 2014). At the two highest doses, clozapine caused increases in heart weight, total body weight, and overall heart to body weight ratios. Similarly, after 4 weeks of treatment in male Wistar rats, clozapine (45 mg/kg/day, p.o.) significantly increased relative heart weight compared to control (Nikolic-Kokic *et al.*, 2018).

    B. Effects on Liver
       1. Rodent Studies
         a. Amodiaquine

In a study of male Wistar rats, amodiaquine was administered either orally (269 µmol/kg [95.7 mg/kg]), intraperitoneally (269 µmol/kg [95.7 mg/kg]), or intramuscularly (2.15, 21.5, 269, or 538 µmol/kg [0.77, 7.7, 95.7, or 191.5 mg/kg]) for 4 days, and liver changes were evaluated from day 5 to day 18 (Clarke *et al.*, 1990). No changes were observed with doses up to 269 µmol/kg (95.7 mg/kg) but following the final intramuscular (i.m.) injection of highest dose (538 µmol/kg [191.5 mg/kg], animals developed a significant increase in serum ALT levels, which remained elevated until day 17. Additionally, hepatic glutathione levels were increased following both the i.p. and i.m. doses, compared to control, but no changes in liver histology were observed.

Using a bacterial fulminant model of hepatitis in C57BL/6 mice, another group demonstrated that amodiaquine had protective effects on the liver (Yokoyama *et al.*, 2007). At 24 hours post-induction, lethality was reduced from 60% to 20% with amodiaquine co-treatment and elevated serum levels of AST and ALT were significantly attenuated at 3 and 6 hours.

In fasted BALB/c mice treated with amodiaquine (180 mg/kg, p.o.), no effects on liver function or morphology were observed, although a moderate decrease in liver GSH levels were observed from 3 to 6 hours (Shimizu *et al.*, 2009). In GSH-depleted mice (induced by L-buthionine-S,R-sulfonimine (BSO)), however, amodiaquine induced a massive increase in plasma ALT levels from 6 hours, peaking at 24 hours, which was accompanied by approximately 60% lethality by 48 hours. Liver histology revealed lipid accumulation in the centrilobular hepatocytes by 6 hours, extensive necrosis with congestion by 24 hours, and inflammatory cell infiltration by 48 hours. Covalent binding of amodiaquine to liver and plasma proteins was doubled in the BSO co-treated group compared to amodiaquine alone. Co-administration of ABT prevented the increases in ALT and lethality observed with the BSO-amodiaquine group.

One study evaluated effects of amodiaquine (6.12 mg/kg, p.o.) administered twice a day in female Wistar rats treated for 3 days (Abolaji *et al.*, 2014). On day 4, slightly elevated plasma ALT, AST, and ALP levels, in addition to increased total plasma proteins, were observed with amodiaquine compared to water-treated controls.

Following 8 days of administration of amodiaquine in male Brown Norway rats (62.5 mg/kg/day, p.o.) or mice (~300-350 mg/kg/day in rodent meal), significant hepatic covalent binding was detected (Lobach and Uetrecht, 2014b). This group also showed that treatment of female C57BL/6 mice with amodiaquine (~200-300 mg/kg/day in rodent meal) for up to 7 weeks led to natural killer cell-mediated liver injury with delayed onset, which resolved despite continued treatment (Metushi *et al.*, 2015). Specifically, serum ALT levels were increased with amodiaquine from weeks 2 to 6, peaking between weeks 3 to 4. Covalent binding of amodiaquine was greater in the centrilobular region of the liver. Covalent binding and liver injury were also observed in male mice, but to a much lower extent.

Additionally, they investigated the impact of immunizing female C57BL/6 mice with amodiaquine-modified hepatic proteins (weekly for 3 weeks, plus 2 week washout) prior to amodiaquine (~200-300 mg/kg/day in rodent meal) treatment and found that this pre-treatment was effective in preventing the ALT spike observed from weeks 3 to 4 with amodiaquine alone (Mak and Uetrecht, 2015). Interestingly, the immunized group had greater leukocyte infiltration in the liver compared to amodiaquine alone. To investigate the role of macrophages in amodiaquine-induced liver injury, we conducted another study using female C57BL/6 wildtype and CCR2$^{-/-}$ mice, but there was no difference in the liver injury (Mak and Uetrecht, 2019). Both genotypes were treated with amodiaquine (~200-300 mg/kg/day in rodent meal).

Moreover, several models of amodiaquine-induced liver injury were investigated using mice and rats (Liu *et al.*, 2016). Amodiaquine (~200-300 mg/kg/day in rodent meal) was fed to male C57BL/6 mice for up to 4 weeks, with or without BSO in the drinking water and a single dose of diethyl maleate to deplete GSH. After 1 week, BSO co-treated animals had significantly lower liver GSH levels compared to amodiaquine alone or control; however, BSO co-treatment paradoxically prevented the amodiaquine-induced ALT peak observed during weeks 2 to 4. Covalent binding of amodiaquine to hepatic proteins was not substantially altered with BSO co-treatment. The BSO phenomena was indicated to have been reproduced in Brown Norway and Wistar rats, although the data was not shown.

Liu et al. (2016) did, however, demonstrate that male Lewis, Wistar, and Brown Norway rats treated with amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) developed elevated serum ALT levels (greatest in Wistar rats) with increased hepatic covalent binding from weeks 2 to 4. Less severe liver injury, as measured by ALT peaks, was observed with female rats. To cause immune suppression, male Brown Norway rats were co-treated with amodiaquine and cyclosporine, which effectively prevented the amodiaquine-induced ALT peak. Conversely, when rats were administered poly I:C prior to amodiaquine to stimulate immune activation, there was an earlier onset of increased ALT. Natural killer cell activation was induced in treated rats by co-administration of retinoic acid, which also exacerbated the amodiaquine-induced ALT increase.

b. Amoxicillin

Serum ALT was increased at day 14 in male Wistar rats administered amoxicillin at a dose of 30 mg/kg/day intraperitoneally (Oyebode *et al.*, 2019).

c. Nevirapine

In male rats administered 6 mg/kg nevirapine for 60 days, serum ALT was increased (Awodele *et al.*, 2015). In another study using male rats, nevirapine was dosed at 32 mg/kg for 4 weeks, an increase in ALT was observed (Adaramoye *et al.*, 2012). Other markers of liver injury were also increased (serum total bilirubin and γ-glutamyl transferase). Necrosis was noted in the liver histology, and liver protein was decreased.

Female Brown Norway rats administered 75 mg/kg nevirapine for 7 days did not show an increase in ALT (Brown *et al.*, 2016), nor at a dose of 200 mg/kg for up to 14 days, although there appeared to be liver injury at day 7 which had resolved by day 14 in this study (Bekker *et al.*, 2012). Both of these studies also tested the effect of co-treatment of nevirapine with a substance that can cause liver injury on its own (galactosamine and lipopolysaccharide (LPS), respectively). Nevirapine was found to attenuate the galactosamine-induced ALT increase. Co-treatment with LPS attenuated the histological findings in the liver caused by nevirapine, although the combination resulted in an increase in ALT not observed with nevirapine treatment alone.

In male C57BL/6 mice, an ALT elevation at week 3 was also observed at a dose of 950 mg/kg administered in food (Sharma *et al.*, 2012). Mild necrosis in the liver was observed by histology.

d. Clozapine

Using a model of liver ischemia/reperfusion (I/R) in male Wistar rats, one group evaluated the protective effects of clozapine (15 mg/kg, subcutaneous (s.c.)) administered immediately following the ischemia phase and again 6 hours into the reperfusion phase (Adachi *et al.*, 2006). Notably, clozapine attenuated I/R-induced plasma ALT and AST increases after 24 hours in comparison to control animals that underwent I/R. Another group similarly showed that clozapine (15 mg/kg, s.c.) pre-treatment at 24 hours, 12 hours and immediately prior to I/R significantly attenuated increases in plasma ALT and AST at 2 hours post-ischemia (El-Mahdy *et al.*, 2013). Moreover, clozapine pre-treatment dampened I/R-mediated vacuolar degeneration of hepatocytes and dilatated central vein pathology and resulted in increased hepatic GSH levels and decreased malondialdehyde (MDA, an end-product of lipid peroxidation) levels, compared to I/R controls.

One group investigated the potential impacts of clozapine in a cecal ligation puncture model of peritoneal sepsis (Machado *et al.*, 2007). After resuscitation, rats were treated with clozapine (25 mg/kg, s.c.) twice a day for 3 days. Clozapine did not attenuate hepatic damage nor was survival significantly improved when compared to the sham-operated control group. Although serum markers of organ dysfunction, including elevated creatine kinase, creatinine, AST, and amylase appeared to be altered in the clozapine-treated group compared to saline-treated controls, statistical comparison of these groups was not included.

At the end of 6 weeks of clozapine (25 or 50 mg/kg/day, p.o. [in rodent meal]) administration in female Lewis rats, clozapine extensively modified proteins in liver subcellular fractions compared to control rats (Gardner *et al.*, 2005). To test whether selenium deficiency enhanced the immunotoxicity of clozapine, female Sprague Dawley rats were maintained on a normal or selenium-deficient diet for 54 days prior to, and during, clozapine (50 mg/kg/day, p.o. in rodent meal) administration for 62 days (Ip and Uetrecht, 2008). At the end of treatment, significant hepatic covalent binding was observed with clozapine treatment, but was not altered by selenium status.

Another group used male Wistar rats to evaluate the effects of 3 weeks of clozapine (20 mg/kg/day, i.p.) with or without social isolation for the duration of treatment (Zlatkovic *et al.*, 2014). Irrespective of isolation conditions, clozapine caused marked infiltration of leukocytes in the hepatic portal triad and increased Kupffer cells and other inflammatory cells in the sinusoids, when compared to saline treated animals. Increased serum ALT and decreased hepatic GSH were also observed in all clozapine-treated groups. Clozapine-mediated increases in hepatic MDA, protein carbonyl content and GST activity were found and were exacerbated by social isolation. In the absence of social isolation, clozapine also caused elevated hepatic levels of cytosolic nitric oxide metabolites.

Hepatotoxicity was also investigated in male Sprague Dawley rats that were given clozapine (25 mg/kg/day, p.o.) with or without pre-treatment of glycyrrhetinic acid for 1 week (Jia *et al.*, 2014). Through the inhibition of 15-hydroxyprostaglandin dehydrogenase and prostaglandin reductase 2, glycyrrhetinic acid prevents the metabolism of active of prostaglandin E2 and F2α, and was proposed to have hepatoprotective activity (Ming and Yin, 2013). Clozapine caused marked increased in serum ALT and AST levels, which were enhanced by glycyrrhetinic acid co-treatment.

2. Clinical Studies
   a. Nevirapine

In a cross-sectional study of patients with HIV and hepatitis C virus coinfection, it was found that both NNRTIs as a class and nevirapine exposure was associated with a decreased rate of fibrosis progression, an effect that was not observed with protease inhibitor exposure (Berenguer *et al.*, 2008). This effect could be due to nevirapine exposure itself and/or due to its effects on the disease states, as HAART-naïve individuals have higher hepatic proinflammatory cytokine mRNA than do HAART-treated individuals (Sitia *et al.*, 2006).

C. Effects on Other Organs
   1. Rodent Studies
      a. Amodiaquine

When amodiaquine (10 mg/kg, i.m.) was administered to fasted male Wistar rats, it caused an increase in parietal cells and a decrease in mucous cells at 50 minutes post-injection (Ajeigbe *et al.*, 2012).

Another group evaluated the effects of amodiaquine (6.12 mg/kg, p.o.) administered twice a day in female Wistar rats treated for 3 days, following which no alterations in plasma levels of electrolytes, creatinine, or urea were observed (Abolaji *et al.*, 2014).

Treatment of female C57BL/6 mice with amodiaquine (~200-300 mg/kg/day in rodent meal) for up to 6 weeks demonstrated that covalent binding of amodiaquine was detectable in the kidney, the red pulp of the spleen, and in the gut (Metushi *et al.*, 2015).

During characterization of various models of amodiaquine-induced liver injury, hypertrophic cells in lung alveolar regions of male Brown Norway rats treated with amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) for 5 weeks, which were not observed in saline-treated controls (Liu *et al.*, 2016).

      b. Clozapine

After cecal ligation, clozapine (25 mg/kg, s.c.) administered twice a day was ineffective in reducing renal, pancreatic, lung, or muscular damage observed in male Wistar rat, although again, statistical comparison of the non-septic clozapine group versus control was not reported (Machado *et al.*, 2007). An anti-glomerular basement membrane (GBM) antibody model of proteinuria in female Wistar rats was used to evaluate the impact of twice a day clozapine (10 mg/kg, s.c.) administration on disease progression after 6 days (Tanda *et al.*, 2007). Compared to the saline and anti-GBM antibody-treated control group, clozapine was ineffective in reducing proteinuria or the formation of crescentic glomeruli in the kidney.

In a male C57BL/6 mice model of arthritis, 2 weeks of clozapine (4 mg/kg/bid, p.o.) markedly protected mice from arthritis symptoms, including an attenuation of ankle thickness from days 2 to 10 (Nent *et al.*, 2013). After 2 weeks, no inflammation was detectable in ankle joint histology in the clozapine group, while the arthritic control group presented with strong inflammation, joint destruction, and periarticular inflammatory processes along the shaft.

Male and female Wistar rats were given clozapine (20 mg/kg/day, p.o. [administered in drinking water]) for 2 months, with or without postnatal exposure to PCP, and it was shown that male non-PCP-exposed clozapine-treated rats had increased leg fat, while female PCP-exposed clozapine-treated rats had decreased total fat content compared to their respective vehicle controls (Nikolic *et al.*, 2017).

In a prenatal methylazoxymethanol (MAM) Sprague Dawley rat model of schizophrenia, where male offspring were treated with clozapine (20 mg/kg/day, i.p.) for 8 days, clozapine ±

MAM exposure did not alter brain levels of nerve growth factor (Fiore *et al.*, 2008). Serum levels of nerve growth factor and entorhinal cortex levels of brain-derived neurotrophic factor (BDNF) were only elevated in the MAM-clozapine group, relative to both the MAM-exposed saline group as well as the non-exposed clozapine group.

Conversely, when male C57BL/6 mice that were treated with PCP, co-treatment of clozapine (6 mg/kg/day, i.p.) resulted in significantly decreased cerebral cortex BDNF levels compared to saline-treated PCP controls after 2 weeks (Barzilay *et al.*, 2011). Following 1 week of clozapine (10 mg/kg/day, p.o.) in male C57BL/6 mice that had been pre-treated with PCP for 2 weeks, clozapine significantly enhanced PCP-mediated increases in prefrontal cortex mRNA expression of GSH peroxidase, glutamate-cysteine ligase modifier subunit, and glutamate-cysteine ligase catalytic subunit mRNA expression versus PCP saline controls (Tran *et al.*, 2017). Clozapine did attenuate PCP-induced GSH decreases and oxidized GSH increases, but clozapine effects in the absence of PCP were not investigated.

In a model of neurotoxicity, where male C57BL/6J mice were administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), treatment with either clozapine (1 mg/kg/day, s.c.) or clozapine *N*-oxide (1 mg/kg/day, s.c.) for 21 days significantly attenuated MPTP-induced neuron loss and reduced microglial activation (as measured by CD11b staining), compared to MPTP controls (Jiang *et al.*, 2016).

One group characterized the impact of 20 days of clozapine (10, 30, or 60 mg/kg/day, p.o. [administered in drinking water]) in a female C57BL/6 mouse model of experimental autoimmune encephalomyelitis (EAE) (Green *et al.*, 2017). At the two highest doses, clozapine significantly attenuated EAE severity scores and at the highest dose, delayed disease onset. No effects of clozapine were observed in the absence of EAE. In a second female C57BL/6 mouse model of EAE, 20 days of clozapine (10 mg/kg/day, p.o.) initiated at disease day 15 did not affect disease progression or severity, but did slightly attenuate EAE-induced white matter loss (Chedrawe *et al.*, 2018).

In another social isolation protocol in male Wistar rats, 3 weeks of clozapine (20 mg/kg/day, i.p.) did not affect cytosolic GSH levels in the hippocampus, but did significantly reduce GSH peroxidase activity, compared to saline controls (Todorovic and Filipovic, 2017b). Both GSH peroxidase and GSH reductase activity and protein levels were reduced with socially isolated clozapine treatment. In the prefrontal cortex, no changes in GSH reductase were observed, but clozapine did reduce GSH levels and GSH peroxidase activity, irrespective of social isolation (Todorovic and Filipovic, 2017a). BDNF levels were also found to be elevated after 30 days of clozapine (10 mg/kg/day, i.p.) in the brains of male CDF-344 rats (Kim *et al.*, 2012). Moreover, numerous changes in the arachidonic acid cascade were reported in the brain, in addition to decreased body weight with this model. With clozapine, the concentrations of prostaglandin E_2 (PGE_2) and COX-1 were decreased, and thromboxane B_2 levels and 5- and 15-lipoxygenases (LOX) mRNA levels were increased, while leukotriene B_4 was unchanged. In a second study by this group, clozapine was also shown to decrease the concentration of unesterified plasma arachidonic acid, and altered the composition of a number of fatty acids in the brain (Modi *et al.*, 2013).

One study evaluated the cardiotoxicity of clozapine (5, 10, and 25 mg/kg/day, i.p.) in male BALB/c mice after up to 2 weeks of treatment (Wang *et al.*, 2008). A dose-dependent increase in myocardial histopathological scores was observed with clozapine compared to saline treated controls, where inflammatory lesions consistent with myocarditis were most severe at day

7 and had begun to resolve by 2 weeks. Co-administration of propranolol significantly dampened the severity of cardiac inflammation observed at 2 weeks.

Cardiac inflammation was also demonstrated in male Wistar rats given clozapine (10, 15, or 25 mg/kg/day, i.p.) for 3 weeks (Abdel-Wahab and Metwally, 2014; Abdel-Wahab *et al.*, 2014). Compared to the saline control group, clozapine caused distinct and dose-dependent inflammatory lesions, interstitial edema, perinuclear vacuolation, focal subendocardial fibrosis, and disorganization and degeneration of the myocardium. Clozapine also increased serum levels of creatine kinase (15 and 25 mg/kg) and LDH (all doses) and reduced cardiac GSH (25 mg/kg) and GSH peroxidase activity (15 and 25 mg/kg). All inflammatory effects were attenuated by co-administration of captopril for the duration of high-dose clozapine treatment.

Additionally, after 4 weeks of treatment in male Wistar rats, clozapine (45 mg/kg/day, p.o.) significantly increased infiltrating leukocytes in the heart and caused an abnormal structure of cardiomyocytes that was not present in the vehicle control group (Nikolic-Kokic *et al.*, 2018).

One study found that clozapine (0.5 mg/kg/day, p.o.) administration to male Sprague Daley rats for 30 days caused noticeable aberrations in kidney, spleen, and heart morphology, and also increased expression of cardiac CYP1A2, CYP3A4, and CYP2C19 mRNA compared to saline control animals (Mohammed *et al.*, 2020). Cardiac histopathological changes induced by clozapine included vascular congestion with intermuscular edema. This was accompanied by severe histopathological lesions in the spleen, as evidenced by hemosiderosis in the red pulp and lymphoid depletion (reduction in the diameter of lymphoid follicles of the white pulp). In the kidney, clozapine-treated animals exhibited numerous hyaline casts in the lumina of the renal tubules, with marked vacuolar and hydropic degeneration in the epithelial linings, focal hemorrhage and vascular congestion. Furthermore, serum levels of cardiac troponin I, LDH, creatinine, blood urea, nitrogen, urea, and uric acid were elevated following clozapine treatment, and serum lysozyme activity levels were decreased. Most biochemical changes were attenuated when clozapine was co-administered with sulpride (another antipsychotic) for the duration of the study.

After 4 weeks of clozapine (20 mg/kg/day, p.o.) treatment in female Wistar rats, multiple cystic atretic follicles were observed with degenerated zona pellucidum and oocyte, vacuolation of cells, and an excessive deposition of collagen fibers, concentrated around the atretic follicles that was not observed in the control group (Khalaf *et al.*, 2019).

In addition to a reduction in spleen weight at all doses of clozapine (1, 5, 10, or 20 mg/kg/day, i.p.), a marked decrease in spleen cellularity and decreased white pulp density were reported following 3 weeks of the clozapine (5, 10, or 20 mg/kg) in female BALB/c mice, compared to saline controls (Abdelrahman *et al.*, 2014). Moreover, when mice were given a single dose of sheep RBCs on after 14 days of clozapine treatment, a dose-dependent reduction in hemagglutination titres was observed at after 21 days. When mice were rechallenged with a second dose of sheep RBCs, clozapine (5, 10, or 20 mg/kg) significantly attenuated footpad swelling. This was accompanied by a decrease in inflammatory infiltrates of leukocytes in both the hypodermis and muscle layers of the foot, observed with the two highest doses of clozapine.

2. Clinical Studies
    a. Clozapine

To investigate cardiovascular complications, a retrospective analysis of 8,000 male and female patients who commenced clozapine therapy was conducted (Kilian *et al.*, 1999). The incidence of myocarditis or cardiomyopathy reportedly due to clozapine administration in this population was 0.23% (20 male and 3 female cases). Of the 15 myocarditis cases, the median

time to onset was 15 days of clozapine (range: 3-21 days) and 6 cases were accompanied by eosinophilia.

Among 38 cases (27 male and 11 female) of clozapine-induced myocarditis, the mean time to onset was 18 ± 2 days (range: 14-33 days) and the mean dose at onset was 232 ± 69 mg/day (range: 50-750 mg/day) (Ronaldson *et al.*, 2010). Eighteen patients also presented with fever up to 6 days prior to detection of elevated serum TRP levels and 20 cases developed eosinophilia in the subsequent week.

In a study of 15 clozapine-naive schizophrenia patients (8 male and 7 female) titrated from 25 mg/day up to 100 mg/day over 4 weeks, a slight increase in serum levels of hsCRP was observed at week 3 and 3 patients developed tachycardia, although no changes in leukocytes or TRP-I were observed (Curto *et al.*, 2015).

D. Effects on Cell Death and Proliferation
1. Rodent Studies
a. Amodiaquine

A dose-dependent loss of spermatogenic cells and disorganization of seminiferous tubule morphology was observed following amodiaquine (5, 10, or 15 mg/kg, p.o.) treatment in male Sprague Dawley rats for 14 days, although undifferentiated spermatogonia remained alive in the testes (Niu *et al.*, 2016). TUNEL staining suggested that these cells had undergone apoptosis, which was supported by increased protein levels of FS-7-associated surface antigen (Fas), Bcl-2-associated X protein (BAX), and caspases-3, -8, and -9 in amodiaquine-treated testes. Blood-testes-barrier damage was also reported, in addition to a significant reduction in the blood-testes-barrier-associated proteins tight junction protein-1, occludin, claudin-11, eppin, and E-cahderin. No changes in serum luteinizing hormone or testosterone were observed. Amodiaquine-induced histological changes were reversed following a 20-day recovery period.

Increased apoptosis, as measured by TUNEL staining, was noted in the livers of male Brown Norway rats treated with amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) for 5 weeks, which was not observed in saline-treated controls (Liu *et al.*, 2016). Likewise, only in amodiaquine-treated animals was cell proliferation in the germinal center of spleen significantly increased at this time. These changes were also preceded by increases in serum osteopontin (peaking at day 12) and serum cytochrome c (peaking at week 4), detected only in the amodiaquine group.

b. Amoxicillin

Caspase-3 and -9 were activated in the liver of male Wistar rats treated with 30 mg/kg/day amoxicillin intraperitoneally once daily for 14 days (Oyebode *et al.*, 2019).

c. Nevirapine

Nevirapine was administered to Sprague-Dawley rats at a dose of 200 mg/kg once daily by oral gavage, and the effect of co-treatment with LPS was examined (Bekker *et al.*, 2012). Apoptosis was noted by histological examination at the 6- and 24-hour endpoints in the nevirapine-treated group, but the liver of the nevirapine and LPS co-treated group appeared normal. At 7 days, the histology of the nevirapine group exhibited centrilobular hepatocellular degeneration, cell swelling, and hepatocyte apoptosis which resolved by days 14 and 21; again, the liver appeared normal in the co-treated group.

In female Brown Norway rats treated with 159 mg/kg/day 12-hydroxynevirapine by oral gavage, transcripts related to apoptosis and autophagy, such as death-associated protein kinase 1 (Dapk1) and Kruppel-like factor 15 (Klf 15), or cell stress, such as lipin1 (Lpin1) and DnaJ

(Hsp40) homologue, subfamily B, member 5 (Dnajb5) were upregulated in the skin 6 hours post-dose (Zhang *et al.*, 2013).

In the liver of Brown Norway rats treated with 150 mg/kg nevirapine for 8 weeks, hepatocytes were noted to have developed necrotic cytoplasm, while some endothelial cells exhibited signs of degeneration (Sastry *et al.*, 2018).

In Wistar rats administered nevirapine for 4 weeks, the live-dead ratio of the sperm was decreased in a dose-dependent manner (Adaramoye *et al.*, 2012). This was confirmed with findings of spermatocyte necrosis observed by histology.

d.   Clozapine

In male Sprague Dawley rats, clozapine (5 mg/kg/day, i.p.) had no effect on ketamine-induced increases in caspase-3 activity in any brain area examined (striatum, hippocampus, or prefrontal cortex) (George *et al.*, 2020). However, clozapine did attenuate the elevated Bax/Bcl-2 ratio and cytochrome c expression induced by ketamine in all brain areas, and also attenuated elevated prefrontal cortical and hippocampal MDA levels.

At 2 hours post-clozapine (10 mg/kg, i.p.) administration in male Sprague Dawley rats, elevated microtubule-associated protein 1 light chain 3-II (an autophagosome marker) staining was observed in the frontal cortex, compared to saline-treated controls (Kim *et al.*, 2018). Increased autophagosome numbers were also demonstrated via transmission electron microscopy at this time. Additionally, autophagy-related protein 5-12 conjugates were increased, further indicating that activation of autophagy had occurred.

Following 21 days of clozapine (20 mg/kg/day, i.m.) administration, Sprague Dawley rats had noticeable neutrophil toxicity, manifested by condensation and subsequent breakdown of chromatin material, although this morphology was not clearly distinguishable in the figures provided in the paper (Wasti *et al.*, 2006).

After 28 days of treatment, another group did not observe any translocation of AIF from the mitochondria to the nucleus in the striatum of male Sprague Dawley rats administered clozapine (30 mg/kg/day, i.p.), suggesting against the possibility of caspase-independent neuronal cell death with clozapine (Skoblenick *et al.*, 2006).

Furthermore, after 28 days of clozapine (10 mg/kg/day, i.p.) treatment in male Sprague Dawley rats, clozapine had no effect on apoptotic markers, including histone-associated DNA fragmentation, Bax/Bcl-2 ratio, or levels of X-linked inhibitor of apoptosis protein in the frontal cortex, compared to saline controls (Jarskog *et al.*, 2007). However, levels of cleaved-caspase 3 protein, in addition to levels of activity of caspase-3, were significantly higher with clozapine.

In contrast, no changes in striatal caspase-3 activity levels were observed in male Wistar rats treated with clozapine (10 mg/kg/day, i.p.) for 21 days (Bishnoi *et al.*, 2011).

In male Wistar rats given clozapine (10, 15, or 25 mg/kg/day, i.p.) for 3 weeks, increased cardiac caspase-3 protein levels were reported (Abdel-Wahab and Metwally, 2014). In a second study by this group, clozapine-induced increases in caspase-3 activity levels and TUNEL staining were observed and were attenuated by co-administration of captopril (Abdel-Wahab *et al.*, 2014).

Following 3 weeks of treatment in male Wistar rats, clozapine (20 mg/kg/day, i.p.) increased focal necroses and apoptotic hepatocytes in liver lobules, with or without the coaddition of social isolation (Zlatkovic *et al.*, 2014). Another study noted acidophilic degeneration (indicative of dying hepatocytes) in male Sprague Dawley rats that had been co-treated with clozapine (25 mg/kg/day, p.o.) and glycyrrhetinic acid for 1 week (Jia *et al.*, 2014).

Another study noted that ovarian antral follicles were characterized by increased apoptotic cells following 4 weeks of clozapine (20 mg/kg/day, p.o.) treatment in female Wistar rats (Khalaf *et al.*, 2019). Additionally, clozapine treatment increased ovarian p53 staining and decreased Ki67 staining in comparison to the water control group.

After 11 days of clozapine (13.5 mg/kg/day, p.o.) administration in male C57BL/6 mice, increased pancreatic apoptosis and decreased proliferation were observed, measured by TUNEL and Ki67 staining, respectively, when compared to vehicle controls (Huang *et al.*, 2012). These effects were exacerbated by clozapine administration in combination with a high fat (60% fat versus standard 12% fat) diet. Members of this group later confirmed these findings, showing that administration of clozapine (13.5 mg.kg/day, p.o.) to male C57BL/6 mice resulted in an increase in apoptosis and a decrease in proliferation in pancreatic islet cells (again, measured by TUNEL and Ki67 staining, respectively), as early as 2 weeks into treatment (Hsu and Fu, 2016).

2. Clinical Studies
a. Nevirapine

To our knowledge, only one study has examined cell death using samples from patients treated with nevirapine. In peripheral blood mononuclear cells, a lesser proportion of total lymphocytes and CD4 T cells was found to be apoptotic in patients receiving nevirapine-containing HAART compared to HAART-naïve, HIV-infected individuals (Karamchand *et al.*, 2008). The proportion of apoptotic total lymphocytes was greater than the proportion of apoptotic CD4 T cells, suggesting that a different lymphocyte subset was undergoing apoptosis. Altogether, this study does not support an increase in total lymphocyte or CD4 T cell apoptosis with nevirapine treatment (in combination with stavudine and lamivudine) when compared to untreated HIV-infected individuals, although an additional comparison to healthy individuals might be informative.

b. Clozapine

To characterize potential pathways of cell death associated with clozapine, one group examined localization of the intramitochondrial protein apoptosis-inducing factor (AIF) in striatal sections collected post-mortem from 4 clozapine-treated schizophrenic patients and 4 healthy controls (Skoblenick *et al.*, 2006). Translocation of AIF from the mitochondria to the nucleus initiates a caspase-independent cell death cascade, however, no translocation was observed in either patients or controls.

E. Effects on Immune Cells
1. Rodent Studies
a. Amodiaquine

Compared to untreated control rats, no effects on phagocytosis activity (measured by carbon particle clearance from blood) were observed when male Wistar rats were treated with amodiaquine (10 mg/kg/day, i.p.) for up to 3 weeks (Elko and Cantrell, 1970). However, amodiaquine did shorten the duration of elevated phagocytosis activity observed in malaria-infected rats.

In the study comparing routes of administration in male Wistar rats, immediately following the final i.m. injection of amodiaquine at the highest dose (538 μmol/kg), animals developed a significant reduction in white blood cells that lasted for 4 days, after which, counts remained higher than baseline until day 14 (Clarke *et al.*, 1990).

Male Wistar rats administered amodiaquine (10 mg/kg/day, p.o.) for 4 days developed a significant increase in fibrinogen and total leukocyte counts compared to saline-treated rats at the study endpoint of 4 weeks (Ajani *et al.*, 2008).

With amodiaquine treatment (6.12 mg/kg, p.o.) twice daily in female Wistar rats, no changes in erythrocyte MDA, GSH, or G6PD levels were reported (Abolaji *et al.*, 2014).

Another group investigated the roles of myeloperoxidase and NADPH oxidase in the covalent binding of amodiaquine to neutrophils using several models: male rats (Brown Norway) and female mice (C57BL/6J wildtype, MPO$^{-/-}$, gp91 phox$^{-/-}$, Rac1$^{-/-}$, Rac2$^{-/-}$, COX1$^{-/-}$, and COX2$^{-/-}$) were administered amodiaquine for up to 8 days via gavage (rats, 62.5 mg/kg/day) or mixed in rodent meal (mice, ~300-350 mg/kg/day, p.o.) (Lobach and Uetrecht, 2014b). In wildtype mice and rats, significant covalent binding was detected in neutrophils after day 1 of treatment, reaching a maximum following day 4. After day 1, significant covalent binding was also observed in the bone marrow of rats and mice; specifically, binding was observed in myeloid cells, lymphoid cells, and megakaryocytes, but not in erythroid cells. Furthermore, amodiaquine appeared to bind both intracellularly as well as to surface proteins. This covalent binding was significantly reduced in neutrophils from amodiaquine-treated MPO$^{-/-}$ mice, and a similar trend emerged in gp91 phox$^{-/-}$ neutrophils, although it did not reach significance. No difference in covalent binding was observed in neutrophils from Rac1$^{-/-}$, Rac2$^{-/-}$, COX1$^{-/-}$, or COX2$^{-/-}$ mice compared to neutrophils from wildtype mice treated with amodiaquine for 8 days, indicating that the oxidative burst pathway, prostaglandin H synthase, or cyclooxygenase are not necessary for covalent binding of amodiaquine in neutrophils.

During amodiaquine (~200-300 mg/kg/day in rodent meal) treatment in female C57BL/6 mice, members of this group found that there was an elevation in lymphocytes in the cervical lymph nodes at week 2, and in the liver and spleen at week 3 of treatment (Metushi *et al.*, 2015). IHC revealed an increase in CD4$^+$, CD8$^+$, CD11b$^+$, F4/80$^+$, CD45R$^+$, and Ki67$^+$ cells in the liver and an increase in CD11b$^+$, Ki67$^+$, F4/80$^+$, and CD45R$^+$ cells in the splenic red pulp with amodiaquine, with maximal increases observed around week 3. Flow cytometry revealed an increased in NK1.1$^+$ and CD8$^+$ cells in the cervical lymph nodes at week 2 in the spleen at weeks 1 and 3, and in the liver at week 3. Amodiaquine treatment of Rag1$^{-/-}$ mice (B- and T-cell deficient) in this study demonstrated an even greater increase liver NK1.1$^+$ cells and ALT, and upon depletion of natural killer cells, the ALT increase was attenuated.

In the immunization protocol by this group, there was expanded white pulp in the spleens of amodiaquine hepatic protein immunized, amodiaquine-treated animals compared to the control or amodiaquine alone treated group at week 3 (Mak and Uetrecht, 2015). Additionally, immunized animals exhibited the highest number of splenic CD11b$^+$/Gr-1$^+$ cells.

Moreover, after week 5 of amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) treatment, this group noted that the livers of treated Wistar rats exhibited focal inflammatory infiltrates with increased ED1$^+$ cells and treated Brown Norway rats displayed hypertrophic Kupffer cells, in addition to increased ED1$^+$, ED2$^+$, and CD45$^+$ leukocytes (Liu *et al.*, 2016). Total liver and lymph node lymphocytes were increased in Brown Norway rats after 3 and 4 weeks of treatment, respectively, driven by increased CD4$^+$ T cells (specifically, T$_H$17 cells in the liver). NK1.1$^+$ cells were also increased in the liver at week 3 and in the lymph nodes and peripheral blood at week 4 of treatment. Peripheral blood, spleen, and lymph node M1 monocytes/macrophages were decreased from weeks 1 to 3 but were increased in the liver at weeks 3 and 4. Activated M2 macrophages were elevated in all investigated organs (spleen, liver, lymph node, peripheral blood) around weeks 3 to 4.

In the study comparing the effects of amodiaquine treatment in female C57BL/6 wildtype and CCR2$^{-/-}$ mice, CD11b^{+}/Gr-1^{+} and NK1.1^{+} cells were increased with amodiaquine in the livers of wildtype-treated animals, but not in CCR2$^{-/-}$ mice (Mak and Uetrecht, 2019).

Another group evaluated the protective effects of amodiaquine in a mouse model of intracerebral hemorrhage (ICH) induced by striatal injection of collagenase VII (Kinoshita *et al.*, 2019). At 3 days, amodiaquine significantly suppressed ICH-induced accumulation of activated microglia/macrophages (Iba1^{+} cells) and also suppressed activation of astrocytes (GFAP^{+} cells) in the perihematomal region, compared to saline-treated ICH controls. Conversely, amodiaquine had no effect on ICH-induced neuron loss or injury volume, although this study did not look at the effects of amodiaquine in the absence of ICH.

b. Amoxicillin

In female B6C3F1 mice dosed with 20, 100, or 500 mg/kg/day amoxicillin by oral gavage for 28 days, some immune changes were noted, but none appeared dose-dependent (Lebrec *et al.*, 1994). WBC counts were noted to be decreased at the highest dose, and there was no change in NK cell activity.

In male Wistar rats administered amoxicillin(30 mg/kg/day, i.p.), WBC counts were increased at day 14 (Oyebode *et al.*, 2019).

Male Sprague-Dawley rats were administered 50 mg/kg amoxicillin by oral gavage 3 hours before induction of lung injury; no change in pulmonary alveolar macrophage function was noted (Tamaoki *et al.*, 1999).

In male Sprague-Dawley rats administered an intratracheal instillation of LPS to induce mucus hypersecretion and administered 40 mg/kg amoxicillin orally for 4 days, there was no difference in leukocyte counts bronchoalveolar lavage fluid (Ou *et al.*, 2008).

c. Nevirapine

Hematological analysis of rats administered 6 mg/kg/day nevirapine orally revealed a decrease in white blood cell (WBC) counts, but no changes in other parameters of erythrocytes or platelets after 60 days (Awodele *et al.*, 2015). In rats administered a higher dose of 200 mg/kg nevirapine orally daily, lymphocytes and platelets were noted to be over the reference range at day 21; however, counts from control animals were not presented (Bekker *et al.*, 2012).

In male Sprague-Dawley rats administered 35–165 µg/kg nevirapine i.p. 4 hours before intravital imaging, decreased rolling velocity and increased rolling flux, adhesion, and emigration was observed (Orden *et al.*, 2014). Leukocyte emigration was confirmed by hematoxylin- and eosin-stained mesenteric tissue, in which a significantly higher number of infiltrated leukocytes, primarily PMNs, were seen.

In a study of hematological parameters of male Wistar rats, different groups were administered 0, 3, 6, 12, or 24 mg/kg drug once daily for up to 25 days; however, the drugs used were a fixed-dose combination of 150 mg lamivudine, 300 mg zidovudine, and 200 mg nevirapine and it was not stated what the dose represented (Nubila *et al.*, 2012). Some changes were found at the individual timepoints, but they did not seem to follow a dose-response pattern. In rats treated with 12 mg/kg, over time, red blood cell (RBC) counts, platelets, and lymphocytes were increased, and MCHC and neutrophils were decreased. Overall, the observed effects were quite small.

In a study using female Brown Norway rats, nevirapine was administered in food at a dose of about 150 mg/kg/day for up to 21 days (Popovic *et al.*, 2006). Ear-draining auricular lymph nodes were analyzed, which were chosen because the ears of these rats have been shown to turn red after about 7 days of nevirapine treatment, preceding the appearance of rash at 14-21

days. Total cell count CD4^{+} T cell, CD8^{+} T cell, B cell, and macrophage counts and percentages were elevated in these lymph nodes of treated rats at days 7, 14, and 21 by flow cytometry. Expression of ICAM-1 and MHC II was increased overall, and MHC II expression was also shown to be increased in B cells and macrophages. Immunohistochemical staining confirmed those findings for ED1 (macrophages), ICAM-1, and MHC I and II at day 7, with a greater increase at day 14. Macrophage infiltration was noted to precede T cell infiltration, which occurred at 14 days.

    d. Clozapine

   In a prenatal MAM schizophrenia model, clozapine (20 mg/kg/day, i.p.) attenuated increased peripheral blood granulocytes in the MAM-exposed group after 8 days (Fiore *et al.*, 2008). In the absence of MAM exposure, clozapine treatment did not have significant effects.

   In male C57BL/6J mice treated with MPTP, co-treatment with clozapine (1 mg/kg/day, s.c.), but not clozapine *N*-oxide (1 mg/kg/day, s.c.), for 3 weeks resulted in a significant decrease in total leukocyte counts compared to the MPTP control group (Jiang *et al.*, 2016). In the absence of MPTP administration, clozapine still caused the decrease in leukocytes, compared to control animals.

   In a female C57BL/6 mouse EAE model, no effect of clozapine (60 mg/kg/day, p.o. [administered in drinking water]) on immune cell populations in the spleen were observed, but clozapine attenuated EAE-induced increases in T cells and macrophages in the spinal cord by day 11 (Green *et al.*, 2017). No effects of clozapine were observed in the absence of EAE.

   In a second EAE model, clozapine (60 mg/kg/day, p.o. [administered in drinking water]) significantly attenuated increases in infiltrating neutrophils and monocytes in the spinal cord, brain, blood, and spleen (Robichon *et al.*, 2020). No significant cell alterations were observed with clozapine in the absence of EAE. In this study, female C57BL/6 mice were also treated with clozapine (60 mg/kg/day, p.o. [administered in drinking water]) for 1 week, following which, animals received a single injection of CCL2 or CCL5 and immune changes in the local draining lymph nodes were evaluated 18 hours later. Compared to CCL2 treated controls, clozapine greatly attenuated increased total cell numbers, T cells, and neutrophils in draining lymph nodes. No changes were noted with CCL5 treatment.

   In a K/BxN mouse arthritis model, clozapine (4 mg/kg/bid, p.o.) did not affect leukocyte counts (Nent *et al.*, 2013). Clozapine (25 mg/kg, s.c.) administered twice a day also did not modulate sepsis-mediated neutrophil infiltration in the liver, lung, or ileum of male Wistar rats, as measured by myeloperoxidase activity (Machado *et al.*, 2007). However, cardiac myeloperoxidase activity was markedly upregulated following administration of male Wistar rats with clozapine (10, 15, or 25 mg/kg/day, i.p.) for 21 days (Abdel-Wahab and Metwally, 2014).

   Following 21 days of clozapine (20 mg/kg/day, i.m.) administration, Sprague Dawley rats showed a marked increase in total WBC and neutrophil counts and a decrease in lymphocyte counts compared to saline-treated controls, while no significant changes in erythrocyte morphology or haematological indices were observed (Wasti *et al.*, 2006).

   Increased blood monocytes, in addition to decreased lymphocytes and total leukocytes, were reported following 3 weeks of clozapine (5, 10, or 20 mg/kg/day, i.p.) in female BALB/c mice (Abdelrahman *et al.*, 2014). At the highest dose, this was also accompanied by a significant reduction in neutrophils and erythrocytes.

   Clozapine (30 mg/kg/day, i.p.) administration in female Sprague Dawley rats resulted in significantly elevated blood neutrophil and monocyte counts, beginning on days 8 and 9, respectively, while no changes in leukocytes or lymphocytes were reported (Lobach and

Uetrecht, 2014a; Ng *et al.*, 2014). A transient neutrophil spike was also reported after day 1. This was accompanied by an expansion of the bone marrow myeloid, but not erythroid or lymphoid, compartment on day 10, as well as an increase in the efflux rate of less mature neutrophils from the bone marrow. Specifically, clozapine caused an increase in granulocyte production and myeloperoxidase-positive cells, concentrated in the paratrabecular zone, and an increase in megakaryocytes, in addition to more prominent sinuses. Clozapine also increased the number of mature cells in the bone marrow, as well as the number of CD18$^+$ and CD11b$^+$ cells.

Although not a rodent study, with clozapine (30 mg/kg/day, s.c.) treatment in female New Zealand White rabbits, the half-life of peripheral blood neutrophils was significantly increased after 2 days, but was significantly decreased after 10 days (Iverson *et al.*, 2010). Absolute neutrophil counts were transiently increased around day 2. Moreover, the efflux rate of neutrophils from the bone marrow was enhanced with clozapine.

In female Sprague Dawley rats treated with clozapine (30 mg/kg/day, i.p.) an increase in covalent binding to neutrophil proteins was observed, plateauing by day 4 (Lobach and Uetrecht, 2014b). No binding to lymphocytes was observed. At the end of 6 weeks of clozapine (40 mg/kg/day, p.o. [in rodent meal]) treatment in female Lewis rats, substantial covalent binding to a 49 kD protein was noted in bone marrow neutrophils that was not present in rats fed normal rodent meal (Gardner *et al.*, 1998).

Following 2 months of clozapine (50 mg/kg/day, p.o. in rodent meal) treatment in female Sprague Dawley rats maintained on a selenium sufficient or deficient diet, no effect on GSH peroxidase activity in whole blood was observed, although significant covalent binding in the bone marrow of clozapine treated animals was detected, irrespective of diet (Ip and Uetrecht, 2008). Weekly peripheral blood leukocyte and neutrophil counts were also unaltered.

2.   Clinical Studies
    a.   Amoxicillin

In children with presumed bacterial or mycoplasmic lower respiratory tract infections treated with 60 mg/kg/day amoxicillin for 10 days, *ex vivo* lymphocyte lytic activity did not change with amoxicillin treatment (Agostoni *et al.*, 1988).

In a phase I clinical trial, healthy male volunteers were given a single dose of 785 mg amoxicillin orally (Gomez-Lus *et al.*, 1998). The killing activity of PMNs isolated from peripheral blood were measured *ex vivo*. Amoxicillin treatment alone did not affect PMN killing, but in combination with serum, bactericidal activity was significantly enhanced, even after amoxicillin concentrations fell below inhibitory concentrations.

In a study designed to examine the effects on immune parameters in healthy individuals, adult males were administered oral amoxicillin (1 g) and clavulanate potassium (125 mg) twice daily for 5 days (Dufour *et al.*, 2005). No changes were observed in white blood cell subsets, NK cell cytokine production, or polynuclear phagocyte activity up to 61 days after starting treatment.

    b.   Nevirapine

Multiple studies involving nevirapine report changes in blood cell counts, but these tend to be in combination with other drugs. Leukopenia responsive to G-CSF treatment has been described, but in these cases zidovudine was part of the treatment regimen which has been suspected of being the causative agent (Yamamoto *et al.*, 2000; Shahar *et al.*, 2004). Similarly, trials of nevirapine and zidovudine combinations for the prevention of perinatal HIV transmission have shown an increase in neutropenia with extended co-treatment of nevirapine and zidovudine (Kumwenda *et al.*, 2008), while neutropenia was attributed to zidovudine

exposure in a study in which some patients were also exposed to single dose nevirapine (Read *et al.*, 2007).

Another example of combination therapy associated with increased risk of blood cell adverse effects is nevirapine and lumefantrine. In an open-label clinical trial in Malawi, patients who were already taking antiretroviral therapy were given lumefantrine in addition to their current therapy, first at half the target dose, then increased to the full dose after 28 days (Banda *et al.*, 2018). Neutropenia was observed in all study groups, but thrombocytopenia was only observed in combination with nevirapine, leading to the recommendation not to titrate the dose of lumefantrine further.

Infants of mothers from the HIV-1 Protective Immunity and Perinatal Exposure Study trial in South Africa were studied up to 12 weeks after birth (Schramm *et al.*, 2010). Infants of HIV-positive mothers received no antiretroviral therapy, single dose nevirapine (the mother was sometimes also given single dose nevirapine at onset of labour if their HIV status was known before delivery), or triple antiretroviral therapy. At birth, nevirapine-treated infants had higher monocyte counts and percentages and basophil counts; these differences did not persist at 6 weeks. Infants whose mothers had a CD4 T cell count >500 cells/µL had higher WBC, monocyte percentages and monocyte and basophil counts but lower lymphocyte and eosinophil percentages if they were exposed to single dose nevirapine than those who were unexposed.

c.   Clozapine

In a retrospective analysis in the United States of 118 hospitalized schizophrenic patients treated with clozapine patients for a minimum of 3 weeks, it was found that female patients were significantly more likely to develop eosinophilia compared to male patients, which was experienced by 23% of female and only 7% of male patients (Banov *et al.*, 1993). Additionally, eosinophilia was always experienced during weeks 3 to 5 of clozapine initiation and resolved with continued treatment. A similar retrospective analysis in a United Kingdom-based population of 160 schizophrenic patients (105 male and 55 female) initiating clozapine therapy was conducted, where 13% of patients (17 male and 4 female) developed eosinophilia between weeks 2 and 4 of clozapine treatment, with peak eosinophil levels reported after 7.5 weeks (range: 3-19 weeks) (Chatterton, 1997). The mean dose at the onset and peak of eosinophilia were 188 mg/day (range: 25-350 mg/day) and 227 mg/day (range: 0-600 mg/day), respectively. All except for 1 patient had resolution of eosinophilia with continued treatment.

Following retrospective analysis of 17 cases of clozapine-induced fever, 4 cases of leukocytosis, 3 cases of bandemia, 2 cases of neutrophilia, 1 case of eosinophilia, and 1 case of monocytosis were noted during the first month of treatment (Tham and Dickson, 2002). Similarly, in a retrospective review of 31 cases (19 male and 12 female) of clozapine-induced fever, 58% of patients had an elevated leukocyte count and 10% of the patients had an elevated eosinophil count (Pui-yin Chung *et al.*, 2008).

In a 6-week study of 14 male and 13 female patients diagnosed with schizophrenia, schizophreniform disorder, or schizoaffective disorder, transient elevations in total leukocyte and granulocyte counts were observed after the second week of treatment, while monocyte and lymphocyte counts remained stable over the course of the study (Pollmacher *et al.*, 1996). Mean clozapine doses were 162 ± 68, 245 ± 115, and 305 ± 160 mg/day at weeks 1, 2, and 6 respectively. Moreover, this group noted increased granulocyte and monocyte counts in a study of 20 schizophrenic patients and these levels were most prominent 2 weeks after commencing clozapine therapy (Pollmacher *et al.*, 1997).

However, an earlier study of 10 schizophrenic patients and 8 healthy controls reported that clozapine did not significantly influence total leukocyte, granulocyte or lymphocyte counts, measured at 1, 2, and 6 weeks of treatment (Pollmacher *et al.*, 1995). Mean clozapine doses were 188 ± 89, 285 ± 138, and 343 ± 169 mg/day, respectively. Likewise, this group did not find any changes in blood cell populations in an investigation of 6 male and 6 female patients with schizophrenia who were monitored for the first 6 weeks of clozapine treatment (Hinze-Selch *et al.*, 2000). In this study, the mean clozapine dose increased from 85 ± 59 mg/day at week 1 to 231 ± 105 mg/day at week 6.

One study reported a negative correlation between monocyte ROS release and the dose of clozapine administered to schizophrenia patients (1 male and 7 female), occurring 3 weeks into treatment, at which time the mean dose was 184 ± 35 mg/day (150–250 mg/day) (Gross *et al.*, 2003). Patients in this study were all started on clozapine at a dose of 50 mg/day, which was subsequently increased by 50 mg/day every 3 days until symptom remediation was observed.

Peaking at 2 weeks of treatment, a significant increase in circulating $CD34^+$ hematopoietic cells, neutrophils and leukocytes was observed in a study of 8 schizophrenia patients starting clozapine therapy (titrated from 50 mg/day up to doses where serum level of 350-600 ng/mL) (Loffler *et al.*, 2010).

F.   Effects on Mediators of Inflammation and Related Concepts
    1.   Rodent Studies
      a.   Amodiaquine

In the bacterial fulminant model of hepatitis, elevated serum levels of TNF-α were significantly dampened at 3 hours with co-administration of amodiaquine (5 mg/kg, s.c.) in C57BL/6 mice (Yokoyama *et al.*, 2007). However, this was not accompanied by any significant changes in increased mRNA levels of TNF-α, IFN-γ, IL-12, or IL-18 in the liver. In the ICH model, amodiaquine significantly suppressed ICH-induced increases in mRNA expression of IL-1β, CXCL2 and CCL2 at 6 hours, compared to vehicle control (Kinoshita *et al.*, 2019).

In another study with amodiaquine (~200-300 mg/kg/day in rodent meal) treatment in female C57BL/6 mice, it was found that serum cytokines IL-1α, IL-12 (p40), and IL-17 were elevated at week 1 with treatment (Metushi *et al.*, 2015). Notably, IL-3, IL-5, IL-10, IL-12(p70), IL-13, IFN-γ, TNF-α, and G-CSF were all significantly downregulated by week 7. Over the first 4 weeks of amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) treatment in male Brown Norway rats, this group also noted spikes in serum levels of IL-2, IL-5, IL-9, IL-12, TGF-β1, and CCL2, which were not observed in saline-treated controls (Liu *et al.*, 2016). After week 5, hepatic RANTES and IL-18 were significantly increased in the amodiaquine-treated group, but IL-2, IL-5, IL-6, and IL-12 levels were significantly decreased compared to control. Following amodiaquine rechallenge, additional increases in serum IL-4, IL-13, and IFN-γ were also observed.

      b.   Amoxicillin

An acute otitis media model using male Sprague-Dawley rats found that treatment with amoxicillin, administered in drinking water to a dose of about 51 mg/kg/day for 5 days, induced changes in osteocalcin (Melhus and Ryan, 2004). Osteocalcin is produced by osteoblasts and is a marker of bone formation. The maximum level of this marker in bullar bone was decreased and delayed compared to untreated animals. No change in expression of IL-6, TNF-α, or IL-10 was noted.

In LPS-induced mucus hypersecretion in male Sprague-Dawley rats, 40 mg/kg amoxicillin administered orally for 4 days did not alter cytokine (IL-1β, IL-8, and TNF-α) levels in bronchoalveolar lavage fluid (Ou *et al.*, 2008).

c. Nevirapine

In rats administered nevirapine and/or LPS, serum IL-2, IFN-γ, and TNF-α were measured (Bekker *et al.*, 2012). Nevirapine treatment did not cause a significant change in serum IL-2 levels up to 24 hours, although co-treatment with LPS attenuated the increase in IL-2 that was observed in the LPS group. No significant difference was observed in serum IFN-γ levels up to 24 hours. Nevirapine treatment caused a significant increase in serum TNF-α levels at 24 hours; this was attenuated with LPS co-treatment. Interestingly, coadministration of nevirapine with LPS may have altered the pharmacokinetics of nevirapine – the 24-hour nevirapine serum level was higher than the 6-hour level with co-treatment, while the opposite was observed with nevirapine alone. With chronic treatment, nevirapine treatment caused a significant increase in serum IL-2 at day 21 compared to nevirapine and LPS, while there was a non-significant increase in serum TNF-α at day 7 in the nevirapine and LPS group compared to the nevirapine group. No changes were observed with IFN-γ. Unfortunately, no results were shown for controls or LPS treatment only. Again, pharmacokinetics differed with LPS coadministration: while nevirapine serum levels were lower at weeks 2 and 3 compared to week 1 with nevirapine only, nevirapine levels were similar at weeks 1, 2, and 3 with co-treatment. The authors note that LPS has been reported to inhibit CYP450 enzymes; they also suggest that LPS has effects on the innate immune system, so the combination of these effects may explain the observations observed in the co-treated groups.

In female Brown Norway rats treated with 150 mg/kg/day nevirapine in food, serum IFN-γ was not changed during the primary exposure up to 21 days, although it was elevated upon rechallenge (Popovic *et al.*, 2006). In a similar study, lymphocytes from auricular lymph nodes of female Brown Norway rats treated for up to 21 days showed increased production of CXCL1, CCL3, IL-10, IL-18, and CCL5 (Chen *et al.*, 2009). In the same animal model but at 6 hours following 150 mg/kg nevirapine administered by gavage, mRNA in the liver showed changes in expression of some immune-related genes, such as Zap70 (associated with control of immune tolerance), FK506 binding protein (Fkbp5 or immunophilin, involved in immunoregulation), or Irgm M; or protein folding, such as FKbp5 and ER degradation enhancer and mannosidase alpha-like 1 (Edem 1) (Zhang *et al.*, 2013). In the skin, 12-hydroxynevirapine (159 mg/kg by gavage) treatment increased expression of over 2,000 genes after 6 hours of treatment, including TRIM63 (a ubiquitin ligase), Fkbp5, IL-22 RA2 (soluble IL-22 antagonist), and S100a7a (a DAMP).

In female mice treated with 3.3 mg/kg/day of nevirapine by oral gavage for 8 weeks, markers of neuroinflammation was characterized in the hippocampus (Zulu *et al.*, 2018). Astrogliosis was detected by increased staining for glial fibrillary acidic protein, and IL-1β and TNF-α were increased. Tenofovir was also found to have a similar effect. These results suggested that nevirapine and tenofovir could induce inflammation in the brain. Nevirapine, but not tenofovir, was also found to decrease brain-derived neurotrophic factor in the hippocampus; the authors speculate that this may be due to a difference in the ability of the two drugs to inhibit DNA polymerase, as tenofovir is a weaker inhibitor.

Cbl-b is an E3 ubiquitin ligase that is involved in regulation of activation in many leukocytes (Lutz-Nicoladoni *et al.*, 2015). In male Cbl-b$^{-/-}$ mice treated with nevirapine in food for 7 days at a dose approximating 950 mg/kg/day, serum IL-6 and IFN-γ were increased with

treatment; the increase was greater if liver necrosis was noted by histology (Sharma *et al.*, 2012). These increases resolved by day 14.

           d. Clozapine

      Pre-treatment with clozapine (5 mg/kg, i.p.) completely prevented the induction of HSP70 immunoreactive cells triggered by PCP in the cingulate cortex of female Sprague Dawley rats, observed at 24 hours (Sharp *et al.*, 1994). Likewise, pre-treatment with clozapine (20 mg/kg, i.p.) significantly reduced PCP-induced increases in hsp70 mRNA levels in cortex at 3 hours (Nakahara *et al.*, 1999). In the absence of PCP treatment, clozapine also decreased hsp70 MRNA in the prefrontal cortex, striatum, and nucleus accumbens. In a similar model, the induction of both c-fos and HSP70 immunoreactive brain cells at 24 hours by dizocilpine was dose-dependently inhibited by clozapine (0.31, 0.62, 1.25 mg/kg, s.c.) pre-treatment in female BKTO mice (O'Neill *et al.*, 1998).

      After 65 days of clozapine (20 mg/kg/day, p.o. [administered in drinking water]), male PCP-exposed clozapine treated rats had increased serum corticosterone concentrations and both male clozapine groups had increased serum TNF-α concentrations, while female PCP-exposed clozapine treated rats had decreased serum corticosterone concentrations and both female clozapine groups had increased serum IL-6 concentrations (Nikolic *et al.*, 2017).

      After 90 minutes, clozapine (30 mg/kg, p.o.) significantly damped LPS- and polyI:C-induced increases in serum TNF-α and IL-6, but further increased IL-10 levels (Sugino *et al.*, 2009). In the absence of LPS or polyI:C, clozapine did not affect levels of proinflammatory mediators, but did increase serum levels of IL-10 compared to saline-treated controls.

      In a male rate hepatic I/R model, clozapine (15 mg/kg, s.c.) pre-treatment significantly reduced hepatic IL-12 (p70) and TNF-α levels compared to I/R control animals at 2 hours post-ischemia (El-Mahdy *et al.*, 2013).

      In a female C57BL/6 mouse model of EAE, clozapine (60 mg/kg/day, p.o. [administered in drinking water]) significantly attenuated increases in CCL2 and CCL5 protein levels in the brain, and mRNA levels in both the brain and spinal cord (Robichon *et al.*, 2020). Clozapine did not affect EAE-induced changes in CCL2 or CCL5 levels in the blood or spleen, nor did clozapine have any effect in the absence of EAE.

      After 2 weeks, clozapine (5 mg/kg/day, i.p.) completely reversed social isolation-induced reductions in plasma IL-4, IL-6, INF-γ, and kynuric acid and elevations in TNF-α, kynurenine, and quinolinic acid in male Sprague Dawley rats (Moller *et al.*, 2013).

      No changes in IL-1β or COX-2 hippocampal protein levels were observed after 3 weeks of clozapine (20 mg/kg/day, i.p.) treatment in male Wistar rats, but decreased TNF-α levels were observed in clozapine-treated rats that were also socially isolated for the duration of the study (Todorovic and Filipovic, 2017b). In the prefrontal cortex, clozapine alone had no effect, but significantly attenuated elevated IL-1β, TNF-α, and COX-2 levels induced by social isolation (Todorovic and Filipovic, 2017a).

      Clozapine (25 mg/kg, s.c.) given twice daily was not effective in reducing sepsis-induced elevations in serum IL-1β, TNF-α, or IL-10 in male Wistar rats (Machado *et al.*, 2007). It appears as though clozapine may have exacerbated the increases in TNF-α and IL-10, but a statistical comparison of clozapine and the appropriate control group was not provided.

      In male Wistar rats, clozapine (2, 8, 32 μmol/kg, s.c. [0.65, 2.61, 10.46 mg/kg]) paradoxically reduced core body temperature by up to 2°C (Oerther and Ahlenius, 2000).

Five hours after administration of clozapine (20 mg/kg, i.p.) to male hooded Long Evans rats, mRNA expression levels of TNF-α were significantly reduced in the prefrontal cortex with treatment but were not altered in other brain regions (Paterson *et al.*, 2006).

Clozapine (30 mg/kg/day, i.p.) administration in female Sprague Dawley rats resulted in transiently elevated serum G-CSF levels at 3 and 6 hours after the first dose and increased CXCL2 expression in the bone marrow on day 10 (Lobach and Uetrecht, 2014a).

No changes in striatal TNF-α, norepinephrine, or dopamine were detected with clozapine (5 or 10 mg/kg/day, i.p.) in male Wistar rats (Bishnoi *et al.*, 2008, 2011). Conversely, another study showed that 2 weeks of clozapine (15 mg/k/day, i.p.) treatment in male C57BL/6 mice caused a moderate increase in cingulate cortex norepinephrine (Ookubo *et al.*, 2013).

In male BALB/c mice, significantly elevated serum levels of epinephrine and norepinephrine were observed from 1 to 2 weeks with clozapine (5, 10, and 25 mg/kg/day, i.p.) (Wang *et al.*, 2008). In the heart tissue, TNF-α levels were also increased with clozapine compared to saline controls but were only measured after 2 weeks of treatment and may have been higher during peak inflammatory lesion observation at day 7. Co-administration of propranolol significantly dampened all increased mediators after 2 weeks.

After 21 days, clozapine (10, 15, or 25 mg/kg/day, i.p.) elevated serum and cardiac 8-oxo-2'-deoxyguanoside levels in male Sprague Dawley rats (Abdel-Wahab and Metwally, 2014; Abdel-Wahab *et al.*, 2014). Clozapine also caused elevations in cardiac MDA, 3-nitrotyrosine, nitric oxide, nitrite, and TNF-α. Elevated levels of serum TNF-α and reduced IL-10 were also reported, and inflammatory effects were attenuated by co-administration of captopril.

After 21 days of treatment in male Wistar rats, clozapine (10 mg/kg/day, i.p.) caused a number differentially-expressed proteins in the nucleus accumbens, including increased levels of HSPA8, HSP75, and GSH synthase (Kedracka-Krok *et al.*, 2016).

Male Sprague Dawley rats treated with clozapine (20 mg/kg/day, i.p.) for 30 days were reported to have reduced cerebrospinal fluid levels of IL-8 versus saline controls, while kynurenic acid levels were unaffected (Larsson *et al.*, 2015)

Clozapine (0.5 mg/kg/day, p.o.) administration for 30 days resulted in increased expression of renal kidney injury molecule-1 and tissue inhibitor of metalloproteinase-1 mRNA (Mohammed *et al.*, 2020). Additionally, serum protein levels of IL-6, IL-1β, TNF-α, and MDA were elevated with clozapine. Except for clozapine-induced MDA increases, all parameters were attenuated by co-administered with sulpride.

2. Clinical Studies
a. Amoxicillin

In healthy adult males administered oral amoxicillin (1 g) and clavulanate potassium (125 mg) twice daily for 5 days, no change in the proportion of TNF-α-expressing CD14^{+} cells in the blood and no change in lactoferrin or lysozyme levels in the serum or feces was observed up to 61 days after beginning treatment (Dufour *et al.*, 2005).

b. Nevirapine

A study examined the activation of T cells isolated from women up to 6 weeks after delivery (Shalekoff *et al.*, 2009). Women were HIV-positive and received single dose nevirapine if HIV status was known before delivery; controls were identified as HIV-positive after delivery and had therefore not received nevirapine. Nevirapine was associated with decreased plasma CCL3 and IL-8, but not IL-15 or TNF-α.

In a study combining patients from three clinical trials in which IL-6 levels in HIV-positive patients were studied, nevirapine exposure was correlated with lower plasma IL-6 levels

compared to efavirenz exposure (IL-6 was observed to be highest in untreated patients) (Borges *et al.*, 2015). Nevirapine treatment is associated with lower HIV RNA levels compared to other NNRTIs, which might help to explain the observed effect. Randomization to protease inhibitor- or NNRTI-based therapies was not performed in these trials.

A study examined the levels of soluble CD14 (sCD14) in plasma of HIV-positive patients in Nantes, France (Allavena *et al.*, 2013). Patients were taking triple antiretroviral therapy including either nevirapine or efavirenz. Patients on nevirapine-based therapy had a median plasma sCD14 level of 1.7 mg/mL, while those on efavirenz-based therapy had a level of 1.9 mg/mL. However, there was no control group and the study was not randomized; patients on efavirenz were significantly younger, had a shorter time on ARV than those on nevirapine. A few comparisons suggested that sCD14 levels were not related to viral load.

A study examined soluble markers of immune activation in HIV-positive mothers and infants, and examined the effect of exposure to nevirapine: some mothers had not received single-dose nevirapine before giving birth, while some had; HIV-negative mother/child pairs were also used as a control (Schramm *et al.*, 2006). Neopterin and soluble L-selectin levels in cord blood in exposed, uninfected infants were significantly higher with maternal nevirapine exposure. No differences in neopterin, soluble L-selectin, or β_2-microglobulin levels were observed in infants infected intrapartum (HIV-negative at birth, but HIV-positive at 6 weeks of age). Neopterin and β_2-microglobulin levels were increased with nevirapine exposure in infants infected *in utero* (HIV-positive at birth). The same markers were measured in maternal plasma drawn at the same time as the cord blood; the only difference was an increase in neopterin levels with nevirapine exposure in mothers whose infants were infected *in utero*. No correlations between maternal and infant levels for any of the three analytes was found, suggesting that immune activation in the infant occurs independent of the maternal environment. The authors suggest that single-dose nevirapine induces T cell anergy, which may aid in preventing HIV-1 replication. Additionally, the authors propose that nevirapine may synergize with HIV-1 to increase immune activation.

Two studies have been performed evaluating the effect of genetic variations in some immune markers. A study examining the effect of CCL3L1 (a CCR5 ligand) gene copies on HIV mother-to-newborn transmission found that maternal nevirapine was associated with decreased spontaneous and phytohemagglutinin-stimulated release of CCL3 in cord blood mononuclear cells from uninfected infants (Kuhn *et al.*, 2007). Additionally, greater infant CCL3L1 gene copies were associated with reduced HIV transmission overall, but this affect was attenuated with maternal single-dose nevirapine exposure. Another study using cohorts in Malawi, South Africa, and Uganda examined multiple chemokine receptor polymorphisms in the context of maternal HIV transmission (Singh *et al.*, 2008). Mother-to-child transmission was reduced with nevirapine treatment with CCR5-59029-G/A or CCR5-59353-T/C, in which both polymorphisms were associated with an increased risk compared to wildtype. Transmission risk was increased with nevirapine treatment for the CX3CR1-745-G/A (249-V/I) (280-T/M) polymorphism. In both cases, nevirapine appears to have an effect on the role of certain cytokines in HIV transmission.

In a study of 11 HIV-positive children initiated on triple therapy with stavudine, ritonavir, and nevirapine for up 24 weeks, responses of blood cells to multiple stimuli were evaluated *ex vivo* (Blazevic *et al.*, 2001). At baseline, peripheral blood mononuclear cells from these children had a lower delayed-type hypersensitivity response to *Candida albicans* compared to peripheral blood mononuclear cells from healthy controls, which increased at week 24.

However, the response to tetanus toxoid was not observed in HIV-positive children at baseline (significantly lower than healthy controls) and did not change at 24 weeks. In evaluating APC function, monocytes from HIV-positive patients produced significantly less IL-12p70 in response to *Staphylococcus aureus* Cowan compared to healthy controls; at 24 weeks, IL-12p70 secretion was increased. However, these effects cannot be attributed to nevirapine therapy alone, as a combination of drugs was administered to these patients; additionally, the effect of treatment and restitution of CD4 T cells also likely plays an important role in these effects.

c.   Clozapine

In a retrospective analysis of 93 schizophrenia or schizoaffective disorder patients (65 male and 28 female) initiated on clozapine in Canada, it was noted that 20% of patients developed fever during first month treatment, lasting an average of 2 weeks (Tham and Dickson, 2002). The majority of fever onset was observed from weeks 1 to 3, although a range from 3 to 26 days was reported. Similarly, in a retrospective review of 227 patients (113 male and 94 female) commencing clozapine therapy in Hong Kong, 14% (19 male and 12 female) developed a fever in first 3 weeks of treatment that lasted around 5 days (range: 1-12 days) (Pui-yin Chung *et al.*, 2008). The mean time to fever onset was 14 days (range: 5-21 days, plus one case at day 47) and mean clozapine dosage at the onset of fever was 166.1 ± 73.2 mg/day (range: 50-300 mg/day).

In a study of 10 schizophrenic patients (7 males and 3 females) and 8 healthy controls (6 males and 2 females), a marked increase in soluble IL-2R levels was observed from weeks 2-6 of treatment, when mean clozapine doses were 285 ± 138 and 343 ± 169 mg/day, respectively (Pollmacher *et al.*, 1995). To characterize these immune changes in more detail, a second 6-week study of 14 male and 13 female patients diagnosed with schizophrenia, schizophreniform disorder, or schizoaffective disorder was conducted (Pollmacher *et al.*, 1996). Compared to pre-clozapine baseline levels, plasma levels of TNF-α, soluble TNF-R p55, soluble TNF-R p75, and soluble IL-2R were elevated for the duration of the study, measured at week 1 (mean clozapine dose: 162 ± 68 mg/day), week 2 (mean clozapine dose: 245 ± 115 mg/day), and week 6 (mean clozapine dose: 305 ± 160 mg/day) of treatment. Additionally, plasma IL-6 levels were transiently increased at week 2 of treatment and 44% of patients developed a fever that lasted up to 6 days.

This group replicated these findings in a study of 6 male and 6 female patients with schizophrenia who, again, were monitored during initiation of clozapine therapy (Hinze-Selch *et al.*, 2000). Soluble TNF-R p55 and p75 were increased with clozapine from weeks 1 to 6 and TNF-α and soluble IL-2R were elevated from week 2 onward. Of 20 schizophrenia patients monitored during the first 6 weeks of clozapine therapy, 55% developed a transient fever and increase in plasma G-CSF levels that was most prominent following 2 weeks of treatment (Pollmacher *et al.*, 1997). Similarly, fever was reported in 47% of schizophrenic patients monitored during the first 2 weeks of clozapine treatment (Hinze-Selch *et al.*, 1998). In this study, mean time to fever onset was 15 ± 5 days (range: 2-25 days) with a mean duration of 2.5 ± 1.3 days (range: 1-6 days). The average clozapine dose increased from 178 ± 57 mg/day at week 1, to 281 ± 111 mg/day at week 2, to 325 ± 152 at week 6.

Another group conducted a study with 17 schizophrenic patients (7 male and 10 female) started on clozapine and sex- and age-matched healthy controls (Monteleone *et al.*, 1997). After 10 weeks (mean clozapine dose: 312 ± 120 mg/day [range: 75-400 mg/day]), no differences in plasma IL-6 levels were observed with clozapine, but TNF-α levels that had been elevated in the schizophrenia group at baseline were dampened to control levels. Cytokine changes were also

evaluated in a study of 23 schizophrenic patients (13 males and 10 females) before and after starting clozapine (Maes *et al.*, 1997). Compared to healthy controls (11 males and 6 females), clozapine-naïve patients showed significantly higher plasma IL-6R and IL-1RA and lower uteroglobin levels. During the first 2 weeks of clozapine treatment, further increases in plasma IL-6 and increased uteroglobin occurred and after 5 weeks. Increased plasma levels of soluble CD8 and IL-1RA were observed.

In a study evaluating cytokine changes in 12 new clozapine users, it was demonstrated that while levels of IL-8 and IL-10 were unchanged, serum levels of soluble CD8 and leukemia inhibitory factor receptor were markedly elevated after 2 months of clozapine treatment, compared to baseline or 4-month treatment levels (Maes *et al.*, 2002). The mean clozapine doses at months 2 and 4 were 358.3 ± 144.3 mg/day and 395.0 ± 101.2 mg/day, respectively. A similar study compared cytokine levels of 22 new clozapine users (16 male and 6 female) to 21 patients who had been maintained on clozapine for greater than 6 months (14 male and 7 female) (Hung *et al.*, 2017). Compared to 6% of old users, 47% of new users developed fever during weeks 1 to 4, lasting an average of 2ays. Although no differences were observed in cytokine levels between new and old clozapine users, compared to new users without fever, plasma levels of TNF-α, INF-γ, IL-2, and IL-6 levels were significantly different in the patients who developed clozapine-induced fever.

Another group conducted a 6-week randomized double-blinded trial to compare the effects of clozapine (mean modal dose: 266.7 ± 77.9 mg/day) and olanzapine (mean modal dose: 21.2 ± 2.5 mg/day) in patients with schizophrenia, schizophreniform disorder or schizoaffective disorder (Kluge *et al.*, 2009). Thirty patients were randomized to either the clozapine (7 male and 8 female) or olanzapine (5 male and 10 female) group. Five patients treated with clozapine developed fever during the first 3 weeks of treatment, which lasted up to 5 days, while no patients treated with olanzapine developed fever. Compared to baseline values, plasma TNF-α, soluble TNFR-2, and soluble IL-2R remained significantly elevated from week 2 to 6 with both treatments and peaked around week 3, although levels of soluble TNFR-2 were markedly higher with clozapine compared to olanzapine. Additionally, IL-6 and soluble TNFR-1 levels were only elevated with clozapine treatment during weeks 2 to 3 and weeks 2 to 6, respectively. Moreover, at week 2 of clozapine treatment, IL-6 and soluble TNFR-2 were significantly higher in patients with fever compared to those without fever. Lastly, plasma leptin levels were significantly increased in females for the entire study, while levels in males were transiently elevated during weeks 2 and 3 with clozapine.

G. Effects on Applicable Signal Transduction Factors
   1. Rodent Studies
    a. Amoxicillin

In male Sprague-Dawley rats administered an intratracheal instillation of LPS to induce mucus hypersecretion and administered 40 mg/kg amoxicillin orally for 4 days, there was no difference in NF-κB expression in bronchial epithelium (Ou *et al.*, 2008).
    b. Clozapine

Using the PCP pre-treatment model in male C57BL/6 mice, it was shown that 1 week of clozapine (10 mg/kg/day, p.o.) administration enhanced PCP-induced decreases in cytosolic Nrf2 and increases in nuclear Nrf2 protein levels in the prefrontal cortex (Tran *et al.*, 2017). However, after day 1, clozapine significantly attenuated PCP-induced mitochondrial translocation of phosphorylated AKT (Tran *et al.*, 2018). In the postnatal model of schizophrenia using male

C57BL/6 mice, it was found that co-administration of clozapine (6 mg/kg/day, i.p.) with PCP greatly decreased the phosphorylation ratio of AKT (also called protein kinase B) in the cerebral cortex compared to vehicle-treated PCP controls, although the phosphorylation ratio of extracellular signal-regulated kinase (ERK) was unchanged (Barzilay *et al.*, 2011).

One study evaluated the impact of 3 weeks of clozapine (1 mg/kg/day, s.c.) pre-treatment on nigral NF-κB levels in male Sprague Dawley rats followed by apomorphine stimulation (Saldana *et al.*, 2006). Clozapine induced a significant decrease in nigral NF-κB p65 and p50 protein levels compared to the apomorphine-stimulated, saline-pre-treated control group. Contrary to this, no changes in striatal NF-κB p65 protein levels were detected following 3 weeks of clozapine (5 or 10 mg/kg/day, i.p.) administration in male Wistar rats (Bishnoi *et al.*, 2008, 2011).

Dose-dependent elevations in cardiac NF-κB p65 were observed following 3 weeks of clozapine (10, 15, or 25 mg/kg/day, i.p.) treatment in male Wistar rats (Abdel-Wahab and Metwally, 2014). A similar outcome was noted in another study, where socially isolated male Wistar rats given clozapine (20 mg/kg/day, i.p.) for 3 weeks had decreased cytosolic and increased nuclear NF-κB p65 protein levels in the liver, versus saline controls (Zlatkovic *et al.*, 2014). This, however, was not observed the prefrontal cortex, where clozapine significantly attenuated social isolation-induced reduced cytosolic and elevated nuclear NF-κB p65 protein levels (Todorovic and Filipovic, 2017a). No changes in cytosolic or nuclear NF-κB p65 hippocampal protein levels were observed in this model (Todorovic and Filipovic, 2017b).

One hour following clozapine (12 mg/kg) administration in female Sprague Dawley rats, hepatic eIF2α phosphorylation was significantly increased, suggesting activation of the protein kinase R-like ER kinase/eukaryotic translation initiation factor 2A (PERK/eIF2α) ER stress axis (Weston-Green *et al.*, 2018). Additionally, in this model, hepatic sphingolipid homeostasis was significantly disrupted. Specifically, hepatic levels of ceramide and sphingolipids were markedly reduced with clozapine, while ceramide synthase and a fatty acid elongase enzyme were increased.

Male Sprague Dawley rats treated with a single dose of clozapine (10 mg/kg, i.p.) showed a marked increase in frontal cortex phospohorylated-Unc-51-like kinase (ULK) 1 (Ser317) and phosphorylated-Beclin1(Ser93) was demonstrated from 2 to 4 hours and an increase in phosphorylated-AMP-activated protein kinase (AMPK) α (Thr172) from 1 to 4 hours, although total levels of these protein were unchanged (Kim *et al.*, 2018). Moreover, AMPK substrates were increased from 1 to 4 hours. Co-treatment of rats with clozapine and the AMPK inhibitor dorsomorphin resulted in attenuation of these increases at 2 hours. Together, the AMPK-ULK1-Beclin1 signaling pathway is responsible for induction of autophagy, where phosphorylation of AMPK leads to its activation and subsequent phosphorylation of ULK, together leading to the activation of Beclin1 and the activation of autophagy components. However, AMPK has been shown to play a role in a number of biochemical pathways, including mitochondrial biogenesis and lipid metabolism (Hardie *et al.*, 2016) and thus, clozapine's impact on AMPK signaling likely extends beyond autophagy.

It was demonstrated that PPARα and LXRα (ligand-activated nuclear hormone receptors that normally control aspects of lipid homeostasis) mRNA levels were significantly decreased at 6 hours following clozapine (25 or 50 mg/kg, i.p.) administration in female Sprague Dawley rats, as compared to vehicle controls (Ferno *et al.*, 2009). Additionally, LXRα target gene cholesterol efflux gene ATP-binding cassette, sub-family A was significantly upregulated at this time. All changes resolved by 48 hours.

One study demonstrated that, when co-administered with ketamine in male Sprague Dawley rats, clozapine (5 mg/kg/day, i.p.) significantly attenuated decreased phosphorylated AKT and phosphorylated GSK-3β and attenuated increased phosphorylated β-catenin in several brain regions, compared to ketamine-treated controls (George *et al.*, 2020).

Thirty minutes following N-desmethylclozapine (20 mg/kg, s.c.) administration in male CD1 mice, increased phosphorylated AKT and phosphorylated AKT-glycogen synthase kinase (GSK) expression levels were reported in the nucleus accumbens, compared to saline controls. This effect was significantly antagonized by pre-treatment with the δ-opioid receptor antagonist naltrindole (Olianas *et al.*, 2011). Using C57BL/6 TLR-2$^{-/-}$ mice, however, it was demonstrated that the increased expression levels of phosphorylated GSK-3α/β observed in TLR-2$^{-/-}$ mice were markedly reduced in several brain regions 1 hour post-clozapine (1 mg/kg, i.p.) administration (Park *et al.*, 2015). Clozapine did not alter increased levels of phosphorylated AKT.

Another study showed that 2 weeks of clozapine (15 mg/k/day, i.p.) treatment in male C57BL/6 mice resulted in a number of brain region-specific changes in histone deacetylases (HDAC) (Ookubo *et al.*, 2013). Specifically, clozapine increased HDAC2, HDAC3, and HDAC8 in the striatum, HDAC2 and HDAC3 in the nucleus accumbens, and HDAC3 in cingulate cortex, compared to vehicle controls. Additionally, acetylated histone H3 protein expression levels were increased in several brain areas with clozapine.

Differences in histone-promoter binding in the frontal cortex were noted following administration of clozapine (10 mg/kg/day, i.p.) in male 129S6/Sv mice for 3 weeks (de la Fuente Revenga *et al.*, 2019). Clozapine induced a significant decrease of lysine-acetylated histone H3 and HDAC2 binding to the metabotropic glutamate 2 receptor, homer protein homolog 1, and glutamate receptor ionotropic, NMDA 1 gene promoter regions of wildtype, but not in clozapine-treated HDAC2$^{-/-}$ mice.

H. Effects on Mitochondria and Oxidative Stress
1. Rodent Studies
a. Amoxicillin

In male Wistar rats, amoxicillin (10 or 30 mg/kg/day, i.p.) was administered once daily for 14 days (Oyebode *et al.*, 2019). The higher dose resulted in significantly increased mitochondrial permeability transition pore opening, enhanced rat liver microsome ATPase activity, cytochrome C release, and increased levels of rat liver microsome malondialdehyde generation.

b. Nevirapine

A study using male CF-1 mice administered 3.3 mg/kg nevirapine by oral gavage for 36 days found that mitochondrial complex IV activity was inhibited in the cerebral cortex, striatum, and hippocampus, but not striatum (Streck *et al.*, 2011).

A study using male albino rats administered 6 mg/kg nevirapine orally for 60 days found no difference in serum catalase, superoxide dismutase, or glutathione (Awodele *et al.*, 2015). Increased serum malondialdehyde was reported, which is used as a measure of lipid peroxidation. This effect was not modulated by co-treatment with antioxidants vitamin C, vitamin E, or jobelyn.

A study of multiple organs in Wistar rats treated with 18 or 36 mg/kg for 4 weeks compared to vehicle control found that nevirapine caused a dose-dependent increase in malondialdehyde in the liver, kidney, and testes, which was accompanied by a decrease in superoxide dismutase and catalase activities and glutathione (Adaramoye *et al.*, 2012).

A number of ultrastructural changes were observed in the liver of female Brown Norway rats treated with 150 mg/kg nevirapine for 8 weeks (Sastry *et al.*, 2018). Degenerate mitochondria were observed in hepatocytes of treated rats. Lipid droplets associated with smooth endoplasmic reticulum cisternae were also observed in these hepatocytes.

In female Brown Norway rats treated with 159 mg/kg/day 12-hydroxynevirapine, transcripts related to mitochondria, including pyruvate dehydrogenase kinase, isozyme 4 (Pdk4), 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (Hmgcs2), and uncoupling protein 3 (Ucp3), were upregulated in the skin 6 hours post-dose (Zhang *et al.*, 2013).

c. Clozapine

One group demonstrated that coadministration of clozapine (10 mg/kg/day, i.p.) in male Sprague Dawley rats with either PCP or methamphetamine for 28 days greatly attenuated the decrease in cytochrome c oxidase activity observed in multiple brain regions observed with PCP or methamphetamine alone (Prince *et al.*, 1997b). Clozapine monotherapy also increased brain cytochrome c oxidase activity (Prince *et al.*, 1997a). Conversely, no changes in complex I activity were observed. Another group evaluated the effects of clozapine (10 mg/kg/day, p.o.) in male C57BL/6 wildtype or p47phox^{-/-} mice that had been pre-treated with PCP (Tran *et al.*, 2018). After day 1, clozapine attenuated PCP-induced mitochondrial changes in the prefrontal cortex of wildtype mice, and attenuated elevations in ROS and protein oxidation in both genotypes. Clozapine in mice without PCP treatment did not appear to have any significant effects.

In male Sprague Dawley rats treated with ketamine, 1 week of clozapine (5 mg/kg/day, i.p.) markedly attenuated decreased hippocampal GSH levels and decreased striatal catalase activity observed in the ketamine control group (George *et al.*, 2020).

One hour following administration of clozapine (25 mg/kg, i.p.) in male Sprague Dawley rats, activating transcription factor 3 (ATF3) mRNA levels in the liver were significantly elevated compared to animals in the water control group (Lauressergues *et al.*, 2012). ATF3 is a sensor of ER stress, which can initiate UPR through several downstream pathways. By 3 hours, ATF3 levels remained elevated, and increases in 3-hydroxy-3-methylglutaryl-coA reductase and tribbles drosophila homolog 3 (a marker of the PERK pathway of ER stress) were also observed.

With social isolation, male Sprague Dawley rats were given clozapine (5 mg/kg/day, i.p.) for 14 days and it was found that clozapine completely reversed social isolation-induced reductions in frontal cortical ATP and elevations in striatal ATP (Moller *et al.*, 2013). However, in another social isolation model in male Wistar, liver cytosolic CuZn-superoxide dismutase protein levels were decreased with clozapine (20 mg/kg/day, i.p.), irrespective of housing status during the study (Zlatkovic *et al.*, 2014).

Compared to controls, male Wistar rats treated with clozapine (25 mg/kg/day, i.p.) for 28 days displayed increased AMP hydrolysis in the striatum, but not in the hippocampus, at 24 hours following the final dose (Lara *et al.*, 2001).

Male Long Evans rats treated with clozapine (10 mg/kg/day, i.p.) for 21 days had neuronal expression of nitric oxide synthase mRNA that was significantly decreased compared to control levels (Bullock *et al.*, 2008).

One group demonstrated that treatment of male Wistar rats with clozapine (25 mg/kg/day, i.p.) for 4 weeks resulted in inhibition of succinate dehydrogenase activity in striatum, but not in other areas of the brain, and cytochrome oxygenase activity was unchanged (Streck *et al.*, 2007).

After 28 days of clozapine (25 mg/kg/day, i.p.) treatment and 3 rest days, no change in mitochondrial superoxide production was observed in several brain regions of male Wistars rats when compared to saline-treated rats (Martins *et al.*, 2008). However, protein carbonyl content (a measure of oxidative damage to proteins) was found to be increased with clozapine in the hippocampus, while thiobarbituric acid reactive substances (TBARS; a measure of lipid peroxidation) were decreased in the cerebral cortex with treatment. Another group investigated similar effects following 21 days of clozapine (10 mg/kg/day, i.p.) administration in male Wistar rats and found increased levels of striatal TBARS and superoxide anion and decreased levels of non-protein thiols and total nitric oxide in comparison to vehicle control rats (Bishnoi *et al.*, 2011).

One group observed multiple stress-related metabolic pathway alterations in prefrontal cortex after 4 weeks of clozapine (21 mg/kg/day, i.p.) administration in male Sprague Dawley rats (Cai *et al.*, 2017). Specifically, clozapine increased creatine, inosine, progesterone, and phosphatidylethanolamine levels, and decreased corticosterone levels in comparison to vehicle controls.

Hippocampal protein levels of malate dehydrogenase and vacuolar ATP synthase were demonstrated to be significantly reduced after 30 days of clozapine (45 mg/kg/day, p.o.) treatment in male Sprague Dawley rats compared to water-treated control animals (La *et al.*, 2006).

After 28 days of treatment in male Wistar rats, clozapine (45 mg/kg/day, p.o.) significantly increased the activity of cardiac SOD1, without affecting protein levels, in comparison to the vehicle control group (Nikolic-Kokic *et al.*, 2018).

After 4 weeks of clozapine (20 mg/kg/day, p.o.) treatment in female Wistar rats, one group noted inhibition of ovarian mitochondrial complex I, but not complex III, activity, decreased SOD and GSH levels, and increased TBARS, and these changes were not observed in controls (Khalaf *et al.*, 2019).

Male Sprague Dawley rats administered clozapine (45 mg/kg/day, p.o.) for 34 days exhibited increased mRNA and protein levels of cytochrome b5 but increased mRNA and decreased protein levels of glial fibrillary acidic protein, shown in mitochondria isolated from the cerebral cortex (Ji *et al.*, 2009). Increases in gene expression of MAPK-activated protein kinase 2 and Ndufv2 (whose encoded protein is an electron transport chain member participating in oxidative phosphorylation) were not observed at the protein level.

One study noted a number of changes in mRNA expression in the brains of male C57BL/6 mice after 31 days of clozapine (10 mg/kg/day, p.o. in rodent meal pellets) versus mice on the control diet (Mehler-Wex *et al.*, 2006). Specifically, isoforms of cytochrome C oxidase, lactate dehydrogenase, ATPase, and mitochondrial uncoupling protein 1 decreased in expression, while phospholipase C increased in expression.

2. Clinical Studies
    a. Nevirapine

A retrospective study of HIV-infected children in Accra, Ghana found that nevirapine exposure was associated with a positive score using the Enquête Périnatale Française, a symptom-based evaluation tool (Langs-Barlow *et al.*, 2013).

A study of black South African patients infected with HIV taking a combination of stavudine, lamivudine, and either efavirenz or nevirapine for 4-24 months found that nevirapine exposure, but not efavirenz exposure, resulted in a significant time-dependent increase in mean total lymphocyte $\Delta\psi m^{low}$ (mitochondrial depolarization) and was also correlated with mean total

lymphocyte apoptosis (Karamchand *et al.*, 2008). However, as control patients were HAART-naïve, it is difficult to isolate the effect of nevirapine alone when it was administered as part of combination drug therapy here.

A cross-sectional study of HIV-infected adults in Tennessee compared exposure for at least 30 days to HAART with or without NNRTI to patients who had not received HAART in the last 3 months (6 months for NNRTIs) on measures of oxidative stress (Redhage *et al.*, 2009). Median plasma F_2-isoprostane levels, a measure of lipid peroxidation, were found to be decreased in the NNRTI-exposed group; there was a trend toward lower levels with nevirapine exposure, but this was not statistically significant.

A prospective cohort study of HIV-uninfected and -infected pregnant women and infant pairs in Cameroon found that nevirapine treatment of HIV-exposed, uninfected infants (compared to HIV-unexposed, uninfected infants) was not associated with a lower mitochondrial to nuclear DNA ratio in dried blood spots at 6 weeks of age, although zidovudine exposure was (Jao *et al.*, 2017).

A multicenter, prospective randomized trial evaluated efficacy and safety of switching to lopinavir-ritonavir and nevirapine versus lopinavir-ritonavir and two NRTIs in HIV-infected adults who had achieved virologic suppression (Negredo *et al.*, 2009). The nevirapine-treated group experienced an increase in mitochondrial to nuclear DNA ratio in peripheral blood mononuclear cells by week 48. There was also an increase in cytochrome c oxidase IV activity at weeks 24 and 48 (presumably also in PBMCs).

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Pharmacological Reviews: The emerging role of the innate immune response in idiosyncratic drug reactions

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Supplemental Data

     A. Effects on Organ Weights
         1. Rodent Studies
            a. Amodiaquine

Compared to untreated control rats, no effects on spleen, liver, or lung weights were observed when male Wistar rats were treated with amodiaquine (10 mg/kg/day, intraperitoneal (i.p.)) for up to 3 weeks (Elko and Cantrell, 1970). A second study also did not find any changes in liver weight during 7 weeks of amodiaquine (~200-300 mg/kg/day in rodent meal) treatment, although spleen weights were significantly increased from weeks 1 to 7, with peak weight at week 3 (Metushi *et al.*, 2015). In contrast, amodiaquine (10 mg/kg/day, per os (p.o.)) treatment of male Wistar rats for 4 days resulted in increased liver weights after 4 weeks compared to saline-treated rats, while heart, spleen, and kidney weights were unaffected (Ajani *et al.*, 2008).

Male Sprague Dawley rats administered amodiaquine (5, 10, or 15 mg/kg, p.o.) for 14 days, following which changes were evaluated up to 34 days (Niu *et al.*, 2016). The testis weights of amodiaquine-treated animals were found to be significantly lower than those of the control animals, although results from this study were presented as pooled groups for each drug dose and included data from 4 different endpoint times.

After 1 week of amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) treatment in male Brown Norway rats, another group noted significantly smaller livers and larger spleens, compared to control animals (Liu *et al.*, 2016).

            b. Amoxicillin

In female B6C3F1 mice dosed with 20, 100, or 500 mg/kg/day amoxicillin by oral gavage for 28 days, there were no changes in spleen weight or cellularity (Lebrec *et al.*, 1994).

            c. Nevirapine

In male rats administered 6 mg/kg nevirapine for 60 days, liver weights appeared slightly decreased compared to those of controls (Awodele *et al.*, 2015). This is unexpected as nevirapine induces liver enzymes, and an increase in liver weight is expected. In contrast, another study using male rats dosed nevirapine at 32 mg/kg for 4 weeks, an increase in liver weights was observed (Adaramoye *et al.*, 2012). The different results may be explained by the different doses used.

            d. Clozapine

To evaluate potential immunotoxicity, female BALB/c mice were treated with clozapine (1, 5, 10, or 20 mg/kg/day, i.p.) for 21 days (Abdelrahman *et al.*, 2014). Clozapine caused a dose-dependent reduction in spleen weight, reductions in both thymus and liver weight at the highest dose, and an overall decrease in body weight at 10 and 20 mg/kg, but no changes in kidney weight.

Conversely, following a week of clozapine treatment (25 mg/kg/day, p.o.) in male Sprague Dawley rats, liver weights were significantly increased compared to controls (Jia *et al.*,

2014). One study investigated the effects of clozapine (10, 15, or 25 mg/kg/day, i.p.) in male Wistar rats after 21 days of treatment (Abdel-Wahab and Metwally, 2014). At the two highest doses, clozapine caused increases in heart weight, total body weight, and overall heart to body weight ratios. Similarly, after 4 weeks of treatment in male Wistar rats, clozapine (45 mg/kg/day, p.o.) significantly increased relative heart weight compared to control (Nikolic-Kokic *et al.*, 2018).

      B. Effects on Liver
          1. Rodent Studies
             a. Amodiaquine

In a study of male Wistar rats, amodiaquine was administered either orally (269 µmol/kg [95.7 mg/kg]), intraperitoneally (269 µmol/kg [95.7 mg/kg]), or intramuscularly (2.15, 21.5, 269, or 538 µmol/kg [0.77, 7.7, 95.7, or 191.5 mg/kg]) for 4 days, and liver changes were evaluated from day 5 to day 18 (Clarke *et al.*, 1990). No changes were observed with doses up to 269 µmol/kg (95.7 mg/kg) but following the final intramuscular (i.m.) injection of highest dose (538 µmol/kg [191.5 mg/kg], animals developed a significant increase in serum ALT levels, which remained elevated until day 17. Additionally, hepatic glutathione levels were increased following both the i.p. and i.m. doses, compared to control, but no changes in liver histology were observed.

Using a bacterial fulminant model of hepatitis in C57BL/6 mice, another group demonstrated that amodiaquine had protective effects on the liver (Yokoyama *et al.*, 2007). At 24 hours post-induction, lethality was reduced from 60% to 20% with amodiaquine co-treatment and elevated serum levels of AST and ALT were significantly attenuated at 3 and 6 hours.

In fasted BALB/c mice treated with amodiaquine (180 mg/kg, p.o.), no effects on liver function or morphology were observed, although a moderate decrease in liver GSH levels were observed from 3 to 6 hours (Shimizu *et al.*, 2009). In GSH-depleted mice (induced by L-buthionine-S,R-sulfonimine (BSO)), however, amodiaquine induced a massive increase in plasma ALT levels from 6 hours, peaking at 24 hours, which was accompanied by approximately 60% lethality by 48 hours. Liver histology revealed lipid accumulation in the centrilobular hepatocytes by 6 hours, extensive necrosis with congestion by 24 hours, and inflammatory cell infiltration by 48 hours. Covalent binding of amodiaquine to liver and plasma proteins was doubled in the BSO co-treated group compared to amodiaquine alone. Co-administration of ABT prevented the increases in ALT and lethality observed with the BSO-amodiaquine group.

One study evaluated effects of amodiaquine (6.12 mg/kg, p.o.) administered twice a day in female Wistar rats treated for 3 days (Abolaji *et al.*, 2014). On day 4, slightly elevated plasma ALT, AST, and ALP levels, in addition to increased total plasma proteins, were observed with amodiaquine compared to water-treated controls.

Following 8 days of administration of amodiaquine in male Brown Norway rats (62.5 mg/kg/day, p.o.) or mice (~300-350 mg/kg/day in rodent meal), significant hepatic covalent binding was detected (Lobach and Uetrecht, 2014b). This group also showed that treatment of female C57BL/6 mice with amodiaquine (~200-300 mg/kg/day in rodent meal) for up to 7 weeks led to natural killer cell-mediated liver injury with delayed onset, which resolved despite continued treatment (Metushi *et al.*, 2015). Specifically, serum ALT levels were increased with amodiaquine from weeks 2 to 6, peaking between weeks 3 to 4. Covalent binding of amodiaquine was greater in the centrilobular region of the liver. Covalent binding and liver injury were also observed in male mice, but to a much lower extent.

Additionally, they investigated the impact of immunizing female C57BL/6 mice with amodiaquine-modified hepatic proteins (weekly for 3 weeks, plus 2 week washout) prior to amodiaquine (~200-300 mg/kg/day in rodent meal) treatment and found that this pre-treatment was effective in preventing the ALT spike observed from weeks 3 to 4 with amodiaquine alone (Mak and Uetrecht, 2015). Interestingly, the immunized group had greater leukocyte infiltration in the liver compared to amodiaquine alone. To investigate the role of macrophages in amodiaquine-induced liver injury, we conducted another study using female C57BL/6 wildtype and CCR2$^{-/-}$ mice, but there was no difference in the liver injury (Mak and Uetrecht, 2019). Both genotypes were treated with amodiaquine (~200-300 mg/kg/day in rodent meal).

Moreover, several models of amodiaquine-induced liver injury were investigated using mice and rats (Liu *et al.*, 2016). Amodiaquine (~200-300 mg/kg/day in rodent meal) was fed to male C57BL/6 mice for up to 4 weeks, with or without BSO in the drinking water and a single dose of diethyl maleate to deplete GSH. After 1 week, BSO co-treated animals had significantly lower liver GSH levels compared to amodiaquine alone or control; however, BSO co-treatment paradoxically prevented the amodiaquine-induced ALT peak observed during weeks 2 to 4. Covalent binding of amodiaquine to hepatic proteins was not substantially altered with BSO co-treatment. The BSO phenomena was indicated to have been reproduced in Brown Norway and Wistar rats, although the data was not shown.

Liu et al. (2016) did, however, demonstrate that male Lewis, Wistar, and Brown Norway rats treated with amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) developed elevated serum ALT levels (greatest in Wistar rats) with increased hepatic covalent binding from weeks 2 to 4. Less severe liver injury, as measured by ALT peaks, was observed with female rats. To cause immune suppression, male Brown Norway rats were co-treated with amodiaquine and cyclosporine, which effectively prevented the amodiaquine-induced ALT peak. Conversely, when rats were administered poly I:C prior to amodiaquine to stimulate immune activation, there was an earlier onset of increased ALT. Natural killer cell activation was induced in treated rats by co-administration of retinoic acid, which also exacerbated the amodiaquine-induced ALT increase.

b. Amoxicillin

Serum ALT was increased at day 14 in male Wistar rats administered amoxicillin at a dose of 30 mg/kg/day intraperitoneally (Oyebode *et al.*, 2019).

c. Nevirapine

In male rats administered 6 mg/kg nevirapine for 60 days, serum ALT was increased (Awodele *et al.*, 2015). In another study using male rats, nevirapine was dosed at 32 mg/kg for 4 weeks, an increase in ALT was observed (Adaramoye *et al.*, 2012). Other markers of liver injury were also increased (serum total bilirubin and γ-glutamyl transferase). Necrosis was noted in the liver histology, and liver protein was decreased.

Female Brown Norway rats administered 75 mg/kg nevirapine for 7 days did not show an increase in ALT (Brown *et al.*, 2016), nor at a dose of 200 mg/kg for up to 14 days, although there appeared to be liver injury at day 7 which had resolved by day 14 in this study (Bekker *et al.*, 2012). Both of these studies also tested the effect of co-treatment of nevirapine with a substance that can cause liver injury on its own (galactosamine and lipopolysaccharide (LPS), respectively). Nevirapine was found to attenuate the galactosamine-induced ALT increase. Co-treatment with LPS attenuated the histological findings in the liver caused by nevirapine, although the combination resulted in an increase in ALT not observed with nevirapine treatment alone.

In male C57BL/6 mice, an ALT elevation at week 3 was also observed at a dose of 950 mg/kg administered in food (Sharma *et al.*, 2012). Mild necrosis in the liver was observed by histology.

d.   Clozapine

Using a model of liver ischemia/reperfusion (I/R) in male Wistar rats, one group evaluated the protective effects of clozapine (15 mg/kg, subcutaneous (s.c.)) administered immediately following the ischemia phase and again 6 hours into the reperfusion phase (Adachi *et al.*, 2006). Notably, clozapine attenuated I/R-induced plasma ALT and AST increases after 24 hours in comparison to control animals that underwent I/R. Another group similarly showed that clozapine (15 mg/kg, s.c.) pre-treatment at 24 hours, 12 hours and immediately prior to I/R significantly attenuated increases in plasma ALT and AST at 2 hours post-ischemia (El-Mahdy *et al.*, 2013). Moreover, clozapine pre-treatment dampened I/R-mediated vacuolar degeneration of hepatocytes and dilatated central vein pathology and resulted in increased hepatic GSH levels and decreased malondialdehyde (MDA, an end-product of lipid peroxidation) levels, compared to I/R controls.

One group investigated the potential impacts of clozapine in a cecal ligation puncture model of peritoneal sepsis (Machado *et al.*, 2007). After resuscitation, rats were treated with clozapine (25 mg/kg, s.c.) twice a day for 3 days. Clozapine did not attenuate hepatic damage nor was survival significantly improved when compared to the sham-operated control group. Although serum markers of organ dysfunction, including elevated creatine kinase, creatinine, AST, and amylase appeared to be altered in the clozapine-treated group compared to saline-treated controls, statistical comparison of these groups was not included.

At the end of 6 weeks of clozapine (25 or 50 mg/kg/day, p.o. [in rodent meal]) administration in female Lewis rats, clozapine extensively modified proteins in liver subcellular fractions compared to control rats (Gardner *et al.*, 2005). To test whether selenium deficiency enhanced the immunotoxicity of clozapine, female Sprague Dawley rats were maintained on a normal or selenium-deficient diet for 54 days prior to, and during, clozapine (50 mg/kg/day, p.o. in rodent meal) administration for 62 days (Ip and Uetrecht, 2008). At the end of treatment, significant hepatic covalent binding was observed with clozapine treatment, but was not altered by selenium status.

Another group used male Wistar rats to evaluate the effects of 3 weeks of clozapine (20 mg/kg/day, i.p.) with or without social isolation for the duration of treatment (Zlatkovic *et al.*, 2014). Irrespective of isolation conditions, clozapine caused marked infiltration of leukocytes in the hepatic portal triad and increased Kupffer cells and other inflammatory cells in the sinusoids, when compared to saline treated animals. Increased serum ALT and decreased hepatic GSH were also observed in all clozapine-treated groups. Clozapine-mediated increases in hepatic MDA, protein carbonyl content and GST activity were found and were exacerbated by social isolation. In the absence of social isolation, clozapine also caused elevated hepatic levels of cytosolic nitric oxide metabolites.

Hepatotoxicity was also investigated in male Sprague Dawley rats that were given clozapine (25 mg/kg/day, p.o.) with or without pre-treatment of glycyrrhetinic acid for 1 week (Jia *et al.*, 2014). Through the inhibition of 15-hydroxyprostaglandin dehydrogenase and prostaglandin reductase 2, glycyrrhetinic acid prevents the metabolism of active of prostaglandin E2 and F2α, and was proposed to have hepatoprotective activity (Ming and Yin, 2013). Clozapine caused marked increased in serum ALT and AST levels, which were enhanced by glycyrrhetinic acid co-treatment.

2. Clinical Studies
    a. Nevirapine

In a cross-sectional study of patients with HIV and hepatitis C virus coinfection, it was found that both NNRTIs as a class and nevirapine exposure was associated with a decreased rate of fibrosis progression, an effect that was not observed with protease inhibitor exposure (Berenguer *et al.*, 2008). This effect could be due to nevirapine exposure itself and/or due to its effects on the disease states, as HAART-naïve individuals have higher hepatic proinflammatory cytokine mRNA than do HAART-treated individuals (Sitia *et al.*, 2006).

C. Effects on Other Organs
    1. Rodent Studies
        a. Amodiaquine

When amodiaquine (10 mg/kg, i.m.) was administered to fasted male Wistar rats, it caused an increase in parietal cells and a decrease in mucous cells at 50 minutes post-injection (Ajeigbe *et al.*, 2012).

Another group evaluated the effects of amodiaquine (6.12 mg/kg, p.o.) administered twice a day in female Wistar rats treated for 3 days, following which no alterations in plasma levels of electrolytes, creatinine, or urea were observed (Abolaji *et al.*, 2014).

Treatment of female C57BL/6 mice with amodiaquine (~200-300 mg/kg/day in rodent meal) for up to 6 weeks demonstrated that covalent binding of amodiaquine was detectable in the kidney, the red pulp of the spleen, and in the gut (Metushi *et al.*, 2015).

During characterization of various models of amodiaquine-induced liver injury, hypertrophic cells in lung alveolar regions of male Brown Norway rats treated with amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) for 5 weeks, which were not observed in saline-treated controls (Liu *et al.*, 2016).

        b. Clozapine

After cecal ligation, clozapine (25 mg/kg, s.c.) administered twice a day was ineffective in reducing renal, pancreatic, lung, or muscular damage observed in male Wistar rat, although again, statistical comparison of the non-septic clozapine group versus control was not reported (Machado *et al.*, 2007). An anti-glomerular basement membrane (GBM) antibody model of proteinuria in female Wistar rats was used to evaluate the impact of twice a day clozapine (10 mg/kg, s.c.) administration on disease progression after 6 days (Tanda *et al.*, 2007). Compared to the saline and anti-GBM antibody-treated control group, clozapine was ineffective in reducing proteinuria or the formation of crescentic glomeruli in the kidney.

In a male C57BL/6 mice model of arthritis, 2 weeks of clozapine (4 mg/kg/bid, p.o.) markedly protected mice from arthritis symptoms, including an attenuation of ankle thickness from days 2 to 10 (Nent *et al.*, 2013). After 2 weeks, no inflammation was detectable in ankle joint histology in the clozapine group, while the arthritic control group presented with strong inflammation, joint destruction, and periarticular inflammatory processes along the shaft.

Male and female Wistar rats were given clozapine (20 mg/kg/day, p.o. [administered in drinking water]) for 2 months, with or without postnatal exposure to PCP, and it was shown that male non-PCP-exposed clozapine-treated rats had increased leg fat, while female PCP-exposed clozapine-treated rats had decreased total fat content compared to their respective vehicle controls (Nikolic *et al.*, 2017).

In a prenatal methylazoxymethanol (MAM) Sprague Dawley rat model of schizophrenia, where male offspring were treated with clozapine (20 mg/kg/day, i.p.) for 8 days, clozapine ±

MAM exposure did not alter brain levels of nerve growth factor (Fiore *et al.*, 2008). Serum levels of nerve growth factor and entorhinal cortex levels of brain-derived neurotrophic factor (BDNF) were only elevated in the MAM-clozapine group, relative to both the MAM-exposed saline group as well as the non-exposed clozapine group.

Conversely, when male C57BL/6 mice that were treated with PCP, co-treatment of clozapine (6 mg/kg/day, i.p.) resulted in significantly decreased cerebral cortex BDNF levels compared to saline-treated PCP controls after 2 weeks (Barzilay *et al.*, 2011). Following 1 week of clozapine (10 mg/kg/day, p.o.) in male C57BL/6 mice that had been pre-treated with PCP for 2 weeks, clozapine significantly enhanced PCP-mediated increases in prefrontal cortex mRNA expression of GSH peroxidase, glutamate-cysteine ligase modifier subunit, and glutamate-cysteine ligase catalytic subunit mRNA expression versus PCP saline controls (Tran *et al.*, 2017). Clozapine did attenuate PCP-induced GSH decreases and oxidized GSH increases, but clozapine effects in the absence of PCP were not investigated.

In a model of neurotoxicity, where male C57BL/6J mice were administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), treatment with either clozapine (1 mg/kg/day, s.c.) or clozapine *N*-oxide (1 mg/kg/day, s.c.) for 21 days significantly attenuated MPTP-induced neuron loss and reduced microglial activation (as measured by CD11b staining), compared to MPTP controls (Jiang *et al.*, 2016).

One group characterized the impact of 20 days of clozapine (10, 30, or 60 mg/kg/day, p.o. [administered in drinking water]) in a female C57BL/6 mouse model of experimental autoimmune encephalomyelitis (EAE) (Green *et al.*, 2017). At the two highest doses, clozapine significantly attenuated EAE severity scores and at the highest dose, delayed disease onset. No effects of clozapine were observed in the absence of EAE. In a second female C57BL/6 mouse model of EAE, 20 days of clozapine (10 mg/kg/day, p.o.) initiated at disease day 15 did not affect disease progression or severity, but did slightly attenuate EAE-induced white matter loss (Chedrawe *et al.*, 2018).

In another social isolation protocol in male Wistar rats, 3 weeks of clozapine (20 mg/kg/day, i.p.) did not affect cytosolic GSH levels in the hippocampus, but did significantly reduce GSH peroxidase activity, compared to saline controls (Todorovic and Filipovic, 2017b). Both GSH peroxidase and GSH reductase activity and protein levels were reduced with socially isolated clozapine treatment. In the prefrontal cortex, no changes in GSH reductase were observed, but clozapine did reduce GSH levels and GSH peroxidase activity, irrespective of social isolation (Todorovic and Filipovic, 2017a). BDNF levels were also found to be elevated after 30 days of clozapine (10 mg/kg/day, i.p.) in the brains of male CDF-344 rats (Kim *et al.*, 2012). Moreover, numerous changes in the arachidonic acid cascade were reported in the brain, in addition to decreased body weight with this model. With clozapine, the concentrations of prostaglandin E_2 (PGE_2) and COX-1 were decreased, and thromboxane B_2 levels and 5- and 15-lipoxygenases (LOX) mRNA levels were increased, while leukotriene B_4 was unchanged. In a second study by this group, clozapine was also shown to decrease the concentration of unesterified plasma arachidonic acid, and altered the composition of a number of fatty acids in the brain (Modi *et al.*, 2013).

One study evaluated the cardiotoxicity of clozapine (5, 10, and 25 mg/kg/day, i.p.) in male BALB/c mice after up to 2 weeks of treatment (Wang *et al.*, 2008). A dose-dependent increase in myocardial histopathological scores was observed with clozapine compared to saline treated controls, where inflammatory lesions consistent with myocarditis were most severe at day

7 and had begun to resolve by 2 weeks. Co-administration of propranolol significantly dampened the severity of cardiac inflammation observed at 2 weeks.

Cardiac inflammation was also demonstrated in male Wistar rats given clozapine (10, 15, or 25 mg/kg/day, i.p.) for 3 weeks (Abdel-Wahab and Metwally, 2014; Abdel-Wahab *et al.*, 2014). Compared to the saline control group, clozapine caused distinct and dose-dependent inflammatory lesions, interstitial edema, perinuclear vacuolation, focal subendocardial fibrosis, and disorganization and degeneration of the myocardium. Clozapine also increased serum levels of creatine kinase (15 and 25 mg/kg) and LDH (all doses) and reduced cardiac GSH (25 mg/kg) and GSH peroxidase activity (15 and 25 mg/kg). All inflammatory effects were attenuated by co-administration of captopril for the duration of high-dose clozapine treatment.

Additionally, after 4 weeks of treatment in male Wistar rats, clozapine (45 mg/kg/day, p.o.) significantly increased infiltrating leukocytes in the heart and caused an abnormal structure of cardiomyocytes that was not present in the vehicle control group (Nikolic-Kokic *et al.*, 2018).

One study found that clozapine (0.5 mg/kg/day, p.o.) administration to male Sprague Daley rats for 30 days caused noticeable aberrations in kidney, spleen, and heart morphology, and also increased expression of cardiac CYP1A2, CYP3A4, and CYP2C19 mRNA compared to saline control animals (Mohammed *et al.*, 2020). Cardiac histopathological changes induced by clozapine included vascular congestion with intermuscular edema. This was accompanied by severe histopathological lesions in the spleen, as evidenced by hemosiderosis in the red pulp and lymphoid depletion (reduction in the diameter of lymphoid follicles of the white pulp). In the kidney, clozapine-treated animals exhibited numerous hyaline casts in the lumina of the renal tubules, with marked vacuolar and hydropic degeneration in the epithelial linings, focal hemorrhage and vascular congestion. Furthermore, serum levels of cardiac troponin I, LDH, creatinine, blood urea, nitrogen, urea, and uric acid were elevated following clozapine treatment, and serum lysozyme activity levels were decreased. Most biochemical changes were attenuated when clozapine was co-administered with sulpride (another antipsychotic) for the duration of the study.

After 4 weeks of clozapine (20 mg/kg/day, p.o.) treatment in female Wistar rats, multiple cystic atretic follicles were observed with degenerated zona pellucidum and oocyte, vacuolation of cells, and an excessive deposition of collagen fibers, concentrated around the atretic follicles that was not observed in the control group (Khalaf *et al.*, 2019).

In addition to a reduction in spleen weight at all doses of clozapine (1, 5, 10, or 20 mg/kg/day, i.p.), a marked decrease in spleen cellularity and decreased white pulp density were reported following 3 weeks of the clozapine (5, 10, or 20 mg/kg) in female BALB/c mice, compared to saline controls (Abdelrahman *et al.*, 2014). Moreover, when mice were given a single dose of sheep RBCs on after 14 days of clozapine treatment, a dose-dependent reduction in hemagglutination titres was observed at after 21 days. When mice were rechallenged with a second dose of sheep RBCs, clozapine (5, 10, or 20 mg/kg) significantly attenuated footpad swelling. This was accompanied by a decrease in inflammatory infiltrates of leukocytes in both the hypodermis and muscle layers of the foot, observed with the two highest doses of clozapine.

2. Clinical Studies
    a. Clozapine

To investigate cardiovascular complications, a retrospective analysis of 8,000 male and female patients who commenced clozapine therapy was conducted (Kilian *et al.*, 1999). The incidence of myocarditis or cardiomyopathy reportedly due to clozapine administration in this population was 0.23% (20 male and 3 female cases). Of the 15 myocarditis cases, the median

time to onset was 15 days of clozapine (range: 3-21 days) and 6 cases were accompanied by eosinophilia.

Among 38 cases (27 male and 11 female) of clozapine-induced myocarditis, the mean time to onset was 18 ± 2 days (range: 14-33 days) and the mean dose at onset was 232 ± 69 mg/day (range: 50-750 mg/day) (Ronaldson *et al.*, 2010). Eighteen patients also presented with fever up to 6 days prior to detection of elevated serum TRP levels and 20 cases developed eosinophilia in the subsequent week.

In a study of 15 clozapine-naive schizophrenia patients (8 male and 7 female) titrated from 25 mg/day up to 100 mg/day over 4 weeks, a slight increase in serum levels of hsCRP was observed at week 3 and 3 patients developed tachycardia, although no changes in leukocytes or TRP-I were observed (Curto *et al.*, 2015).

D.   Effects on Cell Death and Proliferation
1.   Rodent Studies
a.   Amodiaquine

A dose-dependent loss of spermatogenic cells and disorganization of seminiferous tubule morphology was observed following amodiaquine (5, 10, or 15 mg/kg, p.o.) treatment in male Sprague Dawley rats for 14 days, although undifferentiated spermatogonia remained alive in the testes (Niu *et al.*, 2016). TUNEL staining suggested that these cells had undergone apoptosis, which was supported by increased protein levels of FS-7-associated surface antigen (Fas), Bcl-2-associated X protein (BAX), and caspases-3, -8, and -9 in amodiaquine-treated testes. Blood-testes-barrier damage was also reported, in addition to a significant reduction in the blood-testes-barrier-associated proteins tight junction protein-1, occludin, claudin-11, eppin, and E-cahderin. No changes in serum luteinizing hormone or testosterone were observed. Amodiaquine-induced histological changes were reversed following a 20-day recovery period.

Increased apoptosis, as measured by TUNEL staining, was noted in the livers of male Brown Norway rats treated with amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) for 5 weeks, which was not observed in saline-treated controls (Liu *et al.*, 2016). Likewise, only in amodiaquine-treated animals was cell proliferation in the germinal center of spleen significantly increased at this time. These changes were also preceded by increases in serum osteopontin (peaking at day 12) and serum cytochrome c (peaking at week 4), detected only in the amodiaquine group.

b.   Amoxicillin

Caspase-3 and -9 were activated in the liver of male Wistar rats treated with 30 mg/kg/day amoxicillin intraperitoneally once daily for 14 days (Oyebode *et al.*, 2019).

c.   Nevirapine

Nevirapine was administered to Sprague-Dawley rats at a dose of 200 mg/kg once daily by oral gavage, and the effect of co-treatment with LPS was examined (Bekker *et al.*, 2012). Apoptosis was noted by histological examination at the 6- and 24-hour endpoints in the nevirapine-treated group, but the liver of the nevirapine and LPS co-treated group appeared normal. At 7 days, the histology of the nevirapine group exhibited centrilobular hepatocellular degeneration, cell swelling, and hepatocyte apoptosis which resolved by days 14 and 21; again, the liver appeared normal in the co-treated group.

In female Brown Norway rats treated with 159 mg/kg/day 12-hydroxynevirapine by oral gavage, transcripts related to apoptosis and autophagy, such as death-associated protein kinase 1 (Dapk1) and Kruppel-like factor 15 (Klf 15), or cell stress, such as lipin1 (Lpin1) and DnaJ

(Hsp40) homologue, subfamily B, member 5 (Dnajb5) were upregulated in the skin 6 hours post-dose (Zhang *et al.*, 2013).

In the liver of Brown Norway rats treated with 150 mg/kg nevirapine for 8 weeks, hepatocytes were noted to have developed necrotic cytoplasm, while some endothelial cells exhibited signs of degeneration (Sastry *et al.*, 2018).

In Wistar rats administered nevirapine for 4 weeks, the live-dead ratio of the sperm was decreased in a dose-dependent manner (Adaramoye *et al.*, 2012). This was confirmed with findings of spermatocyte necrosis observed by histology.

d. Clozapine

In male Sprague Dawley rats, clozapine (5 mg/kg/day, i.p.) had no effect on ketamine-induced increases in caspase-3 activity in any brain area examined (striatum, hippocampus, or prefrontal cortex) (George *et al.*, 2020). However, clozapine did attenuate the elevated Bax/Bcl-2 ratio and cytochrome c expression induced by ketamine in all brain areas, and also attenuated elevated prefrontal cortical and hippocampal MDA levels.

At 2 hours post-clozapine (10 mg/kg, i.p.) administration in male Sprague Dawley rats, elevated microtubule-associated protein 1 light chain 3-II (an autophagosome marker) staining was observed in the frontal cortex, compared to saline-treated controls (Kim *et al.*, 2018). Increased autophagosome numbers were also demonstrated via transmission electron microscopy at this time. Additionally, autophagy-related protein 5-12 conjugates were increased, further indicating that activation of autophagy had occurred.

Following 21 days of clozapine (20 mg/kg/day, i.m.) administration, Sprague Dawley rats had noticeable neutrophil toxicity, manifested by condensation and subsequent breakdown of chromatin material, although this morphology was not clearly distinguishable in the figures provided in the paper (Wasti *et al.*, 2006).

After 28 days of treatment, another group did not observe any translocation of AIF from the mitochondria to the nucleus in the striatum of male Sprague Dawley rats administered clozapine (30 mg/kg/day, i.p.), suggesting against the possibility of caspase-independent neuronal cell death with clozapine (Skoblenick *et al.*, 2006).

Furthermore, after 28 days of clozapine (10 mg/kg/day, i.p.) treatment in male Sprague Dawley rats, clozapine had no effect on apoptotic markers, including histone-associated DNA fragmentation, Bax/Bcl-2 ratio, or levels of X-linked inhibitor of apoptosis protein in the frontal cortex, compared to saline controls (Jarskog *et al.*, 2007). However, levels of cleaved-caspase 3 protein, in addition to levels of activity of caspase-3, were significantly higher with clozapine.

In contrast, no changes in striatal caspase-3 activity levels were observed in male Wistar rats treated with clozapine (10 mg/kg/day, i.p.) for 21 days (Bishnoi *et al.*, 2011).

In male Wistar rats given clozapine (10, 15, or 25 mg/kg/day, i.p.) for 3 weeks, increased cardiac caspase-3 protein levels were reported (Abdel-Wahab and Metwally, 2014). In a second study by this group, clozapine-induced increases in caspase-3 activity levels and TUNEL staining were observed and were attenuated by co-administration of captopril (Abdel-Wahab *et al.*, 2014).

Following 3 weeks of treatment in male Wistar rats, clozapine (20 mg/kg/day, i.p.) increased focal necroses and apoptotic hepatocytes in liver lobules, with or without the coaddition of social isolation (Zlatkovic *et al.*, 2014). Another study noted acidophilic degeneration (indicative of dying hepatocytes) in male Sprague Dawley rats that had been co-treated with clozapine (25 mg/kg/day, p.o.) and glycyrrhetinic acid for 1 week (Jia *et al.*, 2014).

Another study noted that ovarian antral follicles were characterized by increased apoptotic cells following 4 weeks of clozapine (20 mg/kg/day, p.o.) treatment in female Wistar rats (Khalaf *et al.*, 2019). Additionally, clozapine treatment increased ovarian p53 staining and decreased Ki67 staining in comparison to the water control group.

After 11 days of clozapine (13.5 mg/kg/day, p.o.) administration in male C57BL/6 mice, increased pancreatic apoptosis and decreased proliferation were observed, measured by TUNEL and Ki67 staining, respectively, when compared to vehicle controls (Huang *et al.*, 2012). These effects were exacerbated by clozapine administration in combination with a high fat (60% fat versus standard 12% fat) diet. Members of this group later confirmed these findings, showing that administration of clozapine (13.5 mg.kg/day, p.o.) to male C57BL/6 mice resulted in an increase in apoptosis and a decrease in proliferation in pancreatic islet cells (again, measured by TUNEL and Ki67 staining, respectively), as early as 2 weeks into treatment (Hsu and Fu, 2016).

2.   Clinical Studies
   a.   Nevirapine

To our knowledge, only one study has examined cell death using samples from patients treated with nevirapine. In peripheral blood mononuclear cells, a lesser proportion of total lymphocytes and CD4 T cells was found to be apoptotic in patients receiving nevirapine-containing HAART compared to HAART-naïve, HIV-infected individuals (Karamchand *et al.*, 2008). The proportion of apoptotic total lymphocytes was greater than the proportion of apoptotic CD4 T cells, suggesting that a different lymphocyte subset was undergoing apoptosis. Altogether, this study does not support an increase in total lymphocyte or CD4 T cell apoptosis with nevirapine treatment (in combination with stavudine and lamivudine) when compared to untreated HIV-infected individuals, although an additional comparison to healthy individuals might be informative.

   b.   Clozapine

To characterize potential pathways of cell death associated with clozapine, one group examined localization of the intramitochondrial protein apoptosis-inducing factor (AIF) in striatal sections collected post-mortem from 4 clozapine-treated schizophrenic patients and 4 healthy controls (Skoblenick *et al.*, 2006). Translocation of AIF from the mitochondria to the nucleus initiates a caspase-independent cell death cascade, however, no translocation was observed in either patients or controls.

E.   Effects on Immune Cells
   1.   Rodent Studies
      a.   Amodiaquine

Compared to untreated control rats, no effects on phagocytosis activity (measured by carbon particle clearance from blood) were observed when male Wistar rats were treated with amodiaquine (10 mg/kg/day, i.p.) for up to 3 weeks (Elko and Cantrell, 1970). However, amodiaquine did shorten the duration of elevated phagocytosis activity observed in malaria-infected rats.

In the study comparing routes of administration in male Wistar rats, immediately following the final i.m. injection of amodiaquine at the highest dose (538 μmol/kg), animals developed a significant reduction in white blood cells that lasted for 4 days, after which, counts remained higher than baseline until day 14 (Clarke *et al.*, 1990).

Male Wistar rats administered amodiaquine (10 mg/kg/day, p.o.) for 4 days developed a significant increase in fibrinogen and total leukocyte counts compared to saline-treated rats at the study endpoint of 4 weeks (Ajani *et al.*, 2008).

With amodiaquine treatment (6.12 mg/kg, p.o.) twice daily in female Wistar rats, no changes in erythrocyte MDA, GSH, or G6PD levels were reported (Abolaji *et al.*, 2014).

Another group investigated the roles of myeloperoxidase and NADPH oxidase in the covalent binding of amodiaquine to neutrophils using several models: male rats (Brown Norway) and female mice (C57BL/6J wildtype, MPO$^{-/-}$, gp91 phox$^{-/-}$, Rac1$^{-/-}$, Rac2$^{-/-}$, COX1$^{-/-}$, and COX2$^{-/-}$) were administered amodiaquine for up to 8 days via gavage (rats, 62.5 mg/kg/day) or mixed in rodent meal (mice, ~300-350 mg/kg/day, p.o.) (Lobach and Uetrecht, 2014b). In wildtype mice and rats, significant covalent binding was detected in neutrophils after day 1 of treatment, reaching a maximum following day 4. After day 1, significant covalent binding was also observed in the bone marrow of rats and mice; specifically, binding was observed in myeloid cells, lymphoid cells, and megakaryocytes, but not in erythroid cells. Furthermore, amodiaquine appeared to bind both intracellularly as well as to surface proteins. This covalent binding was significantly reduced in neutrophils from amodiaquine-treated MPO$^{-/-}$ mice, and a similar trend emerged in gp91 phox$^{-/-}$ neutrophils, although it did not reach significance. No difference in covalent binding was observed in neutrophils from Rac1$^{-/-}$, Rac2$^{-/-}$, COX1$^{-/-}$, or COX2$^{-/-}$ mice compared to neutrophils from wildtype mice treated with amodiaquine for 8 days, indicating that the oxidative burst pathway, prostaglandin H synthase, or cyclooxygenase are not necessary for covalent binding of amodiaquine in neutrophils.

During amodiaquine (~200-300 mg/kg/day in rodent meal) treatment in female C57BL/6 mice, members of this group found that there was an elevation in lymphocytes in the cervical lymph nodes at week 2, and in the liver and spleen at week 3 of treatment (Metushi *et al.*, 2015). IHC revealed an increase in CD4$^+$, CD8$^+$, CD11b$^+$, F4/80$^+$, CD45R$^+$, and Ki67$^+$ cells in the liver and an increase in CD11b$^+$, Ki67$^+$, F4/80$^+$, and CD45R$^+$ cells in the splenic red pulp with amodiaquine, with maximal increases observed around week 3. Flow cytometry revealed an increased in NK1.1$^+$ and CD8$^+$ cells in the cervical lymph nodes at week 2 in the spleen at weeks 1 and 3, and in the liver at week 3. Amodiaquine treatment of Rag1$^{-/-}$ mice (B- and T-cell deficient) in this study demonstrated an even greater increase liver NK1.1$^+$ cells and ALT, and upon depletion of natural killer cells, the ALT increase was attenuated.

In the immunization protocol by this group, there was expanded white pulp in the spleens of amodiaquine hepatic protein immunized, amodiaquine-treated animals compared to the control or amodiaquine alone treated group at week 3 (Mak and Uetrecht, 2015). Additionally, immunized animals exhibited the highest number of splenic CD11b$^+$/Gr-1$^+$ cells.

Moreover, after week 5 of amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) treatment, this group noted that the livers of treated Wistar rats exhibited focal inflammatory infiltrates with increased ED1$^+$ cells and treated Brown Norway rats displayed hypertrophic Kupffer cells, in addition to increased ED1$^+$, ED2$^+$, and CD45$^+$ leukocytes (Liu *et al.*, 2016). Total liver and lymph node lymphocytes were increased in Brown Norway rats after 3 and 4 weeks of treatment, respectively, driven by increased CD4$^+$ T cells (specifically, T_H17 cells in the liver). NK1.1$^+$ cells were also increased in the liver at week 3 and in the lymph nodes and peripheral blood at week 4 of treatment. Peripheral blood, spleen, and lymph node M1 monocytes/macrophages were decreased from weeks 1 to 3 but were increased in the liver at weeks 3 and 4. Activated M2 macrophages were elevated in all investigated organs (spleen, liver, lymph node, peripheral blood) around weeks 3 to 4.

In the study comparing the effects of amodiaquine treatment in female C57BL/6 wildtype and CCR2$^{-/-}$ mice, CD11b$^+$/Gr-1$^+$ and NK1.1$^+$ cells were increased with amodiaquine in the livers of wildtype-treated animals, but not in CCR2$^{-/-}$ mice (Mak and Uetrecht, 2019).

Another group evaluated the protective effects of amodiaquine in a mouse model of intracerebral hemorrhage (ICH) induced by striatal injection of collagenase VII (Kinoshita *et al.*, 2019). At 3 days, amodiaquine significantly suppressed ICH-induced accumulation of activated microglia/macrophages (Iba1$^+$ cells) and also suppressed activation of astrocytes (GFAP$^+$ cells) in the perihematomal region, compared to saline-treated ICH controls. Conversely, amodiaquine had no effect on ICH-induced neuron loss or injury volume, although this study did not look at the effects of amodiaquine in the absence of ICH.

b. Amoxicillin

In female B6C3F1 mice dosed with 20, 100, or 500 mg/kg/day amoxicillin by oral gavage for 28 days, some immune changes were noted, but none appeared dose-dependent (Lebrec *et al.*, 1994). WBC counts were noted to be decreased at the highest dose, and there was no change in NK cell activity.

In male Wistar rats administered amoxicillin(30 mg/kg/day, i.p.), WBC counts were increased at day 14 (Oyebode *et al.*, 2019).

Male Sprague-Dawley rats were administered 50 mg/kg amoxicillin by oral gavage 3 hours before induction of lung injury; no change in pulmonary alveolar macrophage function was noted (Tamaoki *et al.*, 1999).

In male Sprague-Dawley rats administered an intratracheal instillation of LPS to induce mucus hypersecretion and administered 40 mg/kg amoxicillin orally for 4 days, there was no difference in leukocyte counts bronchoalveolar lavage fluid (Ou *et al.*, 2008).

c. Nevirapine

Hematological analysis of rats administered 6 mg/kg/day nevirapine orally revealed a decrease in white blood cell (WBC) counts, but no changes in other parameters of erythrocytes or platelets after 60 days (Awodele *et al.*, 2015). In rats administered a higher dose of 200 mg/kg nevirapine orally daily, lymphocytes and platelets were noted to be over the reference range at day 21; however, counts from control animals were not presented (Bekker *et al.*, 2012).

In male Sprague-Dawley rats administered 35–165 μg/kg nevirapine i.p. 4 hours before intravital imaging, decreased rolling velocity and increased rolling flux, adhesion, and emigration was observed (Orden *et al.*, 2014). Leukocyte emigration was confirmed by hematoxylin- and eosin-stained mesenteric tissue, in which a significantly higher number of infiltrated leukocytes, primarily PMNs, were seen.

In a study of hematological parameters of male Wistar rats, different groups were administered 0, 3, 6, 12, or 24 mg/kg drug once daily for up to 25 days; however, the drugs used were a fixed-dose combination of 150 mg lamivudine, 300 mg zidovudine, and 200 mg nevirapine and it was not stated what the dose represented (Nubila *et al.*, 2012). Some changes were found at the individual timepoints, but they did not seem to follow a dose-response pattern. In rats treated with 12 mg/kg, over time, red blood cell (RBC) counts, platelets, and lymphocytes were increased, and MCHC and neutrophils were decreased. Overall, the observed effects were quite small.

In a study using female Brown Norway rats, nevirapine was administered in food at a dose of about 150 mg/kg/day for up to 21 days (Popovic *et al.*, 2006). Ear-draining auricular lymph nodes were analyzed, which were chosen because the ears of these rats have been shown to turn red after about 7 days of nevirapine treatment, preceding the appearance of rash at 14-21

days. Total cell count $CD4^+$ T cell, $CD8^+$ T cell, B cell, and macrophage counts and percentages were elevated in these lymph nodes of treated rats at days 7, 14, and 21 by flow cytometry. Expression of ICAM-1 and MHC II was increased overall, and MHC II expression was also shown to be increased in B cells and macrophages. Immunohistochemical staining confirmed those findings for ED1 (macrophages), ICAM-1, and MHC I and II at day 7, with a greater increase at day 14. Macrophage infiltration was noted to precede T cell infiltration, which occurred at 14 days.

d. Clozapine

In a prenatal MAM schizophrenia model, clozapine (20 mg/kg/day, i.p.) attenuated increased peripheral blood granulocytes in the MAM-exposed group after 8 days (Fiore *et al.*, 2008). In the absence of MAM exposure, clozapine treatment did not have significant effects.

In male C57BL/6J mice treated with MPTP, co-treatment with clozapine (1 mg/kg/day, s.c.), but not clozapine *N*-oxide (1 mg/kg/day, s.c.), for 3 weeks resulted in a significant decrease in total leukocyte counts compared to the MPTP control group (Jiang *et al.*, 2016). In the absence of MPTP administration, clozapine still caused the decrease in leukocytes, compared to control animals.

In a female C57BL/6 mouse EAE model, no effect of clozapine (60 mg/kg/day, p.o. [administered in drinking water]) on immune cell populations in the spleen were observed, but clozapine attenuated EAE-induced increases in T cells and macrophages in the spinal cord by day 11 (Green *et al.*, 2017). No effects of clozapine were observed in the absence of EAE.

In a second EAE model, clozapine (60 mg/kg/day, p.o. [administered in drinking water]) significantly attenuated increases in infiltrating neutrophils and monocytes in the spinal cord, brain, blood, and spleen (Robichon *et al.*, 2020). No significant cell alterations were observed with clozapine in the absence of EAE. In this study, female C57BL/6 mice were also treated with clozapine (60 mg/kg/day, p.o. [administered in drinking water]) for 1 week, following which, animals received a single injection of CCL2 or CCL5 and immune changes in the local draining lymph nodes were evaluated 18 hours later. Compared to CCL2 treated controls, clozapine greatly attenuated increased total cell numbers, T cells, and neutrophils in draining lymph nodes. No changes were noted with CCL5 treatment.

In a K/BxN mouse arthritis model, clozapine (4 mg/kg/bid, p.o.) did not affect leukocyte counts (Nent *et al.*, 2013). Clozapine (25 mg/kg, s.c.) administered twice a day also did not modulate sepsis-mediated neutrophil infiltration in the liver, lung, or ileum of male Wistar rats, as measured by myeloperoxidase activity (Machado *et al.*, 2007). However, cardiac myeloperoxidase activity was markedly upregulated following administration of male Wistar rats with clozapine (10, 15, or 25 mg/kg/day, i.p.) for 21 days (Abdel-Wahab and Metwally, 2014).

Following 21 days of clozapine (20 mg/kg/day, i.m.) administration, Sprague Dawley rats showed a marked increase in total WBC and neutrophil counts and a decrease in lymphocyte counts compared to saline-treated controls, while no significant changes in erythrocyte morphology or haematological indices were observed (Wasti *et al.*, 2006).

Increased blood monocytes, in addition to decreased lymphocytes and total leukocytes, were reported following 3 weeks of clozapine (5, 10, or 20 mg/kg/day, i.p.) in female BALB/c mice (Abdelrahman *et al.*, 2014). At the highest dose, this was also accompanied by a significant reduction in neutrophils and erythrocytes.

Clozapine (30 mg/kg/day, i.p.) administration in female Sprague Dawley rats resulted in significantly elevated blood neutrophil and monocyte counts, beginning on days 8 and 9, respectively, while no changes in leukocytes or lymphocytes were reported (Lobach and

Uetrecht, 2014a; Ng *et al.*, 2014). A transient neutrophil spike was also reported after day 1. This was accompanied by an expansion of the bone marrow myeloid, but not erythroid or lymphoid, compartment on day 10, as well as an increase in the efflux rate of less mature neutrophils from the bone marrow. Specifically, clozapine caused an increase in granulocyte production and myeloperoxidase-positive cells, concentrated in the paratrabecular zone, and an increase in megakaryocytes, in addition to more prominent sinuses. Clozapine also increased the number of mature cells in the bone marrow, as well as the number of CD18$^+$ and CD11b$^+$ cells.

Although not a rodent study, with clozapine (30 mg/kg/day, s.c.) treatment in female New Zealand White rabbits, the half-life of peripheral blood neutrophils was significantly increased after 2 days, but was significantly decreased after 10 days (Iverson *et al.*, 2010). Absolute neutrophil counts were transiently increased around day 2. Moreover, the efflux rate of neutrophils from the bone marrow was enhanced with clozapine.

In female Sprague Dawley rats treated with clozapine (30 mg/kg/day, i.p.) an increase in covalent binding to neutrophil proteins was observed, plateauing by day 4 (Lobach and Uetrecht, 2014b). No binding to lymphocytes was observed. At the end of 6 weeks of clozapine (40 mg/kg/day, p.o. [in rodent meal]) treatment in female Lewis rats, substantial covalent binding to a 49 kD protein was noted in bone marrow neutrophils that was not present in rats fed normal rodent meal (Gardner *et al.*, 1998).

Following 2 months of clozapine (50 mg/kg/day, p.o. in rodent meal) treatment in female Sprague Dawley rats maintained on a selenium sufficient or deficient diet, no effect on GSH peroxidase activity in whole blood was observed, although significant covalent binding in the bone marrow of clozapine treated animals was detected, irrespective of diet (Ip and Uetrecht, 2008). Weekly peripheral blood leukocyte and neutrophil counts were also unaltered.

2. Clinical Studies

a. Amoxicillin

In children with presumed bacterial or mycoplasmic lower respiratory tract infections treated with 60 mg/kg/day amoxicillin for 10 days, *ex vivo* lymphocyte lytic activity did not change with amoxicillin treatment (Agostoni *et al.*, 1988).

In a phase I clinical trial, healthy male volunteers were given a single dose of 785 mg amoxicillin orally (Gomez-Lus *et al.*, 1998). The killing activity of PMNs isolated from peripheral blood were measured *ex vivo*. Amoxicillin treatment alone did not affect PMN killing, but in combination with serum, bactericidal activity was significantly enhanced, even after amoxicillin concentrations fell below inhibitory concentrations.

In a study designed to examine the effects on immune parameters in healthy individuals, adult males were administered oral amoxicillin (1 g) and clavulanate potassium (125 mg) twice daily for 5 days (Dufour *et al.*, 2005). No changes were observed in white blood cell subsets, NK cell cytokine production, or polynuclear phagocyte activity up to 61 days after starting treatment.

b. Nevirapine

Multiple studies involving nevirapine report changes in blood cell counts, but these tend to be in combination with other drugs. Leukopenia responsive to G-CSF treatment has been described, but in these cases zidovudine was part of the treatment regimen which has been suspected of being the causative agent (Yamamoto *et al.*, 2000; Shahar *et al.*, 2004). Similarly, trials of nevirapine and zidovudine combinations for the prevention of perinatal HIV transmission have shown an increase in neutropenia with extended co-treatment of nevirapine and zidovudine (Kumwenda *et al.*, 2008), while neutropenia was attributed to zidovudine

exposure in a study in which some patients were also exposed to single dose nevirapine (Read *et al.*, 2007).

Another example of combination therapy associated with increased risk of blood cell adverse effects is nevirapine and lumefantrine. In an open-label clinical trial in Malawi, patients who were already taking antiretroviral therapy were given lumefantrine in addition to their current therapy, first at half the target dose, then increased to the full dose after 28 days (Banda *et al.*, 2018). Neutropenia was observed in all study groups, but thrombocytopenia was only observed in combination with nevirapine, leading to the recommendation not to titrate the dose of lumefantrine further.

Infants of mothers from the HIV-1 Protective Immunity and Perinatal Exposure Study trial in South Africa were studied up to 12 weeks after birth (Schramm *et al.*, 2010). Infants of HIV-positive mothers received no antiretroviral therapy, single dose nevirapine (the mother was sometimes also given single dose nevirapine at onset of labour if their HIV status was known before delivery), or triple antiretroviral therapy. At birth, nevirapine-treated infants had higher monocyte counts and percentages and basophil counts; these differences did not persist at 6 weeks. Infants whose mothers had a CD4 T cell count >500 cells/µL had higher WBC, monocyte percentages and monocyte and basophil counts but lower lymphocyte and eosinophil percentages if they were exposed to single dose nevirapine than those who were unexposed.

c. Clozapine

In a retrospective analysis in the United States of 118 hospitalized schizophrenic patients treated with clozapine patients for a minimum of 3 weeks, it was found that female patients were significantly more likely to develop eosinophilia compared to male patients, which was experienced by 23% of female and only 7% of male patients (Banov *et al.*, 1993). Additionally, eosinophilia was always experienced during weeks 3 to 5 of clozapine initiation and resolved with continued treatment. A similar retrospective analysis in a United Kingdom-based population of 160 schizophrenic patients (105 male and 55 female) initiating clozapine therapy was conducted, where 13% of patients (17 male and 4 female) developed eosinophilia between weeks 2 and 4 of clozapine treatment, with peak eosinophil levels reported after 7.5 weeks (range: 3-19 weeks) (Chatterton, 1997). The mean dose at the onset and peak of eosinophilia were 188 mg/day (range: 25-350 mg/day) and 227 mg/day (range: 0-600 mg/day), respectively. All except for 1 patient had resolution of eosinophilia with continued treatment.

Following retrospective analysis of 17 cases of clozapine-induced fever, 4 cases of leukocytosis, 3 cases of bandemia, 2 cases of neutrophilia, 1 case of eosinophilia, and 1 case of monocytosis were noted during the first month of treatment (Tham and Dickson, 2002). Similarly, in a retrospective review of 31 cases (19 male and 12 female) of clozapine-induced fever, 58% of patients had an elevated leukocyte count and 10% of the patients had an elevated eosinophil count (Pui-yin Chung *et al.*, 2008).

In a 6-week study of 14 male and 13 female patients diagnosed with schizophrenia, schizophreniform disorder, or schizoaffective disorder, transient elevations in total leukocyte and granulocyte counts were observed after the second week of treatment, while monocyte and lymphocyte counts remained stable over the course of the study (Pollmacher *et al.*, 1996). Mean clozapine doses were 162 ± 68, 245 ± 115, and 305 ± 160 mg/day at weeks 1, 2, and 6 respectively. Moreover, this group noted increased granulocyte and monocyte counts in a study of 20 schizophrenic patients and these levels were most prominent 2 weeks after commencing clozapine therapy (Pollmacher *et al.*, 1997).

However, an earlier study of 10 schizophrenic patients and 8 healthy controls reported that clozapine did not significantly influence total leukocyte, granulocyte or lymphocyte counts, measured at 1, 2, and 6 weeks of treatment (Pollmacher *et al.*, 1995). Mean clozapine doses were 188 ± 89, 285 ± 138, and 343 ± 169 mg/day, respectively. Likewise, this group did not find any changes in blood cell populations in an investigation of 6 male and 6 female patients with schizophrenia who were monitored for the first 6 weeks of clozapine treatment (Hinze-Selch *et al.*, 2000). In this study, the mean clozapine dose increased from 85 ± 59 mg/day at week 1 to 231 ± 105 mg/day at week 6.

One study reported a negative correlation between monocyte ROS release and the dose of clozapine administered to schizophrenia patients (1 male and 7 female), occurring 3 weeks into treatment, at which time the mean dose was 184 ± 35 mg/day (150–250 mg/day) (Gross *et al.*, 2003). Patients in this study were all started on clozapine at a dose of 50 mg/day, which was subsequently increased by 50 mg/day every 3 days until symptom remediation was observed.

Peaking at 2 weeks of treatment, a significant increase in circulating $CD34^+$ hematopoietic cells, neutrophils and leukocytes was observed in a study of 8 schizophrenia patients starting clozapine therapy (titrated from 50 mg/day up to doses where serum level of 350-600 ng/mL) (Loffler *et al.*, 2010).

F.   Effects on Mediators of Inflammation and Related Concepts
1.   Rodent Studies
a.   Amodiaquine

In the bacterial fulminant model of hepatitis, elevated serum levels of TNF-α were significantly dampened at 3 hours with co-administration of amodiaquine (5 mg/kg, s.c.) in C57BL/6 mice (Yokoyama *et al.*, 2007). However, this was not accompanied by any significant changes in increased mRNA levels of TNF-α, IFN-γ, IL-12, or IL-18 in the liver. In the ICH model, amodiaquine significantly suppressed ICH-induced increases in mRNA expression of IL-1β, CXCL2 and CCL2 at 6 hours, compared to vehicle control (Kinoshita *et al.*, 2019).

In another study with amodiaquine (~200-300 mg/kg/day in rodent meal) treatment in female C57BL/6 mice, it was found that serum cytokines IL-1α, IL-12 (p40), and IL-17 were elevated at week 1 with treatment (Metushi *et al.*, 2015). Notably, IL-3, IL-5, IL-10, IL-12(p70), IL-13, IFN-γ, TNF-α, and G-CSF were all significantly downregulated by week 7. Over the first 4 weeks of amodiaquine (62.5 mg/kg/day, 6 days/week, p.o.) treatment in male Brown Norway rats, this group also noted spikes in serum levels of IL-2, IL-5, IL-9, IL-12, TGF-β1, and CCL2, which were not observed in saline-treated controls (Liu *et al.*, 2016). After week 5, hepatic RANTES and IL-18 were significantly increased in the amodiaquine-treated group, but IL-2, IL-5, IL-6, and IL-12 levels were significantly decreased compared to control. Following amodiaquine rechallenge, additional increases in serum IL-4, IL-13, and IFN-γ were also observed.

b.   Amoxicillin

An acute otitis media model using male Sprague-Dawley rats found that treatment with amoxicillin, administered in drinking water to a dose of about 51 mg/kg/day for 5 days, induced changes in osteocalcin (Melhus and Ryan, 2004). Osteocalcin is produced by osteoblasts and is a marker of bone formation. The maximum level of this marker in bullar bone was decreased and delayed compared to untreated animals. No change in expression of IL-6, TNF-α, or IL-10 was noted.

In LPS-induced mucus hypersecretion in male Sprague-Dawley rats, 40 mg/kg amoxicillin administered orally for 4 days did not alter cytokine (IL-1β, IL-8, and TNF-α) levels in bronchoalveolar lavage fluid (Ou *et al.*, 2008).

c. Nevirapine

In rats administered nevirapine and/or LPS, serum IL-2, IFN-γ, and TNF-α were measured (Bekker *et al.*, 2012). Nevirapine treatment did not cause a significant change in serum IL-2 levels up to 24 hours, although co-treatment with LPS attenuated the increase in IL-2 that was observed in the LPS group. No significant difference was observed in serum IFN-γ levels up to 24 hours. Nevirapine treatment caused a significant increase in serum TNF-α levels at 24 hours; this was attenuated with LPS co-treatment. Interestingly, coadministration of nevirapine with LPS may have altered the pharmacokinetics of nevirapine – the 24-hour nevirapine serum level was higher than the 6-hour level with co-treatment, while the opposite was observed with nevirapine alone. With chronic treatment, nevirapine treatment caused a significant increase in serum IL-2 at day 21 compared to nevirapine and LPS, while there was a non-significant increase in serum TNF-α at day 7 in the nevirapine and LPS group compared to the nevirapine group. No changes were observed with IFN-γ. Unfortunately, no results were shown for controls or LPS treatment only. Again, pharmacokinetics differed with LPS coadministration: while nevirapine serum levels were lower at weeks 2 and 3 compared to week 1 with nevirapine only, nevirapine levels were similar at weeks 1, 2, and 3 with co-treatment. The authors note that LPS has been reported to inhibit CYP450 enzymes; they also suggest that LPS has effects on the innate immune system, so the combination of these effects may explain the observations observed in the co-treated groups.

In female Brown Norway rats treated with 150 mg/kg/day nevirapine in food, serum IFN-γ was not changed during the primary exposure up to 21 days, although it was elevated upon rechallenge (Popovic *et al.*, 2006). In a similar study, lymphocytes from auricular lymph nodes of female Brown Norway rats treated for up to 21 days showed increased production of CXCL1, CCL3, IL-10, IL-18, and CCL5 (Chen *et al.*, 2009). In the same animal model but at 6 hours following 150 mg/kg nevirapine administered by gavage, mRNA in the liver showed changes in expression of some immune-related genes, such as Zap70 (associated with control of immune tolerance), FK506 binding protein (Fkbp5 or immunophilin, involved in immunoregulation), or Irgm M; or protein folding, such as FKbp5 and ER degradation enhancer and mannosidase alpha-like 1 (Edem 1) (Zhang *et al.*, 2013). In the skin, 12-hydroxynevirapine (159 mg/kg by gavage) treatment increased expression of over 2,000 genes after 6 hours of treatment, including TRIM63 (a ubiquitin ligase), Fkbp5, IL-22 RA2 (soluble IL-22 antagonist), and S100a7a (a DAMP).

In female mice treated with 3.3 mg/kg/day of nevirapine by oral gavage for 8 weeks, markers of neuroinflammation was characterized in the hippocampus (Zulu *et al.*, 2018). Astrogliosis was detected by increased staining for glial fibrillary acidic protein, and IL-1β and TNF-α were increased. Tenofovir was also found to have a similar effect. These results suggested that nevirapine and tenofovir could induce inflammation in the brain. Nevirapine, but not tenofovir, was also found to decrease brain-derived neurotrophic factor in the hippocampus; the authors speculate that this may be due to a difference in the ability of the two drugs to inhibit DNA polymerase, as tenofovir is a weaker inhibitor.

Cbl-b is an E3 ubiquitin ligase that is involved in regulation of activation in many leukocytes (Lutz-Nicoladoni *et al.*, 2015). In male Cbl-b$^{-/-}$ mice treated with nevirapine in food for 7 days at a dose approximating 950 mg/kg/day, serum IL-6 and IFN-γ were increased with

treatment; the increase was greater if liver necrosis was noted by histology (Sharma *et al.*, 2012). These increases resolved by day 14.

d. Clozapine

Pre-treatment with clozapine (5 mg/kg, i.p.) completely prevented the induction of HSP70 immunoreactive cells triggered by PCP in the cingulate cortex of female Sprague Dawley rats, observed at 24 hours (Sharp *et al.*, 1994). Likewise, pre-treatment with clozapine (20 mg/kg, i.p.) significantly reduced PCP-induced increases in hsp70 mRNA levels in cortex at 3 hours (Nakahara *et al.*, 1999). In the absence of PCP treatment, clozapine also decreased hsp70 MRNA in the prefrontal cortex, striatum, and nucleus accumbens. In a similar model, the induction of both c-fos and HSP70 immunoreactive brain cells at 24 hours by dizocilpine was dose-dependently inhibited by clozapine (0.31, 0.62, 1.25 mg/kg, s.c.) pre-treatment in female BKTO mice (O'Neill *et al.*, 1998).

After 65 days of clozapine (20 mg/kg/day, p.o. [administered in drinking water]), male PCP-exposed clozapine treated rats had increased serum corticosterone concentrations and both male clozapine groups had increased serum TNF-α concentrations, while female PCP-exposed clozapine treated rats had decreased serum corticosterone concentrations and both female clozapine groups had increased serum IL-6 concentrations (Nikolic *et al.*, 2017).

After 90 minutes, clozapine (30 mg/kg, p.o.) significantly damped LPS- and polyI:C-induced increases in serum TNF-α and IL-6, but further increased IL-10 levels (Sugino *et al.*, 2009). In the absence of LPS or polyI:C, clozapine did not affect levels of proinflammatory mediators, but did increase serum levels of IL-10 compared to saline-treated controls.

In a male rate hepatic I/R model, clozapine (15 mg/kg, s.c.) pre-treatment significantly reduced hepatic IL-12 (p70) and TNF-α levels compared to I/R control animals at 2 hours post-ischemia (El-Mahdy *et al.*, 2013).

In a female C57BL/6 mouse model of EAE, clozapine (60 mg/kg/day, p.o. [administered in drinking water]) significantly attenuated increases in CCL2 and CCL5 protein levels in the brain, and mRNA levels in both the brain and spinal cord (Robichon *et al.*, 2020). Clozapine did not affect EAE-induced changes in CCL2 or CCL5 levels in the blood or spleen, nor did clozapine have any effect in the absence of EAE.

After 2 weeks, clozapine (5 mg/kg/day, i.p.) completely reversed social isolation-induced reductions in plasma IL-4, IL-6, INF-γ, and kynuric acid and elevations in TNF-α, kynurenine, and quinolinic acid in male Sprague Dawley rats (Moller *et al.*, 2013).

No changes in IL-1β or COX-2 hippocampal protein levels were observed after 3 weeks of clozapine (20 mg/kg/day, i.p.) treatment in male Wistar rats, but decreased TNF-α levels were observed in clozapine-treated rats that were also socially isolated for the duration of the study (Todorovic and Filipovic, 2017b). In the prefrontal cortex, clozapine alone had no effect, but significantly attenuated elevated IL-1β, TNF-α, and COX-2 levels induced by social isolation (Todorovic and Filipovic, 2017a).

Clozapine (25 mg/kg, s.c.) given twice daily was not effective in reducing sepsis-induced elevations in serum IL-1β, TNF-α, or IL-10 in male Wistar rats (Machado *et al.*, 2007). It appears as though clozapine may have exacerbated the increases in TNF-α and IL-10, but a statistical comparison of clozapine and the appropriate control group was not provided.

In male Wistar rats, clozapine (2, 8, 32 µmol/kg, s.c. [0.65, 2.61, 10.46 mg/kg]) paradoxically reduced core body temperature by up to 2°C (Oerther and Ahlenius, 2000).

Five hours after administration of clozapine (20 mg/kg, i.p.) to male hooded Long Evans rats, mRNA expression levels of TNF-α were significantly reduced in the prefrontal cortex with treatment but were not altered in other brain regions (Paterson *et al.*, 2006).

Clozapine (30 mg/kg/day, i.p.) administration in female Sprague Dawley rats resulted in transiently elevated serum G-CSF levels at 3 and 6 hours after the first dose and increased CXCL2 expression in the bone marrow on day 10 (Lobach and Uetrecht, 2014a).

No changes in striatal TNF-α, norepinephrine, or dopamine were detected with clozapine (5 or 10 mg/kg/day, i.p.) in male Wistar rats (Bishnoi *et al.*, 2008, 2011). Conversely, another study showed that 2 weeks of clozapine (15 mg/k/day, i.p.) treatment in male C57BL/6 mice caused a moderate increase in cingulate cortex norepinephrine (Ookubo *et al.*, 2013).

In male BALB/c mice, significantly elevated serum levels of epinephrine and norepinephrine were observed from 1 to 2 weeks with clozapine (5, 10, and 25 mg/kg/day, i.p.) (Wang *et al.*, 2008). In the heart tissue, TNF-α levels were also increased with clozapine compared to saline controls but were only measured after 2 weeks of treatment and may have been higher during peak inflammatory lesion observation at day 7. Co-administration of propranolol significantly dampened all increased mediators after 2 weeks.

After 21 days, clozapine (10, 15, or 25 mg/kg/day, i.p.) elevated serum and cardiac 8-oxo-2'-deoxyguanoside levels in male Sprague Dawley rats (Abdel-Wahab and Metwally, 2014; Abdel-Wahab *et al.*, 2014). Clozapine also caused elevations in cardiac MDA, 3-nitrotyrosine, nitric oxide, nitrite, and TNF-α. Elevated levels of serum TNF-α and reduced IL-10 were also reported, and inflammatory effects were attenuated by co-administration of captopril.

After 21 days of treatment in male Wistar rats, clozapine (10 mg/kg/day, i.p.) caused a number differentially-expressed proteins in the nucleus accumbens, including increased levels of HSPA8, HSP75, and GSH synthase (Kedracka-Krok *et al.*, 2016).

Male Sprague Dawley rats treated with clozapine (20 mg/kg/day, i.p.) for 30 days were reported to have reduced cerebrospinal fluid levels of IL-8 versus saline controls, while kynurenic acid levels were unaffected (Larsson *et al.*, 2015)

Clozapine (0.5 mg/kg/day, p.o.) administration for 30 days resulted in increased expression of renal kidney injury molecule-1 and tissue inhibitor of metalloproteinase-1 mRNA (Mohammed *et al.*, 2020). Additionally, serum protein levels of IL-6, IL-1β, TNF-α, and MDA were elevated with clozapine. Except for clozapine-induced MDA increases, all parameters were attenuated by co-administered with sulpride.

2. Clinical Studies
   a. Amoxicillin

In healthy adult males administered oral amoxicillin (1 g) and clavulanate potassium (125 mg) twice daily for 5 days, no change in the proportion of TNF-α-expressing $CD14^+$ cells in the blood and no change in lactoferrin or lysozyme levels in the serum or feces was observed up to 61 days after beginning treatment (Dufour *et al.*, 2005).

   b. Nevirapine

A study examined the activation of T cells isolated from women up to 6 weeks after delivery (Shalekoff *et al.*, 2009). Women were HIV-positive and received single dose nevirapine if HIV status was known before delivery; controls were identified as HIV-positive after delivery and had therefore not received nevirapine. Nevirapine was associated with decreased plasma CCL3 and IL-8, but not IL-15 or TNF-α.

In a study combining patients from three clinical trials in which IL-6 levels in HIV-positive patients were studied, nevirapine exposure was correlated with lower plasma IL-6 levels

compared to efavirenz exposure (IL-6 was observed to be highest in untreated patients) (Borges *et al.*, 2015). Nevirapine treatment is associated with lower HIV RNA levels compared to other NNRTIs, which might help to explain the observed effect. Randomization to protease inhibitor- or NNRTI-based therapies was not performed in these trials.

A study examined the levels of soluble CD14 (sCD14) in plasma of HIV-positive patients in Nantes, France (Allavena *et al.*, 2013). Patients were taking triple antiretroviral therapy including either nevirapine or efavirenz. Patients on nevirapine-based therapy had a median plasma sCD14 level of 1.7 mg/mL, while those on efavirenz-based therapy had a level of 1.9 mg/mL. However, there was no control group and the study was not randomized; patients on efavirenz were significantly younger, had a shorter time on ARV than those on nevirapine. A few comparisons suggested that sCD14 levels were not related to viral load.

A study examined soluble markers of immune activation in HIV-positive mothers and infants, and examined the effect of exposure to nevirapine: some mothers had not received single-dose nevirapine before giving birth, while some had; HIV-negative mother/child pairs were also used as a control (Schramm *et al.*, 2006). Neopterin and soluble L-selectin levels in cord blood in exposed, uninfected infants were significantly higher with maternal nevirapine exposure. No differences in neopterin, soluble L-selectin, or β_2-microglobulin levels were observed in infants infected intrapartum (HIV-negative at birth, but HIV-positive at 6 weeks of age). Neopterin and β_2-microglobulin levels were increased with nevirapine exposure in infants infected *in utero* (HIV-positive at birth). The same markers were measured in maternal plasma drawn at the same time as the cord blood; the only difference was an increase in neopterin levels with nevirapine exposure in mothers whose infants were infected *in utero*. No correlations between maternal and infant levels for any of the three analytes was found, suggesting that immune activation in the infant occurs independent of the maternal environment. The authors suggest that single-dose nevirapine induces T cell anergy, which may aid in preventing HIV-1 replication. Additionally, the authors propose that nevirapine may synergize with HIV-1 to increase immune activation.

Two studies have been performed evaluating the effect of genetic variations in some immune markers. A study examining the effect of CCL3L1 (a CCR5 ligand) gene copies on HIV mother-to-newborn transmission found that maternal nevirapine was associated with decreased spontaneous and phytohemagglutinin-stimulated release of CCL3 in cord blood mononuclear cells from uninfected infants (Kuhn *et al.*, 2007). Additionally, greater infant CCL3L1 gene copies were associated with reduced HIV transmission overall, but this affect was attenuated with maternal single-dose nevirapine exposure. Another study using cohorts in Malawi, South Africa, and Uganda examined multiple chemokine receptor polymorphisms in the context of maternal HIV transmission (Singh *et al.*, 2008). Mother-to-child transmission was reduced with nevirapine treatment with CCR5-59029-G/A or CCR5-59353-T/C, in which both polymorphisms were associated with an increased risk compared to wildtype. Transmission risk was increased with nevirapine treatment for the CX3CR1-745-G/A (249-V/I) (280-T/M) polymorphism. In both cases, nevirapine appears to have an effect on the role of certain cytokines in HIV transmission.

In a study of 11 HIV-positive children initiated on triple therapy with stavudine, ritonavir, and nevirapine for up 24 weeks, responses of blood cells to multiple stimuli were evaluated *ex vivo* (Blazevic *et al.*, 2001). At baseline, peripheral blood mononuclear cells from these children had a lower delayed-type hypersensitivity response to *Candida albicans* compared to peripheral blood mononuclear cells from healthy controls, which increased at week 24.

However, the response to tetanus toxoid was not observed in HIV-positive children at baseline (significantly lower than healthy controls) and did not change at 24 weeks. In evaluating APC function, monocytes from HIV-positive patients produced significantly less IL-12p70 in response to *Staphylococcus aureus* Cowan compared to healthy controls; at 24 weeks, IL-12p70 secretion was increased. However, these effects cannot be attributed to nevirapine therapy alone, as a combination of drugs was administered to these patients; additionally, the effect of treatment and restitution of CD4 T cells also likely plays an important role in these effects.

c. Clozapine

In a retrospective analysis of 93 schizophrenia or schizoaffective disorder patients (65 male and 28 female) initiated on clozapine in Canada, it was noted that 20% of patients developed fever during first month treatment, lasting an average of 2 weeks (Tham and Dickson, 2002). The majority of fever onset was observed from weeks 1 to 3, although a range from 3 to 26 days was reported. Similarly, in a retrospective review of 227 patients (113 male and 94 female) commencing clozapine therapy in Hong Kong, 14% (19 male and 12 female) developed a fever in first 3 weeks of treatment that lasted around 5 days (range: 1-12 days) (Pui-yin Chung *et al.*, 2008). The mean time to fever onset was 14 days (range: 5-21 days, plus one case at day 47) and mean clozapine dosage at the onset of fever was 166.1 ± 73.2 mg/day (range: 50-300 mg/day).

In a study of 10 schizophrenic patients (7 males and 3 females) and 8 healthy controls (6 males and 2 females), a marked increase in soluble IL-2R levels was observed from weeks 2-6 of treatment, when mean clozapine doses were 285 ± 138 and 343 ± 169 mg/day, respectively (Pollmacher *et al.*, 1995). To characterize these immune changes in more detail, a second 6-week study of 14 male and 13 female patients diagnosed with schizophrenia, schizophreniform disorder, or schizoaffective disorder was conducted (Pollmacher *et al.*, 1996). Compared to pre-clozapine baseline levels, plasma levels of TNF-α, soluble TNF-R p55, soluble TNF-R p75, and soluble IL-2R were elevated for the duration of the study, measured at week 1 (mean clozapine dose: 162 ± 68 mg/day), week 2 (mean clozapine dose: 245 ± 115 mg/day), and week 6 (mean clozapine dose: 305 ± 160 mg/day) of treatment. Additionally, plasma IL-6 levels were transiently increased at week 2 of treatment and 44% of patients developed a fever that lasted up to 6 days.

This group replicated these findings in a study of 6 male and 6 female patients with schizophrenia who, again, were monitored during initiation of clozapine therapy (Hinze-Selch *et al.*, 2000). Soluble TNF-R p55 and p75 were increased with clozapine from weeks 1 to 6 and TNF-α and soluble IL-2R were elevated from week 2 onward. Of 20 schizophrenia patients monitored during the first 6 weeks of clozapine therapy, 55% developed a transient fever and increase in plasma G-CSF levels that was most prominent following 2 weeks of treatment (Pollmacher *et al.*, 1997). Similarly, fever was reported in 47% of schizophrenic patients monitored during the first 2 weeks of clozapine treatment (Hinze-Selch *et al.*, 1998). In this study, mean time to fever onset was 15 ± 5 days (range: 2-25 days) with a mean duration of 2.5 ± 1.3 days (range: 1-6 days). The average clozapine dose increased from 178 ± 57 mg/day at week 1, to 281 ± 111 mg/day at week 2, to 325 ± 152 at week 6.

Another group conducted a study with 17 schizophrenic patients (7 male and 10 female) started on clozapine and sex- and age-matched healthy controls (Monteleone *et al.*, 1997). After 10 weeks (mean clozapine dose: 312 ± 120 mg/day [range: 75-400 mg/day]), no differences in plasma IL-6 levels were observed with clozapine, but TNF-α levels that had been elevated in the schizophrenia group at baseline were dampened to control levels. Cytokine changes were also

evaluated in a study of 23 schizophrenic patients (13 males and 10 females) before and after starting clozapine (Maes *et al.*, 1997). Compared to healthy controls (11 males and 6 females), clozapine-naïve patients showed significantly higher plasma IL-6R and IL-1RA and lower uteroglobin levels. During the first 2 weeks of clozapine treatment, further increases in plasma IL-6 and increased uteroglobin occurred and after 5 weeks. Increased plasma levels of soluble CD8 and IL-1RA were observed.

In a study evaluating cytokine changes in 12 new clozapine users, it was demonstrated that while levels of IL-8 and IL-10 were unchanged, serum levels of soluble CD8 and leukemia inhibitory factor receptor were markedly elevated after 2 months of clozapine treatment, compared to baseline or 4-month treatment levels (Maes *et al.*, 2002). The mean clozapine doses at months 2 and 4 were 358.3 ± 144.3 mg/day and 395.0 ± 101.2 mg/day, respectively. A similar study compared cytokine levels of 22 new clozapine users (16 male and 6 female) to 21 patients who had been maintained on clozapine for greater than 6 months (14 male and 7 female) (Hung *et al.*, 2017). Compared to 6% of old users, 47% of new users developed fever during weeks 1 to 4, lasting an average of 2ays. Although no differences were observed in cytokine levels between new and old clozapine users, compared to new users without fever, plasma levels of TNF-α, INF-γ, IL-2, and IL-6 levels were significantly different in the patients who developed clozapine-induced fever.

Another group conducted a 6-week randomized double-blinded trial to compare the effects of clozapine (mean modal dose: 266.7 ± 77.9 mg/day) and olanzapine (mean modal dose: 21.2 ± 2.5 mg/day) in patients with schizophrenia, schizophreniform disorder or schizoaffective disorder (Kluge *et al.*, 2009). Thirty patients were randomized to either the clozapine (7 male and 8 female) or olanzapine (5 male and 10 female) group. Five patients treated with clozapine developed fever during the first 3 weeks of treatment, which lasted up to 5 days, while no patients treated with olanzapine developed fever. Compared to baseline values, plasma TNF-α, soluble TNFR-2, and soluble IL-2R remained significantly elevated from week 2 to 6 with both treatments and peaked around week 3, although levels of soluble TNFR-2 were markedly higher with clozapine compared to olanzapine. Additionally, IL-6 and soluble TNFR-1 levels were only elevated with clozapine treatment during weeks 2 to 3 and weeks 2 to 6, respectively. Moreover, at week 2 of clozapine treatment, IL-6 and soluble TNFR-2 were significantly higher in patients with fever compared to those without fever. Lastly, plasma leptin levels were significantly increased in females for the entire study, while levels in males were transiently elevated during weeks 2 and 3 with clozapine.

       G. Effects on Applicable Signal Transduction Factors
          1. Rodent Studies
            a. Amoxicillin

In male Sprague-Dawley rats administered an intratracheal instillation of LPS to induce mucus hypersecretion and administered 40 mg/kg amoxicillin orally for 4 days, there was no difference in NF-κB expression in bronchial epithelium (Ou *et al.*, 2008).
            b. Clozapine

Using the PCP pre-treatment model in male C57BL/6 mice, it was shown that 1 week of clozapine (10 mg/kg/day, p.o.) administration enhanced PCP-induced decreases in cytosolic Nrf2 and increases in nuclear Nrf2 protein levels in the prefrontal cortex (Tran *et al.*, 2017). However, after day 1, clozapine significantly attenuated PCP-induced mitochondrial translocation of phosphorylated AKT (Tran *et al.*, 2018). In the postnatal model of schizophrenia using male

C57BL/6 mice, it was found that co-administration of clozapine (6 mg/kg/day, i.p.) with PCP greatly decreased the phosphorylation ratio of AKT (also called protein kinase B) in the cerebral cortex compared to vehicle-treated PCP controls, although the phosphorylation ratio of extracellular signal-regulated kinase (ERK) was unchanged (Barzilay *et al.*, 2011).

One study evaluated the impact of 3 weeks of clozapine (1 mg/kg/day, s.c.) pre-treatment on nigral NF-κB levels in male Sprague Dawley rats followed by apomorphine stimulation (Saldana *et al.*, 2006). Clozapine induced a significant decrease in nigral NF-κB p65 and p50 protein levels compared to the apomorphine-stimulated, saline-pre-treated control group. Contrary to this, no changes in striatal NF-κB p65 protein levels were detected following 3 weeks of clozapine (5 or 10 mg/kg/day, i.p.) administration in male Wistar rats (Bishnoi *et al.*, 2008, 2011).

Dose-dependent elevations in cardiac NF-κB p65 were observed following 3 weeks of clozapine (10, 15, or 25 mg/kg/day, i.p.) treatment in male Wistar rats (Abdel-Wahab and Metwally, 2014). A similar outcome was noted in another study, where socially isolated male Wistar rats given clozapine (20 mg/kg/day, i.p.) for 3 weeks had decreased cytosolic and increased nuclear NF-κB p65 protein levels in the liver, versus saline controls (Zlatkovic *et al.*, 2014). This, however, was not observed the prefrontal cortex, where clozapine significantly attenuated social isolation-induced reduced cytosolic and elevated nuclear NF-κB p65 protein levels (Todorovic and Filipovic, 2017a). No changes in cytosolic or nuclear NF-κB p65 hippocampal protein levels were observed in this model (Todorovic and Filipovic, 2017b).

One hour following clozapine (12 mg/kg) administration in female Sprague Dawley rats, hepatic eIF2α phosphorylation was significantly increased, suggesting activation of the protein kinase R-like ER kinase/eukaryotic translation initiation factor 2A (PERK/eIF2α) ER stress axis (Weston-Green *et al.*, 2018). Additionally, in this model, hepatic sphingolipid homeostasis was significantly disrupted. Specifically, hepatic levels of ceramide and sphingolipids were markedly reduced with clozapine, while ceramide synthase and a fatty acid elongase enzyme were increased.

Male Sprague Dawley rats treated with a single dose of clozapine (10 mg/kg, i.p.) showed a marked increase in frontal cortex phospohorylated-Unc-51-like kinase (ULK) 1 (Ser317) and phosphorylated-Beclin1(Ser93) was demonstrated from 2 to 4 hours and an increase in phosphorylated-AMP-activated protein kinase (AMPK) α (Thr172) from 1 to 4 hours, although total levels of these protein were unchanged (Kim *et al.*, 2018). Moreover, AMPK substrates were increased from 1 to 4 hours. Co-treatment of rats with clozapine and the AMPK inhibitor dorsomorphin resulted in attenuation of these increases at 2 hours. Together, the AMPK-ULK1-Beclin1 signaling pathway is responsible for induction of autophagy, where phosphorylation of AMPK leads to its activation and subsequent phosphorylation of ULK, together leading to the activation of Beclin1 and the activation of autophagy components. However, AMPK has been shown to play a role in a number of biochemical pathways, including mitochondrial biogenesis and lipid metabolism (Hardie *et al.*, 2016) and thus, clozapine's impact on AMPK signaling likely extends beyond autophagy.

It was demonstrated that PPARα and LXRα (ligand-activated nuclear hormone receptors that normally control aspects of lipid homeostasis) mRNA levels were significantly decreased at 6 hours following clozapine (25 or 50 mg/kg, i.p.) administration in female Sprague Dawley rats, as compared to vehicle controls (Ferno *et al.*, 2009). Additionally, LXRα target gene cholesterol efflux gene ATP-binding cassette, sub-family A was significantly upregulated at this time. All changes resolved by 48 hours.

One study demonstrated that, when co-administered with ketamine in male Sprague Dawley rats, clozapine (5 mg/kg/day, i.p.) significantly attenuated decreased phosphorylated AKT and phosphorylated GSK-3β and attenuated increased phosphorylated β-catenin in several brain regions, compared to ketamine-treated controls (George *et al.*, 2020).

Thirty minutes following N-desmethylclozapine (20 mg/kg, s.c.) administration in male CD1 mice, increased phosphorylated AKT and phosphorylated AKT-glycogen synthase kinase (GSK) expression levels were reported in the nucleus accumbens, compared to saline controls. This effect was significantly antagonized by pre-treatment with the δ-opioid receptor antagonist naltrindole (Olianas *et al.*, 2011). Using C57BL/6 TLR-2$^{-/-}$ mice, however, it was demonstrated that the increased expression levels of phosphorylated GSK-3α/β observed in TLR-2$^{-/-}$ mice were markedly reduced in several brain regions 1 hour post-clozapine (1 mg/kg, i.p.) administration (Park *et al.*, 2015). Clozapine did not alter increased levels of phosphorylated AKT.

Another study showed that 2 weeks of clozapine (15 mg/k/day, i.p.) treatment in male C57BL/6 mice resulted in a number of brain region-specific changes in histone deacetylases (HDAC) (Ookubo *et al.*, 2013). Specifically, clozapine increased HDAC2, HDAC3, and HDAC8 in the striatum, HDAC2 and HDAC3 in the nucleus accumbens, and HDAC3 in cingulate cortex, compared to vehicle controls. Additionally, acetylated histone H3 protein expression levels were increased in several brain areas with clozapine.

Differences in histone-promoter binding in the frontal cortex were noted following administration of clozapine (10 mg/kg/day, i.p.) in male 129S6/Sv mice for 3 weeks (de la Fuente Revenga *et al.*, 2019). Clozapine induced a significant decrease of lysine-acetylated histone H3 and HDAC2 binding to the metabotropic glutamate 2 receptor, homer protein homolog 1, and glutamate receptor ionotropic, NMDA 1 gene promoter regions of wildtype, but not in clozapine-treated HDAC2$^{-/-}$ mice.

H. Effects on Mitochondria and Oxidative Stress
1. Rodent Studies
a. Amoxicillin

In male Wistar rats, amoxicillin (10 or 30 mg/kg/day, i.p.) was administered once daily for 14 days (Oyebode *et al.*, 2019). The higher dose resulted in significantly increased mitochondrial permeability transition pore opening, enhanced rat liver microsome ATPase activity, cytochrome C release, and increased levels of rat liver microsome malondialdehyde generation.

b. Nevirapine

A study using male CF-1 mice administered 3.3 mg/kg nevirapine by oral gavage for 36 days found that mitochondrial complex IV activity was inhibited in the cerebral cortex, striatum, and hippocampus, but not striatum (Streck *et al.*, 2011).

A study using male albino rats administered 6 mg/kg nevirapine orally for 60 days found no difference in serum catalase, superoxide dismutase, or glutathione (Awodele *et al.*, 2015). Increased serum malondialdehyde was reported, which is used as a measure of lipid peroxidation. This effect was not modulated by co-treatment with antioxidants vitamin C, vitamin E, or jobelyn.

A study of multiple organs in Wistar rats treated with 18 or 36 mg/kg for 4 weeks compared to vehicle control found that nevirapine caused a dose-dependent increase in malondialdehyde in the liver, kidney, and testes, which was accompanied by a decrease in superoxide dismutase and catalase activities and glutathione (Adaramoye *et al.*, 2012).

A number of ultrastructural changes were observed in the liver of female Brown Norway rats treated with 150 mg/kg nevirapine for 8 weeks (Sastry *et al.*, 2018). Degenerate mitochondria were observed in hepatocytes of treated rats. Lipid droplets associated with smooth endoplasmic reticulum cisternae were also observed in these hepatocytes.

In female Brown Norway rats treated with 159 mg/kg/day 12-hydroxynevirapine, transcripts related to mitochondria, including pyruvate dehydrogenase kinase, isozyme 4 (Pdk4), 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (Hmgcs2), and uncoupling protein 3 (Ucp3), were upregulated in the skin 6 hours post-dose (Zhang *et al.*, 2013).

   c.   Clozapine

One group demonstrated that coadministration of clozapine (10 mg/kg/day, i.p.) in male Sprague Dawley rats with either PCP or methamphetamine for 28 days greatly attenuated the decrease in cytochrome c oxidase activity observed in multiple brain regions observed with PCP or methamphetamine alone (Prince *et al.*, 1997b). Clozapine monotherapy also increased brain cytochrome c oxidase activity (Prince *et al.*, 1997a). Conversely, no changes in complex I activity were observed. Another group evaluated the effects of clozapine (10 mg/kg/day, p.o.) in male C57BL/6 wildtype or p47phox^{-/-} mice that had been pre-treated with PCP (Tran *et al.*, 2018). After day 1, clozapine attenuated PCP-induced mitochondrial changes in the prefrontal cortex of wildtype mice, and attenuated elevations in ROS and protein oxidation in both genotypes. Clozapine in mice without PCP treatment did not appear to have any significant effects.

In male Sprague Dawley rats treated with ketamine, 1 week of clozapine (5 mg/kg/day, i.p.) markedly attenuated decreased hippocampal GSH levels and decreased striatal catalase activity observed in the ketamine control group (George *et al.*, 2020).

One hour following administration of clozapine (25 mg/kg, i.p.) in male Sprague Dawley rats, activating transcription factor 3 (ATF3) mRNA levels in the liver were significantly elevated compared to animals in the water control group (Lauressergues *et al.*, 2012). ATF3 is a sensor of ER stress, which can initiate UPR through several downstream pathways. By 3 hours, ATF3 levels remained elevated, and increases in 3-hydroxy-3-methylglutaryl-coA reductase and tribbles drosophila homolog 3 (a marker of the PERK pathway of ER stress) were also observed.

With social isolation, male Sprague Dawley rats were given clozapine (5 mg/kg/day, i.p.) for 14 days and it was found that clozapine completely reversed social isolation-induced reductions in frontal cortical ATP and elevations in striatal ATP (Moller *et al.*, 2013). However, in another social isolation model in male Wistar, liver cytosolic CuZn-superoxide dismutase protein levels were decreased with clozapine (20 mg/kg/day, i.p.), irrespective of housing status during the study (Zlatkovic *et al.*, 2014).

Compared to controls, male Wistar rats treated with clozapine (25 mg/kg/day, i.p.) for 28 days displayed increased AMP hydrolysis in the striatum, but not in the hippocampus, at 24 hours following the final dose (Lara *et al.*, 2001).

Male Long Evans rats treated with clozapine (10 mg/kg/day, i.p.) for 21 days had neuronal expression of nitric oxide synthase mRNA that was significantly decreased compared to control levels (Bullock *et al.*, 2008).

One group demonstrated that treatment of male Wistar rats with clozapine (25 mg/kg/day, i.p.) for 4 weeks resulted in inhibition of succinate dehydrogenase activity in striatum, but not in other areas of the brain, and cytochrome oxygenase activity was unchanged (Streck *et al.*, 2007).

After 28 days of clozapine (25 mg/kg/day, i.p.) treatment and 3 rest days, no change in mitochondrial superoxide production was observed in several brain regions of male Wistars rats when compared to saline-treated rats (Martins *et al.*, 2008). However, protein carbonyl content (a measure of oxidative damage to proteins) was found to be increased with clozapine in the hippocampus, while thiobarbituric acid reactive substances (TBARS; a measure of lipid peroxidation) were decreased in the cerebral cortex with treatment. Another group investigated similar effects following 21 days of clozapine (10 mg/kg/day, i.p.) administration in male Wistar rats and found increased levels of striatal TBARS and superoxide anion and decreased levels of non-protein thiols and total nitric oxide in comparison to vehicle control rats (Bishnoi *et al.*, 2011).

One group observed multiple stress-related metabolic pathway alterations in prefrontal cortex after 4 weeks of clozapine (21 mg/kg/day, i.p.) administration in male Sprague Dawley rats (Cai *et al.*, 2017). Specifically, clozapine increased creatine, inosine, progesterone, and phosphatidylethanolamine levels, and decreased corticosterone levels in comparison to vehicle controls.

Hippocampal protein levels of malate dehydrogenase and vacuolar ATP synthase were demonstrated to be significantly reduced after 30 days of clozapine (45 mg/kg/day, p.o.) treatment in male Sprague Dawley rats compared to water-treated control animals (La *et al.*, 2006).

After 28 days of treatment in male Wistar rats, clozapine (45 mg/kg/day, p.o.) significantly increased the activity of cardiac SOD1, without affecting protein levels, in comparison to the vehicle control group (Nikolic-Kokic *et al.*, 2018).

After 4 weeks of clozapine (20 mg/kg/day, p.o.) treatment in female Wistar rats, one group noted inhibition of ovarian mitochondrial complex I, but not complex III, activity, decreased SOD and GSH levels, and increased TBARS, and these changes were not observed in controls (Khalaf *et al.*, 2019).

Male Sprague Dawley rats administered clozapine (45 mg/kg/day, p.o.) for 34 days exhibited increased mRNA and protein levels of cytochrome b5 but increased mRNA and decreased protein levels of glial fibrillary acidic protein, shown in mitochondria isolated from the cerebral cortex (Ji *et al.*, 2009). Increases in gene expression of MAPK-activated protein kinase 2 and Ndufv2 (whose encoded protein is an electron transport chain member participating in oxidative phosphorylation) were not observed at the protein level.

One study noted a number of changes in mRNA expression in the brains of male C57BL/6 mice after 31 days of clozapine (10 mg/kg/day, p.o. in rodent meal pellets) versus mice on the control diet (Mehler-Wex *et al.*, 2006). Specifically, isoforms of cytochrome C oxidase, lactate dehydrogenase, ATPase, and mitochondrial uncoupling protein 1 decreased in expression, while phospholipase C increased in expression.

    2. Clinical Studies
     a. Nevirapine

A retrospective study of HIV-infected children in Accra, Ghana found that nevirapine exposure was associated with a positive score using the Enquête Périnatale Française, a symptom-based evaluation tool (Langs-Barlow *et al.*, 2013).

A study of black South African patients infected with HIV taking a combination of stavudine, lamivudine, and either efavirenz or nevirapine for 4-24 months found that nevirapine exposure, but not efavirenz exposure, resulted in a significant time-dependent increase in mean total lymphocyte $\Delta\psi m^{low}$ (mitochondrial depolarization) and was also correlated with mean total

lymphocyte apoptosis (Karamchand *et al.*, 2008). However, as control patients were HAART-naïve, it is difficult to isolate the effect of nevirapine alone when it was administered as part of combination drug therapy here.

A cross-sectional study of HIV-infected adults in Tennessee compared exposure for at least 30 days to HAART with or without NNRTI to patients who had not received HAART in the last 3 months (6 months for NNRTIs) on measures of oxidative stress (Redhage *et al.*, 2009). Median plasma F_2-isoprostane levels, a measure of lipid peroxidation, were found to be decreased in the NNRTI-exposed group; there was a trend toward lower levels with nevirapine exposure, but this was not statistically significant.

A prospective cohort study of HIV-uninfected and -infected pregnant women and infant pairs in Cameroon found that nevirapine treatment of HIV-exposed, uninfected infants (compared to HIV-unexposed, uninfected infants) was not associated with a lower mitochondrial to nuclear DNA ratio in dried blood spots at 6 weeks of age, although zidovudine exposure was (Jao *et al.*, 2017).

A multicenter, prospective randomized trial evaluated efficacy and safety of switching to lopinavir-ritonavir and nevirapine versus lopinavir-ritonavir and two NRTIs in HIV-infected adults who had achieved virologic suppression (Negredo *et al.*, 2009). The nevirapine-treated group experienced an increase in mitochondrial to nuclear DNA ratio in peripheral blood mononuclear cells by week 48. There was also an increase in cytochrome c oxidase IV activity at weeks 24 and 48 (presumably also in PBMCs).

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