Innate immunity in idiosyncratic drug-induced liver injury: kinetics matters.

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Innate immunity in idiosyncratic drug-induced liver injury: kinetics matters

Het aangeboren afweersysteem in idiosyncratische leverschade door medicijnen: een zaak van kinetiek

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 26 september 2019 des ochtends te 10.30 uur

door

Giulio Giustarini

geboren op 4 mei 1985 te Pisa, Italië

Promotor:	Prof.dr. ir. J. Legler
Copromotoren:	Dr. R.H.H. Pieters Dr. J.J. Smit

"If you're going to try, go all the way.
Otherwise, don't even start.

If you're going to try, go all the way.
this could mean losing girlfriends,
wives, relatives, jobs and
maybe your mind.

Go all the way.
It could mean not eating for 3 or 4 days.
It could mean freezing on a park bench.
It could mean jail,
it could mean derision, mockery, isolation.
Isolation is the gift,
all the others are a test of your endurance,
of how much you really want to do it.
And you'll do it despite rejection and the worst odds
and it will be better than anything else you can imagine.

If you're going to try, go all the way.
There is no other feeling like that.
You will be alone with the gods
and the nights will flame with fire.

Do it, do it, do it.
Do it.

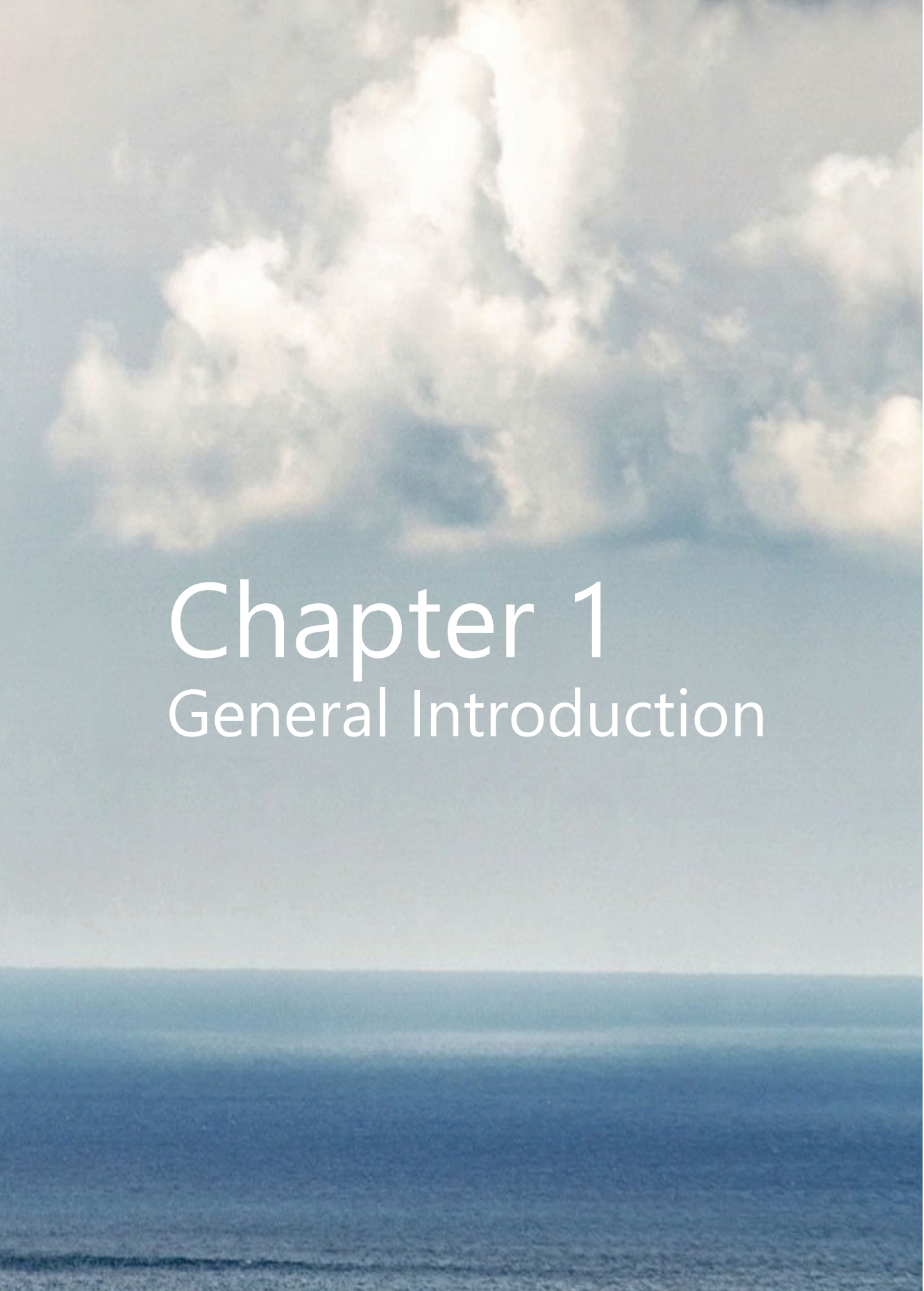
All the way
All the way.

You will ride life straight to perfect laughter,
its the only good fight there is."

Charles Bukowski

To Roberto,
my dad

CONTENTS



Chapter 1
General Introduction

GENERAL INTRODUCTION

1.1 Adverse drug reactions

The use of pharmaceutical compounds does not involve therapeutic effects only, but also a plethora of toxicological effects which may appear with different incidence and severity in humans. Together these toxicological effects are considered adverse drug reactions (ADR).
The World Health Organization defines an ADR as "*a response to a drug which is noxious and unintended, and which occurs at doses normally used in man for the prophylaxis, diagnosis, or therapy of disease, or for the modification of physiological function*"(Who, 1972).
ADR can affect multiple organs, although one of the most often targeted is the liver (Shaw et al., 2010).
The occurrence of ADR represents a major concern for health care systems and pharmaceutical companies all around the world. Indeed, ADR account for 6% of the total hospital admissions in the United Kingdom (Pirmohamed et al., 2004) and more than 10% of the drugs marketed between 1975 and 2000 required interventions regulating or banning their use (Lasser, 2002). The consequence of these interventions includes economical losses for both health care systems and pharmaceutical companies, besides serious discomfort and morbidity in patients. This stresses the need for better defining the mechanisms behind ADR in order to prevent toxic compounds to reach the market or sensitive populations via adequate description on the product leaflet (Lasser, 2002).

1.2 Idiosyncratic Drug-induced liver injury

In the last decades many compounds have been withdrawn from the market or their use has been restricted after high incidence of severe hepatic ADR, so called drug-induced liver injury (DILI) (Hunter et al., 1999; Graham et al., 2003; Lazarczyk et al., 2001; Choi, 2003; Maniar et al., 2006). Two major categories of DILI have been proposed. The first involves intrinsic hepatotoxicity of a particular drug or its metabolites on vital cellular targets of the liver. The second category reflects an idiosyncratic reaction culminating in hepatic inflammation and injury. Whereas intrinsic DILI is usually characterized by dose-dependency and a tight time-relationship between exposure to the drug and onset of the damage, idiosyncratic DILI is not. Among all DILI, particular scientific interest has been raised by idiosyncratic DILI (IDILI), which accounts for 13% of all cases of acute liver failure (Ostapowicz et al., 2002). Although IDILI usually occurs with a relatively low incidence (14-19:100000) these events carry significant morbidity and mortality (Chalasani et al., 2015). Thus, the evaluation of the risk/benefit ratio of drugs associated with this latter type of hepatic reactions has been a common theme in toxicology due to its complexity and undefined mechanisms.

IDILI is defined as hepatic liver injury with unpredictable and non-dose related onset, with variable pathologic features and, in many cases, involves the immune system (Chalasani et al., 2015). In particular, the onset of IDILI is often characterized by leukocyte infiltrates into the hepatic tissue and presence of antibodies against the responsible drug. In addition, IDILI is accompanied by systemic, allergic-like features such as a skin rash, blood eosinophilia and formation of drug–protein adducts, formed by drugs or their metabolites (Licata et al., 2017; Tailor et al., 2015; Chalasani et al., 2015).

IDILI may disappear upon discontinuation of the treatment, but it may also develop into autoimmune disease-like disease with sustained damage in drug-free conditions and presence of autoantibodies (Grell et al.,1995; Schwabe and Brenner, 2006). Based on biochemical criteria and/or histological assessment of biopsies, IDILI can be classified as cholestatic (mostly in elderly) or hepatocellular (mostly in young adults), but a mixed form may occur as well (Chalasani et al., 2015). Evaluation of the ratio between liver-derived enzymes detectable in blood of patients experiencing IDILI, such as alanine aminotransferase (ALT) and alkaline phosphatase (ALP), has been used for the classification of the clinical pattern of these hepatic ADRs (Aithal et al., 2011).

Although some pharmacological classes seem to be more associated than others with the occurrence of IDILI (such as anti-microbials), a chemically diverse list of pharmaceuticals is associated with the onset of these adverse reactions. Moreover, the clinical signature of IDILI varies from drug to drug, in different patient populations (Chalasani et al., 2015; Hayashi and Chalasani, 2012; Suk and Kim, 2012).

1.3 IDILI: risk factors

A number of risks factors has been identified in IDILI by retrospective and prospective studies (Hussaini and Farrington, 2014, 2007; Chalasani and Björnsson, 2010).
Among these risk factors are:

- Age, gender
- Co-morbidities (promoting sterile and non-sterile inflammation, or disruption any physiological balance)
- Human leukocyte antigen polymorphisms
- Metabolic (primary/secondary) and biliary transporter polymorphisms
- Drug interactions (food (alcohol) and nutraceuticals) (Chalasani and Björnsson, 2010; Hussaini and Farrington, 2014, 2007)

1.4 Liver and the immune system: "inflammatory" homeostasis and the balance between tolerance and immunogenicity

The liver is not only the organ responsible for metabolism and excretion of exogenous matter or simply the storage of nutrients. In addition to metabolic and detoxifying properties, the liver also carries out important immunological functions such as presentation of new antigens and clearance of potentially harmful components derived from the gut (Robinson et al., 2016). Hepatocytes, which represent approximatively 80% of the liver volume, are the main cells in charge of metabolic activities. Hepatocytes also intrinsically possess complex innate immunity features like antigen presentation and production of cytokines orchestrating inflammation (Crispe, 2011; Zhou et al., 2016). Non-parenchymal cells (NPCs), that only represent the 6,5% of the total volume of the liver but 40% of the total hepatic cell number, perform pivotal immunological tasks in order to maintain organ and individual homeostasis (Robinson et al., 2016; Tacke et al., 2009).

Among the most common NPCs of the liver are hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells (LSECs). Together, these cells form the linings of hepatic sinusoids which are key sites for antigen presentation to cells homing the liver and clearance of bacterial components originating from the gut. For this reason, the sinusoidal lumen is populated by many other innate and adaptive immune cells which interact with each other and with the NPCs in diverse pathophysiological conditions (Robinson et al., 2016; Tacke et al., 2009). The more abundant innate immune cells in liver are Kupffer cells, which are intra-sinusoidally resident macrophages. These cells are fundamental in liver homeostasis and serve as sentinels against exogenous components by promoting or regulating inflammation (Robinson et al., 2016; Tacke et al., 2009).

Other innate immune cells of the liver are natural killer cells (NKs), monocytes, neutrophils and dendritic cells (DCs) (Robinson et al., 2016; Tacke et al., 2009). Particularly fascinating is the partnership among resident macrophages, monocytes and neutrophils in the context of "homeostatic" inflammation aiming to clear potentially harmful (sterile or non-sterile) elements. The partnership between these cells has been shown to finetune inflammatory mechanisms which are involved in physiological events (Soehnlein and Lindbom, 2010; Robinson et al., 2016). These events in the liver occur due to its proximity to the gut, its major place in blood circulation and its role in metabolism and excretion of exogenous components.

The sequence of events leading to the homeostatic control and resolution of inflammation requires a tight partnership among these innate cells. The phagocyte

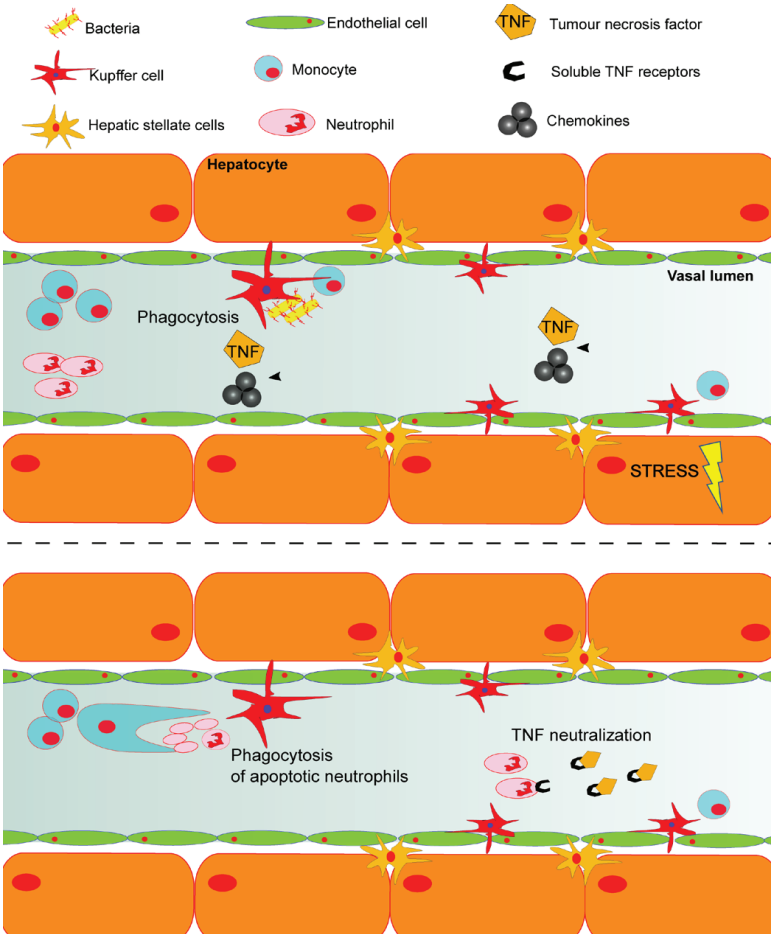


Figure 1 Inflammation in the liver and its resolution. Kupffer cells are liver-resident macrophages which reside into the lumen of hepatic sinusoids. Bacteria or their components entering the blood circulation reach the liver where they are cleared by Kupffer cells. These cells respond to the clearance of bacteria or their components by producing inflammatory cytokines (such as TNF) and chemokines (such as IL-8) with the aim to recruit neutrophils into the liver. A similar inflammatory response by Kupffer cells might also be induced by danger/alarm signals originating from cells experiencing stress. In both scenarios, neutrophils follow the gradient of chemokine concentrations entering the narrow sinusoids of the liver where they release soluble TNF receptors with the attempt to reduce the concentration of this cytokine. The sequestration of neutrophils represents an important event for regulation of inflammation. Neutrophils are very short-lived cells and, at site of inflammation, the phagocytosis of apoptotic neutrophils by Kupffer cells represents the event which marks the change in the production of cytokines from pro-inflammatory (such as TNF) to anti-inflammatory (such as IL-10). Monocytes follow the neutrophils into the liver with the aim to help resident macrophages clearing the apoptotic neutrophils and scavenging harmful cellular components.

partnership is a simplistic illustration of the mechanisms behind onset and regulation of inflammation, but it well describes different roles for each cell type. Resident macrophages and monocytes sense and clear the damaging elements via different mechanisms (such as phagocytosis, scavenger receptors etc.) before alerting other cells by cytokines and other mediators of inflammation. In particular, chemokines recruit leukocytes to the site of inflammation. Neutrophils are among the first to reach the stressed tissue in response to these chemokines (such as IL-8), aiming to help clearing the harmful components and regulating inflammation via direct or indirect mechanisms. Neutrophils are involved in killing and phagocytosis of micro-organisms (bacteria, virus, fungi etc.) but also in the regulation of inflammation via the release of soluble receptors of the key player in inflammation, i.e. TNF (Soehnlein and Lindbom, 2010; Soehnlein et al., 2009). Moreover, it has been recently proven in vitro that macrophage release of TNF is negatively affected by viable neutrophils after stimulation with several mediators of inflammation such as LPS, TNF and IL-1β (Marwick et al., 2018). What exactly is mediating this regulatory effect of neutrophils on macrophages is still a matter of debate.

In addition to the regulation by neutrophils, an indirect mechanism of regulation is represented by the phagocytosis of dying neutrophils by macrophages or monocytes which is considered the first anti-inflammatory event leading to resolution of inflammation (Soehnlein and Lindbom, 2010; Soehnlein et al., 2009). The prompt clearance of dying neutrophils is fundamental to prevent release of neutrophil cellular content and sustaining inflammation (Brauner et al., 2013) (Fig.1). The uptake and/or clearance of the damaged or damaging components is not performed exclusively by Kupffer cells, monocytes and neutrophils and other cells play a role in the uptake process, ensuring redundancy and diversification of this particular immune function. Also DCs, HSCs and LSECs take part in the uptake of diverse potentially harmful elements such as micro-organisms or their derived substances, dying cells, cell debris, soluble macromolecules and colloids (Crispe, 2011; Li et al., 2011).
The uptake and the consequent presentation of antigens in the liver by conventional (DCs and macrophages) or non-conventional (LSEC, HSC) antigen presenting cells (APCs) leads to one of the following two potential fates: tolerance or immunogenicity (Robinson et al., 2016). The liver is unique in terms of exposure to new potential antigens due to its metabolic activity and the collection of mesenteric vein-derived blood. This leaves the liver to face continuous decision making between tolerance or (adaptive) immune activation (Robinson et al., 2016; Doherty, 2016). This dichotomic decision depends on inflammatory mechanisms which participate in initiating the adaptive immunity. Indeed, increased expression of co-stimulatory signals on the membrane of APCs and pro-inflammatory cytokines, originating from stressed/damaged/activated cells, are essential prerequisites for the development of an adaptive immune response (Robinson et al., 2016; Doherty, 2016; Pirmohamed et al., 2002) (Fig.2).

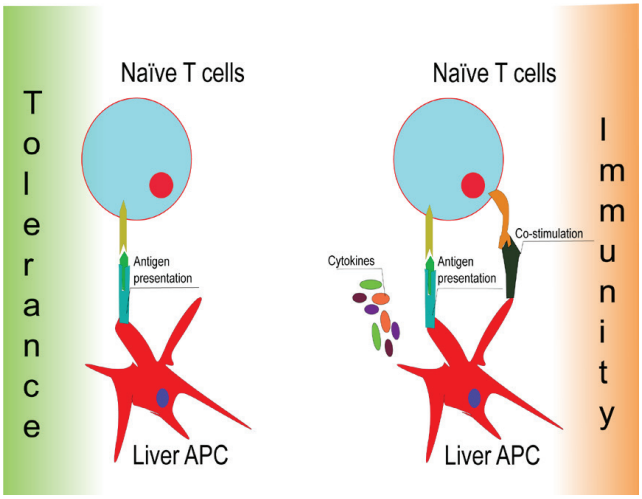


Figure 2
Tolerance and immunity. Antigen presenting cells (APCs) load peptides on the major histocompatibility complex I and II (MHC I/II) in order to present them to naïve T cells. Peptides loaded on MHC I/II that are derived from proteins can activate a specific response against the foreign element after engaging the T cell receptor (TCR) on the surface of naïve T cells, eliciting a so-called signal 1. Tolerance takes place and no activation of T cells occurs if signal 1 remains the only signal. By contrast, if co-stimulatory molecules on APCs are increased and engage their receptors on naïve T cells (signal 2) and, also cytokines are released as result of pattern recognition receptor stimulation (signal 3), a series of events will occur that leads to activation of an adaptive response against the foreign element.

1.5 Liver and TNF: cellular innate immunity in the hepatocytes

1.5.1 TNF and TNF receptors

TNF is a pleiotropic cytokine produced by a variety of immune cells including macrophages/monocytes. TNF can trigger multiple signaling pathways involved in inflammation, proliferation, and apoptosis which together regulate the homeostasis of the liver. (Wullaert et al., 2007).

TNF is synthesized as a 26 kDa membrane bound precursor and then cleaved into its soluble mature form (17 kDa) by TNF-converting enzyme (Idriss and Naismith, 2000). Both TNF forms can transmit their downstream signal through binding to TNF receptor 1 (TNFR1). By contrast, the TNF receptor 2 (TNFR2) is preferentially activated by the non-cleaved form of TNF (Grell et al., 1995). TNFR1/2 receptors exert separate or overlapping downstream signal cascades. The most important difference between the two receptors for TNF is that TNFR1 contains a death domain in the cytoplasmic tail whereas TNFR2 does not (Micheau et al., 2003; Wajant et al., 2003). When soluble TNF binds to its cognate receptor TNFR1, trimerization of this receptor takes place and the silencer of death domain (SODD), which is bound via the receptor's death domain, is released. When SODD is released, the trimeric death domain interacts with the adapter molecule TNF

receptor-associated protein with death domain (TRADD), which contains a similar death domain. The assembly support formed by TNF-R1 and TRADD bears the recruitment of receptor-interacting protein - 1 (RIP1), TNF-receptor-associated factor 2/5 (TRAF2/5), and cellular inhibitors of apoptosis 1/2 (cIAP1/2) to form complex 1 (Vercammen et al., 1998; Micheau et al., 2003) (Fig. 3).
Activation of TNF-R1 leads to the activation of NF-κB, JNK, and p38 through RIP1 and TRAF2/5 leading to the synthesis of anti-apoptotic and pro-inflammatory proteins which mediate survival of the stimulated cells and orchestrate the inflammatory response respectively.

By contrast, activation of caspases and apoptosis is mediated through another molecule called FAS-associated death domain (FADD) capable of binding TRADD (Schwabe and Brenner, 2006). Indeed, subsequently to degradation or dissociation (occurring especially after stimulation) of complex 1 another complex, called complex 2, is formed with the participation of FADD, TRADD, RIP1, TRAF2/5 and procaspase-8 and -10. Complex 2 converts procaspase-8 and -10 to caspase-8 and -10, which initiate apoptosis through caspase-3, -6, and -7 and the mitochondrial death pathway.

If anti-apoptotic signals mediated by TRAF2 and NF-κB are blocked, caspase-8 activates pro-apoptotic members of the Bcl-2 homology (BH)3 domain proteins, mitochondrial depolarization, cytochrome c release, and the executioner caspases (Bradham et al., 1998; Wajant et al., 2003) (Fig.3). TNF-R2 acts differently by exclusively activating proinflammatory pathways and not inducing apoptosis, although TNF-R1- and TNF-R2-induced signals may interact supporting TNF-R1 signaling such as apoptosis (Schwabe and Brenner, 2006). TNF-R2 can participate in the induction of apoptosis mediated by TNF-R1 via at least 4 different mechanisms:

- lowering the availability of TRAF2,
- inducing the degradation of TRAF2 through a cIAP1-mediated mechanism,
- TNF-R2-mediated prolonged JNK activation,
- TNF-R2 induced TNF secretion (Grell et al., 1999).

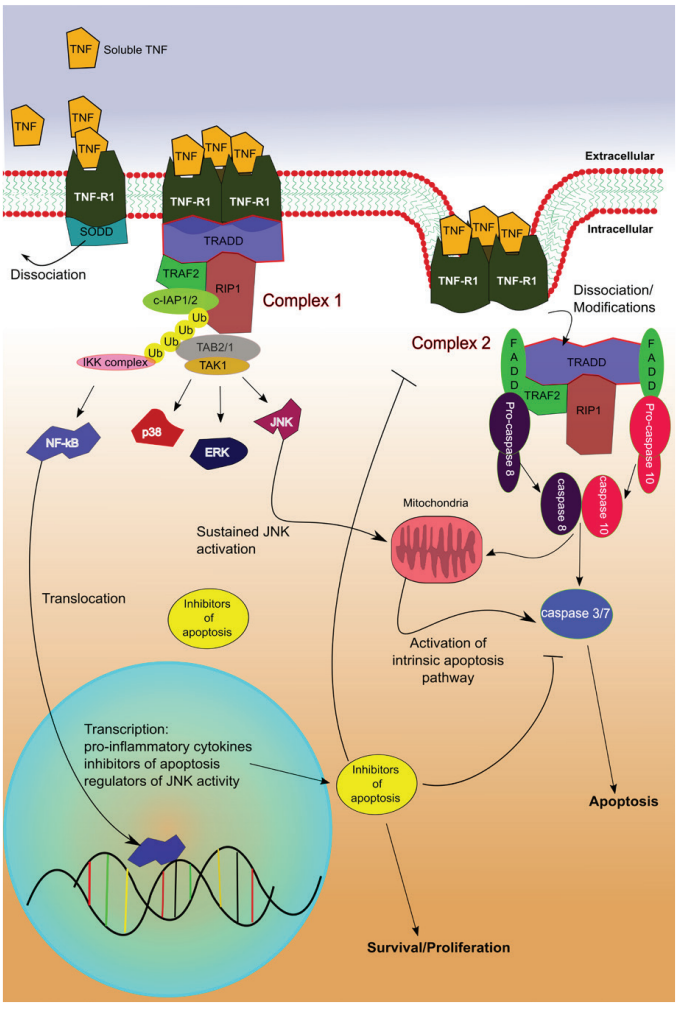


Figure 3

TNF receptor 1 and its intracellular cascade.

TNF receptor 1 (TNFR1) is an ubiquitous membrane receptor which is associated with the silencer of death domain (SODD) in its monomeric inactive form. Upon ligand binding, the TNFR1 forms trimers and releases the SODD, promoting the recruitment of TNF receptor-associated protein with death domain (TRADD). The trimer-TRADD association serves as scaffold for the recruitment of other proteins such as receptor-interacting protein - 1 (RIP1), TNF-receptor-associated factor 2/5 (TRAF2/5), and cellular inhibitors of apoptosis 1/2 (cIAP1/2) to form complex 1. TRAF2 and RIP1 cooperate to recruit the TGF-β activated kinase (TAK)1 complex. TRAF2, an ubiquitin ligase activity, catalyzes the attachment of ubiquitin Lys-63-linked polyubiquitin chains to itself and RIP1. The polyubiquitination of the complex 1 is fundamental for the recruitment of other mediators of TNFR1 functions such as TAK1- binding protein (TAB)2 and TAB3, which mediate the recruitment of TAK1. Activity of TAK1 is fundamental for the activation JNK, p38, ERK and IKK. IKK activation leads to the translocation of the transcription factor NF-κB to the nucleus.

In the nucleus NF-κB promotes the transcription of anti-apoptotic and pro-inflammatory proteins. Inhibitors of apoptosis mainly regulates the activity of JNK and caspases 3/7 in order to promote cell survival. If inhibitors of apoptosis do not exert their activities, prolonged activation of JNK may also occurs leading to activation of apoptosis. After receptor stimulation, TNF induces endocytosis of complex 1 leading to its dissociation and modifications (degradation of c-IAP1/2). This series of events leads to the recruitment of FAS-associated death domain (FADD) by TRADD which mediates the binding of pro-caspases 8 and 10 to form complex 2. The activity of complex 2 induces mitochondrial modifications leading to release of cytochrome C. This event together with cleavage of pro-caspases into caspases mark the initiation of apoptosis.

1.5.2 The balance between NF-κB and JNK in hepatocytes

As mentioned above, TNF is a key mediator of cellular processes such as proliferation, survival and death. In particular, hepatocytes respond to TNF mainly via two opposite responses. Via NF-κB activation TNF increases pro-survival mediators rather than effectors of cell death which are under the regulation of JNK and caspases. It is well-known that activation of TNF-R1 can activate either NF-κB and JNK and the balance between these signaling pathways is responsible for the outcome of the stimulation. TNF-R1, through complex 1, activates both NF-κB and JNK which need to be finely tuned in time and intensity in order to reach the surviving outcome. Disruption of this balance may induce relevant changes in the TNF-induced effects. It is well known that NF-κB inhibition during TNF-R1 activation is associated with increased JNK activity leading to caspase activation in hepatocytes. This evidence revealed how NF-κB mediates its pro-survival effects via the suppression of JNK activity.

1.6 IDILI: the role of the immune system and the MIP-DILI project

Several studies have tried to define the contribution of the immune system in IDILI with the use of in vivo and in vitro models (Adams et al., 2010; Ju and Reilly, 2012; Spahn et al., 2002; Fontana, 2014).

One of the first models to study hepatotoxins was based on the simultaneous administration of ethanol with the liver damaging agents. (Nolan, 2010; Ballet, 2015). This model revealed that an increased susceptibility to hepatotoxins is conferred by the sustained TNF secretion of Kupffer cells due to higher concentration of portal LPS in mice receiving ethanol (Ballet, 2015). Based on this, Prof. Roth's group developed the concept that the increased susceptibility in presence of bacterial or inflammatory stresses may apply also to drugs with IDILI liability. Many IDILI-associated drugs showed overt liver toxicity in these animal models of sterile and non-sterile inflammation. Evidence obtained with the use of animal models pointed to the involvement of the innate immune system in IDILI (Shaw, Beggs, et al., 2009; Deng et al., 2006; Gandhi et al., 2013; Luyendyk, 2003). On the other hand, most of these models not only failed to reproduce all the clinical features of IDILI but also missed to identify or predict hepatic drug toxicity at a pre-clinical level (Kenna and Uetrecht, 2018; Ballet, 2015). Therefore, more mechanistic studies describing the involvement of the immune system in IDILI are needed (Kenna and Uetrecht, 2018) to create better tools for the assessment of liver toxicity early in drug development.

The Innovative Medicines Initiative (IMI) is a public-private partnership aiming to speed up the development of better and safer medicines for patients. Among the several projects with this aim, the EC project called "Mechanism-Based Integrated

Systems for the Prediction of Drug-Induced Liver Injury" (MIP-DILI) set out to provide new mechanistic knowledge on IDILI in order to build a panel of tests for the identification of hepatotoxic compounds at the preclinical assessments. The mechanistic understanding of this multifaceted pathology required different expertise. Metabolism, intrinsic toxicity and activation of both branches of the immune system by drugs or their metabolites were the main areas of investigation in this project.

The project was based on the firm belief that only via a mechanistic understanding of DILI it will be possible to set up a panel of tests in vitro capable of revealing hepatotoxicity of new compounds already at the preclinical assessments. The early identification of hepatotoxic compounds will positively affect costs for pharmaceutical companies and health care systems, nonetheless diminishing the use of animals for toxicological studies.

The main aims of this project were:

- **Work-package 1:** To identify training and testing compounds suitable for mechanistic studies on IDILI
- **Work-package 2 and 3:** To understand the IDILI's molecular and cellular mechanisms in vivo and in vitro with the use of identified training compounds
- Work-package 2 and 3: To validate in vitro and in vivo mechanisms with the use of testing compounds
- **Work-package 4:** To identify and quantify concentrations of a training set of drugs and their metabolites in all systems employed
- **Work-package 5:** To build and validate human relevant predictive tests for the identification of hepatotoxic compounds (in vitro or in silico) via establishment of physiologically-relevant dynamic pathway models that are sensitive to pharmacological perturbations.

The aim entrusted to the laboratory of Immunotoxicology at the Institute for Risk Assessment Sciences (in which the presented research was developed) consisted in the investigation of the role of innate immunity in IDILI.

1.7 Immune-mediated IDILI

Based on the analysis of the identified risk factors (Chapter 1.3) involved in adverse hepatic reactions, in the existing knowledge about IDILI we can find three major, non-mutually exclusive, hypotheses to explain the onset of IDILI in patients:

- The inflammatory stress hypothesis: based on the evidence thatinflammation and consequent activation of the innate immune system may increase the liver toxicity of some pharmaceuticals (Roth et al., 1997, 2017)

- The hapten hypothesis: based on the formation of adducts between drug

or their metabolites with proteins, which are recognized new antigens triggering an adaptive response toward the liver (Zhang et al., 2011)

- The danger hypothesis: hinged on the formation of new antigens in a context of damage/stress resulting in an adaptive response against the liver (Uetrecht, 2007).

1.7.1 The inflammatory stress hypothesis

The inflammatory stress hypothesis is based on the findings that IDILI-associated compounds have been shown to induce liver toxicity in animals in presence of inflammatory stressors. Notably, these stressors may include infections or inflammation, which both can increase the susceptibility of liver injury (Ju and Reilly, 2012).

This knowledge has been used to design animal experiments that combine drug exposure with bacterial components (like LTA or LPS) or with the proximal mediator of LPS, TNF. Among the compounds studied in this set up are amiodarone, chlorpromazine, diclofenac, ranitidine, sulindac, troglitazone and trovafloxacin (TVX) (Gandhi et al., 2013; Lu et al., 2012; Deng et al., 2006; Luyendyk, 2003; Patrick J. Shaw et al., 2009; Patrick J Shaw, Ganey, et al., 2009a, 2009b; Zou et al., 2009). Interestingly, compounds belonging to the same pharmacological class of hepatotoxic drugs did not cause liver injury in these models of infection or inflammation.

The in vivo data on the inflammatory stress hypothesis correspond to some in vitro data. For instance, apoptosis of primary murine hepatocytes or human liver cancer cell line (HepG2) was observed upon incubation to TNF together with the IDILI-associated compounds (Fredriksson et al., 2011; Cosgrove et al., 2009; Lu et al., 2013; Beggs et al., 2014, 2015).

Mainly direct effects of the drugs on cellular innate immunity were described in the studies investigating these models. For instance, drugs like chlorpromazine and diclofenac interfere with intracellular pathways downstream of TNF receptors (Fredriksson et al., 2011; Gandhi et al., 2009) . Whereas the effects of these drugs on cellular innate immunity have been investigated, not many studies clarified direct effects of these drugs on inter-cellular mediators of inflammation like monocytes, Kupffer cells, neutrophils and NK cells.

Direct effects of the drug on the innate immune cells (mainly neutrophils and Kupffer cells and other macrophages) have been proven either involvement in the onset or in the regulation of the damage (Van Hul et al., 2011; Hatano et al., 2008; Holt et al., 2010, 2008; Lee et al., 2016; Ishida et al., 2006; Patrick J Shaw, Ganey, et al., 2009a; Poulsen, Olivero-Verbel, et al., 2014; Poulsen, Albee, et al., 2014). It has been shown that Kupffer cells exert a protective effect with regard to DILI (Ju et

al., 2002; Holt et al., 2010; Yano et al., 2012), despite their capacity to secrete pro-inflammatory cytokines (Robinson et al., 2016; Tacke et al., 2009). Unlike Kupffer cells, neutrophil activation and their release of proteases were associated with exacerbation of the hepatic damage (Patrick J Shaw, Ganey, et al., 2009a; Liu et al., 2006).

Although these models are undoubtedly a step forward in the identification of the mechanisms behind the onset of IDILI, concern has been expressed about their use in pre-clinical studies. Indeed, discrepancy between the relevant pathological features of IDILI observed in clinics and those elicited by these models poses several doubts on the validity of these models (Ballet, 2015).

1.7.2 From hapten to danger hypothesis in IDILI

The hapten hypothesis originated almost 80 years ago when the Nobel laureate Landsteiner noticed that low molecular weight chemicals did not induce an immune response unless they covalently bound to endogenous proteins. This finding was later included in the self/non-self hypothesis describing lymphocyte proliferation and activation as downstream events of the presentation of peptides on major histocompatibility complexes (MHC) by antigen presenting cells (APCs). Peptides exposed on MHC can be distinguished by lymphocyte as self- and non-self-antigens. Upon recognition, lymphocytes respond with tolerance to self and with an immune response to non-self-peptides, in order to eliminate exogenous components.
Compound-protein adducts, also called hapten-carrier complexes, can be presented by MHC and then recognized by lymphocytes as non-self- or neo-antigens (Landsteiner, 1941). Based on this finding, haptens are considered low molecular weight chemicals eliciting an immune response only when these are covalently bound to a protein.

In the context of DILI, reactive species that originate from the metabolism of the administered drug may act as hapten and have the potential to elicit a compound-specific adaptive immune response (Ju and Reilly, 2012). Other studies revealed that small molecules can change the processing of proteins leading to the presentation of normally not presented protein cryptic epitopes (Griem et al., 1998; Diao et al., 2004). Due to the prevalent role of the liver in metabolism of many compounds, this hapten-hypothesis was used to describe potential mechanisms involved in idiosyncratic hepatic drug reactions with allergic-like or autoimmune-like features (Licata, 2016; Kim and Naisbitt, 2016; Tujios and Fontana, 2011; Fontana, 2014; Kullak-Ublick et al., 2017; Castiella et al., 2014; Ju and Reilly, 2012). These IDILI-related features are represented by, skin rashes, antibodies against the drug, autoantibodies, hepatic lymphocyte infiltration and eosinophilia accompanied with organ damage which in some cases may persist even after drug discontinuation (Licata, 2016; Kim and Naisbitt, 2016; Tujios and Fontana, 2011; Fontana, 2014; Kullak-Ublick et al., 2017; Adams et al., 2010). Although this hypothesis remained

intriguing for several years, it was difficult to explain why many neoantigens are tolerated and others elicit immune responses (Uetrecht, 2001; Ju and Reilly, 2012).

1.7.3 Danger hypothesis and IDILI

Almost 20 years ago, the self/non-self hypothesis was challenged by a new concept. Polly Matzinger argued that recognition of a new antigen is not sufficient to activate an adaptive immunity, but that a contextual danger signal must be present to initiate the response against the new antigen (Matzinger, 2002). Danger was later defined as a heterogeneous group of molecules called alarmins or damage-associated molecular patterns (DAMPs), when these are of endogenous origin (e.g. cellular components such as ATP, hyaluronic acid etc) and PAMPs when derived from invading pathogens (e.g. microbial toxins such as LPS or dsDNA, ssRNA) (Fig. 4). These molecules are also called "Signal 0s" for innate immunity and they can be sensed by apposite receptors both at membrane and cytosol level. Among these receptors are Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and absent in melanoma 2-like receptors (ALRs) (Tang et al., 2012).

In view of a unified hypothesis for chemical-induced adverse reactions that may combine all three above-mentioned hypotheses, the reactivity of the drug or its metabolite(s) might be directly linked to the formation of adducts (hapten hypothesis) and/or induction of costimulatory signals (inflammatory stress hypothesis). A key event such as oxidative stress generated during metabolism, or other processes linked to e.g. sterile and non-sterile inflammation can cause the release of alarmins or DAMPs respectively from stressed living and dying cells. In reason of the potential need of damage/stress to induce an adaptive response causing liver injury, it is not surprising that many compounds associated with IDILI in human induce hepatocyte cell death when co-exposed with inflammatory stimuli. Hepatocyte cell death and the consequent release of DAMPs may represent a key step in the process leading to IDILI.

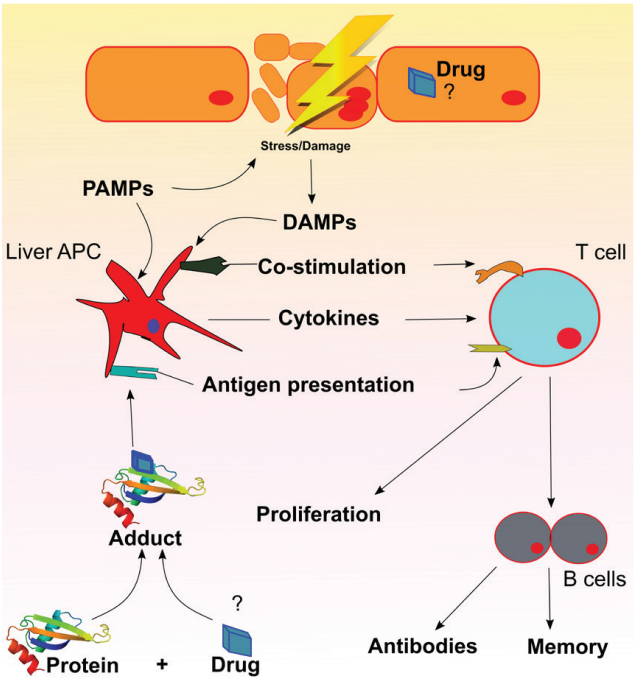


Figure 4
The danger hypothesis in IDILI.
Drugs or their metabolites may form covalent binding with proteins leading to formation of the so-called haptens. Haptens are processed by antigen presenting cells (APCs) and their peptides exposed on major histocompatibility complex I/II for antigen presentation to T cells. The only presentation of the antigen might be not enough to activate an adaptive response. In order to elicit an adaptive response, co-stimulatory signals on APCs and innate cytokines must be present simultaneously to antigen presentation. Danger signals are an essential requirement for the induction of co-stimulatory molecules and innate cytokines by APCs. For this reason, cell damage induced by the drug or its metabolites or pre-existing pathological conditions such as modest hepatic injury or concomitant infection or inflammatory conditions are needed for the activation of adaptive immunity. The activation of the adaptive immunity consists in the proliferation of T cells and the formation of memory B cells and plasma cells producing antibodies against the recognized antigen.

1.7.4 New animal models of IDILI

Many efforts have been spent in the last decade to develop new animal models of IDILI which better resemble the clinical features of this pathology in human. These models would need to include adaptive immune players. But still, models involving adaptive immune responses with matching clinical features of IDILI are rare. New animal models involving adaptive immune responses may represent a tool to clarify the mechanisms involved in IDILI.

A recently developed model for immune-mediated IDILI used genetically modified mice lacking the program death 1 receptor (PD1) or the casitas b lymphoma b receptor (CBL-b). PD-1, CBL-b and also cytotoxic T-lymphocyte-associated protein 4 (CTLA4) are so-called immune checkpoints which are negative regulators of T-cell immune functions. Immune checkpoints are fundamental in the induction of immune tolerance and their inhibition is associated with lymphoproliferative effects (anti-CTLA4 therapy) or less suppression of the TCR-induced activation of lymphocytes (anti-PD1) (Metushi et al., 2015; Seidel et al., 2018).

In the model, the genetically modified mice were treated with anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and amodiaquine. In mice lacking PD1 or CBL-B that received anti-CTLA4 antibody prolonged serum ALT concentrations and liver damage was detected (Metushi et al., 2015). Remarkably, only female mice developed liver injury which was accompanied with hepatic infiltrate of B and T cells (Metushi et al., 2015; Mak and Uetrecht, 2015b). Among the T cells that homed to the liver, CD8 T cells homing the liver were armed to kill as demonstrated by an increased expression of granzyme B and perforin (Metushi et al., 2015). If this model was performed with wild type (C57Bl/6) mice, only mild serum ALT increase was observed, which was accompanied by a hepatic lymphocytic infiltrate, characterized by an increased amount of T regulatory cells expressing PD1 and CTLA4. This data implies that in the wild type mice PD1- and CTLA4-dependent immunoregulation is induced, which will prevent or suppress the drug-induced immune response against the liver (Metushi et al., 2015), although the original danger signal stimulating the adaptive response may still be present.

Other compounds with IDILI liability, such as nevirapine and isoniazid, showed similar effects in this model (Mak and Uetrecht, 2015a). The findings in the model shows the importance of the adaptive immune system in IDILI, but also the importance of the lack of immunoregulatory mechanisms.

Although this represents an important step forward in the identification of the mechanisms involved in IDILI, it needs be demonstrated that pharmaceuticals not associated with IDILI in humans do not elicit liver damage in this model.

1.8 TVX liver toxicity: key role for the innate immune system

1.8.1 Trovafloxacin: an antibiotic withdrawn from the market due to liver toxicity

TVX is a wide spectrum antibiotic belonging to the fluoroquinolone class. It was marketed under the brand-name Trovan™ after Food and Drug Administration (FDA) approval in 1997. The introduction of TVX onto the market was as fast as successful in terms of sells, being administered to more than 2 million people in a very short time frame (Borlak, 2009).

During clinical trials, TVX successfully treated more than 7000 patients with no case of liver injury. However, after approximatively 1 year on the market, TVX was associated with 150 events of liver toxicity of which 14 led to liver failure. TVX-induced liver injury in humans was represented mainly by centrilobular necrosis, increased serum ALT or AST together with increased eosinophil count in blood and liver biopsies. The symptoms of liver injury usually became apparent between1 and 60 days after first administration of the drug and even after ending the treatment. In

the light of these events, the risk/benefit ratio for TVX was re-evaluated, especially because of the unpredictability of the adverse reaction (Medicines and Unit, 1999). After this, TVX was prescribed first under black box warning and later withdrawn from the EU and US market in 1999 and 2006 respectively (Fish and Service, 2014).

1.8.2 Trovafloxacin: molecular structure, pharmacodynamics and pharmacokinetics

TVX, like other fluoroquinolones, exerts its antimicrobial activity via the inhibition of bacterial topoisomerase II/IV and DNA gyrase. These enzymes are fundamental for bacterial DNA replication, recombination and repair (Anderson et al., 2000).

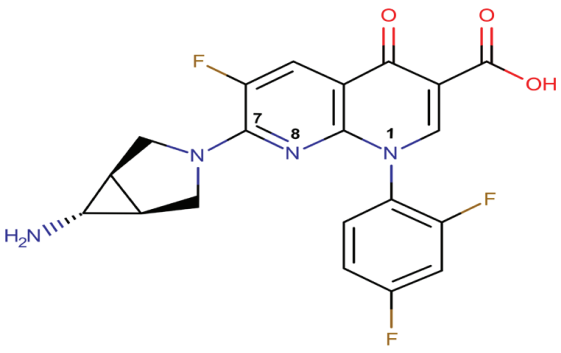


Figure 5
Chemical structure of trovafloxacin.
Trovafloxacin (TVX) is usually categorized as a fluoroquinolone although the nitrogen in position 8 makes this compound a fluoro-naphthyridone. Cyclopropyl-fused pyrrolidine on C-7 and difluoro-phenyl in N-1 confer to TVX the inhibitory activity on Gram+ topoisomerase IV.

When compared with other fluoroquinolones, the chemical structure of TVX presents some peculiarities: a cyclopropyl-fused pyrrolidine on C-7, a difluoro-phenyl in N-1 and a nitrogen atom in position 8 (Fig.5). The first two chemical properties confer to TVX an increased affinity for topoisomerase IV of Gram+ bacteria. Strikingly, the nitrogen atom in position 8 distinguishes TVX from other fluoroquinolones, and therefore the compound could be better defined as a fluoro-naphthyridone (Fig. 5). This peculiarity provides TVX with an increased half-life and bioavailability when compared with quinolone analogues (Brighty and Gootz, 1997). TVX's half-life was estimated to be 10-12 hours at a daily dose of 200 mg/day.

Interestingly, higher concentrations of TVX were observed in pancreas and liver, where the metabolism occurs mainly via phase II reactions and not predominantly via oxidative phase I reactions as for the other fluoroquinolones (Fig. 6). TVX is excreted mainly via the biliary route and almost 55% of the drug is excreted unmodified (Dalvie et al., 1997).

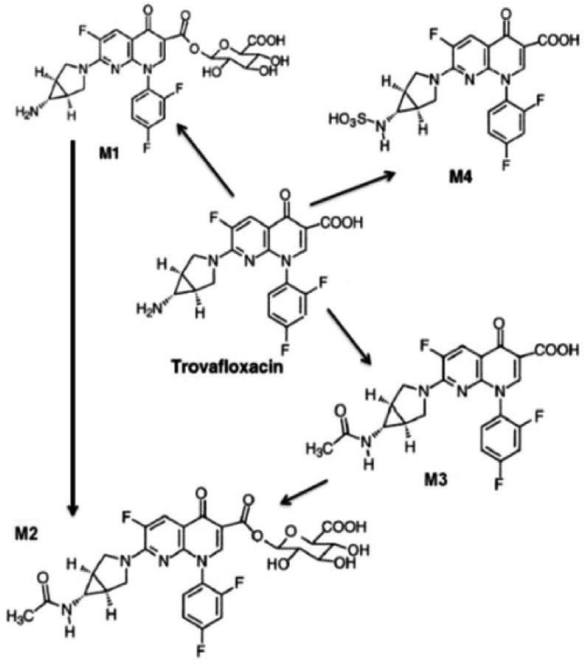


Figure 6
Trovafloxacin metabolism.
Trovafloxacin (TVX) is metabolized mainly via reaction of conjugation (phase II reactions) and not with phase I reactions as for other fluoroquinolones. Glucuronidation of carboxylic group in position 3 is the main metabolic reaction aiming to excrete TVX (M1). This reaction is usually followed by acetylation of the ammine group present in the cyclo-propyl fused pyrrolidine (M2) and this metabolite is the more concentrated metabolite in urine followed by the unchanged drug. The ammine group can also undergo other phase II reactions like sulfonation (M3).

1.8.3 TVX-induced liver injury models: from in vivo to in vitro

As mentioned, Prof. Roth and colleagues developed and characterized the TVX-model of liver injury. The model was initially based on the administration of TVX followed by LPS or peptidoglycan-lipoteichoic acid (LTA) injection. The model revealed how exaggerated production of cytokines (e.g. TNF) or their reduced clearance together with a neutrophil elastase release was fundamental in the development of liver injury by TVX (Shaw et al., 2007; Patrick J Shaw, Ganey, et al., 2009a). Surprisingly, administration of the pharmacological analogue levofloxacin (LVX) in the experimental set up did not elicit liver injury in mice (Shaw et al., 2007; Patrick J Shaw, Ganey, et al., 2009a). Notably, the liver injury observed in this model was dependent on the cytokine TNF as demonstrated by prevention of TVX-induced liver injury after administration of etanercept, a chimeric soluble TNF receptor capable of lowering the free fraction of the cytokine (Shaw et al., 2007).

This use of TNF instead of LPS or LTA shortened and rendered more reproducible the onset of hepatic damage in mice (Patrick J Shaw, Beggs, et al., 2009). TVX+TNF administration resulted in increased serum ALT as early as 3 h, and histopathological modifications of the liver tissue were observed already 4 h after the injection of the cytokine. In this model, greater neutrophil accumulation in the liver was associated

with the observed damage (Patrick J Shaw, Beggs, et al., 2009). In vitro experiments using murine Hepa1c1c7 and human HepG2 hepatoma cell lines confirmed that TVX+TNF decreased the viability of hepatic cell lines (Beggs et al., 2014; Patrick J Shaw, Beggs, et al., 2009). In line with what was observed in vivo, LVX+TNF administration did not affect the viability of the same cells in vitro (Beggs et al., 2014; Patrick J Shaw, Beggs, et al., 2009).

1.8.4 Mechanisms of TVX-induced liver injury

TVX+TNF-induced liver injury appears to be characterized by several toxic pathways which contribute synergistically to the onset of hepatic damage. The pathways of toxicity associated with TVX+TNF liver injury were obtained from in vivo and in vitro studies highlighting a multifaceted mechanistic appearance of TVX+TNF toxicity in liver.

1.8.4.1 The in vivo lessons

The in vivo model, either with the use of LPS or TNF, showed that the innate immune system is deeply involved in the development of TVX-induced liver injury. The observed liver damage in this model was shown to be dependent on three important innate responses which seem to represent segregated pathways of toxicity: 1- thrombin activation; 2- proinflammatory cytokines, such as TNF; 3- neutrophil activation (Patrick J. Shaw et al., 2009; Patrick J Shaw, Ganey, et al., 2009b, 2009a).

With regard to thrombin activation, it has been observed that heparin administration decreased not only the fibrin deposition in the sinusoids, but also the serum levels of ALT in mice treated with TVX+LPS. In contrast, heparin did not decrease the pro-inflammatory cytokine concentrations and neutrophil accumulation in mice treated with TVX+LPS (Patrick J. Shaw et al., 2009).

With regard to the proinflammatory cytokines, inhibition of TNF (via etanercept administration) in both the TVX+LPS and TVX+TNF set up significantly reduced the apoptotic/necrotic areas in the liver and the serum ALT levels. Differently, TNF inhibition was not associated with a reduction in neutrophil accumulation in the liver of mice receiving TVX+LPS but rather led to a greater number of these cells in the liver (Patrick J Shaw, Ganey, et al., 2009b).

Mice lacking neutrophil elastase TVX+LPS elicited significantly lower serum ALT concentrations when compared with wild type mice. This evidence confirms that neutrophil activation is involved in TVX-induced liver injury (Patrick J Shaw, Ganey, et al., 2009a).

1.8.4.2 The in vitro lessons

In vitro experiments characterized the molecular mechanisms behind TVX+TNF-

induced cell death. As mentioned before, the combination of TVX with the cytokine TNF decreased the viability of Hepa1c1c7 and HepG2 (Shaw, Beggs, et al., 2009; Beggs et al., 2014). The increased number of dead cells was not observed when cells were co-incubated with LVX and TNF.

TVX+TNF increased activation of JNK and caspase 3, 8 and 9 in the above-mentioned hepatocyte-like cell lines. In addition, incubation with a caspase inhibitor completely abrogated TVX+TNF-induced cell death, demonstrating that cells were undergoing apoptosis (Patrick J Shaw, Beggs, et al., 2009; Beggs et al., 2014). TVX+TNF-induced cell death was also completely inhibited in presence of the JNK inhibitor SP600125 or the caspase 8 and 9 inhibitors (Patrick J Shaw, Beggs, et al., 2009; Beggs et al., 2014).

It has been demonstrated that TVX inhibited eukaryotic topoisomerase II with the highest affinity in its pharmacological class. The effect of TVX was associated with a peculiar transcriptional profile, similar to that of some other inhibitors of the same enzyme (Reymann and Borlak, 2008; Anderson and Osheroff, 2001). These evidences prompted investigation into the effect of TVX on DNA damage response (DDR). In particular into how DDR might be involved in TVX+TNF-induced apoptosis in HepG2 and in the increased inflammatory response of RAW264.7 cells after TVX+LPS exposure. TVX alone elicited replication stress in HepG2 causing cell cycle blockade in G1 via activation of p21. This was evident also when these cells were exposed to TVX+TNF combination. TVX+TNF co-treatment of HepG2 cells elicited activation of DDR as observed by increased phosphorylation of endogenous protein containing ataxia telangiectasia mutated/ataxia telangiectasia and Rad3 related (ATM/ATR) substrate motifs. Although ATR inhibition reduced TVX+TNF-induced cell death in HepG2, double strand breaks (DSB) showed to be dependent on caspase activation and for this reason secondary to induction of cytotoxicity (Beggs et al., 2015). In line with these findings, TVX with or without TNF increased the formation of ATR foci in HepG2 cells which were dependent on activation of extracellular signal-related kinase (ERK) (Beggs et al., 2015) (Fig. 7).

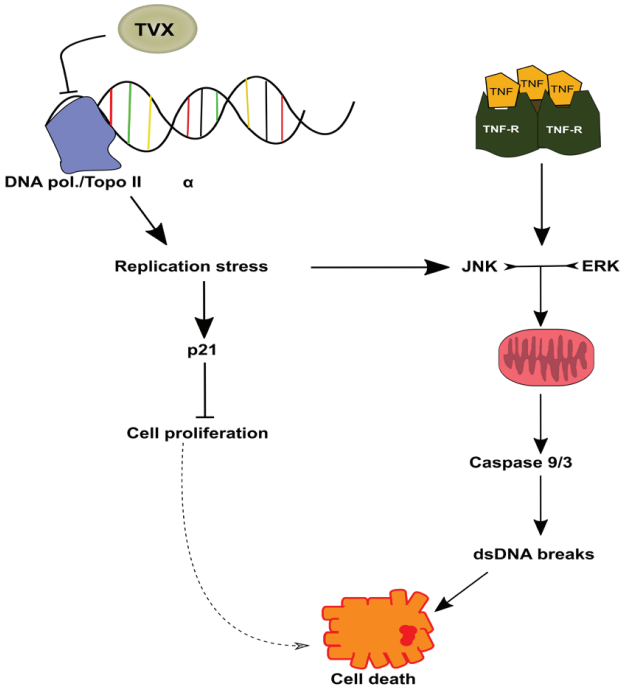


Figure 7
The hypothesis for TVX–TNF interaction in causing inhibition of cell proliferation and death of hepatocytes.
Trovafloxacin (TVX) inhibits topoisomerase IIα potentially causing collision with other polymerases such as DNA polymerase. These events lead to replication stress, cell cycle blockade by p21 with consequent inhibition of proliferation. Replication stress may also activate JNK and ERK pathway. Upon stimulation with only TNF, hepatocytes activate JNK transiently early after receptor activation. The combination of TVX and TNF leads to a prolonged activation of JNK and ERK which causes mitochondrial dysfunctions, caspase 3/9 activation and consequent hepatocyte cell death. This activation of caspases partly explains the mechanisms behind TVX+TNF-induced cell death, although the replication stress response observed in this setting also promotes cell death in a caspase 3 independent manner.

By contrast, TVX alone in RAW264.7 increased pH2AX already 2 h after the incubation with the drug, showing how DNA lesions represent a very early event after TVX exposure. In this case, ATR and ERK sustained the expression of TNF in RAW264.7 cells demonstrating how DNA damage caused changes which affected early expression of cytokines such as TNF (Poulsen, Olivero-Verbel, et al., 2014).
Although DDR is clearly involved in the onset of TVX-mediated apoptosis the exact series of events leading to hepatocellular toxicity has not been assessed yet. Interference of the DDR with TNF or LPS-activated intracellular pathways leading to apoptosis still need to be further characterized.

1.9 Ximelagatran liver toxicity

1.9.1 Ximelagatran: first oral direct thrombin inhibitor withdrawn from market due to IDILI

Ximelagatran (XIM) is an orally administered, direct inhibitor of thrombin which

reached the market at the beginning of 2004 after the approval of the European Medicines Agency (EMA). The drug was marketed by the pharmaceutical company AstraZeneca under the brand ExantaTM.
During short-term phase III clinical trials, no hepatic toxicity was elicited by XIM. However, during long-term clinical trials XIM induced hepatotoxicity and prolonged administration of XIM increased serum ALTs concentration in 7.9% of the patients. Despite this evidence, EMA positively evaluated the risk/benefit ratio of the drug based on: 1- remission of the ALTs alterations, 2- similarity in the frequencies of the total bilirubin elevations and hypersensitivity reactions between XIM-treated and control-treated patients. Upon prolonged XIM treatment patients, experienced increased serum levels of ALT and severe liver injury even after therapy discontinuation. Moreover, monitoring serum ALTs did not reduce the risk of severe liver injuries (Lee et al., 2005; Agnelli et al., 2009). Based on the risk/benefit re-evaluation, XIM was withdrawn from the market in 2006.
So far, the mechanism leading to XIM-induced liver damage is unknown, although an immune component seems reasonable due to the occurrence of the particular ADR after drug therapy discontinuation.

1.9.2 Ximelagatran: molecular structure, pharmacodynamics and pharmacokinetics

Following oral administration, XIM is rapidly absorbed by the small intestine, transferred to the blood and metabolized in the liver. XIM is metabolized to its active form melagatran via formation of two intermediates. In particular, the amidoxime and ester moieties of XIM are respectively reduced or hydrolyzed to form melagatran in two consecutive and interchangeable reactions (Fig. 7). The structure of the pro-drug XIM was intended to increase the oral bioavailability of melagatran. Indeed, whereas bioavailability of melagatran is very low (around 3-7%), modification of the amidoxime and ester moieties led to the 170-fold more lipophilic pro-drug XIM which was used for oral administration. XIM bioavailability in humans is about 20% of the administered drug (Gustafsson et al., 2001) whereas in rodents it is significantly lower, i.e. around 5-10% (Eriksson et al., 2003). Melagatran's half-life is about 2.4 to 4.6 hours and the drug reaches the highest plasma concentration 1.5 to 2.5 hours after oral XIM administration. The drug and its metabolites is excreted primarily via the kidneys (Brighton, 2004).

To date, the reduction of N-hydroxylated compounds such as XIM (Andersson et al., 2008; Krompholz et al., 2012) seems to be mediated by a complex of enzymes which resides in the outer mitochondrial membrane and consists of three components: cytochrome b5, mitochondrial amidoxime reducing component 2 and a cytochrome b5 reductase (Neve et al., 2012).

Melagatran, the active form of XIM, is a direct and competitive inhibitor of thrombin, the central mediator of coagulation. Melagatran structurally resembles a peptide sequence on the α-chain of fibrinogen, which reversibly binds to the

active site of α-thrombin inhibiting its function (Kaplan, 2003). Thus, fibrinogen can no longer be converted by free thrombin into fibrin which participates in the formation of blood clots. The inhibition of fibrinogen shedding to fibrin prevents effectively the activation of coagulation cascade in very serious conditions such as in the prevention of stroke and other thromboembolic complications associated with atrial fibrillation, the prevention of venous thromboembolism especially in patients undergoing knee replacement surgery (Vaughan, 2005; Weitz, 2003).
The use of XIM was supposed to represent an improvement of previous anti-coagulant therapies (vitamin K antagonists and low-molecular weight heparins) which have narrow therapeutic indexes and need continuous monitoring of prothrombin time to prevent bleeding and drug-related deaths (Eriksson et al., 2003).

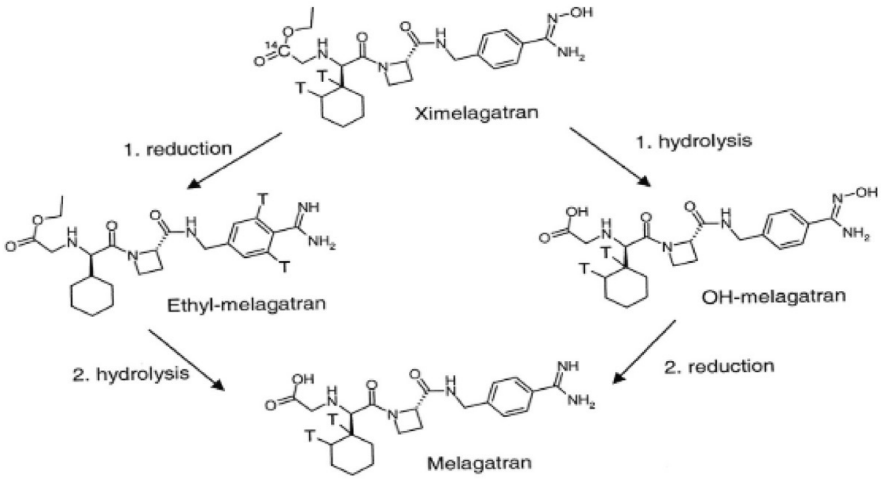


Figure 8

Ximelagatran metabolism.
Ximelagatran (XIM) is metabolized to melagatran in two steps: either by reduction and hydrolysis (left) or by hydrolysis and reduction (right).

1.9.3 Ximelagatran-induced liver injury: the current understanding

As with many other liver-damaging drugs, preclinical toxicology studies did not suggest the hepatotoxic properties of XIM. Although toxicological studies included prolonged administration of the candidate compound in animals for at least 90 days, no evidence of XIM hepatotoxicity was observed, stressing the need for new predictive tests. After marketing, XIM-induced liver injury occurred

between 1 and 6 months after the beginning of the therapy, resulting in liver function abnormalities in a low percentage of patients (<1-2%). Initial evidence of a potentially immunogenic pathogenesis for XIM-induced liver injury was provided by a pharmacogenetic study in 2008, which showed that elevated ALT levels are associated with the MHC alleles DRB1(*)07 and DQA1(*)02 (Kindmark et al., 2008).

Several in vitro models with human hepatoma cell lines or ex-vivo studies with primary human or murine hepatocytes failed to elucidate the cellular mechanism of XIM-induced toxicity (Ainscow et al., 2008; Kenne et al., 2008). These studies focused primarily on the study of direct toxicity to cells. A functional analysis showed that XIM influences mitochondrial respiration and the redox status of cells already in concentrations that do not (yet) influence the survival of hepatocytes. XIM dose-dependently reduces glutathione level of hepatocytes and shifts the redox status towards oxidation. Furthermore, both the basal and the maximum oxygen consumption of mitochondria are reduced by the drug. Damage to mitochondrial respiration, altered redox status, and reduced glutathione levels indicate oxidative stress induced by XIM's metabolites (Neve et al., 2015).

The lab of Prof. Knolle (Technische Universität of München) developed a model of liver injury using XIM as drug. In the XIM IDILI model, exposure to XIM is combined with a virus infection and TNF injection and this unique combination leads to increased ALT levels and mortality in mice even when compared with the pharmacological analogue DAB (unpublished data).

1.10 The kinetics of IDILI: the innate, the adaptive and the damage

Although many studies partly characterized the effects of IDILI-associated compounds using in vivo and in vitro models, the etiology of IDILI still remains unclarified, partly because it is idiosyncratic. Following the recent IDILI hypotheses and the evidence collected in models, the onset of IDILI seems to rely on at least one of the following three key processes: the occurrence of damage/stress, the innate immune activation and an adaptive response against the liver. Although we can speculate with a reasonable margin of confidence that all three main processes are involved in IDILI, it is not yet known how these key players succeed one other to cause the adverse event. It still needs to be determined if the occurrence of the damage/stress to the hepatic parenchyma is prodromal to a cellular-mediated innate response, or if the latter is the cause of the damage. Similarly, we cannot exclude that the damage/stress observed in IDILI in humans may be mediated by the adaptive response against the liver or occurring before the immune activation as hypothesized by the "danger hypothesis". In addition, other factors not related to the drug effects, like sterile and not sterile inflammation, might be involved in this complex mosaic called IDILI. To better characterize IDILI, a kinetic approach to

the investigations of the in vivo and in vitro models is essential to reveal causal relationship of these key events involved in the liver injury.

1.11 Aims and contents

The aim of the work described in this thesis was to clarify the role of innate immunity in the development of IDILI.
For this, we studied the kinetics of innate immunologic changes occurring during the development of liver injury in the well-established TVX and XIM mouse models. We further studied the cellular and molecular mechanisms of TVX with the use of in vitro studies.

With this approach we investigated (Fig. 9):

1- the in vivo kinetics of immunopathological changes in liver, with focus on innate immune cells (Chapter 2 (TVX) and 5 (XIM))

2- the effect of TVX on TNF-induced recruitment of leukocytes into the liver (Chapter 2 and 3),

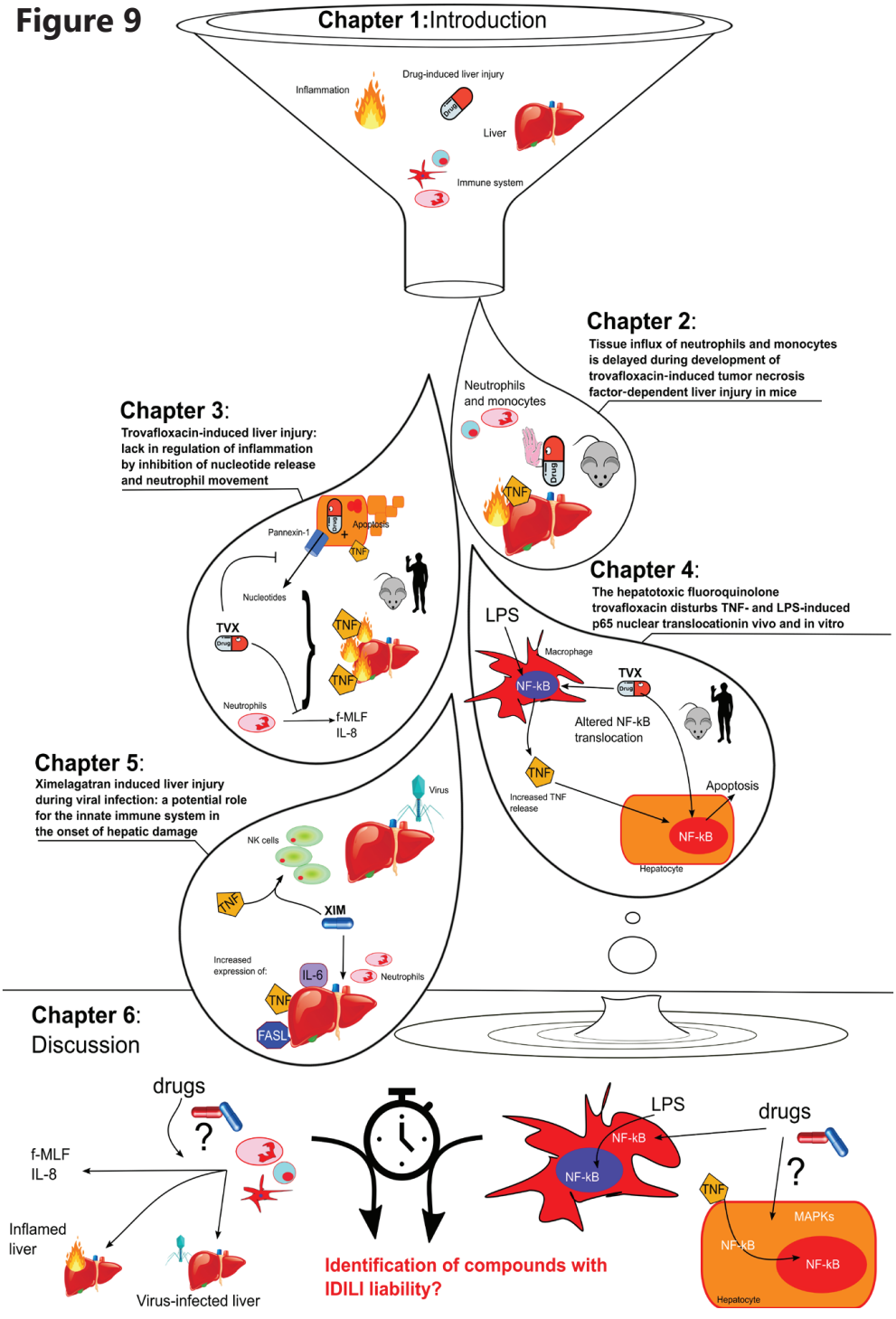
3- the molecular mechanisms involved in TVX-mediated stimulation of TNF- or LPS-induced cellular effects (Chapter 4)

We confirmed the capability of TVX to interact with TNF pathway at the intracellular level disrupting NF-κB and JNK activation at the very early stages after TNFR-1 engagement in vivo and in vitro. Analogously, LPS-induced NF-κB pathway in RAW264.7 cells was also altered and, in particular, first translocation of the transcription factor into the nucleus was prolonged by TVX leading to increased secretion of inflammatory cytokines (Chapter 4).

Moreover, TVX inhibited the early TNF-induced recruitment of neutrophils in vivo leading to increased inflammation in the liver (Chapter 2). In line with this finding, TVX, at concentrations observed in patients, decreased the motility of human neutrophils toward chemoattractants. This may potentially inhibit their movement in the narrow sinusoids delaying their recruitment and increasing the time of permanence in the organ as demonstrated in mice (Chapter 3).

With regard to XIM, we observed that the drug administration in mice may strengthen the immune response against viral infected cells by increasing the number of NK cells and the expression of FASL. Moreover, hepatic TNF and IL-6 expression were also increased by the drug alone showing that mild or regulated liver inflammation may also occur with the only administration of XIM (Chapter 5).

Figure 9


Chapter 1:Introduction
Inflammation
Drug-induced liver injury
Drug
Liver
Immune system
Chapter 2:
Tissue influx of neutrophils and monocytes is delayed during development of trovafloxacin-induced tumor necrosis factor-dependent liver injury in mice
Neutrophils and monocytes
Drug
TNF
Chapter 3:
Trovafloxacin-induced liver injury: lack in regulation of inflammation by inhibition of nucleotide release and neutrophil movement
Pannexin-1
Drug
Apoptosis
TNF
Nucleotides
TVX
Drug
TNF
TNF
Neutrophils
f-MLF
IL-8
Chapter 4:
The hepatotoxic fluoroquinolone trovafloxacin disturbs TNF- and LPS-induced p65 nuclear translocationin vivo and in vitro
LPS
Macrophage
NF-kB
TVX
Drug
Altered NF-kB translocation
TNF
Increased TNF release
Apoptosis
NF-kB
Hepatocyte
Chapter 5:
Ximelagatran induced liver injury during viral infection: a potential role for the innate immune system in the onset of hepatic damage
Virus
NK cells
TNF
XIM
Increased expression of:
IL-6
Neutrophils
TNF
FASL
Chapter 6:
Discussion
drugs
?
f-MLF
IL-8
Inflamed liver
Virus-infected liver
Identification of compounds with IDILI liability?
LPS
NF-kB
NF-kB
drugs
?
TNF
MAPKs
NF-kB
NF-kB
Hepatocyte

References

Adams, D.H. et al. (2010) *Mechanisms of immune-mediated liver injury. Toxicol. Sci., 115, 307–21.*

Agnelli, G. et al. (2009) *Safety assessment of new antithrombotic agents: Lessons from the EXTEND study on ximelagatran. Thromb. Res., 123, 488–497.*

Ainscow, E.K. et al. (2008) *Investigations into the liver effects of ximelagatran using high content screening of primary human hepatocyte cultures. Expert Opin. Drug Saf., 7, 351–365.*

Aithal, G.P. et al. (2011) *Case definition and phenotype standardization in drug-induced liver injury. Clin. Pharmacol. Ther., 89, 806–815.*

Anderson, V.E. et al. (2000) *Action of Quinolones against Staphylococcus aureus Topoisomerase IV: Basis for DNA Cleavage Enhancement †. Biochemistry, 39, 2726–2732.*

Anderson, V.E. and Osheroff, N. (2001) *Type II topoisomerases as targets for quinolone antibacterials: turning Dr. Jekyll into Mr. Hyde. Curr. Pharm. Des., 7, 337–53.*

Andersson, S. et al. (2008) *Characterization and partial purification of the rat and human enzyme systems active in the reduction of N-hydroxymelagatran and benzamidoxime. 33, 570–578.*

Ballet, F. (2015) *Preventing Drug-Induced Liver Injury: How Useful Are Animal Models? Dig. Dis., 33, 477–485.*

Beggs, K.M. et al. (2014) *Molecular Mechanisms of Hepatocellular Apoptosis Induced by Trovafloxacin-Tumor Necrosis Factor-alpha Interaction. Toxicol. Sci., 137, 91–101.*

Beggs, K.M. et al. (2015) *Trovafloxacin-induced replication stress sensitizes HepG2 cells to tumor necrosis factor-alpha-induced cytotoxicity mediated by extracellular signal-regulated kinase and ataxia telangiectasia and Rad3-related. Toxicology, 331, 35–46.*

Borlak, J. (2009) *3.3 Trovafloxacin: A case study of idiosyncratic or iatrogenic liver toxicity-molecular mechanisms and lessons for pharmacotoxicity. Hum. Exp. Toxicol., 28, 119–121.*

Bradham, C.A. et al. (1998) *The Mitochondrial Permeability Transition Is Required for Tumor Necrosis Factor Alpha-Mediated Apoptosis and Cytochrome c Release. Mol. Cell. Biol., 18, 6353–6364.*

Brauner, J.M. et al. (2013) *No littering : the clearance of dead cells and leaking cellular contents and possible pathological complications. OA Arthritis, 1, 1–7.*

Brighton, T.A. (2004) *The direct thrombin inhibitor melagatran/ximelagatran. Med. J. Aust., 181, 432–437.*

Brighty, K.E. and Gootz, T.D. (1997) *The chemistry and biological profile of trovafloxacin. J. Antimicrob. Chemother., 39 Suppl B, 1–14.*

Castiella, A. et al. (2014) *Drug-induced autoimmune liver disease: A diagnostic dilemma of an increasingly reported disease. World J. Hepatol., 6, 160–168.*

Chalasani, N. et al. (2015) *Features and outcomes of 899 patients with drug-induced liver injury: The DILIN prospective study. Gastroenterology, 148, 1340–1352.e7.*

Chalasani, N. and Björnsson, E. (2010) *Risk factors for idiosyncratic drug-induced liver injury. Gastroenterology, 138, 2246–59.*

Choi, S. (2003) *Nefazodone (Serzone) withdrawn because of hepatotoxicity. Cmaj, 169, 1187.*

Cosgrove, B.D. et al. (2009) *Synergistic drug-cytokine induction of hepatocellular death as an in vitro approach for the study of inflammation-associated idiosyncratic drug hepatotoxicity. Toxicol. Appl. Pharmacol., 237, 317–330.*

Crispe, I.N. (2011) *Liver antigen-presenting cells. J. Hepatol., 54, 357–365.*

Dalvie, D.K. et al. (1997) *Excretion and metabolism of trovafloxacin in humans. Drug Metab. Dispos., 25, 423–7.*

Deng, X. et al. (2006) *Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. J. Pharmacol. Exp. Ther., 319, 1191–1199.*

Diao, H. et al. (2004) *Osteopontin as a mediator of NKT cell function in T cell-mediated liver diseases. Immunity, 21, 539–550.*

Doherty, D.G. (2016) *Immunity, tolerance and autoimmunity in the liver: A comprehensive review. J. Autoimmun., 66, 60–75.*

Eriksson, U.G. et al. (2003) *Absorption , Distribution , Metabolism , and Excretion of Ximelagatran , an Oral Direct Thrombin Inhibitor , in Rats , Dogs , and Humans Abstract : 31, 294–305.*

Fish, U.S. and Service, W. (2014) *Federal Register. Fed. Regist., 79, 37617–37925.*

Fontana, R.J. (2014) Pathogenesis of Idiosyncratic Drug-Induced Liver Injury and Clinical Perspectives. Acc. Chem. Res., 146, 914–928.

Fredriksson, L. et al. (2011) *Diclofenac inhibits tumor necrosis factor-α-induced nuclear factor-κB activation causing synergistic hepatocyte apoptosis. Hepatology, 53, 2027–41.*

Gandhi, A. et al. (2013) *Chlorpromazine-induced hepatotoxicity during inflammation is mediated by TIRAP-dependent signaling pathway in mice. Toxicol. Appl. Pharmacol., 266, 430–8.*

Gandhi, A. et al. (2009) *Role of c-Jun N-terminal kinase (JNK) in regulating tumor necrosis factor-alpha (TNF-α) mediated increase of acetaminophen (APAP) and chlorpromazine (CPZ) toxicity in murine hepatocytes. 35.*

Graham, D.J. et al. (2003) *Troglitazone-induced liv-*

er failure: a case study. Am. J. Med., 114, 299–306.
Grell, M. et al. (1999) *Induction of cell death by tumour necrosis factor (TNF) receptor 2, CD40 and CD30: A role for TNF-R1 activation by endogenous membrane-anchored TNF. EMBO J., 18, 3034–3043.*
Grell, M. et al. (1995) *The Transmembrane Form of Tumor Necrosis Factor Is the Prime Activating Ligand of the 80 kDa Tumor Necrosis Factor Receptor. 83, 793–802.*
Griem, P. et al. (1998) *T cell cross-reactivity to heavy metals: Identical cryptic peptides may be presented from protein exposed to different metals. Eur. J. Immunol., 28, 1941–1947.*
Gustafsson, D. et al. (2001) *The direct thrombin inhibitor melagatran and its oral prodrug H 376/95: Intestinal absorption properties, biochemical and pharmacodynamic effects. Thromb. Res., 101, 171–181.*
Hatano, M. et al. (2008) *Effects of Kupffer cell-depletion on Concanavalin A-induced hepatitis. Cell. Immunol., 251, 25–30.*
Hayashi, P.H. and Chalasani, N.P. (2012) *Liver injury in the elderly due to fluoroquinolones: Should these drugs be avoided? Cmaj, 184, 1555–1556.*
Holt, M.P. et al. (2010) *Exacerbation of acetaminophen-induced disturbances of liver sinusoidal endothelial cells in the absence of Kupffer cells in mice. Toxicol. Lett., 194, 34–41.*
Holt, M.P. et al. (2008) *Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. J. Leukoc. Biol., 84, 1410–21.*
Van Hul, N. et al. (2011) *Kupffer cells influence parenchymal invasion and phenotypic orientation, but not the proliferation, of liver progenitor cells in a murine model of liver injury. Am. J. Pathol., 179, 1839–50.*
Hunter, E.B. et al. (1999) *Bromfenac (Duract)-associated hepatic failure requiring liver transplantation. Am. J. Gastroenterol., 94, 2299–2301.*
Hussaini, S.H. and Farrington, E.A. (2007) *Idiosyncratic drug-induced liver injury: an overview. Expert Opin. Drug Saf., 6, 673–684.*
Hussaini, S.H. and Farrington, E.A. (2014) *Idiosyncratic drug-induced liver injury: an update on the 2007 overview. Expert Opin. Drug Saf., 13, 67–81.*
Idriss, H.T. and Naismith, J.H. (2000) *TNFα and the TNF receptor superfamily: Structure-function relationship(s). Microsc. Res. Tech., 50, 184–195.*
Ishida, Y. et al. (2006) *Opposite roles of neutrophils and macrophages in the pathogenesis of acetaminophen-induced acute liver injury. Eur. J. Immunol., 36, 1028–1038.*
Ju, C. et al. (2002) *Protective role of kupffer cells in acetaminophen-induced hepatic injury in mice. Chem. Res. Toxicol., 15, 1504–1513.*
Ju, C. and Reilly, T. (2012) *Role of immune reactions in drug-induced liver injury (DILI). Drug Metab. Rev., 44, 107–15.*
Kaplan, K.L. (2003) *Direct thrombin inhibitors. Expert Opin. Pharmacother., 4, 653–666.*
Kenna, J.G. and Uetrecht, J. (2018) *Do In Vitro Assays Predict Drug Candidate Idiosyncratic Drug-Induced Liver Injury Risk? Drug Metab. Dispos., dmd.118.082719.*
Kenne, K. et al. (2008) *Prediction of drug-induced liver injury in humans by using in vitro methods: The case of ximelagatran. Toxicol. Vitr., 22, 730–746.*
Kim, S.H. and Naisbitt, D.J. (2016) *Update on advances in research on idiosyncratic drug-induced liver injury. Allergy, Asthma Immunol. Res., 8, 3–11.*
Kindmark, a et al. (2008) *Genome-wide pharmacogenetic investigation of a hepatic adverse event without clinical signs of immunopathology suggests an underlying immune pathogenesis. Pharmacogenomics J., 8, 186–95.*
Krompholz, N. et al. (2012) *The mitochondrial amidoxime reducing component (mARC) is involved in detoxification of N-hydroxylated base analogues. Chem. Res. Toxicol., 25, 2443–2450.*
Kullak-Ublick, G.A. et al. (2017) *Drug-induced liver injury: Recent advances in diagnosis and risk assessment. Gut, 66, 1154–1164.*
Landsteiner, K. (1941) *Studies on the Sensitization of Animals With Simple Chemical Compounds: Ix. Skin Sensitization Induced By Injection of Conjugates. J. Exp. Med., 73, 431–438.*
Lasser, K.E. (2002) *Timing of New Black Box Warnings and Withdrawals for Prescription Medications. Jama, 287, 2215.*
Lazarczyk, D.A. et al. (2001) *Trovafloxacin Hepatotoxicity. 46, 925–926.*
Lee, E.-H. et al. (2016) *Immunogenomics reveal molecular circuits of diclofenac induced liver injury in mice. Oncotarget, 7.*
Lee, W.M. et al. (2005) *Hepatic findings in long-term clinical trials of ximelagatran. Drug Saf., 28, 351–370.*
Li, R. et al. (2011) *Role of liver sinusoidal endothelial cells and stabilins in elimination of oxidized low-density lipoproteins. Am. J. Physiol. Liver Physiol., 300, G71–G81.*
Licata, A. (2016) *Adverse drug reactions and organ damage: The liver. Eur. J. Intern. Med., 28, 9–16.*
Licata, A. et al. (2017) DILI and epidemiology. 21, 112–121.
Liu, Z.X. et al. (2006) *Neutrophil depletion protects against murine acetaminophen hepatotoxicity. Hepatology, 43, 1220–1230.*
Lu, J. et al. (2012) *Amiodarone Exposure During Modest Inflammation Induces Idiosyncrasy-like Liver Injury in Rats: Role of Tumor Necrosis Factor-alpha. Toxicol. Sci., 125, 126–133.*
Lu, J. et al. (2013) *Tumor necrosis factor-alpha*

potentiates the cytotoxicity of amiodarone in Hepa1c1c7 cells: roles of caspase activation and oxidative stress. Toxicol. Sci., 131, 164–78.

Luyendyk, J.P. (2003) *Ranitidine Treatment during a Modest Inflammatory Response Precipitates Idiosyncrasy-Like Liver Injury in Rats. J. Pharmacol. Exp. Ther., 307, 9–16.*

Mak, A. and Uetrecht, J. (2015a) *The Combination of Anti-CTLA-4 and PD1–/– Mice Unmasks the Potential of Isoniazid and Nevirapine To Cause Liver Injury. Chem. Res. Toxicol., acs.chemrestox.5b00305.*

Mak, A. and Uetrecht, J. (2015b) *The Role of CD8 T Cells in Amodiaquine-Induced Liver Injury in PD1–/– Mice Cotreated with Anti-CTLA-4. Chem. Res. Toxicol., 150715121837009.*

Maniar, J.K. et al. (2006) *Nevirapine-induced fulminant hepatitis. J. Assoc. Physicians India, 54, 957–8.*

Marwick, J.A. et al. (2018) *Neutrophils induce macrophage anti-inflammatory reprogramming by suppressing NF-κB activation article. Cell Death Dis., 9.*

Matzinger, P. (2002) *The Danger Model : A Renewed Sense of Self. Science (80-.)., 296, 301–305.*

Medicines, H. and Unit, E. (1999) *Public Statement Trovan-Turvel.*

Metushi, I.G. et al. (2015) *Treatment of PD-1-/- mice with amodiaquine and anti-CTLA4 leads to liver injury similar to idiosyncratic liver injury in patients. Hepatology, 61, 1332–1342.*

Micheau, O. et al. (2003) *Induction of TNF Receptor I-Mediated Apoptosis via Two Sequential Signaling Complexes. 114, 181–190.*

Neve, E.P.A. et al. (2012) *Amidoxime reductase system containing cytochrome b5type B (CYB5B) and MOSC2 is of importance for lipid synthesis in adipocyte mitochondria. J. Biol. Chem., 287, 6307–6317.*

Neve, E.P.A. et al. (2015) *Expression and function of mARC: Roles in lipogenesis and metabolic activation of ximelagatran. PLoS One, 10, 1–14.*

Nolan, J.P. (2010) *The role of intestinal endotoxin in liver injury: A long and evolving history. Hepatology, 52, 1829–1835.*

Ostapowicz, G. et al. (2002) *Article Results of a Prospective Study of Acute Liver Failure at 17 Tertiary. 947–955.*

Pirmohamed, M. et al. (2004) *Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. BMJ, 329, 15–19.*

Pirmohamed, M. et al. (2002) *The danger hypothesis--potential role in idiosyncratic drug reactions. Toxicology, 181–182, 55–63.*

Poulsen, K.L., Olivero-Verbel, J., et al. (2014) *Trovafloxacin enhances lipopolysaccharide-stimulated production of tumor necrosis factor-α by macrophages: role of the DNA damage response. J. Pharmacol. Exp. Ther., 350, 164–70.*

Poulsen, K.L., Albee, R.P., et al. (2014) *Trovafloxacin Potentiation of Lipopolysaccharide-Induced Tumor Necrosis Factor Release from RAW 264.7 cells requires ERK and JNK. J. Pharmacol. Exp. Ther.*

Reymann, S. and Borlak, J. (2008) *Topoisomerase II inhibition involves characteristic chromosomal expression patterns. BMC Genomics, 9, 324.*

Robinson, M.W. et al. (2016) *Liver immunology and its role in inflammation and homeostasis. Cell. Mol. Immunol., 13, 267–76.*

Roth, R. a et al. (1997) *Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents? Toxicol. Appl. Pharmacol., 147, 300–11.*

Roth, R.A. et al. (2017) *Idiosyncratic Drug-Induced Liver Injury: Is Drug-Cytokine Interaction the Linchpin? J. Pharmacol. Exp. Ther., 360, 461–470.*

Schwabe, R.F. and Brenner, D.A. (2006) *Mechanisms of Liver Injury . I . TNF-α-induced liver injury : role of IKK , JNK , and ROS pathways. Am J Physiol Gastrointest Liver Physiol, 583–589.*

Seidel, J.A. et al. (2018) *Anti-PD-1 and Anti-CTLA-4 Therapies in Cancer: Mechanisms of Action, Efficacy, and Limitations. Front. Oncol., 8, 1–14.*

Shaw, P.J. et al. (2010) *Idiosyncratic drug-induced liver injury and the role of inflammatory stress with an emphasis on an animal model of trovafloxacin hepatotoxicity. Toxicol. Sci., 118, 7–18.*

Shaw, P.J. et al. (2007) *Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. Toxicol. Sci., 100, 259–66.*

Shaw, P.J. et al. (2009) *The role of the hemostatic system in murine liver injury induced by coexposure to lipopolysaccharide and trovafloxacin, a drug with idiosyncratic liability. Toxicol. Appl. Pharmacol., 236, 293–300.*

Shaw, P.J., Ganey, P.E., et al. (2009a) *Trovafloxacin Enhances the Inflammatory Response to a Gram-Negative or a Gram-Positive Bacterial Stimulus , Resulting in Neutrophil-Dependent Liver Injury in Mice. J Pharmacol Exp Ther, 330, 72–78.*

Shaw, P.J., Beggs, K.M., et al. (2009) *Trovafloxacin enhances TNF-induced inflammatory stress and cell death signaling and reduces TNF clearance in a murine model of idiosyncratic hepatotoxicity. Toxicol. Sci., 111, 288–301.*

Shaw, P.J., Ganey, P.E., et al. (2009b) *Tumor Necrosis Factor α is a Proximal Mediator of Synergistic Hepatotoxicity from Trovafloxacin / Lipopolysaccharide Coexposure. J Pharmacol Exp Ther, 328, 62–68.*

Soehnlein, O. et al. (2009) *Neutrophils launch monocyte extravasation by release of granule proteins. Thromb. Haemost., 102, 198–205.*

Soehnlein, O. and Lindbom, L. (2010) *Phagocyte partnership during the onset and resolution of inflammation. Nat. Rev. Immunol., 10, 427–39.*

Spahn, T.W. et al. (2002) *Induction of colitis in mice deficient of Peyer's patches and mesenteric lymph*

nodes is associated with increased disease severity and formation of colonic lymphoid patches. Am. J. Pathol., 161, 2273–82.

Suk, K.T. and Kim, D.J. (2012) *Drug-induced liver injury: present and future. Clin. Mol. Hepatol., 18, 249–57.*

Tacke, F. et al. (2009) *Inflammatory pathways in liver homeostasis and liver injury. Clin. Rev. Allergy Immunol., 36, 4–12.*

Tailor, A. et al. (2015) *The chemical, genetic and immunological basis of idiosyncratic drug-induced liver injury. Hum. Exp. Toxicol., 34, 1310–1317.*

Tang, D. et al. (2012) *PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol. Rev., 249, 158–75.*

Tujios, S. and Fontana, R.J. (2011) *Mechanisms of drug-induced liver injury: from bedside to bench. Nat. Rev. Gastroenterol. Hepatol., 8, 202–11.*

Uetrecht, J. (2007) *Idiosyncratic Drug Reactions: Current Understanding. Annu. Rev. Pharmacol. Toxicol., 47, 513–539.*

Uetrecht, J. (2001) *Prediction of a new drug's potential to cause idiosyncratic reactions. Curr. Opin. Drug Discov. Devel., 4, 55–9.*

Vaughan, C. (2005) *Ximelagatran (Exanta): alternative to warfarin? Proc. (Bayl. Univ. Med. Cent)., 18, 76–80.*

Vercammen, D. et al. (1998) *Inhibition of Caspases Increases the Sensitivity of L929 Cells to Necrosis Mediated by Tumor Necrosis Factor. J. Exp. Med., 187, 1477–1485.*

Wajant, H. et al. (2003) *Tumor necrosis factor signaling. 45–65.*

Weitz, J.I. (2003) *A novel approach to thrombin inhibition. Thromb. Res., 109, 17–22.*

Who (1972) *International drug monitoring: the role of national centres. Rep. a WHO Meet., 1–25.*

Wullaert, A. et al. (2007) *Hepatic tumor necrosis factor signaling and nuclear factor-κB: Effects on liver homeostasis and beyond. Endocr. Rev., 28, 365–386.*

Yano, A. et al. (2012) *Involvement of immune-related factors in diclofenac-induced acute liver injury in mice. Toxicology, 293, 107–14.*

Zhang, X. et al. (2011) *Involvement of the immune system in idiosyncratic drug reactions. Drug Metab. Pharmacokinet., 26, 47–59.*

Zhou, Z. et al. (2016) *Hepatocytes: A key cell type for innate immunity. Cell. Mol. Immunol., 13, 301–315.*

Zou, W. et al. (2009) *Hepatotoxic interaction of sulindac with lipopolysaccharide: Role of the hemostatic system. Toxicol. Sci., 108, 184–193.*

Chapter 2

Tissue influx of neutrophils and monocytes is delayed during development of trovafloxacin-induced tumor necrosis factor-dependent liver injury in mice

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ABSTRACT

Idiosyncratic drug-induced liver injury (iDILI) has a poorly understood pathogenesis. However, iDILI is often associated with inflammatory stress signals in human patients as well as animal models. Tumor necrosis factor (TNF) and neutrophils play a key role in onset of trovafloxacin (TVX)-induced iDILI, but the exact role of neutrophils and other leukocytes remains to be defined. We therefore set out to study the kinetics of immunological changes during the development of TVX-induced iDILI in the established murine model of acute liver injury induced by administration of TVX and TNF. Initially, TNF stimulated the appearance of leukocytes, in particular neutrophils, into the liver of TVX-treated mice, but even more so in control mice treated with the non-DILI inducing analogue levofloxacin (LVX) or saline as vehicle (Veh). This difference was apparent at 2 hours after TNF administration, but at 4 hours, the relative neutrophil amounts were reduced again in Veh- and LVX-treated mice whereas the amounts in TVX-treated mice remained at the same increased level as at 2 hours. The influx of monocytes/macrophages, which was unaffected in Veh- and LVX-treated mice was markedly reduced or even absent in TVX-treated mice. Unlike controls, mice receiving TVX + TNF display severe hepatotoxicity with clear pathology and apo- ptosis, coagulated hepatic vessels and increased alanine aminotransferase levels and interleukin 6/10 ratios. Findings indicate that TVX delays the acute influx of neutrophils and monocytes/ macrophages. Considering their known anti-inflammatory functions, the disruption of influx of these innate immune cells may hamper the resolution of initial cytotoxic effects of TVX and thus contribute to liver injury development.

INTRODUCTION

Drug-induced liver injury (DILI) is a major concern for healthcare organizations and pharmaceutical companies all over the world, mainly due to the severity and prognosis of these events. Nonetheless, the economic impact associated with the hospitalization of patients and the restricted use of the drug imposed by regulatory agencies cannot be neglected (Chalasani et al., 2015; Fontana et al., 2009). Some forms of DILI are idiosyncratic in nature and, among them, some involve the immune system during the onset and/or the prolongation of the disease. In particular, pharmaceuticals inducing immune-mediated or idiosyncratic DILI (iDILI) are often not detected by available preclinical and clinical tests alike, revealing their toxic features only during post-marketing pharmacovigilance studies (Lee and Senior, 2005; Watkins and Seeff, 2006). For this reason, many efforts have been spent during the last decade to identify new preclinical tests that can predict, early in drug development, the risk for occurrence of iDILI. Several animal models underlined that tumor necrosis factor (TNF) administration in combination with known iDILI-associated compounds discloses the hepatotoxicity of these drugs (Buchweitz, 2002; Deng et al., 2006; Dugan and MacDonald, 2010; Lu et al., 2012; Luyendyk, 2003; Zou et al., 2009). Whether these models reproduce the clinical signature of DILI in patients is still matter of debate. In addition, the role of the immune response triggered by TNF in the development of liver injury is not yet well-defined. For this reason, we investigated the immuno-toxicological effects of trovafloxacin (TVX) in the established mouse model of TVX-induced iDILI using TNF as a surrogate for inflammation.

We emphasized our analyses on the kinetic changes of innate leukocytes in liver, because these leukocytes are known to be involved during the acute phase of inflammation, either as inflammatory or as anti-inflammatory players (Robinson et al., 2016). Characterization of the leukocyte recruitment into the liver will help identifying potential mechanisms involved in the development of iDILI as well as early biomarkers to predict the potential onset of iDILI. Surprisingly, we found that recruitment of neutrophils and monocytes into the liver was delayed by the combination of TVX + TNF when compared with control mice receiving saline (vehicle, Veh) or the pharmacological analogue levofloxacin (LVX) even in the presence of TNF. Kinetics of the immunotoxicological changes in mice receiving TVX + TNF stresses the importance to investigate further the role of innate leukocytes in the development of iDILI.

MATERIALS AND METHODS

Animals

Male, 9–11 week old, C57BL/6 J mice (The Jackson Laboratory, Charles River) were used for all experiments. They were allowed to acclimatize for 1 week in a

12 hour light/dark cycle, and maintained at mean temperature of 23 ± 2°C, 50–55% relative humidity. Acidified drinking water and laboratory food pellets were provided ad libitum. In vivo studies were approved by the Ethics Committee for Animal Experiments of Utrecht University and complied with governmental and international guidelines on animal experimentation.

Chemicals

TVX and LVX were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant murine TNF was purchased from R&D Systems (Minneapolis, MN, USA

Experimental set up

Mice (n = 6) were fasted 7 hours before treatment. TVX (150 mg/kg^{-1}), LVX (375 mg/kg^{-1}) or saline as Veh was administered orally 3 hours before recombinant murine TNF injection (50 µg/kg^{-1}, intra-peritoneally) (Patrick J Shaw, Beggs, et al., 2009). Food was available again immediately after TNF administration. Animals analyzed at time point 0 hours did not receive TNF (Figure 1). Dose of TVX was chosen because it was not associated with either hepatic histopathological modifications or serum alanine aminotransferase (ALT) increase. TVX, at selected dose, was capable of interacting with TNF to cause liver injury (Patrick J Shaw, Ganey, et al., 2009b; Patrick J Shaw, Beggs, et al., 2009). As LVX is prescribed at a dose that is 2.5-fold greater than the dose of TVX to achieve a similar therapeutic effect in clinics (Lubasch et al., 2000), we decided to keep the same ratio among the administered doses of these fluoroquinolones to conform to the experiments performed by Shaw and colleagues (Patrick J Shaw, Ganey, et al., 2009b; Patrick J Shaw, Beggs, et al., 2009) .

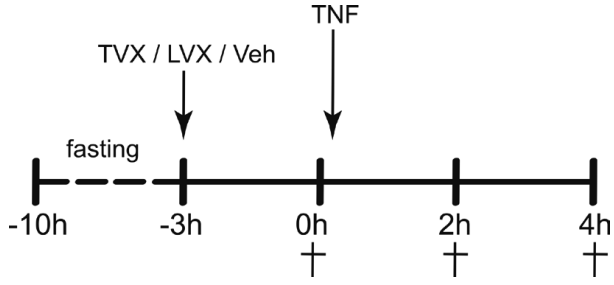


Figure 1

Protocol for the treatment of mice in the TVX + TNF model of drug-induced liver injury.

Mice were fasted for 7 hours and then administered with an intragastric gavage of the drug solution or saline. Three hours later, mice were either killed (time point 0 hours) or received an intraperitoneal injection of TNF. TNF-injected mice were subsequently killed after 2 or 4 hours. LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

Intrahepatic leukocyte isolation

Intrahepatic leukocytes were isolated as previously described by Crispe (Crispe, 2001). Briefly, liver was perfused with 5 ml ice-cold phosphate-buffered saline (PBS) and excised from the animal. Tissue was minced and gently passed through a sieve. The liver slurries were centrifuged, and the pellet resuspended with a collagenase D (0.02%, w/v) and DNAse I (0.002%, w/v) solution. The suspensions were incubated at 37°C for 40 minutes on a reciprocating shaker. Liver leukocytes were isolated by using a 45–67.5% isotonic Percoll density gradient (GE Healthcare, Fisher Scientific, Landsmeer, The Netherlands). Hepatic leukocytes obtained from Veh-only treated mice (without TNF) were used as an internal control. With this, we wanted to rule out small day-by-day differences in the isolation efficiency throughout the experiments.

Plasma alanine aminotransferase and cytokine levels

Blood from inferior vena cava was collected in pre-coated lithium heparin tubes. Plasma was used to determine ALT activity according to the manufacturer's instructions (article no. MAK052; Sigma-Aldrich). TNF, interleukin (IL)-6 and IL-10 were determined in serum samples by sandwich enzyme-linked immunosorbent assay. Antibodies were from eBioscience (Halle-Zoersel, Belgium) and procedures followed according to the manufacturer's instructions.

Histology

Liver and spleen were weighed. Half of the left lateral lobe and half of the spleen was fixed in phosphate-buffered formalin and embedded in paraffin. Five µm sections were cut and stained with hematoxylin– eosin and evaluated in a blind coded fashion by two independent investigators.

Flow cytometry

Leukocytes from spleen were obtained by passing the organ through a 70 µm strainer, after which the collected single cells were washed once. To remove erythrocytes, cell suspensions were incubated with red blood cell lysis buffer (containing NH_4Cl, $KHCO_3$ and Na_2EDTA) for 1 minute, and the remaining cells were washed once with PBS. For flow-cytometric analysis, cells were first stained with LIVE/DEAD® Fixable Dead Cell Stain (Molecular Probes, Invitrogen, Carlsbad, CA, USA) followed by incubation with anti-CD16/CD32 (clone 2.4G2) to block the fragment crystallizable region receptor (FcR). Next cells were stained with fluorescent-labeled antibodies and stored in 1% paraformaldehyde until analysis. The following antibodies were used: anti-CD45.2 Pacific Blue (clone 104; Biolegend, Uithoorn, The Netherlands), anti-LY6G APC (clone 1A8; Biolegend), anti-F4/80 fluorescein isothiocyanate (FITC; clone BM8; eBioscience), anti-CD11b PE and FITC (clone M1/70; eBioscience), anti-Gr1 APC (clone RBG8C5; eBioscience), CD4 PERCP (clone RM4-5; BD Pharmingen), CD8a PERCP (clone 53-6.7; BD Pharmingen, Erembodegem, Belgium), CD3e FITC (clone 145-2C11; eBioscience), NK1.1 PE (clone PK136; eBioscience), CD49b PE-Cy5

(clone DX5; eBioscience), CD86 PERCP (clone GL-1; Biolegend), MHCII FITC (clone M5/114.15.2; eBioscience), PDCA-1 PE (clone eBio129c; eBioscience), CD11c APC (clone N418; eBioscience), CD62L APC and PE (clone MEL- 14; BD Pharmingen), and CD44 PE (clone IM7; eBioscience) in fluorescence-activated cell sorting buffer (PBS containing 0.25% bovine serum albumin, 0.05% NaN3, 0.5 mM EDTA) for 30 minutes at 4°C. Data were acquired by means of fluorescence-activated cell sorting Canto II and analyzed using Weasel flow analysis package (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

Histological TUNEL staining

Immunofluorescence was performed on frozen liver and spleen tissues embedded in optimal cutting temperature compound. Eight µm thick frozen sections were mounted on polylysine-coated glass slides, dried overnight at room temperature and stored at –20°C until use. Briefly, tissue sections were allowed to dry for 2 hours before fixation with 4% formaldehyde in PBS. Terminal deoxynucleotidyl transferase- mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay was performed following procedures for cryopreserved tissues reported in manufacturer's instructions (Roche, Woerden, The Nether- lands). Neutrophil in liver tissue were stained using rat anti-mouse Ly6G (1A8) for 1 hour in PBS/bovine serum albumin 0.1%, after blocking with 5% rat serum for 10 minutes both at room temperature. FITC- conjugated donkey antirat IgG was applied in PBS-Tween20 10% mouse serum for 45 minutes. Nuclei were identified by DAPI. Images were acquired with Olympus BX-60 microscope equipped with Leica CCD camera (Leica DFC425C) (40×, 0.5 objective).

Statistical analyses

Data are presented as means ± standard error of the mean (SEM). Amounts of cells are presented as a percentage of non-TNF and non- drug-treated control animals. Statistical significance for comparisons was determined by one- or two-way analysis of variance with Dunnett's post-hoc test. $P < .05$ was considered statistically significant. All data are analyzed using GraphPad Prism (version 6.07) software (San Diego, CA, USA).

RESULTS

Kinetics of TVX-induced organ changes indicates that liver is affected before TNF exposure and that the spleen is affected as well. We first set out to characterize the kinetics of damage in liver and spleen tissues induced by TVX. Thus, mice received either saline (Veh) or a single oral dose of TVX (150 mg/kg^{-1}) or LVX (375 mg/kg^{-1}) followed by a TNF injection after 3 hours. Mice were killed before TNF injection (0 hours), or 2 or 4 hours after TNF injection (Figure 1).

Injection of TNF caused liver damage in TVX-treated mice, but not in Veh- or LVX-treated mice. The treatments did not affect liver weights (not shown) and liver

damage was evident at 4 hours after TNF injection and associated with infiltration of inflammatory cells around the central veins and by vast areas of necrotic lesions. The necrotic lesions were mainly in the periportal and midzonal regions of the liver lobules (Figure 2B). Higher magnification revealed that TVX + TNF caused apoptosis of hepatocytes, which was confirmed with TUNEL staining to detect DNA fragmentation (Figure 3B). TVX + TNF also induced hepatic blood congestion and increased levels of serum ALT (Figure 2A). None of these effects was present in the livers of Veh- and LVX-treated mice. Liver tissues of TVX-treated mice collected at 2 hours after TNF injection were free from major lesions or leukocytes (data not shown) and serum ALT levels were not enhanced (Figure 2A). Remarkably, TVX without TNF (i.e., 3 hours after TVX administration, t =0 in Figure 1) also caused a modest but clear increase of TUNEL positivity in the hepatocyte nuclei (Figure 3B). However, this was not accompanied by histopathologically apparent hepatic changes (data not shown). Notably, TVX + TNF-induced tissue damage was not restricted tothe liver, as evident from reduction of splenic cell numbers and from focal signs of cell death observed in splenic white pulp by hematoxylin–eosin staining and confirmed by nuclear TUNEL positivity (Figures 2C and 4C). Nuclear TUNEL positivity and apoptotic morphology was also observed in splenocytes of mice treated with TVX at 0 hours (before TNF injection, Figures 4C and 2D respectively).

TNF also caused modest DNA fragmentation in the spleens of Veh- treated mice at 4 hours (Figure 4B), but not in the livers.

Tumor necrosis factor elicits aberrant cytokine production in trovafloxacin-exposed mice.

As the ratio of serum concentrations of IL-6 and IL-10 has been identified as a potential prognostic marker of the severity of different inflammatory conditions, we further investigated the kinetics of these cytokines (de Brito et al., 2016; Nagaki et al., 2000; Stensballe et al., 2009; Taniguchi et al., 1999). We also monitored the serum concentration of TNF, which will be of mixed endogenous and exogenous origin, but in turn can also stimulate secretion of the aforementioned cytokines (Libert et al., 1990; Poll et al., 1997).

TNF administration by itself, but also in combination with LVX, elicited a mild increase in serum levels of TNF, IL-6 and IL-10 when compared with animals not receiving TNF. The combined TVX+TNF treatment caused a profound increase of the serum concentrations of the above-mentioned cytokines when compared with LVX or Veh +TNF (Figure 5). Remarkably, although the serum levels of TNF and IL-10 were already decreasing again in all groups at 4 hours, this was less apparent in the TVX-treated group, which in addition showed further increased levels of IL-6. Consequently, the IL-6/IL-10 ratio was highly increased at 4 hours in TVX-treated mice, when mortality (which occurred in one of six animals between 2 and 4 hours) and severe liver damage were observed. The mild liver damage observed at time point 0 hours, before TNF injection, was not associated with increased serum levels of TNF, IL-6 or IL-10 (Figure 5, time point 0 hours).

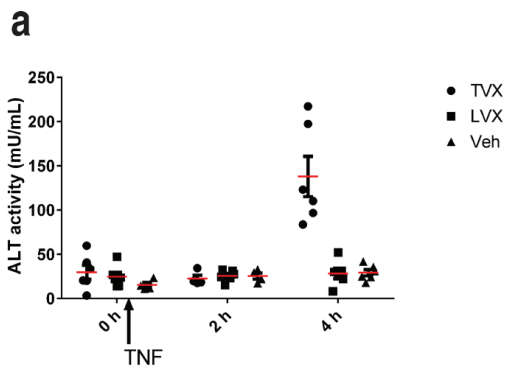
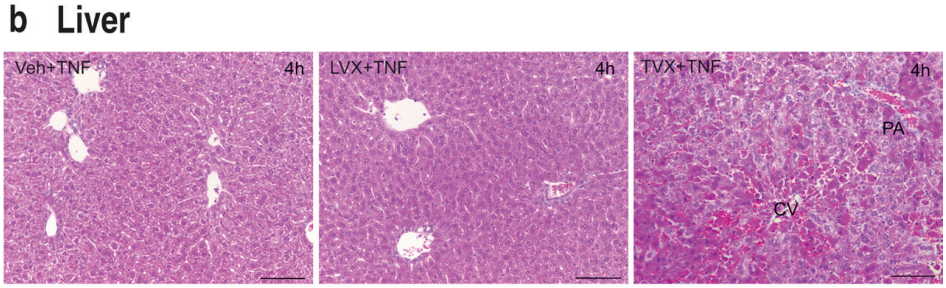
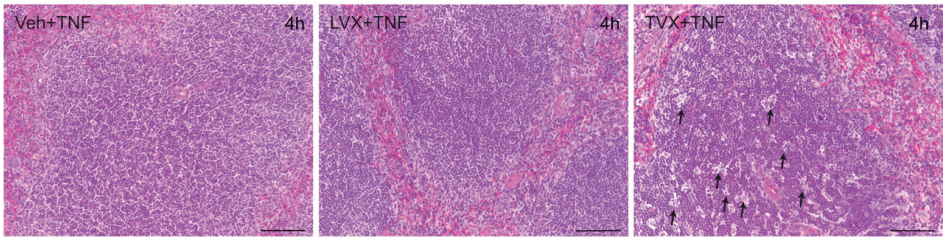


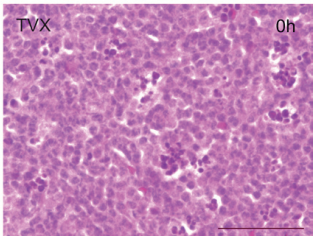
Figure 2

Histology and ALT levels.

Serum ALT levels of TVX-, LVX- or Veh-treated mice. ALT levels were determined by colorimetric assay for ALT activity (A). Hematoxylin–eosin staining of liver and spleen sections taken 4 hours after TNF injection of mice pretreated with TVX, LVX or Veh (B,C). Arrows indicate focal damage observed in spleen. Apoptotic morphology of splenocytes inTVX-treated mice before injection of TNF (0 hour time point) (D).







Representative images selected from six mice per treatment group are shown. Scale bars: (A–C) 100 µm; (D) 50 µm. ALT, alanine aminotransferase; CV, central vein; LVX, levofloxacin; PA, portal area; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

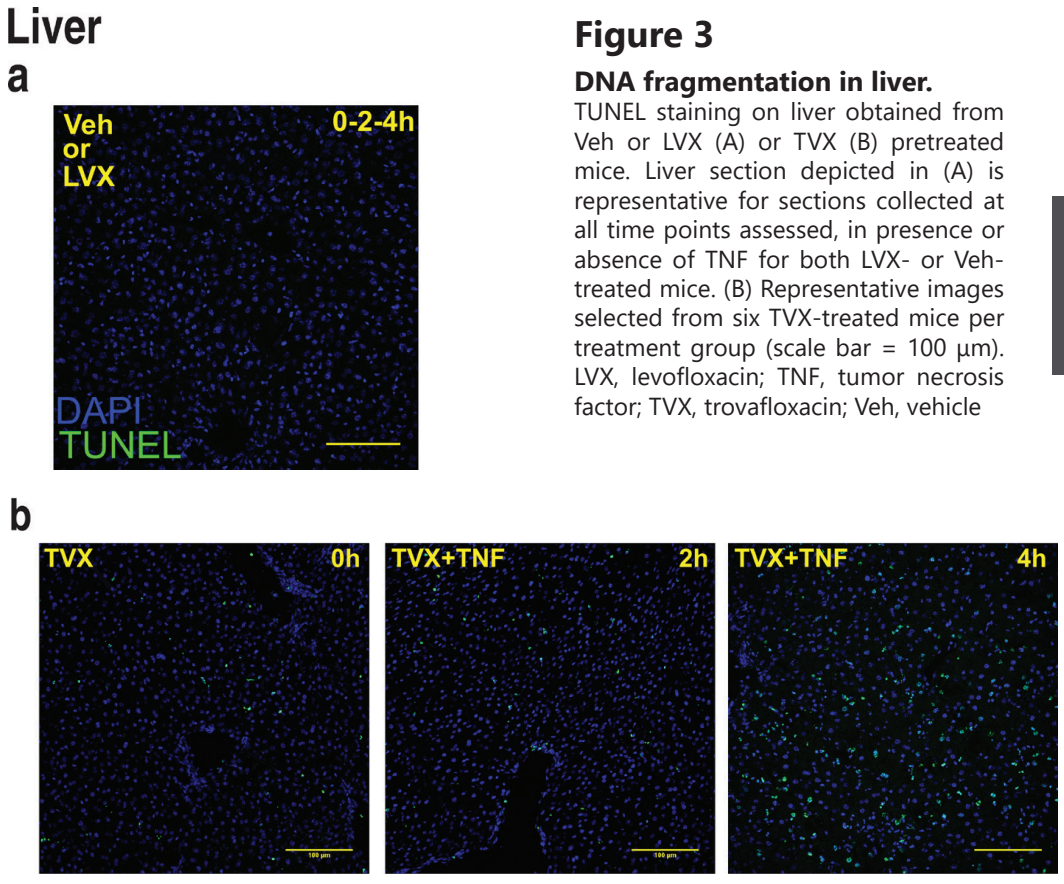


Figure 3

DNA fragmentation in liver.
TUNEL staining on liver obtained from Veh or LVX (A) or TVX (B) pretreated mice. Liver section depicted in (A) is representative for sections collected at all time points assessed, in presence or absence of TNF for both LVX- or Veh-treated mice. (B) Representative images selected from six TVX-treated mice per treatment group (scale bar = 100 µm). LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

Leukocyte influx in liver and spleen of trovafloxacin-treated mice appeared delayed.

Several scientific studies describe the importance of a well-controlled leukocyte recruitment and activity in the regulation of inflammation (Gabrilovich, 2017; Pillay et al., 2013; Soehnlein and Lindbom, 2010). We first assessed the amount of sequestered CD45.2 leukocytes in the liver and spleen induced by the treatments. As expected (Vollmar and Menger, 2009), all the groups receiving TNF showed an increased amount of sequestered leukocytes in the liver with a marked increase at 2 hours after TNF injection.

This increase was reduced again at 4 hours, except in TVX-pretreated mice, where we found a fourfold increase in the amount of leukocytes (CD45.2+ cells) relative to controls (Figure 6B). Remarkably, the presence of leukocytes at 2 hours was significantly lower in TVX-treated groups compared to Veh- or LVX-treated groups (Figure 6B). TVX disturbs leukocyte recruitment in the liver indicating that it causes

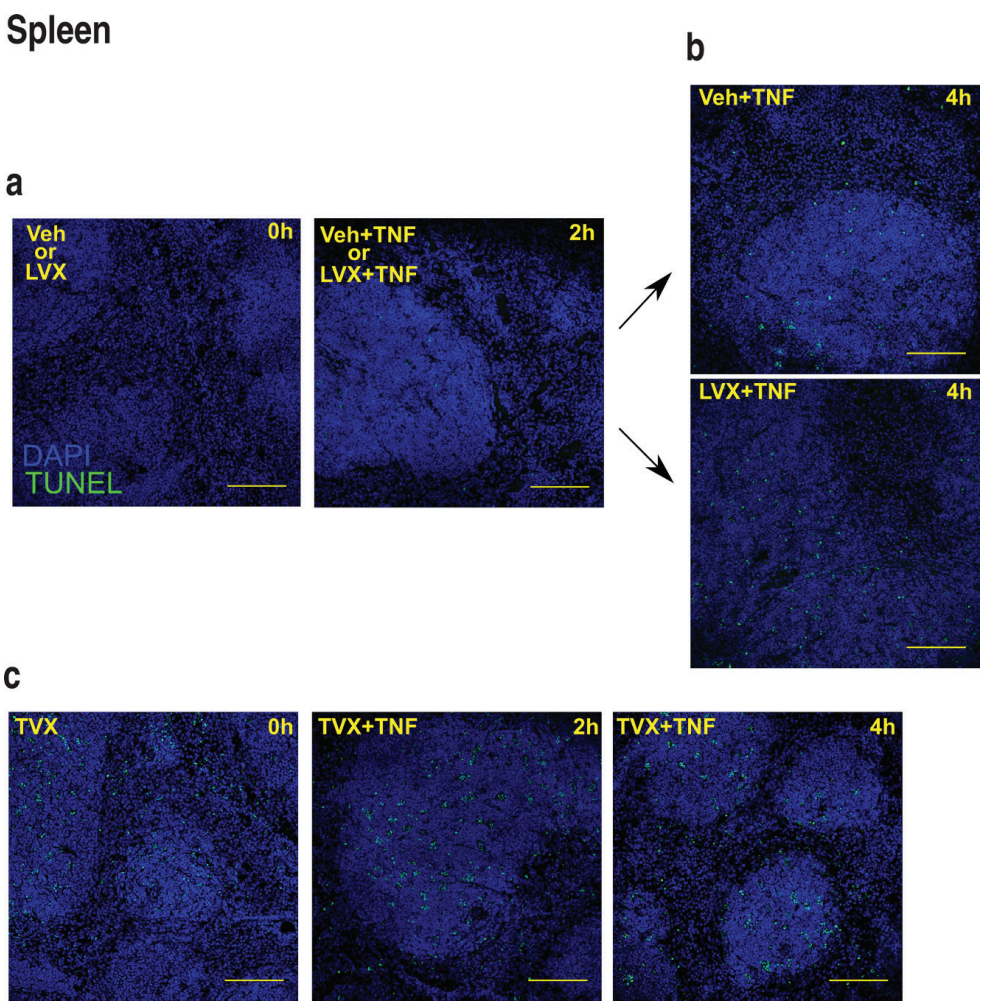


Figure 4

DNA fragmentation in spleen.

TUNEL staining on spleen (A–C) obtained from Veh-, LVX- or TVX-pretreated mice. Spleen sections in (A) and (B) are representative for both Veh- and LVX-treated mice at the time indicated in figure. Four hours after TNF injection, spleens treated with TNF were positive for TUNEL staining independently of the pretreatment (B,C). (C) Representative images selected from six TVX-treated mice per treatment group (scale bar = 100 µm). LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

a delay in the TNF-mediated hepatic sequestration of leukocytes at 2 hours. In addition, LVX and Veh induced an increase in the percentage of viable cells isolated from livers, whereas TVX reduced the viability of the isolated cells to 80% of control at 4 hours (Figure 6B). The amounts and viability of leukocytes in the spleen did not change significantly in control groups but were significantly reduced in TVX-treated mice, again indicating that TVX affects leukocytes also systemically (Figure 6C).

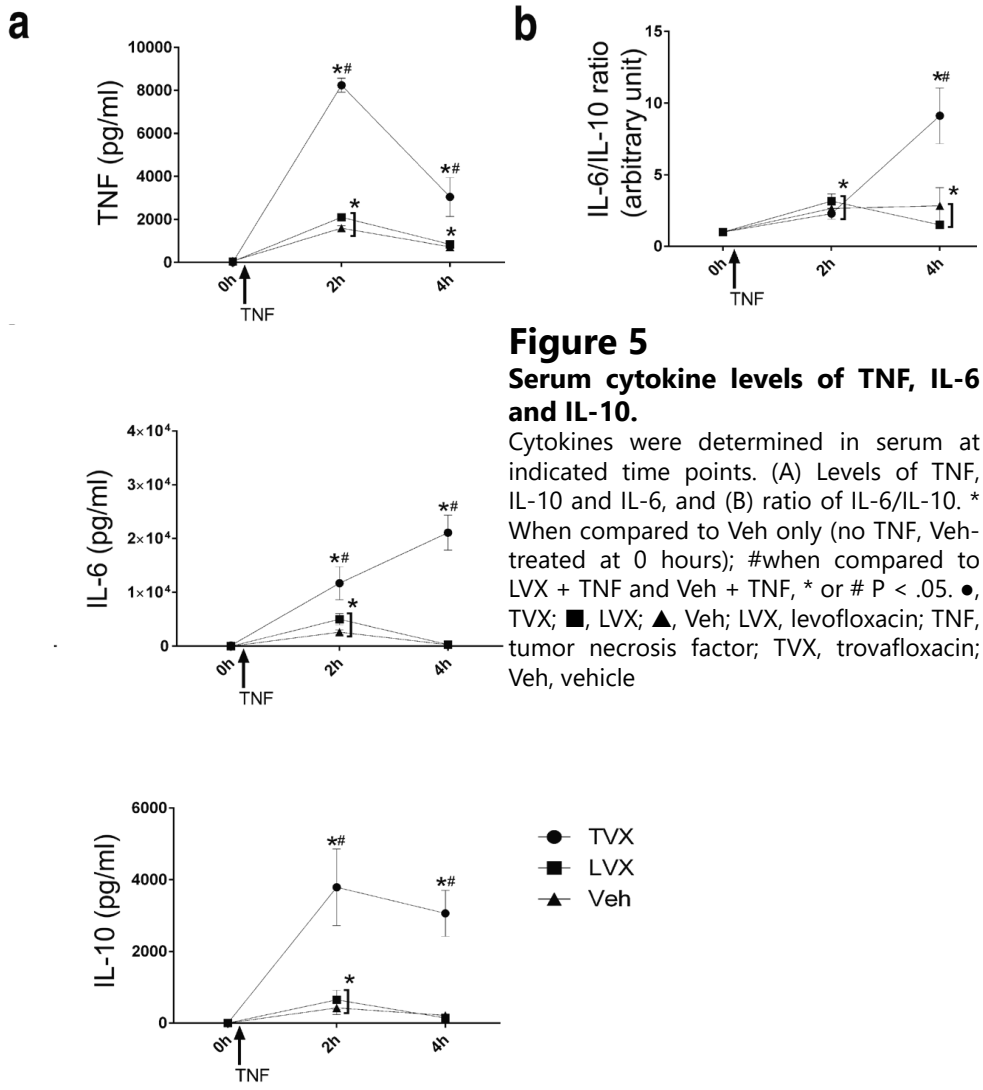


Figure 5

Serum cytokine levels of TNF, IL-6 and IL-10.

Cytokines were determined in serum at indicated time points. (A) Levels of TNF, IL-10 and IL-6, and (B) ratio of IL-6/IL-10. * When compared to Veh only (no TNF, Veh-treated at 0 hours); #when compared to LVX + TNF and Veh + TNF, * or # P < .05. ●, TVX; ■, LVX; ▲, Veh; LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

Trovafloxacin specifically delays liver influx of neutrophils and monocytes

Neutrophils and monocytes are among the first cellular players in the response to an inflammatory trigger (Ribeiro et al., 2015), but their recruitment to the site of inflammation also represents the first anti-inflammatory event (Soehnlein and Lindbom, 2010). For this reason, we focused on further evaluating the amount of these leukocytes in comparison with natural killer/T-cell fractions and other cells (e.g., dendritic cells, B cells). In Table 1, we show the data of the evaluated cell types in various exposure groups, whereas Figure 7 displays the distribution of

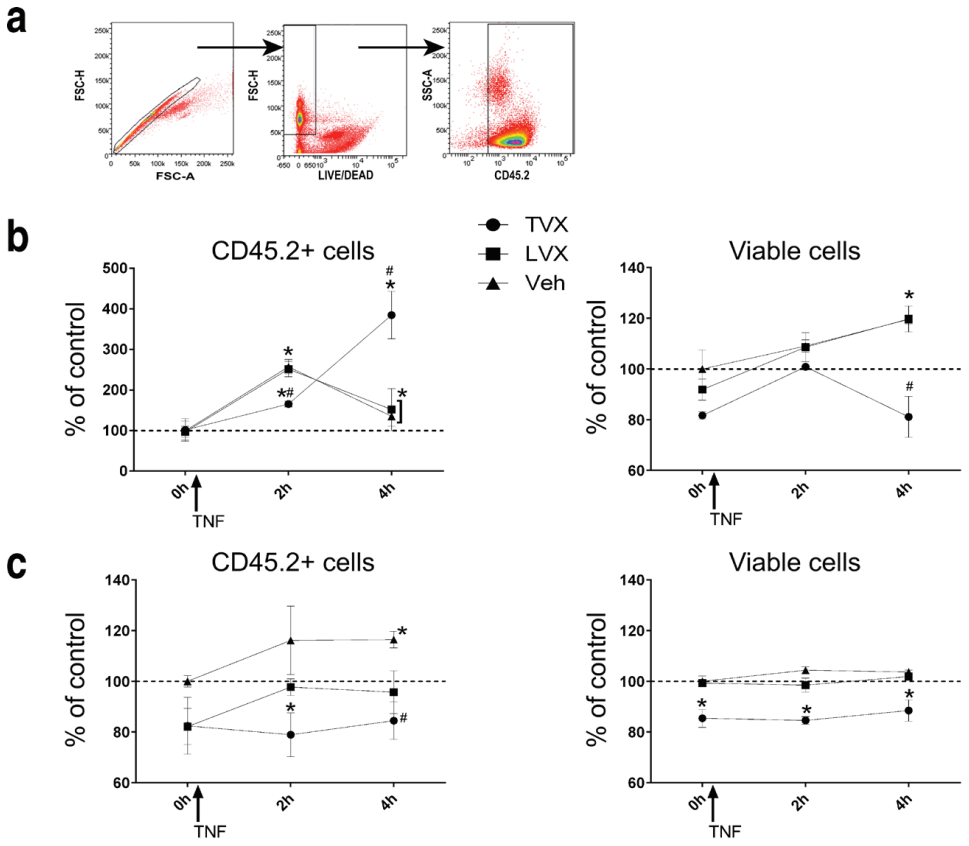


Figure 6

Leukocyte cellularity of liver and spleen.

Amounts and viability of leukocytes isolated from liver (Figure 6B) or spleen (Figure 6C). Leukocytes were characterized as displayed in Figure 5(A); *when compared to Veh only (noTNF, dashed line); #when compared to LVX + TNF and Veh + TNF, * or #P < .05). ●, TVX; ■, LVX; ▲, Veh; LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

the main leukocyte subtypes and other cells. As mentioned in Section 3.2, TNF administration caused a clear change in the percentages of the total leukocyte population present in the liver, and it appeared that these differences between TVX- and LVX- or Veh-treated groups were most remarkable in the neutrophil and monocyte fractions (Figure 7). Notably, all groups receiving TNF displayed a marked increase in the amount of neutrophils (i.e., Ly6G$^+$ cells; Figure 8 and Table 2) at 2 hours. This increase was highest in LVX-treated mice, followed consecutively by Veh- and TVX-treated mice. Remarkably, immunofluorescent staining with the use of anti- Ly6G antibody showed poor positivity on liver sections obtained from TVX + TNF-treated mice 2 hours after TNF injection (Figure 8D), whereas Ly6G$^+$

TABLE 1 Composition of leukocytes isolated from liver: at each time point hepatic lymphocytes were isolated by 45–67.5% isotonic Percoll density gradient and prepared for flow cytometry analysis

			% CD45.2$^+$ cells								
			0 hours			2 hours			4 hours		
Cell type		Phenotype	Veh	LVX	TVX	Veh	LVX	TVX	Veh	LVX	TVX
Lymphocytes	T lymphocytes	CD45.2$^+$/CD3$^+$/NK1.1$^-$	17.50 ± 1.57	17.00 ± 1.63	18.41 ± 0.85	11.78 ± 1.90	5.10 ± 0.79*	8.04 ± 0.24	24.03 ± 1.29	16.14 ± 1.48*	17.12 ± 1.53*
	NK	CD45.2$^+$/CD3$^-$/NK1.1$^+$	12.00 ± 0.48	12.87 ± 1.72	18.89 ± 1.49*	6.28 ± 0.44	3.69 ± 0.59	5.53 ± 0.38	19.78 ± 0.88***	7.23 ± 2.76	14.08 ± 1.93 ***
	NK(T)	CD45.2$^+$/CD3$^+$/NK1.1$^+$	9.28 ± 3.27	13.49 ± 0.53	8.24 ± 1.51	4.52 ± 0.60	2.26 ± 0.20	5.87 ± 0.54	5.74 ± 0.51***	18.67 ± 1.28	7.11 ± 1.78***
Neutrophils		CD45.2$^+$/Ly6G$^+$	4.93 ± 0.69	12.85 ± 0.72	7.76 ± 0.98	50.83 ± 0.92***	76.97 ± 1.56	63.68 ± 1.20***	13.66 ± 2.03	22.46 ± 6.14	33.47 ± 5.76*
Monocytes		CD45.2$^+$/F4-80$^{+\ low}$/CD11b$^{+\ int}$	2.90 ± 0.11	2.69 ± 0.05	1.66 ± 0.01*	2.67 ± 0.14	3.78 ± 0.28	0.48 ± 0.02**	2.38 ± 0.49	2.66 ± 0.49	0.48 ± 0.02**
Others		Non-specific CD45.2$^+$	53.39 ± 4.99	41.10 ± 4.12	45.04 ± 1.94	23.92 ± 0.26	8.20 ± 2.67	16.40 ± 1.45	34.41 ± 1.91	32.84 ± 2.03	27.74 ± 1.82

LVX, levofloxacin; NK, natural killer cells; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle.

Different cell phenotypes are presented in table as percentage of the total CD45.2$^+$ cells (mean ± SEM, *when compared to Veh with or without TNF depending on the time point assessed; **when compared to LVX + TNF and Veh + TNF, ***when compared to LVX + TNF, * or ** or ***$P < .05$).

TABLE 2 Composition of living cells isolated from liver: at each time point hepatic lymphocytes were isolated by 45–67.5% isotonic Percoll density gradient and prepared for flow cytometry analysis

			Number of cells								
			0 hours			2 hours			4 hours		
Cell type		Phenotype	Veh	LVX	TVX	Veh	LVX	TVX	Veh	LVX	TVX
Lymphocytes	T lymphocytes	CD45.2$^+$/CD3$^+$/NK1.1$^-$ (×10^6)	0.25 ± 0.03	0.17 ± 0.03	0.35 ± 0.10	0.60 ± 0.10	0.48 ± 0.10	0.42 ± 0.07	0.26 ± 0.05	0.25 ± 0.03	0.39 ± 0.06
	NK	CD45.2$^+$/CD3$^-$/NK1.1$^+$ (×10^6)	0.26 ± 0.07	0.23 ± 0.05	0.32 ± 0.08	1.05 ± 0.12	0.65 ± 0.13*	0.60 ± 0.05*	0.32 ± 0.04	0.25 ± 0.07	0.48 ± 0.05
	NK(T)	CD45.2$^+$/CD3$^+$/NK1.1$^+$ (×10^6)	0.19 ± 0.09	0.18 ± 0.07	0.14 ± 0.05	0.42 ± 0.07	0.29 ± 0.04	0.42 ± 0.02	0.07 ± 0.02	0.10 ± 0.02	0.19 ± 0.04
Neutrophils		CD45.2$^+$/Ly6G$^+$ (×10^6)	0.07 ± 0.02	0.04 ± 0.01	0.10 ± 0.02	7.39 ± 1.18	9.47 ± 1.11*	4.39 ± 0.48	0.15 ± 0.06	0.43 ± 0.26	3.14 ± 0.39
Monocytes (Gr-1$^+$)		CD45.2$^+$/F4-80$^{+\ low}$/CD11b$^{+\ int}$/Gr-1$^+$ (×10^5)	0.17 ± 0.03	0.11 ± 0.04	0.08 ± 0.04	2.03 ± 1.07	2.91 ± 1.50	0.32 ± 0.16	0.27 ± 0.23	0.33 ± 0.24	0.27 ± 0.19

LVX, levofloxacin; NK, natural killer cells; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle.

Different cell phenotypes were quantified and presented in number as indicated for each cell phenotype (mean ± SEM, *when compared to vehicle with or without TNF depending on the time point assessed; **when compared to LVX + TNF and Veh + TNF, * or **P < .05).

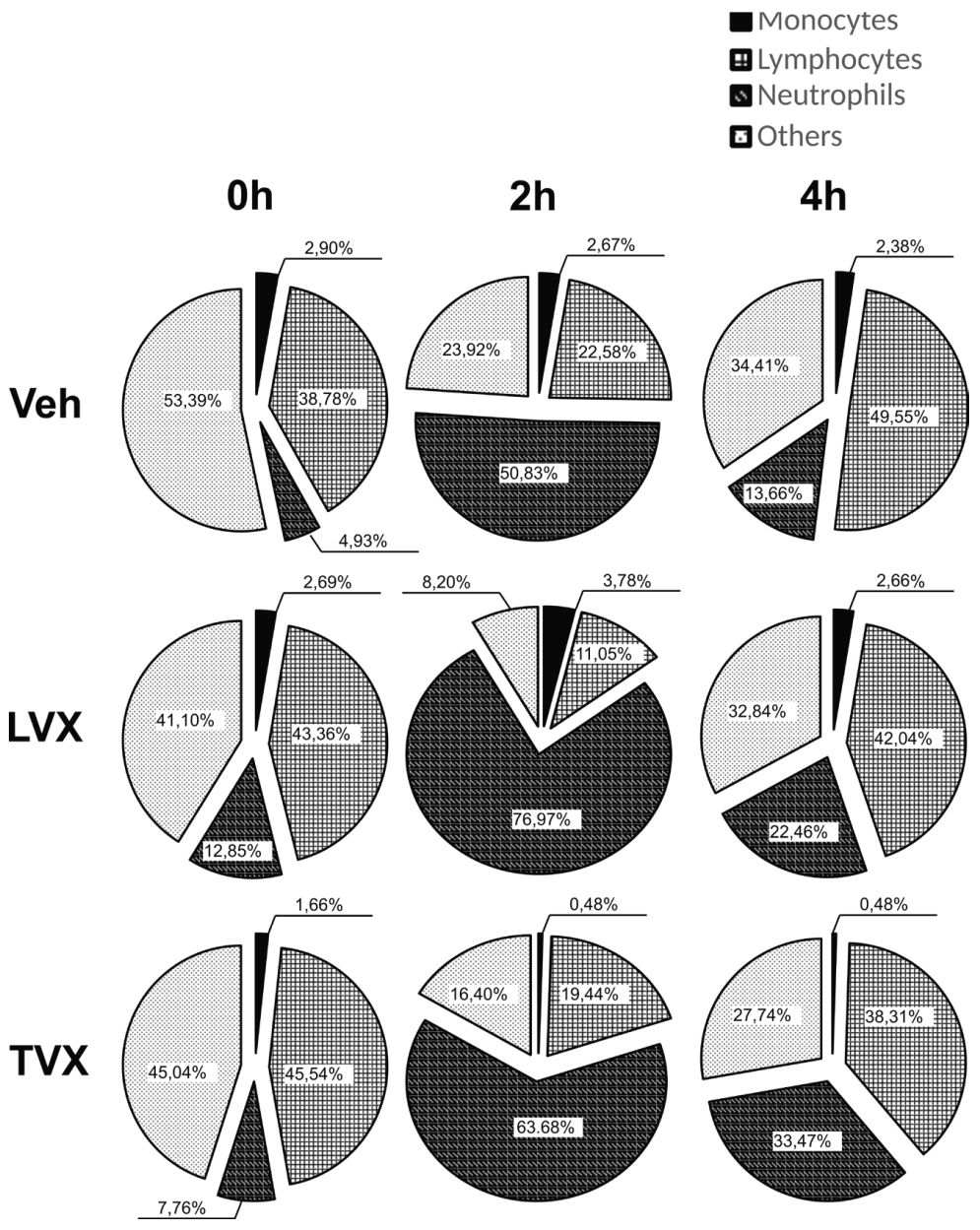
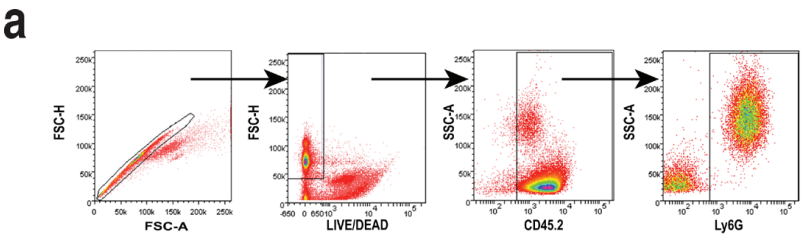
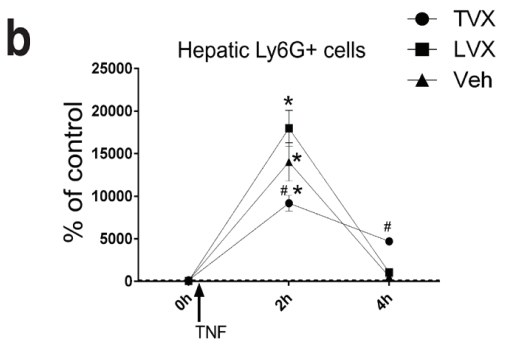


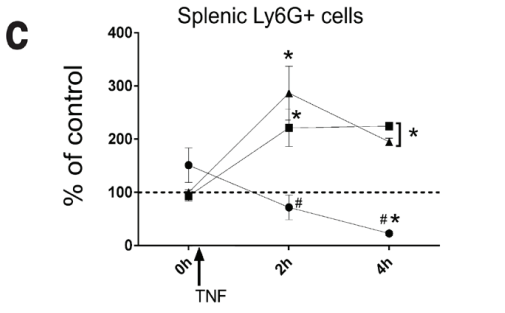
Figure 7

Distribution of various leukocyte subsets.

Distributions in percentage of total leukocytes isolated from livers of TVX-, LVX- or Veh-treated mice at indicated time points. (Others = non-specific CD45.2+ cells.) LVX, levofloxacin; TVX, trovafloxacin; Veh, vehicle







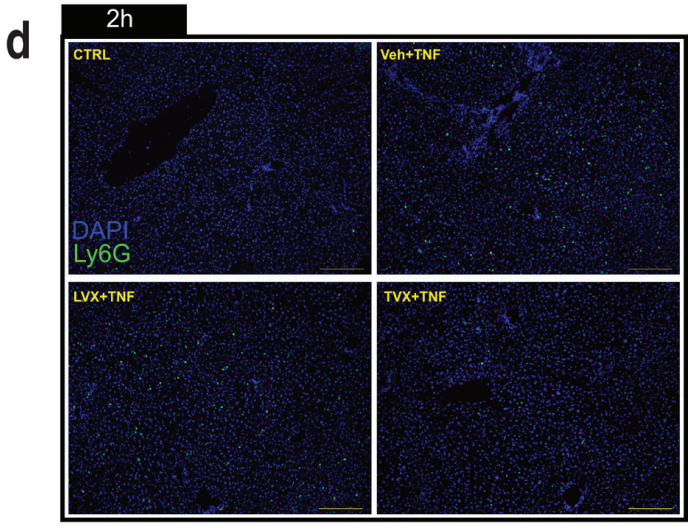


Figure 8

Neutrophil amounts in liver and spleen.

Livers and spleens obtained from mice at each time point indicated were processed to isolate leukocytes. Cells were counted and prepared for flow cytometry. Neutrophils were identified as depicted in Figure 8(A) as $CD45.2^{+}/Ly6G^{+}$ live cells. Amount of cells are presented as percentage of cells counted in samples derived from mice receiving only the Veh (no TNF, dashed line). Flow cytometry data were obtained from experiments performed with four animals per group. Liver sections (8 µm) were obtained from liver of mice (four animals per group) pretreated with TVX, LVX or only the Veh 2 hours after TNF injection. TVX + TNF 2 hours after the cytokine injection showed less $Ly6G^{+}$ cells when compared to the other treatments receivingTNF (Veh + TNF and LVX + TNF); * when compared to Veh only (noTNF, dashed line); #when compared to LVX + TNF and Veh + TNF; * or # $P < .05$) (scale bar = 100 µm). ●, TVX; ■, LVX; ▲, Veh; CTRL, control; LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

cells well populated the hepatic tissue derived from LVX + TNF- and Veh + TNF-treated mice. Four hours after TNF administration, the amount of neutrophils in the liver had returned towards background levels in LVX- and Veh- treated mice. In contrast, however, upon TVX administration, the neutrophil amount was not yet reduced to control levels at 4 hours and even significantly higher when compared to other treatments (Figure 8). The amount of hepatic inflammatory macrophages, identified as $Gr1^{+}/CD11b^{hi}/F4\text{-}80^{lo}$ leukocytes, was clearly increased in LVX- and Veh-treated mice 2 hours after TNF injection (Figure 9B, Table 2) and had returned to background level again at 4 hours. No differences were observed between these two groups of treatment, but again TVX appeared to abrogate the recruitment of these monocytes/macro- phages (Figures 7 and 9B, Table 1) resulting in decreased amounts at both 2 and 4 hours. Remarkably, compared to LVX or Veh, TVX also caused a significant reduction in the number of monocytes/macro- phages already before injection of TNF (Figure 9B, right).

Trovafloxacin also affects tumor necrosis factor-induced neutrophil and monocyte recruitment in the spleen.

The amount of neutrophils isolated from in the spleen was reduced in the TVX-treated mice both at 2 and 4 hours after TNF administration (Figure 8C). In contrast, amounts of splenic neutrophils in Veh- or LVX-treated groups were increased at 2 hours and, different from what we observed in the liver, also at 4 hours. Similarly to what we observed in the liver, TVX also affected amounts of monocytes/macrophages in the spleen. Indeed, the amount of splenic monocytes/ macrophages observed at 4 hours after TNF injection was completely suppressed by the administration of TVX.

DISCUSSION

To determine the role of the immune system in iDILI we focused on the evaluation of kinetics of leukocyte appearance in the liver in the well-established TVX model. Our kinetics data of leukocyte changes in the liver demonstrates that, at 2 hours, TNF induces the strongest neutrophil influx into livers of LVX- and Veh-treated mice whereas in comparison the neutrophil influx was only slightly increased in TVX- treated mice. At 4 hours, however, neutrophil amounts in Veh- of LVX-treated mice were reduced again, whereas those in TVX-exposed mice were still slightly increased, resulting in higher neutrophil amounts than in the other groups at the same time point. This led to us to conclude that TVX may cause a delay in the TNF-induced leukocyte recruitment into the liver.

We excluded the possibility that the decreased number of neutrophils at 2 hours in mice receiving TVX+TNF was due to decreased viability observed in the spleen. Indeed, the number of splenic neutrophils (which may represent the first

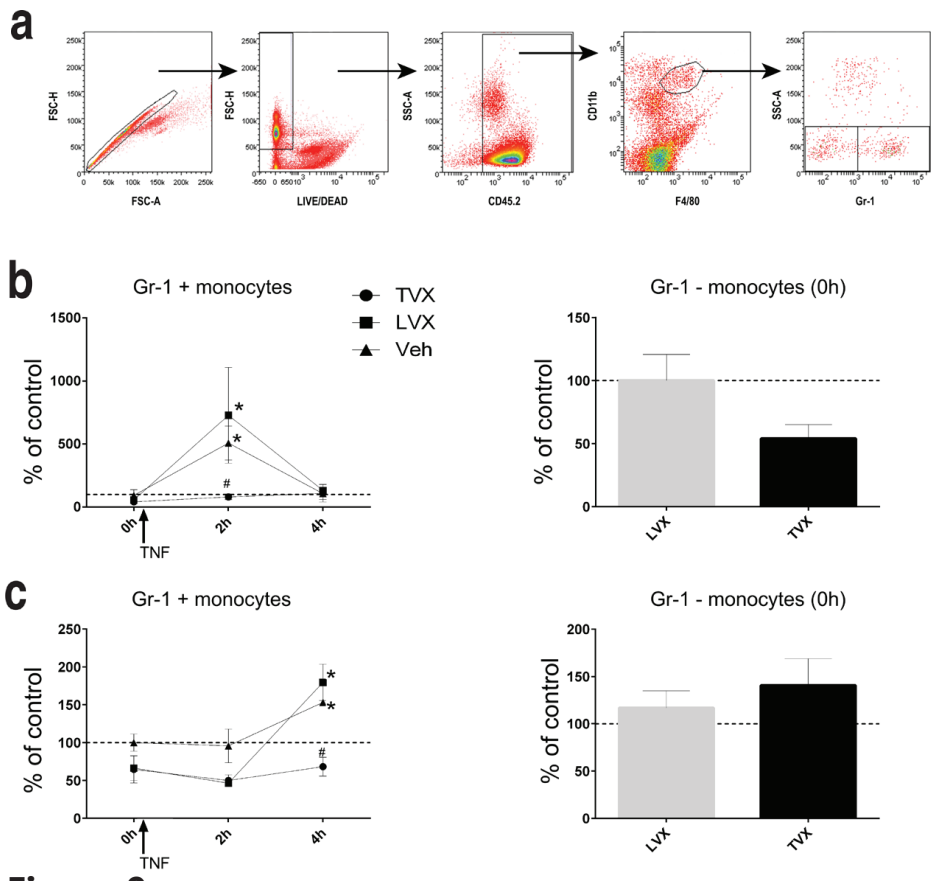


Figure 9

TVX reduced monocyte recruitment into the liver (B) and spleen (C).

Livers obtained from mice at each time point indicated were processed to isolate hepatic leukocytes. Cells were counted and prepared for flow cytometry. Monocytes were identified as depicted in Figure 9(A) (CD45.2+/F4-80+ low/CD11b+ and Gr1+ "classical," Gr1– "non-classical"). Amount of cells are presented as percentage of cells counted in samples derived from mice receiving only the Veh (no TNF). Flow cytometry data were obtained from experiments performed with four animals per group (A). (* when compared to Veh only (no TNF); #when compared to LVX+TNF and Veh+TNF; * or #P < .05). ●, TVX; ■, LVX; ▲, Veh; LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

neutrophils recruited in the inflamed liver due to contiguity of these organs) in TVX-treated animals was even slightly increased when compared with the other treatment groups. In addition, this delaying effect of TVX may also hinder influx of monocytes/macrophages, as these leukocytes were not at all increased in livers of mice treated with TVX, but clearly present in livers of Veh- and LVX-treated mice.

Usually, leukocytes are recruited to the site of inflammation aiming at resolving the damage and the inflammation (Ortega-Gómez et al., 2013; Soehnlein and

Lindbom, 2010). In particular, after the induction of tissue damage, residential hepatocytes and macrophages (Kupffer cells) start producing cytokines to recruit mainly neutrophils and monocytes/macrophages, which in turn release cytokines, reactive oxygen species, proteases but also control cytokine levels via different mechanisms. At the site of inflammation, neutrophils release either soluble TNF receptors, capable of capturing the free fraction of TNF (Lantz et al., 1994; Steinshamn and Bemelmans, 1995), and alpha defensins that inhibit mRNA translation in macrophages (Brook et al., 2016).

Importantly, phagocytosis of apoptotic neutrophils (also called efferocytosis; (Michlewska et al., 2009)) by monocyte/macrophages represents one of the initial anti-inflammatory events capable to regulate inflammation. In particular, it has been demonstrated that impairment of neutrophil sequestration into the liver and lack in their efferocytosis increases the early proinflammatory response (Holub et al., 2009). In addition, depletion of neutrophils in mice with anti-Ly6G antibody led to increased serum concentrations of TNF in response to lipopolysaccharide (LPS), confirming the role for neutrophils in the regulation of inflammation among others by capturing TNF (Daley et al., 2008).

An impairment in the interplay of professional phagocytes may expose an endangered tissue to a higher risk of leakage of intracellular contents due to secondary necrosis (Brauner et al., 2013). Secondary necrosis is a particularly dangerous phenomenon in short living cells such as neutrophils, as harmful mediators released from these cells can establish a vicious circuit of cell death, inflammation and tissue damage (Brauner et al., 2013). Interestingly, depletion of monocytes/macrophages in a model of endotoxemia was associated with a 20-fold increase in secreted TNF by leukocytes (Daley et al., 2008), which is in support of the regulatory role of monocytes/macro- phages on neutrophil activity. Our data may be explained in view of this anti-inflammatory cascade of events. In both control groups, Veh- and LVX-treated groups, no damage (e.g., low ALT, absence of necrosis or apoptosis) was observed and levels of TNF remained very low, whereas a clear, but temporary, influx of neutrophils and of monocyte/ macrophages occurred. Therefore, the mere influx of neutrophils into the liver is not the cause of liver injury but may rather contribute to the prevention of damage. On the other hand, sustained presence of neutrophils combined with hampered influx of monocytes/macrophages may be responsible, at least to a great extent, for the liver injury in case of TVX. We therefore propose that the delay in neutrophil recruitment combined with complete absence of monocyte/ macrophage recruitment represents a key phenomenon in the induction of TVX-mediated liver damage.

The lack in monocyte/macrophage recruitment combined with the neutrophil sequestration in the liver may represent an obstacle to the resolution of inflammation, because TNF-induced apoptotic cells are not effectively cleared (Salamone et al., 2001; Soehnlein and Lindbom, 2010; Tsuchida et al., 1995).

Although the mechanisms behind the TVX-mediated reduction of sequestered neutrophils and monocytes/macrophages into the liver needs further elucidation, current findings could explain the lack in clearance of TNF upon TVX exposure as described in a previous study in this mouse model (Patrick J Shaw, Beggs, et al., 2009). In addition, our findings may be linked to the known pro-coagulatory effects of TVX as coagulation may hamper the influx of leukocytes from the liver blood vessels into the liver tissue. Indeed, previous studies on the model of TVX + LPS-induced liver damage showed that the combination of the fluoroquinolone with the bacterial component increases sinusoidal fibrin deposition in mice. Moreover, mice lacking plasminogen activator-inhibitor 1 and mice pretreated with heparin were less sensitive to TVX + LPS-mediated toxicity, showing a significant decrease in serum ALT and histopathological modification of the liver (Patrick J. Shaw et al., 2009). By contrast, inhibition of thrombin was not associated with a decreased number of hepatic neutrophils in TVX + LPS-treated mice, excluding the possibility that the observed neutrophil accumulation in the liver must rely exclusively on fibrin deposition (Patrick J. Shaw et al., 2009). The increased IL-6/IL-10 ratio, reflecting the inflammatory condition in TVX-induced liver damage, also supports the hypothesis that a disruption in the neutrophil/monocyte partnership occurs in this model. Previously, Shaw et al. 2009 (Patrick J Shaw, Ganey, et al., 2009a, 2009b) have already demonstrated increases of serum levels of the proinflammatory IL-6 and anti-inflammatory IL-10 cytokines in TVX-treated mice, but here we show that kinetics of these cytokines in the serum differs, resulting in the increased IL-6/IL-10 ratio. Several studies (Filardy et al., 2010; Michlewska et al., 2009; Ribeiro-Gomes et al., 2004) show that monocytes/macrophages start producing IL-10 in response to neutrophil efferocytosis. As hepatocytes and possibly Kupffer cells will continue producing IL-6 under TVX(+TNF)-induced conditions, the alteration in the IL-6/IL-10 ratio may represent an additional effect resulting from the disrupted phagocyte partnership.

The TVX-induced suppression in neutrophil and monocyte/macrophage recruitment in spleen at 2 and 4 hours underlines that the effect of TVX is not limited to the liver. It is tentative to speculate that splenic neutrophils migrate to the liver when the damage caused by the TVX + TNF combination takes place. Regardless of this, the effects on the spleen highlight that, despite the peculiar effects of TVX on the liver, leukocyte populations in other organs are also affected. What this means in relation to immuno-toxicological or clinical effects of TVX in other organs such as the skin, is not known.

We also demonstrate that TVX has clear effects before TNF injection, i.e., low level of DNA fragmentation in liver and reduced amounts of macrophages in the spleen. It is known that TVX by itself induces compound-specific effects in hepatocytes (such as DNA damage caused by topoisomerase II inhibition) (Beggs et al., 2015, 2014; Liguori et al., 2008), although the cytotoxic (i.e., apoptosis) effects of TVX in vitro also depend on the presence of TNF (Beggs et al., 2014; Cosgrove et al., 2009). However, we cannot exclude that low levels of TNF, e.g., induced by subtle leakage

of LPS from the gut, are involved in these effects. Nevertheless, although positivity for the TUNEL reaction in this model does not discriminate between apoptosis (potentially induced by TVX and TNF combination) and DNA damage, here we demonstrated to the best of our knowledge for the first time that TVX alone in vivo induced early toxic effects on the liver. Together, our findings indicate that TVX delays the acute influx of neutrophils and monocytes/macrophages. Considering their known anti-inflammatory functions, the disruption of influx of these innate immune cells may hamper the resolution of initial cytotoxic effects of TVX and thus contribute to the development of liver injury.

As already stated before by Shaw and colleagues (Patrick J. Shaw et al., 2009), the combinations of effects by TVX may be the basis of the idiosyncrasy of this particular form of iDILI, and the hampered influx of anti-inflammatory leukocytes may add to the complexity of this disease. In addition to the contribution of neutrophil elastase in the onset of TVX + LPS-induced liver injury as previously demonstrated by Shaw et al. (2009a), our findings delineate a second role for neutrophils in the model of TVX + TNF. In this respect, we believe that early recruitment of neutrophils and monocytes in the inflamed organ aims to resolve inflammation, whereas their prolonged permanence may trigger protease release with consequent tissue damage. For this reason, further investigations to identify mechanisms explaining the observed disruption of leukocyte sequestration may ultimately add to predict idiosyncratic DILI.

References

Beggs, K.M. et al. (2014) *Molecular Mechanisms of Hepatocellular Apoptosis Induced by Trovafloxacin-Tumor Necrosis Factor-alpha Interaction. Toxicol. Sci., 137, 91–101.*

Beggs, K.M. et al. (2015) *Trovafloxacin-induced replication stress sensitizes HepG2 cells to tumor necrosis factor-alpha-induced cytotoxicity mediated by extracellular signal-regulated kinase and ataxia telangiectasia and Rad3-related. Toxicology, 331, 35–46.*

Brauner, J.M. et al. (2013) *No littering : the clearance of dead cells and leaking cellular contents and possible pathological complications. OA Arthritis, 1, 1–7.*

de Brito, R. de C.C.M. et al. (2016) *The balance between the serum levels of IL-6 and IL-10 cytokines discriminates mild and severe acute pneumonia. BMC Pulm. Med., 16, 170.*

Brook, M. et al. (2016) Neutrophil-derived alpha defensins control inflammation by inhibiting macrophage mRNA translation. Proc. Natl. Acad. Sci., 113, 4350–4355.

Buchweitz, J.P. (2002) *Underlying Endotoxemia Augments Toxic Responses to Chlorpromazine: Is There a Relationship to Drug Idiosyncrasy? J. Pharmacol. Exp. Ther., 300, 460–467.*

Chalasani, N. et al. (2015) *Features and outcomes of 899 patients with drug-induced liver injury: The DILIN prospective study. Gastroenterology, 148, 1340-1352.e7.*

Cosgrove, B.D. et al. (2009) *Synergistic drug-cytokine induction of hepatocellular death as an in vitro approach for the study of inflammation-associated idiosyncratic drug hepatotoxicity. Toxicol. Appl. Pharmacol., 237, 317–330.*

Crispe, I.N. (2001) *Isolation of mouse intrahepatic lymphocytes. Curr. Protoc. Immunol., Chapter 3, Unit 3.21.*

Daley, J.M. et al. (2008) *Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. J. Leukoc. Biol., 83, 64–70.*

Deng, X. et al. (2006) *Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. J. Pharmacol. Exp. Ther., 319, 1191–1199.*

Dugan, C. and MacDonald, A. (2010) *A mouse model of severe halothane hepatitis based on human risk factors. J. Pharmacol. Exp. Ther., 333, 364–372.*

Filardy, A. a et al. (2010) *Proinflammatory clearance of apoptotic neutrophils induces an IL-12(low) IL-10(high) regulatory phenotype in macrophages. J. Immunol., 185, 2044–2050.*

Fontana, R.J. et al. (2009) *Drug-induced liver injury network (DILIN) prospective study: Rationale, design and conduct. Drug Saf., 32, 55–68.*

Gabrilovich, D.I. (2017) *Myeloid-Derived Suppressor Cells. Cancer Immunol. Res., 5, 3–8.*

Holub, M. et al. (2009) *Neutrophils sequestered in the liver suppress the proinflammatory response of Kupffer cells to systemic bacterial infection. J. Immunol., 183, 3309–16.*

Lantz, M. et al. (1994) *Adherence of neutrophils induces release of soluble tumor necrosis factor receptor forms. J. Immunol., 152, 1362–1369.*

Lee, W.M. and Senior, J.R. (2005) *Recognizing drug-induced liver injury: current problems, possible solutions. Toxicol. Pathol., 33, 155–164.*

Libert, C. et al. (1990) *Induction of interleukin 6 by human and murine recombinant interleukin 1 in mice. Eur. J. Immunol., 20, 691–4.*

Liguori, M.J. et al. (2008) *Trovafloxacin-Induced Gene Expression Changes in Liver-Derived in Vitro Systems : Comparison of Primary Human Hepatocytes to HepG2 Cells □.*

Lu, J. et al. (2012) *Amiodarone Exposure During Modest Inflammation Induces Idiosyncrasy-like Liver Injury in Rats: Role of Tumor Necrosis Factor-alpha. Toxicol. Sci., 125, 126–133.*

Lubasch, A. et al. (2000) *Comparative pharmacokinetics of ciprofloxacin, gatifloxacin, grepafloxacin, levofloxacin, trovafloxacin, and moxifloxacin after single oral administration in healthy volunteers. Antimicrob. Agents Chemother., 44, 2600–2603.*

Luyendyk, J.P. (2003) *Ranitidine Treatment during a Modest Inflammatory Response Precipitates Idiosyncrasy-Like Liver Injury in Rats. J. Pharmacol. Exp. Ther., 307, 9–16.*

Michlewska, S. et al. (2009) *Macrophage phagocytosis of apoptotic neutrophils is critically regulated by the opposing actions of pro-inflammatory and anti-inflammatory agents: key role for TNF-alpha. FASEB J., 23, 844–54.*

Nagaki, M. et al. (2000) *High levels of serum interleukin-10 and tumor necrosis factor-alpha are associated with fatality in fulminant hepatitis. J. Infect. Dis., 182, 1103–8.*

Ortega-Gómez, A. et al. (2013) *Resolution of inflammation: An integrated view. EMBO Mol. Med., 5, 661–674.*

Pillay, J. et al. (2013) *Immune suppression by neutrophils and granulocytic myeloid-derived suppressor cells: Similarities and differences. Cell. Mol. Life Sci., 70, 3813–3827.*

Poll, T. Van Der et al. (1997) *The role of interleukin-10 in the pathogenesis of bacterial infection. 605–607.*

Ribeiro-Gomes, F.L. et al. (2004) *Macrophage interactions with neutrophils regulate Leishmania major infection. J. Immunol., 172, 4454–4462.*

Ribeiro, D. et al. (2015) *Flavonoids Inhibit COX-1 and COX-2 Enzymes and Cytokine/Chemokine Pro-*

duction in Human Whole Blood. Inflammation, 38, 858–870.

Robinson, M.W. et al. (2016) *Liver immunology and its role in inflammation and homeostasis. Cell. Mol. Immunol., 13, 267–76.*

Salamone, G. et al. (2001) *Promotion of Neutrophil Apoptosis by TNF- . J. Immunol., 166, 3476–3483.*

Shaw, Patrick J. et al. (2009) *The role of the hemostatic system in murine liver injury induced by coexposure to lipopolysaccharide and trovafloxacin, a drug with idiosyncratic liability. Toxicol. Appl. Pharmacol., 236, 293–300.*

Shaw, Patrick J, Ganey, P.E., et al. (2009a) *Trovafloxacin Enhances the Inflammatory Response to a Gram-Negative or a Gram-Positive Bacterial Stimulus , Resulting in Neutrophil-Dependent Liver Injury in Mice. J Pharmacol Exp Ther, 330, 72–78.*

Shaw, Patrick J, Beggs, K.M., et al. (2009) *Trovafloxacin enhances TNF-induced inflammatory stress and cell death signaling and reduces TNF clearance in a murine model of idiosyncratic hepatotoxicity. Toxicol. Sci., 111, 288–301.*

Shaw, Patrick J, Ganey, P.E., et al. (2009b) *Tumor Necrosis Factor α is a Proximal Mediator of Synergistic Hepatotoxicity from Trovafloxacin / Lipopolysaccharide Coexposure. J Pharmacol Exp Ther, 328, 62–68.*

Soehnlein, O. and Lindbom, L. (2010) *Phagocyte partnership during the onset and resolution of inflammation. Nat. Rev. Immunol., 10, 427–439.*

Steinshamn, S. and Bemelmans, M. (1995) Granulocytopenia reduces release of soluble TNF receptor p75 in endotoxin-stimulated mice: a possible mechanism of enhanced TNF activity. Cytokine, 50–6.

Stensballe, J. et al. (2009) *The early IL-6 and IL-10 response in trauma is correlated with injury severity and mortality. Acta Anaesthesiol. Scand., 53, 515–521.*

Taniguchi, T. et al. (1999) *Change in the ratio of interleukin-6 to interleukin-10 predicts a poor outcome in patients with systemic inflammatory response syndrome. Crit. Care Med., 27, 1262–4.*

Tsuchida, H. et al. (1995) *In vivo regulation of rat neutrophil apoptosis occuring spontaneously or induced with TNF-a or cycloheximide. J.Immunol., 154, 2403–2412.*

Vollmar, B. and Menger, M.D. (2009) *The Hepatic Microcirculation : Mechanistic Contributions and Therapeutic Targets in Liver Injury and Repair. 1269–1339.*

Watkins, P.B. and Seeff, L.B. (2006) *Drug-induced liver injury: Summary of a single topic clinical research conference. Hepatology, 43, 618–631.*

Zou, W. et al. (2009) *Hepatotoxic interaction of sulindac with lipopolysaccharide: Role of the hemostatic system. Toxicol. Sci., 108, 184–193.*

Chapter 3

Trovafloxacin-induced liver injury: lack in regulation of inflammation by inhibition of nucleotide release and neutrophil movement

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ABSTRACT

The fluoroquinolone trovafloxacin (TVX) is associated with a high risk of drug-induced liver injury (DILI). Although part of the liver damage by TVX+TNF relies on neutrophils, we have recently demonstrated that liver recruitment of monocytes and neutrophils is delayed by TVX. Here we show that the delayed leukocyte recruitment is caused by a combination of effects which are linked to the capacity of TVX to block the hemichannel pannexin 1. TVX inhibited find-me signal release in apoptotic HepG2 hepatocytes, decelerated freshly isolated human neutrophils toward IL-8 and f-MLF, and decreased the liver expression of ICAM-1. In blood of TVX+TNF-treated mice, we observed an accumulation of activated neutrophils despite an increased MIP-2 release by the liver. Depletion of monocytes and neutrophils caused increased serum concentrations of TNF, IL-6, and MIP-2 in TVX-treated mice as well as in mice treated with the fluoroquinolone levofloxacin, known to have a lower DILI-inducing profile. This supports the idea that early leukocyte recruitment regulates inflammation. In conclusion, disrupted regulation by leukocytes appears to constitute a fundamental step in the onset of TVX-induced liver injury, acting in concert with the capability of TVX to induce hepatocyte cell death. Interference of leukocyte-mediated regulation of inflammation represents a novel mechanism to explain the onset of DILI.

INTRODUCTION

Immune-mediated drug-induced liver injury (i-DILI) is a drug- specific adverse response determined by individual factors, both inherent and environmental. i-DILI is an important socio- economic problem, particularly because it is difficult to predict it preclinically and it becomes apparent in human clinical trials or in clinical practice.

Anti-microbials are an important group of pharmaceuticals linked to i-DILI as they represent almost half of the total number of observed cases (Chalasani et al., 2015). Combined with the urgent need for new safe therapies to counteract resistant microbes, mechanistic knowledge on how anti-microbials cause i-DILI is highly warranted.

The fluoroquinolone antibiotic trovafloxacin (TVX) was withdrawn from the market in 2001 due to a high incidence of DILI, only 3 years after its market introduction. Animal studies investigating the mechanism of toxicity mediated by TVX (Shaw et al., 2007, 2009c) revealed the importance of co-exposure to microbial substances, such as lipopolysaccharide (LPS), as key factor in the development of TVX-induced liver injury. Subsequent studies identified the pro-inflammatory cytokine TNF as a proximal common mediator in the pathogenesis of TVX-induced i-DILI (Shaw et al., 2009d). Also other pharmaceuticals associated with DILI in patients have been demonstrated to cause liver damage in rodents when combined with LPS or TNF (Deng et al., 2006; Gandhi et al., 2013; Lu et al., 2013; Luyendyk et al., 2003).

Studies showing that TVX-induced liver damage is reduced in mice deficient in neutrophil elastase (Shaw et al., 2009c) indicate that activation of neutrophils is crucial in TVX-induced DILI. However, recently we have shown that, although leukocytes are abundantly present when liver damage is apparent, TVX appears to cause a delay of TNF-elicited liver sequestration of neutrophils and monocytes (Giustarini et al., 2018). This finding is of particular significance in view of the recently acknowledged role of these cells in the resolution of inflammation (Soehnlein and Lindbom, 2010).

Importantly, it has also been demonstrated that TVX, at concentrations relevant to those found in the serum of human treated with TVX (≈ 5 mM) (Vincent et al., 1998), blocks the hemi-channel pannexin 1 (PNX1) (Poon et al., 2014). Further studies evaluating the role of immune cells in infection and inflammation identified PNX1 as a key player in the regulation of patho-physiological processes especially involving phagocytes (Lohman et al., 2015). When activated, PNX1 releases small molecules (such as ATP and UTP) capable of stimulating innate cell functions in autocrine and paracrine fashions. These small molecules are considered "find-me" signals attracting immune cells toward dying cells, but are also found to be crucial for the actual migration of leukocytes. In addition, PNX1 blockade has been

shown to reduce the expression of vascular cell adhesion molecule 1, the vascular adhesion molecule for leukocytes (Lohman et al., 2015).

Therefore, we hypothesized that PNX1-dependent exacerbation of inflammatory responses represents a key event in TVX- mediated i-DILI. We investigated whether TVX influences key processes that can be linked to PNX1 interference, eg, release of find-me signals, neutrophil migration, and ICAM-1 expression. Our data shows that TVX, but not its non-iDILI-associated structural homologue levofloxacin (LVX), blocks the PNX1- hemichannel on dying hepatocytes. In addition, data shows that TVX decelerates human neutrophil movements and decreases ICAM-1 expression in vivo. The TVX-induced PNX1 blockade may thus result in a combination of cellular events that provide a novel explanation for the idiosyncrasy of TVX- induced DILI. The idiosyncratic nature of DILI may reside in a kinetics of innate immune responses that may vary per individual. In certain individuals the combination of TVX administration with fluctuant and circadian cytokine concentrations during antimicrobial activity of innate immune responses may increase pro-inflammatory responses due to the suppression of an appropriate regulatory cellular response.

MATERIALS AND METHODS

Animals

Male, 9 to 11-week old, C57BL/6J mice (The Jackson Laboratory, Charles River) were used for all experiments. They were allowed to acclimatize for 1week in a 12-h light/dark cycle and maintained at mean temperature of 23 ± 2 °C, 50–55% relative humidity. Acidified drinking water and laboratory food pellets were provided ad libitum.

In vivo studies were approved by the Ethics Committee for Animal Experiments of Utrecht University and complied with governmental and international guidelines on animal experi- mentation (CCD permission number: AVD108002016503).

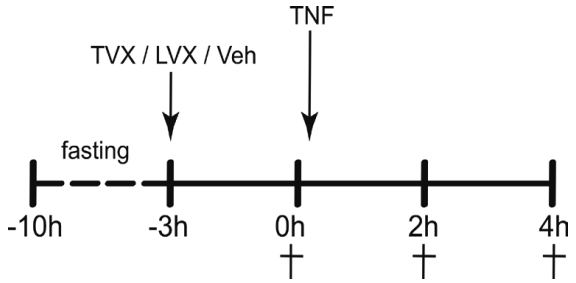

Figure 1

Protocol for the treatment of mice in the TVX+TNF model of DILI.

Mice were fasted for 7 h and then administered with an intragastric gavage of the drug solution or vehicle. Three hours later, mice were either sacrificed to observe the effects of the drugs or intraperitoneal injection of TNF was performed. TNF-injected mice were subsequently sacrificed at 2 and 4 h after the injection with the cytokine.

Human blood cells

Ethical approval for obtaining healthy human volunteer blood was obtained by the institutional ethical review board of the University Medical Centre Utrecht (UMCU) and all subjects provided written informed consent.

Compounds and chemicals

Trovafloxacin (TVX), levofloxacin (LVX), and N-formyl-methionyl-leucyl-phenylalanine (f-MLF) were purchased from Sigma-Aldrich (St. Louis, Missouri). Recombinant murine TNF was purchased from R&D Systems (Minneapolis, Minnesota). IL- 8 (72 aa) was obtained from PeproTech (Rocky Hill, New Jersey).

In vivo experimental set up and depletion of Ly6C1 cells

Mice were fasted 7h prior to treatment. TVX (150 mg/kg), LVX (375 mg/kg), or saline vehicle (Veh) was administered orally 3 h before recombinant murine TNF injection (50 mg/kg, i.p.) (Shaw et al., 2009b). Food was available again immediately after TNF administration. Animals analyzed at time point 0h did not receive TNF (Figure 1). Concentration of TVX was determined by a previous study showing that 150 mg/kg TVX interacts with LPS- induced TNF to increase serum ALT and induce histopathological liver modifications which were not observed with the administration of the drug only (Shaw et al. 2007).

To deplete neutrophils and monocytes/macrophages, mice were treated with anti-Ly6C (200 mg/mouse, i.p., 250 ml) or with the corresponding isotype (IgG2b, 200 mg/mouse, i.p., 250 ml) 72 and 24h before receiving the drug solutions.

Mouse blood, spleen, and intra-hepatic leukocyte isolation

Blood from inferior vena cava was collected in pre-coated lithium heparin tubes and centrifuged for 6 min at 2000 rpm. Intra-hepatic leukocytes were isolated as described previously by Crispe et al. (Crispe, 2001). Briefly, liver was perfused with 5 ml ice-cold PBS and excised from the animal. Tissue was minced and gently passed through a sieve. The liver slurries were centrifuged, and the pellet resuspended with a collagenase D (0.02%, w/v) and DNAse I (0.002%, w/v) solution. The suspensions were incubated at 37°C for 40 min on a reciprocating shaker. Liver leukocytes were isolated by using a 45– 67.5% isotonic Percoll density gradient (GE Healthcare, Fisher Scientific, Landsmeer, The Netherlands). Hepatic leukocytes obtained from Veh-only-treated mice (w/o TNF) were used as an internal control. With this we ruled out small day-by-day differences in the isolation efficiency throughout the experiments. Leukocytes from mouse spleen were obtained forcing the organ to pass through a 70-mm strainer, after which single cells were washed once. To remove erythrocytes, cells were incubated with hypotonic red blood cell lysis buffer (containing NH_4Cl, KHCO3, and Na_2EDTA) for 1 min, and washed once with PBS.

Human neutrophil isolation and 3D chemotaxis migration assay

Neutrophils were isolated as described previously from fresh whole blood by Overbeek et al. (2013). Neutrophil preparations consisted of 95–97% neutrophils as determined by microscopic analysis of cytospin preparations. Neutrophils were resuspended in incubation buffer (20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 1.0 mM $CaCl_2$, 5mM glucose, 0.5% [w/v] HSA) and mixed with fibrinogen (2 mg/ml) and thrombin (2 Units/ml). The final concentration of the cell suspension was 3 x 10^6 cells/ml. For chemotaxis analysis IBIDI 3D chemotaxis slides were used (I-Slide Chemotaxis 3D, ibidiTreat; Integrated BioDiagnostics [IBIDI], Munich, Germany). Cells in fibrin gel mixtures were pipetted in the central channels of the IBIDI 3D slide. The front chambers were filled with f-MLF (10-7 M) or IL-8 (50 ng/ml), whereas the other chamber with negative control incubation buffer. Concentrations of TVX or LVX were tested in combination with both chemo-attractants. The slides were then transferred to an incubator hood (37°C) on a Leica DXMRE microscope and temperature equilibrated for at least 10min, before multi-spot time- lapse imaging at 37°C was performed. Multi-spot time-lapse imaging was performed with a computer-assisted microscopy system (Quantimet for Windows, Qwin), DXMRE microscope, PL fluorostar low power objective lens to a magnification of 5x (Leica, Heidelberg, Germany). Sequences consisted of 100 images per spot with a maximum of 6 revisited spots. The time-lapse intervals were typically 15–25 s. Images were imported into the Optimas image analysis package (Media Cybernetics, Inc. Bethesda, Rockville, Maryland). Custom-made macros (Arithmetic Language for Images, ALI) were used to plot the migrating cells using threshold-based detection and nearest neighbor tracking. Tracks and final vectors of migrating cells were plotted and analyzed for speed, directness, and directionality. Each analysis incorporated several tens to hundreds of tracked cells. Values were normalized for the average migration distance and speed of neutrophils that were not stimulated with chemo-attractants.

Cell culture of HepG2 cells

HepG2 human hepatoblastoma cells (American Type Culture Collection, Manassas, Virginia) were used. Cells were maintained in MEM+Glutamax (Gibco, Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum in 75 cm2 tissue culture-treated flasks. Cells were cultured in a humidified atmosphere composed of 95% air and 5% CO2 and a temperature of 37°C. Cells were passaged twice each week. About 0.25% Trypsin-EDTA was used to detach confluent HepG2 cells from the flask. After plating, cells were allowed 48h to adhere before treatment. TVX and LVX was reconstituted to a stock solution of 200 mM in dimethyl sulfoxide (DMSO) and diluted in culture medium to the desired concentrations. The final DMSO concentrations did not exceed 0.1%. TNF was reconstituted to a stock solution of 100 mg/ml in PBS+BSA 0.1% as indicated by the manufacturer. After incubation with the fluoroquinolones with or without TNF, HepG2 cells were treated with

Trypsin for 5 min before to add complete medium, and after prepared for flow cytometric analysis.

Flow cytometric analyses of liver and blood leukocytes and HepG2 cells

For flow cytometric analysis, cells were first stained with LIVE/ DEAD® Fixable Dead Cell Stain (Molecular Probes, Invitrogen, Carlsbad, California) followed by incubation with anti-CD16/ CD32 (clone 2.4G2) to block the FcR, stained with fluorescently labeled antibodies and stored in 1% paraformaldehyde until analysis. The following antibodies were used: anti-CD45.2 Pacific Blue (clone 104, Biolegend, Uithoorn, The Netherlands), anti-LY6G APC (clone 1A8, Biolegend), anti-F4/80 FITC (clone BM8, eBioscience, Halle-Zoersel, Belgium), anti-CD11b PE and FITC (clone M1/70, eBioscience), anti-Gr1 APC (clone RBG8C5, eBioscience) in fluorescence-activated cell sorting buffer (PBS containing 0.25% BSA, 0.05% NaN_3, 0.5 mM EDTA) for 30 min at 4°C.

HepG2 cells were stained with Annexin V-FITC, 7-AAD, and TO-PRO3 in Annexin V-binding buffer for 10 min at room temperature and immediately placed on ice until flow cytometric analysis. Data were acquired by means of FACS Canto II and analyzed using Weasel flow analysis package (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

Western blot analyses for ICAM-1

Approximately 50 mg of each mouse liver sample was lysed using 500 mL RIPA lysis buffer (Thermo-Fisher scientific, Rockford, Illinois) containing protease inhibitors (Roche Applied Science, Penzberg, Germany) and the total protein concentration was measured by the BCA protein assay kit (Thermo-Fisher scientific, Rockford, Illinois). Standardized protein amounts of boiled samples were isolated by electrophoresis in SDS-PAGE gel 4–10% and electro-transferred onto polyvinylidene difluoride membranes (Bio-Rad, Veenendaal, The Netherlands). Membranes were blocked with immersion in ethanol 100% for 1min and after rehydrated with TBS supplemented with 0.2% Tween (TBS-T) and incubated overnight with polyclonal goat anti-mouse intercellular cellular adhesion molecule 1 (ICAM-1) antibody (1:1000, R&D Systems, Minneapolis, Minnesota). After washing in TBS-T, the membranes were incubated with rabbit anti-goat peroxidase-conjugated secondary antibody (1:5000, Dako, Glostrup, Denmark) for 1 h at room temperature. Finally, blots were washed in TBS-T and once in TBS, incubated with ECL Prime Western Blotting Detection Reagent (Amersham Biosciences, Roosendaal, The Netherlands), and digital images were obtained with the ChemiDoc XRS Quantity One (Bio-Rad Laboratories, Hercules, California). In the next step, the membranes were re-probed with a β-actin antibody (1:4000, Cell Signaling, Massachusetts) to assess the equality of loading. Signal intensities were quantified using ChemiDoc XRS Quantity One (Bio-Rad Laboratories, Hercules, California), and ICAM-1 expression normalized with β-actin and expressed as the

mean fold change in relation to samples obtained from mice receiving only the vehicle.

ELISA for MIP-2, IL6, and TNF

Blood from inferior vena cava was collected in pre-coated lithium heparin tubes. Plasma was used to determine ALT activity following the procedure reported in the manufacturer instruction (art. nr MAK052, Sigma-Aldrich, St. Louis, Missouri). TNF and IL-6 were determined in serum samples by sandwich ELISA. Antibodies were from eBioscience and procedures reported in the manufacturer instruction were followed. MIP-2 protein levels were evaluated in serum and liver tissue lysates obtained as described previously (Matzer et al., 2001). For tissue lysates, total protein concentration was measured with BCA protein assay kit (Thermo-Fisher scientific, Rockford, Illinois). Analysis was per- formed according to manufacturer's protocol (Koma Biotech, Mouse MIP-2 ELISA core kit).

qPCR

For mRNA studies, the medial part of the biggest liver lobe from each mouse (approximately 50mg) was snap frozen in liquid nitrogen and stored at -80°C until RNA isolation. Each sample was suspended in 500 µL RNA InstaPure (Eurogentec) and homogenized using a TissueLyser (Qiagen, Hilden, Germany) for 1 min/25 Hz twice. The homogenized tissue was centrifuged for 10 min at 12 000 x g. The supernatant containing RNA in RNA Insta-Pure was transferred to a new vial and RNA was isolated using phenol-chloroform extraction. The amount of RNA was determined using the NanoDrop 2000 Spectrophotometer (Thermo Scientific). Subsequently, 1 mg of extracted total RNA was reverse transcribed with the iScriptTM cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, California). Quantitative re- verse transcriptase PCR was performed suing a iCycler iQ system (Biorad), and amplification was done using iQ SYBR Green supermix (Biorad) with 0.3 mM final primer concentration. Primer sequences: mouse MIP-2 FW-AAAGTTTGCCT TGACCCTGAAG and RV-CAGTTAGCCTTGCCTTTGTTCAGT. Gene-specific primers for murine MIP-2 were derived from the NCBI GenBank and were manufactured commercially (Eurogentec, Seraing, Belgium). For each sample, mRNA expression was normalized for the detected Ct value of β-actin. Data are expressed as fold increase compared with the control group (vehicle only).

Statistical analyses

Data are presented as means ± standard error of the mean (SEM). Statistical significance for comparisons was determined by one- or two-way ANOVA with Dunnett's post hoc test. A p- value less than .05 was considered statistically significant. All data are analyzed using GraphPad Prism (version 6.07) software (San Diego, California).

RESULTS

TVX decreases "Find Me" signal release from HepG2 apoptotic cells

Recently, Poon et al. (2014) have demonstrated that TVX prevents the release of damage-associated molecular patterns such as ATP, through blockade of PNX1 on early apoptotic cells. Since this action of TVX may be crucial to DILI, we first evaluated the opening of the PNX1 hemichannel on hepatocytes undergoing apoptosis induced by TVX+TNF.

To this end, HepG2 cells were exposed to TVX, LVX (concentration ranges 20–80 mM), or vehicle (Veh) only in presence or absence of TNF for 24 h. Cells were stained with 7-AAD and Annexin V-FITC in order to discriminate viable cells (AnnV-/7-AAD-), early apoptotic cells with unaffected membrane integrity (AnnV+/7-AAD-/TO-PRO3+), permeabilized late apoptotic cells (AnnV+/7-AAD+/TO-PRO3+), and apoptotic bodies (AnnV+/7-AAD-/TO-PRO3+/FSClo)(Figure 2A). The fluorescent dye TO-PRO3 was used to determine the opening of the PNX1 channel since cells are impermeable to TO-PRO3 when PNX1 channel is closed. We thus evaluated the uptake of TO-PRO3 by flow cytometer following the gating strategy displayed in Figure 2A.
Co-incubation with TVX and TNF, but not with LVX or Veh and TNF, was associated with an increased amount of early and late apoptotic cells. TVX+TNF dose-dependently increased the number of early apoptotic cells when compared with the other treatments (Figure 2B, left panel). In these early apoptotic cells, phenotypically characterized as AnnV+/7-AAD-/TO-PRO3+ cells, the median fluorescence intensity (MFI) for TO-PRO3 was decreased dose-dependently by TVX, indicating that TVX indeed blocks the PNX1 hemi channel (Figure 2B, right panel).

TVX reduces human neutrophil migration toward chemo-attractants in vitro

Because the hemichannel PNX1 (and ATP release) is known to be involved in neutrophil movement (Bao et al., 2013; Kukulski et al., 2009), we investigated the effect of TVX on the neutrophil chemotaxis toward gradients of two different chemo-attractants: N-formyl-met-leu-phe (f-MLF) and IL-8.

Incubation of human neutrophils with increasing concentrations of TVX (range 1–10 mM) led to a dose-dependent decrease in neutrophil migration toward f-MLF or IL-8 (Figs. 3A and B, respectively). Incubation with TVX at 5 mM, the Cmax of the fluoroquinolone in human patients after oral therapy (Vincent et al., 1998), resulted in ~50% inhibition of neutrophil vector speed toward both chemo-attractants. No effects on chemotaxis to any of the two chemo-attractants were observed when cells were incubated with 35 mM LVX (Figure 3). To exclude TVX

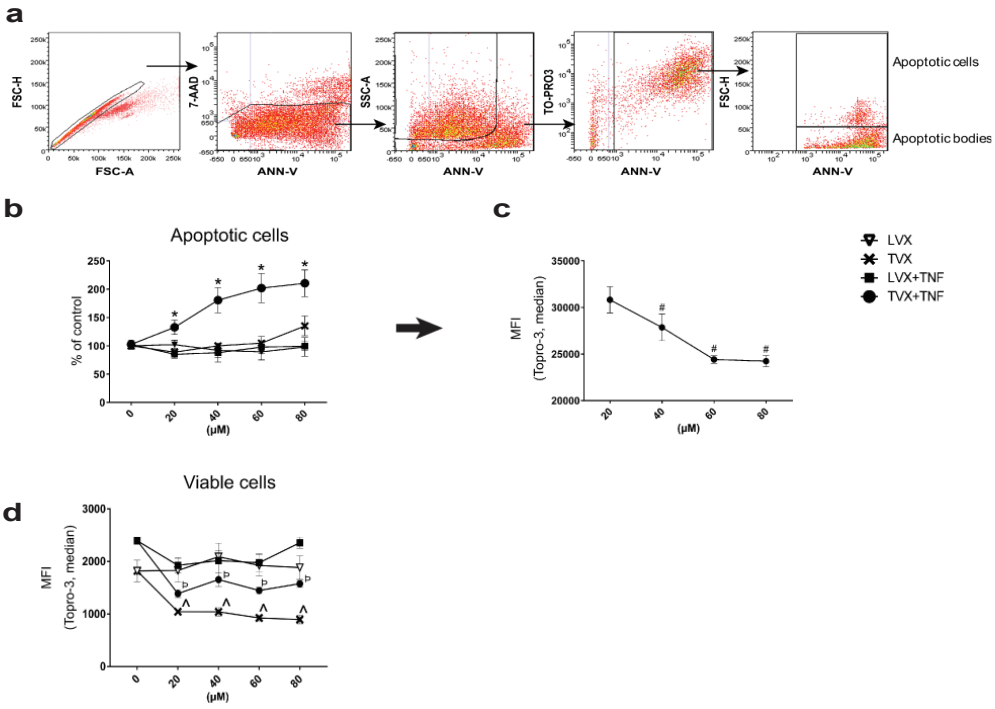

Figure 2

Increased concentration of TVX lowered TO-PRO3 staining in TVX+TNF-induced HepG2 apoptotic cells.

HepG2 cells were incubated for 24 h with TVX, LVX or vehicle with or without TNF. a. Gating strategy of apoptotic cells and bodies b. Number of apoptotic cells are presented as percentage of cells counted in samples derived from HepG2 exposed only to vehicle (no TNF). c. TOPRO-3 median intensity in TVX+TNF-induced apoptotic cells. ●= TVX+TNF, ■= LVX+TNF, ×= TVX, ▼= LVX; d. TOPRO-3 median intensity in viable cells. Data are presented as mean ± SEM; * $p<0.05$ when compared to all the other treatments assessed, # $p < 0.05$ when compared to the lower dose of TVX causing increased amount of apoptotic cells (20 μM); Þ $p<0.05$ when compared to cells exposed to DMSO (indicated as 0 for TVX and LVX), ^ $p<0.05$ when compared to cells exposed to DMSO+TNF (indicated as 0 for TVX+TNF and LVX+TNF), one-way ANOVA followed by Dunnett's post-hoc test.

cytotoxicity as a cause for decreased motility, TVX-treated neutrophils were tested for Annexin V and PI staining 4 h after incubation, and no significant differences in the percentage of viable cells were found with increased concentration of the drug up to 100 mM (Figure 3C).

TVX increases MIP-2 in liver and serum and reduces hepatic ICAM-1 expression

We also investigated the potential indirect (i.e., not direct on neutrophils)

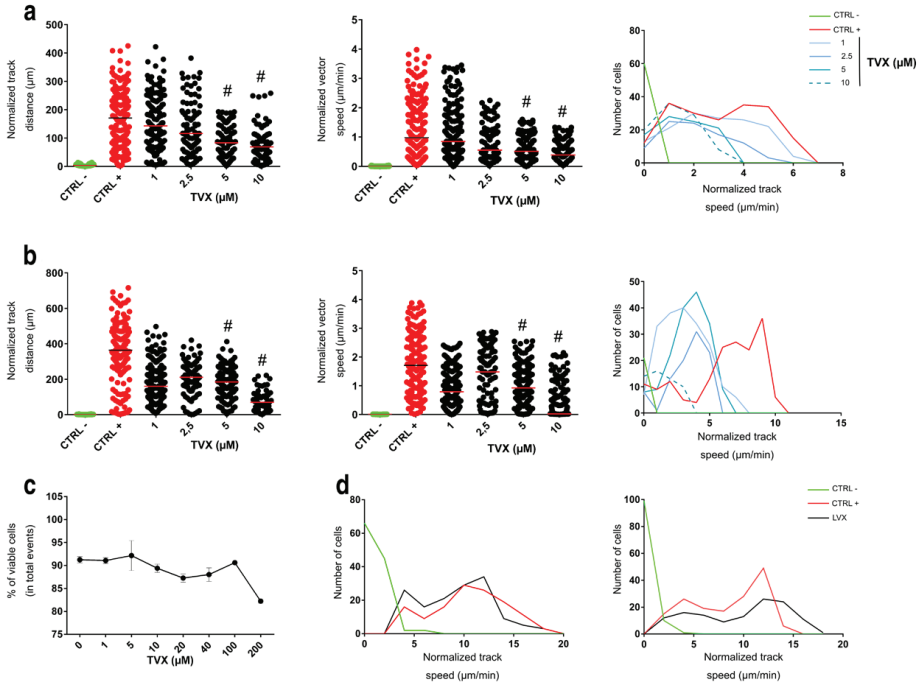

Figure 3

Impairment of human neutrophil chemotaxis toward sterile and non-sterile stimuli by TVX.

Human neutrophils were obtained from healthy donors and immediately isolated and prepared for the 3D functional chemotaxis assay. The migration distance of neutrophils was normalized to the average migration path of neutrophils that were not stimulated with chemoattractants. a-b. Effects of increasing concentration of TVX were assessed on chemotaxis stimulated by f-MLF (a) and IL-8(b) Dose-dependent effects on track distance, vector speed and the distribution of track speeds are depicted. c. Neutrophil were tested for Annexin V and PI positivity 4 h after incubation with TVX. Percentage of double negative (Annexin V and PI) cells calculated in the total amount of analyzed events are presented. d. Effect of LVX (35 µM) on chemotaxis stimulated by f-MLF and IL-8. Distribution of track speeds are depicted. Presented data on chemotaxis are representative results for the experiments which were performed three times. # $p<0.05$ when compared to cells treated with the positive control (CTRL+, a: f-MLF, b: IL-8), one-way ANOVA followed by Dunnett's post-hoc test

mechanisms by which TVX may delay the influx of neutrophils in liver. For this reason, we quantified the amount of the main chemokine for neutrophils MIP-2 and the expression of ICAM-1, which is involved in their trapping in the liver at post-capillary venule level.

In line with the knowledge that PNX1 blockade reduces expression of adhesion

molecules (Lohman et al., 2015) TVX lowered the protein expression of ICAM-1, the receptor of CD11b/ CD18, in liver tissue homogenates at 2 h (Figure 4A) and 4 h (data not shown) after exposure to TNF. LVX treatment led to mildly increased levels of ICAM-1 protein in TNF-treated mice (Figure 4A).

In contrast, the combination of TVX+TNF increased the serum MIP-2 concentrations (the murine functional analogue of the chemokine IL-8) when compared with the other treatments. TNF by itself already increased the serum levels of this cytokine in TVX-, LVX-, and vehicle-treated animals (Figure 4B), but pre-treatment with TVX induced a significant increase of this cytokine compared with the other treatments at both 2 and 4 h after TNF administration. Notably, MIP-2 transcripts and protein levels were already increased in liver homogenates of TVX only-treated mice, so before exposure to TNF (Figure 4C).

TVX induces early neutrophil activation in blood

As observed in our previous study (Giustarini et al., 2018), the number of hepatic neutrophils were decreased early after TNF injection in TVX-treated mice when compared with none- or LVX-treated mice.

Liver- and spleen-isolated neutrophils in all groups had a similar phenotype and activation status (based on CD11b expression) at 2 h after TNF administration. Thereafter, CD11b ex- pression stabilized (Figs. 5A and B) in Veh+TNF- and LVX+TNF- treated mice, but in the TVX+TNF-treated mice the activation status of the liver- and spleen-derived neutrophils was increased (Figs. 5A and B).

Additionally, we checked blood for the presence and activation status of neutrophils. Interestingly, TVX+TNF treatment increased the number of neutrophils in blood at 2 h (Figure 6A), and these neutrophils displayed increased CD11b, and decreased CD62L and CD44 expression when compared with the other treatments (Figure 6A). In addition, flow cytometry of live blood $CD45.2^{+}/Ly6G^{+}$ cells without exclusion of aggregates showed an increased number of multicellular events (identified as $LY6G^{+}/FSC\text{-}W^{hi}$) in mice receiving TVX when compared with Veh- or LVX-treated mice (Figure 6B). This indicates the presence of homotypic and heterotypic neutrophil aggregates.

Effects of Ly6C1 cell depletion on serum levels and hepatic production of cytokines

Using a neutrophil and monocyte depleting antibody (anti-Ly6C clone: RB6-8C5), we investigated whether the lack of these cells after TNF administration affected the inflammatory response, in particular with regard to LVX.

Two hours after the injection of the cytokine (TNF), depletion of neutrophils and monocytes resulted in a significant increase in the serum levels of MIP-2 when

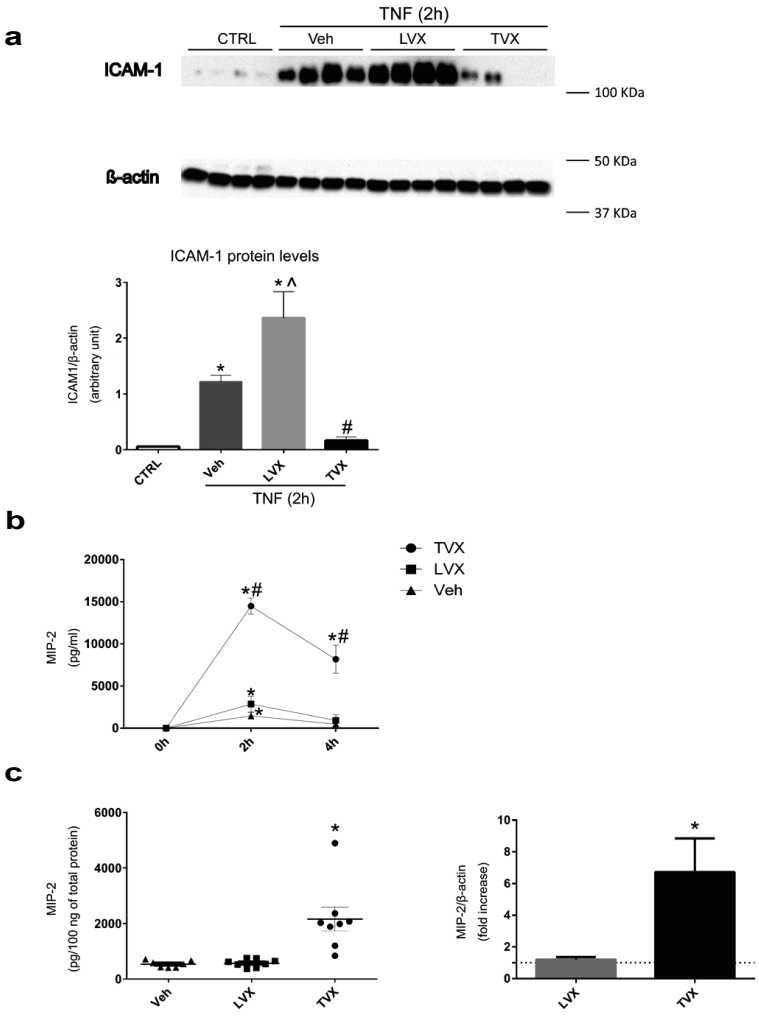

Figure 4

TVX reduced TNF-mediated ICAM-1 expression in the liver and increased serum and hepatic MIP-2 in mice.

Mice (6 per treatment group) were treated with TVX, LVX or vehicle as depicted in figure 1. Blood samples were collected at 0h, 2h and 4h after TNF injection. Liver lysates were obtained 2h after TNF injection. Mice in control group (CTRL) only received the vehicle (no TNF). a. ICAM-1 protein levels in liver lysates. b. MIP-2 levels in serum. c. MIP-2 level in liver lysates. Liver tissue lysates obtained from mice sacrificed at 0 h were used to quantify MIP-2 mRNA by qPCR and protein by ELISA. Total amount of protein was determined by BCA protein assay kit. Data are presented as mean ± SEM.; ●= TVX, ■= LVX, ▲= Veh; * $p<0.05$ when compared to vehicle; # $p<0.05$ when compared to LVX and TNF; ^ $p<0.05$ when compared to Veh+TNF; one-way ANOVA followed by Dunnett's post-hoc test.

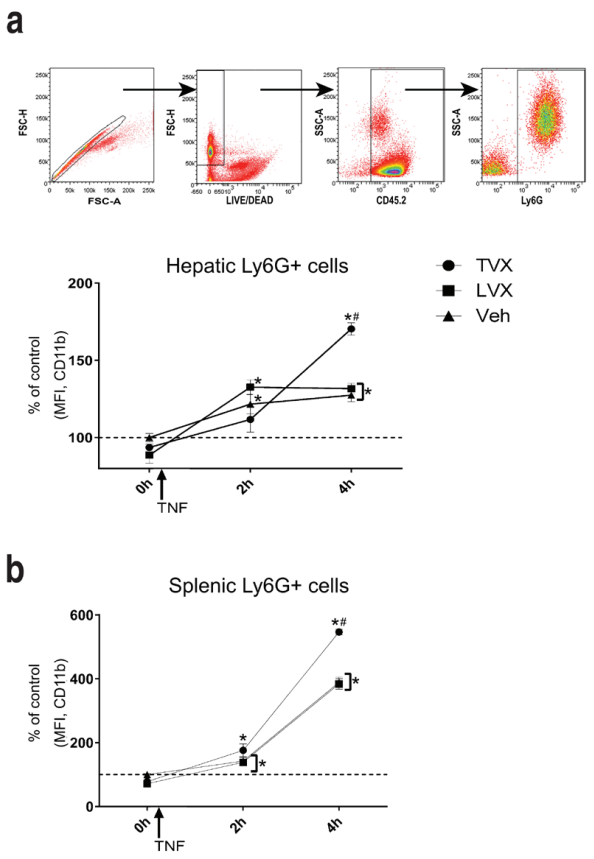

Figure 5

TVX induced intra-hepatic neutrophil activation.

Mice (8 per treatment group) were treated with TVX, LVX or vehicle as depicted in figure 1. 2h after TNF injection livers and spleens were processed to isolate leukocytes. a. Intra-hepatic neutrophils were identified as CD45.2+/Ly6G+ live cells. Median fluorescent intensities (MFI) of CD11b on neutrophils are presented as percentage of the MFI observed in mice receiving only the Veh (no TNF, dashed line). b. Spleen neutrophils were identified as depicted in a and MFI presented as percentage of the MFI observed in mice receiving only the Veh (no TNF, dashed line). Data are presented as mean ± SEM.; ●= TVX, ■= LVX, ▲= Veh; * $p<0.05$ when compared to vehicle; # $p<0.05$ when compared to TNF; one-way ANOVA followed by Dunnett's post-hoc test.

compared with the control (Figure 7A). In addition, depletion of neutrophils and monocytes increased the MIP-2 protein concentrations at 4h in liver homogenates in Veh+PBS and LVX+TNF-treated mice (Figure 7B). Depletion did not affect hepatic MIP-2 concentration in mice receiving Veh+TNF and TVX+TNF (Figure 7B). Together data indicates that lack of Ly6C-positive cells results in increased chemotactic signals.

Depletion of neutrophils and monocytes also influenced IL-6 serum concentrations, and in particular elicited a ≈2-fold increase in IL-6 serum concentration in LVX+TNF- and Veh+TNF-treated mice when compared with mice receiving the isotype antibody.

Depletion did not change IL-6 levels resulting from TVX+TNF and Veh+PBS-treatments (Figure 7A). Notably, the IL-6 levels in depleted LVX+TNF and Veh+TNF-treated mice matched the concentrations observed in mice receiving TVX+TNF.

Serum concentrations of TNF in mice lacking neutrophils and monocytes were

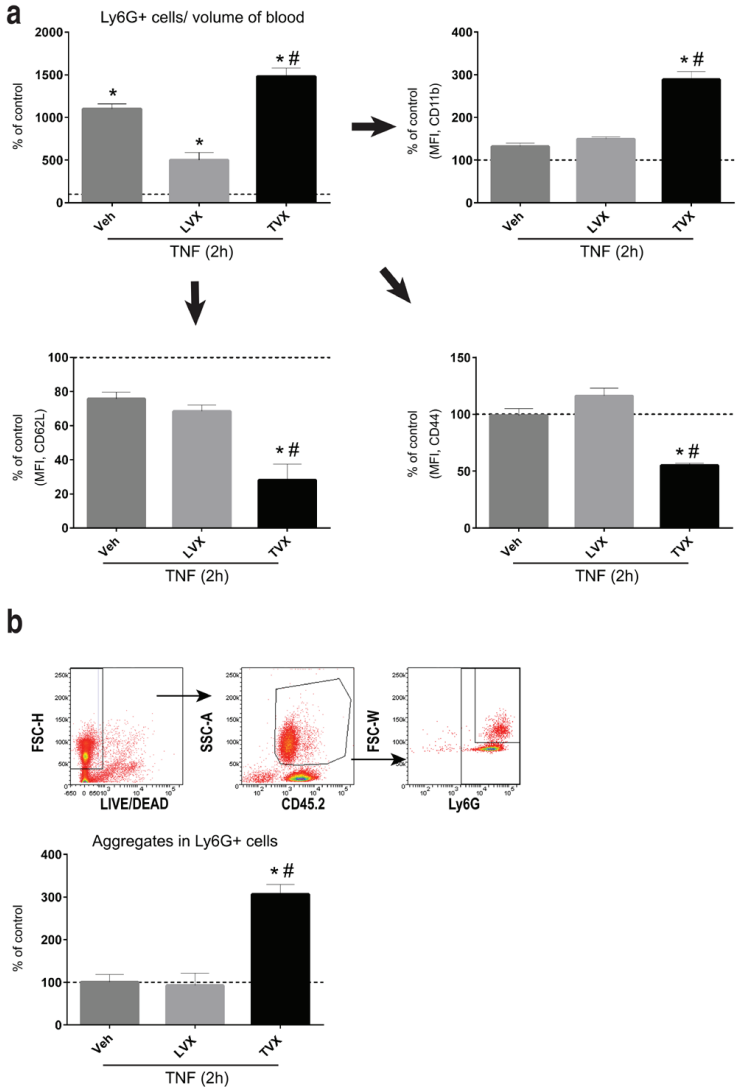

Figure 6

TVX-induced early activation of neutrophils in blood

Mice (8 per treatment group) were treated with TVX, LVX or vehicle as depicted in figure 1. 2h after TNF injection livers and spleens obtained from mice at each time point indicated were processed to isolate leukocytes. Blood was collected from inferior vena cava in heparin-coated tubes. Cells were counted and prepared for flow cytometry. a. Neutrophils were identified as $CD45.2^{+}/Ly6G^{+}$ live cells. Median fluorescent intensities (MFI) of CD11b, CD62L, and CD44 on neutrophils are presented as percentage of the MFI observed in mice receiving only the Veh (no TNF, dashed line). b. $Ly6G^{+}$ aggregates were analyzed by inclusion of doublets, and presented as percentage of mice only receiving the Veh (no TNF, dashed line). Data are presented as mean ± SEM; ●= TVX, ■= LVX, ▲= Veh; * $p<0.05$ when compared to vehicle; # $p<0.05$ when compared to TNF; one-way ANOVA followed by Dunnett's post-hoc test.

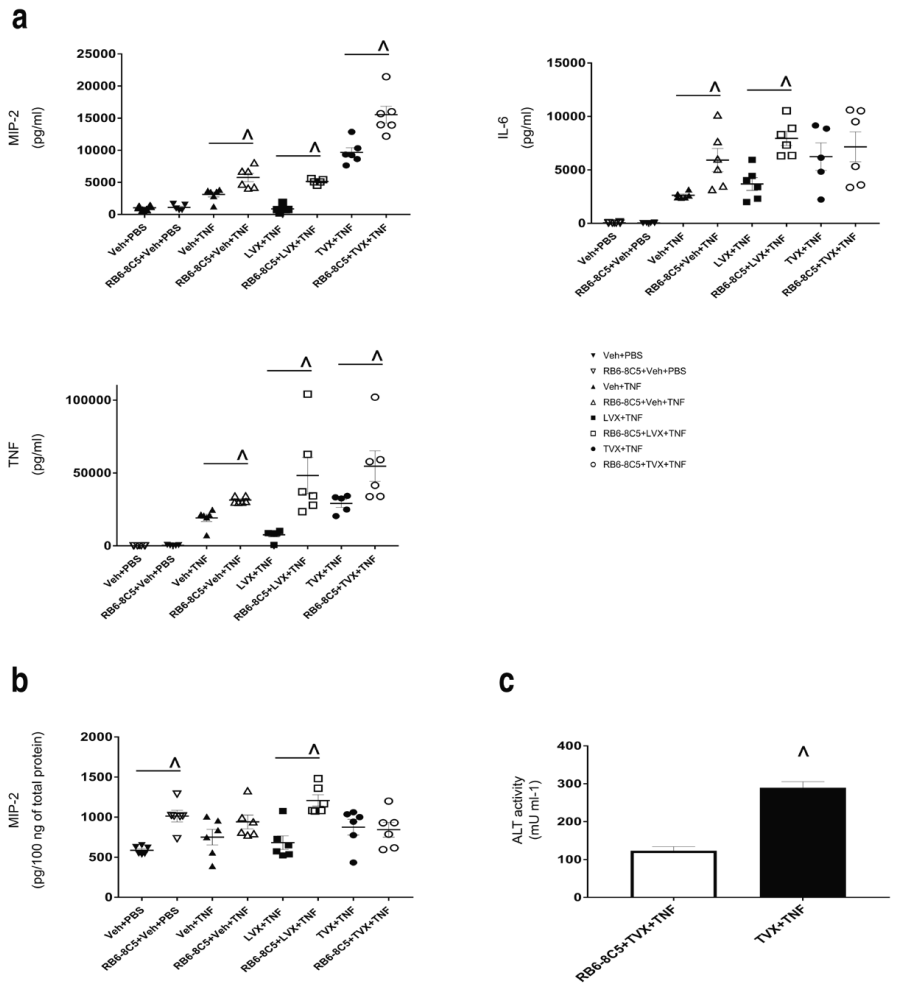

Figure 7

Lack of monocytes and neutrophils led to increased serum and hepatic inflammatory cytokines

Mice were administered with anti-Ly6C (clone: RB6-8C5) or its correspondent isotype (IgG2b) 72 and 24 h before receiving the drug solutions. a. Serum cytokines (MIP-2, IL-6 and TNF) were assessed on serums collected 2 h after TNF injection. b. Hepatic MIP-2 concentration was assessed on liver homogenates obtained from livers excised 4 h after TNF injection. Total amount of protein was determined by BCA protein assay kit. c. Serum ALT levels of depleted (RB6-8C5) and competent TVX+TNF-treated mice were assessed on samples collected 4 h after the injection of TNF. ALT levels were determined by colorimetric assay for ALT activity. Data are presented as mean ± SEM.; ●= TVX +TNF; ○= RB6-8C5+TVX+TNF, ■= LVX+TNF, □= RB6-8C5+LVX+TNF, ▲= Veh+TNF; △ = RB6-8C5+Veh+TNF, ▼= Veh+PBS; ▽ =RB6-8C5+Veh+PBS, ^ $p<0.05$ when compared to neutrophil and monocyte depleted mice receiving the same drug treatment or the correspondent vehicle; one-way ANOVA followed by Dunnett's post-hoc test.

significantly higher than those observed in neutrophil/monocyte-competent TVX-treated mice 1h after exogenous administration of the same cytokine. Both in TVX+TNF-treated and LVX+TNF-treated mice, depletion of Ly6C-positive cells resulted in an increase of serum levels of TNF when compared with the corresponding mice receiving the isotype antibody. This increase was 6-fold in LVX-treated mice and 2-fold in TVX-treated mice (Figure 7A).

Depletion of neutrophils and monocytes significantly decreased serum ALT concentration in TVX+TNF mice (Figure 7C), illustrating that these cells are partly responsible for the ultimate TVX-induced liver injury.

DISCUSSION

In our previous study, we have demonstrated that TVX delays the TNF-elicited influx of neutrophils and monocytes into the liver (Giustarini et al., 2018). Here, we show that TVX blocks the PNX1 hemichannel in apoptotic hepatocytes during TVX+TNF-induced cell death. Additionally, we show that TVX inhibits the chemotactic movement of human neutrophils. Recently, others have shown that TVX is capable of blocking PNX1 which is involved in apoptosis (Poon et al., 2014). In addition, it is known that PNX1 is crucial for optimal migration of leukocytes, neutrophils in particular (Bao et al., 2013; Kukulski et al., 2009). From our current new data, we conclude that blockade of the PNX1 by TVX represents an important event that has a dual effect related to the induction of DILI: it causes a decreased release of "find-me" signals and it hampers phagocyte migration toward attracting stimuli, increasing circulating TNF. The increased concentration of this cytokine, in the light of the capability of TVX to interact with TNF in inducing hepatocyte apoptosis (Beggs et al., 2014; Shaw et al., 2009d), represents a trigger for the development of liver injury.

The impairment in the PNX1-mediated release of molecules (find-me signals) during the mild TVX-induced apoptosis (Giustarini et al., 2018) comprises a potentially toxic mechanism of TVX since the clearance of apoptotic cells relies on the release of "find-me" signals and the subsequent attraction of phagocytes (Chekeni et al., 2010). Usually clearance of apoptotic cells is an immunologically silent process but if the apoptotic cells are not promptly removed, they may undergo secondary necrosis leading to an uncontrolled released of inflammatory danger signals (Brauner et al., 2013). Our conclusion that PNX1 blockade is involved in the delayed sequestration of phagocytes by TVX is also based on the knowledge that phagocytes follow chemotactic gradients (e.g., of chemo-attractants such as f-MLF, IL-8) by means of PNX1-released ATP (Bao et al., 2013; Chen et al., 2010). As a result of the decreased motility of neutrophils in the sinusoids, recruitment will be delayed and the presence of these cells in the narrow vessels of the liver will be prolonged. Moreover, PNX1 blockade has also been shown to reduce expression of adhesion molecules such as vascular cell adhesion molecule 1 (Lohman et al.,

2015), which is in agreement with our finding that TVX directly down-regulates expression of ICAM-1 in liver tissue, reducing the sequestration of leukocytes at the post-capillary venule level, where ICAM-1-dependent sequestration of leukocyte occurs (Kolaczkowska and Kubes, 2013).
In line with the importance of early recruitment of phagocytes in the protection of TVX-induced liver damage, depletion of Ly6C+ cells elicited a greater systemic and hepatic early inflammatory response. Remarkably, depletion of Ly6C+ cells also caused increased inflammatory responses in TNF-only- and LVX+TNF-treated mice. These findings, together with pre- vious works showing a synergistic effect of TVX with inflammatory stimuli (e.g., TNF) causing hepatocyte apoptosis in vitro and in vivo (Beggs et al., 2014; Shaw et al., 2009a,d) confirms our conclusion that delay and lack in the recruitment of neutrophils and monocytes to the site of inflammation is at least partly responsible for the fluoroquinolone-induced hepatotoxicity (Giustarini et al., 2018).

Our conclusion is in line with the consensus that neutrophils and monocytes not only initiate but also regulate inflammation (Soehnlein and Lindbom, 2010). In particular, depletion of neutrophils and monocytes has been associated with an in- crease in LPS- or infection-induced secretion of inflammatory cytokines such as TNF (Daley et al., 2005, 2008; Hewett et al., 1993; Holub et al., 2009; Steinshamn and Bemelmans, 1995). However, the increase of inflammatory cytokines was insufficient to fully elicit LPS-induced liver injury (Hewett et al., 1993), suggesting that neutrophils are still central for the onset of the damage. Although these findings clearly point to a pivotal role for neutrophils and monocytes in the regulation of early TNF-mediated inflammation, we did not formally rule out that depleting antibody administration may have effects which can contribute to the observed cytokine increase. On the other hand, we can rule out the possibility that the increased cytokine levels may be due to apoptotic neutrophils induced by depleting antibody administration. Indeed, as demonstrated by Ren and colleagues, apoptotic neutrophils exert rather an anti-inflammatory effect on LPS-induced inflammation (Ren et al., 2008). In line with this, others (Yu et al., 2007) showed that depletion of neutrophils and monocytes is associated with a protective early gene response to dying neutrophils into the liver rather than with an increased susceptibility to inflammatory cytokine release. This is in line with findings from Shaw et al. (2009b) and our present results on TVX. On the contrary, the hepatic neutrophilia that we observed in mice treated with LVX+TNF did not lead to liver injury (Giustarini et al., 2018), indicating that mere recruitment of neutrophils due to TNF injection is also not sufficient to elicit DILI. We therefore propose that in case of LVX+TNF- or Veh+TNF-treated mice neutrophils are sequestered in organs (such as liver) to regulate inflammation. In contrast, in TVX-treated mice, delayed neutrophils do not inhibit inflammation in the liver, become activated, form aggregates that accumulate in the liver vessels.

Importantly with regard to TVX-induced DILI, initial TVX effects (mild apoptosis, disrupted release of find-me signals, hampered tissue influx, and aggregate

formation of phagocytes) appear to be linked to the intravascular activation of neutrophils (Neelamegham et al., 2000; Simon et al., 1993). Thus, neutrophil aggregates are impeded in their capability to pass the narrow sinusoids and once intravascularly activated (Borregaard et al., 1994; Sheshachalam et al., 2014), they eventually will cause vascular damage and allow late influx of inflammatory leukocytes into the tissue causing DILI, as also shown by Shaw et al. (2009b,c). The observed impairment of sequestration of leukocytes in the liver of TVX-treated mice may also support previous findings showing an impaired clearance of TNF in mice treated with TVX (Shaw et al., 2009a). Possibly, when due to TVX neutrophils and monocytes cannot provide their anti-phlogistic action in the liver, resident macrophages keep on producing TNF (but also other pro-inflammatory cytokines) which, in addition, is not properly eliminated by neutrophil-derived soluble TNF receptors (sTNFR) (Jablonska et al., 1999; Lantz et al., 1994; Shaw et al., 2009a; Steinshamn and Bemelmans, 1995). Indeed, the release of sTNFR by neutrophils and monocytes occurs especially when these cells adhere to surfaces via both integrin and non-integrin-dependent mechanisms (Lantz et al., 1994).

It is also known that activated neutrophils release proteolytic enzymes such as elastase. Elastase has been demonstrated to be crucial in final TVX-induced liver damage (Shaw et al., 2009c) and in the reduction of the IL-8 receptor on the neutrophil surface (Bakele et al., 2014). Also in our experiments, neutrophil activation represents a crucial step in the sequence of events leading to TVX-induced liver damage as confirmed by the reduced serum ALT concentration at 4 h in mice depleted of neutrophils. The intravascular activation of neutrophils as fundamental step in the damage mediated by TVX is also in line with the increased amount of plasminogen activator inhibitor-1 (PAI-1) in mice receiving TVX+LPS (Shaw et al., 2009b), and with the beneficial effects of heparin administration on TVX+LPS-induced DILI. Of note, the decrease of ICAM-1 may be reinforced by increased degranulation of activated neutrophils (Champagne et al., 1998).

Recent observations on the incidence of DILI by antibiotic use emphasize the urgency to clarify the mechanisms underlying fluoroquinolone-induced DILI (Chalasani et al., 2015). In addition, previous studies suggest that the development of TVX-induced liver injury is related to multiple effects of TVX+TNF combination (neutrophils, pro-inflammatory cytokines, and alterations of hemostasis) which seem to represent segregated mechanistic pathways (Shaw et al., 2009b). Interestingly, the mechanism of TVX-induced liver injury involving dysregulation of innate immune responses as proposed here is consistent with the rapid onset of DILI associated with fluoroquinolones in patients (Orman et al., 2011) and explains the liaison between the apparently segregated toxic pathways identified previously by Shaw et al.(2009b). Moreover, other pharmaceuticals (such as diclofenac, chlorpromazine, amiodarone and ranitidine) have been associated with the induction of liver injury and hepatocyte apoptosis when combined with inflammatory mediators in vivo and in vitro, respectively (Deng et al., 2006; Gandhi et al., 2013; Lu et al., 2012; Luyendyk et al., 2003). In our opinion, this indicates

the urge to investigate if other DILI-associated compounds dysregulate early recruitment of phagocytes during inflammation.

With regard to risks of DILI by fluoroquinolones, impairment of innate immune responses in elderly patients (Brubaker et al., 2011) may represent an idiosyncratic factor explaining the higher incidence of fluoroquinolone-induced events in this population (Hayashi and Chalasani, 2012). Our data provides novel insights on leukocyte-mediated regulation of early inflammation and its role in the onset of DILI.

References

Bakele, M. et al. (2014) *An interactive network of elastase, secretases, and PAR-2 protein regulates CXCR1 receptor surface expression on neutrophils. J. Biol. Chem., 289, 20516–20525.*

Bao, Y. et al. (2013) *Pannexin 1 channels link chemoattractant receptor signaling to local excitation and global inhibition responses at the front and back of polarized neutrophils. J. Biol. Chem., 288, 22650–7.*

Beggs, K.M. et al. (2014) *Molecular Mechanisms of Hepatocellular Apoptosis Induced by Trovafloxacin-Tumor Necrosis Factor-alpha Interaction. Toxicol. Sci., 1–11.*

Borregaard, N. et al. (1994) *Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. J. Leukoc. Biol., 56, 80–87.*

Brauner, J.M. et al. (2013) *No littering : the clearance of dead cells and leaking cellular contents and possible pathological complications. OA Arthritis 1, 1–7.*

Brubaker, A.L. et al. (2011) *Age-related Dysregulation of Inflammation and Innate Immunity: Lessons Learned from Rodent Models. Aging Dis., 2, 346–60.*

Chalasani, N. et al. (2015) *Features and outcomes of 899 patients with drug-induced liver injury: The DILIN prospective study. Gastroenterology, 148, 1340–1352.e7.*

Champagne, B. et al. (1998) *Proteolytic Cleavage of ICAM-1 by Human Neutrophil Elastase 1. J. Immunol., 161, 6398–6405.*

Chekeni, F.B. et al. (2010) *Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. Nature, 467, 863–7.*

Chen, Y. et al. (2010) *Purinergic signaling: a fundamental mechanism in neutrophil activation. Sci. Signal., 3, ra45.*

Crispe, I.N. (2001) *Isolation of mouse intrahepatic lymphocytes. Curr. Protoc. Immunol., Chapter 3, Unit 3.21.*

Daley, J.M. et al. (2005) *Transcriptional regulation of TNF-alpha production in neutropenia. Am. J. Physiol. Regul. Integr. Comp. Physiol., 288, R409-12.*

Daley, J.M. et al. (2008) *Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. J. Leukoc. Biol., 83, 64–70.*

Deng, X. et al. (2006) *Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. J. Pharmacol. Exp. Ther., 319, 1191–1199.*

Gandhi, A. et al. (2013) *Chlorpromazine-induced hepatotoxicity during inflammation is mediated by TIRAP-dependent signaling pathway in mice. Toxicol. Appl. Pharmacol., 266, 430–8.*

Giustarini, G. et al. (2018) *Tissue influx of neutrophils and monocytes is delayed during development of trovafloxacin - induced tumor necrosis factor - dependent liver injury in mice. 1–13.*

Hayashi, P.H. and Chalasani, N.P. (2012) *Liver injury in the elderly due to fluoroquinolones: Should these drugs be avoided? Cmaj, 184, 1555–1556.*

Hewett, J.A. et al. (1993) *Relationship between tumor necrosis factor-alpha and neutrophils in endotoxin-induced liver injury. Am. J. Physiol., 265, G1011-5.*

Holub, M. et al. (2009) *Neutrophils sequestered in the liver suppress the proinflammatory response of Kupffer cells to systemic bacterial infection. J. Immunol., 183, 3309–16.*

Jablonska, E. et al. (1999) *Role of neutrophils in release of some cytokines and their soluble receptors. Immunol Lett 70, 191–197.*

Kolaczkowska, E. and Kubes, P. (2013) *Neutrophil recruitment and function in health and inflammation. Nat. Rev. Immunol., 13, 159–75.*

Kukulski, F. et al. (2009) *Extracellular ATP and P2 receptors are required for IL-8 to induce neutrophil migration. Cytokine, 46, 166–170.*

Lantz, M. et al. (1994) *Adherence of neutrophils induces release of soluble tumor necrosis factor receptor forms. J. Immunol., 152, 1362–1369.*

Lohman, A.W. et al. (2015) *Pannexin 1 channels regulate leukocyte emigration through the venous endothelium during acute inflammation. Nat. Commun., 6, 7965.*

Lu, J. et al. (2012) *Amiodarone Exposure During Modest Inflammation Induces Idiosyncrasy-like Liver Injury in Rats: Role of Tumor Necrosis Factor-alpha. Toxicol. Sci., 125, 126–133.*

Lu, J. et al. (2013) *Tumor necrosis factor-alpha potentiates the cytotoxicity of amiodarone in Hepa1c1c7 cells: roles of caspase activation and oxidative stress. Toxicol. Sci., 131, 164–78.*

Luyendyk, J.P. (2003) *Ranitidine Treatment during a Modest Inflammatory Response Precipitates Idiosyncrasy-Like Liver Injury in Rats. J. Pharmacol. Exp. Ther., 307, 9–16.*

Matzer, S.P. et al. (2001) *Constitutive expression of macrophage-inflammatory protein 2 (MIP-2) mRNA in bone marrow gives rise to peripheral neutrophils with preformed MIP-2 protein. J. Immunol., 167, 4635–43.*

Neelamegham, S. et al. (2000) *Shear and Time-Dependent Changes in Mac-1, LFA-1, and ICAM-3 Binding Regulate Neutrophil Homotypic Adhesion. J. Immunol., 164, 3798–3805.*

Orman, E.S. et al. (2011) *Clinical and histopathologic features of fluoroquinolone-induced liver injury. Clin. Gastroenterol. Hepatol., 9, 517–523.e3.*

Overbeek, S.A. et al. (2013) *Cigarette Smoke-Induced Collagen Destruction; Key to Chronic Neutro-*

philic Airway Inflammation? PLoS One, 8.
Poon, I.K.H. et al. (2014) *Unexpected link between an antibiotic, pannexin channels and apoptosis. Nature, 507, 329–34.*
Shaw, P.J. et al. (2007) *Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. Toxicol. Sci., 100, 259–66.*
Shaw, P.J. et al. (2009) *The role of the hemostatic system in murine liver injury induced by coexposure to lipopolysaccharide and trovafloxacin, a drug with idiosyncratic liability. Toxicol. Appl. Pharmacol., 236, 293–300.*
Shaw, P.J., Ganey, P.E., et al. (2009a) *Trovafloxacin Enhances the Inflammatory Response to a Gram-Negative or a Gram-Positive Bacterial Stimulus, Resulting in Neutrophil-Dependent Liver Injury in Mice. J Pharmacol Exp Ther 330, 72–78.*
Shaw, P.J., Beggs, K.M., et al. (2009) *Trovafloxacin enhances TNF-induced inflammatory stress and cell death signaling and reduces TNF clearance in a murine model of idiosyncratic hepatotoxicity. Toxicol. Sci., 111, 288–301.*
Shaw, P.J., Ganey, P.E., et al. (2009b) *Tumor Necrosis Factor α Is a Proximal Mediator of Synergistic Hepatotoxicity from Trovafloxacin / Lipopolysaccharide Coexposure. J Pharmacol Exp Ther 328, 62–68.*
Sheshachalam, A. et al. (2014) *Granule protein processing and regulated secretion in neutrophils. Front. Immunol., 5, 1–11.*
Simon, S.I. et al. (1993) *Beta 2-integrin and L-selectin are obligatory receptors in neutrophil aggregation. Blood, 82, 1097–1106.*
Soehnlein, O. and Lindbom, L. (2010) *Phagocyte partnership during the onset and resolution of inflammation. Nat. Rev. Immunol., 10, 427–439.*
Steinshamn, S. and Bemelmans, M. (1995a) *Granulocytopenia reduces release of soluble TNF receptor p75 in endotoxin-stimulated mice: a possible mechanism of enhanced TNF activity. Cytokine.*
Vincent, J. et al. (1998) *Pharmacokinetics and metabolism of single oral doses of trovafloxacin. Am. J. Surg., 176, 8S–13S*

Chapter 4

The hepatotoxic fluoroquinolone trovafloxacin disturbs TNF- and LPS-induced p65 nuclear translocation in vivo and in vitro.

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ABSTRACT

Idiosyncratic drug-induced liver injury (IDILI) is severe disease that cannot be detected during drug development. It has been shown that hepatotoxicity of some compounds associated with IDILI becomes apparent when these are combined in vivo and in vitro with lipopolysaccharide (LPS) or tumour necrosis factor (TNF). Among these compounds trovafloxacin (TVX), a fluoroquinolone, induced apoptosis in the liver and increased pro-inflammatory cytokines in mice exposed to LPS or TNF. The hepatocyte survival and the cytokine release after TNF/LPS stimulation relies on a pulsatile activation of NF-κB.

We set out to evaluate the dynamic activation of NF-κB in response to TVX+TNF or LPS models, both in vivo and in vitro in mouse and human cells. Remarkably, TVX prolonged the first translocation of p65 (NF-κB subunit) induced by TNF both in vivo (TVX 150 mg/kg, TNF 50 μg/kg) and in vitro (TVX 20 μM, TNF 4 ng/ml). The prolonged p65 translocation caused by TVX was associated with an increased phosphorylation of IKK and MAPKs and accumulation of inhibitors of NF-κB such as IκBα and A20 in HepG2. Coherently, TVX suppressed further TNF-induced NF-κB translocations in HepG2 leading to decreased transcription of ICAM-1 and inhibitors of apoptosis. Moreover, TVX prolonged LPS-induced NF-κB translocation in murine RAW264.7 macrophages increasing the secretion of TNF. In summary, this study presents new, relevant insights into the mechanism of TVX-induced liver injury underlining the resemblance between mouse and human models. In this study we convincingly show that several regularly used toxicity models provide a coherent view of relevant pathways for IDILI.

We propose that assessment of the kinetics of activation of NF-κB and MAPKs is an appropriate tool for the identification of hepatotoxic compounds already during drug development.

INTRODUCTION

Drug-induced liver injury (DILI) is a complex disease of concern to pharmaceutical companies and health care providers (Fontana et al. 2009; Chalasani et al. 2015). The identification of mechanisms behind most idiosyncratic DILI (IDILI) remains a challenge that needs to be solved in order to improve drug safety (Weaver et al. 2017). A prevalent hypothesis to explain the aetiology and patient specific response to certain drugs is represented by the "inflammatory hypothesis" (Roth et al. 2017) proposing that the liver-toxic potential of a number of pharmaceuticals results from a combination of sterile and non-sterile inflammatory stimuli (Buchweitz 2002; Luyendyk 2003; Deng et al. 2006; Zou et al. 2009; Dugan and MacDonald 2010; Lu et al. 2012). The initial insult caused by the combination of drug and inflammatory stimuli as described in this hypothesis is embedded in the so-called "the danger hypothesis" (Matzinger and Kamala 2011). The latter may explain how cellular damage triggers hypersensitivity-like hepatic adverse drug reaction (Pirmohamed et al. 2002).

In the last decade, many studies have underlined the importance of cellular damage and innate immunity in the development of drug-induced liver toxicity (Shaw et al. 2009a; Fredriksson et al. 2011, 2014; Beggs et al. 2014; Herpers et al. 2016; Giustarini et al. 2018a). In particular, tumour necrosis factor (TNF) appears to be involved in the liver toxicity of some DILI-associated compounds but the interactions between TNF signaling and these pharmaceuticals remain to be further elucidated.

Among these DILI-associated compounds the fluoroquinolone trovafloxacin (TVX) is well studied and it is shown that TVX, when administered in combination with lipopolysaccharide (LPS) or TNF to mice induces severe liver toxicity associated with vast apoptotic areas in the liver, increased serum levels of alanine amino transferases (ALT) and pro-inflammatory cytokines (Shaw et al. 2009a, b; Giustarini et al. 2018a). TNF exerts many biological effects that are mediated by engagement of TNF-receptor 1 and TNF-receptor 2. The most important difference between the two receptors for TNF is that the cytoplasmic tail of TNF-R1 contains a protein-protein interaction domain called "death domain" whereas TNF-R2 does not. The death domain interacting with TNF-R1-associated death domain can form two different complexes of signaling proteins called complex I and II (Wullaert et al. 2006). TNF-induced pro-survival signaling occurs via complex I, which mediates activation of NF-κB and MAPK, whereas death signaling via complex II activates caspase 8 (Wullaert et al. 2006). In particular, nuclear translocation of NF-κB (consequent of pathway activation) mediates usually pro-survival effects, especially in hepatocytes regulating activation of JNK. Indeed, inhibition of TNF-induced NF-κB nuclear translocation is associated with sensitization of hepatocytes to cell death via sustained JNK activation which terminates with caspase activation (Liu et al. 2002).

Several other studies have pointed to the importance of NF-κB in the survival of hepatocytes as well as in the synthesis of inflammatory cytokines upon TNF stimulation (Wullaert et al. 2007; Sakai et al. 2017). NF-κB have a similar function in macrophages when exposed to LPS, since Toll-like receptor signaling in liver-resident macrophages increased translocation of NF-κB into the nucleus and consequently transcription of various cytokines (Sakai et al. 2017).

In this study, we wanted to examine whether TVX causes alterations of NF-κB pathway activated by TNF or LPS. Here, we found that TVX perturbs NF-κB -mediated transcription leading to accumulation of inhibitors of NF-κB pathway via a prolonged activation of IKKα/β. This together with the simultaneously prolonged activation and reactivation of MAPKs may explain the observed increase in cytokine production and induction of apoptosis as result of TVX treatment.

MATERIALS AND METHODS

Animals and experimental set up

Male, 9 to 11-week old, C57BL/6J mice (The Jackson Laboratory, Charles River) were used for all experiments. They were allowed to acclimate for 1 week in a 12-h light/dark cycle,mean temperature of 23 ± 2 °C and 50-55% relative humidity. Acidified drinking water and laboratory food pellets were provided ad libitum.

Mice were fasted 7 h prior to treatment. TVX (150 mg/kg), levofloxacin (LVX,375 mg/kg), or saline vehicle (Veh) was administered orally 3 h before recombinant murine TNF injection (50 μg/kg, i.p.) (Shaw et al., 2009b). Food was available again immediately after TNF administration. Animals analyzed at time point 0 h did not receive TNF (Fig.1).

In vivo studies were approved by the Ethics Committee for Animal Experiments of Utrecht University and complied with governmental and international guidelines on animal experimentation.

Chemicals

TVX, LVX and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant murine TNF was purchased from R&DSystems (Minneapolis, MN, USA).

Cell culture

HepG2 human hepatoblastoma cells and RAW264.7 murine monocyte/macrophage cells (American Type Culture Collection, Manassas, VA, USA) were maintained in MEM+Glutamax (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10%

fetal bovine serum (FBS) in 75 cm2 tissue culture treated flasks. Cells were cultured in a humidified atmosphere composed of 95% air and 5% CO2 and a temperature of 37°C. HepG2 cells were passaged twice each week. 0.25% Trypsin-EDTA was used to detach confluent HepG2 cells from the flask. After plating, cells were allowed to adhere 48h before treatment.

TVX and LVX was reconstituted to a stock solution of 200 mM in dimethyl sulfoxide (DMSO) and diluted in culture medium to the desired concentrations. The final DMSO concentrations did not exceed 0.025% (v/v). Recombinant human TNF was reconstituted to a stock solution of 100 mg/mL in PBS+ 0.3% BSA as indicated by the manufacturer (R&DSystems). After incubation with the fluoroquinolones with or without TNF, HepG2 cells were washed with PBS, after treated with cytoplasmic lysis buffer (10mM Hepes; pH 7.9, 10 mM KCl, 0.1mM EDTA, 0.1mM EGTA, 0.4 % NP-40, protease and phosphatase inhibitors) and gently scraped. The suspensions were collected and left on ice for 20 min before centrifugation 13000 rpm for exactly 1 minute at 4°C. Supernatant was collected and immediately stored at -80°C. The pellets were washed 3 times with cytoplasmic lysis buffer without NP-40. Nuclei were resuspended and sonicated in nuclear lysis buffer (20 mM Hepes

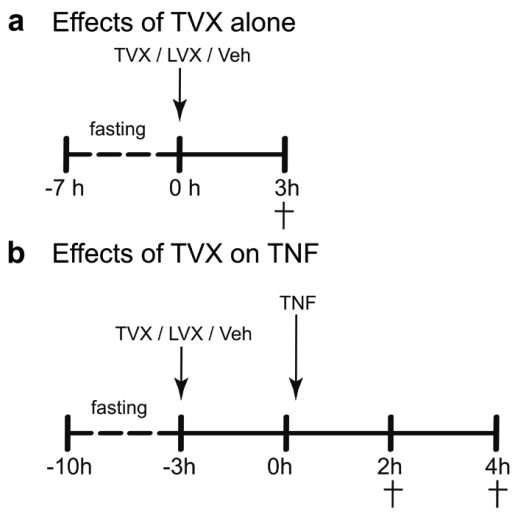


Figure 1

Protocol for the treatment of mice in the TVX+TNF model of drug-induced liver injury

Mice were fasted for 7 hours and then administered with an intragastric gavage of the drug solutions or saline (Veh). Three hours later, mice were either killed to observe the effects of the drugs alone (a) or received an intraperitoneal injection of TNF (b). TNF-injected mice were subsequently killed after 2 and 4 hours. Two hours after TNF was the selected time point to assess the effects of drugs on TNF-induced transcription and p65 nuclear translocation. Livers of mice culled at 4 h were used to confirm the occurrence of the damage. LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

pH 7.9, 25% glycerin, 400 mM NaCl, 1 mM EDTA, 1mM EGTA, 0.8% NP-40, protease and phosphatase inhibitors). Suspension were centrifuged at 13000 rpm for 5 min at 4°C. Supernatant was collected and immediately stored at -80°C.

HepG2 RelA-GFP (NF-κB , p65), A20-GFP BAC and ICAM1 BAC GFP reporter cell lines were previously established and characterized (Wink et al. 2017, 2018). HepG2 BAC GFP reporters were maintained and exposed to drugs in DMEM high glucose

supplemented with 10% (v/v) FBS, 25 U/mL penicillin and 25 µg/mL streptomycin. The cell lines were used between passage 5 and 25. For live cell imaging, the cells were seeded in Greiner black µ-clear 96 wells plates, at 50000 cells per well. After plating, cells were allowed to adhere 48 h before treatment.

RAW264.7 cells were passaged twice each week and a scraper was used to detach them from the flask. LPS was reconstituted to a stock solution of 1 mg/ml in PBS as indicated by manufacturer. After plating 15 x 104 cells in each of the 12 wells, cells were allowed to adhere 48 h before treatment. Drug treatment was similar as with HepG2 cells. RAW264.7 cells were either exposed to the drugs alone for 4 h or for 2 h before receiving LPS (10 ng/ml). Cells for imaging were seeded in IBIDI µ-slide 8 well, washed once with PBS and fixed with 4% formaldehyde, permeabilized with 0.2% triton X-100 in PBS, and blocked with 1.5% normal donkey serum. Polyclonal antibodies against anti-NF-κB p65 (1 µg/well) were applied for 1 h followed by an 1 h incubation with FITC-conjugated donkey anti-rabbit IgG. The presence of cell nucleus was determined with DAPI. After washing with PBS, the coverslips were mounted in SlowFade Diamond (Thermo scientific, Rockford, IL, USA).

Cell death analysis

Induction of apoptosis in real time was quantified using a live cell apoptosis assay previously described (Puigvert et al. 2010). Briefly, binding of annexin V-Alexa633 conjugate to phosphatidyl serine on the membranes of apoptotic cells, intracellular fluorescence of propidium iodide (PI) in cells stained with Hoechst (nuclear staining) was followed in time by a Nikon TiE2000 confocal laser microscope.

Overall cell death (loss of membrane integrity) was determined by lactate dehydrogenase (LDH) release in the medium in essentially the same manner as described (Van de Water et al. 2001).

Microscopy

Accumulation of GFP levels, PI and Hoechst staining was monitored using a Nikon TiE2000 confocal laser microscope (lasers 540, 488 and 408 nm), equipped with an automated stage and perfect focus system at 37 °C with humidified atmosphere and 5% CO2/air mixture. All imaging was similar as previously described. 96-well plate contained one reporter cell line, which was exposed to all the compounds used (TVX, LVX or DMSO). For the ICAM1-GFP reporter experiments, cells were first exposed for 8 h to compound only; next, TNFα was added to all wells, up to a final concentration of 4 ng/ml. Directly after TNFα treatment the live cell imaging was started.
HepG2 RelA-GFP (NF-κB NF-κB , p65) and A20-GFP BAC reporter cell lines were generated and characterized as described previously (Herpers et al. 2016; Wink et al. 2017). Accumulation of GFP levels or nuclear translocation, and Hoechst staining was monitored using a Nikon TiE2000 confocal laser microscope (lasers:

640, 540, 488, and 408 nm). This microscope is equipped with an automated stage and perfect focus system at 37 °C with humidified atmosphere and 5% CO2/air mixture. HepG2 cells were stained with 50 ng/ml Hoechst33342 for 30 min to visualize the nuclei. The Hoechst medium was replaced with exposure medium containing the drugs and a final concentration of 4 ng/ml TNF. To prevent a delay in TNF response in the oscillations of the RelA-GFP reporter, solutions were added at the microscope per well, directly upon imaging of the first image (t = 0).

For RAW264.7 cells images were acquired with Olympus BX-60 microscope equipped with Leica CCD camera (Leica DFC425C) (40×0.5 objective).

Image analysis of fluorescent protein reporter activity

Quantitative image analysis was performed with CellProfiler version 2.1.1 (Kamentsky et al. 2011) with an in house developed CellProfiler module implementing the watershed masked algorithm for segmentation (Yan and Verbeek 2012; Wink et al. 2017). Image analyses results were stored as HDF5 files. Data analysis, quality control and graphics were performed using the in house developed R package h5CellProfiler. For each reporter intensity levels of the GFP signal, the nuclear Hoechst33342 intensity levels and at 24 h the PI staining were measured at the single cell level.

Western blot analyses

Approximately 50 mg of each liver sample was lysed using 500 µL RIPA lysis buffer (Thermo scientific, Rockford, IL, USA) containing protease and phosphatase inhibitors (Roche Applied Science, Penzberg, Germany). The total protein concentration of liver lysates and nuclear and cytoplasmic fractions of cell lines were measured by the BCA protein assay kit (Thermo scientific, Rockford, IL, USA). Standardized protein amounts (30 µg) of boiled samples were analyzed by electrophoresis in SDS-PAGE gel 4-20% or 7.5% and electro-transferred onto polyvinylidene difluoride membranes (Bio-Rad, Veenendaal, The Netherlands). Membranes were blocked with immersion in ethanol 100% for 1 min and afterwards rehydrated with TBS supplemented with 0.2% Tween (TBS-T) and incubated overnight with: anti-human p65 (Abcam, ab16502); anti-human phospho-p65 (Ser536) (Cell signaling Rabbit #3031S); anti-human clathrin HC (Cell signaling, #2410), anti-human lamin A/C (Cell signaling #2032); anti-human IκBα (Cell Signaling, #9242), anti-human phospho-p44/42 (Erk1/2) (Thr202/Tyr204) (Cell Signaling, #9101); anti-human phospho-p38 (Thr180/Tyr182) (Cell Signaling, #9211); anti-human phospho-IκBα (Ser32, clone: 14D4) (Cell signaling, #2859), anti-human phospho-IKKα/β (Ser176/180, clone; 16A6), (Cell signaling, #2697), anti-human phospho-SAPK/JNK (Thr183/Tyr185, clone: 81E11) (Cell Signaling, #4668). After washing in TBS-T, the membranes were incubated with rabbit anti-goat peroxidase-conjugated secondary antibody (1:5000, Dako, Glostrup, Denmark) for 1 h at room temperature. Finally, membranes were washed in TBS-T and once in TBS, incubated with ECL Prime Western Blotting Detection Reagent

(Amersham Biosciences, Roosendaal, The Netherlands), and digital images were obtained with the ChemiDoc XRS Quantity One (Bio-Rad Laboratories, Hercules, CA, USA). In the next step the membranes were re-probed with a β-Actin or Lamin A/C antibody (1:4000, Cell Signaling, MA, USA) to assess the equality of loading. Signal intensities were quantified using ChemiDoc XRS Quantity One (Bio-Rad Laboratories, Hercules, CA, USA), and protein expression normalized with β-Actin.

qPCR

For mRNA studies, the medial part of the biggest liver lobe from each mouse (approximately 50 mg) was snap frozen in liquid nitrogen and stored at −80 °C untill RNA isolation. Each sample was suspended in 500 µL RNA InstaPure (Eurogentec) and homogenized using a TissueLyser (Qiagen, Hilden, Germany) for 1 min/25 Hz twice. The homogenized tissue was centrifuged for 10 min at 12,000 × g. The supernatant, containing RNA in RNA Insta-Pure was transferred to a new vial and RNA was isolated using phenol-chloroform extraction. The amount of RNA was determined using the NanoDrop 2000 Spectrophotometer (ThermoScientific). Subsequently, 1 µg of extracted total RNA was reverse transcribed with the iScriptTM cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative reverse transcriptase PCR was performed suing a iCycler iQ system (Biorad), and amplification was done using iQ SYBR Green supermix (Biorad) with 0.3 µM final primer concentration. Primer sequences:
mouse ICAM-1 FW-GCTACCATCACCGTGTATTCG and RV-TAGCCAGCACCGTGAATGTG;
mouse A20 FW-GAACCAGATTCCATGAAGCAA and RV-CCTGTAGTTCGAGGCATGTC;
mouse IκBα FW-AGGAGTACGAGCAAATGGTG and RV-CGGCTTCTCTTCGTGGATG;
mouse c-IAP1 FW-TTGAGCAGCTGTTGTCCACTTC and RV-GGCCAAAATGCACCACTGT;
mouse c-IAP2 FW-AGGGACCATCAAGGGCACAGTG and RV-TTGGCGGTGTCTCGTGCTATC;
mouse MIP-2 FW- AAAGTTTGCCTTGACCCTGAAG and RV-CAGTTAGCCTTGCCTTTGTTCAGT;
mouse TNF FW-AACGGCATGGATCTCAAAGA and RV-TTTCTCCTGGTATGAGATAGCAAATC; human A20 FW-GCGTTCAGGACACAGACTTG and RV-TTCATCATTCCAGTTCCGAGTATC;
human IκBα FW-GCTGAAGAAGGAGCGGCTACT and RV-TCGTACTCCTCGTCTTTCATGGA;
human IL-8 FW-CTCTTGGCAGCCTTCCTGATT and RV-TATGCACTGACATCTAAGTTCTTTAGCA;
human c-iap1 FW-AGCTGTTGTCAACTTCAGATACCACT and RV-TGTTTCACCAGGTCTCTATTAAAGCC;
human c-IAP2 FW-ACTTGAACAGCTGCTATCCACATC and RV-GTTGCTAGGATTTTTCTCTGAACTGTC.

Gene specific primers were derived from the NCBI GenBank and were manufactured

commercially (Eurogentec, Seraing, Belgium). For each sample, mRNA expression was normalized for the detected Ct value of GAPDH or β-actin. Data are expressed as fold increase compared with the control group (vehicle only).

Statistical analyses

Data are presented as means ± standard error of the mean (SEM). Statistical significance for comparisons was determined by ONE- or TWO-way ANOVA with Dunnett's post-hoc test. A P value less than 0.05 was considered statistically significant. All data are analyzed using GraphPad Prism (version 6.07) software (San Diego, CA, USA).

RESULTS

Co-incubation with Trovafloxacin (TVX) and Tumor Necrosis Factor (TNF) induces apoptosis in HepG2 cells.

HepG2 cells were exposed to TVX (20μM), LVX (50μM) or DMSO (0.01% max) for 24 h either in presence or in absence of TNF (4 ng/ml). TNF concentration used resembles that found in human blood during inflammatory stress (Copeland et al. 2005; Taudorf et al. 2007). Incubation of cells with TVX+TNF, but not with any of the other treatments, showed a gradual increase of Annexin V-staining and an increased leakage of LDH at 24 h. (Fig.2a-b).

TVX disrupts TNF-induced p65 nuclear translocation.

Because of the fundamental role of NF-κB NF-κB in hepatocyte survival after TNF stimulation (Fredriksson et al. 2011) we decided to investigate the effects of TVX and LVX on TNF-induced NF-κB nuclear translocation in the liver of mice and in HepG2 cells. TNF was administered to the mice 3 h after single oral administration of drugs or vehicle (Fig. 1) and 2 h after the cytokine injection liver tissues were collected for immunofluorescence detection of nuclear and cytoplasmic p65 levels. TVX+TNF-treated mice showed a greater number of cells with increased nuclear/cytoplasmic p65 ratio in liver tissue when compared with LVX+TNF- or Veh+TNF-treated mice (Fig.3a-b). Of note, liver tissue of these latter two groups did not show any significant difference in the distribution of p65 nuclear/cytoplasmic ratio values, but all treatments that included TNF significantly increased the number of hepatocytes with a high nuclear/cytoplasmic ratio when compared with Veh+PBS (Fig.3a-b).

HepG2 cells have been shown to reproduce the TVX-induced transcriptional signature as observed in primary human hepatocytes (Liguori et al. 2008), indicating that HepG2 are good model cells to study TVX toxicity. To verify and describe in more detail the effect of TVX on the dynamics of p65 translocation we analyzed the kinetics of nuclear translocation of p65 using genetically modified p65-GFP-

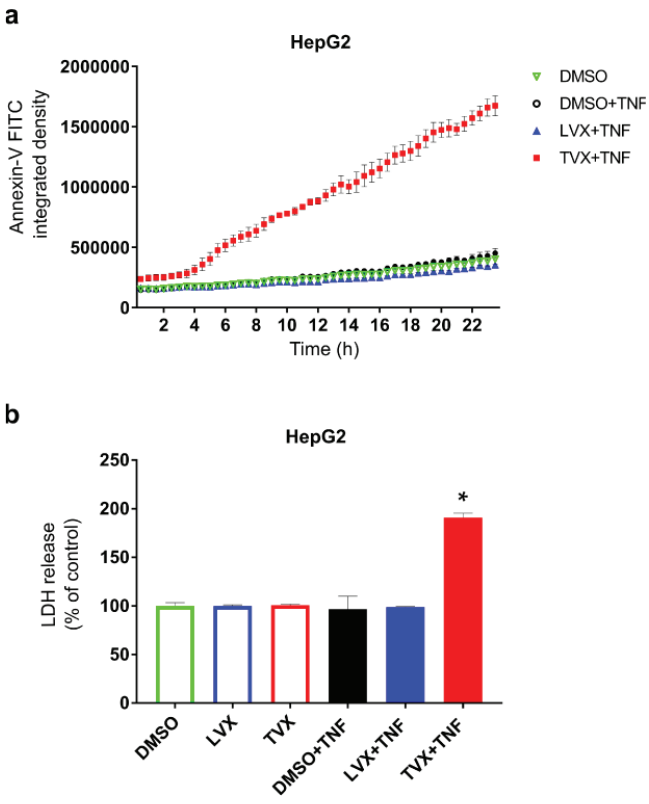


Figure 2

TVX+TNF induced cell death in HepG2

HepG2 cells were incubated for 24 h in presence of TVX, LVX or DMSO with or without TNF (4 ng/ml). (a) Treatment with TVX+TNF induces apoptosis in HepG2 cells (increased annexin V-Alexa633 fluorescence). Presented data are representative results for the experiments which were performed three times (b) Coherently, only TVX+TNF significantly increased the LDH in the supernatant of HepG2 cells, here shown for analyzes 24 h after incubation. Data are presented as mean ± SEM; * $p < 0.05$ when compared with all the other treatments assessed

tagged HepG2 cells. All TNF treatments promoted a clear nuclear translocation of p65, and both LVX- and DMSO-treated cells displayed pulsatile oscillations of the p65 nuclear/cytoplasmic ratio after the exposure to TNF. TVX-treated cells showed a unique and sustained peak of p65 nuclear translocation occurring between 15 and 90 min after treatment, without further oscillations (Fig. 3c).

We confirmed the TVX-induced alterations of p65 translocation in non-mutant HepG2 using quantification of p65 protein levels in nuclear and cytoplasmic extracts of cells treated with DMSO+TNF or TVX+TNF and analyzed by Western blot (Fig. 3d). Simultaneously to nuclear translocation, phosphorylation of p65 occurred on Ser536 in both treatments (Fig.3d), and apart from abrogating further p65 nuclear translocations TVX also lowered its phosphorylation at Ser536 after 120 min to values comparable to levels at 0 min (Fig.3d).

TVX affects TNF-induced expression of NF-κB-related genes.

The translocation of NF-κB is a finely orchestrated process, defining transcription

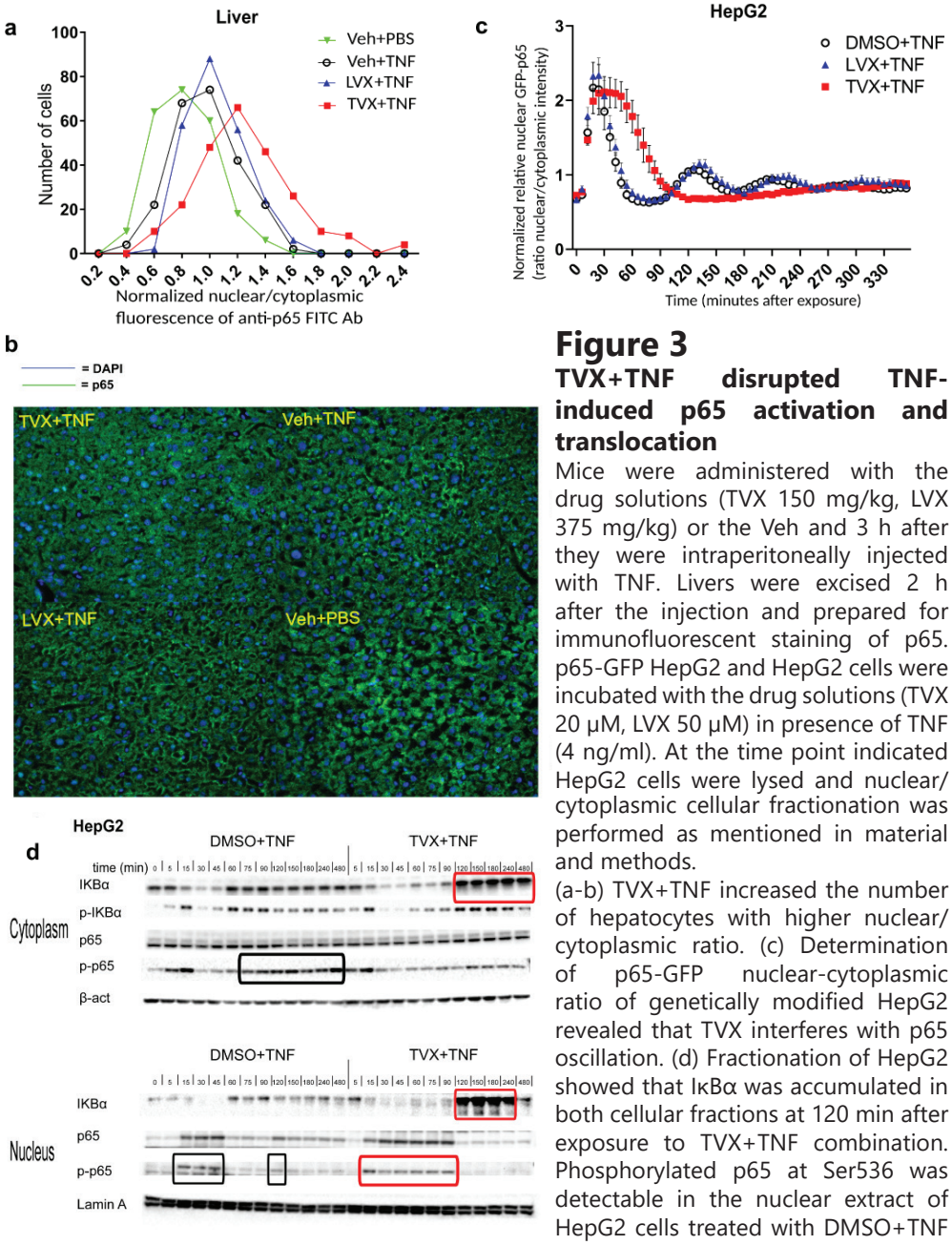


Figure 3

TVX+TNF disrupted TNF-induced p65 activation and translocation

Mice were administered with the drug solutions (TVX 150 mg/kg, LVX 375 mg/kg) or the Veh and 3 h after they were intraperitoneally injected with TNF. Livers were excised 2 h after the injection and prepared for immunofluorescent staining of p65. p65-GFP HepG2 and HepG2 cells were incubated with the drug solutions (TVX 20 μM, LVX 50 μM) in presence of TNF (4 ng/ml). At the time point indicated HepG2 cells were lysed and nuclear/cytoplasmic cellular fractionation was performed as mentioned in material and methods.

(a-b) TVX+TNF increased the number of hepatocytes with higher nuclear/cytoplasmic ratio. (c) Determination of p65-GFP nuclear-cytoplasmic ratio of genetically modified HepG2 revealed that TVX interferes with p65 oscillation. (d) Fractionation of HepG2 showed that IκBα was accumulated in both cellular fractions at 120 min after exposure to TVX+TNF combination. Phosphorylated p65 at Ser536 was detectable in the nuclear extract of HepG2 cells treated with DMSO+TNF between 15 and 45 min, whereas TVX prolonged its nuclear permanence up to 90 min. Representative blots of 3 experiments are presented. Presented data on GFP-p65 and FITC-anti-p65 fluorescence are results of experiments which were performed three times.

of downstream target genes which can be categorized as early (e.g. encoding cytokines and negative regulators of NF-κB pathway), mid (e.g. anti-apoptotic genes) and late (e.g. cell surface receptors) factors (Tian et al. 2005; Zambrano et al. 2016). To obtain further mechanistic insights into the effects of TVX, we set out to examine the kinetics of transcription and translation of NF-κB-related genes (early, middle and late) in presence of TVX+TNF, LVX+TNF and DMSO+TNF.

TVX increases expression of early NF-κB-related factors A20 and IκBα.

Both A20 (TNFAIP3) and IkBα are early NF-κB target genes (Tian et al. 2005). The combination of TVX+TNF caused a higher increase in the transcription of A20 and IκBα in liver homogenates (Fig. 4a) as well as HepG2 cells (Fig. 4c) 2 h after TNF injection or incubation when compared with respective controls (Veh+TNF in vivo and DMSO+TNF in vitro). By contrast, the hepatic protein levels of IκBα were decreased upon TVX+TNF treatment 2 h after TNF when compared to all other groups receiving the cytokine (LVX+TNF, Veh+TNF) (Fig. 4b). In HepG2, - LVX+TNF also elicited a significant increase, in A20 mRNA after 1 h of incubation when compared with cells treated with DMSO+TNF (Fig. 4d). Whereas the LVX+TNF-induced increase of A20 mRNA was not reflected by protein expression of the GFP-A20 in HepG2 cells (Fig. 4e), TVX+TNF induced delayed and sustained A20 expression which was associated with higher expression of GFP-A20 beginning 2 h after incubation (Fig. 4e).

Nuclear and cytoplasmic extracts from TVX+TNF-incubated cells showed an accumulation of IκBα in both cellular fractions. Remarkably, higher and stable levels of IκBα were observed from 2 to 4 h in the nuclear extracts and up to 8 h in the cytoplasmic fractions obtained from TVX+TNF-treated HepG2 (Fig. 3c).

TVX induces different transcriptional regulation of NF-κB-induced inhibitors of apoptosis.

TNF-mediated activation of NF-κB causes expression of anti-apoptotic genes, including the IAP1 family members. Veh+TNF- or LVX+TNF-treatment of mice resulted in increased expression of c-IAP1 in liver homogenates when compared with mice receiving Veh+PBS (Fig. 5a). By contrast, in TVX+TNF- treated mice c-IAP1 mRNA expression was equal to the expression observed in mice receiving Veh+PBS (Fig. 5a). In HepG2 cells, TVX+TNF significantly decreased c-IAP1 gene expression as observed 4 h after the treatment when compared with their respective controls (Fig. 5b). TVX+TNF also decreased XIAP gene expression which was significantly different from controls at 6 h (Fig. 5c). Conversely, c-IAP2 mRNA showed a significant increase in TVX+TNF-treated cells, apparent at 2 h in association with enhanced peak translocation of NF-κB but followed by a rapid decline at 4 h (Fig. 5d). No differences in XIAP and c-IAP2 mRNA expression were observed either in liver and spleen of mice treated with TVX and TNF (data not shown).

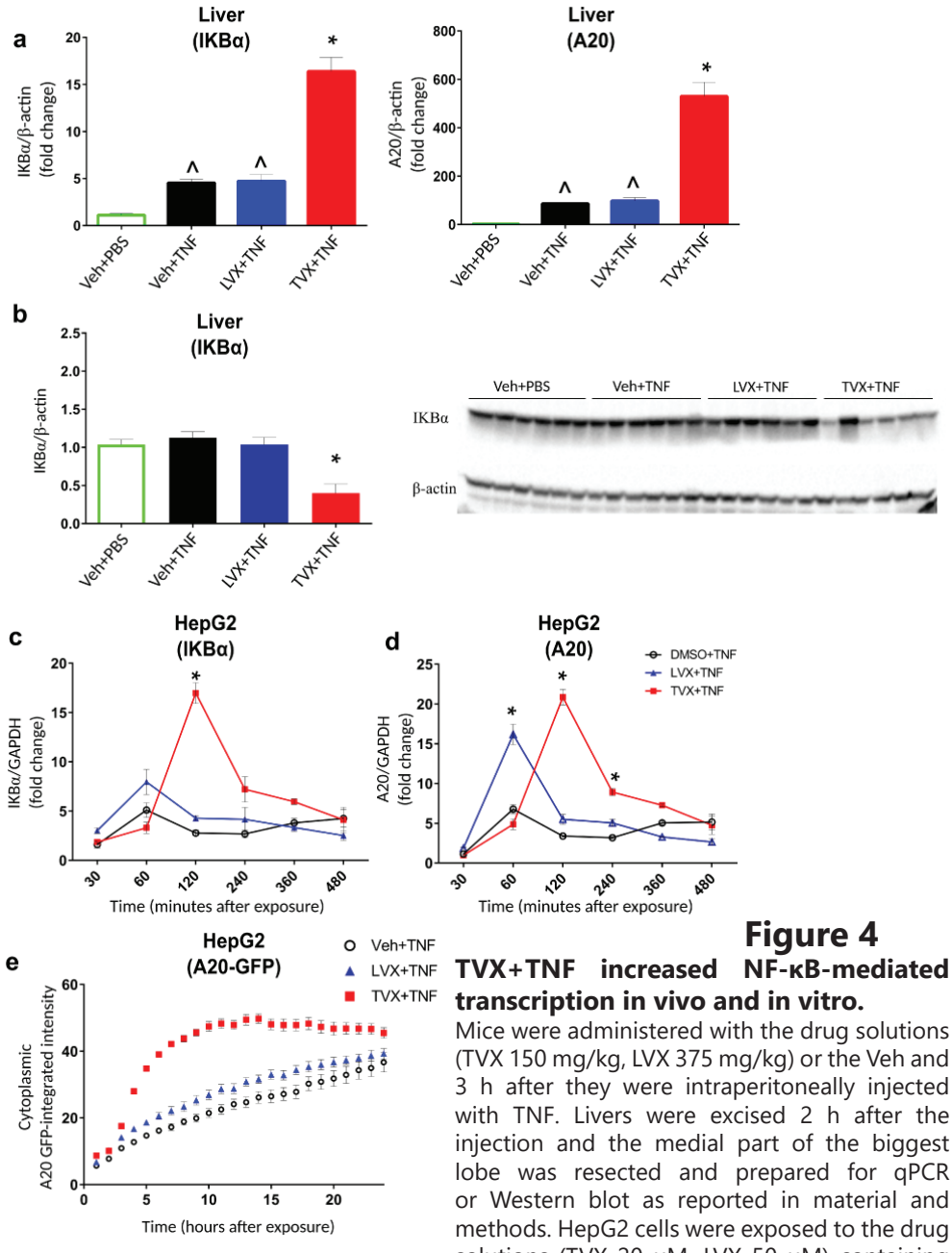


Figure 4

TVX+TNF increased NF-κB-mediated transcription in vivo and in vitro.

Mice were administered with the drug solutions (TVX 150 mg/kg, LVX 375 mg/kg) or the Veh and 3 h after they were intraperitoneally injected with TNF. Livers were excised 2 h after the injection and the medial part of the biggest lobe was resected and prepared for qPCR or Western blot as reported in material and methods. HepG2 cells were exposed to the drug solutions (TVX 20 μM, LVX 50 μM) containing TNF (4 ng/ml) and prepared for qPCR as reported in materials and methods. (a) TVX+TNF significantly increased the TNF-induced mRNA transcription of IκBα (left panel) and A20 (right panel) in liver when compared with LVX+TNF- or Veh+TNF-treated mice. (b) TVX+TNF significantly decreased hepatic IκBα protein levels when compared with the other treatments. (c-d) TVX+TNF significantly increased TNF-induced IκBα (left panel) and A20 (right panel) transcription in HepG2 cells apparent at 2 h after incubation. LVX+TNF significantly increased

the TNF-induced transcription of A20 1 h after the exposure when compared with mice receiving Veh+TNF. (e) TVX+TNF, but not LVX+TNF, significantly enhanced the fluorescence by GFP-A20 HepG2 cells. Fold increase was calculated by comparison with liver or cell lysates were exposed to Veh+PBS or DMSO+PBS. Presented data for GFP-A20 expression are representative results of experiments which were performed three times. All the other data are presented as mean ± SEM; * $p < 0.05$ when compared with all the other treatments assessed at the same time point; ^ $p < 0.05$ when compared with liver homogenates obtained from mice receiving only the Veh without TNF.

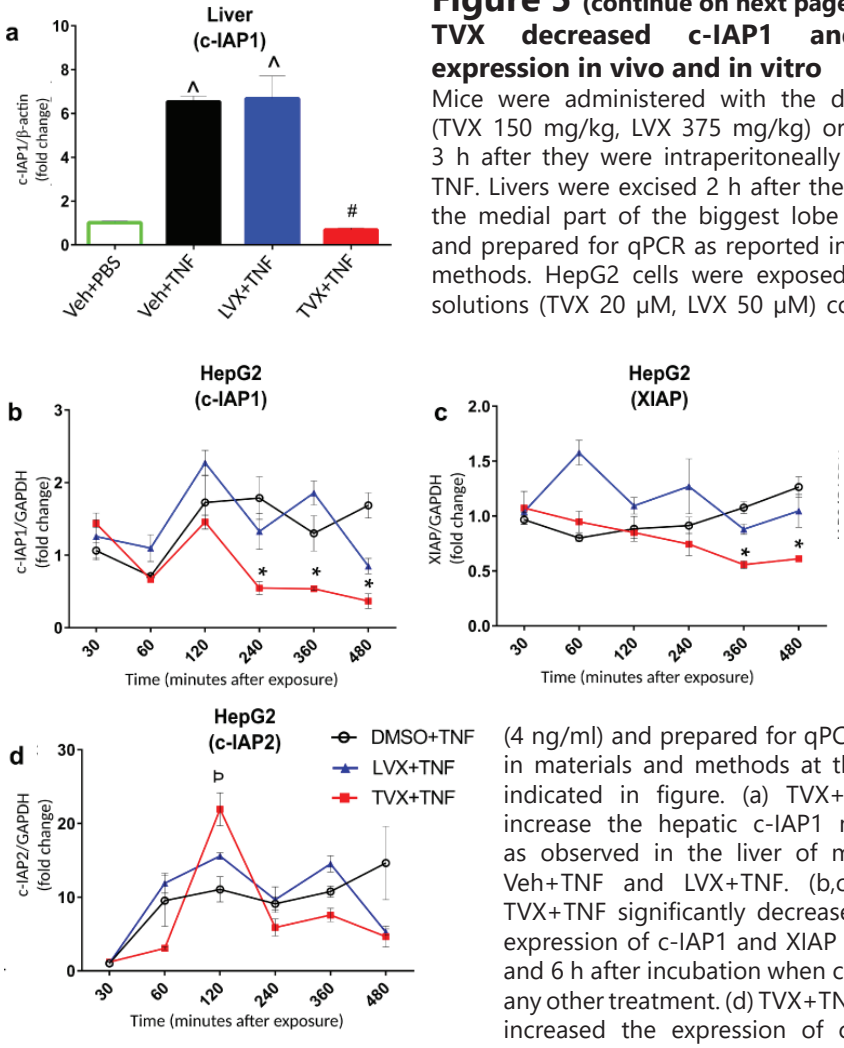
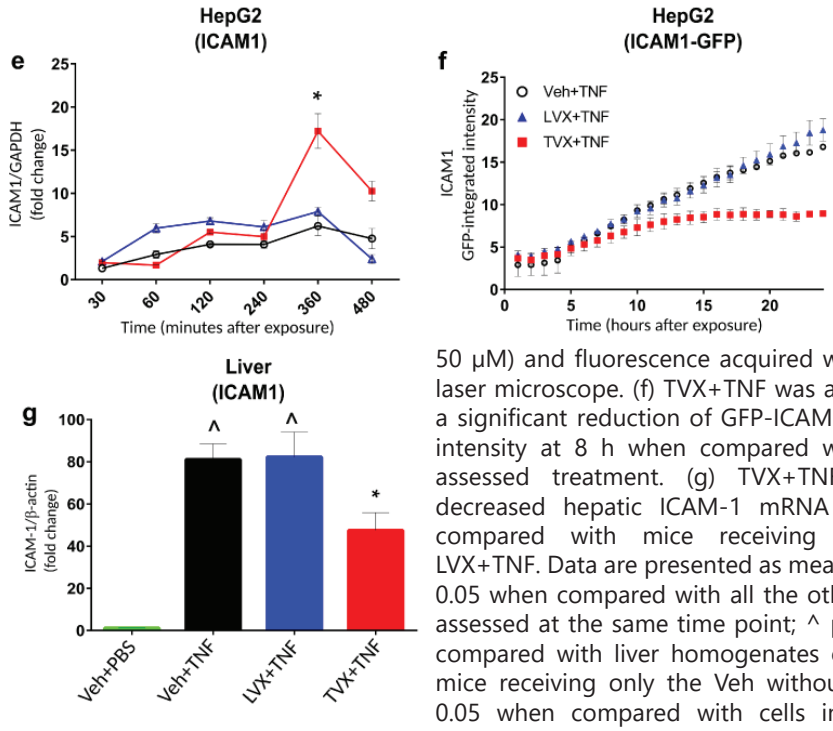


Figure 5 (continue on next page)

TVX decreased c-IAP1 and ICAM-1 expression in vivo and in vitro

Mice were administered with the drug solutions (TVX 150 mg/kg, LVX 375 mg/kg) or the Veh and 3 h after they were intraperitoneally injected with TNF. Livers were excised 2 h after the injection and the medial part of the biggest lobe was resected and prepared for qPCR as reported in material and methods. HepG2 cells were exposed to the drug solutions (TVX 20 μM, LVX 50 μM) containing TNF (4 ng/ml) and prepared for qPCR as reported in materials and methods at the time point indicated in figure. (a) TVX+TNF did not increase the hepatic c-IAP1 mRNA copies as observed in the liver of mice receiving Veh+TNF and LVX+TNF. (b,c) In HepG2, TVX+TNF significantly decreased the mRNA expression of c-IAP1 and XIAP respectively 4 and 6 h after incubation when compared with any other treatment. (d) TVX+TNF significantly increased the expression of c-IAP2 mRNA in HepG2 cells 2 h after incubation when compared with cells exposed to DMSO+TNF. (e) TVX+TNF significantly increased ICAM-1 mRNA 6 h after incubation with HepG2 cells when compared with any other treatment. Fold increase was calculated by comparison with liver or cell lysates that respectively were exposed to Veh+PBS and DMSO+PBS. GFP-ICAM1 modified HepG2 cell were exposed for 24 h to the indicated treatments (TVX 20 μM, LVX



50 μM) and fluorescence acquired with a confocal laser microscope. (f) TVX+TNF was associated with a significant reduction of GFP-ICAM1 fluorescence intensity at 8 h when compared with any other assessed treatment. (g) TVX+TNF significantly decreased hepatic ICAM-1 mRNA copies when compared with mice receiving Veh+TNF or LVX+TNF. Data are presented as mean ± SEM; * $p < 0.05$ when compared with all the other treatments assessed at the same time point; ^ $p < 0.05$ when compared with liver homogenates obtained from mice receiving only the Veh without TNF; Þ $p < 0.05$ when compared with cells incubated with DMSO+TNF.

4

TVX reduces ICAM-1 expression.

ICAM1 is a late NF-κB target gene involved in pro-inflammatory responses (Tian et al. 2005). TVX+TNF increased the number of ICAM-1 mRNA copies when compared with any of the other treatments, with a clear increase at 6 h after treatment (Fig. 5e). By contrast, TVX prevented protein expression of ICAM-1 from 8 h of incubation onward (Fig. 5f, squares). LVX+TNF did not affect the TNF-induced protein expression of GFP-ICAM1 within the 24 h incubation. Importantly, all mice receiving TNF, independently of the drug administered, increased the expression of ICAM-1 mRNA in liver at 2 h time point (Fig. 5g). At the same time point, the TNF-induced ICAM-1 expression was lower in TVX+TNF-treated mice compared to other TNF-treated groups (Fig. 5g). By contrast, no differences in ICAM1 transcription was observed in the spleens of different treatment groups (data not shown).

TVX increases transcription of NF-κB-dependent genes via prolongation of LPS-induced NF-κB translocation in murine RAW264.7 macrophages.

The prolongation of NF-κB translocation observed in presence of TVX prompted

us to investigate if this also occurred in macrophages after LPS stimulation. Indeed, LPS stimulation of e.g. toll-like receptors (TLRs) induces translocation of NF-κB resulting in increased synthesis of TNF (Sakai et al. 2017). LPS stimulation of RAW264.7 cells resulted in a significant increase in mRNA copies of NF-κB-dependent genes A20, IκBα and TNF, 30 min after the exposure with the bacterial components (Fig. 6a-c). Two hours after incubation, TVX+LPS sustained the increased expression of TNF and enhanced A20 and IκBα mRNA transcripts (Fig. 6a-c).

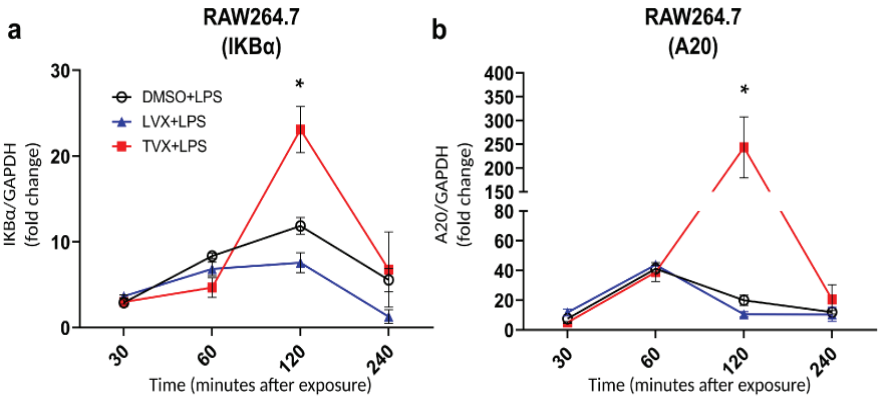
To determine if TVX also affected LPS activation of NF-κB in macrophages we performed an intracellular staining with an antibody against the p65 subunit and assessed its nuclear translocation in RAW264.7 cells. DMSO+LPS and LVX+LPS exerted a peak of translocation of p65 in between 10 and 45 min when the nuclear/cytoplasmic ratio of the fluorescence returned to a lower level (Fig. 6d). TVX+LPS prolonged the first peak of p65 translocation from 10 to 60 min when returned to levels of the ratio which were comparable with the other groups of treatment (Fig. 6d).

TVX prolongs TNF-induced activation of MAPKs and IKKα/β activation in HepG2.

Based on the observed variations of TNF-induced expression of NF-κB related genes in HepG2, mouse liver and the effects of TVX, we wanted to define the kinetics of downstream regulators of TNF-receptor activation, such as IKKα/β and MAPK (JNK1/2, ERK, p38). Of relevance, JNK activation is critical in the synergistic TNF-induced cytotoxicity caused by other toxicants, including diclofenac (Fredriksson et al. 2011; Maiuri et al. 2015) and cisplatin (Benedetti et al. 2013).
TVX+TNF co-incubation led to a prolonged activation of IKKα/β and the other MAPKs (especially JNK1/2 and ERK) when compared with cells incubated with DMSO+TNF (Fig. 7). Phosphorylation of IKKα/β (Fig.7a,c) and JNK1/2 (Fig.7a-b) took place between 15 to 45 min in TNF-treated HepG2 cells, whereas TVX extended their activation up to 90 min (Fig. 7). Cells treated with TVX+TNF showed also a significant increase in ERK and JNK1/2 activation after 8 h when compared with DMSO+TNF-treated cells (Fig. 7a).

TVX alone affects transcription of NF-κB related genes

To better understand the effects of TVX on TNF-induced transcription and pathway activation we tested the effect of TVX at the same time points without TNF both in vivo and in vitro. Remarkably, the hepatic transcription of A20 and IκBα was also increased 3 h after the TVX administration (Fig. 8a and b), and analogously, the protein levels of IκBα (Fig.8c). TVX alone significantly reduced c-IAP1 mRNA expression 3 h after oral administration in liver homogenates when compared with the other assessed treatments (Fig. 8d) but not in spleen (data not shown). A significant decrease of ICAM1 was observed 3 h after drug administration in the



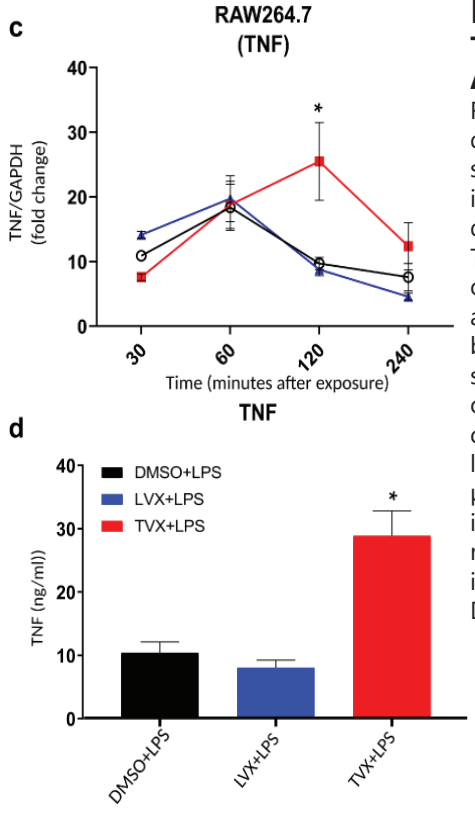
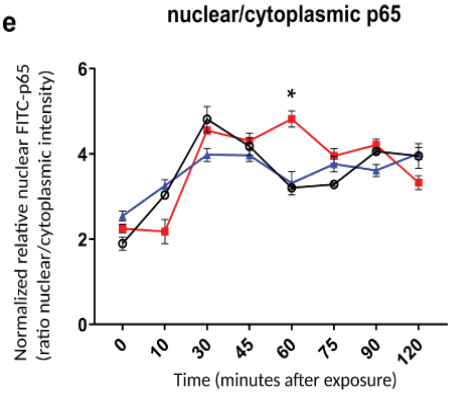


Figure 6

TVX+LPS increased the expression of IκBα, A20 and TNF in RAW264.7 cells

RAW264.7 cells were incubated for 2 h with the drug solutions (TVX 20 μM, LVX 50 μM) and then stimulated with LPS (10 ng/ml). At the time point indicated in figure cells were lysed and prepared for qPCR as reported in material and methods. (a,b,c) TVX+LPS significantly increased the mRNA copies of IKB, A20 and TNF when compared with any other assessed treatment 2 h after the addition of the bacterial component. (d) TVX+LPS increased the secretion of TNF in the supernatant of RAW264.7 cells 4 h after the exposure when compared with cell exposed to Veh+LPS or LVX+LPS. For p65 localization RAW264.7 cells were plated in IBIDI μ-slide 8 well and fixed at the time point showed in figure. (e) Detection of FITC-anti-p65 antibody revealed that TVX+LPS prolonged p65 translocation in the nucleus of RAW264.7 up to 75 min whereas DMSO+LPS and LVX+LPS returned to lower ratio values after 60 min. Presented data for FITC-p65 localization are representative results for the experiments which were performed three times. Data are presented as mean ± SEM; * $p < 0.05$ when compared with all the other treatments assessed at the same time point.



liver of either TVX-treated and LVX-treated mice when compared with the control (Fig. 8e). No differences in XIAP and c-IAP2 mRNA expressions were observed either in liver and spleen 3 h after drug administration (data not shown).

In HepG2, TVX (20 μM) alone already caused a small but clear increase in the transcription of both A20 and IκBα, being significant respectively from 2 and 4 h after incubation onward (Fig. 9a and b). After 2 h of exposure with TVX we also observed by Western blot analysis significant increases in cytoplasmic protein expression of IκBα (Fig. 9c and d) and A20 (Fig. 9e) when compared with DMSO-treated cells. Between 2 and 8 h, protein levels of IκBα and A20 increased in time in TVX-treated cells (Fig.9c-e). LVX itself did not exert any significant effect on the expression of these factors (Fig.9a-h). TVX significantly decreased c-IAP1 gene expression as observed 4 h after the treatment of HepG2 cells when compared with cells receiving DMSO (Fig. 9f). XIAP showed the same tendency to decrease after TVX exposure when compared with cells incubated with DMSO but resulting significantly different at 6 h (Fig. 9g). Incubation of HepG2 cells with TVX, LVX or DMSO did not change either transcription and translation of ICAM-1 gene (data not shown).

TVX exposure in RAW264.7 cells resulted in increased transcription of A20, IκBα but not of TNF (Supplemental data) already 2 h after the incubation with the drugs when compared the same cells incubated with DMSO or LVX.

TVX itself induced p65 translocation in HepG2.

The increased transcription of IκBα and A20 in mice pretreated with TVX before TNF injection prompted us to investigate if TVX by itself already affected p65 translocation. We therefore followed the kinetics of the p65-GFP fluorescence in HepG2 cells for 8 h upon incubation with TVX, LVX or the solvent DMSO in absence of TNF. Data shows that TVX alone induced a profound non-oscillatory p65-GFP translocation into the nucleus beginning immediately after incubation and terminating approximately 120 min after the exposure, whereas LVX or DMSO did not exert any effect on p65 translocation (Fig.10a).

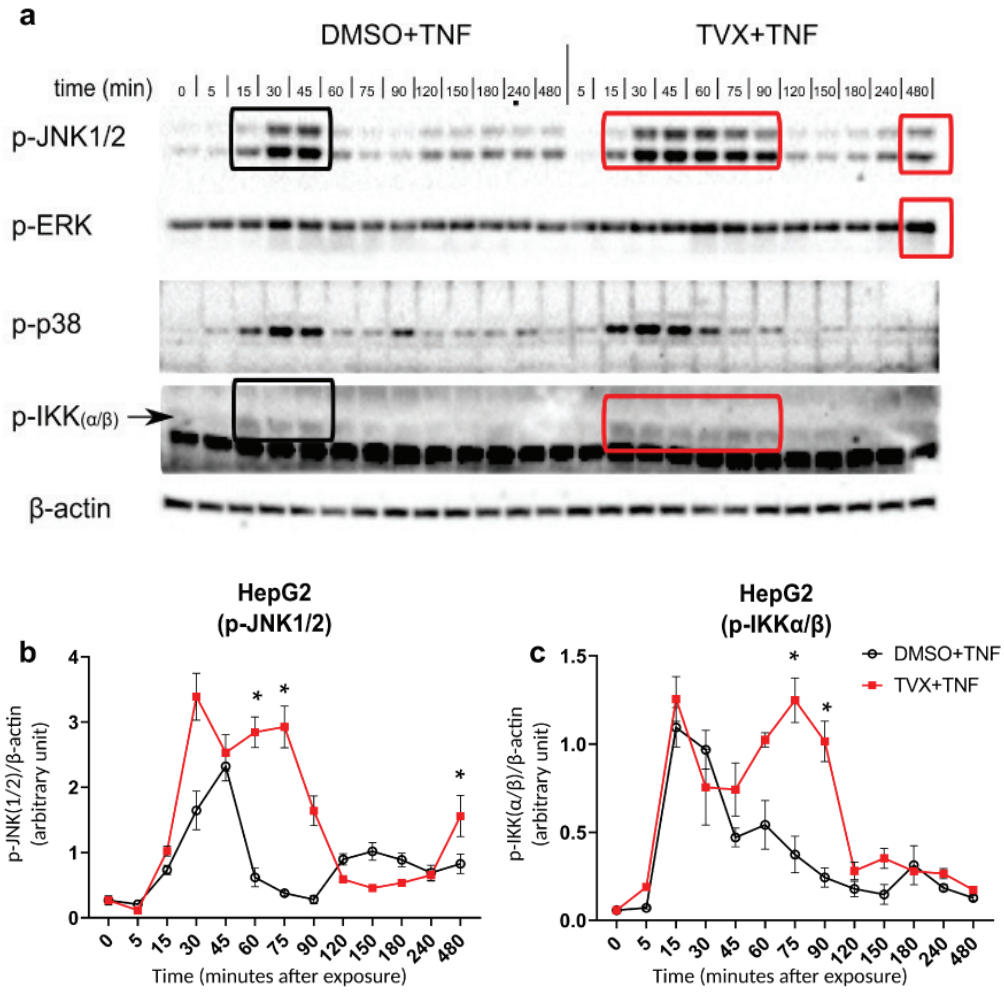


Figure 7

TVX+TNF showed prolonged IKKα/β and JNK1/2 phosphorylation

HepG2 cells were incubated with the drug-cytokine combinations, lysed at the time point indicated in figure and processed as reported in material and methods. (a,b) TVX+TNF mainly prolonged JNK1/2 and (a,c) IKKα/β phosphorylation mediated by TNF receptor activation when compared with cells treated with DMSO+TNF. Representative blots of 3 experiments are presented. Data are presented as mean ± SEM; * $p < 0.05$ when compared with all the other treatments assessed at the same time point.

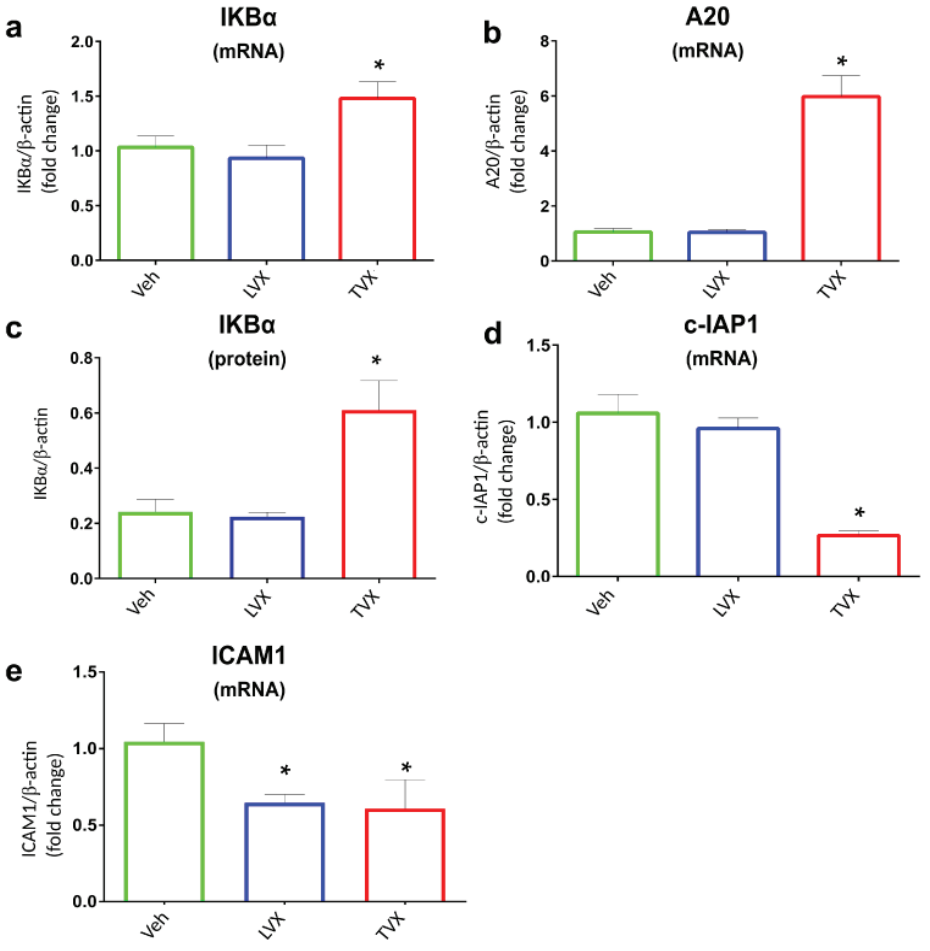


Figure 8

TVX increased hepatic IκBα and A20 expression and reduced those of ICAM-1 and c-IAP1 in vivo

Mice were fasted for 7 h and then orally administered with the drug solutions (TVX 150 mg/kg, LVX 375 mg/kg). Three hours after mice were culled, and the medial part of the biggest lobe was resected and prepared for qPCR or western blot as reported in material and methods. (a,b) TVX administration was associated with an increase of IκBα and A20 mRNA and of (c) IκBα protein expression in liver lysates when compared with those of mice treated with LVX or Veh. (d) TVX significantly reduced the mRNA copies of c-IAP1 in liver lysates when compared with the levels observed in those of mice receiving LVX or Veh. (e) TVX and LVX treatments were associated with a reduced ICAM-1 expression in liver lysates when compared with that observed in mice treated with only Veh. Data are presented as mean ± SEM; * $p < 0.05$ when compared with all the other treatments assessed at the same time point.

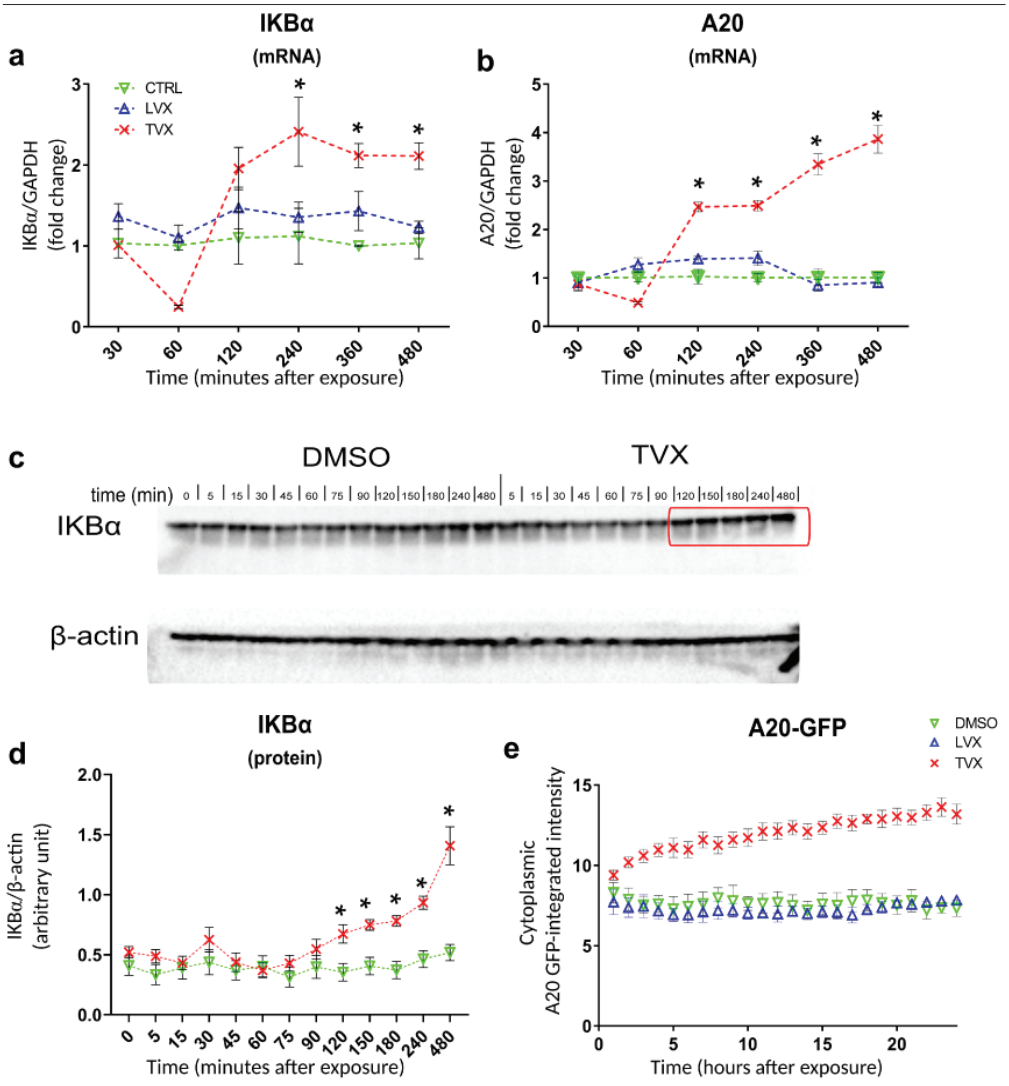
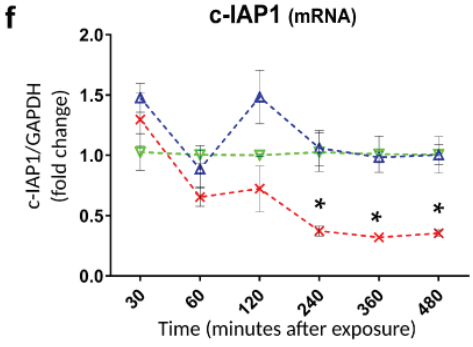


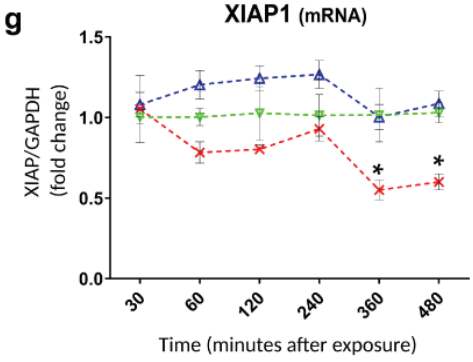
Figure 9 **(continue on the next page)**

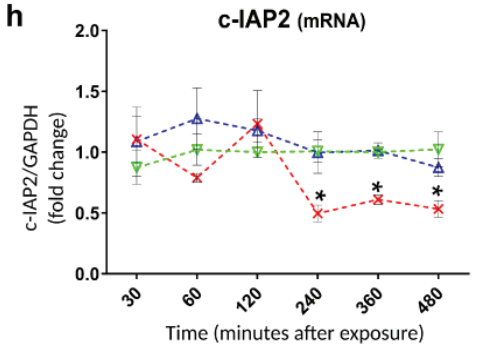
TVX increased expression of IκBα and A20 in HepG2

HepG2 cells were incubated with TVX (20μM), LVX (50μM) and DMSO for the time showed in figure. At each time point cells were lysed and prepared for qPCR or western blot as mentioned in materials and methods. (a-b) TVX increased the mRNA copies of IκBα and A20 respectively 4 and 2 h after drug incubation when compared with cells exposed to DMSO. (c-d) TVX significantly increased the IκBα protein levels in HepG2 2 h after incubation when compared with cells exposed to DMSO. Representative blot is presented. (f-h) TVX significantly decreased the mRNA expression of c-IAP1, XIAP and c-IAP2 in HepG2 at the incubation time indicated in figure, whereas LVX and DMSO did not exert any significant effect. GFP-A20 modified HepG2 were exposed to the drug solutions and then fluorescence was assessed every hour for 24 h. (e) Increased GFP fluorescence was observed in the



cytoplasm of TVX-treated HepG2 during the 24 h of exposure. No significant variance in the cytoplasmic GFP fluorescence was observed in modified HepG2 incubated with LVX or DMSO. Presented data on cytoplasmic A20 expression are representative results for the experiments which were performed three times. Other data are presented as mean ± SEM; * $p < 0.05$ when compared with all the other treatments assessed at the same time point.





DISCUSSION

In order to improve drug safety and prevent new potential hepatotoxic pharmaceuticals reaching the market it is imperative to elucidate possible mechanisms behind the development of DILI. The main challenges are to identify processes that explain (part of) the idiosyncrasy and immune involvement in these adverse reactions.

Although previous experiments on HepG2 and RAW264.7 cells have demonstrated the involvement of DNA Damage Response (DDR) in TVX-induced hepatotoxicity (Poulsen et al. 2014; Beggs et al. 2014) the events leading to the activation of MAPKs and increased synthesis of pro-inflammatory cytokines have not been characterized previously.

In this study, we show that TVX affected several molecular processes by itself, but in particular modulated processes triggered by TNF (in hepatocytes) or by LPS (in macrophages). We demonstrate that unlike the characteristic TNF-induced oscillatory nuclear translocation of NF-κB in HepG2, TVX+TNF combination promoted a single and prolonged translocation of NF-κB into the nucleus. The suppressive effect on p65 translocation was accompanied by enhanced

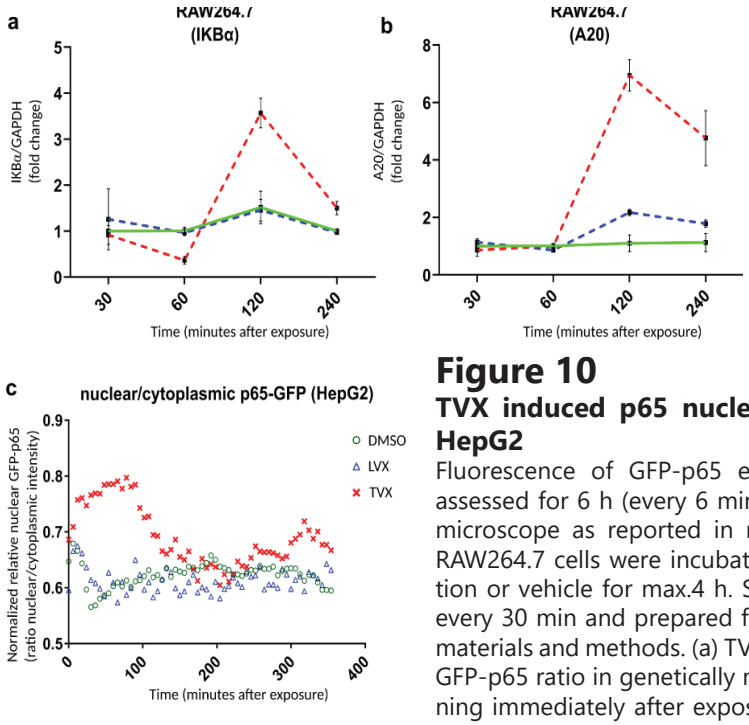


Figure 10

TVX induced p65 nuclear translocation in HepG2

Fluorescence of GFP-p65 expressing HepG2 was assessed for 6 h (every 6 min) with a laser confocal microscope as reported in material and methods. RAW264.7 cells were incubated with the drug solution or vehicle for max.4 h. Samples were collected every 30 min and prepared for qPCR as reported in materials and methods. (a) TVX induced an increased GFP-p65 ratio in genetically modified HepG2 beginning immediately after exposure with the drug and terminating approximatively 120 min after. No effects on GFP-p65 localization was observed with LVX or DMSO treatment. (b-c) TVX significantly increased the expression of IκBα and A20 2 h after incubation. Whereas expression of IKB mRNA in TVX-exposed cells returned to vehicle levels at 4 h, A20 mRNA transcripts were still significantly increased. * $p < 0.05$ when compared with all the other treatments assessed at the same time point. Presented data for GFP-p65 translocation are representative results for the experiments which were performed three times.

accumulation of known NF-κB inhibitors, A20 and IκBα, genes that are activated early after TNF treatment. By contrast, interference with TNF-mediated NF-κB oscillatory translocation by TVX was associated with a decreased transcription of NF-κB-associated middle and late factors (XIAP, c-IAP1 and ICAM1). The prolongation of TNF-induced NF-κB translocation by TVX appears to be due to extended activation of IKKα/β and related to MAPKs activation. Similarly to what we observed in HepG2, TVX also prolonged LPS-induced p65 translocation in RAW264.7 cells and increased expression of A20, IκBα and TNF. We propose that the enhanced early expression of negative regulators of pro-inflammatory signaling suppresses the later expression of anti-apoptotic and pro-inflammatory molecules. In particular the lack of suppressors of apoptosis, such as IAPs, will allow the onset of apoptosis.

The TVX-induced increased mRNA expression of IκBα was associated with elevated

p65 nuclear/cytoplasmic ratio in vivo. The apparent contradiction between in vivo expression of IKBα mRNA and protein prompted us to further characterize the TNF-induced translocation of NF-κB in presence of TVX in vitro in HepG2. TVX+TNF doubled the duration of the first nuclear peak of p65 which was phosphorylated at Ser536. Since phosphorylation of p65 determines its transcriptional activity (Sakurai et al. 1999), it was important to note that p65 was phosphorylated at Ser536 residues for the complete duration of the unique translocation occurring in TVX+TNF treated cells. This evidence well explains the increased protein expression of early NF-κB-dependent genes IκBα and A20.

The aberrant expression of NF-κB inhibitors IκBα and A20 occurring after exposure with TVX+TNF may in turn explains the abrogation of secondary NF-κB oscillations. Other studies have demonstrated that NF-κB translocation and transcription is inhibited by expression of a non-degradable IκBα mutant or by overexpression of native IκBα in hepatocytes in vivo and in vitro (Van Antwerp et al. 1996; Xu et al. 1998; Lavon et al. 2000; Park et al. 2001). Moreover, TNF stimulation of hepatocytes expressing non-degradable IκBα resulted in the prevention of NF-kB translocation and the induction of JNK-mediated apoptosis (Liu et al. 2002). In line with these findings, in our experiments with HepG2 cells the inhibition of NF-kB translocations was followed by reactivation of JNK and ERK which both have been demonstrated to play a fundamental role in TVX+TNF-induced cell death (Beggs et al. 2014, 2015). Although we could not demonstrate the direct causality between these events, it is well-known that inhibition of TNF-induced NF-κB translocation induces cell death via JNK pathway and caspase activation (Liu et al. 2002; Wullaert et al. 2007; Minero et al. 2013). Also, the observed downregulation of XIAP and c-IAP1 match with the cytotoxic effects of TVX as both XIAP and c-IAP1 have been demonstrated to inhibit JNK or apoptosis during stimulation with TNF (Wicovsky et al. 2007) but also in the regulation of the NF-κB response during DNA damage (Jin et al. 2009). In addition, previously it has been demonstrated that mono-phasic, non-oscillatory kinetics of NF-κB translocation, as observed with TVX, is particularly accompanied with decreased expression of late genes (e.g. c-IAP1 and ICAM1) (Tian et al. 2005). All this data, together with the similarities of TVX effects between HepG2 and RAW264.7 stimulated respectively with TNF and LPS, indicates that TVX affects a common downstream molecular target in regulatory pathways of NF-κB and MAPKs. Although further investigations are needed, this study provides strong indications that disruption of TNF-induced NF-κB nuclear translocation occurring in presence of TVX+TNF may drive the JNK-dependent apoptosis.

In our experiments, we observed that TVX alone, at concentrations which resemble those found in patients' livers (Vincent et al. 1997), already activates transcription of NF-κB associated genes like IκBα and A20. In vitro this was preceded by minor and prolonged translocation of p65 NF-κB subunit into the nucleus of HepG2 cells, within the same time frame in which TNF mediates the transcription factor translocation. These results suggest that TVX itself already initiates NF-κB translocation, which may be linked to the observation of apoptotic cells in livers

prior to injection of TNF (Giustarini et al, 2018). This does not exclude the possibility that in vivo hepatotoxic effects of TVX can be reinforced by macrophage activation as result of LPS leaking from the gut (Poulsen et al. 2014).

The presented mechanisms observed for the combined effects of TVX and TNF or LPS exposure provides new insights into the initial steps of TVX-induced liver damage. In addition to the effects observed here, TVX-induced liver damage may also involve lack in regulation of inflammation (Giustarini et al. 2018a, b), and increased formation of apoptotic bodies due to pannexin-1 inhibition (Poon et al. 2014). The possible interplay of these mechanisms exemplifies the complexity of immune-mediated liver injury caused by a combination of inflammatory stressors and drugs like TVX that can reinforce phlogistic processes.

Obviously the "phenotype" of the adverse reaction in mice, which is an acute form of hepatotoxicity remains distinct from the hypersensitivity and DILI features observed in patients (Ballet 2015; Kenna and Uetrecht 2018). Nevertheless, the various key events in TVX-induced adverse reactions, e.g. increased hepatic cell death and macrophage activation, and delayed regulation of inflammation may contribute to sensitization of neo-antigen specific T cells as so-called adjuvant processes. Our data also exemplifies how specific activation kinetics of intracellular cascades may represent an adverse outcome pathway that can allow in vitro identification of aspects of compounds with IDILI liability. The key events we have identified in the etiopathogenesis of TVX-induced liver injury in mice may help to design preclinical in vitro tests to identify specific aspects of drugs with suspected idiosyncratic DILI liability.

References

Van Antwerp, D.J. et al. (1996) *Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science, 274, 787–9.*

Ballet, F. (2015) *Preventing Drug-Induced Liver Injury: How Useful Are Animal Models? Dig. Dis., 33, 477–485.*

Beggs, K.M. et al. (2014) *Molecular Mechanisms of Hepatocellular Apoptosis Induced by Trovafloxacin-Tumor Necrosis Factor-alpha Interaction. Toxicol. Sci., 137, 91–101.*

Beggs, K.M. et al. (2015) *Trovafloxacin-induced replication stress sensitizes HepG2 cells to tumor necrosis factor-alpha-induced cytotoxicity mediated by extracellular signal-regulated kinase and ataxia telangiectasia and Rad3-related. Toxicology, 331, 35–46.*

Benedetti, G. et al. (2013) *TNF-α-mediated NF-κB survival signaling impairment by cisplatin enhances JNK activation allowing synergistic apoptosis of renal proximal tubular cells. Biochem. Pharmacol., 85, 274–286.*

Buchweitz, J.P. (2002) *Underlying Endotoxemia Augments Toxic Responses to Chlorpromazine: Is There a Relationship to Drug Idiosyncrasy? J. Pharmacol. Exp. Ther., 300, 460–467.*

Chalasani, N. et al. (2015) *Features and outcomes of 899 patients with drug-induced liver injury: The DILIN prospective study. Gastroenterology, 148, 1340-1352.e7.*

Copeland, S. et al. (2005) *Acute Inflammatory Response to Endotoxin in Mice and Humans Acute Inflammatory Response to Endotoxin in Mice and Humans. Clin. Diagn. Lab. Immunol., 12, 60–67.*

Deng, X. et al. (2006) *Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. J. Pharmacol. Exp. Ther., 319, 1191–1199.*

Dugan, C. and MacDonald, A. (2010) *A mouse model of severe halothane hepatitis based on human risk factors. J. Pharmacol. Exp. Ther., 333, 364–372.*

Fontana, R.J. et al. (2009) *Drug-induced liver injury network (DILIN) prospective study: Rationale, design and conduct. Drug Saf., 32, 55–68.*

Fredriksson, L. et al. (2011) *Diclofenac inhibits tumor necrosis factor-α-induced nuclear factor-κB activation causing synergistic hepatocyte apoptosis. Hepatology, 53, 2027–41.*

Fredriksson, L. et al. (2014) *Drug-induced endoplasmic reticulum and oxidative stress responses independently sensitize toward TNFα-mediated hepatotoxicity. Toxicol. Sci., 140, 144–159.*

Giustarini, G. et al. (2018) *Tissue influx of neutrophils and monocytes is delayed during development of trovafloxacin - induced tumor necrosis factor - dependent liver injury in mice. J Appl Toxicol, 38, 753–765.*

Giustarini, G. et al. (2019) *Trovafloxacin-Induced Liver Injury: Lack in Regulation of Inflammation by Inhibition of Nucleotide Release and Neutrophil Movement. Toxicol. Sci., 167, 385–396.*

Herpers, B. et al. (2016) *Activation of the Nrf2 response by intrinsic hepatotoxic drugs correlates with suppression of NF-κB activation and sensitizes toward TNFα-induced cytotoxicity. Arch. Toxicol., 90, 1163–1179.*

Jin, H.-S. et al. (2009) *cIAP1, cIAP2, and XIAP act cooperatively via nonredundant pathways to regulate genotoxic stress-induced nuclear factor-kappaB activation. Cancer Res., 69, 1782–91.*

Kenna, J.G. and Uetrecht, J. (2018) *Do In Vitro Assays Predict Drug Candidate Idiosyncratic Drug-Induced Liver Injury Risk? Drug Metab. Dispos., dmd.118.082719.*

Lavon, I. et al. (2000) *High susceptibility to bacterial infection, but no liver dysfunction, in mice compromised for hepatocyte NF-κB activation. Nat. Med., 6, 573–577.*

Liguori, M.J. et al. (2008) *Trovafloxacin-Induced Gene Expression Changes in Liver-Derived in Vitro Systems : Comparison of Primary Human Hepatocytes to HepG2 Cells □.*

Liu, H. et al. (2002) *NF-κB inhibition sensitizes hepatocytes to TNF-induced apoptosis through a sustained activation of JNK and c-Jun. Hepatology, 35, 772–778.*

Lu, J. et al. (2012) *Amiodarone Exposure During Modest Inflammation Induces Idiosyncrasy-like Liver Injury in Rats: Role of Tumor Necrosis Factor-alpha. Toxicol. Sci., 125, 126–133.*

Luyendyk, J.P. (2003) *Ranitidine Treatment during a Modest Inflammatory Response Precipitates Idiosyncrasy-Like Liver Injury in Rats. J. Pharmacol. Exp. Ther., 307, 9–16.*

Maiuri, A.R. et al. (2015) *Cytotoxic synergy between cytokines and NSAIDs associated with idiosyncratic hepatotoxicity is driven by mitogen-activated protein kinases. Toxicol. Sci., 146, 265–280.*

Matzinger, P. and Kamala, T. (2011) *Tissue-based class control: The other side of tolerance. Nat. Rev. Immunol., 11, 221–230.*

Minero, V.G. et al. (2013) *JNK activation is required for TNFα-induced apoptosis in human hepatocarcinoma cells. Int. Immunopharmacol., 17, 92–98.*

Park, G.Y. et al. (2001) *Anti-inflammatory effect of adenovirus-mediated IkappaBalpha overexpression in respiratory epithelial cells. Eur. Respir. J. Off. J. Eur. Soc. Clin. Respir. Physiol., 18, 801–9.*

Pirmohamed, M. et al. (2002) *The danger hypothesis--potential role in idiosyncratic drug reactions. Toxicology, 181–182, 55–63.*

Poon, I.K.H. et al. (2014) *Unexpected link between*

an antibiotic, pannexin channels and apoptosis. Nature, 507, 329–34.

Poulsen, K.L. et al. (2014) *Trovafloxacin enhances lipopolysaccharide-stimulated production of tumor necrosis factor-α by macrophages: role of the DNA damage response. J. Pharmacol. Exp. Ther., 350, 164–70.*

Puigvert, J.C. et al. (2010) *High-throughput live cell imaging of apoptosis. Curr. Protoc. Cell Biol., 1–13.*

Roth, R.A. et al. (2017) *Idiosyncratic Drug-Induced Liver Injury: Is Drug-Cytokine Interaction the Linchpin? J. Pharmacol. Exp. Ther., 360, 461–470.*

Sakai, J. et al. (2017) *Lipopolysaccharide-induced NF-κB nuclear translocation is primarily dependent on MyD88, but TNFα expression requires TRIF and MyD88. Sci. Rep., 7, 1–9.*

Sakurai, H. et al. (1999) *IkB kinases phosphorylate NF-kB p65 subunit on serine 536 in the transactivation domain. J. Biol. Chem., 274, 30353–30356.*

Shaw, P.J. et al. (2009a) *Trovafloxacin Enhances the Inflammatory Response to a Gram-Negative or a Gram-Positive Bacterial Stimulus , Resulting in Neutrophil-Dependent Liver Injury in Mice. J Pharmacol Exp Ther, 330, 72–78.*

Shaw, P.J. et al. (2009b) *Tumor Necrosis Factor α is a Proximal Mediator of Synergistic Hepatotoxicity from Trovafloxacin / Lipopolysaccharide Coexposure. J Pharmacol Exp Ther, 328, 62–68.*

Taudorf, S. et al. (2007) *Human models of low-grade inflammation: Bolus versus continuous infusion of endotoxin. Clin. Vaccine Immunol., 14, 250–255.*

Tian, B. et al. (2005) *A TNF-induced gene expression program under oscillatory NF-kappaB control. BMC Genomics, 6, 137.*

Vincent, J. et al. (1997) *Pharmacokinetics and safety of trovafloxacin in healthy male volunteers following administration of single intravenous doses of the prodrug, alatrofloxacin. J. Antimicrob. Chemother., 39 Suppl B, 75–80.*

Van de Water, B. et al. (2001) *Suppression of chemically induced apoptosis but not necrosis of renal proximal tubular epithelial (LLC-PK1) cells by focal adhesion kinase (FAK). Role of FAK in maintaining focal adhesion organization after acute renal cell injury. J. Biol. Chem., 276, 36183–36193.*

Weaver, R.J. et al. (2017) *Test systems in drug discovery for hazard identification and risk assessment of human drug-induced liver injury. Expert Opin. Drug Metab. Toxicol., 13, 767–782.*

Wicovsky, A. et al. (2007) *Sustained JNK activation in response to tumor necrosis factor is mediated by caspases in a cell type-specific manner. J. Biol. Chem., 282, 2174–83.*

Wink, S. et al. (2018) *Dynamic imaging of adaptive stress response pathway activation for prediction of drug induced liver injury. Arch. Toxicol., 92, 1797–1814.*

Wink, S. et al. (2017) *High-content imaging-based BAC-GFP toxicity pathway reporters to assess chemical adversity liabilities. Arch. Toxicol., 91, 1367–1383.*

Wullaert, A. et al. (2007) *Hepatic tumor necrosis factor signaling and nuclear factor-κB: Effects on liver homeostasis and beyond. Endocr. Rev., 28, 365–386.*

Wullaert, A. et al. (2006) *Mechanisms of crosstalk between TNF-induced NF-kappaB and JNK activation in hepatocytes. Biochem. Pharmacol., 72, 1090–101.*

Xu, Y. et al. (1998) *NF-kappaB inactivation converts a hepatocyte cell line TNF-alpha response from proliferation to apoptosis. Am. J. Physiol., 275, C1058-66.*

Zambrano, S. et al. (2016) *NF-κB oscillations translate into functionally related patterns of gene expression. Elife, 5, 1–38.*

Zou, W. et al. (2009) *Hepatotoxic interaction of sulindac with lipopolysaccharide: Role of the hemostatic system. Toxicol. Sci., 108, 184–193.*

Chapter 5

Ximelagatran-induced liver injury during viral infection: a potential role for the innate immune system in the onset of hepatic damage

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Abstract

Viral infections have been recognized as one of the risk factors involved in the development of idiosyncratic immune mediated drug-induced liver (iDILI). At the present time, the only available animal model of iDILI including a viral component is based on the administration of the thrombin inhibitor ximelagatran (XIM) in liver-specific virally infected mice injected with tumour necrosis factor (TNF) to induce viral hepatitis. This model showed that XIM administration for 7 days increased serum alanine aminotransferases and mortality after TNF injection in infected mice when compared with those receiving the pharmacological analogue dabigatran (DAB) or the vehicle. In this model, we investigated the immunologically relevant changes to characterize the mechanisms involved in liver damage and mortality caused by XIM.

Before viral infection, administration of XIM increased the recruitment of neutrophils and monocytes into the liver congruently with an increased hepatic expression of TNF, IL-6, FAS and FASL which were not observed in mice receiving the other treatments.

Viral infected mice treated with XIM showed an increased percentage of NK and plasmacytoid dendritic cells in liver-isolated leukocytes before and after the cytokine-induced hepatitis.After TNF, hepatic expression of FASL and IL-6 were significantly increased in XIM-treated mice.

This study suggests hitherto unidentified features of XIM to potentiate the anti-viral response of the host leading to increased liver damage and mortality. If the hepatic stress caused by the administration of XIM-only takes part in the potentiation of the anti-viral response remains to be further investigated. A clear understanding of the worsening effects of XIM on TNF-induced liver hepatitis might contribute to the development of better predictive tests for the identification of compounds causing iDILI already at the research and development stage.

Introduction

Immune-mediated drug-induced liver injuries (iDILIs) are hepatic injuries that occur in a very limited percentage of the patient population, but they carry significant mortality and morbidity (Chalasani et al., 2015). Because of these serious adverse events, regulatory agencies regulated or banned the use of drugs associated with such very low incidence of these events in reason of their unpredictable and non-dose related onset (Lasser, 2002; Chalasani et al., 2015). iDILIs might be of idiosyncratic nature and subject to risk factors such as viral and bacterial infections. In fact, studies demonstrated that the probability to experience iDILI is significantly increased in patients with viral infections such as human immunodeficiency and hepatitis B/C virus (den Brinker et al., 2000; Bonacini, 2004; Kim et al., 2016; Chalasani and Björnsson, 2010). Although co-morbidity due to infection seems to represent a recurrent theme in iDILI, the mechanisms behind this association still need to be defined and clarified. In addition, understanding of the series of events leading to the hepatic adverse reactions may help to identify early potential molecular and cellular alerts of hepatotoxicity already during pre-clinical phases of drug development.

Currently, several mouse models of idiosyncratic DILI have been developed combining the administration of sterile (i.e. tumour necrosis factor, TNF) and non-sterile (i.e. lipopolysaccharide, LPS) stimuli with compounds associated with idiosyncratic DILI in man (Deng et al., 2006; Gandhi et al., 2013; Lu et al., 2013; Luyendyk, 2003; Shaw, Beggs, et al., 2009; Shaw, Ganey, et al., 2009). Although these studies have brought an important contribution to the mechanistic understanding of IDILI, doubts have been raised about their validity for the toxicological screening of compounds at the research and development stages (Ballet, 2015).

Recently, a new iDILI-model was introduced that involves the combination of: 1) ximelagatran (XIM), a direct thrombin inhibitor, 2) hepato-specific infection with adenovirus, 3) injection of TNF to induce viral hepatitis. XIM was on the market for only 2 years, between 2004 and 2006, after it was withdrawn from the market due to safety re-evaluation. This evaluation underlined that a significant number of unpredictable and non-dose dependent adverse hepatic reactions were associated with prolonged administration of XIM (Lee et al., 2005; Agnelli et al., 2009). After withdrawal, an immune pathogenesis was hypothesized for XIM-induced liver injury based on the association of DRB1(*)07 and DQA1(*)02 MHC alleles and the increased ALT levels observed in patients treated with the new thrombin inhibitor (Kindmark et al., 2008). In contrast, further studies using XIM-only treatments, failed to recapitulate the occurrence of any liver injury in in vitro and ex vivo models (Ainscow et al., 2008; Kenne et al., 2008). Therefore, a new model may shed lights on possible mechanisms underlying the development of iDILI.

As already mentioned before, the murine model of XIM-induced DILI is based

on the combination of drug administration and acute TNF-induced viral hepatitis. Briefly, independently of the drug treatment, all infected mice receiving TNF experienced increased serum ALT concentrations when compared to mice not receiving the cytokine. However, XIM treatment -induced acute liver failure, mitochondrial alterations, increased serum ALT concentrations and increased mortality when compared to mice treated with dabigatran (DAB, another direct thrombin inhibitor that does not induce DILI in man), or only the vehicle (Veh). Notably, administration of LPS or TNF in conjunction with XIM exposure failed to reproduce the hepatic damage and mortality observed in virus-infected mice.

Here, we set out to examine the involvement of the immune system in this multifactorial model of DILI. We observed that only administration of XIM already increased the percentage of neutrophils and expression of FASL, IL-6 and TNF mRNA in the liver before the viral infection. After viral infection and TNF challenge we observed increased percentages of liver-recruited NK cells and pDCs in the liver of XIM-exposed mice. Our data suggests that XIM initiates changes in innate immune populations in liver that may contribute to liver failure by TNF-induced viral hepatitis.

Material and methods

Experimental set up

Mice (n = 6) were administered with XIM (50 mg/kg), DAB (150 mg/kg) or bi-distilled water (vehicle, Veh) every day for 7 days. The drug solutions or Veh were administered orally with intragastric gavage. 5 days after the beginning of the treatment, mice were injected intravenously (tail vein) with a replication-deficient adenovirus AdGOL ($1x10^9$ PFU/mouse, 150 µl, saline). Mice sacrificed at day 5 did not receive the injection with the virus. At day 7 mice received an intravenous injection of TNF (400 ng/mouse) to induce viral hepatitis. Mice were culled at every time point as indicated in Fig. 1, each time 2 hours after the administration of the drugs or TNF. The drug dosages were adjusted based on two main differences in between human and mice: the different absorption of XIM (Eriksson et al., 2003); the liver/body weight ratio. The percentage of liver mass in mice is about 5% of body weight whereas it is only about 2% in humans (about 1.2 g of liver in 20 g of mouse compared to 1.5 kg of liver on an average weight of 72 kg in humans). Considering these factors, a toxicologically relevant dose of 50 mg XIM/kg in mice was chosen compared to 1 mg XIM/kg in humans.

Virus

Recombinant adenovirus AdGOL were generated as described by Stabenow et al. (2010). AdGOL expresses the green fluorescent protein (GFP), ovalbumin (OVA) and luciferase under the CMV promoter and is not capable of replication due to the deletion of E1 and E3 genes in vivo (Stabenow et al., 2010). Recombinant

adenoviral stocks were grown and purified as described earlier by Sprinzl et al. (2001) (Sprinzl et al., 2001).

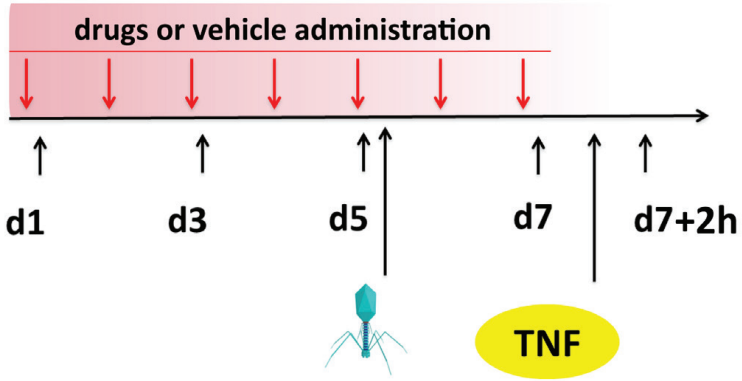


Figure 1

Protocol for the treatment of mice in the model of TNF-induced viral hepatitis
Mice were administered daily with an intragastric gavage of the drug solution or vehicle for 7 days (XIM: 50 mg/kg, DAB: 150 mg/kg, Veh: bi-distilled water). Two hours after the 5th administration of drugs or vehicle, mice were injected intravenously (via tail vein) with a replication-deficient adenovirus AdGOL ($1x10^9$ PFU/mouse, 150 µl, saline). Mice sacrificed at day 5 were not injected with the virus. At day 7, mice received an intravenous injection of TNF (400 ng/mouse) to induce viral hepatitis. Mice were sacrificed at all time points indicated (black arrows) every time 2 h after the administration of the drugs or TNF.

Intrahepatic leukocyte isolation

Intrahepatic leukocytes were isolated as previously described (Crispe, 2001). Briefly, liver was perfused with 5 ml ice-cold phosphate-buffered saline (PBS) and excised from the animal. Tissue was minced and gently passed through a sieve. The liver slurries were centrifuged, and the pellet resuspended with a collagenase D (0.02%, w/v) and DNAse I (0.002%, w/v) solution. The suspensions were incubated at 37°C for 40 minutes on a reciprocating shaker. Liver leukocytes were isolated by using a 45–67.5% isotonic Percoll density gradient (GE Healthcare, Fisher Scientific, Landsmeer, The Netherlands).

Flow cytometry

Leukocytes from spleen were obtained by passing the organ through a 70 µm strainer, after which the collected single cells were washed once. To remove erythrocytes, cell suspensions were incubated with red blood cell lysis buffer (containing NH_4Cl, $KHCO_3$ and Na_2EDTA) for 1 minute, and the remaining cells were washed once with PBS.
For flow-cytometric analysis, liver or spleen cells were incubated with anti-CD16/CD32 (clone 2.4G2) to block the fragment crystallizable region receptor (FcR). Next, cells were stained with fluorescent-labeled antibodies and stored in 1%

paraformaldehyde until analysis. The following antibodies were used: anti-CD45.2 Pacific Blue (clone 104; Biolegend, Uithoorn, The Netherlands), anti-NK1.1 PE (clone PK136; eBioscience), anti-CD3e APC (clone 145-2C11; eBioscience), anti-Gr1 APC (clone RBG8C5; eBioscience), anti-CD11b PE and FITC (clone M1/70; eBioscience), anti-CD4 PERCP (clone RM4-5; BD Pharmingen), anti-CD8a FITC (clone 53-6.7; BD Pharmingen, Erembodegem, Belgium), anti-CD44 PE (clone IM7; eBioscience), anti-CD62L APC (clone MEL- 14; BD Pharmingen), anti-CD11c APC (clone N418; eBioscience), anti-MHCII FITC (clone M5/114.15.2; eBioscience), anti-PDCA-1 PE (clone eBio129c; eBioscience), anti-CD86 PERCP (clone GL-1; Biolegend), anti-F4/80 FITC (clone BM8; eBioscience) and anti-CD49b PE-Cy5 (clone DX5; eBioscience) in fluorescence-activated cell sorting buffer (PBS containing 0.25% bovine serum albumin, 0.05% NaN_3, 0.5 mM EDTA) for 30 minutes at 4°C. Data were acquired by means of fluorescence-activated cell sorting Canto II and analyzed using Weasel flow analysis package (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). The amount of cells was determined by a two-step approach. Total number of isolated-leukocytes of organs were determined with a hemocytometer and multiplied by the volume of the corresponding cell suspension. This total number of cells acquired per organ was multiplied with the percentage of the cell subtype of interest in the CD45 gate to determine the amount of specific cellular subsets.

qPCR

For mRNA studies, the medial part of the biggest liver lobe from each mouse (approximately 50 mg) was snap frozen in liquid nitrogen and stored at 80°C until RNA isolation. Each sample was suspended in 500 µl RNA InstaPure (Eurogentec) and homogenized using a TissueLyser (Qiagen, Hilden, Germany) for 1 min/25Hz twice. The homogenized tissue was centrifuged for 10 minutes at 12000xg. The supernatant containing RNA in RNA Insta-Pure was transferred to a new vial and RNA was isolated using phenol-chloroform extraction. The amount of RNA was determined using the NanoDrop 2000 Spectrophotometer (ThermoScientific). Subsequently, 1 mg of extracted total RNA was reverse transcribed with the iScriptTM cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, California). Quantitative re- verse transcriptase PCR was performed using a iCycler iQ system (Biorad), and amplification was done using iQ SYBR Green supermix (Biorad) with 0.3 mM final primer concentration. Primer sequences:
mouse FAS FW-TCTGGTGCTTGCTGGCTCAC and RV-CCATAGGCGATTTCTGGGAC,
mouse FASL FW-CGGTGGTATTTTTCATGGTTCTGG and RV-CTTGTGGTTTAGGGGCTGGTTGTT,
mouse TNF FW-AACGGCATGGATCTCAAAGA and RV-TTTCTCCTGGTATGAGATAGCAAATC
and mouse IL-6 FW-ATGTTCTCTGGGAAATCGTGGA and RV-TTCCTGATTATATCCAGTTTGGTAGCAT.
Gene-specific primers for TNF, IL-6 and FASL were derived from the NCBI GenBank and were manufactured commercially (Eurogentec, Seraing, Belgium). For each

sample, mRNA expression was normalized for the detected Ct value of b-actin. Data are expressed as fold increase compared with the control group (vehicle only).

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Statistical significance for comparisons was determined by one- or two-way ANOVA with Dunnett's post hoc test. A p- value less than 0.05 was considered statistically significant. All data are analyzed using GraphPad Prism (version 6.07) software (San Diego, California).

RESULTS

Liver and spleen weight during the iDILI model

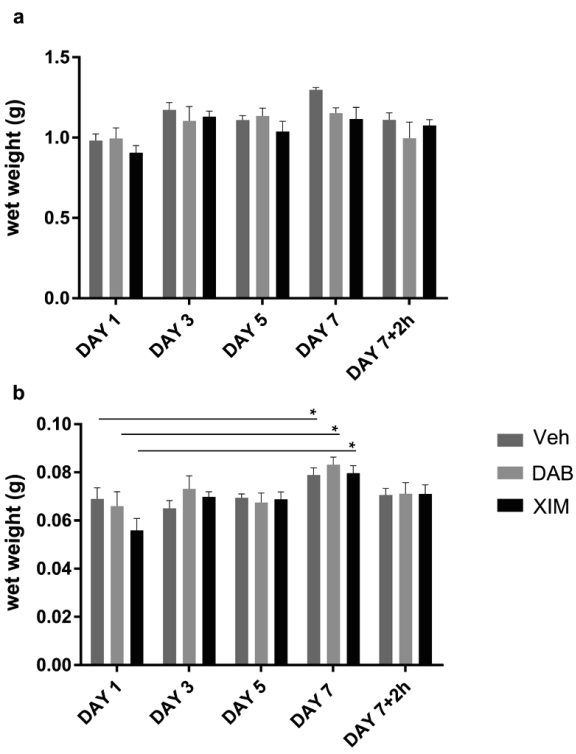


Figure 2

Liver and spleen weights during the iDILI model

Liver (a) and spleen (b) were excised and weighted (after resection of the gall bladder from livers) at every assessed time point indicated in figure. *p < 0.05 when compared with mice from the same group of treatment at day 1; two-way ANOVA followed by Dunnett's post hoc test.

Mice were administered with the drug solution for 7 days and at day 5 injected with AdGOL as depicted in Fig 1. At day 7 (2 days after infection) mice were injected intravenously with TNF and sacrificed 2 h after. Wet weights of liver and spleen were assessed at indicated time points shown in Fig. 2. No significant changes in liver weights were observed throughout the 7 days of the model. By contrast,

spleen weights significantly increased at day 7 (before TNF injection), i.e. 48 h after the infection with AdGOL (Fig.2).

Liver and spleen cellularity during the iDILI model

At each time point, livers and spleens were processed as mentioned in Material and methods. No significant changes in total leukocyte cellularity of the two organs were observed before TNF injection in any of the groups of treatment (Fig.3). However, TNF administration was associated with increased number of liver leukocytes in all assessed groups when compared with mice not receiving the cytokine. XIM and DAB significantly reduced the TNF-mediated recruitment of leukocytes in the liver at day 7 compared with mice treated with Veh and TNF (Fig.3).

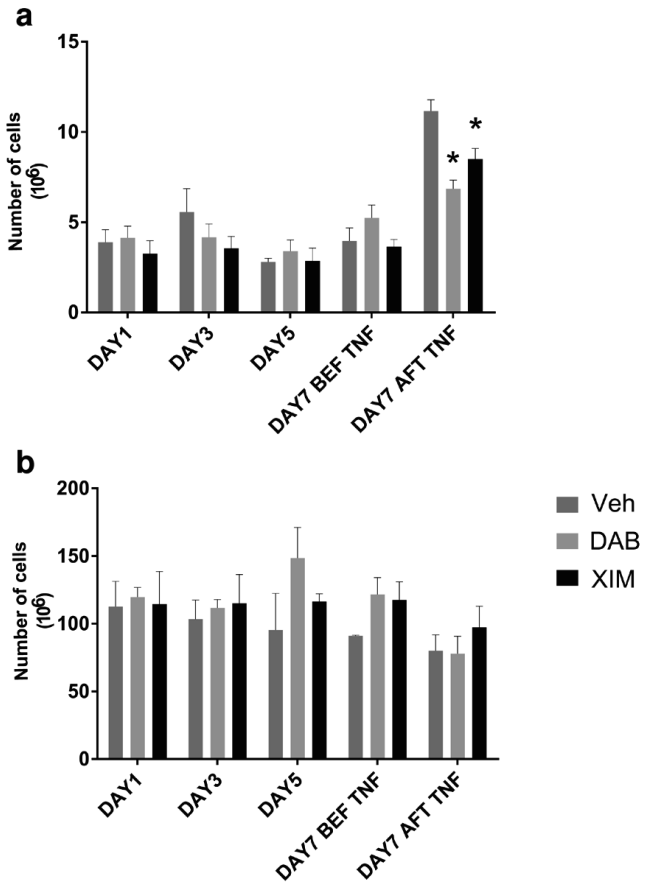


Figure 3

Leukocytes in liver and spleen of mice

Two days after virus infection mice were intravenously injected with TNF, 2 h after receiving the daily drug solution or vehicle. After excision, organs were processed in order to isolate leukocytes from liver and spleen as reported in materials and methods. Cells were counted with the use of a hemocytometer. (a) Both thrombin inhibitors DAB and XIM significantly decreased the number of leukocyte s into the liver after TNF injection when compared to mice receiving Veh. (b) No variation of spleen cellularity was observed during the experiment. * $p < 0.05$ when compared with mice receiving the vehicle at the same time point; one-way ANOVA followed by Dunnett's post hoc test.

XIM treatment caused an early alteration of CD4 and CD8 T cell number in the liver and spleen.

The number and proportion of B ($CD45^+/CD19^+/CD3^-$) or T ($CD45^+/CD4^+$ or $CD8^+/CD3^+$) cells in the liver or spleen were not during the model except for day 1, when XIM administration increased the number of naive CD4+ T cells ($CD45^+/CD4^+/CD3^+/CD62L^+/CD44^-$) in the liver when compared with mice receiving DAB (Fig. 4a). XIM-treated mice showed an increased number of naive CD8+ T cells ($CD45^+/CD8^+/CD3^+/CD62L^+/CD44^-$) in the liver when compared with mice receiving DAB (Fig.4b). At day 1 no differences were observed in numbers of $CD4^+$ and $CD8^+$ T cells between DAB- and Veh-treated mice, in liver or spleen.

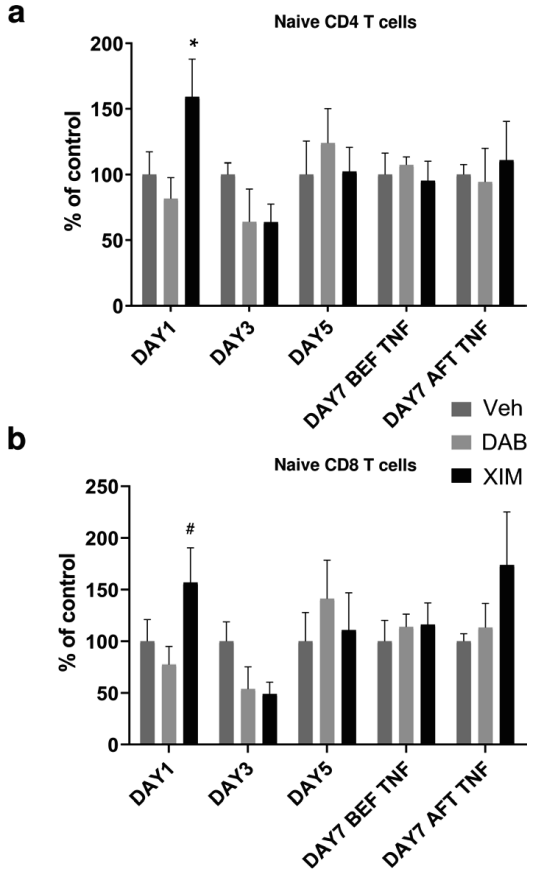


Figure 4

Naïve $CD4^+$ and $CD8^+$ T cells in the liver

Mice were sacrificed 2 h after the daily administration of the drugs/vehicle and TNF. After excision, organs were processed in order to isolate leukocytes from liver and spleen as reported in materials and methods. Naïve $CD4^+$ (a) and $CD8^+$ (b) in the liver are presented as percentage of the number of these cells in the liver of mice receiving the vehicle. * $p < 0.05$ when compared with mice receiving the vehicle at the same time point; # $p < 0.05$ when compared with mice receiving DAB, one-way ANOVA followed by Dunnett's post hoc test.

XIM treatment increased the proportion of NK and pDCs cells in the hepatic leukocyte isolated from virus-infected mice

Although the total number of hepatic NK cells did not differ significantly upon XIM treatment, the proportion in the leucocyte compartment of these NK cells

(CD45.2$^+$/NK1.1$^+$/CD3$^-$) was significantly increased, before and after administration of TNF in infected mice (Fig. 5).
The percentage of pDCs in the population of liver leukocytes was increased in XIM-treated mice at day 3 and after TNF injection when compared with mice receiving Veh (Fig. 5G).

No significant changes were observed in the proportion of splenic NK cells, except at day 1. Remarkably, at that day, administration of XIM was associated with an increased number and percentage of CD49b$^-$ NK cells in the spleen when compared with Veh-treated mice (Fig. 6).

XIM increased the percentage of neutrophils in the liver-isolated leukocytes before infection

XIM administration caused an increase of the percentage of neutrophils (CD45.2^{int}/SSC^{hi}/CD11b$^+$/Gr1$^+$) on day 3 and 5 before the injection of the virus (Fig. 7A). At day 7, after virus infection, all treated groups showed a similar percentage of neutrophils in liver when compared with mice not receiving the viral load. Also, irrespective of the treatment, TNF administration increased both the number and percentage of neutrophils in the CD45.2$^+$ cell population isolated from the liver (Fig. 7A).
Since neutrophil recruitment in the inflamed tissue is usually followed by inflammatory monocytes (CD45.2^{hi}/SSC^{lo}/F4-80^{lo}/CD11b$^+$/Gr-1$^+$) we also evaluated the percentages of inflammatory monocytes in liver and spleen. The percentage of CD45.2$^+$ inflammatory monocytes fluctuated over the different days and the only significant increase in number and percentage of inflammatory monocytes was observed at day 3 in the liver of mice receiving XIM.

XIM administration increased the hepatic expression of TNF, IL-6, FAS and FASL

FASL is a well-known ligand for CD95 death receptor (FAS). The latter is ubiquitously expressed in the liver and its activation leads to apoptosis of the cell. The ligand of CD95, FASL, is expressed by different cells in the liver such as lymphocytes, endothelial cells, hepatic stellate cells and antigen presenting cells. The engagement of FAS by FASL is a mechanism by which activated T and NK cells can kill harmful cells such as virus-infected or cancer cells (Zou et al., 2010; Yoon and Gores, 2002). Interestingly, TNF can increase the expression of FAS on hepatocytes, hereby sensitizing to FASL-induced apoptosis (Faletti et al., 2018). Therefore, we decided to investigate the hepatic expression of key markers of inflammation such as TNF, IL-6 but also FAS and FASL in this model.
XIM administration increased hepatic FAS and FASL mRNA expression when compared with mice receiving DAB or Veh. FAS expression was slightly increased on days 3 and 5, but its transcription returned to levels observed in Veh-treated

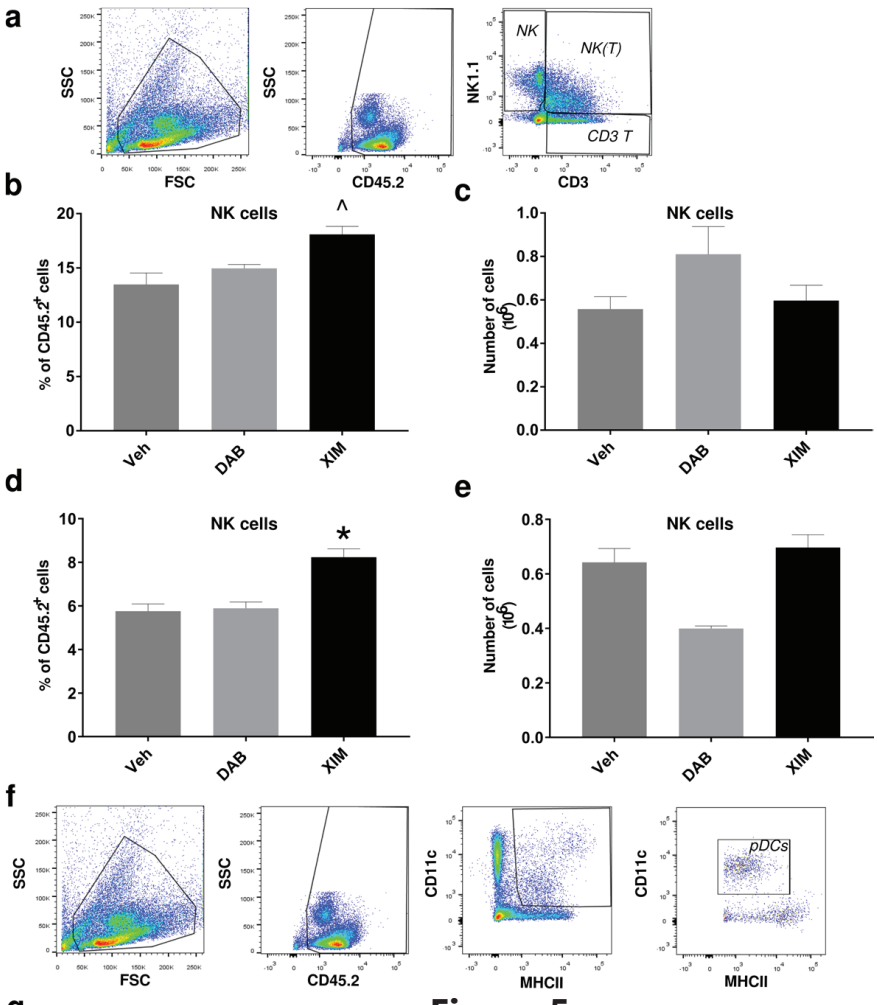


Figure 5

Percentages and number of NK and pDCs in liver during infection

Mice were infected 2 h after the 5th daily administration of the drug solution or vehicle and were sacrificed at day 7 either before and 2 h after the intravenous injection of TNF. Mice were culled, organ excised and prepared for flowcytometry as mentioned in materials and methods. (a) Gate strategy for the identification of liver NK cells. (b) Percentage of NK cells in the CD45.2$^+$ cells isolated from the liver of infected mice at day 7 (before TNF), (c) and the total number of these cells per liver. (d) Percentage of NK cells in the CD45.2$^+$ cells isolated from mice experiencing 2 h after TNF injection, (e) and the total number of NK cells per liver. (f) Gating strategy for the identification of hepatic pDCs. (g) Percentage of pDCs in liver-isolated leukocytes at day 3. * $p < 0.05$ when compared with mice of any other assessed group of treatment; ^ $p < 0.05$ when compared with mice receiving the vehicle; one-way ANOVA followed by Dunnett's post hoc test.

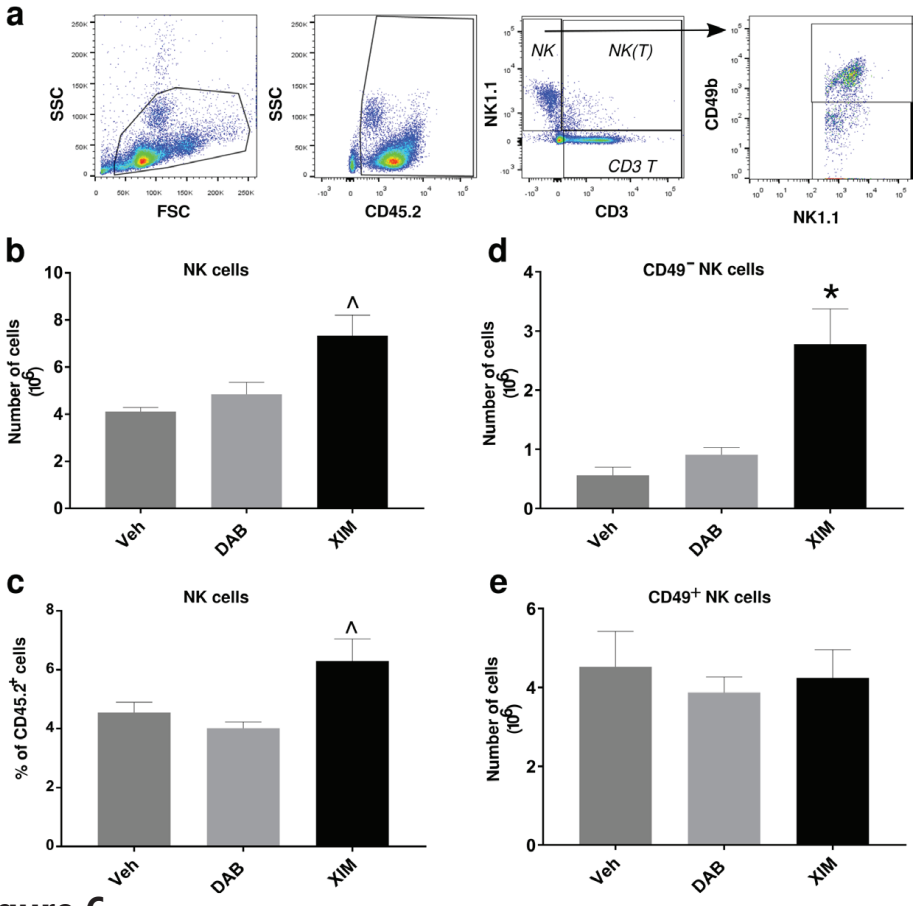


Figure 6

Percentage and number of NK cells and their subtypes in the spleen

Mice were administered daily with an intragastric gavage of the drug solution or vehicle and sacrificed 2 h after in accordance with Fig. 1. Spleens were excised and prepared for flow cytometry as reported in materials and methods. (a) Gating strategy for the identification of NK cells and their CD49b positive and negative subtypes. (b) Percentage of NK cells in the liver-isolated leukocytes and (c) the total number of these cells per spleen 2 h after the first drugs and vehicle administration at day 1. (d-e) Number of $CD49b^-$ (d) and $CD49^+$ (e) NK cell subtype in the spleen 2 h after the first drug and vehicle administration at day 1. * $p < 0.05$ when compared with mice of any other assessed group of treatment; ^ $p < 0.05$ when compared with mice receiving the vehicle; one-way ANOVA followed by Dunnett's post hoc test.

mice at the time points after the infection (Fig. 8A). Differently, the increase of FASL mRNA was gradual, starting at day 5 and resulting in an almost 4-fold increase at day 7 when TNF injection resulted in further (almost 50-fold) increase of the hepatic FASL mRNA expression compared to Veh (Fig. 8B).

Hepatic IL-6 expression in the liver lysates of mice receiving XIM was significantly

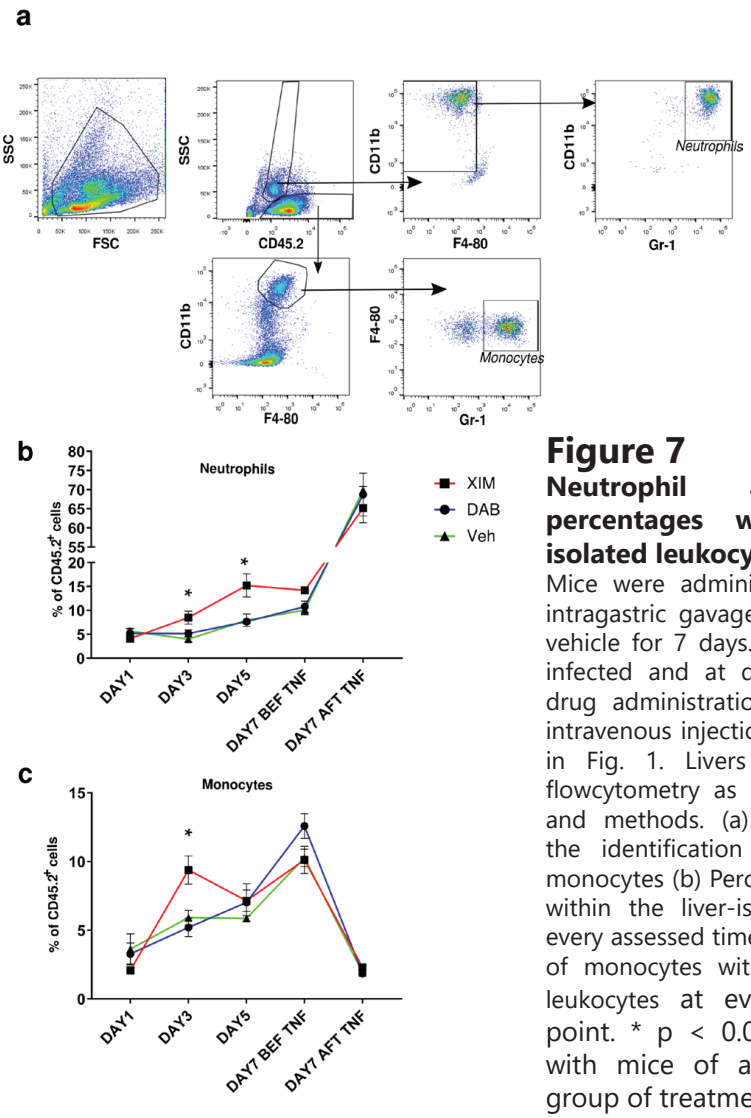


Figure 7

Neutrophil and monocyte percentages within the liver-isolated leukocytes

Mice were administered daily with an intragastric gavage of drug solution or vehicle for 7 days. At day 5 mice were infected and at day 7 (2 h after the drug administration) mice received an intravenous injection of TNF as depicted in Fig. 1. Livers were prepared for flowcytometry as reported in materials and methods. (a) Gating strategy for the identification of neutrophils and monocytes (b) Percentage of neutrophils within the liver-isolated leukocytes at every assessed time point. (c) Percentage of monocytes within the liver-isolated leukocytes at every assessed time point. * $p < 0.05$ when compared with mice of any other assessed group of treatment at the same time point; two-way ANOVA followed by Dunnett's post hoc test

increased at day 3 and 5 when compared to Veh-treated mice. At day 7, before the injection of the cytokine, no difference was observed between hepatic IL-6 mRNA expressions of DAB- and XIM-treated infected mice, but injection of TNF resulted in an increased transcription of IL-6 in the liver of mice receiving XIM when compared with mice receiving DAB or Veh. XIM treatment induced a 3-4 fold increase of TNF mRNA expression at day 1 and day 5 when compared with mice receiving the Veh. All these markers were not significantly different between DAB- and Veh-treated mice.

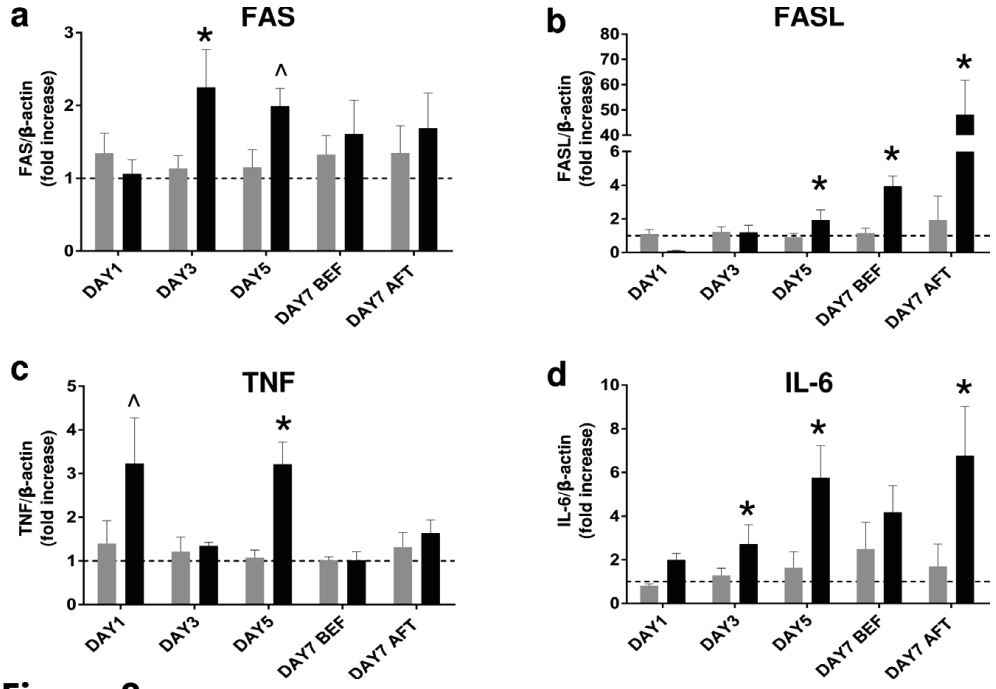


Figure 8

Hepatic expression of TNF, IL-6, FAS and FASL mRNA

Livers were excised and the medial part of the biggest lobe was prepared for qPCR as reported in materials and methods. mRNA expression of (a) FAS, (b) FASL, (c) TNF and (d) IL-6 in the liver of mice at the time point indicated in figure. (* $p < 0.05$ when compared with mice of any other assessed group of treatment at the same time point; ^ $p < 0.05$ when compared with mice receiving the vehicle at the same time point, two-way ANOVA followed by Dunnett's post hoc test.

DISCUSSION

In the last decades, many efforts have been spent investigating the mechanisms behind iDILI and especially trying to develop a reliable and reproducible panel of tests which can identify compounds with hepatotoxic liability already at the first research and development assessments. However, a model capable of reproducing the delayed drug-induced liver damage such as observed in the clinic is still missing. A new mouse TNF-induced viral hepatitis model of iDILI has been developed in the laboratory of Prof. Knolle. The model showed significant increases in mortality and serum ALT levels in mice receiving XIM, a compound associated with iDILI in man, when compared to mice treated with its pharmacological analogue DAB- or with Veh.

Here, we show that XIM-induced liver injury was associated with changes in

the composition of leukocytes in the liver and spleen before and after the viral infection, in particular in the proportions of neutrophils, NK cells and pDCs. These changes were accompanied by an increased hepatic expression of genes involved in either inflammation, like TNF and IL-6, and in cell-mediated cytotoxicity of virus-infected cells, such as FAS and FASL.

We found that XIM significantly increased the percentage of hepatic neutrophils and increased the transcription of TNF and IL-6 in the liver already 3 and 5 days after the first administration of the drug. TNF and IL-6 are key cytokines involved in the acute phase of inflammation with both beneficial and detrimental effects (Scheller et al., 2011; Dong et al., 2016). Similarly, neutrophil sequestration in liver is a hallmark of inflammation. Neutrophil sequestration in the inflamed liver aims to resolve inflammation (Soehnlein and Lindbom, 2010; Holub et al., 2009) although their transmigration into the parenchyma is also associated with tissue destruction (Jaeschke and Hasegawa, 2006). The increased hepatic transcription of the proinflammatory cytokines IL-6 and TNF, together with the increased hepatic leukocyte percentage of neutrophils represent a clear sign of cellular stress in the organ. Our findings showed a hitherto unidentified capability of XIM to increase these markers of inflammation in the liver of mice receiving a daily dose of the drug, although this was not associated with pathological modifications.

In our opinion, the absence of evident damage to the hepatic tissue during the only administration of XIM highlights that the recruitment of the described immune cells and the expression of cytokines might be beneficial for the regulation of inflammation as recently demonstrated in the trovafloxacin model of iDILI. In that model we recently showed that disturbance of early recruitment of neutrophils and monocytes in the inflamed liver is linked to liver damage (Giustarini, Vrisekoop Nienke, et al., 2018). The body of evidence in favor of a regulatory function of the short living neutrophils in inflammation is becoming more consistent. Especially, the role of viable and apoptotic neutrophils in the suppression of the pro-inflammatory activities of especially macrophages has been recently shown in vivo and in vitro (Marwick et al., 2018; Holub et al., 2009; Giustarini, Vrisekoop Nienke, et al., 2018; Souza et al., 2015).

Interestingly, whereas TNF expression was fluctuant, IL-6 expression increased in time, potentially revealing the participation of this cytokine in switching from acute to chronic inflammation. Indeed, IL-6 has been shown to promote the transition from acute to chronic inflammation via the recruitment of monocytes to the site of inflammation (Gabay, 2006). Following this paradigm, the increased expression of IL-6 appeared associated with the recruitment of inflammatory monocytes into the liver. Preliminary experiments performed in the laboratory of Prof. Knolle showed that infection anticipated at day 3 (with TNF-induced viral hepatitis at day 5) was not sufficient to increase mortality and serum ALT levels in mice receiving XIM (unpublished data). This, together with our current findings may suggest that accumulation of stress/damage combined with shifting to chronic

inflammation is essential for the development of poor prognosis and increased severity observed in mice treated with XIM. As already hypothesized for the drug or other environmental factors involved in iDILI, stress/danger signal release may sensitize the hepatocytes to death signals (Seguin and Uetrecht, 2003; Stirnimann et al., 2010). For this reason, the hepatic changes observed by the administration of XIM only, although not associated with histopathological modifications or with increased serum liver enzymes, may have an initiating role in the later development of XIM-induced liver injury.

Notably, XIM administration exerted a slight increase in the percentage of NK cells in liver leukocytes 48 h after the injection of AdGOL (day 7) and 2 h after TNF injection. Both XIM-mediated changes in NK cell percentage were accompanied by an increased expression of FASL in the liver. FASL can be expressed not only by lymphocytes (NK and CD8 T cells) but also by several other cells in the liver and it has been demonstrated that FAS/FASL interaction participates in TNF-induced viral hepatitis (Wohlleber et al., 2012; Zou et al., 2010). In the liver, the clearance of virus-infected cells is sustained by the progressive recruitment of lymphocytes (NK, NK(T) and CD8 T cells) into the organ where they kill infected-hepatocytes via cell-cell interaction (e.g. FAS/FASL) and release of soluble factors (cytokines). The injection of TNF in this model accelerated the sequestration of leukocytes in the liver, as demonstrated by the increased cellularity observed in the liver of mice 2 h after the administration of the cytokine. It is well-known that TNF plays a fundamental role in leukocyte sequestration into the liver and especially elicits the recruitment of neutrophils, monocytes and NK cells in this organ (Vollmar and Menger, 2009; Giustarini, Kruijssen, et al., 2018; Giustarini, Vrisekoop Nienke, et al., 2018; Pilaro et al., 1994). For these reasons, the higher percentage of NK cells together with the increased hepatic expression of FAS and FASL induced by XIM might expose the liver of these mice to increased killing of virus-infected hepatocytes, potentially leading to more severe hepatic damage and poor prognosis as observed after TNF injection (unpublished data). Although further investigations are needed, these findings underline that XIM may potentiate the immune response to viral infection increasing the NK-mediated killing of infected hepatocytes.

Surprisingly, the percentage and amount of $CD49b^-$ NK cells were increased in the spleen of these mice immediately after the first administration of the drug. Although with the available data it is difficult to understand if the presence of liver-specific NK cells in the spleen at day 1 have a role in XIM-induced liver injury, their possible involvement in the liver damage after TNF injection make this finding very interesting for further investigations. Indeed, the fact that these cells have been demonstrated to play a key role in contact hypersensitivity reactions might represent a common feature with skin rashes often observed in man suffering from iDILI (Devarbhavi et al., 2016).

The increased percentage of pDCs might represent another finding pointing to a potential XIM-mediated potentiation of the anti-viral response. pDCs have been

demonstrated to play a fundamental role in the recruitment and activation of NK cells during viral infection (Krug et al., 2004; Takeda et al., 2003). The pDC could also play a role in the increased mortality and serum ALT observed in XIM-treated mice in this model of liver injury.

Since it has been demonstrated that TNF activates apoptosis in infected hepatocytes via stimulation of TNF receptor 1 (Wohlleber et al., 2012) we evaluated the transcription of TNF in response to the injection of the same cytokine. Two hours after the injection of the cytokine no difference in expression of hepatic TNF mRNA was found that could have explained the worsening effects of XIM on liver damage.

In conclusion, the increased percentage of pDCs and NK cell in the liver due to XIM treatment may explain the increased serum ALT levels and the poor prognosis observed in these mice during TNF-induced viral hepatitis (day 7). The increased hepatic expression of FAS and FASL suggests that interaction of these proteins and NK cell-mediated killing of infected hepatocytes are important in XIM-mediated DILI. XIM administration alone induced a certain level of stress in the liver but without evident liver damage, underlying potential regulatory effects for neutrophil and inflammatory monocyte recruitment to the liver. Further investigations of this or similar models with the use of viral infections may further disclose mechanisms involved in iDILI that more closely resemble the clinical signature of these adverse drug reactions in man.

References

Agnelli, G. et al. (2009) *Safety assessment of new antithrombotic agents: Lessons from the EXTEND study on ximelagatran. Thromb. Res., 123, 488–497.*

Ainscow, E.K. et al. (2008) *Investigations into the liver effects of ximelagatran using high content screening of primary human hepatocyte cultures. Expert Opin. Drug Saf., 7, 351–365.*

Ballet, F. (2015) *Preventing Drug-Induced Liver Injury: How Useful Are Animal Models? Dig. Dis., 33, 477–485.*

Bonacini, M. (2004) *Liver Injury during Highly Active Antiretroviral Therapy: The Effect of Hepatitis C Coinfection. Clin. Infect. Dis., 38, S104–S108.*

den Brinker, M. et al. (2000) *Hepatitis B and C virus co-infection and the risk for hepatotoxicity of highly active antiretroviral therapy in HIV-1 infection. AIDS, 14, 2895–902.*

Chalasani, N. et al. (2015) *Features and outcomes of 899 patients with drug-induced liver injury: The DILIN prospective study. Gastroenterology, 148, 1340–1352.e7.*

Chalasani, N. and Björnsson, E. (2010) *Risk factors for idiosyncratic drug-induced liver injury. Gastroenterology, 138, 2246–59.*

Deng, X. et al. (2006) *Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. J. Pharmacol. Exp. Ther., 319, 1191–1199.*

Devarbhavi, H. et al. (2016) *Drug-induced liver injury associated with stevens-Johnson syndrome/ toxic epidermal necrolysis: Patient characteristics, causes, and outcome in 36 cases. Hepatology, 63, 993–999.*

Dong, Y. et al. (2016) *The protective or damaging effect of Tumor necrosis factor - α in acute liver injury is concentration - dependent. Cell Biosci., 4–13.*

Eriksson, U.G. et al. (2003) *Absorption, Distribution , Metabolism , and Excretion of Ximelagatran , an Oral Direct Thrombin Inhibitor , in Rats , Dogs , and Humans Abstract : 31, 294–305.*

Faletti, L. et al. (2018) *TNFα sensitizes hepatocytes to FasL-induced apoptosis by NFκB-mediated Fas upregulation. Cell Death Dis., 9.*

Gabay, C. (2006) *Interleukin-6 and chronic inflammation. 6, 1–6.*

Gandhi, A. et al. (2013) *Chlorpromazine-induced hepatotoxicity during inflammation is mediated by TIRAP-dependent signaling pathway in mice. Toxicol. Appl. Pharmacol., 266, 430–8.*

Giustarini, G., Kruijssen, L., et al. (2018) *Tissue influx of neutrophils and monocytes is delayed during development of trovafloxacin - induced tumor necrosis factor - dependent liver injury in mice. J Appl Toxicol, 38, 753–765.*

Giustarini, G., Vrisekoop Nienke, et al. (2018) *Trovafloxacin-induced liver injury: lack in regulation of inflammation by inhibition of nucleotide release and neutrophil movement. Toxicol. Sci.*

Holub, M. et al. (2009) *Neutrophils sequestered in the liver suppress the proinflammatory response of Kupffer cells to systemic bacterial infection. J. Immunol., 183, 3309–16.*

Jaeschke, H. and Hasegawa, T. (2006) *Role of neutrophils in acute inflammatory liver injury. Liver Int., 26, 912–919.*

Kenne, K. et al. (2008) *Prediction of drug-induced liver injury in humans by using in vitro methods: The case of ximelagatran. Toxicol. Vitr., 22, 730–746.*

Kim, W.S. et al. (2016) *Hepatitis C and not Hepatitis B virus is a risk factor for anti-tuberculosis drug induced liver injury. BMC Infect. Dis., 16, 1–7.*

Kindmark, a et al. (2008) *Genome-wide pharmacogenetic investigation of a hepatic adverse event without clinical signs of immunopathology suggests an underlying immune pathogenesis. Pharmacogenomics J., 8, 186–95.*

Krug, A. et al. (2004) *TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity, 21, 107–119.*

Lasser, K.E. (2002) *Timing of New Black Box Warnings and Withdrawals for Prescription Medications. Jama, 287, 2215.*

Lee, W.M. et al. (2005) *Hepatic findings in long-term clinical trials of ximelagatran. Drug Saf., 28, 351–370.*

Lu, J. et al. (2013) *Tumor necrosis factor-alpha potentiates the cytotoxicity of amiodarone in Hepa1c1c7 cells: roles of caspase activation and oxidative stress. Toxicol. Sci., 131, 164–78.*

Luyendyk, J.P. (2003) *Ranitidine Treatment during a Modest Inflammatory Response Precipitates Idiosyncrasy-Like Liver Injury in Rats. J. Pharmacol. Exp. Ther., 307, 9–16.*

Marwick, J.A. et al. (2018) *Neutrophils induce macrophage anti-inflammatory reprogramming by suppressing NF-κB activation article. Cell Death Dis., 9.*

Pilaro, A.M. et al. (1994) *TNF-alpha is a principal cytokine involved in the recruitment of NK cells to liver parenchyma. J. Immunol., 153, 333–342.*

Scheller, J. et al. (2011) *The pro- and anti-in fl ammatory properties of the cytokine interleukin-6. Biochim. Biophys. Acta, 1813, 878–888.*

Seguin, B. and Uetrecht, J. (2003) *The danger hypothesis applied to idiosyncratic drug reactions. Curr Opin Allergy Clin Immunol, 3, 235–242.*

Shaw, P.J., Beggs, K.M., et al. (2009) *Trovafloxacin enhances TNF-induced inflammatory stress and cell death signaling and reduces TNF clearance in a murine model of idiosyncratic hepatotoxicity. Toxicol. Sci., 111, 288–301.*

Shaw, P.J., Ganey, P.E., et al. (2009) *Tumor Necrosis Factor α is a Proximal Mediator of Synergistic Hepatotoxicity from Trovafloxacin / Lipopolysaccharide Coexposure. J Pharmacol Exp Ther, 328, 62–68.*

Soehnlein, O. and Lindbom, L. (2010) *Phagocyte partnership during the onset and resolution of inflammation. Nat. Rev. Immunol., 10, 427–439.*

Souza, P.R. et al. (2015) *Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis. Sci. Transl. Med., 7, 315ra190-315ra190.*

Sprinzl, M.F. et al. (2001) *Transfer of Hepatitis B Virus Genome by Adenovirus Vectors into Cultured Cells and Mice: Crossing the Species Barrier. J. Virol., 75, 5108–5118.*

Stabenow, D. et al. (2010) *Bioluminescence imaging allows measuring CD8 T cell function in the liver. Hepatology, 51, 1430–7.*

Stirnimann, G. et al. (2010) *Liver injury caused by drugs: An update. Swiss Med. Wkly., 140, 18–24.*

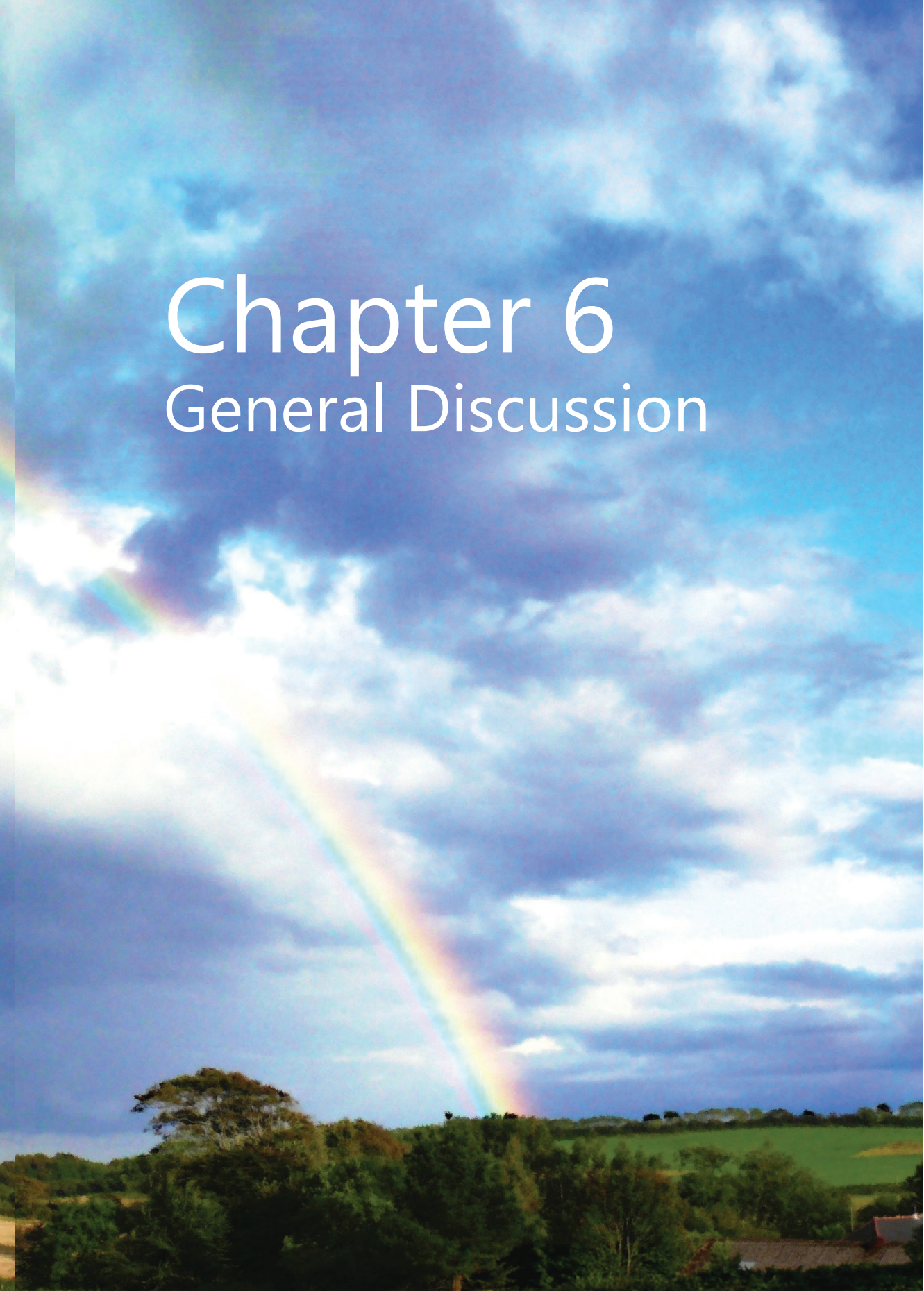
Takeda, K. et al. (2003) Toll-Like Receptors. Annu. Rev. Immunol., 21, 335–376.

Vollmar, B. and Menger, M.D. (2009) *The Hepatic Microcirculation : Mechanistic Contributions and Therapeutic Targets in Liver Injury and Repair. 1269–1339.*

Wohlleber, D. et al. (2012) *TNF-induced target cell killing by CTL activated through cross-presentation. Cell Rep., 2, 478–87.*

Yoon, J.H. and Gores, G.J. (2002) *Death receptor-mediated apoptosis and the liver. J. Hepatol., 37, 400–410.*

Zou, Y. et al. (2010) *Increased Killing of Liver NK Cells by Fas/Fas Ligand and NKG2D/NKG2D Ligand Contributes to Hepatocyte Necrosis in Virus-Induced Liver Failure. J. Immunol., 184, 466–475.*



Chapter 6
General Discussion

GENERAL DISCUSSION

The mechanisms behind idiosyncratic drug-induced liver injury (IDILI) are far from being clear, and reliable pre-clinical tests capable of identifying hepatotoxic compounds already at drug development stage are lacking. Although drugs are extensively tested in vitro and in vivo during pre- and clinical screenings, their use is too often associated with the occurrence of severe hepatic adverse reactions as demonstrated by post-marketing pharmacovigilance studies (Lee and Senior, 2005; Watkins and Seeff, 2006). The occurrence of these hepatic adverse reactions is usually followed by regulation of drug use or their withdrawal from market.

The investigations collected in this thesis were embedded in an EC Innovative Medicines Initiative (IMI)-funded project called **"Mechanism-Based Integrated Systems for the Prediction of Drug-Induced Liver Injury" (MIP-DILI).** This project had the ambition to create better predictive test methods for toxicological screening of drugs.

When we first started these investigations, as part of the Work-package 3 (Chapter 1.6), we did not expect to find a common liver toxicity mechanism for the diverse IDILI-inducing compounds, belonging to several pharmacological classes.

In this thesis, we set up to evaluate the role of innate immunity in the development of IDILI. Innate immunological changes were evaluated in establish models of IDILI using trovafloxacin (TVX) and ximelagatran (XIM).

Based on previously published findings, we expected to contribute to the characterization of IDILI through the investigation of interactions of these pharmaceuticals with inflammatory innate mechanisms. Mechanisms participating in liver homeostasis and pathologies include intra-cellular and inter-cellular innate immune responses of both parenchymal and non-parenchymal cells embedded within the hepatic tissue. We set out to investigate how **pharmaceuticals induce perturbations of inflammatory innate mechanisms in relation to liver injury.**

This approach to study IDILI mechanisms links to the analysis of the hypotheses mentioned in Chapter 1.7. The main hypotheses describing potential immune mechanisms behind the initiation and development of IDILI are non-mutually exclusive and seem to be either additive (like the danger together with the hapten hypothesis) or inclusive (like the inflammatory stress hypothesis with the danger hypothesis) (Pirmohamed et al., 2002; Roth et al., 2017). From this perspective, these apparently exclusive hypotheses seem to converge in a unique postulate, merging the inherent or metabolically acquired reactivity of drugs with cellular stress-inducing capacity of the same drugs or confounding inflammatory substances.

This unified hypothesis incorporates the idea that **episodes of modest**

inflammation during drug therapy may be involved in initiating liver injury and potentially also adaptive responses against drug (or hapten)-protein conjugates. These inflammatory episodes can, for instance, result from mild, subclinical endotoxemia which normally occurs in people (Roth et al., 1997; Ganey and Roth, 2001). In addition, we might also consider that patients receive these treatments for the therapy of very diverse diseases including chronic inflammatory disease or infections.

Importantly however, as mentioned in **Chapter 1.4**, inflammatory mechanisms are also involved in the hepatic homeostatic processes. Interactions of the drugs with this tuning of liver health represent a likely extension of this unified hypothesis that has not been fully considered so far. This hypothesis may explain both the irregular occurrence as well as the low incidence of IDILI as it suggests that multiple immunological and non-immunological checkpoints have to be overcome to elicit a liver failure.

Although clinical investigations on IDILI suggest the involvement of the adaptive immune system in some of these adverse drug reactions (Ju and Reilly, 2012; Kim and Naisbitt, 2016), we were confident that behind a pathological adaptive response we could identify dysregulations of the innate immunity. In fact, based on its own nature, the innate immune system is the apparatus in charge of initial sensing and resolving consequences of cellular stress/damage but also of mounting adaptive responses against harmful agents (Manoury and De Berardinis, 2019). Based on these upstream activities, the innate immune system represents an obligate crossroad for the onset of IDILIs. As a corollary of this reasoning, pharmaceuticals that cause hepatotoxicity in rodents when combined with TNF or LPS (**Chapter 1.7.1**) are legitimate candidates to study innate immunity in relation to IDILI.

Thus, we started with the investigations into the role of innate immune cells using the animal model of TVX-induced liver injury based on co-administration of the fluoroquinolone TVX either with the pleiotropic cytokine TNF or the bacterial component LPS (**Chapter 2, 3 and 4**) as surrogate for inflammation and bacterial infection, respectively. This model was created in the laboratory of Prof. Roth (Michigan State University) and extensively studied in vitro and in vivo.
This model represents a tool to study the effects of TVX on the inflammation caused by pathological and physiological concentrations of TNF, a pleiotropic cytokine involved in non- and sterile inflammation. We have also investigated a new in vivo model of IDILI based on the administration of the thrombin inhibitor XIM during TNF-induced viral hepatitis (**Chapter 5**).

The collected investigations obtained from these models include **the following critical and common points of relevance to underline the proposed unified hypothesis:**

- **Compounds alone elicit liver stress** which is not associated with evident histopathological modifications and increase of markers of liver toxicity (i.e. ALT)
- **Phagocytes, in particular neutrophils and monocytes, sense drug- and TNF-induced stress and, depending on the kinetic of their recruitment, these cells may lead to two opposite fates: regulation of inflammation or damage.**
- **IDILI-associated drugs potentiate innate immune responses to non-sterile and sterile inflammation, and cause tissue damage in an antigen-unspecific manner.**

6.1. Compound-induced stress and immune responses during local and systemic inflammation

Both TVX and XIM showed direct and indirect evidence of hepatocyte stress after exposure to the drugs alone. It was already demonstrated in previous studies that TVX and XIM can respectively cause DNA damage and mitochondrial dysfunctions that were not associated with increased overt cytotoxicity in vitro (Beggs et al., 2014; Ainscow et al., 2008; Kenne et al., 2008).

In our in vivo experiments (**Chapters 2 and 5**), the observed drug-induced stresses resulted in a slight and local increase of inflammation as demonstrated by increased cytokine production and recruitment of neutrophils and NK cells into the liver. Although the stresses observed with administration of the drug alone did not result in overt hepatotoxicity, their occurrence may represent a fundamental step for the onset of IDILI.

The evidence that the drug alone is associated only with signs of organ stress reinforces the idea that IDILI results from a plethora of additional multifactorial influences (García-Cortés et al., 2018) such as sterile and non-sterile inflammation. We found interesting changes in innate immune cells in both animal models (**Chapter 2, 3 and 5**).
For instance, NK cells were enriched in liver and spleen of mice few hours after the administration of both TVX and XIM. Although we cannot clarify the role in the studied models, we have previously observed increase in this subset with some other drugs (ofloxacin, paracetamol, diclofenac, carbamazepine) shortly after administration (Kwast et al., 2016).

In addition, mice exposed to the tested drugs showed increased production of MIP-2, aimed at recruiting neutrophils. It was intriguing to observe that while TVX increased the MIP-2 expression the recruitment of neutrophils into the liver (and spleen) was actually decreased. We further investigated this apparent discrepancy and revealed that the decreased number of neutrophils in presence of increased concentrations of MIP-2 in the liver was due to the inhibitory effect of the fluoroquinolone on human neutrophil motility (**Chapter 3**). In addition to a decreased influx of neutrophils the influx of monocytes into the liver was

completely prevented.

Extravasation of neutrophils in the liver parenchyma is usually associated with tissue damage (Jaeschke and Smith, 1997). By contrast, a regulatory role of these cells has been demonstrated during inflammation in several organs including the liver (Holub et al., 2009; Soehnlein and Lindbom, 2010). The recruitment of neutrophils into the inflamed tissue serves to regulate inflammatory tissue-resident macrophages (Soehnlein and Lindbom, 2010; Holub et al., 2009). In particular, tissue resident macrophages reduce their synthesis of pro-inflammatory cytokines after contact with viable neutrophils (Marwick et al., 2018) or via phagocytosis of apoptotic cells (Soehnlein and Lindbom, 2010; Holub et al., 2009).
Interestingly, the liver-resident macrophages (Kupffer cells, KC) play a fundamental role in the turnover of neutrophils in health and disease (Condliffe et al., 2010) by phagocytizing apoptotic neutrophils. The disruption of neutrophil sequestration by TVX, might render KC more responsive to physiological levels of LPS/TNF in the liver but also to other inflammatory signals. As shown in our and others' studies, the depletion of neutrophils increased the expression of inflammatory cytokines in the liver during the early phase of infection or inflammation (Holub et al., 2009; Giustarini et al., 2019) and transfer of apoptotic neutrophils in mice prior to LPS exposure lowered synthesis of cytokines (Ren et al., 2008).
In addition, as observed for TVX (Giustarini et al., 2019), the decreased motility of neutrophils resulted in a delayed but prolonged presence of these cells in the narrow sinusoids. This potentially associates with their intravascular activation due to increased cytokine releases (especially TNF) during their delayed recruitment.

As several other pharmaceuticals have shown to synergize with TNF both in vivo as well as in vitro (Gandhi et al., 2013; Deng et al., 2006; Lu et al., 2013; Luyendyk, 2003), increased release of TNF due to delayed and prolonged neutrophil recruitment to the liver might represent a new mechanism of IDILI which need to be further investigated.

This new mechanism, presented in Chapter 2 and 3, might be linked to the hypothesis called "failure to adapt". This hypothesis assumes that the severe liver injury may involve a failure in adapting to an injury caused by the drug (Roth et al., 2017). Our current findings may suggest failure to adapt to homeostatic or pathological inflammatory episodes causing increased TNF secretion and IDILI as direct consequence of impaired neutrophil regulatory functions.
It is well known that viable and apoptotic neutrophils may downregulate pro-inflammatory cytokines when sequestered in organs like the liver increasing the secretion of IL-10 by macrophages which, in turn, decrease TNF secretion. For this reason, the early recruitment of neutrophils into organs serves as a signal to switch off the production of proinflammatory cytokines induced by "homeostatic" and pathological inflammatory episodes. In this regard, it is not surprising that patients with genetic low levels of IL-10 secretion seem to be more susceptible to IDILI (Pachkoria et al., 2008; Aithal et al., 2004). In particular, it seems that

the anti-inflammatory rather than the immunoregulatory functions of IL-10 play a fundamental role in the onset of IDILI (Aithal et al., 2004). Among the compounds that were associated with an increased incidence of IDILI in patients with low IL-10 production is diclofenac. Diclofenac and other non-steroidal anti-inflammatory drugs (NSAIDs) also inhibit neutrophil movement but the inhibitory mechanism remain to be further elucidated (Hasçelik et al., 1994).

The fact that NSAIDs are among the pharmaceuticals often associated with the occurrence of DILI (Licata et al., 2017) not only underpins the importance of inflammation in the hepatotoxic synergism elicited by these compounds, but also suggests that molecules with anti-inflammatory features may mask their effects on liver by inhibiting the recruitment of neutrophils or other leukocytes. Indeed, many NSAIDs have been associated with reduced motility of neutrophils, which may affect their recruitment and time of sequestration in the liver, but the exact mechanism behind this effect seems to vary among compounds and it is still under investigation (Díaz-gonzález and Sánchez-madrid, 2016).

In order to create better predictive tests for IDILI, we should overcome the possibility that compounds might mask their toxicity via the reduction of the recruitment of immune cells to the stressed/damaged site (neutrophil count or quantification of their enzymes in organs are commonly used markers for inflammation). More efforts in the identification of "signals 0s", such as DAMPs, may help to create better tools for IDILI toxicity screening.
Neutrophils influx to the site of inflammation is usually followed by the recruitment of monocytes which help resident macrophages scavenging apoptotic neutrophils. If not ingested by monocytes/macrophages, apoptotic neutrophils may experience secondary necrosis which represent a very harmful event. Indeed, release of neutrophil cargo, such as proteases, may cause necrosis and amplification of inflammation (Brauner et al., 2013).
From the observations collected in the model of TVX+TNF, we deduced that the finely regulated partnership of phagocytes was disrupted leading to longer permanence of neutrophils in the liver. This prolonged presence of neutrophils seems to be linked to the disruption of the chemotactic activity of phagocytes and/or the release of apoptosis associated "find me" signals. The inter-cellular regulatory mechanisms of inflammation in IDILI need to be further evaluated and validated with other compounds, although "failure to adapt" hypothesis represents an appealing insight for further investigations. Based on this hypothesis, training compounds are now under investigation with a simplified method for chemotaxis, adherence and invasion using HL60 and RAW264.7 cells. Preliminary data show that at least two other compounds, such as diclofenac and troglitazone, seem to reduce the motility of the tested cell lines toward chemoattractants. Although mechanisms likely differ for each compound, the effect on the migration of phagocytes toward chemoattractant may represent a key event for compounds associated with IDILI in humans.

Chemotaxis of phagocytes and their recruitment in organs is a very complex process depending on the organ and the conditions in which the cells are embedded during the motility phenomenon and drugs may affect this phenomenon at different levels. Proper chemotaxis and organ recruitment rely on finely regulated machinery including: expression of integrins (Fan et al., 2016), activation of a precise intracellular sequelae after chemoattractant or integrin stimulation (Bao et al., 2013; McCormick et al., 2017), shear stress, vessel size and endothelial expression of proteins involved in margination, rolling and crawling/adhesion (Vollmar and Menger, 2009). Any of these components involved in the chemotaxis and organ sequestration of phagocytes might represent a target for pharmaceuticals to establish sustained inflammation.

It is important to underline that disruption of phagocyte activities may require active concentrations of drugs during different phases of the kinetic process of cellular partnership. Apart from the cellular kinetics, we need to consider the pharmacokinetics of the particular drugs. Only at specific intersections between these two kinetics a detrimental outcome might be observed. This should also be considered in setting up predictive in vivo and vitro models. Further evaluations aiming to quantify positive and negative predictivity among training and testing compounds will be needed to evaluate specificity and sensitivity of these tests in the context of IDILI.

In conclusion, the data presented in Chapter 2 and 3 shows a hitherto unidentified role of neutrophils and monocytes in the control of liver homeostasis and in the regulation of inflammation in the context of IDILI. In IDILI neutrophils do not only participate in the destruction of the tissue but also actively and passively regulate inflammation in the liver. Their regulation of inflammation is fundamental to avoid increased release of inflammatory cytokines (i.e. TNF) which are already known to synergize with many IDILI-associated compounds leading to hepatocellular toxicity.
Moreover, the incapability to clear the dead cells (Brauner et al., 2013) or to regulate inflammation in presence of the drugs may play a pivotal role in sustaining systemic or liver inflammation, rendering otherwise occult antigens accessible and, in turn, contributing to setting up the stage for an adaptive response against new antigens.
The evidence collected in chapter 2 and 3 suggest that further investigations should aim at identification of liver-specific DAMPs or cytokine release within the liver in conjunction with the assessment of innate immune cell recruitment into the liver (Fig. 1).

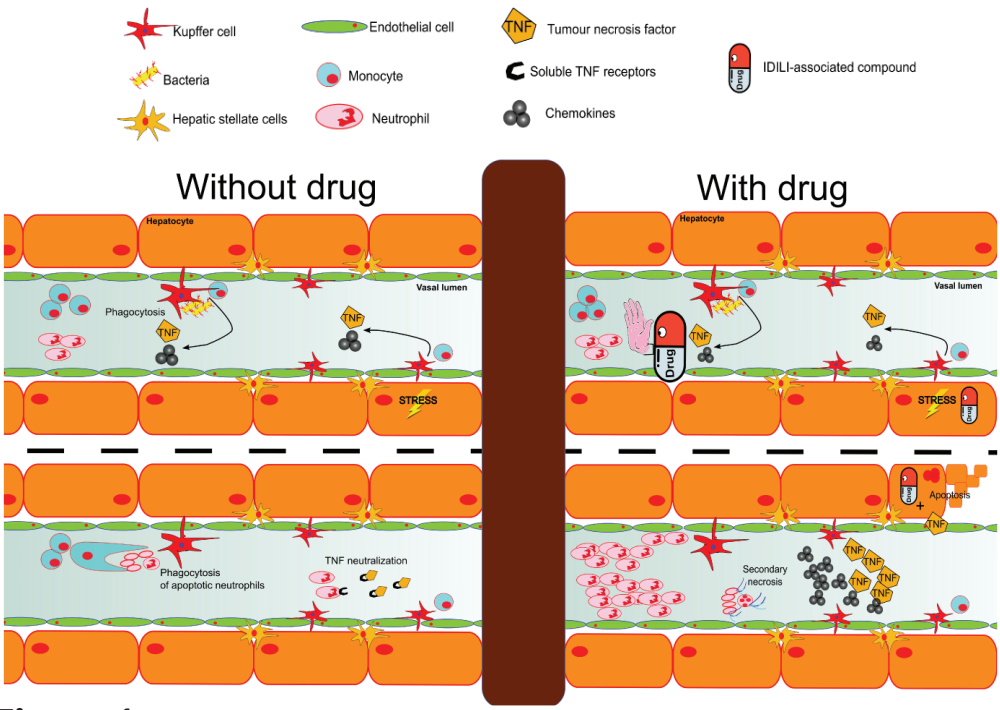


Figure 1

Disruption of neutrophil and monocyte recruitment in the liver increases inflammatory response.

As already explained in Chapter 1.4 neutrophil and monocyte recruitment is fundamental for the regulation of inflammation. Briefly, patrolling monocytes and resident macrophages of the liver (Kupffer cells) sense either cell stress/damage, microbes or their components in the liver and the blood perfusing this organ. In response to these stimuli, Kupffer cells and monocytes start producing cytokines (i.e. TNF) and chemokines (such as IL-8) in order to recruit immune cells into the organ. The first to reach the inflamed organ are neutrophils and monocytes. Viable neutrophils dampen the inflammatory response releasing soluble TNF receptors and via a hitherto unidentified mechanism (Marwick et al., 2018) which is negatively affecting macrophage production of pro-inflammatory mediators. Neutrophils are short living cells and apoptotic neutrophils at inflammation site are promptly phagocyted by macrophages and recruited monocytes. Phagocytosis of apoptotic neutrophils is considered the beginning of inflammation resolution promoting anti-inflammatory functions in macrophages. The prompt clearance of dying neutrophils is fundamental to prevent release of neutrophil cellular content and sustaining inflammation.

Drugs associated with IDILI in humans may negatively affect or delay the recruitment of neutrophils and monocytes during the onset of liver inflammation. As shown for TVX, this will lead to accumulation of these cells in blood, increased pro-inflammatory cytokine (i.e. TNF) and chemokine production in the liver. The increased TNF release due to delayed or impaired neutrophil and monocyte recruitment into the liver could play a role in the induction of the damage mediated by some IDILI-associated drugs which have shown to cause hepatocyte apoptosis when co-administered with TNF or LPS. The impaired movement of phagocytes may also contribute to reinforce inflammation since lack in clearance of apoptotic bodies leads to secondary necrosis and release of dying cell content. In the case of apoptotic neutrophils, the release of their dangerous cargo may directly contribute to the organ damage.

6.2. Drugs potentiate intra- and inter-cellular immune responses during non- and sterile inflammation

Both intracellular and intercellular innate responses in the liver play a fundamental role in organ-specific and systemic responses to pathogens (Peng et al., 2016). Liver-resident macrophages KC have an important role as barrier functions to invasion of pathogenic organisms from the intestine. Physiological and pathological leakage of bacteria or their components into blood has been recognized as one of the potential keystones for the understanding of IDILI (Chen et al., 2015). Bacteria from the gut lumen or their products can reach the liver via the mesenteric veins which collect the blood from the bowels flowing toward the portal vein. The bacteria or their components may vary from patient to patient, depending on the intestinal microbiota and epithelial integrity in bowels (Ju and Tacke, 2016).

The possibility that pharmaceuticals promote an amplification of the intra-cellular innate immune responses toward bacterial components, such as LPS or LTA, has already been identified in vivo and in vitro (Poulsen, Olivero-Verbel, et al., 2014; Poulsen, Albee, et al., 2014; Shaw et al., 2007). Some of the studies showed that TVX increased the synthesis of TNF in mice and RAW264.7 cells exposed to LPS via the activation of ATR (Poulsen, Olivero-Verbel, et al., 2014; Poulsen, Albee, et al., 2014; Shaw et al., 2007).

We aimed to further investigate the effect of TVX on the intracellular pathways activated by LPS in RAW264.7 cells. Data in **Chapter 4** showed that TVX-mediated amplification of NF-kB translocation induced by LPS in RAW264.7 cells may lead to increased TNF release. The potentiation of LPS-induced NF-kB translocation and the related increase in TNF synthesis represents an undoubtful contribution to the development of IDILI. Indeed, the pleiotropic cytokine TNF can activate both proliferation/survival or cell death of hepatocytes depending on the activated intracellular cascades.

As discussed in **Chapters 1.5.2** and **4**, the outcome of TNF-mediated effect on hepatocytes depends on the interplay of very dynamic intracellular pathways such as NF-kB and MAPKs (Papa, S. et al., 2010). Detailed kinetic assessments of the TVX-induced perturbations of pathways triggered by TNF provided a better understanding of the mechanisms involved in hepatocyte cell death due to TVX+TNF exposure. The observed perturbations of pathway kinetics during TNF-stimulation not only better clarified the toxic effects of TVX, but also provided a potential signature for the identification of other compounds with DILI liability. In particular, our studies stress the importance of assessing the kinetics of intracellular pathways downstream of TNF receptors rather than assessing the phenotypic outcome of the cytokine stimulation.

We think that different mechanisms may lead to similar effects on the kinetics

of activation of NF-kB and MAPKs in hepatocytes. Our collaborators from Leiden University developed a panel of genetically modified HepG2 to observe kinetics of different pathways in presence of drugs associated with IDILI in humans (Wink et al., 2017; Herpers et al., 2016). Although this approach may provide useful information on IDILI-inducing compounds, in vivo investigations aiming to observe perturbations caused by pharmaceuticals are needed in addition to their evaluation in vitro. Only through the in vivo-in vitro validation we will be able to identify false positive or artifacts which may disturb the identification of common AOPs.

6.3. Future approaches for assessment of IDILI potential of new pharmaceuticals.

More experiments are needed for the identification of drug effects on the intercellular mechanisms involved in IDILI. As observed in Chapter 5, IDILI-associated drugs may increase the cellular response against viral infection quantitatively and qualitatively. Although these findings need to be further characterized, this evidence may serve to set up experiments involving infected hepatocytes and NK cells. Exacerbated response against viral infections may lead to detrimental effects on infected or non-infected organs. Indeed, as observed in a recent paper by Polakos and colleagues infection occurring in the epithelial cells of the lung is the cause of a "collateral damage" to the liver in some patients (Polakos et al., 2006). Mechanisms behind the liver damage induced by infections still need to be clarified but it seems reasonable that increased expression of FASL and CD40 ligand may play a fundamental role in the onset of damage (Adams and Hubscher, 2006; Polakos et al., 2006). As already demonstrated, hepatocytes are extremely sensitive to FASL-induced cell death and populations of activated lymphocytes seem to home to the liver for clearance after virus eradication (Adams and Hubscher, 2006). These observations linking viral response with the occurrence of liver damage strengthen the need to understand if more than just XIM can increase or exacerbate the immune response against virus. Drug-induced exaggerated immune responses might explain the increased killing potential of lymphocytes. These cells homing the liver may induce organ damage or even failure as "collateral effect" of immune system activation in presence of IDILI-associated compounds.

If further investigations confirm the involvement of these potential mechanisms in the development of IDILI in humans, these new insights might be used to set up a panel of new toxicological tests capable of identifying hepatotoxic compounds already at the preclinical level and to partly decrypt idiosyncrasy in IDILI.
Due to the multifactorial idiosyncratic nature of IDILI, it cannot be expected to capture IDILI-inducing hazard potency of new pharmaceuticals with a single unique test. Rather, a panel of tests will be needed to predict hazard and risk assessment of new pharmaceuticals. This links to the recently developed concept of adverse outcome pathways (AOPs). An AOP is a well-defined series of biological processes

(referred to as molecular initiating events (MIEs) and key events (KEs) leading to an adverse event. AOP allow to define a minimum set of common perturbations needed to elicit an adverse event. In agreement with this definition, different MIE and KE may converge in one AOP leading to the same outcome. Recently, an AOP has been proposed for inflammation (Villeneuve et al, 2018). Based on this AOP and our findings, we strongly believe that groups of compounds associated with IDILI affect different MIEs and KEs of a common AOP and that the latter can be evaluated with specific compounds.

As a derivative, we infer that a combination of tests analyzing effects of compounds on kinetic processes such as phagocyte migration combined with tests analyzing kinetics of relevant intracellular pathways may represent an additional tool to identify hepatotoxic compounds during development of new pharmaceutical entities and to estimate the risk of IDILI.

References

Adams, D.H. and Hubscher, S.G. (2006) *Systemic viral infections and collateral damage in the liver. Am. J. Pathol., 168, 1057–1059.*

Ainscow, E.K. et al. (2008) *Investigations into the liver effects of ximelagatran using high content screening of primary human hepatocyte cultures. Expert Opin. Drug Saf., 7, 351–365.*

Aithal, G.P. et al. (2004) *Hepatic Adducts, Circulating Antibodies, and Cytokine Polymorphisms in Patients with Diclofenac Hepatotoxicity. Hepatology, 39, 1430–1440.*

Bao, Y. et al. (2013) *Pannexin 1 channels link chemoattractant receptor signaling to local excitation and global inhibition responses at the front and back of polarized neutrophils. J. Biol. Chem., 288, 22650–7.*

Beggs, K.M. et al. (2014) *Molecular Mechanisms of Hepatocellular Apoptosis Induced by Trovafloxacin-Tumor Necrosis Factor-alpha Interaction. Toxicol. Sci., 137, 91–101.*

Brauner, J.M. et al. (2013) *No littering : the clearance of dead cells and leaking cellular contents and possible pathological complications. OA Arthritis, 1, 1–7.*

Chen, M. et al. (2015) *Drug-induced liver injury: Interactions between drug properties and host factors. J. Hepatol., 63, 503–514.*

Condliffe, A.M. et al. (2010) *Neutrophil kinetics in health and disease. Trends Immunol., 31, 318–324.*

Deng, X. et al. (2006) *Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. J. Pharmacol. Exp. Ther., 319, 1191–1199.*

Díaz-gonzález, F. and Sánchez-madrid, F. (2016) *NSAIDs : learning new tricks from old drugs. 45, 679–686.*

Fan, Z. et al. (2016) *Neutrophil recruitment limited by high-affinity bent β2 integrin binding ligand in cis. Nat. Commun., 7, 1–14.*

Gandhi, A. et al. (2013) *Chlorpromazine-induced hepatotoxicity during inflammation is mediated by TIRAP-dependent signaling pathway in mice. Toxicol. Appl. Pharmacol., 266, 430–8.*

Ganey, P.E. and Roth, R.A. (2001) *Concurrent inflammation as a determinant of susceptibility to toxicity from xenobiotic agents. Toxicology, 169, 195–208.*

García-Cortés, M. et al. (2018) *Drug-induced liver injury: a safety review. Expert Opin. Drug Saf., 17, 795–804.*

Giustarini, G. et al. (2019) T*rovafloxacin-Induced Liver Injury: Lack in Regulation of Inflammation by Inhibition of Nucleotide Release and Neutrophil Movement. Toxicol. Sci., 167, 385–396.*

Hasçelik, G. et al. (1994) *Effect of Some Anti-Inflammatory Drugs on Human Neutrophil Chemotaxis. J. Int. Med. Res., 22, 100–106.*

Herpers, B. et al. (2016) *Activation of the Nrf2 response by intrinsic hepatotoxic drugs correlates with suppression of NF-κB activation and sensitizes toward TNFα-induced cytotoxicity. Arch. Toxicol., 90, 1163–1179.*

Holub, M. et al. (2009) *Neutrophils sequestered in the liver suppress the proinflammatory response of Kupffer cells to systemic bacterial infection. J. Immunol., 183, 3309–16.*

Jaeschke, H. and Smith, C.W. (1997) *Mechanisms of neutrophil-induced parenchymal cell injury. J. Leukoc. Biol., 61, 647–653.*

Ju, C. and Reilly, T. (2012) *Role of immune reactions in drug-induced liver injury (DILI). Drug Metab. Rev., 44, 107–115.*

Ju, C. and Tacke, F. (2016) *Hepatic macrophages in homeostasis and liver diseases: From pathogenesis to novel therapeutic strategies. Cell. Mol. Immunol., 13, 316–327.*

Kenne, K. et al. (2008) *Prediction of drug-induced liver injury in humans by using in vitro methods: The case of ximelagatran. Toxicol. Vitr., 22, 730–746.*

Kim, S.H. and Naisbitt, D.J. (2016) *Update on advances in research on idiosyncratic drug-induced liver injury. Allergy, Asthma Immunol. Res., 8, 3–11.*

Kwast, L. et al. (2016) *Oral exposure to immunostimulating drugs results in early changes in innate immune parameters in the spleen. J. Immunotoxicol., 13, 535–547.*

Lee, W.M. and Senior, J.R. (2005) *Recognizing drug-induced liver injury: current problems, possible solutions. Toxicol. Pathol., 33, 155–164.*

Licata, A. et al. (2017) *DILI and epidemiology. 21, 112–121.*

Lu, J. et al. (2013) *Tumor necrosis factor-alpha potentiates the cytotoxicity of amiodarone in Hepa1c1c7 cells: roles of caspase activation and oxidative stress. Toxicol. Sci., 131, 164–78.*

Luyendyk, J.P. (2003) *Ranitidine Treatment during a Modest Inflammatory Response Precipitates Idiosyncrasy-Like Liver Injury in Rats. J. Pharmacol. Exp. Ther., 307, 9–16.*

Manoury, B. and De Berardinis, P. (2019) *Bridging Innate and Adaptive Immunity. Front. Immunol., 10, 1–3.*

Marwick, J.A. et al. (2018) *Neutrophils induce macrophage anti-inflammatory reprogramming by suppressing NF-κB activation article. Cell Death Dis., 9.*

McCormick, B. et al. (2017) *Cross-talk between Rho GTPases and PI3K in the neutrophil. Small GTPases, 0, 1–9.*

Pachkoria, K. et al. (2008) A*nalysis of IL-10, IL-4 and TNF-α polymorphisms in drug-induced liver injury (DILI) and its outcome. J. Hepatol., 49,* ***107–114.***

Papa, S. et al. (2010) *Mechanisms of liver disease: The crosstalk between the NF-κB and JNK pathways. Biol Chem., 390, 965–976.*
Peng, H. et al. (2016) *Liver natural killer cells: Subsets and roles in liver immunity. Cell. Mol. Immunol., 13, 328–336.*
Pirmohamed, M. et al. (2002) *The danger hypothesis--potential role in idiosyncratic drug reactions. Toxicology, 181–182, 55–63.*
Polakos, N.K. et al. (2006) *Kupffer cell-dependent hepatitis occurs during influenza infection. Am. J. Pathol., 168, 1169–1178.*
Poulsen, K.L., Olivero-Verbel, J., et al. (2014) T*rovafloxacin enhances lipopolysaccharide-stimulated production of tumor necrosis factor-α by macrophages: role of the DNA damage response. J. Pharmacol. Exp. Ther., 350, 164–70.*
Poulsen, K.L., Albee, R.P., et al. (2014) *Trovafloxacin potentiation of lipopolysaccharide-induced tumor necrosis factor release from RAW 264.7 cells requires extracellular signal-regulated kinase and c-Jun N-Terminal Kinase. J. Pharmacol. Exp. Ther., 349, 185–91.*
Ren, Y. et al. (2008) *Apoptotic cells protect mice against lipopolysaccharide-induced shock. J. Immunol., 180, 4978–85.*
Roth, R.A. et al. (2017) *Idiosyncratic Drug-Induced Liver Injury: Is Drug-Cytokine Interaction the Linchpin? J. Pharmacol. Exp. Ther., 360, 461–470.*
Roth, R.A. et al. (1997) *Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents? Toxicol. Appl. Pharmacol., 147, 300–311.*
Shaw, P.J. et al. (2007) *Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. Toxicol. Sci., 100, 259–66.*
Soehnlein, O. and Lindbom, L. (2010) *Phagocyte partnership during the onset and resolution of inflammation. Nat. Rev. Immunol., 10, 427–439.*
Vollmar, B. and Menger, M.D. (2009) *The Hepatic Microcirculation : Mechanistic Contributions and Therapeutic Targets in Liver Injury and Repair. 1269–1339.*
Watkins, P.B. and Seeff, L.B. (2006) *Drug-induced liver injury: Summary of a single topic clinical research conference. Hepatology, 43, 618–631.*
Wink, S. et al. (2017) *High-content imaging-based BAC-GFP toxicity pathway reporters to assess chemical adversity liabilities. Arch. Toxicol., 91, 1367–1383.*

Chapter 7
Summary

Summary

Adverse drug reactions (ADRs) are complications associated with the use of drugs. The complications may affect several organs of our body with a very diverse impact on people's life. ADRs are a major concern for health care providers, pharmaceutical companies and regulatory agencies. Between 1975 and 2000 many concerns were raised on the approval process of drugs, since almost 10% of the marketed pharmaceuticals were regulated in their use or banned.
Drugs frequently affect the liver causing the so-called "drug-induced liver injury" (DILI). The various types of DILI can be categorized as being intrinsic or idiosyncratic. Intrinsic DILI is defined by a clear dose- and time-dependent relationship with regard to the usage of a particular drug. By contrast, idiosyncratic DILI (IDILI) is not clearly related to the dose of the drug and time of drug administration and is considered to be related to complex immune processes. IDILI may result from a combination of multiple external (e.g. microbial) and individual (e.g. genetic or lifestyle) factors and is therefore a unique condition related to a specific patient. As a result, IDILI can usually not be predicted during the straightforward pre- and clinical toxicity and safety studies that are performed for marketing approval by regulatory agencies. So far, IDILIs became apparent only during post-marketing surveillance, which stresses the importance to set up innovative predictive models for the identification of IDILI alerts that can be used at the early research & development stage of new pharmaceutical leads.

The main aim of the project "Mechanism-Based Integrated Systems for the Prediction of Drug-Induced Liver Injury" (MIP-DILI), funded by Innovative Medicines Initiative (IMI), was therefore to develop a new predictive strategy for the identification of IDILI-causing compounds, using models that are based on mechanisms involved in the onset of liver damage by pharmaceuticals.

In the last decades, it has been proven that overt liver toxicity of IDILI-associated compounds might be observed in animal models when rodents are administrated with a combination of the drug with bacterial components or inflammatory stimuli, such as lipopolysaccharide (LPS) and tumour necrosis factor (TNF) respectively. This evidence suggest that infection or inflammation may take part in the development of IDILI.

IDILI in man is often characterized by involvement of both innate and adaptive components of the immune system. The innate immune system is a complex organization of barriers, such as skin and mucosae, and cells and proteins capable to build an immediate non-specific response against microbes and harmful substances.

The liver does not only metabolize and excrete ingested substances, but also has important immunological functions. Among these are clearance of the bacterial

products originating from the gut flora, regulation of several immune cell turnovers, and the initiation of immune responses toward extraneous and harmful substances for our body.

Liver-resident macrophages play a pivotal role in the immunological functions of the liver. These cells, which belong to the innate immune system, scavenge bacterial components that reach the liver from the gut. Collaboration of these macrophages with other cells of the innate immune system, such as neutrophils and monocytes, is fundamental for keeping the liver in health and to regulate inflammation in this organ. Notably, after clearing bacterial components resident macrophages produce inflammatory signals to recruit neutrophils and monocytes to the liver. In particular neutrophil recruitment stops the production of inflammatory signals by macrophages. Moreover, neutrophils promote the recruitment of monocytes which help resident macrophages to clear dying cells at the site of inflammation. The uptake of dying neutrophils by macrophages/ monocytes will skew macrophages from a pro-inflammatory to anti-inflammatory phenotype, thus ending the inflammatory process. The pivotal role of this innate cell partnership in liver and the involvement of inflammation in the development of IDILI (as underlined by animal models) prompted us to investigate the effects of IDILI-associated drugs on innate responses during the development of liver damage.

We investigated mechanisms of IDILI using two different compounds: trovafloxacin (TVX), an antibiotic, and ximelagatran (XIM), an anti-coagulant, both withdrawn from the market due to the high incidence of unpredicted DILI. For both chemicals complex animal models have been developed that demonstrate clear liver damage. The TVX-model is based on the administration of TVX few hours before the injection of TNF or LPS to simulate inflammation. The XIM-model instead is based on the administration of XIM for 7 days prior to induction of TNF-induced viral hepatitis. In both models we investigated if relevant immunological changes are induced by the above-mentioned drugs and how they mechanistically relate to the onset of liver damage.

In **chapter 1** we investigated several immunological parameters in the mouse TVX-model and compared the effects of TVX with those exerted by the pharmacological analogue levofloxacin (LVX). Drugs were administered orally, 3 h before injection of TNF. As expected, TVX but not LVX or the solvent caused increased serum levels of liver enzymes, increased presence of inflammatory cells (neutrophils and monocytes) and evident pathological modifications, which were prominent already at 4 hours after the administration of TNF. Remarkably, the amount of leukocytes recruited into the liver 2 hours after TVX plus TNF appeared lower when compared to LVX or the solvent, combined with TNF. Thus it appeared that TVX actually impaired the TNF-induced neutrophil and monocyte recruitment into the liver. Nevertheless, increased serum concentrations of TNF, IL-6 and IL-10 were observed at this time point. In summary, TVX appeared to delay and prolong the recruitment

of neutrophils into the liver, suggesting that early recruitment of neutrophils and monocytes into the liver may have a regulatory effect on the inflammation induced by TNF, whereas their prolonged presence in the organ induced liver damage.
The results collected in **chapter 1** prompted us to further investigate the role of neutrophils and monocytes in TVX+TNF-induced liver injury.

In **chapter 2**, we wanted to clarify the mechanism behind the delayed recruitment of neutrophils into the liver. Intriguingly, it was earlier revealed by others that TVX is capable of blocking the efflux of small molecules, such as ATP, from the hemichannel called pannexin-1 (PNX1). These small molecules, release by PNX1 hemichannel during apoptosis, play a fundamental role in the recruitment of phagocytes as so-called "find-me" signals. A disturbed release of "find-me" signals may hamper uptake of dying cells by phagocytes, cause damage and release of cellular content by dead cells. In chapter 2, we observed that hepatocytes dying by TVX+TNF indeed released less "find-me" signals.
Remarkably, the PNX1 hemichannel is also used by neutrophils to direct and propel their migration toward attracting factors. In chapter 2, we demonstrate that TVX reduced the motility of freshly isolated human neutrophils toward attractants such as IL-8, notably at similar concentration as observed in patients' blood. Blocking or the PNX1 hemichannel may thus explain our observation that neutrophils accumulate in liver vessels 2 hours after TNF administration.
In addition, TVX decreased the expression of an important adhesion molecule (ICAM-1) elicited by TNF treatment. Since ICAM-1 is important to arrest monocytes and neutrophils into the liver, this may add to the explanation how TVX decreases the number of hepatic neutrophils and monocytes after TNF.
Furthermore, data in chapter 2 showed that depletion of neutrophils and monocytes in mice prior to administration of TVX and TNF increased the concentrations of TNF in serum and the production by the liver of neutrophil-attracting molecules (e.g. IL8). These findings confirm that recruitment of neutrophils plays an regulatory role in TVX-induced liver toxicity.
Since also other compounds (diclofenac, chlorpromazine, ranitidine etc.) showed the capability to induce hepatocyte apoptosis when co-exposed to TNF, our current data warrant to scrutinize the role of neutrophils and monocytes in DILI induced by these drugs. Studies assessing the effects of IDILI-associated compounds on neutrophil and monocyte function may help to build new predictive tests for the early identification of hepatotoxic compounds.

Although findings obtained in research of chapters 1 and 2 revealed the important role of neutrophils and monocytes in regulation of liver inflammation, the exact mechanism by which TVX causes hepatocyte death also needed to be further clarified. Previously, it was demonstrated that in vitro exposure of macrophages and hepatocytes, respectively to LPS and TNF, in presence of toTVX also caused increased secretion of TNF and apoptotic cell death.
Because the well-known transcription factor NF-kB is important in cell survival and propagation of inflammation after TNF or LPS exposure we decided to investigate if

TVX affected the pathways linked to this transcription factor. TNF and LPS activate NF-kB in hepatocytes and macrophages, respectively. Upon activation NF-kB migrates to the nucleus and activates genes linked to production of pro-survival and pro-inflammatory proteins. Especially in hepatocytes TNF-induced NF-kB activation is fundamental to regulate other pathways centralized around kinases called JNK and ERK which, if over-activated, can induce cell death. These molecular changes, with NF-kB translocation and activation of kinases, are considered part of the so-called cellular stress response.
It was already established before by others that TVX alone prevented replication of hepatocytes and activated JNK and ERK but did not lead to cell death. TNF also activated these kinases, with different kinetics, but again without causing cell death. The combination of TVX- and TNF-mediated activation of these stress responses caused cell death, which could be prevented through the inhibition of JNK and ERK.
In **chapter 3**, we showed TVX prolonged the first migration of NF-kB to the nucleus and prevented further oscillatory nuclear migration of NF-kB that was observed in case of TNF. From previous studies, we knew that oscillatory NF-kB migration is positively regulated by a protein called IKKα/β which contributes to the destruction of IKBα. IKBα negatively regulates nuclear NF-kB migrations whereas NF-kB promotes, like in a loop, the production of IKBα. In TVX+TNF-treated hepatocytes we observed both overproduction of IKBα and prolonged activation of IKKα/β. This matches with prolonged nuclear activity of NF-kB and suppression of further nuclear NF-kB-migrations. Suppression of further NF-kB migrations led to a decreased activation of genes coding for proteins which regulate the stress response. Based on these findings we suggest that the over-activation of JNK mediated by TVX results from NF-kB inhibition. Data together suggests that TVX modulates and boosts TNF signaling in hepatocytes leading to increased cell death.
Also in vivo we found that TVX increased the number of hepatocytes with a high nuclear concentration of NF-kB and in addition caused overproduction of IKBα. TVX also prolonged LPS-induced nuclear migration of NF-kB in macrophages, thus increasing the production of TNF. Both findings are important because they illustrate the translatability of our in vitro findings to the in vivo real-life situation, in which co-exposure of the drug with bacterial components may be the cause of the observed liver injury.
The overall conclusion of chapters 1, 2 and 3 is that TVX, a compound associated with IDILI in man, causes the potentiation of inflammatory responses mainly via two mechanisms: 1-the delayed recruitment of phagocytes and, 2-the interference of intracellular mechanisms downstream of LPS and TNF causing hepatocyte cell death. Performed experiments suggest that kinetic assessment of pathways downstream of TNF and LPS represent a useful approach to identify compounds causing liver injury.

Following the idea that compounds causing IDILI modulate innate immune responses as observed for TVX, we wanted to assess if XIM, another compound

that elicits DILI in mice, has similar activity. For this, we used the recently developed XIM-model showing that XIM increased mortality and liver damage in mice experiencing a TNF-induced viral hepatitis. We set out to investigate XIM-mediated immunological changes in the liver. Data presented in **chapter 4** showed that XIM caused an increase of neutrophils and monocytes during the 5 days preceding the infection of mice. During these 5 days XIM also increased pro-inflammatory molecules like IL-6 and TNF and proteins (FAS and FASL) involved in the killing of hepatocytes by immune cells. Upon infection at day 5 liver of XIM-treated mice became enriched with natural killer (NK) cells, known to kill virus-infected cells via FAS-FASL interaction. These alterations were also observed when mice received TNF at day 7 (48 hours after infection) and the increased expression of FASL and NK cells in the liver indicated that XIM increased hepatocyte killing by NK cells. In support of this, it was observed by others that elimination of NK cells reduced liver damage in mice receiving XIM. In conclusion, we suggest that XIM activated innate cell-killing immune responses within the liver during the 5 days of administration before infection.

In this thesis, we set out to investigate to what extend innate immune players are involved in IDILI using different model drugs. By the results collected in this thesis, we became aware that administration of only these IDILI-associated compounds already caused activation of the innate immune system but without an apparent liver damage. For the latter, additional triggers of innate immune responses are needed, such as viral or bacterial infections or sterile inflammation. The knowledge that IDILI is a complex, multifactorial process links to the idiosyncratic nature of causing IDILI in man.

The study of mechanisms involved in IDILI as part of the IMI-funded MIP-DILI project was directed to design new predictive approaches for identification of IDILI-inducing compounds. Results presented in this thesis confirms the requirement of an integrated approach based on a complementary and modulatory panel of tests to identify such compounds. We suggest that drugs should be at least tested for their effects on the kinetics of neutrophil and monocyte recruitment, the intracellular dynamic responses of NF-kB and MAPKs to TNF/LPS in macrophages/hepatocytes and the potency of the immune responses against viral infections. The integration of the results obtained by these assessments might represent an approach to estimate the risks of compounds to cause IDILI.

Chapter 8
Nederlandse samenvatting

Leverschade door medicijnen

Van veel medicijnen is bekend dat ze ongewenste bijwerkingen hebben, die in het Engels adverse drug reactions (ADR) genoemd worden. Hierbij kan vrijwel elk orgaan betrokken zijn met als resultaat een variatie aan klinische gevolgen, variërend van huidziektes en bloedarmoede tot leveraandoe-ningen. Deze ADR zijn niet alleen een groot probleem voor de patiënt, maar ook voor de farmaceutische industrie. Vanwege ADR is rond de 10% van alle medicijnen die tussen 1975 en 2000 op de markt is verschenen strak gereguleerd in gebruik of teruggehaald van de markt. Dit leidt tot aanzienlijke economische schade, niet alleen voor het bedrijfsleven (het produceren van een nieuw medicijn kan oplopen tot 1 miljard) maar ook de maatschappij (duurdere medicijnen en hogere zorgkosten). Zo is ook het in dit proefschrift gebruikte Ximalagatran, te gebruiken als antistollingsmedicijn, enkele weken na marktintroductie weer van de markt gehaald door de producent (Astra Zeneca).

Het onderzoek beschreven in dit proefschrift is gericht op een specifieke vorm van ADR, "drug-induced liver injury", ook wel afgekort als DILI. We kennen daar verschillende vormen van, met als twee uitersten een vorm van DILI die afhankelijk is van de dosis en een andere vorm die onafhankelijk is van de dosis maar heel erg afhankelijk van persoonlijke eigenschappen. Deze laatste vorm van DILI wordt ook idiosyncratische DILI, of IDILI, genoemd. Een bekend voorbeeld van een medicijn dat dosis-afhankelijke DILI veroorzaakt is paracetamol, waarbij iedereen leverschade zal oplopen als de dosis hoog genoeg is. Daarentegen zal diclofenac als voorbeeld van een medicijn dat IDILI kan veroorzaken, dat slechts in 1 op 10.000 personen doen.
De term idiosyncratisch in dit verband betekent persoonsafhankelijk. Idiosyncratische DILI is dus niet zozeer afhankelijk van (de dosis van) het medicijn maar vooral van persoonlijke omstandigheden van de patiënt. Deze persoonlijke omstandigheden zijn vaak een combinatie is van persoonlijke (genetische) eigenschappen en omgevingsfactoren, zoals microbiële infecties. Dit gegeven maakt het heel moeilijk om het risico voor het ontwikkelen van iDILI te voorspellen met behulp van dierexperimenten (die vaak behoren tot de eerste pre-klinische testen). Immers, dierexperimenten om medicijnen te testen maken gebruik van standaard, genetisch vrijwel homogene proefdieren, meestal ratten of muizen, die onder standaard omstandigheden gehuisvest zijn.
Om de kans op iDILI toch pre-klinisch te kunnen voorspellen is meer kennis nodig over de betrokken mechanismen bij deze aandoening. Deze noodzaak is de aanleiding geweest voor een groot, door de Europese Commissie (EC) en de farmaceutische industrie (verenigd gefinancierd project onder de vlag van het Innovative Medicines Initiatief (IMI). De naam van dit project was "Mechanism-Based Integrated Systems for the Prediction of Drug Induced Liver Injury" (MIP-DILI). Naast dat er in dit project onderzoek naar mechanismen is gedaan, is ook gewerkt aan dierproefvrije methoden die onderdelen van het proces van DILI zouden kunnen voorspellen.

Een deel van het MIP-DILI onderzoek, uitgevoerd bij Universiteit Utrecht, had als doel om meer inzicht te krijgen in hoeverre ontstekingsreacties en componenten van het aangeboren afweersysteem bijdragen aan de ontwikkeling van IDILI en wordt beschreven in de proefschrift.

Betrokkenheid van ontstekingsreacties en het aangeboren afweer systeem bij het ontstaan van IDILI

In de afgelopen jaren is in tal van experimenten naar voren gekomen dat microbiële infecties (zoals door virussen of bacteriën) het ontstaan van leverschade door medicijnen veroorzaken of bevorderen. Een bacteriële component die veel onderzocht is, is lipopolysaccharide (LPS), een component van de celwand van Gram-negatieve bacteriën. Van LPS is bekend dat het de productie van "Tumor Necrosis Factor" (TNF) kan induceren, onder anderen door de macrofagen in de lever.

Deze macrofagen behoren tot het zogenaamde natuurlijke of aangeboren afweersysteem. Naast het aangeboren afweersysteem bestaat er in hogere dieren een "verworven" afweersysteem dat ontstaat na de geboorte, en bestaat uit T en B lymfocyten. T lymfocyten zijn vooral bekend als cellen die heel specifiek op delen van eiwitten reageren die bijvoorbeeld afkomstig zijn van bacteriën. Die delen van eiwitten worden op een speciale manier aan T lymfocyten gepresenteerd door macrofaag-achtige cellen die de bacteriën hebben opgenomen en afgebroken. Eenmaal geactiveerd kunnen T lymfocyten de B lymfocyten aanzetten op antistoffen te maken die de betreffende bacteriën kunnen binden. Door deze binding kunnen andere macrofagen maar ook andere witte bloedcellen (neutrofielen) de bacteriën beter herkennen en aanvallen. Op deze manier maakt het verworven afweersysteem de afweerreactie sneller, specifieker en efficiënter. Maar zonder het aangeboren afweersysteem kan het verworven afweersysteem over het algemeen niet veel aanrichten: zowel de aanzet tot een afweerreactie als de uiteindelijke doding van een bacterie hangt af van cellen van het aangeboren afweersysteem.

In IDILI kunnen beide takken van het afweersysteem een rol spelen. In het beschreven onderzoek zijn we vooral gaan onderzoeken wat de rol is van het aangeboren afweersysteem. Dit omdat naar verwachting ook in IDILI het aangeboren afweersysteem betrokken is bij zowel de aanzet tot, als de ontwikkeling van de uiteindelijke leverschade.

De lever is vooral bekend vanwege de rol in de stofwisseling, maar de lever is ook een belangrijk immunologisch orgaan. De lever bevat heel veel macrofagen (in de lever Kupffer cellen genoemd) en zogenaamde natuurlijke killercellen (NK cellen). Dit is niet vreemd omdat alle bloed vanuit de darm maar ook uit de rest van het lichaam langs de lever stroomt. Dit bloed bevat vreemde stoffen uit de darm zoals onderdelen van micro-organismen (LPS) of dode cellen uit de rest van het lichaam. De macrofagen van de lever houden deze stoffen en cellen tegen en ruimen ze op.

Zo voorkomen de levermacrofagen, samen met andere witte bloedcellen, zoals neutrofielen en monocyten, dat er weefselschade in de lever optreedt ten gevolge van die vreemde stoffen en dode cellen.
Recent is duidelijk geworden dat macrofagen tijdens de verwerking van stoffen en cellen signalen afgeven die de achtereenvolgens neutrofielen en monocyten aantrekken om te helpen. De neutrofielen helpen mee om de schade op te ruimen en raken daarbij zelf beschadigd. De monocyten helpen vervolgens om deze beschadigde neutrofielen op te ruimen. Het eindresultaat is dat er geen of slechts een geringe ontsteking ontstaat en geen ernstige weefselschade optreedt. Een van de ontstekingssignalen is het boven genoemde TNF, en een signaalmolecuul dat betrokken is bij het de stoppen van de reactie is interleukine 10 (IL-10).

Wij hebben in dit proefschrift onderzocht hoe deze samenwerking van witte bloedcellen een rol speelt bij het ontstaan van weefselschade. Daarnaast hebben we ook onderzoek gedaan naar de betrokkenheid van andere aangeboren afweercellen (zoals NK cellen). Verder hebben we onderzocht op welke biochemische processen betrokken zijn bij de beschadiging van levercellen en macrofagen door medicijnen die iDILI kunnen veroorzaken.

De rol van de aangeboren afweercellen in IDILI

We hebben in ons onderzoek twee bekende DILI-medicijnen gebruikt, het antibioticum Trovafloxacin (TVX) en het eerder genoemde Ximalagatran (XIM). Ze zijn beiden kort na de marktintroductie vanwege leverproblemen weer van de markt gehaald. Voor beide medicijnen is gekozen omdat een diermodel met muizen beschikbaar was, en omdat in beide gevallen al bekend was dat het afweersysteem betrokken was.
In het TVX-model, werd de blootstelling aan het medicijn gecombineerd met blootstelling aan TNF. TVX werd eerst eenmalig via maagsonde toegediend. Drie uur later werd TNF ingespoten en 4 uur hierna was er sprake van uitgebreide en ernstige leverschade.

In het XIM-model werden muizen blootgesteld aan het medicijn gedurende 7 dagen, op de vijfde dag werd een virus ingespoten, en op de zevende dag TNF. Twee uur later was er dan duidelijk leverschade te meten.

Voor beide modellen geldt dat de ziekteontwikkeling in de tijd goed bekend was, zodat we in staat waren het verloop van de leverschade en de betrokkenheid van het afweersysteem goed te volgen.

In **hoofdstuk 2** laten we zien dat de leverschade als gevolg van TVX inderdaad snel na TNF injectie optreedt en ook dat een verwant, maar veilig medicijn (Levofloxacin, LVX) geen schade geeft. Twee effecten vielen meteen op. Het eerste was dat er ook al voor de injectie met TNF leverschade zichtbaar was in de vorm van dode levercellen. Het andere, en mogelijk gerelateerde effect was dat de hoeveelheden

NK cellen in de lever al waren toegenomen voor TNF injectie.

De meest opvallende bevinding echter was dat het aantal neutrofielen in de lever van met TVX behandelde dieren na TNF injectie wel was toegenomen maar minder dan in geval van met LVX behandelde dieren. Daarnaast namen monocyten in met TVX behandelde dieren nagenoeg niet toe, terwijl deze goed terug te vinden waren in met LVX behandelde dieren. Na 4 uur waren de neutrofielen in de met TVX behandelde dieren nog steeds hoog, terwijl die in de met LVX behandelde dieren al weer aan het afnemen waren. Het lijkt er dus op dat TVX de instroom van neutrofielen en monocyten uit het bloed verhinderde en vertraagde. Omdat er in LVX dieren geen sprake is van leverschade geven deze resultaten ook aan dat neutrofielen en monocyten inderdaad gezamenlijk zorgen voor het voorkomen van leverschade door TNF. Deze regulerende rol van neutrofielen en monocyten was al min of meer bekend, maar nog niet in het ontstaan van leverschade door een medicijn.

Deze bevindingen waren aanleiding om in **hoofdstuk 3** verder te onderzoeken hoe TVX de verminderde instroom van witte bloedcellen in de lever kan veroorzaken. Rond die tijd werd bekend dat TVX specifiek kan binden aan een molecuul dat aanwezig is op de buitenkant van veel cellen, onder anderen levercellen, neutrofielen en monocyten. Dit molecuul, Pannexin 1 of PNX1 genaamd, is een kanaalvormend molecuul waardoor andere moleculen uit een cel naar buiten geloodst kunnen worden. Dit gebeurt met name als een cel beschadigd is en aan het dood gaan is. Een speciale vorm van celdood is apoptose, een min of meer gecontroleerde vorm van celdood waarbij cellen in stukken uit elkaar vallen en netjes worden opgeruimd. Tijdens dit proces worden moleculen door PNX1 naar buiten gepompt, en trekken neutrofielen en monocyten aan om te helpen bij het opruimen. De functie van PNX1 in beschadigde of stervende levercellen is dus om witte bloedcellen aan te trekken.

PNX1 in neutrofielen en monocyten heeft echter een heel andere functie. In deze cellen is PNX1 betrokken bij de voortbeweging, een proces waarbij ATP wordt uitgescheiden.
Resultaten in **hoofdstuk 3** laten dus zien dat TVX de instroom van witte bloedcellen mogelijk verstoort door het PNX1 kanaal te blokkeren. De bevinding is mogelijk ook van belang voor het begrip van iDILI in het algemeen. Meerdere medicijnen, zoals diclofenac, chlorpromazine en ranitidine, veroorzaken namelijk leverschade in muizen indien gecombineerd met TNF. De effecten die we gevonden hebben zouden daarom ook voor deze medicijnen onderzocht moeten worden om na te gaan in hoeverre onze bevindingen van algemeen belang zijn bij medicijn-geïnduceerde leverschade.

Meestal hebben kleine moleculen niet één specifiek werkingsmechanisme, en dit geldt waarschijnlijk ook voor TVX. Wat we namelijk ook gevonden hebben is dat TVX de hoeveelheid van het molecuul ICAM1 op levercellen verlaagt. Dit

molecuul is eveneens betrokken bij de instroom van witte bloedcellen in de lever. De verlaagde expressie zou in verband kunnen staan met eerder genoemde TVX effecten. We zijn dan ook verder gaan onderzoeken welke moleculaire processen verstoord worden door TVX alleen of in combinatie met TNF. Dat hebben we onderzocht in levercellen, maar ook in macrofagen, **in hoofdstuk 4**. In het laatste geval hebben we gebruik gemaakt van LPS, omdat dit de macrofagen aan kan zetten tot TNF productie.
Zowel TNF (in lever cellen) als LPS (in macrofagen) veroorzaken een activatie van een bekende transcriptiefactor NF-kB. NF-kB gaat van het cytoplasma naar de kern en zorgt, op gen-niveau, voor de productie van moleculen die betrokken zijn bij celdood, maar ook van moleculen die betrokken zijn bij ontstekingsreacties (zoals TNF en ICAM1). Daarnaast beïnvloedt NF-kB de aansturing van aanwezige moleculen JNK en ERK, die ook betrokken zijn bij celdood en overleving.
Wat we gevonden hebben is dat TVX de celdeling van levercellen in vitro verhinderde en zowel JNK als ERK activeerde. Dat gebeurde ook als we cellen met TNF behandelde, en in beide gevallen was er geen sprake van celdood na behandeling. Celdood trad wel op indien TVX en TNF gecombineerd werden toegediend. Als gevolg van de gecombineerde blootstelling werd de migratie van NF-kB van cytoplasma naar de kern verstoord, mogelijk door toegenomen productie van IKBα en A20, moleculen die de werking van NF-kB remmen. De afname van NF-kB zou ook de oorzaak kunnen zijn van de afname van het molecuul ICAM1, activatie van JNK en het ontstaan van apoptose. Vergelijkbare effecten van TVX hebben we gevonden in macrofagen die gestimuleerd werden met LPS.

De bevindingen in de **hoofdstukken 2 en 3** laten zien dat TVX inderdaad op meerdere processen aangrijpt, te weten op de instroom van neutrofielen en macrofagen in de lever en op processen die celdood beïnvloeden. Het is hierbij belangrijk te weten is dat de effecten op cellen (in vitro) bij dezelfde concentratie optreden als die gevonden is in het bloed van mensen die TVX gebruikten.

In **hoofdstuk 5** laten we zien wat er gebeurt met de witte bloedcellen in het XIM diermodel. Door XIM namen de hoeveelheden neutrofielen en monocyten toe in de tijd en zonder de vertraging die we met TVX gevonden hebben. Ook was de expressie van moleculen als TNF, IL-6 en FAS/FASL toegenomen. De toename van TNF en IL-6 duidde op een ontstekingsreactie en de moleculen FAS/FASL, die worden gebruikt door NK cellen, duidden op een verhoging van het aangeboren afweersysteem. Al met al lijkt ook in dit geval dat het medicijn (XIM) het aangeboren afweersysteem beïnvloedt, mogelijk door het induceren van celschade, maar van een verstoorde influx van neutrofielen en monocyten lijkt hierbij geen sprake.

Hoe nu verder?

Het onderzoek beschreven in dit proefschrift geeft allereerst aan dat het ontstaan van iDILI een complex proces is dat sterk afhankelijk is van de stof zelf, maar ook van specifieke omstandigheden die per stof verschillend kunnen zijn. Daarnaast

is duidelijk geworden uit ons onderzoek dat stoffen die iDILI induceren niet noodzakelijk via één uniek mechanisme werken. Dit proefschrift laat zien dat bij TVX in ieder geval twee verschillende mechanismen een rol spelen. De complexiteit van de onderliggende mechanismen gecombineerd met de noodzaak van een immunostimulerende omstandigheid draagt mogelijk bij aan het idiosyncratische karakter van de bestudeerde vormen van iDILI.

Het onderzoek van dit proefschrift, met name ten aanzien van TVX, laat zien dat vrijwel alle effecten die in vivo gevonden zijn terug te voeren zijn naar redelijk eenvoudige in vitro testen. Dit geeft aan dat een combinatie van in vitro testen met humane cellen zoals gebruikt in dit proefschrift wellicht kunnen bijdragen aan het voorspellen van IDILI tijdens de vroege fase van ontwikkeling van nieuwe medicijnen.

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This is what has happened to me. They say that the best way to find yourself is to lose yourself first in the service of others. The service of others is exactly how I interpret Science. A tool to improve our society, at the complete service of others, especially those who suffer. During the first years of my PhD, I understood day by day all my limits and my fears. I could dig into them thanks to the attitude of **Ray**. He always tried to make me responsible for everything during my PhD, although he tried to direct and help me behind the scenes. At the beginning of my PhD I hated him, because I felt alone fighting to have some nice results. I made him aware of my feelings and worries but he already knew what I was going through and, like a good father, he helped me to get back on my feet and motivated me to continue. I could only understand this at the end of my path. Nevertheless, throughout my PhD he has been a good friend. I have had a very good time with him when having a beer or enjoying a party, like between good friends. I was lucky to find you on my way Ray!! And I am even more happy that we will continue working together. Let's grab this science by … the hand.

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And now my **Dalton gang**. The friends who have always been by my side: **Giovanni, Gabriele, Marco**. You are the brothers I found on my path. You all are part of my family as I am part of yours. Actually, we are a family. There has not been a thing that I did not share with you. You were my shelter when I was lost and the pure joy when we wanted to have fun. I would love to have you all here on this day but I know that life had different and important plans for you, and I am even happier to know why you are not here today. For you there are no other words because I have already shared them several times straight to your faces, in our hugs. Someone else got lost during the last two years, but I think you all agree if I say that we will wait for him to be back at our table, to laugh and cheer at our lives.

There wouldn't even have been a beginning of this story, of this PhD, without my family. Let's start from **Nonna Lorenza** who always made any of my dreams come true, but also **Zio Paolo, Zio Vasco and all the Giustarini's, Venturi's and Borsacchi's families.** Thanks to all my Sicilian family: from my grandpa and grandma to my little cousin Giovanni, and everyone in between: **Salvo and Andrea, Maria, Ines, Lucia, Marco, Clara, Luisa, Francesco, my godfather Vincenzo** and all the broad families **Basile, Pipitone and Lo Duca.** Thanks also to the whole new broad family **Mura** but especially to my brother in law **Fabrizio** and his amazing cousin **Silvia**. Thanks to the kind **Alfredo** who is making my Mum so happy. A special thanks to my careful **uncle Nino and auntie Anne**, my amazing cousins **Marco and Alessandro** who I only recently got to know better, finding so many common things between us. **My brother Lorenzo,** so similar but so different. I always pushed you to follow your dreams keeping in mind responsibilities and duties. You will nail your career, I am sure. I received from you an infinite amount of love and support, in any situation. In any place of the world there is no space for loneliness if I think of you. There is no space for weakness if I think of you. We already knew this. **My sister Elisa**, the genius of the family, the person who demonstrated to me that dedication and commitment are the formula to reach any possible goal. You were always a step ahead when any of us in the family needed something. You are an amazing mum and you gave me one of the biggest joys giving birth to that little piece of sugar Alessandro. Undoubtedly you learned how to be a mum from the best one. Yes **Mum**, you are. The glue of our team, let's say a family (siamo una famiglia diciamo). Your dedication with every single problem of the family has been incredible. Capable of crossing mountains and oceans for the good of our family, but also so fragile to lose yourself in a cup of coffee. Especially in the morning. Do you remember when I was home? The beginning of the day was our moment. We set up the things together, destroying any problems, old and new. You taught me what it means "to love". I am not talking about loving a

partner but really to love the humanity beyond differences and beyond difficulties. Every single smile that I see appearing on your face gives me the strength to run back home barefoot. Running yes (although I am terrible at it). The thing I did the most next to studying, since you have left **Dad**. Before your departure, you instilled the desire of knowledge in all of us. The emptiness you left, pushed me to study as much as possible. I could find you there, with your smile without lips, hidden behind your moustache. I could find your wrinkly forehead, sign of your deep thinking. The pure concept of knowledge was crystal clear at our eyes because of you. The knowledge you were so thirsty of. The knowledge as water to satisfy your need. The water you find in this book, only a few droplets. These droplets are the best I could do after collecting knowledge like a waterspout from every person, book, study, talk, story or documentary. This is all I could give till now. Don't be scared, I will not give up. It may seem like I will (as with biking and horse-riding etc.) but I will do my best not to stop.

I promise.

About the author

About the author

Giulio Giustarini was born on May 4th, 1985 in Pisa, Italy. Since he was a child, he was passionate about life sciences, sports and writing small poems. Three passions that grew up along with him. After high-school, due to some unexpected and unpleasant circumstances, he had to stop studying for a couple of years but he managed to work and study in order to obtain his MSc. During the last two years of his master, he joined the laboratory of gastrointestinal pharmacology in the faculty of Medicine at the University of Pisa under the supervision of Prof. C. Blandizzi. Here, he deepened several aspects of pharmacology/toxicology of the gastrointestinal tract with particular regard to chronic inflammatory diseases. In October 2012 he graduated in "Chemistry and pharmaceutical technologies" with the thesis titled "Role of the A2B receptor-adenosine deaminase complex in colonic dysmotility associated with bowel inflammation in rat.". In May 2013, he was selected for a PhD position in Immunotoxicology at the Institute for Risk Assessment Sciences (IRAS) in the faculty of Veterinary Medicine at Utrecht University under the supervision of Dr. Raymond Pieters. In Utrecht, he enjoyed the quiet life of The Netherlands but without forgetting about having some fun with friends who came to visit him. The research presented in this thesis is the result of his work performed with his collaborators at Utrecht University. In more than one occasion he collaborated and visited other laboratories in the Netherlands (Prof. L. Koenderman (UMCU); Prof. B. van de Water (Leiden University)) and in Germany (Prof. P. Knolle, Bonn University (IMMEI); Prof. U. Klingmuller (DKFZ)) where he performed some of the experiments needed for his research. In 2017, while writing his thesis, he joined the laboratory of Prostate Cancer Immunotherapy of Prof. Dasgputa at King's College London studying the mechanisms behind new engineered protein therapies for the treatment of solid tumours. His research goes beyond the lab, as he continues to engage his body by practicing different sports to improve his resilience; and his mind by writing small poems as a way to explore the human feelings which represent for him the real driving force of his research.

List of publications

1. ***Prostate cancer cells enhance IL-15-mediated expansion of NK cells.***
   Sakellariou C, Elhage O, Papaevangelou E, **Giustarini G**, Esteves AM, Smolarek D, Smith RA, Dasgupta P, Galustian C
   BJU Int 2019, doi:10.1111/bju.14893

2. ***Mouse strain differences in response to oral immunotherapy for peanut allergy***
   Wagenaar L, Bol-Schoenmakers M, **Giustarini G**, Garssen J, Smit J, Pieters R
   Immun Inflamm Dis 2019, doi: 10.1002/iid3.242

3. ***Trovafloxacin-induced liver injury: lack in regulation of inflammation by inhibition of nucleotide release and neutrophil movement***
   Giustarini G, Vrisekoop N, Kruijssen L, Wagenaar L, van Staveren S, van Roest M, Bleumink R, Bol-Schoenmakers M, Weaver RJ, Koenderman L, Smit J, and Pieters R
   Toxicol Sci 2018, doi: 10.1093/toxsci/kfy244

4. ***Dietary supplementation with non-digestible oligosaccharides reduces allergic symptoms and supports low dose oral immunotherapy in a peanut allergy mouse model***
   Wagenaar L, Bol-Schoenmakers M, **Giustarini G**, Vonk MM, van Esch BCAM, Knippels LMJ, Garssen J, Smit J, Pieters R.
   Mol Nutr Food Res 2018, doi:10.1002/mnfr.201800369

5. ***Tissue influx of neutrophils and monocytes is delayed during development of trovafloxacin-induced tumor necrosis factor-dependent liver injury in mice***
   Giustarini G, Kruijssen L, van Roest M, Bleumink R, Weaver RJ, Bol-Schoenmakers M, Smit J, Pieters R.
   J Appl Toxicol. 2018; doi: 10.1002/jat.3585

6. ***Anti-inflammatory effect of a novel locally acting A2A receptor agonist in a rat model of oxazolone-induced colitis***
   Antonioli L, El-Tayeb A, Pellegrini C, Fornai M, Awwad O, **Giustarini G,** Natale G, Ryskalin L, Németh ZH, Müller CE, Blandizzi C, Colucci R.
   Purinergic Signal 2018, doi:10.1007/s11302-017-9591-2

7. ***The AMPK enzyme-complex: from the regulation of cellular energy homeostasis to a possible new molecular target in the management of chronic inflammatory disorders***
   Antonioli L, Colucci R, Pellegrini C, **Giustarini G,** Sacco D, Tirotta E, Caputi V, Marsilio I, Giron MC, Németh ZH, Blandizzi C, Fornai M.
   Expert Opin Ther Targets 2016, doi: 10.1517/14728222.2016.1086752.

8. ***Role of the A(2B) receptor-adenosine deaminase complex in colonic dysmotility associated with bowel inflammation in rat***
   Antonioli L, Fornai M, Awwad O, **Giustarini G**, Pellegrini C,Tuccori M, Caputi V, Qesari M, Castagliuolo I, Brun P, Giron MC, Scarpignato C, Blandizzi C,Colucci R.
   Br J Pharmacol 2013, doi: 10.1111/bph.12539

9. ***NSAID-induced enteropathy: are the currently available selective COX-2 inhibitors all the same?***
   Fornai M, Antonioli L, Colucci R, Pellegrini C, **Giustarini G**, Testai L, Martelli A, Matarangasi A, Natale G, Calderone V, Tuccori M, Scarpignato C, Blandizzi C.
   J Pharmacol Exp Ther 2014, doi:10.1124/jpet.113.207118

10. ***The role of purinergic pathways in the pathophysiology of gut diseases: pharmacological modulation and potential therapeutic applications***
    Antonioli L, Colucci R, Pellegrini C, **Giustarini G**, Tuccori M, Blandizzi C, Fornai M.
    Pharmacol Ther 2013, doi:10.1016/j.pharmthera.2013.04.002.

11. ***Drug-Induced taste and smell alterations: a case/non-case evaluation of an Italian database of spontaneous adverse drug reaction reporting***
    Tuccori M, Lapi F, Testi A, Ruggiero E, Moretti U, Vannacci A, Bonaiuti R, Antonioli L, Fornai M, **Giustarini G,** Scollo C, Corona T, Ferrazin F, Sottosanti L, Blandizzi C.
    Drug Saf 2011, doi: 10.2165/11593120-000000000-00000