Dynamics of TNFalpha signaling and drug-related toxicity

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Chapter 7

Discussion and future perspective

Highlights

- The importance of inflammatory stress on the etiology of drug-induced liver injury is increasingly recognized
- Current *in vitro* models do not capture hepatocyte specific immune signaling
- In this thesis, I report both mechanistic insight into hepatocyte specific immune signaling and application for prediction modeling

Idiosyncratic DILI remains an important problem in drug development and safety assessment, since it is difficult to predict DILI liability. Inflammatory stress is considered as an important host factor involved in DILI[208]. Several *in vivo* and *in vitro* models show that inflammatory stress induced by pathogenic or sterile inflammation decreases the toxicity threshold of certain drugs, as discussed in **Chapter 2**[8,16,41,72]. Toxicity is correlated to TNFα levels for diclofenac/LPS-induced hepatotoxicity, and for trovafloxacin proven to be TNFα and TNFR dependent[11,99]. At this moment, several mechanisms of TNFα-induced cell death in different cell types have been recognized[20]. Also drug-induced cell death mechanisms have been extensively investigated, implicating activation of cellular adaptive stress responses as one of the key events[135]. In this thesis, I focused on drug-induced cellular stress responses and their relation to TNFα-induced hepatotoxicity. The interaction between these cellular stress responses and TNFα signaling has been studied previously, by us and others[15,16,43,345].

Although significant studies on drugs and inflammatory stress have shown that drug/cytokine interaction play an important role in DILI, we do not know how drugs interact with signaling downstream of the TNF receptor complex. Furthermore, it has been suggested that the activation of NF-κB and its target genes play an important role in TNFα-induced cytotoxicity[17,42]. The precise process and role of NF-κB activation in drug/TNF-induced hepatotoxicity is unknown. Moreover, it has been shown that also other TNFα-induced pathways play roles in inducing a synergistic drug/TNF apoptosis, including MAP kinases[14]. Drug/TNFα synergistic toxicity has been detected in both PHHs and HepG2 cultures[16,41,120]. In the studies that are presented in this thesis, we thrived to characterize drug-induced skewed TNFα signaling in HepG2 cells (**Chapter 3-5)**. Furthermore, in **Chapter 6** we used our inflammatory stress reporter in addition to our previously characterized fluorescent reporter platform to predict drug-induced liver injury hazard[44].

TNFα-induced NF-κB signaling

One of the best-characterized results of TNFα stimulation is the nuclear translocation of the RelA subunit of the NF-κB protein family. Previously, our lab has shown that this translocation phenotype can be disrupted by diclofenac pre-exposure[42]. To investigate how this phenotype is induced, we generated a stable CMV-driven RelA-GFP reporter. In **Chapter 3**, we used this reporter cell line for live cellular imaging-based siRNA screening, to determine signaling molecules that are involved in the creation of the typical oscillatory phenotype. We focused mainly on the role of kinases and ubiquitinases, both under control as well as drug-exposed conditions. As expected, we determined that various genes that are involved in ubiquitination processes play an essential role in TNFα-induced NF-κB activation[144]. Intriguingly, many candidate genes that delayed the oscillatory phenotype upon knockdown did not increase the toxicity upon drug exposure only, but decreased the cytotoxic potential of TNFα/diclofenac exposure. This underlines the complex relationship between NF-κB activation and cytotoxicity. We identified knockdown of UFD1L and CDK12 (CRK7) as strong inhibitors of the cytotoxic response and delaying RelA translocation under control and drug exposure conditions. Interestingly, CDK12, but not UFD1L knockdown did

increase the TNFα-induced A20 expression massively. UFD1L had already been described as part of the proteosomal targeting complex of IκBα[182]. However, we show here that UFD1L is not involved in IκBα breakdown in TNFα-stimulated HepG2 cells. This emphasizes the importance of cell type specific experiments and cautions against drawing conclusions based on literature in different cell types. CDK12 is known as RNA pol II kinase that phosphorylates Ser2 on the C-terminal domain, leading to improved elongation of RNA transcripts. Remarkably, CDK12 is specifically involved in the induction of stress-induced gene sets, for example upon oxidative and DNA damage stress[198,199]. Here, we suggest that CDK12 is also involved in NF-κB-mediated transcriptional programs. Whether diclofenac/TNFα cytotoxicity is induced by generally skewed CDK12-mediated gene expression or via specific CDK12-mediated inhibition of A20 expression requires more research. As CDK12 is involved in oxidative stress, inflammatory stress and DNA-damage responses, it is a most promising candidate gene to study in relation to iDILI. Especially since CDK12 knockout/knockdown affects gene transcription efficiency only during stress responses[199], CDK12 polymorphisms could form a genetic basis in which certain individuals could be more sensitive to specific drug-induced stress responses.

As shown in **Chapter 3**, delayed TNFα-induced RelA nuclear translocation is not always associated with increased drug/TNFα cytotoxicity. To determine the physiological effects of drug-delayed RelA responses, we tested compound effects on TNFα-induced RelA translocation, target gene induction and cytotoxicity in **Chapter 4**. The compounds chosen were part of the training compound set defined by IMI MIP-DILI[212], plus carbamazepine, a drug we studied extensively previously[16]. Of these compounds, some delayed TNFα-induced translocation (metformin, nefazodone, diclofenac, tolcapone, troglitazone, carbamazepine), while others did not (acetaminophen, flucloxacillin, pioglitazone and entacapone). To investigate TNFα target gene induction, we chose 45 target genes and determined the effect of drug exposure on their TNFα-induced expression kinetics. In general, drug exposure increased TNFα-induced expression of most target genes, not related to a specific kinetic or functional group of target genes. In addition, this increase was not defined by drug-induced delayed TNFα-induced RelA nuclear translocation.

As expected, TNFα-induced target genes that regulate oxidative stress, including SLC7A11, SOD2 and MAFF, were enhanced upon concurrent drug exposure. However, also proinflammatory cytokine and chemokine signaling target genes, including CCL20, IL8, CXCL3, CSF2, CSF3 and CCL5, were generally increased upon combined drug/TNFα exposure compared to TNFα exposure only. The increased expression of these cytokines could influence nearby liver cell populations, but also hepatocytes in an autocrine feedback loop. While the induction of inflammatory molecules has been studied in several experimental models including KCs and other resident immune cells, the excretion by hepatocytes has not been well characterized. In addition, we determined that expression of transcription regulating proteins including IER3, ELF3 and NFKB2 were increased upon drug exposure. The increased expression of these transcription factors complicates the

overall expected physiological outcome, since the hepatocyte-specific role of these transcription factors is currently not entirely clear[263,346].

In other models, combined drug/inflammatory regulator exposure was often directed at KC activation. Direct co-exposure with drug/LPS of human liver slices generally decreased the overall proinflammatory cytokine secretion[113], which was not entirely similar to the results in mouse liver slices[112]. However, as resident immune cells are present in this model, it is likely that the proinflammatory modulator production is at a different scale compared to hepatocyte only produced cytokines. Indeed, no increased cytokine secretion upon compound-only exposure was detectable in mouse or human slices[112,113], in contrast to our results. In a rat KC study, drug-treated KCs increased proinflammatory gene expression upon LPS stimulation, in contrast to LPS stimulation alone[347]. This is more similar to our result in hepatocytes. However, these contradictory outcomes show that it is difficult to extrapolate results between species and models.

It becomes more and more accepted that also hepatocytes are involved in immune regulation on organ and systemic levels[58]. Mice hepatocyte-specific knockout of A20 seem healthy when unchallenged, whit only slightly increased chronic inflammation levels in the liver. However, when challenged with LPS, LPS-induced NF-κB-dependent responses, including expression of IL6 and CCL2 expression are severely enhanced[192]. Thus, altered hepatocyte NF-κB signaling can enhance liver wide inflammatory responses. Therefore, we anticipate that enhanced TNFα-induced NF-κB target gene expression upon drug exposure as shown in this manuscript, could have severe physiological results.

Drug-induced NF-κB signaling

In **Chapter 4** we show that drug exposure by itself also induces TNFα target gene induction in HepG2 cells. Although this induction is partially regulated by RelB and cREL, the induction of target gene expression mainly depends on RelA expression. Interestingly, IL8 (CXCL8) and CCL5 are well-described chemokines, recruiting macrophages and neutrophils (Fig.1). Macrophage and neutrophil recruitment is considered a key event in the etiology of drug-induced liver injury[116,235]. By our knowledge, the regulation of these target genes by drug-induced activity of the whole NF-κB family in hepatocytes has not been described before.

Here, we show that drug-induced RelB expression is independent of RelA transcriptional activity and is strongly associated, and thus likely induced, by oxidative stress. The role of the non-canonical pathway in liver injury is not well characterized, although key protein MAP3K14 (NIK) activity aggravates (TNFα-mediated) liver injury *in vivo*[348,349]. In addition, inhibition of MAP3K14 decreased oxidative stress generation[349]. Indeed, in **Chapter 5** we show that knockdown of MAP3K14 affects drug/TNFα synergism in a drug-specific way. This effect correlates with drug-induced SRXN1 induction. Intriguingly, intricate connections between the classical (RelA) and alternative (RelB) NF-κB pathways exist, also upon TNFα exposure[21,350]. MAP3K14 activity and RelB are not interacting directly, but via several intermediate signalling proteins. Therefore, these results

do not define whether the effect of MAP3K14 knockdown on drug/ TNFα synergism is related to RelB involvement.

Interestingly, Willy *et al*. did also see a NF-κB target gene induction response upon palmitic acid exposure of HepG2 cells, leading to increased cytotoxicity. The cytotoxicity was dependent on the activation of CHOP via IRAK2, which lead to expression of inflammatory regulators, including IL8 and TNFα itself. While IL8 was mainly involved in macrophage recruitment, the CHOP-mediated secretion of TNFα was essential for palmitic acid cytotoxicity. Importantly, this response was not seen in primary mouse hepatocytes, underlining the species-specific regulation of NF-κB activation[345]. Here, we describe drug-induced regulation of NF-κB target gene induction as well. Although we previously and in this thesis showed that CHOP is activated by several drug exposures[16], we do not anticpate CHOP-mediated cytokine secretion. Our drugs are not cytotoxic upon drug only exposure and could not induce detectable TNFα mRNA expression upon drug exposure (data not shown). Therefore, we assume that there is no TNFα excretion by our HepG2 cells upon exposure of drugs alone. However, we anticipate that the increased secretion of inflammatory regulators as we have detected, demonstrate a subtle modulation of the inflammatory response upon drug exposure *in vivo*.

Drug/TNFα-induced cytotoxicity

Already a decade ago, the groups of Roth and Ganey were one of the first to describe synergistic responses in rodents upon low doses drug and LPS[8,72]. Since then, they and others have developed several models to detect inflammatory stress and drug synergistic hepatotoxicity. Roth and colleagues have also demonstrated that TNFα is an essential cytokine in the LPS/drug-induced liver injury *in vivo*[11]. We and others have shown that HepG2 cells also show TNFα/drug synergistic cell death[42,120]. This synergistic cell death is dependent on CHOP expression, MAPK function and the regulation of general protein translation machinery components[14,16]. However, it is still unknown how TNFα stimulation can interact with drug-induced cellular stress responses to increase cytotoxicity. In this thesis we studied both regulators of TNFα-induced RelA nuclear translocation in **Chapter 3** and regulators TNFα/drug synergism in **Chapter 5**. Synergistic cell death seems not strictly correlated with a delayed RelA translocation phenotype, as knockdown of candidate genes can both delay TNFα-induced RelA translocation and inhibit cytotoxicity. Therefore, in **Chapter 5**, we studied several candidate genes that affected TNFα/drug synergistic toxicity with different drug exposures. We detected both generic and compound specific characteristics of the candidate gene effects. Strong generic TNFα/drug synergistic toxicity regulators (A20 and PHF5A) increased cytotoxicity upon knockdown. This knowledge could be used in the development of sensitive safety testing approaches to detect inflammatory cytotoxicity.

The correlation between candidate gene effects under diclofenac and valproic acid exposure suggests a similar mechanism of TNFα toxicity. However, the compound-induced stress responses show stark differences. In conclusion, there must be other, yet unknown,

mechanisms involved in both valproic acid and diclofenac toxicity that synergize with TNFα signaling and affect our candidate gene function.

The effect of candidate gene knockdown on drug/TNFα toxicity was correlated to the drug-induced onset of several stress responses. This effect was most strongly seen with two separate branches of oxidative stress and the DNA damage response (SRXN1/P21 and HMOX1/BTG2). Both branches of these stress responses interact with TNFα and NF-κB signaling differently, according to literature[19,121,351]. Remarkably, both the oxidative stress and DNA damage response are related to target gene expression regulated by candidate gene CDK12 (CRK7)[198,199].

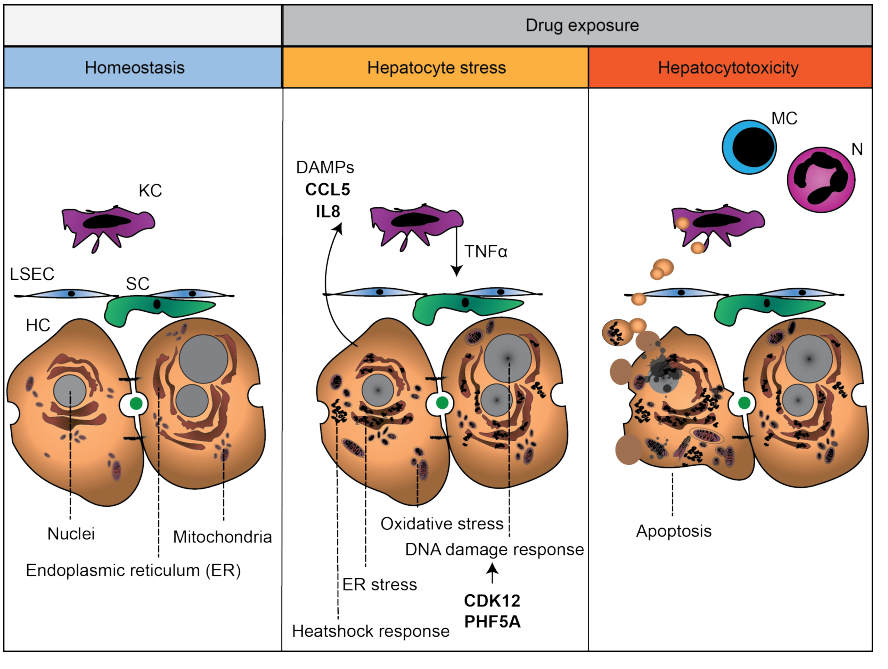

Fig. 1: Overview of drug-induced mechanisms Hepatocytes exposed to drugs induce several responses. In the first panel, hepatocytes are in homeostasis. In the second panel, drugs induce the activation of cellular stress responses. This includes swollen or perturbed mitochondria, increased levels of unfolded/misfolded proteins inside and outside of the endoplasmic reticulum and DNA damage occurring. CDK12 and PHF5A start elongating mRNA transcripts of specific stress-related target genes. The hepatocytes start producing damage associated molecular patterns (DAMPs) and chemokines, including CCL5 and IL8 (as described in this thesis). The DAMPs activate the Kupffer cell, which start to produce high amounts of proinflammatory cytokines, including TNFα. The chemokines start attracting immune cells, including monocytes and neutrophils. In the last panel, the TNFα in combination with the drug-induced responses has led to increased levels of hepatocyte apoptosis. Specific cell types and responses are indicated. KC is Kupffer cell, LSEC is liver sinusoidal endothelial cell, SC is stellate cell, HC is hepatocyte. DAMPs are damage associated molecular patterns. MC is monocyte, N is neutrophil.

This protein function of CDK12 is similar to the RNA pol II Ser2 kinase function of PHF5A, the strongest generic regulator of drug/TNFα toxicity in our study. We determined that the drug/TNFα toxicity effect of both kinases correlated strongly with the SRXN1/P21 branch of stress responses. We anticipate that both CDK12 and PHF5A are essential regulators of drug/TNFα toxicity in hepatocytes and control specific stress-induced transcription programs (Fig.1).

Assessing TNFα signaling in toxicity screening approaches

As discussed in **Chapter 2**, screening approaches that involve addition of TNFα can add valuable information for safety assessment. In **Chapter 6**, we applied a high throughput approach in which we used the previously described BAC-GFP HepG2 reporter platform[44], complemented with the ICAM1-GFP cell line and manual addition of TNFα. Addition of the TNFα-treated ICAM1 reporter increased the mechanistic and predictive power of our stress response reporter platform. The effects of drug exposure on TNFα-induced ICAM1 expression reflects some of the distortion of the inflammatory stress response in hepatocytes by the drug *in vivo*. Clusters of compounds that decrease or increase TNFα-induced ICAM1 expression concurrent with SRXN1 and/or CHOP expression indicate diverse toxicity mechanisms. Furthermore, we show that severe DILI compounds did in general increase adaptive stress responses at a lower exposure concentration than non-severe DILI compounds. Time dynamic, single cell features were distracted for support vector machine-based prediction of liver injury liability. To our best knowledge, this is the first time that high density dynamic temporal data for actual toxicity screening and prediction approaches is used. We anticipate that the detection of more adaptive stress responses, tested with and without inflammatory mediators, will increase the predictive power during *in vitro* safety assessment approaches.

As described in **Chapter 5**, separate branches of individual adaptive stress response pathway correlated strongly with the effects of individual candidate gene knockdowns on drug/TNFα synergism. Therefore, we anticipate that the inclusion of more reporters of the same stress response pathways also improves predictive power. Furthermore we recommend the optimization of the cellular model, for instance by 3D culture, co-culture or the use of stem-cell derived hepatocytes/primary human hepatocytes. To improve the detection of drug-induced immune-related toxicity, culture methods of all of these models should be adjusted to improve the resemblance to liver immunotolerant environment[1]. Furthermore, we anticipate that including hepatocyte-produced pro- and anti-inflammatory factors detection methods in early drug discovery toxicity screening increases the predictive power of *in vitro* safety assessment.

Conclusion and future perspectives

In this thesis I describe the drug-induced perturbations of TNFα signaling in HepG2 cells. The findings in this thesis report several novel processes and candidate genes that were not previously implicated in drug-induced liver injury (some are summarized in Fig. 1). Overall, the importance of inflammatory stress and its detection in

drug toxicity screens for safety assessment is emphasized. The findings in this thesis can be used to develop better predictive models for drug toxicity in early drug development. Furthermore, the knowledge that specific genes are involved in drug/TNFα interactions may, in the end, lead to more specific prescription practice in a personalized medicine approach.

The HepG2 cell line is still one of the most widely used *in vitro* methods in predictive toxicology to test hepatocyte toxicity. The detection of inflammation-induced drug hepatotoxicity is usually not incorporated in the early *in vitro* studies and only detected in animal studies. To lower animal burden and increase early detection of drug-interaction with inflammatory stress signaling pathways, we hypothesized in this thesis that HepG2 cells could be used as an adequate model to study inflammatory stress and drug toxicity. The outcome of these studies show that HepG2 cells are able to reflect many inflammatory signaling characteristics and can be used in predictive screening approaches.

The development of both inflammatory hepatocyte-only and co-culture models would increase our knowledge on inflammatory signaling in the liver substantially. Although primary hepatocytes are currently unsuitable for studying inflammatory signaling[37], evolving isolation and culture methods could increase the usability of this cell type. Furthermore, the development of induced pluripotent stem cells holds the most promising possibilities in the future, for both toxicity testing and personalized medicine strategies. This thesis contributes to the development of inflammatory stress models in the current, but also future testing approaches and clinical practice.