Dysregulation of Human Hepatic Drug Transporters by Proinflammatory Cytokines

Tianran Hao

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science

University of Washington
2023

Committee:
Jashvant D. Unadkat
Kenneth Thummel
Qingcheng Mao (deceased)

Program Authorized to Offer Degree
Pharmaceutics

University of Washington

Abstract

Dysregulation of Human Hepatic Drug Transporters by Proinflammatory Cytokines

Tianran Hao
Chair of the Supervisory Committee
Jashvant D. Unadkat
Department of Pharmaceutics

Proinflammatory cytokines, elevated during inflammation caused by infection and/or autoimmune disorders, result in reduced clearance of drugs eliminated primarily by cytochrome P450 enzymes (CYPs). However, the effect of cytokines on hepatic drug transporter expression or activity has not been well-studied. Here, using plated human hepatocytes (PHHs; n=3 lots), we investigated the effect of interleukin (IL)-6, IL-1β, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), on the mRNA expression and activity of hepatic drug transporters. PHHs were incubated for 72 hours at their pathophysiologically relevant plasma concentrations, both individually (0.01, 0.1, 1, 10 ng/mL) or as a cocktail (i.e., when each was combined at 0.1 or 1 ng/mL). Following cytokine cocktail exposure (1 ng/mL), significant downregulation of mRNA expression of organic anion transporting polypeptide 1B1 (OATP1B1), OATP1B3, sodium/ taurocholate cotransporting polypeptide (NTCP), breast cancer resistance protein (BCRP), P-glycoprotein (P-gp), multidrug and toxin extrusion protein 1 (MATE1), multi-drug resistance protein 2, 3, and 4 (MRP2/3/4) was observed. While the mRNA expression of organic anion transporter 2 (OAT2) and organic cation transporter 1 (OCT1) was downregulated in two lots, it was upregulated in one lot. In agreement (mostly), the 1 ng/mL cytokine cocktail reduced OATP1B1/3, OATP2B1, OAT2, OCT1, and NTCP activity by 75%, 44%, 82%, 47%, and 80%, respectively. Interestingly, upregulation of OAT2 and OCT1 mRNA in one donor did not translate into the same directional change in activity. Although significant inter-lot variability was observed, in general, the above effects, using individual cytokines, could be attributed to IL-1β and IFN- γ.

Table of Contents

List of Figures **Page**

List of Tables **Page**

List of Abbreviations

OATP1B1/3	organic anion transporting polypeptide 1B1/3
OATP2B1	organic anion transporting polypeptide 2B1
BCRP	breast cancer resistance protein
P-gp	P-glycoprotein
MATE1	multidrug and toxin extrusion protein 1
MRP2/3/4	multi-drug resistance protein 2,3, and 4
OAT2	organic anion transporter 2
OCT1	organic cation transporter 1
NTCP	sodium/ taurocholate cotransporting polypeptide
IL-6	interleukin 6
IL-1β	interleukin 1 beta
TNF-α	tumor necrosis factor alpha
IFN-γ	interferon gamma
AUC	area under the curve
ELISA	enzyme-linked immunosorbent assay
HNF	hepatic necrosis factor
CYP	cytochrome P450

Acknowledgements

I would like to express my deep gratitude to Dr. Jashvant Unadkat for his invaluable guidance and for generously sharing his knowledge. I also wish to honor the memory of Dr. Qingcheng Mao; his mentorship has left an indelible mark on my academic and professional growth.

I would also like to thank Dr. Kenneth Thummel for his encouragement and generous contribution.

Lastly, I would like to thank my peers in the Unadkat lab, especially Tim Tsang, Jhohann Benzi, Bob Weber and Mengyue (Melody) Yin for their help and collaboration.

Dedication

To my parents, Hao Quan and Yang Hong, my supporters and role models. Your strength and guidance illuminated my path.

Chapter 1: Introduction

1.1 Altered Drug Pharmacokinetics in Patients during Inflammation

Inflammation stemming from autoimmune diseases or infections is associated with altered drug pharmacokinetics (PK) [1]. Such alterations in PK could lead to the need for dose adjustment. For instance, in patients with rheumatoid arthritis (RA), the area under the plasma concentration-time curve (AUC) of S-verapamil after an oral dose of racemic verapamil (80 mg) was approximately 7-fold higher than that in healthy individuals (Figure 1) [2]. Since S-verapamil is mostly eliminated by the cytochrome P450 enzyme 3A (CYP3A), this result suggests that the activity of CYP3A was downregulated in patients with RA.

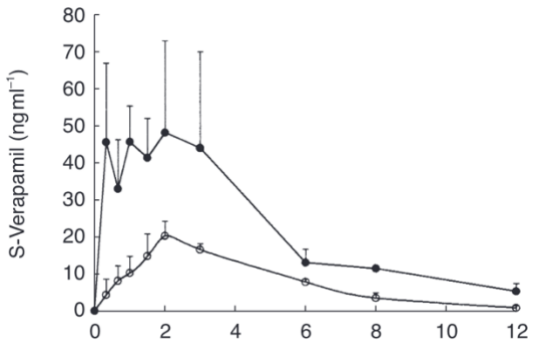


Figure 1. Serum S-Verapamil concentration-time profile in healthy control subjects (open circles) and patients with rheumatoid arthritis (solid circles) after receiving a single 80 mg oral dose of verapamil. Error bars represent S.E. mean (n=8). Reproduced with permission of British Journal of Clinical Pharmacology.

Similarly, patients with active acute pyelonephritis had a significantly lower renal secretory clearance ($CL_{secrection}$) of furosemide compared to that after remission following intravenous antibiotic treatment (Figure 2) [3]. Since furosemide is primarily

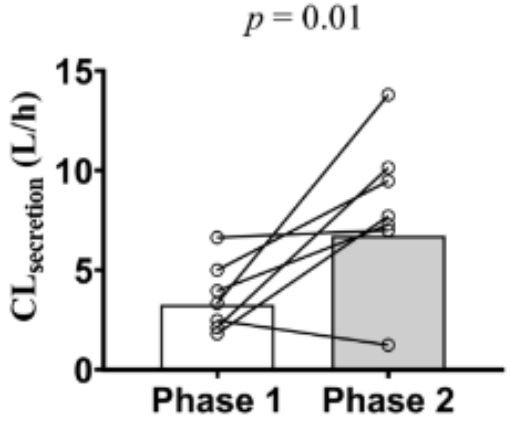


Figure 2. Secretory clearance of furosemide in the presence of (Phase 1) and absence (Phase 2) of acute pyelonephritis in 7 subjects after a single dose (40 mg, p.o.). Data are presented for each individual and the bars represent the geometric mean. The difference in the indicated parameter between Phase 1 and 2 was evaluated using the paired Student's t-test ($p>0.05$).

eliminated from the body by secretion through organic anion transporter 1 and 3 (OAT1 and 3)-mediated transport and by glomerular filtration [4], these data suggest that OAT1 and 3 are downregulated in patients with acute pyelonephritis. For drugs with a narrow therapeutic window, which are candidates for therapeutic drug monitoring, inflammation-induced elevation in drug exposure could lead to deleterious side effects [5].

1.2 Elevated Production of Pro-inflammatory Cytokines During Inflammation

Pro-inflammatory cytokines are small soluble signaling proteins (<40 kDa) that are secreted by immune cells with rather short *in vivo* half-lives ranging from 20 minutes to 6 hours [6]. They are key mediators of inflammatory responses and among these cytokines, interleukin-6 (IL-6), interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α) and interferon-gamma (IFN-γ) act as the principal “conductors” within the cytokine signaling network [7], [8]. Their plasma concentrations are markedly higher in patients with various infectious and autoimmune diseases (Table 1). For acute inflammation, this elevation in plasma pro-inflammatory cytokines promotes the migration of neutrophils and macrophages to the area where the inflammation occurs, which helps with fighting off the infection in the short term [9]. However, failing to resolve acute inflammation will lead to chronic conditions, such as systemic lupus erythematosus (SLE) or Crohn’s disease, which could cause permanent tissue damage and fibrosis [10].

Table 1: Range of proinflammatory cytokines plasma concentrations (pg/mL) in adults with acute and chronic inflammatory conditions.

Disease/ Condition	IL-6	IL-1β	TNF-α	IFN-γ	References
HIV Type 1	1.5-2754.2	1.5-478.6	1.5-389.1	1.5-1862.1	[11]
COVID-19 (severe)	98.7-240.1	30.4-51.2	22.0-121.2	2.9-60.3	[12], [13]
Rheumatoid Arthritis	2.8-177.8	1.6-113.8	1.6-179.85	0.9-17.3	[14]

1.3 Dysregulation of Drug Metabolizing Enzymes (DMEs) by Major Pro-inflammatory Cytokines

In vitro studies have been conducted to investigate the mechanism behind reduced *in vivo* enzyme-mediated drug clearance during inflammation. Pro-inflammatory cytokines downregulate the expression and activity of cytochrome P450 (CYP) enzymes in human hepatocytes (Table 2). Among all the CYP enzymes involved in drug metabolism, CYP3A4 appeared to be most sensitive to cytokine exposure. Additionally, IL-6 and IL-1β seemed to be the main perpetrators of the downregulation in CYP mRNA expression and activity. Although in general CYP activity was less affected than the corresponding mRNA expression, these data indicate altered regulation at the transcriptional level.

Table 2: The impact of proinflammatory cytokines (10 ng/mL) on CYP enzyme expression and activity [15].

	IL-6		IL-1β		TNF-α		IFN-γ	
	mRNA	Activity	mRNA	Activity	mRNA	Activity	mRNA	Activity
CYP3A4	↓98%	↓76%	↓95%	↓90%	↓87%	↓70%	↓75%	NS
CYP1A2	↓27%	↓22%	↓73%	↓65%	↓45%	↓72%	NS	NS
CYP2C9	↓63%	↓65%	↓79%	NS	NS	↓17%	NS	NS
CYP2C19	↓72%	↓35%	↓58%	NS	NS	↓82%	NS	NS

CYP2D6	↓41%	↓39%	↓75%	NS	↓40%	↓42%	NS	NS

1.4 Perturbation of Hepatic Drug Transporter Activity by Major Proinflammatory Cytokines

In vivo evidence suggests that both metabolism and transport-mediated drug clearance are altered during inflammation. However, compared to the substantial evidence of proinflammatory cytokines eliciting significant effect on CYP enzyme activity, their effect on hepatic drug transporter activity is not well characterized. Although a few *in vitro* studies have investigated individual cytokine effects on drug transporter activity and/or mRNA expression, they employed supraphysiological concentrations of cytokines [16]–[18]. In addition, the conditions employed did not replicate those encountered *in vivo*, where plasma concentrations of multiple cytokines are simultaneously elevated. Therefore, the goal of this study was to comprehensively characterize the effects of pro-inflammatory cytokines, both individually and as a cocktail, on the mRNA expression and activity of hepatic transporters using plated human hepatocytes (PHHs).

Chapter 2: Dysregulation of Human Hepatic Drug Transporters by Proinflammatory Cytokines

Chapter 2 has been submitted for publication in Journal of Pharmacology and Experimental Therapeutics

2.1 Introduction

Inflammation caused by autoimmune disorders or infection is associated with altered drug pharmacokinetics (PK) [19]–[23]. These changes, when clinically significant, necessitate a change in the patient's drug dosing regimen [5]. For example, in patients with sepsis, the plasma area under the curve (AUC) of atorvastatin (20 mg PO), a substrate of hepatic OATPs and eliminated primarily by hepatic CYP3A metabolism, was 15-fold higher than that in healthy adults [24]. Such a marked increase in drug exposure will likely increase the risk of myopathy, a side effect that can be fatal without dose reduction [25], [26].

The reduced clearance (CL) of drugs in the presence of inflammation is thought to be mediated by proinflammatory cytokines secreted by innate and adaptive immune cells, including interleukin-6 (IL-6), interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α) and interferon-gamma (IFN-γ) [23]. The plasma concentrations of these cytokines are significantly higher in patients with inflammation or infections than in healthy populations [8]. For example, in patients with acute COVID-19 infection, the plasma concentrations of IL-6 and IL-1β are 200-fold (98.7-240.1 pg/ml) and 50-fold (30.4-51.2 pg/ml), respectively, of those in non-infected controls (Table 1) [13]. Similar patterns have been observed for chronic autoimmune disorders and other infectious diseases [27]–[30]. These elevated cytokine plasma concentrations are associated with reductions in drug metabolizing enzyme (DME) activity *(in vitro and in vivo),* presumably as a consequence of decreased mRNA and protein expression [5], [19]. While much is known about the dysregulation of hepatic DMEs by inflammatory cytokines, their impact on hepatic drug transporter activity, either *in vitro* or *in vivo,* is poorly studied.

A few *in vitro* studies have shown that the mRNA expression and activity of human hepatic drug transporters is downregulated by individual proinflammatory cytokines [16]–[18], however, these studies were conducted at supra-pathophysiological plasma concentrations for only a few cytokines (IL-6: 10 ng/mL; IL-1β: 1 ng/mL; TNF-α: 100 ng/mL; IFN-γ: 10 ng/mL), and not in combination. Importantly, cytokine exposures tested *in vitro* do not replicate those found *in vivo,* where plasma concentrations of multiple cytokines are simultaneously elevated (Table 1) [8], [31]. Therefore, the goal of this study was to comprehensively characterize the effect of IL-6, IL-1β, TNF-α and IFN-γ on mRNA expression of the major influx (e.g. OATPs) and efflux (e.g. P-gp, BCRP) hepatic transporters, followed by quantitation of the activity of the most affected uptake transporters. We accomplished this goal using plated human hepatocytes exposed to the individual cytokines or their fixed combinations (i.e., cocktail) at concentrations which encompass their pathophysiologically relevant plasma concentrations.

2.2 Material and Methods

2.2.1. Chemicals and Reagents

Cytokines, IL-6, TNF-α, IL-1β, and IFN-γ were purchased from R&D systems (Minneapolis, MN). Cryopreserved human hepatocytes, INVITROGRO CP media INVITROGRO HI media and INVITROGRO HT media, were obtained from BioIVT (Westbury, NY). 24-well, 48-well collagen I-coated plates were purchased from Corning (Kennebunk, ME). 96-well collagen I-coated plates and Pierce™ BCA Protein Assay Kit were purchased from Thermo Fisher Scientific (Rockford, IL). Quantikine QuicKit ELISA assays for human IL-6, TNF-α, IL-1β or IFN-γ were obtained from R&D Systems (Minneapolis, MN). RNA Mini-Kit was purchased from Qiagen (Germantown, MD), and

High-Capacity RNA-to-cDNA™ Kit was obtained from Applied Biosystems (Carlsbad, CA). Universal PCR Master Mix and TaqMan probe were purchased from Thermo Fisher Scientific (Rockford, IL). cDNA probes were as follows: OATP1B1 (Hs00272374_m1), OATP1B3 (Hs00251986_m1), OATP2B1 (Hs01030343_m1), OAT2 (Hs00198527_m1), OCT1 (Hs01552829_m1), NTCP (Hs00161820_m1), BCRP (Hs01651967_m1), P-gp (Hs05638872_s1), MATE1 (Hs00217320_m1), MRP2 (Hs00960489_m1), MRP3 (Hs00978452_m1) and MRP4 (Hs00988721_m1). [^{3}H] Estrodial-17β- glucuronide, [^{3}H] estrone-3-sulfate, [^{3}H] cyclic guanosine monophosphate, [^{3}H] taurocholic acid and [^{14}C] metformin were purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). Universal PCR Master Mix and TaqMan probe were purchased from Thermo Fisher Scientific (Rockford, IL).

2.2.2 Primary Human Hepatocyte (PHH) Culture

Cryopreserved PHHs (ADR, YND and ZKF; see Table 3 for demographics) were thawed with INVITROGRO HT medium and seeded into 24-well (for analysis of mRNA), 48-well (for analysis of transporter activity) or 96-well (for cytokine depletion studies) collagen I-coated plates at the density of 0.6×10^6 cells/mL in INVITROGRO CP medium. The medium was switched to INVITROGRO HI medium, with or without cytokine treatment after the cells attached (> 5 hours after seeding).

Table 3: Demographic characteristics of the hepatocyte donors.

Donor	Age	Sex	Race	Hispanic or Latino	Cause of Death	Alcohol Use	Tobacco Use
ADR	38	F	Caucasian	No	Trauma	No	No
YND	37	F	Caucasian	No	Anoxia 2nd to SAH*	Yes	Yes
ZKF	38	F	African American	Yes	Anoxia	Yes	Yes

SAH- Subarachnoid hemorrhage

2.2.3 Proinflammatory Cytokine Exposure

Cytokine stocks (IL-6, TNF-α, IL-1β, and IFN-γ) were prepared according to the manufacturer's instructions. Briefly, the cytokine powder was reconstituted in PBS at a concentration of 100 μg/mL, aliquoted, and stored at -20C. On the day of treatment, the cytokine stock was diluted in the INVITROGRO HI medium to yield the target cytokine concentrations (cocktail, 0.1 and 1 ng/mL and individual, 0.01, 0.1, 1, and 10 ng/mL). The final PBS concentration in the culture medium was < 0.1%. Hepatocytes were incubated in HI medium (24-well, 500μL; 48-well, 200μL, 96-well, 100μL) with and without cytokines (n=3 wells per cytokine concentration) for a total of 72 hours, at 37°C in 5% CO_2, with daily replacement of medium containing freshly diluted stock cytokines.

2.2.4 Isolation of RNA and Quantification of Relative mRNA Expression of Transporters

At the end of the cytokine treatments, total RNA (~ 2.5 μg) was extracted from PHH using RNA Mini-Kit according to manufacturer's instructions, followed by reverse transcription to cDNA. Transporter mRNA expression (OATP1B1, OATP1B3, OATP2B1, OAT2, OCT1, BCRP, P-gp , MATE1, MRP2, MRP3, MRP4 and NTCP) was quantified using qPCR reaction and analyzed as previously described [32]. GUSB mRNA was quantified as the housekeeping control. Where there was low abundance of mRNA (Cq value > 33), the data were not included in analyses. Since plated hepatocytes are not amenable to measurement of efflux transporter activity, only the uptake transporters, where the mRNA expression was downregulated ≥ 50% or induced ≥ 2-fold, were investigated for their change in transporter activity by the 1 ng/mL cytokine cocktail.

2.2.5 Quantification of Drug Uptake Transporter Activity

At the end of cytokine treatment, cells were washed twice with prewarmed Hank's balanced salt solution (HBSS) buffer containing calcium and magnesium. Uptake studies were performed in triplicate with the respective transporter selective substrate, in the absence and presence of the corresponding inhibitor. All the selective substrates ± inhibitors were chosen based on published literature: OATP1B1/3, 25 nM [^{3}H] estrodial-17β- glucuronide ± 200 μM bromsulfthalein (BSP); OATP2B1, 20 nM [^{3}H] estrone-3-sulfate + 5 μM rifampicin (to inhibit OATP1B1/3) ± 200 μM BSP; OAT2, 80 nM [^{3}H] cyclic guanosine monophosphate ± 200 μM ketoprofen; OCT1, 2 μM [^{14}C] metformin ± 500 μM quinidine [33], [34]. NTCP activity was determined by the uptake of 20 nM [^{3}H] taurocholic acid in the presence and absence of Na^{+} where the absence of Na^{+} was interpreted as equivalent to the presence of an inhibitor. Since OATP1B1 activity cannot be distinguished from OATP1B3 activity, their combined activity was measured. The cells were co-incubated with individual substrate ± inhibitor (in HBSS buffer containing calcium and magnesium) for 15 min (preliminary studies indicated that this time point was within the linear range) before washing 3 times with ice-cold HBSS buffer to terminate uptake. Then, 200 μL of 1M NaOH was added to lyse the cells at 37°C for one hour before the lysate was neutralized with 200 μL 1M HCL. Lysate (300 μL) was used for quantification by liquid scintillation counting (PerkinElmer, Waltham, MA). The uptake amount was normalized to the total protein content in each well measured by the BCA assay. Active uptake was calculated by subtracting the passive uptake (with inhibitor) from the total uptake (without inhibitor).

2.2.6 Cytokine Depletion in PHH

Medium was sampled (100 μL) at various time points up to 24 hours after cytokine exposure. The cytokine concentration in each sample was determined using an ELISA kit as per the manufacturer's instructions. Calibrators and quality control samples were prepared using the standards provided in the kit.

2.2.6 Statistical Analyses

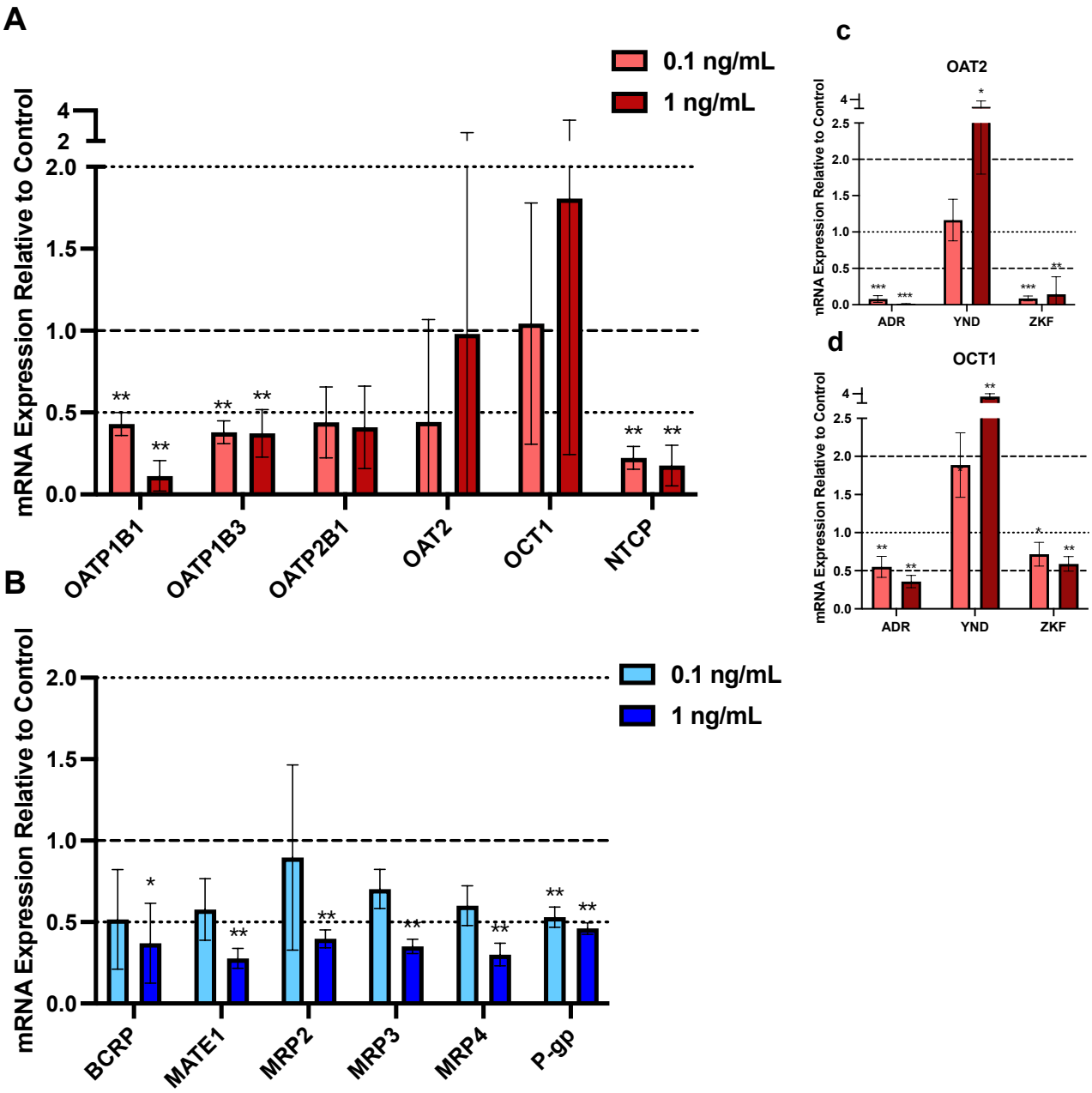
Unless otherwise stated, data are presented as mean ± S.D. Unpaired Students' t test and one-way analysis of variance (ANOVA), followed by post-hoc test (Dunnett's test to correct for multiple comparisons against a single control group or Fisher's LSD test to evaluate differences between individual cytokines relative to their control group) was performed. A p-value less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, La Jolla, CA).

2.3 Results

2.3.1 Cytokine Cocktails Significantly Regulated Hepatic Drug Transporter mRNA Expression

Following cytokine cocktail exposure, the mRNA expression of major hepatic transporters was significantly downregulated in all three lots of hepatocytes tested (except in lot YND where OAT2 and OCT1 were up-regulated) (Fig.5A) with a greater effect, in general, at 1 ng/mL vs. 0.1 ng/mL. The 1 ng/mL cytokine cocktail downregulated mRNA expression of OATP1B1, OATP1B3, and NTCP by 91%, 63% and 82%, respectively (Fig.5A). Downregulation of OATP2B1 mRNA expression was not significant at either 0.1 or 1 ng/mL cytokine cocktail. While the mRNA expression of OAT2 and OCT1 was downregulated in lot ZKF and ADR at both cytokine cocktail concentrations, it was significantly upregulated in lot YND (Fig.5A). The 1 ng/mL cytokine cocktail also downregulated BCRP, MATE1, MRP2/3/4 and P-gp mRNA expression by 68%, 75%, 65%, 67%, 73% and 58%, respectively by (Fig.5B). The 0.1 ng/mL cytokine cocktail did not significantly reduce mRNA expression of the transporters studied, except OATP1B1, OATP1B3, NTCP, OAT2/OCT1 (in only two lots) and P-gp. In general, the downregulation of mRNA expression of uptake transporters was greater than that of the efflux transporters at 0.1 ng/mL cytokine cocktail.

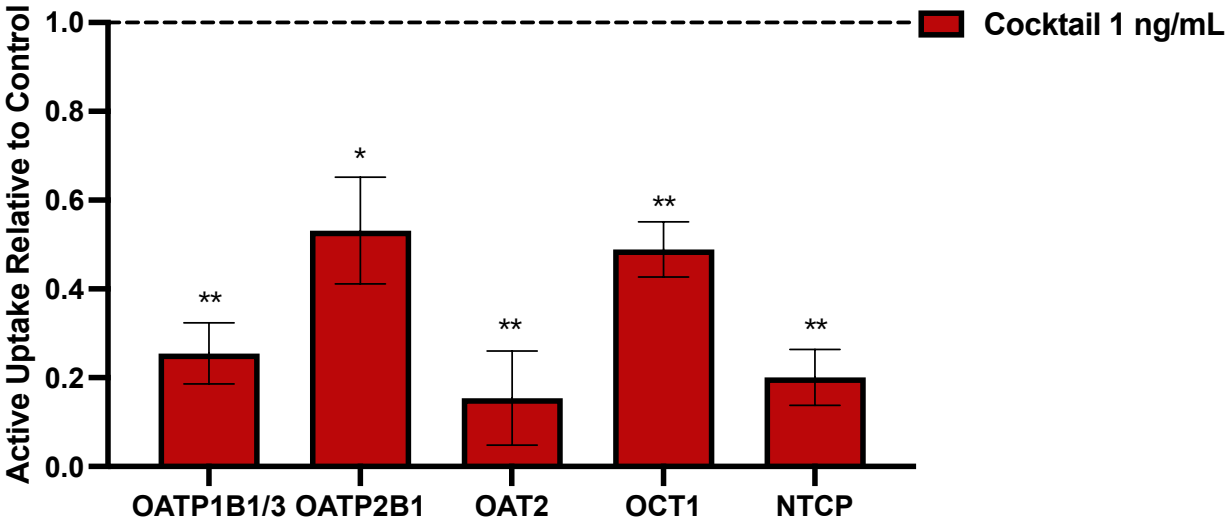
Figure 3


A
0.1 ng/mL
1 ng/mL
mRNA Expression Relative to Control
OATP1B1
OATP1B3
OATP2B1
OAT2
OCT1
NTCP
B
0.1 ng/mL
1 ng/mL
mRNA Expression Relative to Control
BCRP
MATE1
MRP2
MRP3
MRP4
P-gp
c
OAT2
mRNA Expression Relative to Control
ADR
YND
ZKF
d
OCT1
mRNA Expression Relative to Control
ADR
YND
ZKF

2.3.2 Downregulation of Hepatic Drug Uptake Transporter Activity by the 1ng/mL Cytokine Cocktail.

The 1 ng/mL cytokine cocktail downregulated the activity of OATP1B1/3 ≈ OAT2 ≈ NTCP > OATP2B1 ≈ OCT1 by 75%, 82%, 80%, 44%, and 47% respectively (Fig. 4). The degree of downregulation of activity and mRNA expression was generally similar. For example, mRNA expression of OATP1B1 and OATP1B3 was downregulated by 91% and 63%, respectively, resulting in 75% decrease in their combined activity (Fig. 4). Although downregulation of OATP2B1 mRNA was not significant at 1 ng/mL (Fig. 5), its activity was significantly reduced.

Figure 4

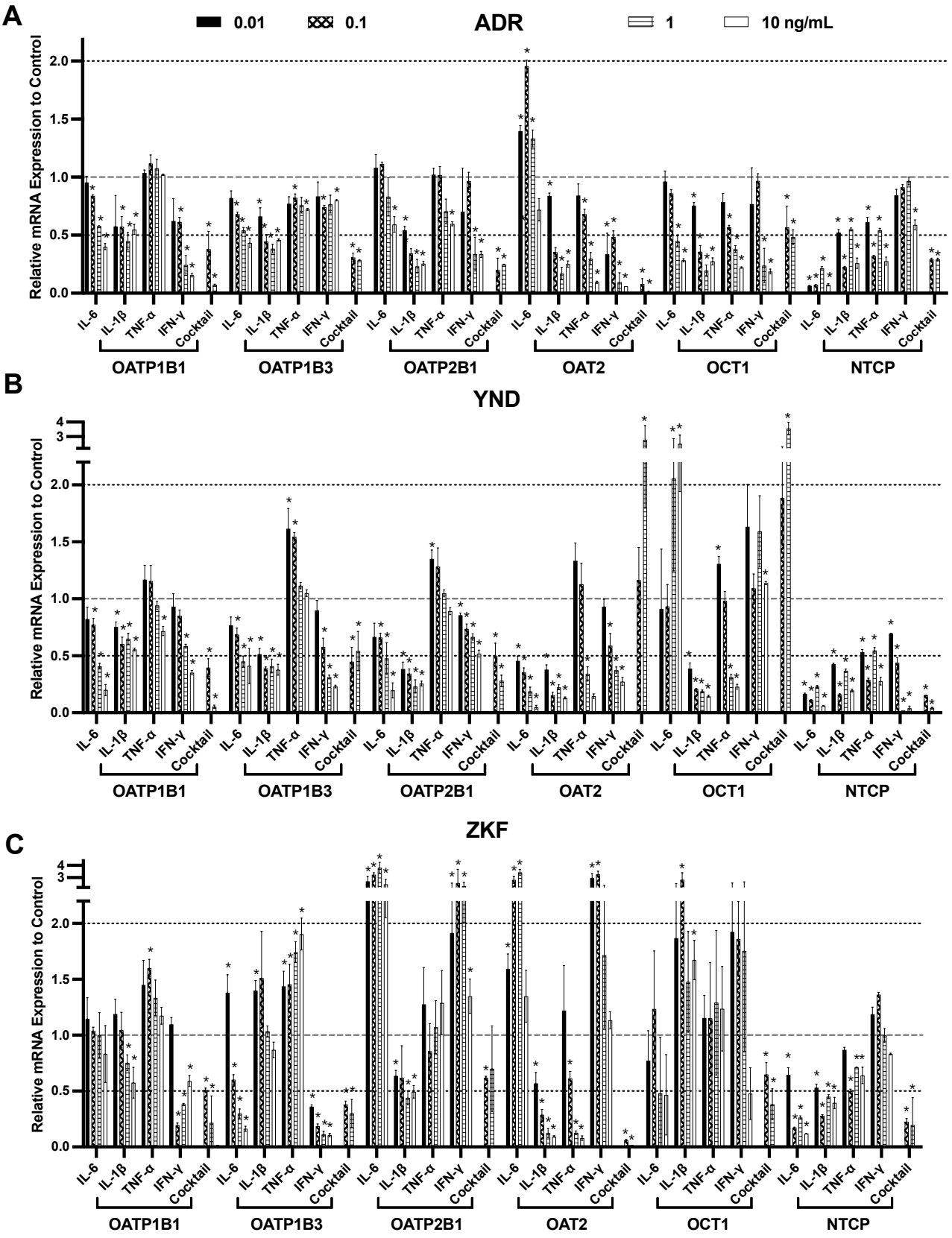


2.3.3 Individual Cytokines Differentially Dysregulated Hepatic Drug Transporter mRNA Expression

Considerable inter-lot variability in the regulation of mRNA expression was observed following exposure to each cytokine. For example, IL-1β downregulated the mRNA expression of OCT1 in lot ADR and YND, yet we observed induction in lot ZKF (Fig. 5).

Moreover, within each lot, each cytokine elicited a variable effect on transporter mRNA expression with no clear pattern emerging. For example, in lot ADR, 1 ng/mL of IL-6 downregulated mRNA expression of OATP1B1, OATP1B3, OATP2B1, OCT1 and NTCP by up to ~75% but induced mRNA expression of OAT2 by ~50% (Fig. 5). Comparable results were reported in a previous study using the same IL-6 concentration (Vee et al., 2009). For IL-1β, all tested drug uptake transporters were markedly down-regulated at 0.1, 1 and 10 ng/mL and appeared to reach near maximal effect at around 0.1 ng/mL. In contrast, TNF-α had either no significant effect (OATP1B1) or a modest effect (OATP1B3/2B1) on mRNA expression of the OATP transporters (Fig. 5). In general, amongst all the four cytokines, IL-6, IL-1β and IFN-γ appeared to be most potent in down-regulating mRNA expression of transporters, while TNF-α elicited a lesser effect. Furthermore, in only a few cases was the effect of the cytokine on mRNA expression concentration-dependent, such as the effect of IL-6 on OATP1B1 mRNA in lot ADR and YND. The highest degree of downregulation was seen with IFN-γ (10 ng/mL) where the mRNA expression of OAT2 was downregulated by up to 90% in lot ADR. Comparing the results of individual cytokines and cytokine cocktail in all three lots, due to the variability, it is difficult to draw a conclusion – which cytokine is the perpetrator of the effect produced on the transporters by the cytokine cocktails. Multiple cytokines likely contribute. For example, within the 1 ng/mL cocktail, IL-1β and IFN-γ appear to be the perpetrator of the downregulation of OATP1B1 mRNA expression.

Figure 5

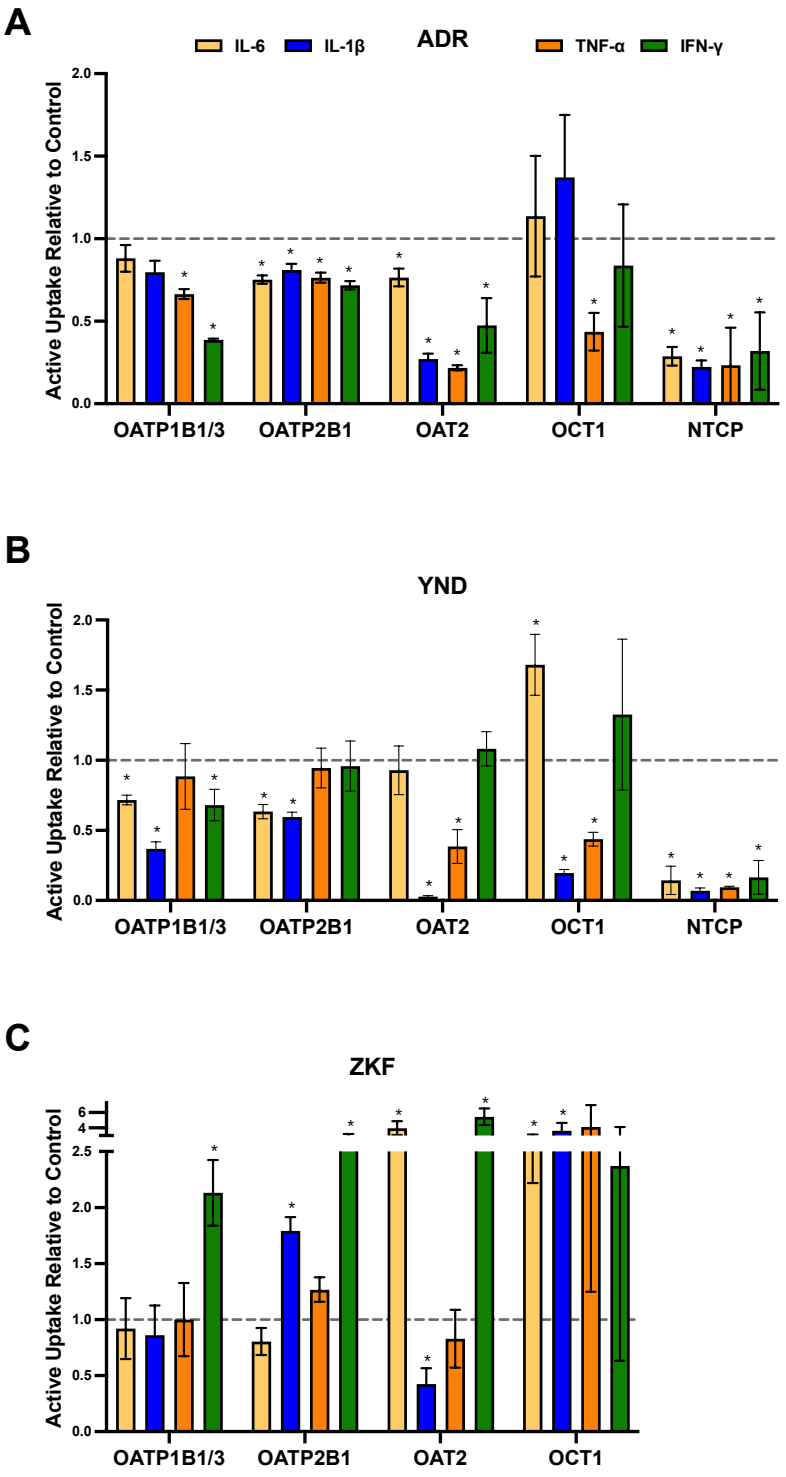

A
0.01
0.1
ADR
1
10 ng/mL
Relative mRNA Expression to Control
IL-6
IL-1β
TNF-α
IFN-γ
Cocktail
OATP1B1
OATP1B3
OATP2B1
OAT2
OCT1
NTCP
B
YND
C
ZKF

2.3.4 Hepatic Drug Uptake Transporter Activity is Differentially Affected by Individual Cytokines

Similar to the mRNA data, significant inter-lot variability was observed when examining the effect of individual cytokines on hepatic transporter activity. For example, while IFN-γ downregulated the activity of OATP1B1/3 in lot ADR and YND, it induced their combined activity in lot ZKF (Fig. 4). In addition, each cytokine showed differential effect on the transporters. In lot YND, TNF-α downregulated OCT1 activity by >50% but showed no significant effects on OATP1B1/3 activity (Fig. 6B). Amongst the four cytokines tested, IL-1β was most potent in down-regulating transporter activity, and thus appeared to be the main perpetrator of the downregulation of activity observed with the 1 ng/mL cocktail. The magnitude of change in activity by the individual cytokines (1 ng/mL) was similar or less than that observed with 1 ng/mL cytokine cocktail. For example, the downregulation of OAT2 activity by IL-1β ranged from 60 to 85% across all three lots, similar to the extent of downregulation caused by the cytokine cocktail at the same concentration (81%). In addition, although the 1 ng/mL cytokine cocktail downregulated all uptake transporter activity, some cytokines individually induced the activity of transporters. For instance, IL-6 and IFN-γ induced the activity of OAT2 by approximately 5-fold in lot ZKF (Fig. 6C), but the cocktail downregulated OAT2 activity by more than 80% in the same lot at the same concentration (Fig. 4). In general, reductions in mRNA expression translated into a corresponding decrease in uptake activity, but to a lesser extent. An exception was in lot ZKF where we observed around 50% and 70%

downregulation in the mRNA expression of OATP1B1 and OATP1B3 by IFN-γ respectively, but their combined activity was induced by up to 2-fold (Fig. 6C).

Figure 6



2.3.5 Cytokines were Differentially Depleted When Incubated with PHH.

To conserve the available hepatocytes, lot ADR and YND were used to test for depletion of the cytokines (in the cocktail or individually) cultured at 1 ng/mL. In all instances, little to no depletion of IL-6 and IL-1β was observed over 24 hours (Fig. 7). In contrast, TNF-α and IFN-γ were depleted by ~50%. Thus, the average concentration (AUC/24 h) of cytokines (as cocktail or individually) that the PHH were exposed to remained ~0.5-1 ng/mL (Fig. 7 and Table 4).

Figure 7

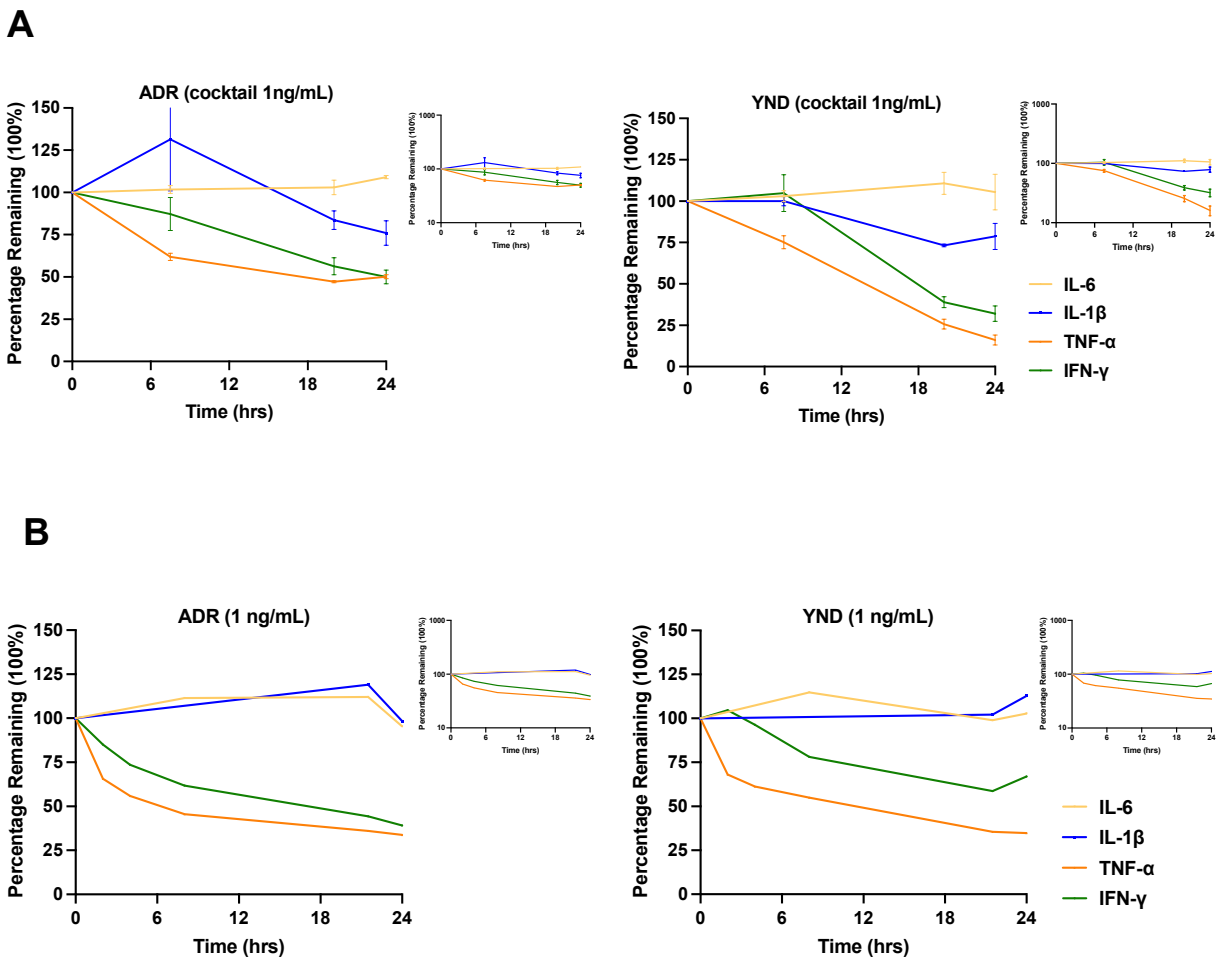


Table 4: Cytokine depletion in cultured PHH. Shown are the estimated average (AUC/ 24h) TNF-α and IFN-γ concentration when incubated with PHH over 24 hours either as part of the cocktail or individually. The nominal (starting) concentration of each cytokine was 1 ng/mL.

	AUC*/ 24h (ng/mL)			
	ADR		YND	
	TNF-α	IFN-γ	TNF-α	IFN-γ
Cocktail	0.69	0.61	0.62	0.77
Individual	0.51	0.63	0.47	0.81

*- AUC was calculated by the trapezoidal rule; data for IL-6 and INF-γ are not shown as they were not depleted.

2.4 Discussion

2.4.1 Overall Results

To our knowledge, this is the first study to investigate the dysregulation of hepatic drug transporters, at the transcriptional and protein activity level, by multiple proinflammatory cytokines (individually or as a cocktail) at their pathophysiologically relevant concentrations. IL-6, TNF-α, IL-1β and IFN-γ were selected in this study as they orchestrate the cytokine network responses of numerous infectious and autoimmune diseases [35]–[39]. However, in these diseases, multiple cytokines are simultaneously elevated, such as IL-6, IL-1β, TNF-α, and IFN-γ in sepsis, and IL-6 and TNF-α in HIV [40]–[42]. Thus, the main focus of our study was the effect of cytokine cocktails on hepatic transporter mRNA expression and activity. Individual cytokines were studied to determine which cytokine(s) was the most likely perpetrator of cocktail effects. Also, previous studies did not measure the *in vitro* depletion of cytokines by hepatocytes and assumed that the nominal concentration was operative. However, if there is severe depletion of the cytokines, the nominal concentration would need to be adjusted to take

such depletion into consideration. We chose to study hepatocytes from premenopausal donors (Table 2) as we are ultimately interested in the combined impact of cytokines and pregnancy-related hormones on the regulation of hepatic transporters. Our study showed that: 1) mRNA expression and activity of the major hepatic drug transporters was significantly altered by exposure to the 1ng/mL cytokine cocktail; 2) among the drug uptake transporters, downregulation in mRNA expression generally translated to a reduction in transporter activity. Parenthetically we note that because selective substrates OAPT1B1 and OATP1B3 are not available, their combined activity was determined; 3) cytokines (cocktail or individually) can not only downregulate but can also upregulate the mRNA expression and activity of transporters, as noted for OATP1B1/3, OAT2 and OCT1; 4) mRNA expression and activity of transporters is differentially sensitive to the effects of individual cytokines, and IL-1β seems to be the major perpetrator; 5) there is substantial inter-donor variability in the effect of cytokines on mRNA expression and activity of transporters; and 6) the depletion of IL-6 and IL-1β by hepatocytes was little to none, while that of TNF-α and IFN-γ was significant, but insufficient to alter the interpretation of our data using the nominal concentration.

2.4.2 Mechanism of Cytokines' Regulatory Effect on Drug Transporters

We found that mRNA expression of drug uptake transporters was, in general, more sensitive to cytokine cocktail exposure than was efflux transporters. Similar findings have been shown with individual cytokine treatments such as IL-6, IL-1β and TNF-α by Le Vee *et al* [18]. At 0.1 or 1 ng/mL, OATP1B1, OATP1B3, OAT2 (in only two lots), OCT1 (in only two lots), and NTCP mRNA expression was significantly downregulated by more than 50% or induced by more than 2-fold (Fig. 1). In addition, considerable

inter-lot variability in response was observed, especially for the effect of the cocktails on mRNA expression of OAT2 and OCT1 (Fig 1c and d). Of the uptake transporters, the average downregulation of mRNA was largest for OATP1B1 (1 ng/mL cytokine cocktail). In contrast, the downregulation of BCRP, MATE1, MRP2, MRP3, MRP4, and P-gp exceeded the 50%/2-fold cut-off only at the higher cytokine concentration (1 ng/mL). We chose this cut-off values because mRNA expression, in general, is a more sensitive readout than activity [43]. Thus, we postulated that a change in mRNA expression that was less than the 50%/2-fold cut-off would not translate into any meaningful change in activity. For this reason, and because the activity of efflux transporters is best studied using sandwich-cultured hepatocytes (not PHH), all subsequent activity studies were focused on the uptake transporters.

There is no clear mechanistic explanation for the differential effect of the cytokines (cocktail or individual) on the mRNA expression of the uptake vs. efflux transporters or the observed significant inter-lot variability (e.g., YND vs ADR or ZKF on the effect on mRNA expression of OAT2 and OCT1). We speculate that lot differences are likely due to differences in gene regulation by epigenetic factors or variation in nuclear transcription factor response elements in the 5-flanking region of the genes. There may also be differences between lots in the ratio of hepatocyte and Kupffer cells; the latter are the “macrophages of the liver” and are capable of producing cytokines. In general, drug and endobiotic transporters are primarily regulated by transcriptional factors, such as nuclear receptors [44]. The increased production of proinflammatory cytokines during inflammatory reactions activates NF-κB [45], which in turn could suppress downstream signaling pathways that regulate transporter gene transcription [44]. Interestingly, with

regard to differential effects on uptake and efflux transporters, OATP1B1, OATP1B3, OATP2B1, and OCT1 all share a regulation mechanism mediated by hepatic nuclear factor HNF4α or HNF1α, while the efflux transporters are subjected to regulation by other nuclear factors [44]. This interplay between cytokines and nuclear receptors warrants additional studies to elucidate the underlying mechanism(s) of the effect of cytokines on transporter mRNA expression and activity. Of particular interest, inherent protective mechanisms of the liver may limit the uptake of toxins via transporters to avoid further stress during inflammatory events [46].

2.4.3 Individual Cytokines vs. Cytokine Cocktail; mRNA expression vs. Activity

Whether the effect of the individual cytokines on mRNA expression was additive, synergistic or antagonistic cannot be readily discerned from our data. To do so, more detailed studies using the method of isoboles would be needed [47]. However, in some instances, the effect appeared to be additive. For example, in lot ADR, the mRNA expression of OATP1B1 by the 1 ng/mL cytokine cocktail was downregulation by >90% while it was downregulated by ~60%, ~50% and ~85%, by the individual cytokines, IL-6, IL-1β and IFN-γ (1 ng/mL), respectively. In some cases, the effect on transporter mRNA expression by the cytokines in the cocktail deviated from additivity. For example, in lot YND, at 1 ng/mL, the mRNA expression of OCT1 was significantly downregulated by IL-1β or TNF-α by ~80%, but it was upregulated (~2-fold) by IL-6. In contrast, the 1 ng/mL cocktail produced ~4-fold upregulation of OCT1 mRNA expression.

We quantified the activity of uptake transporters in the presence of 1 ng/mL cytokine cocktails to determine if the above mRNA changes translated into a change in activity. In general, it did, with some exceptions. For instance, 1 ng/mL of cytokine cocktail

upregulated the mRNA expression of OAT2 and OCT1 in lot YND, but not their functional activity; in fact, the average transporter activity across all three lots for these two transporters were decreased following cytokine cocktail exposure (Fig. 4). It is possible that despite upregulation of mRNA expression, potentially dominating post-translational regulation or reduction in trafficking to the plasma membrane may have resulted in the observed decrease in OAT2 and OCT1 activity. As was the case for the 1 ng/mL cytokine cocktail, changes in mRNA expression in response to the individual cytokines generally resulted in corresponding (but lesser) changes in transporter activity. However, there were exceptions. IL-6 downregulated the mRNA expression of OAT2 in lot YND by ~75% but had no effect on its activity; Interestingly, although IFN-γ transcriptionally downregulated OATP1B1 and OATP1B3 in lot ZKF (Fig. 5C), it significantly upregulated their combined activity (2-fold) (Fig. 6C). This opposite trend was observed in only lot ZFK, but not in the other two tested lots (ADR and YND). Additionally, the activity of other transporters (e.g., OATP2B1, OAT2) was upregulated by several cytokines in only lot ZKF (Fig. 6C). The underlying mechanism for this significant variability between lots in the regulation of transporters is not known. To note, this is the first time that cytokines have been documented to upregulate OAT1B1/3, OAT2B1, OAT2 and OCT1 transporter activity, which could potentially result in increased hepatic drug CL and decreased therapeutic efficacy for certain drugs during inflammation. However, as noted above, these differences could be due to the *in vitro* cell culture system or due to genetic variability in regulatory regions of the affected genes.

Comparing the transporter activity data in the presence of the individual cytokines vs. the cocktail, provided some insights into the cytokine(s) responsible for the changes observed with the cocktail. Of all the transporters tested, IL-1β, followed by IFN-γ, appeared to be the major perpetrators of the observed downregulation in transporter activity observed in the presence of 1 ng/mL cytokine cocktail (Fig. 4). Except in lot ZKF, NTCP was significantly downregulated by all cytokines.

In vivo data are in general agreement with our main findings. During an influenza B outbreak in children, a usual dose of theophylline, an OAT2 substrate [48] which is cleared from the body primarily by CYP1A2 metabolism [49], resulted in toxicity including vomiting and seizures due to decreased clearance of the drug [50]. Although CYP1A2 activity is downregulated during inflammation [51], given that theophylline is a hydrophilic molecule (log P= -0.02), the decrease in OAT2 activity may also have contributed to the observed toxicity. In critically ill sepsis patients receiving oral atorvastatin, an OATP and CYP3A4 substrate, a 15-fold increase (vs. healthy participants) in atorvastatin AUC (for the same dose) was observed [24]. In addition, although 50% of the sepsis patients received concomitant therapy with a CYP3A4 inhibitor, there was no difference in plasma AUC between those that received the inhibitor vs. those that did not. These data suggest that hepatic uptake of atorvastatin by OATPs is the rate-determining step in its hepatic clearance and that this uptake is downregulated during sepsis.

2.4.4 Study Limitations

Our study has some limitations. We conducted it with three hepatocyte lots (from premenopausal females) which demonstrated considerable inter-lot variability. This

hinders our ability to extrapolate to the wider female population or to other populations. Since PHH are not amenable to quantify the activity of the efflux transporters, they were not studied further. We studied only 4 major proinflammatory cytokines, however, there are other cytokines including anti-inflammatory cytokines, such as IL-10, that are also produced during inflammation. Interestingly, IL-10 has been shown to decrease CYP3A activity *in vivo* [52]. Also, we studied the cytokines at their fixed concentration in combination. *In vivo*, the ratio of the plasma concentration of each cytokine to another will vary depending on the time-course of infection or inflammatory condition [53]–[56]. Since our focus was on the effect of cytokines at 1 ng/mL, we studied the depletion of cytokines (for both the cocktail and individual cytokines) at only 1 ng/mL. We assumed that the depletion data obtained at this concentration also applies to the other concentrations.

2.4.5 Summary

In summary, we demonstrated for the first time that proinflammatory cytokines, both individually and as a cocktail, cause significant dysregulation of mRNA expression (influx and efflux) and activity (influx) of major hepatic drug transporters at their pathophysiologically relevant plasma concentrations. In addition, this is the first time that some cytokines have been shown to upregulate the mRNA expression of certain transporters. The mechanism(s) underlying the cytokine-transporter dysregulation is unclear and needs further study but is likely to involve reduced (or increased) mRNA synthesis. However, because a change in mRNA expression did not always translate into altered activity, we conclude that change in *in vitro* transporter activity should be the preferred parameter for *in vitro* to *in vivo* extrapolation. Our data further cements the

notion that altered regulation of transporters by proinflammatory cytokines plays a major role in the observed pharmacokinetic changes (through metabolism and transport) seen in people with inflammation. The magnitude of these changes can be predicted by physiologically based pharmacokinetic models populated by data such as those generated here. The performance of such models can then be evaluated by comparison to the *in vivo* pharmacokinetic observations.

Bibliography

[1] S. M. Seifert, J. R. Castillo-Mancilla, K. M. Erlandson, and P. L. Anderson, "Inflammation and pharmacokinetics: potential implications for HIV-infection," *Expert Opinion on Drug Metabolism & Toxicology*, vol. 13, no. 6, pp. 641–650, Jun. 2017, doi: 10.1080/17425255.2017.1311323.

[2] P. R. Mayo, K. Skeith, A. S. Russell, and F. Jamali, "Decreased dromotropic response to verapamil despite pronounced increased drug concentration in rheumatoid arthritis," *Br J Clin Pharmacol*, vol. 50, no. 6, pp. 605–613, Dec. 2000, doi: 10.1046/j.1365-2125.2000.00314.x.

[3] J. R. de L. Benzi, P. P. dos S. Melli, G. Duarte, J. D. Unadkat, and V. L. Lanchote, "The Impact of Inflammation on the In Vivo Activity of the Renal Transporters OAT1/3 in Pregnant Women Diagnosed with Acute Pyelonephritis," *Pharmaceutics*, vol. 15, no. 10, Art. no. 10, Oct. 2023, doi: 10.3390/pharmaceutics15102427.

[4] R. Chapa *et al.*, "Contribution of Uptake and Efflux Transporters to Oral Pharmacokinetics of Furosemide," *ACS Omega*, vol. 5, no. 51, pp. 32939–32950, Dec. 2020, doi: 10.1021/acsomega.0c03930.

[5] F. Stanke-Labesque, E. Gautier-Veyret, S. Chhun, and R. Guilhaumou, "Inflammation is a major regulator of drug metabolizing enzymes and transporters: Consequences for the personalization of drug treatment," *Pharmacology & Therapeutics*, vol. 215, p. 107627, Nov. 2020, doi: 10.1016/j.pharmthera.2020.107627.

[6] C. Liu, D. Chu, K. Kalantar-Zadeh, J. George, H. A. Young, and G. Liu, "Cytokines: From Clinical Significance to Quantification," *Advanced Science*, vol. 8, no. 15, p. 2004433, 2021, doi: 10.1002/advs.202004433.

[7] C. A. Dinarello, "Proinflammatory Cytokines," *Chest*, vol. 118, no. 2, pp. 503–508, Aug. 2000, doi: 10.1378/chest.118.2.503.

[8] S. Kany, J. T. Vollrath, and B. Relja, "Cytokines in Inflammatory Disease," *Int J Mol Sci*, vol. 20, no. 23, p. 6008, Nov. 2019, doi: 10.3390/ijms20236008.

[9] L. Chen *et al.*, "Inflammatory responses and inflammation-associated diseases in organs," *Oncotarget*, vol. 9, no. 6, pp. 7204–7218, Dec. 2017, doi: 10.18632/oncotarget.23208.

[10] S. Hannoodee and D. N. Nasuruddin, "Acute Inflammatory Response," in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2023. Accessed: Nov. 19, 2023. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK556083/

[11] E. M. Kahle *et al.*, "Plasma Cytokine Levels and Risk of HIV Type 1 (HIV-1) Transmission and Acquisition: A Nested Case-Control Study Among HIV-1–Serodiscordant Couples," *The Journal of Infectious Diseases*, vol. 211, no. 9, pp. 1451–1460, May 2015, doi: 10.1093/infdis/jiu621.

[12] H. Han *et al.*, "Profiling serum cytokines in COVID-19 patients reveals IL-6 and IL-10 are disease severity predictors," *Emerging Microbes & Infections*, vol. 9, no. 1, pp. 1123–1130, Jan. 2020, doi: 10.1080/22221751.2020.1770129.

[13] O. J. McElvaney *et al.*, "Characterization of the Inflammatory Response to Severe COVID-19 Illness," *Am J Respir Crit Care Med*, vol. 202, no. 6, pp. 812–821, Sep. 2020, doi: 10.1164/rccm.202005-1583OC.

[14] O. Selaas, H. H. Nordal, A.-K. Halse, J. G. Brun, R. Jonsson, and K. A. Brokstad, "Serum Markers in Rheumatoid Arthritis: A Longitudinal Study of Patients

Undergoing Infliximab Treatment," *Int J Rheumatol*, vol. 2015, p. 276815, 2015, doi: 10.1155/2015/276815.
[15] L. M. de Jong, W. Jiskoot, J. J. Swen, and M. L. Manson, "Distinct Effects of Inflammation on Cytochrome P450 Regulation and Drug Metabolism: Lessons from Experimental Models and a Potential Role for Pharmacogenetics," *Genes (Basel)*, vol. 11, no. 12, p. 1509, Dec. 2020, doi: 10.3390/genes11121509.
[16] M. Le Vee, P. Gripon, B. Stieger, and O. Fardel, "Down-Regulation of Organic Anion Transporter Expression in Human Hepatocytes Exposed to the Proinflammatory Cytokine Interleukin 1β," *Drug Metab Dispos*, vol. 36, no. 2, pp. 217–222, Feb. 2008, doi: 10.1124/dmd.107.016907.
[17] M. Le Vee, E. Jouan, A. Moreau, and O. Fardel, "Regulation of drug transporter mRNA expression by interferon-γ in primary human hepatocytes: Regulation of drug transporters by interferon-γ," *Fundamental & Clinical Pharmacology*, vol. 25, no. 1, pp. 99–103, Feb. 2011, doi: 10.1111/j.1472-8206.2010.00822.x.
[18] M. L. Vee, V. Lecureur, B. Stieger, and O. Fardel, "Regulation of Drug Transporter Expression in Human Hepatocytes Exposed to the Proinflammatory Cytokines Tumor Necrosis Factor-α or Interleukin-6," *Drug Metab Dispos*, vol. 37, no. 3, pp. 685–693, Mar. 2009, doi: 10.1124/dmd.108.023630.
[19] E. Morgan, "Impact of Infectious and Inflammatory Disease on Cytochrome P450–Mediated Drug Metabolism and Pharmacokinetics," *Clinical Pharmacology & Therapeutics*, vol. 85, no. 4, pp. 434–438, 2009, doi: 10.1038/clpt.2008.302.
[20] A. M. Cressman, V. Petrovic, and M. Piquette-Miller, "Inflammation-mediated changes in drug transporter expression/activity: implications for therapeutic drug response," *Expert Review of Clinical Pharmacology*, vol. 5, no. 1, pp. 69–89, Jan. 2012, doi: 10.1586/ecp.11.66.
[21] R. D. Harvey and E. T. Morgan, "Cancer, Inflammation, and Therapy: Effects on Cytochrome P450–Mediated Drug Metabolism and Implications for Novel Immunotherapeutic Agents," *Clinical Pharmacology & Therapeutics*, vol. 96, no. 4, pp. 449–457, 2014, doi: 10.1038/clpt.2014.143.
[22] R. Evers *et al.*, "Disease-Associated Changes in Drug Transporters May Impact the Pharmacokinetics and/or Toxicity of Drugs: A White Paper from the International Transporter Consortium," *Clin Pharmacol Ther*, vol. 104, no. 5, pp. 900–915, Nov. 2018, doi: 10.1002/cpt.1115.
[23] Y. E. H. Abdallah, S. Chahal, F. Jamali, and S. H. Mahmoud, "Drug-disease interaction: Clinical consequences of inflammation on drugs action and disposition," *J Pharm Pharm Sci*, vol. 26, p. 11137, 2023, doi: 10.3389/jpps.2023.11137.
[24] P. S. Kruger, N. M. Freir, B. Venkatesh, T. A. Robertson, M. S. Roberts, and M. Jones, "A preliminary study of atorvastatin plasma concentrations in critically ill patients with sepsis," *Intensive Care Med*, vol. 35, no. 4, pp. 717–721, Apr. 2009, doi: 10.1007/s00134-008-1358-3.
[25] N. C. Ward, G. F. Watts, and R. H. Eckel, "Statin Toxicity," *Circulation Research*, vol. 124, no. 2, pp. 328–350, Jan. 2019, doi: 10.1161/CIRCRESAHA.118.312782.
[26] W. Abed, M. Abujbara, A. Batieha, and K. Ajlouni, "Statin Induced Myopathy Among Patients Attending the National Center for Diabetes, endocrinology, & genetics," *Ann Med Surg (Lond)*, vol. 74, p. 103304, Feb. 2022, doi: 10.1016/j.amsu.2022.103304.

[27] S. Siebert, A. Tsoukas, J. Robertson, and I. McInnes, “Cytokines as Therapeutic Targets in Rheumatoid Arthritis and Other Inflammatory Diseases,” *Pharmacol Rev*, vol. 67, no. 2, pp. 280–309, Apr. 2015, doi: 10.1124/pr.114.009639.
[28] U. P. Singh *et al.*, “Chemokine and cytokine levels in inflammatory bowel disease patients,” *Cytokine*, vol. 77, pp. 44–49, Jan. 2016, doi: 10.1016/j.cyto.2015.10.008.
[29] M. Kox, N. J. B. Waalders, E. J. Kooistra, J. Gerretsen, and P. Pickkers, “Cytokine Levels in Critically Ill Patients With COVID-19 and Other Conditions,” *JAMA*, vol. 324, no. 15, pp. 1565–1567, Oct. 2020, doi: 10.1001/jama.2020.17052.
[30] A. Santa Cruz *et al.*, “Interleukin-6 Is a Biomarker for the Development of Fatal Severe Acute Respiratory Syndrome Coronavirus 2 Pneumonia,” *Frontiers in Immunology*, vol. 12, 2021, doi: 10.3389/fimmu.2021.613422.
[31] K. Roe, “An inflammation classification system using cytokine parameters,” *Scandinavian Journal of Immunology*, vol. 93, no. 2, p. e12970, 2021, doi: 10.1111/sji.12970.
[32] V. Dixit, N. Hariparsad, F. Li, P. Desai, K. E. Thummel, and J. D. Unadkat, “Cytochrome P450 enzymes and transporters induced by anti-human immunodeficiency virus protease inhibitors in human hepatocytes: implications for predicting clinical drug interactions,” *Drug Metab Dispos*, vol. 35, no. 10, pp. 1853–1859, Oct. 2007, doi: 10.1124/dmd.107.016089.
[33] Y. Bi *et al.*, “Quantitative Contribution of Six Major Transporters to the Hepatic Uptake of Drugs: ‘SLC-Phenotyping’ Using Primary Human Hepatocytes,” *J Pharmacol Exp Ther*, vol. 370, no. 1, pp. 72–83, Jul. 2019, doi: 10.1124/jpet.119.257600.
[34] M. Sachar, V. Kumar, L. C. Gormsen, O. L. Munk, and J. D. Unadkat, “Successful Prediction of Positron Emission Tomography–Imaged Metformin Hepatic Uptake Clearance in Humans Using the Quantitative Proteomics–Informed Relative Expression Factor Approach,” *Drug Metab Dispos*, vol. 48, no. 11, pp. 1210–1216, Nov. 2020, doi: 10.1124/dmd.120.000156.
[35] G. Szabo and T. Csak, “Inflammasomes in liver diseases,” *Journal of Hepatology*, vol. 57, no. 3, pp. 642–654, Sep. 2012, doi: 10.1016/j.jhep.2012.03.035.
[36] J. R. Tisoncik, M. J. Korth, C. P. Simmons, J. Farrar, T. R. Martin, and M. G. Katze, “Into the Eye of the Cytokine Storm,” *Microbiology and Molecular Biology Reviews*, vol. 76, no. 1, pp. 16–32, Mar. 2012, doi: 10.1128/mmbr.05015-11.
[37] B. G. Chousterman, F. K. Swirski, and G. F. Weber, “Cytokine storm and sepsis disease pathogenesis,” *Semin Immunopathol*, vol. 39, no. 5, pp. 517–528, Jul. 2017, doi: 10.1007/s00281-017-0639-8.
[38] A. Shimabukuro-Vornhagen *et al.*, “Cytokine release syndrome,” *j. immunotherapy cancer*, vol. 6, no. 1, p. 56, Dec. 2018, doi: 10.1186/s40425-018-0343-9.
[39] L. Tang, Z. Yin, Y. Hu, and H. Mei, “Controlling Cytokine Storm Is Vital in COVID-19,” *Frontiers in Immunology*, vol. 11, 2020, doi: 10.3389/fimmu.2020.570993.
[40] F. A. Bozza *et al.*, “Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis,” *Critical Care*, vol. 11, no. 2, p. R49, Apr. 2007, doi: 10.1186/cc5783.
[41] F. N. Osuji, C. C. Onyenekwe, J. E. Ahaneku, and N. R. Ukibe, “The effects of highly active antiretroviral therapy on the serum levels of pro-inflammatory and

anti-inflammatory cytokines in HIV infected subjects," *Journal of Biomedical Science*, vol. 25, no. 1, p. 88, Dec. 2018, doi: 10.1186/s12929-018-0490-9.
[42] A. A. Gharamti *et al.*, "Proinflammatory cytokines levels in sepsis and healthy volunteers, and tumor necrosis factor-alpha associated sepsis mortality: A systematic review and meta-analysis," *Cytokine*, vol. 158, p. 156006, Oct. 2022, doi: 10.1016/j.cyto.2022.156006.
[43] I. Papageorgiou, S. Grepper, and J. D. Unadkat, "Induction of Hepatic CYP3A Enzymes by Pregnancy-Related Hormones: Studies in Human Hepatocytes and Hepatic Cell Lines," *Drug Metab Dispos*, vol. 41, no. 2, pp. 281–290, Feb. 2013, doi: 10.1124/dmd.112.049015.
[44] K. L. R. Brouwer *et al.*, "Regulation of Drug Transport Proteins—From Mechanisms to Clinical Impact: A White Paper on Behalf of the International Transporter Consortium," *Clin Pharma and Therapeutics*, p. cpt.2605, May 2022, doi: 10.1002/cpt.2605.
[45] T. Liu, L. Zhang, D. Joo, and S.-C. Sun, "NF-κB signaling in inflammation," *Sig Transduct Target Ther*, vol. 2, no. 1, Art. no. 1, Jul. 2017, doi: 10.1038/sigtrans.2017.23.
[46] M. W. Robinson, C. Harmon, and C. O'Farrelly, "Liver immunology and its role in inflammation and homeostasis," *Cell Mol Immunol*, vol. 13, no. 3, pp. 267–276, May 2016, doi: 10.1038/cmi.2016.3.
[47] R. J. Tallarida, "Quantitative Methods for Assessing Drug Synergism," *Genes & Cancer*, vol. 2, no. 11, pp. 1003–1008, Nov. 2011, doi: 10.1177/1947601912440575.
[48] H. Shen, Y. Lai, and A. D. Rodrigues, "Organic Anion Transporter 2 (OAT2): An Enigmatic Human Solute Carrier," *Drug Metab Dispos*, Jan. 2016, doi: 10.1124/dmd.116.072264.
[49] E. Sutrisna, "The Impact of CYP1A2 and CYP2E1 Genes Polymorphism on Theophylline Response," *J Clin Diagn Res*, vol. 10, no. 11, pp. FE01–FE03, Nov. 2016, doi: 10.7860/JCDR/2016/21067.8914.
[50] M. J. Kraemer, C. T. Furukawa, J. R. Koup, G. G. Shapiro, W. E. Pierson, and C. W. Bierman, "Altered Theophylline Clearance During an Influenza B Outbreak," *Pediatrics*, vol. 69, no. 4, pp. 476–480, Apr. 1982, doi: 10.1542/peds.69.4.476.
[51] C. Lenoir, V. Rollason, J. A. Desmeules, and C. F. Samer, "Influence of Inflammation on Cytochromes P450 Activity in Adults: A Systematic Review of the Literature," *Frontiers in Pharmacology*, vol. 12, 2021, doi: 10.3389/fphar.2021.733935.
[52] J. C. Gorski, S. D. Hall, P. Becker, M. B. Affrime, D. L. Cutler, and B. Haehner-Daniels, "In vivo effects of interleukin-10 on human cytochrome P450 activity," *Clinical Pharmacology & Therapeutics*, vol. 67, no. 1, pp. 32–43, 2000, doi: 10.1067/mcp.2000.103860.
[53] C. K. WONG *et al.*, "Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome," *Clin Exp Immunol*, vol. 136, no. 1, pp. 95–103, Apr. 2004, doi: 10.1111/j.1365-2249.2004.02415.x.
[54] L. ROBERTS *et al.*, "Plasma cytokine levels during acute HIV-1 infection predict HIV disease progression," *AIDS*, vol. 24, no. 6, pp. 819–831, Mar. 2010, doi: 10.1097/QAD.0b013e3283367836.

[55] Y. Liu *et al.*, “Elevated plasma levels of selective cytokines in COVID-19 patients reflect viral load and lung injury,” *National Science Review*, vol. 7, no. 6, pp. 1003–1011, Jun. 2020, doi: 10.1093/nsr/nwaa037.
[56] R. Tortelli *et al.*, “Plasma Inflammatory Cytokines Are Elevated in ALS,” *Frontiers in Neurology*, vol. 11, 2020, doi: 10.3389/fneur.2020.552295.