DMD Fast Forward. Published on December 15, 2008 as doi:10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

Regulation of drug transporter expression in human hepatocytes exposed to the pro-inflammatory cytokines TNF-α or IL-6

Marc LE VEE, Valérie LECUREUR, Bruno STIEGER and Olivier FARDEL

UPRES EA SeRAIC/INSERM U620, IFR140, University of Rennes 1, Rennes, France (M.L.V., V.L., O.F.), Division of Clinical Pharmacology and Toxicology, Department of Medicine, University Hospital, Zurich, Switzerland (B.S.) and Department HITC, Hôpital Pontchaillou, CHU, Rennes, France (O.F.)

Running title: Regulation of human hepatic transporters by TNF-α and IL-6.

Corresponding author: Dr. Olivier FARDEL, UPRES EA SeRAIC/INSERM U620, University of Rennes 1, Faculty of Pharmacy, IFR140, 2 Avenue du Professeur Léon Bernard, 35043 Rennes Cedex, France. Tel.: 33 (0)2 23 23 48 80. Fax. : 33 (0)2 23 23 47 94. E-mail: olivier.fardel@univ-rennes1.fr

Number of text pages: 30
Number of tables: 3
Number of figures: 8
Number of references: 39
Number of words in the:

- Abstract: 235
- Introduction: 416
- Discussion: 1303

The abbreviations used are: ABC, ATP binding cassette; BCRP, breast cancer resistance protein; BSEP, bile salt export pump; CRP, C-reactive protein; IL, interleukin; LPS, lipopolysaccharide; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; NTCP, sodium-taurocholate co-transporting polypeptide; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; RT-qPCR , reverse transcription-quantitative polymerase chain reaction; SLC, solute carrier; TEA, tetra-ethylammonium; TNF-α, tumor necrosis factor-α.

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Tumor necrosis factor (TNF)-α and interleukin (IL)-6 are proinflammatory cytokines known to alter expression of drug transporters in rodent liver. Their effects towards human hepatic transporters remain however poorly characterized. This study was therefore designed to analyze the effects of these cytokines on drug transporter expression in primary human hepatocytes. Exposure to 100 ng/ml TNF-α or 10 ng/ml IL-6 for 48 h was found to down-regulate mRNA levels of major sinusoidal influx transporters, including sodium-taurocholate co-transporting polypeptide (NTCP), organic anion transporting polypeptide (OATP) 1B1, OATP1B3, OATP2B1, organic cation transporter (OCT) 1 and organic anion transporter (OAT) 2. TNF-α and IL-6 concomitantly reduced NTCP and OATP1B1 protein expression and NTCP, OATP and OCT1 transport activities. IL-6, but not TNF-α, was also found to decrease mRNA expression of the canalicular transporters multidrug resistance (MDR) 1 gene, multidrug resistance gene-associated protein (MRP) 2 and breast cancer resistance protein (BCRP); it concomitantly decreased MRP2 and BCRP protein expression. TNF-α, unlike IL-6, markedly reduced bile salt export pump (BSEP) mRNA levels and increased BCRP protein expression. Expression of the sinusoidal MRP3 efflux pump was found to be up-regulated at protein level by both TNF-α and IL-6. Taken together, these data demonstrate that TNF-α and IL-6 similarly altered expression of sinusoidal drug transporters and rather differentially that of canalicular efflux transporters. Such pronounced changes in hepatic transporter expression are likely to contribute to both cholestasis and alterations of pharmacokinetics caused by inflammation in humans.

INTRODUCTION

Inflammation is known to markedly impair hepatic detoxification pathways, and thereby to alter pharmacokinetics of drugs (Slaviero et al., 2003). This has been linked to changes in liver expression of drug metabolizing enzymes such as cytochromes P-450 (Renton, 2004) and, more recently, to that of drug transporters (Geier et al., 2007; Petrovic et al., 2007; Morgan et al., 2008; Teng and Piquette-Miller, 2008). Indeed, treatment by the pro-inflammatory factor lipopolysaccharide (LPS) has been shown to result in a pronounced alteration of hepatic transporter expression in rodents: sinusoidal influx solute-carrier (SLC) transporters such as sodium-taurocholate co-transporting polypeptide (Ntcp) (Slc10a1), organic anion transporting polypeptides (Oatp) 1a1 (Slco1a1) and Oatp1a2 (Slco1a2) and organic cation transporter (Oct) 1 (Slc22a1), and canalicular ATP-binding cassette (ABC) transporters such as multidrug resistance (Mdr) 1a gene/P-glycoprotein (Abcb1a), bile salt export pump (Bsep) (Abcb11) and multidrug resistance-associated protein (Mrp) 2 (Abcc2), are thus markedely down-regulated in liver of endotoxemic rats or mice (Bolder et al., 1997; Piquette-Miller et al., 1998; Vos et al., 1998; Cherrington et al., 2004). Expression of NTCP (SLC10A1) and MRP2 (ABCC2) is similarly decreased in human liver in response to LPS (Elferink et al., 2004).

Pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and IL-6 likely contribute to altered expression of hepatic drug transporters occurring during inflammation (Hartmann et al., 2002). Indeed, administration of IL-1β, TNF-α or IL-6 to rodents resulted in reduced expression of various sinusoidal or canalicular drug transporters (Green et al., 1996; Siewert et al., 2004; Geier et al., 2005). In the same way, treatment of human hepatocytes by IL-1β has recently been shown to alter expression of several major hepatic transporters (Le Vee et al., 2008). Whether TNF-α and IL-6 also affect

human hepatic transporters remains however poorly characterized, even if MDR1 (ABCB1), MRP2 or MRP3 (ABCC3) have been shown to constitute targets for TNF-α or IL-6 in some human hepatoma cell lines (Bohan et al., 2003; Lee and Piquette-Miller, 2003).

The present study was therefore designed to analyze the effects of TNF-α and IL-6 treatment on expression of major human hepatic transporters, using primary human hepatocytes which are well recognized as a convenient cellular model to study regulatory ways of drug detoxification proteins (Gomez-Lechon et al., 2004; Jigorel et al., 2006; Hewitt et al., 2007). Our data indicate that exposure to these cytokines markedly changes the expression profile of drug transporters, thus highlighting the probable contributing role played by these cytokines to the alteration of drug hepatic detoxification commonly observed in humans during inflammation.

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Chemicals and reagents.

Recombinant human TNF-α and IL-6 were provided by R&D Systems (Minneapolis, MN). [^{3}H(G)]taurocholic acid (sp. act. 1.19 Ci/mmol), [6, 7-^{3}H(N)]estrone-3-sulfate (sp. act. 57.3 Ci/mmol) and [1-^{14}C]tetra-ethylammonium (TEA) (sp. act. 2.4 mCi/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA). Probenecid and verapamil were from Sigma Aldrich (Saint-Quentin Fallavier, France). Antibodies against MDR1/P-glycoprotein and breast cancer resistance protein (BCRP) (ABCG2) were from Alexis Biochemicals (Lausen, Switzerland), whereas those against MRP2 and MRP3 were provided by Chemicon International (Temecula, CA). Tetramethyl rhodamine isothiocyanate-labelled secondary antibody against mouse IgG was from Jackson ImmunoResearch (West Grove, PA). All other compounds and reagents were commercial products of the highest purity available. Vehicle for TNF-α and IL-6 was phosphate-buffered saline; control cultures received the same dose of vehicles as treated counterparts.

Cell isolation and culture.

Human hepatocytes were obtained from adult donors undergoing hepatic resection for primary and secondary tumors, via the Biological Resource Center (Rennes, France). Cells were prepared by perfusion of histologically-normal liver fragments using a collagenase solution (Jigorel et al., 2005). They were primary cultured on plastic dishes in Williams'E medium, as already reported (Chouteau et al., 2001; Jigorel et al., 2005; Le Vee et al., 2008). All experimental procedures complied with French laws and regulations and were approved by the National Ethics Committee.

RNA isolation and analysis.

Total RNA was isolated from cells using the TRIzol^{R} reagent (Invitrogen, Cergy-Pontoise, France). RNA was then subjected to reverse transcription-real time quantitative polymerase chain reaction (RT-qPCR) using the fluorescent dye SYBR Green methodology and an ABI Prism 7000 detector (Applied Biosystem, Foster City, CA), as already reported (Jigorel et al., 2005). Gene primers were exactly as previously described (Jigorel et al., 2006; Le Vee et al., 2008). Relative quantification of the steady-state target mRNA levels was calculated after normalization of the total amount of cDNA tested to a 18S endogenous reference.

Western-blot analysis.

Crude membrane extracts were prepared from primary human hepatocytes as previously described (Lecureur et al., 2005; Jigorel et al., 2006). Proteins were then separated on polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. After blocking in Tris-buffered saline containing 4 % bovine serum albumin, membranes were incubated overnight at 4°C with primary antibodies directed against NTCP (Kullak-Ublick et al., 1997), OATP1B1 (SLCO1B1) (Zollner et al., 2003), MDR1, MRP2, MRP3 or BCRP. Peroxydase-conjugated monoclonal antibodies were thereafter used as secondary antibodies. After washing, immuno-labelled proteins were visualized by chemiluminescence. Gel loading and transfer was checked up by staining membranes with Ponceau red. The intensities of stained bands were measured by densitometry using ImageJ 1.40g software (National Institute of Health, Besthesda, MD).

Immunofluorescence studies.

Primary hepatocytes cultured on glass coverslips were first fixed in ice-cold acetone for 10 min. Cells were next incubated for 2 hr with mouse monoclonal antibodies used at 5 µg/ml

and directed against MRP2 or MRP3 or with corresponding isotypic IgG_{2a} or IgG_1 controls. After washing, a tetramethyl rhodamine isothiocyanate-labelled secondary antibody directed against mouse IgG was added for 1 hr and nuclei were subsequently stained with DAPI. Immunofluorescent images were finally detected with an inverted laser scanning confocal microscope DM IRE2 SP2 (Leica, Rueil Malmaison, France) equipped with a 63x NA 1.4 objective lens, using the LCS 3D software (Leica).

Transport assays.

Transport activities due to NTCP, OATPs or OCT1 (SLC22A1) were analyzed through measuring sodium-dependent-intracellular accumulation of the NTCP substrate taurocholate, probenecid-sensitive uptake of the OATP substrate estrone-3-sulfate and verapamil-sensitive uptake of the OCT1 substrate TEA, as previously described (Jigorel et al., 2005). Briefly, cells were incubated at 37°C for 5 min with 0.17 µM [^{3}H]taurocholate in the presence or absence of sodium, with 1.7 nM [^{3}H]estrone-3-sulfate in the presence or absence of the OATP inhibitor probenecid used at 2 mM, or with 40 µM [^{14}C]TEA in the presence or absence of the OCT1 inhibitor verapamil used at 50 µM. After washing in phosphate-buffered saline, cells were lyzed and accumulation of radio-labelled substrates was determined through scintillation counting. Taurocholate accumulation values in the presence of sodium minus accumulation values in the absence of sodium, estrone-3 sulfate uptake values in the absence of probenecid minus uptake values in the presence of probenecid, and TEA uptake values in the absence of verapamil minus uptake values in the presence of verapamil are thought to represent NTCP, OATP and OCT1 activities (Jigorel et al., 2005; Le Vee et al., 2006).

Statistical analysis.

Quantitative data were usually expressed as means ± SEM. They were statistically analyzed using the paired Student's *t* test, ANOVA followed by the Newman-Keuls test or the non-parametric Spearman's rank correlation method. The criterion of significance was $p<0.05$.

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Effects of cytokine treatments on expression of referent inflammation markers.

Primary human hepatocytes from 6 liver donors were exposed to 100 ng/ml TNF-α or 10 ng/ml IL-6 for 8 h, 24 h or 48 h. None of these cytokine treatments exerted toxicity as demonstrated by phase-contrast microscopic examination of the cultures and analysis of cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay (data not shown). As shown in Table 1, TNF-α and IL-6-treatments were shown to markedly increased mRNA expression of IL-8 or C-reactive protein (CRP), respectively, whatever the exposure time, even if the level of induction varied according to hepatocyte populations, especially for CRP up-regulation. Since IL-8 and CRP are considered as prototypical inflammation markers inducible by TNF-α (Osawa et al., 2002) and IL-6 (Zhang et al., 1996), respectively, this most likely indicated that primary human hepatocytes were fully responsive to the cytokines and were thus suitable for investigating TNF-α and IL-6 effects towards drug transporter expression.

Effects of TNF-a and IL-6 treatment on drug transporter mRNA expression.

We first analyzed the effects of TNF-α treatment on SLC and ABC transporter mRNA expression by RT-qPCR. As shown in Fig. 1, TNF-α-treated hepatocytes from 6 individuals exhibited lower mRNA expression of NTCP, OATP2B1 (SLCO2B1), OATP1B1, OATP1B3 (SLCO1B3), OCT1, organic anion transporter (OAT) 2 (SLC22A7) and BSEP when compared to untreated counterparts, whatever the time of treatment (8 h, 24 h or 48 h), whereas mRNA levels of MDR1, MRP2, MRP3, MRP4 (ABCC4) and BCRP remained unchanged. With respect to NTCP, OATP2B1 and OAT2, the time of exposure did not affect the level of expression repression in a major way, i.e. similar reduced levels of transporter

expression were found in hepatocytes exposed to TNF-α for 8 h or 48 h (Fig. 1); by contrast, expressions of OATP1B1, OATP1B3, OCT1 and BSEP in hepatocytes treated with TNF-α for 48 h were lower than those found in counterparts exposed to the cytokine for only 8 h ($p<0.05$), likely indicating that repression increased with exposure time. When considering the repression factor after a 24-h exposure to TNF-α, i.e. the ratio of mRNA levels in untreated hepatocytes versus those found in cytokine-exposed counterparts, BSEP was the most repressed (by a 8-fold factor) among the transporters affected by TNF-α, followed by OAT2 (repression by a 4.7-fold-factor) and OATP1B3 (repression by a 3.6-fold factor) (Table 2). The dose-response relationship for TNF-α effects towards transporter expression was then characterized. For this purpose, we have only analyzed transporters whose mRNA level was decreased by at least 50 % in hepatocytes exposed to 100 ng/ml TNF-α for 48 h, i.e. NTCP, OATP1B1, OATP1B3, OCT1, OAT2 and BSEP (Fig. 1 and Fig. 2). These transporters were similarly markedly down-regulated by 10 ng/ml TNF-α (Fig. 2). TNF-α used at 1 ng/ml was also found to significantly repress transporter expression, although in a more moderate manner (Fig. 2). Lower concentrations of TNF-α such as 0.01 ng/ml or 0.1 ng/ml failed to alter transporter expression; they also did not increase IL-8 mRNA levels, which were, by contrast, hugely induced by TNF-α used at 10 ng/ml or 100 ng/ml (Fig. 2).

We next studied the effects of IL-6 treatment on drug transporter mRNA expression. As shown in Fig. 3, IL-6-exposed human hepatocytes from 6 liver donors exhibited lower mRNA expression of NTCP, OATP2B1, OATP1B1, OATP1B3, OCT1, OAT2, MRP2 and BCRP when compared to untreated counterparts, whatever the time of treatment (8 h, 24 h or 48 h). With respect to OAT2, MRP2 and BCRP, the time of exposure did not affect the level of expression repression in a major way, i.e. similar reduced levels of transporter expression were found in hepatocytes exposed to IL-6 for 8 h or 48 h (Fig. 3); by contrast, expressions of NTCP, OATP2B1, OATP1B1, OATP1B3 and OCT1 in human hepatocytes treated with IL-6

for 48 h were lower than those found in counterparts exposed to the cytokine for only 8 h ($p<0.05$), likely indicating that repression increased with exposure time. IL-6 treatment was also found to moderately repress MDR1 mRNA expression, but only after a 24-h or a 48-h treatment; it also reduced MRP4 mRNA levels, but only for a 8-h and 24-h treatment; by contrast, it did not significantly alter mRNA expression of BSEP and MRP3, whatever the time of treatment (Fig. 3). When considering the repression factor after a 24-h exposure to IL-6, NTCP was the most repressed (by a 18.8-fold factor) among the transporters affected by IL-6, followed by OATP1B1 (repression by a 6.5-fold-factor) and OATP2B1 (repression by a 3.8-fold factor) (Table 2). The dose-response relationship for IL-6 effects towards transporter expression was then characterized. For this purpose, we have only analyzed transporters whose mRNA level was decreased by at least 50 % in hepatocytes exposed to 10 ng/ml IL-6 for 48 h, i.e. NTCP, OATP2B1, OATP1B1, OATP1B3, OCT1 and BCRP (Fig. 3 and Fig. 4). IL-6 used at 1 ng/ml also down-regulated these transporters, but in a more moderate manner, and these repressions reached significant level for only NTCP, OATP2B1, OATP1B1 and OCT1 (Fig. 4); similarly, CRP up-regulation was weaker in response to 1 ng/ml IL-6 than in response to 10 ng/ml IL-6 (Fig. 4). The use of a lower IL-6 concentration such as 0.1 ng/ml failed to alter transporter expression; it also did not alter CRP mRNA levels (Fig. 4).

Effects of TNF-α and IL-6 treatment on drug transporter protein expression.

To determine whether some of the changes in transporter mRNA levels induced by TNF-α or IL-6 treatment also occur at the protein level, we next performed western-blot analysis of crude membranes from cytokine-treated primary human hepatocytes and untreated counterparts. As indicated in Fig. 5, both TNF-α and IL-6 treatments for 48 h failed to obviously alter P-glycoprotein expression, whereas they induced MRP3 expression and reduced NTCP and OATP1B1 levels. IL-6 was moreover shown to reduce MRP2 expression,

which remained unaffected by TNF-α treatment. IL-6 also decreased BCRP expression, which was, by contrast, induced in response to TNF-α (Fig. 5).

Immunofluorescence studies were next performed in order to determine whether cytokine treatment may alter cellular distribution of transporters. A characteristic pattern of MRP2-related fluorescence, restricted to canalicular networks of cultured human hepatocytes as previously reported (Hoffmaster et al., 2004), was similarly found in untreated and TNF-α- and IL-6-exposed human hepatocytes (Fig. 6A). In agreement with previous studies (Konig et al., 1999), MRP3 was shown to be localized to basolateral membranes of untreated hepatocytes and such a cellular location was not altered by TNF-α or IL-6 treatment (Fig. 6B).

Effects of TNF-α and IL-6 treatment on transporter activities.

Owing to the limited availability of human hepatocytes, we focused on the effects of cytokine treatments on activities of NTCP, OATPs and OCT1, whose mRNA expressions were among the most repressed by both TNF-α and IL-6 (Table 2). As shown in Fig. 7, exposure to TNF-α or IL-6 for 48 h resulted in decreased NTCP, OATP and OCT1 transport activity in human primary hepatocytes, when compared to untreated counterparts.

Correlation analysis of cytokine repressing effects on drug transporter mRNA expression in human hepatocytes.

In order to search for a putative correlation between the alterations of drug transporter expression occurring in primary human hepatocytes exposed to TNF-α, IL-6 or IL-1β, drug transporters were ranked according to the down-regulation of their mRNA expression in response to a 24-h treatment by cytokines, from data from Table 2. Data reported for IL-1β effects were from Le Vée et al. (2008); they have been obtained using primary human hepatocytes cultured in the same conditions than those used in the present study. For each

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

treatment, transporters were ranked from the most repressed transporter to the less repressed according to mRNA expression levels. Correlations were analyzed using the Spearman's rank correlation method. Results indicated that the effects of IL-1β were significantly correlated with those of TNF-α and IL-6 (Fig. 8). By contrast, TNF-α effects on drug transporter expression were not correlated with those of IL-6 (Fig. 8).

DISCUSSION

The data reported in the present study demonstrate that the pro-inflammatory cytokines TNF-α and IL-6 can markedly alter drug transporter expression in human hepatocytes. These cytokines were found to act in a dose-dependent manner: Concentrations such as 100 ng/ml (for TNF-α) and 10 ng/ml (for TNF-α and IL-6) were fully active on transporter mRNA levels whereas the use of a lower dose of cytokines (1 ng/ml) remained also active, but in a more moderate manner.

Global effects of the cytokines towards transporters after a 48-h treatment are summarized in Table 3. Specifically, mRNA levels of hepatocyte uptake transporters NTCP, OATP2B1, OATP1B1, OATP1B3, OCT1 and OAT2 are decreased in response to both TNF-α and IL-6 in primary human hepatocytes; moreover, some of these transporters, i.e. NTCP and OATP1B1, are also down-regulated at protein levels whereas NTCP, OATP and OCT1 transport activity are concomitantly reduced. Taken together, these data indicated that these sinusoidal influx transporters, which are well-known, for at least some of them, to be down-modulated by TNF-α and IL-6 in rodent liver (Hartmann et al., 2002; Geier et al., 2005), also constitute major targets of these pro-inflammatory cytokines in human hepatocytes. The down-regulation of NTCP, OATP1B1 and OCT1 mRNA levels in IL-6-exposed primary human hepatocytes from two individuals, recently reported as preliminary data (Nakai et al., 2008), fully supports this conclusion. OAT2 mRNA expression, down-regulated in human hepatocytes exposed to either TNF-α or IL-6, has hovewer been shown to remain unaffected in response to LPS in rat liver (Cherrington et al., 2004), thus suggesting that this anion transporter may be differentially regulated by inflammatory cytokines in human and rodent hepatocytes.

Besides influx SLC transporters, TNF-α and IL-6 also regulate expression of efflux ABC transporters in human hepatocytes. In contrast to SLC transporters, ABC transporters are however rather differentially affected according to the nature of the cytokine (Table 3). Indeed, exposure to IL-6 for 48 h was found to down-regulate mRNA and protein levels of MRP2 and BCRP, whereas TNF-α failed to alter MRP2 mRNA and protein expression and induced protein expression of BCRP. IL-6 was also demonstrated to down-regulate MDR1 mRNA levels in response to a 24-h or 48-h treatment; it moreover diminished MRP4 mRNA expression after a 8-h or 24-h exposure. By contrast, TNF-α failed to alter MDR1 and MRP4 mRNA expression, whatever the exposure time. In addition, BSEP mRNA levels were markedly reduced by TNF-α, but remained unaffected by IL-6. MRP3 was finally the unique ABC transporter whose regulation was similar in both TNF-α- and IL-6-exposed human hepatocytes, i.e. it was up-regulated at protein levels and remained unaffected at mRNA levels. Interestingly, MRP3 has also been shown to be induced at protein levels in human hepatoma HepG2 cells exposed to TNF-α (Bohan et al., 2003). By contrast, MRP2 expression, down-regulated in IL-6-exposed primary human hepatocytes, remained unchanged in IL-6-treated human hepatoma HepG2 cells, whereas MDR1/P-glycoprotein expression was decreased in response to TNF-α in HepG2 cells (Lee and Piquette-Miller, 2003), but not in primary human hepatocytes. Comparison of ABC transporter regulation in cytokine-exposed human hepatocytes with that observed in rodent counterparts also reveals some similarities and some differences: For example, IL-6 decreased MRP2 mRNA levels and failed to significantly alter MRP3 mRNA expression in both primary human hepatocytes and mouse liver, whereas, by contrast, it down-regulated BSEP mRNA levels in mice (Hartmann et al., 2002), but not in human hepatocytes; IL-6 also reduced P-glycoprotein expression in primary rat hepatocytes (Sukhai et al., 2001), but not in human counterparts. In addition, TNF-α reduced BSEP mRNA levels in both human hepatocytes and mouse liver (Geier et al., 2005),

whereas it down-regulated MRP2 and MRP3 mRNA expression in mice (Hartmann et al., 2002), but not in human hepatocytes. Taken together, these data, highlighting some differences with respect to transporter regulatory pathways between human hepatocytes and human hepatoma cells and also between human and rodent hepatocytes, underline the interest of the use of primary human hepatocyte cultures for investigating regulation of drug detoxification proteins in humans, as already reported (Gomez-Lechon et al., 2004).

Cellular and molecular mechanisms responsible for TNF-α- and IL-6-related regulation of transporters in human primary hepatocytes remain to be determined. Transcriptionnal mechanisms may be involved, as already suggested (Sukhai et al., 2000; Geir et al., 2005). In addition, post-transcriptional mechanisms have likely to be considered, especially for the up-regulation of MRP3 expression in response to TNF-α and IL-6 and for that of BCRP in response to TNF-α, which both occur in primary human hepatocytes at protein levels, without concomitant changes in mRNA levels. Cytokine treatment however failed to alter cellular localization of transporters such as MRP2 and MRP3, indicating that putative post-transcriptional regulatory mechanisms triggered by cytokines do not interfere with location of these transporters.

In addition to TNF-α and IL-6, the pro-inflammatory cytokine IL-1β has been shown to markedly alter expression of major SLC and ABC drug transporters in both human and rodent hepatocytes (Geier et al., 2005; Le Vee et al., 2008) (Table 2). This likely indicates that IL-1β, TNF-α and IL-6 may act redundantly on at least some hepatic drug transporters. In the same way, in wild-type and knockout mouse strains lacking TNF-receptor-1, IL-1-receptor or IL-6, LPS administration gives a similar pattern of altered hepatic expression of transporters, thus demonstrating that inhibiting one cytokine-related signaling pathway failed to modify transporter regulation in response to LPS, owing to the putative redondant nature of TNF-α, IL-1β and IL-6 effects towards transporters in mice (Lickteig et al., 2007). Interestingly, using

rank correlation analysis, IL-1β effect towards transporter expression profile was found to be significantly correlated with those of TNF-α and IL-6. This may suggest some interrelationships or crosstalks between IL-1β-signaling pathways and those linked to TNF-α or IL-6, with respect to transporter regulation. It is however noteworthy that TNF-α and IL-6 effects were, by contrast, not correlated, thus favoring the idea that they mobilized independent signaling pathways and that regulatory pathways shared by TNF-α and IL-1β are therefore different from those shared by IL-6 and IL-1β.

Global repressing effects of TNF-α and IL-6 effects toward influx transporters suggest that *in vivo* exposure to each of these cytokines is likely to decrease uptake of drugs by the liver. Moreover, TNF-α, unlike IL-6, may impair bile acid secretion through down-regulating BSEP. Finally, it should be kept in mind that liver inflammation is usually associated with increased production of TNF-α, IL-6 and IL-1β. Therefore, the specific effects of these cytokines toward hepatic transporter expression are likely to add up and to overlapp, thus putatively resulting in a pronounced down-regulation of hepato-biliary transport of drugs and also of bile acids, and thereby contributing in a notable way to alteration of pharmacokinetics and development of cholestasis occuring during liver inflammation in humans (Moseley, 1997; Slaviero et al., 2003). Down-regulation of SLC and ABC transporters in response to LPS in rats is similarly thought to participate to the impaired hepatic uptake and secretion of drugs and the decreased bile salt secretion occurring in endotoxemic rats (Whiting et al., 1995; Bolder et al., 1997). In this context, up-regulation of the sinusoidal efflux transporter MRP3 by TNF-α and IL-6 may be considered as a protective mechanism contributing to prevent accumulation of toxic compounds, including bile acids, into human hepatocytes during inflammatory processes. A similar beneficial role for induction of Mrp3 during cholestasis in rodents has already been proposed (Donner and Keppler, 2001).

In conclusion, the current data demonstrate that the pro-inflammatory cytokines TNF-α and IL-6 can markedly impair expression of both sinusoidal and canalicular drug transporters in human hepatocytes. Such changes, associated with those triggered by IL-1β (Le Vee et al., 2008) and also with the well-established down-regulation of liver drug metabolizing enzymes such as cytochromes P-450 in response to inflammatory cytokines (Aitken and Morgan, 2007), are likely to contribute to alterations of pharmacokinetic features of drugs caused by inflammation in humans.

ACKNOWLEDGEMENTS

We thank Dr S. Dutertre (IFR 140, University of Rennes 1) for helpful advices with respect to immunofluorescence studies.

REFERENCES

Aitken AE and Morgan ET (2007) Gene-specific effects of inflammatory cytokines on cytochrome P450 2C, 2B6 and 3A4 mRNA levels in human hepatocytes. *Drug Metab Dispos* **35:**1687-1693.

Bohan A, Chen WS, Denson LA, Held MA and Boyer JL (2003) Tumor necrosis factor alpha-dependent up-regulation of Lrh-1 and Mrp3(Abcc3) reduces liver injury in obstructive cholestasis. *J Biol Chem* **278:**36688-36698.

Bolder U, Ton-Nu HT, Schteingart CD, Frick E and Hofmann AF (1997) Hepatocyte transport of bile acids and organic anions in endotoxemic rats: impaired uptake and secretion. *Gastroenterology* **112:**214-225.

Cherrington NJ, Slitt AL, Li N and Klaassen CD (2004) Lipopolysaccharide-mediated regulation of hepatic transporter mRNA levels in rats. *Drug Metab Dispos* **32:**734-741.

Chouteau P, Le Seyec J, Cannie I, Nassal M, Guguen-Guillouzo C and Gripon P (2001) A short N-proximal region in the large envelope protein harbors a determinant that contributes to the species specificity of human hepatitis B virus. *J Virol* **75:**11565-11572.

Donner MG and Keppler D (2001) Up-regulation of basolateral multidrug resistance protein 3 (Mrp3) in cholestatic rat liver. *Hepatology* **34:**351-359.

Elferink MG, Olinga P, Draaisma AL, Merema MT, Faber KN, Slooff MJ, Meijer DK and Groothuis GM (2004) LPS-induced downregulation of MRP2 and BSEP in human liver is due to a posttranscriptional process. *Am J Physiol Gastrointest Liver Physiol* **287:**G1008-1016.

Geier A, Dietrich CG, Trauner M and Gartung C (2007) Extrahepatic cholestasis downregulates Oatp1 by TNF-alpha signalling without affecting Oatp2 and Oatp4

expression and sodium-independent bile salt uptake in rat liver. *Liver Int* **27:**1056-1065.

Geier A, Dietrich CG, Voigt S, Ananthanarayanan M, Lammert F, Schmitz A, Trauner M, Wasmuth HE, Boraschi D, Balasubramaniyan N, Suchy FJ, Matern S and Gartung C (2005) Cytokine-dependent regulation of hepatic organic anion transporter gene transactivators in mouse liver. *Am J Physiol Gastrointest Liver Physiol* **289:**G831-841.

Gomez-Lechon MJ, Donato MT, Castell JV and Jover R (2004) Human hepatocytes in primary culture: the choice to investigate drug metabolism in man. *Curr Drug Metab* **5:**443-462.

Green RM, Beier D and Gollan JL (1996) Regulation of hepatocyte bile salt transporters by endotoxin and inflammatory cytokines in rodents. *Gastroenterology* **111:**193-198.

Hartmann G, Cheung AK and Piquette-Miller M (2002) Inflammatory cytokines, but not bile acids, regulate expression of murine hepatic anion transporters in endotoxemia. *J Pharmacol Exp Ther* **303:**273-281.

Hewitt NJ, Lechon MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM and Hengstler JG (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* **39:**159-234.

Hoffmaster KA, Turncliff RZ, LeCluyse EL, Kim RB, Meier PJ and Brouwer KL (2004) P-glycoprotein expression, localization, and function in sandwich-cultured primary rat and human hepatocytes: relevance to the hepatobiliary disposition of a model opioid peptide. *Pharm Res* **21:**1294-1302.

Jigorel E, Le Vee M, Boursier-Neyret C, Bertrand M and Fardel O (2005) Functional expression of sinusoidal drug transporters in primary human and rat hepatocytes. *Drug Metab Dispos* **33:**1418-1422.

Jigorel E, Le Vee M, Boursier-Neyret C, Parmentier Y and Fardel O (2006) Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. *Drug Metab Dispos* **34:**1756-1763.

Konig J, Rost D, Cui Y and Keppler D (1999) Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. *Hepatology* **29:**1156-1163.

Kullak-Ublick GA, Glasa J, Boker C, Oswald M, Grutzner U, Hagenbuch B, Stieger B, Meier PJ, Beuers U, Kramer W, Wess G and Paumgartner G (1997) Chlorambucil-taurocholate is transported by bile acid carriers expressed in human hepatocellular carcinomas. *Gastroenterology* **113:**1295-1305.

Le Vee M, Gripon P, Stieger B and Fardel O (2008) Down-regulation of organic anion transporter expression in human hepatocytes exposed to the proinflammatory cytokine interleukin 1beta. *Drug Metab Dispos* **36:**217-222.

Le Vee M, Jigorel E, Glaise D, Gripon P, Guguen-Guillouzo C and Fardel O (2006) Functional expression of sinusoidal and canalicular hepatic drug transporters in the differentiated human hepatoma HepaRG cell line. *Eur J Pharm Sci* **28:**109-117.

Lecureur V, Ferrec EL, N'Diaye M, Vee ML, Gardyn C, Gilot D and Fardel O (2005) ERK-dependent induction of TNFalpha expression by the environmental contaminant benzo(a)pyrene in primary human macrophages. *FEBS Lett* **579:**1904-1910.

Lee G and Piquette-Miller M (2003) Cytokines alter the expression and activity of the multidrug resistance transporters in human hepatoma cell lines; analysis using RT-PCR and cDNA microarrays. *J Pharm Sci* **92:**2152-2163.

Lickteig AJ, Slitt AL, Arkan MC, Karin M and Cherrington NJ (2007) Differential regulation of hepatic transporters in the absence of tumor necrosis factor-alpha, interleukin-1beta, interleukin-6, and nuclear factor-kappaB in two models of cholestasis. *Drug Metab Dispos* **35:**402-409.

Morgan ET, Goralski KB, Piquette-Miller M, Renton KW, Robertson GR, Chaluvadi MR, Charles KA, Clarke SJ, Kacevska M, Liddle C, Richardson TA, Sharma R and Sinal CJ (2008) Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. *Drug Metab Dispos* **36:**205-216.

Moseley RH (1997) Sepsis-associated cholestasis. *Gastroenterology* **112:**302-306.

Nakai K, Tanaka H, Hanada K, Ogata H, Suzuki F, Kumada H, Miyajima A, Ishida S, Sunouchi M, Habano W, Kamikawa Y, Kubota K, Kita J, Ozawa S and Ohno Y (2008) Decreased expression of cytochrome P450s 1A2, 2E1, and 3A4; and drug transporters Na+-taurocholate cotransporting polypeptide, organic cation transporter 1, and organic anion-transporting peptide-C correlates with the progression of liver fibrosis in chronic hepatitis C patients. *Drug Metab Dispos*.

Osawa Y, Nagaki M, Banno Y, Brenner DA, Asano T, Nozawa Y, Moriwaki H and Nakashima S (2002) Tumor necrosis factor alpha-induced interleukin-8 production via NF-kappaB and phosphatidylinositol 3-kinase/Akt pathways inhibits cell apoptosis in human hepatocytes. *Infect Immun* **70:**6294-6301.

Petrovic V, Teng S and Piquette-Miller M (2007) Regulation of drug transporters during infection and inflammation. *Mol Interv* **7:**99-111.

Piquette-Miller M, Pak A, Kim H, Anari R and Shahzamani A (1998) Decreased expression and activity of P-glycoprotein in rat liver during acute inflammation. *Pharm Res* **15:**706-711.

Renton KW (2004) Cytochrome P450 regulation and drug biotransformation during inflammation and infection. *Curr Drug Metab* **5:**235-243.

Siewert E, Dietrich CG, Lammert F, Heinrich PC, Matern S, Gartung C and Geier A (2004) Interleukin-6 regulates hepatic transporters during acute-phase response. *Biochem Biophys Res Commun* **322:**232-238.

Slaviero KA, Clarke SJ and Rivory LP (2003) Inflammatory response: an unrecognised source of variability in the pharmacokinetics and pharmacodynamics of cancer chemotherapy. *Lancet Oncol* **4:**224-232.

Sukhai M, Yong A, Kalitsky J and Piquette-Miller M (2000) Inflammation and interleukin-6 mediate reductions in the hepatic expression and transcription of the mdr1a and mdr1b Genes. *Mol Cell Biol Res Commun* **4:**248-256.

Sukhai M, Yong A, Pak A and Piquette-Miller M (2001) Decreased expression of P-glycoprotein in interleukin-1beta and interleukin-6 treated rat hepatocytes. *Inflamm Res* **50:**362-370.

Teng S and Piquette-Miller M (2008) Regulation of transporters by nuclear hormone receptors: implications during inflammation. *Mol Pharm* **5:**67-76.

Vos TA, Hooiveld GJ, Koning H, Childs S, Meijer DK, Moshage H, Jansen PL and Muller M (1998) Up-regulation of the multidrug resistance genes, Mrp1 and Mdr1b, and down-regulation of the organic anion transporter, Mrp2, and the bile salt transporter, Spgp, in endotoxemic rat liver. *Hepatology* **28:**1637-1644.

Whiting JF, Green RM, Rosenbluth AB and Gollan JL (1995) Tumor necrosis factor-alpha decreases hepatocyte bile salt uptake and mediates endotoxin-induced cholestasis. *Hepatology* **22:**1273-1278.

Zhang D, Sun M, Samols D and Kushner I (1996) STAT3 participates in transcriptional activation of the C-reactive protein gene by interleukin-6. *J Biol Chem* **271:**9503-9509.

Zollner G, Fickert P, Silbert D, Fuchsbichler A, Marschall HU, Zatloukal K, Denk H and Trauner M (2003) Adaptive changes in hepatobiliary transporter expression in primary biliary cirrhosis. *J Hepatol* **38:**717-727.

FOOTNOTES

Reprint requests should be sent to: Dr. Olivier FARDEL, UPRES EA SeRAIC/INSERM U620, University of Rennes1, Faculty of Pharmacy, IFR140, 2 Avenue du Professeur Léon Bernard, 35043 Rennes Cedex, France. Tel.: 33 (0)2 23 23 48 80. Fax. : 33 (0)2 23 23 47 94. E-mail: olivier.fardel@univ-rennes1.fr

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

LEGENDS TO FIGURES

Fig. 1. Effects of TNF-α on drug transporter mRNA expression in human hepatocytes.

Primary human hepatocytes were either untreated or exposed to 100 ng/ml TNF-α for 8 h, 24 h or 48 h. SLC (upper panel) and ABC (lower panel) transporter mRNA expression was then determined by RT-qPCR, as described under Materials and Methods. Data are expressed for each transporter as percentage of expression found in untreated hepatocytes, arbitrarily set at the value of 100 %. They are the means ± SEM of values from six independent hepatocyte populations. *, $p<0.05$ when compared to untreated cells.

Fig. 2. Dose-response of TNF-α effects towards transporter expression.

Primary human hepatocytes were either untreated or exposed to 0.01, 0.1, 1, 10 or 100 ng/ml TNF-α for 48 h. IL-8 (upper panel) and transporter (lower panel) mRNA expression was then determined by RT-qPCR, as described under Materials and Methods. Data are expressed for IL-8 and each transporter as percentage of expression found in untreated hepatocytes, arbitrarily set at the value of 100 %. They are the means ± SEM of values from three independent hepatocyte populations. *, $p<0.05$ when compared to untreated cells.

Fig. 3. Effects of IL-6 on drug transporter mRNA expression in human hepatocytes.

Primary human hepatocytes were either untreated or exposed to 10 ng/ml IL-6 for 8 h, 24 h or 48 h. SLC (upper panel) and ABC (lower panel) transporter mRNA expression was then determined by RT-qPCR, as described under Materials and Methods. Data are expressed for each transporter as percentage of expression found in untreated hepatocytes, arbitrarily set at the value of 100 %. They are the means ± SEM of values from six independent hepatocyte populations. *, $p<0.05$ when compared to untreated cells.

Fig. 4. Dose-response of IL-6 effects towards transporter expression.

Primary human hepatocytes were either untreated or exposed to 0.1, 1 or 10 ng/ml IL-6 for 48 h. CRP (upper panel) and transporter (lower panel) mRNA expression was then determined by RT-qPCR, as described under Materials and Methods. Data are expressed for CRP and each transporter as percentage of expression found in untreated hepatocytes, arbitrarily set at the value of 100 %. They are the means ± SEM of values from three independent hepatocyte populations. *, $p<0.05$ when compared to untreated cells.

Fig. 5. Effects of TNF-α and IL-6 on drug transporter protein expression in human hepatocytes.

Primary human hepatocytes were either untreated (UNT) or exposed to 100 ng/ml TNF-α or 10 ng/ml IL-6 for 48 h. Transporter protein content was then determined by Western blot analysis. For each transporter, data were quantified by densitometric analysis and expressed relatively to transporter expression found in untreated cells, arbitrarily set at the value of 100 %; they are the means ± SEM of values from four independent hepatocyte populations (upper panel). A representative blot from one hepatocyte population is also shown for each transporter (lower panel). *, $p<0.05$. P-gp, MDR1/P-glycoprotein.

Fig. 6. Effects of TNF-α and IL-6 on MRP2 and MRP3 localization in human hepatocytes.

Primary human hepatocytes were either untreated (UNT) or exposed to 100 ng/ml TNF-α or 10 ng/ml IL-6 for 48 h. MRP2 (A) and MRP3 (B) localizations were then analyzed through immunofluorescence and confocal microscopy using monoclonal antibodies directed against MRP2 and MRP3 and corresponding isotypic controls (IgG_{2a} and IgG_1), as described in Materials and Methods. Arrows indicate MRP2-related red fluorescence of canalicular

membranes of primary hepatocytes (A) or MRP3-related red fluorescence of basolateral membranes of primary hepatocytes (B). Blue fluorescence corresponds to DAPI-stained nuclei. Data shown are representative from immunofluorescence studies performed with three independent hepatocyte populations. Bar = 10 µM.

Fig. 7. Effects of TNF-α and IL-6 on drug transporter activities in human hepatocytes.

Primary human hepatocytes were either untreated (UNT) or exposed to 100 ng/ml TNF-α or 10 ng/ml IL-6 for 48 h. NTCP, OATP and OCT1 activities were then determined using radio-labelled substrates as described under Materials and Methods. Data are expressed relatively to transporter activity found in untreated cells, arbitrarily set at the value of 100 %; they are the means $\pm$ SEM of values from three independent hepatocytes populations. *, $p<0.05$ when compared to untreated cells.

Fig. 8. Rank correlation analysis of cytokine repressing effects on drug transporter mRNA expression in human hepatocytes.

Drug transporters were ranked according to the down-regulation of their mRNA expression in response to a 24-h treatment by cytokines, from data from Table 2. For each treatment, transporters were ranked from the most repressed transporter to the less repressed, through considering the repression factor for each transporter, i.e. the ratio of mRNA levels in untreated hepatocytes versus those found in cytokine-exposed cells. Correlations were analyzed using the Spearman's rank correlation method. Spearman's rank coefficients (ρ) and p values are provided on the right of the correlation graphs.

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1

Effects of TNF-α and IL-6 treatment on expression of referent inflammation markers

	Fold induction (mRNA levels)[a]	
Exposure time to cytokines	IL-8 expression/TNF-α treatment	CRP expression/IL-6 treatment
8 h	88 ± 59[b] (8-214)[c]	8115 ± 7867 (78-20355)
24 h	104 ± 73 (10-271)	15751 ± 14662 (111-31108)
48 h	96 ± 83 (10-344)	11821 ± 11331 (50-30786)

[a]defined as the ratio of mRNA levels in cytokine-treated primary hepatocytes versus those found in untreated counterparts.

[b]expressed as mean ± SEM of values from 6 independent hepatocyte populations.

[c]numbers in parentheses indicate the induction range for the 6 hepatocyte populations analyzed in the study.

TABLE 2

Comparison of cytokine effects on transporter mRNA expression in human hepatocytes

Transporter	Effects of cytokine treatment[a]		
	TNF-α (n=6)	IL-6 (n=6)	IL-1β[d] (n=6)
MDR1	↔ [b]	↓ [c] (1.6)	↓ (1.7)
BSEP	↓ (8.0)	↔	↓ (7.1)
MRP2	↔	↓ (1.8)	↓ (2.3)
MRP3	↔	↔	↓ (1.9)
MRP4	↔	↓ (1.7)	↓ (1.8)
BCRP	↔	↓ (3.2)	↓ (3.7)
NTCP	↓ (2.9)	↓ (18.8)	↓ (11.1)
OATP2B1	↓ (1.7)	↓ (3.8)	↓ (6.0)
OATP1B1	↓ (3.1)	↓ (6.5)	↓ (8.8)
OATP1B3	↓ (3.6)	↓ (3.3)	↓ (8.1)
OCT1	↓ (2.8)	↓ (2.9)	↓ (4.9)
OAT2	↓ (4.7)	↓ (2.5)	ND[e]

[a] Human hepatocytes were exposed to cytokines for 24 h. mRNA transporter expression was then determined by RT-qPCR.

[b] ↔, no significant changes in mRNA levels when compared to untreated control hepatocytes.

[c] ↓, significant repression of mRNA levels when compared to untreated control cells; numbers in parentheses indicate the repression factor, i.e. the ratio of mRNA levels in untreated cells versus those found in cytokine-exposed cells.

[d] Data from Le Vée et al. (2008).

[e] ND, Not done.

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 3

Summary of the effects of TNF-α and IL-6 on human hepatic transporter expression[a]

Transporter	TNF-α			IL-6		
	mRNA	protein	activity	mRNA	protein	activity
MDR1	↔[b]	↔	ND[e]	↓[c]	↔	ND
BSEP	↓	ND	ND	↔	ND	ND
MRP2	↔	↔	ND	↓	↓	ND
MRP3	↔	↑[d]	ND	↔	↑	ND
MRP4	↔	ND	ND	↔	ND	ND
BCRP	↔	↑	ND	↓	↓	ND
NTCP	↓	↓	↓	↓	↓	↓
OATP2B1	↓	ND	↓[f]	↓	ND	↓[f]
OATP1B1	↓	↓	↓[f]	↓	↓	↓[f]
OATP1B3	↓	ND	↓[f]	↓	ND	↓[f]
OCT1	↓	ND	↓	↓	ND	↓
OAT2	↓	ND	ND	↓	ND	ND

[a] Human hepatocytes were exposed to cytokines for 48 h.

[b] ↔, no change.

[c] ↓, significant repression.

[d] ↑, significant induction.

[e] ND, not done.

[f] activity was measured used the pan-OATP substrate estrone-3 sulfate and may therefore not reflect specific OATP2B1-, OATP1B1- or OATP1B3-mediated transport.

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

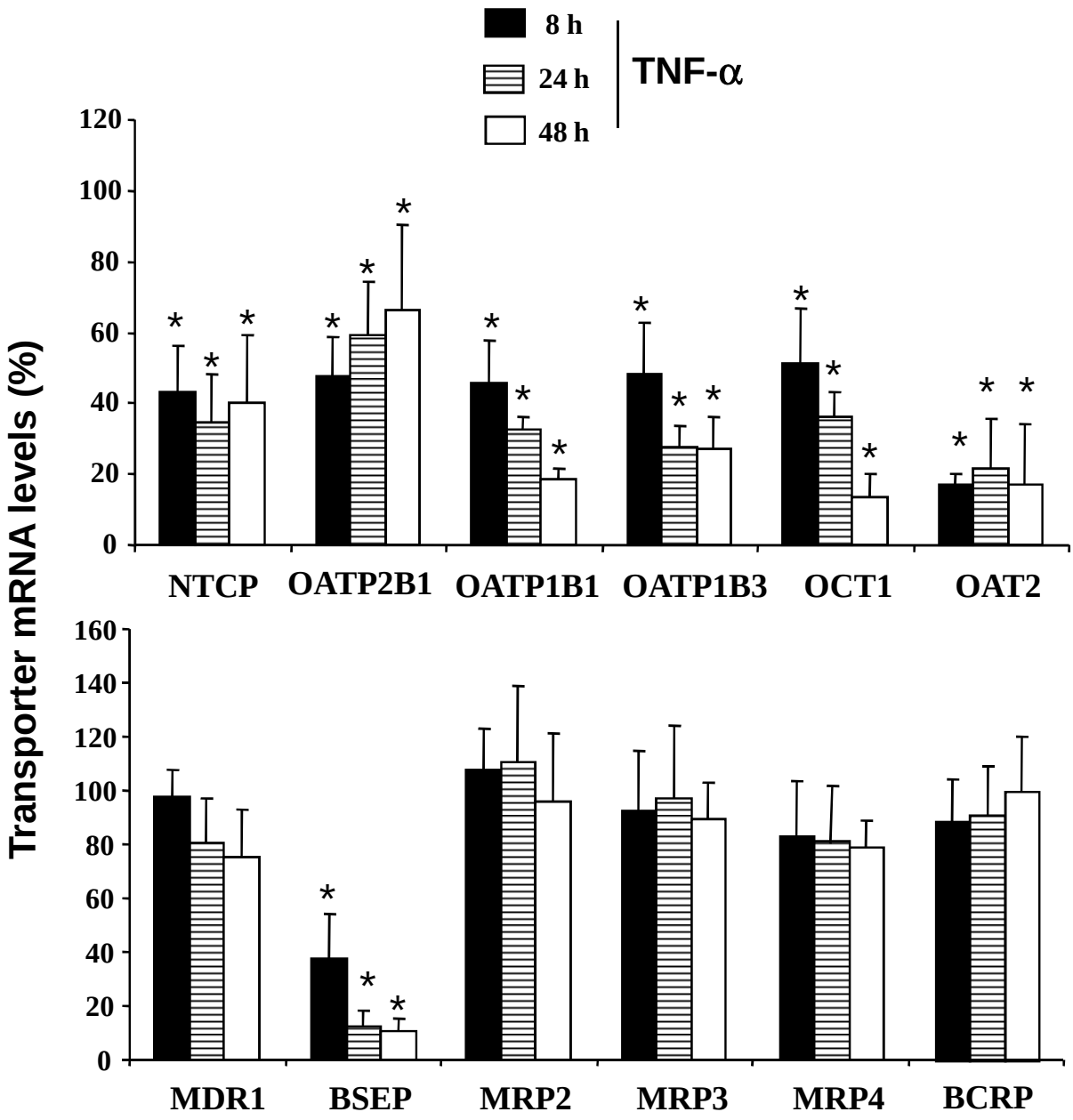


Figure 1

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

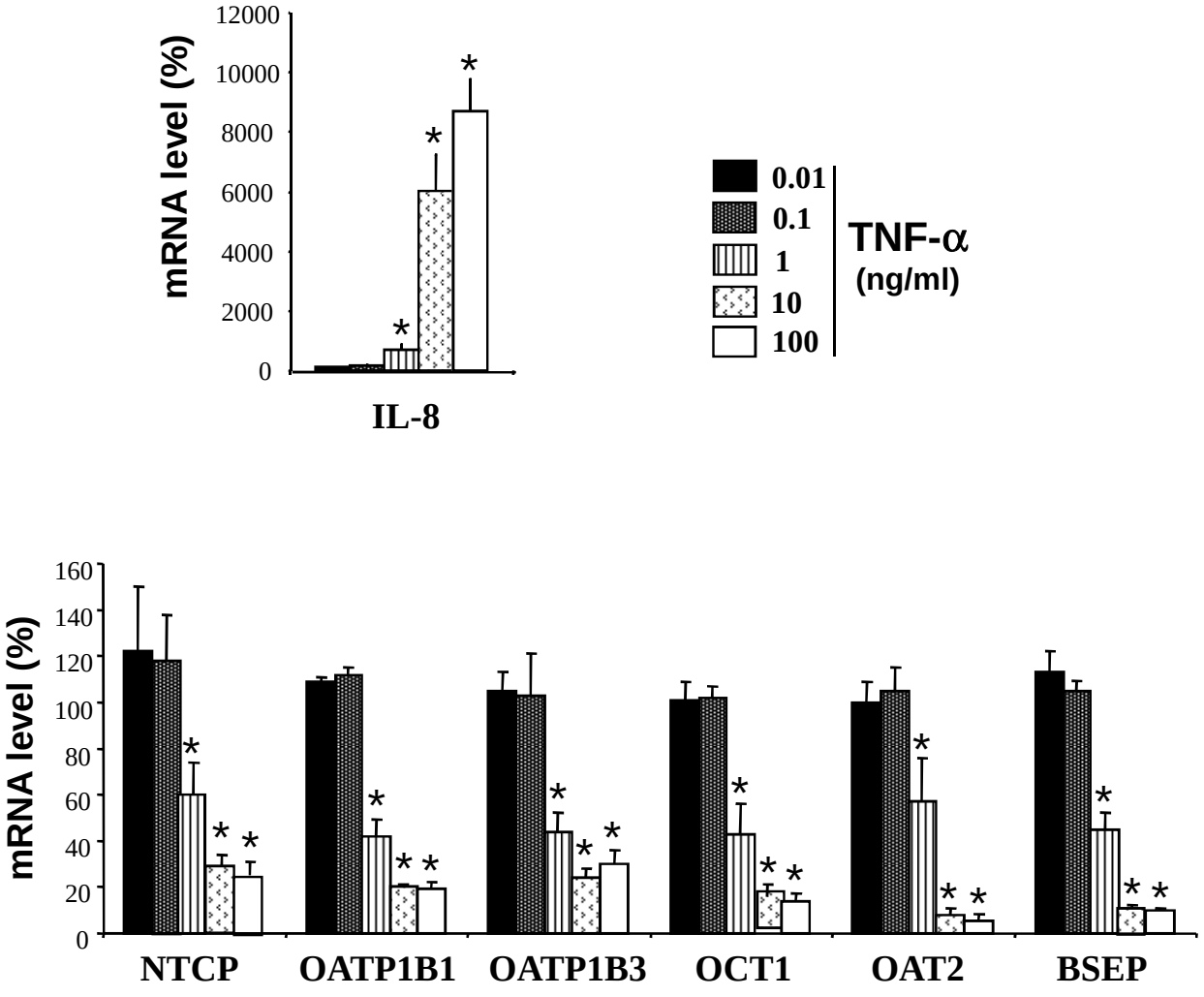


Figure 2

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

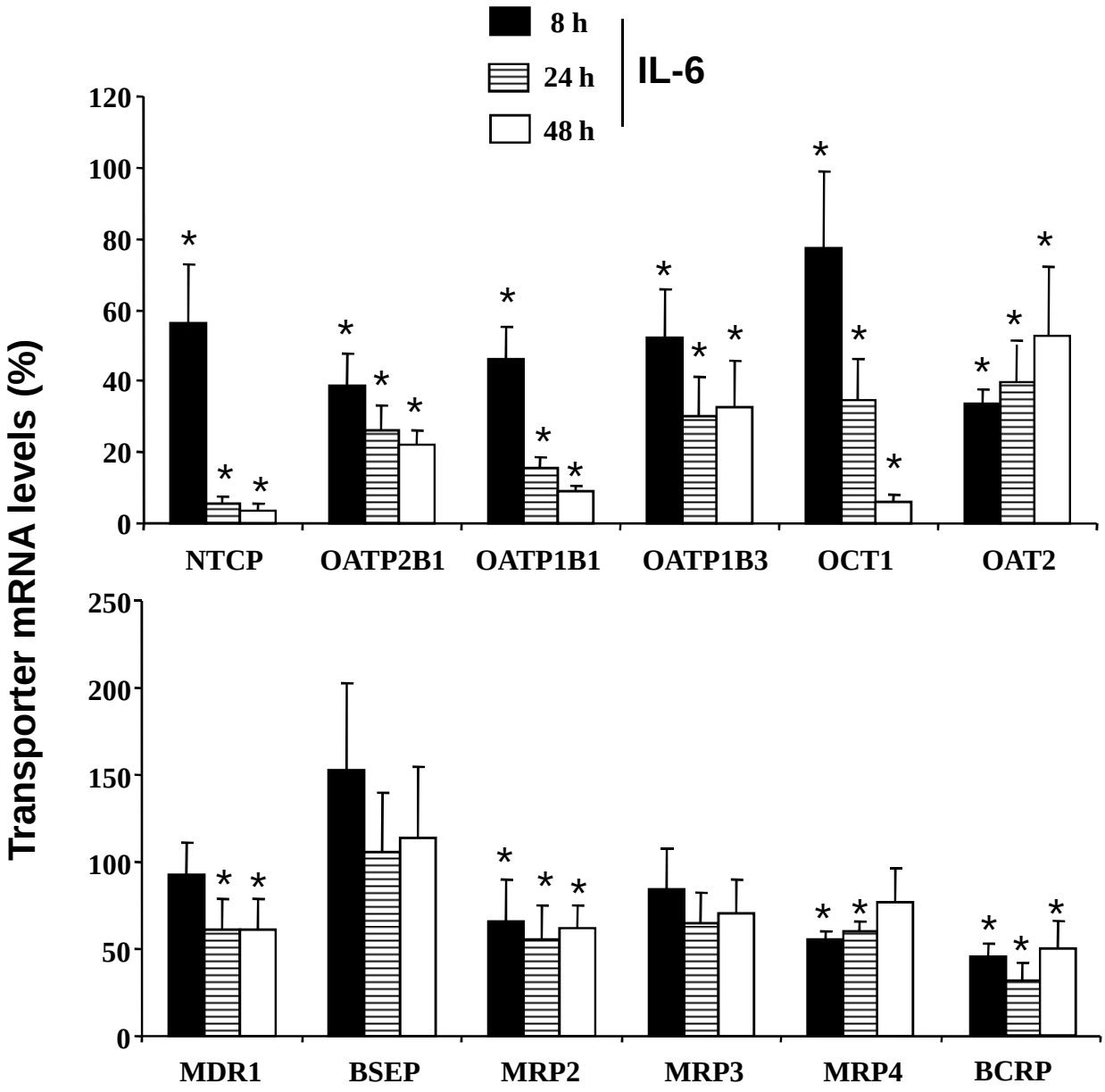


Figure 3

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

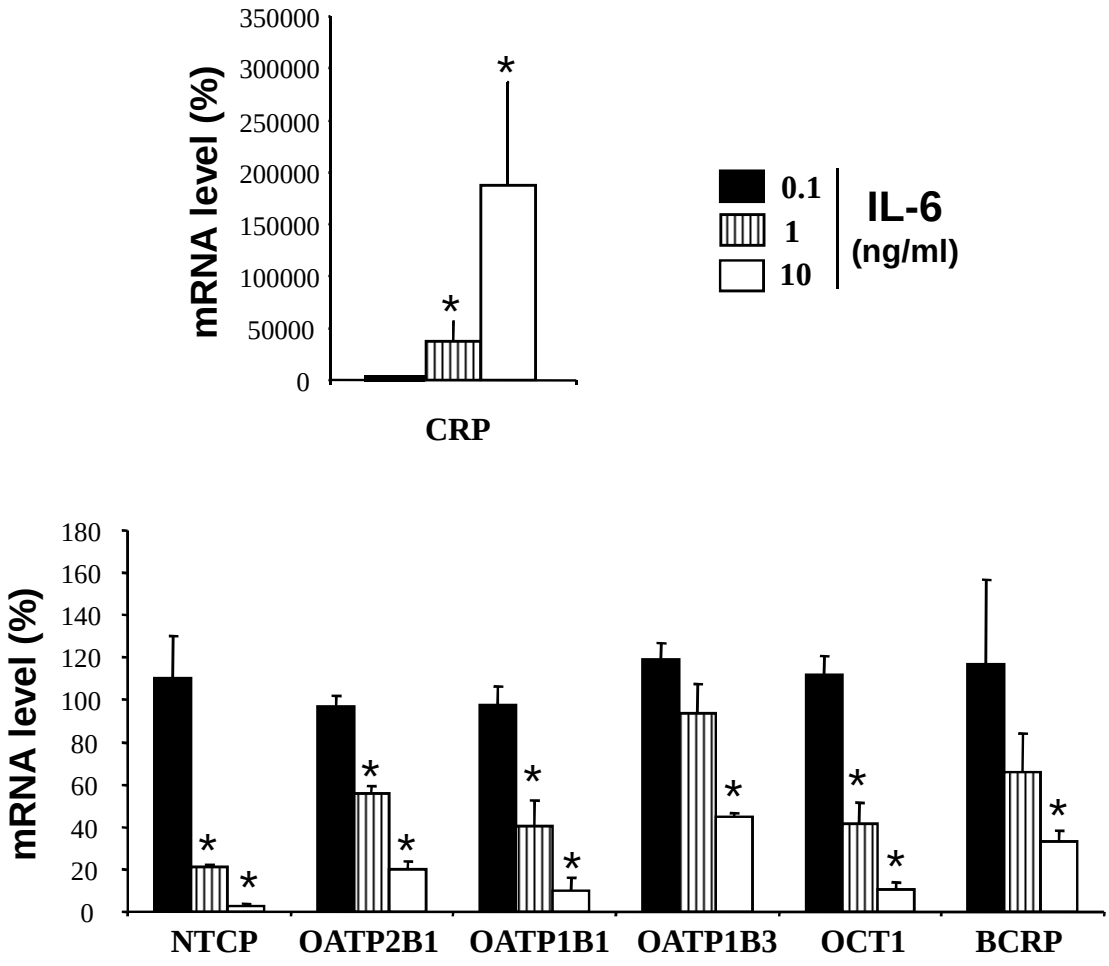


Figure 4

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

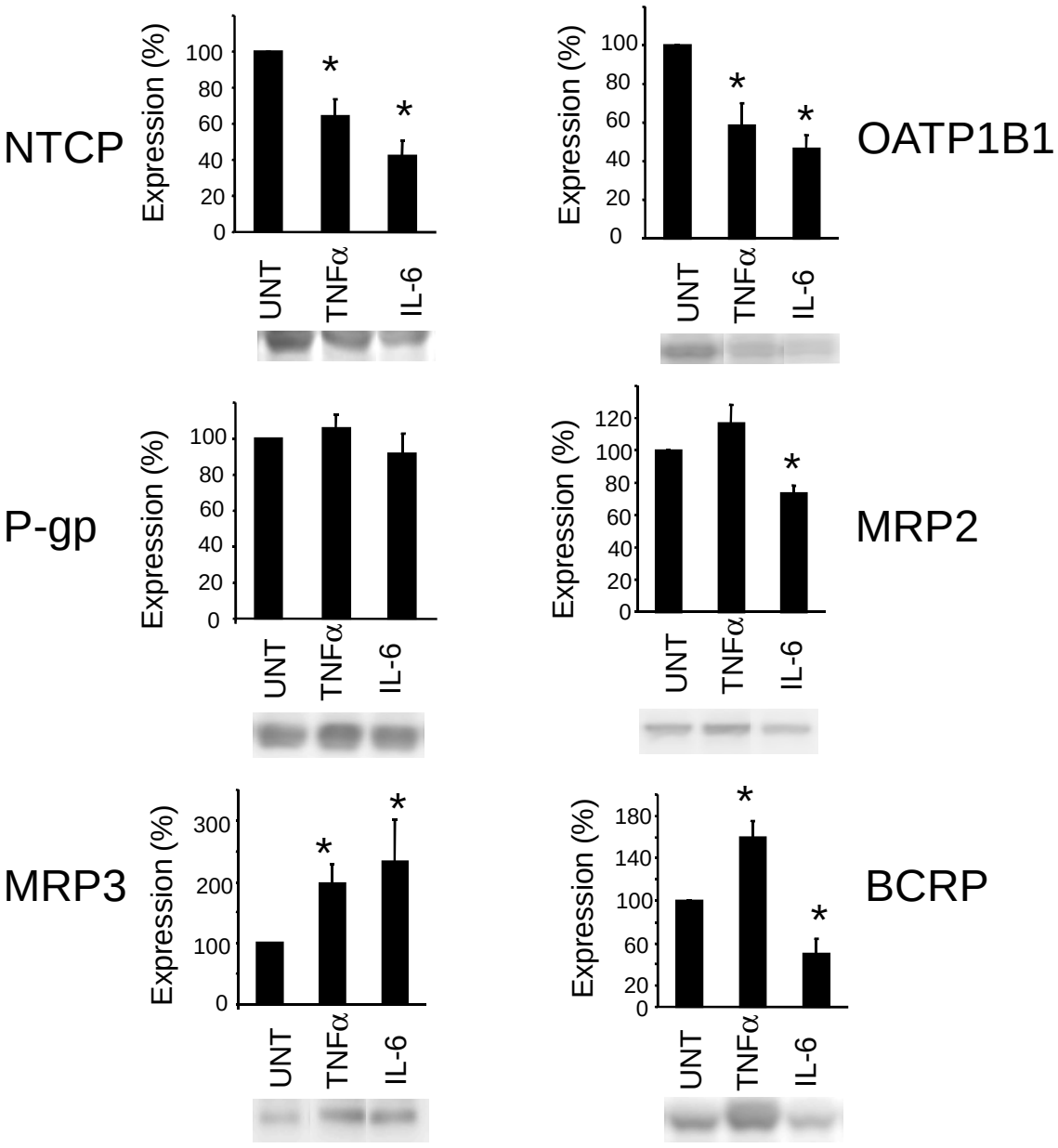


Figure 5

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

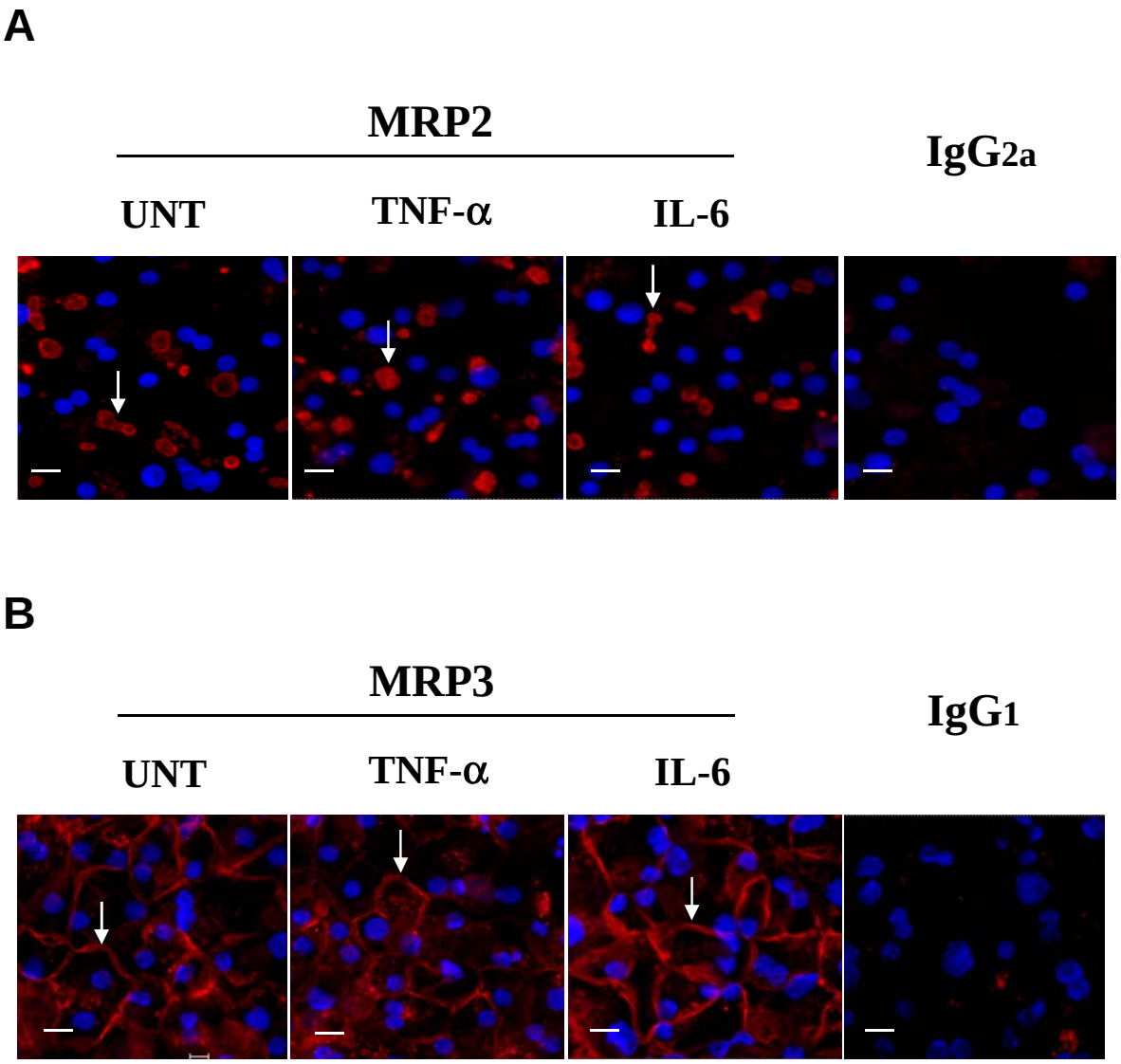


Figure 6

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.

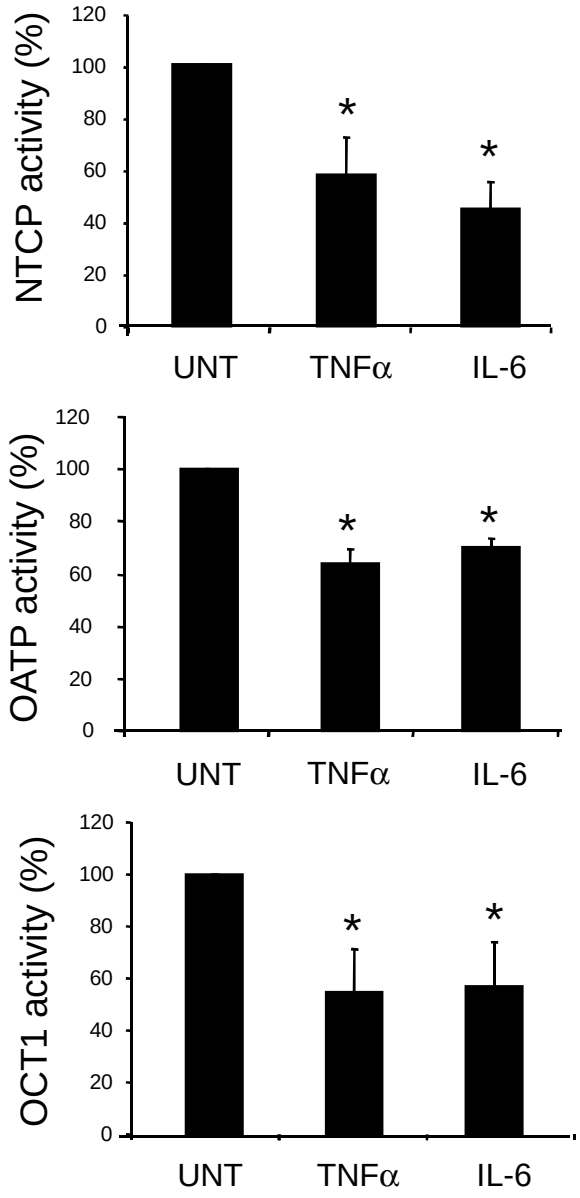
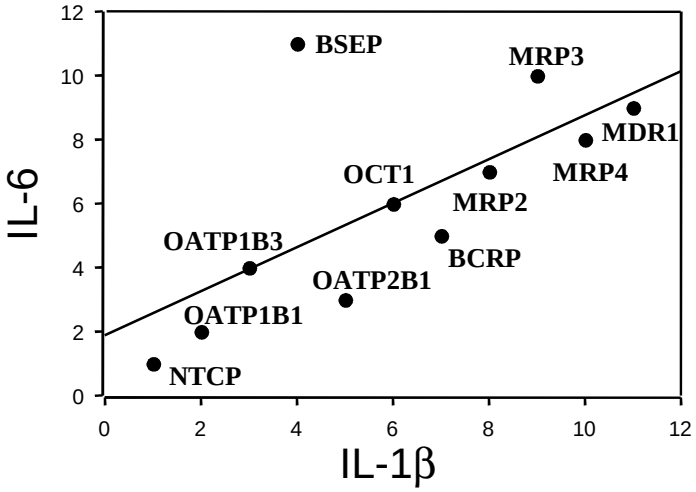
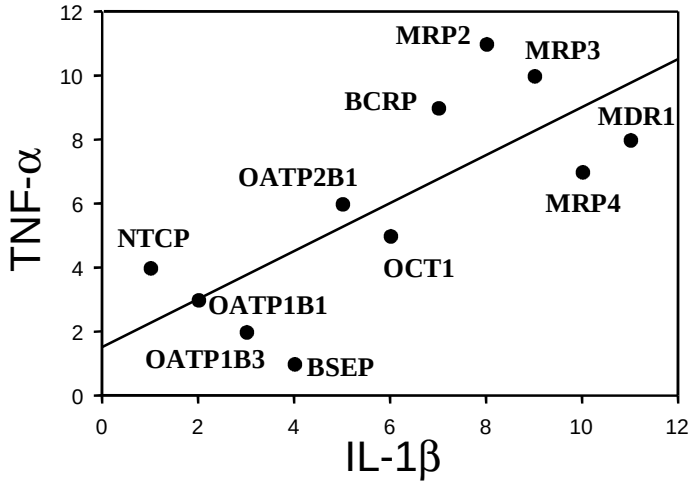


Figure 7

DMD Fast Forward. Published on December 15, 2008 as DOI: 10.1124/dmd.108.023630
This article has not been copyedited and formatted. The final version may differ from this version.





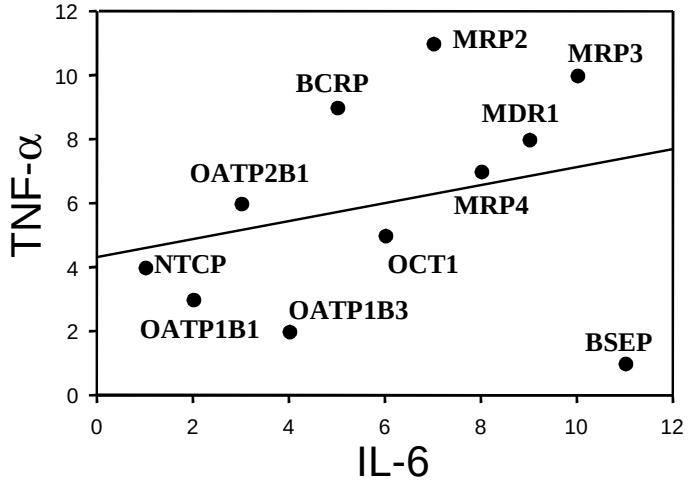


Figure 8