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Establishment of a Hepatocyte-Kupffer Cell Co-Culture Model for Assessment of Proinflammatory Cytokine Effects on Metabolizing Enzymes and Drug Transporters

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ABBREVIATIONS: AAG, alpha-1-acid glycoprotein; IL-1RA , IL-1 receptor antagonist cytokine; CRP, C-reactive protein; DDI, drug-drug interactions; KC, Kupffer cells; Hep:KC, hepatocyte-Kupffer cell; HCM, HepatoPac culture medium; IL-1R1 , IL-1 receptor, type 1; IL-2RB, IL-2 receptor, beta chain; IL-6R, IL-6 receptor, IL-23R, IL-23 receptor; LPS, lipopolysaccharide; MPCC, micropatterned co-culture; SAA2, serum amyloid A2; TP, therapeutic protein

Abstract

Elevated levels of proinflammatory cytokines associated with infection and inflammation can modulate CYP450 enzymes leading to potential disease-drug interactions and altered small molecule drug disposition. We established a human derived hepatocyte-Kupffer cell (Hep:KC) co-culture model to assess the indirect cytokine impact on hepatocytes through stimulation of Kupffer cell-mediated cytokine release and compared this model to hepatocytes alone. Characterization of Hep:KC co-cultures showed an inflammation response following treatment with LPS and IL-6 (indicated by secretion of various cytokines). Additionally, IL-6 exposure up-regulated acute phase proteins (CRP, AAG and SAA2) and down-regulated CYP3A4. Compared to hepatocytes alone, Hep:KC co-cultures showed enhanced IL-1β-mediated effects but less impact from both IL-2 and IL-23. Hep:KC co-cultures treated with IL-1β exhibited a higher release of proinflammatory cytokines, an increased up-regulation of acute phase proteins and a larger extent of metabolic enzyme and transporter suppression. IC_{50} values for IL-1β-mediated CYP3A4 suppression were lower in Hep:KC co-cultures (98.0-144 pg/mL) compared to hepatocytes alone (IC_{50} >5000 pg/mL). CYP suppression was preventable by blocking IL-1β interaction with IL-1R1 using an antagonist cytokine or an anti-IL-1β antibody. Unlike IL-1β, IL-6-mediated effects were comparable between hepatocyte monocultures and Hep:KC co-cultures. IL-2 and IL-23 caused a negligible inflammation response and a minimal inhibition of CYP3A4. In both hepatocyte monocultures and

Hep:KC co-cultures, IL-2RB and IL-23R were undetectable while IL-6R and IL-1R1 were higher in Hep:KC co-cultures. In summary, compared to hepatocytes monocultures, the Hep:KC co-culture system is a more robust *in vitro* model for studying the impact of proinflammatory cytokines on metabolic enzymes.

INTRODUCTION

Therapeutic proteins (TPs) include monoclonal antibodies (mAbs), cytokines, peptides, growth factors, and enzymes. The increased number of TPs in clinical use has raised the question of whether there is a potential for drug-drug interactions (DDIs) with small molecule drugs. Although the mechanisms involved in the disposition of TPs and small molecules are fundamentally different (Lu et al., 2013), regulatory agencies, academics, and pharmaceutical companies have developed an interest in this area. Some TPs have been shown to cause a change in the exposure of small molecules (Mahmood and Green, 2007; Huang et al., 2010; Lee et al., 2010). For example, treatment of rheumatoid arthritis patients with a single 10 mg/kg dose of tocilizumab (an anti-IL-6 receptor mAb) resulted in a 43% reduction in plasma exposure of the CYP3A4 substrate simvastatin (Schmitt et al., 2011). This is most likely due to tocilizumab-mediated normalization of CYP suppression caused by IL-6. TP-DDI effects are not universal, as observed with denosumab inhibition of cytokine RANKL that resulted in no impact on CYP3A4 in women with osteoporosis (Jang et al., 2014).

In general, the focus for TP-DDIs has been on cytokines (e.g., IL-1, IL-6, and TNF-α) and cytokine modulators, as these are known to alter expression levels of various drug metabolizing enzymes and transporters in hepatocyte *in vitro* systems (Aitken et al., 2006; Fardel and Le Vee, 2009; Nguyen et al., 2013).

Cytokines have differential effects on CYP enzymes suggesting the involvement of diverse mechanisms in P450 down-regulation (Gu et al., 2006; Urquhart et al., 2007; Lee et al., 2009; Harvey et al., 2014).

In vitro systems most commonly used for studying effects of TPs on CYP450 enzymes or drug transporters are fresh or cryopreserved primary human hepatocytes (Dickmann et al., 2011; Dallas et al., 2012). Cultured hepatocytes are useful for mechanistic studies related to the impact of a cytokine on gene expression and activity, but there are inherent limitations (Kenny et al., 2013; Evers et al., 2013): (i) Hepatocytes do not reflect the complex interactions between multiple cytokines and cell types that play a role in vivo in inflammatory diseases; (ii) High levels of inter-laboratory and inter-donor variability in cultured hepatocytes make quantitative interpretation of results challenging; and (iii) Hepatocyte mono-cultures are relevant only for studying cytokines with receptors directly expressed on hepatocytes. Based on these limitations, applying *in vitro* data to predict cytokine-mediated DDIs associated with anti-inflammatory TPs is challenging and currently not recommended (Evers et al., 2013).

To address the limitations inherent in hepatocyte monoculture systems, attempts have been made to establish co-culture models by incorporating components of the innate immune system such as Kupffer cells (KC). Sunman et al. (2004) demonstrated in a co-culture system that KC presence caused sustained IL-2 suppression of CYP3A activity in hepatocytes (for 72 hr) compared to 48 hr

suppression observed in hepatocyte monocultures. Conversely, KC did not alter CYP3A activity duration following treatment with IL-1 and IL-6 (positive proinflammatory cytokine controls). Similarly, in co-cultures of parenchymal and non-parenchymal liver cells, Chen et al. (2011) demonstrated the down-regulation of various P450 isoforms and transporters and up-regulation for others. Overall, however, the experience with co-culture systems has been limited and reproducing these observations has been challenging. Attempts at developing standard co-cultures with primary human hepatocytes and KC are limited by the rapid hepatic phenotypic decline. Consequently, the feasibility of standard co-cultures for investigating long-term effects of KC secretions on CYP450 enzymes and other pathways is restricted. In this report, we have established and characterized a long term Hep:KC co-culture model. The model was achieved by incorporating KC to a previously established system of hepatocytes cultured on micropatterned collagen domains of empirically optimized dimensions and supported by mouse 3T3-J2 fibroblasts (Khetani and Bhatia, 2008). The Hep:KC co-culture system provided more stability and allowed the hepatocytes to retain functionality for several weeks, thus enabling the performance of long term studies. In the Hep:KC co-culture system, we demonstrate differential effects on metabolic enzymes and ADME genes by cytokines for which receptors are (i.e., IL-6, IL-1β) or are not (i.e., IL-2, and IL-23) expressed on hepatocytes. We also propose ways in which data generated in this system can be used in assessing risk for TP-DDIs.

MATERIALS AND METHODS

Cell source/ donor demographics

The cryopreserved hepatocytes used in creating the co-cultures were purchased from Celsis (Baltimore, MD) and came from a 31 year old male who died of anoxia (secondary to heroin overdose) and who had a history of marijuana and heroin use. The cryopreserved KC were purchased from Life Technologies and came from a 51 year old male Caucasian who died of blunt injury to the head and was known to have smoked marijuana daily for 36 years. The 3T3-J2 fibroblasts were originally obtained from Howard Green at Harvard University (Rheinwald and Green, 1975). These and additional hepatocyte and KC donors were also used in preliminary assessments. A summary of donor demographics can be found in Supplemental Table 1.

Preparation of human hepatocyte-Kupffer cell co-cultures

Cryopreserved primary human hepatocytes and cryopreserved 3T3-J2 murine embryonic fibroblasts were used in the manufacturing of the micropatterned hepatocyte and fibroblast co-cultures (HepatoPac™, Hepregen Corporation, Medford, MA) in a 96-well format. Briefly, cryopreserved hepatocyte vials were thawed at 37°C for 120 seconds followed by dilution with 50 mL of pre-warmed HepatoPac culture medium (HCM) as described previously (Khetani et al. 2013; Khetani and Bhatia, 2008). The cell suspension was spun at 50x*g* for 5 min. The supernatant was discarded, cells were re-suspended in HCM, and viability was

assessed using trypan blue exclusion (typically 80–95%). Liver-derived nonparenchymal cells, as judged by their size (~10µm in diameter) and morphology (non-polygonal), were consistently found to be less than 1% in these preparations. To create the micropatterned co-culture (MPCC) in 96-well plates, a hepatocyte pattern was first produced by seeding hepatocytes on rat-tail collagen (Corning Biosciences) type I-patterned substrates that mediate selective cell adhesion. The cells were washed with medium 4-6 hr later to remove unattached cells (leaving ~5,000 attached hepatocytes on ~14 collagen-coated islands within each well of a 96-well plate) and incubated in HCM. The 3T3-J2 murine embryonic fibroblasts (up to passage 12) were seeded 18-24 hr later to create co-cultures. Culture medium was replaced every 2 days (~64 µL per well of a 96-well plate.).

Following 7 days of MPCC stabilization, cryopreserved human KC from unmatched donors (Life Technologies, Carlsbad, CA) were thawed and seeded on top of MPCCs at Hep:KC ratios of 1:0 (control cultures without KC addition), 1:0.1 and 1:0.4. These ratios were chosen based upon previous co-culture studies by Sunman, et al. (2004) which approximately represented the ratio of hepatocytes to KCs observed in human liver during the normal physiological (1:0.1) and inflamed state (1:0.4). Unmatched hepatocyte and KC donors were used based upon preliminary assessment showing no obvious deleterious effects to cell cultures when different donors were used for the two cell types (data not shown). Hepatocyte cultures alone and with unmatched KC showed comparable

albumin, urea, and ATP levels (Figure S1 to S5). Cell cultures used within these studies incorporated human MPCC (human hepatocytes plus mouse fibroblast supporting cells) with and without KC. For simplicity, MPCC cultures containing KC are referred to as Hep:KC co-cultures (although technically they are tri-cultures).

Preliminary characterization of Hep:KC co-cultures

Preliminary evaluation of Hep:KC cultures were performed by assessment of hepatocyte metabolic competency in the presence of KC from unmatched donors. KC functions in the co-cultures were also evaluated after 24 hr LPS (lipopolysaccharide) treatment (50 ng/mL; Sigma Aldrich, St. Louis, MO). At the completion of incubation, culture media samples were collected and analyzed for proinflammatory cytokines. Further characterization was performed by treating Hep:KC cultures with cytokines (IL-1β, IL-2, IL-6 and IL-23 [>97% purity by SDS-PAGE]; 0-200 ng/mL; R&D Systems, Minneapolis, MN) in serum-free dosing media. Treatments were repeated every other day (upon initiation of dosing) for 4 days. Concentrations of proinflammatory cytokines were chosen based upon preliminary studies considering toxicity (data not shown) and observed physiological cytokine levels in human plasma. Following stabilization of the co-cultures, dosing was initiated on Day 8 of culture. Supernatants were collected on Day 10 and 12 of culture (corresponding to 2 days post 1st cytokine addition and 2 days post 2nd cytokine addition, respectively) for bioanalyses of non-destructive endpoints (albumin secretion, urea synthesis, and CYP3A4 activity)

to give an indication of cell health. A 4-day cytokine incubation period was chosen based on preliminary studies showing robust effects on metabolic enzymes out to at least 6 days with peaked effects observed after 4 days of cytokine treatment. Sampling timepoints were chosen to cover these 4 days of cytokine treatment. After collection of supernatants on Day 12 of culture, Hep:KC cultures were washed once and incubated in HCM to recover in the absence of cytokine. Supernatants were collected at 6 days post cytokine removal (corresponding to Day 18 of culture) to assess hepatocyte metabolic competence (by measurement of albumin secretion, urea synthesis, and CYP3A4 activity) and cell lysates were evaluated for cytotoxicity (by measurement of cellular ATP) relative to untreated controls. Control Hep:KC cultures were treated with vehicle (0.1% DMSO).

A second set of Hep:KC cultures were also dosed with IL-1β, IL-2, IL-6 and IL-23 (0 – 200 ng/mL) in serum-free dosing media and supernatants were collected following 2 days post 1st cytokine dose (Day 10 of culture) and 2 days post 2nd cytokine dose (Day 12 of culture) and immediately frozen for assessment of secreted proinflammatory cytokines. Control supernatant samples were also collected on Day 8 of culture prior to dosing with cytokines. Cell lysates were obtained at the completion of cytokine treatment on Day 12 and mRNA was isolated for gene expression analyses.

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Estimation of the IC_{50} values for IL-1β and IL-6 inhibition of CYP3A4 activity in human hepatocyte-Kupffer cell co-cultures

The study contained three groups representing basal control (untreated), cytokine-treated, and recovery group. In the basal control group (N=3), on Day 8 of culture, supernatants were collected from all Hep:KC cultures (1:0, 1:0.1, and 1:0.4) maintained in HCM (without cytokines) for assessment of basal levels of albumin, urea, and CYP3A4 activities. Following supernatant collection, cell lysates were processed for evaluation of basal mRNA expression.

In the treatment group, on Day 8 of culture, Hep:KC cultures were dosed with either IL-1β or IL-6 (0.00625, 0.0125, 0.025, 0.050, 0.3125, 0.625, 1.25, 2.5 and 5.0 ng/ml) every other day in serum- free dosing media for a total of 2 doses over 4 days. At Day 10 (2 days post 1st cytokine addition) and Day 12 (2 days post 2nd cytokine addition) supernatants were collected (N=3) for bioanalyses of non-destructive endpoints (albumin secretion, urea synthesis, and CYP3A4 activity). Following supernatant collection on each day, cell lysates (N=3) were processed for evaluation of mRNA expression. Control cells in the treatment group were treated with vehicle (0.1% DMSO) and processed similarly to cytokine-treated cells.

For assessment of recovery from cytokine exposure, Hep:KC cultures were dosed with either IL-1β or IL-6 (0, 50, 5000 pg/mL) on Day 8 and 10 of culture. On Day 12 (following 4 days of cytokine exposure) dosing solutions were

removed and cells were washed 1x and incubated in HCM without cytokine to initiate recovery. Supernatants were collected (N=3) on Day 18 (6 days post recovery) and assayed for albumin secretion, urea synthesis, and CYP3A4 activity. Following supernatant collection, cell lysates (N=3) from Day 18 cultures were processed for mRNA expression analysis. Control cells in the recovery group were treated with vehicle (0.1% DMSO) and processed similarly to cytokine-treated cells.

Evaluation of the effects of anti-IL-1β antibody and IL-1 receptor antagonist on IL-1β-induced inhibition of CYP3A4 activity in human hepatocyte-Kupffer cell co-cultures

The study contained three groups representing untreated (basal control), treated (cytokine co-incubated with either IL-1RA [IL-1 receptor antagonist cytokine] or anti-IL-1β mAb) (R & D Systems) and recovery group. In the basal control group (N=3), on Day 8 of culture, supernatants were collected from all Hep:KC cultures (1:0, 1:0.1, and 1:0.4) maintained in HCM (without cytokines) for assessment of basal levels of albumin, urea, and CYP3A4 activities. Following supernatant collection, cell lysates were processed for evaluation of basal mRNA expression.

For the treatment group, on Day 8 of culture, Hep:KC co-cultures were treated with either anti-IL-1β mAb, IL-1RA or isotype control (mouse IgG1) (R&D Systems) for 2 hr prior to dosing with pro-inflammatory cytokines (either IL-1β or IL-6). Varying concentrations of anti-IL-1β mAb were used to pre-treat both

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Hep:KC 1:0 cultures containing no KC (10, 50, 100 ng/ml) and Hep:KC 1:0.4 cultures (1, 5, 10 ng/ml). Also, varying concentrations of IL-1RA (100, 500, 1000 ng/ml) were used to pre-treat both 1:0 and 1:0.4 Hep:KC cultures. For the isotype control group, all Hep:KC cultures were treated with 100 ng/ml of mouse IgG1. IL-1β study concentrations (2.5 and 0.1 ng/mL) were chosen to be approximately near the IC_{50} of IL-1β-mediated CYP3A4 inhibition in Hep:KC 1:0 and 1:0.4 cultures, respectively. Study concentrations of IL-1RA and anti-IL-1β mAb were selected based upon manufacture's recommendation and preliminary studies centered on reported ED_{50} for IL-1RA and $ND_{50\ of}$ anti-IL-1β mAb (Symons, et al. 1987). Subsequently, the pretreated Hep:KC cultures were co-dosed with varying IL-1β concentrations (0 and 2.5 ng/mL for Hep:KC 1:0 cultures [containing no KC] and 0 and 0.1 ng/ml for Hep:KC 1:0.4 cultures) every other day in serum-free dosing media for a total of 2 doses over 4 days. Similar pretreatment with anti-IL-1β mAb, IL-1RA, and mouse IgG1 and subsequent co-dose with IL-6 (0 and 0.5 ng/mL) were also performed in both Hep:KC 1:0 and 1:0.4 cultures. Co-dosing was also initiated on Day 8 and repeated every other day for a total of 2 doses over 4 days. Concentrations of IL-1β and IL-6 were chosen as approximate of previously determined IC_{50} values for IL-1β-mediated and IL-6-mediated CYP3A4 inhibition (IL-6 was included in this study as a control against nonspecific effects of IL-1RA and anti-IL-1β mAb). On Day 10 and 12 of culture, supernatants were collected (N=3) for bioanalyses of non-destructive endpoints (albumin secretion, urea synthesis, and CYP3A4 activity). Following supernatant collection, cell lysates (N=3) were collected from Day 12 cultures for

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mRNA evaluation. Control cells (those treated with IL-1RA, anti-IL-1β mAb, or mouse IgG1 but no pro-inflammatory cytokines) in the treatment groups were processed similarly to cytokine-treated cells.

Cell culture health and functionality assays

Cellular ATP levels in the cell lysates were measured using the CellTiter-Glo luminescent kits from Promega (Madison, WI) per manufacturer's instructions. In order to calculate hepatocyte- specific responses, cellular ATP levels were also determined in stromal-only cultures (i.e. murine 3T3-J2 fibroblasts). ATP signals in stromal-only control cultures were subtracted from those of Hep:KC cultures to obtain hepatocyte-specific effects.

Urea concentration was assayed using a colorimetric endpoint assay kit using diacetylmonoxime with acid and heat (Stanbio Labs, Boerne, TX).

Albumin content was measured using an enzyme-linked immunosorbent assay (MP Biomedicals, Santa Ana, CA) with horseradish peroxidase detection and 3,3',5,5'-tetramethylbenzidine (Fitzgerald Industries, Acton, MA) as a substrate (Khetani and Bhatia 2008).

CYP3A4 activity was measured by CYP3A4-Glo assay according to manufacturer's protocols (Promega).

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Proinflammatory cytokines released in cell culture media were measured by electrochemiluminescence detection using Human Pro-inflammatory 7-Plex Kit according to manufacturer's protocol (Meso Scale Discovery, Gaithersburg, MD).

Gene Expression Analysis

Cell lysate samples were processed and isolated for total RNA using a RNeasy kit (QIAGEN, Venlo, Limburg) according to manufacturer's protocol. To obtain cDNA, a two-step RT-PCR reaction was conducted by reverse-transcribing an aliquot of total RNA to cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). PCR reactions were then prepared by adding cDNA to a reaction mixture containing TaqMan 2x Universal PCR Master Mix (Applied Biosystems). Custom TaqMan Array microfluidic cards were custom-made by Applied Biosystems and contained probes in triplicate. Samples were applied to low-density microarrays by centrifugation twice for 1 min at 1200*g*. Real-time quantitative PCR was performed using an ABI PRISM 7900 Sequence Detector instrument and Sequence Detector 2.1 software (PerkinElmer Instruments, Shelton, CT). Quantitation of the target cDNA in all samples was normalized to 18S ribosomal RNA (Ct_{target} - Ct_{18S} = ΔCt), and the difference in expression for each target cDNA in treated cultures relative to the amount in control cultures was calculated ($\Delta Ct_{\text{control}}$ -$\Delta Ct_{\text{treated}}$ =$\Delta\Delta Ct$). Fold changes in target gene expression were determined by taking 2 to the power of this number ($2^{-\Delta\Delta Ct}$).

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DATA ANALYSIS

All experiments were run in triplicates and the mean and standard deviation were calculated for each of the doses administered. All data were normalized to vehicle controls. IC_{50} values were determined by nonlinear regression analyses. IC50 values were determined by fitting the dose response curves to a two-parameter equation with variable slope (Equation 1)

$$Y = \frac{100}{1+10^{[LogIC50-X]*HillSlope}} \qquad \text{Equation 1}$$

where Y is the normalized response between 0% and 100% and X is the log (inhibitor concentration). All analyses were performed using GraphPad Prism 5.0 (San Diego, CA).

Statistical Analysis

Data are usually presented as mean ± SD. Statistical analysis was carried out using analysis of variance followed by Tukey's multiple comparison test. A value of $P < 0.05$ was considered statistically significant.

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RESULTS

Characterization of Hep:KC co-cultures

Initial characterization of Hep:KC co-cultures is summarized in Figure S1 to S5. Albumin and urea levels as well as CYP3A4 activity were sustained from Day 7 out to Day 15 (Supplemental Figure 1). Compared to Day 3, CYP3A4 activity increased by 10 to 16 fold between Day 7 and Day 15, the presence of KC had no impact. Following 4 days of cytokine treatment, no changes in ATP levels were observed indicating the absence of cytotoxicity (Supplemental Figure 2 to 5). At the same time, a slight decrease in albumin and urea levels were detected following IL-6 and IL-1β treatment while negligible effects were associated with IL-2 and IL-23. Correspondingly, dramatic down-regulation of CYP3A4 activity was linked to IL-6 and IL-1β treatment but not IL-2 or IL-23 (Figure S2 to S5). Following 6 days of recovery in the absence of cytokine treatment, albumin, urea, and CYP3A4 activity levels returned to levels comparable to untreated cultures (data not shown). In summary, Hep:KC co-cultures were sustainable for at least 2 weeks as evident by the robust detection of albumin, urea, and CYP3A4 activity. Incorporating unmatched hepatocyte and KC donors and treatment with various cytokines did not compromise cell culture viability.

Co-culture of human hepatocytes with Kupffer cells

Hepatocytes were cultured alone (1:0) and with KC at ratios of Hep:KC of 1:0.1 and 1:0.4 representing normal and disease states, respectively. Expression of

the CD163 cell surface marker is reported to be highly restricted to monocytes and macrophages and in liver tissues it has been predominantly observed in KC (Van Gorp, et al., 2010). RT-PCR analysis of Hep:KC cultures showed increasing CD163 expression with increasing Hep:KC ratios (1:0.1 and 1:0.4) compared to hepatocytes alone (1:0), and expression was sustained out to at least Day 15 in culture (Figure 1). These results confirmed that KC remained present in the co-culture model for the entire two-week study period.

Treatment of cell cultures with LPS (50 ng/mL for 24 hr) resulted in substantially higher release of proinflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, TNF-α, and IFN-γ) into culture media compared to untreated cells (Figure 2A-E). Furthermore, the levels of cytokine measured in culture media appeared to be related to the amount of KC in the co-cultures (highest levels were observed with Hep:KC 1:0.4 ratio followed by 1:0.1 and 1:0, respectively). In the absence of LPS stimulation, very low or even undetectable levels of cytokines were observed in culture media. These observations were consistent with previously generated results in our laboratories (data not shown) from separate studies involving co-cultures using various combinations of two different hepatocyte lots and six different KC lots. These studies produced similar effects whereby cytokine secretion (such as IL-6 and TNF-α) was detected following treatment with LPS, and higher KC ratio equated to higher cytokine levels measured in cell culture media. Together, the presence of the KC-specific CD163 cell surface

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marker expression and cytokine release data confirmed that KCs were both present and functional in the co-cultures during the study period.

IL-6 and IL-1β-mediated immune response in Hep:KC co-cultures

Following incubation with either IL-6 or IL-1β (0.05-200 ng/mL), Hep:KC (1:0, 1:0.1 and 1:0.4) culture media was sampled and measured for cytokine release. In general, following 4 days of treatment, the presence of IL-6 stimulated a release of proinflammatory cytokines (IL-8, TNF-α, and INF-γ) in a concentration-dependent manner (i.e. higher cytokine measurements corresponded with higher IL-6 exposure) (Figure 3A-C). For IL-8, higher levels measured in culture media also corresponded with a higher KC ratio. Similarly, exposure to IL-1β also caused a strong release of proinflammatory cytokines (Figure 3D-E). The response appeared to be related to both IL-1β concentrations (i.e. higher IL-1β exposure resulted in higher IFN-γ secretion) and KC ratios (i.e. higher Hep:KC ratios produced higher levels of IL-8, and TNF-α in culture media). These outcomes showed that the Hep:KC co-culture system was responsive to both IL-6 and IL-1β stimulation and that the presence of KC further enhanced this response.

IC_{50} of IL-1β-mediated inhibition of CYP3A4 activity in Hep:KC co-cultures

IC_{50} characterization of the IL-1β-mediated CYP3A4 suppression in Hep:KC cultures (1:0 vs. 1:0.4) was performed at cytokine concentrations (6.25 - 5000 pg/mL) lower than previously studied (50 – 200,000 pg/mL) due to observed

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>50% CYP3A4 suppression at 50 pg/mL (Figure S3). Following IL-1β treatment, a greater extent of CYP3A4 suppression was detected in Hep:KC (1:0.4) co-cultures (at most studied concentrations) compared to hepatocytes alone (Hep:KC of 1:0) (Figure 4A). Correspondingly, IC_{50} was reduced to 98.0 ± 11.9 pg/mL in Hep:KC 1:0.4 co-cultures following 4 days of treatment (compared to IC_{50} >5000 pg/mL observed in Hep:KC 1:0 cultures, assuming that IL-1β is capable of fully suppressing CYP3A4 activity). In contrast, incubation with IL-6 did not show similar IC_{50} shifts (Figure 4C) as values observed for IL-6-mediated CYP3A4 inhibition were not dependent on the presence of KC. Following 6 days of recovery in the absence of either IL-1β or IL-6 treatment, CYP3A4 activity returned to levels similar to untreated controls (Figure 4B and 4D, respectively).

The differences in observed IL-1β- and IL-6-mediated CYP3A4 down-regulation following cytokine treatment may be attributed to variation in levels of receptor expression on hepatocytes and KC (Figure 5A-B). For the IL-6 receptor (IL-6R), results from RT-PCR analysis indicated that the addition of KC to hepatocyte cultures only modestly increased mRNA levels (IL-6R expression in Hep:KC 1:0.1 and 1:0.4 co-cultures were 78%-135% and 88%-151% of 1:0 control cultures, respectively). In contrast, the presence of KC corresponded with a more significant increase in IL-1β receptor, type 1 (IL-1R1) measurements compared to cultures without KC (Hep:KC 1:0) (IL-1R1 mRNA levels in Hep:KC 1:0.1 and 1:0.4 co-cultures were 186%-263% and 284%-376% relative to 1:0 control cultures, respectively).

Specificity of IL-1β effects in Hep:KC co-cultures

The observed suppression of CYP3A4 following cytokine treatment discussed above is not believed to be a general effect seen with all cytokines. Rather, these observations are thought to be specific to only a limited number of cytokines (e.g. IL-1β). The proposed mechanism mediating CYP suppression is most likely a result of direct interaction with cell surface receptor such as IL-1R1. Blocking this interaction with either human interleukin-1 receptor antagonist cytokine (IL-1RA) or mouse anti-human IL-1β (anti-IL-1β) mAb prevented IL-1β-mediated CYP3A4 suppression (Figure 6). IL-1RA is a naturally occurring cytokine that binds to IL-1R type I and II and competitively inhibits its interaction with IL-1α and IL-1β (Gabay, et al. 1997). Conversely, anti-IL-1β mAb binds to the cytokine and prevents it from interacting with surface receptor IL-1R. In the current studies, hepatocyte cultures without KC (Hep:KC 1:0) were co-administered with 2.5 ng/mL of IL-1β (concentration at which ~50% of CYP3A4 suppression was observed) and either IL-1RA (100-1000 ng/mL) or anti-IL-1β mAb (10-100 ng/mL). Likewise, Hep:KC (1:0.4) co-cultures were co-administered with 0.1 ng/mL of IL-1β (~IC_{50} for CYP3A4 suppression in the co-culture) and either IL-1RA (100-1000 ng/mL) or anti-IL-1β mAb (1-10 ng/mL). Results of co-treatment of cell cultures with IL-1β and either IL-1RA or anti-IL-1β mAb are summarized in Figure 6.

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In Hep:KC 1:0 cultures, 4 days of treatment with IL-1β (2.5 ng/mL) with increasing IL-1RA concentrations resulted in normalized CYP 3A4 activity (63%, 87%, and 106% of controls corresponding with 100, 500, and 1000 ng/mL IL-1RA, respectively). The same was observed in Hep:KC (1:0.4) co-cultures following co-administration with both IL-1β (0.1 ng/mL) and IL-1RA (100-1000 ng/mL). Enzyme activity returned to levels (86%-97%) comparable to untreated controls (control cultures were those treated with IL-1RA but not IL-1β). Similarly, when IL-1β was co-incubated with anti-IL-1β mAb (10-100 ng/mL in Hep:KC 1:0 and 1-10 ng/mL in Hep:KC 1:0.4) CYP3A4 activity returned to levels comparable to untreated controls (91-104% and 81-101% of untreated controls in Hep:KC 1:0 and 1:0.4 cultures, respectively). Additionally, cell cultures were also co-treated with mouse IgG1 (100 ng/mL) and IL-1β to control for non-specific isotype effects related to anti-IL-1β mAb. Results showed no effects on IL-1β-mediated CYP3A4 suppression (Figure 6). Control cultures were those treated with mouse IgG1 but untreated with IL-1β. Similar studies with IL-6 (0.5 ng/mL) co-incubation with either IL-1RA or anti-IL-1β mAb did not show any effect on IL-6-associated CYP3A4 down-regulation (data not shown). In summary, results from co-treatment with IL-1RA, anti-IL-1β mAb, and IgG1 isotype suggest that CYP3A4 suppression was mediated through specific interactions of IL-1β with IL-1R1.

IL-2 and IL-23 effects on immune response and CYP3A4 activity in Hep:KC co-cultures

Following 2 and 4 days of treatment with either IL-2 or IL-23 (0.05-200 ng/mL) Hep:KC (1:0, 1:0.1 and 1:0.4) culture media samples were measured for cytokine release. Levels of IL-8, TNF-α, and INF-γ secreted in cell culture media following 4 days of cytokine treatment are summarized in Figure 7. Exposure of Hep:KC cultures to either IL-2 or IL-23 resulted in cytokine release of IL-8 , TNF-α and INF-γ at levels similar to untreated controls (Figure 7A-C). Thus, the release of these cytokines appeared to be less related to IL-2 and IL-23 treatment and more with the presence of KC. These results suggested that the presence of IL-2 and IL-23 was less relevant than IL-1β on stimulating proinflammatory cytokine release in Hep:KC cultures. Not surprisingly, CYP3A4 activity was also less affected by IL-2 and IL-23 treatment (all activity levels remained nearly above 50% relative to untreated controls) (Figure 8). It should be noted that although a modest down-regulation of CYP activity was seen in 1:0 and 1:0.1 Hep:KC cultures, this was not reproducible in 1:0.4 cultures. The minimal interaction of both IL-2 and IL-23 was likely attributed to a lack of cell surface receptor expression (IL-2RB [IL-2 receptor, beta chain] and IL-23R [IL-23 receptor]) on either hepatocytes or KC (Figure 5C and D).

Effects of cytokines on gene expression of metabolism enzymes, drug transporters and acute phase proteins

RT-PCR analysis was performed to evaluate effects of IL-1β on gene expression of various metabolic enzymes, transporters, and acute phase proteins. Results were considered up-regulated or down-regulated when mRNA levels were

≥150% or ≤50% of untreated control, respectively, and observations were concentration-dependent. While translating changes in gene expression to meaningful changes in enzyme activity is difficult, the arbitrary cut-off levels chosen were deemed sufficient to indicate the presence of a cytokine effect that might affect enzyme and transporter activity. Representative dose-response profiles of CYP3A4 and acute phase protein (C-reactive protein [CRP]) following IL-1β and IL-6 treatment are illustrated in Figure 9. A summary of gene expression data for other proteins are summarized in Tables 1, 2, and 3. Gene expression of various Phase I enzymes (CYP1A2, CYP2C8, CYP2C9, CYP3A4) and Phase II enzymes (GSTA1, GSTA2, UGT1A1, and UGT2B7) was generally down-regulated in the presence of IL-1β and IL-6 (Table 1). As illustrated in Figure 9A, a concentration-dependent down-regulation of CYP3A4 mRNA was observed which corresponded with increasing IL-1β exposure (6.25 - 5000 pg/mL). Additionally, Hep:KC (1:0.4) co-cultures produced a greater extent of mRNA suppression compared to cultures without KC (Hep:KC 1:0). Studies with IL-6 (6.25 – 5000 pg/mL) also produced a concentration-dependent down-regulation of CYP3A4 expression (Figure 9C), but showed no KC-dependent effects. Consistent with observed enzyme activity, cultures containing KC (Hep:KC 1:0.4) enhanced IL-1β-mediated gene suppression, but this was not the case for IL-6.

Analogous to metabolic enzymes, gene expression of drug transporters was also down-regulated by the presence of both IL-1β and IL-6. Gene expression results

for various influx and efflux transporters are summarized in Table 2. Following cytokine treatments (6.25 - 5000 pg/mL), similar IL-1β- and IL-6-mediated down-regulation of expression was observed for NTCP, OATP1B3, and OCT1 (mRNA levels were ≤ 50% of untreated control and effects were concentration-dependent). The extent of IL-1β-mediated suppression of these transporters was generally greater in the presence of KC (Hep:KC 1:0.4 co-cultures) than in hepatocytes alone (Hep:KC 1:0 cultures). No KC-dependent effects were observed following IL-6 treatment. Additionally, OATP1B1 and OATP2B1 expression were also affected by IL-1β treatment and the presence of KC further enhanced the impact on OATP1B1.

Treatment with the cytokines IL-1β and IL-6 (6.25 - 5000 pg/mL) resulted in an up-regulation of CRP mRNA expression, indicative of an inflammation response. At the highest concentration studied (5000 pg/mL IL-1β), CRP mRNA levels reached 1133 ± 260% and 53132 ± 15568% of untreated controls in Hep:KC 1:0 and 1:0.4 cultures, respectively (Figure 9B, Table 3). Similar to effects on metabolic enzymes and drug transporters, the presence of KC also enhanced IL-1β-mediated CRP up-regulation. At the highest IL-6 concentration studied (5000 pg/mL) CRP mRNA levels reached 95220 ± 12671% and 80426 ± 25395% of untreated controls in Hep:KC 1:0 and 1:0.4 cultures, respectively (Figure 9D). With IL-6, no obvious differences in effects were seen in the presence and absence of KC.

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In addition to CRP, the gene expression of acute phase proteins alpha-1-acid glycoprotein (AAG) and serum amyloid A2 (SAA2) protein were also evaluated. Incubation of Hep:KC 1:0 and 1:0.4 cultures with IL-1β (6.25 – 5000 pg/mL) also resulted in up-regulation of AAG (effects were enhanced in the presence of KC) and SAA2 (Table 3). Similar treatment with IL-6 (6.25 – 5000 pg/mL) also led to up-regulation of AAG and SAA2 mRNA levels. No differences in effects were detected in cultures with and without KC, however (Table 3).

DISCUSSION

The aim of this work was to establish a viable, functional and long term human Hep:KC co-culture model that is more physiological relevant than hepatocyte monocultures for studying the effects of cytokines and cytokine modulators on drug metabolism enzymes and transporters. The utility of standard co-cultures with human hepatocytes and KC are limited by a short working window (~3 days) and a rapid decline of hepatic functions. The micropatterned hepatocyte system previously established by Khetani and Bhatia (2008) offers flexibility in the timing of KC introduction and experimental design. In this study, KC were incorporated into the micropatterned hepatocyte platform to produce a Hep:KC co-culture model that provided a longer experimental window (~2 weeks) compared to conventional *in vitro* models. The contribution of 3T3 fibroblasts helped to stabilize hepatocyte functions within the system (although their secretory products cannot be formally excluded from having any direct influence on cytokine-mediated responses reported here). Analysis of appropriate hepatocyte-fibroblast control cultures suggested that excretion of different cytokines in the absence of KC was consistent with what would be expected based on literature data. Based on findings that Hep:KC co-cultures were more responsive to LPS and IL-1β compared to hepatocyte monocultures, we feel that this system is a first step towards a more physiologically relevant *in vitro* model to study the effects of cytokines and cytokine modulators on drug metabolism enzymes and transporters.

Characterization of the Hep:KC co-culture model showed sustained KC presence for at least two weeks. During this period, the co-cultures maintained: (i) Their metabolic capacity as indicated by continued production of albumin and urea; (ii) Functionality as demonstrated by sustained CYP3A4 activity; (iii) Capacity to secrete proinflammatory cytokines in response to LPS exposure; and (iv) Reversibility of the effects on CYP3A4 down-regulation after withdrawal of IL-6 or IL-1β. Furthermore, we demonstrated that IL-1β-mediated cytokine release was KC-dependent, whereas IL-6 stimulated cytokine release was not. Additionally, weak cytokine response was observed for IL-2 and IL-23, most likely due to minimal or undetectable IL-2R or IL-23R on either hepatocytes or KC.

The extent of CYP3A4 activity down-regulation observed in Hep:KC cultures by IL-6 (61-97%) was comparable to those reported in numerous studies using human hepatocyte monocultures (20-80%) (Dickmann et al., 2011; Dallas et al., 2012; Nguyen et al., 2013). This indicates that the co-culture model is responding as expected to proinflammatory cytokine stimulation. In this system, IL-1β produced predictable results such as stimulating an immune modulatory response (Figure 3) and down-regulating both CYP3A4 activity and gene expression (Figure 4). To our knowledge, this is the first time in which the incorporation of KC was demonstrated to enhance IL-1β effects (compared to hepatocytes alone). In essence, the addition of another component of the liver to the conventional hepatocyte monoculture model allowed the co-culture system to

more fully capture the impact of IL-1β. As evident, the IC_{50} value for CYP3A4 inhibition in Hep:KC 1:0.4 decreased by greater than 51-fold compared to 1:0 cultures (Figure 4) following IL-1β treatment.

Another advantage of the Hep:KC co-culture model over conventional hepatocyte monocultures is the increased sensitivity to cytokine stimulation thus allowing *in vitro* studies to be performed at physiologically relevant cytokine concentrations. Reported IC_{50} values of IL-1β- and IL-6-mediated CYP inhibition (Figure 4) were within the physiological range of serum IL-1β and IL-6 in patients experiencing inflammation-related diseases. In humans, many proinflammatory cytokines (i.e. IL-1β and IL-6) circulate under normal conditions in the picomolar range and may increase by as much as 1000 fold during infection or trauma (Cannon, 2000). Earlier *in vitro* studies that employed primary hepatocyte monocultures to assess cytokine-CYP450 enzyme interactions often required much higher concentrations of IL-1 and IL-6 (in the ng/mL range) to demonstrate an effect (Sunman et al., 2004; Aitken and Morgan, 2007). In contrast, the IC_{50} values of CYP3A4 inhibition in the Hep:KC system can be observed in the pg/mL range (Figure 4). It is conceivable that the sensitivity of the Hep:KC co-culture model is likely attributed to the KC-mediated release of additional proinflammatory cytokines that can interact with the hepatocytes.

In addition to CYP3A4, effects were also note for other metabolism enzymes and transporters. In general, down-regulation of gene expression associated with

various Phase I and II metabolism enzymes and drug transporters was observed following IL-1β and IL-6 treatment. This is consistent with observations reported in the literature (Nguyen et al., 2013). Gene suppression trends appeared more pronounced in the presence of KC following IL-1β treatment but not IL-6 (Table 1-3), suggesting that the addition of KC increased the capacity of Hep:KC co-cultures to better capture the differential in cytokine impact. For CYP3A4, gene expression down-regulation (Figure 9) corresponded with a decrease in enzyme activity (Figure 4). This is consistent with the previous observations of a simultaneous decrease in activity and expression in the same study using IL-1β (Abdel-Razzak et al., 1993), and IL-6 (Dickmann et al., 2011). It is currently difficult to predict whether these results will lead to important clinical implication due to a lack of clinical TP-DDI data for marketed biologics related to IL-1β (e.g. canakinumab and anakinra). As a result, additional clinical TP-DDI data and more standardized *in vitro* cell culture models across labs are needed before IVIVC can be explored.

Application of the Hep:KC co-culture model to study IL-2 produced conflicting results to observations made by Sunman et al. (2004). Using conventional hepatocytes and KC at similar Hep:KC 1:01 and 1:0.4 ratios, the authors reported sustained IL-2-mediated CYP3A suppression. This discrepancy may be attributed to inter-lab variability associated with differences in experimental conditions, donors used, and/or purity of the hepatocyte/KC cell populations. The negligible impact of IL-2 on CYP3A4 in our co-culture system is consistent with

observed minimal IL-2RB expression which would limit IL-2 interactions leading to diminished cytokine release following treatment. Others have also reported difficulty in measuring IL-2R expression in normal liver compared to those infected with hepatitis C (Morshed et al., 1993).

Elevated IL-23 levels are often associated with psoriasis and rheumatoid arthritis (El-Hadid et al, 2008; Melis et al, 2010). Dallas et al. (2012) previously showed that IL-23 had little impact on CYP3A4 in hepatocyte monocultures. Similar results were confirmed in the current Hep:KC co-culture model whereby IL-23 treatment generated only low levels of CYP3A4 suppression. Together with negligible cell surface receptor expression of IL-23R and minimal stimulation of proinflammatory cytokine release, it can be argued that cytokines with such characteristics may pose less DDI risk with small molecule drugs. However, *in vitro* data should not be interpreted in isolation, but rather in conjunction with clinical evidence to provide a better assessment of TP-DDI risks (i.e. are elevated pro-inflammatory cytokine and CRP levels associated with the disease state) (Evers et al., 2013).

In summary, the current opinion from regulatory agencies is that conventional *in vitro* systems are not yet fully established to provide meaningful prediction of clinical TP-DDI (Kenny et al., 2013; Evers et al., 2013). However, to advance this area of research, the FDA encourages continued research effort and data gathering. Towards this objective, the Hep:KC co-culture system discussed here

offers a potentially improved *in vitro* system compared to conventional hepatocyte monocultures. The Hep:KC system is arguably more physiologically relevant as it incorporates an additional innate immune component (a closer resemblance of the liver than hepatocyte monocultures). This immune component plays an important role in mediating the effects of therapeutic proteins (i.e. cytokines and mAb) on metabolism enzymes and drug transporters. As a result, the more holistic Hep:KC system has the potential to generate a complex network of cross-talk between cytokines and inflammatory mediators, thus making it more sensitive to stimulation by exogenous cytokines. Consequently, *in vitro* studies in Hep:KC co-cultures can be performed at lower, more clinically relevant cytokine concentrations and its effects can be more fully captured (as in the case of IL-1β). Furthermore, the Hep:KC co-cultures can potentially mediate interactions from a broader spectrum of cytokines (i.e. those that lack receptors on hepatocytes but can still indirectly interact through stimulation of KC). While more research is needed to explore the full potential and limitations of this co-culture model, the work presented here demonstrated its value as a tool for providing further understanding into the mechanisms by which cytokines (i.e. IL-1β) interact with hepatocytes and KC to impact metabolic enzymes and drug transporters.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Nguyen, Ukairo, Khetani, McVay, Evers

Conducted experiments: McVay, Kanchagar

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Wrote or contributed to the writing of the manuscript: Nguyen, Ukairo, Khetani, Irrechukwu, Evers

REFERENCES

Abdel-Razzak Z, Loyer P, Fautrel A, Gautier JC, Corcos L, Turlin B, Beaune P, and Guillouzo A (1993) Cytokines down-regulate expression of major cytochrome P-450 enzymes in adult human hepatocytes in primary culture. *Mol Pharmacol* **44**:707-715.

Aitken AE, Richardson TA, and Morgan ET (2006) Regulation of drug metabolizing enzymes and transporters in inflammation. *Annu Rev Pharmacol Toxicol* **46**:123-149.

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.

Cannon JG (2000) Inflammatory Cytokines in nonpathological states. *News Physiol Sci* **15**:298-303.

Chen C, Han YH, Yang Z, and Rodrigues AD (2011) Effect of interferon-α2b on the expression of various drug-metabolizing enzymes and transporters in co-cultures of freshly prepared human primary hepatocytes. *Xenobiotica* **41(6)**:476-485.

Dallas S, Sensenhauser C, Batheja A, Singer M, Markowska M, Zakszewski C, Mamidi R, McMillian M, Han C, Zhou H, and Silva J (2012) De-risking bio-therapeutics for possible drug interactions using cryopreserved human hepatocytes. *Curr Drug Metab* **13**:923-929.

Dickmann LJ, Patel SK, Rock DA, Wienkers LC, and Slatter JG (2011) Effects of interleukin-6 (IL-6) and anti-IL-6 monoclonal antibody on drug-metabolizing enzymes in human hepatocyte culture. *Drug Metab Dispos* **39**:1415–1422.

El-Hadid H, Grace BDM , GheitaT, and Shaker O (2008) Involvement of IL-23 in psoriasis and psoriatic arthritis patients; possible role in pathogenesis. *J Egypt worn Dermatol Soc* **5**:70-75.

Evers R, Dallas S, Dickmann LJ, Fahmi OA, Kenny JR, Kraynov E, Nguyen T, Patel AH, Slatter JG, and Zhang L (2013) Critical review of preclinical approaches to investigate cytochrome P450-mediated therapeutic protein drug-drug interactions and recommendations for best practices: a white paper. *Drug Metab Dispos* **41(9):**1598-1609.

Fardel O and Le Vée M (2009) Regulation of human hepatic drug transporter expression by pro-inflammatory cytokines. *Expert Opin Drug Metab Toxicol* **5(12)**:1469-1481.

Gabay C, Smith MF, Eidlen D, and Arend WP (1997) Interleukin 1 receptor antagonist (IL-1Ra) is an acute-phase protein. *J Clin Invest* **99(12):** 2930–2940.

Gu X, Ke S, Liu D, Sheng T, Thomas PE, Rabson AB, Gallo MA, Xie W, and Tian Y (2006) Role of NF-κB in regulation of PXR-mediated gene expression: A mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. *J Biol Chem* **281(26)**:17882–17889.

Harvey RD, Morgan ET (2014) Cancer, inflammation, and therapy: Effects on cytochrome P450-mediated drug metabolism and implications for novel immunotherapeutic agents. Clin Pharmacol Ther 96(4):449-457.

Huang SM, Zhao H, Lee JI, Reynolds K, Zhang L, Temple R and Lesko LJ (2010) Therapeutic protein–drug interactions and implications for drug development. *Clin Pharmacol Ther* **87(4):**497-503.

Jang G, Kaufman A, Lee E, Hamilton L, Hutton S, Egbuna O and Padhi D (2014) A clinical therapeutic protein drug–drug interaction study: coadministration of denosumab and midazolam in postmenopausal women with osteoporosis. *Pharmacol Res Perspect* **2(2**):e00033.

Kenny JR, Liu MM, Chow AT, Earp JC, Evers R, Slatter JG, Wang DD, Zhang L, and Zhou H (2013) Therapeutic protein drug-drug interactions: navigating the

knowledge gaps-highlights from the 2012 AAPS NBC Roundtable and IQ Consortium/FDA workshop. *AAPS J* **15(4)**:933-940.

Khetani SR and Bhatia SN, (2008) Microscale culture of human liver cells for drug development. *Nat Biotechnol* **26**:120-126.

Khetani SR, Kanchagar C, Ukairo O, Krzyzewski S, Moore A, Shi J, Aoyama S, Aleo M, and Will Y (2013) Use of Micropatterned cocultures to detect compounds that cause drug-induced liver injury in humans. *Toxicol Sci* **132(1):**107–117.

Lee CM, Pohl J, and Morgan ET (2009) Dual mechanisms of CYP3A protein regulation by proinflammatory cytokine stimulation in primary hepatocyte cultures. *Drug Metab Dispos* **37**:865–872.

Lee JI, Zhang L, Men AY, Kenna LA, and Huang SM (2010) CYP-mediated therapeutic protein-drug interactions: Clinical findings, proposed mechanisms and regulatory implications. *Clin Pharmacokinet* **49(5)**:295-310.

Le Vee M, Lecureur V, Stieger B, and Fardel O (2009) Regulation of drug transporter expression in human hepatocytes exposed to the proinflammatory cytokines tumor necrosis factor-α or interleukin-6. *Drug Metab Dispos* **37**:685-693.

Lu D, Girish S, Theil FP, and Joshi A (2013) Pharmacokinetic and pharmacodynamic-based drug interactions for therapeutic proteins , in *Drug-Drug Interactions for Therapeutic Biologics* (Zhou H and Meibohm eds) pp 73-101, Wiley Publisher, New Jersey.

Mahmood I and Green MD (2007) Drug interaction studies of therapeutic proteins or monoclonal antibodies. *J Clin Pharmacol* **47**:1540-1554.

Melis L, Vandooren B, Kruithof E, Jacques P, De Vos M, Mielants H, Verbruggen G, De Keyser F, and Elewaut D (2010) Systemic levels of IL-23 are strongly associated with disease activity in rheumatoid arthritis but not spondyloarthritis. *Ann Rheum Dis* **69(3)**:618–623.

Morshed SA, Fukuma H, Kimura Y, Watanabe S, and Nishioka M (1993) Interferon-γ, interleukin (IL)-2 and IL-2 receptor expressions in hepatitis C virus-infected liver. *Gastroenterol Jpn* **28**(Suppl 5):59-66.

Nguyen T, Kishnani N and Evers (2013) Utility of *in vitro* methods in drug-drug interaction assessment and prediction for therapeutic biologics, in *Drug-Drug Interactions for Therapeutic Biologics* (Zhou H and Meibohm eds) pp 73-101, Wiley Publisher, New Jersey

Rheinwald JG and Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6(3):**331–343.

Schmitt C, Kuhn B, Zhang X, Kivitz AJ, and Grange S (2011) Disease-drug-drug interaction involving tocilizumab and simvastatin in patients with rheumatoid arthritis. *Clin Pharmacol Ther* **89(5)**:735-740.

Sunman JA, Hawke RL, LeCluyse EL, and Kashuba ADM (2004) Kupffer cell-mediated IL-2 suppression of CYP3A activity in hepatocytes. *Drug Metab Dispos* **32**:359-363.

Symons JA, Baron PW, and Rumsby MG (1987) in *Lymphokines and Interferons: A Practical Approach* (Clemens MJ, Morris AG, and Gearing AJH, eds) p272, IRL Press, Oxford.

Urquhart BL, Tirona RG, and Kim RB (2007) Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: Implications for Inter-individual variability in response to drugs. *J Clin Pharmacol* **47(5)**:566-578.

Van Gorp H, Delputte PL, and Nauwynck HJ (2010) Scavenger receptor CD163, a Jack-of-all-trades and potential target for cell-directed therapy. *Mol Immunol* **47:**1650–1660.

FOOTNOTE

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FIGURE LEGENDS:

Figure 1. Kupffer cell CD163 cell surface marker mRNA expression in Hep:KC cultures. Hepatocytes were cultured alone (in micropatterned co-cultures with 3T3-J2 fibroblasts) ± Kupffer cells (KC) (at Hep:KC of 1:0, 1:0.1 and 1:0.4) and RT-PCR analysis was performed on samples from Day 3, 7, 11, and 15 post-KC addition for mRNA expression of CD163. Results were relative to cultures without KCs (Hep:KC 1:0) (n = 3 ± SD). Mean values were significantly different from Hep:KC *1:0 and *#1:0.1 cultures (p-value <0.05).

Figure 2. Cytokine secretion in Hep:KC cultures following LPS treatment. Hepatocytes were cultured alone (in micropatterned co-cultures with 3T3-J2 fibroblasts) ± Kupffer cells (at Hep:KC of 1:0, 1:0.1 and 1:0.4). Secreted proinflammatory cytokines (IL-1β, IL6, IL-8, IL-10, TNF-α, and IFN-γ) were measured in cell culture media 24 hr following exposure to LPS (50 ng/mL). N = 3 ± SD. Mean values were significantly different from *LPS-untreated Hep:KC cultures of matched cell ratios and *#LPS-treated 1:0 cultures (p-value <0.05).

[a] Levels were undetectable.

Figure 3. Cytokine secretion in Hep:KC cultures following IL-6 and IL-1β treatment. Hepatocytes were cultured alone (in micropatterned co-cultures with 3T3-J2 fibroblasts) ± Kupffer cells (at Hep:KC of 1:0, 1:0.1 and 1:0.4). Secreted proinflammatory cytokines such as IL-8, TNF-α, and IFN-γ were measured in cell

culture media 4 days following exposure to IL-6 (A-C) and IL-1β (D-F). N = 3 ± SD. [a] Levels were undetectable.

Figure 4. IC_{50} of CYP3A4 suppression in Hep:KC cultures following IL-1β and IL-6 incubation. Hepatocytes were cultured alone (in micropatterned co-cultures with 3T3-J2 fibroblasts) ± Kupffer cells (at Hep:KC of 1:0 and 1:0.4). Following IL-1β and IL-6 (6.25-5000 pg/mL) incubation, CYP3A4 activity levels were measured using the CYP3A4-Glo assay at 4 days following cytokine exposure (A and C), and 6 days following recovery in cytokine-free media (B and D). IC_{50} values were measured using GraphPad Prism. N = 3 ± SD.

Figure 5. Expression of cytokine receptors in Hep:KC cultures. Hepatocytes were cultured alone ± Kupffer cells (at Hep:KC of 1:0, 1:0.1 and 1:0.4) and RT-PCR analysis was performed on samples from Day 3, 7, 11, and 15 for gene expression of (A) IL-6R, (B) IL-1R1, (C) IL-2RB, and (D) IL-23R. Results were relative to cultures without KC (Hep:KC 1:0) (n = 3 ± SD). Mean values were significantly different from Hep:KC *1:0 and *[#]1:0.1 (p-value <0.05). [a]Levels were undetectable (Ct values for 18s housekeeping gene were consistent across all cell samples but Ct values for IL-2RB were >35 and undetectable for IL-23R).

Figure 6. Effects of interleukin-1 receptor antagonist (IL-1RA) cytokine and anti-IL-1β mAb (αIL-1β) on IL-1β-mediated CYP3A4 suppression. Hepatocytes were cultured alone ± Kupffer cells (at Hep:KC of 1:0 and 1:0.4) and co-dosed with

IL-1β (2.5 ng/mL or 0.1 ng/mL for Hep:KC of 1:0 or 1:0.4, respectively) and either IL-1RA (100-1000 ng/mL) or αIL-1β (10-100 ng/mL). CYP3A4 activity levels were measured using CYP3A4-Glo assay at 4 days following treatment. N = 3 ± SD. *Mean values were significantly different from IL-1β-untreated matched Hep:KC cultures (p-value <0.05).

Figure 7. Cytokine secretion in Hep:KC cultures due to IL-1β, IL-2 and IL-23 exposure. Hepatocytes were cultured alone (in micropatterned co-cultures with 3T3-J2 fibroblasts) ± Kupffer cells (at Hep:KC of 1:0, 1:0.1 and 1:0.4). Secreted proinflammatory cytokines such as IL-8 (A), TNF-α (B) and IFN-γ (C) were measured in cell culture media 4 days following cytokine treatment. N = 3 ± SD. [a] Levels were undetectable.

Figure 8. CYP3A4 activity in Hep:KC cultures following IL-2 and IL-23 treatment. Hepatocytes were cultured alone (in micropatterned co-cultures with 3T3-J2 fibroblasts) ± Kupffer cells (at Hep:KC of 1:0, 1:0.1 and 1:0.4). CYP3A4 activity levels were measured after 4 days of IL-2 (A) and IL-23 (B) treatment. N = 3 ± SD. *Mean values were significantly different from IL-2-untreated Hep:KC cultures of matched cell ratios (p-value <0.05).

Figure 9. Gene expression of CYP3A4 and C-reactive protein (CRP) in Hep:KC cultures following cytokine treatments. Hepatocytes were cultured alone ± Kupffer cells (at Hep:KC of 1:0 and 1:0.4). Following IL-1β (A and B) and IL-6 (C

and D) incubation (6.25-5000 pg/mL), CYP3A4 and CRP mRNA levels were assessed by RT-PCR. N = 3 ± SD.

Table 1. Effects of IL-1β and IL-6 on Metabolism Enzymes mRNA Expression in Hep:KC (1:0 and 1:0.4) Cell Cultures

Metabolism Enzymes	Exposure to IL-1β (6.25 – 5000 pg/mL) - 4 days		Exposure to IL-6 (6.25 – 5000 pg/mL) - 4 days	
	Effects[a]	Effects were Kupffer cell-dependent	Effects[a]	Effects were Kupffer cell-dependent
CYP1A1	↔	-	↔	-
CYP 1A2	↓	Yes	↓	-
CYP2A6	ND	-	ND	-
CYP 2B6	↔	-	↓	-
CYP 2C8	↓	Yes	↓	-
CYP 2C9	↓	Yes	↓	-
CYP 2C18	↔	-	↔	-
CYP 2C19	ND	-	↔	-
CYP 2D6	↔	-	↔	-
CYP2E1	↔	-	↔	-
CYP 3A4	↓	Yes	↓	-
GSTA1	↓[b]	Yes	↓	-
GSTA2	↓[b]	Yes	↓	-
SULT1A1	ND	-	ND	-
SULT1A2	ND	-	ND	-
UGT1A1	↓	Yes	↓	-
UGT2B7	↓[b]	Yes	↓	-

a. Effects were noted as non-effect (↔) or down-regulated (↓) ≤50% of untreated control in a concentration-dependent manner. Samples with Ct value >32 were designated as non-detectable (ND).

b. Effects were observed only in Hep:KC 1:0.4 but not 1:0 cultures.

Table 2. Effects of IL-1β and IL-6 on Uptake and Efflux Transporters mRNA Expression in Hep:KC (1:0 and 1:0.4) Cell Cultures

	Exposure to IL-1β (6.25 – 5000 pg/mL) - 4 days		Exposure to IL-6 (6.25 – 5000 pg/mL) - 4 days	
Transporters	Effects[a]	Effects were Kupffer cell-dependent	Effects[a]	Effects were Kupffer cell-dependent
NTCP	↓[b]	Yes	↓	-
OAT2	↔	-	↔	-
OATP1B1	↓[b]	Yes	↔	-
OATP1B3	↓[b]	Yes	↓	-
OATP2B1	↓	-	↔	-
OCT1	↓	-	↓	-
BCRP	ND	-	ND	-
BSEP	↓	Yes	↓	-
MDR1	↔	-	↔	-
MRP2	↔	-	↔	-
MRP3	↔	-	↔	-
MRP4	ND	-	ND	-

a. Effects were noted as non-effect (↔) or down-regulated (↓) ≤50% of untreated control in a concentration-dependent manner. Samples with Ct value >32 were designated as non-detectable (ND).

b. Effects were observed only in Hep:KC 1:0.4 but not 1:0 cultures.

Table 3. Effects of IL-1β and IL-6 on Acute Phase Proteins mRNA Expression in Hep:KC (1:0 and 1:0.4) Cell Cultures

Proteins[a]	Exposure to IL-1β (6.25 – 5000 pg/mL) - 4 days		Exposure to IL-6 (6.25 – 5000 pg/mL) - 4 days	
	Effects[b] (Highest observed % untreated control)	Effects were Kupffer cell-dependent	Effects[b] (Highest observed % untreated control)	Effects were Kupffer cell-dependent
AAG	↑ 1250 ± 94 (1:0) 1459 ± 412 (1:0.4)	Yes	↑ 1986 ± 170 (1:0) 1024 ± 98 (1:0.4)	-
CRP	↑ 1133 ± 260 (1:0) 53132 ± 15568 (1:0.4)	Yes	↑ 95220 ± 12671 (1:0) 80426 ± 25395 (1:0.4)	-
SAA2	↑ 5057 ± 1181 (1:0) 5319 ± 1688 (1:0.4)	-	↑ 18208 ± 6529 (1:0) 10392 ± 1643 (1:0.4)	-

a. Proteins: alpha-1-acid glycoprotein (AAG), C-reactive protein (CRP); serum amyloid A2 (SAA2)

b. Effects were noted as up-regulation (↑) of ≥150% of untreated control in a concentration-dependent manner.

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Figure 1

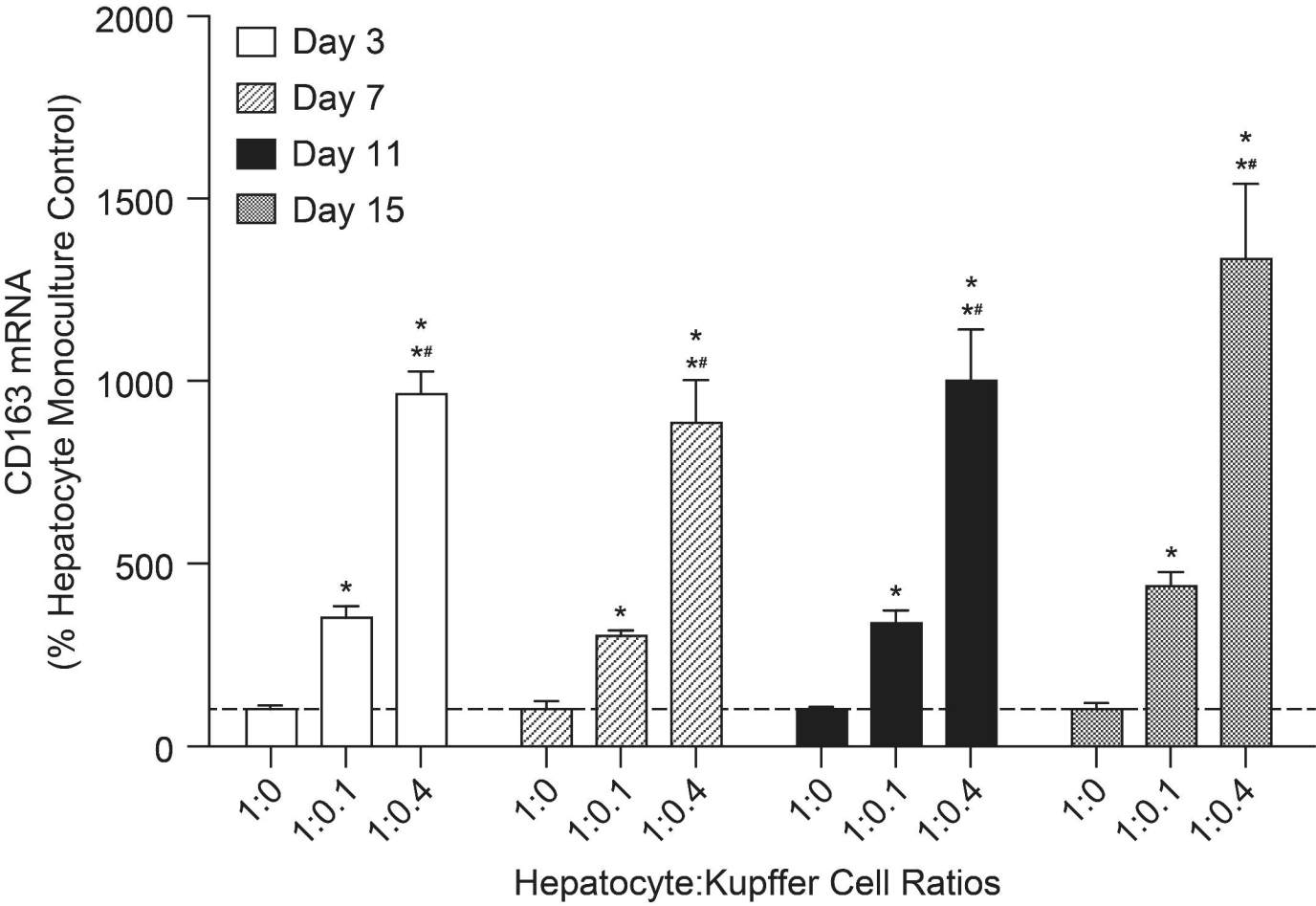

2000
1500
1000
500
0
CD163 mRNA
(% Hepatocyte Monoculture Control)
Day 3
Day 7
Day 11
Day 15
*
*#
1:0
1:0.1
1:0.4
Hepatocyte:Kupffer Cell Ratios

Figure 2

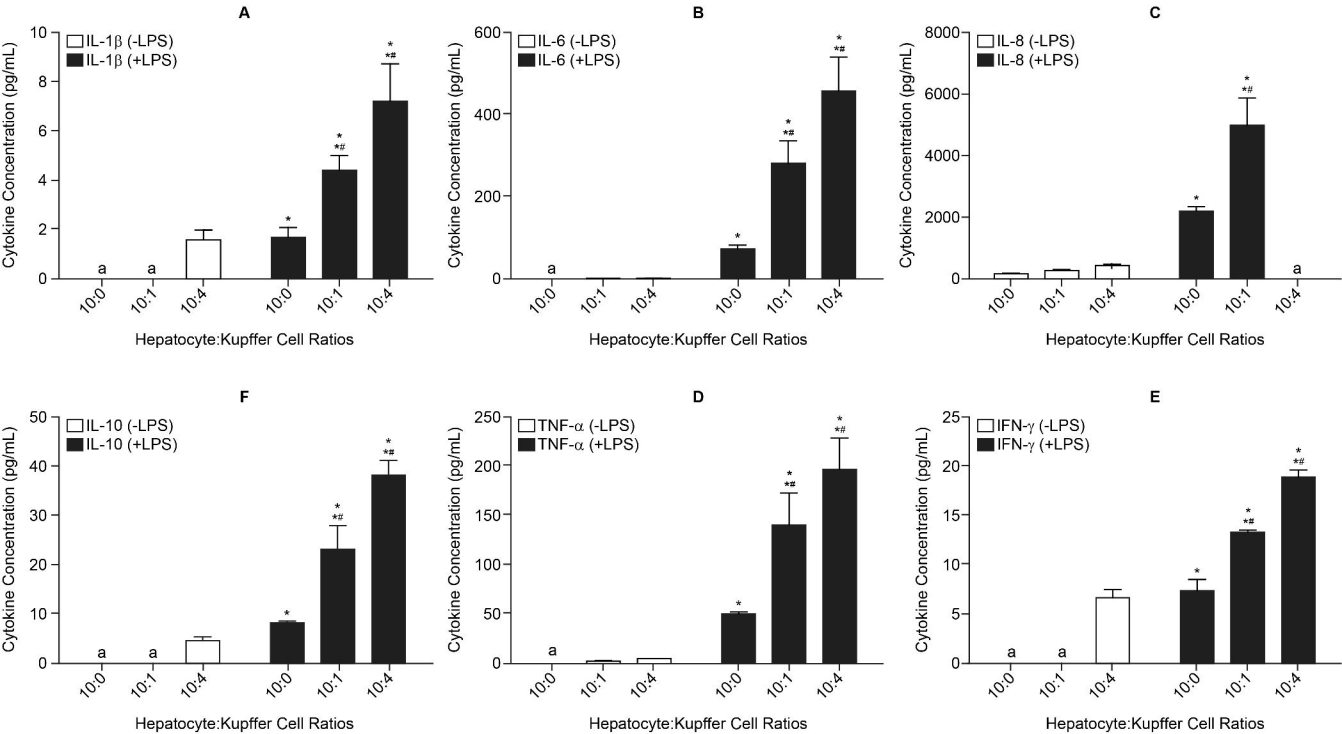

A
IL-1β (-LPS)
IL-1β (+LPS)
Cytokine Concentration (pg/mL)
10
8
6
4
2
0
a
a
*
*#
10:0
10:1
10:4
10:0
10:1
10:4
Hepatocyte:Kupffer Cell Ratios
B
IL-6 (-LPS)
IL-6 (+LPS)
Cytokine Concentration (pg/mL)
600
400
200
0
a
*
*#
10:0
10:1
10:4
10:0
10:1
10:4
Hepatocyte:Kupffer Cell Ratios
C
IL-8 (-LPS)
IL-8 (+LPS)
Cytokine Concentration (pg/mL)
8000
6000
4000
2000
0
*
*#
a
10:0
10:1
10:4
10:0
10:1
10:4
Hepatocyte:Kupffer Cell Ratios
F
IL-10 (-LPS)
IL-10 (+LPS)
Cytokine Concentration (pg/mL)
50
40
30
20
10
0
a
a
*
*#
10:0
10:1
10:4
10:0
10:1
10:4
Hepatocyte:Kupffer Cell Ratios
D
TNF-α (-LPS)
TNF-α (+LPS)
Cytokine Concentration (pg/mL)
250
200
150
100
50
0
a
*
*#
10:0
10:1
10:4
10:0
10:1
10:4
Hepatocyte:Kupffer Cell Ratios
E
IFN-γ (-LPS)
IFN-γ (+LPS)
Cytokine Concentration (pg/mL)
25
20
15
10
5
0
a
a
*
*#
10:0
10:1
10:4
10:0
10:1
10:4
Hepatocyte:Kupffer Cell Ratios

Figure 3

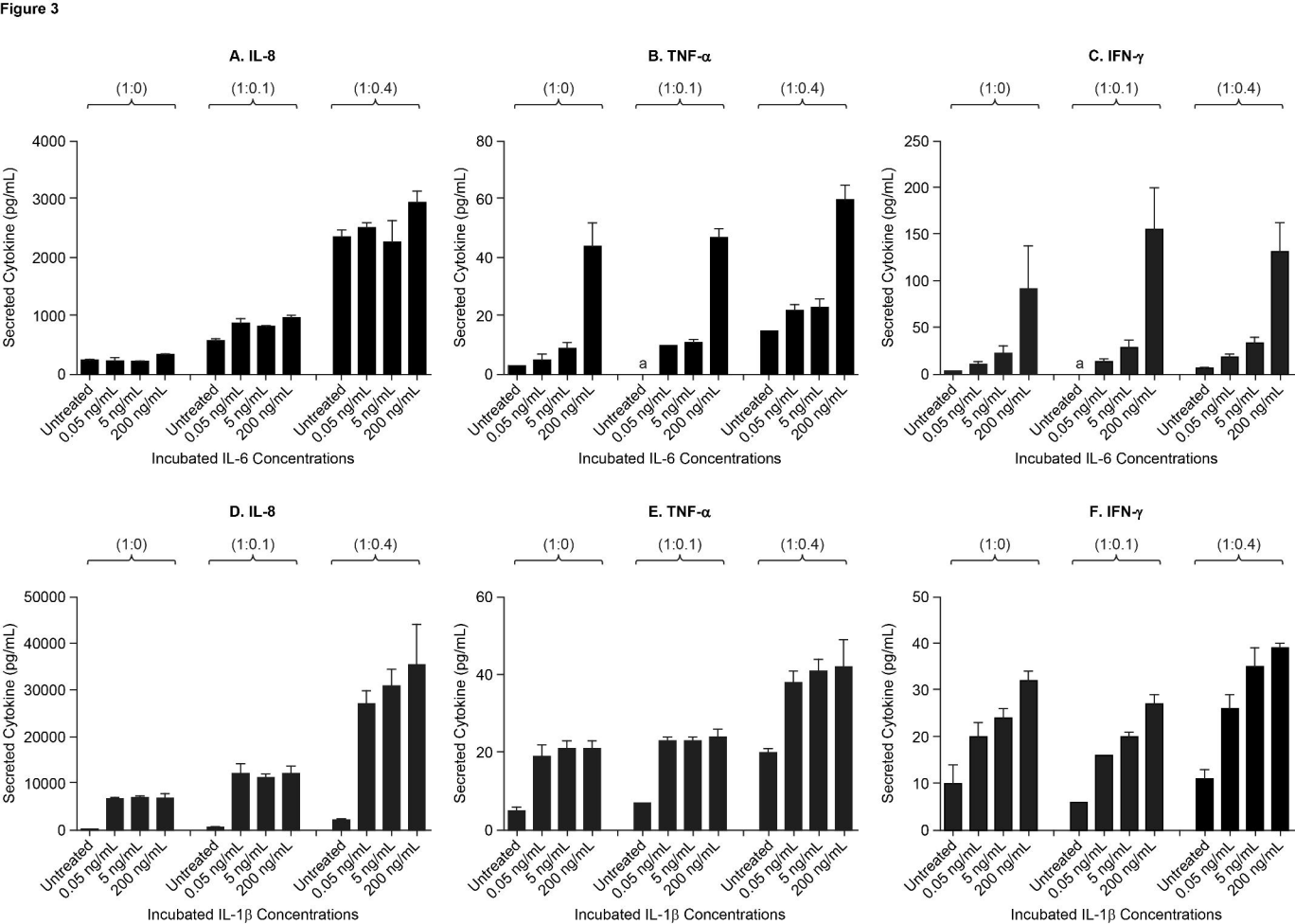
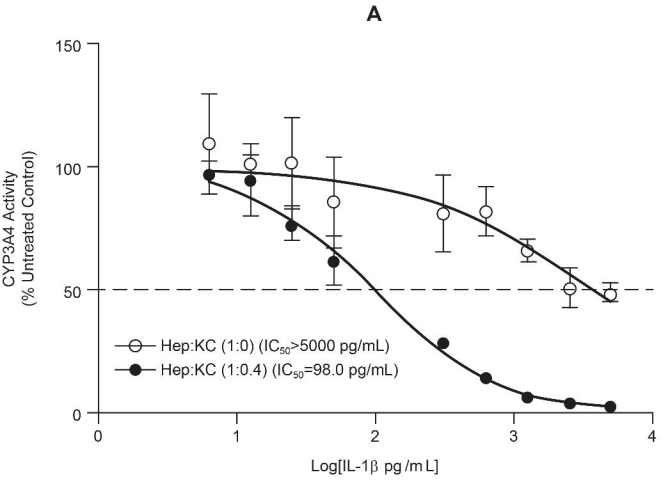
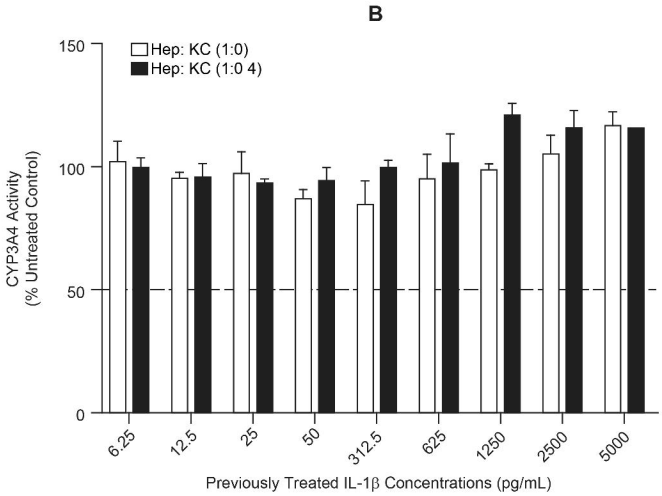
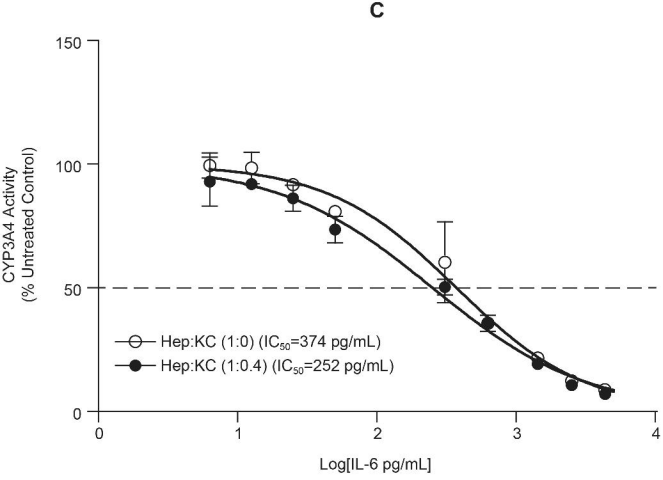

A. IL-8
B. TNF-α
C. IFN-γ
D. IL-8
E. TNF-α
F. IFN-γ
(1:0)
(1:0.1)
(1:0.4)
Secreted Cytokine (pg/mL)
Untreated
0.05 ng/mL
5 ng/mL
200 ng/mL
Incubated IL-6 Concentrations
Incubated IL-1β Concentrations
a

Figure 4


A
150
100
50
0
0
1
2
3
4
CYP3A4 Activity
(% Untreated Control)
Hep:KC (1:0) (IC50>5000 pg/mL)
Hep:KC (1:0.4) (IC50=98.0 pg/mL)
Log[IL-1β pg/mL]


B
Hep: KC (1:0)
Hep: KC (1:0 4)
150
100
50
0
CYP3A4 Activity
(% Untreated Control)
6.25
12.5
25
50
312.5
625
1250
2500
5000
Previously Treated IL-1β Concentrations (pg/mL)


C
150
100
50
0
0
1
2
3
4
CYP3A4 Activity
(% Untreated Control)
Hep:KC (1:0) (IC50=374 pg/mL)
Hep:KC (1:0.4) (IC50=252 pg/mL)
Log[IL-6 pg/mL]

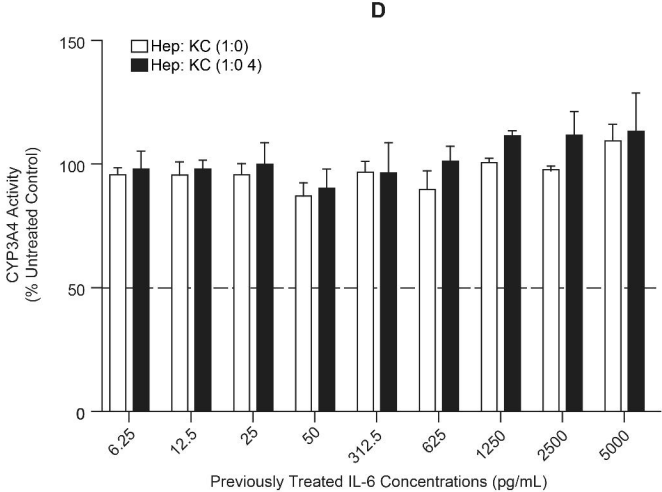

D
Hep: KC (1:0)
Hep: KC (1:0 4)
150
100
50
0
CYP3A4 Activity
(% Untreated Control)
6.25
12.5
25
50
312.5
625
1250
2500
5000
Previously Treated IL-6 Concentrations (pg/mL)

Figure 5

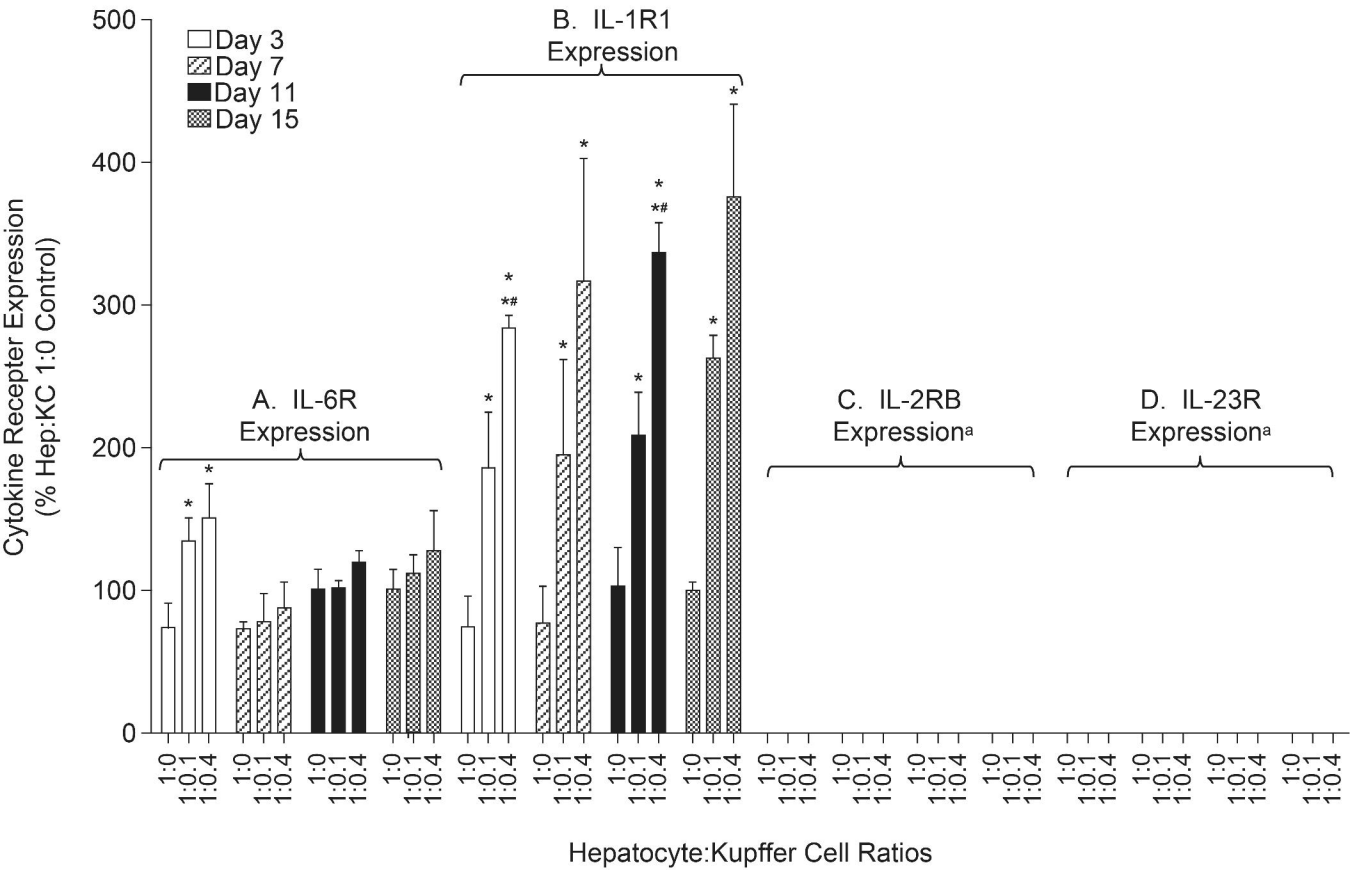

Day 3
Day 7
Day 11
Day 15
A. IL-6R Expression
B. IL-1R1 Expression
C. IL-2RB Expression[a]
D. IL-23R Expression[a]
Cytokine Recepter Expression (% Hep:KC 1:0 Control)
0
100
200
300
400
500
1:0
1:0.1
1:0.4
Hepatocyte:Kupffer Cell Ratios

Figure 6

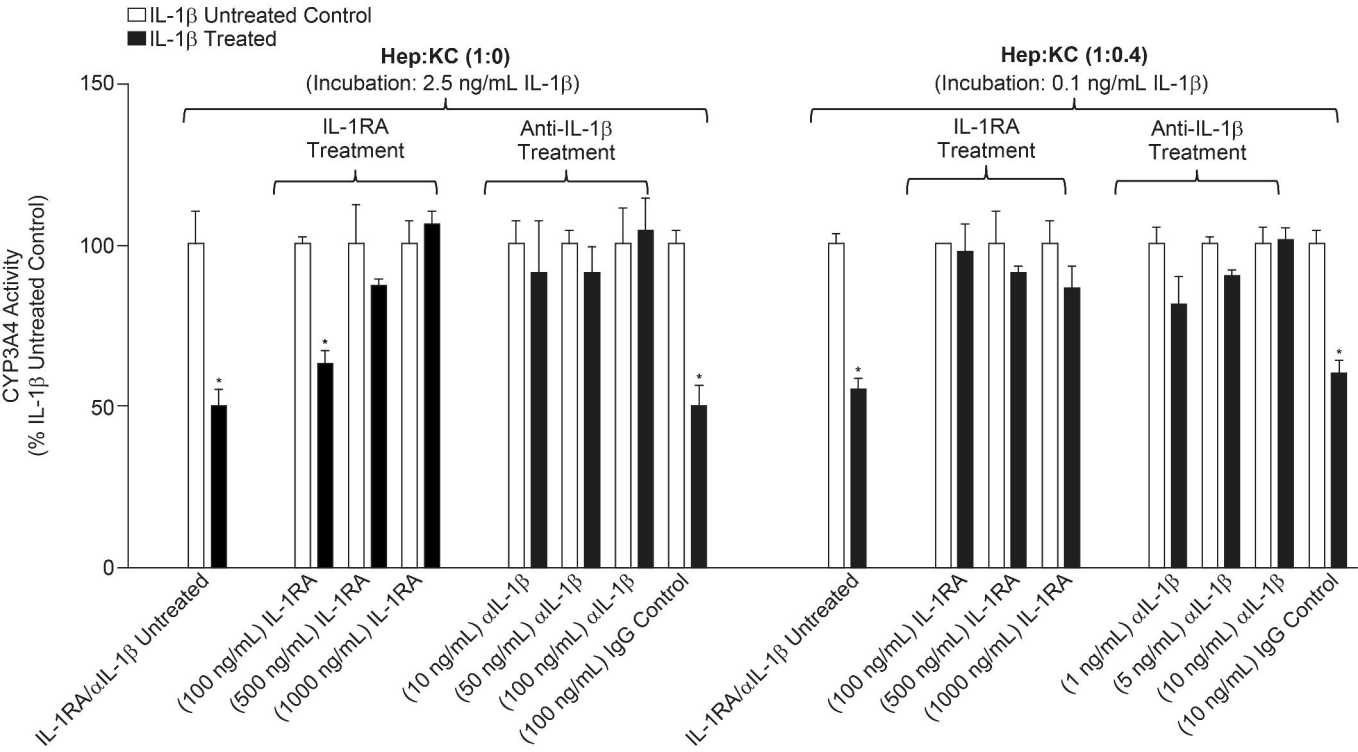

IL-1β Untreated Control
IL-1β Treated
Hep:KC (1:0)
(Incubation: 2.5 ng/mL IL-1β)
Hep:KC (1:0.4)
(Incubation: 0.1 ng/mL IL-1β)
IL-1RA Treatment
Anti-IL-1β Treatment
CYP3A4 Activity
(% IL-1β Untreated Control)
150
100
50
0
IL-1RA/αIL-1β Untreated
(100 ng/mL) IL-1RA
(500 ng/mL) IL-1RA
(1000 ng/mL) IL-1RA
(10 ng/mL) αIL-1β
(50 ng/mL) αIL-1β
(100 ng/mL) αIL-1β
(100 ng/mL) IgG Control
IL-1RA/αIL-1β Untreated
(100 ng/mL) IL-1RA
(500 ng/mL) IL-1RA
(1000 ng/mL) IL-1RA
(1 ng/mL) αIL-1β
(5 ng/mL) αIL-1β
(10 ng/mL) αIL-1β
(10 ng/mL) IgG Control

Figure 7

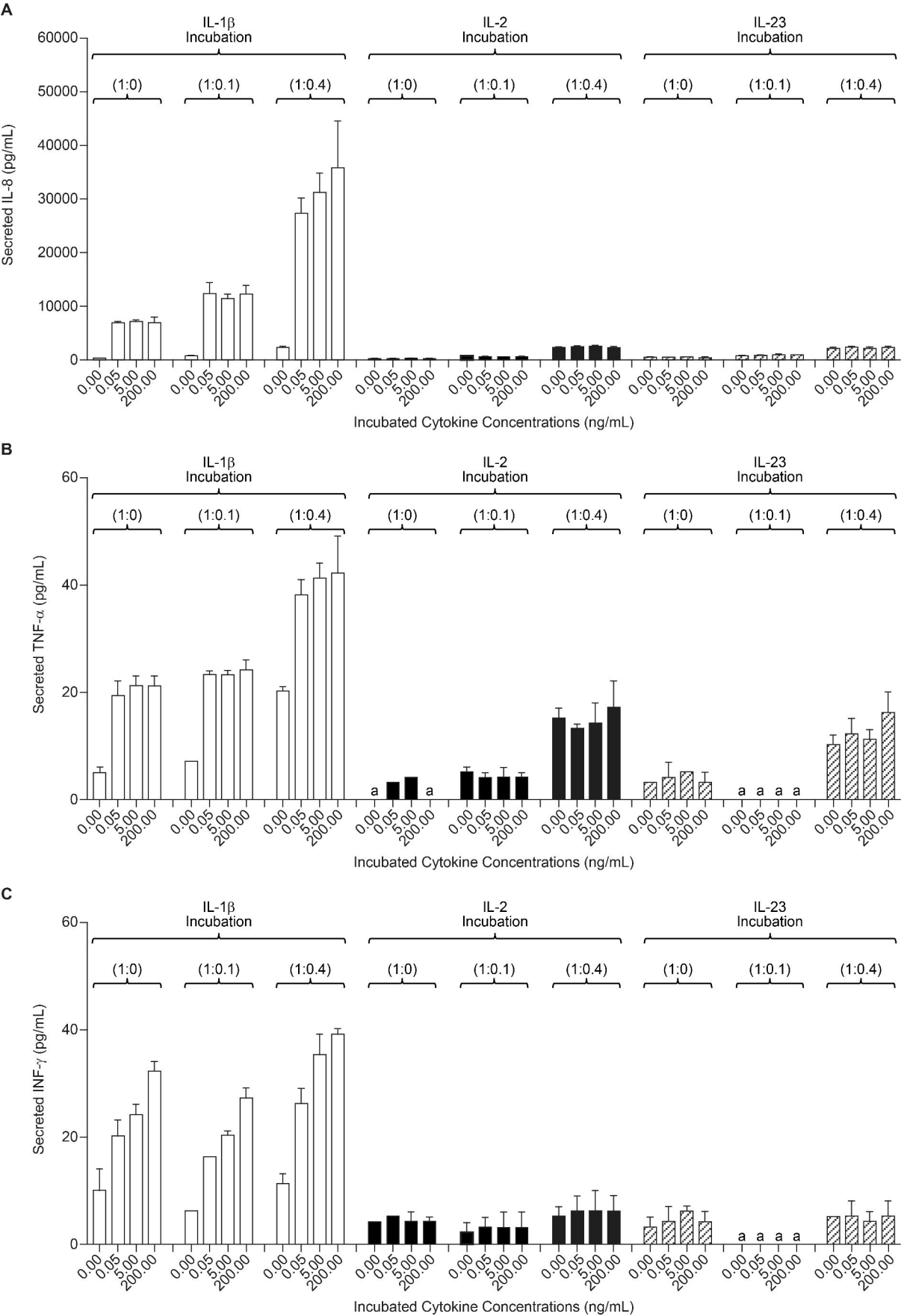

A
IL-1β Incubation
IL-2 Incubation
IL-23 Incubation
(1:0)
(1:0.1)
(1:0.4)
Secreted IL-8 (pg/mL)
60000
50000
40000
30000
20000
10000
0
0.00
0.05
5.00
200.00
Incubated Cytokine Concentrations (ng/mL)
B
Secreted TNF-α (pg/mL)
60
40
20
0
a
Incubated Cytokine Concentrations (ng/mL)
C
Secreted INF-γ (pg/mL)
60
40
20
0
a
Incubated Cytokine Concentrations (ng/mL)

Figure 8

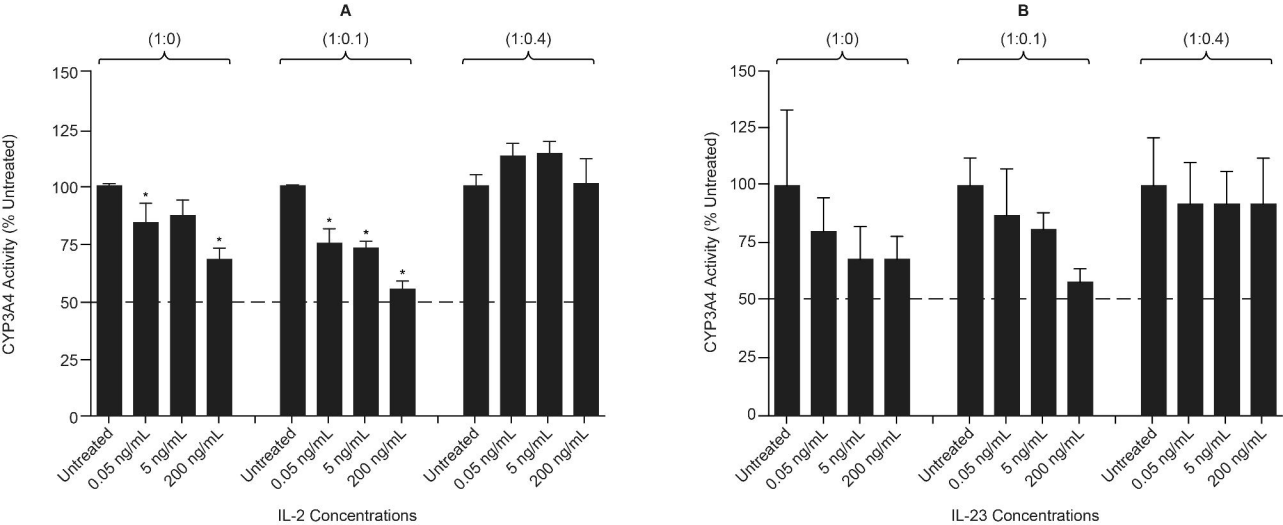
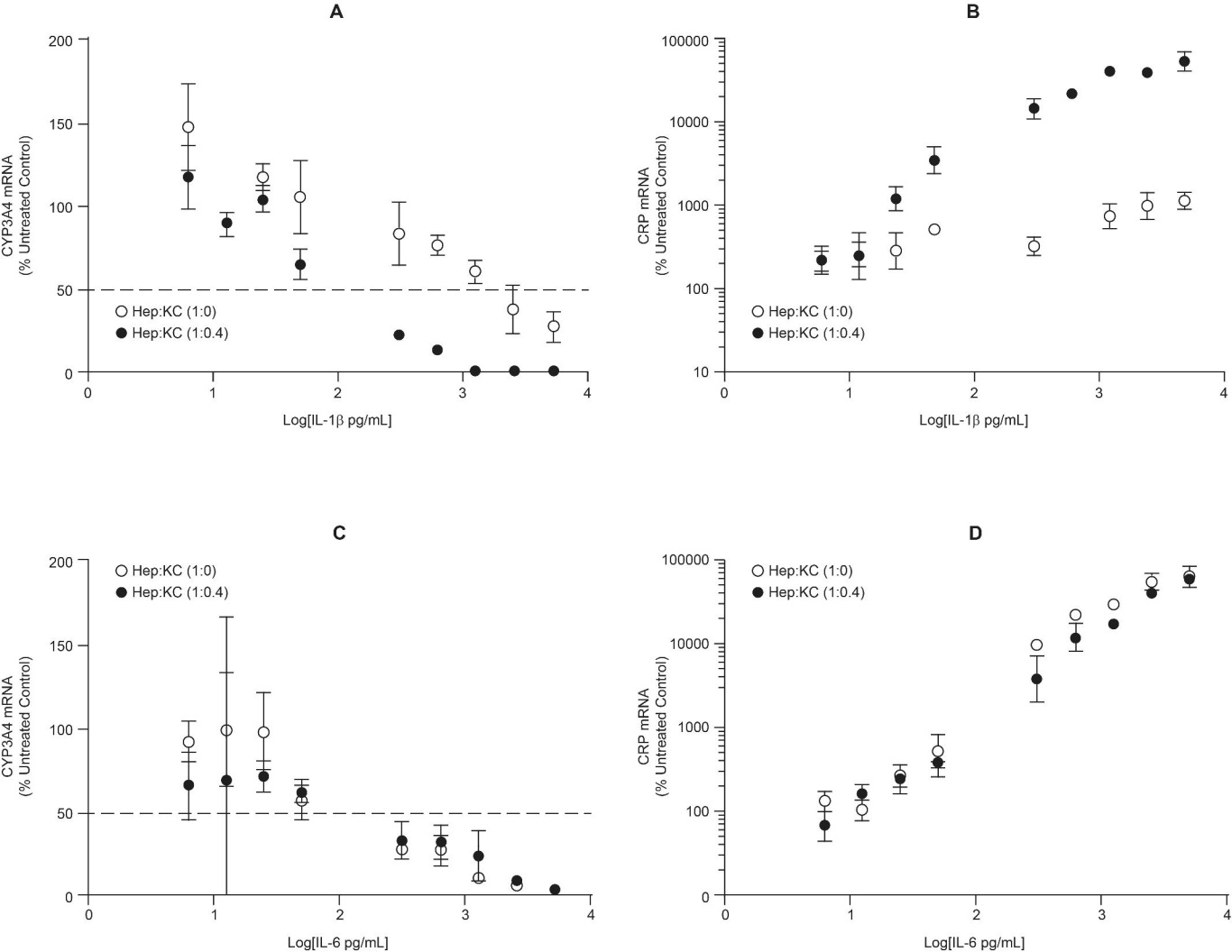

A
(1:0)
(1:0.1)
(1:0.4)
CYP3A4 Activity (% Untreated)
150
125
100
75
50
25
0
Untreated
0.05 ng/mL
5 ng/mL
200 ng/mL
IL-2 Concentrations
B
(1:0)
(1:0.1)
(1:0.4)
CYP3A4 Activity (% Untreated)
Untreated
0.05 ng/mL
5 ng/mL
200 ng/mL
IL-23 Concentrations

Figure 9


A
CYP3A4 mRNA
(% Untreated Control)
200
150
100
50
0
0
1
2
3
4
Log[IL-1β pg/mL]
Hep:KC (1:0)
Hep:KC (1:0.4)
B
CRP mRNA
(% Untreated Control)
100000
10000
1000
100
10
0
1
2
3
4
Log[IL-1β pg/mL]
Hep:KC (1:0)
Hep:KC (1:0.4)
C
CYP3A4 mRNA
(% Untreated Control)
200
150
100
50
0
0
1
2
3
4
Log[IL-6 pg/mL]
Hep:KC (1:0)
Hep:KC (1:0.4)
D
CRP mRNA
(% Untreated Control)
100000
10000
1000
100
0
0
1
2
3
4
Log[IL-6 pg/mL]
Hep:KC (1:0)
Hep:KC (1:0.4)