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An *in vitro* test system to evaluate drug-drug interactions with biologics

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INTRODUCTION

Inflammation, infection, vaccination, and some marketed therapeutic proteins (biologics) are associated with cytokine-mediated suppression (down-regulation) of drug-metabolizing enzymes (DME). Biologics, such as monoclonal antibodies, can trigger the release of pro-inflammatory cytokines (*e.g.* IL-1β, IL-6, INFγ and TNFα); extreme cases of which are known as a cytokine storm. Protein therapeutics may change the clearance of small molecule drugs (SMDs) by affecting DME expression and thereby precipitate drug-drug interactions (DDIs). Potential of biologics to cause DDI is a safety concern recognized by the FDA (Lee *et al*). In the present study, we developed an *in vitro* method to evaluate the potential of biologics to elicit DDIs with SMDs via alteration of DME expression. This method involves treating fresh human blood with a biologic to stimulate the release of pro-inflammatory cytokines from peripheral blood mononuclear cells (PBMCs), after which plasma is prepared and added to primary human hepatocytes co-cultured with Kupffer cells to evaluate effects of biologics on cytochrome P450 (CYP) enzyme expression. *E.coli* lipopolysaccharide (LPS) and murine anti-CD28 monoclonal antibody were evaluated for their potential to perpetrate DDI with SMDs.

MATERIALS & METHODS

***Ex-vivo* stimulation of whole blood:** Four healthy donors gave informed consent to participate in the study. Blood was drawn into 10 mL sodium heparin vacutainers (Becton-Dickinson, Franklin Lakes, NJ), transferred into sterile 50 mL polypropylene tubes (BD Biosciences, San Diego, CA) and aliquoted into sterile polypropylene micro tubes (Sarstedt, Newton, NC) for the incubation. Endotoxin was removed from the sterile normal saline (XenoTech, LLC) with the Endotoxin Removal Gel (Pierce Biotechnology, Rockford, IL). The 5 µg/mL stock solution of lipopolysaccharides from *E.coli* (Sigma-Aldrich, St. Louis, MO) was prepared with purified saline. Type B CpG synthetic oligonucleotide (InvivoGen, San Diego, CA) was resuspended with purified saline at 1 mM.
Anti-CD28 antibody, ANC28.1 (murine IgG1kappa), and iso-type control antibody were purchased from Ancell Corp. (Bayport, MN).
The stimuli were gently mixed with the whole blood and incubated for 24 hrs at 37°C in the atmosphere of 5% CO_2. Plasma was separated by centrifugation at 600 x g for 10 min, aliquoted and stored at -80°C.

Cytokine quantification: Levels of GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70 and TNF-α were measured in the plasma samples with a human cytokine assay kit (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's protocol.

Hepatocyte cultures: Primary human hepatocytes were isolated by a two-step collagenase perfusion method and plated on collagen-coated dishes. Briefly, the hepatocytes were allowed to adapt to culture conditions for two to three days, after which they were treated once daily for three consecutive days either with MCM+ (negative control), IL-6 (positive control, EMD Biosciences, La Jolla, CA), ANC28.1 antibody, LPS, plasma from whole blood stimulated with ANC28.1 or stimulated with LPS or murine IgG1 kappa isotype control.
After 72 hr of treatment CYP enzymatic activities were analyzed in situ and the cells were lysed with TRIzol® reagent (Invitrogen, Carlsbad, CA) for subsequent isolation of RNA.
In situ enzyme activity analysis. Twenty-four hours after the final treatment, the hepatocytes were incubated with a cocktail of phenacetin (100 µM), bupropion (500 µM) and midazolam (30 µM) to measure CYP1A2, CYP2B6 and CYP3A4/5 activity, respectively. Reactions were terminated after 30 minutes with the addition of acetonitrile containing an appropriate internal standard (deuterated metabolite). The metabolites, namely acetaminophen, hydroxybupropion and 1´-hydroxymidazolam, and the corresponding internal standards were quantitated by HPLC-MS/MS.
CYP enzyme mRNA analysis. Total RNA was phase extracted with Trizol® reagent followed by purification with the RNeasy Mini Kit (Qiagen, Valencia, CA). Purified RNA was reverse transcribed to cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) and the Applied Biosystems 7300 Real-Time PCR System AB7300).

RESULTS

Figure 1 shows inter-individual variability and dose-response for nine pro-inflammatory cytokines released from fresh whole human blood cultures in response to LPS (Fig 1A) or CpG (Fig 1B). LPS is a ligand for the Toll-like receptor 4, while CpG signals through human TLR9.

- Inter-individual variability in the quantity of individual cytokines released in response to LPS was in the range of 2- to 3-fold between donors, with an exception of Donor 2 who, on average, had ~25 fold less INF-γ than the other three donors.
- In whole blood samples treated with LPS, a dose-response relationship was observed for all cytokines with an exception of GM-CSF, IL-1B and IL-8. This finding suggests that, for some cytokines, treatment with 5 ng/mL LPS constitutes a saturating dose in our test system.
- Inter-individual variability in the quantity of cytokines released in response to CpG was greater than that observed with LPS; however, unlike in the case of LPS, a dose-response relationship was observed for all cytokines.

Figure 2 examines cytokine response measured on three different occasions in the blood of Donor 2. Blood was treated with 50 ng/mL LPS or the purified saline. For most cytokines the variability in the quantity of cytokines released was in a range of 2- to 3-fold.

Figure 3 compares effects of anti-CD28 antibody (2 µg/mL) or LPS (50 ng/mL) on cytokine release from the blood of Donor 2. The CD28 receptor is found on a surface of T-helper cells and mediates an interaction between T-helper and antigen-presenting cells. This antigen, a target of TGN1412 antibody, is associated with cytokine storm in humans (Suntharalingam, *et al*). Both anti-CD28 antibodies, ANC28.1 and TGN1412, are described as "superagonistic". For most of the cytokines examined, the effect of LPS treatment of human whole blood on cytokine release was dramatically greater (*i.e.* cause the release of higher levels of cytokines) than did treatment with the anti-CD28 antibody. However, IL-2 was notably higher in the plasma from anti-CD28 treated blood, which reached concentrations of 750 ng/mL, as compared to 150 ng/mL in the LPS-treated group.

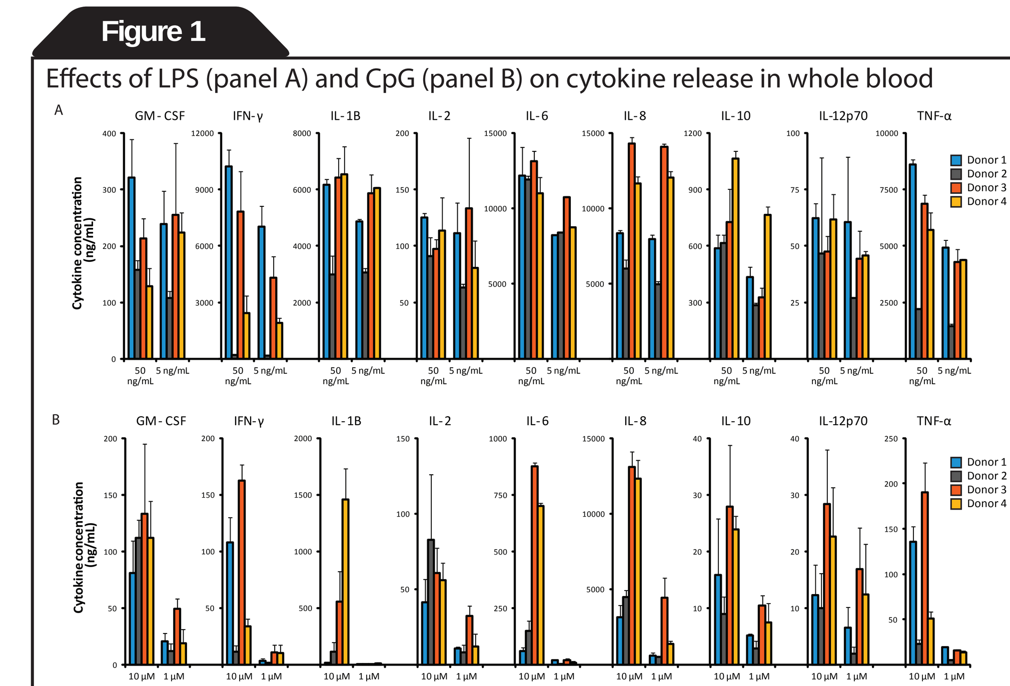
Figure 4 illustrates effects of plasma obtained from the LPS- or control-stimulated whole blood on the activity and mRNA expression of selected CYP enzymes in cultured human hepatocytes. Pretreated (stimulated) plasma [10, 20 or 50% (v/v) of cell culture medium] from Donor 2 was applied to cultured hepatocytes from three donors. LPS binds the CD14/TLR4/MD2 receptor complex which promotes the secretion of pro-inflammatory cytokines.

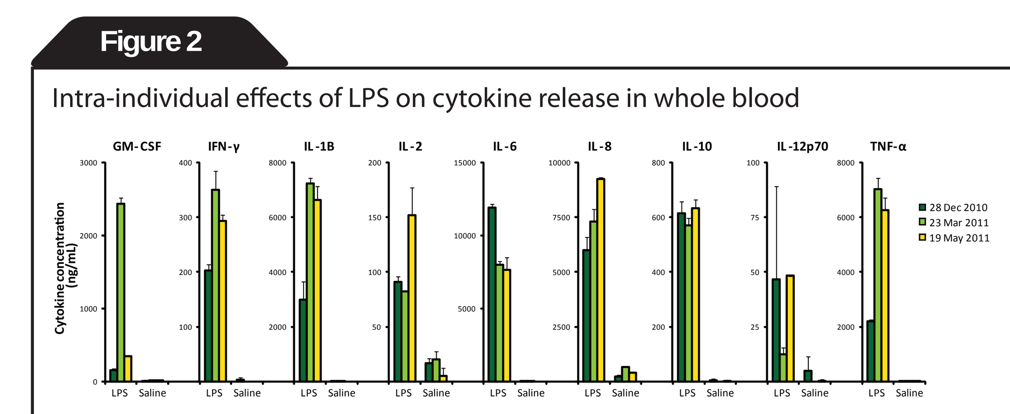
- As expected, direct application of IL-6 to cultured hepatocytes suppressed CYP enzyme activity and mRNA expression and served as a positive control for suppression in this study.
- Treatment of cultured human hepatocytes with plasma from the control (saline-stimulated) blood caused a dose-dependent reduction of both enzymatic activity and the mRNA expression of CYP1A2 and CYP3A4. Interestingly, the same plasma caused a significant dose-dependent increase of bupropion hydroxylase activity and CYP2B6 mRNA. Similar effects of the plasma from the control-stimulated blood were observed in three additional preparations of human hepatocytes (Figure 6).
- Treatment of human hepatocytes with plasma from LPS-stimulated blood reduced both enzymatic activity and the mRNA expression of CYP1A2, CYP2B6 and CYP3A4 as compared to the plasma from the control-stimulated blood. CYP3A4 activity and mRNA expression were most dramatically suppressed (by up to 75 and >99%, respectively) by the "LPS- stimulated plasma". LPS applied directly to the cell culture media suppressed CYP3A4 activity and mRNA expression by up to 70 and 90%, respectively. The observed reduction in CYP activity and mRNA expression following treatment of hepatocyte cultures with LPS represents a direct effect of LPS on the hepatocytes in vitro. However, the suppression of CYP enzymes in hepatocytes following treatment with LPS may be mediated also by the activated hepatic macrophages (i.e. Kupffer cells). This route of CYP suppression was supported by staining of human Kupffer cells in cultures of fresh human hepatocytes.

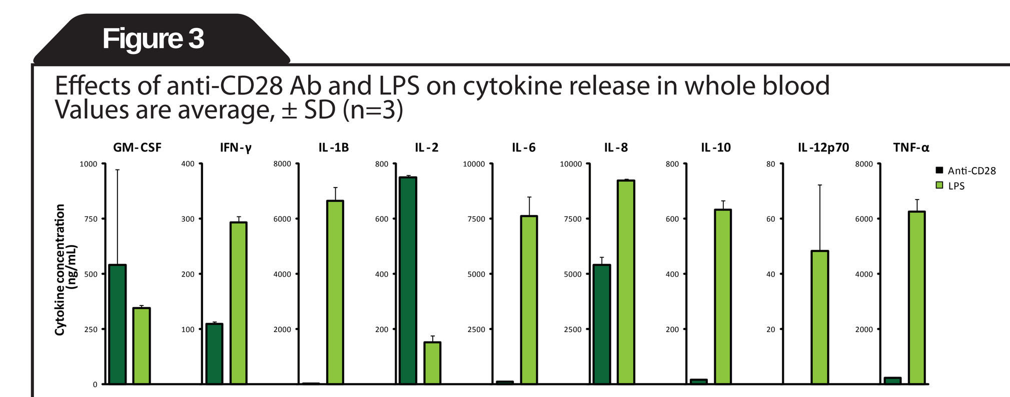
Figure 5 is a representative fluorescence microphotograph of Kupffer cells found in cultures used in this study. Macrophages were stained with fluorescence-tagged anti-CD68 antibody.

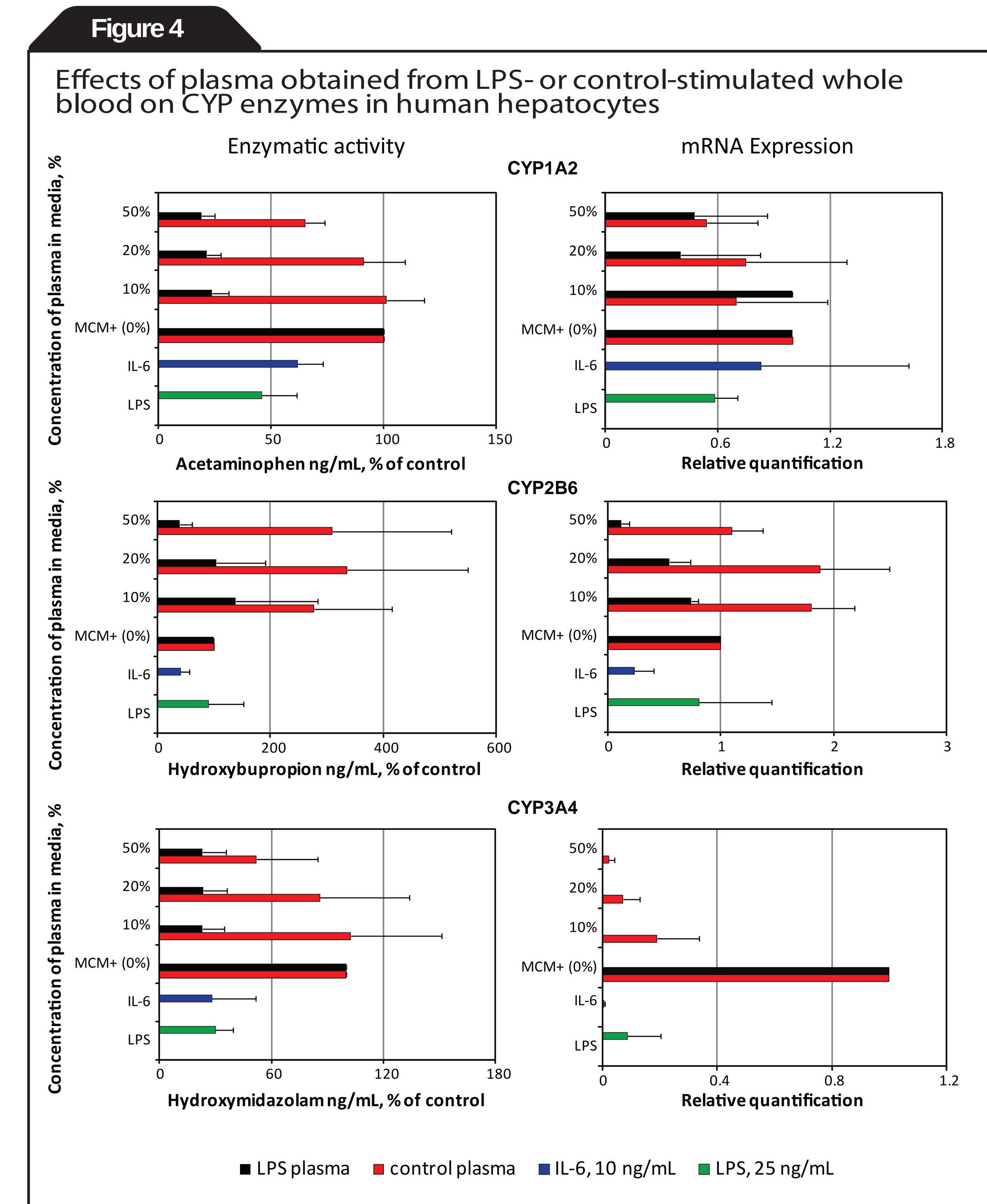
Figure 6 illustrates effects of plasma obtained from the anti-CD28 antibody- or control-stimulated whole blood on the activity and mRNA expression of selected CYP enzymes in cultured human hepatocytes.

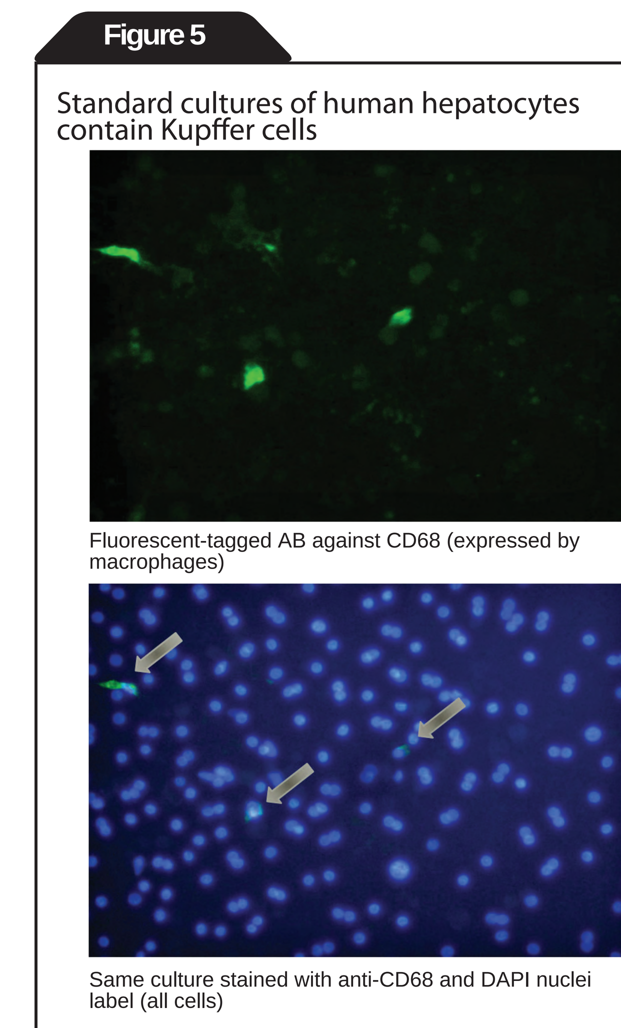
- Treatment of cultured human hepatocytes with plasma from the control (isotype antibody-stimulated) blood caused a dose-dependent reduction of both enzymatic activity and the mRNA expression of CYP1A2 and CYP3A4. Interestingly, the same plasma caused a significant dose-dependent increase of CYP2B6's bupropion hydroxylase activity and the mRNA.
- Treatment of cultured human hepatocytes with plasma from the anti-CD28 antibody-stimulated blood reduced both enzymatic activity and mRNA expression of CYP1A2, CYP2B6 and CYP3A4 compared to control samples. CYP3A4 activity and mRNA expression were suppressed to the largest extent (by up to 70 and 95%, respectively) by the anti-CD28 antibody stimulated plasma. The decrease in CYP activity and mRNA expression was less than that observed in hepatocyte cultures treated with "LPS-stimulated plasma". The observed magnitude of suppression may correlate with the lower concentration of IL-6 in the anti-CD28 antibody stimulated plasma. As expected, IL-2 present at higher concentration in the anti-CD28 antibody-stimulated plasma didn't change expression of CYP enzymes when applied directly to cultured human hepatocytes (data not shown).
- Application of the anti-CD28 antibody directly to the cultured hepatocytes (*i.e.* applied directly to the media) did not have an effect on the activity or mRNA expression of the three CYP enzymes examined. Anti-CD28 antibody exemplifies a biologic drug that requires immune system signaling (*i.e.* via T-cells to exert its effects indirectly on drug metabolism).





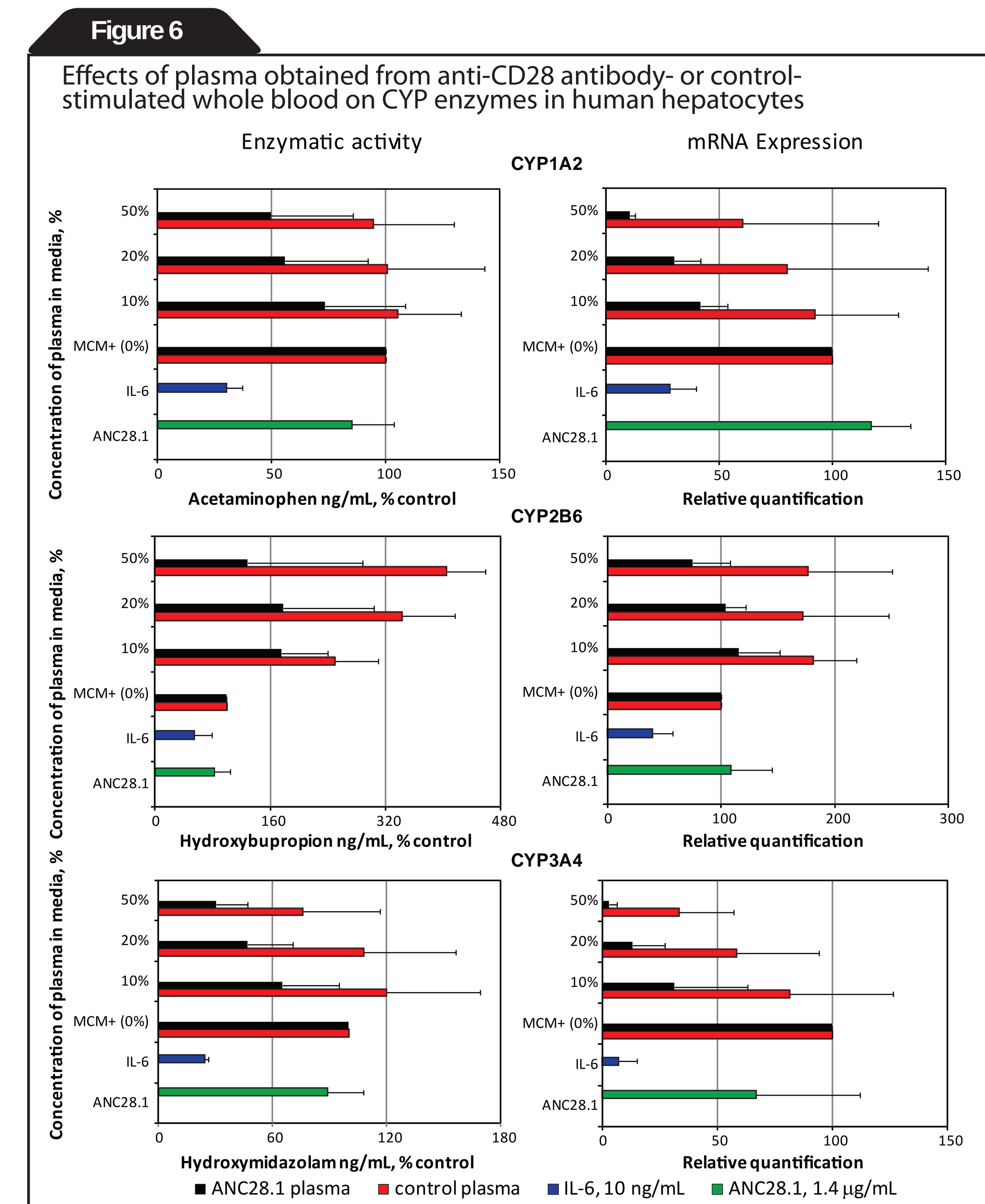






Fluorescent-tagged AB against CD68 (expressed by macrophages)

Same culture stained with anti-CD68 and DAPI nuclei label (all cells)



CONCLUSIONS

In this method, fresh human hepatocytes, co-cultured with Kupffer cells, respond to structurally diverse biologics (*e.g.* lipopolysaccharide, or a monoclonal antibody), in a way that reflects biologics' effects on drug metabolism *in vivo*.

Cytokines released by whole blood cultures in response to stimulation with biologics (*e.g.* lipopolysaccharide, CpG oligonucleotide or a monoclonal antibody) retain their activity following cryopreservation and elicit the anticipated biological response (*i.e.* suppression of CYP activity and mRNA expression) from cultured human hepatocytes under standard cell culture conditions.

The method presented here involving *ex vivo* stimulation of whole blood combined with *in vitro* culture of human hepatocytes has the potential to identify biologics that can precipitate DDI with a small molecule drug through signaling by pro-inflammatory cytokines.

REFERENCES

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