Cytokine-Induced Liver Hepatotoxicity of Trovafloxacin in Co-Culture of Hepatocytes and Kupffer Cells

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Introduction

Immune-mediated chemical-induced hepatotoxicity, i.e. indirect hepatocellular toxicity resulting from immune cells activating liver inflammatory responses, is often overlooked as a potential mode of action due to unavailability of appropriate *in vitro* models. Kupffer cells are the largest population of resident macrophages in the liver and thus play a critical role in immune-mediated hepatotoxicity and liver injury. For this reason, we have established a co-culture system of rat primary hepatocytes and Kupffer cells that can be used to model chemical-induced immune reactions resulting in acute hepatotoxicity. Co-culture of hepatocytes and Kupffer cells may represent a powerful *in vitro* tool to predict adverse liver effects resulting from indirect adaptive immune reactions during chemical exposure.

Methods

Rat hepatocytes and Kupffer cells were obtained from the Hamner Institutes or Life Technologies. Kupffer cells and hepatic co-cultures were cultured in Advanced DMEM supplemented with Penicillin/Streptomycin, GlutaMax, and 15 mM HEPES. For plating media, an additional 10% FBS was used. Cells were analyzed for purity using the Attune Acoustic Focusing Cytometer. Kupffer cell morphological assessments with CD68 and CD163 were performed utilizing standard IHC methodologies. Rat hepatocytes, Kupffer cells, and co-cultures were dosed for 24-72 hr with 1 µg/ml *E. coli* (0127:B8) LPS and various concentrations of TVX. CYP3A activities were measured using P450-Glo Luciferin IPA assay™ , ATP levels were measured using Cell-Titer Glo™ and LDH levels were measured using CytoTox-One™ assay (Promega). Cytokines were assessed by ELISA or Luminex® beads for IL-6 and TNF-α (Life Technologies).

Results

Cytometric analysis of primary Kupffer cells displayed at least 90% purity of two distinctive subpopulations of CD68/CD163 cells. Twenty four, 48, and 72 hr after LPS treatment, cytokine analysis of hepatocyte and Kupffer cell co-cultures for IL-6 and TNF-α was performed to analyze the effect of LPS on hepatic metabolism. Inflammation markedly down-regulated activity of CYP3A 74% and 85% at 48 and 72hr after LPS treatment, respectively. Inhibition of metabolism correlated with IL-6 up-regulation. This response was blunted in co-treatment with LPS and TVX showing a reversal of inhibition of CYP3A activity and significantly lower production of IL-6; however, TNF-α production was unaffected. A subsequent study utilizing LPS and increasing amounts of TVX exhibited the same pattern of reversal of inhibition of CYP3A activity and shifting IL-6/TNF-α ratios in a dose-dependent fashion. ATP and LDH levels correlated with observed necrotic cytotoxicity in hepatocytes after TVX/LPS treatment.

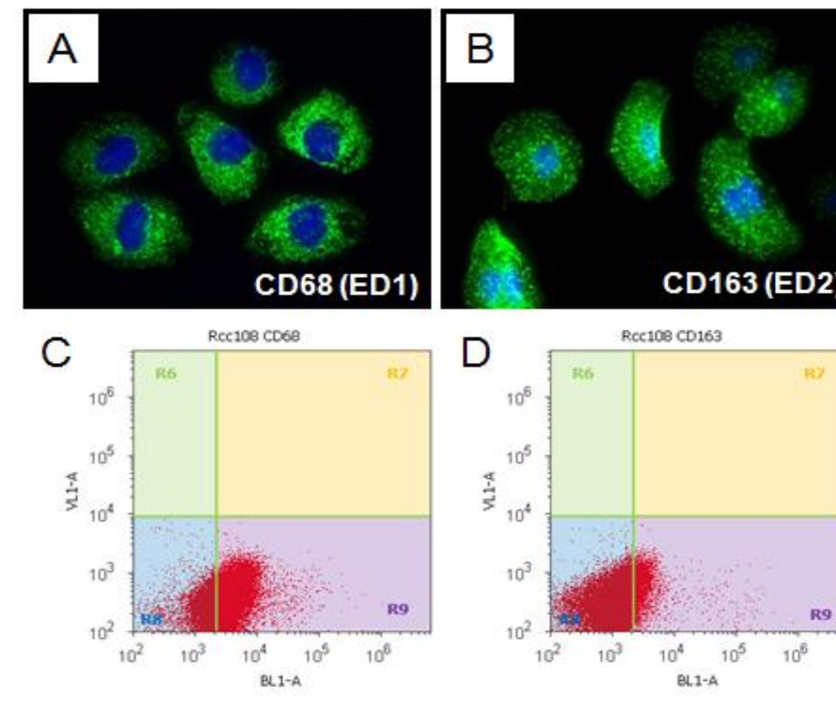

Figure 1. Characterization of isolated and cryopreserved rat Kupffer cells. A. CD68 (ED1) and B. CD163 (ED2) immuno-stained cells that support their macrophage lineage. Additional flow analysis reveals that both markers (CD68 in C and CD163 in D) are represented by the majority of the cells, suggesting very high purity of Kupffer cells (>90%).

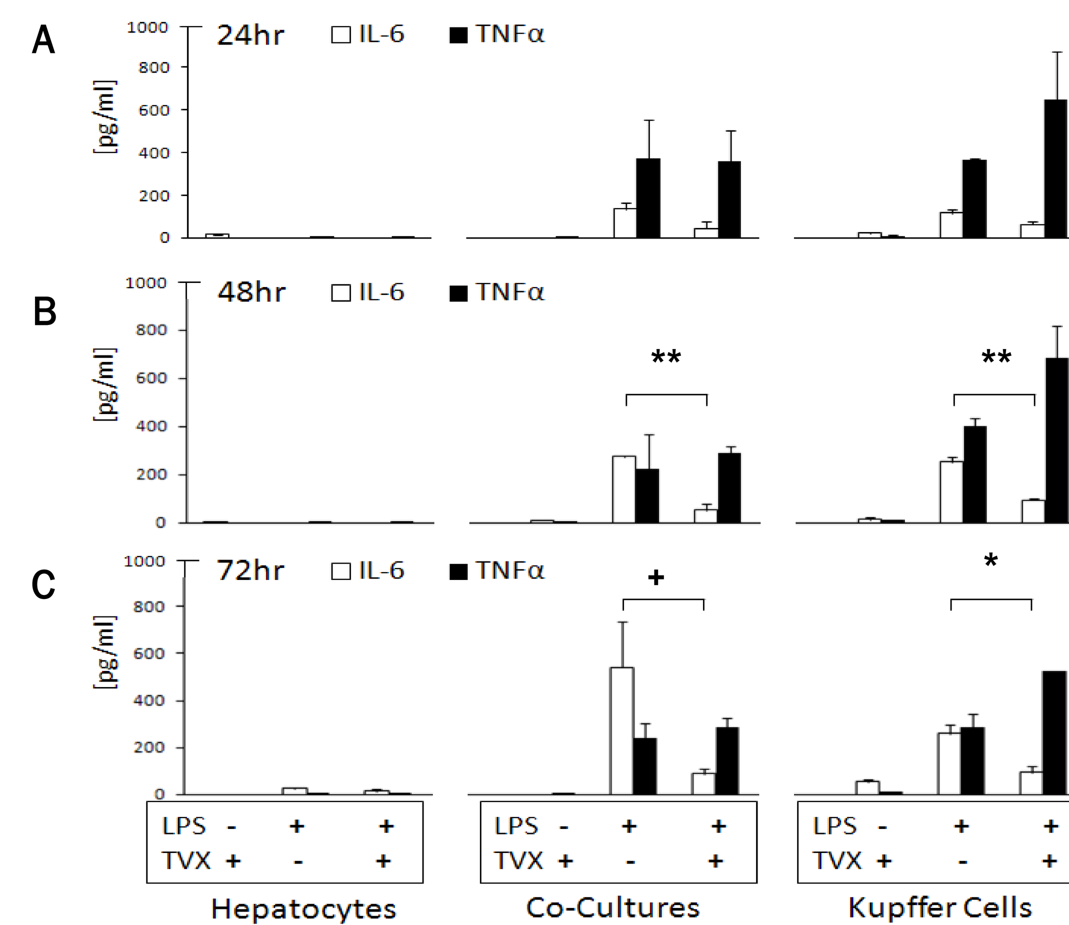

Figure 2. Treatment of co-cultures with LPS (1 µg/ml) and Trovafloxacin (TVX, 50 µM) deregulates cytokine production, leading to an abnormal accumulation of TNF-α and the inhibition of IL-6 production. A. At 24 hr, TVX/LPS and LPS co-culture and Kupffer cells treatment groups display similar ratios of IL-6 to TNF-α production. This ratio changes dramatically at 48 hr (B) and 72 hr (C) where in LPS only treatment, IL-6 increases and TNF-α remains stable, whereas in TVX/LPS, TNF-α remains abnormally high to IL-6 whose production is inhibited. This suggests a reversion of the TNF-α to IL-6 ratio where hepatotoxic TNF-α predominates, and CYP3A modulation by IL-6 is absent. Significance: + P<0.1, * P<0.05, ** P<0.01

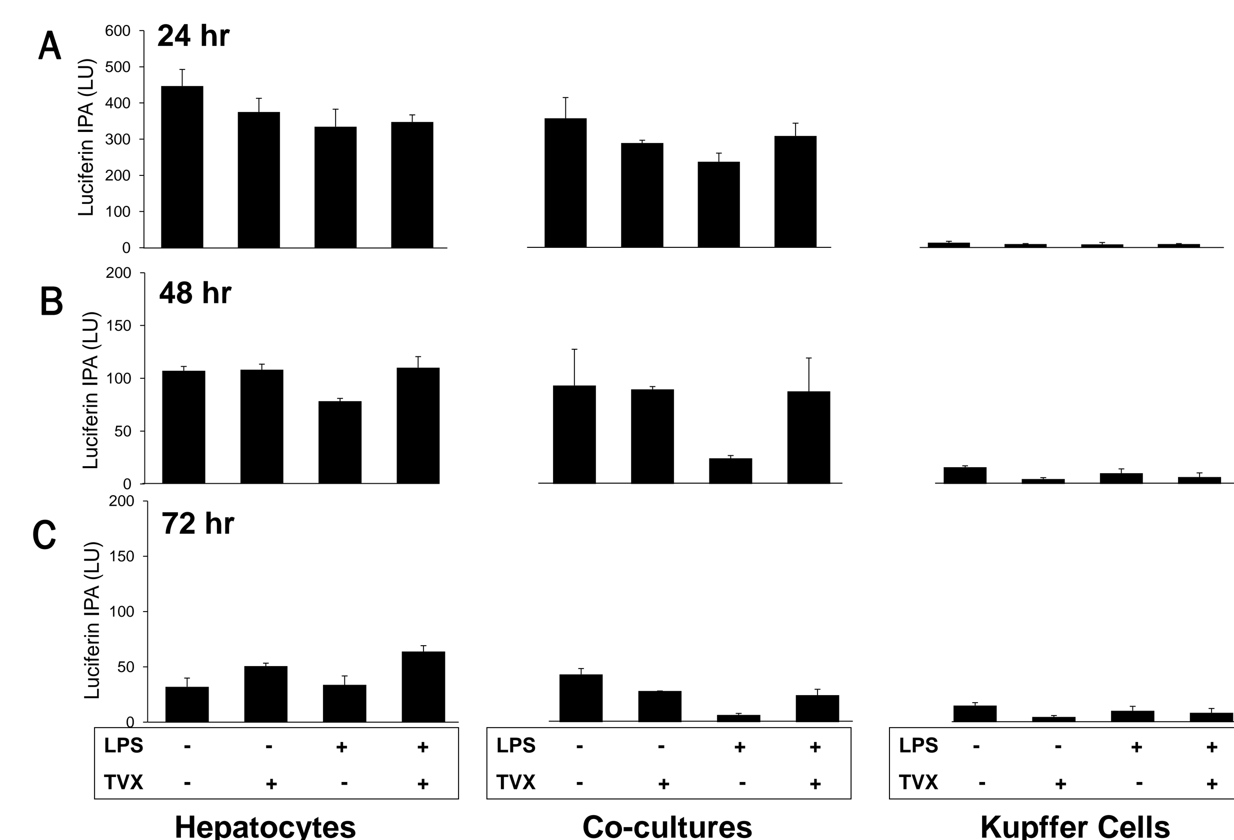

Figure 3. Treatment of co-cultures with LPS (1 µg/ml) results in an inhibition of CYP3A activity which is reversed when after co-treatment with TVX (50 µM). A. At 24 hr LPS co-culture treatment groups begin to show a decrease in CYP3A activity (34% less than control) which is reversed when TVX is added along with the LPS (14% less than control). This pattern of inhibition of activity with LPS then recovery of activity with TVX/LPS continues to a greater degree over 48 hr with 74% inhibition with LPS, 6% inhibition for TVX/LPS (B) and 72 hr with 85% decrease in CYP3A activity with LPS recovering to 44% when TVX is added along with LPS (C) . Inhibition of CYP3A activity by LPS and recovery by adding TVX was not observed in hepatocyte monocultures to the degree observed in co-cultures.

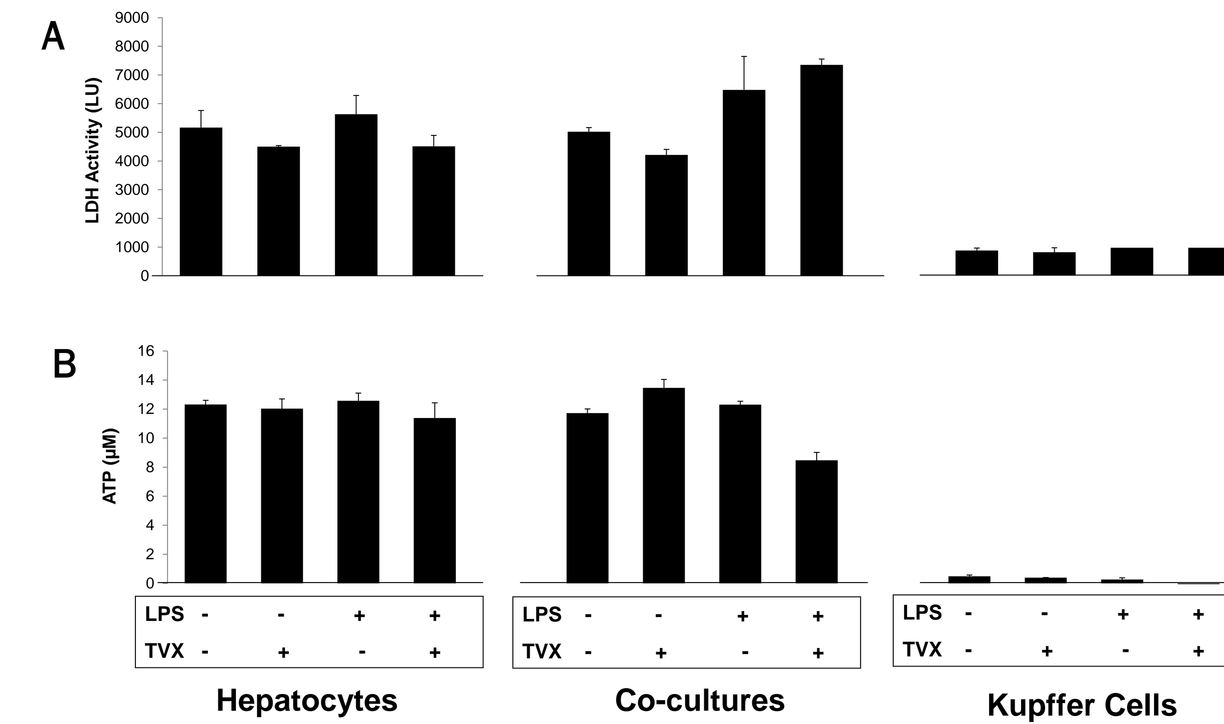

Figure 4. Treatment of co-cultures with LPS (1 µg/ml) and Trovafloxacin (TVX, 50 µM) affects LDH and ATP levels. Representative graph of LDH and ATP levels in hepatocytes, co-cultures, and Kupffer cells after 48 hr of treatment with LPS (1 µg/mL) and/or TVX (50 µM). A. LDH levels in hepatocytes and Kupffer cells show no change after treatment, while treatment of co-cultures with LPS and TVX/LPS result in an increase of 29% and 46% respectively in LDH levels over no treatment controls. B. Similarly, ATP levels were not affected in hepatocytes or Kupffer cells after treatment with LPS and/or TVX, while co-cultures exhibited a drop in ATP (28% compared to no treatment controls) when treated with TVX/LPS. Note that 24 and 72 hr treatments exhibited comparable ATP and LDH results.

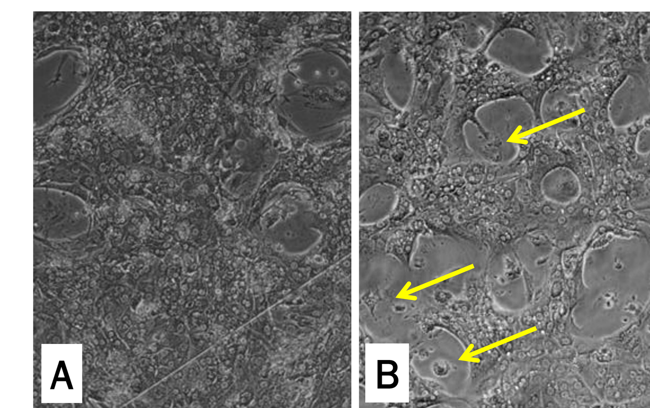

Figure 5: Increased necrotic cell death following 48 hr TVX/LPS treatment. A. Co-cultures treated with LPS (1 µg/mL) only; B. Co-cultures treated with TVX/LPS (50 µM, 1 µg/mL). Note increased appearance of necrotic foci (arrows) as compared with LPS-only group. This suggests the potential involvement of deregulated cytokines in this model of hepatotoxicity.

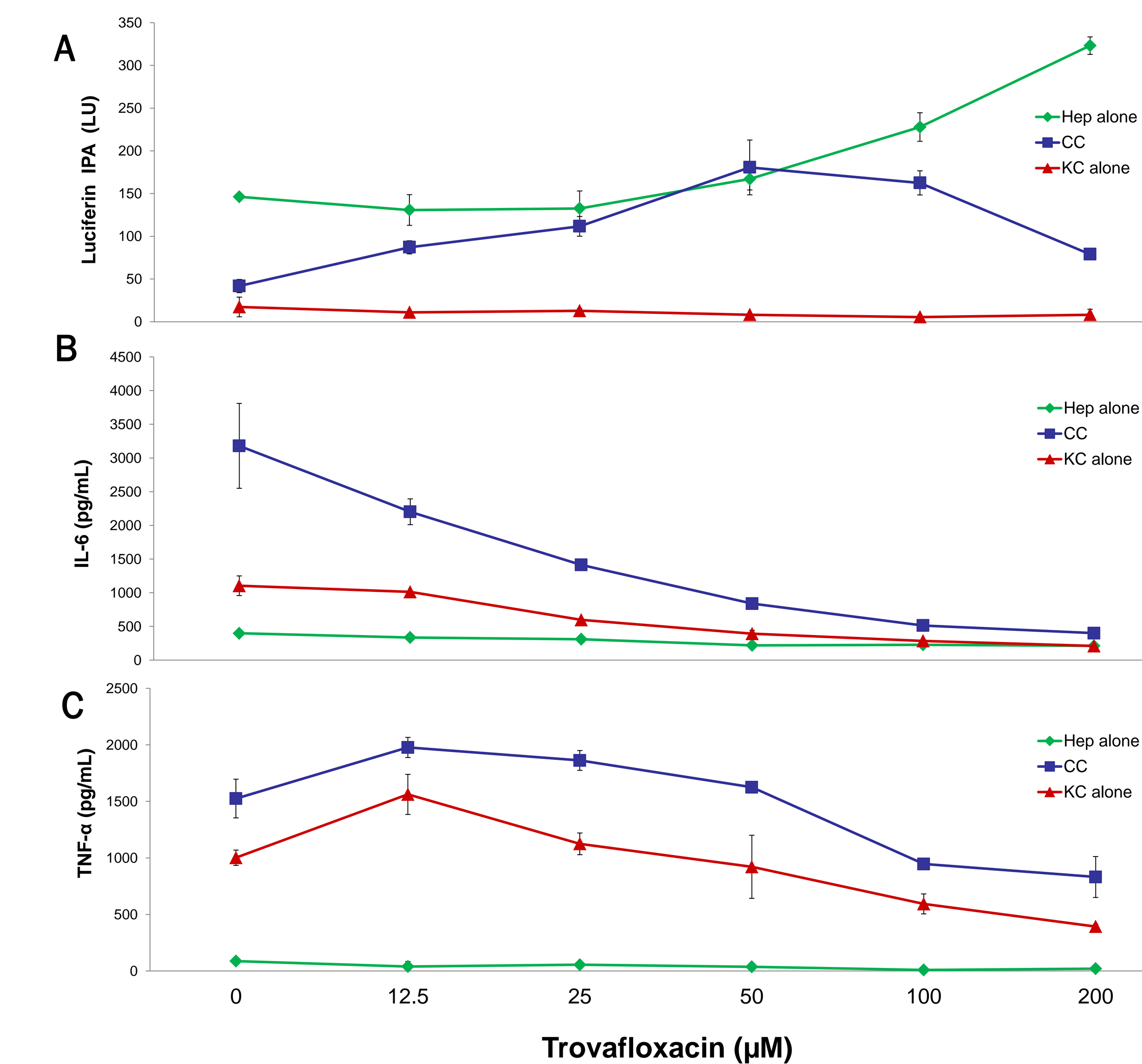

Figure 6. Treatment of co-cultures for 48 hr with LPS (1 µg/ml) and Trovafloxacin (TVX, 0-200µM) deregulates cytokine production, leading to an abnormal accumulation of TNF-α and the inhibition of IL-6 production. A. Increasing amounts of TVX in co-cultures result in a 2-4 fold increase in CYP3A activity over no TVX control with while hepatocyte monocultures exhibit a 1.5 to 2 fold increase of CYP3A activity only at the highest doses of TVX. B. IL-6 levels in co-cultures gradually decline with increasing TVX dose, appearing to cause a reversal of effects of LPS on CYP3A levels in co-cultures. Kupffer cells display a reduced but similar trend in IL-6. C. TNF-α levels show a slight increase and gradual decline with both co-cultures and Kupffer cells, suggesting TNF-α levels predominate over IL-6 levels with increasing TVX dose for co-cultures and Kupffer cells to a lesser degree. Decreasing ATP levels at 50, 100, and 200 µM TVX indicate concurrent cytotoxic effects on these measurements.

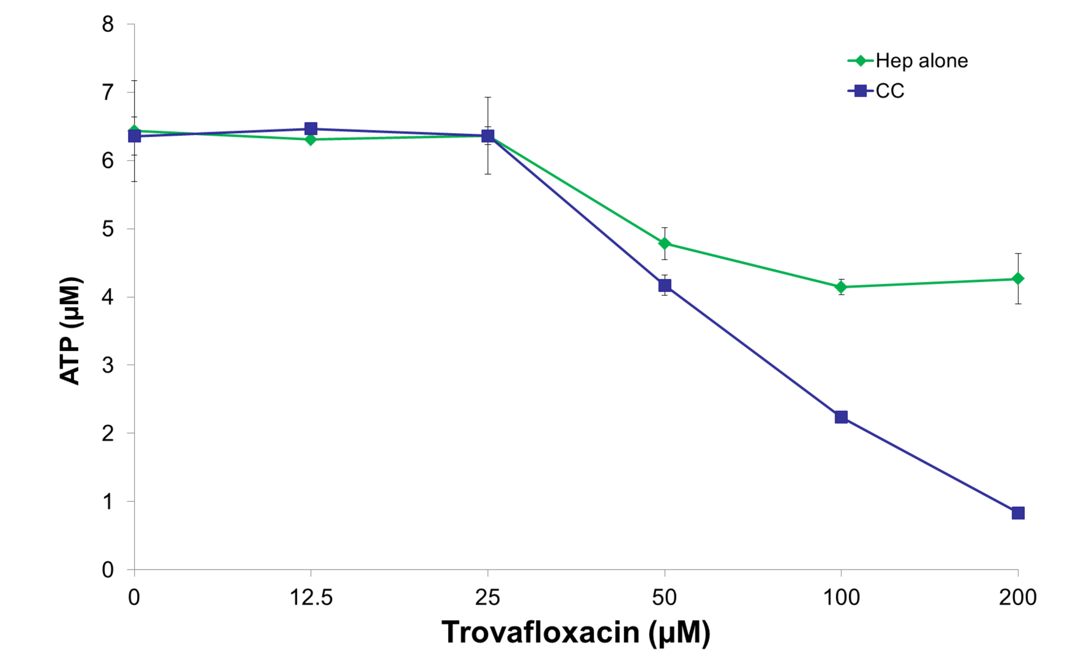

Figure 7. Treatment of co-cultures for 48 hr with LPS (1 µg/ml) and Trovafloxacin (TVX, 0-200 µM) has a dose dependent effect on ATP levels. Graph of ATP levels in hepatocytes and co-cultures after 48 hr of treatment with LPS (1 µg/ml) and TVX (0-200 µM). ATP levels decrease for both cultures at 50 µM TVX and exhibited a much sharper drop in ATP levels for co-cultures alone, likely due to the changing shifts in cytokine profiles with increasing TVX dose. Note that Kupffer cells alone had ATP levels below levels of detection and are not shown.

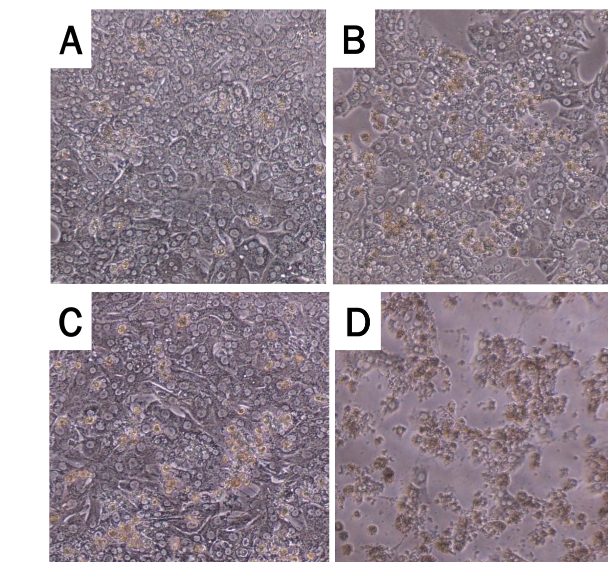

Figure 8: Increased necrotic cell death in co-cultures vs. hepatocytes following 48 hr TVX/LPS treatment. A. Hepatocytes treated with LPS (1 µg/mL) only; B. Hepatocytes treated with TVX/LPS (200 µM, 1 µg/mL). C. Co-cultures treated with LPS (1 µg/mL) only; D. Co-cultures treated with TVX/LPS (200 µM, 1 µg/mL). Co-cultures clearly exhibit effects of increased cytokine levels and decreased ATP levels compared to hepatocyte monocultures.

Conclusion

Co-cultures of hepatocytes and Kupffer cells can be used to predict chemical-induced immune reactions that result in drug hepatotoxicity. As shown in this work, co-cultures showed blunted IL-6 response and increased TNF-α production that resulted in hepatic necrosis and concurrent increases in LDH and decreases in ATP levels indicating cytotoxicity. This data supports use of hepatocytes and Kupffer cells in co-cultures as a powerful *in vitro* tool to evaluate the effects of drug toxicity that are not apparent using in vitro models of monocultured hepatocytes.

References

Available upon request.

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