USE OF HEPATOCYTE AND KUPFFER CELL CO-CULTURE MODELS IN ASSESSMENT OF CYTOCHROME P450 METABOLISM

Scott Heyward and Tara Williamson | BioreclamationIVT | Baltimore, MD

Abstract

Primary human hepatocyte cultures have long been the gold standard for assessment of liver metabolism of xenobiotic compounds. In the liver, the response to infection and inflammation by tissue-associated immune cells can alter gene expression, including cytochrome P450 (CYP) enzymes. Kupffer cells are macrophages which are part of the liver sinusoid and mediate many of these responses. Hepatocyte/Kupffer cell co-cultures offer a more comprehensive model to understand the effects of the liver inflammatory response and macrophage modulation of critical drug metabolizing enzymes for better in vivo correlation. Freshly isolated Sprague Dawley®(SD) rat Kupffer cells and hepatocytes were cultured as separate monolayers and in co-cultures at ratios ranging from physiological (1:16) to highly inflamed (1:2). Trypan blue exclusion was used to assess viability of isolated cells. The phagocytic properties of Kupffer cells are retained in culture. The specific uptake of 1um fluorescently labeled latex microbeads was used to assess purity of the Kupffer cells. Cells were cultured for 24 hours prior to initiating experiment. Bacterial endotoxin, lipopolysaccaride (LPS) was used to stimulate production of cytokine by Kupffer cells. Kupffer/hepatocyte co-cultures, at a ratio of 1:4 respectively, were exposed to increasing concentrations of LPS ranging from 0.1ug/mL to 10ug/mL. Samples were taken 24-hour post-LPS exposure and analyzed for levels of the pro-inflammatory cytokine IL-6 using ELISA. Following normal metabolism induction procedures, hepatocyte only cultures and 1:2, 1:4, 1:8 and 1:16 Kupffer/hepatocyte co-cultures were exposed to known inducers of CYP1A and CYP3A for 48 hours prior to substrate incubation and sample collection. Metabolite formation was determined using LCMS to measure enzyme activity. MTT was run in parallel to monitor viability of cultures throughout experiment. Specific uptake of 1 micron fluorescently labeled microbeads by freshly isolated Kupffer cells showed >90% purity, as indicated by flow cytometry. Isolated Kupffer cells in culture showed positivity for the macrophage marker CD163 (ED2) by immunofluorescence. Cytokine quantitation using ELISA showed increased production of IL-6 (pg/mL) with increasing Kupffer cell content, with the highly inflamed ratio 1:2 producing the highest levels of cytokine. ELISA results also showed a concentration dependent, linear increase in IL-6 production with increasing concentrations of LPS. Cultures were treated with and without LPS for 24 hours followed by induction for 48 hours with dexamethasone and beta-napaflavone. Induction of CYP1A and 3A were assessed by metabolism of protypical substrates. Both 1A and 3A substrate metabolism activity showed significant reduction in CYP gene induction in cultures of Kupffers/hepatocytes. In addition, this reduction was dependant on the ratio of Kupffer cells to hepatocytes. These data indicate that defined in vitro cultures of Kupffer cells and hepatocytes can be obtained and used to better define the impact of immune mediators on liver metabolism.

Introduction

Kupffer cells are the resident macrophage population in the liver, where they reside within the lumen of the liver sinusoid and mediate a number of immune functions. They were first identified by Karl Wilhelm von Kupffer , for whom they are eponymously named (see photo below), though some contention remains.[1,2] Kupffer cells account for approximately 4-8% of the liver cellularity and are the largest pool of macrophages in the body.[3] They are exposed to a significant amount of bacteria, microbial debris and absorbed compounds from the gut due to their primary access to portal vein flow. Kupffer cells have a phagocytic property which allows them to take up large particles, with greater capacity than sinusoidal endothelial cells.[4,5] They are able to respond to bacterial lipopolysacharide(LPS) and virus utilizing toll like receptor(TLR) signaling.[6] They exercise their role in immune mediation through response to immune mediators such as interlukin-2(IL-2) or bacterial endotoxin and concomitant release of IL-6, tumor necrosis factor alpha(TNFα), IL-1, and IL-10.[7,8] The

It has been shown that cytokines can have an effect on the expression of cytochrome P450s in the liver. [9,10] These alterations in expression can have significant metabolic effect on disease states, immune pathology and drug safety. Isolated hepatocytes have long been used for the investigation of a number of hepatic functions *in vitro*. They are used extensively in drug development to model drug clearance, utilizing the intact metabolic and transporter pathways. They are also applied to toxicity using the same intact systems. In order to improve predictive power and explore more complicated questions of drug-drug interaction and toxicity culture systems have been developed which include a broader complement of cell types present in the liver. *In vitro* hepatocyte cultures, co-cultured with other cell types , such as Kupffers are becoming more common. Using these co-culture systems , questions involving the interactions between immune mediators and drug metabolism and toxicity can be explored.[11,12]

In this poster we explore hepatocyte : Kupffer cell co-culture models and explore their effectiveness in modeling immune : metabolic interactions. We explore the ability of both cell types to retain their key functional characteristics. We show the utility of this co-culture model in answering question key to drug development.

Karl Wilhelm von Kupffer (Nov. 14,1829-Dec. 16, 1902)

Methods

Kupffer Cell Isolation: Sprague Dawley rat livers were digested using a standard two step perfusions. Hepatocytes were separated from the non-parenchymal fraction which underwent further proprietary steps to generate a pure population of Kupffer cells. Kupffer cell populations were assessed for purity by cell morphology and their ability to take up fluorescently labeled 1um latex beads (Fluoresbrite® Polychromatic Red Microspheres, Polysciences Inc, Warrigton PA). Co-cultures were generated by recombining cell populations at specified ratios and plated on collagen I coated plates. Cells were cultured with a custom media formulation at 37C and 5%CO2 in a humidified incubator for 24-48 hours prior to addition of LPS or cytochrome p450 inducers.

Kupffer Cell Staining:

Microspheres: Fluoresbrite® Polychromatic Red Microspheres were mixed with cells either in suspension or in plated cells at a concentration of 50 beads/ cell. Cells were incubated with spheres for 1hr to allow uptake, then washed two times with Phosphate Buffer Saline. Visualization was performed with a Guava PCA (Millipore, Billerica, MA), with normalization perfomed using unstained cells. Data was analyzed using FCS Express 4 Plus (De Novo Software, Los Angeles, CA) Plated cells were visualized on a Nikon TE200 scope using phase contrast optics.

ED2 Immunofluorescence: Cells were plated on collagen coated Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) and were cultured in parallel to standard experimental conditions until stained. Cells were washed in PBS and fixed in 4% paraformaldehyde for 10min at room temperature (RT). Cells were washed three times for 5min each in PBS, followed by permeabilization with 0.1% Triton X-100 in PBS for 4min. Cells were rinsed in PBS containing 1% BSA. Staining was performed with a 1hr incubation in the primary antibody, CD163 (ED2) (Santa Cruz Biotechnology Inc., Santa Cruz, CA (all antibodies were obtained here) or a mouse IgG1 isotype control at 4ug/ml. Slides were washed and stained with a goat anti-mouse IgG-FITC antibody at 1:100 for one hour. Slides were coated in mounting solution containing DAPI and imaged on a Nikon TE200 with phase contrast optics

IL-6 ELISA: Supernatents were collected from cell culture wells at the indicated time and treatments and stored frozen until tested. Supernatents were centrifuged at 190xg for 10minutes to pellet cell debris. A Rat IL-6 Elisa kit (RnD Systems, Minneapolis, MN) was used according to manufactures instructions.

Metabolism and Induction: Inducers of CYP450 were incubated in serum free media for 48hrs with media containing inducers replaced daily. Inducer stocks were made with acetonitrile and this was used in the vehicle control. Substrates were added to cultures and incubated for 4 hours before being collected in an equal volume of methanol. Metabolites were analyzed by LCMS. MTT was performed on parallel well to assess viability differences between conditions.

Cytochrome P450 Isoform	Inducer	Substrate	Metabolite Analyzed
CYP3A	50uM Dexamethasone	125uM Testosterone	6-Beta-OH Testosterone
CYP1A	10uM Beta-Naphthaflavone	100uM Phenacetin	Actetaminophen

Kupffer Cell Markers

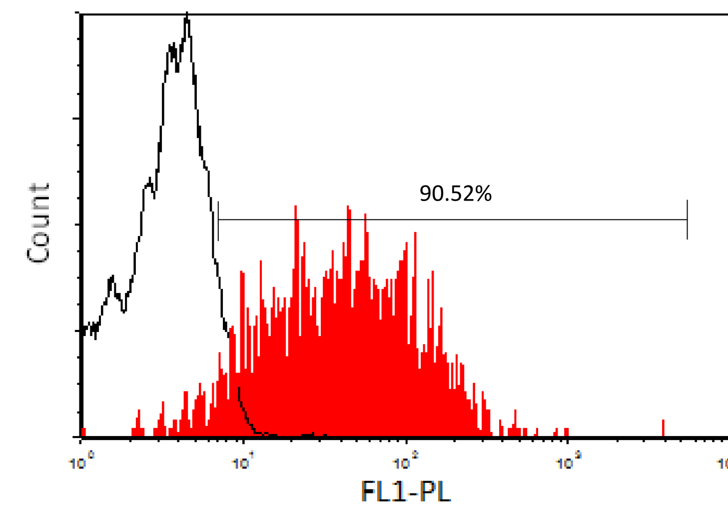

Figure 1. Flow cytometric analysis of the uptake of 1um latex beads by Kupffer cells in freshly isolated rat Kupffer cells.

Fluorescently labeled microbeads which had a mean diameter of 1um were added to cell suspensions at a ratio of 50:1 and allowed to incubate for 1 hour before being washed twice with media prior to analysis. Cells were analyzed on a Guava PCA and compared to unlabeled populations. Histogram plot displays intensity of fluorescence against number of events at that intensity. Difference in gated populations is indicated.

Results: Hepatocytes remained unlabeled (data not shown) while Kupffer cells took up beads with >90% penetrance indicating high purity isolated Kupffer cells. Bead uptake indicates that the natural phagocytic activity of large beads is retained in isolated Kupffer cells.

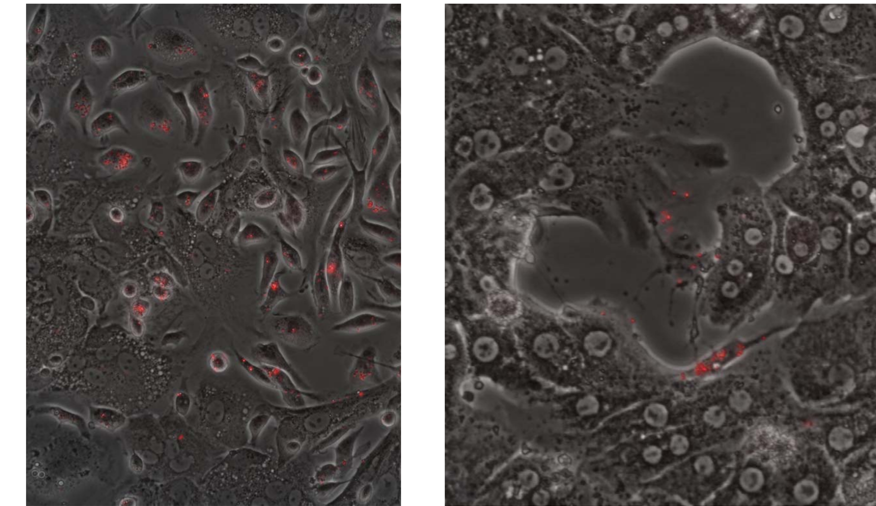

Figure 2. Uptake of 1um latex beads by Kupffer cells in co-culture with hepatocytes.

Kupffer cells were plated with hepatocytes at a ratio of 1:1(left image) or 1:4(right image) and cultured for 72 hours before the addition of fluorescently labeled beads. Beads were added at a ratio of 10:1 with Kupffer cells and allowed to incubate for 1 hour before being washed twice with media. Cells were imaged with both phase contrast and with (filter) then the images merged.

Results: Hepatocytes remained unlabeled while Kupffer cells took up beads with high specificity. Bead uptake indicates that the natural phagocytic activity of large beads is retained in Kupffer cells in culture.

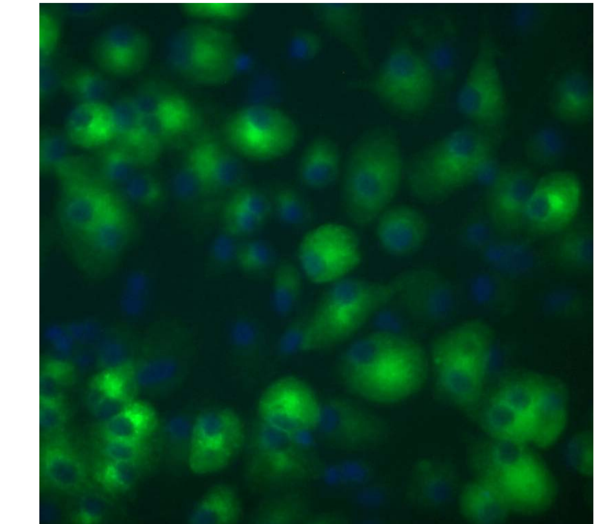

Figure 3. ED2 (CD163) Immunofluorescence in cultured Kupffer cells.

Isolated rat Kupffer cells were plated on chamber slides and cultured for 72 hours prior to fixation with 4% paraformaldehyde. Fixed cells were stained with a mouse anti-rat ED2 antibody and a FITC secondary antibody to mouse IgG1. Isotype control antibody was used on parallel wells and gave negligible background staining. Nuclei were stained blue with a DAPI counterstain. Cells were visualized by fluorescent microscopy and a merged image created.

Results: Cells showed a high proportion of staining confirming the presence of highly enriched Kupffer cells which retain appropriate markers in culture.

LPS Induced IL-6 Synthesis

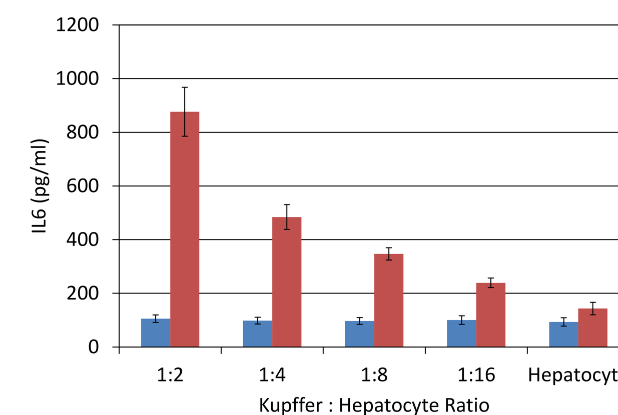

Figure 4. IL-6 secretion by cultured Kupffer : hepatocyte co-cultures in response to LPS.

Hepatocyte and Kupffer co-cultures at varying ratios from 1:2 to 1:16 respectively or hepatocytes alone were incubated for 48hours then treated with LPS at 1ug/ml or with media alone. IL-6 was generated by ELISA and quantitated by the use of a standard curve of IL-6 protein.

Results: Increasing numbers of Kupffer cells with respect to hepatocytes (lower ratios) showed an increasing response to LPS as measured by IL-6 production. The Kupffers cells remain functioning in culture and allow for Kupffer cell contribution to the inflammatory response to be modeled in *in vitro* systems.

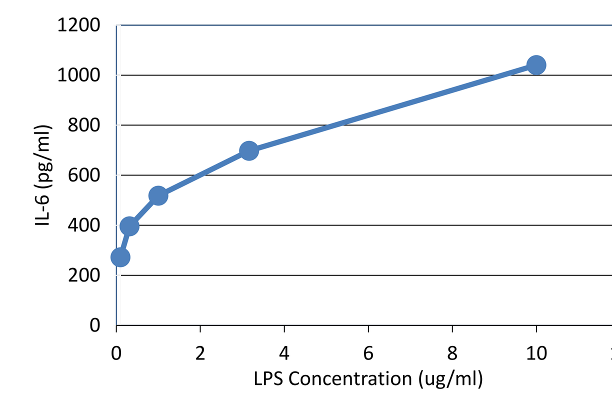

Figure 5. Dose dependant IL-6 secretion by cultured Kupffer : hepatocyte co-cultures in response to LPS.

Hepatocyte and Kupffer co-cultures at a ratio of 1:4 were incubated for 48hours then treated with LPS at concentrations from .1 - 10ug/ml. After 24 hours supernatants were collected and analyzed by ELISA for rat IL-6. Values were quantitated by the use of a standard curve and plotted against concentrations used for stimulation.

Results: The stimulation of Kupffer cells in culture by LPS to produce IL-6 showed dose dependence with saturating properties. This data indicates a functioning Kupffer cell phenotype able to produce cytokines in response to exogenous stimuli (bacterial endotoxin) in a manner similar to that *in vivo*.

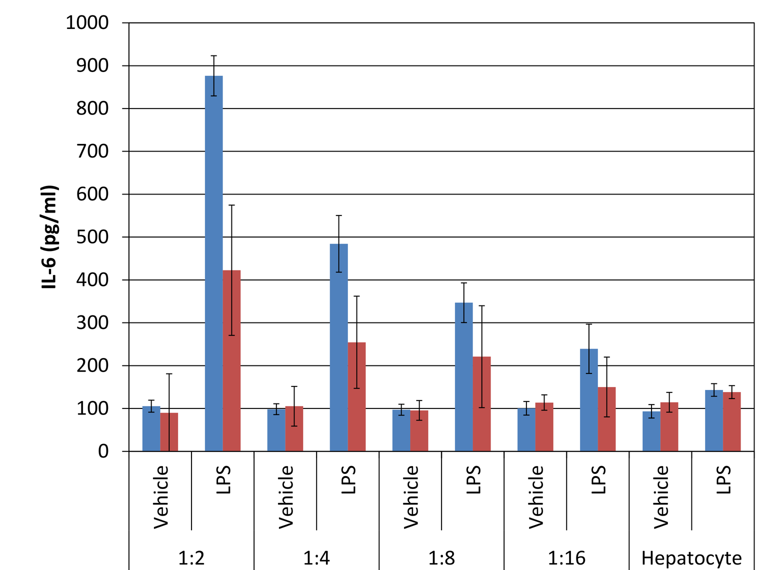

Figure 6. Time dependant secretion of IL-6 by Kupffer : hepatocyte co-cultures in response to LPS.

Hepatocyte and Kupffer co-cultures at varying ratios from 1:2 to 1:16 respectively or hepatocytes alone were incubated for 48 hours then treated with LPS at 1ug/ml or with media alone. Supernatants were collected at 24 hours and media with LPS replaced for an additional 24hour incubation with a final 48 hours post treatment collected. IL-6 was measured by ELISA and quantitated with the use of a standard curve of IL-6 protein.

Results: IL-6 as was shown to increase with increased Kupffer cell content after the first 24 hours of exposure to LPS. The level of IL-6 for each Kupffer: hepatocyte ratio decreases from 24 to 48 hours possibly due to a feedback response. These results show the need for precise experimental design when looking at Kupffer mediated responses.

Kupffer Cell Impact on Metabolism

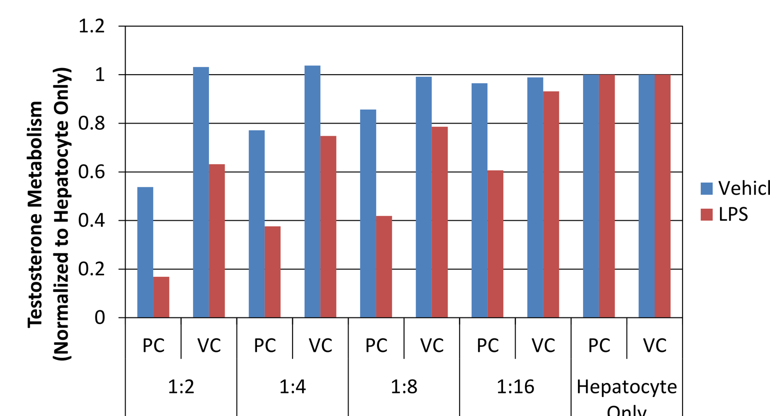

Figure 7. Impact of LPS stimulation of Kupffer : hepatocyte co-cultures on basal and induced CYP3A metabolism.

Hepatocyte and Kupffer co-cultures at varying ratios from 1:2 to 1:16 respectively or hepatocytes alone were incubated for 48 hours then treated with LPS at 1ug/ml (red bars) or with media alone (blue bars). Media was exchanged to include dexamethasone or a vehicle control (acetonitrile) and incubated under these cytochrome p450 inducing conditions for two days. An incubation with 125uM testosterone was performed and production of 6-B-OH testosterone was measured by HPLC. Results were normalized to that in the hepatocyte only wells for each condition.

Results: Kupffer cell cultures showed a reduction in CYP3A expression as measured by testosterone metabolism in a ratio dependant manner with those having the lowest hepatocyte to Kupffer ratio (1:2) showing the greatest impact. Both VC and PC treated with LPS showed larger reduction than the media only treated. Induction in the induced(PC) non-LPS exposed wells also showed a background, ratio dependant, reduction in metabolite formation.

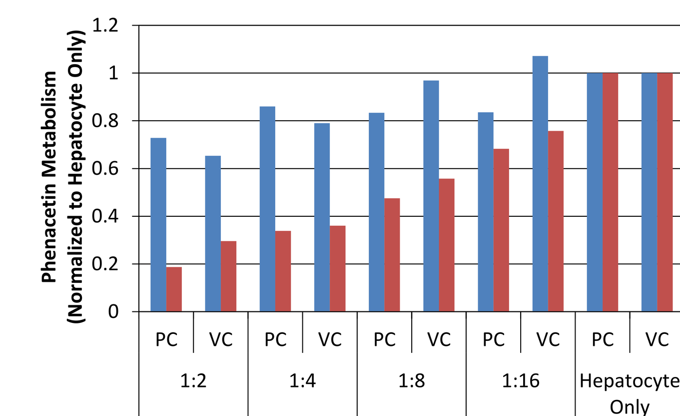

Figure 8. Impact of LPS stimulation of Kupffer : hepatocyte co-cultures on basal and induced CYP1A metabolism.

Hepatocyte and Kupffer co-cultures at varying ratios from 1:2 to 1:16 respectively or hepatocytes alone were incubated for 48 hours then treated with LPS at 1ug/ml (red bars) or with media alone (blue bars). Media was exchanged to include beta-naphthaflavone or a vehicle control (acetonitrile) and incubated under these cytochrome p450 inducing conditions for two days. An incubation with 100uM phenacetin was performed and production of acetaminophen was measured by HPLC. Results were normalized to that in the hepatocyte only wells for each condition.

Results: Kupffer cell cultures showed a reduction in CYP1A expression as measured by phenacetin metabolism in a ratio dependant manner with those having the lowest hepatocyte to Kupffer ratio (1:2) showing the greatest impact. Both VC and PC treated with LPS showed larger reduction than the media only treated. Induction of activity in the non-LPS exposed wells also showed a background ratio dependant reduction in metabolite formation.

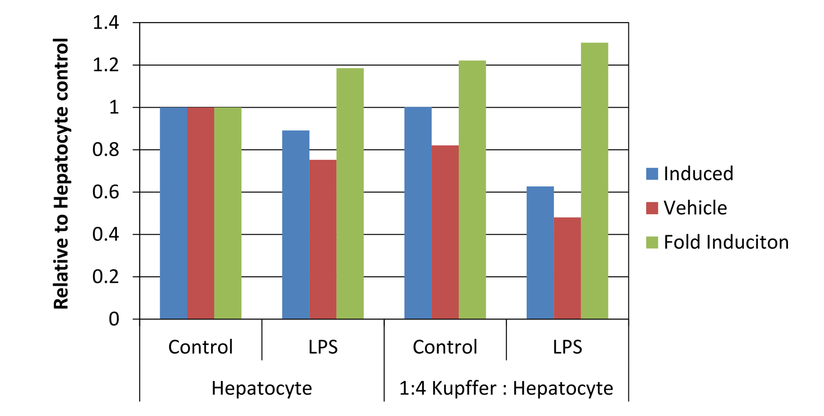

Figure 9. Impact of LPS stimulation of Kupffer : hepatocyte co-cultures on CYP1A metabolism and induction.

Hepatocyte and Kupffer co-cultures at varying ratios from 1:2 to 1:16 respectively or hepatocytes alone were incubated for 48 hours then treated with LPS at 1ug/ml (red bars) or with media alone (blue bars). Media was exchanged to include beta-naphthaflavone or a vehicle control (acetonitrile) and incubated under these cytochrome p450 inducing conditions for two days. An incubation with 100uM phenacetin was performed and production of acetaminophen was measured by HPLC. Results were normalized to that in the hepatocyte only wells for each condition.

Results: CYP1A activity is reduced in co-cultures when exposed to LPS. This reduction occurs in both the presence and absence of inducer. The relative similar change in the induced and vehicle can result in similar fold induction values for LPS treated and un-treated, masking the significant impact on CYP activity.

Conclusions

- **Kupffer cells can be isolated from SD Rat livers at high purity.**
- **Kupffer cells can be visualized using the macrophage specific marker ED2 or fluorescent 1um beads in culture**
- **Functional Co-cultures can be established with Kupffer cells and heptocytes**
- **IL-6 produced by Kupffer cells in repsonse to LPS is dependant on the Kupffer cell content of the co-culture.**
- **Kupffer cell co-cultures produce IL-6 in response to bacterial LPS in a dose dependant manner.**
- **Metabolism of CYP3A and CYP1A substrates is reduced in co-cultures treated with LPS**
- **Reduction of CYP450 metabolism in co-cultures by LPS is present in both cultures untreated and exposed to inducers.**
- **Fold induction can mask cytochrome P450 expression changes in hepatocyte : Kupffer cell co-cultures.**

References

1. Wake K., **Karl Wilhelm Kupffer And His Contributions to Modern Hepatology.** Comparative Hepatology (2004) 3(Suppl):S2.
2. Sródka A., et al. **Browicz or Kupffer Cells?.** Polish Journal of Pathology 2006, 57 (4) : 183–185
3. Hepatology Vol 43, S1: S54-S62
4. Ogawara K. et al. **Hepatic uptake of polystyrene microspheeres in rats: effect of particle size on intrahepatic distribution.** J. Controlled Rlease (1999) 59: 15-22.
5. Kamimoto M. et al. **Uptake ability of hepatic sinusoidal endothelial cells and enhancement by lipopolysaccharide.** Biomedical Research(2005) 26 (3): 99-107
6. Tu Z. et al. **TLR-dependent cross talk between human Kupff er cells and NK cells.** Journal of Experimental Medicine (2008) 205(1): 233-244.
7. Dahle M.K. et al. **The Phosphatidylinositol 3-Kindase/Protein Kinase B Signaling Pathway is Activaed by Kupffer Cell Produciton of Interlukin-6(IL-6) and IL10.** Infection and Immunity (2004) 72(10):5704-5711.
8. Su GL. **Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation.** Am J Physiol Gastrointest LiverPhysiol (2002) 283: G256-G265
9. Aitkin and Morgan. **Gene-Specific Effects of Inflammatory Cytokines on Cytochrome P450 2C, 2B6 and 3A4 mRNA Levels in Human Hepatocytes.** DMD 35:1687–1693, 2007
10. Sunman J.A., et al. **Kupffer Cell Mediated IL-2 Supression of CYP3A Activity in Human Hepatocytes.** Drug Metabolism and Disposition (2004) 32: 359-363.
11. Bilzer M., et. al. **Liver International (2006) : 26: 1175-1186.**
12. Toyoda Y. et al. **Mechanism of Exacerbative Effect of Progesterone on Drug-Induced Liver Injury** Toxicol. Sci. (2012) 126(1): 16-27
13. Porträt, Mit (1903). "Karl v. Kupffer". *Archiv für Mikroskopische Anatomie* **62**: 669. doi:10.1007/BF02985556.