Co-culture of Cryopreserved Primary Rat and Human Kupffer Cells and Hepatocytes to Study APAP Cytotoxicity

Gemma L. Mendel, Todd W. Stewart, Kirsten B. Amaral, Adam M. Farmer, Rachel M. Witek, Manda Edwards, Kimberley Freeman, Erica Deibert, Brian J. Paszkiet, Jonathan P. Jackson, Stephen S. Ferguson, Rafal P. Witek.

Life Technologies, Durham, NC, 27703

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ABSTRACT

Introduction: Kupffer cells (KCs) directly interact with hepatocytes during liver inflammation by modifying immunity and xenobiotic metabolism. KCs are known to suppress the expression of multiple cytochrome P450 enzymes following activation with lipopolysaccharide (LPS). Since KCs are involved in liver diseases that are characterized with inflammation including viral and autoimmune hepatitis and non-alcoholic steatohepatitis (NASH), there is a need for a KCs and hepatocyte co-culture model for drug discovery and ADME/Tox applications. This study hypothesizes that the use of cryopreserved rat and human KCs in co-culture with hepatocytes will recreate liver inflammation and model acetaminophen (APAP) cytotoxicity observed during liver inflammation. **Methods:** Fresh and cryopreserved Kupffer cells were characterized by morphology, plateability, phagocytosis, immunohistochemistry staining for CD68 and CD163, and cytokine release for IL-6 and TNFα by ELISA in response to 24hrs treatment with 1μg/mL LPS. KCs and hepatic co-cultures were cultured in DMEM medium supplemented with non-essential amino acids (NEAA), Insulin-Transferring-Selenium (ITS+) and Penicillin/Streptomycin. For plating and maintenance media, additional 10% or 2% fetal bovine serum (FBS) was used, respectively. Since corticosteroids inhibit inflammation, dexamethasone or hydrocortisone were not used with KCs. For experiments with APAP cytotoxicity, inflammatory ratios of approximately 1:2-1:4 KCs to hepatocytes were used. Co-cultures were cultured for 24 hours and then pre-treated with 1μg/mL LPS for 24, 4 and 0 hours prior to addition of 10mM APAP for additional 24hr in the same media containing LPS. Cells were evaluated for viability using PrestoBlue™ reagent, morphology and cytokine release (e.g. IL-6, TNFα) by ELISA. Real time qRT-PCR was performed to establish gene expression changes in metabolism, inflammation and viability. **Results:** Fresh and cryopreserved rat KCs were found to be similar after comparison of morphology, plateability, culture purity via immunofluorescent staining. The purity of KCs was found to be at 85.6% (±1.9 SE) for CD68 and at 93.5% (±0.4 SE) for CD163, commonly recognized cell surface markers of KCs. Using cytokine panel, activated fresh and cryopreserved co-cultures revealed comparable upregulated expression of IL-1α, IL-1β and IL-2 by at least two fold, and IL-6 IL-10 and TNFα by at least four fold; conversely, they revealed down regulated expression in IL-4 and IL-12 by at least two fold. For the cytotoxicity experiments, viability and morphology data analysis revealed time-dependent hepato-protection in co-cultures at 4 hours pre-treatment with LPS, but not at 0 and 24 hours. A strong correlation was observed with cytokine release of IL-6 and TNFα, and expression of *CYP1A2* and *CYP2E1*. **Conclusions:** Cryopreserved KCs can maintain known characteristics and functions as compared to freshly isolated cells. APAP cytotoxicity data show that these cells can be effectively used for modeling liver inflammation and reveal hepato-protective role of KCs as shown at 4 hour pre-treatment with LPS. Cryopreserved KCs when co-cultured with hepatocytes, provide a powerful *in vitro* ADME/Tox tool to evaluate the effects of xenobiotic metabolism during inflammation.

INTRODUCTION

The largest solid organ in the human body, the liver is responsible for diversity of function with the most important being metabolism, detoxification and protein synthesis. Most of those functions take place within hepatocytes which represent a major cell type of the hepatic parenchyma; however, the liver also contains a large proportion of non-parenchymal cells (NPCs), which include liver sinusoidal endothelial cells, hepatic stellate (ito) cells, cholangiocytes and Kupffer cells. In general, NPCs provide physical and biochemical structure to the liver. Out of all NPCs, Kupffer cells, which are the liver resident macrophages, play an important role in liver physiology and homeostasis by participating in the acute and chronic responses of the liver to toxic compounds during liver inflammation.

In the liver, Kupffer cells are located on the sinusoidal side of hepatic parenchyma and use their stellate like cytoplasmic extensions for direct cell-to-cell contact with hepatocytes. This contact is essential for proper modulation and the development of a fulminant hepatic inflammatory response. In addition to cell contact, activation of Kupffer cells results in the release of a variety of inflammatory cytokines and growth control mediators that suppress the expression of multiple cytochrome P450 enzymes. Two of those factors, Interleukin 6 (IL6) and Tumor Necrosis Factor-a (TNFa) are known to induce the synthesis of acute phase proteins and suppress CYP1A2, CYP2C19 and CYP3A activities. In addition, it has been shown that IL10, an anti-inflammatory cytokine produced during inflammation by Kupffer cells, suppresses CYP2E1, a major P450 involved in APAP metabolism.

MATERIALS AND METHODS

Kupffer cell isolation: Primary rat and human Kupffer cells were isolated using a proprietary enzymatic digestion method developed at Life Technologies. Using this technique, Kupffer cells can be isolated at viabilities higher than 98% and purities of approximately 90% (Rat) and 60% (Human) as determined by immune stain for ED1 (CD68) and ED2 (CD163) markers. Contaminating cells represent populations of endothelial cells, fibroblasts, hepatocytes, and Kupffer cells that did not stain sufficiently to be counted. Isolated Kupffer cells attach within 15-30min and display macrophage morphological characteristics within 24hr. They become activated and ready for experiments within 48hr after plating.

Co-Cultures: Hepatocytes (obtained from Life Technologies, CA) and Kupffer cells were co-cultured in 24-well plates at ratios of 1:2 of Kupffer cells/hepatocytes to approximate physiological inflammatory liver state. Additional 1:4, 1:8 and 1:16 ratios were used to determine linearity of inflammatory response. DMEM supplemented with NEAA, ITS+, 2% (for maintenance) and 10% (for plating) FBS, P/S was used in co-culture and changed every 24 h. No corticosteroids (dexamethasone or hydrocortisone) were used.

Data Analysis: To activate inflammatory response in Kupffer cells, co-cultures were treated with either LPS (1ug/ml), IL2 (200ng/ml) and APAP (10mM). Data analysis was performed using ELISA assays, IHC, qRT-PCR .

RESULTS

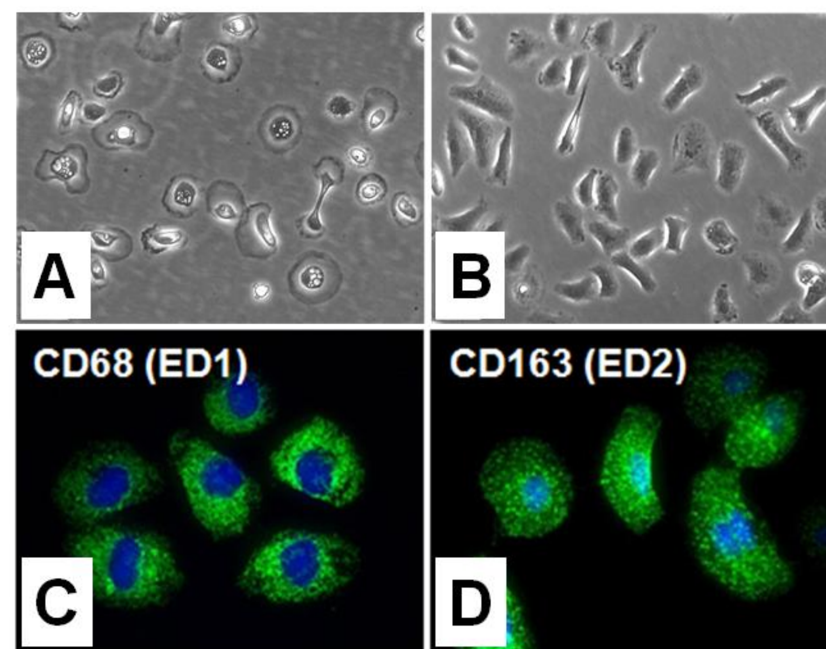
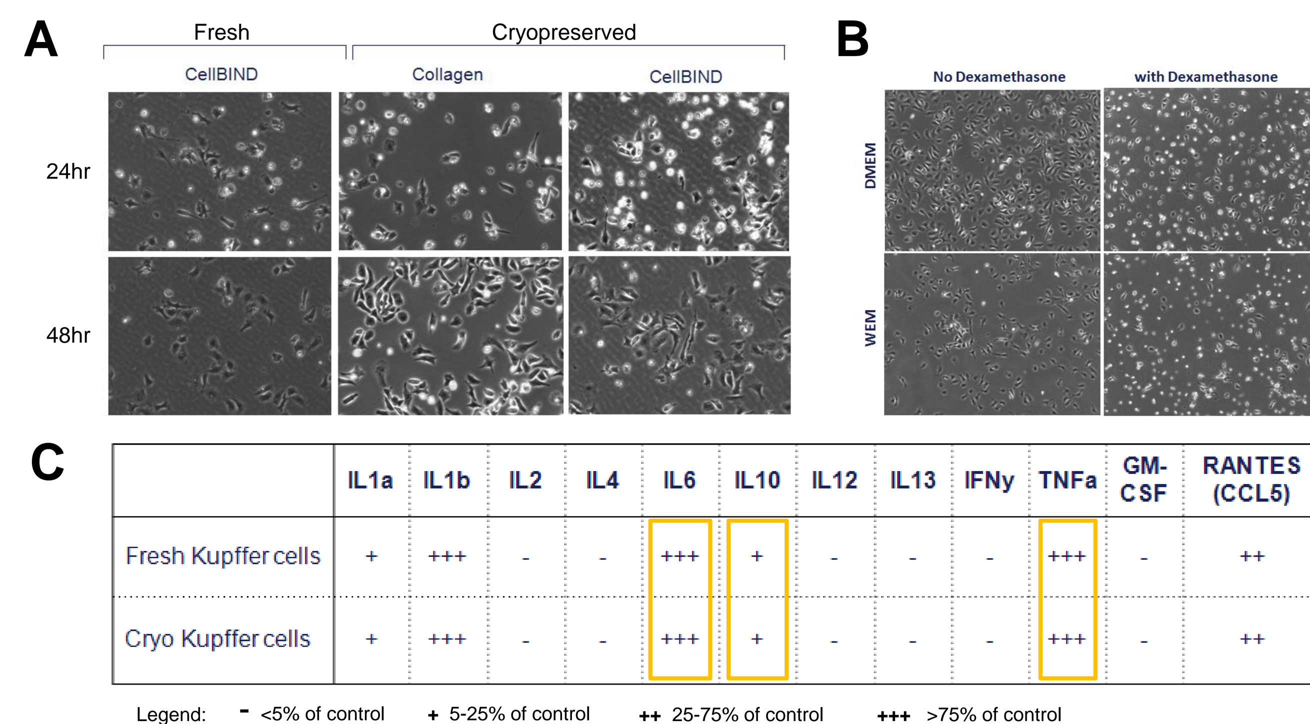


Figure 1. Morphological and immuno-histological characterization of primary rat Kupffer cells. A At 2hr after plating, rat Kupffer cells display fried egg like morphology of immature macrophages; **B** Within 24hr after plating, rat Kupffer cells expand and activate to their mature macrophage-like phenotype; **C-D**. Immune characterization of Kupffer cells at 48hr after plating reveal presence of CD68, a classical marker of macrophages (**C**) and CD163, marker of activated macrophages (**D**).



	IL1a	IL1b	IL2	IL4	IL6	IL10	IL12	IL13	IFNy	TNFa	GM-CSF	RANTES (CCL5)
Fresh Kupffer cells	+	+++	-	-	+++	+	-	-	-	+++	-	++
Cryo Kupffer cells	+	+++	-	-	+++	+	-	-	-	+++	-	++

Legend: - <5% of control + 5-25% of control ++ 25-75% of control +++ >75% of control

Figure 2. Culture of cryopreserved primary Kupffer cells. A Characterization of fresh rat Kupffer cells plated on CellBIND shows morphological similarities with cryopreserved Kupffer cells plated on either CellBIND or Collagen type I; **B.** Fresh and cryopreserved Kupffer cells can be cultured in modified hepatocyte growth medium without dexamethasone. In presence of dexamethasone Kupffer cells detach and die. C. Cytokine panel showing similar inflammatory response of fresh and cryopreserved Kupffer cells following 24hr treatment with LPS. Note high level expression of IL6, TNFa and IL10, major cytokines involved in P450 modulation during liver inflammation.

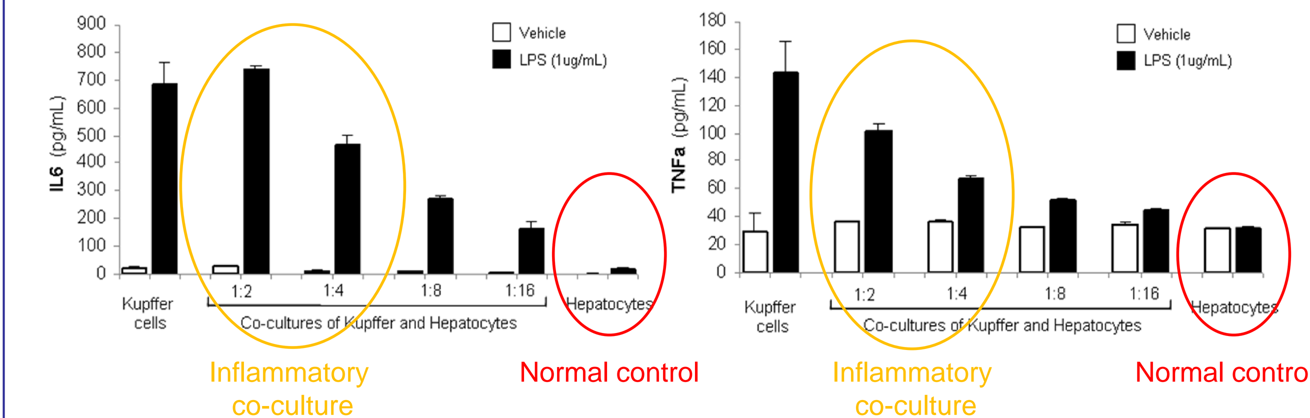


Figure 3. Simulating inflammatory response using progressive production of IL6 and TNFa in co-cultures of Kupffer cells and hepatocytes at different ratios following LPS treatment. Cells were plated at different ratios and treated with LPS (1ug/ml) for 24hr. Progressive increase of IL6 and TNFa indicates that 1:2-1:4 ratios of Kupffer cells to hepatocytes mimics high levels of cytokine production typical to that of liver inflammation. Based on this data, 1:2-1:4 ratios were used to mimic liver inflammatory state, and culture of hepatocytes only was used as control mimicking normal non-inflamed liver.

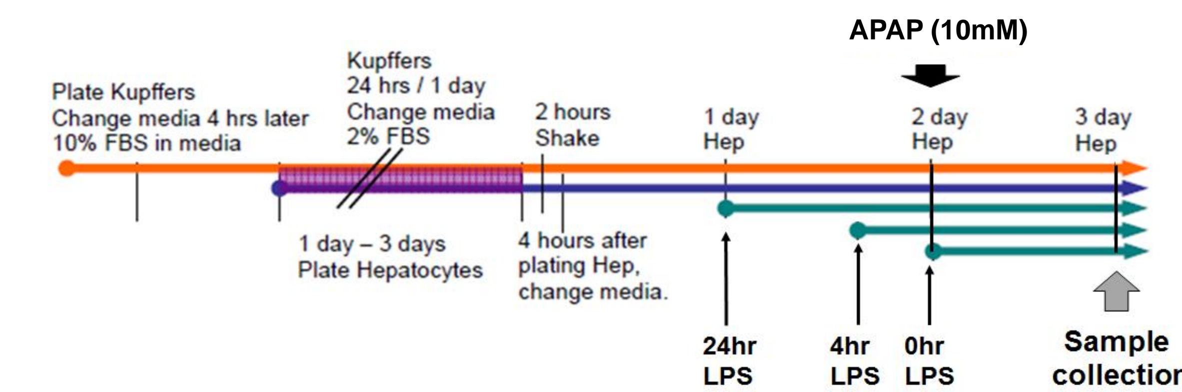
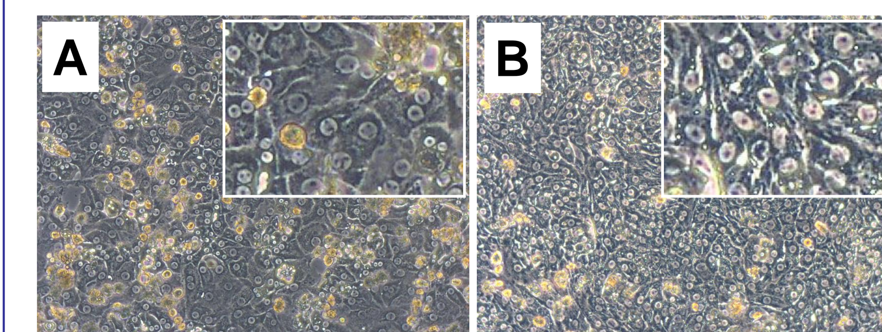
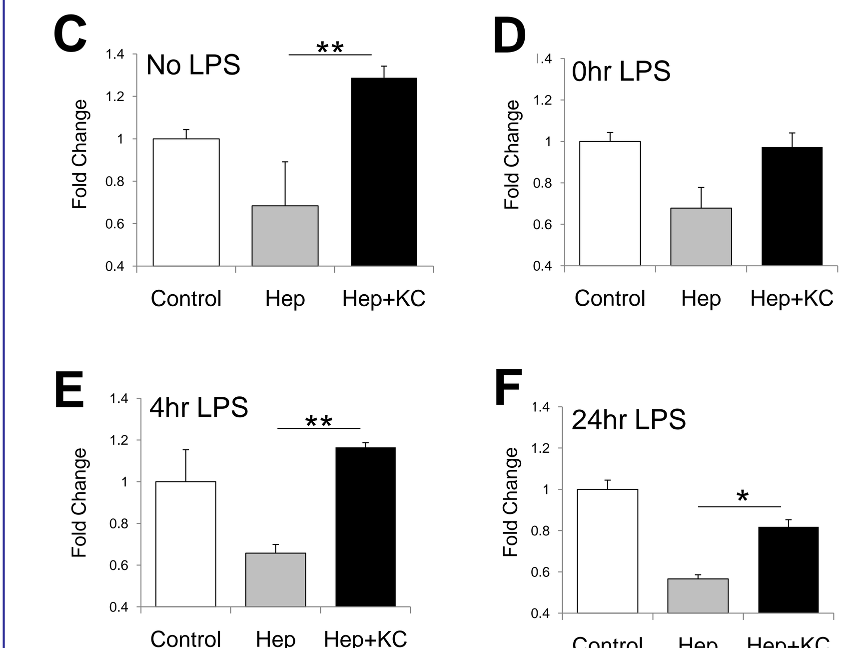


Figure 4. Experimental design for using co-cultures of cryopreserved Kupffer cells and hepatocytes to study APAP cytotoxicity. Following culture of Kupffer cells for 24hr, hepatocytes were plated and allowed to attach for 24hr prior to experiment. At that point, three experimental groups were created. 24hr pretreatment with LPS (1ug/ml) before APAP addition was to simulate cytotoxicity observed during full liver inflammation. 4hr LPS pretreatment was mimicking cytotoxicity in early stages of inflammation and 0hr was simulating cytotoxicity at the start of inflammatory response. Following pretreatment, APAP (10mM) was added in the media also containing LPS (1ug/ml) for additional 24hr of culture. At that point, samples were collected for various analysis.





* p<0.05, ** p<0.01

Figure 5. Morphological and functional assessment of hepatocytes and co-cultures following pre-treatment with LPS and 24hr incubation with APAP. A-B Representative images of hepatocytes only and co-cultures pretreated with LPS for 4hr followed by 24hr incubations with APAP, respectively; **C-F** PrestoBlue™ analysis of each experimental group (no LPS pretreatment, 0, 4, and 24hr LPS pre-treatment) following 24hr incubation with APAP. Each sample is compared to control that used the same pre-treatment condition but was not treated with APAP.
Note, similar observation was obtained using cryopreserved human Kupffer cells when cultured with human hepatocytes.

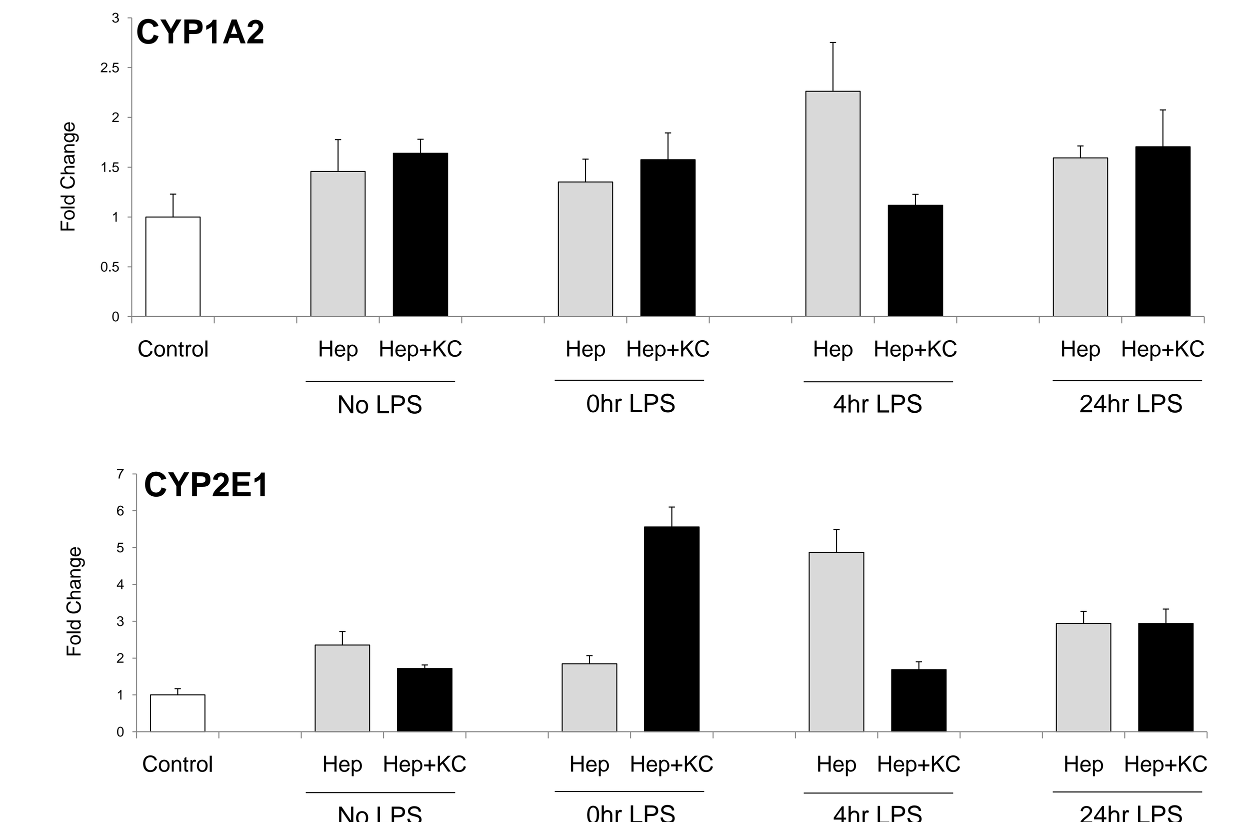


Figure 6. Preliminary real time qRT-PCR assessment of CYP1A2 and CYP2E1 in hepatocytes and co-cultures following pre-treatment with LPS and 24hr incubation with APAP.

TLR4

Fold Change

Control No LPS 0hr LPS 4hr LPS 24hr LPS

Figure 7. Real time qRT-PCR assessment of TLR-4 in co-cultures following pre-treatment with LPS and 24hr incubation with APAP. Note significant increase of TLR-4 mRNA in 4 and 24hr LPS pre-treatment groups suggesting that protective effect in those groups could be modulated through TLR-4 signaling, whereas in co-cultures without LPS or with 0hr pretreatment the protective effect is independent of TLR-4.

* p<0.05, ** p<0.01

CONCLUSIONS

- Cryopreserved Kupffer cells can maintain known characteristics and functions comparable to freshly isolated cells.
- APAP cytotoxicity data show that Kupffer cells can be effectively used for modeling liver inflammation and reveal a hepato-protective role of KCs as shown at 4 hour pre-treatment with LPS
- Current data indicates that there could be two independent hepato-protective mechanisms that Kupffer cells use to decrease APAP cytotoxicity: TLR-4 dependent that acts during inflammation and TLR-4 independent that functions in normal liver without inflammatory stimuli.

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