

Introduction

Kupffer cells, macrophages endogenous to the liver, can modulate hepatic inflammation and injury associated with various pathophysiological and toxicities. Pro-inflammatory cytokines released by activated Kupffer cells, such as TNF- α and IL-6, are associated with up-regulation of acute-phase response proteins and suppression of CYP enzymes. For new biological entities (NBE), particularly modulators of the immune system, evaluating the potential for Kupffer cell activation is an emerging concept in preclinical development. As testing of NBE develops, robust and predictable test systems are necessary, thus the need to evaluate Kupffer cell isolation, culturing and stimulation for *in vitro* applications. In the present study, Kupffer cells were isolated from non-transplantable human livers followed by expansion in culture and evaluation of the cytokine response to endotoxin (E. coli lipopolysaccharide, LPS).

Materials and Methods

Chemicals and Reagents. LPS was purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin (Pen-Strep), and insulin were purchased from Gibco/Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from SAFC (Lexenex, KS). Anti-CD68 and anti-CD163 antibodies were purchased from Abcam (Cambridge, MA). Nuclear stain DAPI (4',6'-diamidino-2-phenylindole, dihydrochloride) was purchased from Thermo Scientific (Lafayette, CO). Trizol reagent was purchased from Invitrogen (Carlsbad, CA). IL-6 and TNF- α primers and probes were purchased from Applied Biosystems (Carlsbad, CA). Enzyme-linked immunosorbent assays (ELISA) for IL-6 and TNF- α were manufactured by AssayMax (St. Charles, MO).

Cell isolation. Kupffer cells were isolated from non-transplantable human livers with a modified two-step Percoll gradient centrifugation method¹. A 100 x g supernatant collected after collagenase digestion of liver was the source of the macrophages. Approximately 1 L of the supernatant was aliquoted into 50 mL tubes and centrifuged at 350 x g for 10 min at 4°C. The supernatant was discarded and the cells resuspended in cold PBS. Cells were centrifuged again 2–3 times (350 x g for 10 min at 4°C) until the supernatant appeared clear. Following the last wash, the cells were suspended in a 50:50 mixture of cold Percoll and PBS and laid at the bottom of a two-step gradient tube beneath layers of 50%, 35% Percoll and PBS. The gradient tubes were centrifuged at 900 x g for 30 min at 4°C. Kupffer cells, contained in the lowermost fraction were washed with PBS and suspended in DMEM. Viable cells were counted with trypan blue.

Cell culture. Isolated Kupffer cells were cultured in multi-well plates (5.3×10^5 cells/cm², Nunc/Thermo Scientific) in DMEM supplemented with FBS, Pen-Strep and insulin for up to 11 days (37°C, 5% CO₂, 95% relative humidity). Cell morphology and confluency were evaluated by light microscopy. At selected times, cells were treated with 5 or 50 µg/mL LPS in DMEM for 24 hr.

Immunocytochemistry. On day 9 or day 11 post-isolation, the expression of CD68 and CD163 macrophage markers were evaluated by immunofluorescent staining. Cells were fixed in ice-cold methanol for 10 min and rinsed with PBS. Wells were blocked with 1% BSA in PBS for one hour and incubated overnight at 4°C with CD68 (1:200) or CD163 (1:100) antibodies. Cells were washed with PBS three times for 10 min between each of the remaining steps. Incubation with an Alexa Fluor® 488-conjugated secondary antibody (1:500) was for 1-2 hr at room temperature. The nuclear stain DAPI (1:1000) was applied for 15 min at room temperature. Cells were covered with 90% glycerol in PBS for storage and imaging.

Analysis of mRNA expression: Total RNA was extracted from cells lysed in TRIzol followed by purification with the RNeasy Mini Kit (Qiagen, Valencia, CA). Purified RNA were reverse transcribed to cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) and the Applied Biosystems 7300 or 7900HT Real-Time PCR System. Relative quantification, based on appropriate calibrators, was calculated with RQ Manager software (Applied Biosystems).

Analysis of protein expression. IL-6 and TNF- α in the supernatant of control and LPS-treated cells were measured by ELISA according to the manufacturer's instructions. Colorimetric measurements were conducted with the Synergy HT microplate reader (Biotek, Winooski, VT).

Results

- **Figure 1.** Isolation of Kupffer cells from four fresh human livers yielded at least 100×10^6 cells each. Cell attachment to culture plates varied between donors, as determined by light microscopy. Approximately 50% of cells from each donor attached in the wells 24 hours post-plating (**A**, **B**) and were mildly flattened (**C**, **D**).
- **Figure 2.** Five days post-plating, Kupffer cells, as illustrated by representative cultures from donor H1160, displayed both circular and/or elongated cell morphology, indicative of early and mature stages of adaptation to cell culture conditions, respectively (**A**, **B**)². Cells expressed scavenger receptor CD68, a general macrophage marker (**C**), and hemoglobin scavenger receptor CD163, a marker of Kupffer cells (**D**). Cells were >90% positive for both CD68 and CD163 markers. Macrophage markers are stained green, and nuclei are stained blue with DAPI for **Figure 2, 3 and 5**.
- **Figure 3.** Eleven days post-plating cultures of Kupffer cells reached >50% confluency and displayed both early/circular and mature/elongated cell morphologies (**A**, **B**). Cells continued to be >90% positive for both CD68 (**C**) and CD163 (**D**) markers.
- **Figure 4.** Eleven days post-plating cultures reached different levels of confluency and Kupffer cells displayed both circular and elongated cell morphologies.
- **Figure 5.** Hepatocytes from donor H1152 exhibited typical morphology and culture confluency of fresh plated primary hepatocytes (**A**). Cultures of fresh (**B**) or cryopreserved (**C**) primary hepatocytes from this donor contained Kupffer cells as demonstrated by positive staining for CD68 or CD163 markers three days post-plating. Cultures of freshly isolated and cryopreserved hepatocytes contained approximately 12% and 6% Kupffer cells, respectively. The freshly cultured hepatocytes contained more Kupffer cells in a mature/elongated stage. Given the ability to isolate Kupffer cells at high purity, it is feasible to increase the presence of Kupffer cells in co-culture to reflect the in vivo ratio of hepatocyte to Kupffer cells.
- **Figure 6 and Table 1.** Effects of treating Kupffer cells with LPS on TNF- α and IL-6 mRNA and protein in vitro. Treatment with up to 50 μ g/mL LPS caused a 5.4-fold increase of mRNA and a 1.6-fold increase of TNF- α protein. Treatment with up to 50 μ g/mL LPS caused a 360-fold increase of mRNA and a 3.0-fold increase of IL-6 protein.

Tables and Figures

Figure 1. Photomicrographs of Kupffer cells one hour and twenty-four hours post-plating. Cells of donor H1171 at one and twenty-four hours post-plating (A, B). Cells of donors H1160 (C) and H1161 (D) at twenty-four hours post-plating.

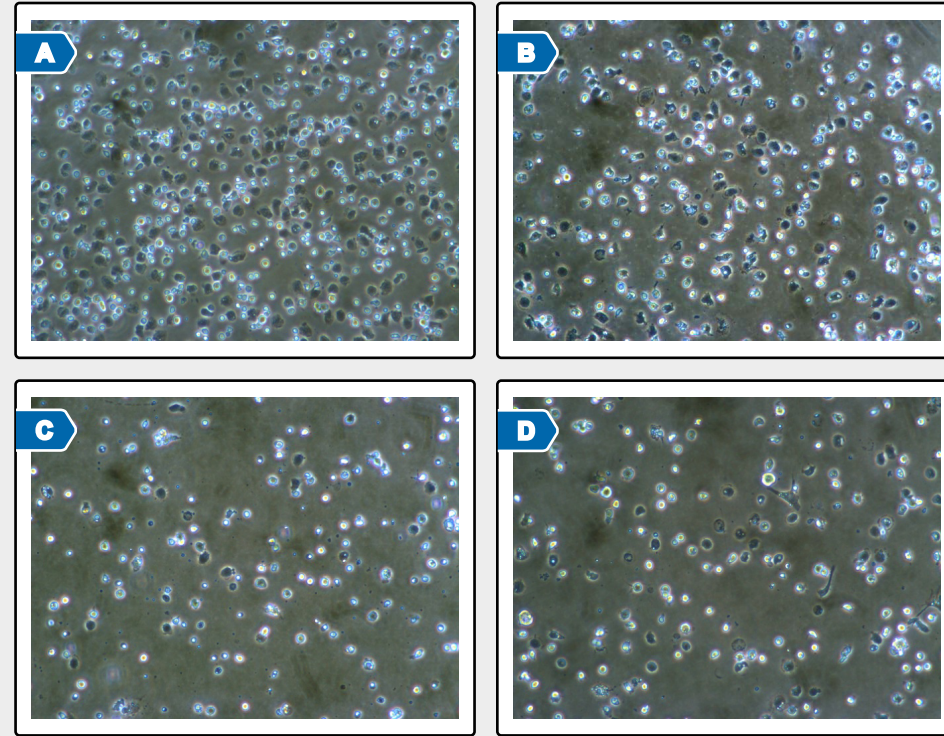


Figure 3. Photomicrographs and immuno-fluorescent staining of Kupffer cells from donor H1160 eleven days post-plating. Cultures continued to display mixed circular and elongated cell morphologies (**A**, 50x; **B**, 100x) magnification. Circular and elongated cells continued to express CD68 (**C**, green) and CD163 (**D**, green) markers (100x). Nuclei are stained blue with DAPI (**C** and **D**).

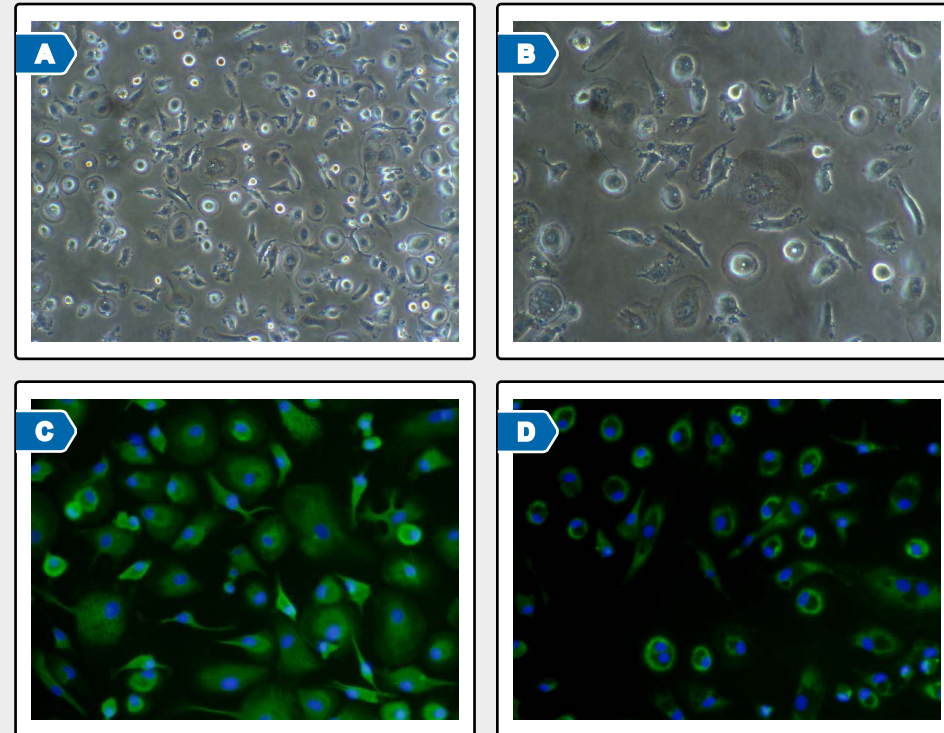


Figure 2. Photomicrographs and immuno-fluorescent staining of Kupffer cells from donor H1160 five days post-plating. Typical mixed morphology culture observed *in vitro* (**A**, 50x; **B**, 100x). Circular and elongated cells expressed CD68 (**C**, green) and CD163 (**D**, green) markers (100x). Nuclei are stained blue with DAPI (**C** and **D**).

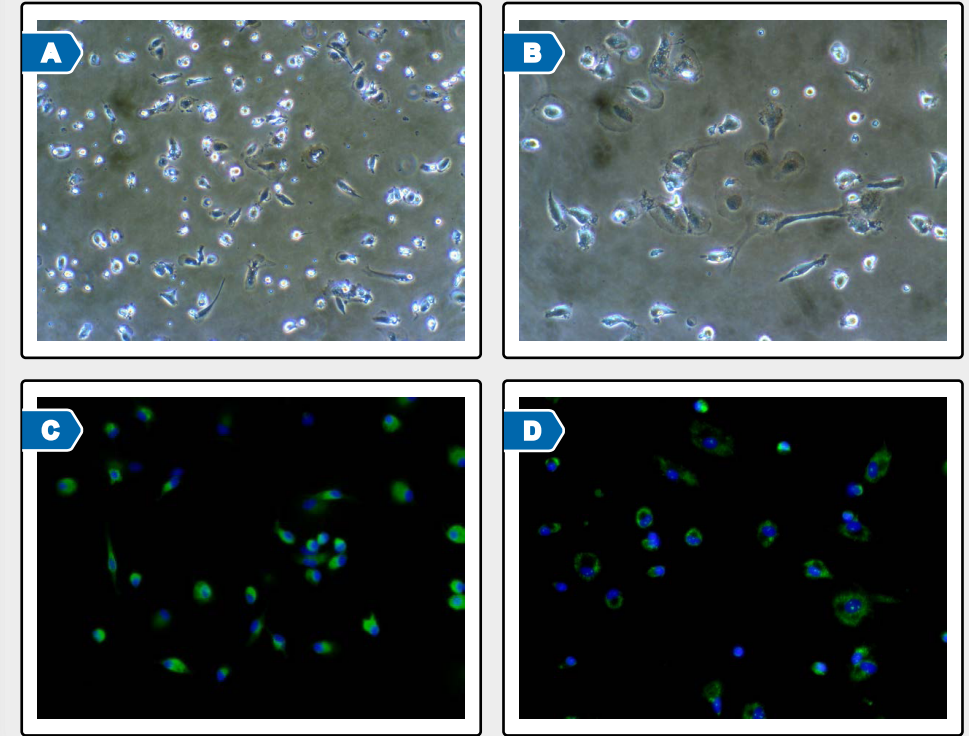


Figure 4. Photomicrographs of Kupffer cell cultures eleven days post-plating. Both circular and elongated cell morphologies were observed in four cultures (100x magnification).

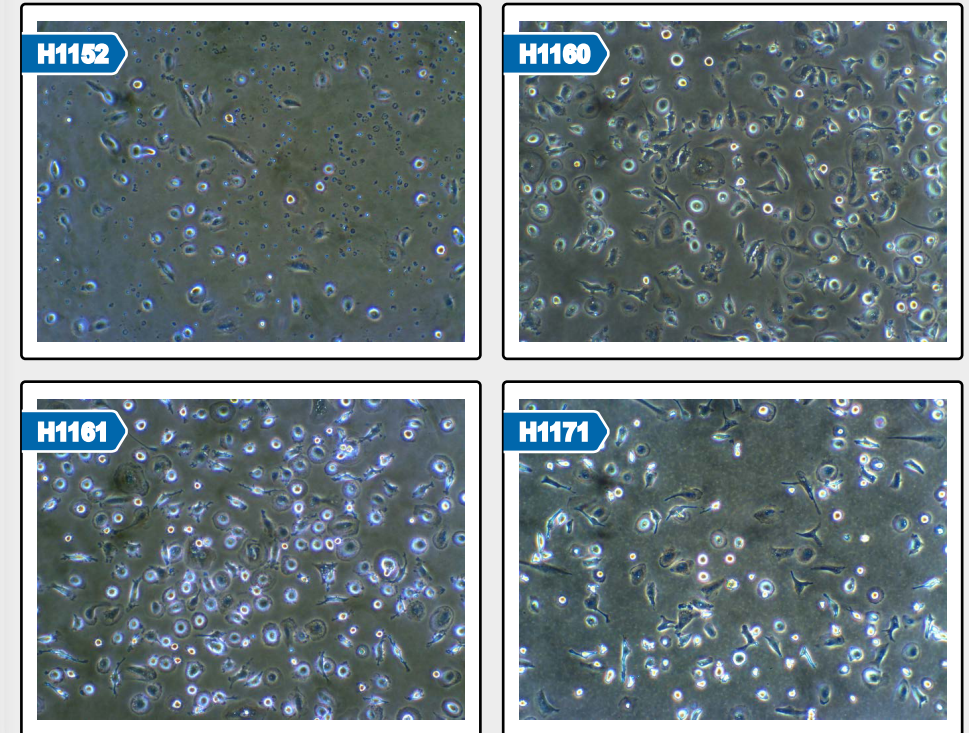


Figure 5. Photomicrographs and immuno-flourescent staining of Kupffer cell present in cultures of fresh and cryopreserved primary plated hepatocytes. Isolated hepatocyte cultures from donor H1152 contained Kupffer cells expressing CD68 marker at day three in culture (**A** and **B**, green). Kupffer cells were also observed among cultured cryopreserved hepatocytes from the same donor (**C**, green). Nuclei are stained blue with DAPI (**B** and **C**).

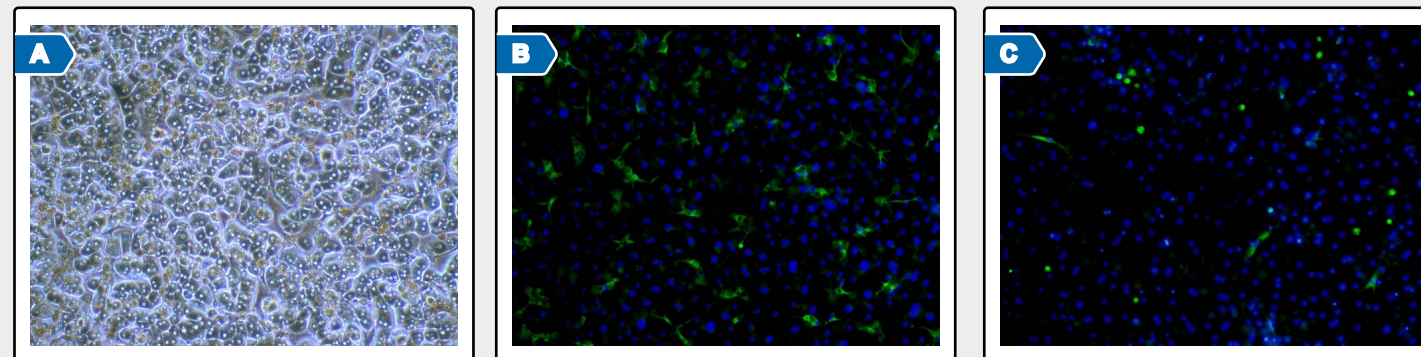


Figure 6. Effects of treating cultured Kupffer cells with LPS on TNF- α and IL-6 mRNA and protein expression

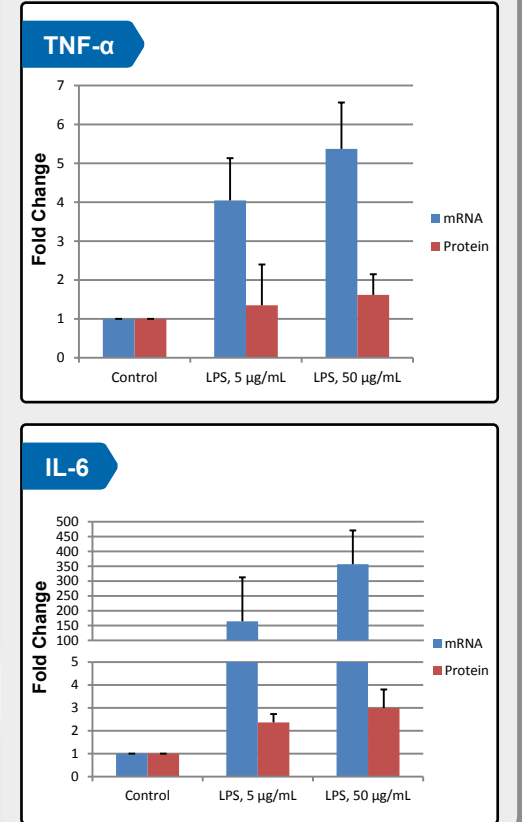


Table 1. Effects of treating cultured Kupffer cells with LPS on TNF- α and IL-6 mRNA and protein expression

	TNF- α		IL-6	
LPS (μ g/mL)	5	50	5	50
Donor	mRNA			
H1152	ND ¹	ND	ND ²	ND
H1160	4.5 ³	6.7	59	230
H1161	4.8	5.2	100	390
H1171	2.8	4.3	330	450
	Protein			
H1152	2.2	ND	2.5	ND
H1160	2.3	2.1	1.8	2.2
H1161	2.1	1.7	2.6	3.8
H1171	1	1	2.6	3.1

¹ Not Determined
² Fold change value was not determined due to control values below level of detection.
³ Values are fold change in comparison to media control; 2 biological samples analyzed in duplicate.
⁴ All data represents day 9 in culture with the exception of H1152 (day 11).

Conclusions

- The method presented here provides a reliable means of isolating human Kupffer cells yielding at least 100×10^6 cells per procedure.
- Cultures of freshly-isolated human Kupffer cells maintained phenotype and responsiveness to endotoxin, a prototypical pro-inflammatory stimulus, for up to 11 days post-isolation. The stimulation of cytokine release observed in cultured human Kupffer cells upon treatment with endotoxin was similar to that observed in the clinical setting³.
- These results suggest that freshly-isolated human Kupffer cells may be a suitable test system for preclinical evaluation of pro-inflammatory effects of new biological or chemical entities.

References

1. Vrochides D et al. (1996) *Hepatology*. **23**(6):1650-1655.
2. Mendel GL et al. (2011) *Drug Metab Rev*. Vol. **43**; S2, P245.
3. Shedlofsky SI et al. (1994) *J Clin Invest*. **94**(6):2209-14.