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Development and optimization of a comprehensive co-culture

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Current research in ADME-toxicology, drug development, physiology and human health increasingly requires the use of physiologically-relevant *in vitro* models that can accurately represent the complexity of interactions between hepatocytes and various non-parenchymal cells (NPCs) in the liver. In this Technical Note, we demonstrate the successful development of a co-culture model of primary hepatocytes with Kupffer cells (KCs), stellate cells (SCs) and liver endothelial cells (LECs). We optimized this model for healthy hepatocyte morphology and function as well as overall NPC functionality. We show that, when including hydrocortisone in the cell culture media and using a Corning® Matrigel® overlay to support hepatocyte health, NPC functionality (cytokine release upon LPS stimulation) is not impaired and the overall health and physiology of the co-culture is maximized. The resulting protocol provides a model system that can be cultured to seven days without a decrease in health or performance and can serve as a physiologically relevant model for a large variety of applications.

Materials

Materials used in this study for establishing the optimized hepatocytes: NPC co-culture are listed in Table 1. Hepatocyte: NPC donor combinations can be found in Table 2. We used two donors that were matched between hepatocytes and Kupffer cells and two that were mismatched to account for donor variation effects.

Methods

Co-cultures were prepared according to protocol instructions found on the Lonza website. Briefly, two separate media were prepared: DMEM Plating Medium (DMEM supplemented with MEM non-essential amino acids, ITS, GlutaMAX™, 1000X Gentamicin/Amphotericin, and hydrocortisone) and DMEM Maintenance Medium (DMEM supplemented with MEM non-essential amino acids, ITS, GlutaMAX™, and 1000X Gentamicin/Amphotericin). Ratios of supplements to each media can be found in the online protocol. Hepatocytes were thawed and plated 1 hour prior to plating NPCs on 24-well collagen coated plates at a density of 5 x 10^5 cells / well using DMEM Plating Medium and placed in an incubator at 37°C and 5% CO_2 until NPCs were added. After an hour, medium was aspirated from each well and NPCs (KCs, SCs, and LECs) were thawed and plated with the hepatocytes using DMEM Plating Medium at the following ratios: 0.25 – 0.29 KC : 1 hepatocyte, 0.13 – 0.15 SC : 1 hepatocyte, and 0.25 – 0.29 LEC : 1 hepatocyte. These ratios generally reflect physiological conditions in the liver[2]. Final volume in each well was brought to 500 µL using DMEM Plating Medium, and cells were returned to the incubator for 1 hour. After this, media and unadhered cells were aspirated from each well and cells were fed 500 µL of DMEM Maintenance Medium before being returned to the incubator for 4 – 5 hours.

At 4 – 5 hours after co-culture establishment, medium was aspirated from each well and 500 µL of a solution of 0.3 mg/mL Matrigel® in DMEM Maintenance Medium was added to each well before returning the plate to the incubator. Medium was aspirated and new DMEM Maintenance Medium was added every day thereafter up to 7 days.

The experiment was carried out in three phases to optimize co-culture conditions. In the first phase, we tested co-culture health and functionality at 4 and 7 day timepoints as well as with and without Matrigel® overlay (for 7 days only) using a single donor (matched hepatocytes : NPCs) and no hydrocortisone in the DMEM Plating Medium. In the second phase, using a 7 day culture, we tested co-culture health and functionality with/without hydrocortisone in the DMEM Plating Medium as well as with/without Matrigel® using a second donor that was mismatched with KCs. Finally, we validated results on 2 final donors (one matched with NPCs, one mismatched).

Hepatocyte morphology (multi-nucleated, polygonal shape with well-defined borders and bile canaliculi) and confluency was assessed by phase contrast imaging.

Hepatocyte health/functionality was quantitatively assessed by measuring albumin release using an ELISA assay. NPC functionality was assessed via ELISA assays for cytokine release after 24 hours of LPS stimulation (1 µg/mL LPS in DMEM Maintenance Medium) starting on day 6. Supernatant was collected and frozen on day 7 until analysis. Cytokines evaluated included TNF-α (KCs), IL-10 (SCs) and CCL2 (LECs).

Results

The first and second rounds of optimization (data not shown) showed that hepatocyte albumin release was higher at 7 days than at 4 days and higher with Matrigel® than without Matrigel®. Hepatocyte morphology was poor in monoculture but appeared healthy in all co-culture conditions. Neither culture duration nor Matrigel® affected NPC cytokine release, indicating that NPC functionality was maintained throughout the culture period and was not disrupted by the Matrigel® overlay. Adding hydrocortisone to the DMEM Plating Medium (which supports hepatocytes but may interfere with NPC cell functionality) resulted in slightly better albumin release, no change in morphology, and higher NPC cytokine release when used in conjunction with Matrigel® overlay, indicating that optimal co-culture conditions should include both hydrocortisone and Matrigel®. Using two further donors (one matched, one mismatched) to validate the optimized protocol above, we found that hepatocyte morphology was maintained across 7 days in co-culture both with and without Matrigel® (Figure 1).

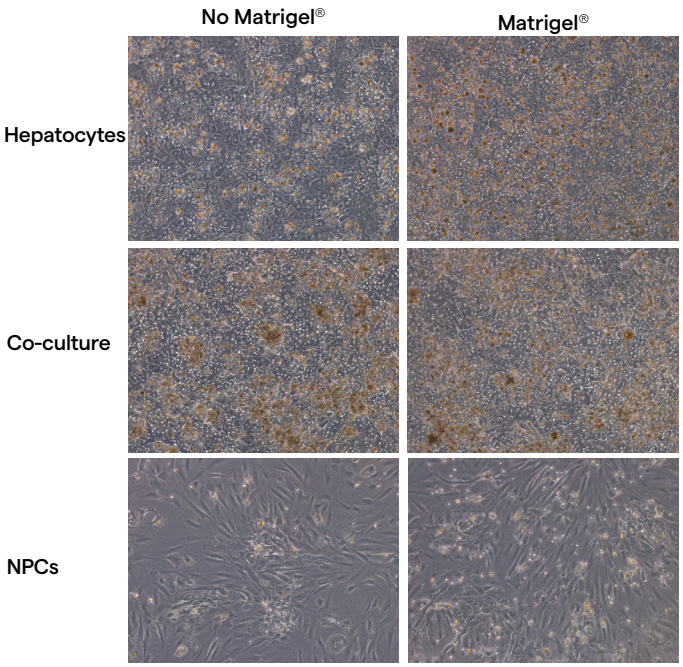


Figure 1.
Cell morphology of hepatocytes, co-cultures, and NPCs at 7 days after co-culture establishment. Donor was mismatched between hepatocytes and NPCs. Normal morphology (polygonal cell shape, bright canaliculi) was observed for hepatocytes in both monoculture and co-culture, with and without Matrigel® overlay for this donor.

In both cases, hepatocytes were polygonal in shape, with bright bile canaliculi apparent in phase contrast. In general, for both donors, albumin production was normal in co-cultures (indicating that co-culture conditions support hepatocyte health) and equal to or slightly higher when using Matrigel® compared to the no Matrigel® treatment (data not shown). In general, when combining data across all four donors, both hepatocyte and NPC health and functionality are well-supported across 7 days in co-culture (Figure 2). Hepatocyte health is further supported by Matrigel® and hydrocortisone in monoculture (Figure 2A), and including these components in co-culture does not impact NPC functionality (Figure 2B – D) as there is no difference in NPC cytokine release in co-culture with or without Matrigel®. Cytokine release after LPS stimulation across all donors is significantly higher in co-culture compared to monoculture for either cell type (Figure 2B – D). Combined with a reduction in albumin release by co-cultured hepatocytes when stimulated with LPS (Figure 2A), this shows that the co-culture model functions as expected and demonstrates an inflammatory response when immune NPCs are stimulated.

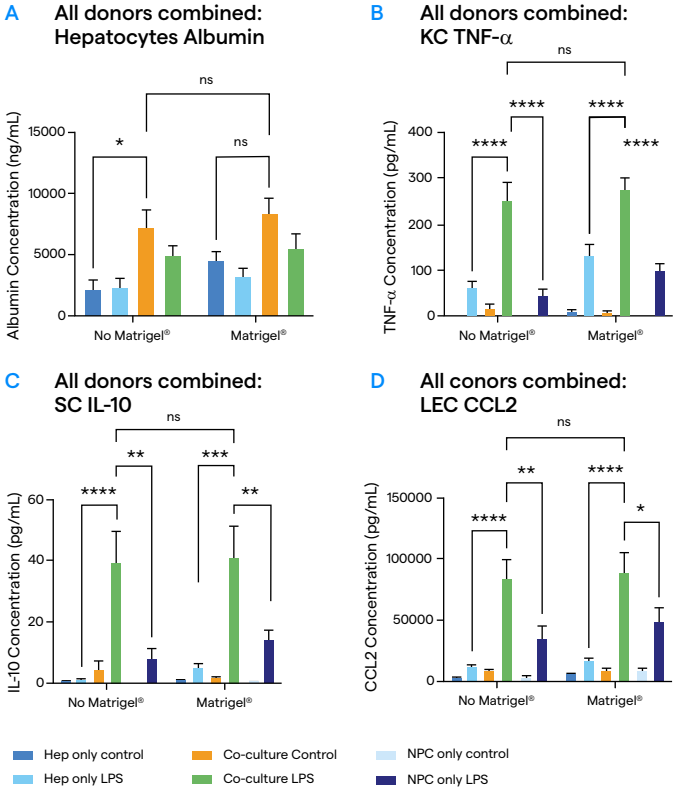


Figure 2.
ELISA assay results for hepatocyte albumin release (A), KC TNF-α cytokine release (B), SC IL-10 cytokine release (C) and LEC CCL2 cytokine release (D) across all donors used in this study. * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001

Conclusion

Our study demonstrates a physiologically-relevant hepatocyte: NPC co-culture that maintains functionality up to 7 days in culture. Although hydrocortisone addition and Matrigel® overlay showed only slight improvements in hepatocyte health and performance under co-culture conditions, the data indicate that both can be used in co-culture as they do not negatively impact NPC health and functionality and are commonly used to support hepatocyte health and morphology. When stimulated by LPS, this model demonstrates an appropriate inflammatory response through high cytokine release from all three NPC types as well as reduced hepatocyte albumin release as would be expected *in-vivo*. Our hepatocyte: NPC co-culture can be used in a large variety of applications, including drug development and toxicology, where understanding the inflammatory crosstalk between hepatocytes and liver NPCs is of vital importance.

References:

1. Hepatocyte and Non-Parenchymal Cell (NPC) 2D Co-Culture Instructions for Use. 2023. LINK.
2. **Ma, Y., *et al.*** (2023) Three-dimensional cell co-culture liver models and their applications in pharmaceutical research. *International Journal of Molecular Sciences* 24: 6248. DOI: 10.3390/ijms24076248.

Catalog no.	Description
HUCPI	Cryopreserved Human Hepatocytes
HLKC-500K	Cryopreserved Human Kupffer Cells
HUCLS-200K	Cryopreserved Human Stellate Cells
HLECP1	Cryopreserved Human Liver-derived Endothelial Cells (LECs)
MCHT50	Hepatocyte Thawing Medium
13-114E	100X MEM NEAA
CC-4083	1000X Gentamicin/Amphotericin
ThermoFisher 31053028	DMEM
ThermoFisher 35050079	GlutaMAX™ supplement
Corning 25-800-CR	ITS 100X
Corning 354408	24-well collagen coated plates
Corning 354234	Corning® Matrigel® (10.1 mg/mL)
StemCell 07925	200X Hydrocortisone 96 µg/mL
	Trypan Blue stain
	PBS

Table 1.
Materials used to create the optimized Hepatocyte:NPC co-culture model.

Donor combination	Matched/mismatched	Hepatocyte donor (HUCPI)	KC donor (HLKC-500K)	SC donor (HUCLS-200K)	LEC donor (HLEP-1)
1	Mismatched	HUM222621	HKC223011	HSC210971	HLE210971
2	Matched	HUM221401	HKC221401	HSC221401	HLE212831
3	Matched	HUM210471	HKC210471	HSC210471	HLE212831
4	Mismatched	HUM220971	HKC221401	HSC222051	HLE212241

Table 2.
Combinations of Hepatocyte and NPC donors used in this study

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