eLife

Reviewed Preprint
v1 • November 14, 2025
Not revised

Reviewed Preprint
v2 • April 30, 2026
Revised by authors

For correspondence:
hyu@a-star.edu.sg
Florent_Ginhoux@a-star.edu.sg

[#] These authors contributed equally

Competing interests: No competing interests declared

Reviewing editor: Simon Yona, The Hebrew University of Jerusalem, Israel

© 2025, Lee et al. This article is distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use and redistribution provided that the original author and source are credited.

Direct contact between iPSC-derived macrophages and hepatocytes drives reciprocal acquisition of Kupffer cell identity and hepatocyte maturation

Christopher Zhe Wei Lee[1,2,3,#], Farah Tasnim[1,4,#], Xiaozhong Huang[1,4,5], Raman Sethi[6], Yoohyun Song[4], Tatsuya Kozaki[2], Sebastiaan De Schepper[2], Nicholas Ang[2], Ivy Low[2], You Yi Hwang[2], Jinmiao Chen[6,7], Hanry Yu[1,4,5,8,9,10], Florent Ginhoux[1,2,3,11,12]

[1]Innovations in Food & Chemical Safety Programme (IFCS), A*STAR, Singapore, Singapore • [2]Singapore Immunology Network, Singapore, Singapore • [3]School of Biological Sciences, Nanyang Technological University, Singapore, Singapore • [4]Institute of Bioengineering and Bioimaging, Singapore, Singapore • [5]Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore • [6]Bioinformatics Institute (BII), Agency for Science, Technology and Research (A*STAR), Singapore, Singapore • [7]Institute of Molecular and Cell Biology (IMCB), Agency for Science, Technology and Research (A*STAR), Singapore, Singapore • [8]NUS Graduate School – Integrative Sciences and Engineering Programme (ISEP), Singapore, Singapore • [9]Mechanobiology Institute, Singapore, Singapore • [10]CAMP IRG, Singapore-MIT Alliance for Research and Technology, Singapore, Singapore • [11]Shanghai Institute of Immunology, Shanghai JiaoTong University School of Medicine, Shanghai, China • [12]Translational Immunology Institute, SingHealth Duke-NUS Academic Medical Centre, Singapore, Singapore

eLife Assessment

This timely and **fundamental** study presents an innovative iPSC based co-culture system to model Kupffer cell-hepatocyte interactions and hepatotoxicity, demonstrating reciprocal acquisition of tissue identity and enhanced hepatocyte maturation. The work is **convincing**, supported by well-executed methodology and functional validation, including physiologically relevant, concentration-dependent hepatotoxic responses. The research approach is promising and of broad interest, further clarification of experimental design and interpretation may strengthen its impact.

https://doi.org/10.7554/eLife.108938.2.sa3

Abstract

Hepatic macrophages play central roles in liver homeostasis, injury, and immune-mediated hepatotoxicity through dynamic crosstalk with hepatocytes. While monocyte-derived macrophages have been widely used in vitro, they do not fully recapitulate the biology of liver-resident Kupffer cells (KCs), which are embryonically derived and maintained locally. Recent advances suggest that induced pluripotent stem cell (iPSC)-derived macrophages (iMacs) more closely resemble embryonic macrophages and may therefore serve as a relevant platform to model KC biology.

Here, we developed a human iPSC-based co-culture system by combining iMacs with iPSC-derived hepatocytes (iHeps) derived from the same donor, enabling direct cell–cell interactions. We hypothesized that such interactions would both enhance hepatocyte maturation and promote KC-like differentiation of iMacs. Indeed, co-culture induced KC-like phenotypes in iMacs and improved functional maturation of iHeps, highlighting the importance of bidirectional cellular communication. Comparative analyses with iMacs cultured in hepatocyte-conditioned media revealed that direct contact provides additional signals beyond soluble factors in driving hepatic

macrophage specialization. Functionally, this co-culture system demonstrated improved physiological relevance, particularly in modeling immune-mediated drug responses, as evidenced by enhanced cytokine production profiles upon exposure to a panel of test compounds.

Overall, this study establishes a novel human iPSC-derived platform that captures key aspects of hepatocyte–macrophage crosstalk, providing a more physiologically relevant model to investigate liver biology and assess immune-mediated drug toxicity.

1. Introduction

Hepatic macrophages play key roles in immune-mediated hepatotoxicity, liver injury and repair [1, 2]. They are impacted by autocrine and paracrine signals from hepatic cells, especially hepatocytes. They also release soluble stress signals in response to external stimuli from foreign particles, leading to macrophage activation and production of cell signalling and stress pathway modulators, including reactive oxygen species and cytokines [3, 4]. In return, hepatic macrophage activation also regulates parenchymal and non-parenchymal cell death and the metabolic activity of hepatocytes [5]. Such crosstalk between liver parenchymal cells (hepatocytes) and non-parenchymal modulators (hepatic macrophages) is critical in modelling liver injury, especially immune-mediated drug-induced responses [6].

Two major populations of hepatic macrophages have been reported: liver-resident Kupffer cells (KCs) and monocyte-derived macrophages (MoMLJs) that infiltrate the liver upon insult [3]. While MoMLJs have been well studied *in vivo* and applied in *in vitro* models [6], in more recent years it has been revealed that KCs are derived from embryonic macrophages that seed the liver early on during development [7][8], and are only minimally replaced by circulating monocytes under steady state conditions [9]. This also implies that human peripheral blood monocytes, the conventional source of primary human macrophages, might not be able to fully recapitulate KC biology. However, it has been demonstrated that murine iPSC-derived macrophages (iMacs) more closely resembled embryonic macrophages than monocyte-derived macrophages [10], suggesting that human iMacs might also serve as a suitable analogue for human embryonic macrophages. Based on these new insights, in our previous work, we differentiated iMacs into iPSC-derived KCs (iKCs) using primary hepatocyte conditioned media (PHCM) to drive iMacs towards iKC identity [11]. Although these iKCs partially resembled primary human KCs (PhKCs) based on the expression of KC specific markers and drug response, PHCM is not readily available and might exhibit differences depending on donor variability of primary hepatocytes. Most importantly, the direct cellular interactions between iMacs and hepatocytes and their potential to impart better reciprocal identity and functionality to each other has not been studied before.

We recently generated macrophage-sufficient brain organoids by coculturing brain organoids with iMacs generated from the same human iPSC line [12]. In such cocultures, iMac differentiated into cells with microglia-like phenotypes and functions. Most importantly, iMac modulated neuronal progenitor cell (NPC) differentiation, limiting NPC proliferation and promoting axonogenesis, profoundly remodelling organoid physiology. Thus, in this study, we hypothesized that iMacs could be directly cultured with iPSC-derived hepatocytes (iHeps) from the same IPSC source to both improve iHep development and maturation as well as impart KC-like identify to the iMacs. Upon development of such a model, we tested its application in detecting immune-mediated responses of seven paradigm compounds. Concurrently, we also cultured iMacs in PHCM for 7 days in a similar way to our previous study [11], comparing the transcriptomic effect of direct contact against soluble factors in the context of hepatic macrophage specialisation. Our results showed improved *in vivo* correlation based on the effects on cytokine production when iMac-derived KCs were used. Altogether, this study highlights the effect of direct crosstalk between hepatocytes and macrophages *in vitro* using iPSCs and provides a new human model to test drug-induced cytokine responses.

2. Materials and Methods

2.1 Maintenance of iPSCs

Human IMR90-iPSCs were obtained from WiCell Research Institute (Madison, WI). The cells were maintained under antibiotic-free conditions in mTESR media (StemCell Technologies, 85850) on Matrigel (Corning, 354234)-coated plates. Cells were passaged using ReLESR (StemCell Technologies, 05872) or Dispase (StemCell Technologies, 07913) as per manufacturer's protocol whenever they reached 80% confluency.

2.2 Differentiation of iPSCs to iHeps

IMR90-iPSCs were differentiated into iPSC-hepatocytes (iHeps) as previously described [13]. Briefly, the iPSCs were cultured to 80% confluency, then made into single cells using Cell Dissociation Buffer (Gibco, 13151014) and seeded at 1.5 x 10^5 cells/cm^2 on the desired well plate format (i.e. 1.5 x 10^6 cells for a 6 well plate) and placed under hypoxic conditions (5% O_2 and 5% CO_2). Growth factors and media used starting from the next day to day 20 are detailed in [11]. Media was changed every day and hypoxic conditions were used during days 5 to15 of differentiation. After 20 days of differentiation, iPSC-derived macrophages (iMacs) were either directly added to the iHeps as described in section 2.5 or iHeps were harvested for seeding into smaller well formats for drug testing. The harvesting protocol has been previously optimized by us and has been detailed in [14].

2.3 Differentiation of iPSCs to iMacs

IMR90-iPSCs were differentiated into iMacs as previously described [10]. Briefly, the iPSCs were cultured to 80% confluency, then passaged using ReLESR. As ReLESR releases the cells as small cell clumps, the passage conditions were optimised to result in roughly 1 x 10^5 cells in each well of a 6 well plate (Day-1). Starting from the next day (Day 0) to day 16, the cells were cultured in Stempro Media, consisting of Stempro-34 SFM (GIBCO, 10639-011), supplemented with 200 ug/mL Human Transferrin (Roche, 10-652-202-001), 1x L-Glut, 1x Pen/Strep, 0.5 mM Ascorbic Acid (Sigma, A4544) and 0.45mM MTG (Sigma, M6145). A full media change was done every other day for the next 16 days, supplemented with the following cytokines: Differentiation Day 0 (5 ng/mL BMP4, 50 ng/mL VEGF, and 2 uM CHIR99021), Differentiation Day 2 (5 ng/mL BMP4, 50 ng/mL VEGF, and 20 ng/mL FGF2), Differentiation Day 4 (15 ng/mL VEGF and 5 ng/mL FGF2), Differentiation Day 6 to 10 (10 ng/mL VEGF, 10 ng/mL FGF2, 50 ng/mL SCF, 30 ng/mL DKK-1 (RnD, 5439-DK), 10 ng/mL IL-6 (RnD, 206-IL), and 20 ng/mL IL-3), Differentiation Day 12 and 14 (10 ng/mL FGF2, 50 ng/mL SCF, 10 ng/mL IL-6, and 20 ng/mL IL-3). From day 16, the cells were cultured in 75% IMDM with Glutamax (GIBCO, 31980-030), 25% F12 (GIBCO, 11765-047), 1x N2 supplement (GIBCO, 17502-048), 1x B27 Supplement (GIBCO, 17504-001), 0.05% BSA (GE Healthcare, SH30574) and 1x Pen/Strep, supplemented with 50ng/ml of CSF-1 (RnD, 216-MC), with a full media change every 3 days. In addition, for the first 8 days of differentiation, the cells were kept in a hypoxic incubator (5% O_2 and 5% CO_2), before being transferred to a standard cell culture incubator for the next 18 days. After transfer to a standard cell culture incubator, floating cells were collected every media change and resuspended back into the culture to retain the hematopoietic progenitors. After 26 days, the floating cells were collected, and the purity of the iMacs was determined by flow cytometry (Supp Fig 1E).

2.4 Differentiation of human peripheral blood monocytes (PBMCs) into monocyte-derived macrophages

Human PBMCs were purified from blood apheresis cones by ficoll gradient (Cytiva, 17-1440-02), before being magnetically sorted with CD14+ microbeads (Miltenyi, 130-050-021). 1 x 10^6 CD14+ cells were then seeded per well of a 6 well plate in RPMI 1640 (Hyclone, SH30027.01) with 10% FBS

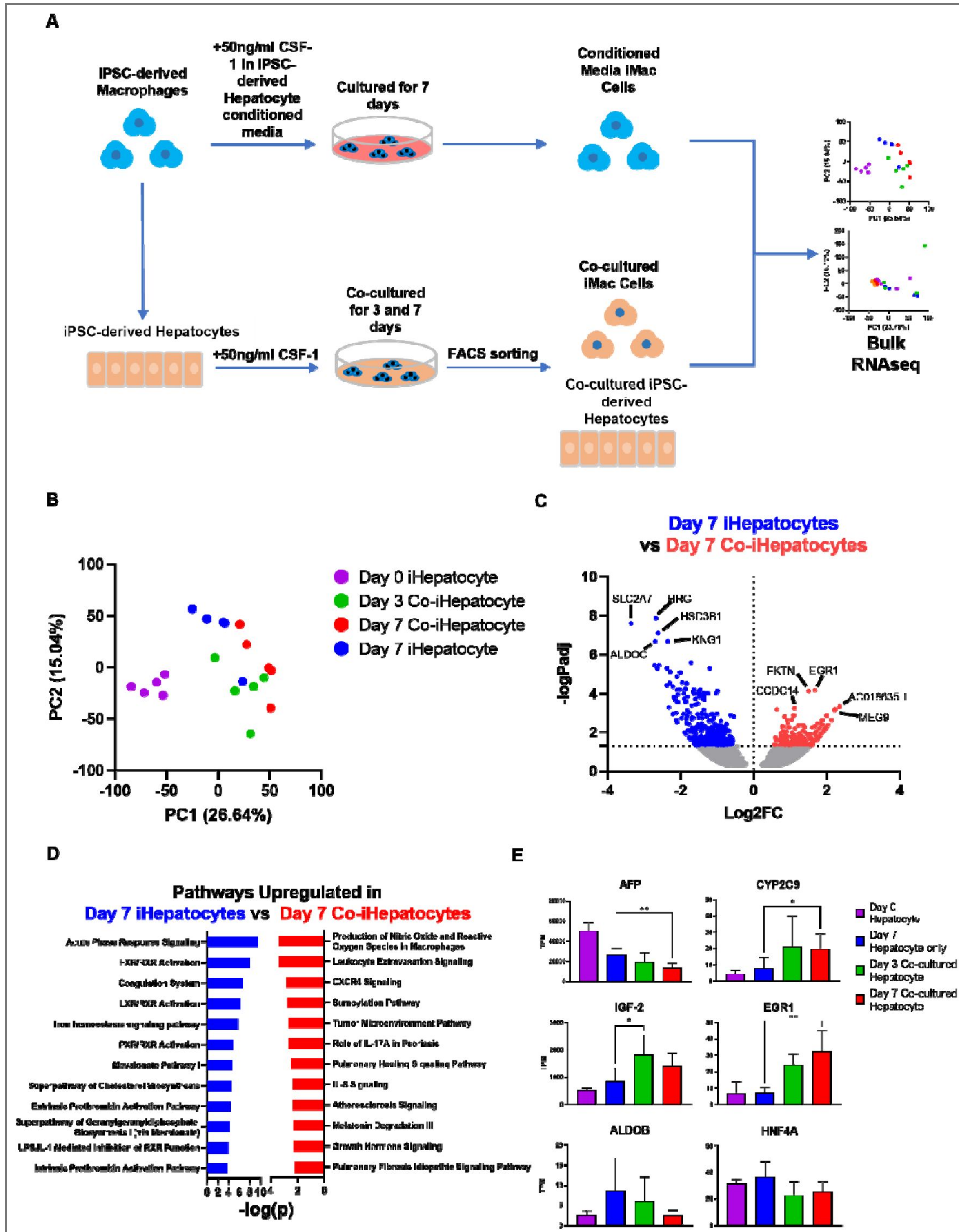


Figure 1. Model development and effects of iMacs on iHeps.

A) Schematic of experimental layout. B) Principal Component Analysis of bulk RNAseq data from Day 0 iHeps, Day 3 co-iHeps, Day 7 co-iHeps and Day 7 iHeps. C) Volcano plot showing differentially-expressed genes between Day 7 iHeps and Day 7 co-iHeps. D) Differentially-expressed upregulated pathways between Day 7 iHeps and Day 7 co-iHeps. E) Gene expression levels of *AFP*, *CYP2C9*, *IGF-2*, *EGR-1*, *ALDOB* and *HNF4A* in Day 0 iHeps, Day 3 co-iHeps, Day 7 co-iHeps and Day 7 iHeps. Student's T test was applied. *p<0.05, **p<0.01

(Biowest, S1810-500), 1x Pen/Strep, 1x Sodium Pyruvate, 1x NEAA and 1x L-Glut with 50ng/ml CSF-1 and cultured for 7 days. The purity of the monocyte-derived macrophages was then determined by flow cytometry.

2.5 Co-culture of iMacs and monocyte-derived macrophages with iHeps

2 x 10^5 iMacs or monocyte-derived macrophages were added to 5 x 10^5 iHeps during a full media change (4ml) per well of a 6 well plate on Day 0. We chose this ratio as it has been previously described that in the adult mouse liver, Kupffer cells can be up to 35% of hepatocyte numbers [15]. This media was comprised of Williams' Medium E containing the following supplements: Penicillin/Streptomycin (10,000 U/mL / (10,000 µg/mL) – final conc = 1%, ITS+ - final conc= 2%, 4mM glutamax, 30mM HEPES buffer. The concentrations of the supplements were double that of standard conditions used due to the infrequent media change (please see section 3.1 for more details). In addition, 50ng/ml of CSF-1 was added to each well. The cells were cultured for 7 days, with a half-media change (2ml) on Day 4 consisting of the same media as described above with 100ng/ml of CSF-1, resulting in a final concentration of 50ng/ml CSF-1 in the well. The cells were collected by digesting with Accutase (StemCell, 07920) for flow cytometry, RNAseq analysis and qPCR. For pharmacological testing, the number of iHeps and iMacs were downscaled to 96-well plates while maintaining the same iHep:iMac ratio (2.5:1).

2.6 Flow cytometry

Standard staining procedures were used to prepare the cells for flow cytometry analysis. Briefly, cells were dissociated into single cells using the different methods described above for the various tissues, before being incubated with 100ul of antibody containing FACS buffer (1% BSA and 4mM EDTA in PBS) per 5 million cells for 20 minutes at 4°C. The cell suspension was then washed with 5ml of FACS buffer, centrifuged at 400g, and the supernatant was removed. The cells were finally resuspended in PBS containing 3uM DAPI (Invitrogen, D1306). Data was acquired by LSRII (BD Bioscience) and analyzed by Flow Jo (Tree Star, Inc.). For cell sorting, cells were sorted using FACS Aria II (BD Bioscience) or FACS Aria III (BD Bioscience). The following antibodies were used: FITC-conjugated anti- human CD14 (Biolegend, 325604), APC/Cy7-conjugated anti-human CD45 (Biolegend, 368516), PE-conjugated anti-human CD163 (R&D Systems, FAB1607P-100), Alexa Fluor 647-conjugated anti-human CX3CR1 (Biolegend, 341608, PE/Cy7-conjugated anti-human CD11b (eBioscience, 25-0118-42).

2.7 Bulk RNA-seq

Total RNA was extracted using Arcturus PicoPure RNA Isolation kit (Thermofisher, KIT0204) according to manufacturer's protocol. All RNAs were analyzed on Labchip GX system (Perkin Elmer, United States) for quality assessment with median RNA Quality Score of 9.15. cDNA libraries were prepared using 2ng of total RNA and 1ul of a 1:50,000 dilution of Ambion ERCC RNA Spike-in Controls (Thermofisher, 4456740) using the SMARTSeq v2 protocol described [16] with the following modifications: 1. Use of 20mM TSO, 2. Use of 250pg of cDNA with 1/5 reaction of Nextera XT kit (Illumina, FC-131). The length distribution of the cDNA libraries was monitored using DNA High Sensitivity Reagent Kit (Perkin Elmer, CLS60672) on the Labchip GX system (Perkin Elmer). All samples were subjected to an indexed PE sequencing run of 2×51 cycles on HiSeq 2000 (Illumina) at 16 samples per lane.

2.8 Bulk RNA-seq analysis

Paired-end raw reads were mapped to GRCh38 human genome build using STAR aligner [17]. Genes were counted for reads that were mapped to genes using featureCounts [18] based on GENCODEv29 gene annotation [19]. Log2 transformed counts per million mapped read (log2CPM) and log2 transformed reads per kilobase per million mapped reads (log2RPKM) were computed using edgeR Bioconductor package [20]. Across all samples, genes with log2CPM inter-quartile range (IQR) less than 0.5 were filtered out from subsequent differential expression gene (DEG)

analysis. DEG analyses for culture condition comparisons were all done using edgeR. Selection of DEGs was done with Benjamini-Hochberg [21] which adjusted P-values <0.05. R function ‘prcomp’ was used to perform Principal Component Analysis (PCA) on log2RPKM values. The pseudobulk values were calculated using the ‘AverageExpression’ function in Seurat. The pearson correlation were calculated using the ‘cor’ function in R. The GO enrichment was calculated using the ‘enrichGO‘ function from the ClusterProfiler package.

2.9 Quantitative real time PCR (qPCR)

Total RNA extracted using RNeasy Plus Micro-kit (Qiagen, 74034) was quantified using a NanoDropTM ND-1000 Spectrophotometer and converted to cDNA using iScript cDNA synthesis kit (Bio-Rad Laboratories, 1708890). qPCR was performed in 7000 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA) with FastStart Universal SYBR Green Master (Rox) (Roche, 04 913 850 001) and primers from GeneCopoeia, Inc. (Rockville, MD, USA). Gyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control. Accession numbers of tested genes are listed in Table below:

Target Gene	Gene Name	Accession
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	NM_001256799.2
AFP	Alpha fetoprotein	NM_000295
AAT	Alpha-1 antitrypsin	NM_001134
ASGPR	Asialoglycoprotein Receptor	NM_001671
ALB	albumin	NM_000477.3
CYP1A2	Cytochrome P450 1A2	NM_000761.5
CYP3A4	Cytochrome P450 3A4	NM_017460.5
CYP2B6	Cytochrome P450 2B6	NM_000767.4
CYP2C9	Cytochrome P450 2C9	NM_000771.3
UGT1A3	UDP-glucuroosyltransferase 1A3	NM_019093.2
GST1A2	Glutathione S-Transferase	NM_000846.3
MRP2	Multidrug resistance-associated protein 2	NM_000392
CD14	Cluster of differentiation 14	NM_001174105.1
CD32	Cluster of differentiation 32	NM_001136219.1
CD68	Cluster of differentiation 68	NM_001251.2
CD163	Cluster of differentiation 163	NM_203416.3
ID1	Inhibitor of DNA-binding protein 1	NM_002165.3
ID3	Inhibitor of DNA-binding protein 3	NM_002167.4

2.10 Enzyme-linked immunosorbent assay (ELISA) for measurement of cytokines

Interleukin-6 (IL-6) levels in the media were measured human IL-6 ELISA kit (Abcam, ab178013) according to manufacturer’s instructions. The cytokine production from dosed cells was normalized with the viability of cells measured from the same well. This ensures that changes in cytokines are not due to changes in cell numbers that might arise upon drug treatment. The

normalized cytokine level was expressed as percentage of DMSO+LPS which allowed better comparison between different batches of experiments. This normalization approach has been used in previous studies [22].

2.11 Cell viability assay

Cell viability was examined with AlamarBlue^{TM} cell viability assay (Thermo Fisher Scientific Inc., Dal1025) according to manufacturer's instructions. Briefly, AlamarBlue was diluted 10-fold using PBS containing 2 mg/mL of glucose and this working solution was added to the cells and incubated for 1 hour. Fluorescence (Ex: 530nm, Em: 590nm) readings were obtained using Tecan Microplate Reader M1000 PRO.

2.12 Drug administration

Following set up of co-culture as described in 2.5, the model was subjected to treatments including 100 ng/ml LPS (Sigma Aldrich, L2654), and drugs (Sigma Aldrich) (with/without LPS) for 48hr. Stock solutions of drugs were prepared in dimethyl sulfoxide (DMSO) and diluted in medium prior to use. Medium containing 0.1% DMSO was used as vehicle control except. After 48 hr, supernatant was collected cytokine measurement and cell viability in the same wells were measured as described in 2.11.

2.13 Statistical analysis

Mean and standard deviation were obtained from at least three independent batches of cells. Unpaired, paired student's T-test and one-way or two-way analysis of variance (ANOVA) were performed accordingly at an overall confidence level of 95% using Prism software (GraphPad, San Diego, CA, USA) and indicated below each figure.

3. Results

3.1 Optimization of culture conditions allow survival and function of iHeps and iMacs

In order to investigate the effects of co-culturing iHeps with iMacs, we first established and optimized a co-culture system utilising IMR90 iPSC-derived iHeps and iMacs (Figure 1A). To ensure survival and functionality of both cell types in our desired culture period (at least one week), certain key factors had to be accounted for: Hepatocytes are high in metabolically activity and often require frequent media change for replenishment of nutrients and removal of waste. On the other hand, it would be ideal to allow the iHeps and iMacs to interact through direct crosstalk as well as through secreted soluble factors without removing these factors from the system via media exchange. To assess the performance of hepatocytes with more infrequent media changes, expression of key hepatic markers was assessed when media was changed every other day, every two days or every three days and compared to a daily media change (Supplementary Figure 1). To compensate for the lower frequency of media change, we tested the effects of 2X supplements; standard media volume (Supplementary Figure 1A) and 2X supplements with 2X standard culture volume (Supplementary Figure 1B). Gene expression was compared as fold change to standard hepatocyte culture conditions (daily media change with 1X supplement and standard media volume). Increasing supplements alone could maintain the expression of ALB (albumin), AAT (alpha-1-antitrypsin), cytochrome P450 (CYP)- 1A2, 3A4, 2B6 and UDP-glucuronosyltransferase (UGT)1A3, but not CYP2C19 (36 - 68% decrease), glutathione S-transferase (GST)A2 (35-66% decrease) and multidrug resistance protein-2 (MRP2; 7 to 18% decrease) (Supplementary Figure 1A). In contrast, combining the increase in supplements with increase in culture volume resulted in maintenance of all hepatic markers (Supplementary Figure 1B). Albumin and urea production were also maintained to 1-1.4 pg/cell/48 hrs and 126-146 pg/cell/48 hrs (Supplementary Figure 1C) which is in the range of albumin and urea production reported by us [11, 14, 22] and others [23–27] previously.

3.2 Co-culture with iMacs improves iHep maturation and development

Cells sorted via flow cytometry on Day 3 and Day 7 (Supplementary Figure 1D, 1E), and the co-cultured hepatocytes (co-iHeps) and co-cultured iMacs (co-iMacs) were then analysed by RNA sequencing in to assess if co-culturing leads to transcriptomic changes reflecting improve differentiation and maturation. We also analysed Day 0 and Day 7 mono-culture controls, as well as iMacs cultured in PHCM for 7 days as control groups.

Principle component analysis (PCA) of the iHep samples revealed that that although the direction of change was the same for both the mono-cultured and co-cultured iHeps, there was a greater degree of change along the PC1 axis for co-iHeps (Figure 1B). We hypothesised that this might be due to the iMacs promoting further maturation and development of the iHeps, and performed a Pearson correlation between the mono-cultured and co-cultured iHeps against *in vivo* fetal liver hepatocytes from a published dataset [28], which revealed that the co-cultured iHeps were better correlated with the *in vivo* hepatocytes than the mono-cultured iHeps (Supplementary Figure 1F). Indeed, looking at the differentially expressed genes (DEGs) between the Day 7 iHeps and Day 7 co-iHeps revealed that the co-iHep upregulated genes associated with tissue morphogenesis and repair like Early Growth Response 1 (*EGR1*) [29], centrosome duplication such as the long non-coding RNA *CCDC14* [30] and angiogenesis-related gene like Maternally Expressed Gene 9 (*MEG9*) [31] (Figure 1C, Supplementary Table 1). On the other hand, iHeps cultured alone upregulated genes that inhibit proliferation and angiogenesis such as Histone Rich Glycoprotein (*HRG*) [32] and Kinogen 1 (*KNG1*) [33] (Figure 1C, Supplementary Table 1). We then performed pathway analysis using Ingenuity and discovered that the top upregulated pathway in Day 7 iHeps was acute phase response signalling, with the associated genes mainly relating to protein production such as Transthyretin (*TTR*) and Serpin Family D Member 1 (*SERPIND1*) (Supplementary Table 2), while the top upregulated pathway in Day 7 co-iHeps was related to nitric oxide and reaction oxygen species production, consisting mainly of metabolic and phosphatase genes like *c-FOS* and Serine/Threonine protein phosphatase 2A regulatory subunit B" beta (*PPP2R3B*) (Figure 1D, Supplementary Table 3). A closer look at the pathways upregulated in Day 7 iHeps and Day 7 co-iHeps also revealed that the Day 7 co-iHeps had a stronger tissue development and anabolic signature, upregulating pathways associated with tumor microenvironment, fibrosis and growth hormone signalling, while the Day 7 iHeps upregulated iron and cholesterol pathways.

Finally, we compared the expression of key genes across all the iHep samples (Figure 1E). In line with our hypothesis that co-culturing iMacs with iHeps would improve the maturation of the iHeps, we found that alpha-fetoprotein (*AFP*), a marker that is upregulated during fetal development and then downregulated as hepatocytes mature [34] followed a similar trend in our culture system, with lower expression in the Day 7 iHeps as compared to the Day 0 iHeps. Furthermore, the expression levels of *AFP* in Day 3 co-iHeps and Day 7 co-iHeps was even lower than that of Day 7 iHeps. Likewise Hepatocyte Nuclear Factor 4 (*HNFA4*), a transcription factor upregulated in hepatic progenitor cells [35], trended toward reduced expression in the co-cultured iHeps, albeit non-significantly. Insulin-like growth factor-2 (*IGF-2*), a key growth factor in fetal development [36], was significantly upregulated in the co-cultured iHeps 3 days after co-culture, with expression diminishing at the 7 day mark. Cytochrome P450 Family 2 Subunit C member 9 (*CYP2C9*), a cytochrome involved in drug metabolism was also significantly upregulated in the co-iHeps, while Aldolase Fructose-Biphosphate B (*ALDOB*), an enzyme whose upregulation is associated with unregulated cell proliferation and poor cancer prognosis [37] had a non-significant trend towards downregulation. Taken together, our data suggest that co-culturing the iHeps with iMacs increased their maturation and promotes improved hepatocyte development.

3.3 Co-culture with iHeps imparts KC-like identity to iMacs

Next, we turned our attention towards the iMacs within our co-culture system to test if they acquired KC features. PCA analysis showed that the co-iMacs clustered much tighter together, while iMacs that were cultured alone or in hepatocyte conditioned media (cond-iMac) were much

more scattered (Figure 2A). This suggests that co-culturing iMacs in direct contact with iHeps may help maintain the identity of the iMacs better, mirroring how *ex vivo* resident tissue macrophages (RTMs) rapidly lose their identity when removed from their tissue environment [38]. DEG analysis of all the iMac groups showed that both the Day 7 iMacs and the Day 7 cond-iMacs shared an activated and inflammatory profile, upregulating genes such as TNFSF18 [39] and ACP5 [40] (Figure 2B, Supplementary Table 4). Day7 iMacs had a more migratory profile, uniquely expressing Chemokine Receptor 7 (*CCR7*) and Chemokine Ligand 5 (*CCL5*), while Day 7 cond-iMacs uniquely expressed Dishevelled Associated Activator of Morphogenesis 2 (*DAAM2*), a developmental regulator of the WNT pathway [41].

As we were interested in how the presence of iHeps in such co-culture model might educate and impart KC-like identity to the iMacs, we looked at the DEGs between Day 0 iMac and Day 7 co-iMacs. Day 0 iMacs were more immunogenic, expressing Proteoglycan 2 Pro Eosinophil Major Basic Protein 2/3 (*PRG2/PRG3*) and Carboxypeptidase A3 (*CPA3*) (Figure 2C). In contrast, Day 7 co-iMacs upregulated the angiogenic Angiopoietin-like 4 (*ANGPTL4*) [42], matching the angiogenic signature observed in the Day 7 co-iHeps. Importantly, Ingenuity pathway analysis revealed that the top pathway in Day 7 co-iMacs is the Liver X Receptor/Farnesoid X Receptor (LXR/RXR) signalling pathway (Figure 2D, Supplementary Table 5), a key regulator of KC identity [43], while the top pathway in Day 0 iMacs is the axonal guidance pathway, consisting of chemokines like C-X-C Motif Chemokine Ligand 12 (*CXCL12*) and C-X-C Motif Chemokine Receptor 4 (*CXCR4*), metalloproteases like ADAM Metallopeptidase with Thrombospondin Type 1 Motif 15 (*ADAMTS15*), and semaphorins like Semaphorin 3C (*SEMA3C*) (Supplementary Table 6). This suggests that co-culturing iMacs with iHeps initiated a KC-specific programme, while the Day 0 iMacs had a more general and unspecified identity lacking environmental cues. In addition, Day 7 co-iMacs also upregulated pathways associated with tissue repair and remodelling, as well as tissue growth factors, while migratory and immune signalling related pathways were upregulated in the Day 0 iMacs (Figure 2D). Altogether, these data suggest that co-culturing iMacs with iHeps is sufficient to impart a more tissue supportive and KC-like identity to the iMacs.

The use of conditioned media as a surrogate of the *in vivo* tissue microenvironment is a popular approach, and we wondered how different iMacs cultured in direct co-culture with iHeps would be from iMacs cultured in conditioned media from primary hepatocytes (PHCM). Comparing the DEGs between Day 7 cond-iMacs and Day 7 co-iMacs, the Day 7 cond-iMacs upregulated immune-related genes such as Toll-Like Receptor 4 (*TLR4*) and *CPA3*, while the Day 7 co-iMacs more highly expressed Fc Gamma Receptor IIIa (*FCGR3A*), which is also upregulated in Kupffer Cells (Figure 2E). Pathway analysis showed that the top upregulated pathway in the Day 7 cond-iMacs was autophagy, which might indicate that conditioned media alone was insufficient to maintain proper macrophage biology in the absence of contact with other cells, although some hepatic related pathways were also upregulated as well (Figure 2F, Supplementary Table 7). On the other hand, the most highly upregulated pathway in the Day 7 co-iMacs was the FXR/RXR pathway, which along with the upregulation of other anabolic pathways related to hepatic health (Supplementary Table 8), suggest that co-culturing rather than conditioned media is superior at inducing KC-like identity in macrophages.

We then looked at the expression of selected key KC genes. Lymphatic Vessel Endothelial Hyaluronan Receptor 1 (*LYVE1*), DNA-binding protein inhibitor ID1 (*ID1*) and DNA-binding protein inhibitor ID3 (*ID3*), which are important markers of KC identity [42]. *ID3* was significantly upregulated in the co-iMacs but not in the cond-iMacs, with a trend toward increased *ID1* and *LYVE1* expression as well (Figure 2G). Retinoid X Receptor Alpha (*RXRA*), the obligate binding partner of LXR [44], was also upregulated in only the co-iMacs. Interestingly, we also found that the co-iMacs upregulated the expression of Heparin Binding EGF Like Growth Factor (*HBEGF*) [45] and Oncostatin M (*OSM*) [46], which are potent effectors of liver regeneration and development.

Finally, we compared the iMacs from our study with *in vivo* embryonic human liver monocytes and KCs [47]. Pearson correlation revealed that regardless of condition the *in vitro* derived iMacs were poorly correlated to the *in vivo* human liver monocyte/macrophages (Supplementary Figure 2A), suggesting that either the co-culture duration was insufficient to induce full KC

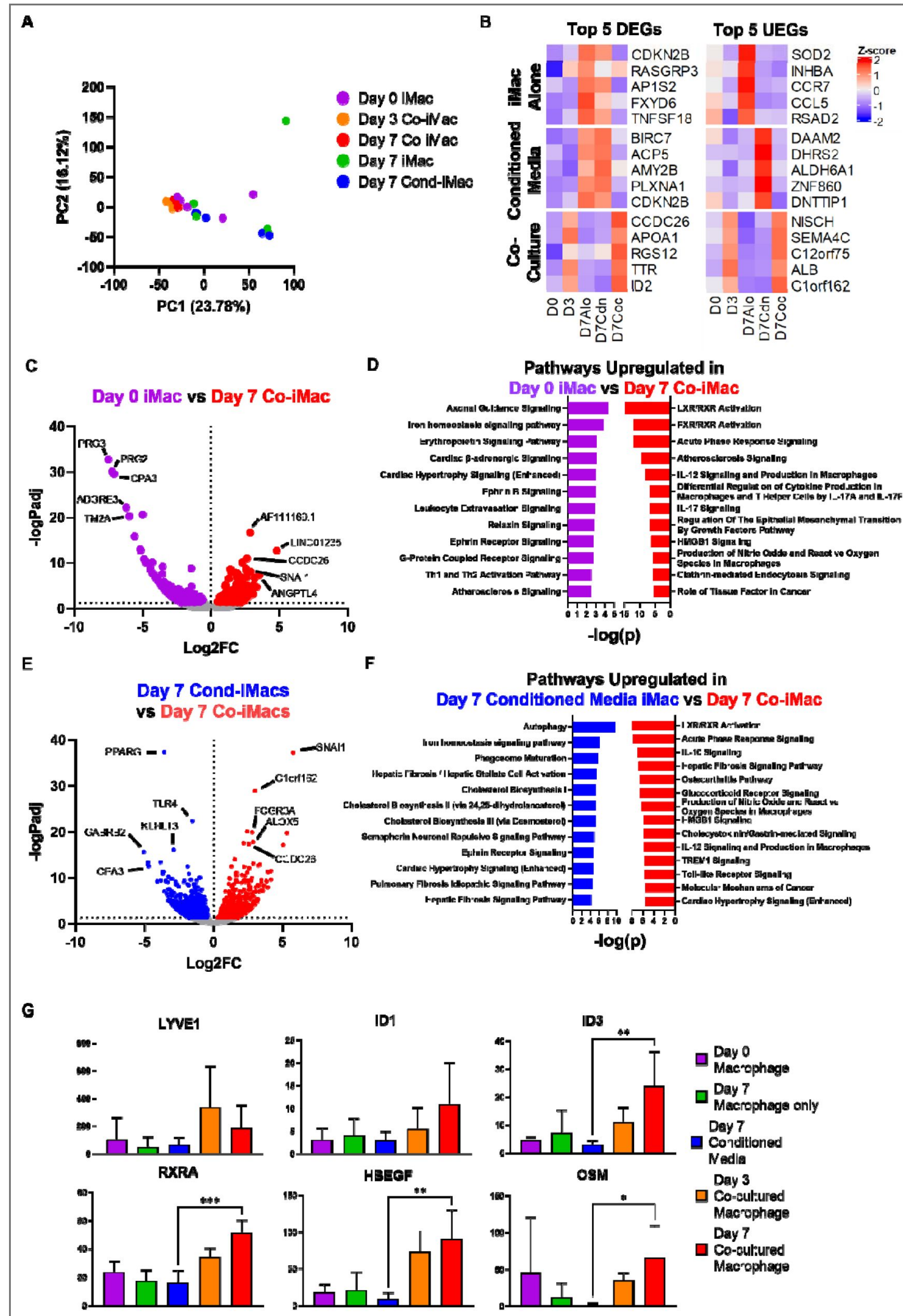


Figure 2. **Effects of iHeps on iMacs.**

A) Principal Component Analysis of bulk RNAseq data from Day 0 iMacs, Day 3 co-iMacs, Day 7 co-iMacs, Day 7 iMacs and Day 7 cond-iMacs. B) Heatmap showing the top 5 differentially and uniquely expressed genes from culturing iMacs alone, with conditioned media (cond-iMacs) and after co-culture with iHepatocytes (Day 7 co-iMacs). C) Volcano Plot showing differentially-expressed genes between Day 0 iMacs and Day 7 co-iMacs. D) Differentially expressed upregulated pathways between Day 0 iMacs and Day 7 co-iMacs. E) Volcano Plot showing differentially-expressed genes between Day 7 cond-iMacs and Day 7 co-iMacs. F) Differentially expressed upregulated pathways between Day 7 cond-iMacs and Day 7 co-iMacs. G) Gene expression levels of *LYVE1*, *ID1*, *ID3*, *RXRA*, *HBEGF* and *OSM* in Day 0 iMacs, Day 3 co-iMacs, Day 7 co-iMacs, Day 7 iMacs and Day 7 cond-iMacs. Student's T test was applied. *p<0.05, **p<0.01, ***p<0.001

commitment or that there might be missing cues from other cell types that were not present in our co-culture. However, pathway analysis on the DEGs between the embryonic human liver macrophages and either the Day 7 cond-iMacs (Supplementary Figure 2B, Supplementary Table 9) or Day 7 co-iMacs (Supplementary Figure 2C, Supplementary Table 10) revealed that pathways relating to cytokine production, sensory development and eye development were differentially regulated between the Day 7 cond-iMacs and embryonic liver macrophages, while there were no significant pathway differences between the Day 7 co-iMacs and embryonic liver macrophages. This suggests that direct co-culture might drive the macrophages to adopt a more tissue developmental or supportive phenotype.

Altogether, the data supports our hypothesis that co-culturing iMacs with iHeps induces a KC-like tissue supportive identity in the iMacs, which in turn promotes the maturation and development of the iHeps.

3.4 Validation of the acquisition of KC-like identity by iMacs and maturation of iHeps in co-culture

We confirmed the observations from the RNA sequencing analysis by analysing gene expression of macrophage and KC-specific markers in Day 3 co-iMacs and Day 7 co-iMacs via RT-PCR. Macrophage markers *CD68*, *CD163*, *CD11b*, *CD32* and *CD14* were all upregulated at Day 3 and Day 7 by 1.8 to 7.6 fold compared to iMac mono-culture control (Figure 3A). Importantly, KC-specific markers *ID1* and *ID3* were upregulated by 3.8 fold and 3.9 fold respectively at Day 3 and by 6.3 fold and 6.5 fold respectively at Day 7 (Figure 3B). This suggests that macrophage phenotype is maintained during the 7 days of co-culture but KC-specific development increases in a time-dependent manner. When Day 7 co-iMacs were compared to iMacs cultured in PHCM (Day 7 cond-iMacs; without the presence of iHeps), macrophage marker upregulation compared to iMac mono-culture control were similar in both conditions (Figure 3C) but KC-specific *ID1* and *ID3* were upregulated to a lower extent in Day 7 cond-iMacs (2.6 fold and 2.5 fold respectively) compared to Day 7 co-iMacs (6.3 fold and 6.5 fold respectively; Figure 3D).

Next, we confirmed the RNA sequence analysis of iHep via RT-PCR. With the exception of Glutathione-S transferase1A2 (*GST1A2*) and Multidrug Resistance Associated Protein 2 (*MRP2*), co-culture with iMacs improved expression of several hepatic marker genes including *ALB*, α-1 antitrypsin (*AAT*), cytochrome P450 enzymes (*CYP1A2*, *CYP3A4*, *CYP2C9*, *CYP2B6*) and UDP-glucuronosyltransferases (*UGT1A3*) by 1.8-53.1 fold at Day 3 and Day 7 of co-culture (Figure 3E). Importantly, *AFP*, a marker that is expressed at high levels in fetal hepatocytes and increases with hepatocyte maturation [30], had 37% lower expression at Day 3 and 37% lower expression in Day 7 iHeps as compared to Day 0 iHeps (Figure 3E). This is consistent with RNA sequencing data that showed a downregulation of *AFP* (Figure 1E).

Overall, validation of the expression of key markers observed as modulated by RNA sequencing analysis confirms that culturing iMacs with iHeps imparts KC-like phenotype in iMacs and supports maturation and function of the iHeps.

3.5 Co-culture model accurately mimics clinical/*in vivo* immune responses of drugs

Drug-induced liver injury (DILI) is a complex and critical clinical problem with an estimated incidence rate of up to 19 cases per 100000 people in western countries [48]. In particular, immune-mediated idiosyncratic drug-induced liver injuries remains especially poorly understood, due in large part to the lack of appropriate human modelling systems [6]. To test the application of the co-culture in detecting immune-mediated response to hepatotoxic drugs, we carefully selected a group of 7 drugs based on the following criteria: 1) confirmed reports of immune/inflammation-associated effects according to 3 databases [49–51]; 2) available clinical data, or *in vivo* responses if the former is unavailable; and 3) known Cmax (maximum serum concentration of drug) to guide the concentration of drug used in the *in vitro* model. Drug concentrations used in our system was no more than 20-fold of its Cmax (Details of selected drugs are summarized in Supplementary

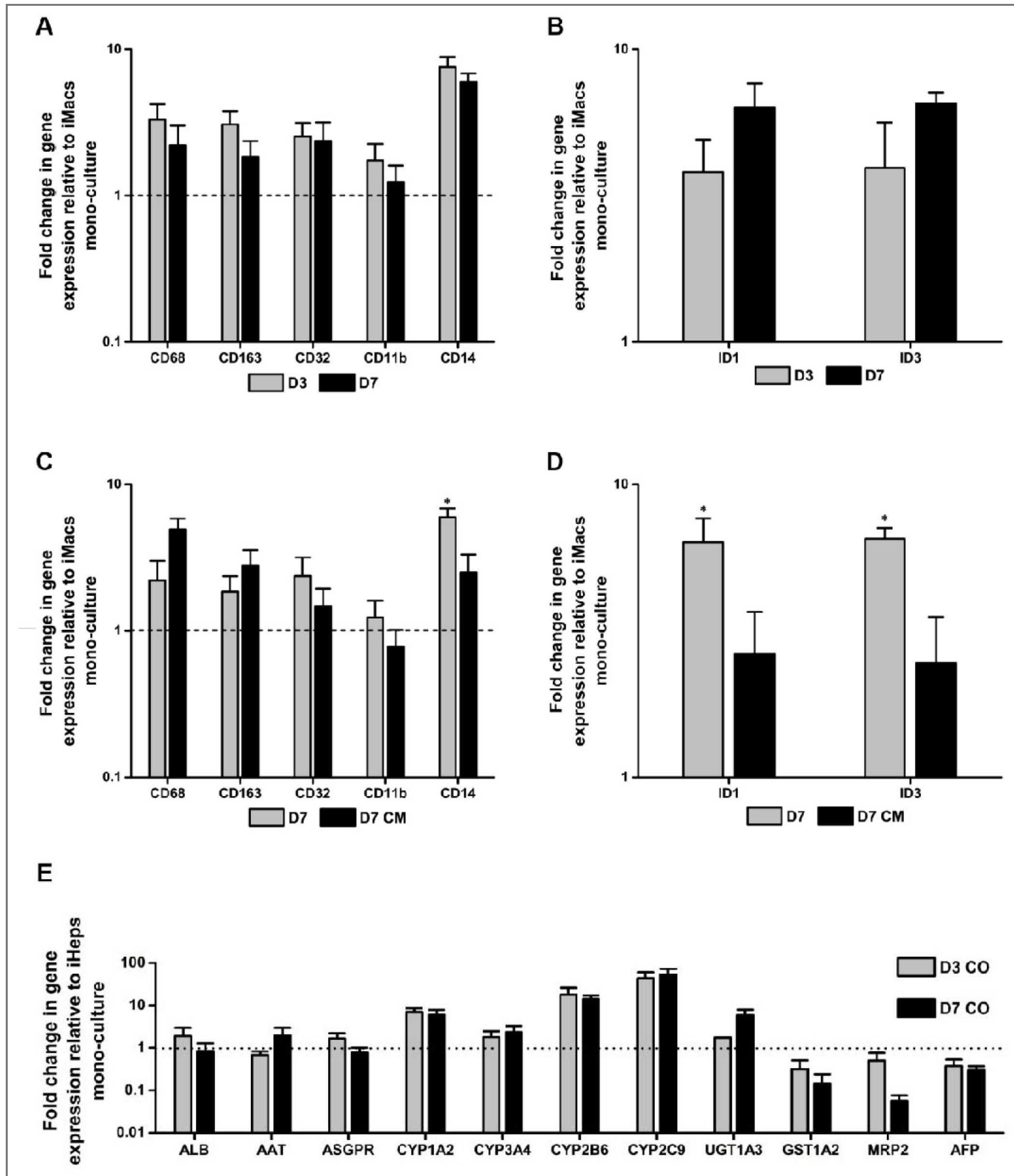


Figure 3. **Gene expression of iMac and iHep markers in co-cultures.**

A) Expression of macrophage markers (left panel) and KC-specific markers (right panel) on day 3 and day 7 of co-culture. B) Expression of macrophage markers (left panel) and KC-specific markers (right panel) on day 7 of co-culture compared to iMac cultured in PHCM. C) Expression of hepatic markers (left panel) on day 3 and day 7 of co-culture. * p<0.05.

Table 11). The drugs included diclofenac (DIC), sulindac (SLD) leflunomide (LFM); amodiaquine (AQ), lamotrigine (LTG), penicillin (PEN), pyrazinamide (PZA). DIC [52], SLD [53] and LFM [54, 55] have been reported to cause a decrease in interleukin-6 (IL-6); whereas AQ and LTG have been reported to cause an increase in IL-6 in patients with drug-induced liver injury [51]. PEN and PZA were selected as negative controls as no immune-mediated effects of these drugs have been reported. The cultures were co-treated with lipopolysaccharide (LPS), which has been reported as an important initiating co-factor in the development of DILI [57].

Our co-culture model could correctly recapitulate IL-6 decrease in DIC (48%, 45% and 18% at 15.7 µM, 50 µM and 150 µM respectively), SLD (46%, 11% and 8% at 67 µM, 200 µM and 600 µM respectively) and LFM (65%, 46%, 38% at 32.5 µM, 125 µM and 500 µM respectively). IL-6 increase upon AQ (157-200 fold) and LTG (155-322 fold) treatment was also correctly recapitulated. No changes in IL-6 level in PEN and PZA-treated samples were observed (Figure 4). Importantly, when iMac-derived iKC-like cells were replaced with blood Monocytes/Macrophages, none of these cytokine changes were recapitulated with the exception of SLD treatment (Figure 5). This shows, from a functional/application perspective, how iMac-iKCs, but not MoMLJs, are indispensable for *in vitro* liver models that can detect immune-mediated changes of hepatotoxicants.

In vivo, KCs have been known to release mediators that are early features of inflammatory responses [56]. They are activated in response to inflammatory stimuli such as bacterial LPS that can damage the liver at large doses in a KC-dependent manner. At smaller doses, LPS activates KCs (without causing liver injury) but sensitizes the liver to a variety of other xenobiotics [57]. In our model, when the co-culture was co-treated with paradigm hepatotoxicants and LPS, drug-dose-dependent changes in cytokine production were observed (Figure 4), suggesting that the system can indeed mimic physiological responses, demonstrating the importance of liver-specific imprinting of macrophages in recapitulating responses in an *in vitro* liver model.

4. Discussion

IPSC-derived tissue cultures have tremendous potential in its ability to generate cells from all intra-embryonic lineages and have greatly expanded both our knowledge of mammalian tissue development and our technical toolbox for therapy and disease modelling [58]. However, a tissue is often more than the sum of its parts, and iPSC-derived tissue models have so far proven to be limited in their ability to effectively recapitulate the functionality and complexities of their presumed organs. In our study, we demonstrated how co-culturing iMacs with iHeps leads to not only acquisition of resident tissue characteristics within the iMac population, but also increased maturation of iHeps. We demonstrated that iPSC-derived Heps can directly impart hepatic macrophage-like identity to iMacs, giving them an iKC-like phenotype and genotype without the need for any additional soluble factors or components present in conditioned media. Application of this model was demonstrated by the ability of the system to mimic clinical IL-6 responses of 5 paradigm immune-mediated hepatotoxicants. Two additional hepatotoxicants with no known immune-mediated responses did not show any response in our model, demonstrating the specificity of the system. Finally, when the immune-mediated drug effects were tested in a system with iHeps and PBMC-derived macrophages, expected cytokine responses were not observed, indicating the importance of iKC-iHep co-culture in predicting drug mediated-immune responses.

Animal models have been used to model immune-mediated liver injury [59–61]; however, consistent liver injury was not observed upon treatment with drugs known to cause immune response in humans, even at high doses [62]. In cases where injury was observed, the characteristics were different from humans and possibly manifested through different mechanisms [61]. Such interspecies variations, contradictory findings as well as cost and ethical issues have spurred the development of *in vitro* models to supplement *in vivo* animal models. *In vitro* models, possibly due to the lack of appropriate human immune cell sources, have focused on soluble factor-driven single hepatocyte cultures in combination with inflammatory mediators or animal cell co-cultures [6]. However, mono-cultures lack cell-cell interactions, and animal *in vitro* models often fail to recapitulate *in vivo* and clinical findings. *In vitro* hepatocyte-KC models have previously been used; however most of them have utilised animal cells [63,64] which often prove

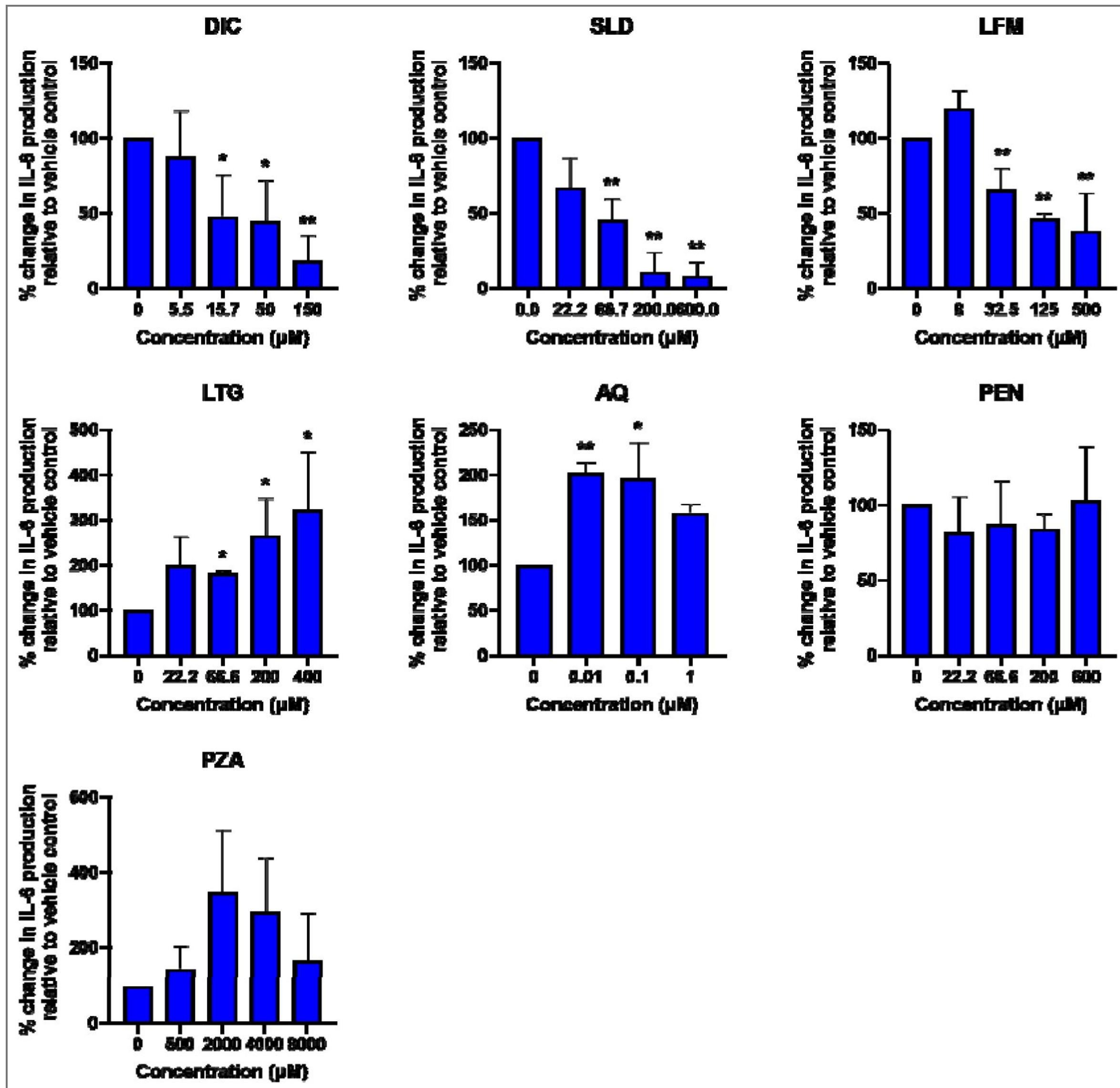


Figure 4. Changes in IL-6 level upon treatment with 7 paradigm compounds when iMac-derived KCs were used.

Cytokine production was assessed in iMac-derived iKC and iHep co-culture treated with the drug stated in the plot title. Data is expressed as the percentage of the LPS-treated vehicle control. Error bars represented S.D., n=3. One-way ANOVA was applied. *p<0.05, **p<0.01 between treatment and vehicle control.

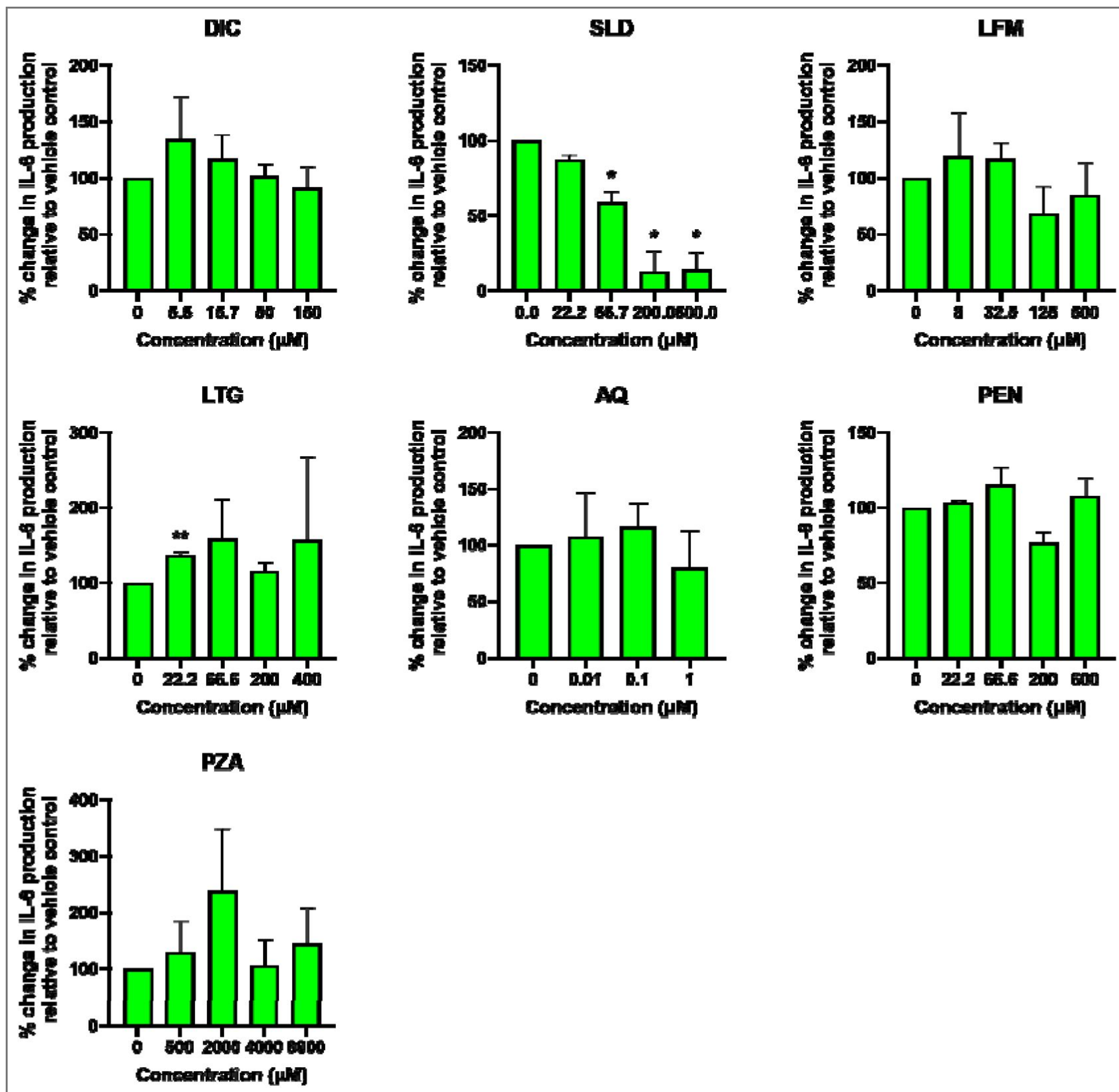


Figure 5. **Changes in IL-6 level upon treatment with 7 paradigm compounds without iMac-derived KCs.**

Cytokine production was assessed in blood monocyte-derived macrophages and iHep co-culture treated with the drug stated in the plot title. Data is expressed as the percentage of the LPS-treated vehicle control. Error bars represented S.D., n=3. One-way ANOVA was applied. *p<0.05, **p<0.01 between treatment and vehicle control.

inadequate in recapitulating *in vivo* or clinical findings. In order to avoid interspecies variations and improve the predictivity of *in vitro* models, appropriate human models still need to be developed and characterized.

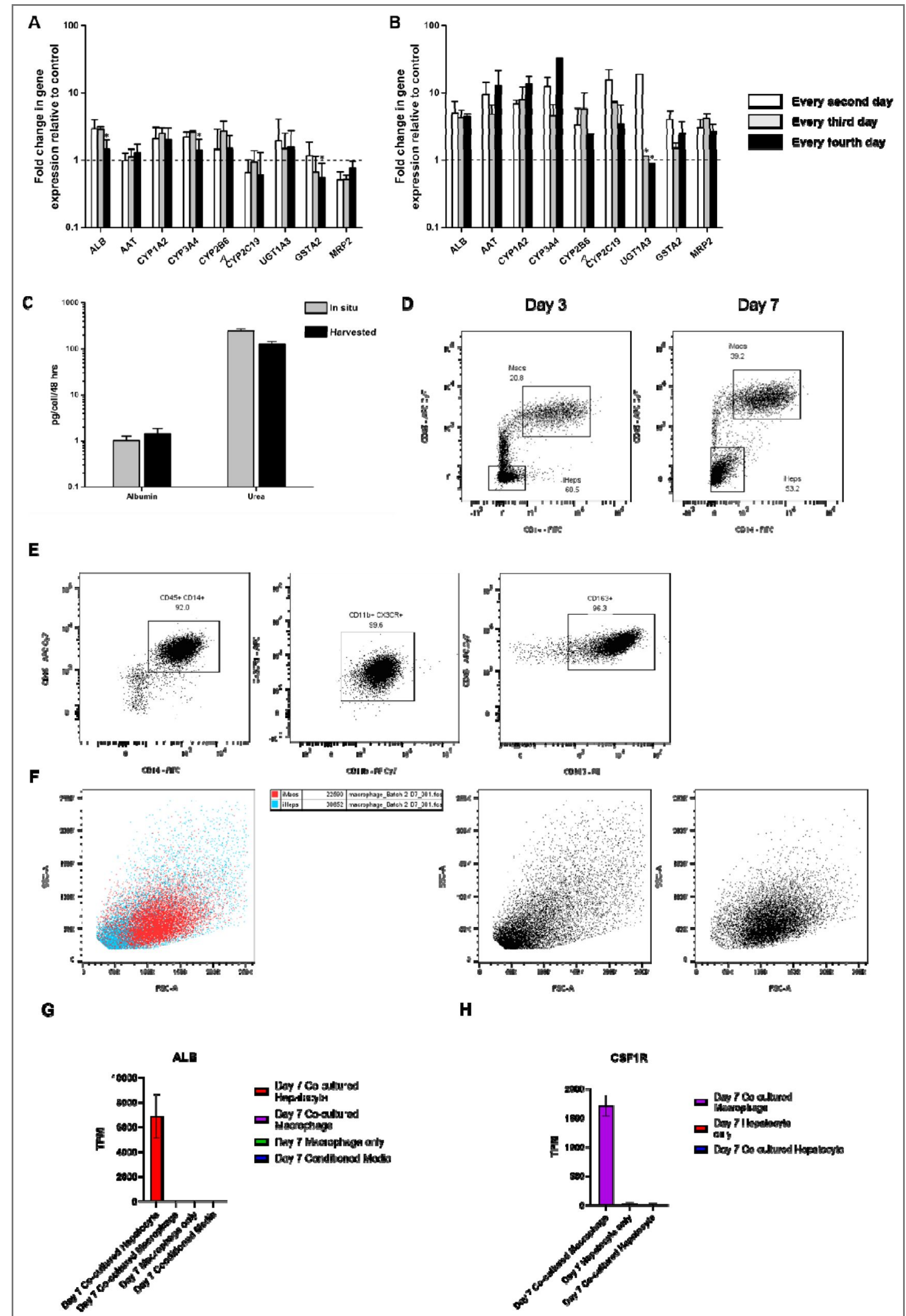
The main challenge in studying human KCs in *in vitro* models is the cell source. The source of commercial primary human KCs (PhKCs) is limited and costly and high donor-to-donor variability is often observed [65]. Alternative human cell sources include THP-1 cells and peripheral monocyte-derived macrophages; however their cytokine profiles are dramatically different from human KCs [66, 67] and they lack liver-specific imprinting [43]. Even when human KCs were used, the work mostly focused on co-culture dependent improvement of hepatocyte function [6, 68], rather than interaction between the two cell-types and immune-mediated effect of drugs. Moreover, these models are often non-isogenic, which further confounds the inflammatory readouts. Hence, in our study, we aimed to first determine if the presence of hepatocytes could impart a tissue-resident-like phenotype to iMacs and drive them to be more Kupffer cell-like, then measured the systemic response to drug treatment compared to using monocyte-derived macrophages. While all tested drugs showed expected changes in cytokine production in the iMac-iHep co-culture (Figure 4), these changes were not recapitulated when the iMacs were replaced by MoMLJs (Figure 5) with the exception of SLD. Interestingly, expected IL-6 increase upon treatment with LFM could not be recapitulated in our previous model of iHep-iKCs co-culture where iKCs were generated using conditioned media from primary hepatocytes (data not shown). However, in our current model, LFM treatment resulted in the expected increase in IL-6, suggesting the potential importance of direct cellular cross-talk in iKCs differentiation and function.

Development of a suitable model system is further complicated by the understanding that KCs are derived from embryonic macrophages that seed the liver early on during development [7–9]. This also implies that conventionally used human peripheral blood MoMLJs might not be able to fully mimic KC biology. Thankfully, iMacs more closely resemble embryonic macrophages than MoMLJs [10] and as demonstrated in our own experiments, were able to acquire features of KCs upon co-culture with iHeps. Soluble factors were only partially responsible for this tissue adaptation, as in contrast to the iMacs that were cultured in hepatocyte conditioned media, the co-cultured iMacs upregulated genes in the LXR/RXR pathway, which has been shown to be important for KC specification and identity [43], further emphasising the importance of cell-cell contact for the acquisition of RTM identity. Furthermore, we also observed that these co-cultured iMacs were upregulating pathways associated with tissue repair and development, which might explain the increased maturity of the iHeps within the co-culture system. This is particularly relevant not only for hepatic model systems, but for other iPSC-derived model systems in general, as one of the key limitations of these systems is the functional and phenotypic immaturity of the differentiated cells [69]. Co-culturing iMacs with these immature iPSC-derived tissue model systems might provide the missing developmental cues needed for more physiologically and functionally relevant patient and disease models as well.

However, the results presented in our study are not without their own limitations as well. We primarily utilised bulk sequencing data in our analysis, and although we have demonstrated that there is minimal contamination between the hepatocyte and macrophage compartments of our co-culture, we are unable to completely rule out the minor possibility. Furthermore, our co-culture model is limited to only hepatocytes and macrophages, and might not fully address the complexity of the liver microenvironment. For instance, our model does not include other important cell types such as hepatic stellate cells [78] or sinusoidal endothelium cells [79]. This could perhaps explain why the day 3 co-cultured iHeps showed greater similarity to their i*n vivo* counterparts than the day 7 co-cultured iHeps, as the two-cell type co-culture alone might be enough to initiate co-development but insufficient to maintain it. Indeed, we observed transient upregulation of *IGF2* in the day 3 co-cultured iHeps (Figure 1E), suggesting the activation of a fetal developmental program. We are still unable to determine if its transient nature is an inherent feature of liver development or due to insufficient signalling. Finally, we also only specifically looked at *IL-6* expression as a systemic readout of liver function within our co-culture system. Although we were

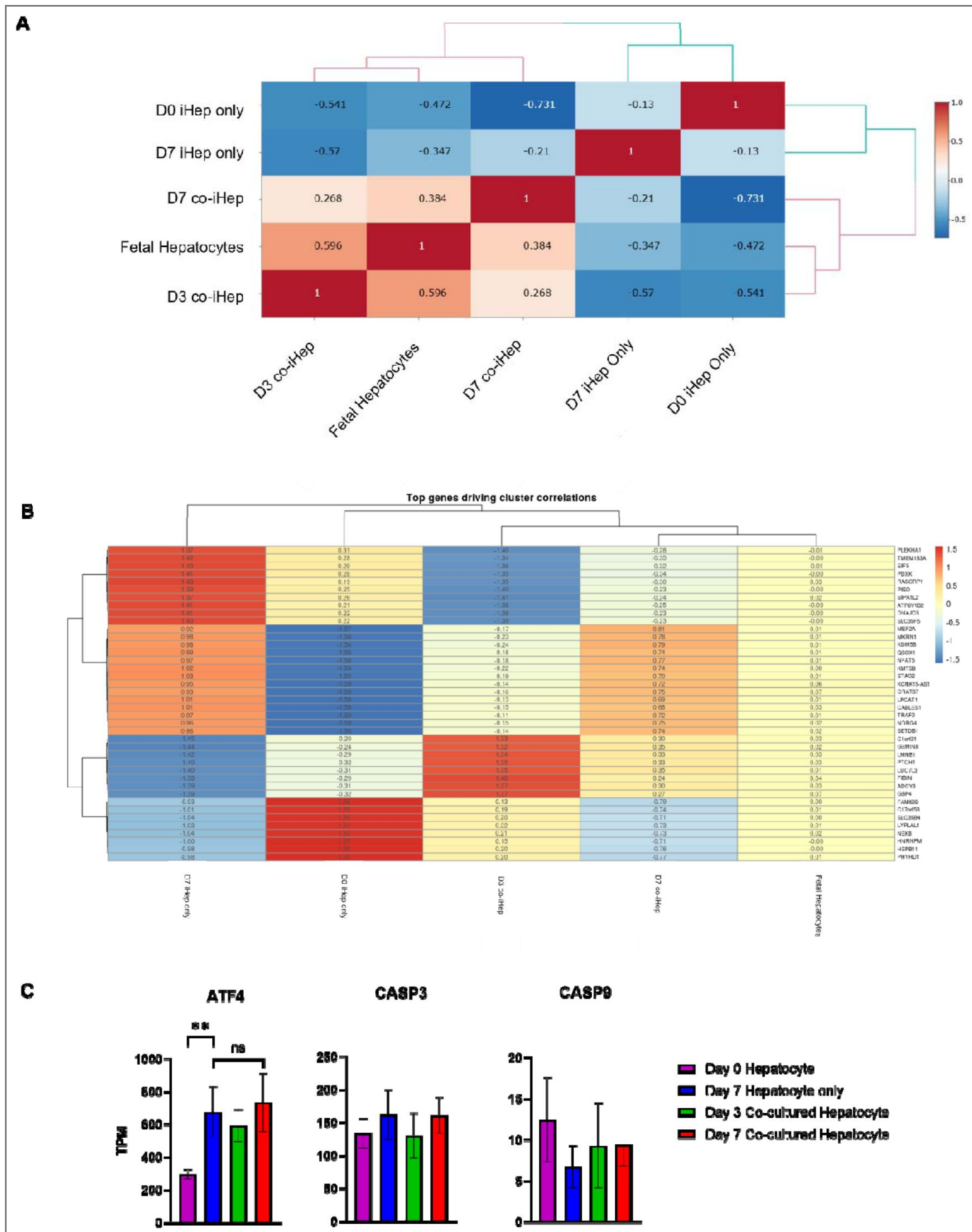
able to recapitulate the differential drug-specific expression of *IL-6* observed in various *in vivo* animal models of DILI [49–55], we did not explore the mechanisms behind the individual drug responses. It is important to note that a simplistic two-cell co-culture model system is still insufficient to fully encompass the complex systemic, metabolic and genetic landscape of a patient-specific condition such as DILI, but should instead serve to highlight the importance of incorporating other secondary cell types into model systems for a more holistic and accurate representation of tissue activity. Nevertheless, we believe that the development of co-culture systems containing multiple distinct cell types are an exciting next step in furthering our understanding of proper tissue specification and maturation, not only as a model of development but also tissue function.

Supplementary figures



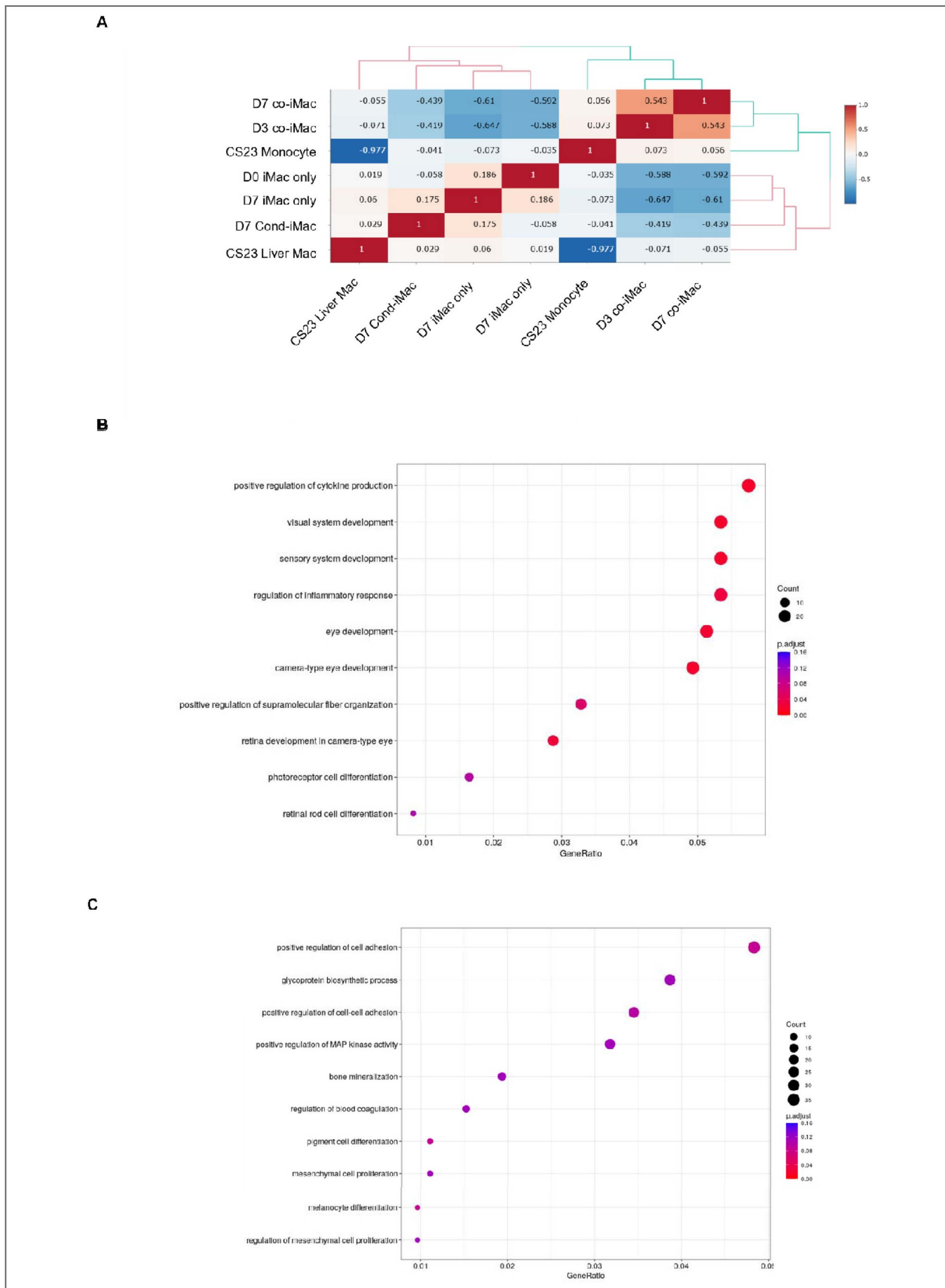
Supplementary Figure 1. Optimisation of co-culture conditions and validation of purity. A & B) Fold change in expression of key hepatocyte genes in normal media volume and supplement concentration (A) or double media volume and supplement concentration (B), normalised to normal hepatocyte culture condition. C) Production of Albumin and Urea in co-culture system after 2 days, normalised to non-co-cultured hepatocytes. D)

Flow cytometry of co-culture 3 and 7 days after co-culture. E) Flow cytometry showing purity of macrophages by CD45, CD14, CD11b, CX3CR1 and CD163 before co-culture. F)Flow cytometry showing relative cellular size and granularity of iMac and iHep populations after co-culture. G) Expression of Albumin in Day 7 co-cultured iHeps vs iMacs. H) Expression of CSF1R in Day 7 co-cultured iMacs vs iHeps.



Supplementary Figure 2. **Correlation of iPSC-derived hepatocytes with or without co-culture with iPSC-derived macrophages.**

A) Pearson correlation of iHeps against fetal hepatocytes from Popescu et al, (2019). B) Top 40 genes influencing Pearson correlation of Supp Fig 2A. C) Expression of ATF4, CASP3 and CASP9 showed no significant upregulation of stress or apoptosis genes after co-culture.



Supplementary Figure 3. Correlation of iMacs with embryonic liver monocytes and macrophages, and pathway analysis of the DEGs.

A) Pearson correlation of iMacs under control, conditioned media or co-culture conditions against embryonic liver monocytes and macrophages from Bian et al, (2020). B) Pathway analysis of DEGs between Day 7 conditioned media iMacs and embryonic liver macrophages. C) Pathway analysis of DEGs between Day 7 co-cultured iMacs and embryonic liver macrophages.

Data availability

Raw data from RNA-seq analysis have been deposited in the NCBI Gene Expression Omnibus under accession number GSE307755.

Additional files

Supplementary Table 1. Differentially Expressed Genes between the iHeps Samples.

Supplementary Table 2. Ingenuity Pathway Analysis for genes upregulated in Day 7 iHeps vs Day 7 co-iHeps.

Supplementary Table 3. Ingenuity Pathway Analysis for genes upregulated in Day 7 co-iHeps vs Day 7 iHeps.

Supplementary Table 4. Differentially Expressed Genes between the iMac Samples.

Supplementary Table 5. Ingenuity Pathway Analysis for genes upregulated in Day 7 co-iMacs vs Day 0 iMacs.

Supplementary Table 6. Ingenuity Pathway Analysis for genes upregulated in Day 0 iMacs vs Day 7 co-iMacs.

Supplementary Table 7. Ingenuity Pathway Analysis for genes upregulated in Day 7 cond-iMacs vs Day 7 co-iMacs.

Supplementary Table 8. Ingenuity Pathway Analysis for genes upregulated in Day 7 co-iMacs vs Day 7 cond-iMacs.

Supplementary Table 9. GO enrichment results for DEGs between Day 7 conditioned media iMacs and embryonic liver macrophages.

Supplementary Table 10. GO enrichment results for DEGs between Day 7 co-cultured iMacs and embryonic liver macrophages.

Supplementary Table 11. Details of drugs used for testing.

Additional information

Author ORCID iDs

Florent Ginhoux: https://orcid.org/0000-0002-2857-7755

References

[1] **Shan Z.**, Ju C. (2020) Hepatic macrophages in liver injury. *Frontiers in immunology* **11**:322 https://doi.org/10.3389/fimmu.2020.00322 | PubMed

[2] **Dixon L.J.**, Barnes M., Tang H., Pritchard M.T., Nagy L.E. (2013) Kupffer cells in the liver. *Comprehensive Physiology* **3**:785 https://doi.org/10.1002/cphy.c120026 | PubMed

[3] **Krenkel O.**, Tacke F. (2017) Liver macrophages in tissue homeostasis and disease. *Nature Reviews Immunology* **17**:306-321 https://doi.org/10.1038/nri.2017.11 | PubMed

[4] **Blériot C.**, Ginhoux F. (2019) Understanding the heterogeneity of resident liver macrophages. *Frontiers in Immunology* **2694** https://doi.org/10.3389/fimmu.2019.02694 | PubMed

[5] **Wen Y.**, Lambrecht J., Ju C., Tacke F. (2021) Hepatic macrophages in liver homeostasis and diseases-diversity, plasticity and therapeutic opportunities. *Cellular & molecular immunology* **18**:45-56 https://doi.org/10.1038/s41423-020-00558-8 | PubMed

[6] **Tasnim F.**, Huang X., Lee C.Z.W., Yu H., Ginhoux F. (2021) Recent Advances in Models of Immune-mediated Drug-Induced Liver Injury. *Frontiers in Toxicology* **3**:22 https://doi.org/10.3389/ftox.2021.605392 | PubMed

[7] **Mass E.,** Ballesteros I., Farlik M., Halbritter F., Günther P., Crozet L., Jacome-Galarza C. E., Händler K., Klughammer J., Kobayashi Y., *et al.* (2016) Specification of tissue-resident macrophages during organogenesis. *Science* **353**:aaf4238 https://doi.org/10.1126/science.aaf4238 | PubMed

[8] **Lee C.Z.W.,** Ginhoux F. (2022) Biology of resident tissue macrophages. *Development* **149**:dev200270 https://doi.org/10.1242/dev.200270 | PubMed

[9] **Scott C. L.,** Zheng F., De Baetselier P., Martens L., Saeys Y., De Prijck S., Lippens S., Abels C., Schoonooghe S., Raes G., *et al.* (2016) Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. *Nature Communications* **7** https://doi.org/10.1038/ncomms10321 | PubMed

[10] **Takata K.,** Kozaki T., Lee C.Z.W., Thion M.S., Otsuka M., Lim S., Utami K.H., Fidan K., Park D.S., Malleret B. (2017) Induced-pluripotent-stem-cell-derived primitive macrophages provide a platform for modeling tissue-resident macrophage differentiation and function. *Immunity* **47**:183-198.e6 https://doi.org/10.1016/j.immuni.2017.06.017 | PubMed

[11] **Tasnim F.,** Xing J., Huang X., Mo S., Wei X., Tan M.-H., Yu H. (2019) Generation of mature kupffer cells from human induced pluripotent stem cells. *Biomaterials* **192**:377-391 https://doi.org/10.1016/j.biomaterials.2018.11.016 | PubMed

[12] **Park D. S.,** Kozaki T., Tiwari S. K., Moreira M., Khalilnezhad A., Torta F., Olivié N., Thiam C. H., Liani O., Silvin A., *et al.* (2023) iPS-cell-derived microglia promote brain organoid maturation via cholesterol transfer. *Nature* **623**:397-405 https://doi.org/10.1038/s41586-023-06713-1 | PubMed

[13] **Cai J.,** Fisher J., Urick A., Wagner T., Cayo K.T.M., Nagaoka M., Duncan S.A. (2015) Protocol for directed differentiation of human pluripotent stem cells toward a hepatocyte fate. *African Scientist* **16**:13-26

[14] **Tasnim F.,** Toh Y.-C., Qu Y., Li H., Phan D., Narmada B.C., Ananthanarayanan A., Mittal N., Meng R.Q., Yu H. (2016) Functionally enhanced human stem cell derived hepatocytes in galactosylated cellulosic sponges for hepatotoxicity testing. *Molecular pharmaceutics* **13**:1947-1957 https://doi.org/10.1021/acs.molpharmaceut.6b00119 | PubMed

[15] **Baratta J.L.,** Ngo A., Lopez B., Kasabwalla N., Longmuir K.J., Robertson R.T (2009) Cellular organization of normal mouse liver: a histological, quantitative immunocytochemical, and fine structural analysis. *Histochemistry and Cell Biology* **131**:713-726 https://doi.org/10.1007/s00418-009-0577-1 | PubMed

[16] **Picelli S.,** Faridani O.R., Björklund Å.K., Winberg G., Sagasser S., Sandberg R. (2014) Full-length RNA-seq from single cells using Smart-seq2. *Nature protocols* **9**:171-181 https://doi.org/10.1038/nprot.2014.006 | PubMed

[17] **Dobin A.,** Davis C.A., Schlesinger F., Drenkow J., Zaleski C., Jha S., Batut P., Chaisson M., Gingeras T.R. (2013) STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**:15-21 https://doi.org/10.1093/bioinformatics/bts635 | PubMed

[18] **Liao Y.,** Smyth G.K., Shi W. (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**:923-930 https://doi.org/10.1093/bioinformatics/btt656 | PubMed

[19] **Harrow J.,** Denoeud F., Frankish A., Reymond A., Chen C.-K., Chrast J., Lagarde J., Gilbert J.G., Storey R., Swarbreck D. (2006) GENCODE: producing a reference annotation for ENCODE. *Genome biology* **7**:1-9 https://doi.org/10.1186/gb-2006-7-s1-s4 | PubMed

[20] **Robinson M.D.,** McCarthy D.J., Smyth G.K. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**:139-140 https://doi.org/10.1093/bioinformatics/btp616 | PubMed

[21] **Benjamini Y.,** Hochberg Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal statistical society: series B (Methodological)* **57**:289-300 https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

[22] **Tasnim F.,** Phan D., Toh Y.-C., Yu H. (2015) Cost-effective differentiation of hepatocyte-like cells from human pluripotent stem cells using small molecules. *Biomaterials* **70**:115-125 https://doi.org/10.1016/j.biomaterials.2015.08.002 | PubMed

[23] **Rose K.A.,** Holman N.S., Green A.M., Andersen M.E., LeCluyse E.L. (2016) Co-culture of hepatocytes and Kupffer cells as an in vitro model of inflammation and drug-induced hepatotoxicity. *Journal of pharmaceutical sciences* **105**:950-964 https://doi.org/10.1016/s0022-3549(15)00192-6 | PubMed

[24] **Shiraki N.,** Yamazoe T., Qin Z., Ohgomori K., Mochitate K., Kume K., Kume S. (2011) Efficient differentiation of embryonic stem cells into hepatic cells in vitro using a feeder-free basement membrane substratum. *PloS one* **6**:e24228 https://doi.org/10.1371/journal.pone.0024228 | PubMed

[25] **Takayama K.,** Kawabata K., Nagamoto Y., Kishimoto K., Tashiro K., Sakurai F., Tachibana M., Kanda K., Hayakawa T., Furue M.K. (2013) 3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing. *Biomaterials* **34**:1781-1789 https://doi.org/10.1016/j.biomaterials.2012.11.029 | PubMed

[26] **Xia L.,** Hong X., Sakban R.B., Qu Y., Singh N.H., McMillian M., Dallas S., Silva J., Sensenhauser C., Zhao S., *et al.* (2016) Cytochrome P450 induction response in tethered spheroids as a three-dimensional human hepatocyte in vitro model. *J Appl Toxicol* **36**:320-9 https://doi.org/10.1002/jat.3189 | PubMed

[27] **Nishimura M.,** Hagi M., Ejiri Y., Kishimoto S., Horie T., Narimatsu S., Naito S. (2010) Secretion of albumin and induction of CYP1A2 and CYP3A4 in novel three-dimensional culture system for human hepatocytes using micro-space plate. *Drug metabolism and pharmacokinetics* **25**:236-242 https://doi.org/10.2133/dmpk.25.236 | PubMed

[28] **Popescu D.,** Botting R. A., Stephenson E., Green K., Webb S., Jardine L., Calderbank E. F., Polanski K., Goh I., Efremova M., *et al.* (2019) Decoding human fetal liver haematopoiesis. *Nature* **574**:365-371 https://doi.org/10.1038/s41586-019-1652-y | PubMed

[29] **Pritchard M.T.,** Cohen J.I., Roychowdhury S., Pratt B.T., Nagy L.E. (2010) Early growth response-1 attenuates liver injury and promotes hepatoprotection after carbon tetrachloride exposure in mice. *Journal of hepatology* **53**:655-662 https://doi.org/10.1016/j.jhep.2010.04.017 | PubMed

[30] **Firat-Karalar E.N.,** Rauniyar N., Yates J.R., Stearns T. (2014) Proximity interactions among centrosome components identify regulators of centriole duplication. *Current biology* **24**:664-670 https://doi.org/10.1016/j.cub.2014.01.067 | PubMed

[31] **Espinosa-Diez C.,** Wilson R., Mukherjee R., Feltham M., Hudson C., Ruhl R., Anand S. (2018) DNA damage dependent hypomethylation regulates the pro-angiogenic LncRNA MEG9. *bioRxiv* https://doi.org/10.1101/442699

[32] **Zhang Q.,** Jiang K., Li Y., Gao D., Sun L., Zhang S., Liu T., Guo K., Liu Y. (2015) Histidine-rich glycoprotein function in hepatocellular carcinoma depends on its N-glycosylation status, and it regulates cell proliferation by inhibiting Erk1/2 phosphorylation. *Oncotarget* **6**:30222 https://doi.org/10.18632/oncotarget.4997 | PubMed

[33] **Colman R.W.,** Jameson B.A., Lin Y., Johnson D., Mousa S.A. (2000) Domain 5 of high molecular weight kininogen (kininostatin) down-regulates endothelial cell proliferation and migration and inhibits angiogenesis, Blood. *The Journal of the American Society of Hematology* **95**:543-550 https://doi.org/10.1182/blood.v95.2.543 | PubMed

[34] **Ang L.T.,** Tan A.K.Y., Autio M.I., Goh S.H., Choo S.H., Lee K.L., Tan J., Pan B., Lee J.J.H., Lum J.J., *et al.* (2018) A Roadmap for Human Liver Differentiation from Pluripotent Stem Cells. *Cell Rep* **22**:2190-2205 https://doi.org/10.1016/j.celrep.2018.01.087 | PubMed

[35] **Chaudhari P.,** Tian L., Deshmukh A., Jang Y.-Y. (2016) Expression kinetics of hepatic progenitor markers in cellular models of human liver development recapitulating hepatocyte and biliary cell fate commitment. *Experimental Biology and Medicine* **241**:1653-1662 https://doi.org/10.1177/1535370216657901 | PubMed

[36] **Agrogiannis G.D.,** Sifakis S., Patsouris E.S., Konstantinidou A.E. (2014) Insulin-like growth factors in embryonic and fetal growth and skeletal development. *Molecular medicine reports* **10**:579-584 https://doi.org/10.3892/mmr.2014.2258 | PubMed

[37] **Chang Y.-C.,** Yang Y.-C., Tien C.-P., Yang C.-J., Hsiao M. (2018) Roles of aldolase family genes in human cancers and diseases. *Trends in Endocrinology & Metabolism* **29**:549-559 https://doi.org/10.1016/j.tem.2018.05.003 | PubMed

[38] **Gosselin D.,** Skola D., Coufal N.G., Holtman I.R., Schlachetzki J.C., Sajti E., Jaeger B.N., O'Connor C., Fitzpatrick C., Pasillas M.P. (2017) An environment-dependent transcriptional network specifies human microglia identity. *Science* 356 https://doi.org/10.1126/science.aal3222 | PubMed

[39] **Croft M.,** Duan W., Choi H., Eun S.-Y., Madireddi S., Mehta A. (2012) TNF superfamily in inflammatory disease: translating basic insights. *Trends in immunology* **33**:144-152 https://doi.org/10.1016/j.it.2011.10.004 | PubMed

[40] **Räisänen S.R.,** Alatalo S.L., Ylipahkala H., Halleen J.M., Cassady A.I., Hume D.A., Väänänen H.K. (2005) Macrophages overexpressing tartrate-resistant acid phosphatase show altered profile of free radical production and enhanced capacity of bacterial killing. *Biochemical and biophysical research communications* **331**:120-126 https://doi.org/10.1016/j.bbrc.2005.03.133 | PubMed

[41] **de Alwis N.,** Beard S., Binder N.K., Pritchard N., Tu'uhevaha J., Walker S.P., Stock O., Groom K., Petersen S., Henry A. (2021) DAAM2 is elevated in the circulation and placenta in pregnancies complicated by fetal growth restriction and is regulated by hypoxia. *Scientific reports* **11**:1-12 https://doi.org/10.1038/s41598-021-84785-7 | PubMed

[42] **Babapoor-Farrokhran S.,** Jee K., Puchner B., Hassan S.J., Xin X., Rodrigues M., Kashiwabuchi F., Ma T., Hu K., Deshpande M. (2015) Angiopoietin-like 4 is a potent angiogenic factor and a novel therapeutic target for patients with proliferative diabetic retinopathy. *Proceedings of the National Academy of Sciences* **112**:E3030-E3039 https://doi.org/10.1073/pnas.1423765112 | PubMed

[43] **Bonnardel J.,** T'Jonck W., Gaublomme D., Browaeys R., Scott C.L., Martens L., Vanneste B., De Prijck S., Nedospasov S.A., Kremer A. (2019) Stellate cells, hepatocytes, and endothelial cells imprint the kupffer cell identity on monocytes colonizing the liver macrophage niche. *Immunity* **51**:638-654.e9 https://doi.org/10.1016/j.immuni.2019.08.017 | PubMed

[44] **Yue L.,** Ye F., Gui C., Luo H., Cai J., Shen J., Chen K., Shen X., Jiang H. (2005) Ligand-binding regulation of LXR/RXR and LXR/PPAR heterodimerizations: SPR technology-based kinetic analysis correlated with molecular dynamics simulation. *Protein science* **14**:812-822 https://doi.org/10.1110/ps.04951405 | PubMed

[45] **Dao D.T.,** Anez-Bustillos L., Adam R.M., Puder M., Bielenberg D.R. (2018) Heparin-binding epidermal growth factor–like growth factor as a critical mediator of tissue repair and regeneration. *The American journal of pathology* **188**:2446-2456 https://doi.org/10.1016/j.ajpath.2018.07.016 | PubMed

[46] **Miyajima A.,** Kinoshita T., Tanaka M., Kamiya A., Mukouyama Y., Hara T. (2000) Role of Oncostatin M in hematopoiesis and liver development. *Cytokine & growth factor reviews* **11**:177-183 https://doi.org/10.1016/s1359-6101(00)00003-4 | PubMed

[47] **Bian Z.,** Gong Y., Huang T., Lee C. Z. W., Bian L., Bai Z., Shi H., Zeng Y., Liu C., He J., *et al.* (2020) Deciphering human macrophage development at single-cell resolution. *Nature* **582** https://doi.org/10.1038/s41586-020-2316-7 | PubMed

[48] **Hoofnagle J. H.,** Björnsson E. S (2019) Drug-Induced Liver Injury — Types and phenotypes. *New England Journal of Medicine* **381**:264-273 https://doi.org/10.1056/nejmra1816149 | PubMed

[49] **Kaplowitz N.** (2005) Idiosyncratic drug hepatotoxicity, Nature reviews. *Drug discovery* **4**:489-99 https://doi.org/10.1038/nrd1750 | PubMed

50. **Ho S.,** McLachlan A., Chen T., Hibbs D., Fois R. (2015) Relationships Between Pharmacovigilance, Molecular, Structural, and Pathway Data: Revealing Mechanisms for Immune-Mediated Drug-Induced Liver Injury, CPT. *Pharmacometrics Systems Pharmacology* **4**:426-441 https://doi.org/10.1002/psp4.56 | PubMed

[51] **Verma S.,** Kaplowitz N. (2009) Diagnosis, management and prevention of drug-induced liver injury. *Gut* **58**:1555-1564 https://doi.org/10.1136/gut.2008.163675 | PubMed

[52] **Mahdy A.,** Galley H.F., Abdel-Wahed M., El-Korny K., Sheta S., Webster N.R. (2002) Differential modulation of interleukin-6 and interleukin-10 by diclofenac in patients undergoing major surgery. *British Journal of Anaesthesia* **88**:797-802 https://doi.org/10.1093/bja/88.6.797 | PubMed

[53] Kang B.-S., Chung E.-Y., Yun Y.-P., Lee M.K., Lee Y.R., Lee K.-S., Min K.R., Kim Y. (2001) Inhibitory effects of anti-inflammatory drugs on interleukin-6 bioactivity. *Biological Pharmaceutical Bulletin* **24**:701-703 https://doi.org/10.1248/bpb.24.701 | PubMed

[54] Yao H.-W., Li J., Chen J.-Q., Xu S.-Y. (2004) Leflunomide attenuates hepatocyte injury by inhibiting Kupffer cells. *World Journal of Gastroenterology* **10**:1608 https://doi.org/10.3748/wjg.v10.i11.1608 | PubMed

[55] Manna S.K., Aggarwal B.B. (1999) Immunosuppressive leflunomide metabolite (A77 1726) blocks TNF-dependent nuclear factor-κB activation and gene expression. *The Journal of Immunology* **162**:2095-2102 https://doi.org/10.4049/jimmunol.162.4.2095

[56] Steuerwald N.M., Foureau D.M., Norton H.J., Zhou J., Parsons J.C., Chalasani N., Fontana R.J., Watkins P.B., Lee W.M., Reddy K.R. (2013) Profiles of serum cytokines in acute drug-induced liver injury and their prognostic significance. *Plos One* **8** https://doi.org/10.1371/journal.pone.0081974 | PubMed

[57] Roberts R.A., Ganey P.E., Ju C., Kamendulis L.M., Rusyn I., Klaunig J.E. (2007) Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis. *Toxicological Sciences* **96**:2-15 https://doi.org/10.1093/toxsci/kfl173 | PubMed

[58] Rowe R. G., Daley G. Q (2019) Induced pluripotent stem cells in disease modelling and drug discovery. *Nature Reviews Genetics* **20**:377-388 https://doi.org/10.1038/s41576-019-0100-z | PubMed

[59] Kuna L., Bozic I., Kizivat T., Bojanic K., Mrso M., Kralj E., Smolic R., Wu G.Y., Smolic M. (2018) Models of Drug Induced Liver Injury (DILI)–Current Issues and Future Perspectives. *Current drug metabolism* **19**:830-838 https://doi.org/10.2174/1389200219666180523095355 | PubMed

[60] Shenton J.M., Chen J., Uetrecht J.P. (2004) Animal models of idiosyncratic drug reactions. *Chemico-biological interactions* **150**:53-70 https://doi.org/10.1016/j.cbi.2004.09.001 | PubMed

[61] Ng W., Lobach A.R., Zhu X., Chen X., Liu F., Metushi I.G., Sharma A., Li J., Cai P., Ip J. (2012) Animal models of idiosyncratic drug reactions. Advances in pharmacology. pp. 81–135 https://doi.org/10.1016/B978-0-12-398339-8.00003-3 | PubMed

[62] Uetrecht J. (2019) Mechanistic studies of idiosyncratic DILI: clinical implications. *Frontiers in pharmacology* **10**:837 https://doi.org/10.3389/fphar.2019.00837 | PubMed

[63] Bonzo J.A., Rose K., Freeman K., Deibert E., Amaral K.B., Ferguson S.S., Andersen M.E., Witek R.P., LeCluyse E.L. (2015) Differential effects of trovafloxacin on TNF-α and IL-6 profiles in a rat hepatocyte–Kupffer cell coculture system. *Applied In Vitro Toxicology* **1**:45-54 https://doi.org/10.1089/aivt.2014.0004

[64] Tukov F.F., Maddox J.F., Amacher D.E., Bobrowski W.F., Roth R.A., Ganey P.E. (2006) Modeling inflammation–drug interactions in vitro: A rat Kupffer cell-hepatocyte coculture system. *Toxicology in vitro* **20**:1488-1499 https://doi.org/10.1016/j.tiv.2006.04.005 | PubMed

[65] Kegel V., Pfeiffer E., Burkhardt B., Liu J.L., Zeilinger K., Nussler A.K., Seehofer D., Damm G. (2015) Subtoxic Concentrations of Hepatotoxic Drugs Lead to Kupffer Cell Activation in a Human In Vitro Liver Model: An Approach to Study DILI. *Mediators Inflamm 2015* **640631** https://doi.org/10.1155/2015/640631 | PubMed

[66] Shaw P.J., Hopfensperger M.J., Ganey P.E., Roth R.A. (2007) Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. *Toxicological Sciences* **100**:259-266 https://doi.org/10.1093/toxsci/kfm218 | PubMed

[67] Kermanizadeh A., Brown D.M., Stone V. (2019) The variances in cytokine production profiles from non-or activated THP-1, Kupffer cell and human blood derived primary macrophages following exposure to either alcohol or a panel of engineered nanomaterials. *Scientific reports* **14**:e0220974 https://doi.org/10.1371/journal.pone.0220974 | PubMed

[68] Novik E., Maguire T.J., Chao P., Cheng K., Yarmush M.L. (2010) A microfluidic hepatic coculture platform for cell-based drug metabolism studies. *Biochemical Pharmacology* **79**:1036-1044 https://doi.org/10.1016/j.bcp.2009.11.010 | PubMed

[69] **Doss M.X.,** Sachinidis A. (2019) Current challenges of iPSC-based disease modeling and therapeutic implications. *Cells* **8**:403 https://doi.org/10.3390/cells8050403 | PubMed

[70] **Chen M.,** Suzuki A., Thakkar S., Yu K., Hu C., Tong W. (2016) DILIrank: the largest reference drug list ranked by the risk for developing drug-induced liver injury in humans. *Drug Discov Today* **21**:648-653 https://doi.org/10.1016/j.drudis.2016.02.015 | PubMed

[71] **Thakkar S.,** Li T., Liu Z., Wu L., Roberts R., Tong W. (2020) Drug-induced liver injury severity and toxicity (DILIst): Binary classification of 1279 drugs by human hepatotoxicity. *Drug discovery today* **25**:201-208 https://doi.org/10.1016/j.drudis.2019.09.022 | PubMed

[72] **Xu J.J.,** Henstock P.V., Dunn M.C., Smith A.R., Chabot J.R., de Graaf D. (2008) Cellular imaging predictions of clinical drug-induced liver injury. *Toxicological sciences: an official journal of the Society of Toxicology* **105**:97-105 https://doi.org/10.1093/toxsci/kfn109 | PubMed

[73] **Zou W.,** Beggs K.M., Sparkenbaugh E.M., Jones A.D., Younis H.S., Roth R.A., Ganey P.E. (2009) Sulindac metabolism and synergy with tumor necrosis factor-α in a drug-inflammation interaction model of idiosyncratic liver injury. *Journal of Pharmacology Experimental Therapeutics* **331**:114-121 https://doi.org/10.1124/jpet.109.156331 | PubMed

[74] **Li J.,** Yao H.-W., Jin Y., Zhang Y.-F., Li C.-Y., Li Y.-H., Xu S.-Y. (2002) Pharmacokinetics of leflunomide in Chinese healthy volunteers. *Acta Pharmacologica Sinica* **23**:551-555 PubMed

[75] **Agabeyoglu I.,** Incecayir T. (2009) Pharmacokinetic Modelling of Lamotrigine from Plasma Concentrations in Healthy Volunteers. *J Bioanal Biomed* **1**:041-045 https://doi.org/10.4172/1948-593x.1000009

[76] **Ademisoye A.A.,** Soyinka J.O., Olawoye S.O., Igbinoba S.I., Olowookere S.A., Ademisoye A.T., Onyeji C.O. (2018) Induction of Amodiaquine Metabolism by Rifampicin Following Concurrent Administration in Healthy Volunteers. *Journal of Exploratory Research in Pharmacology* **3**:71-77 https://doi.org/10.14218/jerp.2017.00024

[77] **Bareggi S.,** Cerutti R., Pirola R., Riva R., Cisternino M. (1987) Clinical pharmacokinetics and metabolism of pyrazinamide in healthy volunteers. *Arzneimittel-Forschung* **37**:849 PubMed

[78] **Martínez García de la Torre R.A.,** Vallverdú J., Xu Z., Ariño S., Ferrer-Lorente R., Zanatto L., Mercado-Gómez M., Aguilar-Bravo B., Ruiz-Blázquez P., Fernandez- Fernandez M., *et al.* (2025) Trajectory analysis of hepatic stellate cell differentiation reveals metabolic regulation of cell commitment and fibrosis. *Nature Communications* **16**:1489 https://doi.org/10.1038/s41467-025-56024-4 | PubMed

[79] **Gage B.K.,** Liu J.C., Innes B.T., MacParland S.A., McGilvray I.D., Bader G.D., Keller G.M (2020) Generation of Functional Liver Sinusoidal Endothelial Cells from Human Pluripotent Stem-Cell-Derived Venous Angioblasts. *Cell Stem Cell* **27**:254-269 https://doi.org/10.1016/j.stem.2020.06.007 | PubMed

Peer reviews

Reviewer #1 (Public review):

The manuscript presents a compelling new in vitro system based on isogenic co-cultures of human iPSC-derived hepatocytes and macrophages, enabling the modelling of hepatic immune responses with unprecedented physiological relevance. The authors show that co-culture leads to enhanced maturation of hepatocytes and tissue-resident macrophage identity, which cannot be achieved through conditioned media alone. Using this system, they functionally validate immune-driven hepatotoxic responses to a panel of drugs and compare the system's predictive power to that of monocyte-derived macrophages. The results underscore the necessity of macrophage-hepatocyte crosstalk for accurate modelling of liver inflammation and drug toxicity in vitro. The manuscript is clearly written and addresses a key limitation in liver organoid systems: the lack of immune complexity and tissue-specific macrophage imprinting.

Strengths:

• Novelty and Relevance: The study presents a highly innovative co-culture system based on isogenic human iPSCs, addressing an unmet need in modelling immune-mediated hepatotoxicity.

• Mechanistic Insight: The reciprocal reprogramming between iHeps and iMacs, including induction of KC-specific pathways and hepatocyte maturation markers, is convincingly demonstrated.

• Functional Readouts: The application of the model to detect IL-6 responses to hepatotoxic compounds enhances its translational relevance.

Weaknesses:

The co-culture model with monocyte-derived macrophages is not fully characterised, making comparisons less informative.

https://doi.org/10.7554/eLife.108938.2.sa2

Reviewer #3 (Public review):

Summary:

In this study, the authors establish a human in vitro liver model by co-culturing induced hepatocyte-like cells (iHEPs) with induced macrophages (iMACs). Through flow cytometry-based sorting of cell populations at days 3 and 7 of co-culture, followed by bulk RNA sequencing, they demonstrate that bidirectional interactions between these two cell types drive functional maturation. Specifically, the presence of iMACs accelerates the hepatic maturation program of iHEPs, while contact-dependent cues from iHEPs enhance the acquisition of Kupffer cell identity in iMACs, indicating that direct cell-cell interactions are critical for establishing tissue-resident macrophage characteristics.

Functionally, the authors show that iMAC-derived Kupffer-like cells respond to pathological stimuli by producing interleukin-6 (IL-6), a hallmark cytokine of hepatic immune activation. When exposed to a panel of clinically relevant hepatotoxic drugs, the co-culture system exhibited concentration-dependent modulation of IL-6 secretion consistent with reported drug-induced liver injury (DILI) phenotypes. Notably, this response was absent when hepatocytes were co-cultured with monocyte-derived macrophages from peripheral blood, underscoring the liver-specific phenotype and functional relevance of the iMAC-derived Kupffer-like cells. Collectively, the study proposes this co-culture platform as a more physiologically relevant model for interrogating macrophage-hepatocyte crosstalk and assessing immune-mediated hepatotoxicity in vitro.

Strengths:

A major strength of this study lies in its systematic dissection of cell-cell interactions within the co-culture system. By isolating each cell type following co-culture and performing comprehensive transcriptomic analyses, the authors provide direct evidence of bidirectional crosstalk between iMACs and iHEPs. The comparison with single-culture controls is particularly valuable, as it clearly demonstrates how co-culture enhances functional maturation and lineage-specific gene expression in both cell types. This approach allows for a more mechanistic understanding of how hepatocyte-macrophage interactions contribute to the acquisition of tissue-specific phenotypes

Weaknesses:

(1) Overreliance on bulk RNA-seq data:

The primary evidence supporting cell maturation is derived from bulk RNA sequencing, which has inherent limitations in resolving heterogeneous cellular states and functional maturation. The conclusions regarding hepatocyte maturation are based largely on increased expression of a subset of CYP genes and decreased AFP levels - markers that, while suggestive, are insufficient on their own to substantiate functional maturation. Additional phenotypic or functional assays (e.g., metabolic activity, protein-level validation) would significantly strengthen these claims.

(2) Insufficient characterization of input cell populations:

The manuscript lacks adequate validation of the cellular identities prior to co-culture. Although the authors reference previously published protocols for generating iHEPs and iMACs, it remains unclear whether the cells used in this study faithfully retain expected lineage characteristics. For example, hepatocyte preparations should be characterized by flow cytometry for ALB and AFP expression, while iMACs should be assessed for canonical macrophage markers such as CD45, CD11b, and CD14 before co-culture. Without these baseline data, it is difficult to interpret the magnitude or significance of any co-culture-induced changes.

(3) Quantitative assessment of IL-6 production is insufficient:

The analysis of drug-induced IL-6 responses is based primarily on relative changes compared to control conditions. However, percentage changes alone are inadequate to capture the biological relevance of these responses. Absolute cytokine production levels - particularly in response to LPS stimulation - should be reported and directly compared to PBMC-derived macrophages to determine whether iMAC-derived Kupffer-like cells exhibit enhanced cytokine output. Moreover, the Methods section should clearly describe how ELISA results were normalized or corrected to account for potential differences in cell number, viability, or culture conditions.

(4) Unclear mechanistic interpretation of IL-6 modulation:

The observed changes in IL-6 production upon drug treatment cannot be interpreted solely as evidence of Kupffer cell-specific functionality. For instance, IL-6 suppression by NSAIDs such as diclofenac is well known to result from altered prostaglandin synthesis due to COX inhibition, while leflunomide's effects are linked to metabolite-induced modulation of immune cell proliferation and broader cytokine networks. These mechanisms are distinct from Kupffer cell identity and may not directly reflect liver-specific macrophage function. Consequently, changes in IL-6 secretion alone - particularly without additional mechanistic evidence or analysis of other cytokines - are insufficient to conclude that co-culture with hepatocytes drives the acquisition of bona fide Kupffer cell maturity.

Reviewers comments to revised manuscript.

The authors successfully established an isogenic, iPSC-derived human liver co-culture model to investigate the role of hepatocyte-macrophage interactions in driving Kupffer cell (KC) identity and hepatocyte maturation. By utilizing a single genetic background, the authors effectively minimized the experimental variability often encountered in non-isogenic systems. A significant highlight of this work is the demonstration that direct co-culture-as opposed to conditioned media alone-is a primary driver for critical KC identity markers such as ID1 and ID3. Furthermore, the model's ability to recapitulate complex clinical IL-6 responses to known hepatotoxicants where standard models have failed underscores its potential utility for early-stage DILI screening. However, there are significant methodological concerns regarding the data analysis. While the study compares four or five distinct experimental groups (e.g., Day 0, Day 7, Day 3 co-culture, and Day 7 co-culture), the authors utilized Student's t-tests for these comparisons. This approach does not account for the

multiple comparisons problem and increases the risk of Type I errors. Additionally, while IL-6 secretion is used as a primary functional readout, the individual mechanisms behind these drug responses were not explored experimentally. Finally, Pearson correlation analysis indicates that the iMacs remain poorly correlated with actual in vivo human embryonic liver macrophages, suggesting that the "imprinting" of true KC identity remains incomplete.

https://doi.org/10.7554/eLife.108938.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *The manuscript presents a compelling new in vitro system based on isogenic co-cultures of human iPSC-derived hepatocytes and macrophages, enabling the modelling of hepatic immune responses with unprecedented physiological relevance. The authors show that co-culture leads to enhanced maturation of hepatocytes and tissue-resident macrophage identity, which cannot be achieved through conditioned media alone. Using this system, they functionally validate immune-driven hepatotoxic responses to a panel of drugs and compare the system's predictive power to that of monocyte-derived macrophages. The results underscore the necessity of macrophage-hepatocyte crosstalk for accurate modelling of liver inflammation and drug toxicity in vitro.*
>
> *The manuscript is clearly written and addresses a key limitation in liver organoid systems: the lack of immune complexity and tissue-specific macrophage imprinting. Nevertheless, several conclusions would benefit from a more careful interpretation of the data, and some important controls or explanations are missing, particularly in the flow cytometry gating strategies, stress marker validation, and cluster interpretations.*
>
> *Strengths:*
>
> *(1) Novelty and Relevance: The study presents a highly innovative co-culture system based on isogenic human iPSCs, addressing an unmet need in modelling immune-mediated hepatotoxicity.*
>
> *(2) Mechanistic Insight: The reciprocal reprogramming between iHeps and iMacs, including induction of KC-specific pathways and hepatocyte maturation markers, is convincingly demonstrated.*
>
> *(3) Functional Readouts: The application of the model to detect IL-6 responses to hepatotoxic compounds enhances its translational relevance.*
>
> *Weaknesses:*
>
> *(1) Several key claims, particularly those derived from PCA plots and DEG analyses, are overinterpreted and require more conservative language or further validation.*

We agree that PCA does not allow for maturation trajectories and mentioned that it was a hypothesis that the co-culture was promoting maturation, which we later validated by looking at the expression of key hepatocyte markers as well as by pearson correlation comparison with fetal hepatocytes.

> *(2) The purity of sorted hepatocytes and macrophages is not convincingly demonstrated; contamination across gates may confound transcriptomic readouts.*

We agree and have highlighted and addressed this limitation in our discussion. Unfortunately, this is a limitation of bulk sequencing that a small amount of contamination might be present, however the TPM values of ALB for example in the iMacs is extremely low especially when compared to the hepatocytes, indicating that the level of contamination is likely to be very low. Likewise, the expression of CSF1R in the co-cultured iHeps is also extremely low. This has been included in Supp Fig 1F and G.

> *(3) Stress response genes and ER stress/apoptosis signatures are not properly assessed, despite being potentially activated in the system.*

This has been included in Supp Fig 2C, where we've included the expression of ATF4, CASP3 and CASP9. Although there's a significant difference in ATF4 expression between Day 0 and Day 7 iHep only/Co-culture, there is no significant difference between the Day 7 iHep only and Day 7 iHep Co-culture. There are no significant differences in CASP3 and CASP9 expression across all the samples.

> *(4) Some figure panels and legends lack statistical annotations, and microscopy validation of morphological changes is missing.*

Although we agree that the morphology changes would be interesting, we think that this question is unfortunately outside of the scope of our question. Although Kupffer cells are in direct contact with hepatocytes, they migrate from the liver parenchyma into the sinusoidal spaces where they primarily reside. We do not think that the morphology would add much to the paper, especially given that this is a 2D model as well.

> *(5) The co-culture model with monocyte-derived macrophages is not fully characterised, making comparisons less informative.*

Although we agree that it would be interesting to look more closely at the monocyte-derived macrophage co-cultures as well, we think that this would be more suited to a future study as the transcriptomic analysis would likely include confounding effects of patient specific transcriptomic changes, and our primary focus was on developing an isogenic co-culture system.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *This study builds on work by Glass and Guilliams showing that mouse Kupffer cells depend on the surrounding cells, including endothelium, hepatocytes, and stellate cells, for their identity. Herein, the authors extend the work to human systems. It nicely highlights why taking monocyte-derived macrophages and pretending they are Kupffer cells is simply misleading.*
>
> *Strengths:*
>
> *Many, including human cells, difficult culture assays, and important new data.*
>
> *Weaknesses:*
>
> *This reviewer identified minor queries only, rather than 'weaknesses' as such.*
>
> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *In this study, the authors establish a human in vitro liver model by co-culturing induced hepatocyte-like cells (iHEPs) with induced macrophages (iMACs). Through flow cytometry-*

> *based sorting of cell populations at days 3 and 7 of co-culture, followed by bulk RNA sequencing, they demonstrate that bidirectional interactions between these two cell types drive functional maturation. Specifically, the presence of iMACs accelerates the hepatic maturation program of iHEPs, while contact-dependent cues from iHEPs enhance the acquisition of Kupffer cell identity in iMACs, indicating that direct cell-cell interactions are critical for establishing tissue-resident macrophage characteristics.*
>
> *Functionally, the authors show that iMAC-derived Kupffer-like cells respond to pathological stimuli by producing interleukin-6 (IL-6), a hallmark cytokine of hepatic immune activation. When exposed to a panel of clinically relevant hepatotoxic drugs, the co-culture system exhibited concentration-dependent modulation of IL-6 secretion consistent with reported drug-induced liver injury (DILI) phenotypes. Notably, this response was absent when hepatocytes were co-cultured with monocyte-derived macrophages from peripheral blood, underscoring the liver-specific phenotype and functional relevance of the iMAC-derived Kupffer-like cells. Collectively, the study proposes this co-culture platform as a more physiologically relevant model for interrogating macrophage-hepatocyte crosstalk and assessing immune-mediated hepatotoxicity in vitro.*
>
> *Strengths:*
>
> *A major strength of this study lies in its systematic dissection of cell-cell interactions within the co-culture system. By isolating each cell type following co-culture and performing comprehensive transcriptomic analyses, the authors provide direct evidence of bidirectional crosstalk between iMACs and iHEPs. The comparison with single-culture controls is particularly valuable, as it clearly demonstrates how co-culture enhances functional maturation and lineage-specific gene expression in both cell types. This approach allows for a more mechanistic understanding of how hepatocyte-macrophage interactions contribute to the acquisition of tissue-specific phenotypes.*
>
> *Weaknesses:*
>
> *(1) Overreliance on bulk RNA-seq data:*
>
> *The primary evidence supporting cell maturation is derived from bulk RNA sequencing, which has inherent limitations in resolving heterogeneous cellular states and functional maturation. The conclusions regarding hepatocyte maturation are based largely on increased expression of a subset of CYP genes and decreased AFP levels - markers that, while suggestive, are insufficient on their own to substantiate functional maturation. Additional phenotypic or functional assays (e.g., metabolic activity, protein-level validation) would significantly strengthen these claims.*

We have added a discussion on the limitations of our study.

> *(2) Insufficient characterization of input cell populations:*
>
> *The manuscript lacks adequate validation of the cellular identities prior to co-culture. Although the authors reference previously published protocols for generating iHEPs and iMACs, it remains unclear whether the cells used in this study faithfully retain expected lineage characteristics. For example, hepatocyte preparations should be characterized by flow cytometry for ALB and AFP expression, while iMACs should be assessed for canonical macrophage markers such as CD45, CD11b, and CD14 before co-culture. Without these baseline data, it is difficult to interpret the magnitude or significance of any co-culture-induced changes.*

We apologise for this oversight, some of the markers were used in determining the purity of the iMacs before co-culture, and we did not end up including these plots for brevity. We have

added the purity plots in Supp Fig 2E now, showing that the iMacs were more than 90% pure before co-culture. We acknowledge the concern about cross-contamination for bulk sequencing, and have added in Supp Fig 2G and H the expression of ALB in the iMac fraction, as well as the expression of CSF1R in the iHep fraction, showing minimal contamination with our gating strategy.

> *(3) Quantitative assessment of IL-6 production is insufficient:*
>
> *The analysis of drug-induced IL-6 responses is based primarily on relative changes compared to control conditions. However, percentage changes alone are inadequate to capture the biological relevance of these responses. Absolute cytokine production levels - particularly in response to LPS stimulation - should be reported and directly compared to PBMC-derived macrophages to determine whether iMAC-derived Kupffer-like cells exhibit enhanced cytokine output. Moreover, the Methods section should clearly describe how ELISA results were normalized or corrected to account for potential differences in cell number, viability, or culture conditions.*

We apologise if this was unclear. The cytokine production from dosed cells was normalized based on the viability of cells measured from the same well.

> *(4) Unclear mechanistic interpretation of IL-6 modulation:*
>
> *The observed changes in IL-6 production upon drug treatment cannot be interpreted solely as evidence of Kupffer cell-specific functionality. For instance, IL-6 suppression by NSAIDs such as diclofenac is well known to result from altered prostaglandin synthesis due to COX inhibition, while leflunomide's effects are linked to metabolite-induced modulation of immune cell proliferation and broader cytokine networks. These mechanisms are distinct from Kupffer cell identity and may not directly reflect liver-specific macrophage function. Consequently, changes in IL-6 secretion alone - particularly without additional mechanistic evidence or analysis of other cytokines - are insufficient to conclude that co-culture with hepatocytes drives the acquisition of bona fide Kupffer cell maturity.*

We fully agree with the reviewer and have highlighted this in our discussion.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) GSE ID for RNA-seq data has not been provided.*

This has been included.

> *(2) Line 291: Can the authors specify what they mean by "state-of-the-art"?*

What we mean here is what others in the field have also recently described. We have rewritten this to be clearer.

> *(3) Lines 299-300: check sentence for grammar mistakes.*

We have rewritten and clarified this.

> *(4) Figure 1B: The PCA does not really allow for following maturation trajectories. Also, all samples (day 3 Co-iHep, day 7 Co-iHep, day 7 iHep) look as if they cluster more or less together. Therefore, the conclusion drawn in lines 303-305 does not hold. Why is day 3 iHep not also shown here?*

We agree that PCA does not allow for maturation trajectories and mentioned that it was a hypothesis that the co-culture was promoting maturation, which we later validated by

looking at the expression of key hepatocyte markers as well as by pearson correlation comparison with fetal hepatocytes.

> *(5) Can the authors show that the cells that they are sorting in the double negative gate are indeed hepatocytes? Typically, these cells are big in cell size; therefore, showing the FSC/SSC gate would also be important.*

We have added the FSC/SSC gate in supp fig. 1E to show that the populations have different sizes.

> *(6) Can the authors provide microscopy pictures of iHeps, iMacs, and the co-cultured cells for the reader to appreciate whether the morphology of cells already changes during the co-culture experiments?*

Although we agree that the morphology changes would be interesting, we think that this question is unfortunately outside of the scope of our question. Although Kupffer cells are in direct contact with hepatocytes, they migrate from the liver parenchyma into the sinusoidal spaces where they primarily reside. We do not think that the morphology would add much to the paper, especially given that this is a 2D model as well.

> *(7) Please show expression of apoptotic and ER stress genes comparing Day7 iHeps and Co-iHeps, since genes such as c-Fos and Ppp2r3b can also be associated with cellular stress.*

This has been included in Supp Fig 2C, where we've included the expression of ATF4, CASP3 and CASP9. Although there's a significant difference in ATF4 expression between Day 0 and Day 7 iHep only/Co-culture, there is no significant difference between the Day 7 iHep only and Day 7 iHep Co-culture. There are no significant differences in CASP3 and CASP9 expression across all the samples.

> *(8) In addition to the genes shown in Figure 1E, could the authors extract a longer gene list of maturing hepatocytes and display them all in bar graphs or heatmaps, or similar? E.g., Albumin expression is shown later, but why not show it already here?*

There are not many differences in the canonical hepatocyte markers, which is why we chose only to show the interesting genes that were different, as seen in the later ALB expression plot where there wasn't a difference in ALB expression after 7 days of co-culture. Instead, we have included a new heatmap in Supp Fig 2B showing the top 40 genes that are contributing to the similarity by pearson correlation.

> *(9) Along these lines, how do the authors ensure that they are culturing only hepatocytes and do not have a mixture of cells that may "dilute" the hepatocyte signature?*

Unfortunately, this is an limitation of our methodology, although the expression of key hepatic markers are routinely confirmed by qPCR to ensure that the majority of the cells are hepatocyte-like.

> *(10) Lines 347-350: similar to the interpretation of the PCA for hepatocytes, this is a completely random interpretation. The expression of ALB in the co-cultured iMacs indicates that there are some hepatocytes that ended up in the macrophage gate.*

We agree and have highlighted and addressed this limitation in our discussion. Unfortunately, this is a limitation of bulk sequencing that a small amount of contamination might be present, however the TPM values of ALB for example in the iMacs is extremely low especially when compared to the hepatocytes, indicating that the level of contamination is likely to be very low. Likewise, the expression of CSF1R in the co-cultured iHeps is also extremely low. This has been included in Supp Fig 1F and G.

> *(11) Figure 2D: Among the pathways shown, there are also stress pathways (acute phase response, HMGB1). Also for these cells, control of apoptotic and ER stress signatures is necessary.*

As mentioned, we have included some stress genes in Supp Fig 2C to address this.

> *(12) Lines 385-386: Why would FCGRA3 indicate tissue residency? Is there literature to support this statement?*

CD16 is a marker often used to distinguish Kupffer cells from the surrounding cells, although it also expressed by non-classical monocytes, we have clarified the text here (Lines 356-357).

> *(13) Figure 3E: ALB and other genes were at the same or even lower levels expressed in D7 compared to D3. Why is that? Are the cells starting to de-differentiate after 7 days? Please discuss.*

This is a very interesting question that we were wondering ourselves as well, although sadly we do not have an answer yet. We hypothesized that this might be due to the activation of cell proliferation/developmental programmes as the cells are kept longer together, as shown by the expression of morphogens like OSM and IGF-2 after co-culture. We have added some discussion for this (Lines 532-540)

> *(14) Line 459: Word "in" is double*

We thank the reviewer for catching this, this has been corrected

> *(15) Figure 5: The findings are interesting, but the co-culture model remains somewhat unclear. Can the authors show, e.g., using qRT-PCR, how hepatocytes are developing in this culture system? If the development with monocyte-derived macrophages is altered, then one would expect that also the cellular response is different.*

We agree with the reviewer, but we think that this question would be better answered in a follow-up study. We were looking to answer if the addition of isogenic iMacs would change the drug response of iHeps, and were using the PBMC-derived macrophages here as a control. A more complete study taking into account the genetic background of the donor PBMC-derived macrophages would be much more informative, but sadly outside of the scope of our present study.

> *(16) Lines 482-484: The authors talk about LPS-treated cultures and refer to Figure 4. However, there is no graph shown for LPS.*

We apologise for being unclear here, but the co-cultures were co-treated with LPS during the drug stimulation assays, as it had been shown that LPS increases the sensitivity of the liver toward hepatotoxic drugs. We have clarified this in the main text (Lines 435-437).

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) It would be nice to add some protein production by the hepatocytes. For example, can they produce albumin or some other protein that can be measured? Perhaps I missed this.*

The protein expression of Albumin and Urea were assessed in the hepatocytes prior to co-culture in Supp Fig 1C; however we did not measure the protein level changes after co-culture as the co-culture would have a significant number of macrophages as well which we thought might affect the readout. Instead, after co-culture the primary analysis was done on the RNA levels of ALB and other cytochrome genes after sorting in Fig 3.

> *(2) Was there an increase in hepatocyte number? Did one cell outgrow the other, or did they maintain numbers?*

The relative proportion of the iHeps remained the same, although we did see an expansion in the iMac population after 7 days by flow cytometry in Fig 1D.

> *(3) What happens if the iMACs and the iHeps are grown in Costar chambers with pore sizes too small to allow for cell contact, but allowing supernatant to be continuously exposed to both cell types?*

We were primarily focused on the acquisition of KC-like phenotype in the iMacs with regards the question of direct contact, which was why we chose to use conditioned iHep media as part of the iMac experimental set up. However, it would be very interesting to see if the converse is also true, and whether secreted factors from the iMacs alone would be sufficient to drive the changes we observed in the iHeps after co-culture in a follow-up study.

> *(4) The discussion could use a brief paragraph on some limitations and what could be added to the co-culture system. For example, could stellate cells and sinusoidal endothelium also impart KC identity? Would growing KCs on endothelium provide a more natural substratum?*

Once again, these are very interesting questions which are unfortunately outside of the scope of our study. However, we have included a short section discussing this in the paper, as we do think that it would be interesting to look at iMacs educated by hepatocyte vs stellate cells for example (Lines 530-536).

> *(5) The axonal guidance pathway in early iMACs is interesting. A recent report in vivo showed that macrophages migrate from the liver parenchyma into the sinusoids in neonates when they are still immature. The process could be chemotaxis, or it could be repulsion by parenchyma. Numerous axonal guidance molecules are repulsive, pushing axons away (robo/slit, etc). The migration of Kupffer cells into sinusoids could be a repulsive rather than a chemoattractant pathway. Did the RNA seq data provide any interesting molecules in this regard?*
>
> ***Reviewer #3 (Recommendations for the authors):***
>
> *This manuscript presents a conceptually well-designed approach to modeling hepatocyte-macrophage crosstalk in vitro. The authors develop a co-culture system aimed at recapitulating key aspects of Kupffer cell (KC) identity and hepatocyte maturation. The data convincingly show that macrophages acquire KC-like features under co-culture conditions. However, several major issues limit the strength of the conclusions, the depth of mechanistic insight, and the translational impact of the work.*
>
> *First, the study relies heavily on bulk RNA-seq data with minimal functional or protein-level validation - particularly for hepatocyte maturation. To substantiate claims of functional maturation, additional assays measuring albumin secretion, urea production, and CYP activity are essential. Furthermore, the omission of zonation-associated markers (e.g., GLUL, CPS1, CYP2E1) leaves a critical gap in assessing whether the iHEPs achieve physiologically relevant functional states.*
>
> *Second, statistical interpretation and reporting are inconsistent. Significant and non-significant findings are frequently conflated, which risks overinterpretation. For instance, the reported reduction in HNF4A expression is not statistically significant, and AFP expression is only significantly reduced in Day 7 co-iHEPs - yet these distinctions are not clearly stated.*

> *Third, although the authors emphasize the role of cell-cell contact in promoting KC identity, no experiments (e.g., transwell separation, adhesion-blocking assays) directly test this claim. As a result, the mechanistic basis for this conclusion remains speculative.*
>
> *Finally, while the data support enhanced macrophage differentiation toward a KC-like phenotype, the evidence that co-culture significantly promotes hepatocyte maturation is far less convincing and requires additional functional, mechanistic, and statistical validation before firm conclusions can be drawn.*
>
> *Minor comments:*
>
> *(1) Methodology: The choice of a 2.5:1 iHEP:iMAC ratio is not justified. This proportion does not reflect physiological hepatocyte-to-KC ratios in vivo and should be either rationalized or benchmarked against native liver composition.*

We admit that the ratio here is on the higher side of things, but it has been previously reported that there can be between 20 to 40 macrophages per 100 hepatocytes (1:5 to 1:2.5) in the adult mouse liver (Baratta et al., 2009), while admittedly in the developing mouse liver the ratio is closer to 1:4 (Lopez et al., 2011). We chose 1:2.5 as we anticipated that not all of the macrophages would be able to attach, and would thus be lost during media change, as evident by the flow cytometry of the co-culture on Day 3 of the co-culture, where only 20% of the cells had clear CD45 and CD14 expression. We have clarified our methodology in paper (Lines 141-143).

> *(2) Effect of iMAC on iHEP (Section 3.2, Supplementary Figure 1E):*
>
> *(2.1) The authors should explain why Day 3 co-cultured iHEPs show stronger transcriptomic similarity to primary hepatocytes than Day 7 cells. Possible biological mechanisms (e.g., transient paracrine signaling or temporal changes in maturation dynamics) should be discussed.*

We have added some discussion for this (Lines 309-311, 536-540).

> *(2.2) The figure legend refers to "fetal hepatocytes," while the correlation map states "hepatocytes." This discrepancy must be clarified. Moreover, if fetal hepatocytes are used as the reference, and the goal is to assess maturation, comparisons to adult hepatocytes are necessary.*

The comparison was done against fetal hepatocytes, and has been clarified in the figure. We chose to use fetal hepatocytes here as it would be unfair to compare iPSC-derived cells that are less than 3 weeks old to adult human tissue, and any similarity or differences between the mono/co-cultures to the adult tissue might be due to the shifting transcriptomic landscape during development. However, we do recognise the nuanced nature of using “maturation” here, and what we mean is that the iPSC-derived cells become more similar to their *in-vivo* counterparts.

> *(2.3) Baseline characterization of both cell types before co-culture is insufficient. For iHEPs, flow cytometry data on ALB and AFP positivity rates should be presented, along with post-co-culture changes. For iMACs, marker expression (CD45, CD11b, CD14) should be shown before and after co-culture. The methods mention CD163, CX3CR1, and CD11b, but these data are absent from the results. Additionally, the gating strategy for cell sorting prior to bulk RNA-seq must be clearly described - including how potential cross-contamination of cell fractions (e.g., macrophages in the hepatocyte population) was excluded.*

We apologise for this oversight, some of the markers were used in determining the purity of the iMacs before co-culture, and we did not end up including these plots for brevity. We have

added the purity plots in Supp Fig 2E now, showing that the iMacs were more than 90% pure before co-culture. We acknowledge the concern about cross-contamination for bulk sequencing, and have added in Supp Fig 2G and H the expression of ALB in the iMac fraction, as well as the expression of CSF1R in the iHep fraction, showing minimal contamination with our gating strategy.

> *(3) IGF2 Expression: The observed upregulation of IGF2, a fetal marker, contradicts the conclusion that co-culture promotes hepatocyte maturation. This inconsistency should be addressed, and possible explanations (e.g., transient fetal-like activation driven by macrophage-derived signals) discussed. The lack of statistical significance for this finding must also be explicitly noted.*

We thank the reviewer for pointing this out. The expression of IGF2 was actually significantly different when comparing the Day 0 Hepatocyte only and Day 7 Hepatocyte only to the Day 3 Co-cultured Hepatocytes, but the significance is lost with the Day 7 co-cultured Hepatocytes. One possible explanation is as the reviewer suggested, that there is a transient program that is activated upon co-culture that is subsequently downregulated. We have updated the figure and text, and added some discussion to reflect this (Lines 309-311, 536-540).

> *(4) Effect of iHEP on iMAC: The reported upregulation of KC-related genes is overstated. Changes in LYVE1 and ID1 are not statistically significant (Figure 2G), yet they are presented as meaningful. Clear separation of statistically significant results from non-significant trends is critical to avoid overinterpretation.*

We apologise for this, as it was never our intention to present these markers as significant, but rather we presented these markers because we thought that these markers would be of interest to the audience. We have clarified the text to reflect that these are trends and non-significant (Lines 367-369).

> *(5) Mimicking In Vivo Clinical Responses:*
>
> *(5.1) The authors' conclusion that IL-6 responses are not recapitulated when iMACs are replaced by monocyte-derived macrophages (MoMs) is not fully supported by the data presented. In fact, the MoM co-cultures exhibit a noticeable trend toward increased IL-6 production (e.g., approximately 150% with LTG at 66.6 μM and 400 μM), suggesting that some degree of responsiveness is retained. To substantiate the claim that the observed cytokine modulation is unique to iKC-containing co-cultures, the authors should perform direct statistical comparisons of absolute IL-6 secretion levels between iKC and MoM co-cultures at each drug concentration. Such analyses are essential to determine whether the differences are statistically significant and biologically meaningful, and to clarify whether the observed effects truly reflect KC-specific functionality rather than general macrophage activation.*
>
> *(5.2) The effects of drug exposure on hepatocytes themselves are not addressed. It is important to evaluate whether the co-culture remains viable under treatment, whether it recovers after drug withdrawal, and whether there is evidence of cytotoxicity or irreversible phenotypic loss.*
>
> *(6) Interpretation of IL-6 Modulation and Model Specificity:*
>
> *The authors show that IL-6 secretion in their co-culture system varies in response to multiple hepatotoxic drugs and parallels some reported clinical trends - notably, a concentration-dependent decrease with diclofenac (DIC) and leflunomide (LFM). They further report that this pattern is not observed in hepatocyte-PBMC-derived macrophage co-cultures, and they conclude that iMAC/iKC-like cells are essential for capturing immune-mediated hepatotoxic responses. However, the data presented do not fully justify such a conclusion. Several key mechanistic issues weaken the interpretation:*

(6.1) Mechanistic ambiguity in the DIC response: The decrease in IL-6 following DIC exposure is most likely attributable to reduced prostaglandin E_2 (PGE_2) production via COX inhibition, which secondarily suppresses IL-6 signaling. This effect is a general pharmacological property of NSAIDs and is not necessarily reflective of Kupffer cell-specific pathways. Direct evidence - such as prostanoid quantification or PGE_2 rescue experiments - is required to establish that the observed effects are liver-specific rather than nonspecific NSAID responses.

(6.2) Pharmacogenetic complexity in the LFM response: LFM-induced hepatotoxicity is highly variable and largely dependent on CYP2C9 polymorphisms, which determine conversion to the active metabolite teriflunomide. Because hepatotoxicity and the associated cytokine responses are not universal among patients, a simplified co-culture model lacking metabolic diversity cannot be assumed to faithfully reproduce patient-specific immune responses. The observed IL-6 suppression could arise from differences in metabolic activation, intracellular exposure, or indirect signaling changes rather than from intrinsic KC-specific mechanisms.

These points significantly undermine the authors' claim that IL-6 modulation provides definitive evidence of model specificity or predictive value. At minimum, the manuscript should (i) explicitly acknowledge these mechanistic limitations, (ii) include supporting data such as prostanoid profiling, CYP2C9 modulation, or teriflunomide quantification, and (iii) temper its claims regarding the model's capacity to recapitulate immune-mediated hepatotoxicity. Without such evidence, the current interpretation risks overstating the functional significance and translational relevance of the co-culture system.

We fully agree with the reviewer and have highlighted this in our discussion (Lines 540 – 551).

https://doi.org/10.7554/eLife.108938.2.sa0