Evaluating the immunotoxicity of CD137-induced agonism on the Emulate Human Liver-Chip

Jake Chaff, Sushma Jadalannagari, Marianne Kanellias, James Velez, Andrew Woodham, Christopher Carman, Lorna Ewart

Emulate, Inc., 27 Drydock Avenue, 02210, Boston, MA, USA

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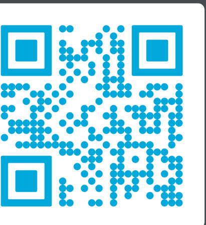


Background & Purpose

The field of cancer immunotherapy is rapidly growing but is challenged by our inability to predict treatment efficacy, patient response and adverse effects. Agonistic antibodies toward T cell co-stimulatory molecules like Urelumab (targeting CD137) are one class of immunotherapy that has shown unparalleled efficacy in murine models of cancer. However, in clinical trials (NCT00309023), Urelumab doses ≥ 1 mg/kg resulted in significant hepatotoxicity (mainly transaminitis), suggesting murine and NHP preclinical assessment failed to predict this clinical safety outcome. While the mechanism of clinical liver toxicity has not been fully elucidated, studies with humanized mice (with human hematopoietic cells) indicate immune-mediated DILI (drug-induced liver injury). Therefore, to model hepatotoxicity associated with Urelumab, we created a novel, immuno-competent human Quad-Culture Liver-Chip using human peripheral blood mononuclear cells (PBMCs) that contain lymphocytes, NK cells and CD137+ monocytes to drive immunotoxicity.

Methods

The Emulate Human Quad-Culture Liver-Chip contains primary human hepatocytes in the epithelial channel, and primary liver non-parenchymal cells (NPCs) such as liver sinusoidal endothelial cells (LSECs), Kupffer cells and stellate cells in the vascular channel (Figure 1). Liver-Chips were seeded and connected to flow according to standard Emulate protocols and were allowed to mature for 6 days (Figure 2B). Urelumab (2 µg/mL) was administered in the absence or presence of resting or activated (3 µg/mL anti-CD3 for 24 h) PBMCs. Anti-CD3 stimulation was included as lymphocytes require T cell receptor (TCR) stimulation to support high CD137 expression (as assessed by flow cytometry). Poly(I:C) was included as a comparator positive control for inflammation and toxicity (Figure 2A).

Pre-labeled PBMCs (CellTracker Red) at 2x10^6 cells/mL were perfused in each channel at 1000 µL/h for 4 h (where applicable). Chips were then treated ± Urelumab for 72 h and subsequent toxicity was evaluated through effluent readouts such as albumin (liver health), ALT (alanine aminotransferase, liver injury), and inflammatory cytokines (IL-6, TNF-α, IFN-γ) at 24, 48 and 72 h post-treatment. PBMC localization was measured using immunofluorescent (IF) imaging of CellTracker Red at 24, 48 and 72 h post-treatment, and cell death was measured through IF imaging of Cytotox Dye at 72 h post-treatment.

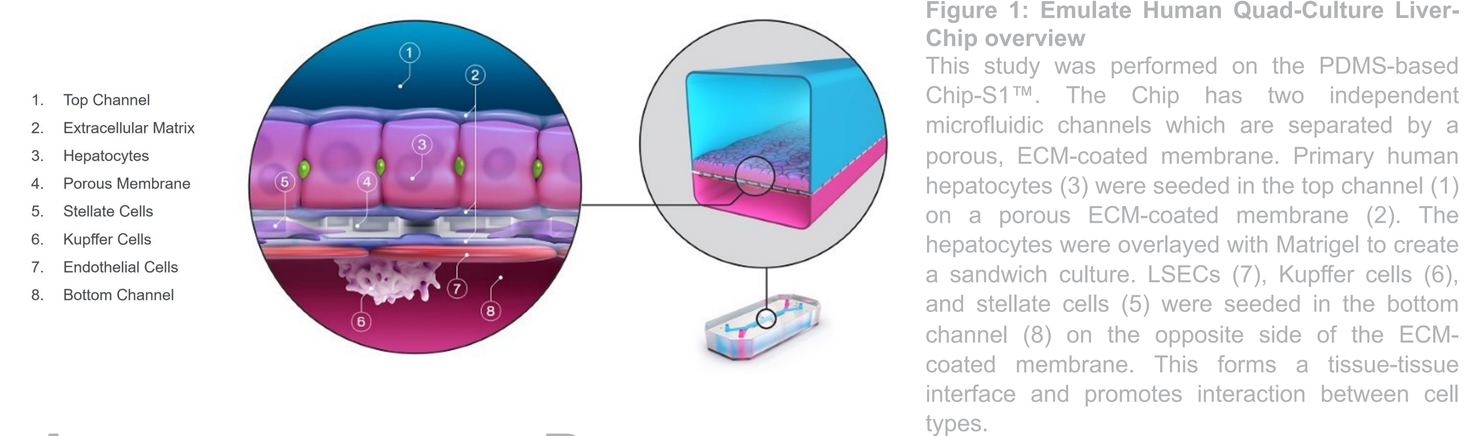
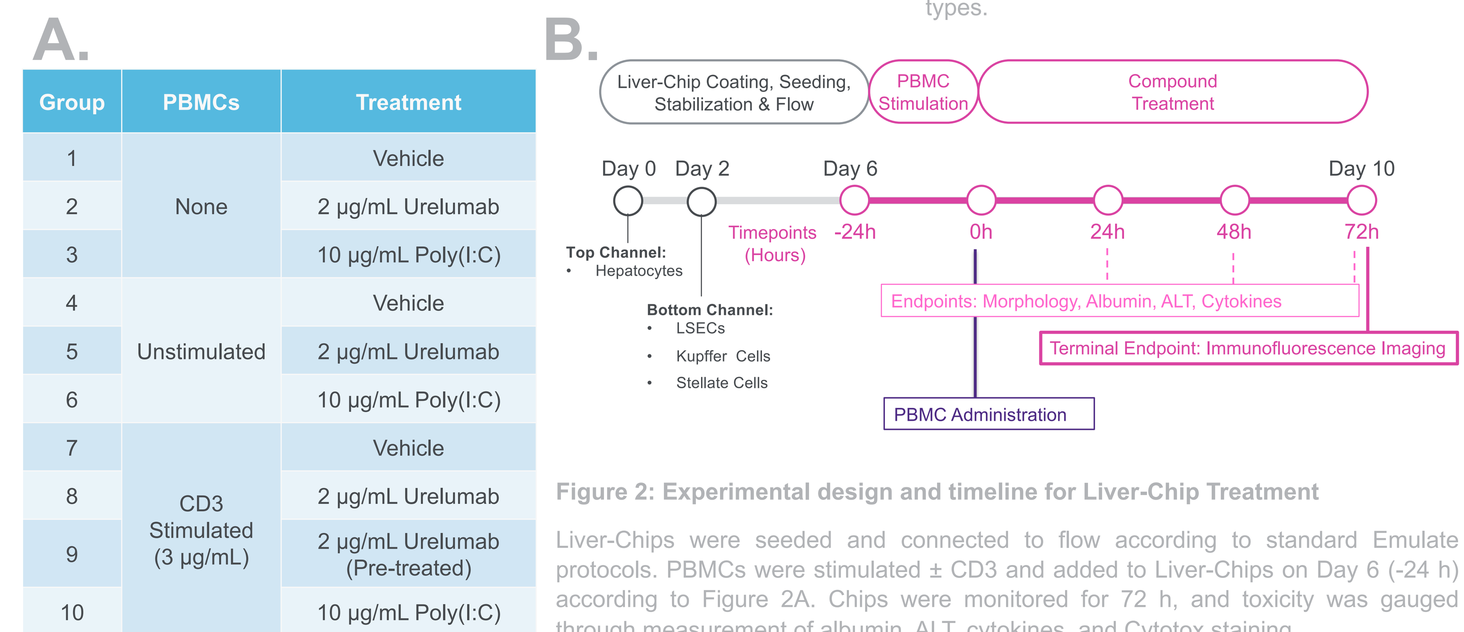


Figure 1: Emulate Human Quad-Culture Liver-Chip overview
This study was performed on the PDMS-based Chip-S1™. The Chip has two independent microfluidic channels which are separated by a porous, ECM-coated membrane. Primary human hepatocytes (3) were seeded in the top channel (1) on a porous ECM-coated membrane (2). The hepatocytes were overlayed with Matrigel to create a sandwich culture. LSECs (7), Kupffer cells (6), and stellate cells (5) were seeded in the bottom channel (8) on the opposite side of the ECM-coated membrane. This forms a tissue-tissue interface and promotes interaction between cell types.



A.

Group	PBMCs	Treatment
1	None	Vehicle
2		2 µg/mL Urelumab
3		10 µg/mL Poly(I:C)
4	Unstimulated	Vehicle
5		2 µg/mL Urelumab
6		10 µg/mL Poly(I:C)
7	CD3 Stimulated (3 µg/mL)	Vehicle
8		2 µg/mL Urelumab
9		2 µg/mL Urelumab (Pre-treated)
10		10 µg/mL Poly(I:C)

Figure 2: Experimental design and timeline for Liver-Chip Treatment

Liver-Chips were seeded and connected to flow according to standard Emulate protocols. PBMCs were stimulated ± CD3 and added to Liver-Chips on Day 6 (-24 h) according to Figure 2A. Chips were monitored for 72 h, and toxicity was gauged through measurement of albumin, ALT, cytokines, and Cytotox staining.

Results

Prior to administration on the Liver-Chip, PBMCs were rested, or stimulated with CD3 for 24 h. When stimulated with CD3, viability was not affected but CD137 was upregulated in subsets of CD4+ and CD8+ T cells, as compared to the unstimulated PBMCs (Figure 3A-B). After stimulation, PBMCs were administered (where applicable) to the Liver-Chips, and near complete coverage of the epithelial channel was observed (Figure 3C). The Liver-Chips which did not receive PBMCs, however, demonstrated healthy morphology. CellTracker staining, revealed that the stimulated PBMCs remained on the Chips more than the unstimulated PBMCs after 72 h of flow and treatment. The onset of toxicity was first observed through the secretion of pro-inflammatory cytokines in response to Urelumab treatment in groups with CD3 stimulated PBMCs (Figure 6A-D). Significant increases in IFN-γ were observed in both the top and bottom channels at 24 h post-treatment. Additionally, a significant increase in TNF-α was observed at 24 h in the top channel, and a significant increase in IL-6 was observed at 24-72 h in the bottom channel. Biomarker investigation showed that the inflammation resulted in the onset of toxicity in groups with CD3 stimulated PBMCs + Urelumab, as significant increases in ALT (Figure 4B) were observed at 48 h post-treatment, followed by decreases in albumin (Figure 4A) at 72 h post-treatment. These observations were further confirmed through Cytotox staining at 72 h, where the highest increase in signal (qualitatively) was observed in groups with CD3-stimulated PBMCs and treated with Urelumab. Although the Emulate Liver-Chip was able to detect immune-mediated DILI as described in clinical trials, there is still room for optimization. Decreased levels of albumin and increased levels of ALT were observed in Chips + PBMCs (especially stimulated) that did not receive treatment, suggesting PBMCs caused baseline toxicity at the tested density. These findings guide the path for future development, in the form of titrating the amount of PBMCs on the Liver-Chip or adapting a protocol to develop a model for immune cell recruitment from the vascular channel of the Chip.

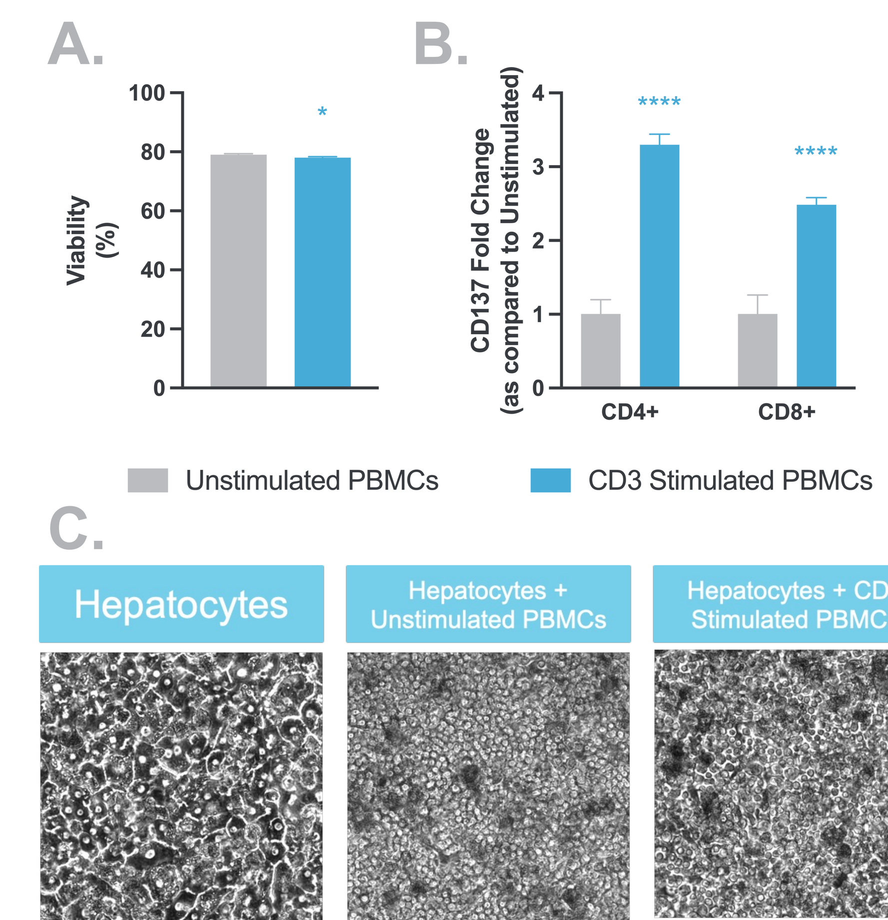


Figure 3: PBMC Stimulation & Attachment
Prior to administration to the Liver-Chips, the (A) viability and (B) upregulation of CD137 was measured on CD3-stimulated PBMCs and compared to unstimulated PBMCs. These results confirmed an expected CD137 upregulation in response to CD3 stimulation. Statistics represented: (A) unpaired t-test, *p<0.05, in comparison to the unstimulated PBMCs, Mean±SD; and (B) two-way ANOVA, ****p<0.0001, in comparison to the unstimulated PBMCs, Fisher's uncorrected LSD Test, Mean±SD. (C) When the PBMCs (8x10^6) were administered to the Chips for 4 h, complete coverage of the hepatocytes was observed, regardless of stimulation. The groups which did not receive PBMCs demonstrated healthy hepatic morphology throughout.

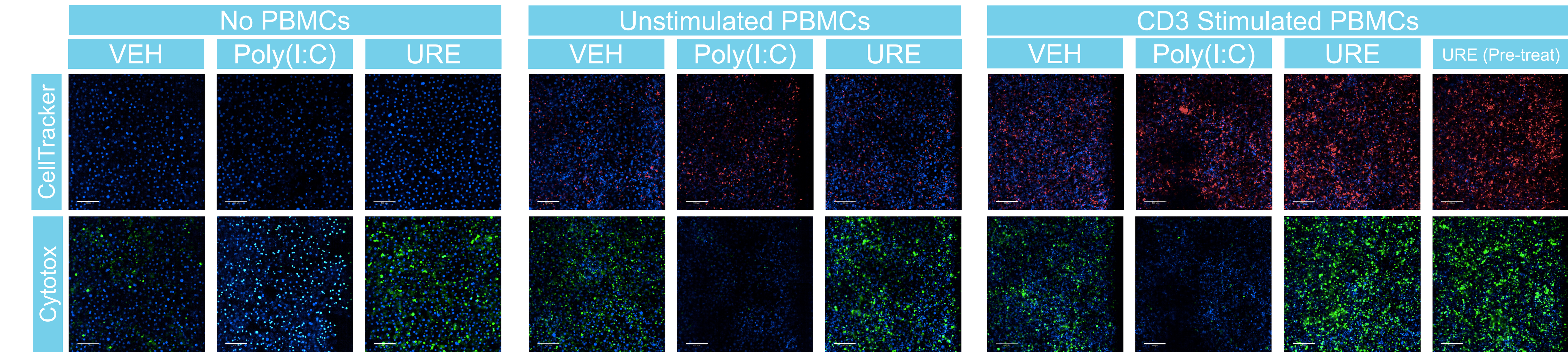


Figure 4: PBMC Localization and Liver-Chip Toxicity
(A) PBMCs were stained with CellTracker prior to administration to the Chips. After 72 h of treatment increased attachment was observed in the stimulated PBMC groups as opposed the unstimulated groups. (B) Similar levels of Cytotox staining were observed between the no PBMC and unstimulated PBMC groups, suggesting minimal toxicity. When treated with Urelumab, CD3 stimulated (CD137^{high}) PBMCs showed an increase in Cytotox signal, suggesting hepatotoxicity. Scale bar = 100 µM

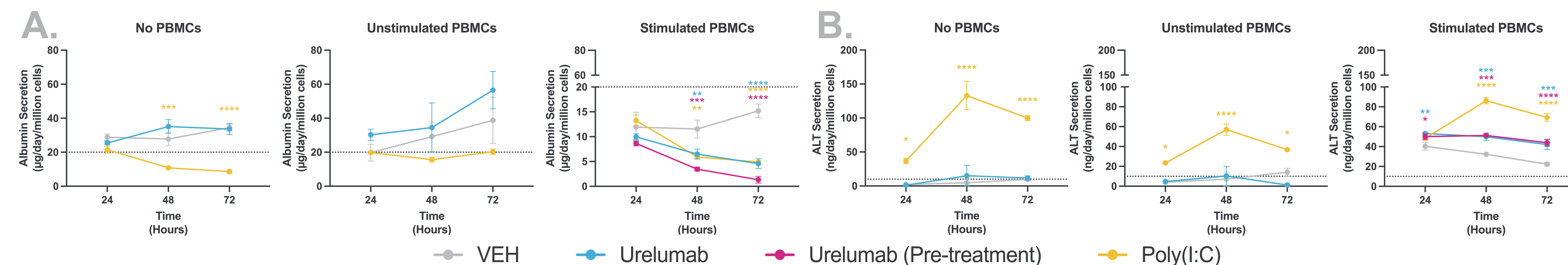


Figure 5: Liver-Chip Biomarker Secretion
(A) Liver-Chip function was gauged by albumin release, with values ≥ 20 µg/day/million cells considered healthy. (B) Toxicity was measured through ALT release and values greater than 10 ng/day/million cells were considered toxic. Together, these results demonstrated that Urelumab only elicited a toxic response in the presence of CD3-stimulated (CD137^{high}) PBMCs. Urelumab treatment had no effect on albumin or ALT release in the absence of PBMCs or in the presence of unstimulated (CD137^{low}) PBMCs. Baseline toxicity was observed (reduced levels of VEH albumin release and elevated levels of VEH ALT release) when stimulated PBMCs were administered to the Chip, suggesting the PBMC seeding density may have been too high.
Statistics represented: Two-way ANOVA, *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001, in comparison to the vehicle control at that timepoint, Fisher's uncorrected LSD test, Mean±SEM.

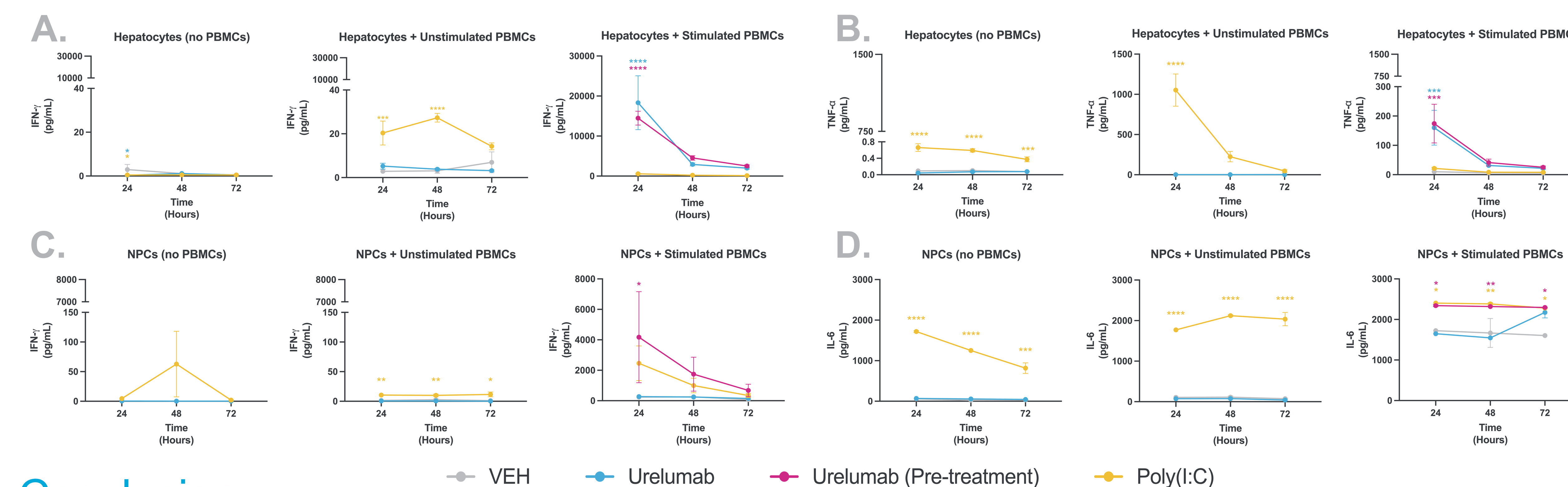


Figure 6: Liver-Chip Cytokine Release
Secreted cytokines were measured at 24, 48, and 72 h post-treatment to determine if Urelumab caused a pro-inflammatory response. IFN-γ was measured from the (A) epithelial and (B) vascular channels. In the epithelial channel, Urelumab treatment caused statistically significant increases in IFN-γ at 24 h in the presence of CD3-stimulated (CD137^{high}) PBMCs only. A similar trend was seen in the vascular channel, except only pre-treated Urelumab caused an increase in IFN-γ. (C) Urelumab treatment caused statistically significant increase in TNF-α release at 24 h, as measured from the epithelial channel, and only in the presence of CD3-stimulated (CD137^{high}) PBMCs. (D) Urelumab treatment caused elevated levels of IL-6, as measured through the vascular channel, for the entirety of the study when pre-treated, or after 72 h of treatment without pre-treatment.

Overall, Urelumab treatment in the absence of PBMCs, or in the presence of unstimulated (CD137^{low}) PBMCs had no biologically relevant impact on IFN-γ, TNF-α, or IL-6 release.

Statistics represented: Two-way ANOVA, *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001, in comparison to the vehicle control at that timepoint, Fisher's uncorrected LSD test, Mean±SEM.

Conclusion

The addition of PBMCs on Emulate Human Liver-Chips demonstrated clinically translatable results and the ability to predict the toxicity of CD137 agonists like Urelumab. These results are a first step in developing a physiologically relevant immuno-competent Liver-Chip, which could be an important preclinical model for immune-mediated DILI caused by emerging immunotherapies.

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