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Modeling therapeutic antibody-small molecule drug-drug interactions using a 3D perfusable human liver co-culture platform

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Abbreviations: AUC, area under the curve; BSA, bovine serum albumin; CI, confidence interval; CRP, C-reactive protein; CYP, cytochrome P450; DDI, drug-drug interaction; DILI, drug-induced liver injury; DMEM, Dulbecco's modified eagle's medium; ELISA, enzyme-linked immunosorbent assay; HC, hydrocortisone; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; KC, Kupffer cell; LC, liquid chromatography; LV, lovastatin; MS, mass spectrometry; PBPK, physiologically-based pharmacokinetic; PBS, phosphate buffered saline; PK, pharmacokinetics; sIL-6R, soluble IL-6 receptor; SVA, simvastatin hydroxy acid; SVS, simvastatin; WEM, William's E medium

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Abstract

Traditional *in vitro* human liver cell culture models lose key hepatic functions such as metabolic activity during short-term culture. Advanced 3D liver co-culture platforms offer the potential for extended hepatocyte functionality, allowing for the study of more complex biological interactions that can improve and refine human drug safety evaluation. Here, we utilize a perfusion flow 3D microreactor platform for the co-culture of cryopreserved primary human hepatocytes and Kupffer cells to study the regulation of CYP3A4 activity by chronic IL-6-mediated inflammation over two weeks. Hepatocyte cultures remained stable over two weeks, with consistent albumin production and basal IL-6 levels. Direct IL-6 stimulation that mimics an inflammatory state induced a dose-dependent suppression of CYP3A4 activity, an increase in C-reactive protein (CRP) secretion, and a decrease in shed soluble IL-6R levels, indicating expected hepatic IL-6 bioactivity. Tocilizumab, an anti-IL-6R monoclonal antibody used to treat rheumatoid arthritis, has been demonstrated clinically to impact small molecule drug pharmacokinetics by modulating cytochrome P450 enzyme activities, an effect not observed in traditional hepatic cultures. We have now recapitulated the clinical observation in a 3D bioreactor system. Tocilizumab was shown to de-suppress CYP3A4 activity while reducing CRP concentration after 72 hours in the continued presence of IL-6. This change in CYP3A4 activity decreased the half-life and AUC_{last} of the small molecule CYP3A4 substrate simvastatin hydroxy acid, measured before and after tocilizumab treatment. We conclude that next-generation *in vitro* liver culture platforms are well-suited for these types of long-term treatment studies and show promise for improved drug safety assessment.

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Introduction

Prediction of drug safety in humans remains one of the biggest challenges facing the drug development industry. In particular, preclinical *in vivo* models fall short of recapitulating human liver biology sufficiently well to anticipate complex forms of drug-induced liver injury (DILI) or to forecast clinical pharmacokinetic (PK) properties when liver function is modulated (Navarro, et al. 2006, Peters 2005). In response, researchers have focused on developing human-based *in vitro* liver platforms in an effort to improve accurate prediction of drug safety. Primary human hepatocytes cultured on flat, collagen-coated plates are widely used for PK and toxicity studies, but in that culture format the cells lose key hepatic functions, such as metabolic activity, after only a few days in culture. Recently, there have been several platforms introduced that offer the potential for long-term, multi-week primary human hepatocyte culture, which would provide researchers the ability to model more complex interactions involving chronic dosing effects, low clearance compounds and drug accumulation, human-specific metabolite identification, DILI, and the effects of chronic inflammation (Ballard, et al. 2015, Chao, et al. 2009, Kostadinova, et al. 2013, Messner, et al. 2013). There are several approaches that can increase *in vitro* hepatic performance and concomitant physiological relevance including 3D cell culture, application of media flow and shear conditions to mimic natural tissue, and co-cultures with liver non-parenchymal cell types such as Kupffer cells (KCs), liver sinusoidal endothelial cells, and hepatic stellate cells (Ebrahimkhani, et al. 2014, LeCluyse, et al. 2012).

Hepatocytes express cytochrome P450 (CYP) enzymes, which are critical to the phase I metabolism of many xenobiotics. In particular, CYP3A4 is primarily responsible for the

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metabolism of over 50% of marketed small molecule drugs (Ingelman-Sundberg 2004).

Inflammation is known to down-regulate CYP activity, a normal adaptation to a stressed state mediated by transcription factors such as PXR, CAR, and NF- κ B as well as reactive oxygen species (Morgan, et al. 2008). Inflammatory cytokines have been linked to the suppression of specific CYP enzymes (Huang, et al 2010, Zhou, et al. 2014), which has potential implications for modulating drug exposure and thus safety. Interleukin 6 (IL-6) is an inflammatory cytokine elevated in a number of diseases such as rheumatoid arthritis, inflammatory bowel disease, diabetes, and several forms of cancer (Ishihara, et al. 2002, Heikkila, et al. 2008). The regulation of IL-6 is an important aspect of normal physiology. In the liver, Kupffer cells secrete IL-6 locally in response to inflammatory stimuli such as lipopolysaccharide (LPS) that can travel to the liver from the intestinal microbiota (Xu, et al. 2004), but hepatocytes are also capable of secreting IL-6 (Norris, et al. 2013). Hepatocytes respond to systemically high levels of IL-6 caused by inflammatory diseases through the IL-6 receptor (IL-6R) (Morgan, et al. 2008). This receptor exists in both a cell membrane-bound form and a shed soluble form (sIL-6R), both of which bind IL-6 with equivalent affinity and mediate the same downstream signaling cascade (Rose-John 2012, Scheller, et al. 2014). C-reactive protein (CRP), an acute phase protein secreted by hepatocytes in response to IL-6, is commonly used as an *in vitro* marker of IL-6 signaling in hepatocytes (Bode, et al. 2012, Nguyen, et al. 2015).

In traditional culture models, IL-6 has been shown to decrease activity or expression for a number of CYP enzymes in hepatocytes including CYP3A4 (Dickmann, et al. 2011, Huang, et al 2010, Jover, et al. 2002). However, conventional *in vitro* liver platforms do not allow for the study of complex inflammation-related DDIs because of their limited capacity for long-term

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culture. Dickmann, et al. were able to demonstrate IL-6-based modulation of CYP activities using hepatocytes on collagen-coated plates, but these effects were limited to 96 hours of treatment or less. This short time scale prevents the analysis of changes in small molecule pharmacokinetics before and after CYP modulation with inflammatory/anti-inflammatory stimuli in the same cell populations over time to determine whether there is a functional consequence of those changes in apparent CYP activity. Those studies also utilized an anti-IL-6 neutralizing antibody co-dosed with IL-6 over 72 hours to prevent CYP suppression. Long-term culture would allow for both phases of the process to be observed, first suppression by cytokine application, followed by de-suppression of CYPs in hepatocytes.

Cytokines such as IL-6 continue to be targets of large molecule-based therapies for chronic inflammatory conditions. For example, tocilizumab is an anti-IL-6R monoclonal antibody approved to treat rheumatoid arthritis and juvenile idiopathic arthritis. Because it interferes with cytokine signaling and its downstream effects on CYP expression levels, tocilizumab has the potential to de-suppress CYP activity in the liver (Lee, et al. 2010, Morgan, et al. 2009). Tocilizumab has been shown clinically to increase CYP3A4 activity in rheumatoid arthritis patients and alter corresponding small molecule PK, particularly of a common medication for that patient population, simvastatin. The pro-drug simvastatin (SVS) and its biologically active form simvastatin hydroxy acid (SVA) are part of the statin family of HMG CoA reductase inhibitors used to treat high cholesterol. Statins are metabolized primarily by CYP3A4 and both SVS and SVA showed reduced exposure in rheumatoid arthritis patients after tocilizumab administration (Schmitt, et al. 2011). FDA guidance has encouraged the exploration of these

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types of large molecule-small molecule drug-drug interactions (DDI) to support regulatory filings and improve human drug safety evaluation (US FDA CDER 2012).

Here, we utilize the LiverChip™, a next-generation *in vitro* hepatic culture system, to study the effect of chronic IL-6 inflammation on primary human hepatocyte/KC co-cultures. Cells in the LiverChip™ are cultured within 3D micro-channels in a scaffold exposed to continuous media perfusion flow used to simulate a liver sinusoid (Powers, et al. 2002, Sivaramann, et al. 2005, Hwa, et al 2007, Domansky, et al. 2010). The LiverChip™ has been previously reported to preserve human hepatocyte metabolic activity (Vivares, et al. 2014) and to model hepatic inflammation (Sarkar, et al. 2015, Yu, et al. 2015), but to date, human hepatocyte data have been limited to one week of culture. In these studies, we use the LiverChip™ to directly demonstrate both an IL-6-based reduction in CYP3A4 activity and a tocilizumab-mediated effect on simvastatin hydroxy acid exposure through manipulation of CYP3A4 activity over a period of two weeks.

Materials and Methods

Optima™ LC/MS-grade water, acetonitrile, methanol, and acetic acid were obtained from Thermo Fisher Scientific (Waltham, MA). LC/MS-grade ammonium acetate, simvastatin (SVS, CAS: 79902-63-9), and rifampicin (CAS: 13292-46-1) were obtained from Sigma-Aldrich (St. Louis, MO). Simvastatin hydroxy-acid (SVA, CAS:139893-43-9) and lovastatin (LV, CAS: 75330-75-5) were purchased from Toronto Research Chemicals (Toronto, ON). Recombinant human IL-6 (Cat #206-IL) was obtained from R&D Systems (Minneapolis, MN).

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LiverChip™ platform

The LiverChip™ was obtained from CN Bio Innovations (Hertfordshire, UK). Each platform contains twelve bioreactors with a polysulfone cell culture plate secured to a pneumatic plate (see Figure 1A). Bioreactors hold a collagen-coated polystyrene scaffold with 300 cylindrical micro-channels of diameter 300 μm and height 250 μm for cell culture (see Figure 1B). Fluid flow is controlled by pneumatic pumping through the scaffold utilizing a thin polyurethane membrane between the cell culture and pneumatic plates (see Figure 1C). All LiverChip™ experiments were performed in a humidified incubator at 37 °C with a 5% CO₂ atmosphere under a 1 μL/sec flow rate.

Human hepatocyte-Kupffer cell co-cultures

Cryopreserved human hepatocytes and Kupffer cells (KCs) were purchased from Thermo Fisher Scientific. Donor information is shown in Supplemental Table 1. After thawing, hepatocytes were gently transferred to 50 mL warm cryopreserved hepatocyte recovery medium (CHRM[®], Thermo Fisher Scientific), centrifuged at 100xg for 10 minutes, and re-suspended in Advanced DMEM containing 5% FBS, 4 μg/mL insulin, 2 mM GlutaMax, 15 mM HEPES, and 1% pen/strep (seeding medium). After thawing, KCs were transferred to ice cold seeding medium, centrifuged at 400xg for 4 minutes at 4 °C, and re-suspended in cold seeding medium. Viable cells were quantified using a trypan blue exclusion assay. Viability was greater than 90% for all cell lots. Each reactor well of the LiverChip™ was seeded with 6.0 x 10⁵ viable hepatocytes and 6.0 x 10⁴ viable KCs in 1.6 mL of seeding medium. After seeding, flow was set to 1.0 μL/sec in the down direction for eight hours, allowing cells to attach to the microchannel scaffolds. After eight hours, flow was reversed to 1.0 μL/sec in the up direction for the duration of the

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experiment to allow for medium re-oxygenation along the bioreactor surface channel in each well. On culture day one (24 hours after seeding), medium in each well was replaced with Advanced DMEM containing 6.25 $\mu\text{g}/\text{mL}$ insulin, 6.25 $\mu\text{g}/\text{mL}$ transferrin, 6.25 $\mu\text{g}/\text{mL}$ selenous acid, 1.25 mg/mL BSA, 5.25 $\mu\text{g}/\text{mL}$ linoleic acid, 2 mM GlutaMax, 15 mM HEPES, and 0.5% pen/strep (hepatocyte maintenance supplement, Thermo Fisher Scientific). On culture day three, medium was replaced by Williams E Medium containing maintenance supplement and 100 nM hydrocortisone (maintenance medium), which was subsequently changed every 48 hours. Medium was sampled, aliquoted and stored at $-80\text{ }^{\circ}\text{C}$ prior to analysis.

CYP3A activity

CYP3A4 enzyme activity was measured using a P450-GloTM kit with a Luciferin-IPA substrate (Promega, Madison, WI). Substrate was diluted 1:1000 to 3 μM in 1.6 mL maintenance medium and added to each bioreactor after a medium change. After one hour of incubation at $37\text{ }^{\circ}\text{C}$, media were collected from each reactor and 50 μL was added to a white-walled 96 well plate. 50 μL of standards, prepared using a serial dilution of click beetle luciferin potassium salt (Promega E1601), were also added to the 96 well plate. 50 μL of luciferin detection reagent was added to all samples and standards, after which the plate was covered and incubated for 20 minutes at room temperature. Luminescence was measured using a SpectraMax L plate reader (Molecular Devices, Sunnyvale, CA).

Imaging

Scaffolds were carefully removed from the LiverChipTM cell culture plate and washed with PBS. Scaffolds were fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA)

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in PBS for one hour at room temperature. After fixation, cells were permeabilized with 0.1% Triton X-100 (Sigma Aldrich, St. Louis, MO) in PBS then stained with a 1:200 dilution of Alexa488 Phalloidin (Thermo Fisher Scientific) for 30 min. Scaffolds were counterstained with Hoechst's dye (Sigma-Aldrich) for 5 min. Scaffolds confocal reconstructions were acquired on an Olympus Fluoview 1000 scanning confocal microscope, using the 10x and 20x objectives. Images were processed using MetaMorph software (Molecular Devices, Sunnyvale, CA).

Protein Analysis

Human albumin secretion was measured using an ELISA kit purchased from Bethyl Laboratories (Montgomery, TX). IL-6, CRP, and sIL-6R alpha were measured using human Quantikine ELISA kits (R&D Systems). Assays were developed according to manufacturer's instructions. Absorbance was measured on a SpectraMax M3 plate reader (Molecular Devices).

IL-6 stimulation studies

Triplicate wells of hepatocytes and Kupffer cells cultured in the LiverChipTM were induced with 10 μ M rifampicin on culture day 5 along with 1, 10, 50, 100, 500, 1000, 5000, or 10000 pg/mL recombinant human IL-6. Media were collected and refreshed with the same components on culture day 7. CYP3A4 was measured on culture day 9 after 96 hours of stimulation. CRP and sIL-6R alpha were measured in exchanged media by ELISA.

Tocilizumab-simvastatin hydroxy acid interaction studies

Hepatocytes and Kupffer cells were cultured in the LiverChipTM for fourteen days. Six sample groups were run in triplicate for each of three hepatocyte lots. All wells were induced with 10

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μM rifampicin starting on culture day 5. During the first treatment period of days 5-9, sample groups 2, 4, and 6 were stimulated with 1 ng/mL recombinant human IL-6. On culture day 7, all wells were treated with 909 ng/mL simvastatin hydroxy acid (SVA) in 2.0 mL of hepatocyte maintenance medium. This SVA concentration was chosen such that the unbound fraction of drug was set to 10x human serum C_{max} . Final DMSO media concentrations for wells dosed with rifampicin with or without SVA was set to 0.1%. 50 μL of media was removed at 0.5, 1, 4, 6, 8, 10, 24, and 48 hours and stored at $-80\text{ }^{\circ}\text{C}$ for LC-MS/MS analysis of SVA. On culture day 9, CYP3A4 enzyme activity was measured.

During the second treatment period of days 9-14, groups 1 and 6 received no treatment, group 2 was treated only with 1 ng/mL IL-6, group 5 was treated only with 1.6 μM pharmaceutical grade tocilizumab (Actemra[®], Genentech, South San Francisco, CA), and groups 3-4 were co-treated with both 1 ng/mL IL-6 and 1.6 μM tocilizumab. This tocilizumab concentration was chosen to represent human serum C_{max} . Media were replaced on culture day 12 to allow for 72 hours of tocilizumab treatment prior to SVA addition. SVA was dosed to all groups along with corresponding IL-6/tocilizumab treatments on day 12 and sampled similarly to the day 7 dose. CYP3A4 enzyme activity was measured on culture day 14.

Simvastatin hydroxy acid sample preparation.

50 μL media samples containing SVA were thawed and spiked with 5 μL of 100 ng/mL lovastatin (LV) as an internal standard. 200 μL of ice cold 50:50 methanol/acetonitrile was added to each sample, which was then vortexed and placed on ice for 15 min. Samples were then spun down at 2000xg at $4\text{ }^{\circ}\text{C}$ for 15 min. Supernatants were removed and used for LC-MS/MS

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analysis. Standards were prepared in a similar manner, first spiking SVA and LV into hepatocyte maintenance medium with final concentrations ranging from 50-1200 ng/mL SVA prior to protein extraction.

Simvastatin hydroxy acid LC-MS/MS analysis

An UltiMate[®] 3000 LC system (Thermo Fisher Scientific) was connected to a Hypersil Gold[™] UHPLC column (50 mm x 2.1 mm; 1.9 μ m particle size, Thermo Fisher Scientific) and maintained at ambient temperature (~23 °C). The sample injection volume was 20 μ L and samples were processed using isocratic elution for 10 minutes with a loading pump flow rate of 150 μ L/min using 75:25 (% v/v) acetonitrile:100 mM ammonium acetate (adjusted pH to 5 with glacial acetic acid), adapted from the method described by Ahmed, et al. 2012. The autosampler was maintained at 4°C. Analytes were detected using a Q Exactive[™] Hybrid Quadrupole-Orbitrap[™] Mass Spectrometer (Thermo Fisher Scientific) equipped with a HESI-II Probe. The HESI-II probe settings were determined using manufacturer suggested settings at a flow rate of 150 μ L/min with sheath gas flow rate = 40, aux gas flow rate= 10, sweep gas flow rate= 2, spray voltage= 3.5 kV, capillary temperature= 250 °C, S-lens RF level 65, and heater temp= 300 °C. Scan parameters were optimized with full-scan MS data collection from time = 0-10 minutes and MS/MS data also collected from time = 1-3 minutes and operating in positive ion mode. Full MS-SIM scans were operated in positive ion mode with 70 K resolution, 1.0e6 AGC target, and a scan range of 100-500 m/z; while targeted MS/MS scans operated in positive ion mode with 17.5 K resolution, 1.0e5 AGC target, 4.0 m/z isolation window, and 10.0 NCE. The analytes were quantitated using selected reaction monitoring (SRM) by detecting the [M+H]⁺ precursor to product ion transitions. The precursor/product ion transitions were observed at m/z 419.28 /

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199.15 for simvastatin (SVS), 437.29 / 303.20 for simvastatin hydroxy acid (SVA), and 405.26 / 199.15 for lovastatin (LOV). Quantitation was performed using Thermo Xcalibur™ Quan Browser v2.2 using lovastatin as an internal standard with ICIS peak detection, minimum peak height (S/N) = 3.0, quadratic curve fitting, and $1/X^2$ weighting.

Statistical Analyses

Statistical analyses of CYP3A4 activity, SVA half-life, and SVA AUC_{last} were performed using a one-way ANOVA with Dunnett's post-hoc test in GraphPad Prism software version 7.0.

Results

Hepatocyte/Kupffer cell dynamic co-culture characterization

Hepatocytes and KCs seeded into LiverChip™ bioreactors attached to the collagen-coated polystyrene scaffold inserts and downward flow after seeding helped direct them into the scaffold micro-channels. Co-culture micro-tissue morphology is shown in Figure 2 by confocal reconstruction. By culture day seven, micro-tissues several cell layers thick in the radial direction have formed inside the channels, and these tissues extend the 250 μm channel depth. Gaps in the tissue allow for fluid flow through the scaffold. Cells are also present on the top surface of the scaffolds. Kupffer cells co-cultured in this format have previously been demonstrated to be interspersed amongst the hepatocytes (Wheeler, et al. 2014).

Figure 3A shows the hepatocyte albumin production profile as a function of culture time for hepatocyte donor HU8160. Albumin synthesis is a widely accepted marker of hepatocyte activity

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for *in vitro* cultures. Albumin accumulates between media changes, so the measured albumin concentrations shown are from the end of those 24-48 hour periods of accumulation and are normalized per day. Albumin production began at low levels prior to day three before subsequently increasing and stabilizing by five days. Figure 3B shows that for hepatocyte donor HU8160, basal levels of inflammation, as measured by IL-6 concentration began high with a mean concentration of 96.0 +/- 46.5 pg/mL before decreasing and stabilizing to healthy human physiological serum levels (<10 pg/mL) by seven days.

IL-6 stimulation studies

CYP3A4 activity, CRP secretion, and levels of sIL-6R all showed a strong dose response to IL-6 for hepatocyte donor HU8160 (see Figure 4). CYP3A4 activity decreased with increasing IL-6 exposure over 96 hours, showing a sigmoidal relationship with an EC₅₀ of 463 pg/mL IL-6 (Figure 4A). As a result, for subsequent studies modulating CYP3A4 with IL-6, an IL-6 concentration of 1 ng/mL was chosen to saturate the response. CRP, being an acute phase protein, showed an increase in secretion over 48 hours in response to higher levels of IL-6, with a sigmoidal relationship and an EC₅₀ of 354 pg/mL IL-6 (Figure 4B). sIL-6R receptor levels showed a first order decrease in response to increasing IL-6 concentration over 48 hours (Figure 4C), further demonstrating the bioactivity of IL-6 on the cellular system (Rose-John 2012).

Tocilizumab effects on CYP3A4 activity

In order to parse the ability of the *in vitro* liver microreactor system to capture the crucial features of known human responses to tocilizumab in patients who also take simvastatin, we designed an experiment involving six samples groups with two sequential periods of treatment,

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mimicking inflammation and resolution of inflammation by tocilizumab treatment, as shown schematically in Figure 5. For each sample group, relative CYP3A4 activities for hepatocyte lot HU8160 are represented as a ratio between the activities measured at the ends of the second treatment period (days 12-14) to the first treatment period (days 7-9) and are plotted in Figure 6. For hepatocytes not treated throughout the experiment, there is no change in CYP3A4 activity between these two culture periods. Under inflammatory conditions of 1 ng/mL IL-6 throughout the experiment, CYP3A4 activity is depressed (Figure 4A) but there is no change in the activity measured between treatment periods, so the activity ratio is close to 1. Similarly, CYP3A4 activity was unchanged in cultures that received no treatment in the early period and were subsequently administered 1 ng/mL IL-6 along with 1.6 μ M tocilizumab (anti-IL-6R). These results indicate that tocilizumab can block IL-6 signaling in this culture system, since the addition of IL-6 as an inflammatory cue would have otherwise decreased the ratio of CYP3A4 activity for day 14 (inflammation cue + inflammation blocker) relative to day 9 (no treatment). A no treatment phase followed by tocilizumab alone did not impact CYP3A4, showing that the effect in the presence of IL-6 was specific to inflammatory cytokine inhibition. Initial treatment with IL-6 followed by co-administration of tocilizumab with IL-6 resulted in de-suppression of CYP3A4, and a corresponding 2.0 \pm 0.5 (* p < 0.05)-fold increase in the activity ratio on day 14 relative to day 9. Initial treatment with IL-6 followed by a wash-out period also de-suppressed CYP3A4 activity with a mean effect ratio of 1.9 \pm 0.5 (* p < 0.05). In total, these results demonstrate that the culture system is capable of multiple forms of physiological adaptation to inflammation.

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Testing in a second hepatocyte donor, HU8163 (Supplemental Figure 1), confirmed the previous results. Similar patterns of CYP3A4 de-suppression occurred with IL-6 after tocilizumab treatment, with a mean effect ratio of 1.7 +/- 0.1 (*p < 0.05), and for the removal of IL-6, with a mean effect ratio of 1.9 +/- 0.1 (*p < 0.05). In a third hepatocyte donor, HU8196, the IL-6-only controls surprisingly resulted in CYP3A4 de-suppression, with higher activity levels measured at the end of the experiment resulting in an effect ratio approaching that of tocilizumab addition (Supplemental Figure 2). However, sIL-6R alpha levels for that donor approached zero after seven days of IL-6 treatment (Supplemental Figure 3), indicating that continued stimulation may have resulted in receptor loss and lack of consistent IL-6 signaling through the pro-inflammatory sIL-6R over the course of the experiment. Tocilizumab treatment for this donor increased sIL-6R alpha levels, suggesting that treatment reduces receptor internalization and possibly enhances receptor shedding.

Tocilizumab effects on CRP

CRP, as a marker of the acute phase response to inflammation, should increase with IL-6 application. Indeed, after 48 hours, sample groups stratify into increasing or decreasing CRP levels based on the respective presence or absence of a 1 ng/mL IL-6 stimulus, as illustrated in Figure 7 for each of the six sample groups in the tocilizumab experiment for hepatocyte donor HU8160. High levels of IL-6 produced an initial four-fold increase in CRP, while hepatocytes with no inflammatory stimulus throughout the experiment produced CRP levels that decreased and approached zero over time, which is consistent with the basal IL-6 decrease seen in Figure 3B. High CRP was maintained for hepatocytes dosed only with IL-6 throughout the experiment while treatment with tocilizumab caused CRP levels to decrease within two days with near-basal

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levels of CRP measured three days after application of antibody. This pattern was mirrored in samples where IL-6 was removed from the system, providing further evidence that tocilizumab successfully inhibited pro-inflammatory IL-6 signaling. These results were corroborated in hepatocyte donor HU8163 (Supplemental Figure 4). Fold changes for that donor were even higher due to lower basal levels of CRP.

Simvastatin hydroxy acid metabolism in the LiverChipTM

With IL-6-mediated CYP3A4 suppression and subsequent tocilizumab-induced CYP3A4 de-suppression established in the LiverChipTM, we turned our attention to measuring simvastatin hydroxy acid (SVA) metabolism in this model with the aim of directly demonstrating the impact of tocilizumab on SVA pharmacokinetics, as observed in human patients. SVA concentration was measured from bioreactor samples using an LC-MS/MS method with lovastatin (LV) as an internal standard. Supplemental Figure 5 shows a representative LC elution profile for productions of SVS, SVA, and LV, which all demonstrated distinct elution times. For bioreactor studies, interconversion from SVA to the simvastatin lactone form was negligible (<1%) and freeze-thaw did not significantly impact measurements (data not shown). Supplemental Figure 6 shows representative parent and product ion spectra for SVA and LV with expected ion peaks identified for SVA and LV (Li, et al. 2006a). The linear range of detection for this assay was 50-1200 ng/mL SVA (data not shown). MS peaks associated with known phase I metabolites of SVA such as 3'-hydroxy SVA, 6'-exomethylene SVA, and 3',5'-dihydrodiol SVA were not detected in this platform, likely due to large bioreactor media volumes diluting their signals below background (data not shown).

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In hepatocyte maintenance medium, SVA protein binding was measured to be 66% using an ultracentrifugation protocol (Nakai, et al. 2003). An SVA dose of 909 ng/mL was chosen for bioreactor studies to set the free drug fraction in the media to 10x the reported average human serum C_{max} (30.9 ng/mL) for an 80 mg simvastatin dose (Pharmapendium). Figure 8 shows SVA concentration profiles from hepatocyte lot HU8160 under normal and inflammatory conditions during treatment periods starting on day 7 (Figure 8A) and day 12 (Figure 8B). Actual measured concentration at time zero and for no cell controls is slightly less than 909 ng/mL due to an expected small loss in recovery (Ahmed, et al. 2012). SVA half-life was found to be 5.0 +/- 0.3 hours on day 7 and 4.9 +/- 0.3 hours on day 12. Under inflammatory conditions with 1 ng/mL IL-6, mean SVA half-life was increased to 6.4 +/- 1.2 hours on day 7 and 6.8 +/- 1.5 hours on day 9, consistent with decreasing CYP3A4 activity. SVA AUC_{last} was 4841.0 +/- 142.8 ng·hr/mL on day 7 and 4961.2 +/- 127.5 ng·hr/mL on day 12. Under inflammatory conditions, SVA AUC_{last} was 5688.7 +/- 831.2 ng·hr/mL on day 7 and 6034.7 +/- 889.7 ng·hr/mL on day 12. Increasing half-life and AUC_{last} under inflammatory conditions is consistent with CYP3A4 depression.

Tocilizumab-simvastatin hydroxy acid interaction

For the LiverChipTM experiment detailed in Figure 5, simvastatin hydroxy acid was co-dosed during two treatment periods and media were repeatedly sampled over the next 48 hours for measurements of how tocilizumab impacts SVA pharmacokinetics in the microreactor. Half-life and AUC_{last} results for hepatocyte donor HU8160 are shown in Figure 9. Each parameter is represented as a ratio to demonstrate changes induced by tocilizumab. Co-administration of tocilizumab after an initial IL-6 treatment reduced mean SVA half-life ratio by 29.8% (*p <

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0.05) (see Figure 9A) and reduced mean AUC_{last} ratio by 21.0% compared to IL-6 treatment alone (* $p < 0.05$) (see Figure 9B). Similar effect sizes were observed for hepatocyte donor HU8163, where tocilizumab decreased mean SVA half-life ratio by 20.6% (Supplemental Figure 7A) and mean AUC_{last} ratio by 19.0% compared to IL-6 treatment alone (* $p < 0.05$) (Supplemental Figure 7B).

Discussion

Dynamic flow culture and *in vitro* 3D liver tissue engineering have been proposed as solutions to overcome limitations in the functional capacity of primary human hepatocytes (Dash, et al. 2009, Ebrahimkhani, et al. 2014, LeCluyse, et al. 2012). These methods have shown promise in the evaluation of hepatic drug metabolism, DILI, and pharmacokinetics (Ballard, et al. 2015, Chao, et al. 2009, Kostadinova, et al. 2013, Messner, et al. 2013). The LiverChipTM platform offers continuous perfusion of oxygenated media through a scaffold containing micro-channels for 3D cell culture under flow. Human hepatocytes cultured in the LiverChipTM have previously been shown to express essential metabolic enzymes over one week of culture (Vivares, et al. 2014), and co-cultures with Kupffer cells recapitulated key behaviors under inflammatory conditions such as cytokine secretion in the presence of LPS (Sarkar, et al. 2015). Here, we have demonstrated hepatocyte functionality and the ability to successfully modulate cytochrome P450 activities in response to an IL-6 inflammatory stimulus over two weeks of culture in the LiverChipTM. In contrast, previous *in vitro* studies involving IL-6-mediated CYP3A4 suppression in primary human hepatocytes were limited to 96 hours (Dickmann, et al. 2011). As a result, those 2D cultures were unable to show subsequent CYP de-suppression induced by interfering

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with inflammatory cytokine signaling, an important effect that has been demonstrated clinically using tocilizumab (Schmitt, et al. 2011). The LiverChip™ platform and its capacity for long-term culture offer the opportunity to directly recapitulate this clinically-relevant tocilizumab-mediated CYP de-suppression and its impact on small molecule PK *in vitro*.

In LiverChip™ cultures, both albumin and basal IL-6 stabilized over time, with albumin production increasing after three days, and basal inflammation decreasing to healthy physiological levels (<10 pg/mL) after one week. This indicates an initial period of adaptation to the culture after cryopreserved hepatocytes were seeded. Similar patterns for decreases in the clinical liver injury markers aspartate aminotransferase and alanine aminotransferase were observed for freshly isolated human hepatocytes in the LiverChip™ along similar time scales, although these were not measured in our study (Wheeler, et al. 2014).

IL-6 induced a dose-dependent decrease in CYP3A4 activity and an increase in CRP synthesis. These effects were similar to those observed for hepatocytes in 2D culture (Dickmann, et al. 2011). Our results have shown that the IL-6 EC₅₀ for CYP3A4 and CRP effects fell in the clinical range for inflammatory diseases (10-1500 pg/mL; Machavaram, et al. 2013), as did the 1 ng/mL IL-6 dose subsequently chosen for chronic inflammation studies. Kupffer cells were included to build a more complete hepatic cellular microenvironment, and numerous studies have shown their impact on the behavior of *in vitro* liver systems, such as enhanced IL-1β-mediated cytokine secretion, acute phase protein production, and CYP suppression (Hoebe, et al. 2001, Nguyen, et al. 2015, Rose, et al. 2016, Sarkar, et al. 2015, Sunman, et al. 2004). There are other mechanisms mediated by IL-6 that could potentially be modeled in the LiverChip™ system

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such as drug transporter activity. Exploring additional aspects of cytokine signaling in the LiverChip™ is a logical extension of the current work.

Notably, in our studies dexamethasone, a potent synthetic anti-inflammatory steroid, was not added to seeding or maintenance medium, a common practice to boost hepatocyte-specific gene expression and activity. We instead used hydrocortisone (HC), a naturally-occurring corticosteroid, in maintenance medium beginning on culture day 3. 100 nM HC is a physiologically-relevant concentration, and HC metabolism in LiverChip™ co-cultures has previously been characterized (Sarkar, et al. 2015). The absence of HC reduced basal CYP3A4 activity to low levels after one week (data not shown). Our studies also utilized rifampicin as a CYP3A4 induction agent to increase the dynamic range of CYP3A4 activity. Rifampicin acts on pregnane X receptor (PXR), the nuclear receptor that controls the bulk of CYP3A4 transcription (Li, et al. 2006b). PXR is the same receptor acted upon by IL-6 signaling (Yang, et al. 2010), and rifampicin has been previously used to augment the effects of inflammatory agents on CYP3A4 in hepatocytes (Gu, et al. 2006, Pascussi, et al. 2000).

sIL-6R showed an IL-6 dose-dependent decrease. In this platform, membrane resident IL-6 receptors on hepatocytes are likely outnumbered by high concentrations of soluble receptors (Nesbit, et al. 1992) due to a high medium volume to tissue ratio. Membrane and soluble receptors of IL-6 have equivalent affinity for IL-6 (Scheller, et al. 2014), and are both inhibited by tocilizumab (Mihara, et al. 2005), making the soluble receptors the key mediators of IL-6 signaling in the LiverChip™. This fits with the observed decrease in sIL-6R upon IL-6 treatment. In order for IL-6 to be used as a chronic pro-inflammatory stimulus in long-term

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cultures, sIL-6R must not be depleted over the course of the experiment. We observed that when sIL-6R was depleted for hepatocyte donor HU8196 following IL-6 administration, this contributed to a CYP3A4 result in disagreement with other hepatocyte donors.

In our studies, LiverChipTM culture was used to mimic a large molecule-small molecule DDI, specifically the clinically-demonstrated impact of tocilizumab on CYP3A4 and simvastatin hydroxy acid (SVA) metabolism (Schmitt, et al. 2011). High systemic levels of IL-6 in rheumatoid arthritis patients decrease CYP3A4 activity in the liver, but when tocilizumab is administered, IL-6 signaling decreases, leading to a de-suppression of CYP3A4 activity. Schmitt, et al. reported pharmacokinetic results for the prodrug simvastatin (SVS) and its biologically active HMG CoA reductase inhibitor form SVA before and after two weeks of tocilizumab treatment. Clinically, tocilizumab reduced SVA AUC_{last} by 39% (24-51% 90% CI) and SVS AUC_{last} by 57% (45-66% 90% CI). No significant changes in half-life of SVS or SVA after tocilizumab were reported, with SVA t_{1/2} of 5 +/-1 hours before tocilizumab and 4 +/-2 hours after tocilizumab. For our studies we focused on SVA because it is the biologically active form, and because in cell culture media the SVS parent undergoes a pH-dependent non-enzymatic conversion to SVA (Ahmed, et al. 2012), which confounds its analysis. Non-specific SVA interconversion back to the SV lactone form was negligible in our system (<1%), and metabolic products of SVA were not detected likely due to large bioreactor media volumes diluting low metabolite signals. However, the media volume in this platform was also advantageous because it allowed for repeated PK sampling over 48 hours post-SVA treatment.

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To mimic the tocilizumab-SVA clinical study *in vitro*, hepatocytes were treated with 10x human serum C_{\max} SVA under inflammatory conditions before and after the addition of tocilizumab at its human serum C_{\max} . SVA AUC_{last} was measured and compared for both treatment periods, showing decreases of 20.6% and 21.0% after tocilizumab treatment in two hepatocyte donors. This effect was statistically significant compared to controls but fell just under the 90% CI for the reported clinical tocilizumab effect. CYP3A4 activity showed a 70-90% increase across donors after tocilizumab addition to IL-6 inflamed hepatocytes. This assay uses a substrate at its CYP3A4 enzyme K_m , whereas for SVA a concentration of 2.1 μM was used, well below its K_m of 50-80 μM (Prueksaritanont, et al. 2002), indicating a relatively low enzyme activity rate. This SVA concentration was chosen based on physiological relevance balanced with LC-MS/MS assay range of detection, which may help to explain why the effect size for SVA PK after tocilizumab was not larger. The biological activity of tocilizumab on IL-6 signaling in hepatocytes was confirmed using CRP, which increased under inflammatory conditions and abruptly decreased after tocilizumab addition, mimicking clinical findings (Nishimoto, et al. 2008).

In studies with primary cells, donor variability is always a concern. Cryopreserved human hepatocytes are advantageous because they provide inter-experimental consistency. Cytochrome P450 metabolic enzyme activity is highly donor-dependent, with a 30-40 fold observed variation in CYP3A activity (Tracy, et al. 2015). Attempting to capture the whole population spectrum of human hepatocytes *in vitro* would likely prove challenging and costly. While it is possible to use hepatocyte donor cocktails for short-term cultures in an attempt to represent a population average, these pooled hepatocytes are not necessarily amenable to long-term culture because of

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difficulties controlling cell numbers over time. As an alternative, physiologically-based pharmacokinetic (PBPK) models have been used for simulation of IL-6 mediated CYP phenomena (Machavaram, et al. 2013, Xu, et al. 2015), but notably these models often rely on parameters generated from *in vitro* datasets.

To our knowledge, the studies presented here have been the first to directly demonstrate the clinically observed tocilizumab-SVA interaction in an *in vitro* liver culture model, which was made possible due to the extended culture times allowed by a 3D perfusion bioreactor platform. This proof of concept study illustrates the power of next-generation cell culture technologies for the prediction of clinically relevant drug-drug interactions. Advanced *in vitro* systems in combination with *in silico* modeling approaches continue to drive improvements in the early drug development process. As these *in vitro* models increase in complexity and approach greater physiological relevance, their application should continue to expand from drug PK and toxicity evaluations to pharmacodynamic and human disease models (Ananthanarayanan, et al. 2014, Verneti, et al. 2015, Wheeler, et al. 2014).

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Authorship Contributions

Participated in research design: Long, Dunn, Hamadeh, Afshari, McBride, and Griffith

Conducted experiments: Long and Cosgrove

Contributed new reagents or analytic tools: Cosgrove and Dunn

Performed data analysis: Long, Cosgrove and Stolz

Wrote or contributed to the writing of the manuscript: Long, Cosgrove, Dunn, Stolz, Hamadeh, Afshari, McBride, Griffith

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Footnotes

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Figure Legends

Figure 1 The LiverChip™ platform. (A) A cell culture plate (yellow) is attached to a pneumatic plate (gray) forming twelve fluidically isolated bioreactors per platform. (B) A collagen-coated polystyrene scaffold containing micro-channels is placed into each bioreactor for cell culture. Scaffold diameter is 1 cm. (C) Bioreactor cross-section schematic showing a thin membrane separating the lower pneumatic plate and the upper cell culture plate that houses the scaffold. Media is pumped through the scaffold and recirculated along a surface channel.

Figure 2 Representative low magnification (A) and high magnification (B) confocal reconstructions of hepatocyte micro-tissue morphology in a LiverChip™ scaffold after seven days of culture stained for f-actin (green) and Hoechst (blue). Scale bars = 100 μm

Figure 3 (A) Albumin production by hepatocytes in the LiverChip™ over two weeks of culture. (B) Basal IL-6 profile for hepatocyte-Kupffer cell co-cultures in the Liverchip™. Data points represent mean +/- SD for n = 3 technical replicates for hepatocyte donor HU8160.

Figure 4 Dose responses of IL-6 on CYP3A4 activity (A), C-reactive protein (CRP) secretion (B), and soluble IL-6 receptor (sIL-6R) shedding (C). Data points represent mean +/- SD for n = 3 technical replicates for hepatocyte donor HU8160.

Figure 5 Tocilizumab-simvastatin interaction study design. A two week experiment with six samples groups +/- in triplicate. This experiment was repeated with three hepatocyte lots. Two

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dosing periods were used in this study to measure the effects of different treatments in succession on simvastatin hydroxy acid (SVA) pharmacokinetics and CYP3A4 activity.

Treatments in the first dosing period from days 5-9 were either +/- 1 ng/mL IL-6. Treatments in the second dosing period from days 9-14 included IL-6 and 1.6 μ M tocilizumab either separately or dosed together. 10x C_{max} simvastatin hydroxy acid (SVA) was co-dosed with other treatments on days 7 and 12, with repeated media sampling taking place over the subsequent 48 hours for pharmacokinetic measurements. CYP3A4 activity assays were run after sampling on days 9 and 14.

Figure 6 Ratio of CYP3A4 activities between day 14 and day 9 per sample group of tocilizumab/simvastatin interaction study. Columns represent mean +/- SD for n = 3 technical replicates per group for hepatocyte donor HU8160. (* p<0.05 compared to IL-6/IL-6 treatment)

Figure 7 Fold changes in CRP secretion over time. Data points represent mean +/- SD for n = 3 technical replicates per group for hepatocyte donor HU8160.

Figure 8 Simvastatin hydroxy acid (SVA) concentration profiles in the LiverChipTM after treatment on culture day 7 (A) and culture day 12 (B). Data points represent mean +/- SD for n = 3 technical replicates per group for hepatocyte donor HU8160.

Figure 9 Tocilizumab effect on simvastatin hydroxy acid (SVA) half-life (A) and AUC_{last} (B). Columns represent mean +/- SD for n = 3 technical replicates per group for hepatocyte donor HU8160. (*p < 0.05 compared to IL-6/IL-6 treatment)

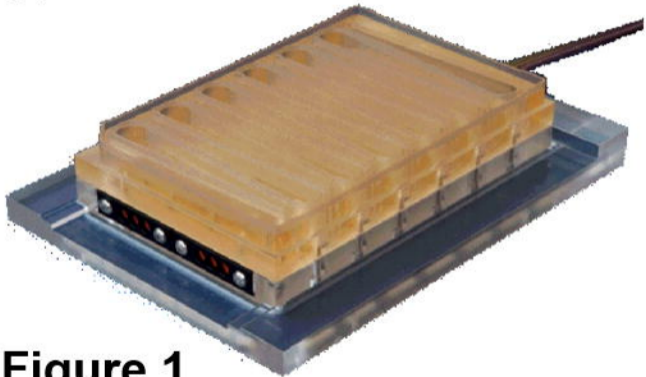
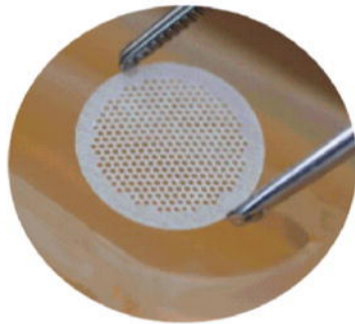
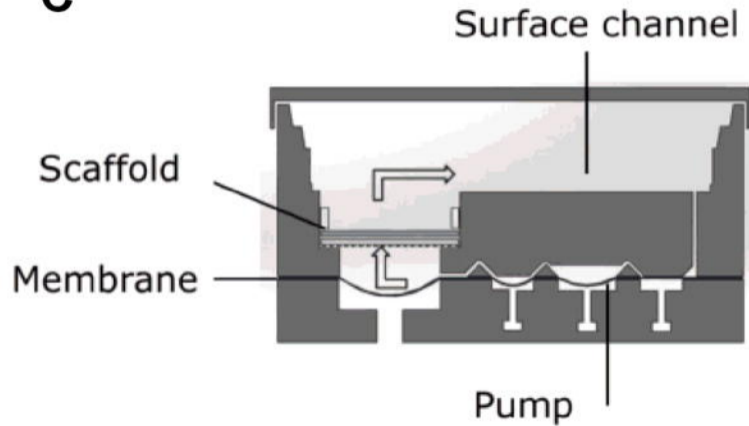
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Figure 2

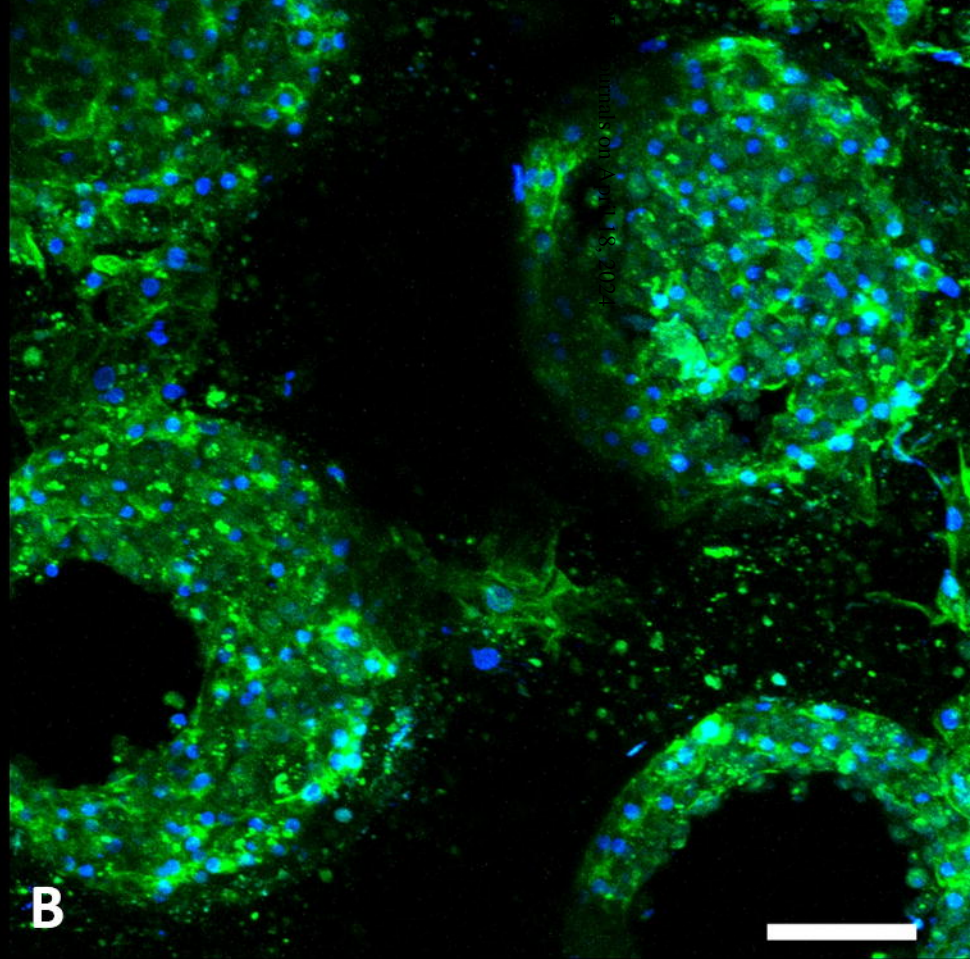
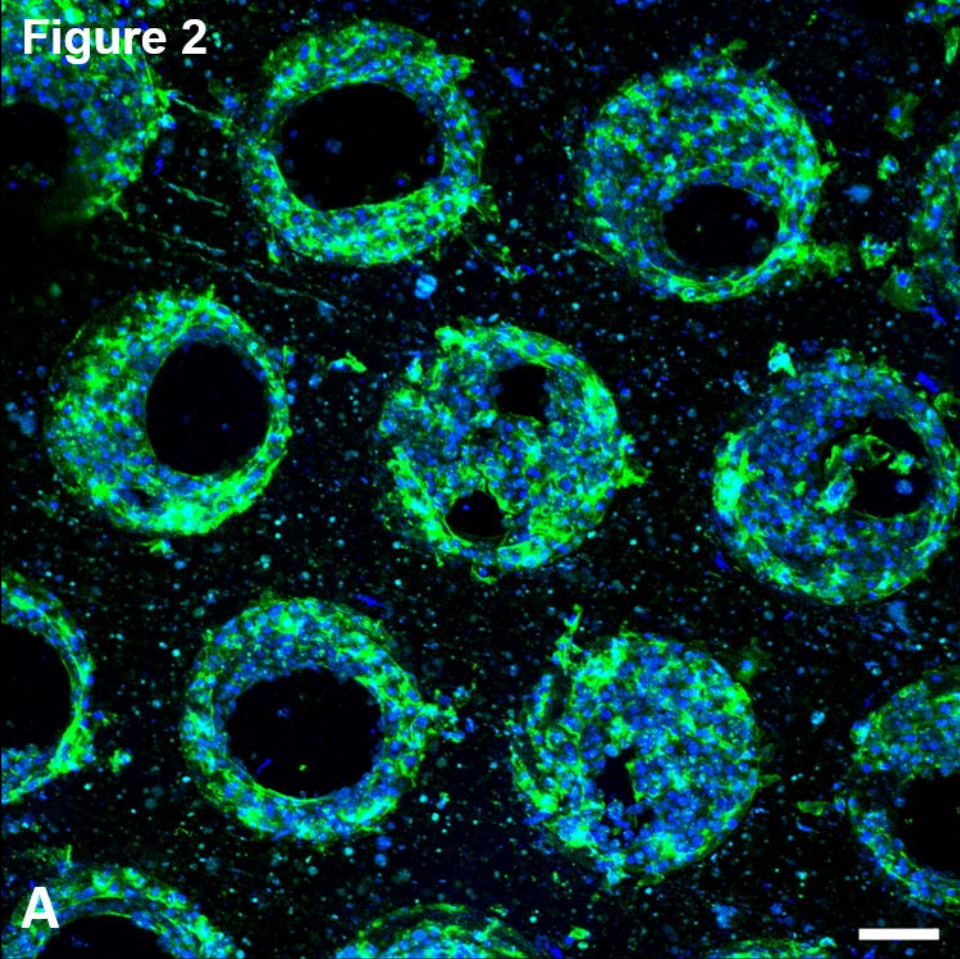


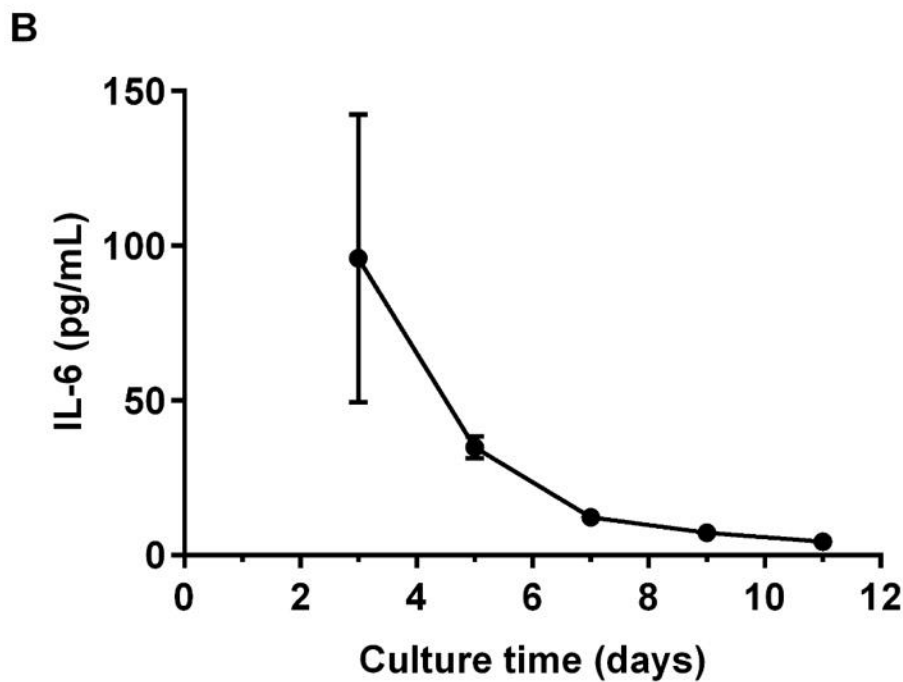
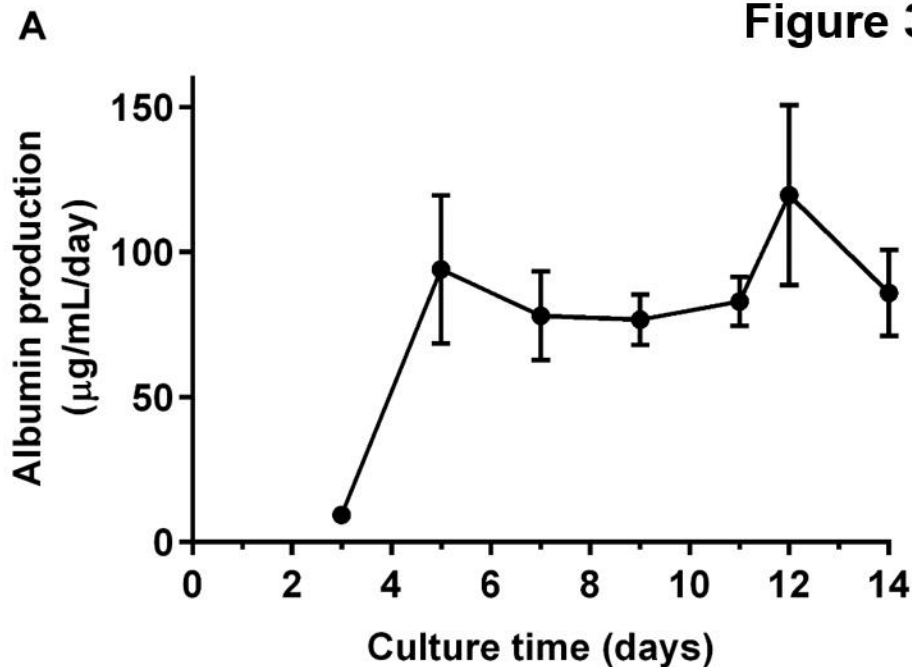
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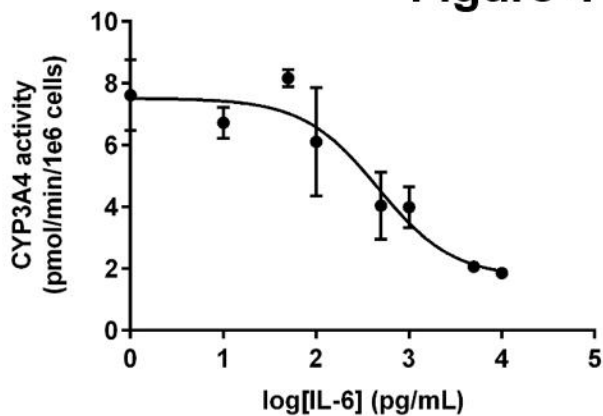
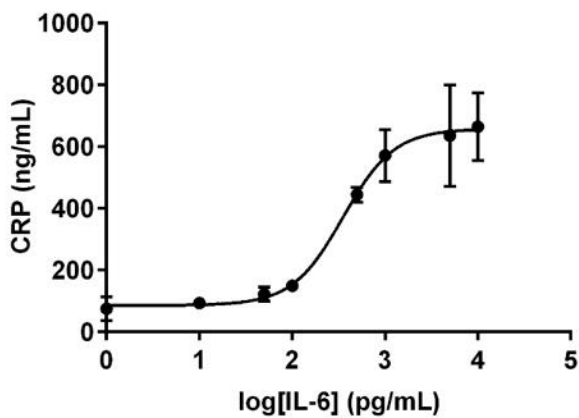
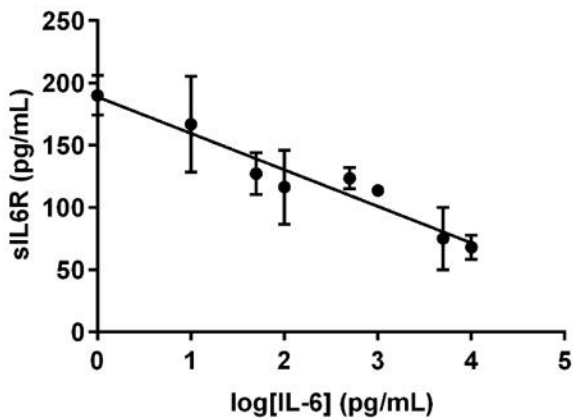
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Figure 5

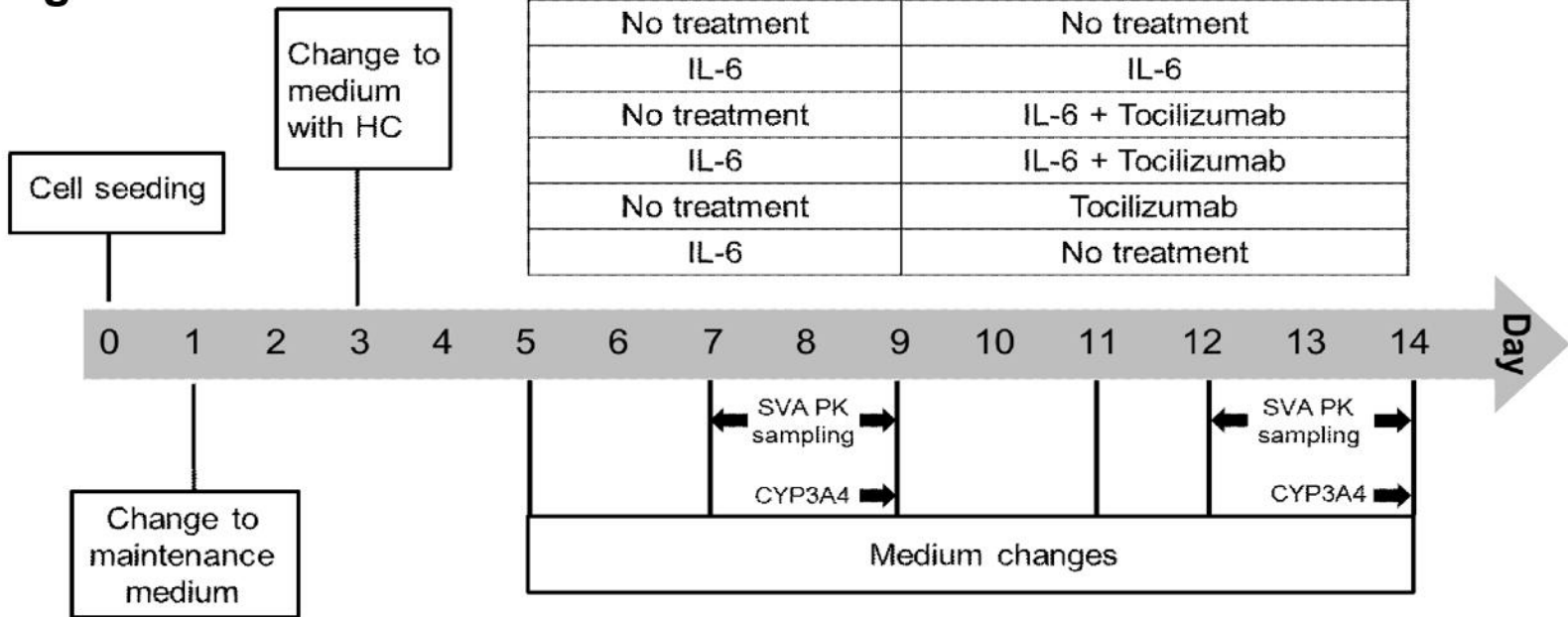


Figure 6

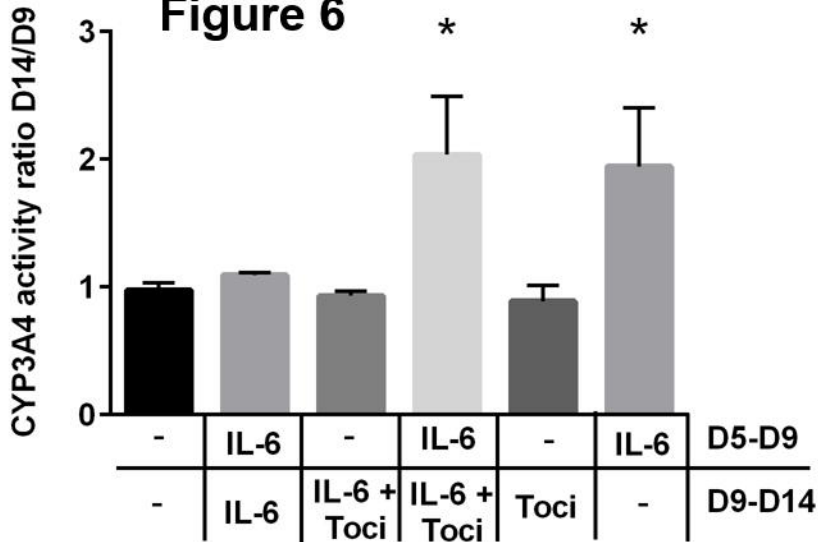
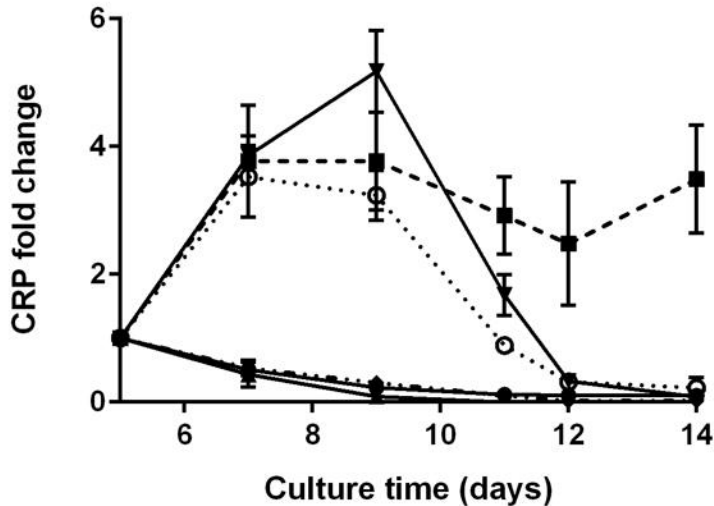


Figure 7



	D5-9	D9-14
●	-	-
■	IL-6	IL-6
▲	-	IL-6 + Toci
▼	IL-6	IL-6 + Toci
◆	-	Toci
⊙	IL-6	-

Figure 8

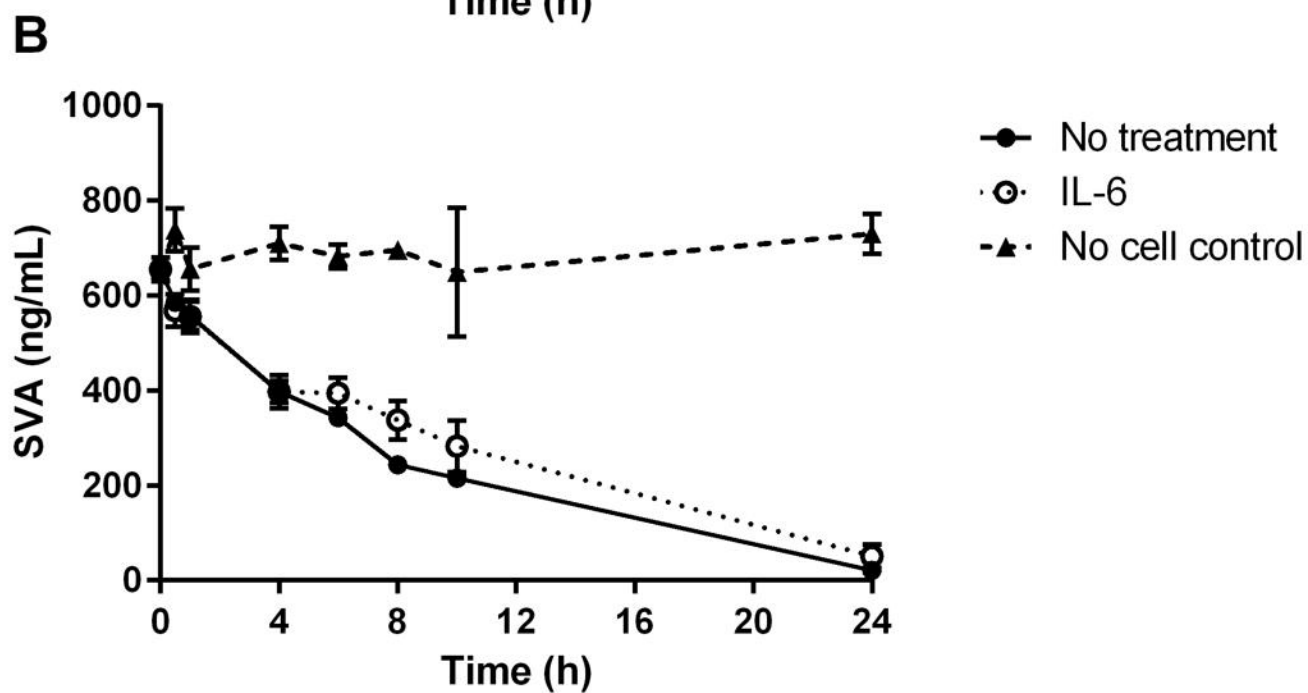
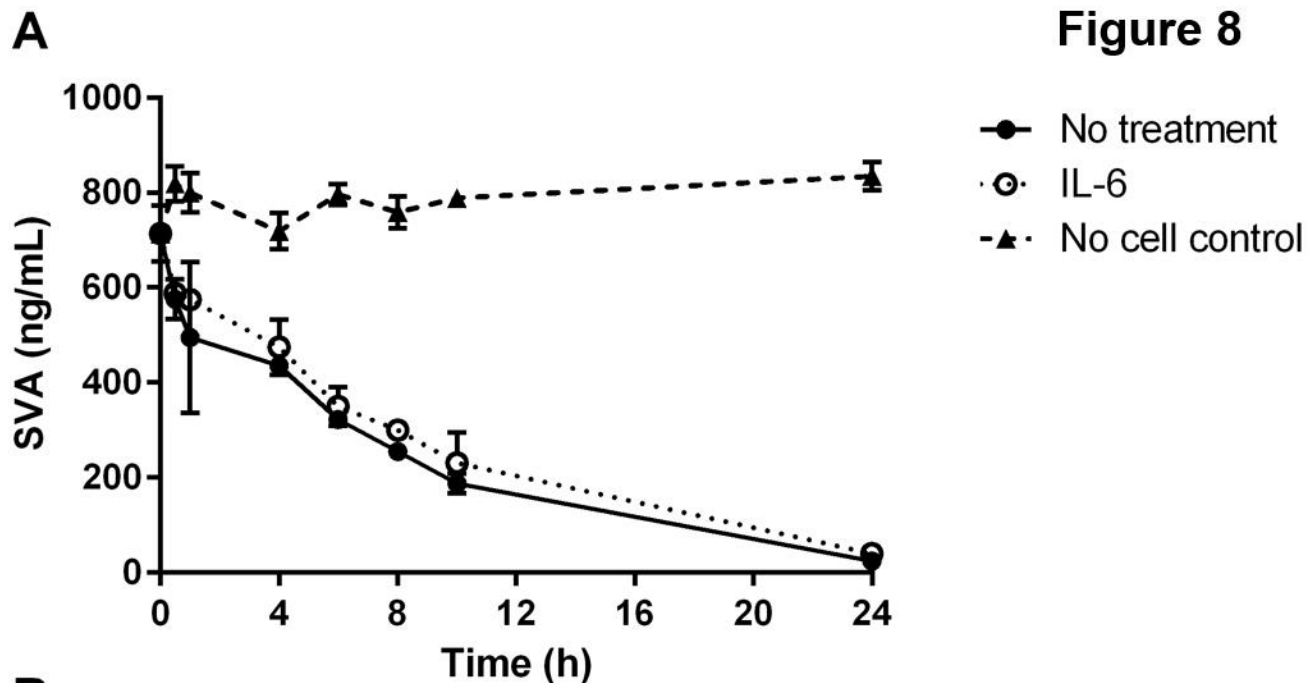
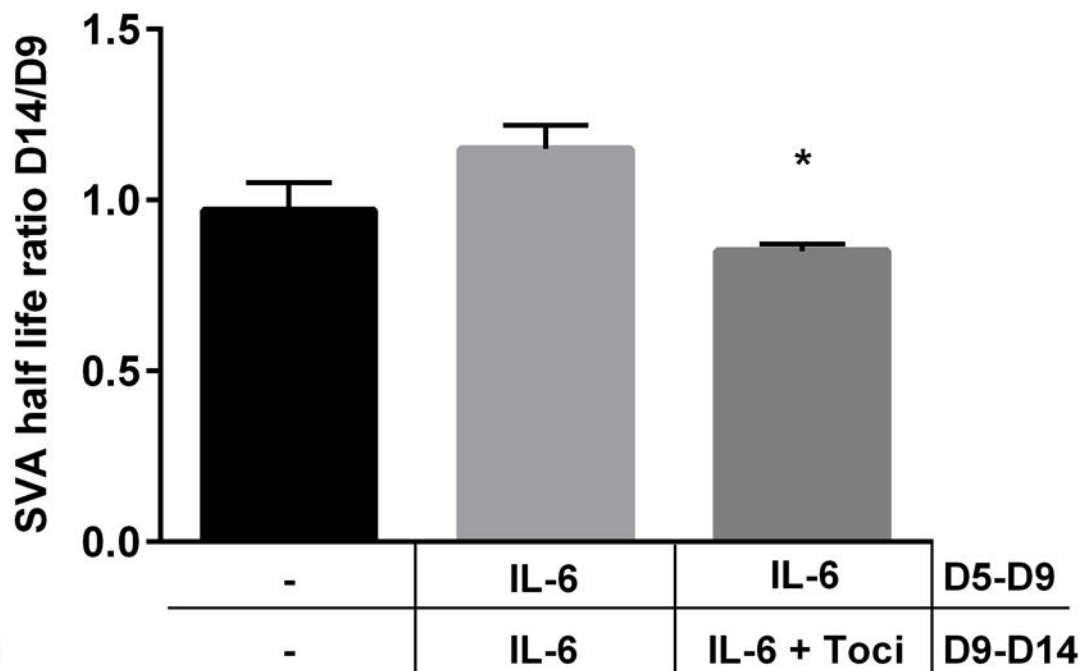


Figure 9

A



B

