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Silencing porcine CMAH and GGTA1 genes significantly reduces xenogeneic consumption of human platelets by porcine livers

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Abstract

Background—A profound thrombocytopenia limits hepatic xenotransplantation in the pig-to-primate model. Porcine livers also have shown the ability to phagocytose human platelets in the absence of immune-mediate injury. Recently, inactivation of the porcine ASGR1 gene has been shown to decrease this phenomenon. Inactivating GGTA1 and CMAH genes has reduced the antibody-mediated barrier to xenotransplantation; herein we describe the effect that these modifications have on xenogeneic consumption of human platelets in the absence of immune-mediated graft injury.

Methods—WT, ASGR1$^{-/-}$, GGTA1$^{-/-}$, and GGTA1$^{-/-}$CMAH$^{-/-}$ knockout pigs were compared for their xenogeneic hepatic consumption of human platelets. An in vitro assay was established to measure the association of human platelets with liver sinusoidal endothelial cells (LSECs) by immunohistochemistry. Perfusion models were used to measure human platelet uptake in livers from WT, ASGR1$^{-/-}$, GGTA1$^{-/-}$, and GGTA1$^{-/-}$ CMAH$^{-/-}$ pigs.

Results—GGTA1$^{-/-}$, CMAH$^{-/-}$ LSECs exhibited reduced levels of human platelet binding in vitro, when compared to GGTA1$^{-/-}$ and WT LSECs. In a continuous perfusion model, GGTA1$^{-/-}$ CMAH$^{-/-}$ livers consumed fewer human platelets than GGTA1$^{-/-}$ and WT livers. GGTA1$^{-/-}$ CMAH$^{-/-}$ livers also consumed fewer human platelets than ASGR1$^{-/-}$ livers in a single pass model.

Conclusions—Silencing the porcine carbohydrate genes necessary to avoid antibody-mediated rejection in a pig-to-human model also reduces the xenogeneic consumption of human platelets by the porcine liver. The combination of these genetic modifications may be an effective strategy to limit the thrombocytopenia associated with pig-to-human hepatic xenotransplantation.

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Author Contributions:

This manuscript has been revised and approved by all authors. JRB, LLP, MT and AJT drafted this article and developed study concepts. PL and JLE performed gene manipulations, pig cloning and genotyping. JRB, RLB, LLP, GRM and JML performed data collection and data analysis.

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Introduction

The most pressing issue affecting patients awaiting transplant is a growing dearth of available allografts. While peak transplanted volumes for all solid organs has plateaued, each year the number of patients added to solid-organ wait lists increases.[1] As recently stated by Salomon et al., from the AST/ASTS workshop on increasing organ donation: “our current system of organ donation is not meeting the growing demand”. [2] Xenotransplantation of genetically modified porcine organs is now positioned to be a clinically-viable answer to this growing problem. Advances in genetic engineering have rapidly increased the pace at which model organs may be created and tested. [3] Recently, a triple knockout model has reduced human antibody binding below the threshold at which acute antibody-mediated rejection (AMR) would not be expected. [4] It is from this position of immunologic equivalence, that it is now important to examine organ-specific barriers to clinical xenotransplantation.

The use of porcine livers for xenotransplantation is limited by a dramatic thrombocytopenia when studied in a pig-to-primate model. [5] As described by Burlak et al., [6] porcine sinusoidal endothelial cells (LSEC) and Kupffer cells (KC) also remove *human* platelets from circulation in the absence of immunologic injury or sheer-stress activation. Recently, gene silencing has been used to limit this thrombocytopenia. As described by Paris et al., [7] removing the asialoglycoprotein receptor 1 protein from the porcine livers significantly reduces the amount of human platelet uptake. Although carbohydrate reductions have proved essential to limiting AMR in a pig-to-human model, little attention has been afforded to the effect that these modifications may have on hepatic platelet consumption. Silencing of the GGTA1 gene, which facilitates cell surface expression of the Galα(1,3)Gal (αGal) xenoantigen, and the CMAH gene which allows expression of the N-Glycolylneuraminic acid (Neu5Gc) xenoantigen may help avoid early xenogeneic AMR;[8] it is therefore important to understand the effect that these modifications have on the human thromboregulatory system. Herein we describe the effect that silencing the porcine GGTA1 and CMAH genes has on the consumption of human platelets by a porcine liver.

Methods

Genetically modified pigs

The GGTA1$^{-/-}$, GGTA1$^{-/-}$CMAH$^{-/-}$ and ASGR1$^{-/-}$ pigs used in this study have been described previously.[7,9] Briefly, the same parental background was used across all animals to limit variability from external loci. GGTA1 and CMAH gene silencing was accomplished by CRISPR/Cas9-directed mutagenesis as described by Li et al. [9] ASGR1 silencing was effected by TALEN- directed mutagenesis as described by Paris et al. [710] The Institutional Biosafety and Institutional Animal Care and Use Committee at Indiana University School of Medicine approved the use of animals in this research.

Platelet Isolation and Staining

One unit of expired human platelets was purchased from a local blood bank. The platelets were centrifuged (5 minutes at 5,000×g). The pellet was resuspended in PBS/ACD 50:1 and

washed twice. 2×10^{11} platelets were obtained by count on hemocytometer, of which 25 percent were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Grand Island, NY) as described. [6] Both labeled and unlabeled platelets were added to a total of 1L modified Krebs solution 2.0g/L D-glucose, 0.141 g/L $MgSO_4$, 0.16 g/L KH_2PO_4, 0.35 g/L KCl, 6.9 g/L NaCl, 2.10 g/L $NaHCO_3$, 0.37g/L $CaCl_2$, 2.38 g/L HEPES, 1U/mL Heparin (Sagent Pharmaceuticals, Schaumburg, IL) pH 7.2-7.4 and then perfused through a Capiox BT05 bubble trap (Terumo Medical, Tokyo, Japan) to remove aggregated platelets prior to perfusion. Platelets were again counted to account for platelet loss and resuspended at a concentration of 1×10^{10}/L in modified Krebs solution. A total of 10L of platelet suspension was prepared and was warmed to 37 °C until perfusion.

Perfusion of Livers

Livers were procured from genetically modified animals and flushed at time of procurement with HTK solution (Essential Pharmaceuticals, Ewing, NJ, USA). Organs were removed from cold preservation within 2 hours and cannulated to achieve definitive control of the portal vein, hepatic artery and suprahepatic inferior vena cava and were warmed to a controlled temperature range of 35-37 °C by perfusion with warmed Kreb's solution. Livers were perfused in an oxygenated and pulsatile circuit as previously described. [7] For the single-pass model, sampling occurred simultaneously from the hepatic artery, portal vein and suprahepatic inferior vena cava; samples were collected every 30 seconds for 10 minutes. A sample from the vena cava port was collected at time 0 to calculate a background fluorescence which was subtracted from all samples. For the continuous-pass model, a time zero sample of platelets was obtained prior to the introduction of platelets, and samples were taken from a central reservoir at reported time points and immediately fixed 1:1 in 10 percent formalin solution prior to counting.

Measuring Xenogeneic Human Platelet Consumption

For the continuous-pass model, each of the time point samples were counted using a hemocytometer and total available platelets in the perfusion system were calculated. These values were compared to input platelet amounts and percent remaining available platelets were calculated. Results were subjected to analysis by ANOVA with repeated measures and Tukey's post test to determine significance. Samples obtained from single-pass hepatic perfusions were analyzed by measuring the fluorescence (Ex: 485, Em: 525) of 200uL perfusate loaded into 96 well micro clear plates (Greiner Bio One, Monroe, NC, USA) on a Spectramax M2 plate reader (Molecular Devices Corp., Sunnyvale, CA, USA). Percent maximum fluorescence was calculated by comparing individual time points to input fluorescence. Percent maximum values were analyzed on Prism software (Graphpad Software Inc., La Jolla, CA). Inclusion criteria for statistical analysis required that arterial and portal vein fluorescence reached at least 90% of input fluorescence to suggest adequate circulation of labeled platelets through the system. Comparison of platelet quantities in the perfusate were made once the platelet levels had plateaued indicating that the system had reached a steady state. Statistical significance was determined by unpaired t-test of percent fluorescence values obtained after inclusion criteria were met.

Staining and Confocal Microscopy

For histologic analysis by in vitro assay, primary WT, GGTA1$^{-/-}$, ASGR1$^{-/-}$ and CMAH$^{-/-}$GGTA1$^{-/-}$ LSEC lines were isolated as previously described. [6] Cells were cultured for 4 days post isolation at 37 °C and 5% CO_2 in clear-bottomed cell culture-treated microscopy slide wells (100, 000 cells/well initial concentration). Fresh human platelets were isolated from a healthy donor and labeled with carboxyfluorescein succinimidyl ester (CFSE) as described above. Four million CFSE-labeled platelets were added to experimental wells containing primary endothelial cells and media. Slides were incubated with platelets for 60 minutes. Duplicate wells for each group were washed with 150 μL of PBS three times then fixed with 50 μL of 4% paraformaldehyde–PBS solution for 20 min. To facilitate immunohistochemical visualization, and confirmation of platelet deposition within ex vivo platelet-perfused livers, punch biopsies were obtained at the conclusion of perfusions, sectioned at 4um and then fixed with 50 μL of 4% paraformaldehyde–PBS solution for 20 min. Cells and tissues were stained with goat anti-Pig CD31 1:100 (R and D systems, Minneapolis, MN) to visualize the endothelial surface, followed by three PBS washes and bovine anti-goat IgG DyLight 649 (Jackson Immuno Research Laboratories Inc., West Grove, PA). Slides were incubated with secondary antibody for approximately 1 h and then washed three times with PBS. DAPI (Invitrogen, Grand Island, NY) was added to all slides for 1 min as a nuclear stain followed by two PBS washes. Slides were mounted in ProLong Gold (Invitrogen, Grand Island, NY). Confocal microscopy was performed using an Olympus FV1000 (Olympus America Inc., Center Valley, PA, USA).

Results

Analysis of In Vitro Human Platelet Uptake

CFSE-labeled human platelets associate less with isolated porcine liver endothelial cells from the GGTA1$^{-/-}$CMAH$^{-/-}$ background compared to GGTA1$^{-/-}$ and WT cells. The ratio of human platelets (green) bound by liver endothelial cells (red) was measured by colocalization of platelet per CD31+ cell for all 16 fields viewed per cohort. The median LSECs per field was 58 and was not significantly different across cohorts (p=0.32). When subjected to statistical analysis by ANOVA with Tukey's multiple comparison test, GGTA1$^{-/-}$CMAH$^{-/-}$ LSECs bound significantly fewer human platelets when compared to to GGTA1$^{-/-}$ (0.13 vs 0.34 platelets per LSEC p=0.002, n=16) and WT (0.13 vs 0.37 platelets per LSEC p=0.002 n=16) cells; ASGR1$^{-/-}$ LSECs exhibited significantly less platelet binding than GGTA1$^{-/-}$ (0.17 vs 0.34 platelets per LSEC p=0.013, n=16) and WT (0.17 vs 0.37 platelets per LSEC p=0.009, n=16) cells. Although GGTA1$^{-/-}$CMAH$^{-/-}$ LSECs appeared to associated fewer human platelets than ASGR1$^{-/-}$ LSECs, the difference failed to reach statistical significance (0.13 vs 0.17 platelets per LSEC p= 0.8, n=16). Cells from GGTA1$^{-/-}$ and wild type livers exhibited similar levels of platelet retention (0.34 vs 0.37 platelets per LSEC p=0.99, n=16). The images in Figure 1 are representative of all images captured (n= 16 for each cohort).

Analysis of Ex Vivo Perfusion with Human Platelets

Because of the reduced interaction of human platelets with GGTA1$^{-/-}$CMAH$^{-/-}$ porcine liver sinusoidal endothelial cells, perfusion studies were performed to determine if the

removal of human platelets from a perfusion circuit was also minimized. In a continuous-pass model of platelet uptake, GGTA1$^{-/-}$CMAH$^{-/-}$ livers consumed fewer platelets than GGTA1$^{-/-}$ ($p<0.0001$) and WT ($p<0.0001$) livers at every time point (Figure 2). We have previously demonstrated that eliminating the asialoglycoprotein receptor from pig livers reduced human platelet consumption when compared to WT; [7] when compared within this analysis, these findings were confirmed ($p<0.0001$). Although a trend towards lower platelet uptake was noticed when comparing GGTA1$^{-/-}$CMAH$^{-/-}$ to ASGR1$^{-/-}$ livers, the difference failed to reach statistical significance at two of the time points within this model ($p<0.161$). Representative immunochemical histology obtained post continuous pass perfusion is shown in Figure 2B and confirms that the loss of platelets through the system is a function of hepatic deposition.

To better characterize the difference between GGTA1$^{-/-}$CMAH$^{-/-}$ livers and ASGR1$^{-/-}$ livers, we sought to increase the sensitivity of our perfusion model. Though continuous pass circuits are sufficient for demonstrating large differences in platelet uptake, it was insufficient to reliably measure more subtle differences. Consequently, we developed a "single pass" perfusion circuit to examine the loss of platelets following transit through the organ a single time (SDC Figure 1). Serial samples were collected until the input (artery and portal vein,) and the output (vena cava) reached a plateau, indicating steady state had been reached (Figure 3). After plateau phase was achieved, data from time points 390, 420 and 450 seconds were averaged (Figure 3D); suprahepatic inferior vena cava fluorescence reached 95.4% (SEM=0.590) of initial sample fluorescence for GGTA1$^{-/-}$ CMAH$^{-/-}$ livers compared to 83.9% (SEM=0.513) in ASGR1$^{-/-}$ livers during steady-state. ($P= 0.001$, GGTA1$^{-/-}$ CMAH$^{-/-}$ n=3, ASGR1$^{-/-}$ n= 4)

Discussion

Xenotransplantation of genetically modified porcine organs offers an imminent answer to the growing shortage of available allografts for transplantation. With the recent success of new knockout models, the antibody barrier to clinical application has effectively been crossed. [4] Prior to human trials, it is important to understand the physiologic compatibility of these organs with the human thromboregulatory system. Pig-to-primate and pig-to-human models of hepatic xenotransplantation are challenged by early thrombocytopenia. Although activated human platelets will interact with all porcine endothelial cells, the porcine liver is uniquely capable of removing *unactivated* platelets from circulation. [6,11] Though the mechanism remains poorly understood, it appears that porcine asialoglycopritein receptor and Von Willebrand factor may contribute to this interaction. [12-15] Recent efforts to reduce xenogeneic thrombocytopenia in a pig-to-primate model have also exposed the importance of limiting immune-mediated graft injury. [16,17] Judicious immunosuppression has prevented thrombocytopenia following pig-to-primate xenotransplantation of the porcine heart and kidney. [18,19] Given this information, it is possible that a combination of immunosuppression and the reduced antigenicity of newer knockout porcine donors alone may facilitate success for renal or cardiac xenografts. Owed to the inherently phagocytic tendency of the liver, further modifications will be necessary to avoid thrombocytopenia with hepatic xenografts.

The porcine ASGR1 gene produces the asialoglycoprotein receptor, which is known to bind and clear human platelets. Paris et al. previously demonstrated that ASGR1$^{-/-}$ livers consume fewer human platelets than WT livers, making them a more appropriate comparison for the present study. [7] GGTA1 and CMAH genes produce carbohydrates on the porcine cell surface; humans and primates have inactivated these genes over the course of evolution and therefore carry pre-formed antibodies against the products. Silencing these carbohydrate genes results in a favorable crossmatch against human sera, and therefore will be necessary for clinical application of pig-to-human xenotransplantation. [8] Surprisingly, these same modifications that have reduced the xenoantigen barrier also reduce the consumption of human platelets by the porcine liver, even in the absence of immune-mediated injury. Elimination of the CMAH gene appears to provide the majority of protection from platelet consumption.

Though the relationship between carbohydrate profiles and hepatic platelet uptake is still being defined, the ability of carbohydrates to affect hepatic recognition and transport has been recognized for over 30 years. [20] More recently, hepatic lectins and surface carbohydrates have been implicated in the process of hepatic platelet consumption. [21-23] Although the specific role of Neu5Gc (CMAH gene product) remains unknown, its induced expression on human atherosclerotic plaques [24] and cancer cells [25] in vivo suggests a link between altered N-glycan structure and human platelet adhesion. It is plausible that the induced expression of Neu5Gc in human solid-organ malignancies is partially responsible for the tumor-induced prothrombotic state, and that Neu5Gc-mediated aggregation is responsible for the human platelet's supportive role in tumor metastasis. [26]

Humanizing the porcine carbohydrate profile significantly reduces xenogeneic consumption of human platelets by the porcine liver. Simultaneous silencing of the GGTA1 and CMAH genes outperformed both GGTA1$^{-/-}$ and ASGR1$^{-/-}$ livers with respect to reduction in human platelet uptake. It is likely that a combination of carbohydrate and receptor protein knockout strategies will limit the xenogeneic thrombocytopenia associated with pig-to-human hepatic transplantation and facilitate clinical application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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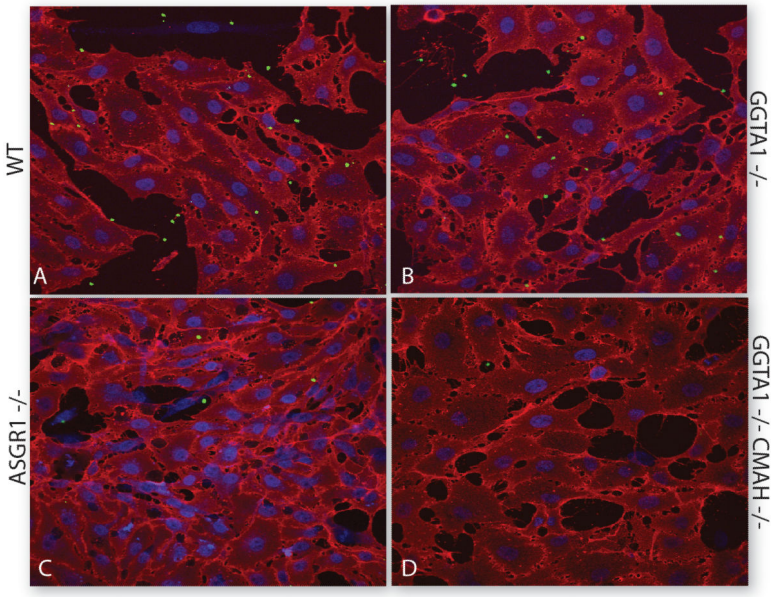

Figure 1. Human platelet accumulation by GGTA1$^{-/-}$CMAH$^{-/-}$, GGTA1$^{-/-}$, ASGR1$^{-/-}$, and WT liver sinusoidal endothelial cells in vitro

Primary WT, GGTA1$^{-/-}$, ASGR1$^{-/-}$ and CMAH$^{-/-}$GGTA1$^{-/-}$ LSEC lines were isolated and cultured for 4 days. Human platelets were isolated from a healthy donor and labeled with carboxyfluorescein succinimidyl ester (CFSE). Labeled Platelets (Green) were added to experimental wells containing primary endothelial cells. Cells were stained with anti-Pig CD31 (Red) to visualize the endothelial surface; and DAPI (Blue) was added to all slides as a nuclear stain. Confocal microscopy was performed using an Olympus FV1000. The relationships between human platelets and GGTA1$^{-/-}$CMAH$^{-/-}$ or GGTA1$^{-/-}$ livers seen in the ex vivo perfusion model was upheld by in vitro analysis. Based on colocalization analysis, CFSE-labeled human platelets associated less with porcine liver endothelial cells from the GGTA1$^{-/-}$CMAH$^{-/-}$ background compared to WT (p=0.002) and GGTA1$^{-/-}$ (p=0.002) cells; ASGR1$^{-/-}$ LSECs exhibited significantly less platelet binding than GGTA1$^{-/-}$ (p=0.013) and WT (p=0.009) cells. Although GGTA1$^{-/-}$ CMAH$^{-/-}$ LSECs appeared to associate fewer human platelets than ASGR1$^{-/-}$ LSECs, the difference failed to reach statistical significance (p= 0.8). Figure is representative of all images captured (n= 16 for each cohort).

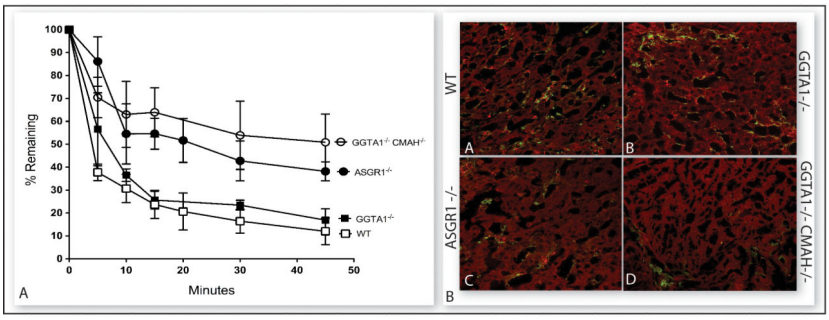

Figure 2. A Continuous-pass model for the xenogenic consumption of human platelets by modified porcine livers

(A) In a continuous-pass model of platelet uptake, both GGTA1$^{-/-}$ CMAH$^{-/-}$ and ASGR1$^{-/-}$ livers consumed fewer platelets than GGTA1$^{-/-}$ or WT livers ($p<0.0001$ for all comparisons). Platelet disappearance from the system was less at each measured time point after time zero. Although a trend towards lower platelet uptake was noticed when comparing GGTA1$^{-/-}$CMAH$^{-/-}$ to ASGR1$^{-/-}$ livers, the difference failed to reach statistical significance at two of the time points within this model ($p<0.161$). (GGTA1$^{-/-}$ n=2, GGTA1$^{-/-}$ CMAH$^{-/-}$ n=3, WT n=3 and ASGR1$^{-/-}$ n=3). The ASGR1$^{-/-}$ data was published previously and used here with permission. [7] (B) Representative immunochemical histology obtained from post continuous pass perfusion hepatic tissue sectioning is shown and confirms that the loss of platelets through the system is a function of hepatic deposition. Labeled Platelets (Green) were added to experimental wells containing primary endothelial cells. Cells were stained with anti-Pig CD31 (Red) to visualize the endothelial surface. Confocal microscopy was performed using an Olympus FV1000. Images are representative of all samples viewed (n= 6 for each perfusion).

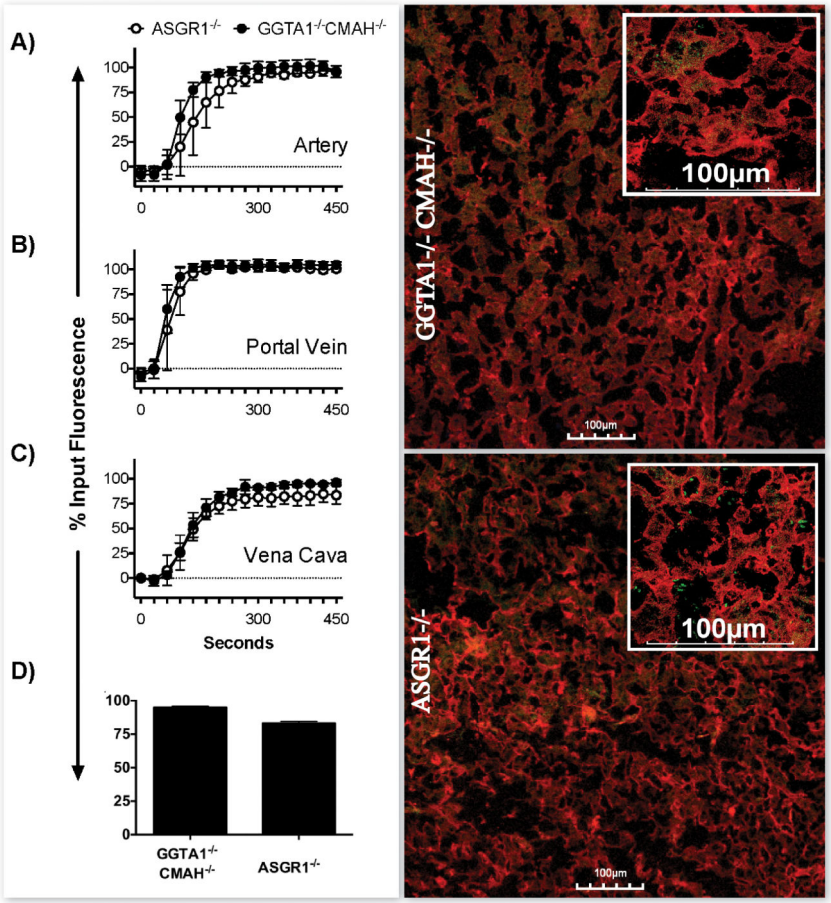

Figure 3. A single-pass model of human platelet uptake for GGTA1$^{-/-}$ CMAH$^{-/-}$ and ASGR1$^{-/-}$ livers

To further characterize a trends noted within a continuous pass model, livers of a GGTA1$^{-/-}$ CMAH$^{-/-}$ background were compared to ASGR1$^{-/-}$ livers in a more sensitive single pass model. Livers of a GGTA1$^{-/-}$ CMAH$^{-/-}$ background consumed significantly fewer platelets than ASGR1$^{-/-}$ livers in a single-pass model of human platelet uptake. After the addition of CFSE-labeled human platelets into the circuit diagramed in SDC Figure 1B, samples were obtained simultaneously from the hepatic artery (A), portal vein (B), and suprahepatic inferior vena cava (C), over time and measured for fluorescence. Each sample was measured against the pre-perfusion fluorescence measurement to achieve a percent input fluorescence value. (D) After plateau phase was achieved, data from time points 390, 420 and 450 seconds were averaged; suprahepatic inferior vena cava fluorescence reached 95.4% (SEM=0.590) of initial sample fluorescence for GGTA1$^{-/-}$ CMAH$^{-/-}$ livers compared to 83.9% (SEM=0.513) in ASGR1$^{-/-}$ livers during steady-state. (P= 0.001, GGTA1$^{-/-}$ CMAH$^{-/-}$ n=3, ASGR1$^{-/-}$ n= 4) Representative immunochemical histology obtained post single pass perfusion is shown at figure right and confirms that the loss of platelets through the system is a function of hepatic deposition.