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# Regeneration and Cell Recruitment in an Improved Heterotopic Auxiliary Partial Liver Transplantation (APLT) Model in the Rat

**Yoshihiro Ono, MD, PhD**[1,★], **Angelica Pérez-Gutiérrez, MD**[1,★], **Mladen I. Yovchev, PhD**[1], **Kentaro Matsubara, MD, PhD**[2], **Shinichiro Yokota, MD**[3], **Jorge Guzman-Lepe, MD**[1], **Kan Handa, MD, PhD**[1], **Alexandra Collin de l'Hortet, PhD**[1], **Angus W. Thomson, PhD, DSc**[3], **David A. Geller, MD**[3], **Hiroshi Yagi, MD, PhD**[2], **Michael Oertel, PhD**[1,4], and **Alejandro Soto-Gutierrez, MD, PhD**[1,3,4]

[1]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

[2]Department of Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

[3]Thomas E Starzl Transplantation Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

[4]McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA

## Abstract

**Background—**Auxiliary partial liver transplantation (APLT) in humans is a therapeutic modality used especially to treat liver failure in children or congenital metabolic disease. Animal models of APLT have helped to explore therapeutic options. Though many groups have suggested improvements, standardizing the surgical procedure has been challenging. Additionally, the question of whether graft livers are reconstituted by recipient-derived cells after transplantation has been controversial. The aim of this study was to improve experimental APLT in rats and to assess cell recruitment in the liver grafts.

Corresponding author: Alejandro Soto-Gutierrez, MD, PhD, Department of Pathology, University of Pittsburgh, 200 Lothrop Street, S-BST 423, Pittsburgh, PA 15261, USA, Phone: (412) 648-0064; Fax: (412) 648-1916, sotogutierreza@upmc.edu.

★Equal Contribution

**Author contributions**

All authors have met the following 4 criteria.

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work;
- Drafting the work or revising it critically for important intellectual content;
- Final approval of the version to be published;
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved;

As specific contributions, Y.O., K.M., H.Y. and A.S-G., designed the study; Y.O., S.Y., and K.M. performed the APLT surgical model; Y.O, A. P-G. and J. G-L performed immunohistochemistry and immunofluorescence staining; Y.O., K.H., A.P-G., J. G-L, A.C.I., K.M., H.Y., M.O., and A.S-G. analyzed and interpreted data; M.I.Y and M.O., breed and prepared F344 rats expressing green fluorescence protein; Y.O., A.P-G., S.Y., K.M., M.I.Y, D.A.G., A.W.T., H.Y., M.O., A.S-G. prepared the manuscript; A.S-G. obtained funding for these studies. All authors revised the manuscript.

**Disclosures**

There is no authors' conflict of interest to disclose as described by the ICMJE

**Methods—**To inhibit recipient liver regeneration and to promote graft regeneration, we treated recipients with retrorsine and added arterial anastomosis. Using green fluorescence protein transgenic rats as recipients, we examined liver resident cell recruitment within graft livers by immunofluorescence co-staining.

**Results—**In the improved APLT model, we achieved well-regenerated grafts that could maintain regeneration for at least 4 weeks. Regarding the cell recruitment, there was no evidence of recipient-derived hepatocyte, cholangiocyte or hepatic stellate cell recruitment into the graft. Macrophages/monocytes, however, were consistently recruited into the graft and increased over time, which might be related to inflammatory responses. Very few endothelial cells showed co-localization of markers.

**Conclusions—**We have successfully established an improved rat APLT model with arterial anastomosis as a standard technique. Using this model, we have characterized cell recruitment into the regenerating grafts.

## Introduction

Since the first report of auxiliary partial liver transplantation (APLT) in 1965[1], many groups have adopted this procedure. The results of APLT in humans during the initial 2 decades were discouraging[2]. In the 1990s, however, successful auxiliary partial orthotopic liver transplantation (APOLT) cases were documented in hepatic failure[3,4] and metabolic liver disease[5]. Subsequently, several case series were reported[6–10]. Many of these achieved favorable results leading to recommendation of APLT, especially in childhood hepatic failure, due to its beneficial long-term effect on life expectancy and the possibility of immunosuppressive drug withdrawal. Some reports, however, suggested that APOLT was associated with higher mortality and morbidity rates compared with orthotopic liver transplantation (OLT)[8,11]. While APOLT has exhibited more rejection than OLT[12,13], the exact mechanism underlying this phenomenon has yet to be elucidated. It has been postulated that resident macrophages (Kupffer) cells in native livers might play a role in promoting rejection in APLT[13]. Vascular and bile duct complications caused by technical problems or incorrect clinical indications have also been implicated in these relatively unfavorable results of APOLT[10]. As a result, APOLT has been performed by only a limited number of institutions[6,7,14,15].

Auxiliary partial liver transplantation (APLT) with portal vein (PV) and arterial anastomosis in rats was introduced by Lee and Edgington in 1968[16]. Hess et al[17] improved the model by adding bile duct reconstruction of the graft and ligation of the native bile duct resulting in long-term graft survival. Although this APLT model was first reported more than 4nine decades ago, due to technical difficulty and relatively poor outcomes, it has not been used commonly as an experimental model. Several groups have tried to improve the model. Thus Yoo et al[18] reported that PV reconstruction without hepatic arterial inflow could sustain the graft after APLT. Muller et al[19] explored the PV arterialization technique and Marni et al[20] proposed a cuff method to simplify the procedure and decrease operation time, achieving an improved survival rate. Overall, however, both technical difficulties and immunological challenges[12,13] have precluded general application of the model.

To overcome these obstacles, recently we developed a simple technique of APLT in the rat that recapitulates many physiological effects of human procedures (see Matsubara et al, 2015)[21] by incorporating standardized techniques for PV, bile duct reconstruction and the use of retrorsine (RS) (a pyrrolizidine alkaloid that blocks the hepatocyte cell cycle)[22] before transplantation to allow regenerative advantage to the donor liver. Thus, providing for the first time, a new tool to study compensatory regeneration in transplanted liver grafts[21].

With regard to cell repopulation in liver transplants, there are several reports that bone marrow (BM)-derived cells play an important role in renewal and repair during liver regeneration[23–27]. These studies suggest that hematopoietic stem cells are recruited to the graft and transdifferentiate into hepatic parenchymal or endothelial cells. However, these interpretations have been questioned following reports of spontaneous fusion of BM cells with other cell types[28,29]. Thus the role of BM cell recruitment or transdifferentiation in hepatic cell renewal remains controversial.

Establishment of an improved rat APLT model as a standard procedure would aid investigation of clinically relevant APLT and cell liver regeneration/immune cell recruitment. Here, based on our previously developed APLT model, we have improved the graft function and regeneration by incorporating arterial anastomosis and modifying bile duct reconstruction, and we characterized cell recruitment into the graft during regeneration.

## Materials and Methods

### Rat strains and care

Outbred Sprague-Dawley (SD) male rats were obtained from Charles River Laboratories and used for auxiliary liver transplantation as both donors and recipients. Nagase analbuminemic rats (NAR) of SD background were also used as recipients, Inbred Fisher (F)344 male rats (Charles River Laboratories) were used as donors and homozygous F344 rats expressing green fluorescence protein (GFP) under the ubiquitin C promoter (line 307; F344-Tg(EGFP) F455/Rrrc) obtained from the Rat Resource and Research Center (University of Missouri, Columbia, MO) used as recipients. For APLT, 200 – 350g rats were used as donors or recipients. The animals were maintained in accordance with the guidelines established by the Committee on Laboratory Resources, National Institutes of Health and experiments were conducted under an Institutional Animal Care and Use Committee - approved protocol.

### Experimental groups

Five groups were constituted (total, n=28): groups 1 and 2, SD recipients transplanted from SD donors (each group, n=8); groups 3 and 4, NAR recipients transplanted from SD donors (each group; n = 3). To evaluate cell recruitment into the liver graft, F344 GFP transgenic (tg) rats were used as recipients and F344 rats as donors (group 5; n = 6). Group 1 and 2 rats were euthanized at 2 and 4 weeks and group 3 and 4 rats at 4 weeks after APLT. Group 5 rats were euthanized at 1, 2 and 4 weeks postoperatively. Retrorsine (RS) (Sigma Chemical Co., St. Louis, MO) was administered to graft recipients in groups 1, 3 and 5 to inhibit the mitosis of host native hepatocytes, as previously described[21, 22]. Briefly, 2 intra-peritoneal injections of 30 mg/kg RS were administered to rats weighing 90 – 140 g, 2 weeks apart.

Four weeks after the second injection, APLT with 1/3 hepatectomy was performed in each rat.

## Donor surgery

Donor surgery was performed as described[21]. We improved the previous model by adding arterial anastomosis. The celiac artery (CeA) were skeletonized, the side branches (left gastric artery, splenic artery and gastroduodenal artery) were ligated and divided. An arterial stent made from 22 or 24-gauge catheter (BD) was inserted into the celiac artery from the proximal side and secured in place with 7-0 silk.

## Recipient surgery (see Supplementary Video online)

After laparotomy, right nephrectomy and IVC anastomosis (Figure 1A) was performed as described[21]. For arterial anastomosis (Figure 1B), the operating table was rotated 45° anti-clockwise. The graft CeA was anastomosed to the recipient right renal artery (RA) using a 22 or 24 G stent. Just after completion of arterial anastomosis, the IVC clamp and arterial clamp were removed and the graft re-perfused with arterial flow. At the same time, the proximal side of the PV was clamped to avoid backflow bleeding. The graft SMV was then anastomosed to the recipient PV/SMV confluence by end-to-side anastomosis (Figure 1C). After completing the PV anastomosis, the vascular clamp on the PV was released and PV flow provided to the graft. Following reperfusion, the PV between the pyloric vein and the PV anastomosis was ligated completely using 6-0 silk to increase inflow to the graft. After vascular anastomoses, a left lateral lobectomy (1/3 hepatectomy) of the native liver was performed.

The bile duct was reconstructed using choledocho-duodenostomy (Figure 1D). Two stitches were placed between the bile duct and the duodenal wall and the graft bile duct inserted into the recipient duodenum.

## Postoperative management

Following surgery, 1.0 mg/kg tacrolimus was injected intramuscularly each day until the end of the experiment[30,31].

## Assessment and definition of “graft weight ratio”

To evaluate graft regeneration, the following formulae were applied:

pre GRWR = preoperative graft weight/recipient body weight (at APLT surgery);

post GRWR = postoperative graft weight/recipient body weight (at euthanasia);

“graft weight ratio” = post GRWR/pre GRWR

## Serum albumin levels

Serum albumin levels were determined in serum samples obtained from NAR (group 3 and 4) 1, 3, 7, 14, 21 and 28 days after APLT by enzyme-linked immunosorbent assay (ELISA) (Bethyl Lab, Montgomery, TX).

### Immunohistochemistry

Epithelial cell adhesion molecule (EpCAM) expression was assessed in 5 μm sections, prepared from 4% paraformaldehyde-fixed, paraffin-embedded tissue. After Ag retrieval in citrate buffer, the samples were incubated with rabbit anti-EpCAM (Abcam ab71916) 1:500. For immunoperoxidase detection, the Vectastatin ABC kit Elite PK-6101 rabbit IgG and ImmPACT DAB peroxidase substrate kit SK-4105 were used.

### Immunofluorescence staining

For albumin and albumin/Ki67 co-staining, paraffin sections were incubated with primary Abs: sheep anti-albumin (Bethyl A110-134A) 1:100 and mouse anti-Ki67 (BD 550906) 1:50 at 4°C overnight. Alexa Fluor 488 anti-mouse IgG (Invitrogen) 1:250 and Jackson Cy3 anti-sheep 1:100 were used as secondary Abs. To assess proliferation, positive and negative Ki-67 hepatocytes were counted in 5 fields per section in each group at ×400 magnification and positive hepatocytes expressed as a percentage.

For GFP co-staining together with cell surface markers, sections were fixed in 2% paraformaldehyde, followed by 30% sucrose for 24 hr each, then snap frozen. Four μm sections were post-fixed in 4% paraformaldehyde, followed by incubation with primary Abs at 4°C overnight. The primary Abs and dilutions were: rabbit anti-GFP (Novus NB600-308), 1:1000; goat anti-GFP (Abcam ab5450), 1:1000; sheep anti-albumin (Bethyl A110-134A), 1:100; rabbit anti-EpCAM (Abcam ab71916), 1:100; rabbit anti-von Willebrand factor (vWF) (Abcam ab6994), 1:200; mouse anti-alpha smooth muscle actin (αSMA) (Abcam ab7817), 1:100; and mouse anti-CD68 (Serotec MCA341GA), 1:100. The secondary Abs were: Alexa Fluor 488 anti-rabbit/goat IgG (Invitrogen), 1:250; Alexa Fluor 594 anti-sheep IgG (Invitrogen), 1:250 and Jackson Cy3 anti-rabbit/mouse, 1:100. Sections were mounted using Vectashield mounting medium with 4,6-diamino-2-phenylindole (Vector) counterstaining of cell nuclei.

### Statistics

All statistical analyses were performed using SPSS® version 23.0 (IBM, Armonk, New York,) and the Mann-Whitney U test was used for analysis of continuous variables. $P \leq 0.05$ was considered statistically significant. Data are expressed as means ± SEM.

## Results

### Technologic refinements and surgical outcomes

After establishing the surgical model, 54 APLTs were performed. The recipient mortality rate was 25.9 % (n=14) and the morbidity rate was 30.0 % (n=12). The causes of mortality were graft congestion (42.7 %, n=6), bleeding (21.4 %, n=3), major bile leakage (14.3 %, n=2) and other causes (14.3 %, n=2). Causes of morbidity were bile obstruction (83.3 %, n=10) and localized bile leakage (16.7 %, n=2). Bile leakage and obstruction were the main complications of this procedure. The use of a stent for bile duct reconstruction produced a bile stone on the duodenal side of the stent in long-term survival cases; therefore the reconstruction method was modified (see bile duct reconstruction in the Materials and Methods). Arterial anastomosis was added to increase blood supply to the bile duct wall and

decreased the morbidity rate of the choledocho-duodenostomy. Successful cases (Figure 1E and F, n=28) were defined as having a “graft weight ratio” > 1.0 and histologically less bile duct proliferation compared with complicated cases. In our experience, PV anastomosis completed within 30 min minimizes intestinal congestion or PV thrombosis and reduces mortality.

### Histological assessment of graft livers after APLT

To assess the condition of the grafts, bile duct (EpCAM) and hepatocyte (albumin) marker expression was examined at 14 and 28 postoperative days. Graft livers showed high bile duct proliferation in complicated cases (n = 10). In successful cases (n = 28), although some bile duct proliferation was evident, the bile duct proportion in the parenchymal space was < 20 % (Figure 2A). There was no observable difference in bile duct proliferation between 2 and 4 weeks after surgery.

### Serum albumin levels in analbuminemic rats with APLT graft

In groups 3 (n= 3) and 4 (n= 3), NARs were used as recipients to allow assessment of graft function by serum albumin measurement. Recipient sera were collected 1, 3, 7, 14, 21 and 28 days after APLT and ELISA performed. Serum albumin levels increased continuously for 4 weeks and reached 2/3 of the normal control level (serum albumin level of SD rats). There is no detected difference between the groups 3 (RS+) and 4 (RS−) in this study (Figure 2B).

### Regenerative capacity of the APLT graft

Graft regenerative capacity was assessed in RS (groups 1; n= 8 and 3; n= 3) versus non-RS (groups 2; n= 8 and 4; n= 3) groups. Ki67 and albumin co-staining was performed on graft and native livers with RS treatment 2 weeks after APLT (Figure 3A). Ki67 analyses showed that the proportion of $Ki67^+$ cells in the native liver was constantly low 2 and 4 weeks after APLT in both the RS and non-RS group (Figure 3B, left panel). Compared to native livers, $Ki67^+$ cells in the grafts increased 2 weeks after APLT to almost the same level in both groups 1 (RS+; SD recipient) and 2 (RS−; SD recipient), indicating that the grafts in both groups were regenerating at 2 weeks. Four weeks after APLT, $Ki67^+$ cells in the grafts of group 3 (RS+; NAR recipient) were significantly higher than those in group 4 (RS−; NAR recipient) ($p = 0.009$), suggesting that RS treatment was necessary for prolonged regeneration in the graft (Figure 3B, right panel). One reason that native livers regenerated less in the non-RS group could be decreased portal flow to the native liver because of PV ligation (Figure 1C).

“Graft weight ratios” in the RS treatment groups were significantly higher than those in non-RS groups, especially at postoperative week 4 ($p = 0.05$), while non-RS group ratios decreased at 4 weeks after increasing at 2 weeks (Figure 3C). These results also indicate that RS treatment could prolong graft regeneration for at least 4 weeks. The total liver weight to body weight ratios were consistently in the range of 3.5 – 4.0 % for all groups.

### Migration of recipient-derived $GFP^+$ cells into the graft liver

To assess cell recruitment to the APLT, $GFP^-$ liver grafts were transplanted into RS-treated GFP tg rats. To evaluate cell recruitment from the host to graft livers, markers for

hepatocytes (Alb), cholangiocytes (EpCAM), endothelial cells (vWF), hepatic stellate cells (αSMA) and Kupffer cells/monocytes (CD68) were co-stained with GFP immunofluorescence. Since all recipient native liver cells were $GFP^+$, they co-localized with these different markers (Figure 4). In contrast, in the liver grafts, some $GFP^+$ cells were found, mainly in perivascular areas. We detected co-expression of GFP and CD68 at 1, 2 and 4 postoperative weeks (POW) (Figure 4 and 5); first with a perivascular distribution, then after 4 weeks, with a more homogenous distribution in the parenchyma. These co-expressing cells increased over time (Figure 6). At 4 weeks postsurgery, very few graft endothelial cells co-expressed GFP and vWF (Figures 4 and 5).

## Discussion

Several reports have addressed technical issues concerning heterotopic APLT in the rat. Thus, Yoo et al[18] suggested that PV reconstruction without arterial anastomosis could be sufficient to sustain partial liver transplants long-term. However, graft survival in their study was low (<50% by 30 postoperative days) and they also ligated the native bile duct; consequently the native liver shrank, similarly to the procedure of Hess et al with a survival rate of only 44.4%[17]. Others have reported portal arterialization[19] producing a survival rate at 14 days of 77.7%; however, in these studies, hepatocytes did not survive long-term with arterial blood flow alone[32,33]. Marni et al[20] also achieved a high survival rate of 80% at 2 weeks and 73.3% at 8 weeks by using a cuff technique for PV and IVC anastomosis. However, in their study arterial anastomosis was not performed, and the morbidity rate and histological assessment of the graft were not reported. The survival rate in the present study was 74% at 4 weeks.

The procedure in the present study was adopted from a model previously developed by Matsubara et al[21] and incorporates similar characteristics to clinical APLT and maintained healthy conditions in both the graft and native livers for at least 4 POW with a morbidity rate of 30%. When the native livers were treated with RS, the grafts increased their regeneration rates and compensated for native liver disability. Indeed, grafts in the RS-treated group maintained regenerative function for at least 4 weeks, which was longer than in the non-RS treated group.

In the current study, the impairment of native liver regeneration by RS treatment continued for 4 weeks. If native livers are healthy, both native and graft livers orchestrate a “hepatostat” balance, that ensures liver weight is controlled for the performance of its homeostatic functions[34]. In the present model, 1/3 of the donor liver was transplanted and 1/3 hepatectomy was performed on the recipient. As a result, total liver volume was maintained without massive hepatocyte loss. If the hepatostat was driven mainly by the hepatic mass, the liver would not regenerate, but even in the non-RS treatment group, the graft liver regenerated within 2 POW to the same level as in the RS-treated group (Figure 3).

Liver regeneration after hepatectomy usually completes 1 week after surgery[35,36], however, the grafts in our model maintained their regenerative capacity for at least 2 weeks. This may be because the graft received adequate PV blood flow essential for liver regeneration[37,38] compared with the native liver, that had reduced portal venous flow (only pyloric venous

flow remaining), and likely retained the stimulus for regeneration. Recent studies[39,40] suggest that portal pressure plays an important role in modulating liver regeneration. In the present study, portal flow was completely re-directed to the graft, thus, maintaining an adequate portal pressure for its regeneration. Future studies could be directed to elucidate the role of portal pressure on regulating graft regeneration in this model by modulating the extent of portal ligation.

On the other hand, although graft regeneration in the non-RS treated group did not continue for 4 weeks, serum albumin levels increased continuously during this period. Even if there were differences in graft size, albumin production was not significantly different between the non-RS and RS groups. This may be due to the fact that the liver grafts already had a large liver mass (nearly 40% of a whole liver) able to produce albumin and also since albumin has a long half-life (approximately 20 days) and could accumulate over time. However, additional APLTs will have to be performed in greater numbers on analbuminemic rats in the future to study significant differences in the capacity to produce serum albumin. Moreover, previously, other APLT models, with only portal arterialized flow, have been used for bioengineered liver grafts implantation[41,42]. The advantage of the present model is that it ensures both arterial and PV flow, mimicking physiologically the same conditions as in the native liver, thus, representing an adequate APLT model to test the function and regeneration of bioengineered livers.

Many investigators have attempted to elucidate the mechanistic basis of liver regeneration. In regard to cell recruitment into the liver, Wang et al[43,44] suggested that sinusoidal endothelial cells that transdifferentiated from BM cells could repopulate chronically-injured livers, whereas Okabayashi et al[45] reported that host BM stem cells could differentiate into hepatocytes under specific conditions. Hepatocytes are assumed to have the capacity to transdifferentiate into cholangiocytes[46,47], but whether and how BM cells can constitute hepatic cells remains controversial[48]. Here, we investigated cell origin/cell migration into the regenerating liver graft by performing immunofluorescence co-staining. Markers specific for hepatocytes, cholangiocytes, endothelial cells, stellate cells and Kupffer cells/monocytes were used to determine the origin of cells within the APLT. There was no evidence of hepatocyte, cholangiocyte or hepatic stellate cell migration into the grafts. $CD68^+$ cells (Kupffer cells/monocytes) however, were consistently recruited into the grafts and increased over time from a perivascular to a parenchymal sinusoid location.

Recent studies have revealed that Kupffer cells are derived from embryonic progenitors in the yolk sac and maintained by self-renewal *in situ*[49,50]. Thus, the recruitment of $CD68^+$ cells into the graft might be related to inflammatory responses of circulating blood monocytes. Very few cells exhibited co-expression of vWF and GFP only in the cytoplasm, which could be the result of endothelial cell phagocytosis. Our finding supports previously published evidence[29] that only inflammatory cells are detected in human liver grafts. However, the limitation of this study is that we have investigated only liver resident cells and have not evaluated the significance of liver stem cells, nor the role of other cells that were recruited into the graft. Also, this study has focused on early regeneration and not long-term regeneration. Further investigation should be conducted to elucidate the mechanistic basis of liver regeneration.

An important aspect affecting the outcome after APLT in the clinics is graft atrophy due to early and late acute graft rejection. Different contributor factors have been associated to especially late acute rejection, for instance, lowering immunosuppression[51], native liver affecting allograft tolerance[12,13,19], competition of metabolic function and/or portal circulation between the graft and the native liver[52]. However, the etiologies of this issue have not been clearly elucidated. Although in these particular experimental settings, we did not investigate the immunological rejection of the liver graft. The described APLT model could be used for future experiments to study graft rejection and the role of immune cells (eg T cells, NK cells, granulocytes, dendritic cells).

In summary, we have successfully established a rat APLT model with arterial anastomosis. Using this model, we have evaluated cell recruitment into the graft. Of the graft cells post-APLT, only macrophages/monocytes were recruited into the grafts and these findings support a self-renewal ability of the liver. This improved model may prove valuable in addressing questions related to liver regeneration, recruitment, immunology and transplantation of bioengineered livers.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **αSMA** | alpha smooth muscle actin |
| **APLT** | auxiliary partial liver transplantation |
| **BM** | bone marrow |
| **CeA** | celiac artery |
| **ELISA** | enzyme-linked immunosorbent assay |
| **EpCAM** | epithelial cell adhesion molecule |
| **GFP** | green fluorescence protein |
| **GRWR** | graft-recipient weight ratio |

| | |
|---|---|
| **IHIVC** | infra-hepatic inferior vena cava |
| **IVC** | inferior vena cava |
| **NAR** | Nagase analbuminemic rats |
| **OLT** | orthotopic liver transplantation |
| **PBS** | phosphate-buffered saline |
| **POW** | postoperative weeks |
| **PV** | portal vein |
| **RA** | renal artery |
| **RS** | retrorsine |
| **SD** | Sprague-Dawley |
| **SHIVC** | supra-hepatic inferior vena cava |
| **SMA** | superior mesenteric artery |
| **SMV** | superior mesenteric vein |
| **tg** | transgenic |
| **vWF** | von Willebrand factor |

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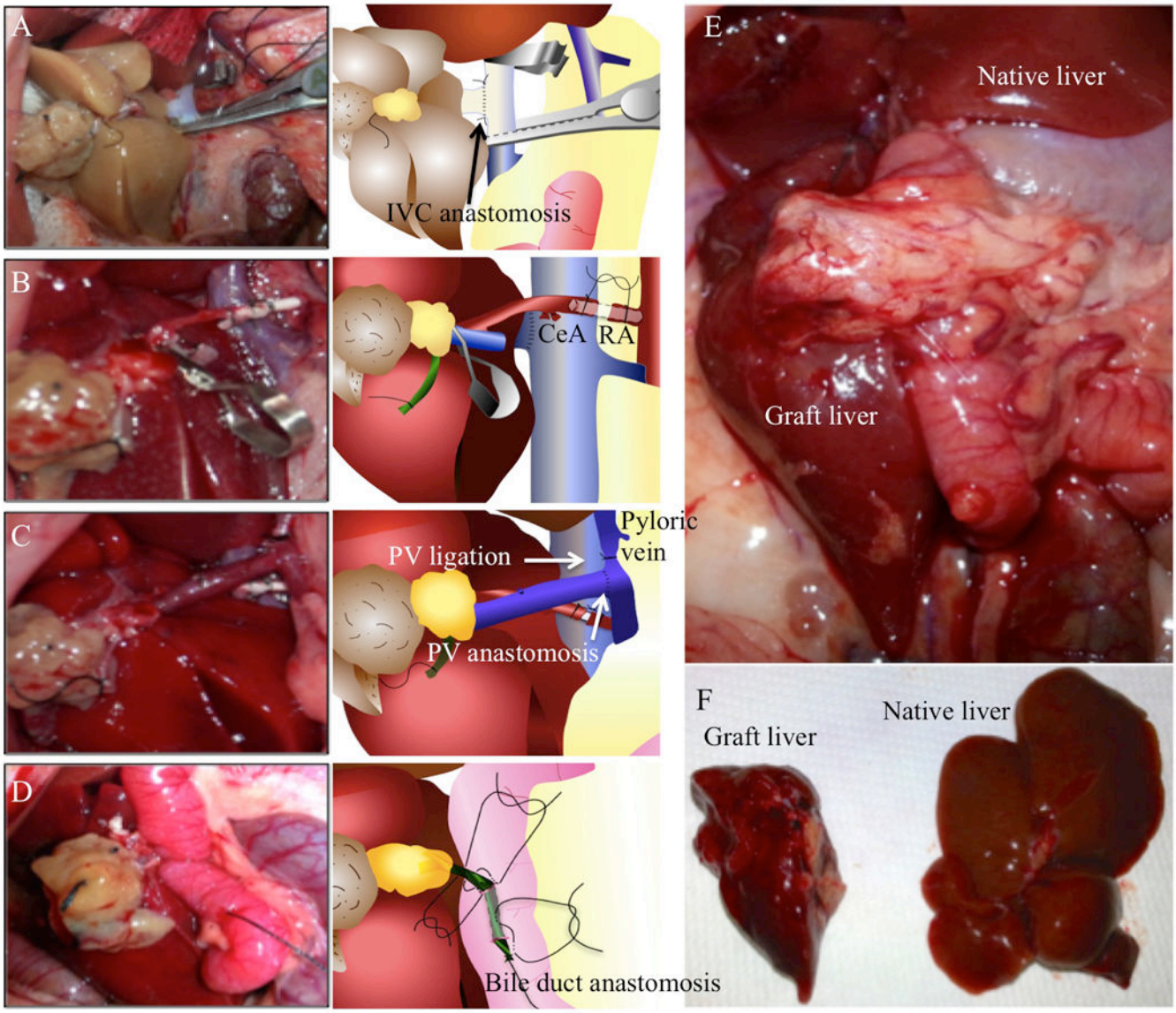


**Figure 1. Images and schematic drawings of each anastomosis during APLT in the rat and postoperative appearance of the graft and native liver**

(A) IVC anastomosis. After clamping the proximal and distal ends of the IHIVC, the anterior wall of the IHIVC was cut to make a 7 mm vertical incision, as close to the ligated right renal vein as possible, for the IVC anastomosis. A stay suture was placed with 10-0 nylon at the distal end before beginning the continuous suture. Prior to completion of the anastomosis, saline was injected slowly using a L-shaped injector into the IHIVC anastomosis to remove any air trapped inside. (B) Arterial anastomosis. The thread tied to the right RA was held and pulled to the left side of the recipient. The proximal side of the right RA was clamped and the anterior wall of the artery cut with scissors and the blood inside the artery washed out. Then, the arterial stent tied on the graft celiac artery was inserted into the recipient's right RA. The arterial stent was ligated in place with 7-0 silk to complete the arterial anastomosis. (C) PV anastomosis. The inside of the graft PV was washed and 2 stay sutures placed from outside to inside on each edge of the graft SMV

using 10-0 nylon. The superior mesenteric artery (SMA) was clamped during PV anastomosis to minimize intestinal congestion. The splenic vein, the PV and SMV were clamped, to provide space for the anastomosis. The PV was cut about 4 mm on the opposite side of the splenic vein for anastomosis. An end-to-side anastomosis was performed between the graft SMV and the recipient's PV. (D) Bile duct reconstruction. A small stitch was placed on the duodenal wall and on the right side wall of the graft bile duct close to the hilum using 10-0 nylon. Then, another small stitch was placed from the duodenum to the left side of the bile duct, 2 mm proximal to the previous suture. A 24-gauge needle was inserted into the duodenum 1 cm distal from the sutures and exited from the duodenal lumen between the sutures to introduce the bile duct into the duodenum. The anterior wall of the bile duct was then cut to create an opening for bile drainage. The bile duct was pulled with the bile duct opening inside the duodenum to avoid bile leakage and the 10-0 sutures tied to connect the duodenum and the bile duct. One Lembert suture was placed on the duodenum to close the insertion hole from the 24-gauge needle. (E) Appearance of the graft and native livers *in situ* 4 weeks after surgery. The liver graft has regenerated well and is similar macroscopically to the native liver. (F) Appearance of the liver graft (left) and native liver (right) following excision 4 weeks after surgery.

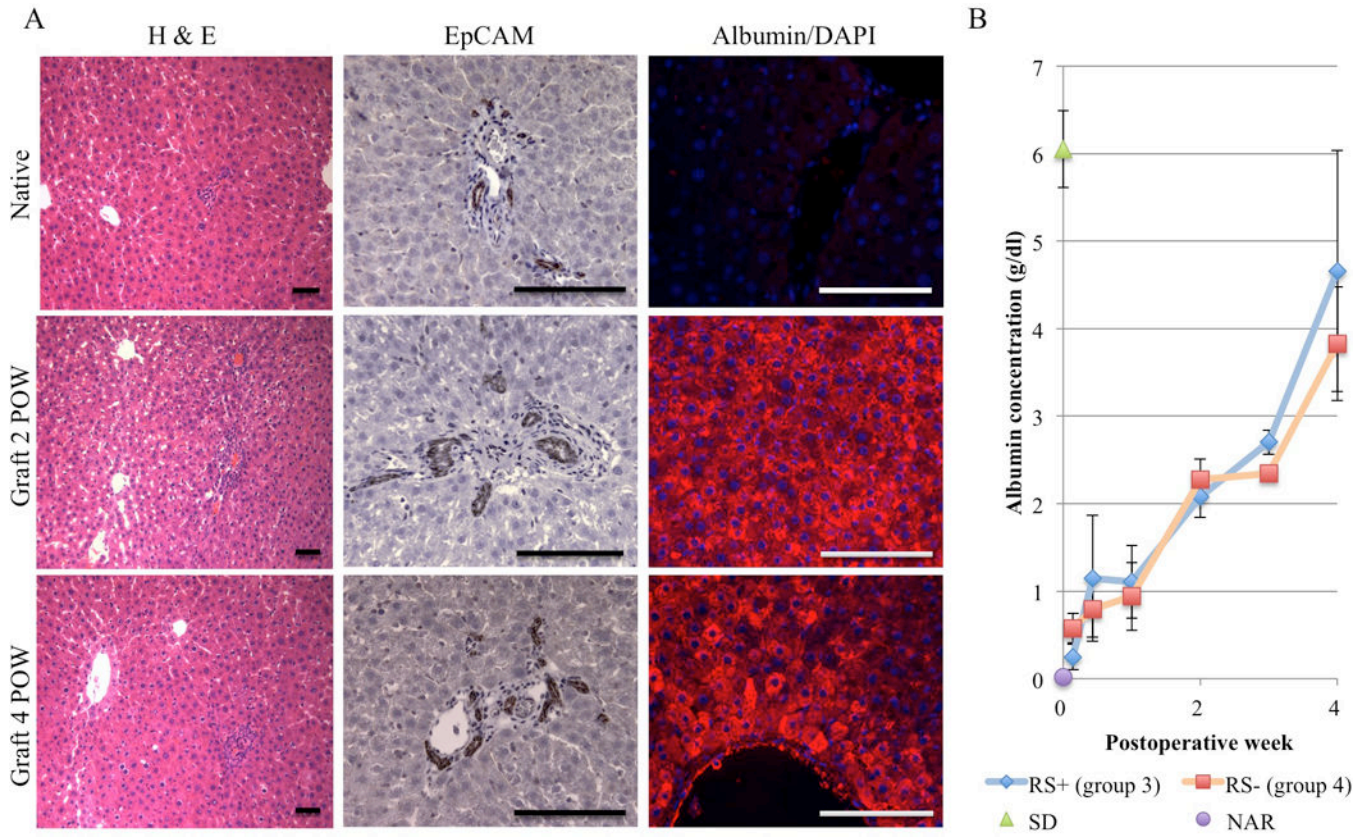


**Figure 2. Histological appearance of liver graft and assessment of graft function**

(A) H & E staining and immunohistochemical analyses of EpCAM and albumin expression in NAR native liver as a control and in the liver graft at 2 and 4 postoperative weeks (POW; representatives of groups 1 and 3). Scale bar = 50 μm. (B) Time course of serum albumin levels determined by ELISA in NAR recipient rats with normal SD grafts. Data show post-transplant RS+ NAR (group 3; n = 3) and RS− NAR (group 4; n = 3) albumin levels over time. As controls normal SD (n = 3) and NAR (n = 3) albumin levels are also shown at 0 POW. Values are shown as means ± SEM.

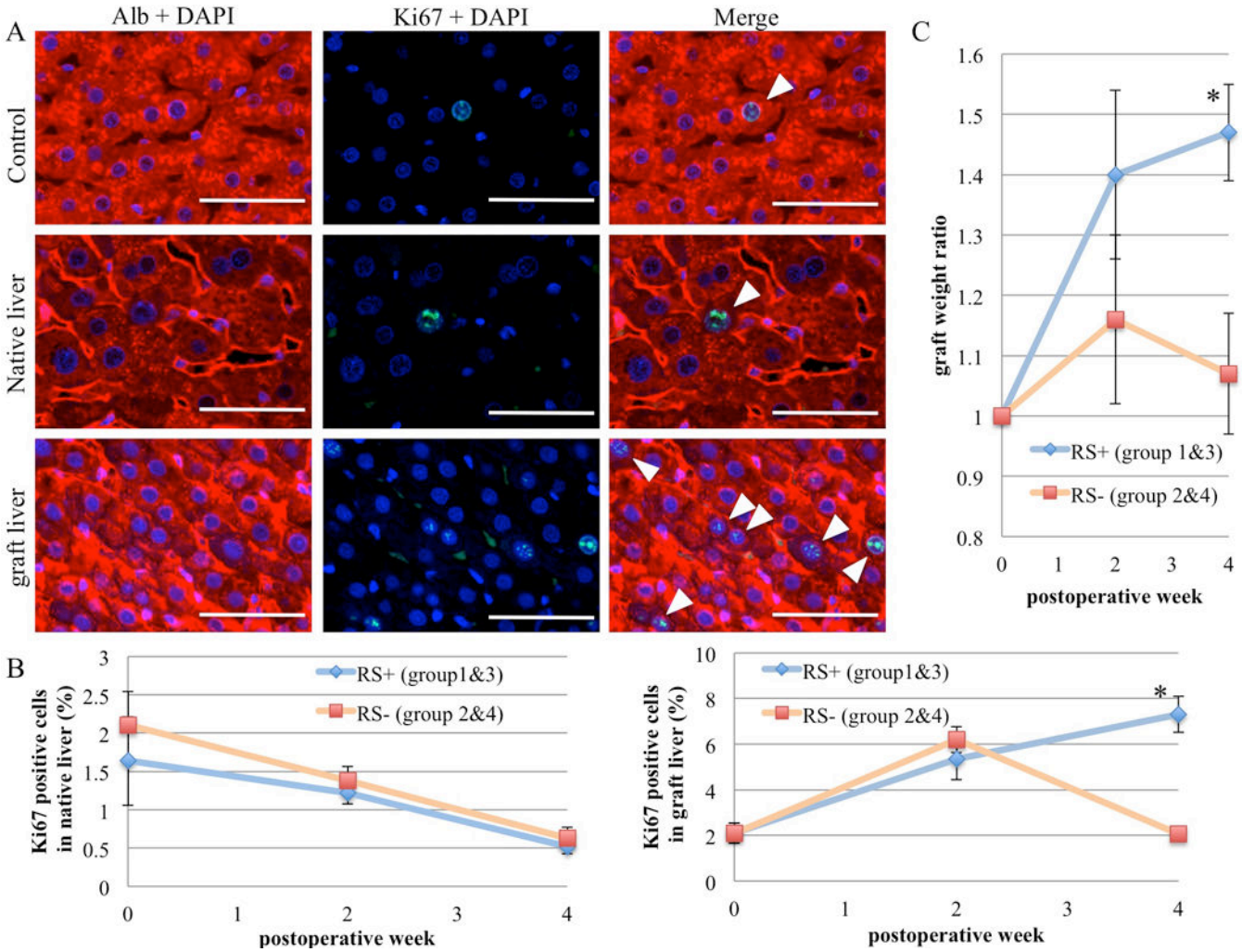


**Figure 3. Assessment of the regenerative capacity of the transplanted liver in RS-treated (group 1 and 3) versus non RS-treated recipients (group 2 and 4)**

(A) Ki67/Albumin co-immunofluorescence staining in the graft and native liver with RS treatment 2 weeks after APLT. White arrowheads indicate Ki67$^+$ cells. Scale bars = 50 μm. (B) To assess proliferation, Ki67 positive and negative hepatocytes were counted in 5 high power fields of each group at ×400 magnification and positive hepatocytes expressed as a percentage of total hepatocytes. (C) “Graft weight ratio” at 2 and 4 weeks after APLT comparing the RS and non RS-treated groups. Values are means ± SEM. *, P ≤ 0.05.

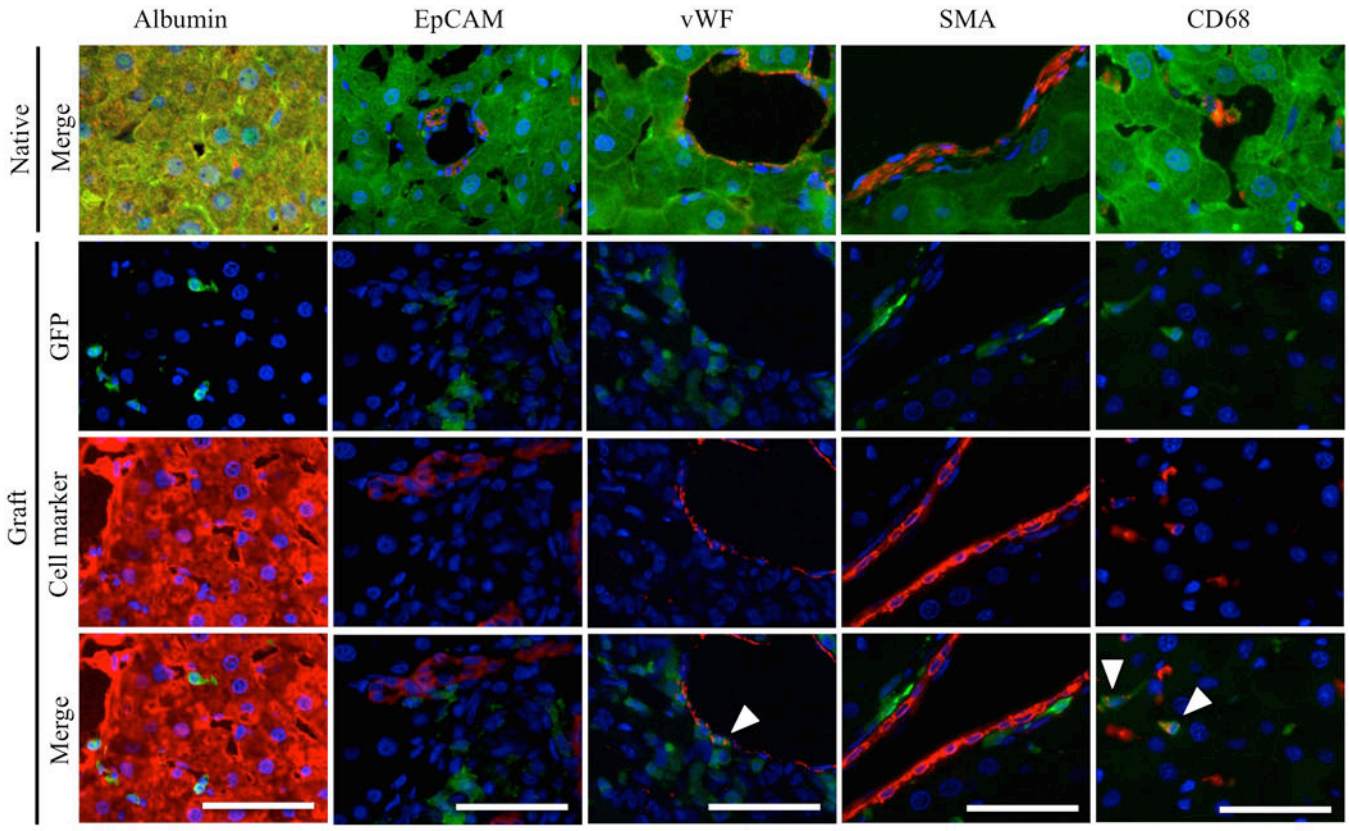


**Figure 4. Detection of host-derived cells in liver grafts**

Staining was performed on native livers and liver grafts for hepatocytes (Alb), cholangiocytes (EpCAM), sinusoidal and vessel endothelial cells (vWF), hepatic stellate cells (αSMA) and Kupffer cells/monocytes (CD68), together with GFP immunofluorescence, 4 weeks after APLT. White arrowheads indicate co-localization. Scale bars = 50μm.

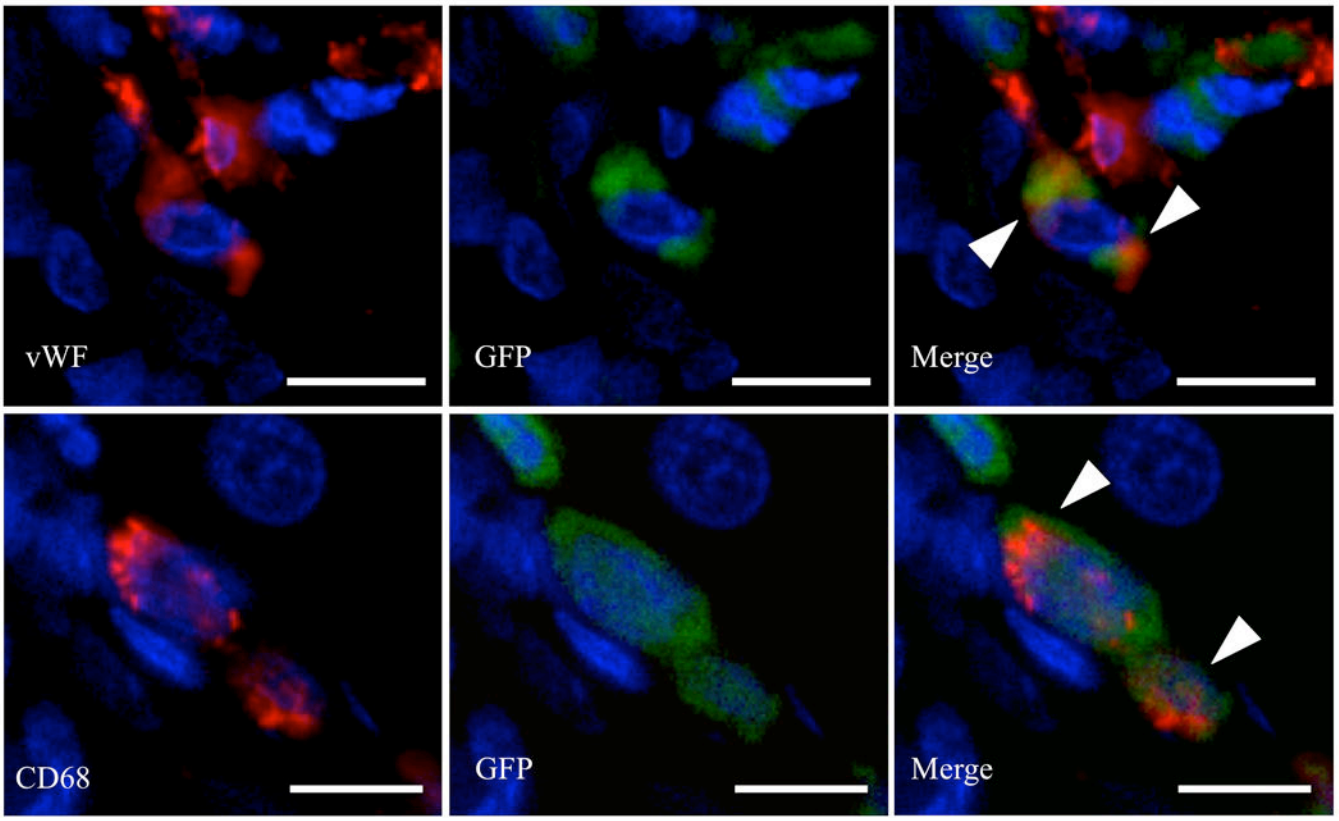


**Figure 5. Co-localization of GFP and CD68 or vWF immunofluorescence staining in liver grafts 4 weeks after transplantation**

White arrowheads indicate co-localization. Scale bars = 10μm.

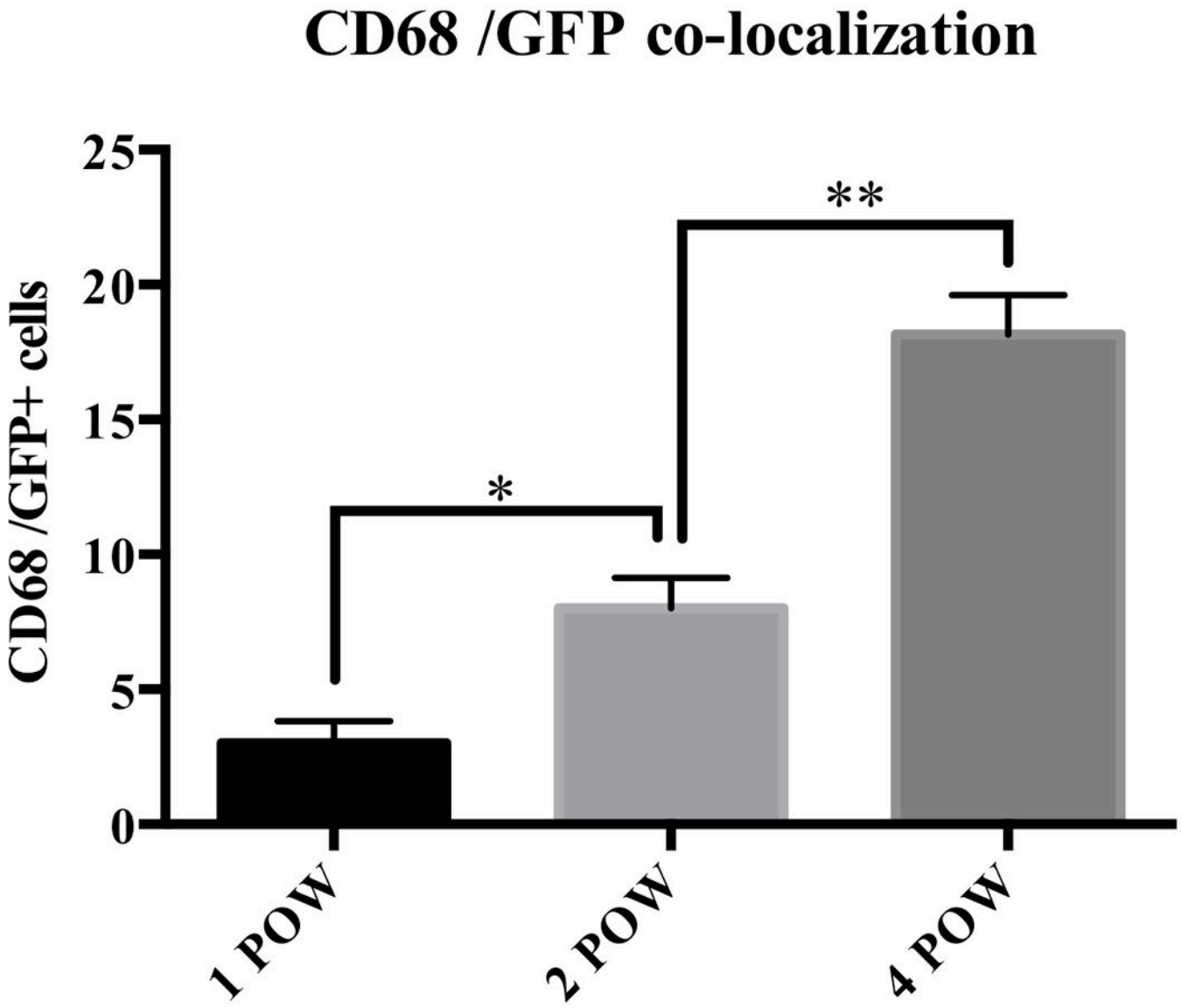


**Figure 6. Host-derived CD68$^+$/GFP$^+$ cells increase over time after APLT**

To assess co-localization of CD68$^+$/GFP$^+$ cells in the liver graft, double-positive cells were counted in 5 fields per representative of each group at ×200 magnification. These co-expressing cells increased over time (1POW; 3.0 ± 0.82, 2POW; 8.0 ± 1.1, 4POW; 18 ± 1.4 cells). POW = 4. Scale bars = 50μm. Values are shown as means ± SEM. *, P = 0.0238, **, P = 0.0043.