Transforming the spleen into a liver-like organ in vivo

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Regenerating human organs remains an unmet medical challenge. Suitable transplants are scarce, while engineered tissues have a long way to go toward clinical use. Here, we demonstrate a different strategy that successfully transformed an existing, functionally dispensable organ to regenerate another functionally vital one in the body. Specifically, we injected a tumor extract into the mouse spleen to remodel its tissue structure into an immunosuppressive and proregenerative microenvironment. We implanted autologous, allogeneic, or xenogeneic liver cells (either primary or immortalized), which survived and proliferated in the remodeled spleen, without exerting adverse reverse responses. Notably, the allografted primary liver cells exerted typical hepatic functions to rescue the host mice from severe liver damages including 90% hepatectomy. Our approach shows its competence in overcoming the key challenges in tissue regeneration, including insufficient transplants, immune rejection, and poor vascularization. It may be ready for translation into new therapies to regenerate large, complex human tissue/organs.

INTRODUCTION

The shortage of human organs suitable for transplantation is a global challenge (1). Tissue engineering has shown promise for growing tissues in vitro to replace dysfunctional organs in vivo (2). However, its application in regenerating large functional organs has achieved little clinical success (3). One major reason is that those organs have abundant, well-orchestrated blood vasculature, which is too complex for the current techniques (e.g., co-delivering angiogenic agents) to recapitulate in engineered tissues (4). Poor vascularization has become the critical limitation accounting for the unsatisfactory performance of engineered tissue transplants in the body (5).

We proposed here the approach of “transforming,” instead of “transplanting” or “engineering,” an organ to solve the problem. It aimed to reconstruct an existing organ in the body to develop the function of another, dysfunctional one. An existing organ offers extensive, interconnected vasculature that is difficult to mimic in engineered tissues and provides a cell-adhesive microenvironment (6, 7). Furthermore, the organ to be transformed should be functionally dispensable and large enough so that it can adequately perform the function of a vital, large organ (like the liver). Evidence in multiple aspects suggested that the spleen would be such a suitable organ for transformation. First, as a large, peripheral lymphoid organ, the spleen is essentially a vestigial hematopoietic organ (8). Its functions could largely be compensated by the lymph nodes (for lymphoid) and the liver/kidney (for blood filtration). Second, the spleen is relatively large (>170 cm³ in adult humans) and has abundant blood supply from the hepatic artery (9). Third, patients who received splenectomies do not suffer from severe conditions (10), further confirming its dispensability.

To validate our hypothesis, and aimed at liver regeneration, we devised a two-step method to transform a mouse spleen into a functioning liver. In the first step (“remodeling”), through repeated injection of a tumor extract, the supernatant of a tumor tissue homogenate [solute tumor homogenate (STH)], we remodeled the tissue matrix of the spleen to support the growth of epithelium and establish an immunosuppressive microenvironment. In the second step (“transplantation”), on the basis of our previous findings that transspecies cell lines could grow—but be confined—in the established immunosuppressive niche, we transplanted several auto-, allo-, and xenograft liver cells into the remodeled spleen. These cells grew and functioned in the host; in particular, the allograft primary cells exerted comprehensive liver-specific functions to support the survival of the mice suffering from 90% hepatectomy or drug-induced liver failure. Our results suggest that we have transformed the spleen into an organ that functions as a liver.

RESULTS

Remodeling of the spleen in mice

We transformed the spleen into a functioning liver in two steps: remodeling the spleen tissue and transplanting liver cells into the remodeled spleen. The transplanted liver cells—autograft, allograft, or xenograft—should survive, grow, and perform the physiological functions of the liver in the mouse body (“hepatization”).

Before these two steps, we performed a preparatory, but essential, surgical operation to translocate the spleen to facilitate subsequent repeated intraspleen injections. Specifically, we moved the murine spleen from its original site in the abdominal cavity to a subcutaneous location (Fig. 1A), with the surgery recorded in movie S1 and further illustrated in fig. S1A. Successful translocation of the spleen could be confirmed by both micro-computed tomography (micro-CT) (Fig. 1B) and direct observation in the euthanized mice (Fig. 1C). Seven days after the operation, the animals showed a satisfactory recovery, with no notable weight loss (fig. S1B) or physiological abnormalities (table S1). The translocated spleen maintained its natural size, morphology (Fig. 1C), and weight [expressed as spleen weight/body weight (SW/BW); Fig. 1D], and its gene expression was the same as that of the normal spleen (Fig. 1E). Meanwhile, the weight, histology, and zonation of the liver, as well as the flow of the liver portal venous, were not altered after the spleen translocation (fig. S1, C to F). In accordance with our design, the translocated...
Fig. 1. Spleen remodeling. (A) Illustration of spleen remodeling. (B) The spleen (white dotted) before and after translocation (micro-CT). (C) Gross view of a normal and translocated spleen. (D) Mean SW/BW of normal and translocated spleens 7 days after translocation. (E) Microarray analysis of gene expression between normal and translocated spleens. IRCs, immune-related cytokines. (F to O) The translocated spleens injected with phosphate-buffered saline (PBS) or STH three times over 7 days with their (F) gross view; (G) SW/BW; (H) gene expression compared by microarray; (I) expression of typical genes of ECM, chemokines, growth factors (GFs), and cytokines; (J) contents of hydroxyproline, COL1, and COL4, plus the hardness of the spleens (n = 8 for COL4); (K) hematoxylin and eosin (H&E) and (L) Masson’s trichrome staining; (M) expression of COL1, COL4, and α-smooth muscle actin (α-SMA) (inset scale bar, 200 μm); (N) levels of growth factors and cytokines [enzyme-linked immunosorbent assay (ELISA); values normalized to PBS group]; and (O) three-dimensional (3D) reconstructed micro-CT images showing vascularization, with the vessel area measured. (P) Average proportion of different cell populations in the spleens treated with PBS or STH. Images are representative of three independent experiments. Results are shown as means ± SEM (n = 5 unless otherwise noted). Statistics: (D, G, J, N, and O) Student’s t test. TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; TGF-β1, transforming growth factor-β1; EGF, epidermal growth factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor. Photo credit: Lintao Wang, Nanjing University.
spleen could be easily accessed for subcutaneous injections (movie S2 and fig. S1G).

Then, we started to perform remodeling as the first step of the organ transformation. We used STH to remodel the tissue matrix of the translocated spleen for two purposes: (i) suppress immune rejection for accommodating allo-/xenograft liver cells and (ii) increase extracellular matrix (ECM) production (which is low in the spleen) to support epithelial development. We prepared STH from four different allograft tumor models—S180 (sarcoma), Hepa1-6 (hepatoma), 4T1 (breast cancer), and B16-F10 (melanoma)—which are all murine cancer cell lines implanted in mice. We compared the activity of all STH samples to promote interleukin-10 (IL-10) expression in mouse bone marrow–derived macrophages and enhance type 1 collagen (COL1) production in mouse embryonic fibroblasts (MEFs). Among them, the STH generated from the S180 model outperformed other samples in inducing the expression of the two genes (fig. S2, A and B). Thus, we selected the S180-derived STH (termed as “STH”) in the following experiments.

We analyzed the protein content of STH by liquid chromatography–mass spectrometry (LC-MS) and several cytokines by enzyme-linked immunosorbent assay (ELISA; table S5 and fig. S2C). The concentrations of IL-10 and transforming growth factor–β1 (TGF-β1) were high according to the ELISA results. We injected STH into the spleen three times, at days 7, 10, and 14 after the translocation operation. Each time, we injected 50 μl, which is the maximal value that can be injected to the spleen, to five separate sites (10 μl at each site), as illustrated in fig. S1 (H and I). At day 17, the mice were euthanized, and the spleens were collected for analysis. The three injections of STH markedly changed the characteristics of the spleen tissue in various aspects. First, the size of the STH-treated spleens was enlarged (Fig. 1F), and their average weight was about twice of that of the phosphate-buffered saline (PBS)–treated ones (Fig. 1G). Second, the expression of more than 4800 genes in the STH-treated spleen was far different from that in the normal spleen (Fig. 1H). Cluster analysis based on the variations highlighted in Fig. 1 (E and H) uncovered that most notable alterations occurred to the genes encoding ECM molecules, chemokines, growth factors, and immune-related cytokines (Fig. 1I). Third, the ECM composition was also changed, in agreement with the above outcomes of gene analysis. The hydroxyproline content of the STH-treated spleen was much higher than that of the control spleens because the content of COL1 in the spleens was increased, although that of COL4 remained unchanged, which made of the control spleens because the content of COL1 in the spleens in agreement with the above outcomes of gene analysis. The hydroxyproline content, reflecting the concentration of collagen, in the STH-treated spleen was comparable to that of normal liver tissue (fig. S2D) (12).

Cytokine profiling further highlighted the radical changes in the matrix microenvironment in the remodeled spleen. Examination of key cytokines by ELISA (Fig. 1N) revealed that vascular endothelial growth factor (VEGF) was among the most up-regulated ones, indicating enhanced angiogenesis in the transformed spleen. This was further confirmed by micro-CT that showed a higher density of blood vessels in the STH-treated spleen than in the normal one (Fig. 1O). Meanwhile, the elevated variation of TGF-β1, IL-10, and interferon-γ (IFN-γ; together with the histological changes) indicated a marked change in the cellular composition of the spleen. We observed a notable decrease in the number of both T and B cells, as well as an increase in that of fibroblasts, in the STH-treated spleen (Fig. 1P and fig. S2E). Macrophages, a key regulator of tissue microenvironment, not only increased in number but also expressed more CD206, an M2 polarization marker, in the STH-treated spleen (fig. S2E) (13). T regulatory (Treg) cells, one of the most important immune suppressors, also increased in number in the STH-treated spleen (fig. S2F) (14). Depletion of Treg cells or macrophages abolished the STH’s protecting effect (fig. S2G). Meanwhile, any injections after the third did not further change the cell composition in the spleen (Fig. 1P), suggesting that three injections were enough for remodeling.

To validate the effect of STH in changing the phenotype of spleen cells, we isolated total spleen cells, treated them with STH ex vivo, and analyzed their gene expression profiles. We found that the STH treatment markedly changed the expression of key genes in spleen cells (fig. S2H); for instance, the levels of collagens, fibroblast growth factor (FGF), epidermal growth factor (EGF), VEGF, and IL-10 were significantly up-regulated, and the expression of IL-12 and tumor necrosis factor–α (TNF-α) decreased. Furthermore, we purified the spleen macrophages for the same treatment and analysis; the outcomes showed a clear decrease in the expression of typical inflammation-related genes, such as IL-12a, nitric oxide synthase 2, TNF-α, IL-6, and Toll-like receptor, as well as an increase in that of anti-inflammatory cytokines such as IL-10 and TGF-β1 (fig. S2I). Further measurement of key cytokines by quantitative reverse transcription polymerase chain reaction (RT-PCR) (fig. S2J) demonstrated that more spleen macrophages switched from an M1 to an M2 phenotype after STH treatment for 18 hours (15). These results highlighted the direct effect of STH in inducing an immunosuppressive phenotypic change in spleen cells.

Such effect of STH, especially its capacity in inducing immunosuppression, led us to investigate its safety for use. To do so, first, we fluorescently labeled TGF-β1 (the most abundant cytokine in STH), injected it into the spleen at the same dose as in STH, and traced its distribution. Most signals were located around the injection sites in the spleen (fig. S3A), suggesting that TGF-β1 at this dose was immediately captured by the local cells. Second, we isolated macrophages from different organs of both normal mice and those whose spleen had been treated with STH for 24 weeks. The macrophages from the same organ—except the spleen—of the treated or untreated mice expressed IL-10 and TGF-β1 to a similar extent (fig. S3B). In addition, histological analysis of the other organs (the liver, lung, kidney, and heart) revealed no abnormalities (fig. S3C). Third, because M2 macrophages were reported to promote liver fibrosis, we examined the influence of STH on liver fibrosis in a mouse model induced by CCl4. Nevertheless, injection of STH into the spleen did not affect the process of fibrosis (fig. S3D). Fourth, the long-term (24 weeks) injection of STH did not cause any adverse changes in the main
fluorescence-activated cell sorting (FACS) data showed that CD4+ cells in the spleens 7 days after the transplantation of HepG2 cells. The in the remodeled spleen maintained a much lower level compared that of the IgG and IgM in both animal groups (Fig. 2C).


cal cytokine indicators for immune rejection, was consistent with very different from what was observed in the PBS-treated mice, gradually increased in the next few days (Fig. 2C). This trend was spleen were unchanged in the first 3 days after transplantation and pathways related to immune rejection were lower in STH-remodeled of Genes and Genomes (KEGG) analyses. The levels of key genes/

The transplanted cells included the following: (i) autograft liver cells (autoHEPs), (ii) allograft mouse liver cells (alloHEPs), (iii) xenograft human primary liver cells (hHEPs), and (iv) xenograft human induced pluripotent stem cell (iPSC)-derived liver cells (hiPS-HEPs). For most groups, we monitored the cell growth for 8 weeks after their transplantation; for one group of alloHEPs, we continued STH injection for 24 weeks to evaluate their long-term growth.

For autoHEPs, the hepatocytes harvested from the same recipient mice, they grew larger tissue (gross view; fig. S6A) and proliferated faster [5-ethyl-2’-deoxyuridine (EdU) staining; fig. S6C] in the spleens remodeled by STH than in those treated with PBS.

For alloHEPs, we derived primary murine hepatocytes from green fluorescent protein (GFP) transgenic or luciferase transgenic mice for transplantation. These cells settled well and proliferated in the STH-remodeled spleen, as examined by cell counting (Fig. 3B), spleen weighing (expressed as SW/BW; Fig. 3C), EdU staining (fig. S6G), and bioluminescence imaging (Fig. 3D). In addition, they formed neotissue in the remodeled spleen, as evidenced by histological analysis (Fig. 3E and fig. S6D), morphology (Fig. 3F), and GFP immunostaining (Fig. 3G). Meanwhile, we traced the formation of bile duct (fig. S6E) and found that hepatic stellate cells took part in liver-like microstructure formation (fig. S6F). Furthermore, through the long-term (24 weeks) development, these alloHEPs expanded to occupy most space in the spleen (Fig. 3H) and developed the typical hepatic structure including the bile ducts (Fig. 3I) and liver zonation [indicated by the specific distribution of glutamine synthetase (GS); Fig. 3J].

Establishment of an immunosuppressive microenvironment in the remodeled spleen

Transplantation of allogeneic or xenogeneic cells into immuno-

competent hosts can trigger acute immune rejection leading to transplant failure (16). Before testing the transplantation of liver cells of various origins, we evaluated whether the STH-remodeled spleen could provide an immunosuppressive microenvironment to accommodate xenograft cells.

We injected HepG2 cells (1 × 106), a human cancer cell line, into the STH-remodeled spleen in C57BL/6 mice (Fig. 2A). First, we examined the gene expression profile in the spleens by microarray 8 hours after the transplantation, followed by Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. The levels of key genes/ pathways related to immune rejection were lower in STH-remodeled spleens than in PBS-treated ones, both having received HGF xenograft (Fig. 2B). In addition, the immunoglobulin G (IgG) and IgM concentrations in the blood from the mice with the remodeled spleen were unchanged in the first 3 days after transplantation and gradually increased in the next few days (Fig. 2C). This trend was very different from what was observed in the PBS-treated mice, where both IgG and IgM increased immediately upon cell transplantation. The change in the levels of TNF-α and IFN-γ, two typical cytokine indicators for immune rejection, was consistent with that of the IgG and IgM in both animal groups (Fig. 2C).

Next, we assessed the change in CD4+ and CD8+ T cell proportions in the spleens 7 days after the transplantation of HepG2 cells. The fluorescence-activated cell sorting (FACS) data showed that CD4+ cells in the remodeled spleen maintained a much lower level compared with the control group; the proportion of CD8+ T cells, although starting to increase 1 day after transplantation, was still lower than that in the normal spleen most of the time (Fig. 2D). In addition, histological analysis of the spleen sections 4 days after transplantation indicated that inflammatory responses were minimal in the STH-transformed spleens but obvious in the PBS-treated control group (fig. S5A).

Then, we continued to monitor immunosuppression in the transformed spleen for a longer period of 30 days, with STH injected every 4 days (schemed in Fig. 2E). As a control group, injection of STH was ceased 14 days after cell implantation. We analyzed the T cell profiles (fig. S5B) and cytokine expression (Fig. 2F). We further evaluated CD8+ T cell activation in an OT-1 mouse model, in which the CD8+ T cells express a T cell receptor recognizing the SIINFEKL peptide of ovalbumin (OVA) (Fig. 2G) (17). Upon the stimulation of OVA-expressing HepG2 (OVA-HepG2) cells, the CD8+ T cell activation (as marked by the expression of IFN-γ) was significantly lower in the STH-remodeled spleen than in the PBS-treated one, as indicated by the expression of IFN-γ in spleen cells (Fig. 2H) and the ratio of IFN-γ/CD8+ T cells (Fig. 2I). As long as STH was injected, the CD8+ T cells remained inactivated; once STH injection was ceased, these cells became activated (Fig. 2, J and K). These results suggested that continued STH injection was both effective and necessary to maintain the immunosuppressive environment in the spleen following the transplantation of the liver cells. In addition, the increased blood vessels in the spleen seemed unchanged after the end of STH injection (fig. S5C).

Together, these data demonstrated that the immunosuppressive niche established by STH treatment prepared the transformed spleen for accommodating xenograft cells. The transplanted human hepatoma did not trigger acute immune rejection.

Transplanting auto-/allo-/xenograft liver cells in the remodeled spleens in mice

As demonstrated above, continued STH injection (once every 4 days) could maintain an immunosuppressive niche in the remodeled spleen. We set out to comprehensively investigate the growth of autologous, allogeneic, and xenogeneic liver cells in this niche. As illustrated in Fig. 3A, after translocating and remodelling the spleen, we transplanted four different types of liver cells into the site and continued STH injection for another 8 weeks before sample collection and analysis. The transplanted cells included the following: (i) autograft liver cells (autoHEPs), (ii) allograft mouse liver cells (alloHEPs), (iii) xenograft human primary liver cells (hHEPs), and (iv) xenograft human induced pluripotent stem cell (iPSC)–derived liver cells (hiPS-HEPs). For most groups, we monitored the cell growth for 8 weeks after their transplantation; for one group of alloHEPs, we continued STH injection for 24 weeks to evaluate their long-term growth.

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Fig. 2. Establishment of immunosuppression in the remodeled spleen. (A) Scheme of xenografting HepG2 cells into the spleen. (B) Genome-wide microarray profiling of the spleen 8 hours after HepG2 transplantation. Signaling pathways (KEGG, left) and gene expression (heat map, right) related to immune rejection in STH/PBS-treated spleens. NOO, nucleotide oligomerization domain; JAK-STAT, Janus kinase–signal transducers and activators of transcription. (C) Serum concentration of immunoglobulins and cytokines and (D) CD4+CD8+ T cell proportion in peripheral blood (statistics on the right) in mice 1 week after HepG2 transplantation. (E) Scheme of assessing long-term rejection of xenografted HepG2, with (F) the determination of growth factors and cytokines in the spleen. (G) Scheme of analyzing the response in OT-1 mice, whose (H) expression level of IFN-γ by spleen cells 4 days after stimulation with OVA-expressing HepG2 (OVA-HepG2) cells and (I) CD8+ T cell proportion expressing IFN-γ in the spleen (statistics on the right). Furthermore, OT-1 mice with OVA-HepG2 cell transplantation and long-term STH treatment were analyzed against the group with STH injection ceased for 14 days: (J) IFN-γ expression by the spleen cells and (K) CD8+ T cell proportion expressing IFN-γ. Images are representative of three independent experiments. Results are shown as means ± SEM (n = 5). Statistics: (F) one-way and (C and D) two-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparisons post hoc test and (H to K) Student’s t test.
For xenograft cells, both hHEPs and hiPS-HEPs showed desirable settlement and growth in the STH-remodeled (but not the PBS-treated) spleen, as validated by quantification of human genomic DNA (gDNA; Fig. 3K), SW/BW (Fig. 3L), as well as measurement of the human albumin content in the spleen (Fig. 3M). Meanwhile, both cells were proliferating in the remodeled spleen, while hiPS-HEP proliferated faster than the primary human liver cells, according to the outcomes from the EdU staining (fig. S6G). Moreover, they generated xenograft tissues in the remodeled spleen, as evidenced by morphological observation (Fig. 3N), histological staining (H&E; Fig. 3O).
Growth of xenograft hepatoma cells in the remodeled spleen in mice

On the basis of the above finding that transspecies hepatocytes could grow well in the STH-remodeled spleen with minimal rejection, we speculated on generating a hepatic tissue in the remodeled spleen with xenograft hepatoma cells, which would be valuable in solving the problem of the shortage of primary cells for transplantation. As indicated in Fig. 4A, we transplanted HepG2 cells into the translocated and STH-remodeled spleen and continued STH injection for another 8 weeks before sample collection and analysis. Our pilot experiment suggested $1 \times 10^6$ to be the minimal number of HepG2 cells that could steadily grow in the remodeled spleen (fig. S8A).

We first used luciferase-labeled HepG2 cells, which enabled us to monitor their growth under a whole-body fluorescent imaging system. Through an 8-week observation, we found that the fluorescent signal steadily increased in the STH-remodeled spleen than in that treated with PBS. One injection of $1 \times 10^6$ McA-RH7777 cells could expand the implanted alloHEPs or xenograft hiPS-HEPs disappeared from the spleen only 2 weeks after we stopped STH injection (fig. S6H). Furthermore, STH treatment did not induce fibrosis in the implanted hepatocytes (fig. S7A), and long-term injection of STH did not change the properties of new hepatocytes (fig. S7B).

Together, the above data validated that the STH-transformed spleens in mice supported the survival, proliferation, and development of auto-, allo-, and xenograft hepatic cells, including human primary liver cells.

Function of hepatic cells in the remodeled spleen in mice: Evidence of hepatization

We lastly evaluated whether the allo-/xenograft liver cells growing in the transformed spleen could perform the functions of the liver. The alloHEPs, growing in the STH-transformed spleens for 8 weeks, exerted the typical hepatic functions of synthesizing lipids and glycogen, as illustrated by the Oil Red O staining and the periodic acid–Schiff (PAS) staining (Fig. 5A). To more critically assess the hepatic function of the transformed spleen, we performed 90% hepatectomy (fig. S9, A and B) in the mice implanted with alloHEPs and evaluated whether the transformed spleens could recover the essential liver functions to save the animals from death. First, an indocyanine green (ICG) clearance test (Fig. 5B) and follow-up observation of the frozen sections of the spleen (Fig. 5C) revealed that the transformed spleen restored the liver’s uptaking capacity. Second, the mice, either with STH treatment but without cell transplantation or with PBS treatment followed by cell transplantation, all died within 48 hours after the hepatectomy. Only the mice with the STH-remodeled spleen and alloHEPs transplantation could all survive through the study period (Fig. 5D).

We noticed that the hepatectomy procedure itself stimulated the growth of both the remnant liver and alloHEPs in the spleens. In the STH + alloHEPs group, the volume of the remnant 10% liver tissue reached about 30% size of a normal liver 7 days after the operation. The transformed spleen also doubled its size compared with that in the mice without hepatectomy (fig. S9, D and F). Meanwhile, the liver-like microstructures that included bile ducts, blood vessels, and hepatic stellate cells could be observed in the transformed spleen (fig. S9C). Although 30% liver tissue could support the animal to survive, we assumed that the mice needed to rely on the hepatic...
function provided by the transformed spleen to survive after the first 48 hours. To validate this assumption, we carried out small interfering RNA (siRNA) inhibition of c-Met after the hepatectomy, which suppressed hepatocyte proliferation in both the liver residue and the transformed spleen but found that it did not change the survival rate of the mice with alloHEPs (fig. S9, D to L). This outcome supported that the survival of these mice depended on the function of alloHEPs in the transformed spleen.

Xenograft human cells (HepG2 or hiPS-HEP) were then implanted to the STH-transformed spleen in mice. We first profiled the expression of the main genes involved in the metabolic pathways specific to the liver, in comparison with that of the normal liver and hepatoma tissue harvested from patients with hepatoma (table S6). The results demonstrated the expression of these genes in the transformed spleens with HepG2 cells and hiPS-HEP cells (Fig. 5E). The gene expression profile was closer to that in the normal human liver than...
in the hepatoma tissue even in the HepG2-derived tissue. Second, as albumin production is one key function of the hepatocytes to maintain the blood osmotic pressure and other important functions, we examined the serum HSA using human-specific ELISA. As shown in Fig. 5F, HSA was detected in the mouse serum, and its concentration increased as HepG2 cells grew. Although hiPS-HEP and hip proliferated slower than HepG2 cells in the spleens, we could detect HSA in the mouse serum (Fig. 5G and fig. S10A). Third, because human liver cells are more capable of modifying debrisoquine (DB) into 4-hydroxydebrisoquine (4OHDB) than mouse liver cells, we injected DB into the mice with human hepatocyte–grafted spleens \(^\text{(18)}\). We observed a significantly higher serum level of 4OHDB/DB in these mice than in the control group (Fig. 5H), suggesting that the liver tissues generated by the human cell lines exhibited metabolic functions. Fourth, the Oil Red O and PAS staining (Fig. 5I) demonstrated that the hepatic tissue generated in the spleen had the typical liver function of synthesizing lipids and glycogen. Fifth, the transformed spleens transplanted with the human liver cells also developed the ability to take up ICG (Fig. 5J). Last, after 90% hepatectomy, 70% of the mice with the STH-remodeled spleen implanted with human primary hepatocytes from patients survived, whereas the control mice (i) with STH treatment and without hepatocyte transplantation or (ii) with PBS-treated spleen and grafted hepatocytes all died within 48 hours after the hepatectomy (fig. S10B).

To further test the ability of the transformed spleen in performing comprehensive functions of the liver, we again used the rat hepatic cell line McA-RH7777. One important feature of these cells is that they can resist the \(\alpha\)-galactosamine hydrochloride (\(\alpha\)-Gal)–induced apoptosis \(^\text{(19, 20)}\). We confirmed that they grew well and expressed hepatic markers in the transformed spleen; two genes were particularly obvious, prothrombin and GS, which are related to the key liver functions (prothrombin for the coagulation function and GS for the blood ammonia control) in maintaining the whole physiological

![Fig. 5. Function of the hepatized spleen.](image-url)
homeostasis and the liver of the animals (Fig. 5K) (21, 22). We developed a fulminant liver failure model induced by d-Gal 8 weeks after the cell implantation (23). The induction was successful as indicated by the serum alanine aminotransferase (ALT) and aspartate transaminase (AST) level, histological examination of the liver tissue, and the massive animal death within 12 hours. First, both groups of mice with the McA-RH7777 cell hepatized spleens and the control mice developed massive liver injury, evidenced by the H&E staining of the liver tissue and the serum level of ALT (>150 U/liter) and AST (>90 U/liter) (fig. S10, C and D), while the tissue formed by the rat hepatic cells in the transformed spleen remained relatively intact under the challenge from d-Gal (Fig. 5L). Furthermore, the key parameters reflecting the liver functions, including coagulation time (Fig. 5M) and ammonia blood concentration (Fig. 5N), were better in the STH-transformed mice than in the control group (24). In addition, half of the mice (50%) with transformed spleen and xenograft liver tissue survived after the induction of liver failure, while all the animals in the other two groups died within 12 hours (Fig. 5O).

Our strategy resonates with other attempts to support the extrahepatic engraftment of liver cells (33). These attempts provided valuable insights; however, those capillary vessels were far from the large, mature vasculature demanded for the development of most large tissue/organs (including the liver). Here, our method uses and enhances the naturally developed vasculature in the spleen, which is abundant, large, and well connected to the main circulation. As the data showed, the spleen vasculature enabled the implanted liver cells of different origins to grow and fully integrate with the host body. Such improvement is key to the overall efficacy of the transformed organ. An engineered or transplanted tissue is of physiological significance only when its volume, cell number, and functional capacity are comparable to those of its native counterpart (34). Our data showed that the transformed tissue was comparable to the native liver in size and accommodated enough functioning liver cells. It not only exhibited standard liver functions (e.g., albumin production and drug metabolism) but also, more importantly, rescued the mice from two types of extreme damages: fulminant liver failure and 90% hepatectomy. To the best of our knowledge, few previous studies have achieved this level of effectiveness.

Note that the spleen-transformed “liver” differs from the native liver in terms of blood supply, which may cause several consequences. The former harnesses the artery-rich splenic vasculature to support the transplanted hepatocytes, while the latter mainly relies on the portal blood with lower oxygen tension (35). Although our data showed that the transplanted hepatocytes could exert the main hepatic functions in the remodeled spleen, it is worth investigating whether the blood with possibly higher oxygen tension poses any subtle or long-term impact on the hepatocytes. Meanwhile, the native liver receives intestine-derived growth factors and other nutrients from the portal blood and filters harmful substances before they enter the main circulation. In the transformed organ, the spleen has no direct access to the portal blood; hence, the growth of the grafted hepatocytes is possibly supported by growth factors that are delivered from the main circulation or produced by the spleen (36). In addition, the protection effect of the native liver as a reticuloendothelial organ may be weakened because the portal blood that may contain intestine-derived toxins is not immediately filtered; we speculate that these toxins can still be digested by the spleen-transformed liver tissue.

DISCUSSION
Cell-based approaches to regenerate large tissue/organs face several major challenges, including an insufficient number of therapeutic cells, immune rejection to allo-/xenografts, and poor vascularization of the transplants (16). In this study, we demonstrated a completely different strategy of tissue regeneration by transforming an existing organ—the spleen—into a “new” one that performed the function of the liver in the mouse body.

This transformation builds on the remodeling of the spleen into an immune-privileged transplant bed to support xenogeneic liver cells to grow and function. We chose to remodel an existing organ, instead of delivering an engineered scaffold, because the former has inherent advantages such as highly organized vasculature, versatile stromal cells, sophisticated ECM structure, and well-formed communication with the host body, which remain hard to recapitulate in most current scaffolds (25). Our strategy resonates with other excellent studies demonstrating the benefits of using an existing organ for growing different tissue—the liver for pancreatic islets (26), lymph nodes for hepatocytes (27), and, notably, kidney for hepatocytes (28–31). The present study has further extended the application of this concept, aimed at enhancing both the efficacy and feasibility of this approach. First, we chose the spleen for remodeling because this organ is functionally dispensable and well connected to the main circulation. Meanwhile, we emphasized on modifying the stromal tissue microenvironment in the spleen. This remodeling has proven effective, which increased fibroblasts and collagen content and made the organ larger, stiffer, and more suitable for anchorage-dependent epithelial cell growth. The remodeling also induced the macrophages into an anti-inflammatory, proradregenerative phenotype and promoted vascularization in the spleen (32). As a result, the remodeled spleen facilitated the colonization, proliferation, and function of the xenografted liver cells.

Our findings further highlighted the importance of vascularization for tissue transplantation. Inadequate blood supply is a major hurdle for in vivo settlement of engineered tissue constructs, which encounter poor survival, function, and integration with the host. Previous studies delivered growth factors to induce angiogenesis and create a capillary bed that could support the extrahepatic engraftment of liver cells (33). These attempts provided valuable insights; however, those capillary vessels were far from the large, mature vasculature demanded for the development of most large tissue/organs (including the liver). Here, our method uses and enhances the naturally developed vasculature in the spleen, which is abundant, large, and well connected to the main circulation. As the data showed, the spleen vasculature enabled the implanted liver cells of different origins to grow and fully integrate with the host body. Such improvement is key to the overall efficacy of the transformed organ. An engineered or transplanted tissue is of physiological significance only when its volume, cell number, and functional capacity are comparable to those of its native counterpart (34). Our data showed that the transformed tissue was comparable to the native liver in size and accommodated enough functioning liver cells. It not only exhibited standard liver functions (e.g., albumin production and drug metabolism) but also, more importantly, rescued the mice from two types of extreme damages: fulminant liver failure and 90% hepatectomy. To the best of our knowledge, few previous studies have achieved this level of effectiveness.
Although no abnormalities were observed in the animals, it is vital to assess the safety of this remodeling. Special attention has been paid to two parts of our approach. First, we invented a protocol of translocating the spleen to a subcutaneous site, which, as the preparatory step, brought great convenience to the whole practice. We performed comprehensive tests to confirm that the animals with translocated spleens were physiologically normal, suggesting that this procedure had no adverse effects. In addition, this procedure will not be performed if our approach is translated clinically because laparoscopy and other interventional technologies can provide surgeons with more convenience for operations on human patients. Thus, this procedure is unlikely to become a safety concern. Furthermore, although excessive tissue fibrosis might be tumorigenic, it is not a concern in our study because the extent of fibrosis induced by STH injection was much lower than that in a typical pathological scenario (e.g., liver fibrosis or pulmonary fibrosis). Neoplastic lesion-induced tumorigenesis in the spleen should be unlikely on the basis of our 24-week monitoring of these mice. Second, STH is used to remodel the spleen. Although no adverse effects of STH were found at all stages of our study, a replacement of clearer content will still be pursued. To address this issue, we demonstrated that a cocktail of cytokines might have the potential to replace STH in the future, although its efficacy needs substantial improvement and its safety requires long-term investigation.

Several factors must be considered for the future translation of this approach. First, liver diseases such as hepatitis and cirrhosis can cause substantial hypersplenism and other splenic changes, and they are among the major causes of liver failure. Therefore, further optimization of this approach in larger animals with representative kinds of liver diseases and splenic abnormalities will be of translational significance. In addition, inflammatory reactions are expected to be common under pathological circumstances and can lead to splenic enlargement; hence, anti-inflammatory treatment may be considered to aid the transformation of the spleen. Furthermore, strategies should be planned for more complex and specific clinical scenarios. For instance, for patients with immunodeficiency conditions or virus-infected diseases, the viability or function of the transplanted cells may be compromised. Customized manipulations, such as adjusting the extent of immunosuppression and exploiting genetic modification, need to be devised. Similarly, for patients with acute liver failure, the overall time of the spleen-transforming process should be shortened.

MATERIALS AND METHODS

Mice
C57BL/6 mice were purchased from Vital River Laboratories (Beijing, China). C57BL/6 GFP mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). OT-1 T cell receptor transgenic mice [C57BL/6-Tg(TcraTcrb)1100Mjb/J] were a gift from L. Li (Nanjing University, China). Luciferase transgenic receptor transgenic mice [C57BL/6-Tg (TcraTcrb) 1100Mjb/J] were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). OT-1 T cell-Gt (ROSA) 26Sor em1(CAG-Luc-EGFP) Smoc

Human liver tissue samples
Surgically resected paired hepatoma and normal adjacent tissues were collected from patients who had undergone surgery to remove localized liver tumors at the Affiliated Hospital of Jiangnan University (Wuxi, China). The surgically resected tissues were quickly frozen in liquid nitrogen until analysis or immediately digested and prepared for hepatocyte transplantation. The study was approved by the Human Research Ethics Committee of Nanjing University Affiliated Drum Tower Hospital (Nanjing, China) and Jiangnan University (Wuxi, China). The information about the clinical sample donors is listed in table S6.

Cell preparation

Hepatocyte preparation
Mouse primary hepatocytes for allograft transplantation were obtained using a two-step collagenase perfusion assay as reported before (27). Mouse primary hepatocytes for autograft transplantation and human primary hepatocytes hHEPs were harvested from the digestion of liver tissues with collagenase IV and deoxyribonuclease I (both from Sigma-Aldrich, St. Louis, MO, USA). hiPS-HEPS (Y10050, Takara Bio Inc., Shiga, Japan) were thawed according to the manufacturer’s instruction. Two kinds of hepatoma cell lines, human hepatoma HepG2 (Institute of Biochemistry and Cell Biology, Shanghai, China) and rat hepatoma McA-RH7777 (Cobioer Technology Ltd., Nanjing, China), were respectively cultured in Dulbecco’s modified Eagle’s medium (DMEM) and RPMI 1640 (both from Thermo Fisher Scientific, Waltham, MA, USA) containing 10% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT, USA). For bioluminescent imaging, HepG2 cells were stably transfected by lentiviruses packed with the luciferase gene (GenePharma, Shanghai, China) to obtain the luciferase transgenic hepatocytes. For in vivo tracing, detection, and quantification, HepG2 cells and McA-RH7777 cells were transfected by lentiviruses packed with the GFP gene (GenePharma) to obtain the GFP-positive hepatocytes. For immune tolerance test in OT-1 transgenic mice, HepG2 cells expressing membrane-bound OVA (OVA-HepG2) were constructed by using a lentivirus to introduce the full-length OVA protein linked to the transmembrane region of H-2D^{b} (GenePharma) (37). To isolate hepatocytes from hepatized spleens, the spleens were digested with Accumax solution (Sigma-Aldrich) to generate cell suspension. The cell suspension was filtered through 40-μm cell strainers (BD Biosciences, San Diego, CA, USA) to obtain single cells. After red blood cell lysis, the pure hepatocytes were obtained using Percoll gradient method (38).

Nonhepatic cell preparation
Primary bone marrow cells obtained from 6-week-old C57BL/6 mice were cultured and differentiated into bone marrow–derived macrophages in DMEM containing 10% (v/v) heat-inactivated FBS (HyClone), mice granulocyte-macrophage colony-stimulating factor (10 ng/ml) and mice macrophage colony-stimulating factor (5 ng/ml; both from PeproTech, Rocky Hill, NJ, USA), and penicillin (50 U/ml) and streptomycin (50 μg/ml; both from Beyotime Biotechnology, Shanghai, China) (39). Primary embryonic fibroblasts (MEFs) were isolated, cultured, and purified from mouse fetus of C57BL/6 mice at the 13.5-day gestational age (40). The MEFs were cryopreserved at passage 1 and used from passages 3 to 5 for all experiments. Primary peripheral blood monocytes and macrophages from different organs were isolated following the manufacturer’s instructions (TBD Science, Tianjin, China). For isolation of splenocytes, the spleens were excised and placed in Hanks’ balanced salt solution (Thermo Fisher Scientific).
...and macerated using frosted glass slides. Cells were repeatedly aspirated with a sterile Pasteur pipette until a single-cell suspension was obtained. For isolation of splenic macrophages, after the single-splenocyte suspension was obtained, macrophages were separated using murine splenic macrophage isolation kit (TBD Science) according to the manufacturer’s instructions.

**Acquisition and analysis of STH**

To acquire STH, four types of tumor cells—mouse sarcoma cell line 7180 cells, mouse hepatoma cell line Hepa1-6 cells, mouse breast carcinoma cell line 4T1 cells, and mouse melanoma cell line B16-F10 cells (obtained from Institute of Biochemistry and Cell Biology)—were prepared and transplanted into mice (41). Briefly, 1 × 10^6 cells were transplanted by subcutaneous injections into the left armpits of the animals. The tumors were harvested after 1 month and homogenized in equal volume of ice-cold PBS (pH 7.4) to obtain 50% (w/v) homogenate. The homogenate was then centrifuged at 12,000 rpm for 20 min at 4°C to remove the insoluble tissue fragments, and the supernatant was collected. The steps of centrifugation and collection were repeated three times to obtain the final STH for subsequent use.

To analyze the composition of STH, the solution was subjected to label-free LC-MS through TripleTOF 5600 (AB SCIEX, Framingham, MA, USA) (42). Briefly, the samples were reduced with dl-dithiothreitol, alkylated with iodoacetamide, washed with tetramethylammonium bromide, digested with porcine sequencing-grade trypsin (LC-MS grade; all from Sigma-Aldrich), and subjected to LC-MS analysis. Samples were performed in three technical replicates. Identification of peptides and proteins from continuum LC-MS data was performed using the ProteinPilot 4.5 software (AB SCIEX).

**Remodeling of the spleen tissue**

For translocation of the spleen, the surgical operation illustrated in Fig. 1A, fig. S1, and movie S1 was designed by referring to a method of removing the spleen (43). The detailed process was as follows: (i) The mice were anesthetized with pentobarbital sodium (100 mg/kg of body weight) via intraperitoneal injection. (ii) The fur of mice was wetted with 70% ethanol and prevented from entering the peritoneum. (iii) A 2.5-cm-long skin incision midway between the last rib and the hip joint and a 1- to 2-cm incision in the peritoneal wall were made on the left side with the scissors. (iv) The spleen in the abdominal cavity was slightly pulled onto the exterior surface of the peritoneum and both the mesentry and connective tissue around blood vessels were cut away. (v) The peritoneal wall and the skin were closed with two separate sutures and three sutures, respectively, (vi) Any blood was removed from the spleen. The mice were returned to a clean cage until the anesthesia wore off.

To remodel the spleen, the precooled PBS, STH, or cytokine cocktail containing mouse TGF-β1, IL-10, and VEGF (all from PeproTech) was injected into the translocated spleen after the mouse was anesthetized. The detailed method of injection is shown in fig. S3 and movie S2. In short, PBS, STH, or cytokine cocktail (total of 50 μl per mouse) was injected into the spleen at five independent sites using a 31-gauge needle (BD Biosciences) every 4 days for the indicated times. After injection, the needle holes were pressed for 5 min with medical cotton ball to prevent leakage.

**Histological analyses**

**H&E staining, Masson’s trichrome staining, and Sirius Red staining**

Tissues were fixed in 4% paraformaldehyde (PFA) for 1 hour, processed, and embedded in paraffin. The sections (5 μm thick) were prepared and stained according to the manufacturer’s instructions (Jiancheng Bioengineering Institute, Nanjing, China).

**PAS and Oil Red O staining**

Spleen tissues were immediately embedded in optimal cutting temperature (OCT; Leica Microsystems, Buffalo Grove, IL, USA) medium and sectioned. The sections were stained following the manufacturer’s instructions (Jiancheng Bioengineering Institute). The stained sections were photographed at different magnification times under a BX51 microscope (Olympus, Tokyo, Japan).
Fluorescence in situ hybridization
The sections (5 μm) were deparaffinized, rehydrated, enzymatically disintegrated, and hybridized with human-specific GAPDH and rat-specific GAPDH (Abiocenter, Beijing, China; listed in table S9) according to the manufacturer’s instructions. Briefly, the sections were pretreated with pretreatment solution for 2 hours at 38°C and hybridized at 40°C overnight, followed by 4,6-diamidino-2-phenylindole (DAPI; Beyotime Biotechnology) for nuclear staining.

Immunostaining
Tissues were fixed in 4% PFA for 1 hour, processed, and embedded in paraffin or embedded in OCT medium. Sections (5 to 10 μm) were mounted on poly-L-lysine-coated glass slides (Citotest, Haimen, China). The sections with antigen retrieval were blocked with 5% bovine serum albumin (BSA) containing 3%o Triton X-100 for 1 hour and then stained with primary antibody at 4°C overnight. Next, the sections were incubated with the corresponding fluorescent secondary antibody (Thermo Fisher Scientific) for 45 min at room temperature, followed by DAPI staining for nuclei. Fluorescence including GFP bioluminescence was visualized under a Leica TCS SP8 confocal microscope (Leica Microsystems).

EdU cell proliferation assay in vivo
Mice received an intraperitoneal injection of EdU (5 mg/kg of body weight; RiboBio, Guangzhou, China). After 96 hours, the spleen was harvested, embedded in OCT, and sectioned. Staining of the EdU-labeled spleen was performed using Cell-Light Apollo Stain Kit (RiboBio) according to the manufacturer’s instructions. Sections were counterstained with DAPI and viewed under a Leica TCS SP8 confocal microscope.

ICG clearance test
Mice received an intravenous injection of ICG (10 mg/kg of body weight) 12 hours after 90% hepatectomy. After 15 min, the blood samples were collected, allowed to coagulate at room temperature, and centrifuged to obtain serum. The concentration of ICG in serum was analyzed using Varioskan LUX (Thermo Fisher Scientific) at 800 nm. Besides, to further detect the ICG uptake by hepatic spleens, the spleens were quickly removed. The spleen was immediately embedded in OCT and sectioned. Followed by three washes with PBS, the sections were photographed under a BX51 microscope.

Assessment of drug metabolism activity
DB (Sigma-Aldrich), which was metabolized to 4OHD (Enzo Life Sciences, Farmingdale, NY, USA) in humans but negligible in mice (18), was orally administrated (2 mg/kg of body weight) to the mice with remodeled spleens and transplanted human hepatocytes (HepG2 or hiPS-HEP) with untreated mice as control. Blood samples from the mice were collected 8 hours after drug administration. Plasma was separated by centrifugation from the blood. An internal standard (niflumic acid, Sigma-Aldrich; 1 μM) in 100 μl of methanol was added to 5 μl of plasma and centrifuged (15,000 rpm, 4°C, 5 min). The supernatant was subjected to LC–tandem MS (1290 Infinity LC/6400 QQQ MS, Agilent Technologies, Santa Clara, CA, USA). The sampling volume was 10 μl. The mobile phase was a water:acetonitrile (6:4) solution. In the first and second analysis of MS, the turbo gas was maintained at 300° and 250°C and the flow rate was set as 10 and 7 liters/min, respectively. The ionspray voltage was 3500 V; the m/z (mass/charge ratio) transition (Q1/Q3) ratios for DB, 4OHD, and internal standard were 176.5/134.2, 192.6/132.1, and 283.2/245.4, respectively. The metabolic ratios were determined by dividing $\frac{AUC_{8h}}{C_{t}}$ (the area under the curve at 8 hours) of 4OHD by that of DB.

Microarray analysis
Five cell/spleen samples from three independent experiments were pooled according to individual groups. For most experiments, total RNA was extracted with TRIzol (Thermo Fisher Scientific). The quantity and quality of RNA were measured by NanoDrop ND-1000 (Thermo Fisher Scientific). RNA integrity was assessed by standard denaturing agarose gel electrophoresis. The RNA sample for gene expression profiling was hybridized on the Whole Mouse Genome Oligo Microarray (4x44 K, Agilent Technologies) and analyzed with KangCheng Biotechnology (Shanghai, China). The scatterplot with highlighted genes was produced using ggplot 2. Hierarchical clustering was performed using Cluster 3.0, and the result was viewed by using Java TreeView. The “significantly differential” genes were sorted by setting a twofold change. KEGG pathway analysis was performed in the standard enrichment computation method. After KEGG pathway analysis, the pathways of interest and related genes were displayed in a histogram and heat map.

Polymerase chain reaction
gDNA was isolated from the spleen tissue using a DNA extraction kit (Generay, Shanghai, China). The total RNA was extracted with TRIzol. The quantity and quality of DNA/RNA were measured by NanoDrop Lite (Thermo Fisher Scientific). For RT-PCR, 1 μg of RNA was reversely transcribed into cdNA with PrimeScript RT Master Mix (Takara Bio Inc.). PCR was performed using AmpliTaq Gold Fast PCR Master Mix (Thermo Fisher Scientific) on Applied Biosystems ProFlex PCR (Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) was performed with SYBR Premix Ex Taq on an ABI 7500 fast real-time PCR system (Applied Biosystems, Waltham, MA, USA). Each sample was analyzed in triplicate within one test and repeated for three independent tests. Primer sequences are listed in table S7. For gDNA quantification, the standard curve was generated by qPCR analysis of commercial standard gDNA (Sangon). The quantity of gDNA was calculated by its interpolation in the standard curve. For qPCR analysis, the level of each target gene in each sample was normalized by subtracting the mean Ct value of β-actin gene from the mean Ct value of the target gene ($Ct_{target gene} - C_{\text{mean } \beta\text{-actin gene}}$). The difference in the level of each normalized target gene was obtained ($\Delta Ct_{\text{experimental group}} - \Delta Ct_{\text{control group}}$), and the fold difference was calculated using the equation $2^{-\Delta \Delta Ct}$.

Flow cytometry
To analyze blood samples, the whole blood was collected in K$_2$EDTA collection tubes (Terumo Medical, Somerset, NJ, USA). To analyze spleen samples, the tissue was digested with Accumax solution to generate cell suspension. After red blood cell lysis, cells were blocked with 2% BSA and incubated with the fluorescence-conjugated antibodies in the dark for 30 min at 4°C. For intracellular cytokine staining, splenocytes were incubated in a cell culture incubator for 10 hours with phorbol 12-myristate 13-acetate (50 ng/ml), ionomycin (750 ng/ml), and brefeldin A (10 μg/ml; all from Sigma-Aldrich). Surface staining was performed as described above. After surface staining, the cells were resuspended in a fixation/permeabilization solution (Cytofix/ Cytoperm Kit, Multi Sciences, Hangzhou, China). The intracellular cytokine staining was performed according to the manufacturer’s protocol. To analyze the frequencies of T$_{reg}$ cells in the spleens, samples
were prepared and detected following the manufacturer’s protocol (Mouse Regulatory T Cell Staining Kit, Multi Sciences). To determine the total number of GFP-positive hepatocytes in the spleen, total cells were resuspended in 1 ml of 2% BSA and mixed well after red blood cell lysing. Then, 100 μl of the mixture was transferred to a new test tube to determine the number of GFP-positive hepatocytes. Each sample was measured three times, and the total number of GFP-positive hepatocytes was calculated by multiplying the average of the three results by 10. Flow cytometry data were acquired on FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, San Diego, CA, USA). For sorting the GFP-positive hepatocytes in the spleen, a FACS machine (FACSAria II, BD Biosciences) was used to separate the negative cells and the GFP-positive after red blood cell lysing.

**Enzyme-linked immunosorbent assay**

Blood samples were collected, allowed to coagulate at room temperature, and centrifuged to obtain serum. The spleen was homogenized in equal volume of PBS to obtain 50% (w/v) homogenate and then centrifuged at 12,000 rpm for 10 min at 4°C to harvest supernatants. The levels of HSA, TNF-α, IL-1β, IFN-γ, IL-10, IL-12p70, TGF-β, IL-1β, VEGF, EGF, hepatocyte growth factor, IgG, IgM, COL1, COL4, phosphorylated–extracellular signal–regulated kinase 1/2 (p-ERK 1/2), and ERK 1/2 were measured using corresponding ELISA quantitation kits (Abcam, Cambridge, UK) according to the manufacturer’s instructions.

**Western blot**

Proteins were extracted from tissue homogenate in radioimmuno precipitation assay buffer (Beyotime Biotechnology) with a protease inhibitor (Sigma-Aldrich) and spun at 6000g for 10 min at 4°C. The lysate was resolved by SDS–polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane using Mini PROTEAN Tetra Electrophoresis System (Bio-Rad, CA, USA), and probed. Horseradish peroxidase (HRP)–linked anti-rabbit (Jackson ImmunoResearch, West Grove, PA, USA) was used, and the HRP signal was visualized with 4200SF (Tanon, Shanghai, China).

**Hardness testing**

The hardness of the spleen was measured using an elasticity-measuring instrument equipped with a coil spring (CL-150SL Type A Durometer, ASKER, Kyoto, Japan). To offer adequate thickness, five spleen samples were stacked together for the measurement. A constant force was applied to tissue samples, and the mean value representing three individual tests was recorded.

**Quantitation assay of the hydroxyproline content**

The content of hydroxyproline in tissue was measured using a hydroxyproline assay kit (Jiancheng Bioengineering Institute) to quantify collagen content following the manufacturer’s protocol. The data are expressed as hydroxyproline (in micrograms)/tissue wet weight (in grams).

**Doppler measurements**

Each mouse was examined in supine position after a rest of 15 min to avoid influence of posture and exercise. All subjects were scanned on a micro-ultrasound system with a 24-MHz transducer (Vevo 2100, VisualSonics, Canada). B-mode was used to display anatomical structures in two-dimensional (2D) grayscale image. Color Doppler mode was used to display blood flow in the portal vein. Pulse-wave Doppler mode was used to quantify the blood flow through the vessel.

**Synthesis and use of a fluorescent conjugated TGF-β1**

To assess the biodistribution of TGF-β1, TGF-β1 (PeproTech) was labeled with fluorescein isothiocyanate (FITC; Sigma-Aldrich). Briefly, FITC (1 mg/ml) was dissolved in ethyl alcohol and then was added to 0.05 M PBS (pH 7.2) containing TGF-β1 (1 mg/ml) at a ratio of 1:100 w/w (dye:protein). After 24 hours of stirring under light protection, the FITC-labeled TGF-β1 was purified and concentrated to 25 mg/ml using Amicon Ultra-4 device (3000 molecular weight cut-off; Sigma-Aldrich). Thirty minutes after intrasplenic injection of the FITC-labeled TGF-β1, the spleen was removed and immediately embedded in OCT for sectioning. The sections were photographed using a Leica TCS SP8 confocal microscope.

**Synthesis of a fluorescent conjugated bile acid**

To trace the flow of bile from the spleen, a fluorescence-conjugated bile acid analog was prepared. Briefly, a green fluorescent compound, N-butyl-4-(2-hydroxyethylamino)-1,8-naphtalimide (NNOH), was synthesized with a reported method (47). NNOH (7.5 mg/ml) was dissolved in dimethylformamide. Then, cholic acid (20.4 mg/ml), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (9.55 mg/ml), and 4-dimethylaminopyridine (1.25 mg/ml) from Sigma-Aldrich were added. The mixture was stirred at room temperature overnight. The solution was evaporated under vacuum. The residue was dissolved in CH2Cl2, washed with distilled water and brine, dried with MgSO4, and purified by silica gel column chromatography (48).

**Bioluminescence imaging**

IVIS Lumina XR System (Caliper Life Sciences, Hopkinton, MA, USA) was used to detect the donor hepatocytes (luciferase transgenic) and the direction of bile flow from the spleen in vivo. To test hepatocyte proliferation, the fluorescence intensity gated over the total was measured and analyzed using Living Image software (Caliper Life Sciences) after intraperitoneal injection of luciferin substrate (300 mg/kg of body weight; Promega, Madison, WI, USA). For tracing the bile flow from the spleen, the fluorescence intensity gated over the total body was detected and analyzed after intrasplenic injection of the fluorescence-conjugated bile acid (1 mg/kg of body weight).

**Micro–computed tomography**

The mice were examined with micro–CT for the regenerated tissue and vascular network in the spleen. The animals were perfused with 1 ml of iohexol (GE Healthcare AS, Munich, Germany) and euthanized 5 min after the injection of the contrast agent. The mice were scanned using the Skyscan 1176 (Bruker micro-CT, Brussels, Belgium) system at a resolution of 12.59 μm and a rotation step of 0.9830. The system comprised two metalceramic tubes equipped with a fixed Al filter (0.5 mm) and two digital x-ray cameras (1280 by 1024 pixels). Images were acquired at 50 kV and 455 μA. The 3D images of the vessels were reconstructed using CTvol (Bruker micro-CT), and the blood vessel density in the spleen was determined using CTan (Bruker micro-CT).

**Prothrombin time measurement**

Blood samples were obtained in a vacuum blood collection tube. Prothrombin time was measured with a Sysmex CS-5100 System (Siemens Healthineers, Erlangen, Germany).
Blood ammonia assessment

Blood ammonia in serum was quantified using the corresponding kit according to the manufacturer's instruction (Jiancheng Bioengineering Institute).

Assessment of multiple organ damage

AST, ALT, blood urea nitrogen, creatine kinase, and lactate dehydrogenase in the serum were evaluated using corresponding kits according to the manufacturer’s instructions (Jiancheng Bioengineering Institute).

Blood routine examination

Blood samples were collected and subjected to the counting for platelets, leukocytes, erythrocytes, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, thrombocytocrit, and mean platelet volume on Sysmex xe-5000 (Siemens Healthiners).

Statistical evaluation

Data are shown as means ± SEM. All data were normally distributed. Statistical analysis was performed using Prism Software (GraphPad, USA). Student’s t test and one-way and two-way analysis of variance (ANOVA) were performed, followed by Bonferroni’s multiple comparison test and log-rank test. Results were considered significant at P < 0.05.

SUPPLEMENTARY MATERIALS

Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/24/eaaz9974/DC1

View request a protocol for this paper from Bio-protocol.

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