Decoding the single-cell landscape and intercellular crosstalk in the transplanted liver: a 4-dimension mouse model

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Key words

liver transplantation; graft remodeling; crosstalk; single-cell RNA sequencing; cytometry by time-of-flight

Abbreviations

LT, liver transplantation; scRNA-seq, single-cell RNA sequencing; CyTOF, cytometry by time-of-flight; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IRI, liver ischemia-reperfusion injury; H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; tSNE, t-distributed stochastic neighbor embedding; GO, Gene Ontology; GSVA, gene set variation analysis; PCA, principal component analysis; NK, natural killer; DCs, dendritic cells; DEGs, differentially expressed genes; GDCs, graft-derived cells; RDCs, recipient-derived cells

ABSTRACT

Graft remodeling after transplantation maintains graft functionality and determines graft survival. However, a comprehensive understanding of cellular diversity and interplay during graft remodeling remains to be fully characterized. In this study, we established a well tolerant C57BL/6 to C57BL/6 orthotopic liver transplantation (LT) mice model and observed two stages of graft recovery including an acute phase and a steady phase. We next performed single-cell RNA sequencing (scRNA-seq) and cytometry by time-of-flight (CyTOF) and recorded the cellular hierarchy in the transplanted liver during the two stages. Besides the dynamic change of cell proportion, it was notable that recipient-derived cells took over the transplanted liver in most cell types (e.g., B cells, T cells, dendritic cells, granulocytes and monocytes) except CD206⁺ MerTK⁺ macrophages and CD161⁺ CD49a⁺ CD49b⁻ natural killer cells. We then focused on macrophages and captured 5 distinct transcriptional signatures to define novel subclusters. Using a ligand-receptor interaction strategy, we identified specific macrophage-hepatocyte interactions during the acute and stable phases, causing metabolic remodeling in the transplanted liver. Our results delineated a 4-dimension cell atlas (type-proportion-source-time) of the transplanted liver, which sheds light on the physiological process of liver graft maintenance and graft-recipient crosstalk.

INTRODUCTION

Liver transplantation (LT) is the most effective salvage treatment for various end-stage liver diseases [1]. With the development of the transplant technique and immunosuppressant in the last decades, LT has become a routine surgical procedure with a high success rate [2]. Long-term survival and improved quality of life are the current purposes after LT [3, 4]. In liver recipients, long-term graft survival mostly relies on the prompt recovery of graft function and avoidance of post-transplant complications such as graft dysfunction, rejection, biliary complications and metabolic disorders [5-7].

Graft remodeling has been considered a complex and well-orchestrated process that involves the cellular composition and functional state from multiple lineages [8]. In LT, immune cells derived from the recipient's peripheral blood invade through the hepatic sinusoid and move towards the interstitial space, where interplay with graft-derived cells to further reorganize immune and metabolic homeostasis [9, 10]. During the early post-LT period, parameters reflecting liver injury and inflammatory response would be predictors for prognosis. For example, alanine aminotransferase (ALT) and γ -GT levels on postoperative day 1 have been considered as independent predictors of early allograft dysfunction [11]. A combined detection of IL-10, IL-17, and CXCL10 hold an excellent prediction property for acute rejection [12]. Therefore, a deep understanding of graft remodeling including the recipient-graft interplay is essential to promote the recovery of the transplanted liver and prevent post-transplant complications.

The single-cell approach has emerged as a powerful tool to deconstruct the composition and function of complex tissues at single-cell resolution [13]. Recently, single-cell RNA sequencing (scRNA-seq) has been applied to dissect the complex landscapes of organogenesis, including liver development [14] and decidualization [15]. The advent of the high-throughput single-cell profiling technology provides a foundation for "transplanted liver atlas". In this study, we employed scRNA-seq and cytometry by time-of-flight (CyTOF) to comprehensively reveal the cell states and sources involved in liver graft remodeling. We then uncovered an interplay between hepatocytes and macrophages using ligand-receptor interactions based on the transcriptome data [16]. Our analyses constructed a detailed molecular and cellular map and revealed complex intercellular crosstalk in the transplanted liver.

MATERIALS AND METHODS

Animals treatments

Male 8-12 weeks old wild-type C57BL/6 mice and C57BL/6-Tg (CAG-EGFP) mice were purchased from Shanghai Model Organisms Center. All mice were housed at 20°C with a 12:12 light-dark cycle in a specific pathogen-free animal facility and fed ad libitum with a normal chow diet. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee and the Ethics Committee of Zhejiang University. Livers from C57BL/6-Tg (CAG-EGFP) mice (all tissue

expressing eGFP) used as donors and wild-type C57BL/6 mice were used as recipients for the orthotopic LT model. Surgery was performed as we described before [17]. After the postoperative restoration of temperature and rehydration, mice were sent to the individually ventilated cages for housing. The transplanted liver and serum samples were collected at 3H, 6H, 12H, 1D, 3D, 7D, and 14D post-LT. Biochemical parameters including ALT and aspartate aminotransferase (AST) were measured with a BioVision system. The severity of liver ischemia-reperfusion injury (IRI) was graded by modified Suzuki's histological criteria [18].

Immunohistochemistry

Immunohistochemistry was performed as we described previously [19]. Liver specimens (4μm) were stained with hematoxylin and eosin (H&E). Two independent pathologists reviewed and confirmed the pathohistological features of all the samples. For staining of neutrophils and macrophages, anti-Ly6g (Abcam, USA, ab238132) and anti-F4/80 (Bio-rad, USA, MCA497R) were used. For terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, liver specimens were stained using the In Situ Cell Death Detection Kit (Roche, USA, 11684817910) according to the manufacturer's protocol. Labeled cells were counted with 5 randomly selected 400× fields from each sample.

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA, 15596018) and quantitative real-time PCR was performed using the HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme Biotech, China, Q221-01) in an Applied Biosystems QuantStudio 5 Real-Time PCR Instrument (Applied Biosystem, USA) as we described previously [17]. The detailed primer information was provided in [Table S1](#).

Tissue dissociation and preparation

Liver tissues were obtained from pre- and post-LT period. The tissues were transferred into sterile RNase-free culture dishes containing an appropriate amount of calcium-free and magnesium-free PBS on ice and cut into 0.5 mm³ pieces. Then the tissues were washed with PBS and dissociated into single cells in dissociation solution (0.35% collagenase IV, 2 mg/ml papain, 120 Units/ml DNase I) in 37°C water baths with shaking at 100 rpm for 20 min. Digestion was terminated by PBS containing 10% fetal bovine serum (V/V). The suspensions were passed through 100 μm cell sieves (Corning, Japan, 431752) and then 40 μm cell sieves (Corning, Japan, 431750). The flow-through was centrifuged and resuspended in 5 ml of red blood cell lysis buffer (Invitrogen, USA, 00-4300) for 10 min. After incubation, the suspension was centrifuged at 300 g for 5 min at 4°C. For scRNA-seq, the overall cell viability was confirmed by trypan blue (over 85%), single-cell suspensions were counted using a Countess II Automated Cell Counter (Invitrogen, USA, 00-4300), and concentration adjusted to 700-1200 cells/μl.

CyTOF

Liver single-cell suspensions were processed for CyTOF analysis according to the standard protocol

as we described previously [17]. Briefly, the single-cell suspensions were labeled by 194Pt for 5 min to distinguish live or dead and incubated with Fc blocking mix for 20 min at room temperature to block Fc receptors. Then the single-cell suspensions were stained for 30 min with an antibody mix panel (Table S2), followed by DNA Intercalator-Ir overnight staining. Intracellular markers were stained using an intracellular antibody mix for 30 min. Finally, the single cells were washed with PBS, and the signals were detected using a CyTOF system (Fluidigm, USA). The types of immune cells were identified via t-distributed stochastic neighbor embedding (tSNE), followed by density clustering [20]

scRNA-seq

Liver single-cell suspensions were loaded into the 10x Chromium Controller, and the 10x Genomics Chromium Single Cell 3' kit (V3) was applied according to the manufacturer's protocol. Libraries were sequenced on an Illumina NovaSeq 6000 sequencing system. We used FastQC to perform basic statistics on the quality of the raw reads. Raw reads were demultiplexed and mapped to the reference genome by 10x Genomics Cell Ranger pipeline (<https://support.10xgenomics.com/single-cell-geneexpression/software/pipelines/latest/what-is-cell-ranger>). All downstream single-cell analyses were performed using Cell Ranger (version 3.1.0) and Seurat (version 3.2.0) unless mentioned specifically. In brief, for each gene and each cell barcode (filtered by CellRanger), unique molecule identifiers were counted to construct digital expression matrices. Secondary filtration by Seurat: A gene with expression in more than 3 cells was considered as expressed, and each cell was required to have at least 200 expressed genes. We removed low-quality cells with > 20% mitochondrial RNA content and included cells with > 200 and < 6000 genes expressed per cell. Seurat was used to normalizing data, dimensionality reduction, clustering and differential expression analysis. To eliminate batch effects, a reference Seurat object was generated by identifying anchor genes between samples as described previously [14]. For clustering, highly variable genes were selected and the principal components based on those genes were used to build a graph. The tSNE analysis was used for dimension reduction and visualization of gene expression. For differential expression analysis, marker genes for each cluster were determined by comparing the cells of each cluster to all other cells using the FindAllMarkers function in Seurat. For all comparisons between groups and clusters, only genes expressed by at least 10% of cells were included. Gene Ontology (GO) enrichment analysis of marker genes was implemented by the clusterProfiler R package (version 3.16.1), in which gene length bias was corrected. GO terms with corrected *P* value less than 0.05 were considered significantly enriched by marker genes. gene set variation analysis (GSVA) analysis of distinct clusters was performed by the GSVA R package (version 1.36.2)[21].

Cell-cell interplay analysis

To enable a systematic analysis of cell-cell communication, we applied nichenetr package (version 1.0.0) to predict cell-cell interplay as previously reported [22]. NicheNet could predict ligands of

sender cells that are the most active in affecting gene expression of receiver cells, through the correlation of ligand activity with genes characterized as targets in a prior model that integrates existing knowledge on ligand-to-target signaling path [16]. Using macrophages and hepatocytes subsets, we applied NicheNet to predict ligands that might influence transcription during hepatocytes metabolic remodeling. In the integration analysis, hepatocytes were defined as the receiver population and all macrophage subclusters were defined as potential sender cells. In separate analyses, target macrophage subclusters were defined as potential sender cells. Gene sets of interest were defined based on metabolic-related genes in MSigDB gene sets, including regulation of glucose metabolic process, insulin signaling pathway and lipid homeostasis (Table.S3). A background gene set includes all other genes expressed in at least 10% of cells in hepatocytes. Activities of ligands were ranked by calculating Pearson correlation coefficients of the ligand-target regulatory potential scores for each ligand and the target indicator vector, which defines a gene as present or absent within the gene set of interest. The circlize R package (version 0.4.10) was used to represent the regulatory networks between the predicted ligands in distinct macrophage subclusters and their receptors and targets expressed in hepatocytes. Cytoscape (version 3.8.0) was used to depict the regulatory networks between the predicted ligands in the target macrophage subclusters and their targets expressed in hepatocytes.

RESULTS

Liver graft recovery following transplantation

Liver graft injury is inevitable due to multiple factors including IRI and surgical stress. To monitor the liver graft recovery after transplantation, we established an orthotopic LT mice model and collected serum and liver tissue samples during the perioperative period (Pre-LT, 3H, 6H, 12H, 1D, 3D, 7D and 14D post-LT; n = 3/group, Fig.1A). We observed sharply increased ALT and AST levels at 3H post-LT. From 3H to 1D, serum ALT and AST levels gradually decreased and maintained steady after 1D (Fig.1B). We next assessed the morphology organization of transplanted livers using histopathology methods. H&E staining showed the destruction of hepatic architecture as presented by sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration. Suzuki's histological grading of hepatocellular damage displayed the highest score at 1D (Fig.1C). We further found consistent results in hepatocellular necrosis/apoptosis assessment (Fig.1D). Moreover, we assessed inflammatory cell infiltration using immunohistology and observed that neutrophils and macrophages maintained at high levels during the first day and reduced thereafter (Fig.1E). The cytokine and endothelial cell activation marker expression patterns showed a similar trend with inflammatory cells (Fig.1F). The results suggested an acute injury of graft function within 1D (acute phase) and a steady recovery of graft function after 3D (stable phase).

Mapping cellular hierarchy in the transplanted liver

To map the cellular diversity and the dynamic change during the perioperative period, we generated scRNA-seq profiles from 6 liver samples (pre-LT, 3H, 6H, 12H, 3D and 7D post-LT) using 10x Genomics sequencing (Fig.2A). We obtained a total of 22,119 single-cell transcriptomes for analysis. We conducted gene expression normalization, principal component analysis (PCA), and tSNE. Finally, all liver cells could be assigned to 10 distinct types based on marker gene expression and scMCA (<http://bis.zju.edu.cn/MCA/blast.html>). They were granulocyte (38.8%, marked with *Ly6g*), T cells (14.5%, marked with *Cd3d*), macrophages (13.1%, marked with *Adgre1*), endothelial cells (9.4%, marked with *Bmp2*), B cells (8.3%, marked with *Cd79a*), dendritic cells (DCs, 6.4%, marked with *Itgax*), natural killer (NK) cells (5.9%, marked with *Klrb1c*), hepatocytes (2.1%, marked with *Apoa1*), biliary epithelial cells (1.0%, marked with *Krt19*) and fibroblast (0.4%, marked with *Acta2*, Fig. 2B). The differentially expressed genes (DEGs) and GO enrichment analysis further confirmed the cell identity (Fig. 2C and Fig.S1A). Moreover, we observed a significant variation in the proportion of immune cells during the perioperative period (Fig.2D). For instance, granulocytes increased rapidly from 8.1% to 78.3% at 6H post-LT and macrophages increased from 9.7% to 29.7% at 12H post-LT, indicating an IRI-induced inflammatory response. Both T cells and B cells dropped to less than 15% after 6H post-LT, suggesting a tolerance in our model. (Fig.2D and Fig.S1B).

The cellular source in the transplanted liver

To explore the comprehensive graft-recipient interaction following LT, we used an eGFP-labeled donor mouse to distinguish graft-derived cells from recipient-derived cells. The efficiency of eGFP fluorescence was 90% according our previous report [17]. We acquired liver tissue samples at pre-LT, 12H, 1D, 3D, 7D and 14D post-LT and purified CD45⁺ liver nonparenchymal cells for CyTOF analysis (Fig. 3A), which comprises 42 antibodies. Cells were initially gated and t-SNE visualized to distinct populations. A schematic clustering explanation is depicted in Fig. 3B. Among the 29 clusters, No. 5 could not be categorized and the other 28 were defined as 7 cell types including B cells (2 clusters, marked with CD19), T cells (5 clusters, marked with CD3), DCs (5 clusters, marked with CD11c), NK cells (3 clusters, marked with NKp46 or CD49b), macrophages (8 clusters, marked with F4/80), granulocytes (2 clusters, marked with LY6G) and monocytes (3 clusters, marked with LY6C; Fig. 3C). We observed that graft-derived cells (GDCs) dramatically decreased from 100% to 11%, whereas the recipient-derived cells (RDCs) sharply increased from 0% to 89% within the first day post-LT (Fig. 3D). The cell source maintained a steady level from 1D to 14D. RDCs accounted for most cells at 14D post-LT (26/29 clusters) (Fig. 3E). Of note, two M2 macrophage clusters (cluster 1 and cluster 23; F4/80⁺ CD206⁺ MerTK⁺) and one NK cell cluster (cluster 16; CD161⁺ CD49a⁺ CD49b⁺) were composed of around half GDCs at the stable phase (Fig. 3F).

Metabolic remodeling in the transplanted liver

Considering the specific source and vital role of the macrophage in hepatic homeostasis particularly

glucose and lipid metabolism, we focused on the metabolic remodeling by analyzing the macrophage-hepatocyte interplay. First, we re-analyzed the scRNA-seq data of macrophages and stratified them into 5 subclusters with distinct transcriptomic signatures (Fig. 4A). We identified the function of different subclusters using GSVA, which showed that subclusters 0, 1, 2, 3 and 4 were associated with pro-inflammation, ribosome and proteasome, metabolism, immune regulation and allograft rejection, respectively (Fig. 4B). We found that subcluster 0 and 1 expressed high levels of *Ly6c2* and *Ccr2* (immature monocyte-derived macrophages genes), while subcluster 3 expressed low level *Ccr2* and high level of *Cx3cr1* (mature monocyte-derived macrophages genes). Subcluster 2 expressed a certain number of *Adgre1*, *Clec4f* and *Timd4* (Kupffer cells genes). Moreover, we assumed that subcluster 0 and 1 belonged to the M1 phenotype due to the pro-inflammatory markers such as *Tnf*, *Ccl2* and *Ccl9*, while subcluster 2 belonged to the M2 phenotype due to the prominent expression of M2 markers such as *Mrc1* (coding CD206), *Cd163* and *Mertk* (Fig. 4C). We observed the dynamic change of different subclusters in the acute and stable phases. For instance, subcluster 0 sharply increased in the acute phase and decreased in the stable phase, whereas subcluster 2 dramatically decreased in the acute phase and partially recovered in the stable phase (Fig. 4D).

We should also note that subcluster 2 was comprised of both graft- and recipient-derived cells (F4/80⁺ CD206⁺ MerTK⁺ Macrophages) according to the CyTOF findings. In subcluster 2, the genes involved in anti-fibrosis and metabolism such as *Cd5l* [23], *Timd4* [24], *Clec2* [25] and *Pltp* [26] were significantly up-regulated and the genes related to inflammation such as *Tnf*, *Il1b* and *Cxcl2* [27] were significantly down-regulated compared to other macrophages (Fig. 4E). Furthermore, a comparison of subcluster 2 in the acute and stable phases with that at pre-LT revealed distinct transcriptional programs (Fig. 4F). Compared with pre-LT, genes highly expressed in subcluster 2 in the acute phase were enriched in cytokine receptors and extracellular vesicles. In the stable phase, highly expressed genes were mainly enriched in cytoplasmic translation and ribosome.

Second, we analyzed the metabolic activity of hepatocytes using GSVA and found decreased gluconeogenesis, insulin signaling and lipid metabolic process whereas increased glycolysis in the acute phase. The changed glucose and lipid metabolism well recovered in the stable phase (Fig. 5A). Next, we performed NicheNet, which could show ligand-receptor interactions and ligand-to-target signaling paths [16], to analyze the macrophage-hepatocyte interplay. A circle plot was designed to show the interaction between the receptors and target genes in hepatocytes and the most active ligands in macrophages (Fig. 5B). Macrophage subcluster 0, 1, 2 and 4 presented potential specific ligands that regulate insulin signal, glucose and lipid metabolic genes in hepatocytes. For example, *Ceacam1*, *Cxcl2*, *Tnf* and *Itgam* were most expressed in subcluster 0, while *Cxcl13*, *Cxcl16*, *ApoE* and *Nampt* were expressed in subcluster 2.

Based on the abundant crosstalk between subcluster 0 / 2 and hepatocytes, we isolated the 3 populations and further predicted the specific ligand-receptor interactions. Ligands activity analysis showed the top 10 ligands in subcluster 0 and 2 (Fig. 5C). For instance, *Tnf*, coding cytokine involved

in hepatocyte cell death as well as insulin resistance and lipid accumulation [28, 29], was mainly expressed by subcluster 0. The expression of *Tnf* increased significantly in the acute phase and recovered in the stable phase (Fig.5D). In addition, hepatocytes strongly expressed Tnf receptor genes *tnfrsf1a* and *tnfrsf1b* in the acute phase, suggesting an enhanced Tnf-Tnf receptor interaction in the acute phase (Fig.5E). Target genes prediction showed that *Tnf* could regulate different types of metabolic genes, including glucose metabolism genes (*Sirt1*, *Ppara*, *Akt1*, etc.), lipid metabolism genes (*Cebpa*, *Nr1h2*, *Apoa1*, etc.) and insulin pathway genes (*Pck1*, *Socs3*, *Ptprt1*, etc.; Fig.5F). For another example, *Apoe*, coding apolipoprotein E that involved in lipid and lipoprotein metabolism, was mainly expressed by subcluster 2. The expression of *Apoe* and Apoe receptor genes, such as *Sor11* and *Ldlr*, decreased in the acute phase and increased in the stable phase, suggesting an enhanced Apoe-Apoe receptor interaction in the stable phase. Furthermore, target genes prediction showed that *Apoe* mainly regulated lipid metabolism-related genes in hepatocytes such as *Srebf1*, *Abca1*, *Apoa1*, *Apoa2*, *Apob*, *Igf1*, *Ldlr*, etc. Other ligands such as *Ceacam1*, *Rtn4*, *C3*, *Cxcl2*, *Cxcl13*, *Timp1*, *Calml1* and *Tgfb1*, were also predicted to regulate a series of metabolic genes in hepatocytes and ultimately contribute to the changes in metabolism-related genes during the perioperative period.

DISCUSSION

The occurrence of primary nonfunction, graft dysfunction or rejection dramatically reduced both graft and recipient survival. Therefore, graft recovery during the 7-10 days after LT greatly determines the outcome [30]. In this study, we established a tolerant mice model to understand the physiological process of graft recovery and graft-recipient interaction during the early post-LT period. According to the dynamically detected biochemical and pathological parameters, we proposed a two-phase theory of graft recovery following LT, which includes an acute phase and a stable phase. Acute phase was represented by sharply increased hepatocyte damage and inflammatory cell infiltration, occurring at very early stage typically within 24h following LT. The emergence of the peaks of aminotransferases in our model was consistent with most of previous studies [18, 31]. Also, the graft injury would be affected by multiple factors such as ischemia time and perfusate, causing diversity in graft recovery [32, 33]. Stable phase showed a well recovered liver function after the acute graft injury, which probably started from 3D after LT. Then we conducted a single cell technology for the in-depth understanding of graft remodeling in both acute and stable phases.

We described the transcriptomic landscape of the transplanted liver and depicted the cellular atlas, including the cell types and proportion. The diversity of liver parenchymal and non-parenchymal populations was consistent with previous studies both in mice [34] and human [21]. Given the distinct cell types, we found that the cellular composition of the transplanted liver has changed dramatically during the perioperative period. The persistently elevated inflammatory cells (granulocytes and macrophages) in the transplanted liver throughout the acute and stable phases revealed an IRI-induced inflammatory response, which has been associated with the development of post-transplant

complications such as tumor recurrence, diabetes, hyperlipidemia and fibrosis [35]. In addition, the transplanted liver in the stable phase had decreased proportions of T cell and B cell, which were known to promote rejection [36], indicating tolerance in the stable phase.

We next focused on the graft-recipient source in distinct cell types. Our previous pioneering study using the same strategy and focusing on the monocyte-derived cells has revealed that RDCs took over the majority of cells, including monocytes, DCs, M0 and M1 macrophages. This study further explored the cellular source of various nonparenchymal (e.g., macrophages, NK cells, DCs, endothelial cells, T cells and B cells) in the transplanted liver. The results were consistent with our previous findings that GDCs disappeared in a short time and RDCs made the major contribution to Kupffer cells pool. The result challenged the classical theory that Kupffer cells are locally proliferate and self-renew [37] at least under the circumstance of LT. Furthermore, not only the monocytes-derived cells, most nonparenchymal cells were refreshed by RDCs at steady phase, suggesting a pivotal role of graft-recipient crosstalk during graft remodeling. It was very interesting to find that GDCs were still accounted for a half of proportion in certain cell populations including $F4/80^+ CD206^+ MerTK^+$ Kupffer cells and $CD161^+ CD49a^+ CD49b^-$ NK cells. $F4/80^+ CD206^+ MerTK^+$ Kupffer cells have been associated with the resolution of inflammation and the compromise repair after ischemia-reperfusion [38, 39], as well as the improved hepatic insulin sensitivity [40]. However, these macrophages with an anti-inflammatory and reparative phenotype have been revealed to drive hepatocellular carcinoma progression and metastasis [37]. In addition, a recent study on $CD49a^+$ liver-resident NK have showed that a role for $CD49a^+$ NK cells in the negative regulation of immune responses and the development of hepatocellular carcinoma[41]. Investigation of diseases associated with Kupffer cells and NK cells after LT (e.g., liver fibrosis [42], rejection[43] and hepatocellular carcinoma [44]) should consider both the graft and recipient genetic backgrounds.

Our previous studies in both large human cohorts [45, 46] and mice models [19] demonstrated an imbalanced hepatic metabolic homeostasis after LT. This study not only proved that hepatocytes showed a metabolic reprogram after LT including insulin resistance, glucose and lipid metabolic homeostasis, but also revealed a comprehensive interplay between macrophage and hepatocytes in the transplanted liver. In previous studies, scRNA-seq analysis has been used to elucidate the diversity of macrophages in human and mouse livers [47, 48]. In this study, we defined 5 macrophage subclusters in the transplanted liver and preliminarily explored their functional transformation at different phases, such as subcluster 0 with high pro-inflammatory activity in the acute phase and subcluster 4 with suppressed rejection activity in the stable phase. Further exploring the interplay between liver macrophages and hepatocytes, macrophages subcluster 0 and subcluster 2 hold the most abundant ligand-receptor pairs and excellent regulatory potential, suggesting an effectively regulation in the metabolic remodeling. For instance, the Tnf - Tnf receptor pair in the acute phase was significantly enhanced, which have been found to be crucial for the development of metabolic disorders [49]. In addition, an enhanced $Apoe$ - $Apoe$ receptor pair, promoting the clearance of remnants of

triglyceride-rich lipoproteins[50], was found to contribute to the stable phase. Our scRNA-seq data and functional analysis showed that the metabolic remodeling of the transplanted liver was a very complex process, involving a regulatory network of a series of ligands and receptors between macrophages and hepatocytes.

The study had both advantages and limitations. First, to the best of our knowledge, this is the first study to map a 4-dimension cell atlas of the transplanted liver based on a well tolerant LT mice model. The open source website construction is in progress and the datasets would be available for further analyses such as the comparison with the rejection model or marginal graft model. Second, our finding shed a light on the intrahepatic donor-recipient crosstalk, which is the key to understand liver transplant immunology and pathophysiology. For instance, the F4/80⁺ CD206⁺ MerTK⁺ hepatic macrophages had both donor- and recipient-sources. Since this subclass involves in liver fibrosis, nonalcoholic steatohepatitis and tumor recurrence, both donor and recipient genetic background potentially affect the long-term survival of recipients [37, 51]. The predicted ligand-receptor interactions need to be further proved and more cellular interplays need to be explored.

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DISCLOSURE

Q.J. is a member of PLT Tech Inc.

AUTHOR CONTRIBUTIONS

Q.L. designed the research; H.H., S.F., T.R., and H.C. performed the research; H.H., X.Z and Q.J. analyzed the data; H.H. and R.C. wrote the manuscript; Q.L. reviewed the manuscript.

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FIGURE LEGENDS

Figure 1 Liver graft recovery in orthotopic liver transplantation mice model. (A) Experimental design in mice. (B) Serum ALT and AST levels in mice. (C) H&E staining and Suzuki's histological grading of transplanted livers. (D) TUNEL staining of transplanted livers. (E) Neutrophils (LY6G+) and macrophages (F4/80+) infiltration in transplanted livers. (F) The expression of cytokine and endothelial cell activation markers in transplanted livers. ALT, alanine aminotransferase; AST, aspartate aminotransferase; H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

Figure 2 The cellular hierarchy in the transplanted liver. (A) Schematic diagram of the scRNA-seq analysis workflow. (B) tSNE plots for the cell type identification of single cells (left); Violin plots showing marker genes for 10 distinct cell types (right). (C) Heatmap showing the top 5 DEGs in each cell type. (D) Bar plots showing the proportion of immune cells at pre-LT, 3H, 6H, 12H, 3D, and 7D post-LT. scRNA-seq, single-cell RNA sequencing; tSNE, t-distributed stochastic neighbor embedding; DEGs, differentially expressed genes; LT, liver transplantation

Figure 3 The cellular source in the transplanted liver. (A) Schematic diagram of the CyTOF analysis workflow. (B) tSNE plots for the cell type identification of single cells. (C) Heatmap showing the expression levels of markers in distinct clusters. (D) tSNE and pie plots showing the proportion of GDCs and RDCs at pre-LT, 12H, 1D, 3D, 7D and 14D post-LT. (E) Bar plots showing the proportion of GDCs and RDCs in distinct clusters at 14D post-LT. (F) Bar plots showing the proportion of GDCs and RDCs in cluster 1, 23 and 16 at pre-LT, 12H, 1D, 3D, 7D and 14D post-LT. CyTOF, cytometry by time-of-flight; tSNE, t-distributed stochastic neighbor embedding; GDCs, graft-derived cells; RDCs, recipient-derived cells; LT, liver transplantation

Figure 4 Distinct macrophage subclusters in the transplanted liver. (A) tSNE plots of total macrophages (left); Heatmap showing the top 30 DEGs for each subcluster (right). (B) Pathway activity (scored by GSVA) in 5 subclusters. (C) tSNE plots color-coded for the expression (gray to red) of marker genes for the distinct subclusters. The red circle showed subcluster 2. (D) tSNE and bar plots showing the proportion of distinct subclusters at pre-LT, acute phase and stable phase. (E) Volcano plot exhibiting DEGs between subcluster 2 and other macrophages. (F) Scatterplots showing genes upregulated in either acute phase and/or stable phase of subcluster 2 (left); Bar plots showing enriched GO terms of the genes overexpressed by subcluster 2 in the acute phase and stable phase (right). tSNE, t-distributed stochastic neighbor embedding; DEGs, differentially expressed genes; GSVA, gene set variation analysis; LT, liver transplantation; GO, Gene Ontology.

Figure 5 Macrophage-hepatocyte interplay in the transplanted liver. (A) Heatmap showing the metabolic-related pathway activity (scored by GSVA) in hepatocytes. (B) Circle plot showing links between ① predicted ligands from subcluster 0, subcluster 1, subcluster 2, subcluster 4 or common, ② their associated receptors found on hepatocytes and ③ metabolic genes potentially targeted by the ligand-receptor pairs. (C) Potential upstream ligands in subcluster 0 and subcluster 2; (D) Bubble plot showing the expression of potential upstream ligands in subcluster 0 and subcluster 2 at pre-LT, acute phase and stable phase; (E) Cube plot showing the potential receptors expressed by hepatocytes associated with each potential ligands, and their expression at pre-LT, acute phase and stable phase. (F) Network plot showing links between predicted ligands and metabolic genes. GSVA, gene set variation analysis; LT, liver transplantation.

SUPPLEMENTAL

Figure S1 Function and proportion of each cell type. (A) GO analysis of DEGs in distinct cell types. (B) tSNE plots of distinct cell types at pre-LT, 3H, 6H, 12H, 3D, and 7D post-LT. GO, Gene Ontology; DEGs, differentially expressed genes; tSNE, t-distributed stochastic neighbor embedding; LT, liver transplantation

Table S1 List of primers used.

Target	Forward primer	Reverse primer
Tnfa	5'-CCCTCACACTCAGATCATCTTCT-3'	5'-GCTACGACGTGGGCTACAG-3'
Il1b	5'-GCAACTGTTCCCTGAACTCAACT-3'	5'-ATCTTTTGGGGTCCGTCAACT-3'
Il10	5'-GCTCTTACTGACTGGCATGAG-3'	5'-CGCAGCTCTAGGAGCATGTG-3'
Ifng	5'-ATGAACGCTACACACTGCATC-3'	5'-CCATCCTTTTGCCAGTTCCTC-3'
E-selectin	5'-AGTCTAGCGCCTGGATGAAA-3'	5'-ATCGCCACCAGATGTGTGTA-3'
P-selectin	5'-GTCCACGGAGAGTTTGGTGT-3'	5'-AAGTGGTGTTCGGACCAAAG-3'
Vcam1	5'-AAGAGAACCCAGGTGGAGGT-3'	5'-ACCTAGCGAGGCAAACAAGA-3'
Icam1	5'-CGAAGGTGGTTCTTCTGAGC-3'	5'-GTCTGCTGAGACCCCTCTTG-3'
Gapdh	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'

Table S2 Antibody panel

Label	Marker	Label	Marker	Label	Marker	Label	Marker	Label	Marker
89Y	CD45	147Sm	Ly6G	156Gd	CD1d	165Ho	CD161	174Yb	CTLA4
115In	CD3	148Nd	Ly6C	157Gd	CD335	166Er	Arginase1(Intra)	175Lu	TCRab
139La	CD44	149Sm	CX3CR1	158Gd	CD19	167Er	CD16	176Yb	MerTK
141Pr	CD192	150Nd	CD49b	159Tb	F4/80	168Er	FoxP3(Intra)	197Au	CD4
142Nd	MHCII	151Eu	EGFP(Intra)	160Gd	CD274	169Tm	CD127	198Pt	CD8a
143Nd	CD366	152Sm	CD11c	161Dy	iNOS(Intra)	170Er	CD49a	209Bi	CD11b
144Nd	Tbet(Intra)	153Eu	TCRgd	162Dy	CD206(Intra)	171Yb	GATA3(Intra)		
145Nd	GR1	154Sm	CD62L	163Dy	CD25	172Yb	CD279		
146Nd	TER119	155Gd	CD80	164Dy	RORgt(Intra)	173Yb	CD115		

Table.S3 Gene sets of interest

Genes	Sets
Acacb	Glucose metabolic process
Actn3	Glucose metabolic process
Adipoq	Glucose metabolic process
Adipor1	Glucose metabolic process
Akt1	Glucose metabolic process
Akt2	Glucose metabolic process
Arpp19	Glucose metabolic process
Bad	Glucose metabolic process
C1qtnf1	Glucose metabolic process
C1qtnf12	Glucose metabolic process
C1qtnf3	Glucose metabolic process
Clk2	Glucose metabolic process
Cox11	Glucose metabolic process
Cry1	Glucose metabolic process
Ddb1	Glucose metabolic process
Dgat2	Glucose metabolic process
Dgkq	Glucose metabolic process
Dusp12	Glucose metabolic process
Dyrk2	Glucose metabolic process
Enpp1	Glucose metabolic process
Ep300	Glucose metabolic process
Epm2aip1	Glucose metabolic process
Erfe	Glucose metabolic process
Esrrb	Glucose metabolic process
Fam3a	Glucose metabolic process
Fam3c	Glucose metabolic process
Fbp1	Glucose metabolic process

Foxa2	Glucose metabolic process
Foxk1	Glucose metabolic process
Foxk2	Glucose metabolic process
Foxo1	Glucose metabolic process
Gcg	Glucose metabolic process
Gcgr	Glucose metabolic process
Gck	Glucose metabolic process
Gckr	Glucose metabolic process
Gnb3	Glucose metabolic process
Gnmt	Glucose metabolic process
Gpld1	Glucose metabolic process
Gpt	Glucose metabolic process
Gsk3a	Glucose metabolic process
Gsk3b	Glucose metabolic process
Igf1	Glucose metabolic process
Igf2	Glucose metabolic process
Igfbp3	Glucose metabolic process
Igfbp4	Glucose metabolic process
Inpp5k	Glucose metabolic process
Ins2	Glucose metabolic process
Insr	Glucose metabolic process
Irs1	Glucose metabolic process
Irs2	Glucose metabolic process
Kat2a	Glucose metabolic process
Kat2b	Glucose metabolic process
Khk	Glucose metabolic process
Lcmt1	Glucose metabolic process
Lep	Glucose metabolic process
Lepr	Glucose metabolic process

Maea	Glucose metabolic process
Midn	Glucose metabolic process
Mlycd	Glucose metabolic process
Mst1	Glucose metabolic process
Mtor	Glucose metabolic process
Ncoa2	Glucose metabolic process
Nfe2l1	Glucose metabolic process
Nkx1-1	Glucose metabolic process
Nln	Glucose metabolic process
Ogt	Glucose metabolic process
Pask	Glucose metabolic process
Pdk1	Glucose metabolic process
Pdk2	Glucose metabolic process
Pdk3	Glucose metabolic process
Pdk4	Glucose metabolic process
Pfkfb1	Glucose metabolic process
Pfkfb2	Glucose metabolic process
Pgp	Glucose metabolic process
Phkg2	Glucose metabolic process
Phlda2	Glucose metabolic process
Pmaip1	Glucose metabolic process
Pomc	Glucose metabolic process
Ppara	Glucose metabolic process
Ppargc1a	Glucose metabolic process
Ppp1ca	Glucose metabolic process
Ppp1cb	Glucose metabolic process
Ppp1r3a	Glucose metabolic process
Ppp1r3b	Glucose metabolic process
Ppp1r3c	Glucose metabolic process

Ppp1r3d	Glucose metabolic process
Ppp1r3e	Glucose metabolic process
Ppp1r3f	Glucose metabolic process
Ppp1r3g	Glucose metabolic process
Ppp4r3b	Glucose metabolic process
Park2	Glucose metabolic process
Pth	Glucose metabolic process
Ptpn2	Glucose metabolic process
Ranbp2	Glucose metabolic process
Rgn	Glucose metabolic process
Rora	Glucose metabolic process
Rorc	Glucose metabolic process
Rubcnl	Glucose metabolic process
Sdhaf3	Glucose metabolic process
Selenos	Glucose metabolic process
Serpina12	Glucose metabolic process
Sesn2	Glucose metabolic process
Sik1	Glucose metabolic process
Sirt1	Glucose metabolic process
Sirt7	Glucose metabolic process
Slc25a12	Glucose metabolic process
Slc35b4	Glucose metabolic process
Slc45a3	Glucose metabolic process
Soga1	Glucose metabolic process
Sorbs1	Glucose metabolic process
Src	Glucose metabolic process
Tff3	Glucose metabolic process
Tigar	Glucose metabolic process
Trp53	Glucose metabolic process

Usp7	Glucose metabolic process
Wdr5	Glucose metabolic process
Zmpste24	Glucose metabolic process
Acaca	Insulin signaling pathway
Akt3	Insulin signaling pathway
Araf	Insulin signaling pathway
Braf	Insulin signaling pathway
Calm1	Insulin signaling pathway
Calm2	Insulin signaling pathway
Calm13	Insulin signaling pathway
Calm4	Insulin signaling pathway
Cbl	Insulin signaling pathway
Cblb	Insulin signaling pathway
Cblc	Insulin signaling pathway
Crk	Insulin signaling pathway
Crkl	Insulin signaling pathway
Eif4e	Insulin signaling pathway
Eif4e1b	Insulin signaling pathway
Eif4e2	Insulin signaling pathway
Eif4ebp1	Insulin signaling pathway
Elk1	Insulin signaling pathway
Exoc7	Insulin signaling pathway
Fasn	Insulin signaling pathway
Fbp2	Insulin signaling pathway
Flot1	Insulin signaling pathway
Flot2	Insulin signaling pathway
G6pc	Insulin signaling pathway
G6pc2	Insulin signaling pathway
Grb2	Insulin signaling pathway

Gys1	Insulin signaling pathway
Gys2	Insulin signaling pathway
Hk1	Insulin signaling pathway
Hk2	Insulin signaling pathway
Hk3	Insulin signaling pathway
Hras	Insulin signaling pathway
Ikbkb	Insulin signaling pathway
Inpp5d	Insulin signaling pathway
Irs4	Insulin signaling pathway
Kras	Insulin signaling pathway
Lipe	Insulin signaling pathway
Map2k1	Insulin signaling pathway
Map2k2	Insulin signaling pathway
Mapk1	Insulin signaling pathway
Mapk10	Insulin signaling pathway
Mapk3	Insulin signaling pathway
Mapk8	Insulin signaling pathway
Mapk9	Insulin signaling pathway
Mknk1	Insulin signaling pathway
Mknk2	Insulin signaling pathway
Nras	Insulin signaling pathway
Pck1	Insulin signaling pathway
Pck2	Insulin signaling pathway
Pde3a	Insulin signaling pathway
Pde3b	Insulin signaling pathway
Pdpk1	Insulin signaling pathway
Phka1	Insulin signaling pathway
Phka2	Insulin signaling pathway
Phkb	Insulin signaling pathway

Phkg1	Insulin signaling pathway
Pik3ca	Insulin signaling pathway
Pik3cb	Insulin signaling pathway
Pik3cd	Insulin signaling pathway
Pik3cg	Insulin signaling pathway
Pik3r1	Insulin signaling pathway
Pik3r2	Insulin signaling pathway
Pik3r3	Insulin signaling pathway
Pik3r5	Insulin signaling pathway
Pklr	Insulin signaling pathway
Gm5601	Insulin signaling pathway
Prkaa1	Insulin signaling pathway
Prkaa2	Insulin signaling pathway
Prkab1	Insulin signaling pathway
Prkab2	Insulin signaling pathway
Prkaca	Insulin signaling pathway
Prkacb	Insulin signaling pathway
Prkag1	Insulin signaling pathway
Prkag2	Insulin signaling pathway
Prkag3	Insulin signaling pathway
Prkar1a	Insulin signaling pathway
Prkar1b	Insulin signaling pathway
Prkar2a	Insulin signaling pathway
Prkar2b	Insulin signaling pathway
Prkci	Insulin signaling pathway
Prkcz	Insulin signaling pathway
Prkx	Insulin signaling pathway
Ptpn1	Insulin signaling pathway
Ptprf	Insulin signaling pathway

Pygb	Insulin signaling pathway
Pygl	Insulin signaling pathway
Pygm	Insulin signaling pathway
Raf1	Insulin signaling pathway
Rapgef1	Insulin signaling pathway
Rheb	Insulin signaling pathway
Rhoq	Insulin signaling pathway
Rps6	Insulin signaling pathway
Rps6kb1	Insulin signaling pathway
Rps6kb2	Insulin signaling pathway
Rptor	Insulin signaling pathway
Sh2b2	Insulin signaling pathway
Shc1	Insulin signaling pathway
Shc2	Insulin signaling pathway
Shc3	Insulin signaling pathway
Shc4	Insulin signaling pathway
Slc2a4	Insulin signaling pathway
Socs1	Insulin signaling pathway
Socs2	Insulin signaling pathway
Socs3	Insulin signaling pathway
Socs4	Insulin signaling pathway
Sos1	Insulin signaling pathway
Sos2	Insulin signaling pathway
Srebf1	Insulin signaling pathway
Trip10	Insulin signaling pathway
Tsc1	Insulin signaling pathway
Tsc2	Insulin signaling pathway
Abca1	Lipid homeostasis
Abca12	Lipid homeostasis

Abca2	Lipid homeostasis
Abcb11	Lipid homeostasis
Abcb4	Lipid homeostasis
Abcd1	Lipid homeostasis
Abcg1	Lipid homeostasis
Abcg5	Lipid homeostasis
Abcg8	Lipid homeostasis
Abhd4	Lipid homeostasis
Abhd5	Lipid homeostasis
Abhd8	Lipid homeostasis
Acox1	Lipid homeostasis
Acox2	Lipid homeostasis
Acox3	Lipid homeostasis
Acox1	Lipid homeostasis
Acsml	Lipid homeostasis
Acsml3	Lipid homeostasis
Adck1	Lipid homeostasis
Adora1	Lipid homeostasis
Akr1c21	Lipid homeostasis
Angptl3	Lipid homeostasis
Angptl4	Lipid homeostasis
Angptl8	Lipid homeostasis
Apoa1	Lipid homeostasis
Apoa2	Lipid homeostasis
Apoa4	Lipid homeostasis
Apoa5	Lipid homeostasis
Apob	Lipid homeostasis
Apoc2	Lipid homeostasis
Apoc3	Lipid homeostasis

Apoc4	Lipid homeostasis
Apoe	Lipid homeostasis
Apom	Lipid homeostasis
Asgr2	Lipid homeostasis
Atp13a2	Lipid homeostasis
Cav1	Lipid homeostasis
Cav3	Lipid homeostasis
Cd24a	Lipid homeostasis
Cebpa	Lipid homeostasis
Ces1d	Lipid homeostasis
Commd9	Lipid homeostasis
Cyp39a1	Lipid homeostasis
Cyp7a1	Lipid homeostasis
Cyp7b1	Lipid homeostasis
Ddx3x	Lipid homeostasis
Dgat1	Lipid homeostasis
Disp3	Lipid homeostasis
Ehd1	Lipid homeostasis
Enpp7	Lipid homeostasis
Ephx2	Lipid homeostasis
Fabp3	Lipid homeostasis
Fabp4	Lipid homeostasis
Fbxw7	Lipid homeostasis
Fgfr4	Lipid homeostasis
Fitm2	Lipid homeostasis
Gip	Lipid homeostasis
Got1	Lipid homeostasis
Gpam	Lipid homeostasis
Gpihbp1	Lipid homeostasis

Gramd1b	Lipid homeostasis
Hdac9	Lipid homeostasis
Hnf4a	Lipid homeostasis
Il18	Lipid homeostasis
Insig1	Lipid homeostasis
Lamtor1	Lipid homeostasis
Lcat	Lipid homeostasis
Ldlr	Lipid homeostasis
Ldlrap1	Lipid homeostasis
Lima1	Lipid homeostasis
Lipc	Lipid homeostasis
Lipg	Lipid homeostasis
Lpl	Lipid homeostasis
Lrp5	Lipid homeostasis
Mall	Lipid homeostasis
Malrd1	Lipid homeostasis
Med13	Lipid homeostasis
Mlxipl	Lipid homeostasis
Mttp	Lipid homeostasis
Myliip	Lipid homeostasis
Npc1	Lipid homeostasis
Npc2	Lipid homeostasis
Nr1d1	Lipid homeostasis
Nr1d2	Lipid homeostasis
Nr1h2	Lipid homeostasis
Nr1h3	Lipid homeostasis
Nr1h4	Lipid homeostasis
Nr1i2	Lipid homeostasis
Nr1i3	Lipid homeostasis

Nr5a2	Lipid homeostasis
Nus1	Lipid homeostasis
Ormdl1	Lipid homeostasis
Ormdl2	Lipid homeostasis
Ormdl3	Lipid homeostasis
Pcsk9	Lipid homeostasis
Pla2g10	Lipid homeostasis
Pla2g12b	Lipid homeostasis
Plscr3	Lipid homeostasis
Pnpla1	Lipid homeostasis
Pnpla2	Lipid homeostasis
Pnpla3	Lipid homeostasis
Pnpla5	Lipid homeostasis
Pnpla8	Lipid homeostasis
Pold1	Lipid homeostasis
Pparg	Lipid homeostasis
Raly	Lipid homeostasis
Rbp1	Lipid homeostasis
Rcn3	Lipid homeostasis
Rtn4	Lipid homeostasis
Scarb1	Lipid homeostasis
Sec24a	Lipid homeostasis
Slc25a27	Lipid homeostasis
Soat1	Lipid homeostasis
Soat2	Lipid homeostasis
Srebf2	Lipid homeostasis
Thada	Lipid homeostasis
Tlcd1	Lipid homeostasis
Tlcd2	Lipid homeostasis

Tm6sf2	Lipid homeostasis
Tmem97	Lipid homeostasis
Ttc39b	Lipid homeostasis
Usf1	Lipid homeostasis
Usf2	Lipid homeostasis
Vdr	Lipid homeostasis
Xbp1	Lipid homeostasis
Zbtb20	Lipid homeostasis