Achieving permanent transplant tolerance via reverse chimeras in orthotopic liver transplantation

gaofeng Tang
zhengzhou university
huibo zhao
Guoyong Chen (chenguoyong@zzu.edu.cn)

Research Article

Keywords:

Posted Date: October 28th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2212066/v1

Abstract

The severe side-effects and toxicities associated with lifelong immunosuppression after organ transplantation necessitate the quest for immunological tolerance, it is a durable immunosuppression-free state in which the transplanted allograft functions well without chronic rejection occurrence, and there is no transplant tolerance protocol available clinically. Here we show that this tolerance, during the transition from hypertrophy to hyperplasia upon liver regeneration, is acquired early and maintained permanently in orthotopic rat half-size liver transplantation (LT) while host bone marrow stem cells are mobilized and short-course immunosuppression is administered. Compared with whole and half-size LT as controls, survival for more than 500 days was observed in tolerant rats, the liver allograft worked better and its microstructures remained normal without chronic rejection. Sex-mismatch LT revealed that the host bone marrow stem cells repopulated the allograft to create reverse chimeras with host Y chromosomes in female donor livers and the increased host to donor ratio over time. However, a donor specific hyporesponse was not achieved through skin transplantation and skin allograft challenge did not elicit the rejection of liver allografts. Our finding that transplant tolerance achieved via reverse chimeras bypasses the complex immune system simplifies its induction clinically and facilitates its translation and application in human

Background

LT is well accepted as the only life-saving treatment for end-stage liver diseases[1]. After LT, the standard-of-care is a calcineurin inhibitor-based multidrug regimen with a narrow therapeutic window to protect the graft against immune destruction. In the upper of this window, side-effects and toxicities are associated with infection, de novo malignancy and metabolic syndrome[1,2], and it was reported that approximately 80% of LT recipients were over-immunosuppressed[3]; in the lower of this window, the allograft loss develops insidiously due to immune injury from acute or chronic rejection. No optimized immunosuppression regimen is universally applicable, resulting in disreputably adverse effects; a lower quality of life and cost burdens[2].Big data revealed that for decades the recipients had hardly gained longer-term survival after LT due to immunosuppression[4]. Transplant tolerance can circumvent major histocompatibility complex (MHC) barriers and permit complete cessation of immunosuppressants[5]. In 1969, Calne first realized liver allograft tolerance in 12 of 55 cases using a partial liver that was heterotopically placed in pigs[6]. Great immunological progress has advanced our understanding of rejection as a bidirectional immune response; the predominant dogma of tolerance induction is to train or educate immunity of the host to accept the graft[7-9]. Currently it is agreed that the creation of hematopoietic cell chimerism originating from the donor has the potential to induce transplant tolerance through myoablative preconditioning, this procedure will incur life-threatening complications such as engraftment syndrome, infections and graft versus host disease[10,11], and maintaining macrochimerism still presents immerse challenges due to immune destruction by the host[12]. Transplant rejection can reoccur after years of graft tolerance, sometimes following infection[13].

Feng et al. reported a higher rate of spontaneous tolerance of parental partial allografts in highly selective pediatric recipients; this report suggested that liver regeneration may be attributable to tolerance[14]. The liver has a unique regenerative capacity to restore his native mass following major resection; this phenomenon is neglected in tolerance induction. Liver regeneration is mediated by three sources: hepatocytes (accounting for 80% of parenchymal cells), liver-resident progenitors (<2%) and extrahepatic stem cells. Generally, the premise of intrahepatocytes involving in regeneration is that they are intact upon hepatectomy; when intrahepatic cell proliferation is refrained, extrahepatic stem cells contribute to this process and reinstitute the partial graft into reverse chimeras[15-17]. Here we deployed unique liver regeneration to operationally achieve transplant tolerance in the setting of half LT.

Experimental Design And Groupings

To establish a protocol for transplant tolerance induction and potentially clinical application, we performed reduced-size (30, 50, 70%) allograft LT to stimulate liver regeneration in our study and the smaller liver can better trigger it. A 30% liver graft is highly responsible for small-size syndrome and was abolished in our study. According to the references and our pilot study, we comparatively performed whole and 50% LT administered with cyclosporine A (CSA) and recombinant human granulocyte stimulating factor (r-GSF) to confirm the effect of liver regeneration of the smaller graft[18]; 50% LT with CSA served as the control to determine the effect of bone marrow stem cells (Table 1, Extended Data Table 1, Extended Data Fig.1). The experiments were conducted in compliance with the standards and rules for animals set by the Institutional Animal Care Committee of Henan Provincial People's Hospital.

Survival, functions and structures of rat liver allografts

Clinically, transplant tolerance is defined as survival of an allograft without chronic rejection in the absence of any immunosuppression for more than 1 year (2 months for small animals). For nonhuman organ transplantation, graft survival is equivalent to host survival. In this study, we calculated graft survival time from the date of transplantation to the day when chronic rejection was confirmed according to the Banff criteria[19]. The core of transplant tolerance is to assess whether the allograft functions well and normal structures are maintained after complete withdrawal of immunosuppression. Liver allograft survival, functional and histological examinations were primary end-point foci. Glutamic-pyruvic transaminase (ALT) and glutamic-oxaloacetic transaminase (AST) are routine parameters used to evaluate liver functions. We monitored survival and liver functions in all rats; notably, our results showed that tolerant rats lived significantly longer than rats in control groups (p=0.000, Fig.1). At 14 days (d) post-LT, ALT and AST levels gradually returned to normal in all groups; afterward two values remained normal in tolerant rats, and were lower than those in the 2 controls (Fig.1). Hematoxylin-eosin (H&E) staining revealed that chronic rejection and fibrosis developed within 2 months in the controls, and that no acute or chronic rejection or fibrosis occurred in tolerant rats, in which normal structures of the liver grafts with mild ductular reaction scored 1 were present (Fig.2, Extended Data Fig.2). Immunoglubin G which is a biomarker for humorous immunity was stained positive in the controls; CK19, as a biomarker of bile duct proliferation, was detected more positive in the control groups than in the tolerant rats (Fig.2).

Liver allograft remodeling during liver regeneration

To further gain insight into the underlying mechanism of liver regeneration in the context of tolerance, we evaluated whether and how host bone marrow stem cells migrated and repopulated the allograft. 5-Bromo-2-deoxyuridine BrdU was used to examine liver regeneration. In the Ctrl-1 group, liver regeneration almost discontinued; while it was relatively common in the tolerant and the Ctrl-2 groups but was generally much less frequent (Extended Data Fig.3). $CD3^+$ lymphocytes were pronounced in immune rejection (Ctrl-1 and Ctrl-2 groups). $CD34^+$, which is a biomarker of bone marrow stem cells, was used to evaluate whether host-derived stem cells repopulated after r-GSF mobilization. Immunohistochemical staining showed that $CD34^+$ was most notable in tolerant rats, and that it was more pronounced in the Ctrl-2 group than that in the Ctrl-1 group, indicating bone marrow stem cell repopulation was significant. CD133 was not detected in all rats. $CD44^+$ was stained more positive in controls than in the tolerant rats, suggesting that chronic injury was ongoing (Fig.3).

Sex-mismatch liver transplantation

Additionally, we performed sex-mismatch LT from female Lewis rats (XX chromosomes) to male BN rats (XY) to assess host bone marrow stem cell repopulation[20] (Table 1). Quantitative polymerase chain reaction (q-PCR) showed that the reverse chimeras were robust with the host to donor ratio increased over time (Fig.4).

Combining the histological examinations and sex-mismatch LT, we concluded that host bone marrow stem cells were mobilized and repopulated in the donor liver graft to generate reverse chimeras in tolerant rats, and that host inflammatory cells infiltrated into the donor grafts due to immune rejection in the control groups.

Donor-specific immune responses

Lastly we performed skin transplantation at least 1 month after LT to evaluate whether a donor-specific immune hyporesponse occurred. In 3 groups, the skin allograft hardened and sloughed off in 2 weeks and a robust immune reaction occurred to reject the skin allograft; this response indicated a split tolerance, revealing that a donor-specific immune hyporesponse did not occur (Fig. 5).

Discussion

Clinically spontaneous tolerances have been reported sparsely in recipients several years following LT, especially for pediatric LT recipients in whom partial or reduced-size grafts were used[6,14,21], it was suggestive that the unique liver regeneration phenomenon was the underlying mechanism of tolerance. Sun et al. confirmed that plerixafor-mobilized host bone marrow stem cells reinstituted the graft to prolong survival in a half-size graft LT model from *Dark*-Agouti (DA) to Lewis rats, they noted that $CD133^+$ (not $CD34^+$) stem cells migrated and repopulated in the allograft, and their conclusion was that self-perpetuating antigen specific immunosuppression rather than transplant tolerance was attributable to the

long survival[20,22].We speculated that the half liver graft underwent a longer duration of regeneration in DA rats because this rat is a poor metabolizer that lacks cytochrome P450 subfamilies CYP2D1 and CYP2D2[23], this model exhibits the strongest rejection resembling human LT; insufficient tacrolimus use (0.1 mg/kg, four times in total in the first week) was the fundamental cause of chronic immune injury, resulting in compromised hepatocyte proliferation capacity; the small hepatocytes which are one of hepatic progenitors, started to proliferate and eventually fibrosis ensued, their study provided the clue as to that the injuries were ongoing in the allografts and liver regeneration or remodeling of the graft was taking place continuously. The small hepatocyte's specific biomarker $CD44^+$ was detected negative in tolerant rats but positive in the control groups in our findings[24].

We do not directly intervene in the immune system of the host and achieve permanent transplant tolerance completely in accordance with the Banff criteria first in the solid organ[19]. Why is the smaller liver allograft harbored well by the host upon the synergistic effect of liver regeneration and bone marrow stem cells? We propose that a series of events after smaller liver graft implantation played pivotal roles in tolerance induction in our study. One immediate outcome is channeling the entire portal blood flow through half vessels to increase portal blood pressure and provide initiating signals for liver regeneration[25]. Upon ischemia reperfusion injury, hepatocytes would first undergo growth or hypertrophy to compensate and their volume would increase by 50% in a few days which was evidenced and supported by reduced BrdU levels in the half allografts in our study. During the hypertrophy stage, subtle and dynamic variations in the spatial conformation of MHC molecules changed the interaction between MHC and antigen peptides and led to the failure of the recognition of MHC molecule and antigen peptide binding complexes through exquisite T-cell receptor's docking[26.27]. Bone marrow stem cells can be mobilized commonly with r-GSF and migrate, repopulate and differentiate in the half allograft to generate reverse chimeras that the host bone marrow stem cells (not inflammatory cells) were much in presence of the graft and never be destroyed or deleted[28,29]; mesenchymal stem cells are immunosuppressive and downregulate the expression of costimulatory molecules, inhibiting the differentiation of dendritic cells from $CD34^+$ progenitors and reducing proinflammatory cytokine secretion[30].As a result, tissue repair improved quickly, and the allograft was tolerated. This finding was consistent with the Danger Model[31]. After a hypertrophy switches to hyperplasia, the changed spatial conformation of the MHC molecules is not restored to their original state because of the exquisite docking, resultantly immune recognition does not occur. It resembles a growing fetus in utero with dynamic conformation change of the MHC molecules which leads to fetomaternal tolerance while the placenta works as a haematopoietic organ[32]; and perhaps this is one reason for split tolerance and why skin allograft challenge did not result in the liver allograft rejection.

In our study, transplant tolerance was achieved in 100% of the hosts, the allograft exhibited almost completely normal microstructures for more than one year, hepatic infiltration in tolerant rats was much less mild than the results reported by Taubert or Todo[9,33].Of note, our protocol is of high clinical relevance because it targets the allograft and the manipulations are ready to perform: reduced-size or split LT is extensively conducted in many centers, host-derived bone marrow stem cells are mobilized clinically,

short-course and lower dose immunosuppressant use confer fewer impacts on the host, and the side-effects and toxicities associated with immunosuppression can be waived. In view of liver regeneration to induce tolerance, the proliferative index can be used as a biomarker to predict the tolerant state. In addition, the graft can be divided into 2 halves which are used for 2 recipients; the paradigms of one liver for two recipients will theoretically double the donor supplies for those on the waitlist for LT.

References

1. Jain, A. et al. Long-term survival after liver transplantation in 4,000 consecutive patients at a single center. *Ann. Surgery*. **232**,490-500 (2000).

2. Simone, P. D. et al. Modification of immunosuppressive therapy as risk factor for complications after liver transplantation. *Best. Pract. Res. Clin. Gastroenterol*. **31**,199-209 (2017).

3. Londoño,M.C. et al. Immunosuppression minimization vs. complete drug withdrawal in liver transplantation. *J. Hepatol*. **59**, 872-879 (2013).

4. Rana, A. et al. No gains in long-term survival after liver transplantation over the past three decades. *Ann. Surg*. **269**, 20-27 (2019).

5. Billingham, R. E., Brent, L. & Medawar, P. B. Actively acquired tolerance of foreign cells. *Nature***172**,603-606 (1953).

6. Calne, R.Y. et al. Induction of immunological tolerance by porcine liver allografts. *Nature***223**,472-476 (1969).

7. Houssin, D. et al. Specific transplantation tolerance induced by spontaneously tolerated liver allograft in inbred strains of rats. *Transplantation***29**,418-419 (1980).

8. Cvetkovski, F. et al. Strategies for liver transplantation tolerance. *Int. J. Mol. Sci*. **22**,2253 (2021).

9. Todo, S.& Yamashita, K . Anti-donor regulatory T cell therapy in liver transplantation. *Hum. Immunol*. **79**,288-293 (2018).

10. Starzl, T.E. et al. Cell migration and chimerism after whole-organ transplantation: the basis of graft acceptance. *Hepatology***17**, 1127-1152 (1993).

11.Yan, X. et al. Delayed donor bone marrow infusion induces liver transplant tolerance. *Transplantation***101**, 1056-1066 (2017).

12. Chaudhry, S. et al. Transient-mixed chimerism with nonmyeloablative conditioning does not induce liver allograft tolerance in nonhuman primates. *Transplantation***104**,1580-1590 (2020).

13. Brouard, S. et al. The natural history of clinical operational tolerance after kidney transplantation through twenty-seven cases. *Am. J. Transplant*. **12**, 296-3307 (2012).

14. Feng, S. et al. Complete immunosuppression withdrawal and subsequent allograft function among pediatric recipients of parental living donor liver transplants. *JAMA*. **307**, 283-293 (2012).

15. Nagy, P. et al. Reconstitution of liver mass via cellular hypertrophy in the rat. *Hepatology* **33**, 339-345(2001).

16. Petersen, B. E. et al. Bone marrow as a potential source of hepatic oval cells. *Science***284**,1168-1170 (1999).

17. Russo, F. P. et al .The bone marrow functionally contributes to liver fibrosis. *Gastroenterology***130**,1807-1821 (2006).

18. Shimizu, A. et al. Chronic antibody-mediated responses may mediate chronic rejection in rat orthotopic liver transplantation. *Transplant. Proc.***45**, 1743-1747 (2013).

19. A. J. Demetris, et al. 2016 Comprehensive Update of the Banff Working Group on Liver Allograft Pathology: Introduction of Antibody-Mediated Rejection. *Am. J. Transplant*. **16,** 2816-2835 (2016).

20. Sun, Z.L. et al. Recruitment of host progenitor cells in rat liver transplants. *Hepatology***49**,587-597 (2009).

21. Oike, F. et al. Complete withdrawal of immunosuppression in living donor liver transplantation. *Transplant Proc.***34**, 1521 (2002).

22. Okabayashi, T. A. et al. Mobilization of host stem cells enables long-term liver transplant acceptance in a strongly rejecting rat strain combination. *Am. J. Transplant*. **11**, 2046-2056 (2011).

23.Yamamoto,Y. et al. Molecular basis of the Dark Agouti rat drug oxidation polymorphism: importance of CYP2D1 and CYP2D2. *Pharmacogenetics*. **8**,73-82 (1998).

24. Fukasawa, H. et al. Morphological characterization of small hepatocytes after bile duct ligation in chicken. *Poult. Sci*. **98**,717-721 (2019).

25.Michalopoulos, G.K. & Bhushan, B. Liver regeneration: biological and pathological mechanisms and implications. *Nat. Rev. Gastroenterol. Hepatol.***18,** 40-55 (2021).

26. Matot, I. et al. Impaired liver regeneration after hepatectomy and bleeding is associated with a shift from hepatocyte proliferation to hypertrophy. *FASEB. J.* **31**, 5283-5295 (2017).

27. Yanaka, S. et al. Peptide-dependent conformational fluctuation determines the stability of the human leukocyte antigen class I complex. *J. Biol. Chem*. **289**, 24680-24690 (2014).

28. Sun, Z.L. & Williams, G.M. Host stem cells repopulate liver allografts: reverse chimerism. *Chimerism***2**, 120-122 (2011).

29. Hove,W.R.et al. Extensive chimerism in liver transplants: vascular endothelium, bile duct epithelium, and hepatocytes. *Liver. Transpl.* **9,** 552-556 (2003).

30. Watanabe, Y., Tsuchiya, A.& Terai, S. The development of mesenchymal stem cell therapy in the present, and the perspective of cell-free therapy in the future.*Clin. Mol. Hepatol.* **27**, 70-80 (2021).

31. Matzinger, P. The danger model: a renewed sense of self. *Science***296**, 301-305 (2002).

32. Ottersbach, K & Dzierzak, E. The placenta as a haematopoietic organ. *Int. J. Dev.Biol.***54**,1099-1106 (2010).

33.Taubert, R. et al. Hepatic infiltrates in operational tolerant patients after liver transplantation show enrichment of regulatory T cells before proinflammatory genes are downregulated. *Am. J. Transplant.* **16**, 1285-1293 (2016).

Methods

Data reporting

The sample size was predetermined according to the references without statistical methods[5, 34, 35]. The experiments were randomized and the investigators were blinded during data analysis for all experiments.

Rats

Rats (200-300 g weight) including Sprague Dawley (SD), Lewis and Brown Norway (BN) rats served as donors and recipients, they were purchased from Beijing Vital River Laboratory Animal Technology Corporation. All rats were housed and cared for in a temperature and light-controlled environment with free access to food and bottled water and the rats were fasted for 12 hours before LT. All experiments were conducted in compliance with the standards for animal use and care set by the Institutional Animal Care Committee of Henan Provincial People's Hospital and were approved by the Ethics Committee of Henan Provincial People's Hospital (No:HNTCMDW-20190304).

Orthotopic rat LT and experimental groupings

Rat LT was performed by one surgeon under a microscope. After opening the abdomen with a modified mask for isoflurane inhalation anesthesia we perfused the liver grafts first via the aorta with 5 ml of heparinized (50 U/mL) normal saline and then through the portal vein with 10 ml of cold lactated Ringer solution containing dexamethasone (24 mg/l). The cold storage time was less than 3 hours. The recipient's native liver was explanted out and the allograft was orthotopically implanted, followed by

suprahepatic vena cava anastomosis with an 8-0 running suture. The two-cuff technique was used to reconnect the portal vein and the infrahepatic vena cava[36,37]. Arterial reconnection was made with a stent[38]. Biliary continuity was achieved by a polyethylene stent. The recipients were monitored daily for mobility, weight, posture, and urine color. A 50% liver graft consists of removal of the left lateral lobe, left portion of the middle lobe and caudate lobes; for a 70% graft, the left lateral lobe was removed[39] (Table 1, Extended Data Table.1, Extended Data Fig.1).

To formulate tolerance induction processes and investigate the potential mechanism of liver regeneration, we performed LT from Lewis to BN rats in the different groups, which is a well-established model of strong acute rejection. Whole LT was performed to compare half-size allograft LT with daily subcutaneous injection of CSA at 2 mg/kg for 9 d and r-GSF at 200 U/kg for 5 d and study the significance of liver regeneration. Half LT was performed with injection of CSA to emphasize the contribution of host bone marrow stem cells to the regenerating liver compared with the tolerant group (Table 1). 70% graft LT was performed for comparison with 50% graft LT to determine graft size (Extended Data Table 1). Other rat LT was performed to provide the controls for the histology images (Extended Data Fig.2).

Skin transplantation

We selected inbred Lewis rats as skin donors and post-LT BN rats as recipients for skin transplantation. A 1.5 cm × 1.5 cm full thickness allograft was prepared from the donor abdomen, and a graft bed (1.5 cm × 1.5 cm) was prepared on the back of the recipient rat[40]. When the skin allograft was attached to the back of the recipient with interrupted sutures of 5-0 silk, we covered the allograft with a protective tape and made the first inspection 3 days later and daily thereafter. Rejection was defined as a red–brown color, hard consistency, and necrosis and sloughing in the skin allograft.

Blood collection and liver sample preparation

After the subject rat was euthanized, the abdominal cavity was opened through a midline incision, the intestine was pulled out to expose the abdominal aorta, a blood collection needle pierced the aorta and was connected to a negative air pressure tube containing ethylenediaminetetraacetic acid (EDTA) for anticoagulation, and then the tube was inverted several times to prevent blood clotting. The plasma was collected at 2000 g centrifuge for 10 minutes (min) and the aliquots were labeled and cryopreserved until use. For spectrometry, the corresponding parameters were set on the automatic biochemical analyzer, the results were obtained after plasma was loaded, and ALT and AST levels were measured through the Substrate Method.

The liver allograft was perfused with normal saline through the mesenteric vein and extracted. The graft was weighed and cut into several small parts, and then immerged in 10% neutral paraformaldehyde solution for later use.

Liver histology

H&E and Masson trichrome staining were performed as previously described. Tissues were fixed in 10% neutral buffered formalin, and tissue processing, sectioning and staining was performed by Wuhan Servicebio Technology Ltd. The 3-μm paraffin sections were deparaffinized in xylene three times and rehydrated in a graded alcohol series. The slides were placed in citric acid (pH6.0) and heated several times for antigen epitope retrieval. Endogenous peroxidase was inhibited with 3% hydrogen peroxide and incubated for 25 min at room temperature in the dark. For CD3 staining, the slides were incubated first with normal goat serum, primary antibody was applied at a dilution of 1:1000 and incubated at 4 °C overnight, and the corresponding secondary antibody to primary antibody (hydrogen-peroxide oxidoreductase label) was applied to the slides and incubated at room temperature for 50 min. After being slightly dried, the slide was colored with freshly-prepared diaminobenzidine chromogen solution. Counterstaining was performed with hematoxylin. This immunohistochemistry staining procedure was also applied to CD44 (1:400), CD34 (1:500), CD133 (1:500), CK19 (1:2000) and IgG (1:500) (Servicebio Inc, Wuhan, China). Tissue sections were scanned and analyzed by Pannoramic confocal 3DHISTECH and with analyzed with Caseviewer software (The Digital Pathology Company, Budapest, Hungary).

Evaluation of liver regeneration

We studied hepatocyte replication by measuring the incorporation of BrdU with immunohistochemistry. BrdU (50 mg/kg) was injected intraperitoneally 24 hours before the liver tissue was harvested. The liver tissues were then cut into small pieces that were fixed in 10% neutral formalin, embedded in paraffin and sectioned. BrdU-incorporated hepatocytes were detected with an immunochemical system to monitor cell proliferation with a monoclonal anti-BrdU cell proliferation kit[41-45] (Extended Data Fig.3), (GB12051, Servicebio Inc. Wuhan, China).

q-PCR analysis of rat Y chromosomes

Total DNA was extracted from isolated cells by using a TIANamp genomic DNA Kit (DP304-03, Hefei, China) according to the manufacturer's protocol. The primer sets to amply rat Y chromosome were 5-ATTTATGGTGTGGTCCCGTGGAGA-3 and 5-TTCTGGTTCTTGGAGGACTGGTGT-3[20]. The primer sets for control amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5-AGGGAAATCGTGCGTGAC-3 and 5-CATACCCAAGAAGGAAGGCT-3. All primers used in our study were synthetized and purchased from General Biol Inc (Hefei,China). Twenty microliters (μl) of PCR reaction solution contained 10 μl of 2×ChamQ Universal SYBR qPCR Master Mix (Vazyme, NJ, USA),1 μl of cDNA, 0.4 μl of forward primer, 0.4 μl of reverse primer, 8.2 μl of RNase Free double distilled water. The thermal cycling conditions started with one cycle at 95 °C for 10 min. This was followed by 40 cycles at 95 °C for 10 seconds(s), 58 °C for 30 s, 72°C for 30 s, and 72 °C for final extension for 30 s. PCR products were electrophoresed on 2.5% agarose gels and visualized with ethidium bromide immunofluorescence staining (Zvast-bio Inc, Nanchang, China).

FISH

To further assess host bone marrow stem cell repopulation, we performed FISH of X and Y-chromosomes using rat a chromosome X Point Probe (Cat. No. FRWC-20P, Creative Bioarray Inc, USA) and a Y Point Probe (Cat.FRWC-21P, Creative Bioarray Inc, USA) according to the manufacturer's protocol. The tissue slides were placed in 100% xylene for 5 min and this process was repeated 3 times. The slides were rehydrated in a graded alcohol series. After that, the slides were rinsed in distilled water for 1 min and in phosphate buffered saline (PBS) for 5 min, and then heated in distilled water at 100 °C for 15 min. The tissue was treated with pepsin solution for 10 min and rinsed in PBS. The slides were dehydrated by incubating in a gradient ethanol series each for 1 min and then air dried. Denaturing solution was equilibrated in an 88±2 °C water bath for approximately 30 min and the slides were immersed in the denaturing solution for 5 min. We dehydrated slides immediately in a gradient ethanol series each for 2 min, and air dried slides again. We denatured the FISH probes (10 μl of probe for each slide) in 88 °C for 5 min and kept the probes at 37 °C for 2 min. Finally we applied the denatured probes to the slides, applied a cover-slip, sealed them with rubber cement, and then hybridized them overnight at 40 °C in a humidified chamber. The rubber cement and cover slips were removed, and the slides were immersed in saline sodium citrate buffer (SSC)/0.3% NP-40 (Nonidet P-40) lysis buffer at 74±1 °C for 4 min. Then the slides were immersed in 2×SSC/0.3% NP-40 at room temperature for 5 min. Dehydrate slides by incubating slides in a gradient ethanol series each for 1 min and air dried the slides in the dark. Cell nuclei were stained blue with 4,6-diamidino-2-phenylindole (DAPI). Tissue sections were analyzed by confocal fluorescence microscopy (Creative Bioarray Inc, New York, USA).

Statistics and reproducibility

Statistical analysis was performed for survival curves generated by Kaplan-Meier analysis using GraphPad Prism 8. $p \leq 0.05$ was set as significant difference.

Data availability

All data generated and supporting the findings of this study are available within the paper, Additional information and materials will be made available upon request.

34.Shimizu, Y. et al. Restoration of tolerance to rat hepatic allografts by spleen-derived passenger leukocytes. *Transpl. Int*. **9**,593-595 (1996).

35. Dresske, B., Lin, X., Huang, D.S., Zhou, X.& Fändrich, F. Spontaneous tolerance: experience with the rat liver transplant model. *Hum. Immunol*. **63**, 853-861 (2002).

36. Lee, S., Charters, A.C., Chandler, J.G.& Orloff, M.J. A technique for orthotopic liver transplantation in the rat. *Transplantation*. **16**,664-669 (1973).

37. Zhao, H.B. et al. Revisiting orthotopic rat liver transplant. *Exp Clin Transplant*. **9**, 956-962 (2021).

38. Zhou, S.T. et al. New method of stent-facilitated arterial reconstruction for orthotopic mouse liver transplantation. *J. Surg. Res*. **18**7, 297-301 (2014).

39. Madrahimov, N., Dirsch, O., Broelsch, C.& Dahmen, U. Marginal hepatectomy in the rat: from anatomy to surgery. *Ann. Surg*. **244**,89-98 (2006).

40. Barker, C. F. & Billingham, R. E. Skeletal muscle as a privileged site for orthotopic skin allografts. *J. Exp. Med*. **138**, 289-299 (1973).

41. Forbes, S. J. et al. A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. *Gastroenterology* **126**,955-963(2004).

42.Dalakas, E. et al. Bone marrow stem cells contribute to alcohol liver fibrosis in humans. *Stem. Cells. Dev*. **19**,1417-1425 (2010).

43. Chen, Y.H. et al. Contribution of mature hepatocytes to small hepatocyte-like progenitor cells in retrorsine-exposed rats with chimeric livers. *Hepatology*. **57,**1215-1224 (2013).

44. Higashiyama, R. et al. Negligible contribution of bone marrow-derived cells to collagen production during hepatic fibrogenesis in mice. *Gastroenterology* **137**,1459-1466 (2009).

45. Assy, N. & Minuk, G.Y. Liver regeneration: Methods for monitoring and their applications. *J. Hepatol*. **26**, 945-952 (1997).

Declarations

Acknowledgement: We are grateful to Professor Gang Chen (Tongji hospital Wuhan) for the potential mechanism of our study and to Dr Yabing Huang and Dr Zhanwei Zhang for additional consultation with pathology. This work was sponsored by the National Natural Science Foundation of China (No.U2004124) and the Doctor Initiative Fund.

Authors' contributions: S.T.Z. conceived, designed and performed OLT and experiments, wrote the manuscript and finalized the study. G.F.T. performed experiments and discussed the draft. H.B.Z. performed some experiments. S.D.W. performed the data analysis. X.Y.G cared animals. F.Z.L. discussed the project. D.L. collected the data. H.G. designed experiments and performed the pathological examinations. G.Y.C. funded and discussed the study.

There is no interest declaration.

†Corresponding authors: zcguo@tjh.tjmu.edu.cn (H.G), chenguoyong@zzu.edu.cn (G.Y.C)

Tables

Groupings	Graft	Donor (M/F#)	Host (M)	Treatment	Survival (d)
Lewis→BN (Ctrl-1)	100%	5/5	10	CSA+r-GSF	123,72,61,65,43,58#.56#,12#,7 ,7
Lewis→BN (Ctrl-2)	50%	6/5	11	CSA	163,97,157,89,68,59,57#.54#,12# 7 ,7
Lewis→BN (Tolerant)	50%	12/6	18	CSA+r-GSF	153,167,189,243,237,365,341,113,508, 438,410 ,406 ,62#,12#,49#,7 ,7 ,8

Table 1. **Experimental groupings for graft size and sex, treatment and survival.** Male/Female: M/F, female donors served sex-mismatch LT; Cyclosporine A: CSA; recombinant human granulocyte stimulating factor: r-GSF ;# represents sex-mismatch LT to determine reverse chimeras. The deadline day for our study was June 20, 2022.

Figures

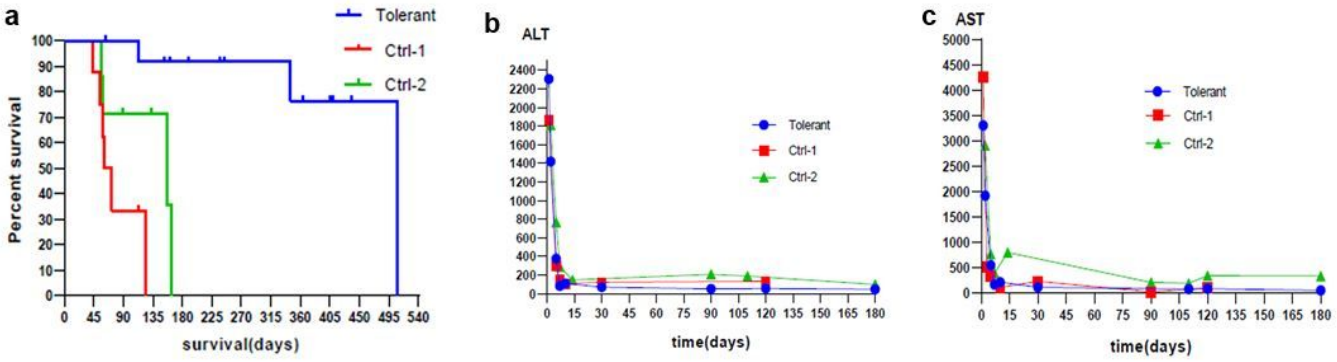

Figure 1

Survival and liver functions of the recipient rats in 3 groups. a. Survival curves were generated by Kaplan-Meier analysis. The endpoint of survival time for all rats for this study was June 20. 2022, the survival time of less than 15 d in control groups, or 60 d in the tolerant group was not included in survival curves. There were significant differences between groups (p=0.0001). b and c. The line chart showing ALT and AST, both are parameters of liver function (both normal value range 60-240 u).

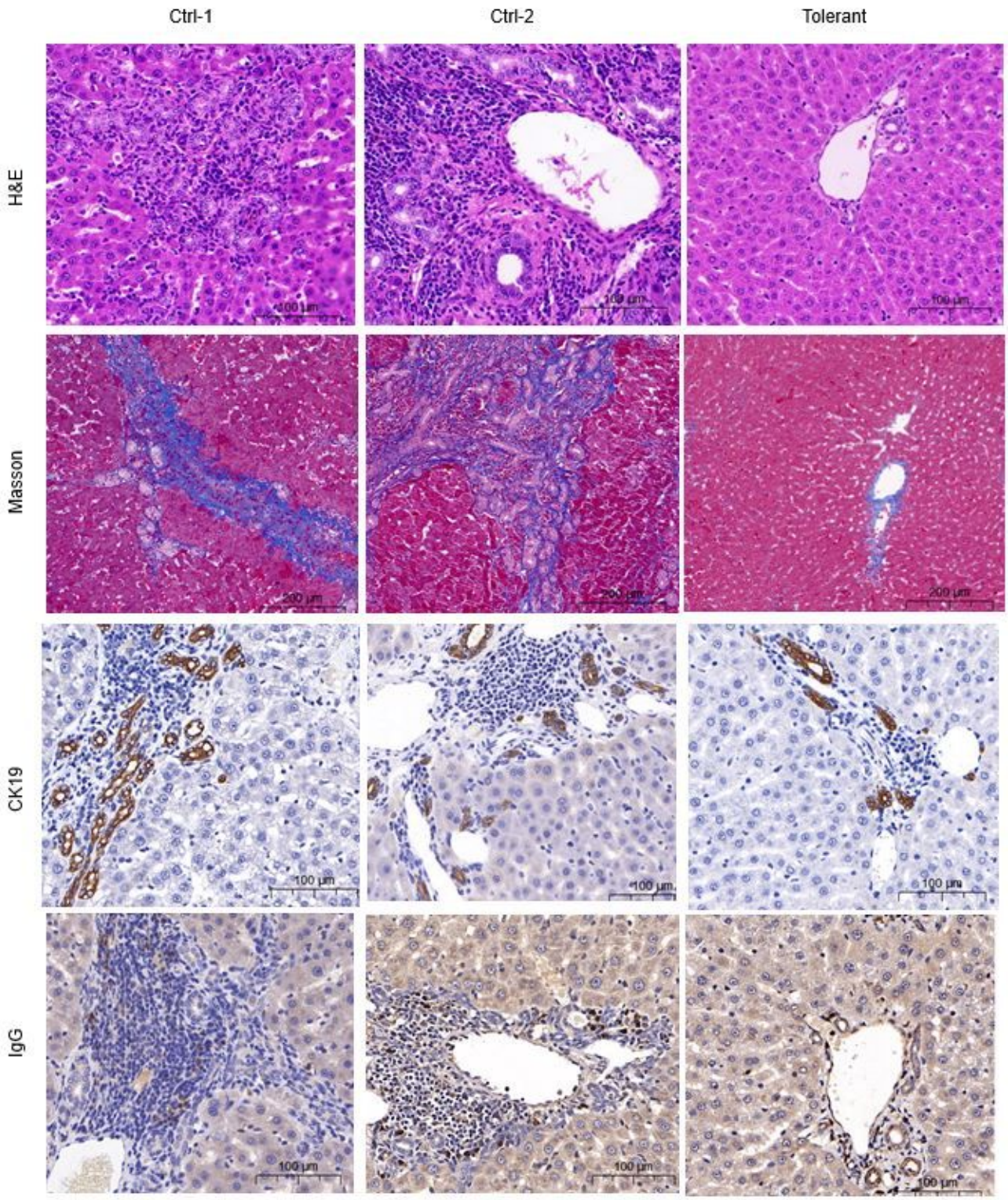

Figure 2

Histological examinations of liver allografts in 3 groups. The liver grafts were procured at the end-point time (due to chronic rejection). H&E staining in Ctrl-1 (56 d), Ctrl-2 (54 d) and tolerant groups (438 d); scale bar 100 μm. Masson trichrome staining showed thatfibrosis was more remarkable in Ctrl-1 (59 d) or Ctrl-2 group (57 d) than that in tolerant group (438 d); scale bar 200 μm. CK19 was most remarkable in

Ctrl-1 (123 d) and Ctrl-2 groups (157 d), not remarkable in the tolerant rats(365 d); scale bar 100 μm, IgG for immunoglobulin G deposits (Ctrl-1,65 d; Ctrl-2, 61 d; Tolerant, 341 d); scale bar 100 μm.

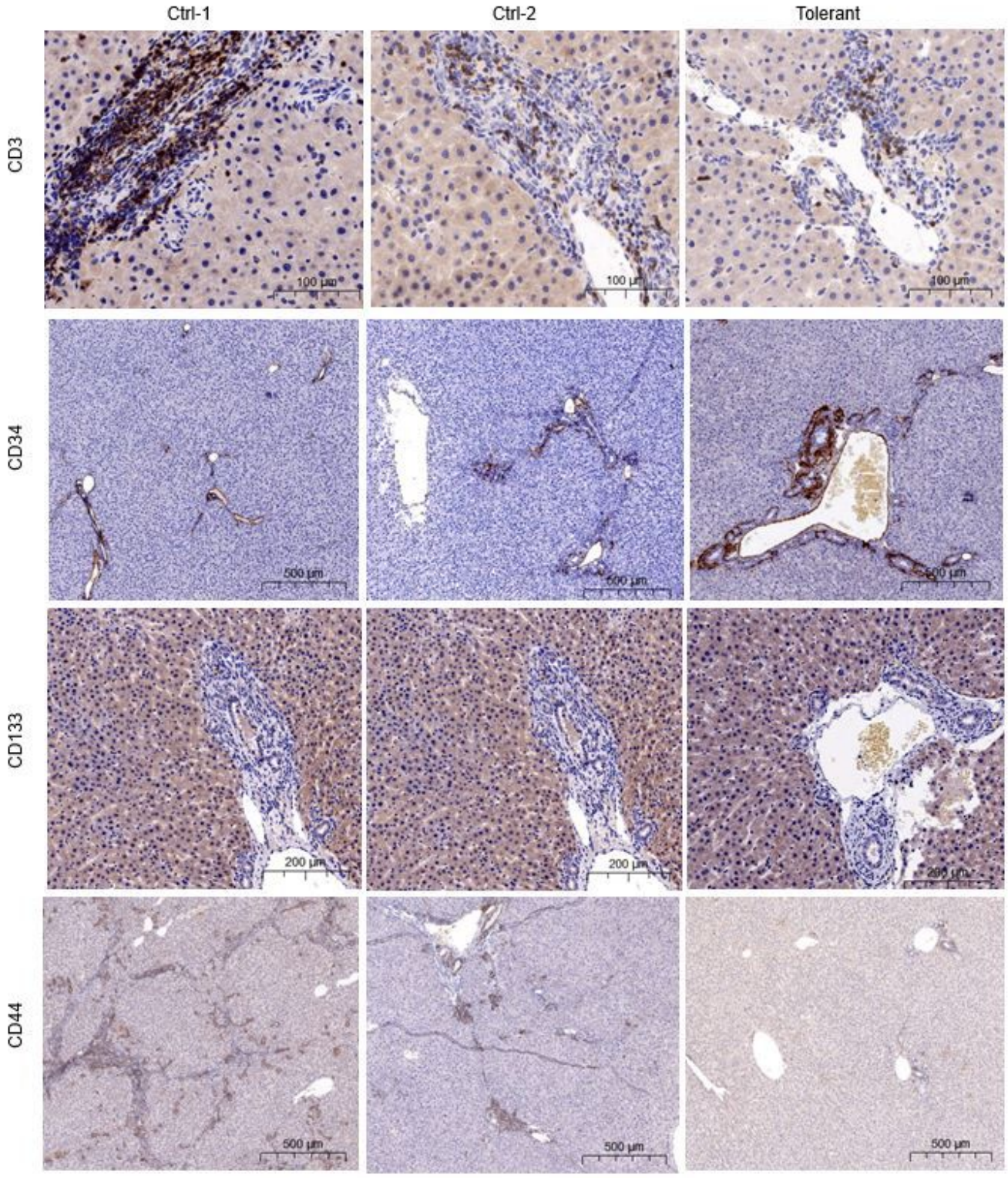

Figure 3

Immunohistochemical stainings of the allografts. CD3 was stained for immune rejection; CD34 and CD133 were stained for bone marrow stem cells. $CD3^{+}$ lymphocytes were pronounced in immune

rejection (Ctrl-1,123 d and Ctrl-2,121 d), less in tolerant rats (365 d), scale bar 100 μm. CD34 was notable in the tolerant rats(7 d), more notable in the Ctrl-2 group (7 d) than that in the Ctrl-1 group (6 d), scale bar 500 μm. CD133 was negative in 3 groups (Ctrl-1, 6 d), (Ctrl-2,7 d), (Tolerant,7 d),scale bar 200 μm. $CD44^{+}$ stained for small hepatocyte proliferation and more notable in the control groups than that in tolerant group, scale bar 500 μm.

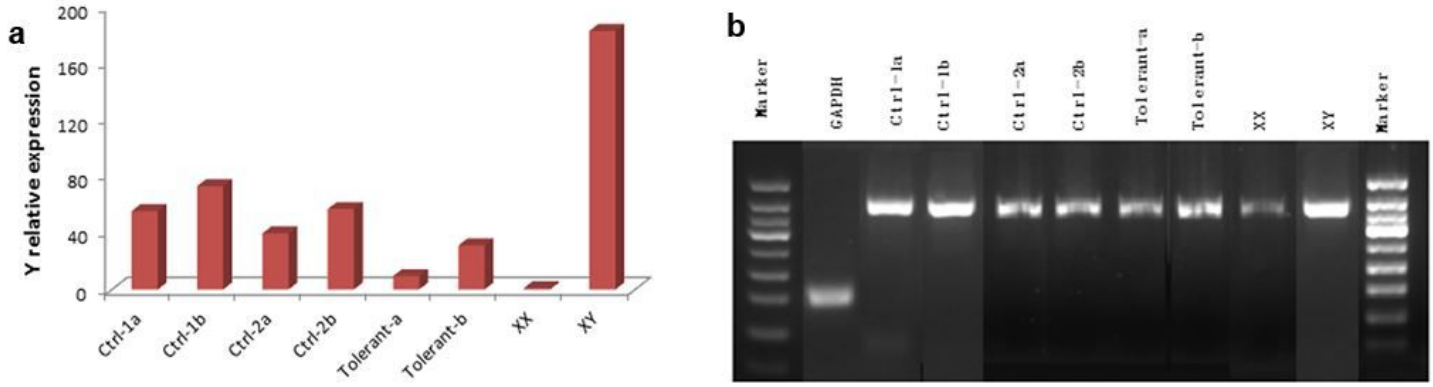

Figure 4

Host bone marrow stem cell repopulation. a. PCR revealed host Y chromosomes in the female liver grafts, In the tolerant group, the host to donor ratio (reverse chimera) increased over time due to host bone marrow stem cell reinstitution. Tolerant-a for the tolerant group shortly after immunosuppression cessation (early) and Tolerant-b for the rats after 30 d post-LT (delayed); for the control groups, this ratio increased due to inflammatory cell infiltration derived from rejections, Ctrl-1a for the Ctrl-1 group rats in 10-14 d after LT (early), Ctrl-1b for the rats after 30 day post-LT (delayed); Ctrl-2a for the Ctrl-2 group rats in 10-14 d after LT, Ctrl-2b for the rats after 30 day post-LT(delayed). **b.** The presence of the reverse chimeras was supported by southern blot for analysis of complemented DNA of the Y chromosomes. **c.** The FISH result showed host chromosomes in the donor liver graft. X chromosomes (green), Y chromosomes (red).

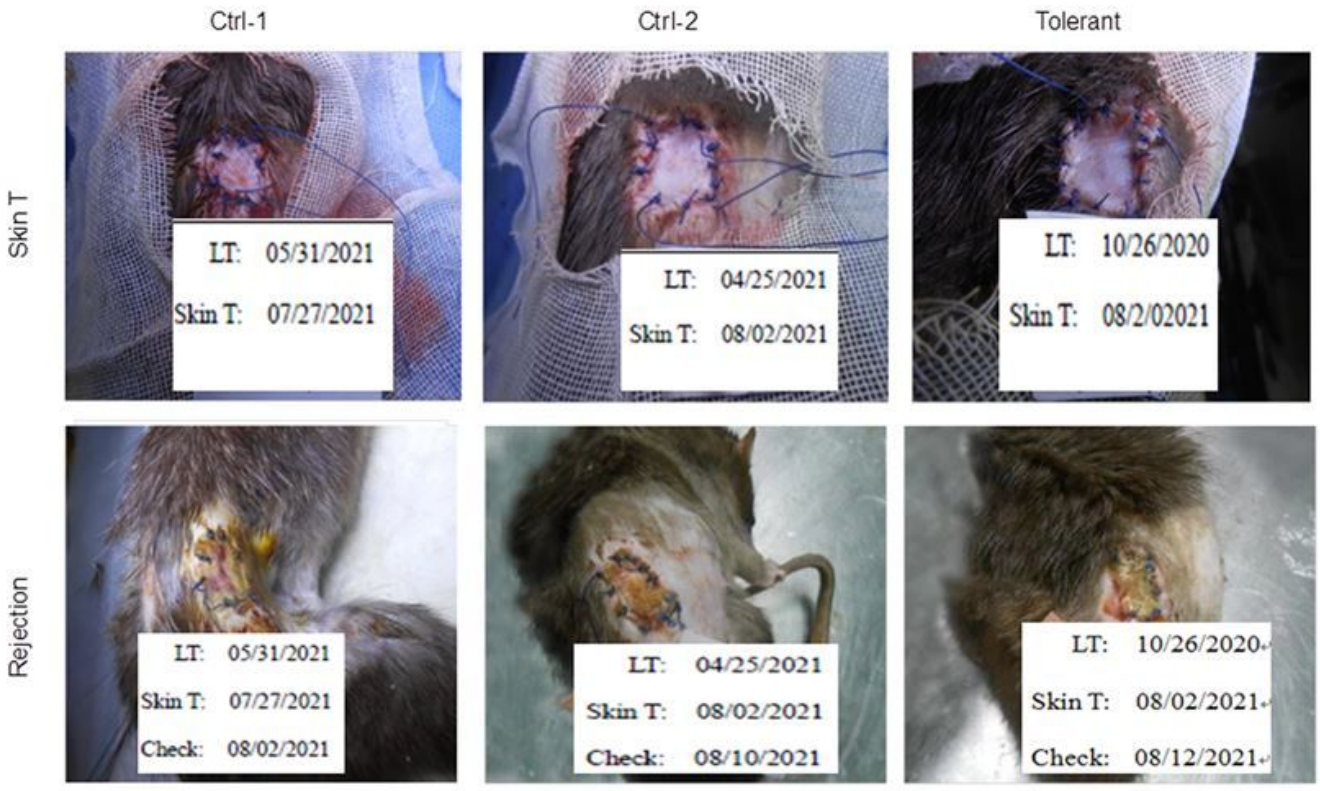

Figure 5

Split tolerance of skin graftstransplantedat least one month after LT in 3 groups. Donor skin grafts started to harden 4 d after skin graftingand sloughed off in 14 d in 3 groups, indicating strong rejection of the skin grafts.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.jpg
- figureS2.jpg
- figureS3.jpg
- ExtendedDataTable1.docx