Graft-Versus-Tumor Effect in Major Histocompatibility Complex–Mismatched Mouse Liver Transplantation

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Liver transplantation (LT) is currently considered an important method in treating hepatocellular carcinoma (HCC) and an alternative treatment for other liver malignancies. Here, we demonstrated that the graft-versus-tumor (GVT) effect exists in allogeneic liver transplantation (allo LT). Recipient-derived T cells played a critical role in the GVT process of allo LT, as demonstrated by extensive infiltration and significant activation of recipient T cells in the tumor after surgery. Moreover, this process was related to donor-derived T/B cells by improving the immune microenvironment in the tumor, as demonstrated by elevated levels of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-6, IL-16, chemokine (C-X-C motif) ligand 10 (CXCL10), and CXCL11 and decreased levels of IL-10 and IL-4 at tumor sites. Additionally, tacrolimus (FK506) treatment inhibited the GVT effect on allo LT. Donor liver-derived T/B cells infiltrate extrahepatic tumors to trigger a strong T-cell-mediated immune response and thus improve the tumor immune microenvironment.

Allogeneic hematopoietic stem cell transplantation (allo HSCT) has become an effective treatment option for a variety of hematologic malignancies or solid tumors.[1,2] One of the distinctive characteristics of allo HSCT is that the stem cell graft contains immunologically competent donor lymphocytes that are capable of mediating a reaction against malignant tumor cells, referred to as the graft-versus-tumor (GVT) effect, which could potentially eradicate disease and induce remission in hematological cancer patients who have relapsed.[3,4] Major histocompatibility complex (MHC) and/or minor histocompatibility antigen mismatch is the target of alloreactive T cells, which are mediators of GVT and graft-versus-host disease (GVHD) after allo HSCT.[5,6] Thus, exploitation of MHC-mismatched allo HSCT is an appropriate way to reduce relapse. In addition, although donor T cells are considered the principal mediators of the antitumor effect following allo HSCT,[7,8] some other observations have also confirmed that recipient-derived immune cells may also play a role as key effectors.[9-11]

Liver transplantation (LT) has much in common with hematopoietic stem cell transplantation (HSCT).

Abbreviations: allo HSCT, allogeneic hematopoietic stem cell transplantation; allo LT, allogeneic liver transplantation; CTC, circulating tumor cell; CXCL, chemokine (C-X-C motif) ligand; DAPI, 4′,6-diamidino-2-phenylindole; DLI, donor lymphocyte infusion; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; FK-506, tacrolimus; GRZB, granzyme B; GVHD, graft-versus-host disease; GVT, graft-versus-tumor; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; HPF, high-power field; HSCT, hematopoietic stem cell transplantation; IFNγ, interferon γ; IL, interleukin; LT, liver transplantation; MHC, major histocompatibility complex; NK, natural killer; NS, not significant; PBS, phosphate-buffered saline; PFR, perforin;

First, mixed chimeras of donor and recipient immune cells are present after both LT and HSCT.[12] There is a migration of donor immune cells after LT, which may be the cause of spontaneous immune tolerance.[13] Second, the mixed chimeras partly change the immune function of recipients after LT.[14,15] The immune tolerance of the liver graft appears to protect other organs to a certain extent in combined organ transplantation. Transfer of hepatitis B virus–specific immunity from immunized living liver donors to recipients was also confirmed. Third, although GVHD exists after LT, it is far more infrequent (0.4%-2% of cases) compared with that after stem cell transplant.[16,17] On the basis of this evidence, we speculate that the liver graft may affect the immune microenvironment of the tumor and even lead to the GVT effect similar to HSCT.

Here, we evaluated the antitumor effects of liver grafts using a mouse LT model. The CT-26 mouse colon cancer cell line was used as a model of an extrahepatic tumor to investigate the role and mechanism of GVT in LT. Our study demonstrated that allogeneic liver grafts induced a strong immune response and promoted tumor apoptosis. Notably, donor liver-derived immune cells, especially MHC-mismatched T/B cells, migrated into tumors early after mouse allogeneic liver transplantation (allo LT) and then induced immigration and activation of the recipient T cells, which eventually exerted antitumor effects. Our findings provide the first evidence that the GVT effect also exists in LT. Rational use of this effect, combined with appropriate human leukocyte antigen (HLA)–matching principles and donor lymphocyte infusion (DLI) therapy, may improve the prognosis of LT for malignant tumors and reduce postoperative recurrence.

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Shusen Zheng and Sheng Yan conceived and designed the study. Dongdong Yu, Lidong Wang, and Chenwei Cui performed the experiments and acquired the data. Jinhua Zhang and Hui Li conducted the animal experiments. Yang Tian, Yan Wang, and Yaohui Zhang analyzed and interpreted the data. Tianchun Wu and Lin Zhou drafted the manuscript.

Additional supporting information may be found in the online version of this article.

Materials and Methods

MICE

Male wild-type (WT) BALB/c (H2^d) mice, male WT C57BL/6 (H2^b) mice, female WT BALB/c (H2^d) mice, male Recombination Activating Gene 1 (RAG1)$^{-/-}$ C57BL/6 (H2^b) mice, male enhanced green fluorescent protein (EGFP)$^+$ transgenic C57BL/6 mice that ubiquitously express EGFP, and BALB/c nude mice aged 6-8 weeks were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All animals were kept in the Laboratory Animal Center of Zhejiang University (Hangzhou, China). Animal feeding practices and all experiments involving animals were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the Animal Ethics Committee of Zhejiang University.

CELL LINES AND CELL CULTURE

The BALB/c mouse mammary carcinoma cell line (EMT-6) and the BALB/c mouse colon cancer cell line (CT-26) were purchased from the China Center for Type Culture Collection (Shanghai, China). EMT-6 cells were cultured in Dulbecco's modified Eagle's medium (10566, Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Gibco),

1% penicillin-streptomycin, and 1% glutamine. CT-26 cells were cultured in Roswell Park Memorial Institute 1640 medium (Gibco) supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% glutamine.

ALLOGENEIC AND SYNGENEIC ORTHOTOPIC LT IN MICE

WT C57BL/6 mice weighing 24-26 g were used as donors, and WT BALB/c mice weighing 26-28 g were used as recipients. All of the mouse orthotopic LTs of C57BL/6 (H2^b)–BALB/c (H2^d), ie, allo LT, and BALB/c (H2^d)–BALB/c (H2^d), ie, syngeneic liver transplantation (syn LT), were performed by 2 proficient surgeons who were licensed for animal surgery using the improved double cuff method as described previously.[18] The warm ischemia/cold ischemia time of each transplantation was strictly controlled to eliminate the deviation caused by ischemia/reperfusion injury. After the postoperative recovery of body temperature and rehydration, mice were sent to the independent ventilation cage facility for further housing. The donor and recipient mice were both male in the experimental models using the CT-26 cell line, whereas the donor mice were male and the recipient mice were female in the experimental models using the EMT-6 cell line.

MOUSE TUMOR MODELS

BALB/c mice were given subcutaneous injections with a count of 5×10^6 EMT-6 cells or CT-26 cells in 0.1-mL Hank's balanced salt solution (SH30030, HyClone, Logan, Utah, USA) immediately after transplantation. Detailed methods for all mouse models can be found in the Supporting Methods, and the information on survival and rejection in the different strain combinations employed in our experiments can be found in Supporting Table 1.

In the CT-26 models, tumors were measured 2-dimensionally with Vernier calipers every day, and tumor volume was calculated as (length × width^2)/2. In the EMT-6 model, tumors were measured 2-dimensionally with Vernier calipers every other day. Body weights were measured at least 3 times every week. Mice were killed when tumor volume approached 1000 mm^3, weight loss exceeded 20%, or the tumors began to ulcerate.

TACROLIMUS TREATMENT

The tacrolimus (FK-506) stock (Fujisawa Pharmaceutical, Osaka, Japan) was dissolved with phosphate-buffered saline (PBS) to obtain a working concentration of 0.08 mg/mL. In allo LT with the FK-506 treatment models, recipients were given intraperitoneal injection with FK-506 1.0 mg/kg/day every day after transplantation.[19-21]

DLI TREATMENT

A count of 2×10^7 lymphocytes isolated from the liver of donor C57BL/6 mice was resuspended with 0.1 mL of PBS and injected into the tail vein of BALB/c donor mice immediately and at 2 days after transplantation.

ISOLATION OF LEUKOCYTES FROM DIFFERENT ORGANS AND FLOW CYTOMETRY ANALYSIS

Spleens, lungs, blood, and tumors were harvested, and the leukocytes were prepared as described.[19] The antibodies used for flow cytometry are described in the Supporting Methods.

Acquisition was performed on Canto II flow cytometry (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences). Analysis was performed using FlowJo 7.6.1 software (Tree Star, Inc., Ashland, OR). All results were pooled from at least 3 independent experiments (n = 3 for each model in each independent experiment). Isotype control and single-color control were set in each experiment. When measuring intracellular factors, the isolated leukocytes were cocultured with Leukocyte Activation Cocktail with BD GolgiPlug (BD Biosciences, San Jose, CA) for 6 hours. The biomarkers of cell classification were as follows: natural killer (NK) cells (CD3$^-$ CD49b$^+$), T cells (CD45$^+$ CD3$^+$), B cells (CD19$^+$ CD3$^-$), Kupffer cells, and macrophages (CD3$^-$ SCC-A^{LOW} F4/80$^+$). T cells were further classified as CD4$^+$ and CD8$^+$ groups. CD4$^+$ T cells were then divided into regulatory T cells (Tregs; CD4$^+$ CD25$^+$ forkhead box P3$^+$) and T helper 1 (T$_h$1; CD4$^+$ interferon γ [IFNγ]$^+$) and T helper 2 (T$_h$2; CD4$^+$ interleukin [IL] 4$^+$).

IMMUNOFLUORESCENT STAINING

For immunofluorescent staining, organs and tissues were embedded in paraffin and sectioned into 4-μm slides and dewaxed. Antigen retrieval and goat serum blocking were first performed, and then paraffin-embedded tissue sections were incubated with

anti-CD45, anti-Ki-67, and anti-CD31 (Abcam, Cambridge, UK) overnight at 4°C followed by staining with Alexa Fluor 488–labeled goat anti-mouse or anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA) for 2 hours at room temperature. Images were taken under a fluorescent microscope or confocal microscopy at a magnification of 400×.

MULTIFACTOR DETECTION

We used Bio-Plex Pro Mouse Chemokine Panel 33-Plex (#12002231, Bio-rad, Hercules, CA) to detect the contents of various factors in tumor tissues.

STATISTICS

All of the data were presented as mean ± standard deviation (SD) and analyzed using GraphPad Prism, version 5.0.1 (GraphPad Prism Software, Inc., La Jolla, CA). Student t test was used to assess data obtained from different models, and a 2-sided $P < 0.05$ was considered to be statistically significant.

Results

ALLOGENEIC MICE LT INHIBITED EXTRAHEPATIC TUMOR GROWTH

To examine whether allo LT can exert an antitumor effect, colon tumor mouse models were established by subcutaneous injection of CT-26 cells (5×10^6) into the right axilla of BALB/c mice immediately after transplantation. Syn LT and sham operations were carried out as normal controls (Fig. 1A). Tumor growth kinetics of syn LT and sham models had no significant difference ($P > 0.05$), indicating that LT was not relevant to GVT (Fig. 1B). However, significantly stronger tumor suppression was shown in allo LT models compared with the syn LT models ($P < 0.05$; Fig. 1B,C). To further study this GVT effect, tumor tissues were harvested 7 days after surgery for the assessment of tumor apoptosis, proliferation, and angiogenesis by performing Ki-67, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL), and CD31 immunofluorescent staining. As expected, the proportion of TUNEL+ tumor cells in allo LT models was significantly higher than in syn LT and sham models ($P < 0.001$; Fig. 1D). However, tumor proliferation

and angiogenesis showed no difference among these models ($P > 0.05$; Supporting Fig. 1). In addition, similar tumor inhibition effects were observed in EMT-6 experiments (Supporting Fig. 2). These results indicated that allo LT has a powerful effect on tumor inhibition. However, the underlying mechanisms required further investigations.

DONOR T/B CELLS PARTICIPATED IN GVT PROCESS OF ALLO LT

CT-26 (H2d) and EMT-6 (H2d) tumors grew progressively in syngeneic hosts (BALB/c mice) but were rejected by allogeneic hosts (C57BL/6 mice; Supporting Fig. 3). This indicated that H2d was immunogenic in immunocompetent allogeneic hosts. Thus, we first focused our research on allogeneic donor leukocytes. To trace the migration of donor-derived leukocytes after transplantation in allo LT models, allogeneic liver grafts from EGFP+ C57BL/6 mouse donors were transplanted into WT BALB/c mice recipients, which allowed us to easily distinguish donor leukocytes (CD45+ EGFP+) by flow cytometry. Donor leukocytes could be found in the tumor at 6 hours and gradually decreased over time up to 4 days after allo LT (Fig. 2B). Similar results were also found in peripheral blood and other organs of mice recipients (Fig. 2B). In terms of cell classification, these donor tumor-infiltrating leukocytes (TILs) were mainly T/B cells with a small number of NK and other cells (Fig. 2C). These results demonstrated that donor leukocytes migrated very rapidly into the recipient tumor after allo LT.

Although donor TILs were detected in tumors early after LT, there was no direct evidence of their involvement in the GVT process. To determine the immunologic function of donor leukocytes in the GVT effect, allogeneic livers from lethally irradiated C57BL/6 donors (donor leukocytes deleted) were transplanted into WT BALB/c recipients. Significant increases in tumor weight and decreased tumor apoptosis were observed in irradiated mouse models compared with allo LT models at 7 days after transplantation ($P < 0.001$; Fig. 3A,B). Because there was no donor leukocyte migration in peripheral blood of recipients 4 days after LT (Fig. 2B), tumor cells were injected at 4 days after allo LT, and tumor growth was measured to reconfirm the immunologic function of donor leukocytes in the GVT effect. A significant increase in tumor growth was observed in this model compared

with allo LT models at 7 days after tumor implantation ($P < 0.001$; Fig. 3A,B). These results indicated that the donor TIL participated in the GVT process.

To further investigate the functional subsets of donor-derived leukocytes, we performed allo LT with RAG1$^{-/-}$ C57BL/6 mice donors, NK cells, or Kupffer cell–deleted C57BL/6 mice donors and WT BALB/c mice recipients. Antibodies of different concentration gradients were injected into donor mice preoperatively to confirm the efficient concentration for leukocyte clearance. The clearance efficiency of 20-µg anti-asialo monosialotetrahexosylganglioside antibody was >95%, and the efficiency of 300-µL

clodronate liposomes was >70% within 5 days after LT (Supporting Fig. 4A,B).

A significant increase in tumor growth and reduction in tumor apoptosis were observed in RAG1$^{-/-}$ mouse models compared with allo LT models at 7 days after transplantation ($P < 0.001$; Fig. 3C,D). However, no difference in tumor weight and tumor apoptosis was detected between the NK cell and Kupffer cell deletion models compared with the allo LT models ($P > 0.05$; Fig. 3C,D). To restore donor leukocytes to infiltrate the tumor in the RAG1$^{-/-}$ model, 2×10^7 C57BL/c donor-derived hepatic cells were injected through the tail vein of recipients immediately after LT. Tumor
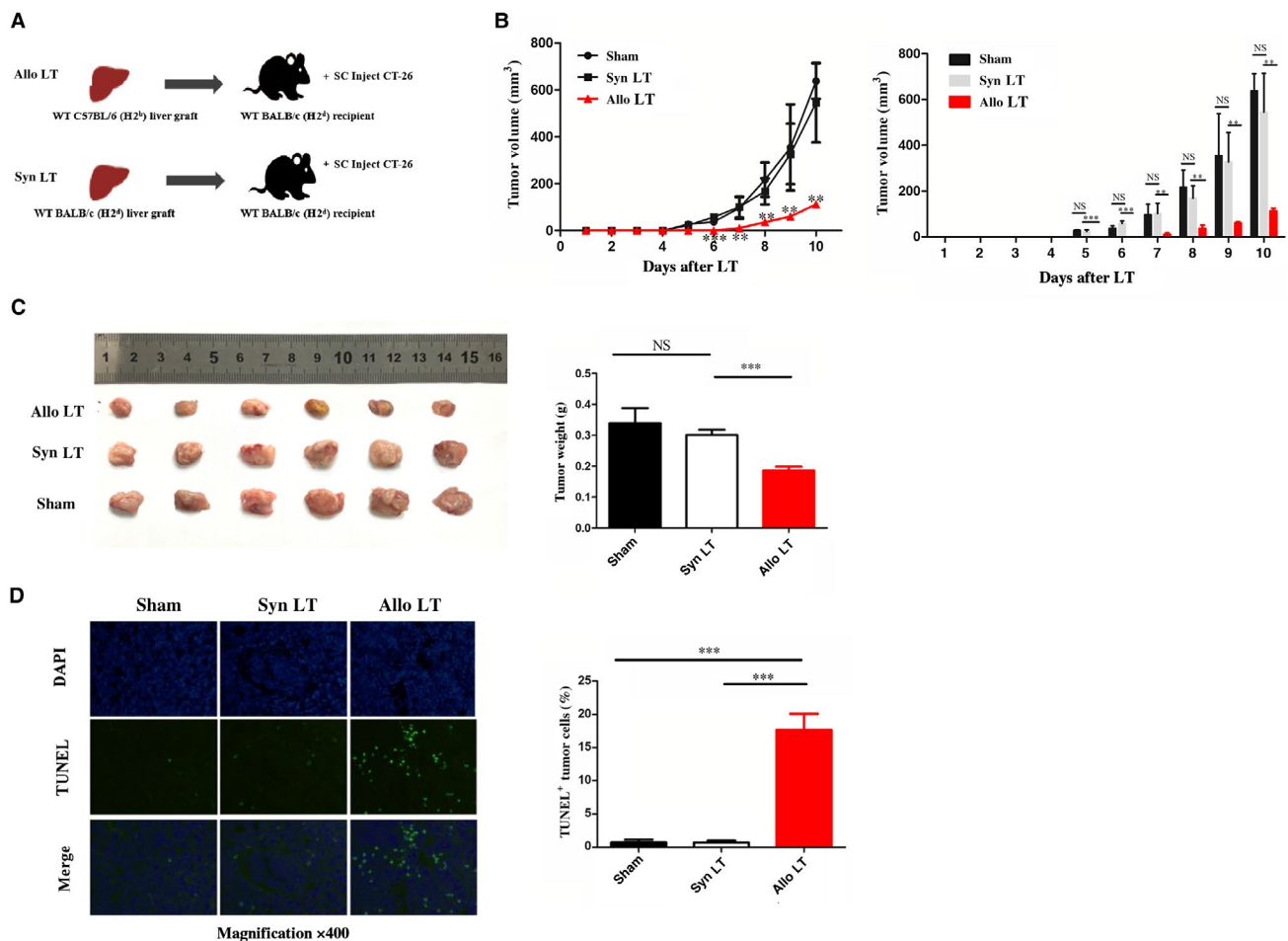

FIG. 1. Tumor growth after liver transplantation in mice. (A) Schematic diagram for mouse liver transplantation models. (B) Short-term tumor growth curve after surgery in allo LT, syn LT, or sham models. Tumor volumes were calculated on day 10 after implantation. Significant tumor suppression was observed between allo LT and syn LT models (2-tailed t test; n = 6). (C) Measurement of tumor weight at 7 days after implantation. A significant difference was obtained between allo LT and syn LT models (2-tailed t test; n = 6). (D) Apoptosis of tumor cells was detected by TUNEL (green) staining (n = 6). Representative images from 1 experiment are shown at a magnification of 400×. The percentage of TUNEL$^+$ tumor cells in total cells was counted and calculated for statistical analysis. Results are presented as mean ± SD for each model. **$P < 0.01$, ***$P < 0.001$.
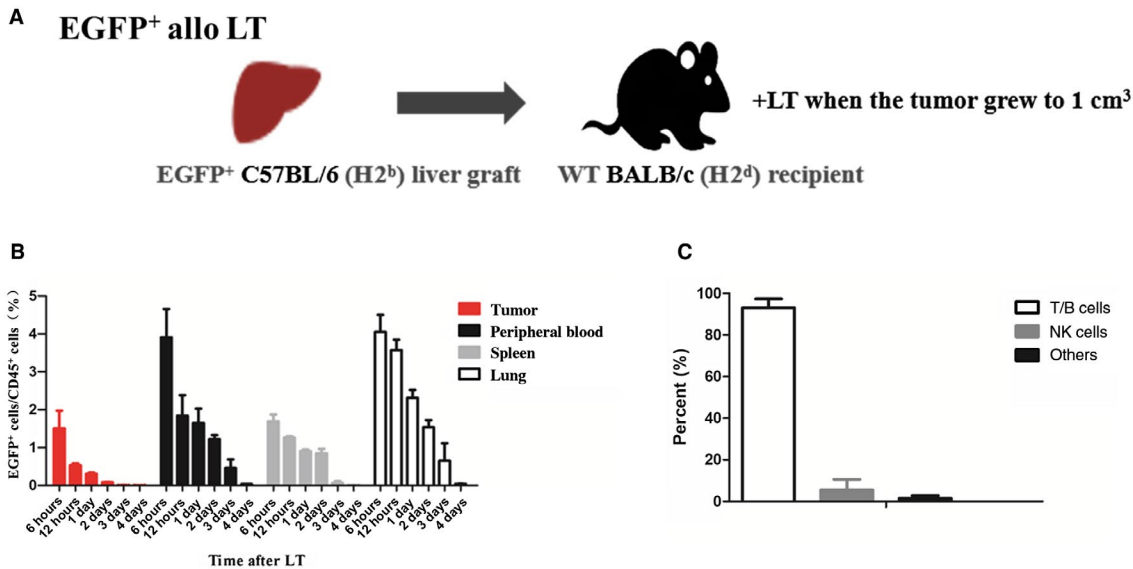
FIG. 2. Migration, distribution, and composition of donor-derived leukocytes after transplantation in allo LT models. (A) Schematic diagram for mouse liver transplantation models of EGFP⁺ graft. (B) Analysis of the migration of donor-derived leukocytes (EGFP⁺ cells). (C) Analysis of the composition of tumor-infiltrating donor cells at 6 hours after LT.

growth was significantly inhibited in these models compared with untreated RAG1$^{-/-}$ models and was close to allo LT models ($P < 0.001$; Fig. 3C,D). These results suggested that donor T/B cells are important for the GVT process.

RECIPIENT T CELLS PLAYED A CRITICAL ROLE IN THE GVT PROCESS OF ALLO LT

As reported in previous studies, recipient-derived immune cells played a critical role in the antitumor effect of allo HSCT and DLI.[20] The previous flow cytometry results confirmed that TILs were almost exclusively derived from the recipient 4 days after transplantation, suggesting that recipient immune cells may participate in the antitumor responses observed after allo LT (Fig. 2B). To investigate whether there is an active recruitment of leukocytes to primary tumor sites in allo LT models, we examined the level of leukocyte infiltration in tumor tissues. As expected, we found that the number of TILs at 7 days after transplantation was significantly higher in allo LT models compared with syn LT models or sham models ($P < 0.001$; Fig. 4A). Particularly, the results of flow cytometry analysis demonstrated that TILs were mostly T cells 2 days after surgery in the allo LT group (Fig. 4B). Moreover,

we found no significant change in the proportion of T cells in peripheral blood after LT ($P > 0.05$), indicating that the increasing of TILs was independent (Fig. 4B). By exploring T cell–activating biomarkers CD25, CD69, and CD44 with flow cytometry, we found that tumor-infiltrating T cells in the allo LT model showed relatively more activity than the syn LT group, manifested by an increasing proportion of CD69⁺, CD25⁺, or CD44⁺ T cells ($P < 0.05$; Fig. 5A). Additionally, there were fewer CD4⁺ T cells in allo LT models than in the syn LT models at 4 days after transplantation. Meanwhile, there were more CD8⁺ T cells compared with syn LT models ($P < 0.05$). However, no significant difference was found at 7 and 10 days after LT. The proportion of granzyme B (GRZB)⁺ or perforin (PFR)⁺ CD8⁺ T cells was significantly higher in allo LT models than in syn LT models at 4, 7, and 10 days after LT ($P < 0.05$; Fig. 5B). Flow cytometry analysis of T cells in tumor tissues demonstrated a significantly increased proportion of T$_h$1 cells and a reduced proportion of T$_h$2 and Tregs in tumor tissues from allo LT models compared with the syn LT models at 4, 7, and 10 days after LT, suggesting that allo LT reshaped the tumor microenvironment ($P < 0.05$; Fig. 5C). Analysis of cytokines and chemokines in tumor tissues from allo LT models also showed the improvement of the immune microenvironment by significantly
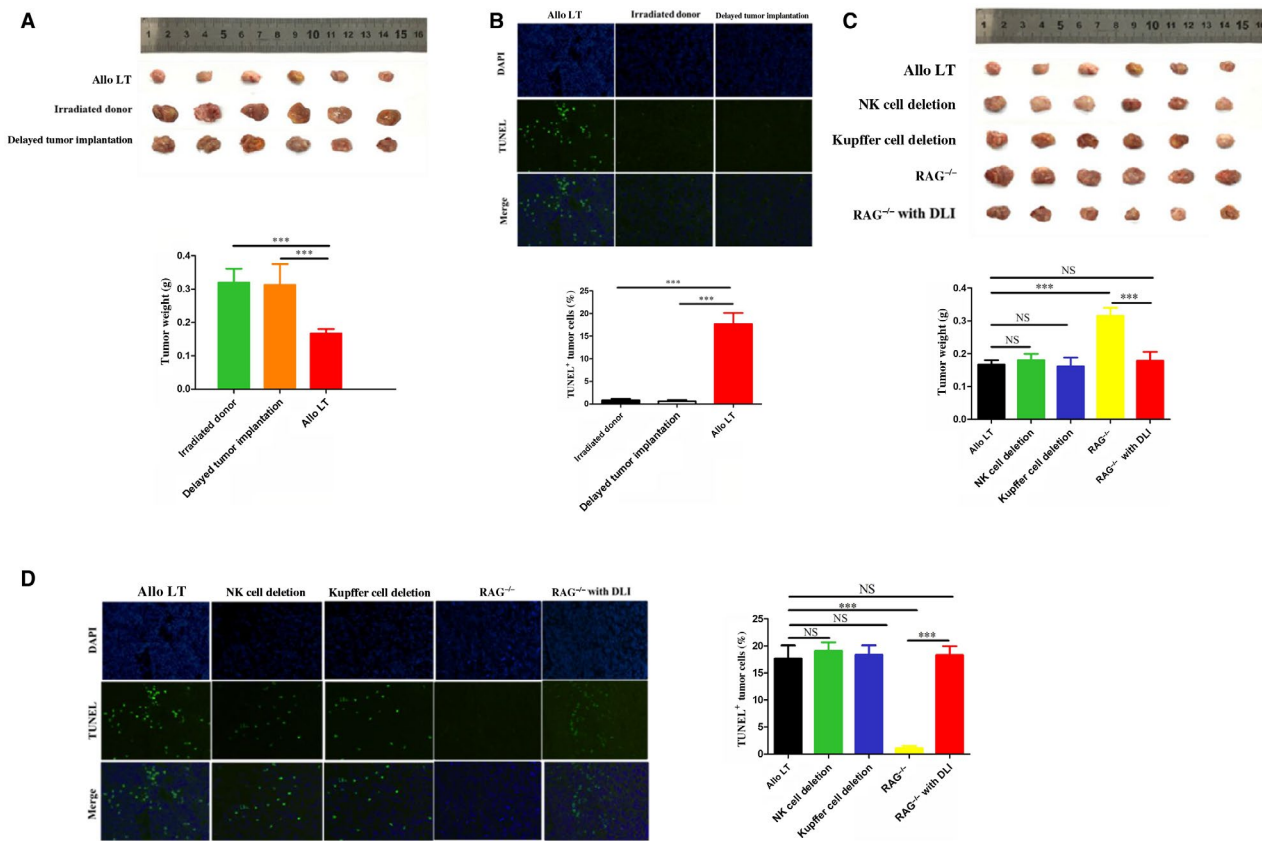
FIG. 3. Donor T/B cells participated in the GVT process of allo LT. (A) A comparison of tumor weight at 7 days after LT with irradiated donor, delayed tumor implantation, and allo LT models. A significant difference was achieved between allo LT and irradiated donor or delayed tumor implantation models (2-tailed t test; n = 6). (B) Apoptosis of tumor cells was detected by TUNEL (green) staining in irradiated donors and in the delayed tumor implantation and allo LT models. Representative images from 1 experiment are shown at a magnification of 400×. The percentage of TUNEL+ tumor cells in the total cells was counted and calculated for statistical analysis. (C) Cross comparison of tumor weight at 7 days after LT with allo LT, NK cell deletion, Kupffer cell deletion, RAG1$^{-/-}$ graft, and RAG1$^{-/-}$ graft with DLI models. A significant difference was achieved between allo LT and RAG1$^{-/-}$ graft models (2-tailed t test; n = 6). (D) Apoptosis of tumor cells was detected by TUNEL (green) staining in allo LT, NK cell deletion, Kupffer cell deletion, RAG1$^{-/-}$ graft, and RAG1$^{-/-}$ graft with DLI models. Results are presented as mean ± SD for each model. ***$P < 0.001$.

elevated levels of IFNγ, IL2, and tumor necrosis factor α (TNF-α); decreased levels of IL10 and IL4; and significantly elevated levels of T cell–related chemokines (IL16, chemokine [C-X-C motif] ligand [CXCL] 10, CXCL11) compared with syn LT models at 4, 7, and 10 days after LT ($P < 0.05$; Fig. 5D). These findings supported the conclusion that allo LT improved the tumor immune microenvironment.

Given the possibility that recipient T cells exerted an antitumor effect in allo LT mouse models, we next performed transplantation of allogeneic livers from WT C57BL/6 donors into immunodeficient BALB/c nude mice (H2^d), a model with T cell deficiency due to the lack of thymus. A significant increase in tumor weight and

reduction in tumor apoptosis were observed in this model compared with allo LT models at 7 days after transplantation ($P < 0.001$; Fig. 5E), indicating that recipient T cells participate in the GVT process of allo LT.

EXTENSIVE INFILTRATION OF RECIPIENT-ACTIVATED T CELLS WAS RELATED TO DONOR T/B CELLS

Acute rejection after allo LT could activate syngeneic effector T cells (H2^d), which may also enhance the antitumor effect in recipient mice. To determine whether rejection could induce antitumor reactions in recipient
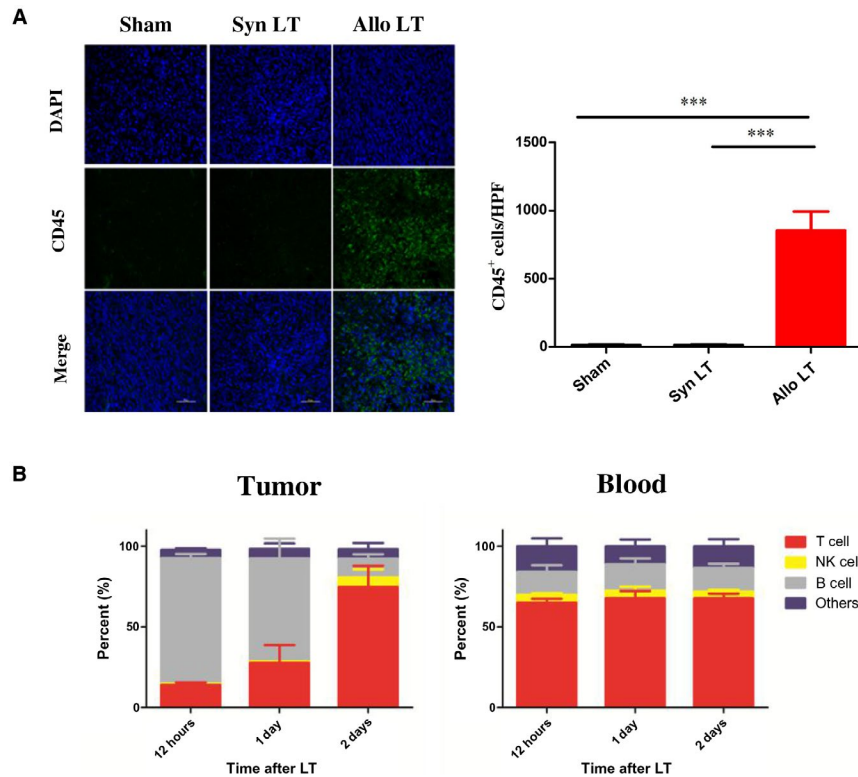
FIG. 4. A large number of T cells infiltrated the tumor after allo LT. (A) TILs were detected by CD45 (green) staining in the allo LT, syn LT, or sham models. The counts of CD45+ cells/HPF were counted and calculated for statistical analysis. The counts of TILs at 7 days after transplantation were significantly higher in allo LT models compared with syn LT or sham models. (B) Peripheral blood and tumor leukocyte classification. Results are presented as mean ± SD for each model. ***$P < 0.001$.

mice, we transplanted allogeneic livers from lethally irradiated C57BL/6 donors (donor leukocytes deleted) into WT BALB/c recipients. Although significant increased activation of recipient T cells in the spleen was found compared with syn LT models ($P < 0.05$; Supporting Fig. 5), the similar tumor weight, tumor apoptosis, infiltration, and activation of recipient TILs was found between irradiated mouse models and syn LT models ($P > 0.05$; Fig. 6A-D). These results suggested that acute rejection had limited antitumor effects after allo LT.

Considering the key role of donor leukocytes in GVT, we investigated the relationship between donor leukocytes and recipient T cells. Although similar activation of recipient T cells in the spleen was found between irradiated mouse models and allo LT models ($P > 0.05$; Supporting Fig. 5), significantly reduced infiltration of TILs and activation of recipient T cells in the tumor were observed in irradiated mouse models compared with allo LT models at 7 days

after LT ($P < 0.001$), suggesting that donor leukocytes could activate recipient T cells (Fig. 6C,D). To further investigate which subsets of donor leukocytes play the role, we used RAG1$^{-/-}$ C57BL/6 mice, NK cell mice, or Kupffer cell deletion C57BL/6 mice as a source of allogeneic donor livers transplanted into WT BALB/c recipients. Significantly reduced infiltration of TILs and activation of recipient T cells in the tumor were observed in RAG1$^{-/-}$ mouse models compared with allo LT models at 7 days after LT ($P < 0.01$; Fig. 6C,D). Moreover, significantly increased infiltration of TILs and activation of recipient T cells were observed in RAG1$^{-/-}$ mouse models with DLI treatment compared with untreated RAG1$^{-/-}$ models ($P < 0.01$; Fig. 6C,D).

These results suggested that the infiltration of TILs and activation of recipient T cells were related to the infiltration of donor T/B cells (Fig. 6C,D). Contrarily, similar infiltration of TILs and activation of recipient T cells were found between NK cell or
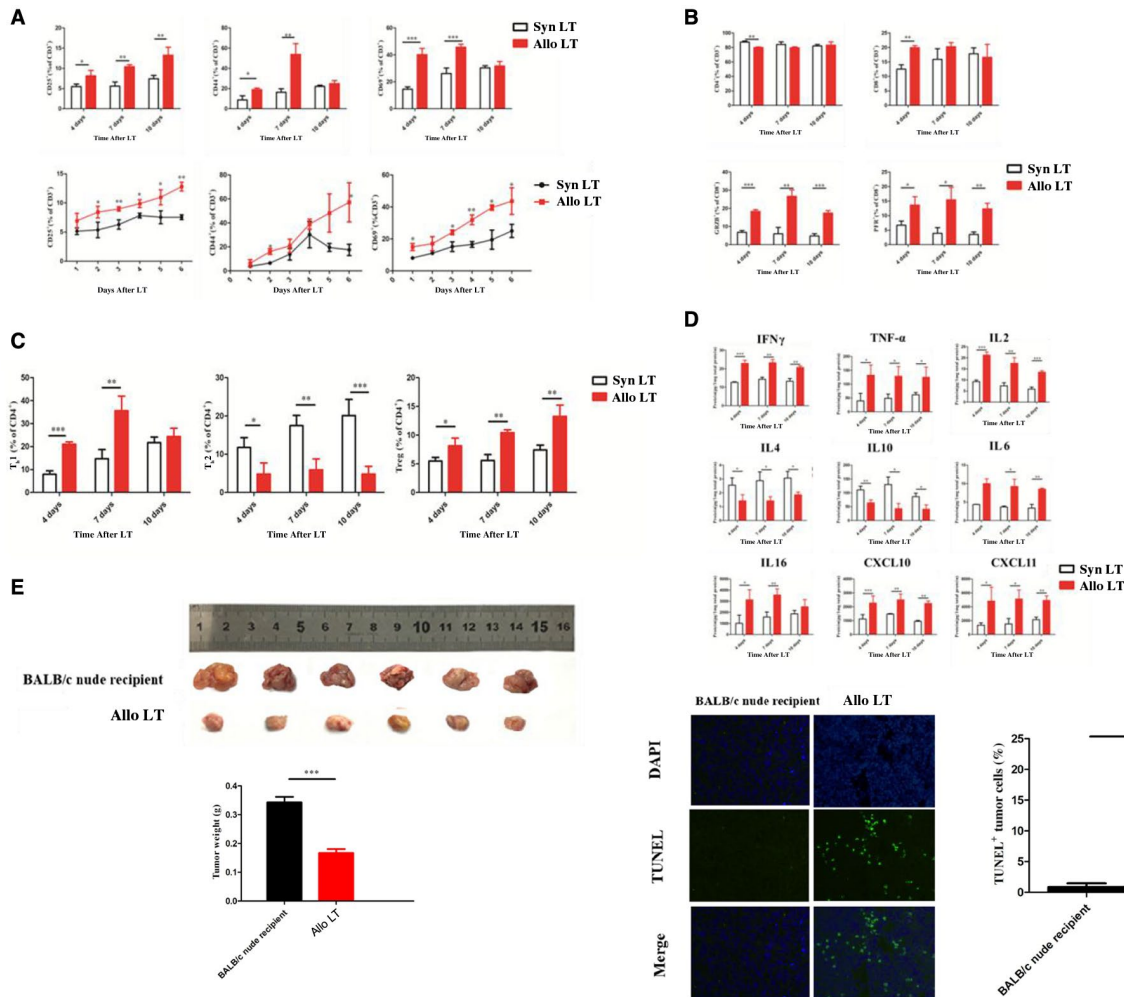
FIG. 5. Activation and classification of tumor-infiltrating T lymphocytes. (A) Tumor-infiltrating T lymphocyte activation was detected with CD25, CD69, and CD44 markers by flow cytometry analysis. (B) Tumor-infiltrating T lymphocytes are divided into CD4$^+$ T cells and CD8$^+$ T cells. Meanwhile, CD8$^+$ T cell activation was detected by GRZB and PFR markers. (C) The proportion of T_h1, T_h2, and Tregs in CD4$^+$ T cells was detected. All indicators were compared in the syn LT and allo LT models (2-tailed t test; n = 3). (D) T cell–associated cytokines and chemokines in tumor tissue were compared in the syn LT and allo LT models. A significant difference was achieved between the allo LT and syn LT models (2-tailed t test; n = 3). (E) Comparison of tumor growth at 7 days after LT with BALB/c nude recipient and allo LT models. Results are presented as mean ± SD for each model. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Kupffer cell deletion models and allo LT models at 7 days after LT ($P > 0.05$), suggesting that neither donor NK cells nor donor Kupffer cells could affect the infiltration of TILs and activation of recipient T cells. In addition, significantly decreased levels of IFNγ, IL2, and TNF-α and T cell–related chemokines (IL16, CXCL10, and CXCL11) as well as elevated levels of IL10 and IL4 were observed in RAG1$^{-/-}$ mouse models compared with allo LT models at 7 days after LT ($P < 0.05$; Fig. 6E), suggesting that donor T/B cells could improve the tumor immune microenvironment.

FK-506 TREATMENT INHIBITED THE GVT EFFECT ON ALLO LT

FK-506 is a conventional immunosuppressant widely used in clinical LT, which is effective in not only preventing acute rejection but also prolonging allograft survival time by inhibiting the function of T cells. Therefore, we investigated whether FK-506 treatment inhibited GVT effects on allo LT. Significant increases in tumor weight and reductions in tumor apoptosis were observed in allo LT mouse models with FK-506 treatment compared with allo LT models
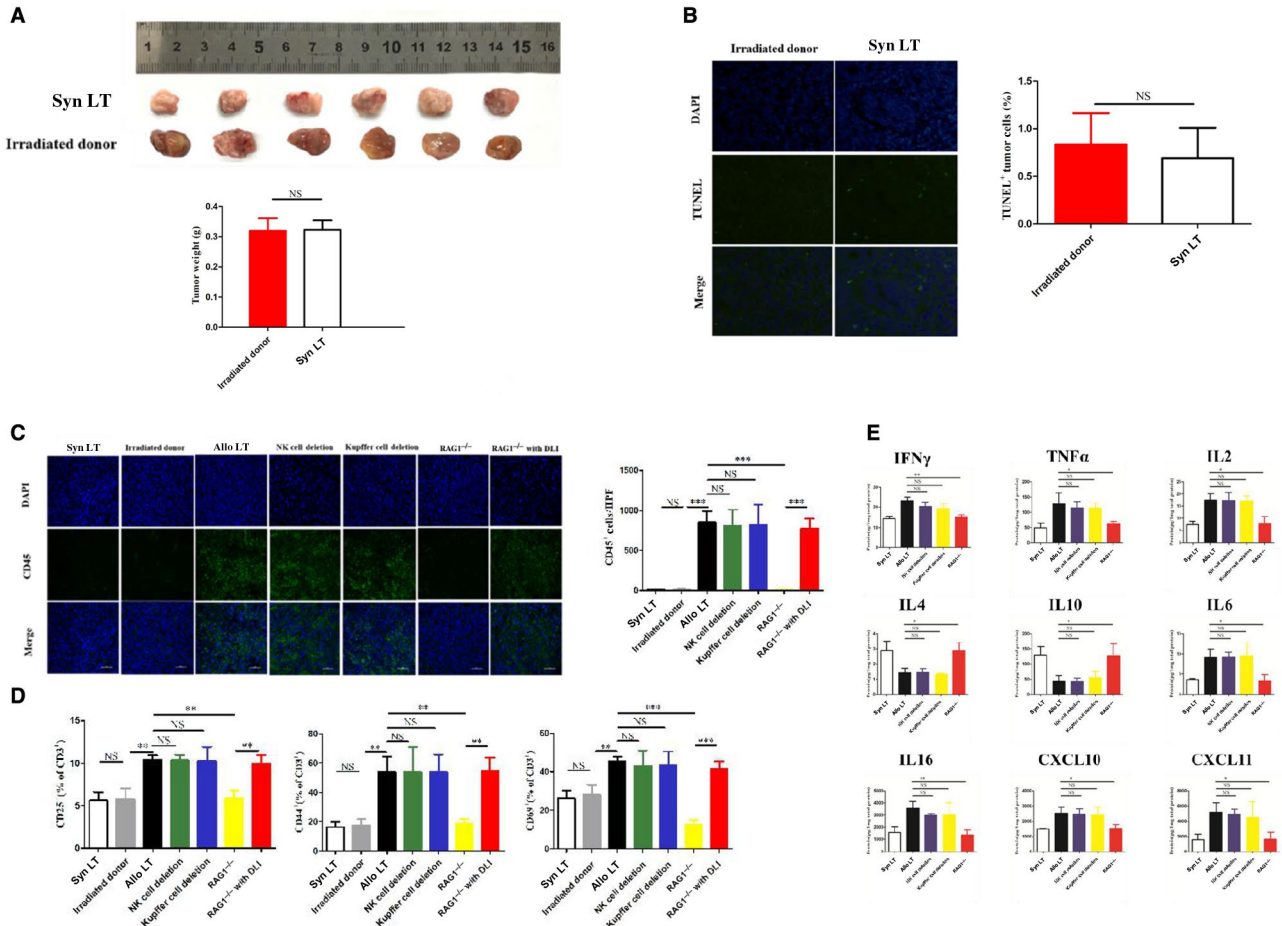
FIG. 6. Extensive infiltration of recipient-activated T cells was related to donor T/B cells. (A) Comparison of tumor weight at 7 days after LT with irradiated donor and syn LT models. No significant difference was achieved (2-tailed t test; n = 6). (B) Comparison of tumor apoptosis at 7 days after LT with irradiated donor and syn LT models. No significant difference was achieved (2-tailed t test; n = 6). (C) TILs were detected by CD45 (green) staining in syn LT, irradiated donor, allo LT, NK cell deletion, Kupffer cell deletion, RAG1$^{-/-}$ graft, and RAG1$^{-/-}$ graft with DLI models. The counts of CD45$^+$ cells/HPF were counted and calculated for statistical analysis. (D) Tumor-infiltrating T lymphocyte activation was detected with CD25, CD69, and CD44 markers by flow cytometry analysis in syn LT, irradiated donor, allo LT, NK cell deletion, Kupffer cell deletion, RAG1$^{-/-}$ graft, and RAG1$^{-/-}$ graft with DLI models. (E) T cell–associated cytokines and chemokines in tumor tissue were compared in syn LT, irradiated donor, allo LT, NK cell deletion, Kupffer cell deletion, RAG1$^{-/-}$ graft, and RAG1$^{-/-}$ graft with DLI models. Results are presented as mean ± SD for each model. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

without FK-506 treatment at 7 days after transplantation ($P < 0.01$; Fig. 7A,B). Moreover, a significantly reduced number of TILs and activated recipient T cells in the tumor were observed in allo LT mouse models with FK-506 treatment compared with allo LT models at 7 days after transplantation ($P < 0.05$; Fig. 7C,D). These results suggest that FK-506 treatment will inhibit the GVT effect on allo LT. In addition, significant reductions in tumor weight and increases in tumor apoptosis were also observed in this model compared with syn LT models at 7 days after transplantation ($P < 0.01$; Fig. 7A,B), suggesting that allo LT with FK-506 treatment still has GVT effects, although relatively weak.

Discussion

Allo HSCT from an HLA-compatible donor has been widely used to treat hematologic malignancies.[21] Allo HSCT not only replaces the marrow affected by the disease but also exerts an immune GVT effect
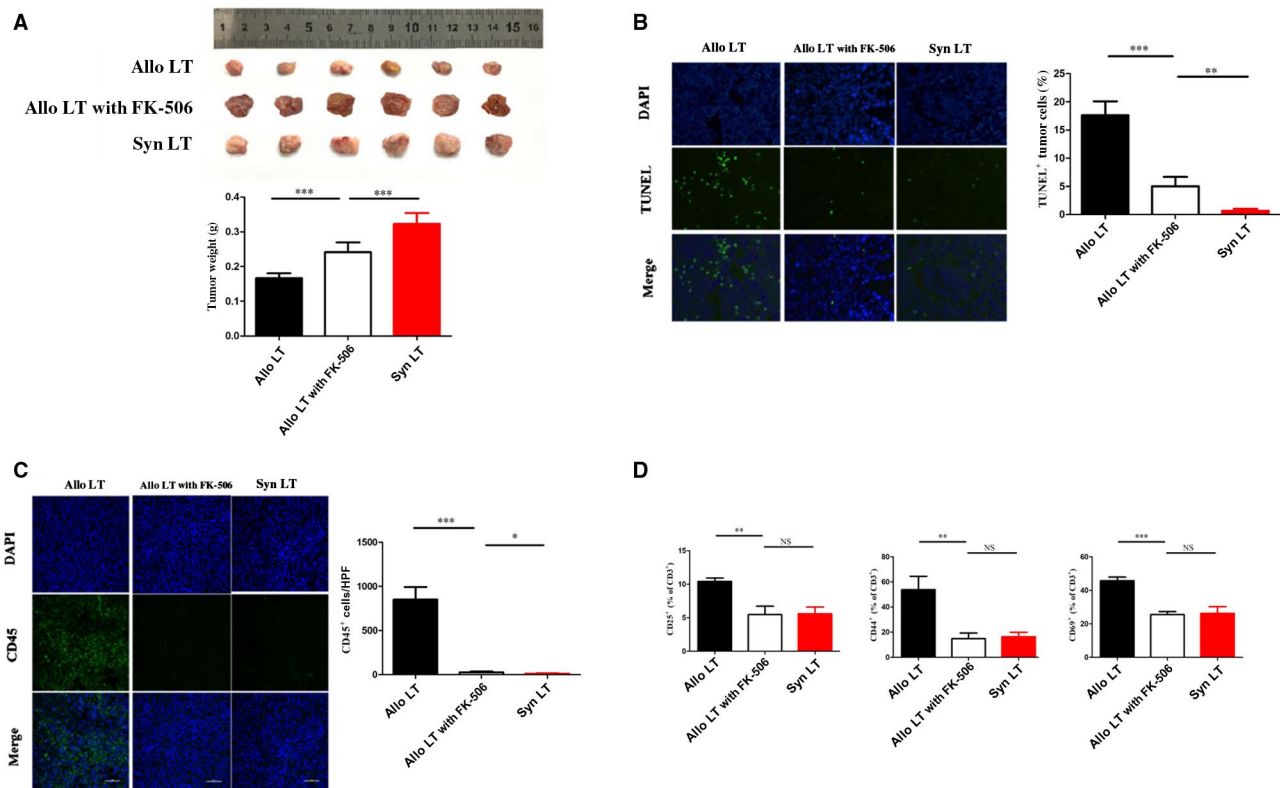
FIG. 7. FK-506 treatment inhibited the GVT effect on allo LT. (A) Comparison of tumor weight at 7 days after LT with allo LT with FK-506 and syn LT or allo LT models. (B) Comparison of tumor apoptosis at 7 days after allo LT with FK-506 and syn LT or allo LT models. (C) TILs were detected by CD45 (green) staining in allo LT with FK-506 and syn LT or allo LT models. (D) Tumor-infiltrating T lymphocyte activation was detected with CD25, CD69, and CD44 markers by flow cytometry analysis in allo LT with FK-506 and syn LT or allo LT models. Results are presented as mean ± SD for each model. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

mediated by donor lymphocytes.[22] The development of nonmyeloablative conditioning regimens before allo HSCT has allowed this therapy to be used in elderly and disabled patients.[23] This allogeneic GVT effect has been observed in a number of patients with renal, breast, colorectal, ovarian, and pancreatic cancer treated with allo HSCT.[20,24] In general, the tumor response is associated with the development of acute and chronic GVT disease.[25,26]

The liver is rich in immune cells as well and has unique immune characteristics.[27] A liver graft may not only take on new functions by transplantation but also may bring the immune characteristics of the donor to the recipient by carrying donor-derived immune cells.[13,15] Our previous research found that immune changes in MHC-mismatched donor livers caused by rejection may accelerate the growth of intrahepatic tumors after LT.[28] However, the role of rejection and

migration of donor-derived immune cells in extrahepatic tumors has not been studied.

This study showed that allo LT had a strong anti-extrahepatic tumor effect in the recipient. However, unlike the mechanism of GVT in bone marrow transplantation, donor-derived immune cells were not the main force of the antitumor effect in allo LT. Infiltration of allogeneic liver-derived leukocytes changed the immune microenvironment of extrahepatic tumors, which resulted in an increase in the levels of IL2, IFNγ, and TNF-α (the activation cytokines related to T cells) and an increase in the levels of CXCL10, CXCL11, IL6, and IL16 (the chemokines related to T cells). These led to the wide infiltration and activation of recipient-derived T cells, which promoted tumor apoptosis and the antitumor effect. Similar results were found in DLI treatment studies.[29,30] Low doses (approximately 4%) of peripheral blood DLI induced

renal tumor regression. This proportion is like that of donor-derived leukocytes in peripheral blood in the early stage of allo LT. However, previous studies did not reveal the mechanism of how low-dose DLI inhibited tumor growth, even though it was attributed to the role of donor-derived leukocytes. Our study demonstrated that chimeras with such a small number of donor-derived leukocytes could decrease tumor growth.

Both HLA-mismatched HSCT and DLI have a stronger antitumor effect than HLA-matched HSCT, but they may also bring a greater risk of GVHD, one of the fatal complications after HSCT.[31-34] Therefore, efforts have been made over the last 2 decades to explore the difference between the mechanism of GVT and GVHD and to design strategies to separate GVT from GVHD.[35,36] One important difference is that the threshold number of T cells triggering GVT is relatively lower. This assumption is supported by clinical experience that low-dose DLI is generally sufficient to reduce the risk of relapse of the tumor without GVHD.[29,30] Similarly, considering the low level of donor-derived immune cells in chimerism, MHC-mismatched LT may have an appreciable antitumor effect without the occurrence of GVHD. Our study showed that in mouse models, the number of donor-derived immune cells after LT just exceeded the threshold of triggering the GVT effect but would not cause GVHD (no GVHD was found in this mouse model). With the decrease of GVHD incidence, the GVT effect of LT was much weaker than that of HSCT but was still observable in our mouse model.

Surprisingly, our study confirmed that recipient-derived cells were also involved in the GVT response in LT. Although previous studies of GVT considered donor-derived immune cells as the major mediator of the antitumor effect in allo HSCT, growing evidence has been shown to support that recipient-derived immune cells also played an important role in the anti-solid tumor effect. Some studies showed that recipient leukocyte infusion may lead to a host-versus-graft reaction, which was associated with recipient-derived T cells, resulting in full donor graft rejection and a significant anti-leukemia response without a risk for GVHD.[11] Another indirect evidence showed that graft antitumor immunity could be achieved even when the allogeneic donor cells were already fully rejected: Sustained antitumor response was seen in 50% of rechallenged longterm survivors of animals that underwent allogeneic lymphocyte infusion.[37] In addition, in a mouse model of treating renal cell carcinoma

by inducing low donor chimerism to trigger antitumor effects through DLI, the TILs were almost exclusively of recipient origin.[29]

The results of our study and previous studies demonstrated the limited effect of inactivated recipient-derived immune cells on tumors.[38] However, when recipient-derived cells were activated, they had a strong antitumor effect on recipient-derived tumors (Supporting Fig. 6). In our allo LT mouse model, we found there were 2 factors that led to the activation of recipient leukocytes. One was the immune rejection, and the other was the infiltration of donor-derived leukocytes into the tumor. Rejection led to the activation of peripheral immune cells, especially T cells, which may enhance the antitumor response, but was not the key factor that caused the activation of GVT-related recipient cells. When we used RAG1$^{-/-}$ mouse models or irradiated mouse models (with rejection but without donor cell infiltration), spleen recipient–derived leukocytes were activated but did not cause a significant antitumor effect.

These results suggested that rejection had a limited effect on recipient T cell–mediated antitumor response. However, when we removed tumor-infiltrating donor-derived T/B cells through RAG1$^{-/-}$ mouse models, the activation degree of tumor-infiltrating T cells was significantly reduced. Then, after increasing the infiltration of donor-derived lymphocytes in tumors by intravenous infusion, the activation of tumor-infiltrating T cells was significantly elevated and the antitumor effect was enhanced, suggesting that donor lymphocyte infiltration was the main activating factor of the antitumor reaction. A similar antitumor immune response was found following DLI in mixed chimeras as well. Colvin et al. reported that immediately after donor-derived cell infusion, a cytokine storm was induced, which was thought to break immune tolerance of recipient immune cells toward tumors in patients with various hematologic malignancies.[39]

Although previous studies have confirmed that LT is an excellent treatment option for patients with hepatocellular carcinoma (HCC) in accordance with the Milan criteria, it is not recommended for those who exceed Milan criteria or suffer from other liver malignancies (metastatic tumors, intrahepatic cholangiocarcinoma, and so on).[40] Two possible risks need to be assumed for these patients. First, circulating tumor cells (CTCs) after LT increased the risk of tumor recurrence.[41] Second, for metastatic tumors, whether the primary tumor is completely resected affected the

prognosis of patients. Previous studies have analyzed various solid tumors and found that T cell infiltration was an important factor affecting prognosis.[42-44] In patients with early colorectal cancer, the presence of intratumoral or peritumoral CD8^{+} T cells affected tumor recurrence. In addition, because many studies focused on the role of NK cells in CTC clearance, some reports provided circumstantial evidence for the correlation between CTC and T cell immunity.[45] In this study, we confirmed that allo LT changed the distribution of T cells inside and outside the tumor. Such changes in T cell distribution and activation may be beneficial to patients in the early stage after LT.

Although allo LT mice showed a significant antitumor effect, clinical application still requires further research. First, considering that a strict match of HLA is not required in clinical LT, HLA-mismatched LT may be performed to reduce the postoperative recurrence of HCC. However, more clinical studies are required to verify the effect of HLA-mismatched LT on HCC patients. Second, the usage of traditional immunosuppressive agents after LT will significantly affect T cell function, which will surely impact the GVT effect. This is the reason that there is no significant GVT effect in clinical LT. Therefore, how to balance rejection and the GVT effect with an appropriate regimen of immunosuppressors needs further study. In summary, the GVT effect of an allogeneic liver graft indeed existed in mice LT. Our results demonstrated the key roles of donor- and recipient-derived immune cells in the GVT effect. Broadening the therapeutic strategy of LT for malignant liver tumors could be beneficial.

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