



Decreased Foxp3 and function of Tregs caused immune imbalance and liver injury in patients with autoimmune liver diseases post-liver transplantation

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Background: Autoimmune liver diseases (AILD) is a type of autoimmune disease which may cause end-stage liver failure and require liver transplantation. Regulatory T cells (Tregs) play an irreplaceable role in maintaining immunological homeostasis.

Methods: In this study, we made a comparative analysis of the immune balance and graft function between AILD patients' post-transplantation and the patients who have had liver failure with hepatitis B virus (HBV) infection post-transplantation. Immune cell phenotype of two groups were analyzed. We sorted CD4+CD25+CD127⁻Tregs both *in vitro* and *vivo* and did TSDR methylation status assay to explore further possible mechanisms.

Results: Our data showed that there is a worse prognosis with severe graft function in liver transplant patients with AILD compared to patients with HBV-induced liver failure. Immune cell phenotype analysis showed that more Tregs could be detected in AILD patients compared with HBV patients' post-transplantation. We sorted CD4+CD25+CD127⁻Tregs *in vivo* and showed that Tregs presented decreased function both *in vitro* and *vivo*. Mechanism study also proved that modulation of the phosphorylation level of STAT1 and STAT3 as well as the methylation level of TSDR in Foxp3 might partially result in the function loss of Tregs.

Conclusions: These results suggest that loss of Foxp3 expression and suppressive function of Tregs may be the critical factor that causes graft loss for liver transplant patients after AILD.

Keywords: Autoimmune liver diseases (AILD); liver transplantation; liver injury; Treg; immune balance

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Introduction

Currently, liver transplantation is the most effective treatment for acute and chronic liver failure when medical options are no longer available (1,2). Autoimmune liver diseases (AILD) is a type of autoimmune disease mainly composed of autoimmune hepatitis (AIH), primary

sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) which may cause end-stage liver failure and require liver transplantation. In the USA, 24% of liver transplant patients are patients with AILD (3).

Regulatory T cells (Tregs) play an irreplaceable role in maintaining immunological homeostasis. Two main subsets

of Tregs have been well described: thymus-derived natural Tregs (nTregs) and induced Tregs (iTregs). Interleukin (IL)-2 and transforming growth factor (TGF)- β can induce the formation of iTregs from CD4⁺ naïve T cells *in vitro* (4). Tregs play a key role in maintaining the balance and tolerance in transplant patients and sustaining the graft function in patients. Tregs have been proven to be important in regulating alloreactive immunity after solid organ transplantation in several studies (5,6). For example, Treg frequency is related to the long-term graft survival one year after kidney transplantation (7). Tregs tend to decline after transplantation, but their suppressive capacity is maintained or even enhanced against donor antigens (8). Studies in kidney transplant recipients indicated increased FoxP3 expression negatively correlated with acute rejection incidence (9), Louis observed reduced Treg frequencies in peripheral blood during chronic rejection (10). Similarly, Tregs after bone marrow transplantation had been related to acute and chronic graft-versus-host disease (11,12), with reduced Treg frequencies possibly being involved in the development of chronic graft-versus-host disease (13-15). However, Specific post-transplant issues in patients transplanted for AILD are the recurrence of the original disease and a higher rate of rejection, which may both impact on survival (16).

Here, we demonstrate that low Foxp3 expression and unstable functionality may be the cause of the immune imbalance and decreasing of graft function. Improving the function of Tregs may be a novel therapeutic strategy to prolong the graft function and benefit to those post-transplantation patients with AILD.

Methods

Patients

Sixty-nine patients with liver transplantation during between June 2012 and February 2019 who had had a clear clinical diagnosis of failure, including hepatitis B virus (HBV)-induced liver cirrhosis or AILD, were used as research subjects. Indications of LT were the complications of end-stage liver diseases, including hepatic encephalopathy, upper gastrointestinal hemorrhage, spontaneous bacterial peritonitis, hepatorenal syndrome and refractory ascites. The local ethics committee approved the study. Informed consent was obtained from all participants.

Specimen collection

When the patients were admitted to the hospital, we

collected fasting blood at 7:00 in the morning without any treatments. These samples would be used for biochemical indicators and immune cell phenotype determination.

For the immune cell phenotype determination, peripheral blood mononuclear cells (PBMCs) were surface-stained with CD8 to evaluate the proportion of CD8⁺T cells. For Tregs analysis, PBMCs were stained with CD4, CD25, and CD127, while CD4⁺CD25⁺CD127⁻ were considered Tregs.

For the function analysis of Treg, PBMCs were prepared from heparinized venous blood of transplant patients from both groups by Ficoll-Hypaque density gradient centrifugation. Human nTregs were sorted from PBMCs by gating on CD4⁺CD25^{bright}CD127⁻ cells (>97% purity). nTregs were activated and expanded with anti-CD3/CD28 beads (1 bead to 3 cells) and IL-2 (300 U/mL) for 10 days.

Observation indicators

Biochemical indicators for liver function such as ALT and AST, was detected through Beckman Coulter AU5800 automatic biochemical analysis system. The phenotype of the immune cells for the patient was analyzed through FACS.

Suppressive assays *in vitro*

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized venous blood of healthy adult volunteers by Ficoll-Hypaque density gradient centrifugation. PBMC were labeled with CFSE (InVitrogen). Anti-CD3 mAb-coated beads (Dyna) were added in a 1:1 ratio (bead: PBMC), and nTregs were added at different ratios. Finally, cultures were incubated at 37 °C. On day 4, cells were stained with anti-CD8 APC. Data were acquired and analyzed using the proliferation platform in FlowJo, and the suppression index was determined using Division Index (17).

Flow cytometry

For extracellular staining, harvested cells were washed and incubated in PBS containing 1% FBS containing the below fluorochrome-conjugated antibodies in a flow tube. For intracellular staining of cytokines, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL, Biogems), ionomycin calcium salt (1 μ g/mL, Biogems), and brefeldin A (5 μ g/mL, Biogems) for 6 h. Then, cells were stained with surface markers and further fixed/permeabilized (BioLegend) and stained for intracellular protein. Human-

specific monoclonal antibodies used for flow cytometry included CD4 (A161A1), CD25 (BC96), Foxp3 (206D), CD45RA (HI100), purchased from BioLegend, and CD127 (A019D5) purchased from BD Pharmingen. Sample detection was performed by MACSQuant Analyzer 10 (Miltenyi Biotec, Germany) and data were analyzed with FlowJo software.

Xeno-GVHD model generation

NOD/SCID/IL2r common γ chain^{-/-}(NOG) mice were obtained from Jackson Laboratory. The mice were bred and housed under specific pathogen-free conditions in microisolator cages and given unrestricted access to autoclaved food and sterile water. Animals of both sexes were used for experiments at 8–12 weeks of age. The mice received a single dose of 200 cGy gamma irradiation from a linear accelerator before the injection of human PBMC on the same day (18). All experiments were performed according to the guidelines of the Institutional Animal Committee of Nanjing Medical University.

Western blot analysis

Proteins were extracted from harvested cells, and their concentration was determined by the BCA assay (Pierce). Protein samples (30 μ g) were resolved by SDS-PAGE and transferred to a PVDF membrane. The following antibodies were used: STAT1 (Cell signaling technology, #9170), STAT3 (Santa Cruz biotechnology, SC-8019), p-STAT1 (Cell signaling technology, #7649), and p-STAT3 (Santa Cruz biotechnology, SC-81523). The results were visualized with Kodak autoradiography film (Kodak XAR film).

TSDR methylation status assay

Genomic DNA was isolated using the Mammalian Genomic DNA Extraction kit (Beyotime) and processed by using the EZ DNA Methylation-Direct kit (Zymo Research) according to the manufacturer's protocol. Purified bisulfite-treated DNA was used in bisulfite sequencing PCR with the following a pair of TSDR amplification PCR primers: 5'-TTG GGT TAA GTT TGT TGT AGG ATA G-3' and 5'-ATC TAA ACC CTA TTA TCA CAA CCC C-3'. The PCR products were purified and cloned into pMD-18T vector (Takara) and single clones were selected for sequencing. All sequencing results of bisulfite-converted TSDR region were analyzed on BDPC DNA methylation

analysis platform, and the average methylation status of 11 CpG sites in TSDR region was statistically analyzed.

Statistical analysis

All research data were imported into excel form, statistical analysis was performed using SPSS software, and charts were made with Graphpad 6.0. The counting data obtained from the study were uniformly processed by paired *t*-test analysis, linear regression analysis and nonlinear regression analysis. Differences in Kaplan-Meier survival curves were analyzed by the log-rank test. Analyze the data with a statistical difference of $P < 0.05$ or $P < 0.01$.

Results

Patient information

A total of 69 patients who underwent liver transplantation in The Liver Transplantation Center of the First Affiliated Hospital of Nanjing Medical University from June 2012 to February 2019 were included in the study, which comprised 41 males and 28 females. The mean age of the patients was 52.7 years; 4.3% of the patients were younger than 30 years old, while 20.3% were older than 60 years. Among the patients, 47 patients have had a hepatic viral infection (68.1%) while 22 had autoimmune (31.9%) disease, which caused liver failure before transplantation. Furthermore, 17.4% of the patients presented abnormal hepatic function (ALT or TBIL > 1.5 folds of the local laboratory upper limit of normal) (*Table 1*).

Liver function and immune cell phenotype were modulated for patients after liver transplantation

The serum ALT and TBIL levels were considered as important markers of liver graft function post-liver transplantation (19,20) and correlated with the extent of hepatocellular necrosis (21) and biliary tract functionality (22,23). Thus, we analyzed both the ALT and TBIL of the patients' post-transplantation from the HBV group or the AILD group. Our data showed that AILD presented higher levels of ALT, AST and TBIL compared with the HBV group (ALT: 90.8 ± 61.9 to 38.5 ± 31.7 , TBIL: 76.3 ± 143.5 to 9.9 ± 5.1 , P value < 0.001) (*Figure 1A*). Moreover, we also investigated the percentage of the patients with abnormal ALT or TBIL in a different group, which indicated that there was both a higher ALT and TBIL level in the AILD

Table 1 Patient information for the subjects

Patients information	Number	Proportion (%)
Gender		
Male	41	59.4
Female	28	40.6
Age (years)		
<30	3	4.3
31–60	52	75.4
>60	14	20.3
Average	52.7±9.5	
Pathogen		
Autoimmune liver disease	22	31.9
AIH	10	14.5
PBC	6	8.7
PSC	6	8.7
Hepatitis B cirrhosis	47	68.1
Liver dysfunction		
Present	10	14.5
Absent	59	85.5
MELD score		
Autoimmune liver disease	16.4±6.5	
Hepatitis B cirrhosis	15.1±6.0	
Months after surgery		
Average	41.6±22.2	
Total	69	100

AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

group compared to the HBV group (ALT: 27.3% to 8.5%, TBIL: 18.2% to 4.4%, P value <0.001) (*Figure 1B*). We also analyzed both the ALB, INR and Cr of the patients post-transplantation from the HBV group and the AILD group. Our data showed that AILD presented higher levels of INR compared with the HBV group, lower levels of ALB compared with the HBV group and the levels of Cr had no statistical difference in two groups (*Figure S1*).

We also tested immune cell phenotypes like CD8 and Tregs for different groups. The unique structure of the hepatic tissue allows direct activation of alloreactive naïve CD8 T cells within the liver graft itself, long-term donor chimerism, Tregs and soluble allo-MHC molecules secreted

by the liver allograft (24). Tregs, mainly CD4⁺CD25⁺FoxP3⁺ T cells, are responsible for the maintenance of allograft tolerance following transplantation of various organs (25). No significant difference was observed in CD8 T cells for different groups (*Figure 1C*); surprisingly, we observed higher Tregs in the AILD group compared with the HBV group (*Figure 1D*). CD8/Treg can also be an essential marker for immune balance and tolerance (26,27). Thus we calculated the level of CD8/Treg from different groups, which proved that CD8/Treg was significantly decreased in the AILD group (2.3±1.1) compared with the HBV group (4.4±1.9) (*Figure 1E*).

Treg and CD8/Treg fully corrected to the graft function in AILD patients post-transplantation

We verified CD8, Treg, and CD8/Treg to the graft function, including ALT and TBIL. While no correlation was observed between CD8 and TBIL, CD8 showed a negative correlation to ALT (*Figure 2A*), Treg showed a strong positive correlation to ALT or TBIL in the AILD group post-transplantation (*Figure 2B*). Furthermore, a negative correlation was also detected between CD8/Treg to ALT or TBIL (*Figure 2C*). We also analyzed the relative correlation level in the HBV group; no significant correlation was detected (*Figure S2A,B,C*).

AILD-Treg expresses less Foxp3 and is not stable during culturing compared to HBV-Treg

Previous data surprisingly determined the percentage of CD4⁺CD25⁺CD127⁻ subset was negatively correlated with graft function in ALID patients post-transplantation. It was previously thought that Tregs play an essential role in maintaining immune balance and graft function during organ transplantation (25). Thus, we isolated nTregs from AILD and HBV groups through sorting CD4⁺CD25^{bright}CD127⁻ cells (>97% purity) from PBMCs of the patients. As Foxp3 is the key marker for Tregs (28), we evaluated the expression of Foxp3 from both groups. Our data showed that Tregs from the AILD group (AILD-Tregs) presented lower Foxp3 expression compared with Tregs from the HBV group (HBV-Tregs) (*Figure 3A,B*). We also expanded the Tregs for further experience and analyzed the stability of Foxp3 expression for both Treg subsets. Tregs were cultured with the addition of IL-2, anti-CD3/CD28 beads for 10 days, and IL-2 and culture medium were added every three days to maintain the concentration of cells during culture. The results were that both Tregs

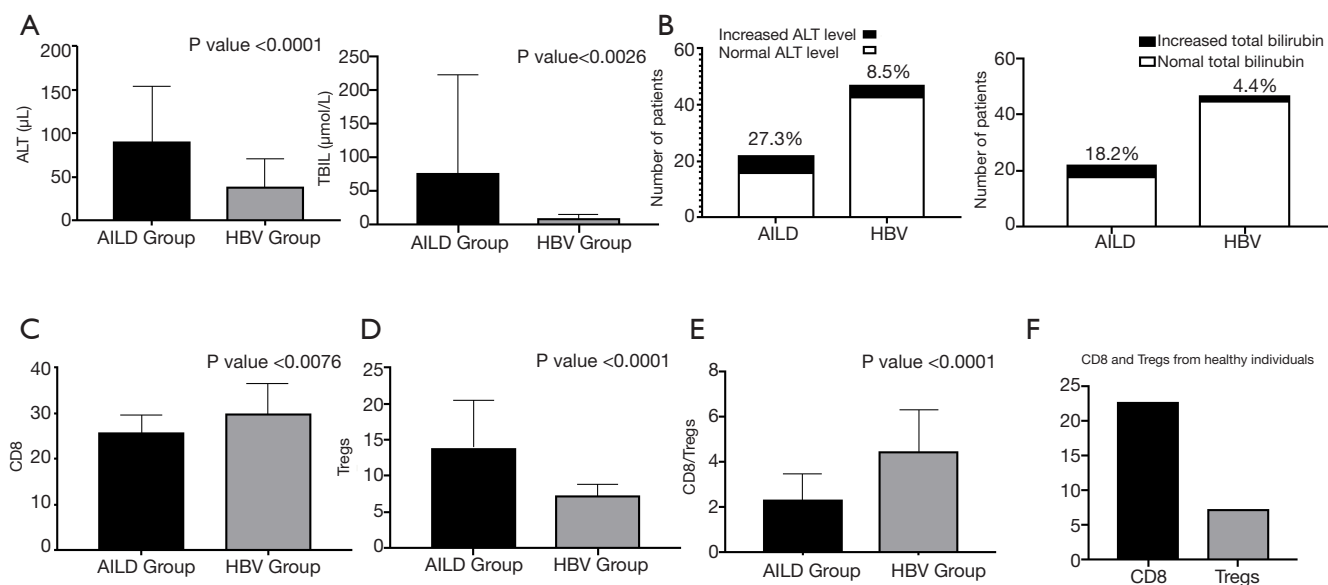


Figure 1 Liver function and immune cell phenotype were modulated for patients after liver transplantation. (A) The levels of ALT and TBIL were evaluated from the serum of liver transplantation patients; (B) the percentage of abnormal ALT or TBIL levels between both groups; the proportion of CD8 (C) and Tregs (D) from different groups were analyzed through FACS; (E) the ratio of CD8/Treg is shown; (F) the proportion of CD8 and Tregs from healthy individuals is shown. The bar shows the mean ± SEM of the levels of indicated proteins. The difference was analyzed by *t*-test. ALT, alanine aminotransferase; TBIL, total bilirubin.

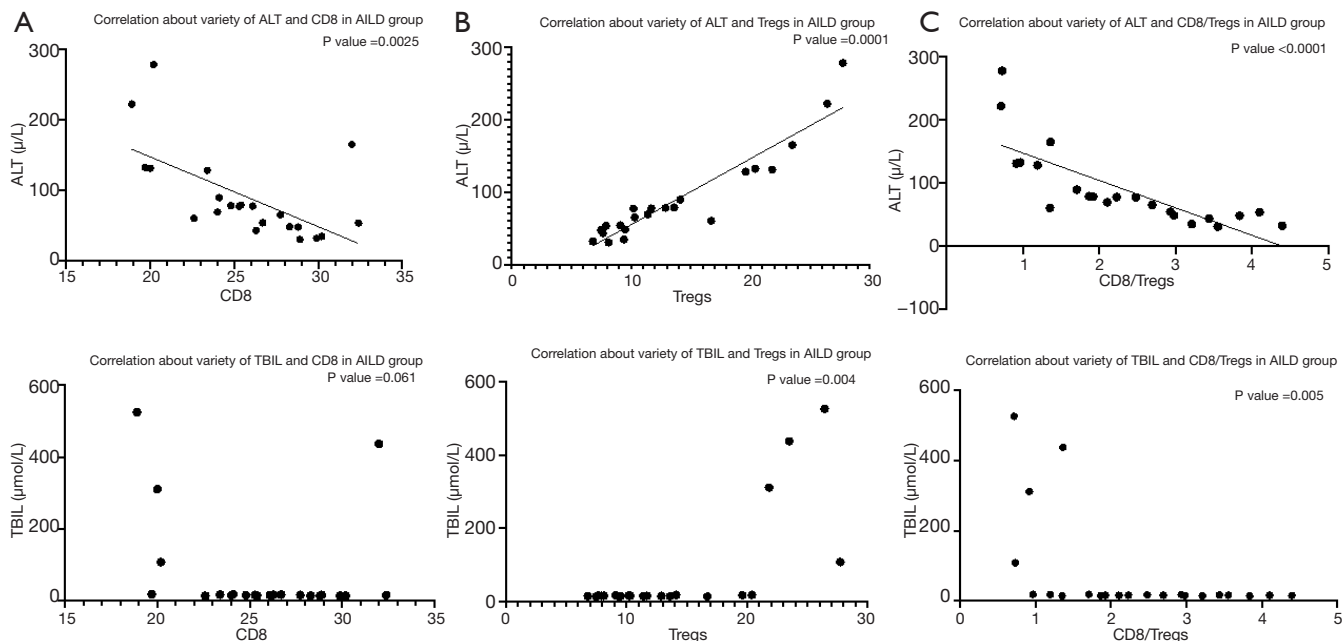


Figure 2 Treg and CD8/Treg fully corrected to the graft function in AILD patients' post-transplantation. Linear regression analysis was used to assess the correlation between the changing of CD8 (A), Treg (B), and CD8/Treg (C) to the graft function such as ALT or TBIL for the AILD patients' post-transplantation. AILD, autoimmune liver disease; ALT, alanine aminotransferase; TBIL, total bilirubin.

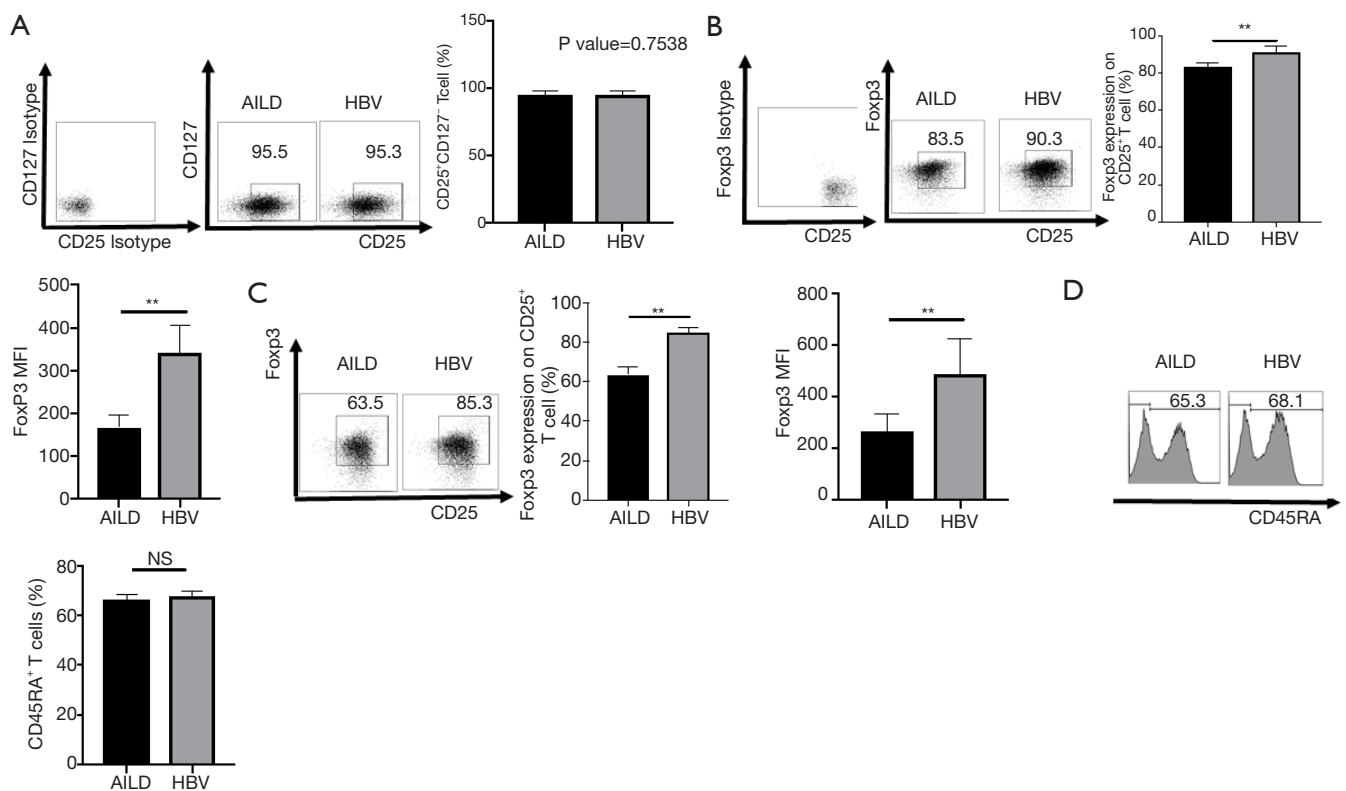


Figure 3 AILD-Treg expresses less Foxp3 and is unstable during culturing compared with HBV-Treg. Representative flow plot for Foxp3 expression of AILD-Treg (A,B) and HBV-Treg (C) on day 0 and day 10. The CD45RA staining showed that the percentage of CD45RA⁺ T cells in two groups had no statistical difference (D). Data are mean \pm SEM of five independent experiments. **, $P < 0.01$. AILD, autoimmune liver disease; HBV, hepatitis B virus.

loss Foxp3 expression while AILD-Tregs decreased more Foxp3 compared with HBV-Tregs (Figure 3C). The CD45RA staining showed that the percentage of CD45RA⁺ T cells in two groups had no statistical difference (Figure 3D).

Tregs presented lower suppressive ability both in vitro and in vivo in AILD group compared with HBV group post-transplantation

PBMC isolated nTreg from AILD or HBV group were cultured for 10 days, and then, cells were harvested. CFSE co-culture assays were performed to estimate the suppressive ability of the two Treg subsets. nTreg were co-cultured with CFSE-labeled fresh PBMC in the presence of anti-CD3 beads. HBV-Tregs showed stronger suppressive activity in any ratio contrasting to AILD-Tregs (Figure 4A). We next analyzed the immunosuppressive ability of nTreg from both groups *in vivo* in a mouse xenogeneic

GVHD model. Co-transfer of HBV-Tregs with PMBCs significantly prolonged mouse survival and delayed severe weight loss compared to the AILD-Treg group (Figure 4B), and pathological examination proved there was more critical tissue damage and infiltration in the small intestine, liver, and kidney in the AILD-Treg groups compared with the HBV-Treg group (Figure 4C).

Higher STAT1 and STAT3 phosphorylation and CpG methylation was detected in AILD-nTreg compared with HBV-nTreg

To further examine the underline mechanism by which AILD-Tregs express lower Foxp3 and are unstable, and not as functional as HBV-Tregs *in vitro* and *in vivo*, we investigated the expression and activation of the downstream signaling during inflammatory cytokine stimulation. It has been well recognized that IL-1 and/or IL-6 exert their effects through the activation of STAT1 and STAT3, respectively (29-31).

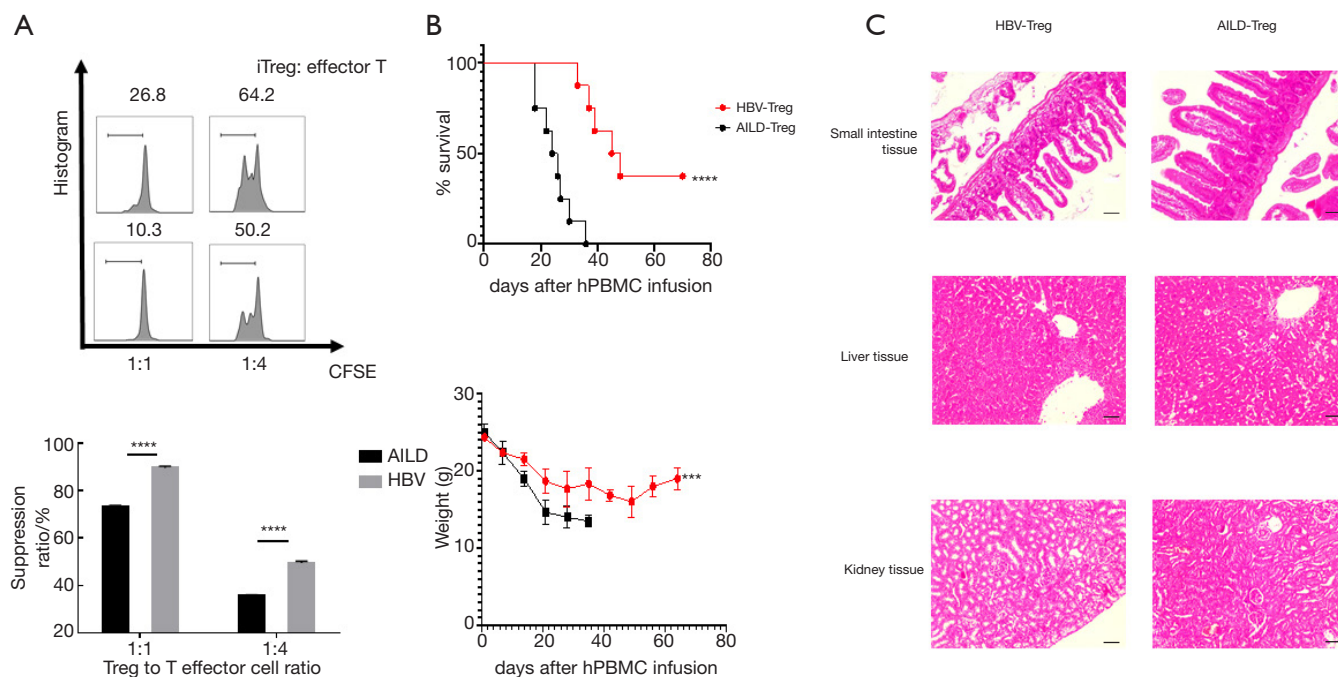


Figure 4 Tregs presented lower suppressive ability both *in vitro* and *in vivo* in the AILD group compared with the HBV group post-transplantation. (A) CFSE-labeled PBMCs were cocultured with both nTreg subsets with the addition of anti-CD3 mAb-coated beads for three days. Cells were stained with anti-human-CD8 antibody, and the suppressive activity of various primed cells subsets on CFSE-labeled CD8+ cells at different T suppressor to T effector ratios is shown. The result is representative of three separate experiments. (B) Survival and average weight of GVHD mice are shown. (C) Representative pathological figures for the intestines, liver, and kidney are shown. Kaplan-Meier survival curves depict the percentage of live mice (B). The weight loss data are presented as the mean \pm SEM from two independent experiments (B). Scale bars: 200 μ m. ***, $P < 0.001$; ****, $P < 0.0001$. AILD, autoimmune liver disease; HBV, hepatitis B virus.

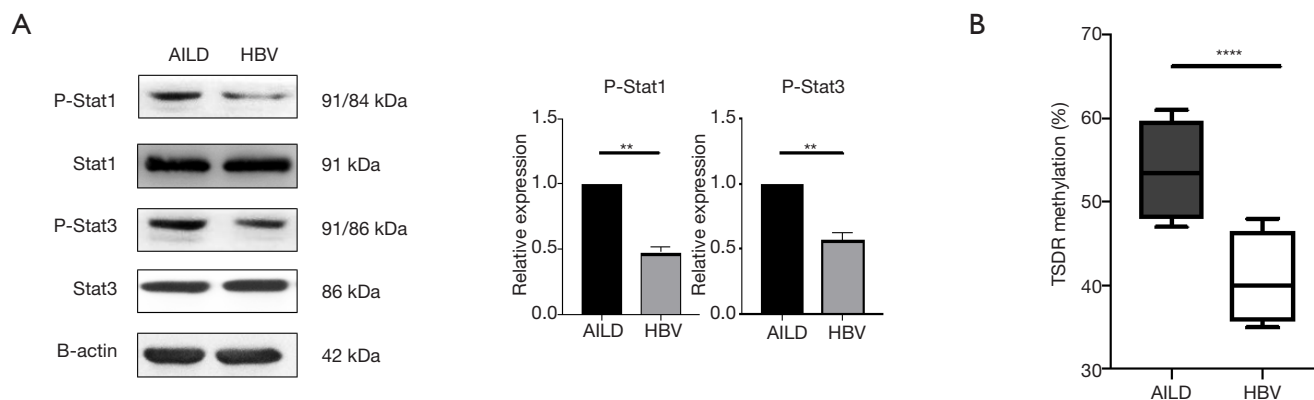


Figure 5 Higher STAT1 and STAT3 phosphorylation and CpG methylation were detected in AILD-nTreg compared with HBV-nTreg. (A) The phosphorylated STAT1 and STAT3 of expanded nTregs from both groups were determined by Western blot. Data are representative of four separate experiments with similar results. (B) The methylation status of CpG motifs of TSDR of Foxp3 locus in aforementioned harvested nTregs was detected with bisulfite sequencing PCR. Percentages are the average methylation within the TSDR. Data are mean \pm SEM of five independent experiments. **, $P < 0.01$; ****, $P < 0.0001$. AILD, autoimmune liver disease.

We observed that AILD-Tregs markedly improved STAT1 and STAT3 activation compared to HBV-Tregs (Figure 5A). The epigenetic regulation in the *Foxp3* locus leading to demethylation of CpG islands in the region of *Foxp3* locus is considered to be an essential hallmark for the stability and functionality of *Foxp3*⁺ Tregs (32). We used bisulfite sequence analysis to examine the methylation status of both nTreg. As expected, a higher methylation level was detected in AILD-nTreg (Figure 5B).

Discussion

AILD is a type of autoimmune disease that may cause liver injury or failure. Evidence for the loss of central immune tolerance in AIH comes from murine studies (33-35), and inducing non-specific T-cell activation in mice has been shown to result in T-cell-mediated liver injury (36). In the development of PBC, specific loss of immune tolerance to a mitochondrial antigen, the lipoyl domain of the immunodominant E2 component of pyruvate dehydrogenase (PDC-E2) subunit is archetypal (37). In PSC, there is a clear T-cell predominant infiltrate (38) but little knowledge to date of relevant auto-antigenic triggers. There is no curative treatment for all three disorders, and a considerable number of patients eventually progress to an end-stage liver disease requiring liver transplantation (LT). LT, in this context, has a favorable overall outcome with the current patient and graft survival exceeding 80% at 5 years (16).

In this study, we made an analysis comparing the immune balance and graft function between AILD patients' post-transplantation and the patients who have had a liver failure with HBV infection post-transplantation. Our data showed that there is a lower prognosis with severe graft function in liver transplant patients with AILD compared to those patients with HBV-induced liver failure. Immune cell phenotype analysis showed that more Tregs could be detected in AILD patients compared with HBV patients' post-transplantation. We sorted CD4⁺CD25⁺CD127⁻ Tregs *in vivo* and showed that Tregs presented decreased function both *in vitro* and *vivo*. Mechanism study also proved that modulation of the phosphorylation level of STAT1 and STAT3 in addition to the methylation level of TSDR in *Foxp3* might have partially resulted in the expression and function loss of Tregs. These results suggest that function loss of Tregs may be the critical factor that causes graft loss for liver transplant patients after AILD.

Our clinic study data verified that CD8⁺/Treg positive correlated to the graft function post-transplantation while

Treg negatively correlated to the graft function. This data is conflicted with the previous result indicating that Tregs protect and reduce liver failure in AILD (39). As the low percentage of *Foxp3*⁺ Tregs in the blood, we usually evaluated the CD4⁺CD25⁺CD127⁻ Tregs to evaluate the *Foxp3* expression in the clinic. However, CD4⁺CD25⁺CD127⁻ Tregs may not be suitable for presenting the percentage of *Foxp3*⁺ cells in AILD patients post-transplantation due to CD4⁺CD25⁺CD127⁻ Tregs lost *Foxp3* expression *in vivo*; in addition to further analyzing the *Foxp3* expression and suppressive function *in vivo* and *in vitro*, this is why we sorted the Tregs from AILD patients. The experimental data proved that Tregs were not functional in AILD patients due to low expression of *Foxp3*, while our published data indicated that low *Foxp3*⁺ Tregs might be able to induce instead of suppressing inflammatory reactions. Meanwhile, increasing low functional Tregs may aggravate the inflammatory disorder, which finally leads to graft function loss. Besides, we found that CD8⁺ T cells is positive correlated to the graft function post-transplantation, the result is surprising, but we still need more investigation on the function of the improved CD8⁺ T cells.

Previously, several studies had already estimated mechanisms that may be related to Treg function and stability *in vitro* and *vivo*. Liu found that increased phosphorylation of STAT3 resulted in *Foxp3* and IL-10 expression in Tregs (40). Keohane reported JAK induces silencing of T Helper cytokine secretion and a profound reduction in T regulatory cells (41). Harusato demonstrated NFκB activation and STAT transcription might also regulate the induction of Tregs (42).

We proved that AILD-Tregs expressed higher STAT1 and STAT3 phosphorylation and CpG methylation compared with HBV-nTreg. This may partly explain why lower *Foxp3* expression and Treg function was observed in AILD-Tregs but not HBV-Tregs.

Our findings in this study present a new understanding of Tregs and immune balance in AILD patients' post-transplantation. Further characterization of the mechanisms underlying the conversion and function of plastic *Foxp3*⁺ T cells in AILD patients is needed for the development of new therapeutic strategies to preserve graft function and reduce liver injury for patients with autoimmune diseases post-liver transplantation.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm.2020.03.203>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Institutional Animal Committee of Nanjing Medical University.

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Supplementary

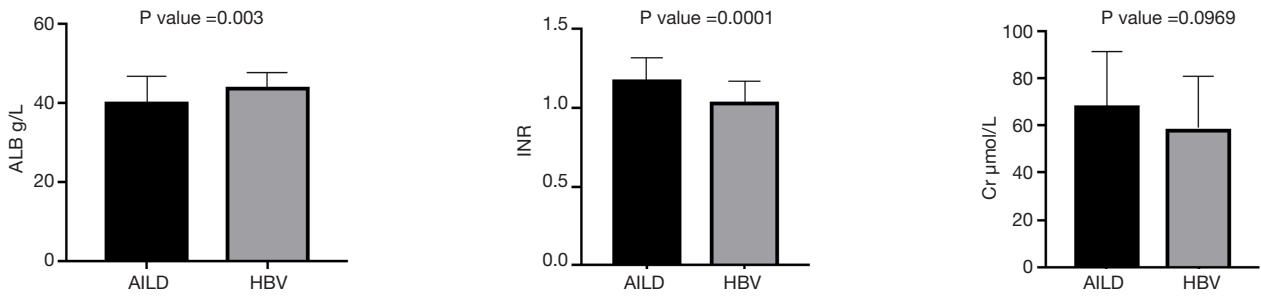


Figure S1 Other liver function indicators including albumin, INR and creatinine were modulated for patients after liver transplantation. The levels of ALB, INR and Cr were evaluated from the serum of liver transplantation patients; the bar shows the mean \pm SEM of the levels of indicated proteins. The difference was analyzed by *t*-test.

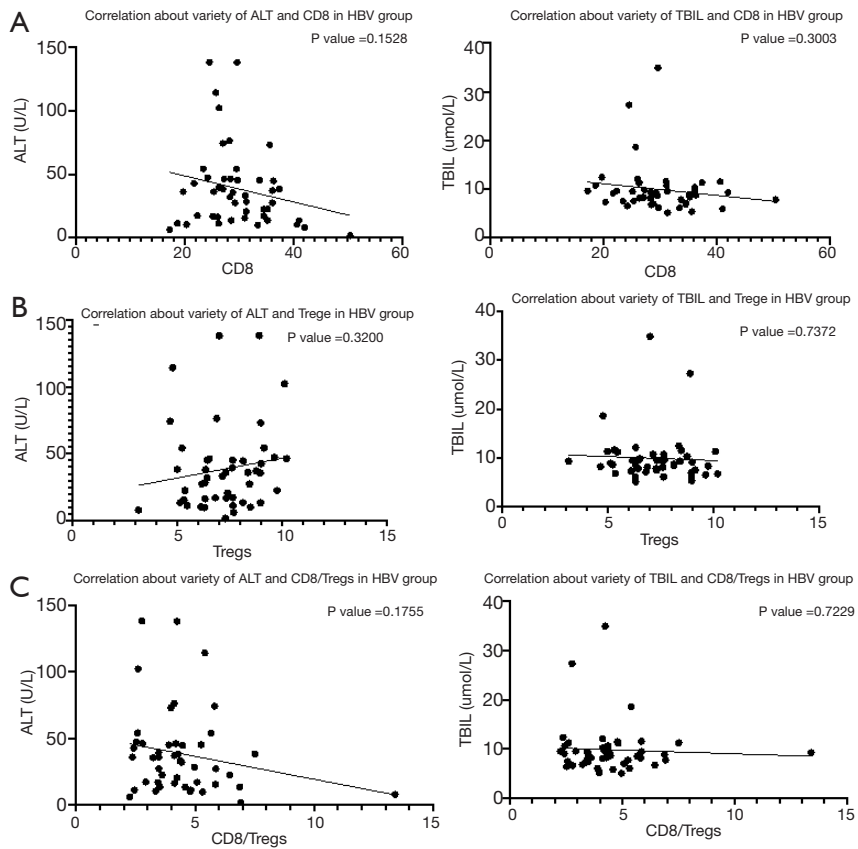


Figure S2 No correlation was observed between Treg and CD8/Treg to the graft function in HBV patients' post-transplantation. Linear regression analysis was used to assess the correlation between the changing of CD8 (A), Treg (B), and CD8/Treg (C) to the graft function such as ALT or TBIL for the HBV patients' post-transplantation. ALT, alanine aminotransferase; TBIL, total bilirubin; HBV, hepatitis B virus.