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Circulating CXCR3-CCR6-CXCR5$^+$CD4$^+$ T cells are associated with acute allograft rejection in liver transplantation

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ABSTRACT

Circulating T follicular helper (cTFH) cells have been demonstrated to be involved in B-cell-mediated alloreactive responses in kidney and liver transplantation; however, whether these cells are involved in acute liver allograft rejection after liver transplantation, and which subsets are involved, remains to be clarified. The present study aimed to investigate the profiles of cTFH cells in acute liver allograft rejection, including the C–X–C motif receptor 3 (CXCR3)$^+$ chemokine receptor 6 (CCR6)$^-$ subset, the CXCR3$^-$CCR6$^-$ subset, and the CXCR3$^-$CCR6$^+$ subset. Twelve liver transplant patients with acute rejection (AR) and 20 with no acute rejection (NAR) were enrolled in the study. The results showed that the proportion of CXCR3-CCR6-CXCR5$^+$CD4$^+$ T cells was significantly increased and the proportion of CXCR3$^-$CCR6$^+$CXCR5$^+$CD4$^+$ T cells was significantly decreased in patients with AR compared with patients with NAR. In addition, the proportion of CXCR3-CCR6-CXCR5$^+$CD4$^+$ T cells was positively correlated with the proportion of B cells in patients with AR. The level of serum interleukin (IL)-21 was higher in the AR group than in the NAR groups. Furthermore, the proportion of CXCR3-CCR6-CXCR5$^+$CD4$^+$ T cells was positively correlated with alanine amino transferase (ALT), whereas the proportion of CXCR3$^-$CCR6$^+$ CXCR5$^+$CD4$^+$ T cells was negatively correlated with ALT. B cells and TFH cells were detected in follicular-like structures in liver allograft tissues from patients with AR. These results suggest that CXCR3-CCR6-CXCR5$^+$CD4$^+$ T cells may be involved in acute allograft rejection after liver transplantation.

1. Introduction

Previous studies have demonstrated that T helper (Th)1/Th2 cells [1,2], natural killer (NK) cells [3], natural killer T (NKT) cells [4,5], γδ T cells [6], Foxp3$^+$ regulatory T cells (Treg) and Th17 cells [7] are involved in alloreactivity in organ transplantation. Recently, T follicular helper (TFH) cells have been reported to be involved in allograft rejection in kidney transplantation both in vitro and in vivo [8]. TFH cells are a heterogeneous population of CD4$^+$ Th cells that are active in secondary lymphoid organs and are phenotypically characterized by expression of follicular-homing CXC chemokine receptor 5 (CXCR5), inducible costimulator (ICOS), programmed death 1 (PD-1) and B cell lymphoma 6 (Bcl-6) [9,10]. Differentiated TFH cells also produce interleukin (IL)-21, and express IL-21 receptor (IL-21R). IL-21R is expressed on a variety of immune cells, including T cells, B cells, NK cells and dendritic cells, as well as non-immune cells (e.g. endothelial cells and epithelial cells) [11,12]. Through the autocrine and paracrine systems, IL-21 amplifies TFH cell-mediated immune responses, and regulates the activation, proliferation and survival of CD4$^+$ T cells and B cells. It mediates B cell differentiation into immunoglobulin (Ig)-producing plasmablasts, plasma cells and memory B cells [13,14] when IL-21 binds to IL-21R, which activates the protein tyrosine kinases, signal transducers and transcription activators in the Janus kinase (JAK) family [15].

Human circulating TFH (cTFH) cells can be categorized into three distinct subsets: CXCR3$^+$ chemokine receptor 6 (CCR6)$^-$, CXCR3$^-$CCR6$^-$,

Abbreviations: ALT, alanine amino transferase; AR, acute rejection; cTFH, circulating T follicular helper; FACS, fluorescence-activated cell sorting; NAR, no acute rejection

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Table 1
Baseline characteristics of the patients.

	Acute rejection (AR)	No acute rejection (NAR)
Gender (male/female)	11/1	17/3
Age (years)		
median	44	50
range	38–69	35–66
HBsAg-positive	10	16
Time since graft (months)	3	3
CD4 counts		
Median	503	608
range	422–946	451–1098

and CXCR3$^-$CCR6$^+$ [16]. CXCR3-CCR6-CXCR5$^+$CD4$^+$ T cells and CXCR3-CCR6$^+$CXCR5$^+$CD4$^+$ T cells, but not CXCR3$^+$CCR6-CXCR5$^+$CD4$^+$ T cells, can induce naive B cells to produce Igs by secreting IL-21 [16]. cTFH cells have been found in increased numbers in human and murine models of systemic autoimmune diseases such as primary biliary cirrhosis [17], rheumatoid arthritis [18,19] and autoimmune hepatitis [20,21]. A previous study suggested that cTFH cells may be involved in alloreactive responses following human liver transplantation by promoting B cell differentiation into plasmablasts and plasma cells [22]. However, it remains unclear whether cTFH cells are involved in acute allograft reject after liver transplantation, and which subsets are responsible if so. In this study, for the first time, the characteristic profiles of the cTFH cell subsets are investigated in patients who had acute allograft rejection after liver transplantation.

2. Materials and methods

2.1. Patients

Thirty-two liver transplant patients were enrolled in this study. All patients had received a first cadaveric liver transplant with an identical or compatible blood-group graft between April 1, 2015 and January 31, 2017 at Beijing 302 Hospital. Based on clinical manifestations, biochemical indicators and pathologic diagnosis, the patients were divided into an acute rejection (AR) group (n = 12) and a no acute rejection (NAR) group (n = 20). The characteristics of the enrolled patients are shown in Table 1. AR was established at the time of sample collection using histopathologic diagnosis with the Banff classification system

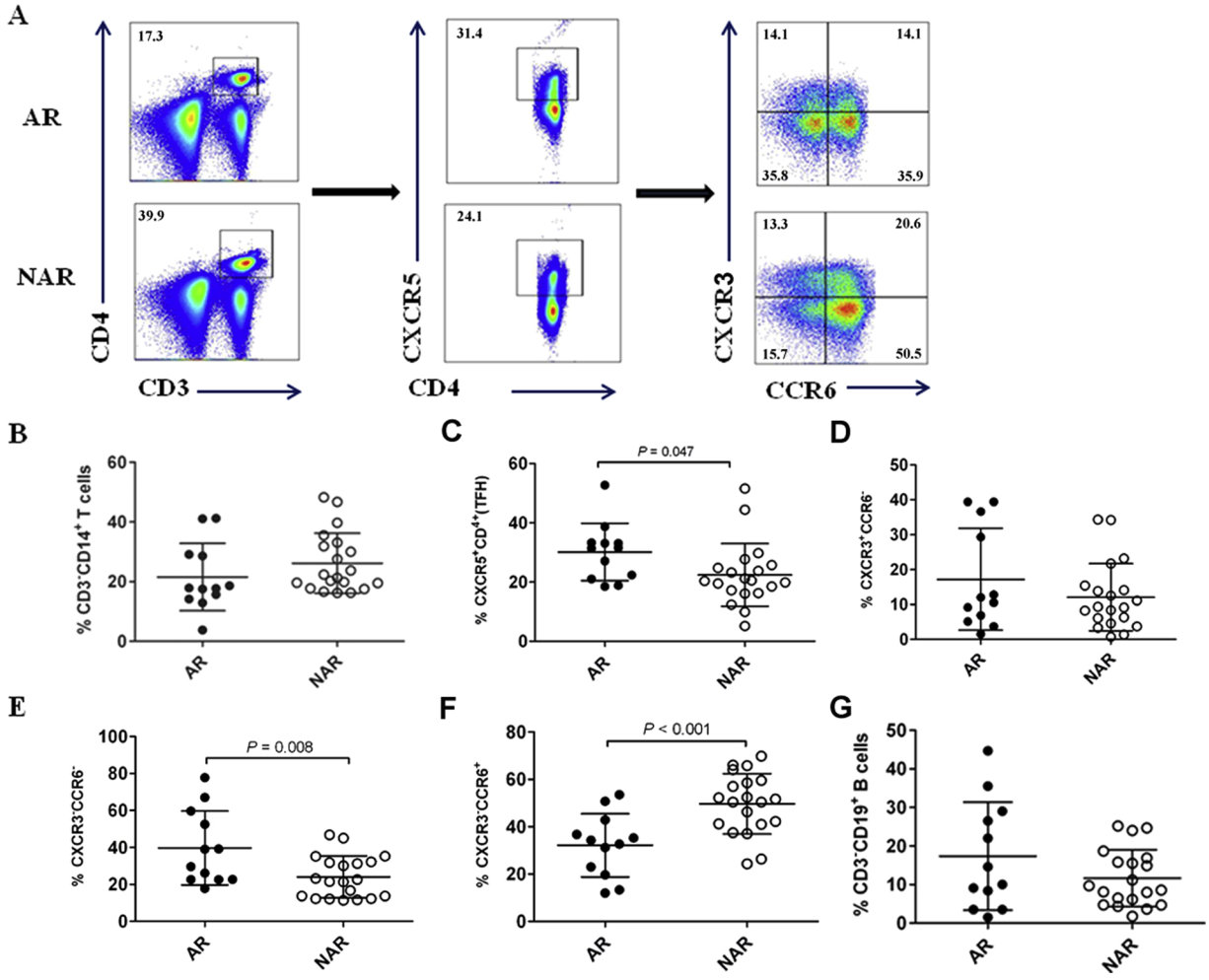

Fig. 1. The proportions of circulating T follicular helper cell subsets in liver transplant patients.
(A) A typical example of the FACS strategy used to obtain CXCR5$^+$CD4$^+$ TFH cell subsets.
(B) The proportions of the CD3$^+$CD4$^+$ T cells in liver transplant patients.
(C) The proportions of the CXCR5$^+$CD4$^+$ TFH cells in liver transplant patients.
(D) The proportions of the CXCR3$^+$CCR6$^-$ subset in liver transplant patients.
(E) The proportions of the CXCR3$^-$CCR6- subset in liver transplant patients.
(F) The proportions of the CXCR3$^-$CCR6$^+$ subset in liver transplant patients.
(G) The proportions of the CD3$^-$CD19$^+$ B cells in liver transplant patients.
AR, acute rejection; FACS, fluorescence-activated cell sorting; NAR, no acute rejection.

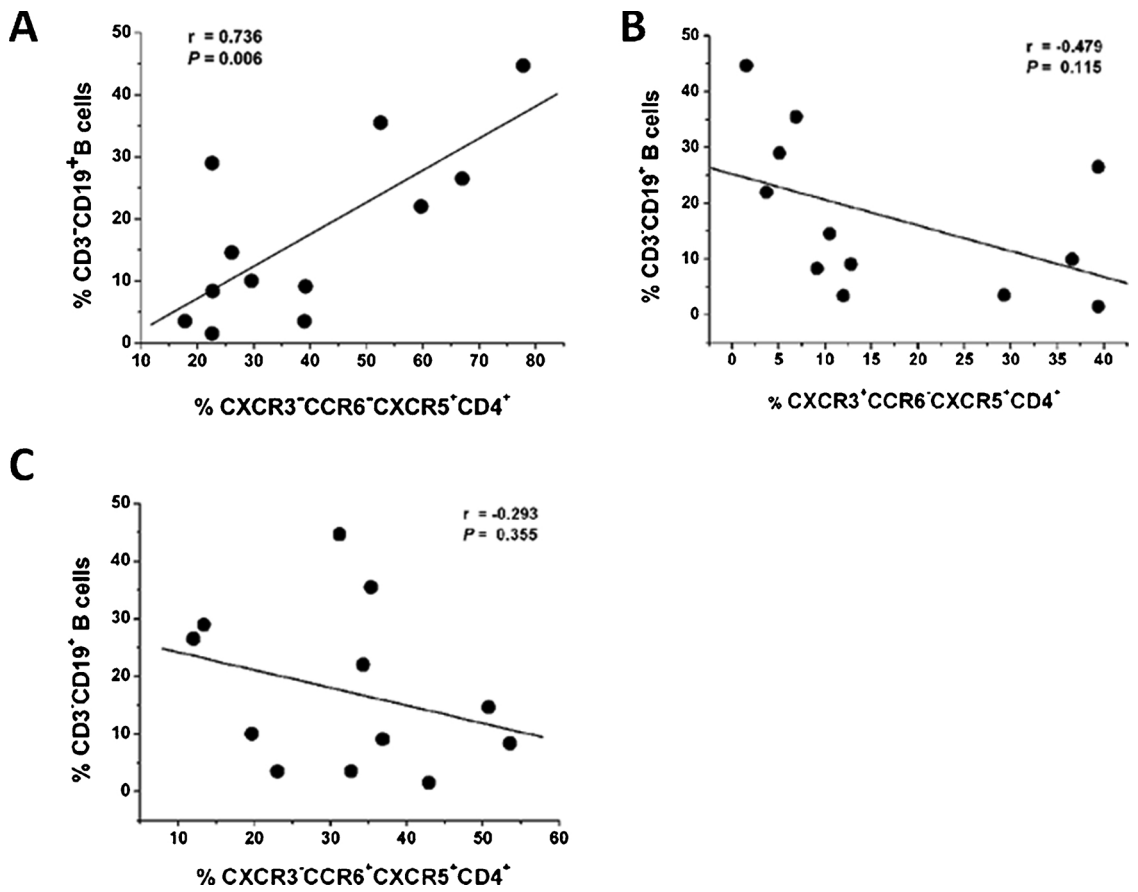

Fig. 2. The relationships between the proportions of circulating T follicular helper cells and B cells in the enrolled patients. The relationships between the proportion of $CD3^{-}CD19^{+}$ B cell and the proportion of.
(A) $CXCR3^{-}CCR6^{-}CXCR5^{+}CD4^{+}$ T cells.
(B) $CXCR3^{+}CCR6^{-}CXCR5^{+}CD4^{+}$ T cells.
(C) $CXCR3^{-}CCR6^{+}CXCR5^{+}CD4^{+}$ T cells.

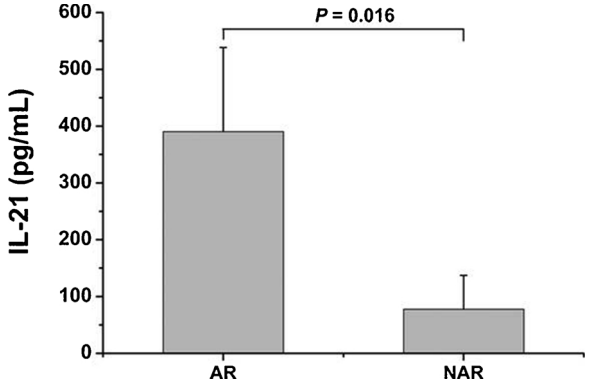

Fig. 3. Serum IL-21 levels in liver transplant patients.
AR, acute rejection; IL, interleukin; NAR, no acute rejection.

[23]. NAR was defined as the absence of any clinically active disease at the time of sample collection. All patients underwent induction therapy with basiliximab, and received glucocorticoid, tacrolimus (FK506) and mycophenolate mofetil triple immunosuppressive therapy in the early postoperative period. The glucocorticoid dose was gradually reduced in the 3 months after surgery until it was withdrawn. At this point, depending on the condition of patient, FK506 was administered alone or in combination with mycophenolate mofetil. If AR was diagnosed, the immunosuppressive agent doses were adjusted. Patients with hepatitis B virus infection received prophylactic therapy with hepatitis B Ig plus nucleoside or nucleotide analogues. Patients were followed up at Beijing 302 Hospital. The study protocol was approved by the institutional review board of Beijing 302 Hospital and written informed consent was obtained from each subject.

2.2. Sample processing

Blood samples anticoagulated with heparin were centrifuged at 1500 rpm for 10 min, after which the plasma was collected and stored at −80 °C. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using the Ficoll-Hypaque technique (GE Healthcare, Munich, Germany).

Paraffin-embedded liver tissue samples were obtained either from ultrasonically guided needle liver biopsies or by excision from the donor livers.

2.3. Flow cytometry

To differentiate between cTFH cell subsets and B cells, PBMCs at 1×10^{6}/tube were stained with the following fluorochrome-conjugated antibodies: brilliant violet 510 (BV510)-anti-CD3 and phycoerythrin (PE)-anti-CD19 (both from Biolegend, San Diego, CA, USA), and peridinin chlorophyll protein (PerCP)-anti-CD4, Alexa Fluor 488-anti-CXCR5, allophycocyanin (APC)-anti-CXCR3 and BV421-anti − CCR6 (all from BD Bioscience, San Jose, CA, USA). The PBMCs were stained at room temperature for 20 min. After being washed with phosphate buffered saline, the cells were counted using a FACSVerse flow cytometer (BD Bioscience).

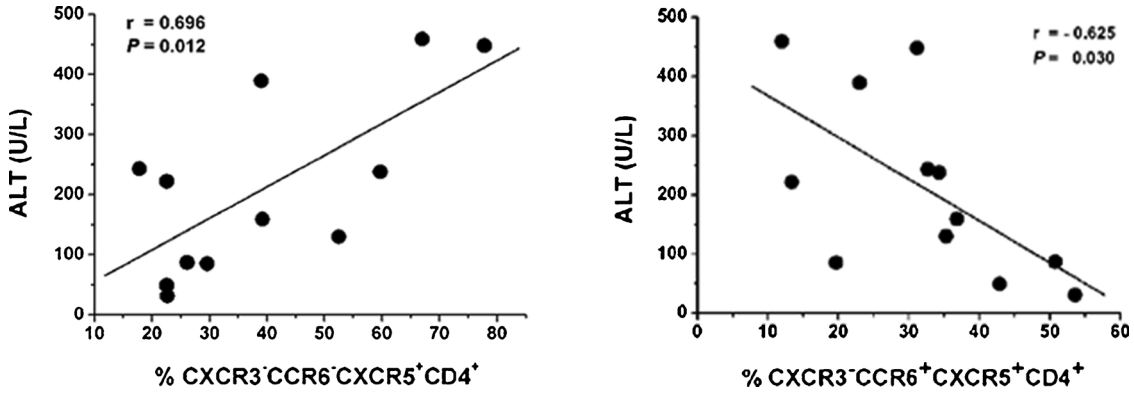

Fig. 4. The relationships between the proportions of circulating T follicular helper cells and biochemical indice of liver injury in patients with acute rejection. ALT, alanine amino transferase.

2.4. Enzyme-linked immunosorbent assay

Serum IL-21 was measured by enzyme-linked immunosorbent assay (ELISA) according to the kit manufacturer's protocol (eBioscience, San Diego, CA). Samples were tested in duplicate along with standards and calibrator controls provided by the manufacturer.

2.5. Immunohistochemistry

Biopsies from donor livers and allografts from patients with AR were stained to enable observation of TFH cells. Formalin-fixed and paraffin-embedded liver tissues were cut into 4-μm sections. The sections were incubated overnight at 4 °C with primary monoclonal antibodies, including anti-CD38, anti-CD20, anti-CD4 (Zhongshan Goldenbridge Biotech, Beijing, China) and anti-Bcl-6 (Abcam, Cambridge, UK), and then incubated with biotinylated goat anti-rat or goat anti-rabbit amplifiers (Zhongshan Goldenbridge Biotech, Beijing, China). Sections of tonsil were used as positive controls. Double-positive labelling with Bcl-6 and PD-1 identified TFH cells.

2.6. Statistical analysis

All data were analyzed using SPSS 19.0 for Windows software (IBM, Armonk, NY, USA). Data are presented as means ± standard deviation. Baseline clinical characteristics were compared between groups using the chi-squared test for categorical variables and the Wilcoxon-rank-sum test for continuous variables. Comparisons between two groups were made using the Mann–Whitney *U* test. Relationships between two variables were evaluated using the Spearman rank correlation test. For all tests, a two-sided *P* value of < 0.05 was considered significant.

3. Results

3.1. cTFH cell profiles in liver transplant recipients

To investigate the profiles of cTFH cells, PBMCs were analyzed by flow cytometry (Fig. 1A). The proportion of $CD3^+CD4^+$ T cells in the AR group (21.5 ± 10.8%) was no significant difference compared with the NAR group (26.2 ± 9.8%) ($P = 0.235$) (Fig. 1B). The proportion of $CXCR5^+CD4^+$ cTFH cells in the AR group (30.1 ± 9.3%) was significantly higher than that in the NAR group (22.4 ± 10.3%) ($P = 0.047$) (Fig. 1C). The proportion of $CXCR3^+CCR6^-$ subset in the AR group (17.2 ± 14.0%) was no significant difference compared with the NAR group (12.1 ± 9.7%) ($P = 0.241$) (Fig. 1D). The proportion of the $CXCR3^-CCR6^-$ subset was higher in the AR group (39.7 ± 19.2%) than in the NAR group (24.0 ± 11.3%) ($P = 0.008$) (Fig. 1E). However, the $CXCR3^-CCR6^+$ subset occurred significantly less frequently in the AR group (32.1 ± 12.8%) compared with the NAR group (49.7 ± 12.8%) ($P < 0.001$) (Fig. 1F). We also examined the proportion of $CD3^-CD19^+$ B cells in the enrolled patients, and found that the proportions of these cells tended to be higher in the AR group (17.4 ± 13.4%) than in the NAR group (11.7 ± 7.4%), but this difference was not significant ($P = 0.140$) (Fig. 1G).

3.2. The proportions of $CXCR3^-CCR6^-CXCR5^+CD4^+$ T cells were correlated with the proportion of B cells

The relationships between the proportions of the cTFH cell subsets and B cells in all enrolled patients were analyzed. In AR group, the proportions of $CXCR3^-CCR6^-CXCR5^+$ $CD4^+$ subset were positively correlated with the proportion of B cells (r = 0.736, $P = 0.006$) (Fig. 2A); however, the proportions of the $CXCR3^+CCR6^-CXCR5^+CD4^+$ (r = -0.479, $P = 0.115$) and $CXCR3^-CCR6^+CXCR5^+CD4^+$ subsets (r = -0.293, $P = 0.355$) were not significantly correlated with the proportion of B cells (Fig. 2B, C). In NAR group, the proportions of the cTFH cell subsets were not significantly correlated with the proportion of B cells (data not shown).

3.3. Serum IL-21 was elevated in patients with acute rejection

Because cTFH cells can promote differentiation of B cells into plasmablasts in an IL-21-dependent manner in liver transplantation, we analyzed the level of serum IL-21 in the study participants. The results showed that the level of serum IL-21 in the AR group (390.5 ± 147.9 pg/mL) was significantly higher than that in the NAR group (77.8 ± 59.5 pg/mL) ($P = 0.016$) (Fig. 3).

3.4. The relationships between the proportions of cTFH cells and biochemical indice of liver injury

Little is known about the relationships between cTFH cells and liver injury in liver transplant recipients. We therefore investigated correlations between the proportion of cTFH cell subsets and indice of liver damage, such as alanine amino transferase (ALT), in liver transplant patients with acute rejection. We found that the proportion of the $CXCR3^-CCR6^-CXCR5^+CD4^+$ subset was positively correlated with ALT (r = 0.696, $P = 0.012$) (Fig. 4A). Surprisingly, the proportion of the $CXCR3^-CCR6^+CXCR5^+CD4^+$ subset was negatively correlated with ALT (r = -0.625, $P = 0.030$) (Fig. 4B), whereas the proportion of the $CXCR3^+CCR6^-CXCR5^+CD4^+$ subset was not significantly correlated with ALT (data not shown).

3.5. TFH cells were present in follicular-like structures in patients with acute rejection

Samples of allograft liver tissue from patients with AR and from the donor's livers were stained to examine the distribution of TFH cells by

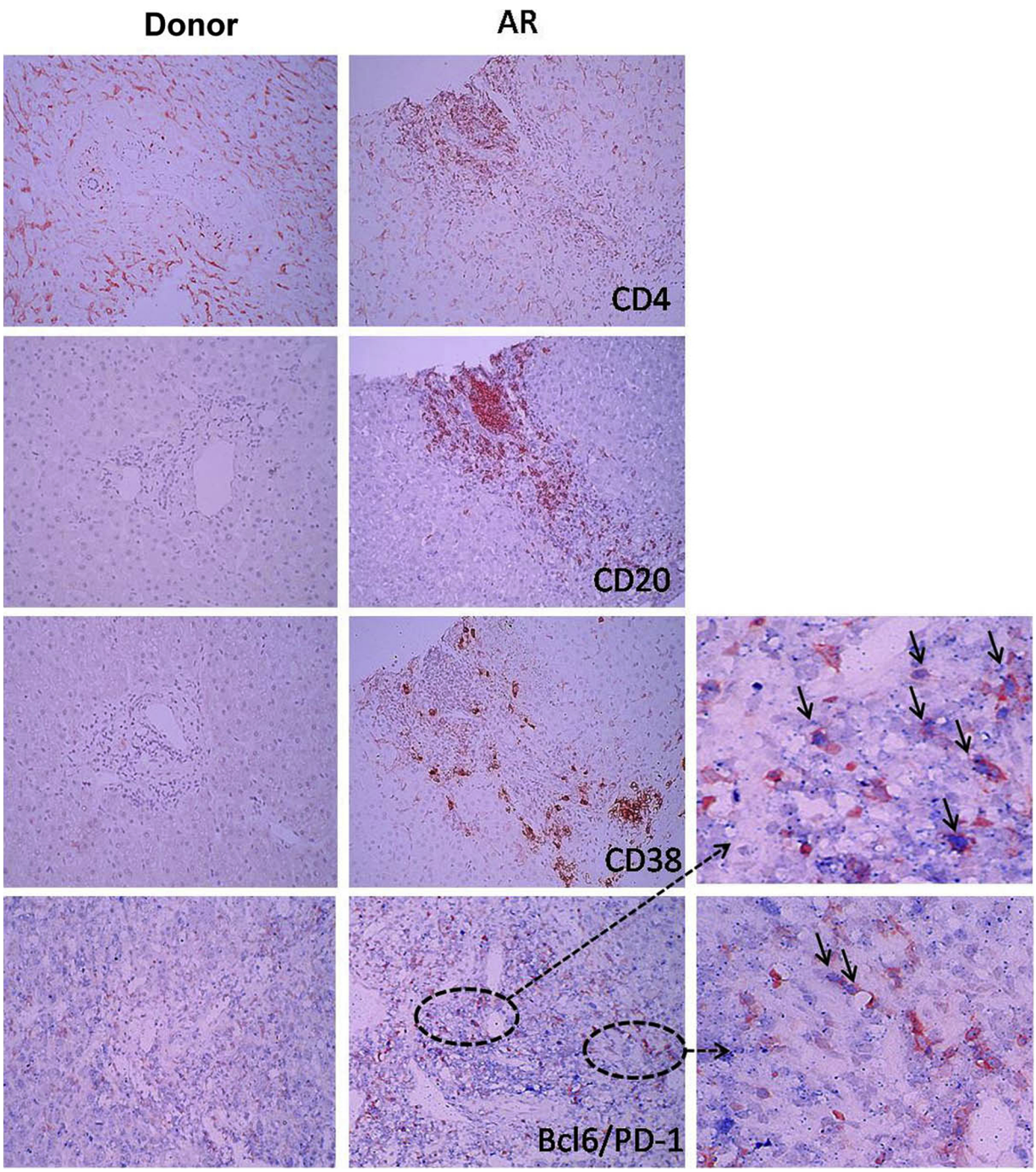

Fig. 5. T follicular helper cell distribution in donor liver samples and liver allograft samples from patients with acute rejection. Staining for $CD4^+$ T cells, $CD20^+$ B cells, $CD38^+$ plasmablasts and PD-$1^+$$CXCR5^+$ TFH cells in donor liver samples and liver allograft samples from patients with AR. The arrows indicate the Bcl-6^+PD-1^+ TFH cells. AR, acute rejection; TFH, T follicular helper.

semi-quantitative immunohistochemical analysis. $CD4^+$ T cells, $CD20^+$ B cells, $CD38^+$ plasmablasts and Bcl-6^+ PD-1^+ TFH cells were observed in follicular-like structures in liver allograft tissue samples from patients with AR, but few was found in the donor liver tissues. These results indicate that TFH cells may be involved in acute allograft rejection after liver transplantation (Fig. 5).

4. Discussion

Despite the use of potent immunosuppressive agents, acute rejection remains an obstacle to long-term allograft survival. Several immune cell types have been reported to be involved in alloreactive responses after organ transplantation [1–7]. Based on a previous study which indicated that $CXCR5^+CD4^+$ TFH cells are associated with this response [22], in this study, we explored the relationship between TFH cell subsets and acute liver allograft rejection. The major finding of the study is that the $CXCR3^-CCR6^-$ and $CXCR3^-CCR6^+$ subsets, but not the $CXCR3^+CCR6^-$ subset, may be involved in acute allograft rejection after liver transplantation. The data showed that in patients with AR, the proportion of $CXCR3^-CCR6^-CXCR5^+CD4^+$ T cells was significantly increased, whereas the proportion of $CXCR3^-CCR6^+CXCR5^+CD4^+$ T cells was significantly decreased. In addition, the proportion of $CXCR3^-CCR6^-CXCR5^+CD4^+$ T cells was positively correlated with the proportion of B cells and ALT, whereas the proportion of $CXCR3^-CCR6^+CXCR5^+CD4^+$ T cells was

negatively correlated with ALT. Our study has for the first time demonstrated the characteristic profiles of the TFH cell subsets in liver transplantation, and highlighted the important role of cTFH cells in the alloreactive response during AR.

TFH cells play a crucial role in promoting B cell maturation and in the production of antibodies in response to foreign antigens [24]. In organ transplantation, TFH cells have been found to play an important role in acute rejection after kidney transplantation by inducing B cell differentiation and Ig production. A previous study also found that cTFH cells are involved in alloreactive responses following liver transplantation via promoting the differentiation of B cells into plasmablasts and plasma cells. This may be mediated by the IL-21 secreted by cTFH cells, as IL-21 is essential for B cell activation and expansion, and plasma cell generation [16,25,26].

Expression of IL-21 and the IL-21R by T cells plays an important role in acute cardiac rejection, and blockade of the IL-21 pathway has been shown to inhibit the expansion of donor antigen-activated T cells [27]. A previous study found that the proportion of IL-21-producing cTFH cells declined, and the capacity of cTFH cells to promote B cell differentiation reduced, after liver transplantation in recipients with normal liver function [22]. However, in acute liver allograft rejection, a characteristic pattern of IL-21-producing cTFH cells remains to be determined. Previous studies have reported that the $CXCR3^+CCR6^-CXCR5^+CD4^+$ T cell is not efficient at promoting B cell differentiation [28,29], but that $CXCR3^-CCR6^-CXCR5^+CD4^+$ and $CXCR3^-CCR6^+CXCR5^+CD4^+$ T cells display an efficient helper response for the promotion of B cell differentiation and Ig secretion [16]. Elevated the proportion of $CXCR3^-CCR6^-CXCR5^+CD4^+$ T cell has been shown to be positively correlated with serum IgG4 levels and $IgG4^+$ plasmablast counts in patients with IgG4-related disease; however, $CXCR3^+CCR6^-CXCR5^+CD4^+$ and $CXCR3^-CCR6^+CXCR5^+CD4^+$ T cells showed no such correlation [30]. In the current study, in patients with AR, the proportion of $CXCR3^-CCR6^-CXCR5^+CD4^+$ T cells and serum IL-21 levels were significantly increased when compared with people with normal liver function. This suggests that IL-21 secreted by $CXCR3^-CCR6^-CXCR5^+CD4^+$ T cells may be associated with liver allograft rejection. de Graav et al. [8] reported that TFH cells co-localized with B cells and Igs in follicular-like structures in kidney biopsies from patients with acute rejection; in the present study, $CD20^+$ B cells and $CD38^+$ plasmablasts co-localized with TFH cells in follicular-like structures in liver allograft biopsy samples from patients with acute rejection. Examination indicated that these cells interacted physically at the graft site to regulate B cell function. In addition, the present study revealed that the proportion of $CXCR3^-CCR6^-CXCR5^+CD4^+$ T cells was positively correlated with the proportion of $CD3^-CD19^+$ B cells in patients with AR. Clinically, we found that the proportion of $CXCR3^-CCR6^-CXCR5^+CD4^+$ T cells was positively correlated with biochemical indice of liver damage such as ALT; in contrast, the proportion of $CXCR3^-CCR6^+CXCR5^+CD4^+$ T cells was negatively correlated with ALT. Taken together, these data indicate that $CXCR3^-CCR6^-CXCR5^+CD4^+$ T cells may promote acute allograft rejection after liver transplantation, and that $CXCR3^-CCR6^+CXCR5^+CD4^+$ T cells may suppress it; however, the underlying mechanisms of this remains to be demonstrated.

Conflict of interest

The authors declare no potential conflicts of interest.

Acknowledgements

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